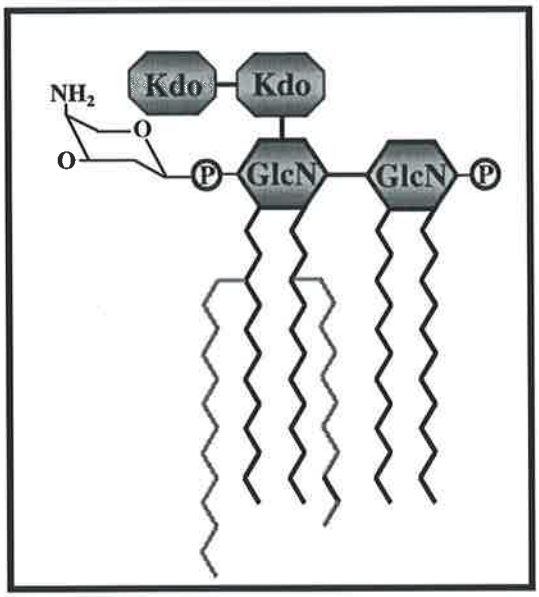
Molecular characterisation of *Salmonella typhi* PhoP/Q regulated genes



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Abstract

Salmonella typhi, the causative agent of typhoid fever in humans is highly host-adapted and is unable to cause this disease in animals. An important part of *Salmonella* pathogenesis is the ability of the bacterium to survive and grow in macrophages. The PhoP-PhoQ (PhoP/Q) two component regulatory system which has been shown to be involved with virulence and survival in macrophages, both activates (*pag/pqa*) and represses (*prg/pqr*) genes located around the chromosome, in response to environment signals (extracellular Mg^{2+} levels and pH). A large number of *pags/prg*s involved with virulence have been identified in *Salmonella*, eg survival in macrophages, invasion of mammalian cells, protein secretion, and antimicrobial peptide resistance. The PhoP/Q operon has been shown to regulate another two component regulatory system, PmrA-PmrB (PmrA/B), which influences lipid A structural modification and polymyxin B resistance. PhoP/Q regulated genes have also been shown to be involved in housekeeping functions.

This project involved comparisons of *phoP/Q* regulation in *S. typhi* and *S. typhimurium* and detailed analysis of the seven *pqa/pqr*::Mu*dJ* insertion mutants isolated previously (Baker, 1993). Comparison of *S. typhi* and *S .typhimurium* wildtype, *phoP12*, ($PhoP^-$) (PhoP null mutations in which are *pqr/prg* genes are up-regulated and *pqa/pag*s are down-regulated) and *phoP24* ($PhoP^c$) (PhoP constitutive mutations in which *pqa/pag* genes are upregulated and *pqr/prg*s are downregulated) strains showed differences in both the number and apparent molecular mass of PhoP/Q regulated proteins, indicating that *S. typhi* and *S. typhimurium* may have different PhoP/Q regulated proteins and/or that their regulation may be different. This latter statement correlates with the fact that a difference in levels of a *Salmonella pag* protein (PhoN) encoding a non-specific acid phosphatase was noted with lower levels of PhoN being seen for *S. typhi* compared to *S. typhimurium*. Higher levels of PhoN activity were found in

Salmonella PhoP$^-$ strains complemented with *phoP/Q* on medium copy number plasmids compared to chromosomal *phoP/Q* backgrounds and when the seven *S. typhi pqa/pqr*::Mu*dJ* mutants were tested for β-galactosidase activity in PhoP$^-$, PhoP$^+$ and PhoP^c chromosomal backgrounds, the levels of β-galactosidase were found to be lower in the latter compared to the plasmid complemented strains. Due to this decreased regulation in the chromosomal background, only five *S. typhi pqa/pqr*::Mu*dJ* mutants were found to be significantly regulated by *phoP/Q* and only these strains were analysed further.

Both *S. typhi* and *S. typhimurium* were tested against a range of antimicrobial agents (protamine, melittin, and polymyxin B) and *S. typhi* was found to be approximately 10-fold more sensitive to these antimicrobial agents than *S. typhimurium*. Both *S. typhi and S. typhimurium* PhoP^c strains had an increased resistance compared to the respective wildtype strain. The *S. typhi* PhoP/Q regulated *pqa/pqr*::Mu*dJ* insertion mutants (*pqaA*::Mu*dJ*, *pqaB*::Mu*dJ*, *pqaD*::Mu*dJ*, *pqrA*::Mu*dJ*, and *pqrB*::Mu*dJ*) were also tested against these peptides and the *pqaB*::Mu*dJ* mutant was found to be sensitive to the antimicrobial peptide melittin and the antibiotic polymyxin B.

The *S. typhi pqa/pqr*::Mu*dJ* fusions were cloned and sequenced to find the insertion point of the Mu*dJ* transposon. Partial sequencing of the *S. typhi pqaD*::Mu*dJ*, *pqrA*::Mu*dJ*, and *pqrB*::Mu*dJ* insertion mutants indicated that *pqaD*, *pqrA* and *pqrB* had strong homology to known *E. coli* and *Salmonella* housekeeping genes. PqaD had ~95% homology to the *E. coli* DeoA protein which is involved with the pyrimidine salvage pathway. PqrA had ~95% homology to the *E. coli* GcvA protein which is the glycine activator protein, and PqrB had ~80% homology to the *E. coli* Rsd protein, a stationary phase protein which forms a complex with σ^{70}.

The *S. typhi pqaA* gene was isolated, sequenced, and found to lie downstream of a 413 aa protein which had strong homology to proteins which belong to the multidrug efflux family. The *S. typhi pqaA* gene was found to be *Salmonella* specific, and to be located on a 30.4 kb

DNA region flanked by DNA with significant similarity to *E. coli* K12 and *S. typhimurium* DNA, indicating that PqaA may be encoded on a currently unidentified Salmonella Pathogenicity Island. The 3' end of the *S. typhi pqaA* DNA region was found to encode Dcp (dipeptidyl carboxypeptidase), which is located at 32.5 minutes on the *S. typhimurium* chromosome. The *S. typhi pqaA* gene encodes a 518 aa (59.3 kDa) protein which was confirmed by overexpression and L-[S^{35}] methionine labelling experiments, and had no homology to other bacterial genes when compared to the sequence databases with Blast 2.0 N/X/P programs.. PqaA is a highly hydrophilic protein and predicted by computer analysis to be a cytoplasmic membrane anchored protein, and it was confirmed by construction of PqaA::LacZ protein fusions that the majority of the protein is located in the cytoplasm.

A cell culture based assay involving the human/monocyte macrophage cell line U937 has been developed as an alternative to an animal model for *S. typhi* due to the host specificity of this pathogen and the limitations of the current animal models. This cell line was chosen because macrophages have been shown to be crucial for *Salmonella* pathogenesis. *S. typhi* wildtype and both null and constitutive *phoP/Q* mutants were assayed for their ability to invade and grow in U937 cells. It was found that the *S. typhi* $PhoP^-$ mutant was growth restricted in the phorbol myristate acetate (PMA) differentiated U937 (PMA-U937) cells, this growth defect was able to be complemented by a low copy number plasmid carrying the *S. typhi*miurum *phoP/Q* genes . Additionally the *S. typhi* $PhoP^c$ mutant showed an invasion defect with the PMA-U937 cells. Neither of the *phoP/Q* mutants were growth impaired in HeLa cells. The behaviour of *Salmonella typhimurium* wildtype, $PhoP^-$ and $PhoP^C$ mutants in the PMA-U937 cells was also investigated. All three strains grew in the cells, indicating that the $PhoP^-$ mediated growth restriction in the PMA-U937 cells was *S. typhi* specific. PMA-U937 cells were used to test the five *S. typhi,* PhoP/Q regulated, Mu*dJ* insertion mutants for invasion and growth restriction. Although all five mutants tested were unaffected for invasion, one Mu*dJ* insertion mutant (*pqaB*::Mu*dJ*) showed a growth defect compared to

wild-type *S. typhi*. The *S. typhi pqaB* gene was isolated, sequenced and showed 98% identity to the fifth gene in a *S. typhimurium* PmrA/B regulated operon necessary for 4-amino-arabinose modifications of lipid A and polymyxin B resistance. It was found that the *S. typhi pqaB*::Mu*d*J fusion was regulated by PmrA-PmrB and that the *pqaB* mutant was sensitive to polymyxin B (as mentioned previously). A defined *pqaB*::Kan mutation (designed so as to not affect downstream genes in the operon) in the *S. typhi* wildtype background also showed a growth deficiency in the PMA-U937 cell assay and sensitivity to melittin similar to the *pqaB*::Mu*d*J mutation. The same defined *pqaB*::Kan mutation in the *S. typhi* PhoP^{c} background showed an increased sensitivity to polymyxin B as expected.

The LPS of *S. typhi* and *S. typhimurium* wildtype, PhoP^{-} and PhoP^{c} mutants were compared by SDS-PAGE gel and silver staining. Differences in the LPS profile between the two *Salmonella* species were observed, and shown to be affected differently by the PhoP^{c} mutation. Additionally, the *pqaB*::Mu*d*J mutation affected *S. typhi* LPS.

The differences between *S. typhi* and *S. typhimurium* LPS, *phoP/Q* regulated proteins, differential susceptibility to antimicrobial peptides and polymyxin B, and net growth in PMA-U937 cells suggest that differences between *S. typhi* and *S. typhimurium phoP/Q* regulation of proteins and LPS chain length/modifications may impact on the relative virulence of *Salmonella* species in different hosts.

This work contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited in the University library, being available for loan and photocopying.

Sarah Jane Baker

I dedicate this thesis to my family, especially my parents Valerie and Daryl Baker

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Publications

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Abbreviations

A:	adenine
A_{260}:	absorbance at 260 nm
aa:	amino acid
Amp:	ampicillin
ATP:	adenosine-5'-triphosphate
β-gal:	β-galactosidase
bp:	base pair
C:	cytosine
Cml:	chloramphenicol
CTP:	cytosine-5'-triphosphate
C-terminal:	carboxy terminal
DIG:	digoxigenin
DNA:	deoxyribonucleic acid
DNase:	deoxyribonuclease
dNTP:	deoxyribonucleoside triphosphate
ddNTP:	dideoxyribonucleoside triphosphate
DMF:	dimethyl formamide
DTT:	dithiothreitol
EDTA:	ethylene-diamine-tetra-acetic acid
EGTA:	ethylene-bis(oxyethylenenitrilo)tetra-acetic acid
EtBr:	ethidium bromide
FBS:	foetal bovine serum
4AA:	4-amino-arabinose
G:	guanine
gbD:	Genbank DNA sequence accession number (National Center for Biotechnology Information)
gbP:	Genbank protein sequence accession number (National Center for Biotechnology Information)
Gm:	gentamycin
GTP:	guanine-5'-triphosphate
IPCR:	inverse polymerase chain reaction
IPTG:	isopropyl-β-D-thiogalactopyranoside
IS:	insertion sequence
kb:	kilobase pairs
kDa:	kilodalton
KDO:	keto-3-deoxy-D-manno-octulosonic acid

Kan: kanamycin

LA: luria agar

LacZ: β-galactosidase

LB: luria broth

LBON: LB without NaCl

LDH: lactate dehydrogenase

LPS: lipopolysaccharide

MCS: multiple cloning site

mg: milligram

ml: millilitre

MM: M9 minimal media

mM: millimolar

min: minute

mRNA: messenger RNA

MOMP: Major Outer Membrane Protein

mQ: milli Q water

NA: nutrient agar

nt: nucleotide

N-terminal: amino terminal

OD: optical density

OM: outer membrane

ORF: open reading frame

PAGE: polacrylamide gel electrophoresis

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PEG: polyethylene glycol –8000

PhoA: alkaline phosphatase

PhoP$^-$: *phoP* null mutant phenotype

PhoP$^+$: *phoP* wildtype phenotype

PhoP^c: *phoP* constitutive mutant phenotype

***phoP*$^-$:** *phoP* null mutant genotype

***phoP*$^+$:** phoP wildtype genotype

***phoP*^{c}:** phoP constitutive mutant genotype

***pag*:** *phoP/Q* activated gene

PMA: phorbol-12-myristate acetate

pmol: picomolar

PmB:	polymyxin B
***prg*:**	*phoP/Q* repressed gene
***pqa*:**	*phoP/Q* activated gene (*S. typhi*)
***pqr*:**	*phoP/Q* repressed gene (*S. typhi*)
^{R}:	resistant
RBS:	ribosome binding site
rfb:	O-antigen biosynthesis genes
RNA:	ribonucleic acid
RNase:	ribonuclease
rpm:	revolutions per minute
RT:	room temperature
^{S}:	sensitive
SD:	Shine-Dalgarno
SDS:	sodium dodecyl sulphate
Spc:	spectinomycin
SSP-PCR:	single specific primer polymerase chain reaction
SS:	single stranded
Strep:	streptomycin
T:	thymine
Tet:	tetracycline
Tc:	tetracycline
TEMED:	N,N,N',N'-tetramethyl-ethylene-diamine
Tn:	transposon
Tris:	Tris (hydroxymethyl) aminomethane
TTP:	thymine-5'-triphosphate
ts:	temperature sensitive
U:	uracil
µg:	microgram
µl:	microlitre
UV:	ultraviolet
V:	voltage
v/v:	volume per volume
w/v:	weight per volume
X-gal:	5-bromo-4-chloro-3-indolyl-β-galactopyranoside
X-pho:	5-bromo-4-chloro-3-indolylphosphate

Chapter 1

Introduction

1.1 Salmonellae

Salmonella belongs to the family Enterobacteriaceae, and are Gram negative, non-spore forming rods (Hook, 1990). Most *Salmonellae* are motile due to peritrichous flagella with the exception of the gallinarum-pullorum serotype (Hook, 1990). The *Salmonella* genus contains more than 2,200 serovars (serotypes) (Le Minor *et al.*, 1982), distinguished by the Kauffmann-White scheme (Kauffmann, 1954). The Kauffmann-White scheme is based on the antigenically distinguished variations in lipopolysaccharide components (somatic O antigens) of the cell wall and flagella protein (H) antigens which provide each *Salmonella* species with its own unique antigenic combination (Kauffmann, 1954). *Salmonella* strains identified by this scheme have been traditionally given their own species names such as *Salmonella typhimurium* and *Salmonella typhi* (Farmer *et al.*, 1984; Le Minor & Popoff, 1988; Old, 1990). With the advent of DNA studies, *Salmonellae* have been reclassified as one species which is divided into six distinct subgroups. Most of the disease causing *Salmonellae* (>99%) belong to subgroup 1 (Farmer *et al.*, 1984; Hook, 1990). Due to the reclassification of *Salmonella* into one species; *S. enterica*, strains historically referred to as *S. typhimurium* should be written as *Salmonella* enterica, var Typhimurium ((Le Minor & Popoff, 1987). However this method of classification becomes very cumbersome and unwieldy in scientific communications and it was proposed that each *Salmonella* strain should be reported with its common serotype name (Le Minor *et al.*, 1982) and this was advocated by the U.S. Centre for Disease Control from the beginning of 1983 (Farmer *et al.*, 1985; Farmer *et al.*, 1984). This

system of practical nomenclature will be used throughout this communication; ie; *Salmonella* serotypes will be written as if they are species e.g. *Salmonella typhi*.

1.2 Host range of *Salmonella*

Salmonella strains have been isolated from nearly all animal species including domesticated animals such as: cows, dogs, donkeys, pigs, sheep, poultry and other birds; reptiles, such as lizards and snakes; and higher mammalian species, such as chimpanzees and humans (Bennett & Hook, 1959). The "broad-host" range species such as *S. typhimurium* cause non-typhoidal Salmonellosis in humans and the principal source is the reservoir of infection in animals, although person to person transmission can occur (Bennett & Hook, 1959). Other strains of *Salmonella* have a very narrow host range and tend to be "species-specific" including *S. typhi*, the causative agent of typhoid fever. Other *Salmonella* strains such as *S. paratyphi* A & C, *S. paratyphi* B and *S. sendai* can also cause a typhoidal-like fever in humans (known as para-typhoid fever) (Hook, 1990). The only known reservoir for strains such as *S. typhi* is man and although experimental studies have shown that chimpanzees and other animals such as mice can be infected with *S. typhi*, it is not known to occur in nature (Edsall *et al.*, 1960). However, some *S. paratyphi* bacterial infections in animals have very occasionally been observed in nature (Rubin & Weinstein, 1977). Other serotypes are host-adapted to animals, e.g. birds are the major reservoirs of *S. gallinarum-pullarum* infections (Rubin & Weinstein, 1977). Most studies on the human pathogenic species of *Salmonella* causing typhoid and para-typhoid fever have therefore been limited by the lack of good animal models. Consequently *S. typhimurium* strains, which cause a typhoid-like fever in mice have been widely studied, and the results used as a model for *S. typhi* infections in humans. Recent advances in tissue culture studies using human-derived cells and cell-lines have enabled a more thorough investigation of *S. typhi* virulence factors and the genetic analysis of the host-

range determinants of *Salmonella* will be discussed in Section 1.13. Although *S. typhi* is the main focus of this communication, studies on *S. typhimurium* will be discussed and conclusions drawn where appropriate for *S. typhi*.

1.3 Disease state of *Salmonella*

1.3.1 Gastroenteritis

Non-typhoidal *Salmonellae* infections in humans (including *S. typhimurium* and *S. enteriditis*) are usually due to contaminated food or water via the faecal/oral route. After ingestion of the contaminated substance (containing as little as 10^5-10^6 *Salmonella*), the infectious bacteria must survive the low pH (~ pH 2.0) of the gastric acids. Symptoms normally appear within 6-24 h and can last up to a week (Hook, 1990). Symptoms usually include nausea and vomiting which is then followed by abdominal pain and diarrhoea (fluid secretion). The fluid secretion is thought to be due to an influx of polymorphonuclear cells (PMNs) to the *Salmonella*-infected intestinal tissue and release of prostaglandins from the PMNs (Ginannella *et al.*, 1975). Cytotoxins and enterotoxins such as the *Salmonella* Stn protein which has been shown to cause fluid accumulation *in vitro* (Prasad *et al.*, 1992) may also be involved in fluid secretion. Occasionally *Salmonella* infections can produce bloody diarrhoea and one cytotoxin characterised in *S. typhimurium* (Cyx) has been linked to this symptom (Libby *et al.*, 1990). However, as most of the potential *Salmonella* cytotoxins and endotoxins have not been purified and their role in diarrhoea has not been elucidated (Darwin & Miller, 1999), therefore they will not be discussed any further in this thesis. In some gastroenteritis cases fever may also occur and the severity of the pain and diarrhoea will differ from person to person. In very severe cases the infection can become systemic although this is normally only seen in infants or immuno-compromised individuals, such as cancer and

AIDS patients. A small percentage of patients may become chronic carriers and continue to shed bacteria for more than a year after the symptoms subside (Abigail & Dixie, 1994).

1.3.2 Typhoid fever

The systemic diseases known as typhoid fever or para-typhoid fever (caused by *S. typhi* and *S. paratyphi* strains, respectively) is usually initiated due to contaminated food or water (via the faecal/oral route). A large number of organisms are usually needed (~10^6-10^9 organisms) in healthy humans although extremely virulent bacilli and health-compromised patients can lower the dose needed for infection (Hook, 1961; Hook, 1990; Hornick *et al.*, 1970; Rubin & Weinstein, 1977). The incubation time for *S. typhi* ranges from one week to a month after infection (Abigail & Dixie, 1994). The infectious bacteria must survive the low pH (~ pH 2) of the gastric acids and they penetrate through to the intestine via the M cells and Peyers patches to the mesenteric nodes where they are able to infect mononuclear cells such as neutrophils and macrophages. After infection of the mesenteric lymph nodes they spread systemically, infecting the spleen, liver and bloodstream in large numbers. It has been suggested that they are able to survive and replicate in macrophages within the spleen and liver, creating a "safe-haven" and protection from the immune system (Edwards *et al.*, 2000). For 2-3 weeks at this stage, the patient can suffer high fever, a flushed appearance, anorexia, and other symptoms, such as chills, convulsions, and delirium can also occur. The high fever is usually implicated as being due to the lipopolysaccharide components (endotoxins) of the bacterial cell wall. Healthy volunteers injected with these endotoxins produced symptoms of headaches, fever, abdominal pain and malaise similar to those observed in a typhoidal infection (Hornick *et al.*, 1970). Finally, the bacteria move from the liver into the gall bladder and are shed in bile, at which stage they may re-enter the intestine. In morbid infections severe ulceration of the intestines may occur and the disease is usually fatal at this stage.

Other patients may carry bacteria in their gallbladders for years (chronic carriers) and could therefore shed bacilli in their faeces for years with no other outward symptoms (Hook, 1990). Approximately 1-3% of typhoid fever sufferers may become chronic carriers, with a higher percentage of these being older patients and women (Hook, 1990). In the pre-antimicrobials era, ~ 12-16% of typhoid fever victims died.

1.4 History and Epidemiology

Salmonella infections worldwide are still classified as a major health problem by the World Health Organisation (WHO), with the incidence of non-typhoidal *Salmonellosis* increasing. Typhoid fever infections are decreasing worldwide but approximately 16.6 million cases of typhoid fever occurring annually were reported in 1995, and of these infections, nearly 600,000 resulted in death (Pang *et al.*, 1995). Non-typhoidal salmonellosis caused 1.3 billion cases of acute gastroenteritis, and 3 million deaths occur annually as a result. More non-typhoidal salmonellosis cases in recent years have been caused by *S. enteriditis* than *S. typhimurium* (Pang *et al.*, 1995).

Typhoid fever infections occur mainly in developing countries, and become a serious consideration whenever water supplies are limited and the possibility of contamination of those water sources available. The history of typhoid fever in the Australian goldfields in the late 1800's, early 1900's has been extensively reviewed (Whittington, 1988) and demonstrates how severe water limitation and poor sanitary conditions enable the disease to spread in epidemic proportions. The conditions that the gold miners and their families experienced in these times and the results are analogous to times of drought, famine and overcrowded living conditions in third world countries today. Although the causative agent of typhoid fever was first demonstrated by Erbeth in 1880 and was isolated by Fafflay in 1884 (Gaffley, 1884), debates raged for years on how it was transmitted. Physicians in the late 1800's did agree that

the bacilli were the causative agents but they did not understand how it was transmitted by contaminated water as they found the levels of bacteria detected were extremely low. A report by the Australian Medical Gazette (1895) on "Typhoid fever at Coolgardie" (one of the Western Australian goldfields) supported the theory that it was transmitted by night air. It was reported that "the majority of those on the goldfield drink nothing but unadulterated condensed water, nether less, great numbers of them are attacked by this fever. From these considerations, the conclusion is justified that the usual mode of infection is by inhalation of bacillus laden air". A previous report, by Colonel Surgeon Dr. A. R. Wayles in 1882 had reported a link between typhoid fever and poor sanitation factors. However due to limited water supplies, miners in Coolgardie constructed small dams to catch water whenever it rained, and due to living conditions this water was easily polluted. The pollution of the rain-water was clarified by Dr. Chas H. Hill, the Health Officer at Coolgardie (Western Mail, 1895) reporting that around soaks in the hills, a collection of human faecal deposits could be found. He also noted that at the time nearly every bush and tree surrounding the mining communities had human faecal matter hidden behind it. Water drainage from these soaks and hills was collected by residents further down whenever it rained, and limited water supplies combined with these habits were undoubtedly the cause of annual epidemics from 1892-1910 in the Western Australian Goldfields.

Typhoid fever had been established as epidemic in Australia by 1852 occuring annually. The disease was a world wide infectious disease endemic to many countries at this time including Iceland and Ireland. Typhoid fever was also common in the cities at this time and a high incidence rate was assisted by miners leaving the goldfields, not only in Western Australia, but also in New South Wales and Victoria when they suspected they had contracted the deadly fever and returning to hospital care in the major cities. Even widely read treatises such as those published by Dr. Ellis in 1886 (Eastern Districts Chronicle, 1896) to help prevent typhoid fever by stating that "the obvious simple way to avoid the disease is to see

that all the water used for any domestic purpose whatsoever has all the germs in it properly destroyed". It was also stated clearly for the public that "Water must be boiled for at least 10 minutes to be effective. But... it is not uncommon for people to be careful to drink only tea because it is made with boiled water, while they dilute their milk with un-boiled water, or put up their drinking vessels to drain after having washed them with germ-laden water". Despite these recommendations, typhoid fever continued to be a major problem for the Coolgardie and Koolgoorie goldfields until water piped in from the Munderly Weir, carrying fresh water inland for 351 miles, reached them at the start of 1903.

Outbreaks of typhoid fever are not just limited to areas of poor water quality or low-sanitization. In 1885, an epidemic was traced back to one person in Plymouth, Pennsylvania. A man, ill with typhoid fever during winter, dumped the contaminated contents of his bedpan directly into the snow outside his cabin all winter long. Once spring came, the snow melted and carried with it all the freshly thawed *Salmonella* bacteria that had been preserved into a nearby stream. Upstream, 8000 of the population of Plymouth who drew their water supplies directly from this stream were infected and 114 died (Galishoff, 1983).

Typhoid fever is still endemic in many undeveloped nations and most cases in developed nations are infections bought back by travellers (Ryan *et al.*, 1989). As most *Salmonella* infections for typhoid fever are due to contaminated food/water, another source is often contaminated fish and crustaceans, especially those consumed raw such as oysters. In the early part of the 20th century in the United States, oysters and other shellfish were harvested from contaminated waters polluted by sewerage washed down in the rainy season. The aviation pioneer Wilbur Wright died of typhoid fever contracted from contaminated shellfish in 1912 at the age of 45 (McCarthy, 1987). Sewerage treatment and good sanitation for shellfish produce has almost eliminated this source of *Salmonella* contamination in the US and other developed countries (Earampamoorthy & Koff, 1975). However in other parts of the world, problems still occur. The fish markets of Coimbatere, South India had fish and

crustaceans collected and analysed over a two-year period (1990-1992). It was found that 14.25% of the fish and 17.39% of crustaceans were contaminated by *Salmonella* species, including *S. typhi, S. paratyphi* B and *S. typhimurium* (Hatha, 1997).

Another major source of infection over the years have been those that have recovered from typhoid fever and become carriers. One of the most famous carriers of typhoid fever was Mary Mallon, a professional cook in New York. Health officials diagnosed her as being a carrier after tracing back a number of typhoid cases to her. A gallbladder operation was offered (the only known way of treating chronic carriers before antibiotics were discovered) which she refused. After imprisonment for three years, she was released and ignoring the warning to change professions, changed her name instead and went back to being a cook in hotels, restaurants and hospitals. Many more people caught typhoid fever before she was re-apprehended and imprisoned for life. Consequently she is known in history books as "Typhoid Mary" (Abigail & Dixie, 1994).

1.5 Treatment for *Salmonellosis*

Treatment for *Salmonella* infections is based on the type and severity of the disease symptoms. Transient infections or short-term gastroenteritis infections are usually treated with symptomatic therapy including fluid and electrolyte replacement rather than antibiotics, and symptoms usually clear up in a short time. Patients who are suffering from bacteraemia or enteric fever may benefit greatly from antibiotic treatment with chloramphenicol or ampicillin. (Hook, 1990). Enteric fever may be also be treated effectively by trimethoprim-sulfamethoxazole, or some of the third-generation cephalosporins, and quinolones if the *Salmonella* strains prove to be resistant to chloramphenicol or ampicillin. The chronic carrier state is more effectively treated by ampicillin/amoxicillin rather than chloramphenicol (Hook, 1990).

1.6 *Salmonella* and Multiple Drug Resistance

Since the advent of antibiotic treatment, death rates from typhoid fever (estimated to be approximately 12-16%) have dropped dramatically, especially after the introduction of chloramphenicol in 1948 (Germanier, 1984). Recently however, multi-drug resistant *Salmonella* species responsible for both typhoid and non-typhoid salmonellosis have been detected throughout the world (including China, Egypt, India, Korea, Latin America, Nigeria, Pakistan, the Philippines and Vietnam) hampering effective treatment (Pang *et al.*, 1995). In the Indian subcontinent, 50-70% of the *S. typhi* strains isolated have been shown to be resistant to chloramphenicol (previously the "gold standard" agent (Islam *et al.*, 1993) and other antibiotics (Pang *et al.*, 1995). R plasmids; large transferable, conjugatable plasmids (40-120 kb) carrying genes for multiple antibiotic resistance (R factors) have been identified as the cause of this spread. When 20 *S. typhi* isolates were examined from typhoid cases in the 1989-1990 epidemic in Calcutta, India 84% of them carried R plasmids. The *S. typhi* strains carrying these plasmids had resistance to chloramphenicol, ampicillin, tetracycline and streptomycin (however they were still sensitive to nalidixic acid and ciprofloacin) (Karmaker *et al.*, 1991). In an outbreak in Tehran, Iran in 1995, a 91.2 megadalton plasmid was detected carrying resistance to the above antibiotics as well as trimethroprim-sulphamethoxazole (Bahrmand & Velayati, 1997). In 1991, a letter to the Lancet recommended that physicians should consider ciprofloxacin as the drug of choice for any patients which had recently returned from travel to India, Pakistan or the Arabian Gulf as 20% of the patients treated in the UK for *S. typhi* infection had been infected with chloramphenicol resistant strains (Rowe *et al.*, 1991). Even treatment by ciprofloxacin may be limited in the future as isolates from an outbreak of *S. typhi* in 1997 in Tajikistan were shown to be resistant to ciprofloxacin, although it was chromosomally and not yet plasmid encoded (Hampton *et al.*, 1998).

Suggested reasons for the emergence of these multi-drug resistant strains include: overuse of antibiotics in animals (DuPont & Steele, 1987), the excessive and inappropriate use of antibiotics by medical professionals (particularly in hospital situations) and unskilled practitioners distributing inappropriate, poor/degraded quality and counterfeit antibiotics, mainly in developing countries (Okeke *et al.*, 1999). Travellers to developing countries may also be exposed to residents who are asymptomatic carriers of antibiotic-resistant, potentially pathogenic organisms (Murray *et al.*, 1990; Woolfson *et al.*, 1997). The overcrowded and unhygienic living conditions in these countries may encourage the spread of these organisms between people, and the exchange of resistance genes between the bacteria (Okeke *et al.*, 1999). As the speed and ease of world travel increases, this means that these multidrug-resistant *Salmonellae* can potentially spread very rapidly to distant regions. Increased epidemiological surveillance combined with the appropriate usage of antibiotics as recommended by the World Health Organisation (Couper, 1997) is needed to stop the continuing escalation of antimicrobial resistant *Salmonellae* (& other pathogenic bacteria) in developing countries, and the resultant spread to developed countries (Okeke *et al.*, 1999; Pang *et al.*, 1995).

1.7 Genetic determinants of *Salmonella* virulence and regulatory factors

Salmonella virulence determinants are encoded at distal parts all around the *Salmonella* chromosome, however many of the *Salmonella* virulence genes are clustered into distinct loci called pathogenicity islands (PIs). *Salmonella* are predicted to have evolved from a distant *E. coli* ancestor by the horizontal transfer of these PIs (Groisman & Ochman, 1997). Pathogenicity islands have been defined as regions of DNA inserted into the bacterial chromosome, encoding genes which are found in pathogenic bacteria but absent from phylogenetically related non-pathogenic organisms, therefore conferring virulence-associated

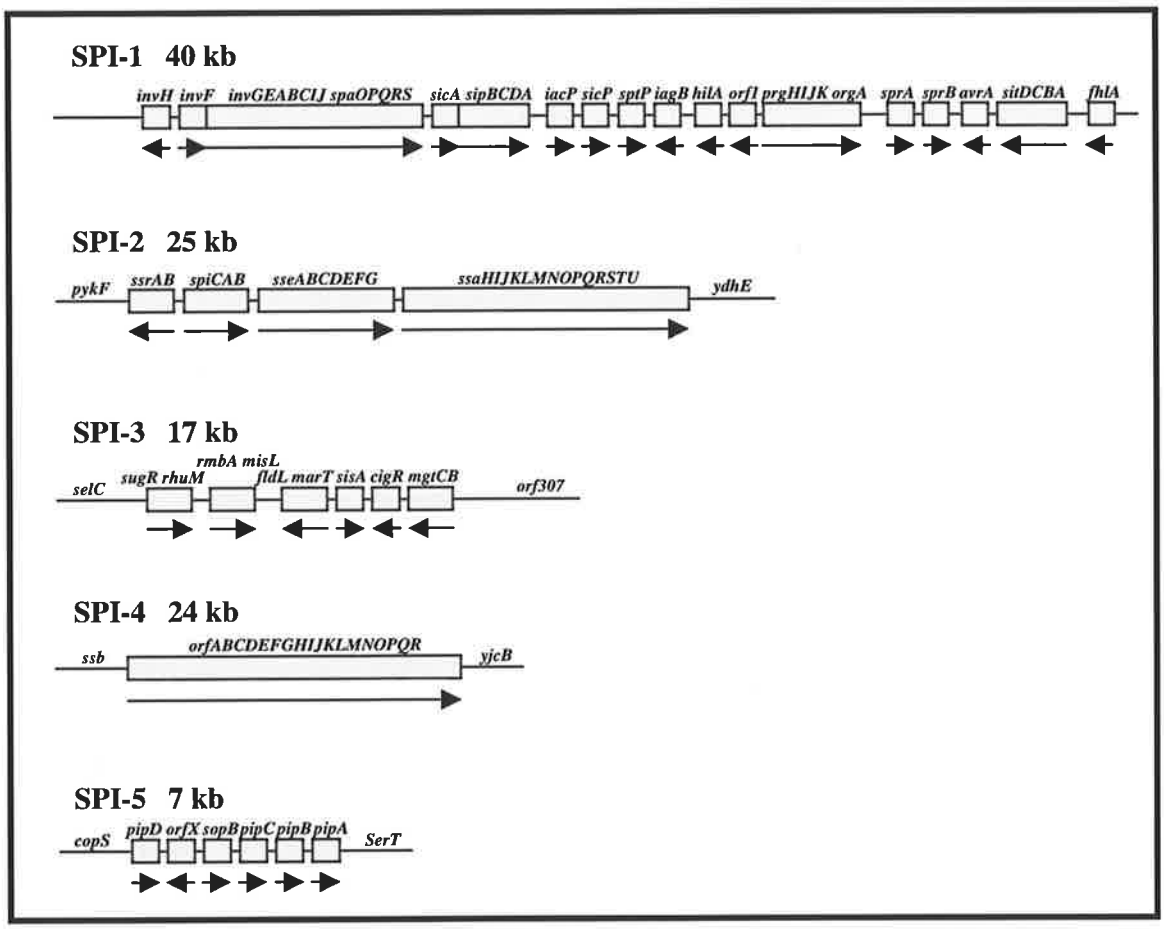
functions upon the host chromosome (Mills *et al.*, 1995). The DNA content (G + C %) of these pathogenicity island regions is generally lower for *Salmonella* compared to the normal host chromosome G + C % content (~52%) (Hensel *et al.*, 1997). The PIs also tend to be located in "genetic hot spots" of integration such as tRNA genes and often have a bacteriophage or transposon-like structure correlating with horizontal gene transfer (Bäumler, 1997). A general outline of the *Salmonella* pathogenicity islands will be discussed in the next section and a brief overview of the known regulators. This will be followed by Section 1.8 outlining the determinants of *Salmonella* pathogenicity corresponding approximately to the disease process.

1.7.1 *Salmonella* Pathogenicity Islands (SPI)

So far five pathogenicity islands have been identified in *S. typhimurium* (designated SPI-1 to SPI-5), although more may be identified in the future when the genome sequencing of *S. typhimurium* (Genome Sequencing Center at Washington University, started 1998, (http://genome.wustl.edu/gsc/Projects/bacterial/salmonella.info.shtml)) and *S. typhi* (Sanger Center, 1999) is completed. SPI-1 (~ 40 kb) encodes a type III secretion system located at 63 centisomes on the *S. typhimurium* chromosome (Mills *et al.*, 1995), and is involved with protein secretion and invasion of mammalian cells (Galán, 1996). SP1-2 (~ 40kb) encodes a second type III secretion system located at 31 centisomes and is required for later stages of *Salmonella* systemic disease, including macrophage survival (Ochman *et al.*, 1996; Shea *et al.*, 1996). SP1-3 (~17 kb) is located at 82 centisomes on the *S. typhimurium* chromosome and is essential for intramacrophage survival and the ability to grow in Mg^{2+}-limiting conditions (Blanc Potard & Groisman, 1997). SP1-4 (~25 kb) is located at 92 centisomes and is predicted to be involved with toxin secretion and intramacrophage survival (Wong *et al.*, 1998). SP1-5 (~ 7.5 kb) is located at 20 centisomes on the *S. typhimurium* chromosome and

may be important for gastroenteritis rather than systemic salmonellosis (Wood *et al.*, 1998). The *S. typhimurium* genes encoded on these pathogenicity islands are numerous (Figure 1.1), have been the subject of many insightful studies, and have been reviewed extensively (particularly SPI-1) (Collazo & Galán, 1997; Galán, 1999). Therefore, only the characterised *S. typhimurium* pathogenicity island genes and encoded proteins relevant to the general background for this study will be discussed in the text (when appropriate), rather than reviewing all five pathogenicity islands in detail.

Figure 1.1: Schematic diagram of *Salmonella* Pathogenicity Islands



This figure shows the genetic organisation of the *Salmonella* pathogenicity islands. Genes are outlined in boxes and the direction of transcription is indicated by arrows (→). This Figure is modified from (Sirard *et al.*, 1999).

1.7.2 Regulatory Factors of *Salmonella* virulence determinants

Pathogenic organisms such as *Salmonella* have to respond to a large range of environmental conditions during the infection process. Virulence genes are usually subject to tightly controlled and coordinate regulation by regulatory factors, which respond to a large variety of environmental signals and are able to activate and/or repress virulence genes (Cotter & Miller, 1998; Soncini & Groisman, 1996). The *Salmonella* chromosome encodes a large number of regulators, including several two-component regulatory systems consisting of a transcriptional regulator and an environmental sensor-histidine kinase (Miller, 1991). These two-component regulators include PhoP/Q, SirA/unidentified sensor, RcsB/RcsC, PmrA/PmrB, SsrB/SsrA and EnvZ/OmpR which are all involved with bacterial invasion of eukaryotic cells and intracellular survival (Ahmer *et al.*, 1999; Arricau *et al.*, 1998; Gunn & Miller, 1996; Johnston *et al.*, 1996; Mills *et al.*, 1998). These systems are able to control other regulatory genes as part of a "regulatory cascade", involving other regulators such as HilA (Bajaj *et al.*, 1996). Other global regulators include the alternative sigma factor σ^S (*rpoS* locus), which is involved with stationary-phase stress responses (Fang *et al.*, 1992) and SlyA which is involved with systemic salmonellosis (Watson *et al.*, 1999). Many studies have dealt with analysis of these regulators, and the environmental factors that they are responsive to. The only regulators and environmental signals that will be discussed in depth in this communication are PhoP/Q and regulators involved with the PhoP/Q regulatory cascade. Other *Salmonella* regulators, the environmental signals they respond to, and the genes they regulate will be mentioned when appropriate within the text.

1.8 Virulence determinants of *Salmonella*

1.8.1 Acid Tolerance Response

During the infective lifecycle of *Salmonellae*, the bacteria are exposed to organic and inorganic (low pH) acid conditions at many stages, including the environment (pond water, decaying faecal matter, industrial waste and decaying organic matter) and during the infection process (extreme low pH in the stomach, volatile fatty acids present in the intestine and faeces) as well as low pH within macrophage phagolysosomes (Bearson *et al.*, 1997; Lin *et al.*, 1995).

The first line of host defence is gastric acidity (Gianella *et al.*, 1972; Peterson *et al.*, 1989) and it has been shown that *S. typhimurium* have greater survival at a low pH (pH 3.0) after exposure to short-chain fatty acids such as acetate and propionate (Kwon & Ricke, 1998) which are used as preservatives in food products such as meat carcasses, salad dressing and mayonnaise (Cherrington *et al.*, 1991; Dorsa, 1997). This may help explain why the infectivity dose of *Salmonella* may be greatly decreased (from ~10^5 to 10^2-10^3 organisms) when injested as an inoculum on food sources such as milk chocolate, chedder cheese and hamburger, which may contain these preservatives (Blaser & Newman, 1982). It has also been found that inoculating *Salmonella* on a solid food source such as ground beef or egg white enhanced the ability to survive under strong acidic conditions (pH 2.5), indicating that a solid food source may affect survival of *Salmonella* when encountering stomach contents (Waterman & Small, 1998).

The survival of *S. typhimurium* in extreme acid conditions is due to a complex system called the acid tolerance response (ATR) (Foster & Hall, 1990). The ATR in *S. typhimurium* was first demonstrated when it was shown that adapting *Salmonella* bacteria to mild (pH 5.8) or moderate (pH 4.4) could enable the cells to survive in very acidic (pH 3.3) conditions (Foster & Hall, 1990). Over 50 proteins called Acid Shock Proteins (ASP's), are synthesised

during the ATR process, (Bearson *et al.*, 1997; Foster, 1991; Lee *et al.*, 1994). These acid-shock proteins are involved in a number of systems in both exponential and stationary growth phases of *Salmonella*. The systems are classified as either σ^{38} (an alternative sigma factor encoded by *rpoS* which is important for stationary growth phase physiology (Lange & Hengge-Aronis, 1991; Prince *et al.*, 1994)) -dependent or, -independent. The stationary phase σ^{38}-dependent system is not induced by acidity (as levels of σ^{38} are already increased as the bacteria enter stationary phase) and appears to be part of a general stress resistance, which also improves acid tolerance (Lee *et al.*, 1994; Lee *et al.*, 1995). The stationary phase σ^{38}-independent system is induced by acid and 15 ASP's are noted to be upregulated by 2D-SDS-PAGE gel analysis (Foster, 1991; Lee *et al.*, 1994). The σ^{38}-dependent systems in exponential phase growth are induced by acid shock (inducing 8 ASPs), and RpoS is needed to sustain an ATR response, as *rpoS* mutants are only transiently able to induce an ATR (Lee *et al.*, 1995; Wilmes-Riesenberg *et al.*, 1997). The *S. typhimurium* mouse virulence gene *mviA* product (MviA) has been reported to negatively regulate the RpoS protein. MviA has significant homology to the response regulatory family of regulators and is thought to be involved in modulating the levels of σ^{38} at the translational level (Bearson *et al.*, 1996). Other "global" regulators are also involved with log-phase σ^{38}-independent ATR response include the major iron regulatory protein Fur (appearing to sense intracellular pH independently of its ability to sense iron and involved in the induction of 8 ASPs (Foster & Hall, 1992; Hall & Foster, 1996)), and the Ada protein. The latter is involved with the adaptive response of *E. coli* to alkylating agents, but its role in the acid tolerance response is not yet known (Hakura *et al.*, 1991); both factors are involved with the organic acid stress response (Bearson *et al.*, 1998). An RpoS-independent system involved with resistance to inorganic acid (low pH) is controlled by the *S. typhimurium* PhoP/Q two-component regulator (Bearson *et al.*, 1998) (discussed in Section 1.9.3).

1.8.2 *Salmonella* adhesion to eukaryotic cells

Fimbriae and pili encoded by *Salmonella* are involved with adhesion to eukaryotic cells. *S. typhimurium* has genes for many types of fimbriae and pili including: Type 1 fimbiae (FIM) (encoded by the *fimAICDHF* operon at centisome 15; (Collinson *et al.*, 1996)), virulence plasmid encoded (PE) fimbriae (*pefBACD* encoded on the virulence plasmid (Friedrich *et al.*, 1993)), long polar (LP) fimbriae (encoded by *lpfABCDE* at centisome 80 (Bäumler *et al.*, 1996)), and thin aggregative fimbriae (curli) (encoded by *agfBAC* on centisome 26, (Sukupolvi *et al.*, 1997)). Recently, two more putative fimbrial operons were identified. The putative fimbrial gene cluster (*saf*) was identified in *S. enterica* subgroup 1 (on centisome 7 (Folkesson *et al.*, 1999) and the novel *S. typhimurium* fimbial operon *stfACDEFG* was located at centisome 5 (Emmerth *et al.*, 1999).

S. typhi do not contain the *pef*, *lpf* or *stf* operons and according do not produce either PEF or LPF fimbriae. *S. typhi* does produce SEF14 fimbriae (located on a pathogenicity island) which are predicted to be involved in adhesion to macrophage cells (Edwards *et al.*, 2000) and recently another putative novel *S. typhi* fimbrial operon (*tcf*) was identified (Folkesson *et al.*, 1999). The presence of four or more fimbrial systems in *Salmonella* suggests that attachment to eukaryotic cells may be an important factor in *Salmonella* pathogenesis, especially if the fimbriae are involved in binding to certain types of cells (such as SEF14 and macrophages). The redundancy of fimbrial operons has made it difficult to assess the role of each type *in vitro* and *in vivo*. Future studies with multiple fimbrial mutations and tissue culture assays with defined tissue culture cell lines should help identify the roles that particular fimbriae or pili have in *Salmonella* pathogenesis (Darwin & Miller, 1999).

1.8.3 *Salmonella* invasion of eukaryotic cells

An important part of *S. typhimurium* pathogenesis is the invasion and entry of "non-phagocytic" eukaryotic cells. Over 25 invasion genes have been identified which are required for *S. typhimurium* cell invasion (Altmeyer *et al.*, 1993; Behlau & Miller, 1993; Collazo *et al.*, 1995; Galán & Curtiss, 1989A; Ginocchio & Galán, 1995; Hermant *et al.*, 1995; Hueck *et al.*, 1995; Jones & Falkow, 1994; Kaniga *et al.*, 1994; Kaniga *et al.*, 1995A; Kaniga *et al.*, 1995B; Lee *et al.*, 1992). *S. typhimurium* invasion is characterised by "membrane ruffling" where the epithelial cell plasma membrane near the bacteria begins to elongate, swell and bud, distorting the cell surface (Galán, 1994). The bacteria are engulfed within a membrane-bound vesicle, resulting in internalisation of the bacteria-containing vesicle into the host cell. There is also rearrangements of cytoskeletal proteins such as polymerised actin, producing loose strings of actin filaments which accumulate near the organism. At the same time, there is aggregation of host cell surface proteins associated with the cytoskeleton. *Salmonella* host signals normally involved with uptake of growth factors such as calcium and inositol fluxes, are also triggered during invasion, indicating that *Salmonella* involves normal host cell functions during invasion (Galán, 1994)

1.8.3.1 Genetic determinants of *Salmonella* invasion

A large number of the *Salmonella* invasion genes have been found to be located on the *Salmonella* pathogenicity island SPI-1 which encodes a Type III secretion system. A brief outline of Type III secretion systems and the other three protein secretion pathways will be given below, followed by a more detailed outline of the genes involved in *Salmonella* invasion and protein secretion.

1.8.3.1.1 Protein secretion systems in *Salmonella*

Four pathways of protein secretion (Types I-IV) have been described for Gram-negative bacteria (Pugsley, 1993; Salmond & Reeves, 1993).

Type II and Type IV secretion pathways involve transport across the inner membrane to the periplasm via the *sec* system. The *sec* pathway in *E. coli* involves a complex of proteins including: a number of cytoplasmic inner membrane proteins, a cytoplasmic membrane-associated ATPase (providing the energy for export), a chaperone that binds to pre-secretory target proteins and the periplasmic signal peptidase as well as a number of accessory proteins required for normal function. A signature of sec-dependent protein export is the presence of a short (~ 30 aa), highly hydrophobic, amino-terminal signal sequence in the secreted protein (Murphy & Beckwith, 1996; Pugsley, 1993). Type II secretion proteins require an additional set of inner and outer membrane proteins to be transported across the outer membrane (e.g. *Kebsiella oxytoca* pullulanase secretion requires 14 additional secretion factors) (Pugsley, 1993). However Type IV secretion proteins (including the vacuolating cytotoxin of *Helicobacter pylori*) are "autotransporters", encoding the information needed for transport across the outer membrane within the secreted protein (Finlay & Falkow, 1997). Type I secretion proteins (including the *E. coli* alpha-hemolysin) are sec-independent and require three secretory proteins: an inner membrane transport ATPase (ABC: protein for ATP-binding cassette) which provides the energy for the secretion, an outer membrane protein and a membrane fusion protein which is anchored in the inner membrane spanning the periplasmic space. The Type I secreted protein and secretory apparatus are usually found clustered on the chromosome (Fath & Kolter, 1993).

The third secretion pathway is the Type III secretion system which is generally defined by three characteristics. Firstly, proteins secreted by the Type III system do not have a typical amino-terminal sequence characteristic of proteins exported in a sec-dependent manner and therefore the system is sec-independent. However, some usage of the sec system for the

assembly of the Type III secretion apparatus does probably occur, as several components of the secretion apparatus do have sec-characteristic amino-terminal signal sequences (Hueck, 1998). Secondly, the Type III export machinery directs the translocation of the target proteins through two membranes without cleavage of their amino termini, and finally an inducing extracellular signal is required for complete activation of the secretary apparatus (these signals will be discussed later). The Type III secretion apparatus is usually composed of ~ 20 proteins encoded by genes which tend to be clustered together on the chromosome. Most of these 20 proteins are located in the inner membrane and a cytoplasmic ATPase is needed which is likely to be membrane associated. As there is no amino-terminal processing of the secreted proteins and the amino-terminal part of the proteins do not share any recognisable stuctural similarities, it has recently been proposed from studies on *Yersinia* Type III secretions systems that the 5' region of the mRNA contains the secretion signal residues (Anderson & Schneewind, 1997). The secreted proteins also require small cytoplasmic proteins with chaperone abilities to protect the secreted proteins from premature interaction with later parts of the secretion system components (Hueck, 1998)

Salmonellae are unique in being the only bacterial species so far to have two type III secretion systems identified, located in SPI-1 and SPI-2. SPI-1 is needed for initial penetration of the intestinal mucosa, whilst SPI-2 is necessary for later stages of systemic infection (Hueck, 1998). The Type III secretion systems are triggered when the pathogen comes into close contact with eukaryotic cells, inducing synthesis of the secretion apparatus and effector molecules. These effector molecules cause changes in host cell function which facilitate the pathogens ability to survive and replicate. Only genes that have been well characterised and assigned a function (defined or putative) will be discussed below.

1.8.3.2 *S. typhimurium* invasion and SPI-1

Salmonella invasion of eukaryotic cells is characterised by "membrane ruffling", dramatic cytoskeletal arrangements and internalisation of the bacteria via membrane-bound vacuoles formed from the membrane ruffles termed macropinocytosis. Effector proteins involved with this process are encoded on SPI-1 (Figure 1.1) and involved with a Type III secretion system. The effector proteins involved with the "membrane ruffling" include the distantly located SopE protein (Hardt *et al.*, 1998A; Mirold *et al.*, 1999), which after translocation into the host cell, acts as a guanine nucleotide exchange factor (GNEF) for small cellular molecules Rac and Cdc42 (GTPases), converting them from inactive GDP-bound to active GTP-bound forms leading to membrane ruffling (Hardt *et al.*, 1998A; Hardt *et al.*, 1998B). After invasion the SptP protein reverses the process by acting as a GTPase-activating protein (GAP) returning the Rac and Cdc42 active proteins to their GDP-bound states which within minutes leads to the return of the cell membrane to its normal state (Fu & Galán, 1999). The dramatic cytoskeletal rearrangements are due to the SipA and SipC proteins which are able to bind actin and bundle it into tight parallel bundles, resulting in pronounced outward extended membrane ruffles, which facilitate bacterial uptake (Hayward & Koronakis, 1999; Zhou *et al.*, 1999A; Zhou *et al.*, 1999B). The effector proteins mentioned above are translocated via a "needle complex" on the bacterial envelope, which spans both membranes and has two identifiable domains: a cylindrical base and an apparently hollow, thin needle-like structure. Three proteins encoded on SPI-1 have been identified in the needle complex so far; InvG (Kaniga *et al.*, 1994) and the lipoproteins PrgH and PrgK (Kubori *et al.*, 1998; Pegues *et al.*, 1995).

The SPI-1 Type III secretory system also includes structural genes encoding proteins involved with the secretory process. These include inner membrane proteins such as the putative channel InvA (Galán *et al.*, 1992; Ginocchio & Galán, 1995), SpaP, SpaQ and SpaR (potential membrane spanning proteins), and SpaS (Collazo & Galán, 1996; Groisman &

Ochman, 1993). The InvC protein is known to have ATPase activity and is suggested to be the energizer for the translocation process (Collazo & Galán, 1997; Eichelberg *et al.*, 1994). Accessory proteins of the secretory apparatus include chaperones, which assist in the translocation of the secreted proteins by helping to maintain confirmation and/or by inhibiting degradation (Collazo & Galán, 1997). SicA is the chaperone for the SipB, SipC and SipD proteins (Collazo & Galán, 1997; Kaniga *et al.*, 1995B) and the InvI is the putative chaperone for *invJ* and *spaO* (Collazo *et al.*, 1995). SicP is the specific chaperone for the effector protein SptP (Fu & Galán, 1998). The InvH helper lipoprotein is required for the localisation of InvG (a putative channel forming a ring-like multimer in the outer membrane) (Crago & Koronakis, 1998). The oxygen regulated, *orgA* encoded protein (Jones & Falkow, 1994) is likely to be involved in protein translocation but its exact function is not yet known (Collazo & Galán, 1997). InvJ, SpaO and SipD are secreted proteins involved in the secretion of effector molecules such as the Sip proteins (Collazo & Galán, 1996; Collazo *et al.*, 1995; Kaniga *et al.*, 1995A; Li *et al.*, 1995).

1.8.3.2.1 Regulation of the SPI-1 invasion genes

The regulation of the SPI-1 encoded invasion genes is extremely complex. Several regulators encoded within the SPI-1 locus include HilA (a member of the OmpR-ToxR family) (Bajaj *et al.*, 1995) and InvF (a member of the AraC family) (Kaniga *et al.*, 1994). HilA activates the transcription of the SPI-1 secretion apparatus components and activates the InvF regulator (Bajaj *et al.*, 1996). Expression of the genes encoding the effector proteins is regulated differentially by both InvF and HilA (Eichelberg *et al.*, 1999). The system is also influenced by global regulators including the PhoP/Q two component regulatory system (Behlau & Miller, 1993; Pegues *et al.*, 1995), the flagella-associated sigma factor FliA (σ^{28}) (Eichelberg *et al.*, 1999), the SirA response regulator (Ahmer *et al.*, 1999; Johnston *et al.*,

1996), SirB and SirC (an AraC-like regulator) (Rakeman *et al.*, 1999). Two additional transcriptional regulatory factors have recently been identified: SprA (an AraC-like regulator) and SprB (Eichelberg *et al.*, 1999). In *S. typhi* the RcsB-RcsC regulatory system (responsive to low osmilarity) is also involved (Arricau *et al.*, 1998). The regulation of this secretion pathway is complex and in *S. typhimurium* may also be affected by growth phase, oxygen, osmilarity and pH (Collazo & Galán, 1997). Due to space constraints, only regulation which has been shown to involve *S. typhimurium* PhoP/Q will be discussed further.

1.8.3.3 *S. typhi* and epithelial cell invasion

S. typhi have been shown to invade cultured epithelial cells with similar features as *S. typhimurium*. Membrane ruffling, cytoskeletal rearrangements, macropinocytosis (indicating spacious vacuole formation) and aggregation of host-cell-surface proteins such as Class I-MHC heavy chain was observed (Mills & Finlay, 1994). The *S. typhi* bacteria replicated intracellularly at a similar level to *S. typhimurium*. The main difference noted between *S. typhi* and *S. typhimurium* cell invasion was that only one to two *S. typhi* organisms were observed with each membrane ruffle and as such *S. typhi* invaded at a lower level overall than *S. typhimurium* (Mills & Finlay, 1994). This reduced invasion level was found to be due to a physical limitation on *S. typhi* entry into epithelial cells, suggesting that a limited number of *S. typhi* receptors and entry sites exist per cell (Huang *et al.*, 1998). Recently, *S. typhi* was shown to use the cystic fibrosis transmembrane conductance regulator (CFTR) as a receptor for internalisation into eukaryotic cells but *S. typhimurium* was shown not to use this receptor (Pier *et al.*, 1998). *S. typhi* invasion has also been shown to depend on motility (Liu *et al.*, 1988) and is affected by osmilarity and growth phase (Leclerc *et al.*, 1998; Tartera & Metcalf, 1993).

1.8.4 *Salmonella* and M Cells

After surviving the gastric stomach acids, the next stage of the *Salmonella* infection involves the penetration of the intestinal mucosa by entering the follicle-associated epithelium (FAE), which overlies gut-associated lymphoid tissues (ileal Peyer's patches). *Salmonella* enter the FAE by adhering to, invading and destroying specialised epithelial cells in the FAE called M cells, allowing bacterial entry into the reticuloendothelial system (Jones, 1996). M cells are involved in sampling and delivering antigens to the underlying lymphoid tissue as part of the mucosal immune protection system (Siebers & Finlay, 1996). The *S. typhimurium* M cell invasion process in the mouse model causes cell death and depolarisation of the intestinal epithelium, leading to bacterial invasion of enterocytes and penetration into deeper tissue (Clark *et al.*, 1994; Jensen *et al.*, 1998; Jones *et al.*, 1994). *S. typhi* bacteria have also been shown to invade and destroy M cells in ligated ileal loops in mice which correlates with characteristic autopsy findings in typhoid fever victims (Kohbata *et al.*, 1986). Non-invasive *S. typhimurium* mutants unable to enter and destroy M cells have been shown to be avirulent (Kohbata *et al.*, 1986; Penheiter *et al.*, 1997), indicating that the invasion and destruction of M cells is an important part of *Salmonella* virulence. A number of systems are involved with the M cell adherence, invasion and destruction. Adherence to M cells by *S. typhimurium* involves the chromosomally encoded *lpfABCDE* fimbrial operon (Bäumler & Heffron, 1995; Bäumler *et al.*, 1996). The *S. typhimurium* sigma factor RpoS is involved with the regulation of adherence to and colonization of murine Peyer's patches and also FAE destruction (Nickerson & Curtiss, 1997) The *inv* locus (encoded on the SPI-1 pathogenicity island) is also involved with M cell invasion and FAE destruction (Clark *et al.*, 1998; Clark *et al.*, 1996) and recently, it has been shown that DNA adenine methylase (Dam) mutants of *S. typhimurium* are non-cytotoxic for M cells, unable to invade enterocytes and are defective in non-phagocytic cell invasion, and that Dam may play a role in the regulation of SPI-1 genes Type III secretion genes (Garcia-del Portillo *et al.*, 1999). The *S. typhimurium* SlyA virulence regulator (Libby

et al., 1994) is not involved with invasion or colonization but is necessary for the destruction of M cells and survival in the murine Peyer's patch, however the *slyA* regulated genes involved have not yet been identified (Daniels *et al.*, 1996; Watson *et al.*, 1999).

1.8.5 *Salmonella* and transepithelial Signalling of polymorphonuclear cells (PMNS)

Non-typhoidal serotypes of *Salmonella* such as *S. typhimurium* are able to stimulate an intense intestinal inflammatory response consisting mainly of neutrophils (polymorphonuclear leucocytes [PMNs]) which migrate across the epithelial lining of the intestine (McCormick *et al.*, 1993; Takeuchi, 1967). It was shown that transepithelial signalling to PMN's required *S. typhimurium* adhesion to the epithelial cell apical membrane and consequent protein synthesis in both bacteria and epithelial cells (McCormick *et al.*, 1995). This contact induces the production of the potent PMN chemotactic peptide IL-8 from the basolateral surface of the epithelial cells and PEEC (pathogen-elicited epithelial chemoattractant) from the apical cell surface (McCormick *et al.*, 1995; McCormick *et al.*, 1998). Although the invasion defective *S. typhimurium* mutants, *phoP*^{c}, *hilA* and *invA* have been shown to be incapable of inducing the PMN transmigration (McCormick *et al.*, 1995), recently *S. typhimurium* internalisation was shown to be not linked to the signalling method for PMN movement but is probably reliant on the SPI-I Type III secretion system that the above genes are involved with. A candidate for the effector protein is SopB which is encoded on SP1-5 (Wood *et al.*, 1998), as mutations in the *S. dublin sopB* gene exhibited approximately 50% decrease in fluid secretion and PMN influx in calf ileal mucosa (Galyov *et al.*, 1997), although the mutants showed wildtype invasion levels. Interestingly, it was noted during these studies that host restricted *Salmonella* serotypes such as *S. typhi* and *S. paratyphi* failed to induce PMN transepithelial migration in the human-cell culture model (McCormick *et al.*, 1995).

1.8.6 *Salmonella* and interactions with host defence cells

After surviving the gastric acids, penetration and invasion of M cells and other epithelial cells of the ilial mucosal layer, *Salmonella* come into contact with phagocytic cells such as dendritic cells (DCs), macrophages and neutrophils. *S. typhimurium* have been shown to associate with DC's in ligated loops of mouse ileal intestine (Hopkins, 1997; Valdivia & Falkow, 1996). It has also been proposed that DCs may be a major niche for *Salmonella*, facilitating bacterial dissemination throughout the lymphatic and reticulo-endothelial system (Sirard *et al.*, 1999), however little work has been done on this area and it will not be further discussed here. *Salmonella* are able to survive in macrophages (discussed in Section 1.8.6.2 and Section 1.9.7), however neutrophils potentially make up part of the major host immune defences against *Salmonella*.

1.8.6.1 *Salmonella* and neutrophils

Neutrophils are a major part of the host immune system and kill bacteria by both oxygen-dependent and oxygen-independent mechanisms. Neutrophils generate superoxide anion (O_2^-) and other species derived from it such as H_2O_2 via a single-electron transfer from NADPH to molecular oxygen in the oxygen-dependent mechanisms (Segal & Abo, 1993). Patients who suffer chronic granulomatous disease (CGD) lack neutrophils capable of generating the respiratory burst and have high mortality rates due to bacterial infection, indicating that this is an important part of the immune defenses against bacteria (Royer-Pokora *et al.*, 1986). "Neutropenic mice" (depleted of neutrophils by the granulocyte-depleting monoclonal antibody RB6-8C5) are far more susceptible to *Salmonella* infections than wildtype mice, and bacteria were found to grow to exceedingly high numbers in the liver and spleen, indicating that neutrophils and neutrophils-mediated host defences play a major role in defence against *Salmonella* infections (Conlan, 1996; Conlan, 1997). A "safe-site" for *S. typhimurium* has

previously been proposed to be within splenic cells (Dunlap *et al.*, 1991), and localized within the liver sinusoid associated macrophages called Kupffer cells in the liver, where they were shown to be able to survive and multiply intracellularly (Nnalue *et al.*, 1992). Further discussion on this topic will be done in Section 1.8.6.2.

A recent study which looked at the *in vivo* blockage of nitric oxide (NO) in mice by the addition of the inducible NOS (iNOS) inhibitor aminoguanidine sulphate to their drinking water, found that it also blocked the influx of neutrophils and macrophages into the spleens and resulted in 90% mortality of mice to a highly attenuated *aroA* vaccine strain of *S. typhimurium* (SL3235 (MacFarlane *et al.*, 1999A)). Hence it may be suggested that part of the susceptibility to the vaccine *S. typhimurium* strain was more likely to be due to the lack of neutrophils rather than lack of macrophages in the influx of PMN's into the spleen.

1.8.6.2 *Salmonella* and macrophage invasion and survival

Macrophages are a major line of host defense against invading organisms and are able to engulf and kill non-pathogenic (and some pathogenic) bacteria. *Salmonella* however are able to survive and replicate within macrophages, evading the formidable defences (Beaman & Beaman, 1984; Fields *et al.*, 1986B). These defences include: oxygen-independent (phagolysome-lysosome fusion, vacuole acidification, digestive enzymes and antibacterial cationic proteins), and oxygen-dependent (reactive oxygen metabolites, superoxide anions, hydroxyl radicals and hydrogen peroxide, all generated via the respiratory burst mechanisms) (Beaman & Beaman, 1984).

S. typhimurium strains have been shown to survive in a wide variety of macrophage types and it was found that the ability of *S. typhimurium* to survive and replicate varies with the source of the macrophage (Buchmeier & Heffron, 1989). Tissue culture cell lines such as the J774 macrophage cell line were the most permissive for *S. typhimurium* growth, survival of *S.*

typhimurium was at a moderate level in splenic and bone-marrow derived macrophages, and peritoneal macrophages were the least permissive for growth, being the most efficient killers of the bacteria (Buchmeier & Heffron, 1989). This correlates with the fact that different macrophage populations located in different areas may have different mechanisms to deal with invading bacteria. The type of host macrophage may also have an effect on *Salmonella* intracellular survival and may be related to host specificity for *Salmonella* as virulent *S. typhi* strains were shown to be able to survive in J774 macrophage cells lines and human monocytes-derived macrophages but not mouse peritoneal macrophages (Vladoianu *et al.*, 1990). Recent studies also indicated that *S. typhimurium* strains have a distinct advantage compared to *S. typhi* for survival in murine macrophages and conversely *S. typhi* survive better than *S. typhimurium* in human-derived macrophages (Schwan *et al.*, 2000). Further discussion on the topic of host specificity and macrophage survival will be continued in Section 1.13.

S. typhimurium enter the macrophage either by conventional phagocytosis leading to close-fitting phagosomes surrounding the bacteria or as an invasive step characterised by generalized membrane ruffling, and macropinocytosis leading (Section 1.8.3) to the bacteria being enclosed in a spacious phagosome (Alpuche Aranda *et al.*, 1994). After entry into the macrophage the *S. typhimurium* bacteria are enclosed within phagosomes, the phagosomes may then fuse with a lysosome to create a phagolysosomes exposing the bacteria to lysosomal degradative enzymes and other host defences such as increased acidification. *S. typhimurium* has been shown to inhibit acidification of the phagosomes which may hinder these host defences (Alpuche Aranda *et al.*, 1992). It has also been found that *S. typhimurium* can inhibit the normal phagosomes-lysosome fusion (Buchmeier & Heffron, 1991; Ishibashi & Arai, 1990), and another study has found that all *S. typhimurium* containing phagosomes fuse with lysosomes (Oh *et al.*, 1996), however these contradictory reports may be explained by the markers used to detect phagolysosomes. Over 75% of the phagosomes that the *S.*

typhimurium bacteria are contained within are divergent from the normal degradative pathway of the macrophage as they do contain some lysosomal markers such as LAMP1 and lysosomal acid phosphatase but not cathepsin L, or the mannose-6- phosphate receptor. Heat-killed *Salmonella* taken up by normal macrophage phagocytosis were shown to be contained within degradative phagolysomal compartments positive for all the markers mentioned above (Oh *et al.*, 1996; Rathman *et al.*, 1997). The *Salmonella*-specific vacuoles are accessible to fluid phase markers, and have been shown to contain calnexin, an endoplasmic reticulum marker, indicating that *Salmonella* may control the fusion of vesicles from the secretory pathway and only select some lysosome membrane components (Mills & Finlay, 1998; Oh *et al.*, 1996; Rathman *et al.*, 1997). Therefore *Salmonella* may survive within macrophages by interfering with normal cellular trafficking and thus hinder maturation of *Salmonella*-containing phagosomes. The SPI-2 encoded SpiC effector protein has been shown to interfere with phagosome-lysosome fusion and normal trafficking of non-*Salmonella* containing vesicle compartments within macrophages, substantiating this idea (Uchiya *et al.*, 1999).

The *Salmonella* induced "non-degrative" phagosomes and normal macrophage phagolysosomes may correlate with reports that two populations of intracellular bacteria can be seen within U937 cells after invasion by *S. typhimurium*, one static and the other rapidly dividing (Abshire & Heffron, 1993) with one population being degraded by the normal macrophage defences and the other representing the *S. typhimurium* intracellular survival strategy (Rathman *et al.*, 1997). Correlating with this proposal, a recent report showed that *S. enterica* SEF14 fimbrial mutants are unable to survive in activated macrophages, indicating a role for the fimbriae in attachment and/or invasion of the macrophages to help direct uptake of *S. enteriditis* to a "safe" intracellular compartment to enable survival and replication of the bacteria (Edwards *et al.*, 2000).

Salmonella have also been shown to be cytotoxic for macrophages and cause apoptosis (Chen *et al.*, 1996; Monack *et al.*, 1996). Invasive *S. typhimurium* bacteria (causing the

characteristic membrane ruffling and spacious phagosomes formation and grown under optimal conditions for invasion) cause optimal killing of the host macrophage in J774A.1 and RAW1264.7 macrophage cell lines, and murine bone-marrow derived macrophages. Mutations preventing invasion and growth conditions which lead to lower invasion abrogate this effect (Chen *et al.*, 1996; Monack *et al.*, 1996). The killing of the host macrophage when the bacteria are grown under optimal condition for invasion, is likely to be due to the SPI-1 encoded invasin SipB which has been shown to bind to caspase-1 in macrophages, activating the macrophage's apoptotic machinery (Hersh *et al.*, 1999). *Salmonella* induced apoptosis in macrophages has also been linked to growth phase as during the transition from the exponential to stationary growth phase, *Salmonella* bacteria induced apoptosis in more than 90% of infection macrophages (Lundberg *et al.*, 1999). Other host-adapted bacteria such as *S. typhi*, *S. gallinarum* and *S. dublin* were also shown to be toxic for cultured J774A.1 murine macrophages, so this toxicity for macrophages is likely to be a common virulence factor (Chen *et al.*, 1996), however it has been shown recently that *S. typhi* causes less cytotoxicity to both mouse and human-derived macrophage cell lines than *S. typhimurium* (Schwan *et al.*, 2000). The role of macrophage cytoxicity in the *Salmonella* infection process is not yet known. It may aid the bacteria in evasion of the host immune system by killing the host macrophage before it can send out cytokine signals to activate further immune defences, release from the macrophage in order to invade other cells or alternatively it may be a host-related defence system to eject the bacteria from the macrophages and allow exposure to other host-defences such as neutrophils (Monack *et al.*, 1996). The latter case may be more likely as apoptosis of macrophages has been shown to trigger inflammation via the SP1-1 encoded *sipB* invasion, which activates the proinflammatory protease capase-1 in macrophages, this then activates the processing of secreted IL-1β which is involved with the recruitment of inflammatory cells (Hersh *et al.*, 1999).

S. typhimurium mutants that are no longer able to survive in the macrophage have been isolated and have also been shown to be less virulent in the murine mouse model than their parental strain (Fields *et al.*, 1986A). The macrophage survival mutants were affected in many different genes including: those affecting LPS alterations, colony morphology, auxotrophy, complement sensitivity, oxidative stress sensitivity, and motility (Fields *et al.*, 1986B). Other mutations in genes which affected macrophage survival include: the *recA* and *recBC* genes (involved with repair of DNA damage) (Buchmeier *et al.*, 1993), the *htrA* (heat shock gene) virulence gene (Bäumler *et al.*, 1994) and genes encoded on the SPI-2 pathogenicity island (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Ochman *et al.*, 1996). The variety of macrophage survival mutants is not surprising when the large number of proteins induced by bacterial entry into macrophages (over 40) and repressed (over 100) (Abshire & Heffron 1993; Buchmeier & Heffron, 1990). Further analysis and comparison of these induced proteins showed that many of them are specifically induced/repressed in the macrophage environment compared to entry into epithelial cells (Burns-Keliher *et al.*, 1998). One regulatory locus relevant to this thesis that has been shown to be extensively involved with macrophage survival is the *S. typhimurium* PhoP/Q locus (Fields *et al.*, 1986B) (discussed in Section 1.9.7).

1.8.7 *Salmonella* and antimicrobial proteins/agents resistance

Resistance to host antimicrobial agents and proteins is an important part of *Salmonella* pathogenesis. These antimicrobial peptides/proteins have been isolated from a diverse variety of insects, amphibians and mammals (Boman, 1995). The peptides are part of the innate immune response against microbial infections, fungi and protozoa (Groisman, 1994). Evidence suggesting that these antimicrobial peptides protect the host from microbial infections include: the fact that defensins make up to 5-7% of the total cellular protein in

neutrophils and macrophages; that patients who have a clinical condition, "specific granule deficiency" almost completely lack defensins and have frequent/severe microbial infections (Lehrer *et al.*, 1993) and the genetic loci encoding defensins are located near the loci encoding lysozymes (part of macrophage defences) in mouse and human chromosomes (Groisman, 1994). Over 40 antimicrobial agents and peptides have so far been discovered (Boman, 1995) including: the polymyxins (pentacationic amphiphathic lipopeptide antibiotics with a hepapeptide ring and fatty acid tail) (Storm *et al.*, 1977); polylysines (Vaara, 1992); protamine (strongly cationic protein from salmon sperm (Vaara & Vaara, 1983). There are also other small cationic proteins such as defensins (29-34 residues, 3 to 10 net positive charges), cecropins (31-39 residues with 4 to 8 net positive charges), magainins (23 residues with 3-4 net positive charges) and melittin (26 residues, 6 net positive charges), which are present in mammalian phagocyte cells, insect hemolymph, frog skin and bee venom respectively (Vaara, 1992). A bacterial/permeability-increasing (BPI) cationic antimicrobial protein from neutrophils has also been isolated (Qi *et al.*, 1995). The small cationic proteins (defensin, cecropin, magainin and melittin), have been shown to form channels in artificial membranes and form α-helices in hydrophobic conditions (Christensen *et al.*, 1988; Duclohier *et al.*, 1989; Kagan *et al.*, 1990; Tosteson & Tosteson, 1984)). Most of these antimicrobial agents (including magainin, cecropin and melittin) act on Gram-negative bacteria via a similar mechanism, binding to and permeabilizing the outer membrane, crossing to reach the inner cytoplasmic membrane and lysing the cell (Boman, 1995; Vaara, 1992). Protamine has been found to have an antibacterial effect without causing cell lysis or cytoplasmic membrane permeabilization by disrupting energization and inhibiting nutrient uptake (Aspedon & Groisman, 1996).

A major part of the natural bacterial resistance are the lipopolysaccharides (LPS) in the OM of Gram-negative bacteria; polymyxins, defensins and magainin have all been demonstrated to bind to LPS (Vaara, 1992). *Salmonella* resistance determinants to these

antimicrobial agents/peptides has been hypothetically categorized into four parts (Groisman, 1994), and resistance mechanisms have not yet been found for all of these types. The first resistance mechanism is to prevent the peptides from reaching the membrane targets; the second is to destroy/inhibit biological activity of the peptide; the third is to affect the ability of the peptide to insert and generate channels in the membranes; the fourth is to restore the physiological balance of the cell.

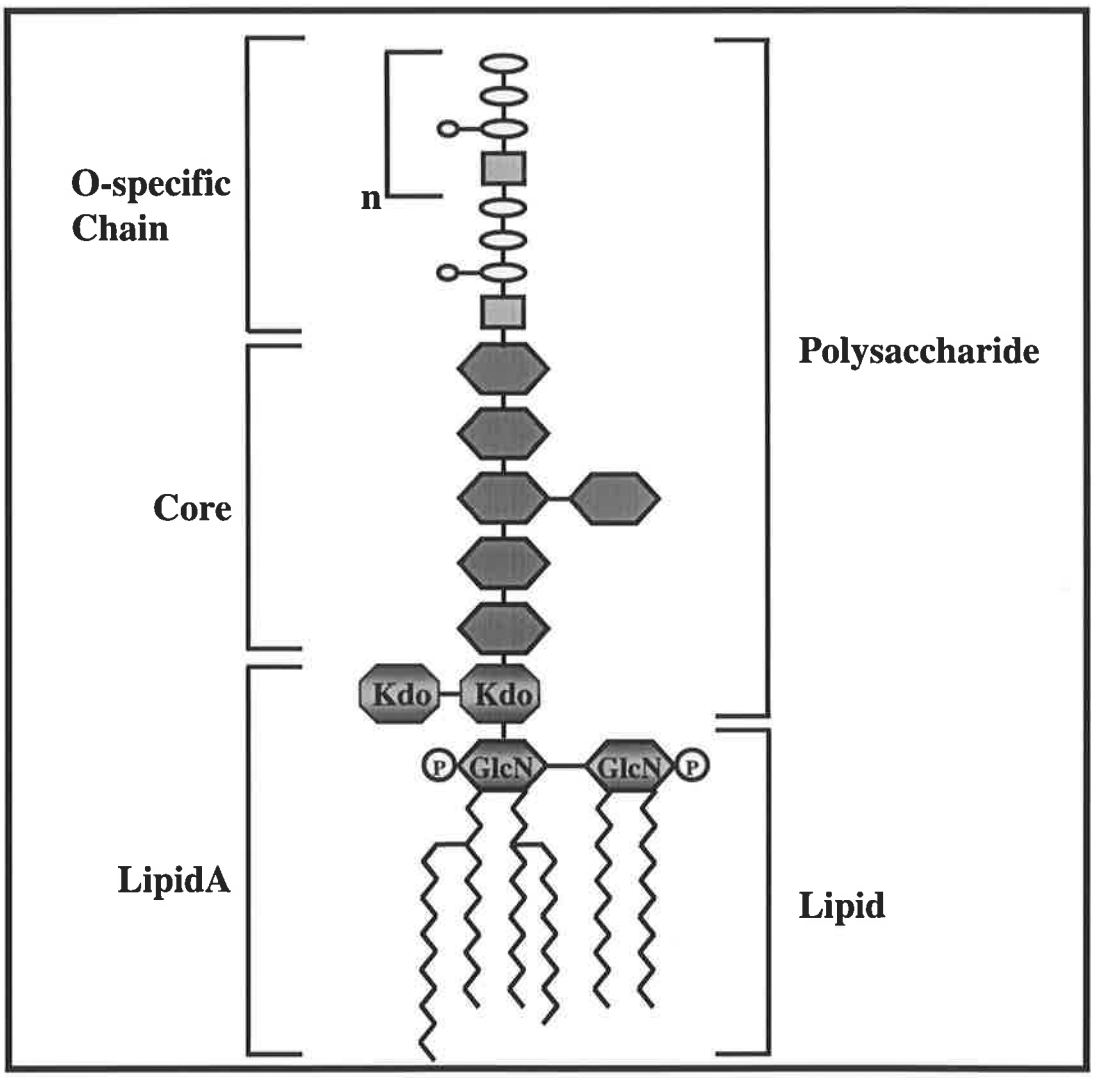
Gram-negative bacterial LPS is the first defence mechanism against cationic peptides and the ability of peptides to bind *E. coli* LPS has been correlated with microbial activity (Scott *et al.*, 1999). Other mutations affecting the LPS negative charge in physiological conditions are also involved with resistance, such as modifications to the lipid A as seen in *S. typhimurium pmrA* mutants (discussed in Section 1.9.11). Resistance to protamine has been demonstrated via the *S. typhimurium* SapABCDE (ATP-binding cassette) (Parra Lopez *et al.*, 1993), which may be involved with the transport of protamine into the cytoplasm where the peptides can be degraded by cellular proteases (Groisman, 1994). Recently, the *E. coli* protease OmpT was found to degrade protamine on the external face of the outer membrane (Stumpe *et al.*, 1998). The *S. typhimurium* PhoP/Q locus has been found to be extensively involved with many types of peptide resistance (Fields *et al.*, 1989; Groisman *et al.*, 1992B; Miller *et al.*, 1990) and this is discussed in Section 1.9.8.

1.8.8 *Salmonella* and Lipopolysaccharide (LPS)

A major part of *Salmonella* pathogenesis is due its LPS. The LPS molecule consists of three components: lipid A, the core oligosaccharide and the O-antigen (Figure 1.2). The core oligosaccharide is covently linked to the glucosamine containing lipid A molecule (situated in the lipid bilayer of the outer membrane) by keto-3-deoxy-D-mannose-octulosonic acid (KDO) (Schmidt *et al.*, 1969). The O antigen is linked to the core oligosaccharide and is typically

composed of oligosaccharide repeat units. These sugars include commonly occurring neutral sugars, aminosugars and sometimes other unusual sugars such as 6-deoxyhexoses (Raetz, 1996).

Figure 1.2: Schematic structure of lipopolysaccharide



The lipid A, core oligosaccharide and the O-specific chain are indicated on the left hand side of the figure. Individual O-repeats are indicated, n represents the number of repeating units. The polysaccharide and lipid component of the molecule are shown in brackets n the right side of the figure. Abbreviations; Kdo: 3-deoxy-D-*manno*-octulonic acid, Gln: D-glucosamine. Diagram adapted from Raetz, 1996.

The structural determinants (sugar composition, linkages between the sugar molecules and the sugar and/or non-sugar substitutions) of O antigen side chains provide the basis for the serological classification of *Enterobacteracae* according to the Kaufmann-white scheme

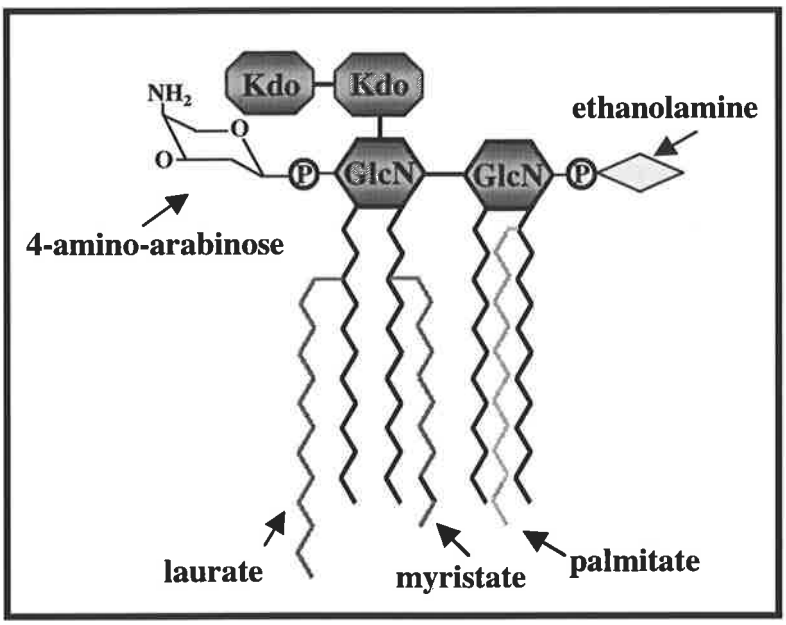
(Kauffmann, 1954). O antigen is also the immunodominant portion of the LPS molecule (Whitfield, 1995) and relatively minor differences in O-antigen structure can be detected immunologically. Wildtype strains of *Salmonella* which contain a complete LPS molecule are called smooth strains (forming smooth colonies on solid media) whereas rough strains, (forming rough colonies on solid media) are unable to synthesise LPS molecules with complete O antigen chains. The rough mutants are characterised by the structure or chemotype of the incomplete LPS that they synthesise and the various gene mutations responsible (Raetz, 1996). The degree of polymerisation of O-side chains in *S. typhimurium* ranges considerably from 0-40 repeat units after analysis of cell envelopes and isolated LPS by SDS polyacrylamide gel electrophoresis (Lesse *et al.*, 1990; Schnaitman & Klena, 1993). At least 50 genes have been identified that are involved in the biosynthesis and assembly of LPS (Schnaitman & Klena, 1993). The synthesis of the lipid A molecule involves the *lpxA* genes (Anderson *et al.*, 1985), the *waa/rfa* cluster is involved with core biosynthesis (Klena *et al.*, 1992), *rfb/wba* with the O-antigen (Jiang *et al.*, 1991) and polymerisation (chain length) is encoded for by *rfc/wzy* and *rol/wzz* (Raetz, 1996).

1.8.9 Lipid A

Lipid A is the hydrophobic anchor for LPS in the Gram-negative outer membrane and is the bioactive component (endotoxin) responsible for many of the symptoms associated with typhoid fever and systemic Salmonellosis (Section 1.3.2) (Hornick *et al.*, 1970). Lipid A consists of a backbone of a β,1-6-linked disaccharide of glucosamine that is phosphorylated at the 0-1 position of the reducing glucosamine and the 0-4 position of the non-reducing residue (Figure 1.3) (Raetz, 1996). The lipid A molecule backbone is acylated by four (R)-3-hydroxy fatty acids at positions 0-2, 0-3, 0-2' and 0-3' (Figure 1.3) (Raetz, 1996). In the last steps of lipid A synthesis, laurate and myristate residues are incorporated into the distal unit of lipidA,

generating the acyloxyacyl moieties (Figure 1.3). The genes responsible for the laurate and myristate addition are *waaM* (*htrB*) and *waaN* (*msbB*) respectively (Brozek & Raetz, 1990; Clementz *et al.*, 1996; Clementz *et al.*, 1997). Mutations in *waaN* and *waaM* have been shown to affect *Salmonella* virulence (Jones *et al.*, 1997; Khan *et al.*, 1998). The *waaM* mutant was severely limited in either its ability to colonise mouse organs or cause systemic disease in mice (Jones *et al.*, 1997). The *waaN* mutant was able to infect mice and grow at the same rate as the wildtype until bacteria had reached extremely high counts, but most mice were then able to clear the organism and survive, indicating a role for lethality for lipid A. The *waaN* mutants were also shown to have an altered O antigen length compared to the wild type strain, indicating a possible role for lipid A in O antigen chain length determination (Khan *et al.*, 1998).

Figure 1.3: Schematic structure of Lipid A



This schematic figure shows the lipid A molecule with the acyloxyacyl moieties laurate and myristate and their postions shown. Additional modifications to the lipid A molecule, including ethanolamine, 4-amino-arabinose and palmitate are also shown. This diagram was adapted from Raetz, 1996 and Darveau, 1998.

Modifications to the lipid A may also play a role in host responses to bacterial infections as it has been shown that *E. coli* lipid A lacking both the acyloxyacyl-linked (secondary) fatty acids, laurate and myristate failed to elicit the production of inflammatory mediators from host cells both *in vitro* and *in vivo* (Lynn & Golenbock, 1992; Munford & Hall, 1986; Pohlman *et al.*, 1987). The most characterised host response LPS activation pathway involves the binding of LPS to the serum protein LPS-binding protein (LBP) which is then able to transfer the LPS to CD14. The CD14 can be either membrane bound on myeloid cells, or in a soluble form which is involved with the activation of non-myeloid cells (Pugin *et al.*, 1993; Wright *et al.*, 1990). It has been suggested that the recognition of lipid A structural details probably occurs after CD14 binding by a host cell LPS recognition protein (Darveau, 1998). Recently a Toll-like receptor 4 (Tir4) has been shown to function as the transmembrane component of the LPS receptor complex (it is not yet known whether complexed LPS-CD14 is involved with binding to this receptor but it seems likely (Beutler, 2000)). It has been found that mouse macrophages respond equally well to lipid A and tetra-acyl lipid A (has no secondary acyl chains) whereas human monocytic cells only respond well to the intact lipid A molecule (Golenbock *et al.*, 1991; Poltorak *et al.*, 2000). In experiments expressing either human or murine Tlr4 in macrophages devoid of endogenous Tir4 gene expression, it was shown that the species origin of Tlr4 determined whether a response to tetra-acyl lipid A could be made. Human Tlr4 was able to discriminate between intact lipid A and tetra-acyl lipid A whilst mouse Tlr4 conferred a response to both lipid A molecules (Poltorak *et al.*, 2000).

Therefore modifications to lipid A such as 4-amino-arabinose (4AA), ethanolamine, 2-hydroxy myristate and palmitate (Figure 1.3) which have been found to be regulated by PhoP/Q and involved in virulence related phenotypes (discussed in Section 1.9.8 and 1.9.11) may be also involved with host specificity (Section 1.13).

1.9 The S. typhimurium phoP/Q locus

S. typhimurium phoP mutants have been shown to be involved with virulence, intramacrophage survival and antimicrobial peptide resistance (Fields *et al.*, 1989; Fields *et al.*, 1986B), and early studies demonstrated that mutations in *phoP* affected expression of the *phoN* gene product (non-specific acid phosphatase) suggesting that PhoP was a regulatory protein (Kier *et al.*, 1979). The *S. typhimurium phoP* locus contains two genes *phoP* and *phoQ*, which overlap by one nucleotide forming an operon, located at 25 minutes next to the *purB* gene (purine biosynthesis) on the chromosome (Miller *et al.*, 1989). PhoP encodes a protein of 224 aa (Groisman *et al.*, 1989; Miller *et al.*, 1989) and PhoQ encodes a 487 aa protein (Miller *et al.*, 1989). The PhoP/Q operon has strong homology to other bacterial two-component regulatory systems which consist of an environmental sensor kinase (responding to environmental signals), autophosphorylating at a histidine residue and then transferring the phosphate to the amino-terminal domain (at an aspartate residue) of a transcriptional regulator protein which is then able to activate/repress other genes on the bacterial chromosome (Miller, 1991). PhoP has strong homology to transcriptional activators such as OmpR and PhoB (Makino *et al.*, 1986A; Mizuno *et al.*, 1982), and PhoQ to environmental sensors such as EnvZ and PhoR (Makino *et al.*, 1986B; Mizuno *et al.*, 1982).

The *phoP* and *phoQ* genes have been reported to be highly conserved between *S. typhi* and *S. typhimurium* (Miller *et al.*, 1993) and oligonucleotides designed from the *S. typhimurium phoP* gene were able to PCR a 299 bp product from *S. typhimurium*, *S. typhi*, *S. paratyphi A*, *S. enteriditis*, *S. choleraesuis*, *S. gallinarum*, *S. pullorum* and *S. arizonae* indicate that the *phoP* gene is conserved across many *Salmonella* species (Way *et al.*, 1993). *S. typhimurium phoP* homologs have been detected in other Gram-negative bacteria (*Escherichia coli*, *Shigella flexneri*, *Citrobacter freundii*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Serratia marsescens*, *Proteus mirabilis*, *Erwinia herbicola*

and *Yersinia pestis*) by stringent Southern hybridisation analysis (Groisman *et al.*, 1989). Recently a *phoP/Q* homologue has also been reported in *Pseudomonas aeruginosa* (Macfarlane *et al.*, 1999B).

The *E. coli* K-12 *phoP/phoQ* operon has been cloned and sequenced (Kasahara *et al.*, 1992) and was located to 25 min on the *E. coli* K-12 linkage map. The *E. coli phoP/Q* genes also form an operon in *E. coli* (overlapping by 1 bp) and encode proteins of 223 and 486 aa respectively. The *E. coli* PhoP protein is 93% identical to *S. typhimurium* and the PhoQ protein is 86% identical with predicted protein structures similar to *S. typhimurium* PhoP and PhoQ proteins (Groisman *et al.*, 1992C; Kasahara *et al.*, 1992). The *E. coli phoP* gene has been reported to complement *S. typhimurium phoP* mutants for the production of non-specific acid phosphatase (Groisman *et al.*, 1992C).

Two *S. typhimurium phoP/Q* genotypes have been reported from studies with the *S. typhimurium phoP/Q* locus, *phoP* null mutants derived from chemical mutagenesis (*phoP12* (Kier *et al.*, 1979)), random transposon insertion (Fields *et al.*, 1986A) and defined mutagenesis (Miller *et al.*, 1989) all demonstrated similar phenotypes and will be collectively referred to as $PhoP^-$ strains in the rest of this thesis. A *S. typhimurium phoQ* constitutive mutant isolated by chemical mutagenesis (*phoP24*, (Kier *et al.*, 1979)) and found to possess a single amino acid substitution ($Thr^{48}\rightarrow Ile$) in the periplasmic domain of PhoQ (Gunn *et al.*, 1996) will be described as $PhoP^c$. Wildtype *Salmonella* strains containing an intact *phoP/Q* locus will be described as $PhoP^+$ strains.

1.9.1 Mutations in *Salmonella* PhoP/Q regulated genes and phenotypic effects

By two-dimensional gel electrophoresis the *S. typhimurium* PhoP/Q have been shown to affect the expression of over 40 different proteins (Buchmeier & Heffron, 1990; Miller &

Mekalanos, 1990). In *E. coli* the *phoP/Q* locus has been reported to affect the expression of at least 50 protein species (Kasahara *et al.*, 1992), indicating that PhoP/Q is a global regulator in both *S. typhimurium* and *E. coli*. The *S. typhimurium* PhoP protein is able to activate and repress genes and these genes have been named *pag*s (PhoP activated genes) and *prg*s (PhoP repressed genes) (Miller *et al.*, 1989). *S. typhimurium* PhoP$^-$ (null) mutants always have *pag*s downregulated and *prg*s upregulated whereas PhoP^c (constitutive) have *pag*s upregulated and *prg*s downregulated (Miller, 1991).

A large number of *S. typhimurium* phoP/Q regulated genes have been identified and mutations in these genes affect a large number of *Salmonella* phenotypes including: non-specific acid phosphatase production, acid tolerance, bile resistance, invasion of mammalian cells, macrophage survival and spacious phagosomes formation, antimicrobial peptide/agent resistance, protease production, macrophage antigen presentation, lipid A modifications, magnesium transport and growth under Mg^{2+}-limiting conditions. The *S. typhimurium* repressed genes are listed in Table 1.1a and the known *S. typhimurium phoP/Q* activated proteins, genes and operons are listed in Table 1.1b.

Table 1.1a: PhoP/Q repressed genes

Genes	Function/Cellular location	Reference
iviXVII (pdu)	1,2-propanediol utilization	Heithoff *et al.*, (1999A)
prgA	function unknown/envelope protein	Behlau & Miller, (1993)
prgB	function unknown/envelope protein	Behlau & Miller, (1993)
prgC	function unknown/envelope protein	Behlau & Miller, (1993)
prgD	function unknown/envelope protein	Behlau & Miller, (1993)
prgHIJK	invasion of eukaryotic cells	Behlau & Miller, (1993)
psgA	unknown	Soncini *et al.*, (1996)
psgB	unknown	Soncini *et al.*, (1996)
psgC	unknown	Soncini *et al.*, (1996)
psgD	unknown	Soncini *et al.*, (1996)
psgE	unknown	Soncini *et al.*, (1996)
psgF	unknown	Soncini *et al.*, (1996)
psgG	unknown	Soncini *et al.*, (1996)

Table 1.1a shows the *S. typhimurium* PhoP/Q repressed genes that have been identified so far Table 1.1b shows the *S. typhimurium* PhoP/Q activated proteins, genes and operons that have been identified so far. As many of the PhoP/Q genes have been isolated by different groups, and given different names, genes are listed in alphabetical order (followed by other gene names if appropriate). References correspond to the first identification of the gene and further analysis of the PhoP/Q regulated proteins, genes and operons is discussed in the text when appropriate.

Table 1.1b: *S. typhimurium* PhoP/Q activated proteins, genes and operons

Protein	Function/Cellular location	Reference
ASP6	unknown acid shock protein	Bearson *et al.*, (1998)
ASP15	unknown acid shock protein	Bearson *et al.*, (1998)
ASP52	unknown acid shock protein	Bearson *et al.*, (1998)
ASP29	(PhoP) acid shock protein	Bearson *et al.*, (1998)
Protease1	unknown	Adams *et al.*, (1999)
Protease2	unknown (dibasic amino acid recognition motif)	Adams *et al.*, (1999)

Genes	Function/Cellular location	Reference
iviVI-A	adhesin like	Heithoff *et al.*, (1997)
iviXVI	aldehyde dehydrogenase like	Heithoff *et al.*, (1999A)
mgtA	putative P-type ATPase (magnesium import)	Garcia Vescovi *et al.*, (1996)
mgtB	putative P-type ATPase (magnesium import)	Soncini *et al.*, (1996)
mgtC	macrophage survival	Garcia Vescovi *et al.*, (1996)
pagA/ugd/pmrE	UDP-glucose dehydrogenase, upregulated in macrophages upregulated by PmrA/B	Miller *et al.*, (1989)
pagB/pmrC/psiD	upregulated in macrophages	Miller *et al.*, (1989)
pagC	macrophage survival	Miller *et al.*, (1989)
pagD	macropage survival	Belden & Miller, (1994)
pagE	unknown	Belden & Miller, (1994)
pagF	unknown	Belden & Miller, (1994)
pagG	unknown	Belden & Miller, (1994)
pagH/pmrG	homology to *E. coli* AIS protein Regulated by PmrA/B	Belden & Miller, (1994)
pagI	unknown	Belden & Miller, (1994)
pagJ	similar to *pagK*	Belden & Miller, (1994)
pagK	hydrophobic protein (plus signal sequence) *S. typhimurium* and *S. enteriditis* specific	Belden & Miller, (1994)
pagL	unknown	Belden & Miller, (1994)
pagM	60 aa + signal seq	Belden & Miller, (1994)
pagN	putative transcriptional regulator, *Salmonella* specific	Belden & Miller, (1994)
pagO	homology to an ORF on Yersinia virulence plasmid, integral membrane protein (plus signal sequence)	Belden & Miller, (1994)
pagP	palmitate addition to lipidA	Belden & Miller, (1994)
pbgB	*mar* locus	Soncini *et al.*, (1996)
pbgC	unknown	Soncini *et al.*, (1996)
pbgD	unknown	Soncini *et al.*, (1996)
pbgE	4-AA addition to lipid A, regulated by PmrA/B	Soncini *et al.*, (1996)
pbgF	unknown	Soncini *et al.*, (1996)
pbgM	unknown	Soncini *et al.*, (1996)
pbgO	unknown	Soncini *et al.*, (1996)
pbgP	4-AA addition to lipid A, regulated by PmrA/B	Soncini *et al.*, (1996)
pbgW	unknown	Soncini *et al.*, (1996)
pbgX	unknown	Soncini *et al.*, (1996)
pbgY	unknown	Soncini *et al.*, (1996)
pcgD	unknown	Soncini *et al.*, (1996)
pcgF	unknown	Garcia Vescovi *et al.*, (1996)
pcgG	unknown	Garcia Vescovi *et al.*, (1996)
pcgH	unknown	Soncini *et al.*, (1996)
pcgJ	unknown	Soncini *et al.*, (1996)
pcgL	D-Ala-D-Ala dipeptidase, *Salmonella* specific	Hilbert *et al.*, (1999)
pcgN	unknown	Soncini *et al.*, (1996)
pcgP	unknown	Soncini *et al.*, (1996)
pcgQ	unknown	Soncini *et al.*, (1996)
phoN	non-specific acid phosphatase	Kier *et al.*, (1979)
phoP	transcriptional regulator, autoreglated	Soncini *et al.*, (1995)
psiD/pagB/pmrC	unknown, PmrA/B regulated	Groisman *et al.*, (1989)
pmrA	response regulator	Roland *et al.*, (1993)
pmrB	environmental sensor	Roland *et al.*, (1993)
pmrC/pagB/psiD	unknown function	Roland *et al.*, (1993)
pmrE/pagA/ugd	UDP-glucose dehydrogenase	Gunn *et al.*, (1998B)
pmrG/pagH	homology to *E. coli* AIS protein, regulated by PmrA/B	Gunn *et al.*, (1998B)
spvB	systemic infection; located - *Salmonella* virulence plasmid	Heithoff *et al.*, (1997)
ugd/pagA/pmrE	UDP-glucose dehydrogenase	Garcia Vescovi *et al.*, (1996)
ugtL	unknown, *Salmonella* specific	Hilbert *et al.*, (1999)
Operons		
pbgPE/pmrF	4-amino-arabinonse addition, Regulated by PmrA/B	Soncini *et al.*, (1996)
pmrF/pbgPE	4-amino-arabinonse addition, Regulated by PmrA/B	Gunn *et al.*, (1998B)

The regulation of the *S. typhimurium phoP/Q* operon and *phoP/Q* regulated genes is complex and will be discussed in Section 1.10, after the phenotypic effects of *Salmonella phoP/Q* mutations and the identification of *phoP/Q* regulated genes have been described.

1.9.2 Non-specific acid phosphatase production

S. typhimurium encodes 3 acid phosphatases. These perplasmic phosphatases include an acid hexose phosphatase, a cyclic 2'3'-nucleotide phosphodiesterase and a non-specific acid phosphatase (Kier *et al.*, 1977). Both the acid hexose phosphatase and the cyclic 2'3'-nucleotide phosphodiesterae are regulated by the cyclic AMP-catabolite repression system. (Kier *et al.*, 1977). The non-specific acid phosphatase production was not affected by mutations in the cyclic AMP pathway indicating novel regulation, and mutations in *S. typhimurium* leading to a lack of non-specific acid phosphatase production were mapped by Hfr crosses to two unlinked loci; *phoN* (co-transducible with *purA* at 93 minutes) and *phoP* (co-transducible with *purB* at 25 minutes) (Kier *et al.*, 1979). A mutant in the *phoP* locus (*phoP12*) totally lacked acid phosphatase production whereas another mutation (*phoP24*) had greatly increased production of acid phosphatase, and this mutation also mapped to the *phoP* locus (Kier *et al.*, 1979). Further analysis of the *phoN* gene indicated that *phoN* does encode the structural gene for non-specific acid phosphatase, encoding a 26 kDa protein (Kasahara *et al.*, 1991). PhoP/Q was shown to positively regulate *phoN*, indicating that *phoN* is a *pag* gene (Table 1.1b) (Groisman *et al.*, 1989; Miller *et al.*, 1989). Analysis of mutants have indicated that *phoN* has no role in virulence (Fields *et al.*, 1989), however it is useful in *Salmonella* studies on the *phoP/Q* locus, as it allows discrimination between $PhoP^-$, $PhoP^+$ and $PhoP^c$ strains (Section 3.6.3).

1.9.3 Acid tolerance response

S. typhimurium has a complex adaptive system called the acid tolerance response (ATR) (Section 1.8.1). The *S. typhimurium* PhoP/Q regulon has been recently shown to control an RpoS-independent acid tolerance system involved with inorganic (low pH) survival (Bearson *et al.*, 1998). The *S. typhimurium* PhoP$^-$ mutant was originally reported to have some adaptive ATR responses and to have normal growth in moderately low pH media (pH 5.8) but was 1000-fold more sensitive to low pH (inorganic acid) than wildtype PhoP$^+$ strains (Foster & Hall, 1990). Later, this extreme sensitivity to acid was found to be due to the fact that the study had been performed with a strain which was also *rpoS* in addition to *phoP*. However, a more recent study demonstrated that a *phoP* mutation in an *rpoS*$^+$ cell has a small (~10-fold) but reproducible effect on acid tolerance, indicating an RpoS-independent system (Bearson *et al.*, 1998). During the shift from pH 7.7 to a moderate pH of 4.4, *S. typhimurium* produces 51 ASP's (seen by 2D gel electrophoresis) and a PhoP$^-$ mutant has been shown to lack four induced ASP's (Table 1.1b) from this set compared to wildtype strains. The PhoP protein itself has been shown to be one of these four ASP's but the identity of the other three are unknown at this stage (Bearson *et al.*, 1998). Both *phoP* and *phoQ* are needed for the induction of this inorganic acid tolerance (Bearson *et al.*, 1998) and no *S. typhimurium* PhoP/Q genes (apart from *phoP* itself) have been identified which are involved in the ATR response. Changes in pH levels have also been shown to play an important role in the regulation of the *S. typhimurium* PhoP/Q operon and PhoP/Q regulated genes (Bearson *et al.*, 1998; Soncini & Groisman, 1996) (discussed in Section 1.10).

1.9.4 Bile resistance

During Salmonellosis, *Salmonella* bacteria come into contact with bile. Bile salts are detergents made in the liver and then secreted into and stored within the gall bladder. *Salmonella* organisms are resistant to bile salts, a phenotype that is often used in the isolation and detection of these organisms (e.g. MacConkey agar). The outer membrane of Gram-negative bacteria is involved with the resistance to bile salts and changes in LPS and membrane proteins have been shown to affect bile salt tolerance (Nikaido, 1976; Sukupolvi & Vaara, 1989). In the chronic carrier state of Salmonellosis, affected individuals carry *Salmonella* bacteria within the gallbladder where the bacteria are likely to come into contact with bile (Hook, 1990). *Salmonella* resistance to bile salts has also been shown to require the PhoP/Q regulon (Van Velkinburgh & Gunn, 1999). *S. typhi* and *S. typhimurium* $PhoP^-$ mutants showed decreased survival to bile salts whereas $PhoP^c$ strains showed increased resistance. The PhoP/Q resistance was shown to be specifically to the bile components deoxycholate and conjugated forms of chenodeoxycholate (Van Velkinburgh & Gunn, 1999). *Salmonella* involved in a systemic infection are likely to come into contact with bile containing dihydroxy deoxycholate and chenodeoxycholate bile salts for which the PhoP/Q regulon plays a major role in resistance (Van Velkinburgh & Gunn, 1999). The growth of *S. typhi* and *S. typhimurium* in either bile or deoxycholate showed an increase/decrease in a number of proteins which correlated with many PhoP/Q activated or repressed products seen by 1D or 2D gel electrophoresis. None of the known *Salmonella* PhoP/Q regulated genes (including those known to modify *Salmonella* LPS), were found to be responsible for this resistance, however two PhoP/Q repressed genes *prgC* and *prgH* were shown to be transcriptionally repressed (independent of PhoP/Q and bacterial growth stage) by medium containing bile. *S. typhimurium* strains demonstrated an increased resistance compared to comparative *S. typhi* strains to the crude ox bile extract used in this study (Van Velkinburgh & Gunn, 1999).

1.9.5 Magnesium transport

S. typhimurium encodes three Mg^{2+} transport systems: CorA (a 40 kDa protein) which is constitutively expressed and mediates both the influx and efflux of Mg^{2+} (Hmiel *et al.*, 1989; Smith *et al.*, 1993A) and the MgtA and MgtB loci which encode putative P-type ATPases (Smith *et al.*, 1993A; Smith *et al.*, 1993B). Mutations in *mgtA* were shown to have a small affect on intramacrophage survival (Blanc Potard & Groisman, 1997). The *S. typhimurium* MgtA and MgtB proteins are produced under Mg^{2+} limiting conditions and mediate only the influx of magnesium (Tao *et al.*, 1998). The *mgtCB* operon is located on SPI-3, encoding the 102 kDa MgtB Mg^{2+} transport protein (Blanc Potard & Groisman, 1997) and 22.5 KDa hydrophobic MgtC protein, involved with mouse virulence and long-term survival in macrophages (Blanc Potard & Groisman, 1997; Tao *et al.*, 1998). The MgtC protein is not involved with magnesium transport and its function in intramacrophage survival is unknown. It has been shown that expression of the MgtA and MgtB proteins requires a functional *corA* gene (Moncrief & Maguire, 1998). Both the *S. typhimurium mgtA* and *mgtCB* loci are regulated by PhoP/Q (Table 1.1), with MgtA and MgtB required for growth in liquid low-Mg^{2+} (micromolar) environments (Soncini *et al.*, 1996). Mutations in *phoP*, *mgtA* or the *mgtCB* loci also affected bacterial cell morphology in reduced-[Mg^{2+}] liquid media producing single cells at stationary phase 10 to 20 times larger than that of the parental wild type strain and these mutants were no longer able to form colonies on solid media at this stage. *S. typhimurium* $PhoP^-$ mutants are unable to form colonies on low-Mg^{2+} solid media and mutants in a number of PhoP/Q regulated genes (*pcgP*, *pcgD*, *pbgP*, *pbgM*, *pbgE* and *ugd*) (Table 1.1b) also showed this growth deficiency on solid media. However they showed no growth defect in liquid low-[Mg^{2+}] media (Soncini *et al.*, 1996). Magnesium has been shown to stabilize the Gram-negative bacterial outer membrane by interacting with phosphate groups in the LPS (Coughlin *et al.*, 1983) and in low Mg^{2+} environments, the charge repulsion between LPS molecules within and between cells is predicted to increase. It has been predicted that

this change in charge repulsion affected cell morphology and bacterial colony formation (Soncini *et al.*, 1996). Extracellular magnesium levels are also involved in the regulation of *S. typhimurium phoP/Q* and *phoP/Q* regulated genes (Section 1.10).

1.9.6 Invasion of mammalian cells

S. typhimurium PhoP^c bacteria were found to be invasion deficient as was the *S. typhimurium prgH* mutant (Table 1.1a) (Behlau & Miller, 1993). The *prgH* gene was found to be part of an operon (*prgHIJK*), involved with protein secretion and located in the *S. typhimurium* SPI-1 locus (Figure 1.1) (Johnston *et al.*, 1996; Pegues *et al.*, 1995). PrgH and PrgK have been found to be part of the SPI-1 Type III secretion translocation "needle complex" which is needed for translocation of the Type III secretion system effector proteins (Kubori *et al.*, 1998). However the function of the PrgI and PrgJ proteins is currently unknown. The *prgHIJK* operon has been found to be upregulated by HilA (which is repressed by PhoP/Q) and needs SirA for complete expression (Bajaj *et al.*, 1995; Bajaj *et al.*, 1996; Johnston *et al.*, 1996).

1.9.7 Macrophage survival

S. typhimurium bacterial survival in macrophages has been strongly correlated with virulence (Fields *et al.*, 1986A). A bank of 83 *S. typhimurium* Tn*10* mutants were shown to be unable to survive in mouse peritoneal macrophages and of these insertions, three were found to be in the *phoP* gene (Fields *et al.*, 1989). One of these *phoP* mutants was later found to be unable to survive in J774 macrophage-like cells, bone marrow derived and splenic macrophages (Buchmeier & Heffron, 1989). The *phoP* gene was found to be the first gene of an operon (*phoP/Q*) (Groisman *et al.*, 1989; Miller *et al.*, 1989) and both the *phoP* and *phoQ*

genes are needed for survival in macrophages (Miller *et al.*, 1989). The *S. typhimurium* PhoP protein was shown to be induced in macrophages (Buchmeier & Heffron, 1990) and a *phoP*$^-$ mutant strain failed to induce at least 9 other macrophage-induced proteins (Buchmeier & Heffron, 1990), confirming that *phoP* is part of a regulatory locus. The *S. typhimurium* PhoP^c mutant has also been reported to have decreased survival in macrophages, indicating that both *pags* and *prgs* are needed in bacterial survival in macrophages (Miller & Mekalanos, 1990).

1.9.7.1 PagC

A *phoP*/Q activated gene (*pagC*) (Table 1.1b), has been shown to be involved with macrophage survival and mouse virulence, but not defensin resistance (Miller *et al.*, 1989). The *S. typhimurium pagC* gene encodes an 188 aa membrane protein which shows similarity to the other virulence-related outer membrane proteins including: the *ail* encoded eucaryote cell invasion protein of *Yersinia enterocolitical*, the *lom* encoded protein of bacteriophage lambda (Pulkkinen & Miller, 1991), and the virulence plasmid complement resistance gene *rck* (Heffernan *et al.*, 1992). The *pagC* gene is not involved in invasion however, and a *pagC* mutant cannot be complemented by *ail*, therefore indicating structural homology to this protein only (Miller *et al.*, 1992). PagC::Tn*phoA* fusions have been shown to be strongly up-regulated (~ 100-fold) within *Salmonella*-infected bone marrow derived macrophages but not epithelial cells (Alpuche Aranda *et al.*, 1992), indicating the gene product specifically acts in macrophages however However the actual function of *pagC* during survival in macrophages by *Salmonellae* has not been defined.

1.9.7.2 Other PhoP/Q regulated genes

Two other virulence associated *pag* genes identified at the same time as *pagC* are *pagA* and *pagB* (Miller *et al.*, 1989). These have also been shown to be strongly upregulated in

macrophages (Alpuche Aranda *et al.*, 1992), however these genes were shown not to be involved in survival in the mouse peritoneal macrophage model (Miller *et al.*, 1989). PagA encodes UDP-glucose dehydrogenase (Gunn *et al.*, 1998B), and *pagB* is the first gene (*pmrC*) of the *S. typhimurium* PhoP/Q regulated *pmrC/pmrA/pmrB* operon encoding a second two component regulatory system (Table 1.1b). As both these loci are involved with PhoP/Q regulated lipid A modification and protection against antimicrobial agents (Section 1.9.11) it is not surprising that they are upregulated during *Salmonella* infection of macrophages. A further 13 *S. typhimurium* PhoP/Q regulated *pag* loci have been identified (*pagD-pagP*) which encode membrane or secreted proteins (Table 1.1) (Belden & Miller, 1994). All of these *pag*::Tn*phoA* fusion mutants showed decreased survival in macrophages and affected *Salmonella* virulence in mice (Belden & Miller, 1994). However defined mutants for each of these *pag* genes need to be tested as a defined mutant in *pagD* (encoded next to and transcribed divergently from *pagC*) was later found not to be involved with virulence (Gunn *et al.*, 1995) and defined mutants in *pagJ*, *pagK* and *pagM* did not attenutate virulence (Gunn *et al.*, 1998A). While most of these *pag* loci encode novel proteins, *pagH* had similarity to an *E. coli* aluminium induced gene (*ais*), *pagO*; homology to a product of the *Yersinia* virulence plamid (*yadA*), and *pagP*: homology to CrcA (necessary for *E. coli* resistance to camphor) (Gunn *et al.*, 1998A). The *S. typhimurium pagJ* and *pagK* genes were also found to be highly similar to each other (possibly indicating a redundancy in PhoP/Q regulated genes) and were found to be unique to *S. typhimurium* and *S. enteriditis* strains by Southern hybridisation analysis(Gunn *et al.*, 1998A).

1.9.7.3 The *S. typhimurium* spv locus

S. typhimurium strains carry a large cryptic plasmid (~100 kb) called the virulence plasmid which has been found in many other *Salmonella* strains involved with systemic

infection except in *S. typhi*. The virulence plasmid has been shown to be responsible for spreading infection beyond the intestines to deeper tissues such as spleen and liver and is involved in intracellular multiplication in these organs (Gulig, 1990). Encoded on the virulence plasmid is the *spv* locus and all species of *S. typhimurium* that cause systemic infection in mice contain the *spv* locus as do *S. enterica* species which cause systemic infection in cattle (Gulig *et al.*, 1993; Roudier *et al.*, 1990; Wallis *et al.*, 1995). The *spv* gene locus encodes five genes, *spvR* and the *spvABCD* operon (Gulig *et al.*, 1993) and has been shown to be induced within macrophages (Fierer *et al.*, 1993; Rhen *et al.*, 1993). The *spvB* gene from *S. dublin* has been reported to be co-ordinately regulated by PhoP/Q and RpoS (Fang *et al.*, 1991; Heithoff *et al.*, 1997).

1.9.7.4 Spacious phagosomes (SP) formation

Invasion of *S. typhimurium* mouse macrophages by *Salmonella* involves generalized membrane ruffling and macropinocytosis as described previously (Section 1.8.3). It has been noted that *Salmonella* inside macrophages are enclosed within spacious phagosomes morphologically similar to macropinosomes (Alpuche Aranda *et al.*, 1994). *Salmonella* was shown to persist in these spacious phagosomes which can enlarge by fusion with macropinosomes or other phagosomes and take a long time to shrink (Alpuche Aranda *et al.*, 1994). *S. typhimurium* PhoP^c bacterial mutants have previously been shown to be less invasive than wildtype strains, induce less macropinocytosis and SP's (inducement is at a similar level as heat-killed bacteria) and show decreased survival early after phagocytosis (~ 1h) (Alpuche Aranda *et al.*, 1995) . The PhoP^c bacteria are more likely to be enclosed within macrophages in close-fitting phagosomes consistent with that expected for normal receptor-mediated phagocytosis by phagocytic cells (Alpuche Aranda *et al.*, 1995), indicating that PhoP/Q regulated genes are involved in the formation of these SP's. Both PhoP$^-$ and PhoP^c *S.*

typhimurium mutants have been shown to have decreased intramacrophage survival, therefore suggesting that both *pags* and *prgs* are needed (Miller & Mekalanos, 1990). The shrinkage of the SP's for wildtype *S. typhimurium* bacterial infection correlates with the activation of PhoP/Q upregulated gene (*pag*) expression in the macrophage (~4-6 h after infection) (Alpuche Aranda *et al.*, 1992) and the PhoP$^-$ mutants only demonstrate growth deficiency in macrophages at this later time (Alpuche Aranda *et al.*, 1995). These results indicate that *S. typhimurium prg* gene expression is needed in the early stages of macrophage infection for invasion and SP formation, and *pag* gene expression is switched on later, after shrinkage and acidification of the SP, up-regulating the expression of genes needed for the latter stages of bacterial macrophage survival (Alpuche Aranda *et al.*, 1995). It has been suggested that the SP formation allows dilution of toxic compounds produced by the macrophage, and delay of the phagolysomal fusion and acidification gives the bacteria time to express genes needed for survival in the intracellular environment (Alpuche Aranda *et al.*, 1995).

1.9.8 PhoP/Q mutations and antimicrobial cationic proteins

The *S. typhimurium phoP*/Q locus has been shown to be involved with resistance to microbicidal proteins from mammalian phagocytic cells (Fields *et al.*, 1989; Groisman *et al.*, 1992B; Miller *et al.*, 1990). *S. typhimurium* PhoP$^-$ mutants were originally found to be extremely sensitive to crude extracts from human neutrophils and rabbit peritoneal macrophages, and purified rabbit defensin NP-1 (Fields *et al.*, 1989). Further studies showed that *S. typhimurium* PhoP$^-$ mutants were sensitive to protamine, frog magainin 2, pig cecropin P1 and the insect venom-derived peptides mastoparan and melittin (Groisman *et al.*, 1992B) and the human intestinal defensin 5 (Porter *et al.*, 1997). Both *phoP* and *phoQ* are needed for defensin resistance (Miller *et al.*, 1990) and the *S. typhimurium pagP* gene (Table 1.1b) has been shown to be required for the addition of palmitate to lipid A (Figure 1.3) and general

resistance to the cationic peptides (Guo *et al.*, 1998) (Section 1.8.7; Section 1.8.9). The PhoP/Q operon has also been shown to be involved in the upregulation of two unknown proteases, one of which which recognises a dibasic amino acid motif characteristic of the omptin protease family (Adams *et al.*, 1999). It was proposed that these proteases may contribute to *Salmonella* resistance to cationic antimicrobial proteins (similar to the OmpT protease which hydrolyzes the antimicrobial peptide protamine) (Adams *et al.*, 1999; Stumpe *et al.*, 1998). Recently, a *S. typhimurium phoP/Q* activated gene: *pcgL* (Table 1.1b) has been found to encode a D-Ala-D-Ala dipeptidase which is specific to *Salmonella* strains. As yet the function of this protein is unknown in *Salmonella* pathogenesis but it may be involved in resistance to a toxic compound encountered by *Salmonella* in the environment as it has not been shown to be involved in virulence (Hilbert *et al.*, 1999).

1.9.9 Antigen presentation

A major part of the stimulation of the host humoral and cell-mediated immune response to bacterial infections is antigen processing and presentation by specialised cells of the immune system. Macrophages play a major role in stimulating these immune responses as they are phagocytic, express bactericidal defences and are "professional" antigen presenting cells, displaying the processed antigens on both MHC-I and MHC-II (Harding & Geuze, 1993; Unanue, 1984). It has been found that the efficiency of antigen processing from *S. typhimurium* by macrophages is affected by bacterial viability (dead bacteria are processed much more efficiently than live) and epitope abundance (more epitopes give better processing) (Wick *et al.*, 1994). LPS phenotype may also be involved as rough strains of *S. typhimurium* are processed more efficiently than smooth strains, although this may be due to increased uptake of the LPS mutants by macrophages (Wick *et al.*, 1994). The *S. typhimurium* PhoP/Q regulon also affects the ability of activated macrophages to process and

present *S. typhimurium* antigens (Wick *et al.*, 1995). *S. typhimurium* PhoP$^-$ strains have been shown to be processed much more efficiently than wildtype bacteria, and PhoP^c mutants to be processed less efficiently than wildtype strains. Killing of the bacteria allowed wildtype strains to be processed as efficiently as PhoP$^-$ strains (Wick *et al.*, 1995). These results indicate a role for *S. typhimurium* PhoP/Q regulated genes in the inhibition of specific immunity, specifically the processing and presentation of antigens. As yet it is unknown which PhoP/Q regulated gene(s) are involved in this process.

1.9.10 *S. typhimurium* PmrA/B locus

1.9.10.1 PhoP/Q and PmrA/B

The *S. typhimurium* PhoP/Q regulator has been shown to regulate another two component regulatory system called PmrA/B (Gunn & Miller, 1996; Soncini & Groisman, 1996). The *pmrA/B* genes are located at 93.5 min on the *S. typhimurium* chromosome and are encoded in an operon containing three genes (Roland *et al.*, 1993). The first gene of the operon encodes *pmrC* (previously identified as the *phoP*/Q upregulated genes *pagB* and *psiD* (Table 1.1b) (Groisman *et al.*, 1989; Miller *et al.*, 1989)). The second gene encodes *pmrA*, which has homology to a number of DNA binding response regulators whilst the third is *pmrB* which shows homology to a number of autophosphorylatable histidine kinase sensor proteins (Roland *et al.*, 1993). An *S. typhimurium pmrA* constitutive mutant (*pmrA*505)(due to a single nt change in *pmrA* resulting a His to Arg substitution in the PmrA protein) has been shown to have increased resistance to the antibiotic polymyxin B (Makela *et al.*, 1978). The *pmrA*505 mutant also showed increased resistance to the cationic antimicrobial human neutrophil proteins CAP37 and CAP57 as well as polylysine and protamine (Shafer *et al.*, 1984; Spitznagel, 1990; Vaara, 1981A). *S. typhimurium* PmrA^c strains also possessed an increased level of phosphate substitution on the LPS core (due to modifications of the lipid A region

with ethanolamine) and a high degree of substitution of the 4' phosphate of lipid A by 4-aminoarabinose (4AA) (Figure 1.3) (Helander *et al.*, 1994; Vaara, 1981B, Vaara *et al.*, 1981), which are thought to decrease the negative charge of LPS, therefore reducing the binding of the cationic antimicrobial agents (Gunn & Miller, 1996).

1.9.10.2 PmrA/B regulated genes

The PmrA/B operon has been shown to regulate a number of genes (identified independently by different groups) located at distant sites on the *S. typhimurium* chromosome (Table 1.1b). The *S. typhimurium* PmrA/B regulated *ugd* gene encoding UDP-glucose dehydrogenase (Morona *et al.*, 1995) was originally identified as a *phoP/Q* activated locus (*pagA)* (Miller *et al.*, 1989), and was recently identified as being PmrA/B regulated by two groups: (*ugd:* Soncini & Groisman, 1996); *pmrE:* Gunn *et al.*, 1998B)). A seven gene operon regulated by PmrA/B was identified by two groups (*pbgPE* locus: Soncini & Groisman, 1996); *pmrF* locus: Gunn *et al.*, 1998B)). This operon was identified as being involved with polymyxin resistance (Groisman *et al.*, 1997; Gunn *et al.*, 1998B), responsible for the presence of 4-AA on lipid A (Gunn *et al.*, 1998B) and growth on low [Mg^{2+}] containing solid media (Groisman *et al.*, 1997). The *phoP/Q* regulated gene *pagH* (Belden & Miller, 1994), which has homology to the *E. coli* aluminium induced Ais protein, was renamed *pmrG* by Gunn *et al.*, (1998B) when it was found to also be PmrA/B regulated. Other unlinked loci regulated by PmrA/B include *pbgM*, *pcgD*, and *pcgP* (Soncini & Groisman, 1996). The *pmrD* gene is located at the end of the *S. typhimurium pbgPE/pmrF* locus and confers polymyxin B resistance in *S. typhimurium* when expressed in multiple copies (Roland *et al.*, 1994), however it is not yet known whether it is regulated by PmrA/B.

1.9.10.3 Regulation of the PmrA/B operon and regulated genes

The PmrA/B locus has been shown to be transcriptionally autoregulated and when examined, the locus was found to contain two promoters, P1 and P2 (Gunn & Miller, 1996). P1 is a weak constitutive promoter located within the *pmrC* coding sequence, directs transcription of *pmrA/B,* and is not affected by the presence/absence of either PhoP/Q or PmrA/B. The second promoter P2 is located upstream of the *pmrC* gene and is upregulated by PmrA/B and PhoP/Q (Soncini & Groisman, 1996). Further characterization of the *S. typhimurium* PmrA/B regulated genes identified a conserved sequence in the transcription start sites of three PmrA regulated genes (*pmrC*, *pmrG* and the *pbgPE/pmrF* operon) (Wosten & Groisman, 1999). DNA footprinting analysis identified a 16 base pair inverted repeat sequence (5' –TTAAKTTCTTAADGTT) to which his-tagged PmrA protein could bind. PmrA could not be demonstrated to bind to any part of the *ugd* promoter sequence and sequence analysis showed that this region of DNA did not contain the repeat sequence, indicating indirect regulation of *ugd* by PmrA via another regulator (as yet unknown). It has been suggested that a PmrA dimer activates transcription of the divergent *pmrG* and *pbgPE* promoters by binding to a single site in the *pmrG-pbgPE* promoter intergenic region (Wosten & Groisman, 1999).

1.9.11 Lipid A modifications

The *S. typhimurium* PhoP/Q regulon has been found to regulate structural modifications of lipid A. *S. typhimurium* PhoP^c strains were found to have an addition of 4AA to lipid A phosphate groups, replacement of the lipid A acyl group myristate with 2-OH myristate and the formation of heptaacylated lipid A by the addition of palmitate (Guo *et al.*, 1997). Lipid A is the major signalling component of *S. typhimurium* LPS that stimulates cytokine release in the host (Tokada & Kotani, 1992) and the 4AA and 2-hydroxymyristate substituted lipid A

was found to decrease expression of the adhesion molecule E-selectin by endothelial cells and tumor necrosis factor-α (TNF-α) by adherent monocytes, compared to wildtype lipid A (Guo *et al.*, 1997). *S. typhimurium* PmrA constitutive mutants have previously been found to have a high level of substitution of LPS phosphates by 2-aminoethanol (ethanolamine) and 4AA which is associated with an increase in resistance to polymyxin B (Vaara *et al.*, 1981). Similar substitutions on the lipid A of *E. coli* polymyxin B resistant mutants have also been found (Nummila *et al.*, 1995). Two *S. typhimurium* PmrA/B regulated loci have been shown to be responsible for the modification of lipid A with 4AA (Gunn *et al.*, 1998B). The two loci responsible for this modification are the *ugd/pmrE* gene encoding UDP-glucose dehydrogenase and the *pmrF/pgbPE* locus (Gunn *et al.*, 1998B). The formation of heptaacylated lipid A by the addition of palmitate was found to be due to the *S. typhimurium* PhoP/Q regulated *pagP* gene which has been suggested to encode an acyltransferase or a nonenzymatic inner membrane protein complex component essential for the transfer of palmitate to lipidA and is involved with cationic antimicrobial peptide resistance (Guo *et al.*, 1998).

1.10 How PhoP/Q regulates expression of *phoP/Q* regulated genes

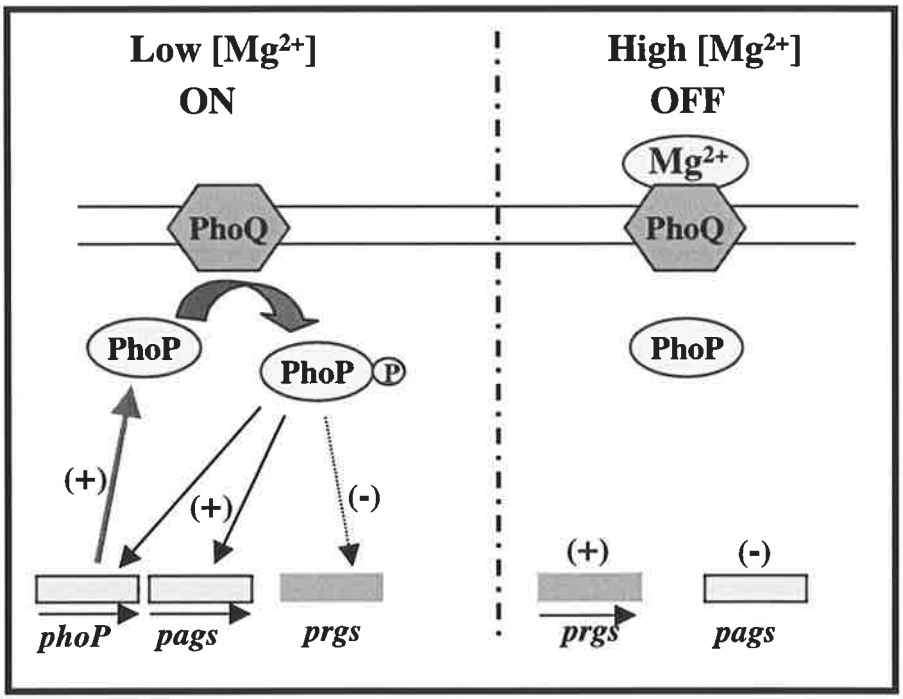
In a study on isolation of *S. typhimurium phoP/Q* regulated genes it was discovered that the *phoP/Q* operon is transcriptionally autoregulated (Soncini *et al.*, 1995). Wildtype *S. typhimurium* strains have been shown to produce two different transcripts from the *phoP/Q* locus in exponential growth and the levels of both are decreased in stationary phase cells. Primer extension experiments identified two distinct transcriptional start sites differing by 11 nucleotides corresponding to two promoters which were named *phoP*p1 (dependant on *phoP* and *phoQ* for activity) and *phoP*p2 (*phoP/Q* independent but stimulated by PhoP activation)

(Soncini *et al.*, 1995). A model of *S. typhimurium* PhoP/Q regulation follows. In a repressing environment, *phoP/Q* is transcribed from *phoP*p2 at a basal level and when the bacteria encounter activating environmental conditions, PhoQ autophosphorylates, then phosphorylates PhoP, activating transcription from *phoP*p2 (and to a lesser extent *phoP*p1), which would then lead to the activation of *S. typhimurium pag* genes and repression of *prg* genes. A change in environmental conditions back to the repressing conditions would then result in a return to the basal level of *phoP/Q* expression (by a mechanism not yet known) and the consequent down-regulation of *pag* genes and expression of *prg* genes (Soncini *et al.*, 1995).

One of the environmental conditions affecting *S. typhimurium phoP/Q* regulation has been shown to be extracellular Mg^{2+} concentrations (Figure 1.4) (Garcia Vescovi *et al.*, 1996). Mg^{2+} is a membrane, ribosome, and LPS stabiliser, and is essential in reactions that require ATP (Reinhart, 1988), and this was the first time it has been shown to be involved as a signal for a regulatory system. The identification of two PhoP/Q regulated high affinity Mg^{2+} transporters (MgtA and MgtB), whose expression was known to be upregulated in low-Mg^{2+} media (Snavely *et al.*, 1991) led to Mg^{2+} ~~concentrations~~ and PhoP/Q mediated gene regulation being investigated (Garcia Vescovi *et al.*, 1996). It was then shown that the *phoP*p1 (*phoP/Q* dependent) transcript was produced in low-Mg^{2+} media, indicating that Mg^{2+} controls the positive feedback loop regulating *phoP/Q* (Garcia Vescovi *et al.*, 1996). High-Mg^{2+} media was also shown to render wildtype *Salmonella* phenotypically $PhoP^-$, indicated by extreme sensitivity to the antimicrobial peptide magainin 2 (due to a decrease in *pag* gene expression) (Garcia Vescovi *et al.*, 1996). The *S. typhimurium* PhoQ protein was then shown to directly bind Mg^{2+} ions to a region on the large periplasmic loop which is rich in acidic amino acids, and by doing so to change PhoQ confirmation. This highly acidic amino acid region was specific, as a chimera protein harbouring the periplasmic region of EnvZ fused to the PhoQ cytoplasmic tail lost the capacity to bind to Mg^{2+}. Ca^{2+} ions were also able to bind and cause

a conformational change in PhoQ, however the amount needed was higher than normal physiological conditions (Garcia Vescovi *et al.*, 1997; Garcia Vescovi *et al.*, 1996). The expression of many *S. typhimurium* PhoP/Q regulated genes was also affected by Mg^{2+} concentration (Groisman *et al.*, 1997; Soncini *et al.*, 1996).

Figure 1.4: PhoP/Q regulation and Mg^{2+} levels



This figure shows the regulation of *phoP/Q* and *phoP/Q* regulated genes by Mg^{2+} levels. In an environment with high Mg^{2+} levels ('Off' state), Mg^{2+} ions are able to bind to PhoQ, inhibiting kinase activity. Therefore no phosphorylated PhoP (PhoP-P) is produced, allowing *prg*s to be transcribed and to decreased *pag* gene expression. In an environment with low Mg^{2+} levels ('On' state), Mg^{2+} no longer binds to PhoQ therefore enabling PhoQ to autophosphorylate, which is followed by the phosphorylation of PhoP. Phosphorylated PhoP is then involved with activation of *pag*s transcription (including *phoP* itself) and the repression of *prg*s. Diagram adapted from Garcia Vescovi *et al.*, (1996) and Bearson *et al.*, (1998).

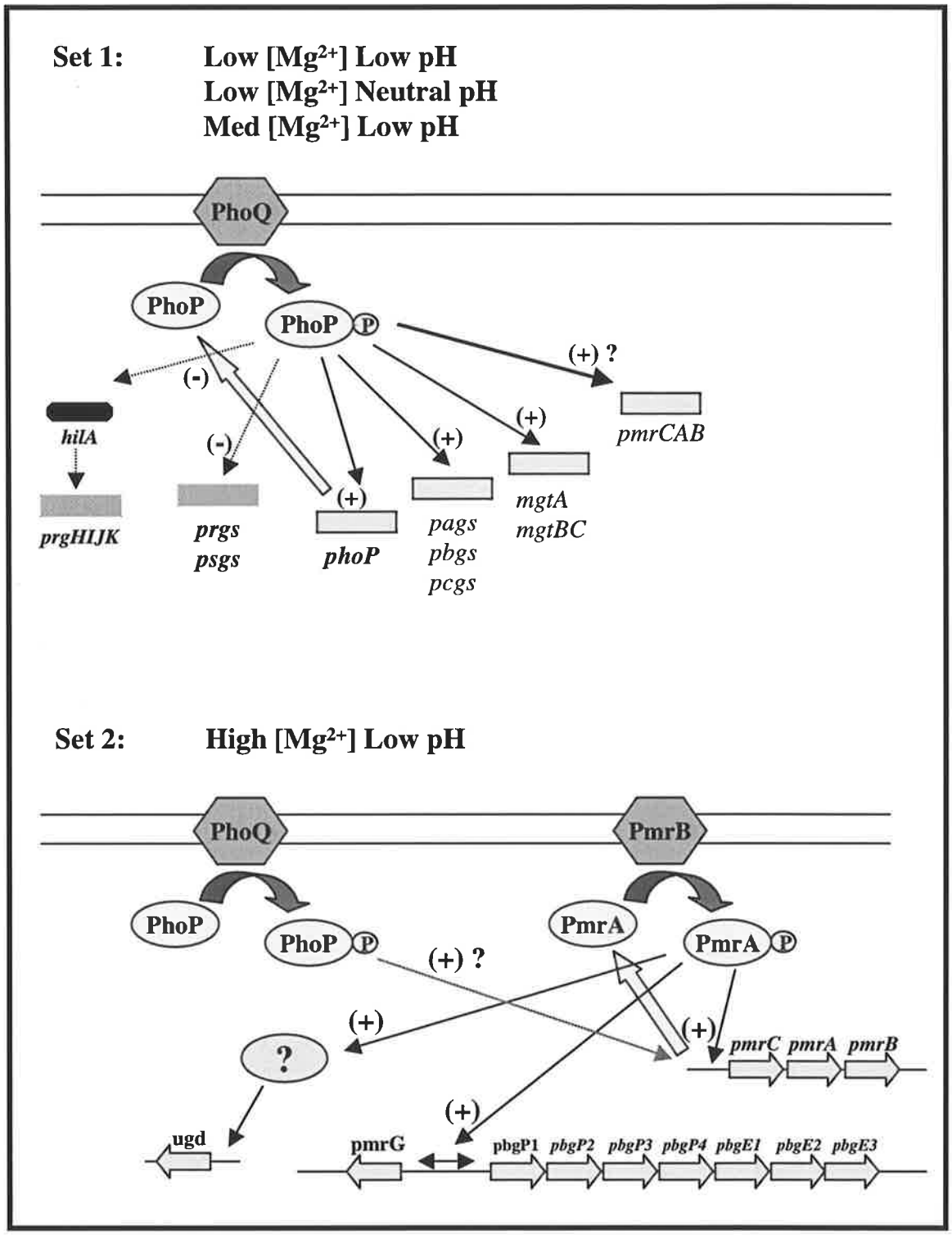
Another environmental factor involved in the regulation of *S. typhimurium* PhoP/Q regulated genes is extracellular pH levels. PhoP was shown to be induced by acid shock (Bearson *et al.*, 1998) while in rich media (high-Mg^{2+}), and it is not apparent whether *phoQ* senses pH independently of Mg^{2+} or whether pH affects the interaction between the PhoQ-Mg^{2+} binding site and Mg^{2+} (Bearson *et al.*, 1998). While acid-induced expression of some

phoP/Q-dependent genes requires PmrA (*pbgP* and *pmrC*), others are PmrA independent (*mgtB* and *phoP*), or require both PhoP and PmrA (*pagA/ugd*). These studies indicate that while *phoP*/Q is regulated by Mg^{2+} levels, low pH may trigger *phoP* activation even in a high Mg^{2+} environment. Two models were proposed by Bearson *et al.*, (1998), one for strictly PhoP/Q regulated genes and the other for genes needing both PhoP/Q and PmrA. These models are represented in Figure 1.5.

Both sets of *S. typhimurium* PhoP/Q regulated genes are affected by extracellular [Mg^{2+}] and [H^+] levels, however Set 1 needs only phorphorylated PhoP (PhoP-P) for induction/repression and Set 2 needs both PhoP-P and phosphorylated PmrA (PmrA-P). The scenario involving high Mg^{2+} and alkaline (high) pH, is not shown in Figure 1.5; this would result in low levels of PhoP-P, upregulation of *prg* genes and down regulation of *pag* genes. It was proposed that the amount of phosphorylated PhoP (PhoP-P) varies in response to different levels of Mg^{2+} sensed by PhoQ. The levels of Mg^{2+} could also be affected by higher or lower H^+ concentrations, therefore affecting the levels of phosphorylated PhoP (PhoP-P). Different PhoP/Q regulated genes respond to different levels of PhoP-P, such that genes that only need a little PhoP-P for induction/repression, may respond to either low pH or low Mg^{2+} environmental conditions. PhoP/Q regulated genes requiring high amounts of PhoP-P are only induced under extremely low Mg2+ concentrations at neutral or low pH, or more moderate Mg^{2+} levels in a low pH environment (Bearson *et al.*, 1998).

The second set involves the PmrA/B operon and PmrA/B regulated genes. The second PmrA/B promoter (P2) has been shown to be upregulated by PmrA/B expression (Gunn & Miller, 1996) and repressed by high levels of Mg^{2+} and Ca^{2+} in the presence of wildtype PhoP/Q (Soncini & Groisman, 1996). Other PmrA activated genes (*pbgPE/pmrF*, *pmrC* and *ugd*) have also been shown to be responsive to decreased levels of Mg^{2+} and Ca^{2+} in the presence of wildtype PhoP/Q as well as being activated by mild acid pH in a PhoP/Q independent manner (Wosten & Groisman, 1999).

Figure 1.5: Schematic model of *Salmonella phoP/Q* gene regulation (with Mg^{2+} and pH)



This schematic diagram shows the models for Mg^{2+} and pH involvement in regulation of *S. typhimurium phoP/Q* regulated genes. The actual details of the amplification responses are outlined in the text. Solid arrows represent induction of genes and dashed lines represent repression. This diagram is adapted from Wosten & Groisman, (1999).

Therefore at alkaline (high) pH, the activation of PmrA/B regulated genes is dependent on PhoP/Q and controlled by the levels of Mg^{2+}, but in acidic (low pH) conditions, the regulation of the PmrA/B regulated genes may become PhoP/Q-independent (stimulated by H^+ only) or be reliant on very small amounts of PhoP-P to induce the PmrA/B system. Induction of the PmrA/B system would then result in increased production of PmrA-P, which activates the *pmrA/B* locus, amplifying the pH signal and initiating the regulatory cascade (Bearson *et al.*, 1998; Wosten & Groisman, 1999). It is not yet known how PhoP/Q activates PmrA/B in low Mg^{2+} conditions (indicated by '?' in Figure 1.5, Set 1 and Set 2), *phoQ* carried on a plasmid under the control of an inducible promoter could not restore a PmrA regulated gene expression in a *phoP* null strain, indicating that PmrA is not directly phosphorylated by PhoQ (Soncini & Groisman, 1996).

1.10.1 *In vivo* expressed PhoP/Q regulated genes

A genetic system IVET (*in vivo* expression technology) has been used to identify *Salmonella* genes that are expressed *in vivo* (Mahan *et al.*, 1993A; Mahan *et al.*, 1993B). Many of the isolated genes have no known function and show no significant homology to entries in the DNA databases but some of the isolated genes were shown to be PhoP/Q regulated. These genes included: *phoP*, *pmrB*, *spvB*, *mgtA*, *mgtB* and previously unidentified PhoP/Q regulated genes: *ivi*VI-A (an adhesin like *pag* gene) *ivi*XVI (an aldehyde dehydrogenase-like *pag*) and one *prg*: *ivi*XVII [*pdu*] (involved with 1,2-propanediol utilization) (Table 1.1a and 1.1b); (Heithoff *et al.*, 1999A; Heithoff *et al.*, 1997). The seven *S. typhimurium pag*s were shown to be induced by low pH and low Mg^{2+}, correlating with activation by the PhoP/Q system and all were induced (though at different levels) upon entry into mammalian cell lines (Heithoff *et al.*, 1999A). The *prg ivi*XVII (*pdu*) gene was found to respond to increased iron levels in the absence of *phoP* (Heithoff *et al.*, 1999A). The PhoP/Q

regulated *in vivo* expressed genes were recovered from sites after intragastric and intraperitoneal infections, indicating that *phoP/Q* has a role at both early and late stages of infection (Heithoff *et al.*, 1999A; Heithoff *et al.*, 1997). The isolation of many *S. typhimurium phoP/Q* regulated *ivi* genes correlates with the fact that PhoP/Q is a global regulator in *Salmonella* and plays an important role in pathogenesis.

1.11 Vaccine studies

With the increase in recent years of multi-drug resistant *Salmonella* strains (Section 1.6), *Salmonella* vaccine development has become increasingly more important. Avirulent *Salmonella* strains have also been widely advocated as carrier strains for antigens from other enteric pathogens and other sources (such as viruses), as these live attenuated strains can be administered orally, and induce significant levels of mucosal immunity as well as humoral and cellular immune responses (Cárdenas & Clements, 1992; Curtiss *et al.*, 1993). Many publications and reviews on this topic have been presented over recent years (Cárdenas & Clements, 1992; Dougan, 1994; Hackett, 1990; Sirard *et al.*, 1999), and due to space limitations and the fact that this thesis is concerned with *S. typhi* and *Salmonella* PhoP/Q regulated genes in general, only the current *S. typhi* vaccines and the potential of *Salmonella phoP/Q* mutants as candidate vaccine and antigen-carrier strains will be presented here.

1.11.1 Killed bacteria and Vi-antigen typhoid vaccines

Killed vaccines against typhoid fever given orally have been shown to give no side affects but induce only low levels of circulating and secretory antibodies and give insignificant levels of protection against challenge (Germanier, 1984), indicating that either live or protein (antigen) vaccines are a better strategy against typhoid fever infection.

Many *S. typhi* strains produce a capsular polysaccharide called the Vi antigen which is highly immunogenic (Robbins & Robbins, 1984), and strains producing the Vi antigen have been shown to be more virulent than Vi-negative strains in human volunteers (Hornick *et al.*, 1970). The Vi antigen is encoded by two chromosomal loci in *S. typhi*, *viaA* and *viaB* (Johnson *et al.*, 1965). The ViaA region encodes the transcriptional regulatory gene *rcs* and Vi capsular expression is controlled by the two component regulatory systems RcsB/RcsC and OmpR/EnvZ and by the positive regulator TviA (Houng *et al.*, 1992; Pickard *et al.*, 1994; Virlogeux *et al.*, 1996). The ViaB region was found to contain 11 ORF, three of which encode Vi antigen structural genes (*vipA*, *vipB* and *vipC*) and five encoding genes specific for Vi antigen export (*vexA*, *vexB*, *vexC*, *vexD*, *vexE*) (Hashimoto *et al.*, 1993). Although use of the Vi antigen as a vaccine confers 65-70% protection (similar to the *S. typhi* Ty21a vaccine), it is limited by age-related immunogenicity (doesn't protect against typhoid fever in infants and young children) and the immunogenicity is T-cell independent (re-injection at any age does not induce a booster effect) (Keitel *et al.*, 1994; Landy, 1954). Therefore, conjugate-Vi protein vaccines have been developed which have been shown to induce higher and longer-lasting levels of antibodies than Vi alone in adults and are being evaluated in children (Kossaczka *et al.*, 1997). The other major problem with the use of the Vi antigen as a vaccine is, although *S. typhi* Vi-positive strains may be more virulent, there are *S. typhi* Vi-negative strains which can cause typhoid fever and this vaccine offers no protection against these organisms.

1.11.2 *S. typhi* live oral vaccines

The development of an effective vaccine for *S. typhi* has been limited by the lack of good animal models due to the host specificity of *S. typhi*. Studies with avirulent *S. typhimurium* strains with mutations affecting virulence such as *galE* (defined galactose epimerase-less),

Δ*aroC* Δ*aroD* (aromatic pathway mutations) or the Δ*cya* Δ*crp* (cyclic AMP pathway) gave mutants which attenuated virulence in mice. However, it was found that although analogous *S. typhi* strains were highly immunogenic in humans, they were also reactogenic at high doses (although the Δ*aroC* Δ*aroD* strain was satisfactory at low doses)(Hone *et al.*, 1988; Tacket *et al.*, 1992).

The only live attenuated *S. typhi* vaccine currently available is the galactose epimerase-deficient (*galE*) mutant designated Ty21a. The *S. typhi* Ty21a strain was developed by mutatgenesis of *S. typhi* Ty2 with the alkylating agent nitrosoguanidine (Germainier & Fürer, 1975) and is genetically undefined but known to be Vi-negative and stable (no reversions have ever been noted). *S. typhi* Ty21a produces rough LPS when grown without galactose (Germainier & Fürer, 1975), and has been shown to be an *rpoS* mutant (Robbe-Saule *et al.*, 1995). Two preparations of live Ty21a vaccine are commercially available, an enteric-coated capsule and liquid formation. The liquid preparation was found to be superior in a recent field trial; three doses (every other day schedule) showed that the liquid gave 77% protection over three years and 78% over five years of follow up (compared to the capsule: 67% protection over three years and 62% protection over seven years of follow up) (Levine *et al.*, 1999). Despite this efficiency, groups worldwide are continually looking for a better vaccine strain, which can be given as a single dose vaccine, has defined mutations and confers greater protection and a longer immune status. The *S. typhi* Ty21a strain has also been used with varying success as a carrier strain for heterologous antigens from *Shigella sonnei* and *S. flexneri* (Formal *et al.*, 1981; Tramont *et al.*, 1984), enteropathogenic *E. coli* (Clemens & El-Morshidy, 1984) and *Vibrio cholerae* (Tacket *et al.*, 1990).

1.12 *Salmonella* and PhoP/Q mutants as vaccine strains

Potential live, attenuated *Salmonella* vaccines include *Salmonella* strains with mutations in the *phoP*/Q locus. *S. typhimurium* PhoP$^-$ mutants were found to be avirulent in mice (Fields *et al.*, 1986A) and produce a protective immune response in mice (Galán & Curtiss, 1989B; Miller *et al.*, 1990). *S. typhimurium* PhoP^c mutant strains are also very effective as live vaccines in the mouse typhoid fever model with as few as 15 *S. typhimurium* PhoP^c bacteria protecting mice against challenge with virulent organisms. The protectiveness of the PhoP^c bacteria indicates that *phoP*/Q activated gene (*pag*) products are important antigens in mouse typhoid fever (Miller *et al.*, 1993). Although potentially a good vaccine, the *phoP*^{c} mutation is due to a single aa change in PhoQ (Gunn *et al.*, 1996) and can revert to a wildtype phenotype during infection therefore limiting its vaccine potential (Miller *et al.*, 1993). Mutations in *phoP*/Q regulated genes such as *prgH* (affecting invasion) in PhoP$^-$ strains were shown to further attenuate virulence compared to PhoP$^-$ strains, and it has been proposed that potentially foreign antigens could be expressed from within *pag* loci chromosomal positions (such as *pagC* which are downregulated in PhoP$^-$ strains but not completely turned off) rather than other loci such as *aroA*, to limit overattenuation of the vaccine strains (Miller *et al.*, 1993). A recent study which used an attenutated *S. typhimurium ΔaroAD* mutant to carry a plasmid construct with the C fragment of tetanus toxin under the control of the *pagC* promoter found that stable, highly immunogenic, high-level expression of the antigen was found, indicating that *phoP/Q* regulated gene promoters may be useful for *in vivo* expression of heterologous antigens (Dunstan *et al.*, 1999). The *S. typhi phoP*/Q, *pagC* and *prgH* genes are highly homologous to *S. typhimurium* (Garcia Vescovi *et al.*, 1997) and a *phoP*/Q deleted *S. typhi* Ty2 (Ty800) has been shown to be a safe and immunogenic typhoid vaccine in humans which even at high doses is not reactogenic (Hohmann *et al.*, 1996A). Studies with an Δ*aroA* Δ*phoP/Q* deleted strain showed it to be highly attenuated and poorly immunogenic, and

therefore over-attenuated for used as a vaccine (Hohmann *et al.*, 1996B). These results are indicate that Ty800 is an extremely promising single-dose oral typhoid vaccine candidate, as high doses were not reactogenic, lower doses were immunogenic and no bacteraemia occurred. Large numbers of vaccine-specific IgA-secreting cells were also detected, indicating the stimulation of mucosal immunity and potential capability for delivering heterologous antigens (possibly produced from foreign genes inserted into deleted *phoP*/Q regulated gene chromosomal locations) to the gastrointestinal immune system (Hohmann *et al.*, 1996A). Processing and presentation of these heterologous antigens is also likely to increased in a $PhoP^-$ background vaccine strain (compared to $PhoP^+$ strains), as *phoP/Q* activated genes have been shown to reduce normal macrophage antigen processing and presentation (Section 1.9.9) (Wick *et al.*, 1995).

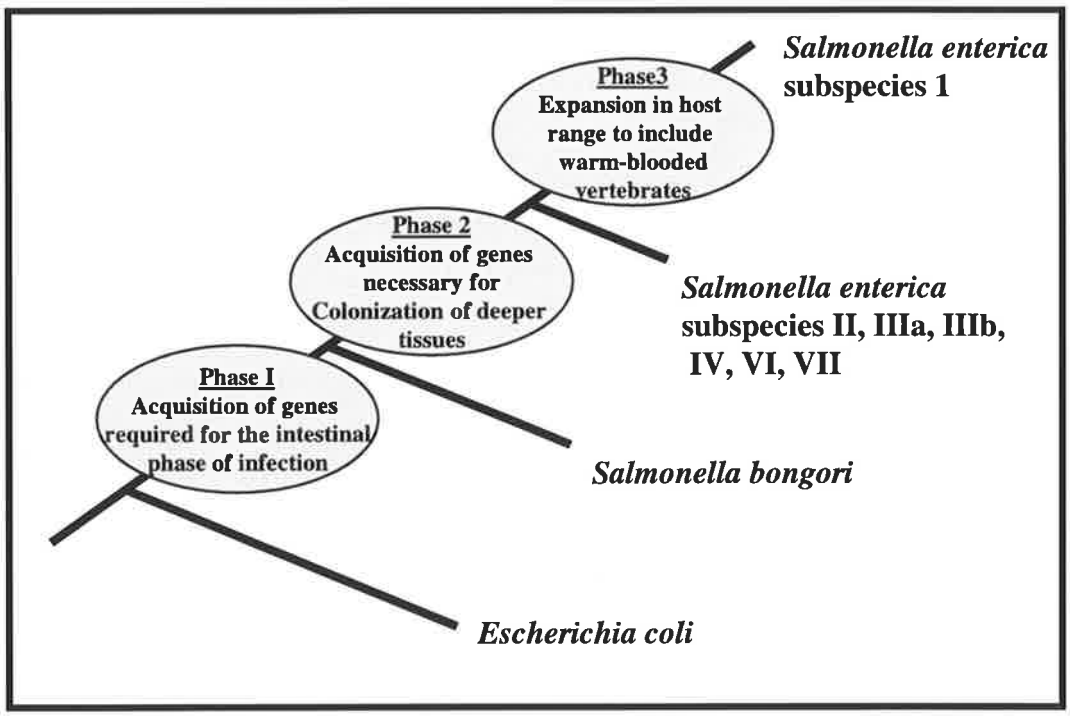
1.13 Genetic determinants of *Salmonella* host specificity

Salmonella serotypes differ greatly in their host range and degree of host adaptation. It has been suggested that the genus *Salmonella* evolved and obtained virulence phenotypes in three phases (Figure 1.6) (Bäumler, 1997). The first phase involved acquisition of the *Salmonella* pathogenicity island I (SPI-1) (Figure 1.1), encoding virulence factors (such as invasion) used by all *Salmonella* serotypes during the intestinal phase of infection (Galán, 1996; Mills *et al.*, 1995), induction of neutrophil recruitment (Galyov *et al.*, 1997; McCormick *et al.*, 1995) and secretion of intestinal fluid (Galyov *et al.*, 1997). The second phase bought about two distinct lineages of *Salmonella*: *S. enterica* and *S. bongori* (Le Minor & Popoff, 1987; Reeves *et al.*, 1989) and the acquisition of a second pathogenicity island (SPI-2) (Figure 1.1; Figure 1.6) in *S. enterica* serotypes, contributing to systemic infection and pathogenesis (Hensel *et al.*, 1997; Ochman & Groisman, 1996). Finally the lineage of *S. enterica* was thought to have branched into 6 groups with subgroup 1 containing most of the

pathogenic species for warm-blooded vertebrates, and most of the other subgroups are generally isolated from reptiles (Farmer *et al.*, 1984).

The ability to cause systemic disease in a particular host is likely to be due to a complex phenotype caused by many different genes. Subtractive hybridisation recently has shown that 20% of the genome of *S. typhimurium* is not present in *S. typhi* and vice versa (Lan & Reeves, 1996), and as the *S. typhimurium* genome can be estimated to contain approximately 4,400 genes, this means serovar specific DNA may encode up to 880 genes, some or all of which may be involved with host-range (Bäumler *et al.*, 1998).

Figure 1.6: Model for the evolution of virulence in the genus *Salmonella*



This figure shows the three phases in which virulence evolved in the genus *Salmonella* since its divergence from the *E. coli* lineage. The phylogenetic tree is not drawn to scale. This diagram was based on Fig 1 in Bäumler *et al.*, (1998).

Studies on transepithelial signalling to neutrophils by *Salmonellae* showed that the host restricted typhoid and paratyphoid causing strains *S. typhi* and *S. paratyphi* failed to induce polymorphonuclear leukocyte (PMN) migration across the epithelial lining of the intestine and

do not cause gastroenteriditis in humans (McCormick *et al.*, 1995), indicating that the lack of some genes and signalling mechanisms may also be involved in host specificity.

Different cell adhesin molecules may also be involved in host specificity of different *Salmonella* strains. *S. typhimurium* has been found to contain many different fimbrial operons compared to *S. typhi* and vice versa (Section 1.8.2). These fimbriae may be involved in binding to different cell receptors. For example, *S. typhi* has been shown to use the cystic fibrosis transmembrane conductance regulator (CFTR) for entry into epithelial cells but *S. typhimurium* does not (Pier *et al.*, 1998), and the SEF14 fimbriae which are encoded and expressed in *S. enteriditis* and *S. typhi* but not *S. typhimurium* have been shown to be involved in uptake and survival in macrophages (Edwards *et al.*, 2000).

It has been found that host specificity of *Salmonella* infections in chickens and mice is expressed primarily at the reticuloendothelial system *in vivo* (Barrow *et al.*, 1994). Most of the host-specific *Salmonella* serotypes were able to colonise and invade host tissues at the same level but showed a difference in ability to survive and multiply in the visceral (particularly spleen and liver) organs (Barrow *et al.*, 1994). A major part of the reticuloendothelial immune defence system is the lymph node tissues containing phagocytic cells. It has been proposed that *Salmonella* serotypes which infect warm-blooded vertebrates encounter macrophages in the intestinal mucosa of mammals, and the bacteria must have evolved and acquired resources to be able to breach these defences in order to cause a systemic infection (Bäumler *et al.*, 1998). Correlating with this theory, it has been shown that the human-adapted *S. typhi* species are able to survive in human macrophages but not murine macrophages (Ishibashi & Arai, 1996; Vladoianu *et al.*, 1990) and *S. typhi* but not *S. typhimurium*, resisted intracellular killing by human monocyte-derived macrophages (Ishibashi & Arai, 1996). A recent study showed that *S. typhimurium* strains demonstrated enhanced survival in murine peritoneal macrophages, compared with a significant decline in viable *S. typhi* bacteria. Conversely, *S. typhi* strains were able to survive at a level up to 100-

fold higher in elutriated human macrophages. The same study also showed that *S. typhi* strains caused significantly less macrophage apoptosis than *S. typhimurium*, indicating that host specificity may be linked not only with survival in macrophages but the elicited damage caused to a specific host's macrophage cells (Schwan *et al.*, 2000). This also correlates with the proposal that *Salmonella* use liver and spleen macrophages as a "safe-haven", allowing intracellular replication, persistence of bacteria and protection from host defences such as neutrophils (Edwards *et al.*, 2000; Nnalue *et al.*, 1992). Spacious phagosomes formation within mouse macrophages also correlates with *Salmonella* serotype pathogenicity and host susceptibility as *S. typhimurium* strains pathogenic for mice were able to hold open SP in murine macrophages (correlating with survival in macrophages), whereas serotypes non-pathogenic for mice (including *S. typhi*) had SP's that shrank quickly after entry (Alpuche Aranda *et al.*, 1995). Spacious phagosomes formation has also been correlated to the bacterial method of entry, again indicating a role for adhesins such as SEF14 fimbriae (Alpuche Aranda *et al.*, 1995).

The lipid A part of *Salmonella* LPS has been found to be responsible for stimulation of cytokine release in the host and therefore host immune responses (Takada & Kotani, 1992). Structural modifications of the Lipid A molecule by such as 4AA substitution and additions of fatty acid chains such as 2-hydroxy-myristate have been shown to affect host cytokine expression (Guo *et al.*, 1997). Recently, a transmembrane component of the LPS receptor molecule on macrophages, the Toll-like receptor 4 (Tlr4) has been shown to respond differently to deactylated forms of Lipid A depending on the species origin of Tlr4 (Beutler, 2000; Golenbock *et al.*, 1991; Poltorak *et al.*, 2000). This implies that modifications to the lipid A molecule by bacteria may influence the host immune response and that these responses in different hosts may be involved with bacterial host specificity.

As the *S. typhimurium phoP/Q* genes have been shown to be involved in SP formation in macrophages (Alpuche Aranda *et al.*, 1994), bacterial intramacrophage survival (Fields *et al.*,

1989; Groisman *et al.*, 1989; Miller *et al.*, 1989), and lipid A modifications (Gunn *et al.*, 1998B; Guo *et al.*, 1997; Guo *et al.*, 1998), a reasonable hypothesis is that the *Salmonella phoP/Q* operon may regulate genes involved with host specificity.

1.14 Previous Studies

Previous studies have mainly concentrated on isolating and characterising *S. typhimurium* PhoP/Q regulated genes. Due to the homology of the PhoP/Q locus in *S. typhi* to *S. typhimurium*, a study was undertaken which involved the isolation and characterisation of PhoP/Q genes in *S. typhi* (Baker, 1993). *S. typhi* Ty2 *phoP12* (PhoP$^-$) and *phoP24* , *purB*::Tn*10* (PhoP^c, *purB*::Tn*10*) mutants, and *S. typhimurium* C5 *phoP12* (PhoP$^-$) and *phoP24* (PhoP^c) strains were constructed. The PhoP$^-$ strains (*S. typhimurium* - RMA1010, *S. typhi* - RMA1030) were mutagenised with a P22 lysate propagated on *S. typhimurium* TT10288 to produce random Mu*dJ* insertions. Kanamycin (Kan) resistant transductants, were selected on MacConkey/lactose agar containing Kan. Colonies which were Lac$^+$ (having Mu*dJ* inserted in a site which had promoter activity) and Lac$^-$ (having Mu*dJ* inserted at a site with little or no promoter activity) were picked at random, patched on a grid array and mated on plates with *E. coli* RMA1049 to introduce the plasmid pRMSB3 containing the *phoP*^{c} allele.

Approximately 4500 Lac$^-$ and 2000 Lac$^+$ *S. typhi and S. typhimurium* colonies were screened. One of the *S. typhimurium* Lac$^+$ colonies showed down-regulation (reduced β-galactosidase activity) indicating a *prg*::Mu*dJ* mutation. Amongst the *S. typhi* Lac$^-$ colonies, 5 *pag*::Mu*dJ* insertion mutants were detected using MacConkey agar indicator plates. Due to the low recovery of *pag/prg*::Mu*dJ* mutants, it was decided to use LA plates containing X-gal instead of MacConkey agar, and in a preliminary experiment one *S. typhi prg*::Mu*dJ* insertion mutant was found. Southern hybridization analysis was undertaken using *Bgl*II digested

chromosomal DNA derived from the mutant strains (*S. typhi pag*::Mu*dJ* insertions [*pqaA*::Mu*dJ*, *pqaB*::Mu*dJ* *pqaC*:Mu*dJ* *pqaD*::Mu*dJ* *pqaE*::Mu*dJ*], *S. typhi prg*::Mu*dJ* [*pqrB*::Mu*dJ*] insertion, and the *S. typhimurium prg*::Mu*dJ* [*pqaA*::Mu*dJ*], which was hybridised with a DIG-11-dUTP labelled *lacZ* gene. The results indicated that the mutants had single, random insertions of Mu*dJ*, and that the insertions were in different sized restriction fragments (Baker, 1993). β-galactosidase assays were also conducted with the *S. typhi* and *S. typhimurium* Mu*dJ* insertion mutants in the PhoP$^-$ background complemented with pRMSB2 (*phoP/Q*$^+$) and pRMSB3 (*phoP/Q*^{c}) to examine regulation of expression, and all appeared to be regulated by PhoP/Q by this assay (Baker, 1993). A small fragment of the *S. typhi pqaA* gene was also isolated (Baker, 1993). The mutants were transferred into *S. typhi* Ty2 and PhoP^c strains by Vi phage transduction and further analysis of the mutants involved assaying for cationic peptide sensitivity. *S. typhi* Ty2 was found to be sensitive to a lower concentration of the antimicrobial peptide melittin (7.5 μg/ml) than concentrations reported for *S. typhimurium* (20 μg/ml; Groisman *et al.*, 1992B) and two of the *S. typhi* mutants (*pqaA* and *pqaB*) were shown to be sensitive to the antimicrobial peptide melittin (Daniels, 1994).

1.15 Aims of project

The aims of this project were to:

Compare PhoP/Q regulated genes in *S. typhi* and *S. typhimurium*

Identify previously isolated *S. typhi* and *S. typhimurium* PhoP/Q regulated genes

Analyse invasion and growth of *Salmonella* wildtype, *phoP/Q* mutant strains and *S. typhi pqa/pqr*::Mu*dJ* insertion mutants in a tissue culture cell model

Isolate and characterise the *S. typhi pqaA* and *pqaB* genes

Chapter 2

Materials and Methods

2.1 Bacterial strains and plasmids

E. coli, S. typhimurium and *S. typhi* strains used in this study are listed in Tables 2.1, 2.2 and 2.3 respectively. The *S. typhimurium* C5, PhoP$^-$ and PhoP^c strains and all the *S. typhi* strains (Ty2, PhoP$^-$, PhoP^c and *pqa/pqr*::Mu*dJ* fusion strains) were serotyped by the *Salmonella* Reference Laboratory (IMVS, Adelaide) and found to be identical to the wildtype strains. Plasmids constructed and the cloning vectors used during this study are listed in Tables 2.4.

2.2 Bacterial growth media

2.2.1 Liquid growth media

Bacterial cultivation used the following media. General growth media was Oxoid, No 2 Nutrient Broth (NB), which consisted of Oxoid-beef extract (3 g/l), Oxoidpeptone (5 g/l) and sodium chloride (NaCl) (5 g/l). Luria-Bertani (LB) broth consisted of Difco Bacto-Tryptone (10 g/l), Difco Bacto Yeast Extract (5 g/l) and NaCl (5 g/l) (Miller, 1972). LBON broth medium (Luria-Bertani medium without NaCl) used for protein overexpression was composed of Bacto-Tryptone (10 g/l), Bacto Yeast Extract (5 g/l) and sodium hydroxide (NaOH) was used to adjust the pH to 7.0 (Bhandari & Gowriskhankar, 1997). MacConkey Broth consisted of Difco MacConkey Lactose Broth (35 g/l).

Table 2.1: *Escherichia coli* strains used in this study

	Escherichia coli K-12 strains	
Strain	Characteristics	Source/Construction
DH5	F⁻, *supE-44*, λ⁻, *thi-1, gyrA-96, rec-A1, end-A1, rel-A1, hsd-R17*(r_k-, m_k+)	Bethesda Research Laboratories USA
DH5α	F-ϕ80, U169, *supE-44*,λ⁻, *thi-1*, lacZΔM15Δ(*lac*ZYA/*argF*), *gyrA-96, rec-A1, end-A1, rel-A1, hsd-R17*(r_k-, m_k+)	Bethesda Research Laboratories USA
E2096	DH5 + pGP1-2	Laboratory collection
GJ1158	*ompT, hsdS, gal, dcm,* ΔmalAp510(Ap510/malP::proUp-T7 RNAP)	Bhandari & Gowrishankar, (1997)
S17-1	*pro, res*⁻, Mod⁺, RP4-2-Tc::Mu-Km::*Tn7*, recA⁻, Tp^R, Sm^R	U. Priefer
RMA1049	S17-1 + pRMSB3	Baker, (1993)
RMA1055	S17-1 + pSUP203	Baker, (1993)
RMA1076	S17-1 + pRMSB1	Baker, (1993)
RMA1082	DH5α + pRMSB10	This study (Section 3.7.2)
RMA1121	DH5α + pRMSB19	This study (Section 5.4.2)
RMA1261	DH5α + pRMSB20	This study (Section 3.8.1)
RMA1262	DH5α + pRMSB21	This study (Section 6.2.1)
RMA1266	DH5α + pRMSB25	This study (Section 3.8.1.2)
RMA1280	DH5α + pRMSB38	This study (Section 5.4.2)
RMA1281	DH5α + pRMSB39	This study (Section 5.4.2)
RMA1282	DH5α + pRMSB40	This study (Section 5.4.2)
RMA1285	DH5α + pRMSB43	This study (Section 5.4.2)
RMA1286	DH5α + pRMSB44	This study (Section 5.4.2)
RMA1287	DH5α + pRMSB45	This study (Section 5.4.2)
RMA1289	DH5α + pRMSB47	This study (Section 5.4.2)
RMA1292	DH5α + pRMSB50	This study (Section 6.2.2)
RMA1295	DH5α + pRMSB53	This study (Section 6.2.2)
RMA1298	DH5α + pRMSB56	This study (Section 6.2.2)
RMA1299	DH5α + pRMSB57	This study (Section 6.2.2)
RMA1300	DH5α + pRMSB58	This study (Section 3.8.2)
RMA1324	DH5α + pRMSB90	This study (Section 5.8.5)
RMA1331	DH5α + pRMSB87	This study (Section 6.9)
RMA1332	DH5α + pRMSB88	This study (Section 5.8.8)
RMA1347	DH5α + pRMSB91	This study (Section 5.8.5)
RMA1349	DH5α + pRMSB92	This study (Section 5.8.5)
RMA1351	DH5α + pRMSB71	This study (Section 5.8.8)
RMA1353	DH5α + pRMSB73	This study (Section 5.8.8)
RMA1354	DH5α + pRMSB74	This study (Section 5.8.8)
RMA1356	DH5α + pRMSB76	This study (Section 6.9)
RMA1359	DH5α + pRMSB79	This study (Section 6.9)
RMA1360	DH5α + pRMSB80	This study (Section 6.9)
RMA1362	DH5α + pRMSB82	This study (Section 5.8.3)
RMA1364	DH5α + pRMSB84	This study (Section 5.8.3)
RMA1365	DH5α + pRMSB85	This study (Section 5.8.3)
RMA1368	GJ1158 + pET11BYZ	This study (Section 5.8.3)
RMA1369	GJ1158 + pRMSB82	This study (Section 5.8.3)
RMA1371	E2096 + pWSK29	This study (Section 5.8.3)
RMA1373	E2096 + pRMSB84	This study (Section 5.8.3)
RMA1374	E2096 + pRMSB85	This study (Section 5.8.3)
RMA1376	E2096 + pRMSB82	This study (Section 5.8.3)
RMA1377	E2096 + pET11BYZ	This study (Section 5.8.3)
RMA1390	GJ1158 + pWSK29	This study (Section 5.8.3)
RMA1392	GJ1158 + pRMSB84	This study (Section 5.8.3)
RMA1393	GJ1158 + pRMSB85	This study (Section 5.8.3)
RMA1403	DH5α + pRMSB93	This study (Section 5.8.5)
RMA1405	DH5α + pRMSB94	This study (Section 5.8.5)
RMA1407	DH5α + pRMSB95	This study (Section 5.8.5)

Table 2.2: *Salmonella typhimurium* strains used in this study

Strain	*Salmonella typhimurium* strains Characteristics	Source/Construction
C5	wildtype	Laboratory Collection
EX730	LT2	P. Reeves, University of Sydney
EX2000	LT2 *tryC2, metA22, Hl-bnml, H2-enx, fla-66, rpsL12, xyl-404met, E55-l, hsdSA29, ilv-452, hsdSB121,leu-3121, galE856*	R. Morona, University of Adelaide
JSG421	*pmrA*::Tn10*d*	Gunn *et al.*, (1998B)
P9133	LT2	P. Reeves, University of Sydney
RMA1000	TA2362 *purB*::Tn*10 phoP12*	Baker, (1993)
RMA1004	TA2367 *purB*::Tn*10 phoP24*	Baker, (1993)
RMA1006	EX2000 *purB*::Tn*10 phoP12*	Baker, (1993)
RMA1008	C5 *purB*::Tn*10 phoP12*	Baker, (1993)
RMA1010	C5 PhoP$^-$	Baker, (1993)
RMA1017	EX2000 PhoP^{-+}	Baker *et al.*, (1997)
RMA1022	RMA1010 *purB*::Tn*10 phoP24*	Baker *et al.*, (1997)
RMA1024	C5 *phoP24*, PhoP^c	Baker *et al.*, (1997)
RMA1095	C5 *pqrA*::Mu*dJ*	This study (Section 3.7.1)[a]
RMA1106	RMA1010 *pqrA*::Mu*dJ*	Baker, (1993)
RMA1144	RMA1010 + pSUP203	Baker *et al.*, (1997)
RMA1146	RMA1010 + pRMSB1	Baker *et al.*, (1997)
RMA1148	RMA1010 + pRMSB3	Baker *et al.*, (1997)
TA2362	LT2 *phoP12* (PhoP$^-$)	Kier *et al.*, (1979)
TA2367	LT2 *phoP24* (PhoP^c)	Kier *et al.*, (1979)
RMA1276	RMA1017 + pRMSB34	This study (Section 3.4)
RMA1278	RMA1017 + pRMSB36	This study (Section 3.4)
RMA1382	RMA1017 + pCL1921	This study (Section 3.4)
TT10288	LT2 *hisD*9953::Mu*dJ* *hisA*9944::Mu*dI*	Hughes & Roth, (1988)

[a]Transduction with P22 HT*int*105 propagated on *S. typhimurium* RMA1106 and selected for Km resistance

Table 2.3: *Salmonella typhi* strains used in this study

Strain	*Salmonella typhi* strains Characteristics	Source
RMA1030	Ty2 *phoP12*, PhoP$^-$	Baker *et al.*, (1997)
RMA1032	RMA1030 *purB*::Tn*10 phoP24*	Baker *et al.*, (1997)
RMA1090	Ty2 *phoP24*, PhoP^c	This study (Section 3.3)
RMA1102	RMA1030 *pqaC*::MudJ	Baker *et al.*, (1997)
RMA1104	RMA1030 *pqaE*::MudJ	Baker *et al.*, (1997)
RMA1110	RMA1030 *pqaA*::MudJ	Baker *et al.*, (1997)
RMA1112	RMA1030 *pqaD*::MudJ	Baker *et al.*, (1997)
RMA1116	RMA1030 *pqrB*::MudJ	Baker *et al.*, (1997)
RMA1126	RMA1030 *pqaB*::MudJ	Baker *et al.*, (1997)
RMA1164	Ty2 + pSUP203	This study Section 4.5.3
RMA1166	Ty2 + pRMSB1	This study (Section 4.5.3)
RMA1168	RMA1030 + pSUP203	This study (Section 3.6.3)
RMA1170	RMA1030 + pRMSB1	This study (Section 3.6.3)
RMA1174	RMA1182 + pSUP203	This study (Section 3.7.3)
RMA1176	RMA1182 + pRMSB3	This study (Section 3.7.3)
RMA1180	Ty2 *pqrA*::MudJ	This study (Section 3.7.1)[a]
RMA1182	RMA1030 *pqrA*::MudJ	This study (Section 3.7.1)[a]
RMA1184	RMA1090 *pqrA*::MudJ	This study (Section 3.7.1)[a]
RMA1200	RMA1126 + pSUP203	Baker *et al.*, (1997)
RMA1202	RMA1126 + pRMSB3	Baker *et al.*, (1997)
RMA1213	RMA1102 + pSUP203	Baker *et al.*, (1997)
RMA1214	RMA1102 + pRMSB3	Baker *et al.*, (1997)
RMA1216	RMA1104 + pSUP203	Baker *et al.*, (1997)
RMA1218	RMA1104 + pRMSB3	Baker *et al.*, (1997)
RMA1220	RMA1110 + pSUP203	Baker *et al.*, (1997)
RMA1222	RMA1110 + pRMSB3	Baker *et al.*, (1997)
RMA1228	RMA1112 + pSUP203	Baker *et al.*, (1997)
RMA1230	RMA1112 + pRMSB3	Baker *et al.*, (1997)
RMA1232	RMA1116 + pSUP203	Baker *et al.*, (1997)
RMA1234	RMA1116 + pRMSB3	Baker *et al.*, (1997)
RMA1242	RMA1102 + pRMSB1	Baker *et al.*, (1997)
RMA1244	RMA1104 + pRMSB1	Baker *et al.*, (1997)
RMA1250	RMA1110 + pRMSB1	Baker *et al.*, (1997)
RMA1252	RMA1112 + pRMSB1	Baker *et al.*, (1997)
RMA1256	RMA1116 + pRMSB1	Baker *et al.*, (1997)
RMA1258	RMA1126 + pRMSB1	Baker *et al.*, (1997)
RMA1307	RMA2326 *pmrA*::Tn10*d*	This study (Section 6.6)[b]
RMA1309	RMA2526 *pmrA*::Tn10*d*	This study (Section 6.6)[b]
RMA1325	RMA1030 + pCL1921	This study (Section 3.4)
RMA1327	RMA1030 + pRMSB34	This study (Section 3.4)
RMA1329	RMA1030 + pRMSB36	This study (Section 3.4)
RMA1333	Ty2 + pRMSB87	This study (Section 6.9)
RMA1338	RMA2510 *pmrA*::Tn*10*	This study (Section 5.8.1)[b]
RMA1340	RMA2512 *pmrA*::Tn*10*	This study (Section 3.9)[b]
RMA1343	RMA2516 *pmrA*::Tn*10*	This study (Section 3.9)[b]
RMA1344	RMA1184 *pmrA*::Tn*10*	This study (Section 3.9)[b]
RMA1395	Ty2 + pRMSB88	This study (Section 5.8.8)
RMA1396	Ty2 + pRMSB88	This study (Section 5.8.8)
RMA1398	RMA1090 + pRMSB87	This study (Section 6.9)
RMA1399	Ty2 *pqaB*::Kan	This study (Section 6.9)
RMA1400	Ty2 *pqaB*::Kan	This study (Section 6.9)
RMA1401	RMA1090 *pqaB*::Kan	This study (Section 6.9)
RMA1402	RMA1090 *pqaB*::Kan	This study (Section 6.9)
RMA2302	Ty2 *pqaC*::MudJ	Baker *et al.*, (1997)
RMA2304	Ty2 *pqaA*::MudJ	Baker *et al.*, (1997)
RMA2310	Ty2 *pqaA*::MudJ	Baker *et al.*, (1997)
RMA2312	Ty2 *pqaD*::MudJ	Baker *et al.*, (1997)
RMA2316	Ty2 *pqrB*::MudJ	Baker *et al.*, (1997)
RMA2326	Ty2 *pqaB*::MudJ	Baker *et al.*, (1997)
RMA2502	RMA1090 *pqaC*::MudJ	Baker *et al.*, (1997)
RMA2504	RMA1090 *pqaE*::MudJ	Baker *et al.*, (1997)
RMA2510	RMA1090 *pqaA*::MudJ	Baker *et al.*, (1997)
RMA2512	RMA1090 *pqaD*::MudJ	Baker *et al.*, (1997)
RMA2516	RMA1090 *pqrB*::MudJ	Baker *et al.*, (1997)
RMA2526	RMA1090 *pqaB*::MudJ	Baker *et al.*, (1997)
Ty2	wildtype	Hone *et al.*, 1988

[a]Transduction with P22 HT*int*105 propagated on *S. typhimurium* RMA1106 and selected for Kan resistance
[b]Transduction with P22 HT*int*105 propagated on *S. typhimurium* JSG421 and selected for Tet resistance

Table 2.4: Plasmid and cloning vectors used in this study

Plasmid	Characteristics	Source/Reference
pBC- KS$^+$	Cml^R derivative of pBluescriptKS$^+$	Stratagene
pBluescript KS$^+$	Amp^R	Stratagene
pBluescript SK$^-$	Amp^R	Stratagene
pCACTUS	suicide vector with temperature sensistive replicon, Cml^R	Laboratory collection, C. Clark
pCB267	lacZ	Schneider & Beck, (1986)
pCL1921	Spec^R	Lerner & Inouye, (1990)
pET11BYZ	overexpression vector, Amp^R	Daniels, *et al.*, (1998)
pGEM-T	Amp^R	Promega
pGP1-2	T7 RNA polymerase, Kan^R	Tabor & Richardson, 1985)
pSB315	*aphT* (*kan* lacking terminator) cartridge, Kan^R	Galán *et al*, (1992)
pWKS130	low copy number pBluescript KS$^+$ derivative, Amp^R	Wang & Kushner, (1991)
pWSK129	low copy number pBluescript SK$^+$ derivative, Amp^R	Wang & Kushner, (1991)
pSUP203	Amp^R, Cml^R, Tet^R	Simon *et al.*, (1983)
pUTKm	*kan* (from Tn5)	Herrero *et al.*, (1990)
pRMCD28	*E. coli 'phoA* in pWSK29	Daniels, *et al.*, (1998)
pRMCD70	*E. coli 'lacZ* in pWSK29	Daniels, *et al.*, (1998)
pRMSB1	*phoP/Q*$^+$ in pSUP203	Baker *et al.*, 1997
pRMSB2	*phoP/Q*$^+$ in pBluescript KS$^+$	Baker *et al.*, 1997
pRMSB3	*phoP/Q*^{c} in pSUP203	Baker *et al.*, 1997
pRMSB4	*phoP/Q*^{c} in pBluescript KS$^+$	Baker *et al.*, 1997
pRMSB5	0.6 kb *pqaA*:MudJ fragment in pBluescriptKS$^+$	Baker, (1993)
pRMSB10	Km in pCB267	This study (Section 3.7.2)
pRMSB19	2.92 kb *Hind*III/*Eco*RV *pqaA* fragment in pBluescript KS$^+$	This study (Section 5.4.2)
pRMSB20	1.25 kb *Mlu*I *pqrA* IPCR fragment in pGEMT	This study (Section 3.8.1.3)
PRMSB21	0.65 kb *Mlu*I *pqaB* IPCR fragment	This study (Section 6.2.1)
pRMSB25	0.95 kb *Mlu*I *pqa*D IPCR fragment in pGEMT	This study (Section 3.8.1.2)
pRMSB34	*phoP/Q*$^+$ in pCL1921	This study (Section 3.4)
pRMSB36	*phoP/Q*$^+$ in pCL1921	This study (Section 3.4)
pRMSB38	2.73 kb pRMSB19 nested deletion	This study (Section 5.4.2)
pRMSB39	2.27 kb pRMSB19 nested deletion	This study (Section 5.4.2)
pRMSB40	2.05 kb pRMSB19 nested deletion	This study (Section 5.4.2)
pRMSB43	1.79 kb pRMSB19 nested deletion	This study (Section 5.4.2)
pRMSB44	1.55 kb pRMSB19 nested deletion	This study (Section 5.4.2)
pRMSB45	1.25 kb pRMSB19 nested deletion	This study (Section 5.4.2)
pRMSB47	0.70 kb pRMSB19 nested deletion	This study (Section 5.4.2)
pRMSB50	0.93 kb *Hind*III/*Cla*I *pqaB* fragment in pGEMT	This study (Section 6.2.2)
pRMSB53	2.9 kb *Eco*RV *pqaB* IPCR fragment in pGEMT	This study (Section 6.2.2)
pRMSB56	0.87 kb *Apa*I/*Eco*RV fragment from pRMSB53 in pBC	This study (Section 6.2.2)
pRMSB57	1.42 kb *Not*I/*Eco*RV fragment from pRMSB53 in pBC	This study (Section 6.2.2)
pRMSB58	0.84 kb *Hind*III/*Cla*I *pqrB* SSPCR fragment in pGEMT	This study (Section 3.8.2)
pRMSB71	0.65 kb *pqaA* fragment in pBC	This study (Section 5.?)
pRMSB73	0.83 kb *pqaA* fragment in pRMSB71	This study (Section 5.?)
pRMSB74	*aphT/kan Pst*I fragment from pSB315 in pRMSB73 (Cml^R, Kan^R)	This study (Section 5.?)
pRMSB76	0.8 kb *Hinc*II/*Pst*I fragment from pRMSB50 in *Sma*I/*Pst*I pBC	This study (Section 6.9)
pRMSB79	1.1 kb HincII/ClaI fragment from pRMSB57 in *Eco*RV/ClaI pRMSB76	This study (Section 6.9)
pRMSB80	*aphT/kan Pst*I fragment from pSB315 in pRMSB79	This study (Section 6.9)
pRMSB82	1.62 kb *pqaA* in pET11BYZ	This study (Section 5.?)
pRMSB84	1.62 kb *pqaA* in pWKS30	This study (Section 5.?)
pRMSB85	1.62 kb *pqaA* in pWKS30	This study (Section 5.?)
pRMSB87	*Sac*I/*Sal*I *pqaB*::Kan from pRMSB80 in pCACTUS	This study (Section 6.9)
pRMSB88	*Sac*I/*Sal*I *pqaA*::Kan from pRMSB74 in pCACTUS	This study (Section 5.8.8)
pRMSB90	0.65 kb *Xba*I/*Eco*RI *pqaA* fragment in pRMCD70	This study (Section 5.?)
pRMSB91	1.15 kb *Xba*I/*Eco*RI *pqaA* fragment in pRMCD70	This study (Section 5.?)
pRMSB92	1.70 kb *Xba*I/*Eco*RI *pqaA* fragment in pRMCD70	This study (Section 5.?)
pRMSB93	0.65 kb *Xba*I/*Eco*RI *pqaA* fragment in pRMCD28	This study (Section 5.?)
pRMSB94	1.15 kb *Xba*I/*Eco*RI *pqaA* fragment in pRMCD28	This study (Section 5.?)
pRMSB95	1.70 kb *Xba*I/*Eco*RI *pqaA* fragment in pRMCD28	This study (Section 5.?)

M9 minimal media (MM) was made with M9 salts (0.48 M Na_2HPO_4, 22 mM KH_2PO_4, 19 mM NH_4Cl, 8.6 mM NaCl, pH 6.5) and 0.5% (w/v) glucose. Amino acids (cysteine, isoleucine, leucine, lysine, methionine, tryptophan and valine) were added to the MM at a final concentration of 20 μg/ml, vitamin B1 was used at 1 μg/ml and adenine at 20 μg/ml when required. N-Buffer media consisted of N buffer (5 mM KCl, 7.5 mM $(NH_4)_2.SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 0.1 M Tris-HCl pH 7.4) (Snavely *et al.*, 1991), 22 mM glucose, 1% (w/v) casamino acids (Difco), magnesium chloride ($MgCl_2$) (final concentrations 40 or 200 μM), cysteine and tryptophan (20 μg/ml each) and vitamin B1 (1 μg/ml).

2.2.2 Solidified growth media

Solidified growth media used in this study included: Nutrient Agar (NA), which consisted of NB with the addition of 15 g/l Difco Bacto-Agar. Luria Agar (LA) consisted of LB with the addition of Difco Bacto Agar 15 g/l. LBON Agar (LBONA), LBON media with the addition of Difco Bacto Agar, 15 g/l. Minimal media Agar (MMA) consisted of MM with the addition of Difco Bacto Agar 15 g/l. MacConkey lactose media (MacConkey/Lactose agar) consisted of Difco MacConkey Agar (40 g/l) and 0.2% (w/v) lactose. N-Buffer solidified media consisted of N buffer with the addition of 15 g/l agarose (Progen). Ethylenebis(oxyethylenenitrilo)tetra-acetic acid (EGTA) (BDH) and Ethylenediaminetetra-acetic acid (EDTA) (BDH) were added at 0.1M when needed

2.2.3 Solidifed colour indicator and/or selection media

Solidified colour indicator and/or selection media included; X-pho plates: MMA or LA with the addition of 5-bromo-4-chloro-3-indolylphosphate (X-pho) (Roche) freshly dissolved in dimethyl formamide (DMF) at a final concentration of 40 μg/ml, X-gal plates; LA with the

addition of 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (Roche) dissolved in DMF and added at a final concentration of 40 μg/ml. Protamine sulphate plates were LA plus various concentrations of protamine sulfate (Sigma) freshly dissolved in phosphate buffered saline (PBS) and used on the same day they were poured. XLD medium (Oxoid) was used at 53 g/l for selective isolation of *Salmonellae*. ABI plates used for blue/white colour selection consisted of LA containing X-gal (40μg/ml), IPTG (1 mM) and Amp at 100 μg/ml.

2.2.4 Antibiotics

When needed, antibiotics were added to media at the appropriate concentrations: ampicillin (Amp) was added at 50 μg/ml or 100 μg/ml for increased selection; chloramphenicol (Cml), 25 μg/ml (dissolved in 100% ethanol); kanamycin (Kan) 50 μg/ml, spectinomycin (Spec) (40 μg/ml) and tetracycline (Tet) (dissolved in 50% (v/v) ethanol) 10 μg/ml.

2.3 Maintenance of bacterial strains

Bacterial strains in routine use were either stored as a suspension of freshly grown bacteria at -70°C in 1% Difco Bactopeptone, containing 30% (v/v) glycerol, or lyophilized. Strains were streaked from a loopful of glycerol suspension prior to use onto the appropriate solidified media and incubated overnight at 37°C unless otherwise indicated.

Liquid bacterial cultures were grown in 20 ml McCartney bottles at 37°C with moderate aeration unless otherwise stated.

2.4 Chemicals and Reagents

All chemicals and reagents used in this study were Analar grade and will be listed below in alphabetical order followed by the supply company in brackets [].

Acrylamide [Bio-Rad], agarose (High gelling temperature (HGT) and low melting point (LMT)) [Seakem], ammonium persulphate [Bio-Rad], antibiotics (ampicillin and kanamycin sulphate, chloramphenicol, spectinomycin sulphate, tetracycline) [BDH Chemicals, Roche, Sigma]. Antimicrobial agents (dyes and detergents) [Sigma, BDH Chemicals, Glaxo, or Calbiochem], 2'-deoxynucleotides (dNTP's) [Roche] and dithiothreitol (DTT) [Sigma]. Ethanol [BDH Chemicals], ethylene-diamine-tetra-acetic-acid, disodium salt (EDTA) [Ajax Chemicals], ethylene-glycol-bis-(b-amino-ethylether) N, N, N', N'-tetra-acetic-acid (EGTA) [Ajax Chemicals]. Herring sperm DNA [Sigma], mineral oil (Primol 352) [Esso], phenol [BDH Chemicals], propan-2-ol (isoproponol) [BDH Chemicals], sodium dodecyl sulphate (SDS) [BDH Chemicals], sucrose [BDH Chemicals]. TEMED (N,N,N',N'-Tetramethyl-ethylenediamine [Sigma], Tris (Trisma base) [Roche], ultra pure N,N'-methylene bis-acrylamide [BRL], urea [BRL]. Other chemicals were from Ajax, BDH Chemicals or Sigma.

Milli Q water was water purified using a Milli Q water purification system (Millipore Corp.) with a measured resistance to conductivity of 18 MΩ/cm.

2.5 DNA Methods

2.5.1 Isolation of DNA

2.5.1.1 Chromosomal DNA isolation

A rapid method of chromosomal DNA isolation was used for both *E. coli* and *Salmonella* bacterial strains (Christopher Clark, personal communication). The chromosomal DNA was prepared from 10 ml of 16-18 h cultures grown in 20 ml McCartney bottles with aeration in LB. The cultures were centrifuged (5 K, 10 min, IEC Centra 4X centrifuge), resuspended in 3

ml of saline and vortexed with an equal volume of phenol (Tris saturated) for 2 min. After vortexing, the mixture was centrifuged (1.5 K, 5 min) and the aqueous layer transferred to a fresh McCartney bottle containing 3 ml of isopropanol. The DNA was precipitated by rolling the bottle vigorously between ones hands and the spooled DNA washed with 70% (w/v) ethanol and resuspended in 1 ml of 10 mM Tris-HCl, 1 mM EDTA pH 7.5, or MQ.

2.5.1.2 Plasmid DNA isolation

The same procedure was used for the isolation of low, medium and high copy number vectors. An overnight 10 ml culture of the desired bacterial strain, grown in LB with the appropriate antibiotics added, was centrifuged at 5 K in an IEC Centra 4X centrifuge. The supernatant was decanted and the resulting pellet vortexed in the small amount of media remaining, then transferred to an 1.5 ml reaction tube. 300 µl of solution P1 (50 mM Tris-HCl, 10 mM EDTA, 100 µg/ml RNaseA, final pH 8.0) (Qiagen) was added, followed by 300 µl of solution P2 (0.2 M NaOH, 1% (w/v) SDS) (Qiagen) and the 1.5 ml reaction tube was gently inverted and incubated at RT for five min. After the 5 min incubation, 300 µl of ice cold solution P3 (3.0 M KAc, pH 5.5) (Qiagen) was added, the reaction tube inverted gently several times and incubated on ice for 10 min until a white precipitate formed. The reaction tube was then centrifuged at 15K rpm for 10 min (Heraeus Biofuge 15) and the supernatant was transferred into a fresh reaction tube. 400 µl of chloroform was then added to the supernatant, vortexed briefly and centrifuged at RT, 15K rpm for 1 min. The aqueous layer was then transferred to a fresh reaction tube and the chloroform extraction repeated. The aqueous layer was transferred to another reaction tube, 0.7 volumes of isopropanol added and incubated on ice for 5 min (high copy number plasmids) or 15 min (low copy number plasmids). The reaction tube was then centrifuged for 15 min at 15K rpm, (Heraeus Biofuge 15), the supernatant decanted off and the pellet washed with 500 µl of 70% ethanol. The

pellet was then centrifuged at 15K rpm for 5 min, and the ethanol decanted. Finally the pellet was dessicated and resuspended in 50-100 µl MQ (medium to high copy number plasmids) or 20 µl MQ (low copy number plasmids).

2.5.1.3 Small scale plasmid isolation for rapid screening of cloned inserts

A small scale plasmid isolation to screen many potential cloned inserts was used. A 96 well microtitre tray containing 200 µl of LB + antibiotic per well was inoculated with the potential clones and incubated at 37°C with agitation overnight or until a heavy growth was visible. The bacterial cells were then pelleted by centrifugation at 5K rpm for 10 min (Haraeus Labofuge 400R), the supernatant discarded and the plates blotted on paper. The pellets were resuspended in 10 µl of 1 x TE (0.01 M Tris HCl, 0.001 M EDTA, pH 8.0), mixed with 5 µl of 10% (w/v) SDS and then 50 µl of 0.5 M NaOH, 1.5 M NaCl was added and mixed. The trays were then centrifuged at 5K rpm for 10 min in a Haraeus Labofuge 400R and 5 µl of the lysate was transferred to Hybond N membrane for Southern blotting analysis (Section 2.7).

2.5.2 Analysis of DNA

2.5.2.1 DNA quantification

The concentration of DNA in a solution was determined by measurement of absorption at 260 nm which assumes that an A_{260} of 1.0 is equal to 50 µg DNA/ml (Miller, 1972).

2.5.2.2 Calculation of restriction fragment size

The sizes of the DNA restriction fragments was calculation by comparison of their relative mobility with that of *Eco*RI digested *Bacillus subtilis* bacteriphage SPP1 DNA. The

fragment sizes of the digested SPP1 DNA were: 8.5, 7.35, 6.10, 4.84, 3.59, 2.81, 1.95, 1.86, 4.51, 1.16, 0.98, 0.72, 0.48, 0.36 and 0.09 kb (Franzon & Manning, 1986). The size of larger DNA fragments was calculated by comparing their migration with *Bgl*II digested lambda phage DNA: 22.01, 13.29, 9.68, 2.39 and 0.65 kb.

2.5.2.3 Restriction endonuclease digestion of DNA

Restriction endonucleases were purchased from Amersham, Roche, New England Biolabs (NEB) or Pharmacia and used according to the supplier's instructions with the supplied buffers from the manufacturers of the enzyme. 0.1-0.5 µg of DNA or purified restriction fragments were incubated with 2 units of each of the appropriate restriction enzymes in a final volume of 20-50 µl at 37°C (or 25°C when appropriate (e.g. *Apa*I)), for a minimum of 2 h. The reactions were terminated by heating at 65-80°C for 10 min to inactivate the restriction enzymes. Prior to loading on a gel, one tenth volume of tracking dye (15% (w/v) glycerol, 0.1% (w/v) bromophenol blue, 0.1 mg/ml RNASE A) was added.

2.5.2.4 Analytical and preparative separation of restriction fragments

Electrophoresis of DNA was carried out at room temperature on horizontal, 0.7-1.5% (w/v) agarose gels (Seakem HGT). Gels were electrophoresed at 100 V in either 1 x TBE (67 mM Tris base, 22 mM boric acid, 2 mM EDTA, final pH 8.0) buffer or 1 x TAE (40 mM Tris acetate, 2 mM EDTA) buffer. The gels were stained in distilled water containing 2 µg/ml ethidium bromide after electrophoresis. The DNA bands were visualised by trans-illumination with UV light and photographed using a Tracktel gel documentation system together with a Mitsubishi video copy processor or Polaroid 667 positive film.

2.5.2.5 Isolation of specific DNA fragments

For isolation of specific DNA fragments, electrophoresis was carried out on a 0.8% low melting point Seaplaque agarose to separate the DNA fragments. Plasmid, Restriction enzyme and PCR DNA fragments were recovered from excised bands with the QIAquickGel Extraction Kit (Quiagen) according to the manufacturer's instructions. Chromosomal DNA fragments containing surrounding DNA size bands in comparison with SPP1 *Eco*RI digested size markers were excised from the gel (e.g. a 3 kb chromosomal DNA fragment was isolated from an excised gel slice which contained chromosomal DNA, cut between SPP1 bands 5 (3.59 kb) and 6 (2.81 kb)). The excised gel slice was placed in dialysis tubing with 1 ml of 1 x TAE buffer, which was then positioned in an electrophoretic tank filled with 1 x TAE buffer. After electrophoresis of 100V for 1 h, the DNA was assumed to have moved out of the gel slice and be contained within the buffer in the dialysis tubing. The chromosomal DNA in the buffer was then precipitated with 1/10 volume of 3 M NaAc (pH 5.5) and two volumes of 100 % ethanol, pelleted by centrifugation at 15K rpm for 15 min (Heraeus Biofuge 15), the pellet washed with 70% (v/v) ethanol, dessicated and resuspended in 50 µl of MQ.

2.5.2.6 Generation of step-wise deletions (nested deletions)

Uni-directional deletions were generated in plasmid DNA using the Erase-a-base kit from Promega. Briefly 2.5 µg of pRMSB19 plasmid DNA were digested with *Hind*III (providing a 5' overhang sensitive to exonuclease III) and *Apa*I (generating an exonuclease III resistant 3' overhang), and treated with exonuclease III, then treated with klenow and S1-nuclease, and the plasmids were then relegated with T4 DNA ligase. After transformation into DH5α, Amp^R transformants were screened for the extent of the deletion by plasmid preparation and restriction enzyme analysis. Sequencing of the nested deletion plasmids was carried out using

dye terminator sequencing reactions (Section 2.5.2.7.1) using the M13 reverse oligonucleotide (Table 2.5).

2.5.2.7 DNA Sequencing

2.5.2.7.1 Sequencing using dye-labelled oligonucleotides

DNA sequence was determined using dye-terminator sequencing kits from Perkin Elmer. Plasmid DNA was prepared as outlined in Section 2.5.1.2, and PCR products were purified by Quiaquick purification spin columns (Quiagen) according to the manufacturers instructions. The sequencing reaction was carried out in a 0.5 ml reaction tube (Perkin Elmer), 1-2 µg of template DNA and 3.2 pmol of oligonucleotide were added to 9.5 µl of Ready reaction mix (Applied Biosynthesis) and the final volume of 20 µl made up with MQ water. The reaction mix was then briefly centrifuged, overlaid with light mineral oil (Nujol, Perkin Elmer) and subjected to 25 cycles of 96°C 30 sec; 50°C 15 sec; 60°C 4 min in a DNA thermal cycler (Perkin Elmer). The reaction mix was then transferred to a fresh 1.5 ml reaction tube, 2 µl of 3 M sodium acetate and 50 µl of ice cold 100% ethanol were added and the DNA was precipitated for 2 h at -20°C. The precipitated DNA was then centrifuged at 15K rpm for 15 min (Heraeus Biofuge 15), and the pellet washed with 70% (v/v) ethanol, dessicated and stored at -20°C.

2.5.2.7.2 Analysis of DNA Sequences

The DNA was sequenced by the Sequencing Laboratory, I.M.V.S., Adelaide South Australia on a 373A automated sequencer and the raw sequence analysed by an Applied Biosystems Seq Ed program version 6.0. Further analysis of the raw sequence data involved DNASIS, PROSIS (Hitachi Software Engineering) and BLAST 2.0 (Altschul *et al.*, 1997).

2.5.3 Amplification of DNA

2.5.3.1 Oligodeoxynucleotides

Most of the oligodeoxynucleotides (oligonucleotides) used in this study were synthesised on an Applied Biosystems 318A DNA synthesiser in the trityl-off mode by the Institute of Medical and Vetinary Sciences (I.M.V.S), Adelaide, South Australia. Purification before use required the addition of butanol (1 ml) to 100 µl of the oligodeoxynucleotide (previously stored at -20°C), the mixture was vortexed for 1 min, then centrifuged at room temperature (RT) for 5 min at 15K rpm (Heraeus Biofuge 15). The supernatant was then decanted and the pellet dessicated. Following the addition of 100 µl of MQ water, the oligodeoxynucleotide was stored at -20°C until needed. The remaining oligodeoxynucleotides were purchased from Bresatec, Adelaide which were obtained as lyophilised DNA and were then resuspended in sterile MQ to a concentration of 10 mM and stored at -20°C until needed. Oligodeoxynucleotides used in this study are listed in Table 2.5.

Table 2.5: Oligodeoxynucleotides used in this study

Oligo Name	Sequence (5′-3′)	Strain/plasmid/ Transposon	Gene	Location (nt)	Source
#M13F	GTAAAACGACGGCCAGT	pBluescript KS$^+$	MCS	616-600	Stratagene
#M13R	CAGGAAACAGCTATGACC	pBluescript KS$^+$	MCS	808-826	Stratagene
#800	GGATCTTTTTGCGCACTGACAAAAAAA	Mu*dJ*	MuR	235-215	Metcalf *et al.*, (1990) (gbD: M38276; Figure 5.1)
#801	*Eco*RI *Hind*III GATCC**GAATTC**AAGCTTGAATTCG	Ligatable oligonucleotide	-	-	Willoughby *et al.*, (1991)
#832	TAGTGTCTGACTACCACCTG	*S. typhi*	*pqaA*	2230-2211	This study (Figure 5.11)
#1046	GTGTGATTTGTCTCATGTTCTC	*S. typhi*	*pqaA*	2120-2141	This study (Figure 5.11)
#2103	*Eco*RI CG**GAATTC**GTGACAGCTTGGTTGTT	Mu*dJ*	Mu*dJL*	252-228	Priess *et al.*, (1987) (gbD: M64097; Figure 3.5)
#2104	*Bam*HI CG**GGATCC**AGGCATACCATCAGC	Mu*dJ*	Mu*dJL*	857-835	Priess *et al.*, (1987) (gbD: M64097; Figure 3.5)
#2105	CGATAACAACCAAGCTGTC	Mu*dJ*	Mu*dJL*	224-242	Priess *et al.*, (1987) (gbD: M64097; Figure 3.5)
#2106	GGCTGCTGATGGTATGCCT	Mu*dJ*	Mu*dJL*	843-861	Priess *et al.*, (1987) (gbD: M64097; Figure 3.5)
#2275	ACTGCCGATCGAGAAGATGATG	*S. typhimurium*	*hisD*	1150-1169	Carlomagno *et al.*, (1988) (gbD: X13464)
#2276	GCAGACTGATACGGTACCAG	*S. typhimurium*	*hisD*	2655-2636	Carlomagno *et al.*, (1988) (gbD: X13464)
#2308	TCGCATTTATCGTGAAACGCTTTCG	Mu*dJR*	MuR	46-22	Metcalf *et al.*, (1990) (M38276; Figure 5.1)
#2317	CCCTACTACCAGCAAGGC	*S. typhi*	*pqaA*	2835-2852	This study (Figure 5.11)

Oligo Name	Sequence (5'-3')	Strain/plasmid/ Transposon	Gene	Location (nt)	Source
#2353	GGAAGAAGGGAGTAATAATGC	*S. typhi*	*pqaA*	1920-1900	This study (Figure 5.11)
#2430	CTCATCTATGCCTTGCTGG	*S. typhi*	*pqaA*	2861-2843	This study (Figure 5.11)
#2431	AATATTGGCTCACCCAACC	*S. typhi*	*pqaA*	1500-1482	This study (Figure 5.11)
#2485	TACCGCCTAAACCAATGCAG	*S. typhi*	*pqaA*	1110-1091	This study (Figure 5.11)
#2511	CTGTATGGCGTCTGGATCG	*S. typhi*	*pqaA*	1770-1788	This study (Figure 5.11)
#2512	CGATTCGCCATGAGCGATG	*S. typhi*	*pqaB*	1416-1434	This study (Figure 6.9)
#2513	CACGATCCAGTCGCCAGAC	*S. typhi*	*pqaB*	833-815	This study (Figure 6.9)
#2548	GATAATCGGGATCGTCG	*S. typhi*	*pqaB*	1736-1752	This study (Figure 6.9)
#2549	CAGTACCGACATCACAGC	*S. typhi*	*pqaA*	770-753	This study (Figure 5.11)
#2602	TCTGCGTATTCATCACATTG	*S. typhi*	*pqaA*	453-472	This study (Figure 5.11)
#2603	CGGATATCTTAACGGATCTATTATTTG	*S. typhi*	*pqaA*	2922-2895	This study (Figure 5.11)
#2604	CCCAAGCTTTGTCAGAAAGAAATTAG	*S. typhi*	*pqaA*	1-23	This study (Figure 5.11)
#2605	TTAGCTATCCTGATGTACAG	*S. typhi*	*pqaB*	2128-2147	This study (Figure 6.9)
#2606	GCAGCACGCTCACCACC	*S. typhi*	*pqaB*	1260-1244	This study (Figure 6.9)
#2607	AACAGTCTGGGTCAGTGG	*S. typhi*	*pqaB*	888-905	This study (Figure 6.9)
#2618	CCATCGTAATATCAGTGAGTG	*S. typhi*	*pqaA*	2446-2466	This study (Figure 5.11)
#2631	GCAGAACCTGTTCATGCC	*S. typhi*	*pqaB*	2594-2577	This study (Figure 6.9)
#2632	CCTTCCGACTTCCGCATG	*S. typhi*	*pqaB*	496-481	This study (Figure 6.9)
#2633	CAGCGATTGCCGTGGAAC	*S. typhi*	*pqaB*	294-310	This study (Figure 6.9)
#2634	CTGGCATGAACAGGTTCTG	*S. typhi*	*pqaB*	2575-2593	This study (Figure 6.9)
#2636	GCGACGGTAAGAGCGCCAG	*E. coli*	*deoA*	8722-8740	Blattner *et al.*, (1997) (gbD: AE000508)
#2637	GATGCCGAATGAGTCCAGC	*E. coli*	*deoA*	10292-10273	Blattner *et al.*, (1997) (gbD: AE000508)

Oligo Name	Sequence (5'-3')	Strain/plasmid/ Transposon	Gene	Location (nt)	Source
#2640	AATGAAGCCGCTAATGGCG	*E. coli*	*gcvA*	1287-1269	Wilson & Stauffer, (1994) (gbD: U01030)
#2641	TCACCAGAACACGCATTCC	*E. coli*	*gcvA*	127-145	Wilson & Stauffer, (1994) (gbD: U01030)
#2642	ATACAGCCATCGATCACGAA	*S. typhi (plasmid)*	*pqrB*	138-156	pRMSB58 (Section 3.8.2)
#2643	CGTTGTTGTAGACATAGCTC	*S. typhi*	*pqrB*	414-394	pRMSB58 (Section 3.8.2)
#2648	CTCCTGACGATGTTGGTTG	*S. typhi*	*pqaB*	2204-2186	This study (Figure 6.9)
#2649	ACGTTATGCAGGACGAAGC	*S. typhi*	*pqaB*	1680-1663	This study (Figure 6.9)
#2650	TCAGCTATTGCCGATCCTG	*S. typhi*	*pqaA*	2712-2730	This study (Figure 6.9)
#2791	GGTTGACGCGCAAGCCATC	*S. typhimurium*	*pmrD*	7935-7917	Gunn *et al.*, (1998B) (gbD: AF036677)
#2813	CTTCGTACCAGACAATACTCG	*S. typhi*	*pqaA*	2990-3007	This study (Figure 5.11)
#2814	TAGCTTCCCAACCTGTCTTC	*S. typhi*	*pqaA*	3351-3332	This study (Figure 5.11)
#2815	GTGATTTCCGTTATGCCTGTG	*S. typhi*	*pqaA*	3241-3261	This study (Figure 5.11)
#2869	GATATCCTGCTGGCCGGCA	*S. typhi*	*pqaB*	1-19	This study (Figure 5.11)
#2891	AAGAAATCATCGCCACTTGC	*S. typhi*	*pqaA*	2989-2970	This study (Figure 5.11)
#2930	AAGAAATCATCGCCACTTGC	*S. typhimurium*	ORF4	4649-4666	Gunn *et al.*, (1998B) (gbD: AF036677)
#2964	CGTTCTAACCACAAGATAGG	*S. typhi*	*pqaA*	3603-3622	This study (Figure 5.11)
#2965	CGATCCATATAGTTGAGCATG	*S. typhi*	*pqaA*	3759-3739	This study (Figure 5.11)
#2986	*Nde*I TGTACTTATAT**CATATG**TTTAAGAGG	*S. typhi*	*pqaA*	1939-1964	This study (Figure 5.11)
#2987	*Bam*HI CG**GGATCC**ATCGCAAGCGTGATGG	*S. typhi*	*pqaA*	3569-3546	This study (Figure 5.11)
#2988	*Xba*I GC**TCTAGA**TCTGGTGACGGCAATG	*S. typhi*	*pqaA*	1809-1832	This study (Figure 5.11)

Oligo Name	Sequence (5'-3')	Strain/plasmid/ Transposon	Gene	Location (nt)	Source
#2989	*Hind*III CCC**AAGCTT**GGTCTGGTGACGGC	*S. typhi*	*pqaA*	1806-1828	This study (Figure 5.11)
#3001	*Sma*I ACACATC**CCCGGG**CCGTGAAG	*S. typhi*	*pqaB*	627-646	This study (Figure 6.9)
#3002	*Pst*I TGCTTC**CTGCAG**AGCTGACCG	*S. typhi*	*pqaB*	2388-2368	This study (Figure 6.9)
#3003	*Hind*III CGCA**AAGCTT**ATCAGTCCCCAC	*S. typhi*	*pqaB*	2691-2670	This study (Figure 6.9)
#3004	*Eco*RI GCTG**GAATTC**CTGCGACGAATGG	*S. typhimuriumm*	*pmrD*	7962-7940	Gunn *et al.*, (1998B) (gbD: AF036677)
#3066	*Xba*I TGT**TCTAGA**CGTATTTATGGAAATTATG	*S. typhi*	*pqaA*	1954-1981	This study (Figure 5.11)
#3067	*Eco*RI CAC**GAATTC**TGCTTGCCAGTGTTTGC	*S. typhi*	*pqaA*	2436-2411	This study (Figure 5.11)
#3068	*Eco*RI TTTCG**GAATTC**CAAGGCGGGGTTGG	*S. typhi*	*pqaA*	2957-2933	This study (Figure 5.11)
#3069	*Eco*RI CGG**GAATTC**CGTTTTCCCCTAATCC	*S. typhi*	*pqaA*	3504-3480	This study (Figure 5.11)

2.5.3.2 Polymerase Chain Reaction (PCR) protocol

The procedure used in this study for PCR is based on a protocol for the generation of PCR products with cohesive ends (Saiki & Gelfand, 1989). The PCR reaction was performed in 0.5 ml reaction tubes in a volume of 50 or 100 µl (depending on the amount of product to be generated: 50 µl for high product yielding reactions, 100 µl for low) containing a final concentration of 1 x PCR buffer (1.5 mM $MgCl_2$, 10 mM Tris pH 8.4, 50 mM KCl), 200 µM each deoxynucleoside triphosphate (dNTP), 100 pmol each oligodeoxynucleotide (oligonucleotide), 0.001-1 µg DNA template, 2 U Taq polymerase (Cetus) and MQ: up to 50 or 100 µl. The reagents were centrifuged briefly before being overlaid with a drop of light mineral oil (Nujol, Perkin Elmer).

The PCR reactions were conducted using a Perkin Elmer thermal cycler and initial PCR reactions always had

25 cycles amplification with;	Denaturation:	95°C for 30 sec
	Annealing:	50°C for 30 sec
	Extension:	72°C for 2 min

The annealing temperatures were raised to increase specificity when needed and the extension time was adjusted by an increase or decrease of approximately 1 min per 1 kb of product.

Following PCR, 10 µl was taken for analysis on an agarose gel, if the reaction was satisfactory, the remaining PCR product was removed from under the mineral oil and purified using the Qiagen PCR purification kit according to the manufacturers protocol and resuspended in 50 µl of elution buffer at all times.

2.5.3.3 Inverse PCR (IPCR)

Inverse PCR (IPCR) was used during this study to amplify fragments of DNA from the ends of known regions (Ochman *et al.*, 1988). A Southern hybridisation was performed with the bacterial chromosome digested with random restriction enzymes and digestions giving a 1 to 4 kb fragment were selected. 10 µg of bacterial chromosomal DNA was then digested with the appropriate enzyme in a total volume of 100 µl and after digestion the restriction enzymes were inactivated by heating at 65°C for 20 min. Circularisation and ligation of the DNA fragment occurred by diluting the digested DNA 1/10 or 1/100 in a 1.5 ml reaction tube, with 5 units of T4 DNA ligase and 1 x Ligation buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 0.6 mM ATP, 10 mM DTT) in a final volume of 100 µl for 16 h at 4°C. Amplification of the IPCR fragment was then achieved using a PCR reaction with oligonucleotides reading out of the known gene fragment.

2.5.3.4 Single specific oligonucleotide polymerase chain reaction (SSP PCR)

The SSP-PCR method was developed (Shyamala & Ames, 1993) to help amplify DNA fragments for which sequence is only available at one end and involves using plasmids with a multiple cloning site (MCS) containing a large number of unique restriction enzyme sites (in this study the pBluescript KS^+ or KS^- plasmids (Stratagene) were used). The bacterial chromosome was digested with appropriate enzymes (e.g. those contained in the pBluescript MCS and shown by Southern analysis to give a target DNA fragment between 0.5 and 4.0 kb) and ligated to similarly digested pBluescript KS^+ or KS^-. This ligation mix is then used as a template for a PCR reaction and performed with a specific oligodeoxynucleotide and either the #M13F or #M13R oligonucleotide (Table 2.5) for pBluescript KS^+ or KS^- (Stratagene), depending on the enzymes used for the original digestions and the ligated chromosomal fragment orientation.

2.5.4 Manipulation of DNA

2.5.4.1 *In vitro* cloning of restriction digested DNA fragments

DNA to be subcloned was cleaved with either single or double restriction enzyme digests. The enzyme was then inactivated by heating for 20 min at 65-80°C. The digested DNA or purified DNA fragment was then combined with similarly digested vector DNA at a molar ratio of 3:1 (insert:vector), then ligated with 2 units of T4 DNA ligase and 1 x ligation buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 0.6 mM ATP, 10 mM DTT) in a volume of 20-50 µl for 16 h at 4°C. The ligated DNA was then used directly for chemical transformation of *E. coli* or *Salmonella* strains or precipitated with 1/10 volume 3 M NaAc, two volumes of 100% ethanol, washed with 70% ethanol (v/v), dessicated and resuspended in MQ for electroporation of *E. coli* or *Salmonella* strains.

2.5.4.2 *In vitro* cloning of PCR amplified DNA fragments

Both purified (Section 2.5.2.5) and unpurified PCR products were directly ligated to the vector pGEM-T (Promega) according to the manufacturers instructions. The ligated DNA was then used directly for the transformation or electroporation of *E. coli* strains.

2.6 DNA transfer procedures

2.6.1 Conjugation

All conjugations between *E. coli* and *Salmonella* strains were performed in a liquid conjugation. Recipient and donor cells were grown overnight in LB (with the addition of antibiotics when appropriate), centrifuged 4 K rpm, 10 min at RT in an IEC Centra 4X centrifuge and resuspended in 10 ml of LB. Donor cells were added to recipient cells at a ratio of 1:10 (0.4:4 ml) in McCartney bottles and incubated at 37°C, standing for 1 h. The

mixture was then vortexed vigorously for 30 sec, and 100 µl of neat or 1/10 dilution were plated out onto selective plates.

2.6.2 Electroporation, Chemical transformation and Competent Cells

2.6.2.1 Preparation of Chemically competent cells

"Super-competent" cells were prepared according to the method as follows: A 10 ml cuture of *E. coli* K-12 bacteria were grown overnight in 20 ml McCartney bottles at 37°C, subcultured 1/20 into fresh 10 ml LB and cultured until ~ 4 x 10^8 cells/ml were obtained. The cells were chilled for 10 min, centrifuged at 5 K rpm for 5 min in an IEC Centra 4X centrifuge and resuspended in 10 ml of ice cold Solution α (30 mM KAc, 100 mM KCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol (v/v)). The solution was then recentrifuged at 5 K rpm for 5 min in an IEC Centra 4X centrifuge and the pellet resuspended in 1 ml of Solution β (10 mM MOPS, 75 MM $CaCl_2$, 10 mM KCl, 15% glycerol (v/v)) and left on ice for 1-2 h. The competent cells were then aliquoted (100 µl per tube) into 1.5 ml reaction tubes and either used immediately or snap frozen in a dry ice/ethanol bath and stored at -70°C until needed.

2.6.2.2 Preparation of Electro-competent cells

Electro-competent *E. coli* and *Salmonella* bacterial cells were prepared according to the Bio-Rad protocol. Initially, 0.5 ml of an overnight broth of bacteria was subcultured into 10 ml LB and incubated at 37°C with agitation until the cells reached an OD_{600} 0.5-0.8. The cells were allowed to cool briefly on ice before being centrifuged (5 K rpm for 5 min in an IEC Centra 4X centrifuge) and resuspended in 10 ml of ice-cold 10% glycerol (v/v). The 10% glycerol wash was repeated and the bacterial cells resuspended in 1 ml of 10% glycerol and

kept on ice if being used the same day or 200 µl aliqots in 1.5 ml reaction tubes were snap-frozen with dry ice/ethanol and stored at -70°C until needed.

2.6.2.3 Chemical Transformation

Chemical transformation procedures were performed according to the standard method of Brown *et al.*, (1979). *E. coli* K-12 and *Salmonella* strains were made competent as described above in Section 2.6.2.1. 100 µl of competent cells were mixed with 1-2 µg of DNA in a 1.5 ml reaction tube and left on ice for 20 min. The mixture was then heated in a 37°C waterbath for 3 min, placed back on ice for 10 min before adding 1 ml of LB and incubating in the 37°C waterbath for 1-2 h. The mixture was then centrifuged at 15 K rpm for 1 min (Heraeus Biofuge 15), resuspended in 100 µl of LB and plated on the appropriate selection plates.

2.6.2.4 Electroporation

100-200 µl of electro-competent cells (prepared as described in Section 2.6.2.2) were mixed with 1-2 µl of plasmid DNA (in sterile MQ water) in a sterile 1.5 ml reaction tube on ice and then transferred to an ice-cold sterile *E. coli* Pulser™ cuvette (0.2 cm electrode gap, Bio-Rad). The Bio-Rad gene pulser was set at 25µF and the pulse controller at 200Ω. The *E. coli* and *Salmonella* cells were pulsed at 2.5 kV with time-constraints of 4.5-4.7 msec. Immediately after electroporation, 1 ml of LB was added to the cuvette, the contents mixed and transferred to a sterile McCartney bottle. After incubation at 30°C or 37°C for 60-90 min with agitation the electroporated cells were transferred to a sterile 1.5 ml reaction tube and centrifuged at 15 K rpm for 1 min (Heraeus Biofuge 15). The supernatant was then discarded and the pellet gently resupended in 200 µl of sterile LB and plated onto NA or LA plates containing the appropriate antibiotics.

2.6.3 Transduction

2.6.3.1 P22 phage propation

P22 phage propagation was as follows: *Salmonella* bacteria strains grown overnight in 10 ml LB with aeration at 37°C were diluted 1/10 in LB and grown in the same conditions for 1.5-2 h (early exponential phase). 0.1 ml of 1 M $MgSO_4$ and 0.1 ml of $CaCl_2$ were added to the cultures, then 0.1 ml of P22 stock (~5 x 10^9 pfu/ml) was added. The cultures were then shaken at 37°C for 3-5 h or until lysis of cells had occurred. 0.1 ml of chloroform was added, the cultures were vortexed vigourously and centrifuged at 5 K rpm for 5 min in an IEC Centra 4X centrifuge to remove cell debris. The supernatant (containing the propagated phage) was decanted into a fresh McCartney bottle and either used immediately or stored at 4°C until needed.

2.6.3.2 Transduction with P22 bacteriophage

Overnight cultures of recipient *Salmonella* strains were grown in 10 ml LB at 37°C with agitation, diluted 1/10 in LB and grown for 4-5 h at 37°C with aeration. Bacterial cells were then centrifuged for 10 min at 5 K rpm in an IEC Centra 4X centrifuge and the pellets resuspended in 2 ml LB. 0.1 ml of 1 M $MgSO_4$ and 0.01 ml of 1 M $CaCl_2$ was added to both suspensions. Then 0.1 ml of P22 phage stock was added to one culture and 0.1 ml of LB added to the other (control), the cultures were incubated at 37°C (standing) for 10 min. 0.01 ml of 10 mM EGTA was added and the mixture incubated for a further 10 min at 37°C, followed by centrifugation in a bench centrifuge (4 K rpm, 10 min) and the pellet resuspended in 1 ml LB. 0.1 ml was then plated onto the appropriate selection plates and incubated for 18-24 h. Control reactions underwent exactly the same procedure apart from the fact that 0.1 ml of LB was used instead of P22 phage stock. A loopful of phage stock was streaked onto selection plates to ascertain that P22 phage stock was not contaminated.

2.7 Southern hybridisation blotting procedure

All Southern hybridisation blotting procedures used during this study, were performed with the non-radioactive DIG-11-dUTP method (Roche).

2.7.1 Labelling of Digoxigenin-11-dUTP probes

2.7.1.1 Random primed labelling

Random primed labelling with the Digoxigenin-11-dUTP (DIG) (Roche) labelled probes were prepared according to the Manufacturer's protocol. Single stranded (SS) DNA from 5 µl of a plasmid preparation (Section 2.5.1.2) or 3 µl of digested, purified DNA (Section 2.5.2.5) was prepared by boiling at 100°C for 10 min and then snap frozen with dry ice/ethanol. 2 µl of 10 x hexanucleotide mix (Roche) and 2 µl of dNTP labelling mix (Roche), 1 µl Klenow enzyme and MQ water up to 20 µl were then added to the SS DNA and the mixture incubated overnight at 37°C. The reaction was stopped by the addition of 0.2 M EDTA pH 8.0 (2 µl) and the DNA precipitated with 2 µl 4 M LiCl and 50 µl of cold ethanol at -20°C for 2 h. The labelled DNA probe was collected by centrifugation at 15 K rpm for 15 min (Heraeus Biofuge 15) washed with 70% (v/v) ethanol, dessicated and resuspended in 50 µl of MQ water and stored at -20°C until needed.

2.7.1.2 DIG PCR labelling

DIG-labelled DNA probes were also prepared by PCR according to the protocol from Roche. A PCR reaction was set up using 1 x PCR buffer (1.5 mM $MgCl_2$, 10 mM Tris pH 8.4, 50 mM KCl), 1 x dNTP DIG labelling mix, 100 pmol each oligodeoxynucleotide (oligonucleotide), 0.001-1 µg DNA template, 2 U Taq polymerase (Cetus) and MQ up to 50 µl. The 1 x dNTP mixture used consisted of 20 µM dATP, dCTP, dGTP and 19 µM dTTP with the additon of 1 µM DIG-11-dUTP. As DIG-11-dUTP is a larger molecule than dTTP

which it replaces at random in the PCR reaction, the products were electrophoresed on an agarose gel and compared to the same PCR products made with the "normal" dNTP mixture and compared. If the product had been labelled correctly with DIG-11-dUTP then is tended to run slightly higher and was considered labelled. The DIG product was then precipitated with 6 µl 4 M LiCl and 100 µl of cold ethanol at -20°C for 2 h. The labelled DNA probe was collected by centrifugation (15 min at 15 K rpm in a Heraeus Biofuge 15), and the pellet washed with 70% (v/v) ethanol, dessicated and resuspended in 50 µl of MQ water and stored at -20°C until needed.

2.7.2 Preparation and transfer of DNA from gel electrophoresis onto nylon membrane

Digested chromosomal DNA samples and appropriate markers were electrophoresed overnight on a 0.7% (w/v) agarose TAE gel, stained with EtBr and photographed with 667 polaroid film against a ruler. The agarose gel was then denatured by soaking in several volumes of 1.5 M NaCl and 0.5 M NaOH solution for one hour with slow agitation. The denatured gel was neutralised using several volumes of 1 M Tris-HCl (pH 8.0) containing 1.5 M NaCl for one hour with slow agitation. Transfer of the DNA to a Hybond N nylon membrane (Amersham) occurred by overnight transfer at RT using capillary action as described by Maniatis (1982) with 10 x SSC (1.5 m NaCl, 0.15 Na citrate, pH 7.0) as the transfer buffer. After transfer the DNA was fixed onto the nylon by placing the membrane (DNA side up) on Whatmann soaked with 0.4 M NaOH for 20 min. The membrane was then washed briefly in 5 x SSC, air-dried and either used immediately or stored between two pieces of Whatmann at 4°C until needed.

2.7.3 Transfer of small scale plasmid preparations onto nylon membranes for Southern analysis

DNA from small scale plasmid preparations (Section 2.5.1.3) (5 µl) was spotted onto Hybond N nylon membranes (Amersham), air-dried and then fixed by placing the membrane onto blotting paper soaked with 0.4 M NaOH for 20 min. The membrane was then washed briefly in 5 x SSC, air-dried and either used immediately or stored between two pieces of Whatmann blotting paper at 4°C until needed.

2.7.4 Prehybridisation and hybridisation of the probe

The filters were prehybridised in 20-25 ml of alternative hybridisation solution (5 x SSPE (Maniatis, 1982), 50% formamide (v/v), 1% skim milk (w/v), 7% SDS (w/v) and 250 µg/ml SS herring sperm DNA (Sigma)) for a minimum of 2 h. Either fresh DIG-labelled probe was preheated at 95°C for 5 min and then added to the prehybridisation solution or the prehybridisation solution was drained and replaced by a previously used DIG labelled probe solution (heated at 65°C for 10 min before use). The probe solution was left on the filters overnight at 42°C. High stringency washes were performed with all Southern hybridisations perfomed in this study; two 10 min washes with 5 x SSC containing 0.1% (w/v) SDS at RT were followed by 2 x 20 min with 5 x SSC containing 0.1% (w/v) SDS at 65°C were used.

2.7.5 Enhanced chemiluminescence (ECL) detection

Detection of the probed DNA by ECL detection was performed by washing the hybridised filters briefly in Buffer 1 (0.1 M Tris HCl, 0.15 M NaCl, final pH 7.5) and then incubating in 5% skim milk (w/v) in Buffer 1 for a minimum of 30 min at RT with agitation. These filters were then washed briefly in Buffer 1 and DIG antibody-conjugate (POD) (Roche), diluted 1/5000 in Buffer 1 (with excess skim milk) and left to incubate for a

minimum of 30 min at RT with agitation. Unbound Ab-conjugate was removed after the incubation by washing 4 x 5 min with Buffer 1 followed by one 15 min wash with 1 x PBS. The PBS was then drained off and 5 ml of ECL detection solution (Roche) was added to the filter in a clean plastic bag and sealed for one minute. After 1 min the ECL solution was drained off and the filter detected by exposure with audioradiography film (Kodak XR-100), starting with 2-5 min exposures, and increasing if necessary. The film was developed in D19 (Kodak) for 2-5 min before being washed and fixed for 5 min in Hypan rapid fixer (Ilford).

2.7.6 Colorimetric Detection

Detection of the probed DNA by colorimetric detection was performed by briefly washing the hybridised filters in Buffer 1 and then incubating in 5% skim milk (w/v) in Buffer 1 for a minimum of 30 min at RT with agitation. These filters were then washed briefly in Buffer 1, and DIG antibody-conjugate (AP) (Roche), diluted 1/5000 in Buffer 1 was added and left to incubate for a minimum of 30 min at RT with agitation. Unbound Ab-conjugate was removed after the incubation by washing 2 x 10 min with Buffer 1. The filter was then incubated in 1 x Buffer 3 (0.1 M Tris-HCl, 0.1 m NaCl, 0.05 M $MgCl_2$, final pH 9.5) for a minimum of 10 min and detected with 10 ml of freshly prepared colour solution. The colour solution was prepared by the addition of 45 µl of nitroblue-toluidine (NBT) (75 mg/ml) and 35 µl of the substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP) (50 mg/ml) in 10 ml of Buffer 3. The filter was allowed to develop in the dark with no agitation and was stopped by the removal of the colour solution and addition of 100 ml of 1 x TE.

2.8 SDS polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10 to 20 % polyacrylamide gels (Lugtenberg *et al.*, 1975) using a modification of the procedure as described by Achtman *et al.*, (1979) to separate either proteins or lipopolysaccharide.

2.8.1 Preparation and staining of whole cell protein samples for SDS polyacrylamide gel electrophoresis

Samples of whole cells were prepared for analysis by resuspending 1 ml of a pelleted overnight or mid-exponential phase culture in 100 µl of 1 x sample buffer (25 mm Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 15% (w/v) bromophenol blue). Before loading, the samples were heated to 100°C for 5 min and gels were normally electrophoresed at 150-180 V for 2.5-3 h (10-15% gels) or 13 mA constant current for 16 h (10-15% gels). The proteins were stained with gentle agitation overnight at room temperature in 0.06% (w/v) Coomasie Brilliant Blue G250 (dissoved in 5% (v/v) perchloric acid). Several changes of 5% (v/v) acetic acid with gently agitation for 24 h effectively destained the gels.

Low molecular weight (LMW) size markers (Pharmacia) were phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-Lactalbumin (14.4 kDa).

2.8.2 Protein overexpression using the T7 RNA polymerase and temperature induced or NaCl expression systems

2.8.2.1 Temperature induced overexpression of proteins

The T7 RNA polymerase overexpression system with temperature induction was used with the *E. coli* strain E2098 and is essentially the method described by Tabor & Richardson

(1985). Plasmids containing the gene of interest under the control of the T7 RNA polymerase promoter were transformed into E2096 harbouring the plasmid pGP1-2 (carries the T7 RNA polymerase under the control of the lambda P_L promoter (Tabor & Richardson, 1985). Single colonies were innoculated into 10 ml of LB broth containing Amp (50 μg/ml) and Kan (50 μg/ml) and grown with aeration at 30°C for 16 h. The culture was then subcultured 1/10 and incubated at 30°C with constant aeration until an OD of A_{600} of 0.6 was reached. A final volume of 0.4 M (isopropyl-β-D-thiogalactopyranoside (IPTG) was added to strains containing pET11bYZ-based and pBluescript-based plasmids, and the cultures were incubated at 42°C for 20 min, then shifted to 37°C for a further 1-3 h. One ml of cells were then pelleted (15 K rpm, 2 min Heraeus Biofuge 15), and resuspended in 200 μl of sample buffer (Section 2.8.1). The samples were heated at 100°C for 5 min prior to electrophoresis on SDS-polyacrylamide gels (Section 2.8.1)

2.8.2.2 NaCl expression system

The NaCl protein overexpression system was based on the procedure outlined by Bhandari & Gownshankar (1997). Plasmids containing the gene of interest under the control of the T7 RNA polymerase promoter were transformed into GJ1158. Single colonies were innoculated into 10 ml of LBON broth containing Amp (50 μg/ml) and Kan (50 μg/ml) and grown with aeration at 30°C for 16 h. The culture was then subcultured 1/10 and incubated at 37°C with constant aeration until an OD of A_{600} of 0.7-0.8 was reached. A final volume of 0.4 M IPTG was added to strains containing pET11bYZ-based and pBluescript-based plasmids and a final concentration of 0.3 M NaCl was added. The cultures were then incubated at 37°C for a further 1-3 h. One ml of cells were then pelleted (15 K rpm, 2 min Heraeus Biofuge 15), and resuspended in 200 μl of sample buffer (Section 2.8.1). The samples were heated at 100°C for 5 min prior to electrophoresis on SDS-polyacrylamide gels (Section 2.8.1).

2.8.3 L-[S^{35}]-methionine labelled proteins

Radioactive labelling of proteins was essentially performed by the method of Tabor & Richardson (1985) with the *E. coli* E2096 strain containing expression vectors. Single colonies were innoculated into 10 ml of LB broth containing Amp (50 µg/ml) and Kan (50 µg/ml) and grown with aeration at 30°C for 16 h. The culture was then subcultured 1/10 and incubated at 30°C with constant aeration until an OD of A600 0.6 was reached. The cells were then pelleted (15 k rpm, 10 min, IEC Centra X centrifuge) and washed 3 times in M9 media before being resuspended in 1 ml of M9 media supplemented with 0.2 mg/ml $MgSO_4$, 0.5% (w/v) glucose and 50 mg/ml thiamine HCl, and incubated at 30°C for 1 h. After the incubation, cells were pelleted again and resuspended in 1 ml of overexpression media (1:50 Methionine Assay Media (MAM) (Difco):M9 media supplemented with 0.2 mg/ml $MgSO_4$, 0.5% (w/v) glucose and 50 mg/ml thiamine HCl. A final volume of 0.4 M IPTG was added to strains containing pET11bYZ-based and pBluescript-based plasmids and the cultures were then incubated at 42°C for 20 min to induce the pGP1-2 P_L promoter by the inactivation of the repressor cI_{ts}, allowing the expression of the T7 RNA polymerase from the I_{pL} promoter. After the 20 min, rifampicin was added at a final concentration of 200 µg/ml to inactivate the *E. coli* host RNA polymerase and the 42°C incubation was continued for another 10 min. The culture was then transferred to 37°C for 2 h, followed by pulsing for 5 min at 30°C with 5 µCi L-[^{35}S]-methionine (Amersham) after expression at 37°C. The cells were then pelleted, (15 K rpm, 1 min, Heraeus Biofuge 15) and resuspended in 120 ml of 1 x SDS sample buffer (Section 2.8.1). Samples were heated at 100°C for 5 min prior to electrophoresis on SDS polyacrylamide gels.

The SDS-PAGE gels containing the L-[^{35}S]-methionine-labelled protein samples were dried on Whatman 3MM chromatography paper at 60°C for 2 h on a Bio-Rad gel drier after Coomassie G250 staining. The gels were then subjected to autoradiography which was

perfomed at -70°C for 1-7 days without intensifying screens using Kodak XR-100 film and developed in D19 (Kodak) for 2-5 min before being washed and fixed for 5 min in Hypan rapid fixer (Ilford).

2.9 Lipopolysaccharide (LPS) methods

All reagents used for LPS preparation, gel electrophoresis and silver staining were analar grade and distilled, deionized water which had been passed through a series of Millipore filters (MQ) and had a conductivity of not more than 18 Mega ohms/cm (MQ water) was used to rinse all glassware and in the preparation of solutions.

2.9.1 LPS isolation

Lipopolysaccharide (LPS) was isolated from *Salmonella* strains by the method of Hitchcock & Brown (1983). Small scale LPS preparations from 1.5 ml of overnight *Salmonella* cultures grown in LB at 37°C which was centrifuged at 15 k rpm for 1 min (Heraeus Biofuge 15). The pellet was then resuspended in 50 µl of lysing buffer (2% (w/v) SDS, 4% (w/v) β-mercaptoethanol, 10% (v/v) glycerol, 1 M Tris-HCl pH 6.8, and 0.1% (w/v) bromophenol blue) and heated at 100°C for 10 min. 10 µl of Proteinase K (25 mg/ml) was then added to each sample and incubated at 60°C for 4-16 h. Samples were stored at -20°C.

2.9.2 LPS gel electrophoresis and silver staining

The LPS samples prepared as in Section 2.9.1 were heated at 100°C for 5 min and loaded onto a 20% SDS-PAGE gel and electrophoresed for 20 h at 12 mA constant current. After electrophoresis, silver staining of the SDS-polyacrylamide gel containing LPS was performed using a modified method of Tsai & Frasch, (1992). The SDS-polyacrylamide gel was fixed for 2 h in 40% (v/v) ethanol, 10% acetic acid, and then oxidized for 5 min with 0.7% (w/v)

perchloric acid in 40% (v/v) ethanol, 10% (v/v) acetic acid. After three 15 min washes with MQ water the gel was stained for 10 min in a solution containing 28 ml 0.1 M NaOH, 2 ml concentrated NH_4OH and 5 ml 20% (w/v) $AgNO_3$ in a total volume of 150 ml. The gel was then washed three times for 10 min with MQ and developed in a freshly prepared 500 ml solution containing 50 mg citric acid and 0.5 ml formamide. The citric acid was dissolved in MQ and heated to 37°C prior to use and formaldehyde was added just before the detection solution was added to the gel. The detection reaction was stopped by the addition of 0.2% (v/v) acetic acid for 20 min, after this the developed gel was photographed and then stored in the dark in MQ water.

2.10 β-galactosidase assays

β-galactosidase activity was measured using a combination of two previous methods (Miller, 1972; Bignon *et al.*, 1993). Cultures were grown in LB at 37°C with aeration for 16 h. They were cooled at for 20 min and their OD_{600} was measured (Pharmacia LKB-Ultraspec Plus Spectrophotometer). Dilutions were made using cold LB so as all cultures had an OD_{600} of 0.550-0.850. Diluted cultures were then aliquoted (300 µl) into 96 well flat-bottomed microtitre trays and the OD_{600} read immediately. In a microfuge tube, diluted culture (500 µl) was added to 500 µl of Z buffer (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1 mM $MgSO_4.7H_2O$, 50 mM β-mercaptoethanol adjusted to pH 7.0 and stored at 4°C). Cells were opened by addition of 2 drops of chloroform and 1 drop of SDS (0.1% w/v) and vortexing for 10 seconds. Aliquots (100 µl) of the bacterial lysates were placed in duplicate well of a 96 well microtitre tray. The tray was incubated at 28°C for 15 minutes and the reaction started by addition of 2-nitrophenyl-β-D-galactopyranoside (ONPG) (Roche) (200 µl) (0.7 mg/ml in Z buffer) to each well, with mixing. Optical densities were recorded at 410 nm

(O-nitrophenol colour change) and 570 nm (cell debris). Enzyme units were calculated for each strain at the time point when the OD_{410} first exceeded 0.6 using the following equation.

$$\beta\text{-galactosidase Units} = \frac{1000 \times (OD_{410} - 1.75 \times OD_{570})}{\text{Time} \times \text{Volume} \times OD_{600}}$$

2.11 Antimicrobial agent assays

2.11.1 Polymyxyin B (PmB) assays

Bacteria were grown for 16 h in LB at 37°C with aeration were subcultured (1:20) in LB and grown to mid-exponential phase. These cultures were then diluted to approximately 1 x 10^4 bacteria/ml and chilled. Aliquots (100 µl) of diluted culture were added to a 96 well microtitre tray (Corning wells catalogue no. 250850-96). 100 µl of appropriately diluted PmB (0.2-0.6 µg/ml) in phosphate buffered saline (PBS) pH 7.5, were added to each well. Controls consisted of bacteria (100 µl) and PBS (100 µl). The microtitre trays were incubated at 37°C for 1 h after which they were placed on ice. Plates (LA) were spread with 50 µl (neat) from each well; the plates were incubated overnight at 37°C for 18 h, and the colonies were counted. The data is presented as percentage survival relative to the control with no added PmB.

2.11.2 Melittin Assays

Bacteria were grown for 16 h in LB were subcultured (1:20) in LB and grown to mid-exponential phase. These cultures were then finally diluted 1 in 20,000 to give ~ 1 x 10^5 cells/ml. Aliquots (50 µl) of diluted culture were added to a 96 well microtitre tray (Corning cell wells catalogue # 250850-96). 50 µl of melittin in 1 x PBS at double final strength (15 µg/ml) was added to each well. Controls consisted of bacteria (50 µl) and PBS (pH 7.5) (50

µl) only, and peptide (50 µl) and PBS (50 µl) only. Microtitre trays were incubated with agitation at 37°C for 1 h after which they were placed on ice. Duplicate plates (LA) were spread with ten-fold dilutions from each well, the plates were incubated overnight at 37°C; the colonies were then counted. The data is presented as percentage survival relative to the control with no added peptide. Stock solutions of melittin (Roche or Sigma) were made by dissolving appropriate amounts of solid in PBS and used within 48 h.

2.12 Tissue Culture

2.12.1 Maintenance of cell lines

The U937 monocyte cell line (obtained from Professor L. Ashman, Hanson Cancer Research Centre, Adelaide) was maintained in RPMI-1640 media containing 10% (v/v) fetal bovine serum (FBS), 1% (w/v) glutamine with 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL) (RPMI maintenance media) at 37°C with 5% CO_2. HeLa cells (human epithelioid carcinoma cells - ATCC CCL2) were maintained in MEM (Minimal Eagles salt media) containing 10% (v/v) FBS, 1% (w/v) glutamine with 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL) (MEM maintenance media). Tissue culture assay media was the same as the maintenance media for each cell type but did not contain any antibiotics.

2.12.2 Differentiation of U937 cells and preparation of HeLa cells for bacterial invasion and survival assays

The PMA-differentiated U937 cell assay is based on the previous assay used by Hone *et al.*, (1992). Differentiation of U937 cells with phorbol myristate acetate (PMA) (Sigma) to obtain monocyte-derived macrophages involved washing U937 cells grown to a high density, and seeding in 24 multi-well plates (Corning, #25820) at approximately $1x10^6$ cells/well with

6 ng/ml PMA (dissolved in DMSO) in RPMI maintenance media. Cells were differentiated with PMA for 72 h before being washed 3 times with phosphate buffered saline (PBS) prior to the assay.

HeLa cells were grown to semi-confluency and seeded in a 24 well tray. After incubation for 24 h (cells were now ~1 x 10^6 cells/well), the cells were washed 3 times with PBS prior to the assay.

2.12.3 Staining of PMA-U937 and U937 cells

When stained with Diff-Quik (Lab Aids [LP-64851])(a modification of the Wright Stain technique), the monocytic U937 cells stain with a violet lobated nucleus and sky blue cytoplasm whereas the PMA-U937 cells stain with a dark blue nuclueus, pale pink cytoplasm and reddish lilac granules. When photographed in black and white, the U937 cells appear as single pale grey cells with a defined nucleus, whereas the PMA-U937 cells are slightly larger, stain a darker grey with a defined nucleus and form "clumps" of cells (Section 4.4). The PMA-U937 cells are adhesive on glass after the 72 h differentiation, and were fixed and stained on a cover slip whereas the U937 cells are non-adhesive and were centrifuged onto a glass slide to be fixed and stained.

2.12.4 Bacterial invasion and survival assays

All bacterial strains were grown for 16 h in LB at 37°C with aeration, subcultured 1/20 into 10 ml LB and grown 2.5 h until bacteria had reached 1 x 10^9 cells/ml. Bacteria were then washed and diluted in the appropriate tissue culture media (RPMI or MEM assay media) to be added to the tissue culture cells.

2.12.4.1 U937 cell assay I and II

The U937 cell assay I, involved the *S. typhi* bacteria (1 x 10^7 bacteria/ml) incubated with 1 x 10^6 U937 cells/ml in a final volume of 6 ml in a 10 ml centrifuge tube (62.9924.284, Sarstedt) and incubated for 2½ h to allow invasion of the U937 cells by the bacteria. The cells were then centrifuged and the pellet resuspended in 6 ml of RPMI media + 0.2 mg/ml of gentamicin for one hour, washed three times with PBS and resuspended in 5 ml of RPMI + 10 µg/ml gentamicin. One ml was taken off before the final wash, washed once and lysed with 0.5 ml of 0.1% Triton in PBS for the 0 h timepoint. Then 1 ml was sampled, washed and lysed for the 6, 18 and 24 h timepoints. All bacteria (including initial inoculum) were diluted in saline and plated out for viable cell counts on LA plates. After 18 h incubation, the colonies were then counted and the number of cfu/ml at each timepoint were calculated.

The U937 cell assay II was the same as the U937 cell Assay I except that after the 0 h timepoint, the U937 cells were transferred into fresh 10 ml tubes in 1 ml aliquots for the 6, 18, and 24 h timepoints.

2.12.4.2 HeLa cells and PMA-U937 assay

After 1 ml of bacteria (*S. typhi* 10^7 bacteria/ml, *S. typhimurium* 10^6 bacteria/ml were added to the HeLa and PMA-U937 cells in the 24 multi-well plates (*S. typhi* strains were added at a ratio of 10:1 and *S. typhimurium* strains at 1:1 (bacteria:tissue culture cells)), invasion was allowed to occur for 2 h, the supernatant with bacteria were removed and the cells washed once with 1 ml of PBS, and the appropriate media containing 0.2 mg/ml gentamicin (Gibco BRL) was added for 1 h. Cells were then washed 3 times with 1 ml PBS and the 0 h timepoint taken by lysing cells with 0.5 ml of 0.1% (v/v) Triton X-100 in PBS. All remaining wells had the appropriate assay media containing 10 µg/ml gentamicin added for the remainder of the assay. Subsequent timepoints were taken at 6, 18 and 24 h. All

bacteria (including initial inoculum) were diluted in saline and plated out for viable cell counts on LA plates. After 18 h incubation, the colonies were then counted and the number of cfu/ml at each timepoint were calculated.

2.12.5 LDH assay

Survival of the tissue culture cells during the bacterial infection assays was determined using the CytoTox 96^{TM} Non-Radioactive Cytotoxicity assay (Promega). Infected and control cells were washed 3 times with PBS, as for lysis for bacterial counts, but were then lysed with 200 µl of lysis buffer according to the manufacturers instructions and assayed accordingly. A standard LDH curve was performed with each assay to calculate the amount of LDH units/well present at 0 and 24 h after bacterial infection.

2.13 Statistical Analysis

Statistical analysis was performed using Student's two-tailed t-test for independent means.

Chapter 3

Characterisation of *S. typhi* and *S. typhimurium* PhoP/Q regulated genes

3.1 Introduction

An important part of *Salmonella* virulence involves the *Salmonella* two-component regulatory system (PhoP/Q) (Garcia Vescovi *et al.*, 1994; Groisman & Saier, 1990; Miller, 1991). A large number of *S. typhimurium phoP/Q* regulated genes termed *pag* (*phoP/Q* activated genes) and *prg* (*phoP/Q* repressed genes) have been identified. A point mutation in *phoQ* (*phoP24*) results in a constitutive phenotype ($PhoP^c$); the expression of *pag* genes is increased and the expression of *prg* genes is decreased (Gunn *et al.*, 1996; Miller & Mekalanos, 1990). A mutation in *phoP* (*phoP12*) resulted in a null phenotype ($PhoP^-$); the expression of *prg* genes is increased and the expression of *pag* genes is decreased (Miller & Mekalanos, 1990)

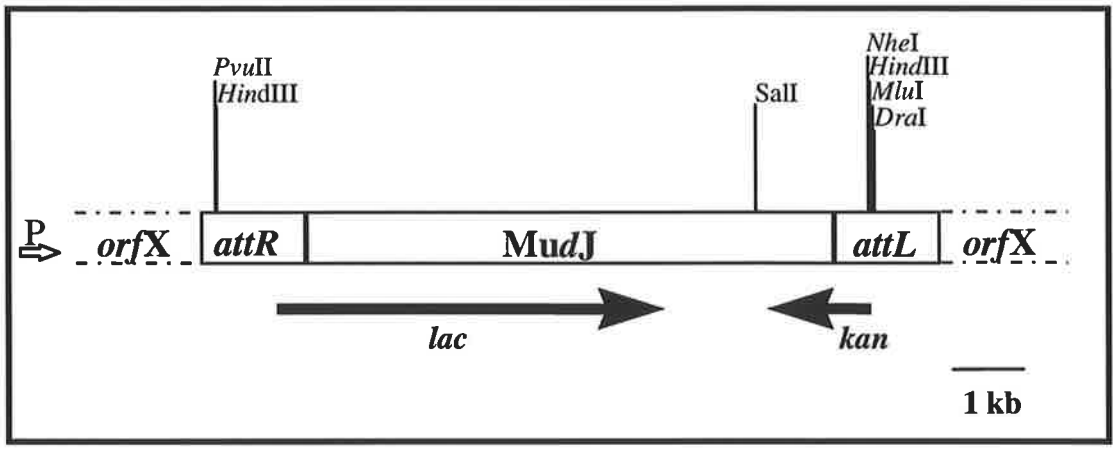
As the *Salmonella* PhoP/Q regulon had only been studied in detail in *S. typhimurium* before this project was undertaken, I wished to identify and characterise PhoP/Q regulated genes in *S. typhi*. *S. typhimurium* $PhoP^-$ and $PhoP^c$ strains have been shown to be attenuated in mice (Miller *et al.*, 1989; Miller & Mekalanos, 1990), indicating that the *S. typhimurium* PhoP/Q regulon is involved with *S. typhimurium* virulence in the murine typhoid model. Therefore by studying the PhoP/Q regulon and *phoP/Q* regulated genes in *S. typhi*, it was hoped that these studies would further the understanding of *S. typhi* pathogenesis.

In a previous study, *S. typhi phoP12* ($PhoP^-$), and *S. typhimurium phoP12* ($PhoP^-$) and *phoP24* ($PhoP^c$) mutant strains were constructed and a screening system based on Mu*dJ*

transposon mutagenesis to identify PhoP/Q regulated genes in *S. typhi* and *S. typhimurium* was devised (Baker, 1993).

The Mu*d*J transposon used in this work was randomly inserted into the *S. typhi* PhoP$^-$ mutant strain using transitory *cis* complementation (Hughes & Roth, 1988) via P22 phage. The Mu*d*J transposon consists of 11.3 kb of DNA containing the Mu phage attachment sites (*attR* and *attL*, allowing the transposon to insert into DNA), a promoterless *lac* operon and an antibiotic kanamycin resistance cartridge. When the Mu*d*J transposon is inserted into the correct orientation in the bacterial chromosome, transcription from a promoter near the RHS of the Mu*d*J insertion can proceed into Mu*d*J to allow expression of the promotorless *lacZYA* operon (Casadaban & Cohen, 1979; Castilho *et al.*, 1984) (verified by the activity of LacZ in β-galactosidase assays). A complete sequence of the Mu*d*J transposon is not available in any sequence database but sequence from the Mu left end (Priess *et al.*, 1987) (gbD: M64097) and MuR (Metcalf *et al.*, 1990) (gbD: M33723) were available. Using these sequences a schematic diagram of Mu*d*J (Figure 3.1) was constructed. Figure 3.1 shows the Mu*d*J transposon insertion into a bacterial chromosomal gene (*orfX*) in the correct orientation to give a transcriptionally active *lac* operon driven by the *orfX* chromosomal gene promoter (indicated by P and an open arrow showing transcription direction). In this thesis the LHS of Mu*d*J containing the *attL* site (Bacteriophage Mu left end, (Priess *et al.*, 1987) and the RHS of Mu*d*J containing the *attR* site (MuR, (Metcalf *et al.*, 1990)) will be referred to as MudJL and MudJR respectively. All figures depicting a chromosomal Mu*d*J transposon insertion will show the Mu*d*J transposon as inserted in a transcriptionally active *lac* orientation, and nucleotide labelling will start from nt 1 of the edge of the Mu*d*J transposon insertion end with respect to either Mu*d*JL or Mu*d*JR (depending on which transposon end is currently being described).

Figure 3.1 Schematic diagram of the Mu*d*J transposon



This figure shows a schematic diagram of the Mu*d*J transposon. The transposon is depicted as in a transcriptionally active *lac* operon orientation, driven by the chromosomal gene (designated *orfX* in dashed lines) promoter labelled with a P and open arrow. The *attR* and *attL* sites needed for transposition are labelled, and the *lac* operon and kanamycin cartridge (*kan*) which have solid arrows indicating the direction of transcription. Relevant restriction enzyme site positions are also marked.

3.2 Nomenclature for *S. typhi pag/prg* genes

As many *S. typhimurium* PhoP/Q regulated genes have been identified and named as *pags/prg*s, to avoid confusion in the literature it was decided to name the *S. typhi pag* genes as *pqa* (Pho*P/Q a*ctivated genes), and *prg* genes as *pqr* (Pho*P/Q r*epressed genes) (Baker *et al.*, 1997). The *Salmonella pqa/pqr*::Mu*d*J insertion mutations used in this study were: *pqaA*::Mu*d*J, *pqaB*::Mu*d*J, *pqaC*::Mu*d*J, *pqaD*::Mu*d*J, *pqaE*::Mu*d*J, *pqrA*::Mu*d*J and *pqrB*::Mu*d*J.

3.3 Construction of the *S. typhi* PhoP^c mutant

At the start of this project only the *S. typhimurium* PhoP$^-$, PhoP^c and *S. typhi* PhoP$^-$ strains had been constructed and the *S. typhi* PhoP^c mutant (RMA1032) still contained the *pur*B::Tn*10* insertion (Baker, 1993). As the *pur*B::Tn*10* mutation may have had an unknown affect on future assays, an *S. typhi* PhoP^c, PurB$^+$ strain was needed. The *pur*B::Tn*10* mutation had previously been shown to be unstable when streaked out on minimal media, therefore the

S. typhi RMA1032 strain was repeatedly streaked on MM (containing cysteine, tryptophan and vitamin B1) until the *purB* mutation was lost and a PhoP^c, Tet sensitive strain was isolated. This *S. typhi* PhoP^c strain (RMA1090) was confirmed to be Tet sensitive, an adenine prototroph and gave darker blue colonies than the *S. typhi* Ty2 wildtype strain on LA containing X-pho plates.

3.4 Complementation of the *S. typhimurium* and *S. typhi* PhoP$^-$ mutants

Previously (Baker, 1993), the *phoP/Q*$^+$ and *phoP/Q*^{c} alleles were amplified by PCR with oligonucleotides (#667 and #688 containing *Eco*RI and *Hind*III restriction enzyme sites respectively (Table 2.5)) from chromosomal DNA of *S. typhimurium* LT2 EX730 and *S. typhimurium* LT2 TA2367 respectively. The PCR products were cloned into the *Eco*RI/*Hind*III digested medium copy number, mobilisable plasmid pSUP203 (Simon *et al.*, 1983) to give plasmids pRMSB1 (*phoP/Q*$^+$) and pRMSB3 (*phoP/Q*^{c} (Baker *et al.*, 1997). The DNA fragments containing the *phoP*$^+$ and *phoP24* genes, respectively were then subcloned with *Eco*RI and *Hind*III into similarly digested pBluescript KS$^-$, (Baker *et al.*, 1997). It was found that the pBluescript KS$^-$ *phoP/Q*$^+$ (pRMSB2) and *phoP/Q*^{c} (pRMSB4) plasmids could not be maintained in *Salmonella* as *S. typhi* and *S. typhimurium* PhoP$^-$ strains carrying pRMSB2 and pRMSB4 grew poorly and gave very small colonies that could only be found in the depot areas in plate streakouts (Baker, 1993), indicating that multi-copy *phoP/Q* affects *Salmonella* growth.

For this study it was necessary to clone the *phoP/Q* genes onto a low copy number plasmid for complementation experiments (Section 4.5.3). Therefore the pRMSB2 and pRMSB4 plasmids were digested with *Bam*HI and *Hind*III enzymes to isolate the *phoP/Q* wildtype and constitutive alleles which were then ligated to the similarly digested, low copy number plasmid, pCL1921 (Lerner & Inouye, 1990). After transformation of the *S.*

typhimurium PhoP$^-$ strain RMA1017, blue colonies (indicating PhoN activity) were selected on LA containing spectinomycin (Spc) and X-pho. The resulting plasmids pRMSB34 (*phoP/Q*$^+$) and pRMSB36 (*phoP/Q*^{c}) and the parental plasmid pCL1921 were then electroporated into *S. typhi* PhoP$^-$ mutants, to create RMA1325 (RMA1030 + pCL1921), RMA1327 (RMA1030 + pRMSB34) and RMA1329 (RMA1030 + pRMSB36). Unlike the high copy number pBluescript clones (pRMSB2 and pRMSB4), it was found that these low copy number plasmids carrying *phoP/Q*$^+$ and *phoP/Q*^{c} alleles could easily be maintained within the *S. typhi* host strains, giving "normal" sized colonies and growth streak-out patterns comparable to wildtype *S. typhi* Ty2 on agar plates.

3.5 Magnesium dependent growth of *S. typhi* strains

It has been previously reported, that *S. typhimurium phoP* mutants were unable to grow on N-buffer solidified media containing 40 μM Mg^{2+} (Garcia Vescovi *et al.*, 1996). When *S. typhi* was tested for growth on N-Buffer solidified media, it was found that growth occurred on media with no added Mg^{2+} (Table 3.1). When EGTA and EDTA at 0.1 mM final concentration were added to the N-buffer media to chelate background Mg^{2+}, *S. typhi* PhoP$^-$, PhoP$^+$ and PhoP^c strains were unable to grow on these plates. The addition of 40 μM $MgCl_2$ to these N-buffer plates containing 0.1 mM EGTA and 0.1 mM EDTA allowed growth of PhoP$^+$ and PhoP^c strains but not the PhoP$^-$ strain, and the addition of 200 μM $MgCl_2$ to this medium allowed growth for all three strains (Table 3.1). The *S. typhi pqa/pqr*::Mu*dJ* insertion mutants (Section 3.6) all grew identically to *S. typhi* PhoP$^+$ on the these plates (data not shown). Therefore these results indicate that *phoP/Q* regulates Mg^{2+} dependent growth in *S. typhi* as it does in *S. typhimurium*.

Table 3.1: Growth of *S. typhi* strains on N-buffer agarose plates containg Mg^{2+}

	S. typhi PhoP/Q background[a]		
N-buffer agarose + additives	RMA1030 (PhoP$^-$)	Ty2 (PhoP$^+$)	RMA1090 (PhoP^c)
No added Mg^{2+}	+	+	+
No added Mg^{2+} + 0.1 mM EDTA + 0.1 mM EGTA	-	-	-
40 µM Mg^{2+} + 0.1 mM EDTA + 0.1 mM EGTA	-	+	+
200 µM Mg^{2+} + 0.1 mM EDTA + 0.1 mM EGTA	+	+	+

[a] Indicated strains were streaked on N-buffer agarose plates with the additives indicated
+: growth occurred, -: no growth occurred

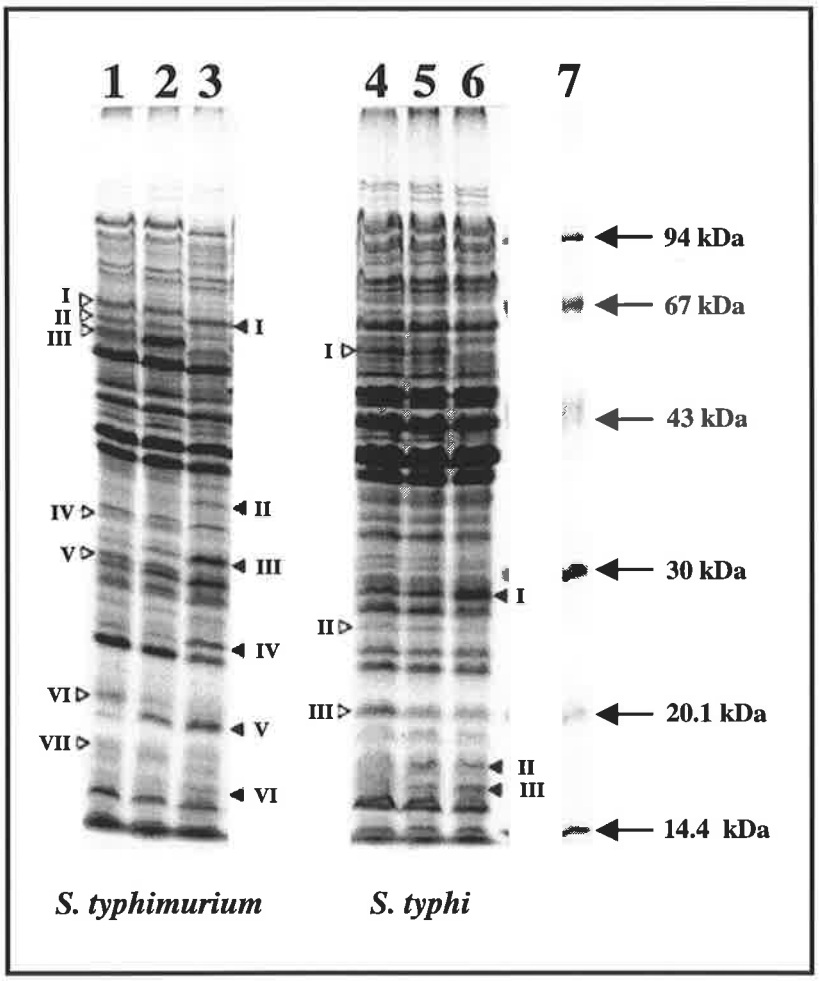
3.6 Comparison of *S. typhi* and *S. typhimurium* PhoP$^-$, PhoP$^+$ and PhoP^c strains

3.6.1 Comparison of *S. typhi* and *S. typhimurium* PhoP/Q regulated proteins

The *phoP/Q* locus regulates the production of 40-50 proteins, as seen by 2D-SDS-PAGE (Miller & Mekalanos, 1990). The whole cell protein profile of *S. typhimurium*, *S. typhi* and their respective PhoP$^-$ and PhoP^c derivatives was compared by SDS-PAGE. A number of proteins (*S. typhimurium*: 13, *S. typhi*: 6, Table 3.2) were observed whose production was dependent on the *phoP* allele present as indicated in Figure 3.2; *S. typhimurium* PhoP$^-$ (RMA1010) had new proteins (Prg's), and *S. typhimurium* PhoP^c (RMA1024) had new proteins (Pag's) relative to *S. typhimurium* PhoP$^+$. PhoP/Q regulated proteins were also detected in *S. typhi* (Figure 3.2, lanes 5, 6). Interestingly, many of the PhoP/Q regulated proteins detected have different relative molecular sizes when *S. typhi* and *S. typhimurium* are compared, and *S. typhi* had fewer detectable PhoP/Q regulated proteins than *S. typhimurium* (Figure 3.2, Table 3.2) using this system. This result suggested that *S. typhi* has several

PhoP/Q regulated genes that are different to *S. typhimurium*, and/or that some of the genes may be expressed at different levels between the *Salmonella* species.

Figure 3.2: Whole cell proteins of *S. typhimurium* C5 and *S. typhi* Ty2 PhoP$^+$, PhoP$^-$ and PhoP^c derivatives.



Whole cell proteins were prepared as described in (Section 2.8.1). 20 µl samples (~ 1x 10^8 cells) were electrophoresed on an SDS 15% polyacrylamide gel and stained with Coomassie Blue. Prg proteins are represented by open triangles (Δ) and Pag proteins by closed triangles (▲). The samples in the lanes are; Lane 1: *S. typhimurium* RMA1010 (PhoP$^-$), Lane 2: *S. typhimurium* C5 (PhoP$^+$), Lane 3: *S. typhimurium* RMA1024 (PhoP^c), Lane 4: *S. typhi* RMA1030 (PhoP$^-$), Lane 5: *S. typhi* Ty2 (PhoP$^+$), Lane 6: *S. typhi* RMA1090 (PhoP^c), Lane 7: Low molecular weight markers (Pharmacia) (Section 2.8.1).

Table 3.2: Apparent molecular weights of *S. typhi* and *S. typhimurium* PhoP/Q regulated proteins

S. typhimurium PhoP/Q regulated proteins				*S. typhi* PhoP/Q regulated proteins			
Pag No.	Size (kDa)	Prg No.	Size (kDa)	Pag No.	Size (kDa)	Prg No.	Size (kDa)
I	69.3	I	61.3	I	54.0	I	28.6
II	64.3	II	34.2	II	26.3	II	17.4
III	59.9	III	30.6	III	20.5	III	16.3
IV	34.0	IV	24.7				
V	31.3	V	19.5				
VI	21.7	VI	16.1				
VII	18.7						

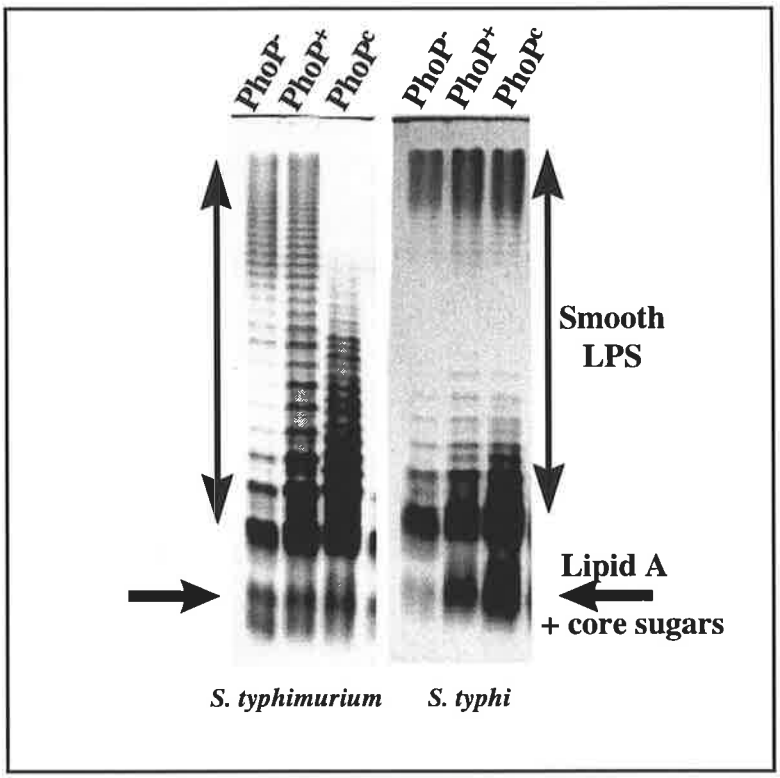
Proteins tabled are derived from Figure 3.2. Protein sizes were calculated using Pharmacia low molecular weight molecular weight markers (kDa) (Section 2.8.1)

3.6.2 Comparison of *S. typhi* and *S. typhimurium* PhoP$^-$, PhoP$^+$ and PhoP^c lipopolysaccharide (LPS)

Previous studies had stated that the *Salmonella* PhoP/Q: PhoP$^-$ and PhoP^c mutations did not affect the O-antigen of LPS on silver stained gels (Galán & Curtiss, 1989B; Miller & Mekalanos, 1990). However, recently it was reported that the PhoP^c mutation affected the ratio of O-antigen sugars to core sugars in *S. typhmurium* strains (Guo *et al.*, 1997). Therefore it was decided to examine the LPS produced by *S. typhi* and *S. typhimurium* wildtype, PhoP$^-$ and PhoP^c strains (Figure 3.3).

As shown in Figure 3.3, the LPS profile of *S. typhi* Ty2 strains had O-antigen chains with a larger model chain length than the LPS of *S. typhimurium* C5. *S. typhi* Ty2 and *S. typhimurium* C5 wildtype strains showed minor differences compared to that of their respective isogenic PhoP$^-$ mutants. However the LPS of the *S. typhimurium* PhoP^c (RMA1024) strain had a dramatically shorter O-antigen chain length compared to the wildtype and PhoP$^-$ strains (Figure 3.3). There was little difference between LPS of the *S. typhi* PhoP^c (RMA1090), wildtype (Ty2) and PhoP$^-$ (RMA1030) strains.

Figure 3.3: Comparison of *S. typhi* and *S. typhimurium* PhoP$^-$, PhoP$^+$ and PhoP^c LPS.



Samples (representing 10^8 cells) were prepared from 18 h cultures and electrophoresed on an SDS-20% polyacrylamide gel, then subjected to silver staining to detect LPS (Section 2.9). The strains in each lane are: *S. typhimurium* RMA1010 (PhoP$^-$), *S. typhimurium* C5 (PhoP$^+$), *S. typhimurium* RMA1024 (PhoP^c), *S. typhi* RMA1030 (PhoP$^-$), *S. typhi* Ty2 (PhoP$^+$) and *S. typhi* RMA1090 (PhoP^c). Smooth LPS: O-antigen chains; LipidA + core sugar molecules are indicated.

The data presented here does not agree with earlier work (Miller & Mekalanos, 1990), but does agree with Guo *et al*, (1997). Figure 3.3 also shows that the LPS of *S. typhi* Ty2 has O-antigen chains with a larger modal chain length than the LPS of *S. typhimurium* C5. All of the *S. typhi pqa/pqr*::Mu*d*J insertion mutants discussed below in section 3.6, had their LPS profile examined and only one of them (the *pqa*B::Mu*d*J mutant) showed any differences compared to the parental strains and this result is presented in Section 6.8.

3.6.3 Comparison of *S. typhi* and *S. typhimurium phoN* regulation on indicator plates

Comparison of the growth of *S. typhimurium* and *S. typhi* PhoP$^+$ and PhoP^c derivatives on X-pho plates showed that the *S. typhimurium* strains gave darker blue colonies than *S. typhi* strains (Table 3.3). This observation suggested that either the *S. typhi* PhoN (non-specific acid phosphatase) has lower activity compared to *S. typhimurium,* or that *S. typhi phoN* expression is not up-regulated by PhoP/Q to the same level as in *S. typhimurium*. The *S. typhi* PhoP$^-$ derivatives gave white colonies on LA + X-pho plates as expected since expression of *phoN* is upregulated by PhoP/Q (Kier *et al.*, 1979; Miller *et al.*, 1989).

Table 3.3: PhoN regulation in *S. typhimurium* and *S. typhi* strains on indicator plates

	PhoP/Q Phenotype		
	LA + X-pho plates[b]		
Strain[a]	PhoP$^-$	PhoP$^+$	PhoP^c
S. typhi Ty2	-	+	++
S. typhimurium C5	-	++	+++
	PhoP$^-$ + pSUP203	PhoP$^-$ + pRMSB1	PhoP$^-$ + pRMSB3
S. typhi RMA1030	-	++	+++
S. typhimurium RMA1010	-	+++	++++
	PhoP$^-$ + pCL1921	PhoP$^-$ + pRMSB34	PhoP$^-$ + pRMSB36
S. typhi Ty2	-	+	++

[a]Strains used are listed in Tables 2.2 and 2.3
[b]Colonies were scored at the single colony level;

-	White
+	Pale blue with white outline
++	Pale blue
+++	Blue with white outline
++++	Intense blue

On LA + X-gal indicator plates, *S. typhi* and *S. typhimurium* strains that have a low amount of PhoN activity gave white colonies whilst those that express high amounts of PhoN activity gave intensely coloured blue colonies. Other strains had colony colours ranging between these two extremes.

Plasmids pRMSB1 (*phoP/Q*$^{+}$) and pRMSB3 (*phoP/Q*^{c}) were able to complement *S. typhi* PhoP^{-} (RMA1030) and *S. typhimurium* PhoP^{-} (RMA1010) strains. The colonies of these complemented strains were a darker blue colour on X-pho plates than the strains with chromosomal *phoP/Q*$^{+}$or *phoP/Q*^{c} alleles (Table 3.3). The pRMSB3 (*phoP/Q*^{c}) complemented strains showed blue colour development after ~ 14 h growth on X-pho plates compared to the pRMSB1 (*phoP/Q*$^{+}$) complemented strains (taking ~ 24 h to produce blue colonies). The PhoN expression for the *S. typhi* complemented strains with the low copy number pCL1921 based plasmids, pRMSB34 (*phoP/Q*$^{+}$) and pRMSB36 (*phoP/Q*^{c}) gave blue colonies that were similar to their respective PhoP^{+} (Ty2) and PhoP^{c} (RMA1090) strains with chromosomally encoded *phoP/Q* genes. A combination of gene dosage due to plasmid copy number and the constitutive mutation in *phoQ* (*phoQ*^{c}) gave the greatest upregulation of *phoN* for both *S. typhimurium* and *S. typhi* strains (ie, the most intensely coloured blue colonies, Table 3.3).

3.6.4 Comparison of *S. typhi* and *S. typhimurium* PhoP^{-}, PhoP^{+} and PhoP^{c} strains: sensitivity to protamine

Defensin-like cationic proteins have been shown to have anti-bacterial activity and both *phoP* and *phoQ* derivatives of *S. typhimurium* have been shown to be hypersensitive to defensins and a variety of antimicrobial peptides (Groisman *et al.*, 1992B; Miller *et al.*, 1990). *S. typhi* and *S. typhimurium* PhoP^{-}, PhoP^{+} and PhoP^{c} strains were tested on for sensitivity to protamine on LA containing this antimicrobial peptide (Table 3.4). It was found that the *S. typhimurium* strains had a greater resistance to protamine than *S. typhi* (Table 3.4) and that the *S. typhimurium* PhoP^{-} strain (RMA1010) had increased sensitivity to protamine compared to the wildtype strain (C5) than the comparable *S. typhi* strains (RMA1030 and Ty2).

Table 3.4: Growth of *S. typhi* and *S. typhimurium* strains on protamine sulfate plates

	LA with protamine sulfate (mg/ml)				
Strain[a]	0.5	0.75	1.0	1.5	2.0
Ty2 ($PhoP^+$)	+	+	-	-	-
RMA1030 ($PhoP^-$)	+	-	-	-	-
RMA1090 ($PhoP^c$)	+	+	-	-	-
C5 ($PhoP^+$)	+	+	+	+	-
RMA1010 ($PhoP^-$)	+	+	-	-	-
RMA1024 ($PhoP^c$)	+	+	+	+	-

[a] All strains can be found in Tables 2.2 and 2.3.
The *S.typhi* and *S. typhimurium* strains were plated for single colonies from liquid cultures grown for 16 h with aeration. +: colonies grew -: no colonies grew

The *S. typhi pqa*::Mu*dJ* insertion mutants (Section 3.6) were also assayed on the LA containing protamine sulphate plates, however none of the mutants showed an increase in sensitivity to protamine compared to the wildtype strain (data not shown).

3.6.5 Comparison of *S. typhi* and *S. typhimurium* $PhoP^-$, $PhoP^+$ and $PhoP^c$ strains: sensitivity to polymyxin B

During the course of this study, *S. typhimurium phoP* mutants were shown to be sensitive to the antibiotic polymyxin B (Gunn *et al.*, 1998B). Therefore I decided to test the *S. typhi* and *S. typhimurium* $PhoP^-$, $PhoP^+$ and $PhoP^c$ strains against varying concentrations of polymyxin B. The assay was based on a MIC (Minimal Inhibitory Concentration) assay (Roland *et al.*, 1994) as outlined in Section 2.11.1. The *S. typhi* strains were found to be more sensitive to polymyxin B than the *S. typhimurium* strains and the *S. typhimurium* $PhoP^-$ strain (RMA1010) had increased sensitivity to polymyxin B compared to the wildtype strain (C5) than the comparable *S. typhi* strains (RMA1030 and Ty2). One of the *S. typhi pqa/pqr*::Mu*dJ* insertion mutants (Section 3.6) showed sensitivity compared to the wildtype strain (data not

shown). The mutant had the *pqa*B::Mu*dJ* insertion and further analysis of polymyxin B sensitivity is reported in Section 6.7.

Table 3.5: Survival of *S. typhi* and *S. typhimurium* PhoP$^-$, PhoP$^+$ and PhoP^c strains with Polymyxin B

	Polymyxin B (μg/ml)						
Strain[a]	0.1	0.2	0.4	0.6	0.8	1	5
RMA1030 (PhoP$^-$)	+	+/-	-	-	-	-	-
Ty2 (PhoP$^+$)	+	+	+/-	-	-	-	-
RMA1090 (PhoP^c)	+	+	+	+/-	-	-	-
RMA1010 (PhoP$^-$)	+	+	+/-	-	-	-	-
C5 (PhoP$^+$)	+	+	+	+	+	+	-
RMA1024 (PhoP^c)	+	+	+	+	+	+	-

[a]All strains can be found in Tables 2.2 and 2.3.
Polymyxin B assay results were based on three assays; +: growth after 20 h; -: no growth after 24 h; +/-: growth in at least one assay after 20 h.

3.7 Analysis of *Salmonella pqa/pqr*::Mu*dJ* insertion mutants

3.7.1 Construction of *S. typhi pqrA*::Mu*dJ* mutants in PhoP$^-$, PhoP$^+$ and PhoP^c chromosomal backgrounds

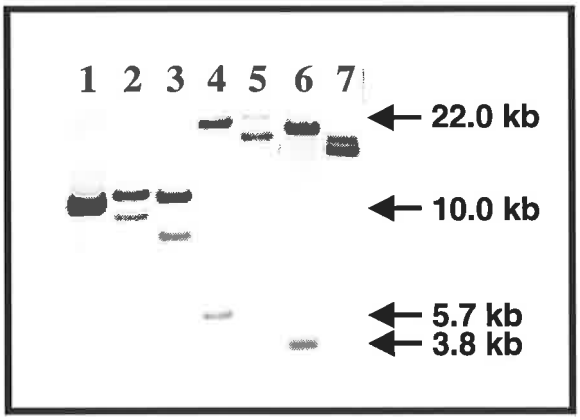
The *pqr*A::Mu*dJ* mutation was initially isolated in an *S. typhimurium* background (Baker, 1993), therefore to enable comparative assays with the other *S. typhi pqa/pqr*::Mu*dJ* insertion mutants, the mutation from *S. typhimurium* RMA1106 was moved into *S. typhi* Ty2 PhoP$^+$, PhoP$^-$ and PhoP^c strains by P22 transduction (Table 2.3) to give the *S. typhi pqaA*::Mu*dJ* strains RMA1182, RMA1180 and RMA1184, respectively. The *pqrA*::Mu*dJ* mutation was also moved into the *S. typhimurium* C5 strain by the same method to give RMA1095 (Table 2.2).

3.7.2 Southern hybridisation analysis of the *S. typhi* Mu*d*J insertion mutants with a *lacZ*/Tn*5* Kan cartridge probe

Previous analysis of the seven *S. typhi phoP/Q* regulated Mu*d*J insertion mutants (*pqaA*::Mu*d*J, *pqaB*::Mu*d*J, *pqaC*::Mu*d*J, *pqaD*::Mu*d*J, *pqaE*::Mu*d*J, *pqrA*::Mu*d*J and *pqrB*::Mu*d*J) included Southern hybridisation with a DIG-11-dUTP labelled LacZ probe using the pCB267 plasmid (Schneider & Beck, 1986) as a template, and chromosomal DNA from each of the mutants digested with *Bgl*II. The results indicated that the Mu*d*J insertions were in different genes, however it was possible that some of the insertions may have been in different places within the same genes or that a double transposon insertion may have occurred (Baker, 1993). To verify that the seven *S. typhi phoP/Q* regulated Mu*d*J insertion mutants had single transposon insertions, a plasmid carrying both the *lacZ* and Kan^R genes to be used as a probe was constructed. The Tn*5* Kan cartridge from pUTKm (Herrero *et al.*, 1990) was isolated by a *Bgl*II/*Sal*I digest and ligated to similarly digested pCB267 (Schneider & Beck, 1986). The ligation was transformed into DH5α and transformants were selected on NA containing Kan and Amp. The resulting plasmid was confirmed to have the expected structure, named pRMSB10 (in RMA1082) and labelled by the random primer method with DIG-11-dUTP (Section 2.7.1.1) to be used as a *lacZ*/Kan probe.

Chromosomal DNA from the seven Mu*d*J insertion strains was then digested with *Sal*I (as a unique *Sal*I site lies between the *lacZ* and Kan genes in Mu*d*J, Figure 3.1), electrophoresed on a 0.7% (w/v) TAE agarose gel, transferred to nylon and probed with DIG-11-dUTP-labelled pRMSB10 (Section 2.7). As can be seen in Figure 3.4, only two bands could be seen for each strain (a doublet can be seen for *S. typhi* RMA1102 in lane 1, Figure 3.4), indicating single, random insertions of Mu*d*J into different *phoP/Q* regulated genes.

Figure 3.4: Southern hybridisation analysis of the *S. typhi pqa/pqr*::Mu*d*J insertion mutants



The *S. typhi pqa/pqr*::Mu*d*J chromosomal DNA was digested with *Sal*I and probed with a DIG-11-dUTP labelled plasmid (pRMSB10) (Section 2.7), detecting both the *lacZ* gene and Kan cartridge within Mu*d*J. The sizes of the bands detected were calculated in kb by comparison with EcoRI digested SPP1 and BglII digested λ DNA (Section 2.5.2.2). All strains can be found in Table 2.2. The *S. typhi* chromosomal DNA samples in the lanes are; Lane 1: RMA1102 (*pqaC*::Mu*d*J), Lane2: RMA1104 (*pqaE*::Mu*d*J), Lane 3: RMA1180 (*pqrA*::Mu*d*J), Lane 4: RMA1110 (*pqaA*::Mu*d*J), Lane 5: RMA1112 (*pqaD*::Mu*d*J), Lane 6: RMA1116 (*pqrB*::Mu*d*J), Lane 7: RMA1126 (*pqaB*::Mu*d*J)

3.7.3 β-galactosidase assay of *S. typhi pqa/pqr*::Mu*d*J mutants on indicator plates

The initial assessment for PhoP/Q regulation of the *S. typhi pqa/pqr*::Mu*d*J mutants was performed on LacZ indicator plates with the *pqa/pqr*::Mu*d*J mutations in a PhoP$^-$ background and complemented by pRMSB1 (*phoP/Q*$^+$) and pRMSB3 (*phoP/Q*^{c}) (Baker, 1993). The *S. typhi pqa/pqr*::Mu*d*J strains with the insertion mutations in PhoP$^-$, PhoP$^+$ and PhoP^c chromosomal backgrounds were streaked onto either MacConkey lactose plates containing Kan or LA plates containing X-gal and Kan, and assessed for β-galactosidase production by the colour of the single colonies (Table 3.6). *S. typhi* PhoP$^-$ *pqa/pqr*::Mu*d*J mutants harbouring plasmids pSUP203, pRMSB1 (pSUP203 + *phoP*$^+$)and pRMSB3 (pSUP203 + *phoP*^{c}) were also assessed for comparison (Table 3.6).

Table 3.6: Analysis of β-galactosidase activity of *pqa/pqr::*Mu*dJ* mutants on LacZ indicator plates.

	PhoP/Q chromosomal background[a]			PhoP/Q plasmid complemented background[a]		
pqa/pqr::Mu*dJ* insertion	PhoP$^-$	PhoP$^+$	PhoP^c	PhoP$^-$ + pSUP203	PhoP + pRMSB1	PhoP$^-$ + pRMSB3
	MacConkey lactose agar plates[b]					
pqaA::Mu*dJ*	-	+	++++	-	+	++++
pqaB::Mu*dJ*	-	++++	++++	-	++++	++++
*pqaC::*Mu*dJ*	-	-	-	-	++	+++
pqaD::Mu*dJ*	+	++	++	+	++	++++
pqaE::Mu*dJ*	-	-	++	-	++	+++
pqrA::Mu*dJ*	++++	++++	+++	++++	++++	++
	X-gal Luria agar plates[c]					
pqrB::Mu*dJ*	+++	++	+	++++	+++	++

[a]Strains used are listed in Table 2.3

[b]Colonies were scored at the single colony level;

-	White
+	Pale pink
++	Red with white outline
+++	Solid red/pink
++++	Bright pink plus pinkish halo

On MacConkey lactose indicator plates, *S. typhi pqa/pqr*::Mu*dJ* strains that have a low amount of β-galactosidase (LacZ) gave white-pale pink colonies whilst those that express high amounts gave intensely coloured red or pink colonies with a pinkish halo surrounding the colony for those expressing the highest amount of β-galactosidase (LacZ). Other strains had colony colours ranging between these two extremes.

[c]Colonies were scored at the single colony level with;

-	White
+	Pale blue
++	Blue with white outline
+++	Solid blue
++++	Intense dark blue

On LA containing X-gal indicator plates, *S. typhi pqa/pqr*::Mu*dJ* strains that have a low amount of β-galactosidase (LacZ) gave white colonies whilst those that express high amounts gave intensely coloured blue colonies. Other strains had colony phenotypes ranging between these two extremes.

Strong up-regulation of *pqa*::Mu*dJ* fusions and down-regulation of *pqr*::Mu*dJ* fusions (by observation of LacZ activity) in the PhoP^c backgrounds compared to the PhoP$^-$ and PhoP$^+$ backgrounds for all seven *pqa/pqr*::Mu*dJ* fusions was observed (Table 3.6). The plasmid

complemented strains showed a greater increase/decrease in regulation than the strains containing chromosomal *phoP/Q*$^{+}$ and *phoP/Q*^{c} alleles. This correlates with increased regulation of PhoN activity by plasmid located *phoP/Q* noted above in Section 3.6.3.

3.7.4 β-galactosidase assays of *S. typhi pqa/pqr*::Mu*d*J strains grown in liquid culture

The *S. typhi pqa/pqr*::Mu*d*J mutants were grown to stationary phase in liquid media (LB) and the level of β-galactosidase (LacZ) was determined (Table 3.7) (Section 2.10). Attempts were made to grow the *S. typhi pqa/pqr*::Mu*d*J mutants in MacConkey lactose broth to correlate the assays better with the plate data. However it was found that although *S. typhi* PhoP^{+} (Ty2) and PhoP^{c} (RMA1090) strains could grow in this media, the PhoP^{-} (RMA1010) strain could not. This growth situation correlates with the fact that the PhoP/Q regulon is involved with bile resistance (Van Velkinburgh & Gunn, 1999) and that MacConkey broth contains Bacto Oxgal (5 g/l) which contains bile salts that are likely to be at too high a concentration for the growth of *S. typhi* PhoP^{-} strains. The *S. typhi* PhoP^{-} were able to grow on MacConkey lactose plates however, possibly because this is a slightly different media and contains Bacto Bile Salts no 3 (1.5 g/l) and therefore may have a lower concentration of bile salts than the broth media, allowing the *S. typhi* PhoP^{-} strains to grow. Therefore the β-galactosidase assays were only performed on bacteria grown in LB

The β-galactosidase assay results of liquid media grown strains (Table 3.7) correlated with those obtained with solidified media for five of the mutant strains (Table 3.6), however the results for *S. typhi* with the *pqaE*::Mu*d*J and *pqaC*::Mu*d*J insertion mutations did not correlate as well. As plasmid complemented strains appeared to have an increased *phoP/Q* regulation on plates, these two mutants were then assayed using liquid media grown cultures using the plasmid complemented strains (the *pqa*::Mu*d*J insertion in a PhoP^{-} mutant background complemented with pSUP203, pRMSB1 (*phoP/Q*$^{+}$) and pRMSB3

(*phoP/Q*^{c}))(Table 3.8). The results in Table 3.8, although suggesting that *phoP/Q* regulation was occurring, were not statistically significant and therefore no further analysis of the *S. typhi* *pqaC*::Mu*dJ* and *pqaE*::Mu*dJ* insertion mutants was undertaken in this study.

Table 3.7: β-galactosidase (LacZ) enzyme assays for *pqa/pqr*::Mu*dJ* mutants

	PhoP/Q chromosomal background[a]			
pqa/pqr::Mu*dJ* insertion	PhoP$^-$ (RMA1030)	PhoP$^+$ (Ty2)	PhoP^c (RMA1090)	PhoP$^-$:PhoP^c Regulation
pqaA::Mu*dJ*	174 ± 22.4	462 ± 98.2	1867 ± 161.9	↑ 10.7 fold
pqaB::Mu*dJ*	77 ± 14.5	645 ± 89.3	1113 ± 80.9	↑ 14.5 fold
pqaC::Mu*dJ*	166 ± 11.0	153 ± 17.1	163 ± 7.59	↑ 1.0 fold
pqaD::Mu*dJ*	289 ± 36.1	446 ± 79.5	458 ± 56.1	↑ 1.6 fold
pqaE::Mu*dJ*	97 ± 16.6	120 ± 18.8	140 ± 29.0	↑ 1.4 fold
pqrA::Mu*dJ*	1040 ± 46.8	1007 ± 64.9	374 ± 49.0	↓ 2.8 fold
pqrB::Mu*dJ*	465 ± 85.9	375 ± 64.5	206 ± 6.4	↓ 2.3 fold

[a] Strains used are listed in Table 2.3
The indicated strains were grown to stationary phase (16 h growth with aeration) and β-galactosidase (LacZ) activity was determined (Section 2.10). The PhoP$^-$:PhoP^c increased/decreased PhoP/Q regulation ratio, the mean value and the standard deviation (SD) were calculated from 3-4 repeat experiments.

Table 3.8: β-galactosidase assays for plasmid complemented *S. typhi* *pqa*::Mu*dJ* mutants.

	PhoP/Q Plasmid complemented background[a]		
pqa::Mu*dJ* insertion	PhoP$^-$ + pSUP203	PhoP$^-$ + pRMSB1	PhoP$^-$ + pRMSB3
pqaC::Mu*dJ*	101 ± 4.2	117 ± 7.8	164 ± 25.5
pqaE::Mu*dJ*	60 ± 8.5	103 ± 2.8	118 ± 21.9

[a] Strains used are listed in Table 2.3
The indicated strains were grown to stationary phase (16 h growth with aeration) and β-galactosidase (LacZ) expression was analysed by liquid enzyme assays (Section 2.10). The mean value and the standard deviation (SD) were calculated from repeated 3-4 experiments.

3.8 Identification of the *S. typhi pqa/pqr* genes

Analysis of the Mu*d*J insertions in the *S. typhi pqaA*::Mu*d*J, *pqaB*::Mu*d*J, *pqaD*::Mu*d*J, *pqrA*::Mu*d*J and *pqrB*::Mu*d*J fusions in PhoP$^-$, PhoP$^+$ and PhoP^c backgrounds showed that they appeared to have single, unique Mu*d*J insertions and β-galactosidase assays clearly showed that they were all PhoP/Q regulated (Section 3.7). The next stage of this study was to identify the *S. typhi pqa/pqr* genes by isolating DNA sequences adjacent to the Mu*d*J transposon insertion from each of the fusion mutants and find whether the genes had homology to known or were novel *Salmonella* PhoP/Q regulated genes. The sequence identification of the *S. typhi pqaD*, *pqrA* and *pqrB* mutants is described in the following sections. Sequence identification of the *S. typhi pqaA* and *pqa*B mutants and further characterisation of these genes will be discussed in Chapters 5 and 6, respectively. Two methods were used to isolate flanking DNA from the Mu*d*J mutants. The first involved Inverse PCR (IPCR) (Ochman *et al.*, 1988) based on the LHS of the Mu*d*J transposon and was used to identify the *S. typhi pqaD* and *pqrA* genes (Section 3.8.1). The second method used the Single Sided Primer PCR (SSP-PCR) method (Shyamala & Ames, 1993) which was used to identify the *S. typhi pqrB* gene (Section 3.8.2)

3.8.1 Identification of the *S. typhi pqaD* and *pqrA* genes with inverse PCR using Mu*d*JL

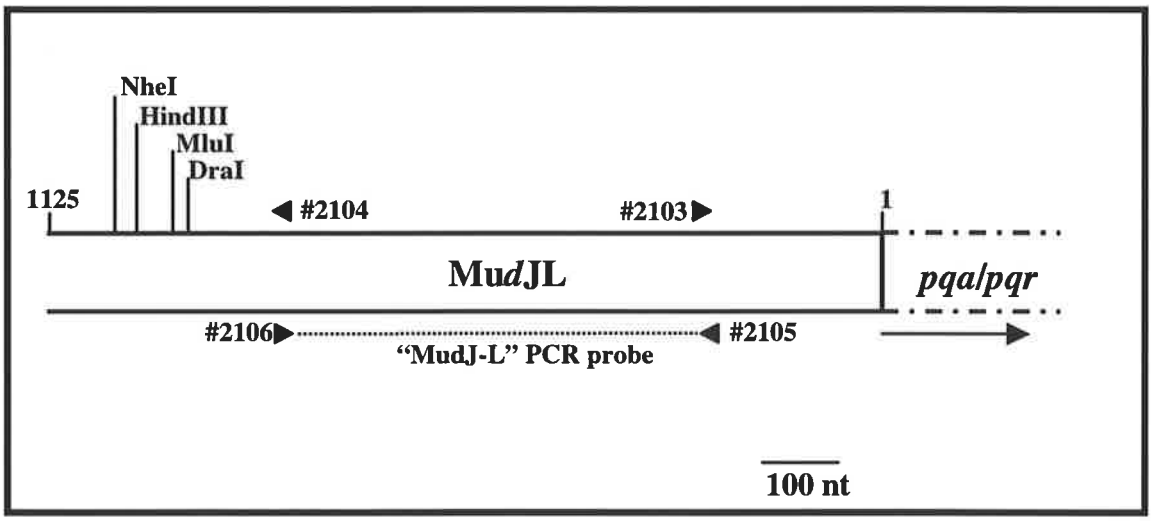
3.8.1.1 Outline of the Mu*d*JL IPCR method

The first method used to identify the sequence of the *S. typhi pqaD* and *pqrA* genes was IPCR based on the sequence of the left hand side (LHS) of the Mu*d*J transposon (Mu*d*JL) insertion directly adjacent to the *S. typhi* genes. Mu*d*JL is represented in Figure 3.5, which outlines the position of the oligonucleotides and restriction enzyme sites used for the IPCR. As the entire Mu*d*J transposon is over 11.3 kb in size (Castilho *et al.*, 1984) (Figure 3.1),

Figure 3.5 represents 1.125 kb of Mu*dJ*, and the end of the LHS of the transposon is labelled as nt 1. Four oligonucleotides #2103, #2104, #2105 and #2106 (Table 2.5) were designed to help identify the *S. typhi pqa/pqr* sequences.

The oligonucleotides #2103 and #2104 were designed to read out of the Mu*dJ* transposon during the IPCR reaction. Oligonucleotides #2105 and #2106 were designed to PCR amplify a section of Mu*dJL* to create a "Mu*dJ*-L" DIG-11-dUTP, PCR-labelled, 619 nt probe for Southern hybridisation analysis of the *S. typhi pqa/pqr*::Mu*dJ* insertion mutants (Section 2.7). Four restriction enzyme sites located 100-150 nt distant from the #2104 oligonucleotide (*Dra*I, *Mlu*I, *Hind*III and *Nhe*I), were used for the isolation of the fragments of DNA for the IPCR reaction template.

Figure 3.5: Schematic diagram of Mu*dJL*



The LHS of the Mu*dJ* transposon (Priess *et al.*, 1987) (gbD: M64097) is outlined in a solid line and labelled 1 - 1125 nt from the LHS edge. The *S. typhi pqa/pqr* gene adjacent to the transposon insertion is represented as a dashed line with an arrow indicating the direction of transcription. Oligonucleotides and their positions; #2103 (nt 251-228), #2104 (nt 835-857 nt), #2105 (nt 224-242) and #2106 (nt 861-843) are represented by arrow heads (▲) representing the direction of extension from the 3' end, restriction enzyme sites; *Dra*I (nt 935), *Mlu*I (nt 953), *Hind*III (nt 1002) and *Nhe*I (nt 1031) and their relative positions in the transposon are also shown.

S. typhi RMA2312 (*pqaD*::Mu*dJ*), RMA2316 (*pqrB*::Mu*dJ*) and RMA1182 (*pqrA*::Mu*dJ*) chromosomal DNA was digested with the enzymes *Dra*I, *Mlu*I, *Hind*III and *Nhe*I, electrophoresed on a 0.7% (w/v) TAE agarose gel, transferred to nylon and probed with the DIG-11-dUTP labelled "Mu*dJ*-L" probe (Figure 3.5; Section 2.7). The Southern hybridisation band sizes for the *D*raI, *Mlu*I and *Hind*III digests are represented in Table 3.9 as are the expected IPCR band sizes.

Table 3.9: Southern hybridisation analysis of *S. typhi pqa/pqr*::Mu*dJ* chromosomals with the "Mu*dJ*-left" probe

	Restriction enzyme					
	*Dra*I		*Mlu*I		*Hind*III	
Strains[a]	Southern band size (kb)	Predicted IPCR product size (kb)	Southern band size (kb)	IPCR product size (kb)	Southern band size (kb)	Predicted IPCR product size (kb)
RMA2312 (*pqaD*::Mu*dJ*)	3.2	2.6	1.55	0.95	2.0	1.4
RMA1182 (*pqrA*::Mu*dJ*)	2.95	2.35	1.85	1.25	9	8.4
RMA2316 (*pqrB*::Mu*dJ*)	8.1	7.5	9	8.4	>9	>9

[a] All strains are represented in Table 2.3
Chromosomal digests were probed with "Mu*dJ*-L" DIG-11-dUTP labelled probe (Section 2.7) and the sizes of the bands are given in kb and were calculated compared to *Eco*RI digested SPP1 markers (Section 2.5.2.2). The IPCR product size expected is ~ 600 bp less than the Southern hybridisation band.

DNA fragments between 1 and 3 kb were judged to be suitable for the IPCR reaction, which would give an IPCR reaction product of ~600 nt smaller in size than the corresponding Southern hybridisation band (Table 3.9) as the oligonucleotides #2103 and #2104 extend away from the section of the Mu*dJ* transposon used for the probe (Figure 3.5). The results (Table 3.9) indicated that the *Mlu*I and *Hind*III fragments could be used for inverse PCR with RMA2312 (*pqaD*::Mu*dJ*) DNA. A suitable sized fragment was also obtained for the *Mlu*I digest for RMA1182 (*pqrA*::Mu*dJ*). The RMA2316 (*pqrB*::Mu*dJ*) strain gave large fragments

(> 8 kb) for all three digests indicating that the IPCR method could not be used, therefore the SSP-PCR method was used to obtain sequence for this gene (Section 3.8.2).

3.8.1.2 Identification of the *S. typhi pqaD* gene

Southern analysis of the RMA2312 (*pqaD*::Mu*dJ*) chromosome digested with *Mlu*I and *Hind*III gave predicted fragments of 0.95 kb (*Mlu*I) and 1.4 kb (*Hind*III). RMA2312 chromosomal DNA was digested with *Mlu*I and *Hind*III, diluted for circularisation and religated, the ligation mixture was then used as a template for the IPCR with the oligonucleotides #2103 and #2104 (Section 2.5.3.3). Both the *Mlu*I and *Hind*III ligation mixtures gave an IPCR product of expected size and these PCR products were ligated to pGEMT and transformed into DH5α, plated out onto NA + Amp plates and transformants screened for the correct insertion. Only the *Mlu*I IPCR product was cloned, the resultant plasmid was named pRMSB25 and was sequenced with #2103 using the dye-terminator reaction (Section 2.5.2.6). The sequence from the oligonucleotide #2103 was analysed with BlastN/X 2.0 (Altschul *et al.*, 1997). The expected Mu*dJ* LHS sequence was found at the beginning of the sequence (Figure 3.6a) and after the end of the transposon, the *S. typhi pqaD* gene showed 80% homology at the DNA level (data not shown) and ~ 91% homology at the amino acid level to the *E. coli* K12 *deoA* gene (Figure 3.6b). The *E. coli* K12 *deoA* gene is located in the MG1655 section 398 ((nt 8877-10199) (AE000508) (Blattner *et al.*, 1997)) of the complete *E. coli* K12 genome and encodes a 440 aa protein. It was determined that the Mu*dJ* transposon had inserted between nt 538 and 539 of the *deoA* gene sequence, hence the *S. typhi pqaD*::Mu*dJ* mutation is an insertion in the *S. typhi deoA* gene.

The *deoA* gene is located at min 99.5 on the *E. coli* chromosome, with the corresponding location at 99.0 min on the *S. typhimurium* chromosome (Sanderson & Roth, 1988). DeoA is thymidine phosphorylase which catalyses the reversible phosphorolysis of pyrimidine

deoxyribonucleosides except for 4-amino-substituted compounds such as deoxycytidine and is involved with the pyrimidine salvage pathway. The *deoA* gene is encoded within the deo operon (*deoC*, *deoA*, *deoB* and *deoD*) which has been shown to be negatively regulated by CytR and DeoR and positively regulated by the cAMP-CRP complex (Cohen & Wolfenden, 1971; Hammer-Jespersen, 1983).

Figure 3.6a: BlastN 2.0 analysis of pRMSB25 insert – Mu*d*JL homology

```
pRMSB25:   1 ttttttgaagctgttattgaaatgatttgcagtgtttttgtttttcgtattttcaatgaa 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MudJL:   213 ttttttgaagctgttattgaaatgatttgcagtgtttttgtttttcgtattttcaatgaa 154

pRMSB25:  61 acaaaagcaatttttttactatctttctcgtttcattgattaacgactaaaatttgcacta 120
             |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
MudJL:   153 acaaaagcaatttttttactatctttcgcgtttcattgattaacgactaaaatttgcacta 94

pRMSB25: 121 caggcttgcaagccccaccaaatctaatcccatcagatcccgaataatccaatgtcctcc 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MudJL:    93 caggcttgcaagccccaccaaatctaatcccatcagatcccgaataatccaatgtcctcc 34

pRMSB25: 181 cggtttntttcgtacttcaagtgaatcaataca 213
             |||||| |||||||||||||||||||||||||
MudJL:    33 cggtttttttcgtacttcaagtgaatcaataca 1
```

Figure 3.6b: BlastX 2.0 analysis of pRMSB25 insert – DeoA homology

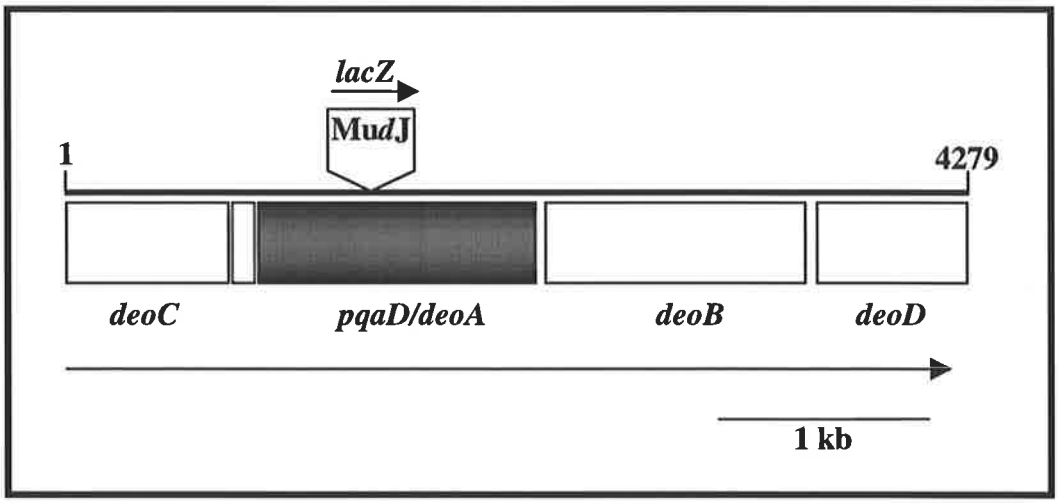
```
pRMSB25: 215 PLITGSILAKKLAEGLDALVMDVKVGSGAFMPTYELSEALAETIVGVANGAGVRTTALLT 394
             PLIT SILAKKLAEGLDALVMDVKVGSGAFMPTYELSEALAE IVGVANGAGVRTTALLT
DeoA:    181 PLITASILAKKLAEGLDALVMDVKVGSGAFMPTYELSEALAEAIVGVANGAGVRTTALLT 240

Query:   395 DMNQVLASSAGNAVEVREAVQFLTGEYRNPRLFDITMALCVEMLISGQLAKRDTQTRAKL 574
             DMNQVLASSAGNAVEVREAVQFLTGEYRNPRLFD+TMALCVEMLISG+LAK D + RAKL
DeoA:    241 DMNQVLASSAGNAVEVREAVQFLTGEYRNPRLFDVTMALCVEMLISGKLAKDDAEARAKL 300

Query:   575 QGVL 586
             Q VL
DeoA:    301 QAVL 304
```

This figure shows the pRMSB25 insert sequence analysed with Blast 2.0 (Altschul *et al.*, 1997) and the sequence homology (a) to Mu*d*J left (gbD: M64097) (Priess *et al.*, 1987) and (b) to the *E. coli* DeoA protein (gbP: P07650) (Burland *et al.*, 1995), (gbD: AE000508) (Blattner *et al.*, 1997).

Figure 3.7 shows the Mu*d*J insertion in the *pqaD/deoA* gene, adjacent genes to *deoA* and their direction of transcription. Computer analysis of the 4.58 kb *E. coli* DNA sequence used in Figure 3.7 with BlastN 2.0 against the Sanger Centre *S. typhi* sequencing project released sequence contigs (Sanger Centre, 1999) gave 87% DNA identity between the entire 4.58 kb of

E. coli and *S. typhi* sequence, confirming that the *deoA* gene and adjacent genes are arranged in the same way in *S. typhi*.

Figure 3.7: The *E. coli deo* operon and Mu*d*J insertion

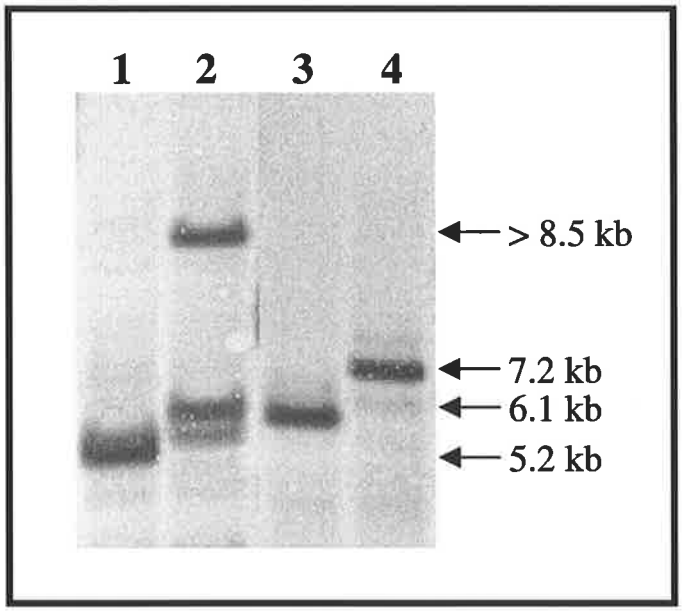


This figure is based on sequence from *E. coli* (gbD: AE000508; Blattner *et al.*, 1997) and shows the *E. coli deo* operon and direction of transcription of the operon (→). The four genes of the *deo* operon are labelled with the small intergenic region between *deoC* and *deoA* shown (dotted box). The Mu*d*J transposon insertion into the *S. typhi pqaD*/*E. coli deoA* gene at 538 nt into the *deoA* gene is also shown.

Confirmation of the *pqaD/deoA* Mu*d*J transposon insertion involved Southern hybridisation analysis with a 1.57 kb *E. coli deoA* gene PCR DIG-11-dUTP labelled probe using oligonucleotides (#2636: nt 8722-8740 and #2637: nt 10292-10273), designed from the *E. coli deoA* sequence (Blattner *et al.*, 1997) (gbD: AE000508) (Table 2.5).

S. typhi Ty2 and RMA2312 (*pqaD*::Mu*d*J) chromosomal DNA was digested with a number of enzymes and probed with the PCR DIG-dUTP labelled *deoA* probe The *Eco*RV and *Hinc*II digests gave the most prominent differences in band sizes between the two strains (Figure 3.8), and the motility of the bands seen for *S. typhi* RMA2312 chromosomal DNA are clearly different compared to the wildtype *S. typhi* DNA, confirming that the Mu*d*J insertion in RMA2312 is contained within the *S. typhi deoA* gene.

Figure 3.8: Southern hybridisation analysis of the *S. typhi* RMA2312 Mu*dJ* insertion mutant



Chromosomal DNA from *S. typhi* Ty2 and *S. typhi* RMA2312 *(pqaD*::Mu*dJ*) was digested with *Eco*RV or *Hinc*II and probed by Southern hybridisation analysis with the DIG-11-dUTP labelled "*deoA*" probe (Section 2.7). The sizes of the bands detected were calculated in kb by comparison with *Eco*RI digested SPP1 markers (Section 2.5.2.2) and the lanes are numbered and contain; Lane 1: *S. typhi* Ty2 [*Eco*RV], Lane 2: *S. typhi* RMA2312 [*Eco*RV], Lane 3: *S. typhi* Ty2 [*Hinc*II], Lane 4: *S. typhi* RMA2312 [*Hinc*II]

The *E. coli deo* operon was also found to have two REP (repetitive extragenic palindromic) elements located in the intergenic region between *deoA* and *deoC*. These REP elements have previously been suggested to form a strong stem loop structure, affecting the translation of *deoA* mRNA. This is consistent with the fact that *deoC* is expressed at a higher level than the distal *deoA*, *deoB* and *deoD* genes, therefore possibly acting as a transcriptional attenuator (Valentin-Hansen *et al.*, 1982). These REP sequences are also found in other intergenic regions on the *E. coli* K12 chromome, including upstream of *livK* (LivK: a leucine-specific binding protein (LS-BP)) (gbD: JO5516) which is involved with the transport of branched-chain amino acids into the cytoplasm (Landick & Oxender, 1985), upstream of *sucA* (SucA: a 2-oxoglutarate dehydrogenase) (gbD: AE111175) involved with energy metabolism of carbon and the TCA cycle (Darlison & Guest, 1984) and upstream of *dppB* (DppB: a transmembrane protein) (gbD: L08399) in the *dpp* locus which encodes a dipeptide transporter

system (Olsen *et al.*, 1991). None of these genes (*livK*, *sucA* or *dppB*) have been shown to be regulated by *phoP/Q* and time constraints prevented further study of these REP elements and their potential involvement in PhoP/Q regulation.

3.8.1.3 Identification of the *S. typhi pqrA* gene

Southern analysis with the "Mu*d*J-left" probe of the RMA1182 *pqrA*::Mu*d*J chromosomal DNA digested with *Mlu*I gave a predicted IPCR product of 1.25 kb (Table 3.9). Therefore, the chromosomal DNA was digested with *Mlu*I, diluted for circularisation, relegated, and used as a template for an IPCR reaction with oligonucleotides #2103 and #2104 (Section 2.5.3.3). A PCR product of the expected size was obtained, ligated to pGEMT, transformed into DH5α, and Amp resistant transformants were screened for the correct insert. The insert in the resultant plasmid pRMSB20 was then sequenced with the oligonucleotide #2103 and dye-terminator sequencing (Section 2.5.2.7.1). The sequence was analysed by BlastN/X 2.0 (Altschul *et al.*, 1997) and gave the expected homology to the left end of Mu*d*J (Figure 3.9a). After the end of the Mu*d*J transposon, the DNA sequence showed 82% homology to the *E. coli gcvA* gene at the DNA level (data not shown) and 72% at the amino acid level (Figure 3.9b).

The *E. coli gcvA* gene is located in the MG1655 section 254 ((nt 7719-8636) (gbD: AE000364) (Blattner *et al.*, 1997)) of the complete genome and encodes a 305 aa protein. It was determined that the Mu*d*J transposon had inserted between nt 561 and 562 of the *gcvA* gene sequence, hence the *S. typhi pqrA*::Mu*d*J mutation is in the *S. typhi gcvA* gene. GcvA is located at min 63.3 min on the *E. coli* K12 chromosome with the corresponding position at 65.1 min on the *S. typhimurium* chromosome (Sanderson *et al.*, 1995) .

Figure 3.9a: BlastN 2.0 analysis of pRMSB20 insert – Mu*d*JL homology

```
pRMSB20:  76 actatctttctcctttcattgattcaccactactatctccactacccgcttgcaagcccc 135
           |||||||||| | |||||||||| || ||||  || | ||||||  ||||||||||||
MudJL:   137 actatctttcgcgtttcattgattaacgactaaaatttgcactacaggcttgcaagcccc 78

pRMSB20: 136 accaaatcttatcccatcagatcccgaataatccaatgtcctcccggtttttttcgtact 195
           ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
MudJL:    77 accaaatctaatcccatcagatcccgaataatccaatgtcctcccggtttttttcgtact 18


pRMSB20: 196 tcaagtgaatcaataca 212
           ||||||||||||||||
MudJL:    17 tcaagtgaatcaataca 1
```

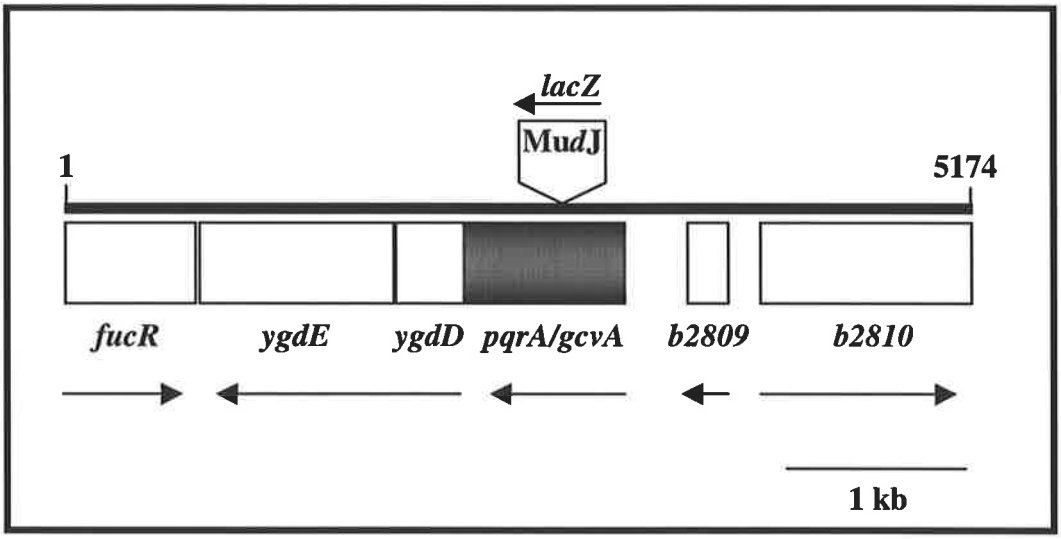
Figure 3.9b: BlastX 2.0 analysis of pRMSB20 insert – DeoA homology

```
pRMSB20:  213 LTGEKPLKTPEDLAKHTLLHDASRRDWQAYTRQLGLNHINVQQGPIFTTARWCYRPLFTD 392
              LTGEKPLKTPEDLAKHTLLHDASRRDWQ YTRQLGLNHINVQQGPIF+ +     +
GcvA:     174 LTGEKPLKTPEDLAKHTLLHDASRRDWQTYTRQLGLNHINVQQGPIFSHSAMVLQAAIHG 233

Query:    393 RG-SRWRIMLWRI*N*TGRLVCPFNDVLVSKNAFYLVCHDSQAELG 527
              +G +     ++ +      GRLVCPFNDVLVSKNAFYLVCHDSQAELG
GcvA:     234 QGVALANNVMAQSEIEAGRLVCPFNDVLVSKNAFYLVCHDSQAELG 279
```

This figure shows the pRMSB20 insert sequence analysed with Blast 2.0 (Altschul *et al.*, 1997) and the sequence homology (a) to Mu*d*J left (gbD: M64097) (Priess *et al.*, 1987) and (b) to the *E. coli* GcvA protein (gbP: U01030) (Wilson & Stauffer, 1994), (gbD: AE000364) (Blattner *et al.*, 1997)

The *E. coli gcvA* gene encodes a glycine cleavage activator protein which is a LysR type transcriptional regulator protein (Wilson & Stauffer, 1994). GcvA has been shown to activate the *gcv* operon in the presence of glycine and represses in the presence of purines (Wilson & Stauffer, 1994). Figure 3.10 shows the Mu*d*J insertion in the *pqrA/gcvA* gene, genes adjacent to *gcvA* and their direction of transcription. Computer analysis of the 6.47 kb *E. coli* DNA sequence used in Figure 3.10 with BlastN 2.0 against the Sanger Centre *S. typhi* sequencing project released sequence contigs (Sanger Centre, 1999)] gave 82% DNA identity between the entire 6.47 kb of *E. coli* and *S. typhi* sequence, confirming that the *gcvA* gene and adjacent genes are arranged in the same way in *S. typhi*.

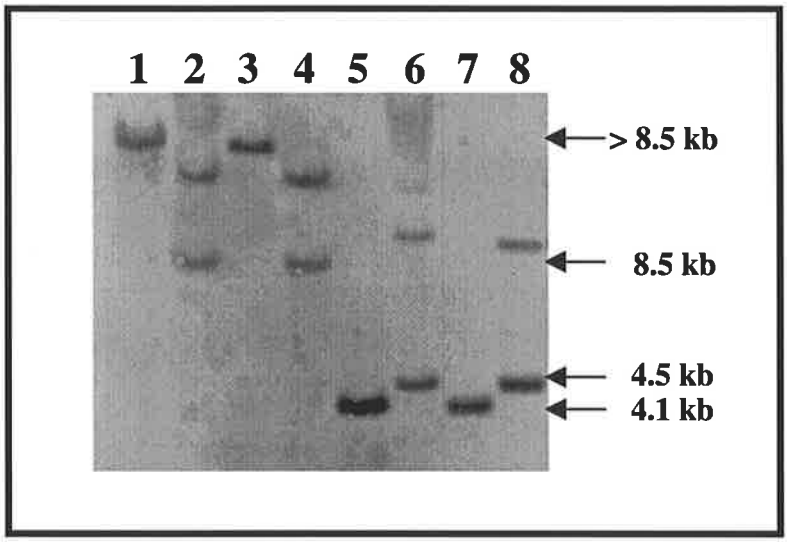
Figure 3.10 The *E. coli gcvA* gene, Mu*dJ* insertion and flanking genes.



This figure is based on sequence from *E. coli* (gbD: AE000364) (Blattner *et al.*, 1997) and shows the *E. coli gcvA* gene (shaded box) which lies downstream of the *fucR* gene and is flanked by *E. coli* genes encoding proteins with no known homologies. The direction of transcription of all the genes are indicated by arrows. The Mu*dJ* transposon insertion into the *S. typhi pqrA/E. coli gcvA* gene at 561 bp is also shown.

Confirmation of the *S. typhi pqrA/gcvA* Mu*dJ* transposon insertion involved Southern hybridisation analysis with a 1.14 kb *E. coli gcvA* gene PCR DIG-11-dUTP-labelled probe, made using oligonucleotides (#2640: nt 1287-1269 and #2641: nt 127-145) (Table 2.5), designed from the *E. coli gcvA* sequence (gbD: AE000364) (Blattner *et al.*, 1997). *S. typhi* Ty2 , *S. typhimurium* C5, RMA2312 and RMA1195 (*pqrA*::Mu*dJ*) chromosomal DNA were digested with a number of enzymes and probed with the PCR DIG-dUTP labelled *deoA* probe (Section 2.7). The *Eco*RV and *Hind*III digests gave the most prominent differences in band sizes between the strains (Figure 3.11) and two bands can be seen for the *S. typhi* and *S. typhimurium pqrA*::Mu*dJ* insertion mutants chromosomal DNA compared to the wildtype *S. typhi* DNA, confirming that the Mu*dJ* insertions in RMA1182 and RMA1095 are contained within the *Salmonella gcvA* genes.

Figure 3.11: Southern hybridisation analysis of the *S. typhi* and *S. typhimurium* pqrA Mu*dJ* insertion mutants



Chromosomal DNA from the *Salmonella* strains listed below were digested with either *Hind*III or *Eco*RV enzymes and subjected to Southern hybridisation analysis with the DIG-11-dUTP labelled "*gcvA*" probe (Section 2.7). The sizes of the bands detected were calculated in kb by comparison with *Eco*RI digested SPP1 markers (Section 2.5.2.2). All strains can be found in Table 2.2 and Table 2.3. The *Salmonella* chromosomal DNA samples in the lanes are; Lane 1: *S. typhi* Ty2 [*Hind*III], Lane2: *S. typhi* RMA1182 (*pqrA*::Mu*dJ*) [*Hind*III], Lane 3: *S. typhimurium* C5 [*Hind*III], Lane 4: *S. typhimurium* RMA1195 (*pqrA*::Mu*dJ*) [*Hind*III], Lane 5: *S. typhi* Ty2 [*Eco*RV], Lane 6: *S. typhi* RMA1182 (*pqrA*::Mu*dJ*) [*Eco*RV], Lane 7: *S. typhimurium* C5 [*Eco*RV], Lane 8: *S. typhimurium* RMA1195 (*pqrA*::Mu*dJ*) [*Eco*RV]

3.8.2 Identification of the *S. typhi pqrB* gene

Southern hybridisation analysis with the "Mu*dJ*-left" probe showed that the *Dra*I, *Mlu*I and *Hind*III fragments for the *S. typhi pqrB*::Mu*dJ* DNA (Section 3.8.1, Table 3.9) were too large for an IPCR reaction. Therefore it was decided to use the Single Specific Primer Polymerase Chain Reaction (SSP-PCR) method to obtain sequence for the *pqrB* gene. The SSP-PCR method (Shyamala & Ames, 1993) can be used to amplify DNA fragments for which sequence is only available at one end (Section 2.5.3.4). *Hind*III was chosen as the "base enzyme" for the SSP-PCR method as it gave a large fragment of over 9 kb with the "Mu*dJ*-left" probe and was known to restrict at just over 1 kb into the LHS of the Mu*dJ* transposon (Figure 3.5). The *S. typhi pqrB*::Mu*dJ* (RMA2316) chromosomal DNA was double digested with *Hind*III and a variety of enzymes that are known to cut once within the

pBluescript multiple cloning site, electrophoresed on a 0.7% (w/v) TAE gel, transferred to nylon and probed with DIG-11-dUTP " Mu*d*JL" PCR probe (Section 2.7). The double digests which gave bands between 1 and 3 kb are listed below in Table 3.10. The expected SSP-PCR size is approximately 0.75 bp less than the fragment size from the Southern analysis, as the specific primer used was #2103 (located ~ 250 bp into the LHS of Mu*d*J, Figure 3.5) which is 0.75 kb distant from the *Hind*III site in the LHS of Mu*d*J (Figure 3.5).

Table 3.10: Southern hybridisation analysis of *S. typhi pqrB*::Mu*d*J (RMA2316) chromosomal double digests with the "Mu*d*J-left" probe.

Digest	Band Size (kb)	Predicted SSP-PCR Size (kb)
*Hind*III/*Bam*HI	1.40	0.65
*Hind*III/*Cla*I	1.56	0.81
*Hind*III/*Eco*RI	2.27	1.52
*Hind*III/*Hinc*II	1.53	0.78
*Hind*III/*Sal*I	2.49	1.74

Chromosomal DNA from RMA2316 was digested with the indicated enzymes and probed with the DIG-11-dUTP labelled "Mu*d*J-left" probe (Section 2.7). Sizes were calculated in kb by comparison with *Eco*RI digested SPP1 marker DNA (Section 2.5.2.2).

The double digests chosen for the SSP-PCR reaction were *Hind*III/*Eco*RI, *Hind*III/*Cla*I and *Hind*III/*Hinc*II as they showed the predicted SSP-PCR product size to be between 0.7 kb and 2 kb (Table 3.10). *S. typhi pqrB*::Mu*d*J (RMA2316) chromosomal DNA was digested with the respective enzymes and ligated to similarly digested pBluescript KS^+ plasmid DNA. The ligation mix was then used as a template for the SSP-PCR reaction with either oligonucleotides #2103 and #M13 F (*Hind*III/*Eco*RI) or oligonucleotides #2103 and # M13 R (*Hind*III/*Cla*I, *Hind*III/*Hinc*II), according to the orientation of the fragment to be ligated into the plasmid (Section 2.5.3.4). The *Hind*III/*Cla*I SSP-PCR reaction produced a product of the expected size (~0.81 kb), this product was ligated to pGEMT and transformed into DH5α. Amp resistant transformants were screened for the correct insert, the resultant plasmid was named pRMSB58, and the insert was sequenced with the oligonucleotide #2103 using dye-

terminator sequencing (Section 2.5.2.7.1). The sequences were analysed by BlastN/X 2.0 (Altschul *et al.*, 1997) and the expected Mu*dJ* homology was found at the beginning of the sequence obtained (Figure 3.12 a). The pRMSB58 sequence after the Mu*dJL* DNA gave ~70% homology at the amino acid level to the *E. coli* K12 Rsd protein and 86% DNA homology to the corresponding *rsd* gene from the *S. typhimurium* STMF1 fragment (Figure 3.12 b).

Figure 3.12a: BlastN 2.0 analysis of pRMSB58 insert – Mu*dJL* homology

```
pRMSB58:   1 tttttactatctttcgcgtttcattgaataacgaataaaatttggactacagggttggaa 60
             |||||||||||||||||||||||||||| |||||| |||||||| |||||||| ||| ||
MudJL:   142 tttttactatctttcgcgtttcattgattaacgactaaaatttgcactacaggcttgcaa 83

pRMSB58:  61 gccccaccaaatctaatcccatcagattccgaataatccaatgtcctcccgggttttttc 120
             |||||||||||||||||||||||||| ||||||||||||||||||||||| |||||||
MudJL:    82 gccccaccaaatctaatcccatcagatcccgaataatccaatgtcctcccggttttttttc 23

pRMSB58: 121 gtacttcaagtgaatcaataca 142
             |||||||||||||||||||||
MudJL:    22 gtacttcaagtgaatcaataca 1
```

Figure 3.12b: BlastN/X 2.0 analysis of pRMSB20 insert – *rsd* and Rsd homology

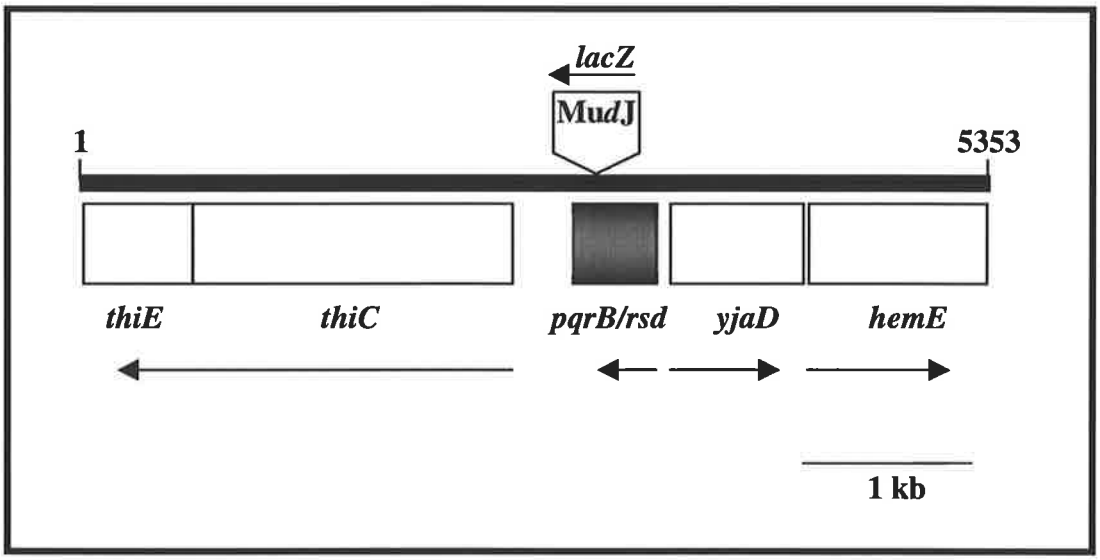
```
pRMSB58:   143 gccatcgatcacgaaaattgtctcgaatttcaacaagctctgtcaaatatcggggaaacg 202
               |||||||||||||| |||||||||||||||||||||||||||| ||||||| ||| ||
STMF1:   27616 gccatcgatcacgataattgtctcgaatttcaacaagctctgtcagatatcggcgaagcg 27557

pRMSB58:   203 ctgaaaggggctttgtgctggnnnnnnngctgaatatgctggtgtttgacgcgaaagca 262
               ||| |||   |||||||||||       ||||| |||||||||||||||||| | |||
STMF1:   27556 ctggaagcccgctttgtgctggaagacaagctgattatgctggtgtttgacgcg-atgca 27498

pRMSB58:   263 cgaaggggccaaaaatcaaacgtcccgcttgaattctgcgcgaataacaggttgtttact 322
               ||| || ||| | | ||||||||||||||||| |||||||||| |||| ||| |||||||
STMF1:   27497 cgacggcgcc-agagtcaaacgtcccgcttgagttctgcgcgagtaacgggtagtttact 27439

pRMSB58:   323 tcagcccccctcttcgga 339
               ||||||||||||||||||
STMF1:   27438 tcagcccccctcttcgga 27422


pRMSB58:       134 INTAIDHENCLEFQQALSNIGETLKGGFVLEKKLNMLVFDA 256
                   + TAIDH+N LEFQQ LS+IGE L+  FVLE KL +LV DA
E. coli Rsd:   111 LETAIDHDNYLEFQQVLSDIGEALEARFVLEDKLILLVLDA 151
```

This figure shows the pRMSB58 insert sequence analysed with Blast 2.0 (Altschul, *et al.*, 1997) and the DNA sequence homology (a) to Mu*dJ* left (gbD: M64097) (Priess, *et al.*, 1987) and (b) DNA homology to the *rsd* gene in the *S. typhimurium* STMF1 fragment (gbD: AF170176) (Latreille, 2000) and amino acid homology to the *E. coli* Rsd protein (gbP: P31690) (Jishage & Ishihama, 1998), (gbD: AE000473) (Blattner, *et al.*, 1997)

The *rsd* gene is located at 90 min on the *E. coli* K12 chromosome in Section 363 (bp 6694-7170) (gbD: AE000473) (Blattner *et al.*, 1997)) and encodes a 158 aa protein. The corresponding position of the *S. typhimurium* chromosome is 90.5 min (Sanderson & Roth, 1988). The Mu*dJ* insertion was found to be between nucleotides 148 and 149 of the *S. typhimurium rsd* gene sequence, hence the *S. typhi pqrB*::Mu*dJ* insertion is in the *S. typhi rsd* gene. The *E. coli* Rsd protein has been shown to be expressed at the beginning of stationary phase of growth, binds to the σ^{70} subunit and is proposed to be a regulator that facilitates the switching of the σ subunit on RNA polymerase from σ^{70} to σ^{s} during the transition from exponential to stationary phase (Jishage & Ishihama, 1998; Jishage & Ishihama, 1999).

Figure 3.13 shows the Mu*dJ* insertion in the *S. typhimurium pqrB/rsd* gene, genes adjacent to *rsd* and their direction of transcription.

Figure 3.13: The *S. typhi rsd* gene, Mu*dJ* insertion and flanking genes.

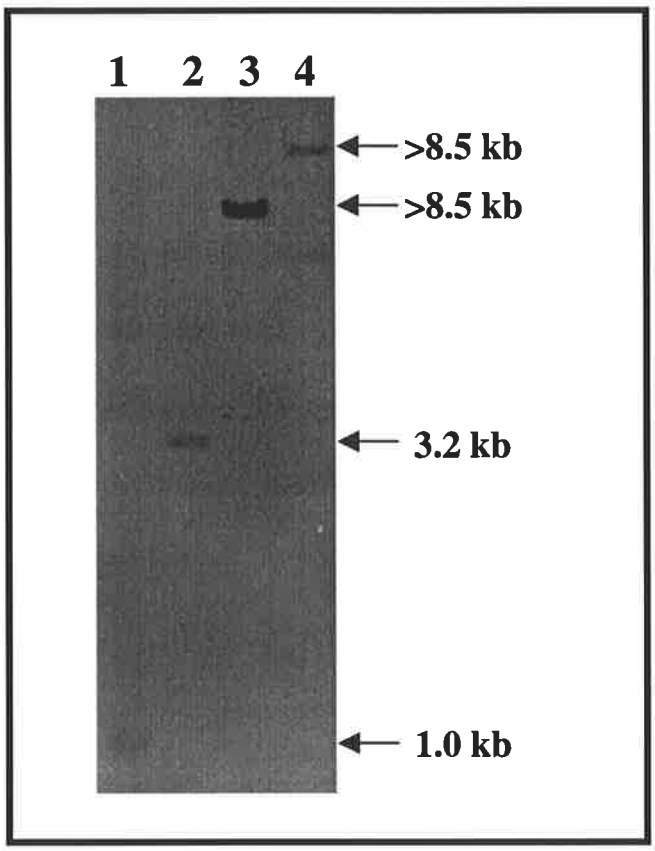


This figure is based on sequence from the *S. typhimurium* STMF1 fragment (gbD: AF170176) ((Latreille, 2000) and shows the *Salmonella rsd* gene (shaded box) which lies downstream of the *thi* operon (the first two genes of the operon, *thiE* and *thiC* are shown), followed by an *S. typhimurium* gene encoding a protein with no known function, and *hemE*. The direction of transcription of all the genes are indicated by arrows. The Mu*dJ* transposon insertion (and direction of *lacZ* transcription) between 148 and 149 nt of the *S. typhi pqrB/rsd* gene is also shown.

Analysis of the 5.34 kb *S. typhimurium* DNA sequence used in Figure 3.7 with Blast 2.0 N against the Sanger Centre *S. typhi* sequencing project released sequence contigs (Sanger Centre, 1999) gave 96% DNA identity between the entire 5.34 kb of *S. typhimurium* and *S. typhi* sequence, confirming that the *rsd* gene and adjacent genes are arranged in the same way in *S. typhi*.

Confirmation of the *S. typhi pqaB/rsd* Mu*dJ* insertion was more difficult than for *pqaD/deoA* and *pqrA/gcvA*, as when this part of the study was undertaken, only protein sequence was available for the *E. coli* Rsd protein. The sequence of the *rsd* gene for *E. coli* (gbD: AE000473) and *S. typhimurium* (gbD: AF170176) was only released after this part of the study had been completed. Therefore confirmation of the *S. typhi pqrB/rsd* Mu*dJ* transposon insertion involved Southern hybridisation analysis with a 0.72 kb *S. typhi rsd* gene PCR DIG-11-dUTP labelled probe using oligonucleotides (#2642 and #2643, designed from the *S. typhi pqrB/rsd* sequence (pRMSB58; Table 2.5) using *S. typhi* Ty2 chromosomal DNA as the template for the PCR labelling reaction (Section 2.7.1.2). *S. typhi* Ty2 and RMA2316 chromosomal DNA were digested with a number of enzymes and probed with the PCR DIG-dUTP labelled *"pqrB"* probe. The restriction enzymes *Hinc*II and *Sac*II gave the most prominent differences in band sizes between the strains (Figure 3.14), and the motility of the bands seen for *S. typhi* RMA2316 compared to the wildtype Ty2 confirms that the Mu*dJ* insertion is within the *S. typhi rsd* gene.

Figure 3.14: Southern hybridisation analysis of the *S. typhi pqrB*::Mu*dJ* insertion mutants



Chromosomal DNA from the *S. typhi* Ty2 and RMA2316 (*pqrB*::Mu*dJ*) strains listed below were digested with either *Hinc*II or *Sac*I restriction enzymes and analysed by Southern hybridisation analysis with the DIG-11-dUTP labelled "*pqrB*" probe (Section 2.7). The sizes of the bands detected were calculated in kb by comparison with *Eco*RI digested SPP1 markers (Section 2.5.2.2). The *Salmonella* chromosomal DNA samples in the lanes are; Lane 1: *S. typhi* Ty2 [*Hinc*II], Lane 2: *S. typhi* RMA2316 (*pqrB*::Mu*dJ*) [*Hinc*II], Lane 3: *S. typhi* Ty2 [*Sac*I], Lane 4: *S. typhi* RMA2316 (*pqrB*::Mu*dJ*) [*Sac*I]

3.9 Analysis of PmrA/B regulation for *S. typhi pqrA*, *pqrB* and *pqaD* genes

The *S. typhimurium phoP/Q* regulated *ugd, pmrG*, and *pmrF* genes have been shown to be strongly regulated by PmrA/B (Gunn *et al.*, 1998B). Therefore, to test whether the *S. typhi pqrA/gcvA*, *pqrB/rsd* and *pqaD/deoA* were also regulated by PmrA/B, a *pmrA*-null mutation (*pmrA*::Tn*10*d) from the *S. typhimurium* strain JSG421 was transduced by P22 phage (Section 2.6.3.2) into *S. typhi* PhoP^c *pqrA*::Mu*dJ*, *pqrB*::Mu*dJ* and *pqaD*::Mu*dJ* (RMA1184, RMA2516 and RMA2512) strains to give RMA1344, RMA1343 and RMA1340 respectively.

None of the three resulting *S. typhi* PhoP^c, *pqa/pqr*::Mu*dJ*, *pmrA*::Tn*10*d strains (RMA1344, RMA1343 and RMA1340) showed any difference in LacZ activity on LA containing X-gal plates compared to the parental *S. typhi* PhoP^c *pqa/pqr*::Mu*dJ* strains, therefore indicating that the *S. typhi pqrA/gcvA*, *pqrB/rsd* and *pqaD/deoA* genes were not regulated by PmrA/B.

3.10 Analysis of the promoter regions of *S. typhi phoP/Q* regulated genes

As three *S. typhi* genes previously unknown to be *phoP/Q* regulated genes were identified in Section 3.8, I wished to compare the promoter regions of these genes to the promoter regions of known *S. typhimurium phoP/Q* regulated genes to determine if there were any similarities. The known *S. typhimurium phoP/Q* regulated genes included: the *phoP/Q* activated genes; *mgtA* (gbD: STU07843) (Snavely *et al.*, 1991), *mgtB* and *mgtC* (gbD: STYMGTBC) (Snavely *et al.*, 1991), *pagC* (gbD: STYPAGC) (Pulkkinen & Miller, 1991), *pagD* (gbD: STU31849) (Gunn *et al.*, 1995), *pagJ* (gbD: AF013776) ((Gunn *et al.*, 1998A), *pagK*, *pagM* and *pagO* (gbD: AF013775) (Gunn *et al.*, 1998A), *pagP* (gbD: AF057021) (Guo *et al.*, 1998), *pcgL* & *ugtL* (gbD: AF120672) (Hilbert *et al.*, 1999), *phoN* (gbD: STPHONG) (Kasahara *et al.*, 1991) and *phoP* (gbD: M24424, M25241) (Groisman *et al.*, 1989; Miller, *et al.*, 1989). The promoter regions of the known *S. typhimurium phoP/Q* repressed genes *prgHIJK* (gbD: STU21676) (Pegues *et al.*, 1995) and *hilA* (gbD: STU25352) (Bajaj *et al.*, 1996) were also used. Although extensive comparison of these promoter regions by BlastN/X 2.0 (Altschul *et al.*, 1997) and DNASIS was performed, no significant DNA sequence similarities between the *phoP/Q* regulated genes promoter regions were found.

3.11 Summary

In this chapter, the *S. typhi* Ty2 and *S. typhimurium* C5 and *phoP/Q* mutant (PhoP$^-$ and PhoP^c) strains were analysed and compared for any differences in phoP/Q regulated genes.

Initially, differences were noted in the level of PhoN activity between the two strains with *S. typhi* strains having lower levels of non-specific acid phosphatase than *S. typhimurium* strains. Complementation of the *S. typhi* and *S. typhimurium* PhoP$^-$ strains with plasmids containing *S. typhimurium* phoP/Q genes gave greater PhoN activity than chromosomal alleles (Section 3.6.3). The magnesium dependency of *S. typhi* strains was analysed and the *S. typhi* PhoP$^-$ strains were shown to have a greater requirement for Mg^{2+} than the wildtype strains (Section 3.5).

The whole cell proteins produced by *S. typhi*, *S. typhimurium* and respective PhoP$^-$ and PhoP^c derivatives were compared, and it was found that *S. typhi* Pag and Prg proteins were fewer in number and had a different apparent molecular mass than *S. typhimurium* Pag and Prg proteins (Section 3.6.1).

Analysis of *S. typhi* sensitivity to polymyxin B and protamine showed that *S. typhi* PhoP$^-$ mutants were extremely sensitive to both antimicrobial agents when compared to the wildtype strain and that *S. typhi* had increased sensitivity when compared to *S. typhimurium* strains (Section 3.6.4; Section 3.6.5).

The LPS of *S. typhi* Ty2, PhoP$^-$ and PhoP^c and *S. typhimurium* C5, PhoP$^-$ and PhoP^c derivatives was examined on an SDS-PAGE gel (Section 3.6.2). Differences were noted between *S. typhimurium* wildtype and PhoP$^-$, and PhoP^c LPS which were not seen for *S. typhi*, and the effect on LPS will be discussed in Chapter 7.

The *S. typhi pqaD* gene was identified as *deoA*, the *pqrA* gene as *gcv*A and the *pqrB* gene as *rsd* (Section 3.8). The *gcvA* and *rsd* genes are involved with regulation in *Salmonella*, analysis of the promoter regions of these genes and *deoA* found no consensus region in common with other known *Salmonella* PhoP/Q regulated genes (Section 3.9). The possible role of PhoP/Q mediated regulation of *deoA*, *gcvA* and *rsd* will be discussed further in Chapter 7.

Chapter 4

Growth of *Salmonella typhi* in tissue culture cells

4.1 Introduction

An important part of *Salmonella* pathogenesis is the ability of the bacterium to survive and multiply in macrophages (Buchmeier & Heffron, 1989; Fields *et al.*, 1986A). The *S. typhimurium* PhoP/Q two component regulatory system has been shown to function in *S. typhimurium* survival in macrophages (Buchmeier &Heffron, 1989; Fields *et al.*, 1989) and invasion of mammalian cells (Behlau & Miller, 1993; Johnston *et al.*, 1996; Pegues *et al.*, 1995). *Salmonella* PhoP/Q regulated genes and survival in macrophages from particular host species have also been implicated in host specificity (Section 1.13).

As *S. typhimurium* $PhoP^-$ and $PhoP^c$ mutants have been previously shown to have decreased survival in macrophage cell lines, a monocyte-derived human cell line was used to assess the invasion and growth of *S. typhi* and *S. typhimurium* wildtype, *phoP/Q* mutants and five *S. typhi pqa/pqr*::Mu*dJ* insertion mutants. The use of a monocytic cell line that can be differentiated to macrophage-like cells has a number of advantages over assays with human blood derived monocytes. In addition to cost benefits, the genetic homogeneity of cells lines minimises phenotype variation between experiments and allows the cells to be activated to a defined state enabling more reproducible results. The monocytic cell line chosen for this study was the U937 cell line which is a continuous, suspension cell line displaying many of the characteristics of monocytic cells such as receptors for Fc and C3 and the capacity for lysozyme production and strong esterase activity (Ralph *et al.*, 1976; Sundström & Nilsson, 1976). The U937 cell line is able to be differentiated to macrophage-like cells with phorbol esters such as phorbol myristate acetate (PMA) (discussed below).

Monocytic U937 cells have been used previously for the *in vitro* characterization of potential live oral vaccine *S. typhi* mutant strains (Dragunsky *et al.*, 1989; Dragunsky *et al.*, 1990). PMA and other phorbol ester differentiated U937 cells have been used for analysis of proteins produced by *S. typhimurium* during growth within macrophages (Abshire & Heffron, 1993B), growth rates of *S. typhimurium* within macrophages (Abshire & Heffron, 1993A), antimicrobial macrophage defenses against *S. typhimurium* (Château & Caravano, 1993), infection of macrophages by *Legionella pneumophila* (Cianciotto *et al.*, 1989; Pearlman *et al.*, 1988) and tumor necrosis factor (TNFα) secretion from macrophage-like cells after *S. typhi* infection (Hone *et al.*, 1992; Hone *et al.*, 1993). The monocytic U937 cells (Sundström & Nilsson, 1976) can be differentiated into macrophage-like cells by 72 h of PMA treatment (Ascensao & Mickman, 1984), which stimulates protein kinase C-dependent signalling (Tsutsumi *et al.*, 1993; Ways *et al.*, 1994)). The PMA-differentiated U937 cells express many of the normal mononuclear phagocyte characteristics, including more extensive cytoplasm and cytoskeletal rearrangements (Hass *et al.*, 1989), increased protein synthesis (Lacraz *et al.*, 1994), decreased mitosis (e.g. decreased cell proliferation), increased phagocytosis and increased HLA-DR and Fc-γ receptor expression (Klein *et al.*, 1990; Minta & Pambrun, 1985; Nambu *et al.*, 1989; Radzun *et al.*, 1983). The most dramatic marker of PMA-differentiated U937 cells (PMA-U937) is the expression of adhesion molecules (structurally related glycoproteins that function in cell-substrate and cell-cell adhesion reactions of lymphoid cells) normally expressed on tissue macrophages, including CR3 (Mac-1; CD11b/CD18), CR4 (150/95; CD11c/CD18) and LFA-1 (Miller *et al.*, 1986) (Pedrinaci *et al.*, 1989; Sitrin *et al.*, 1998). After PMA differentiation, U937 cells produce all three of these adhesion molecules with similar expression levels as tissue macrophages (Miller *et al.*, 1986; Radzun *et al.*, 1983). PMA-U937 cells have two different adhesion patterns; approximately 50% of the cell population form aggregates or "clumps" (cell-cell adhesion) and the other 50% adhere to and spread on the substrate (in this case tissue culture wells) (cell-substrate adhesion) (Hass *et al.*,

1989). The cell-cell aggregation is mediated by LFA-1/ICAM-1 interaction (Miller *et al.*, 1986).

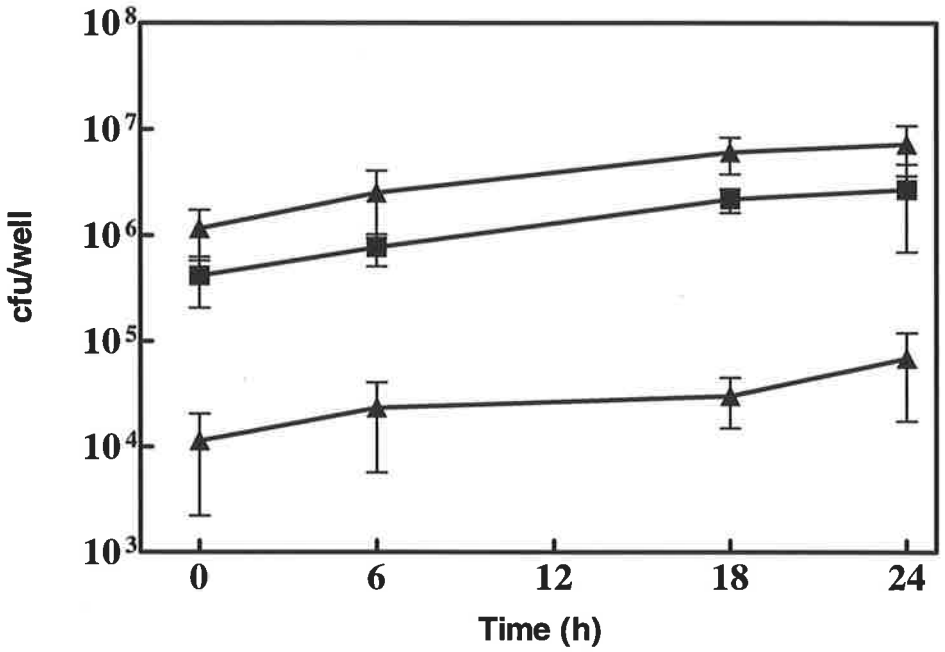
In this chapter the PMA-U937 assay was used to study the invasion and intracellular growth of *S. typhi* and *S. typhimurium* wildtype, *phoP/Q* mutant derivatives and the *S. typhi* *pqa/pqr* Mu*d*J insertion mutants. The *S. typhi* strains were also assayed in an HeLa cell (epithelial) line to assess non-specific defects in invasion and/or intracellular growth. All experiments used non-opsonised bacteria.

4.2 Invasion and growth of *S. typhi* PhoP$^+$, PhoP$^-$ and PhoP^c strains in HeLa cells

S. typhimurium phoP/Q mutations are known to affect survival of the bacteria in macrophages but not epithelial cells (Fields *et al.*, 1989; Galán & Curtiss, 1989B; Miller & Mekalanos, 1990) therefore the *S. typhi* PhoP$^-$ PhoP$^+$, and PhoP^c bacterial strains ability to invade and grow in an epithelial HeLa (non-macrophage) cell-line was investigated (Section 2.12.4.2) (Figure 4.1, Table 4.1). Both *S. typhi* Ty2 and RMA1030 (PhoP$^-$) strains invaded approximately 10-fold more efficiently than RMA1090 (PhoP^c) (Table 4.1). This result correlated well with data on an *S. typhimurium* PhoP^c mutant which had previously been shown to be invasion deficient (Behlau & Miller, 1993). Strain RMA1030 invaded better (~ 2 fold) than the wildtype strain (Figure 4.1, Table 4.1), suggesting that the method used to grow the bacteria for the assay was not optimal for invasion (discussed in Section 4.7). All three strains grew to give a Growth Index (GI) (Table 4.1) of 6. This GI correlated well with previously reported growth levels for *S. typhi* intracellular growth in HeLa cells (Mills & Finlay, 1994) and indicates that the PhoP$^-$ and PhoP^c mutations do not affect growth of *S. typhi* in HeLa cells. The results for the *S. typhi* RMA1030 strains concurs with data reported for *S. typhimurium* PhoP$^-$ strain which showed no intracellular growth deficiency when

compared to the wildtype strain in an epithelial (Henle-407) cell line (Galán & Curtiss, 1989B).

Figure 4.1 Net growth of *S. typhi* strains in HeLa cells



This figure shows the net growth characteristics of *S. typhi* Ty2 (■), RMA1030 (PhoP$^-$) (●) and RMA1090 (PhoP^c) (▲) strains after invasion of HeLa cells. *S. typhi* strains were added at a ratio of 10:1 (bacteria:HeLa cells) and each point represents the arithmetic mean of 4 assays expressed as viability of cfu/well ± SD at a given time in hours.

Table 4.1: Invasion and Growth Index (GI) of *S. typhi* strains in HeLa cells

Bacterial strains[a]	Invasion[b]	GI[c]
Ty2 (PhoP$^+$)	1.97 ± 1.05	6
RMA1030 (PhoP$^-$)	4.60 ± 0.55	6
RMA1090 (PhoP^c)	0.05 ± 0.03	6

[a]All bacterial strains can be found in Table 2.3
[b]Invasion percentages were calculated by dividing the number of bacteria at time 0 after gentamicin treatment by the number of bacteria initially added, then multiplied by 100
[c]Growth Index (GI) was calculated by the number of bacteria at timepoint 24 h divided by the number of bacteria at time 0 h

4.3 U937 cell assay

4.3.1 Optimisation of the U937 cell assay

The *S. typhi* Ty2 strain and isogenic PhoP$^-$ (RMA1030) and PhoP^c (RMA1090) derivatives were assessed for their ability to invade and grow inside undifferentiated U937 cells in a total volume of 6 ml during the assay (Section 2.12.4.1). It was noted that the cells remained in a large volume throughout the entire assay. Large culture volumes may potentially affect the growth of Salmonella bacteria strains within U937 cells by limiting O_2 and CO_2 gas diffusion. Therefore, to address this, a second experiment was performed in which, 1 ml of cells were aliquoted into separate 10 ml tubes after the 6 h timepoint to improve the surface area to volume ratio, and used for the 18 and 24 h timepoints. Comparison of the *S. typhi* bacterial growth between those cells left in the large volume (6 ml) and those in the 1 ml samples, indicated a significant growth difference (Table 4.2). Similar differences between the growth of the *S. typhi* PhoP$^-$ and PhoP^c strains in 6 ml versus 1 ml volumes were also noted (data not shown). Therefore future assays using U937 cells were performed in a maximum volume of 1 ml for any timepoints taken after the 0 h timepoint (U937 cell assay II, Section 2.12.4.1) (e.g. invasions were still be performed in a 6 ml volume but after this cells wer aliquoted into 1 ml volumes in separate 10 ml tubes for the later timepoints).

Table 4.2: *S. typhi* Ty2 Growth Index (GI) in U937 cell assay with volume differences

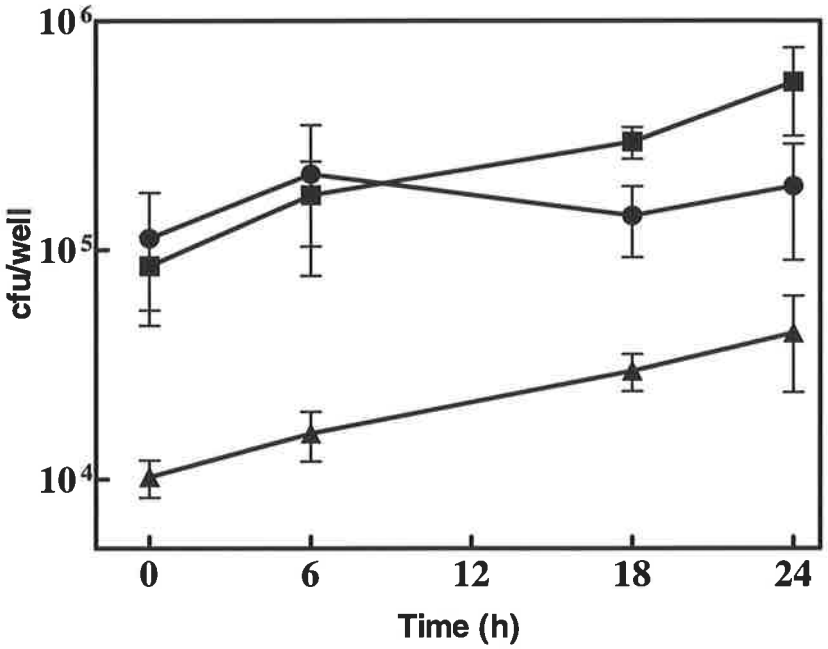
Time (h)	6 ml volume (GI)[a]	1 ml Volume (GI)[a]
0	1	1
6	1	1
18	7	24
24	7	35

[a]Growth Index (GI) was calculated by the number of bacteria at timepoint 24 h divided by the number of bacteria at time 0 h.

4.3.2 Invasion and growth of *S. typhi* strains in U937 cells

S. typhi strain Ty2 and the isogenic PhoP$^-$ (RMA1030) and PhoP^c (RMA1090) derivatives were assessed for their ability to invade and grow inside U937 cells (Figure 4.2) (Section 2.12.4.1). The overall level of invasion (Table 4.3) was lower when compared to the HeLa cell invasion (Table 4.1) levels. This is likely to be because undifferentiated U937 cells are a suspension cell line, and bacteria and cells were not centrifuged to allow extended close contact, so less bacteria may have come into contact with the tissue culture cells. This low invasion also correlates with previous data (Dragunsky *et al.*, 1989) of low invasion levels with U937 cells, and the fact that the U937 cells are monocytic in nature and have low intrinsic phagocytic ability.

Figure 4.2: Net growth of *S. typhi* strains in U937 cells



This figure shows the net growth characteristics of *S. typhi* Ty2 (■, RMA1030 (PhoP$^-$) (●) and RMA1090 (PhoP^c) (▲) strains after invasion of U937 cells. *S. typhi* strains were added at a ratio of 10:1 (bacteria:cells) and each point represents the arithmetic mean of 4 assays expressed as viability of cfu/well ± SD at a given time in hours.

Table 4.3: Invasion and Growth Index (GI) of *S. typhi* strains in U937 cells

Bacterial strains[a]	Invasion[b]	GI[c]
Ty2 ($PhoP^+$)	0.33 ± 0.03	6
RMA1030 ($PhoP^-$)	0.34 ± 0.09	2
RMA1090 ($PhoP^c$)	0.08 ± 0.04	4

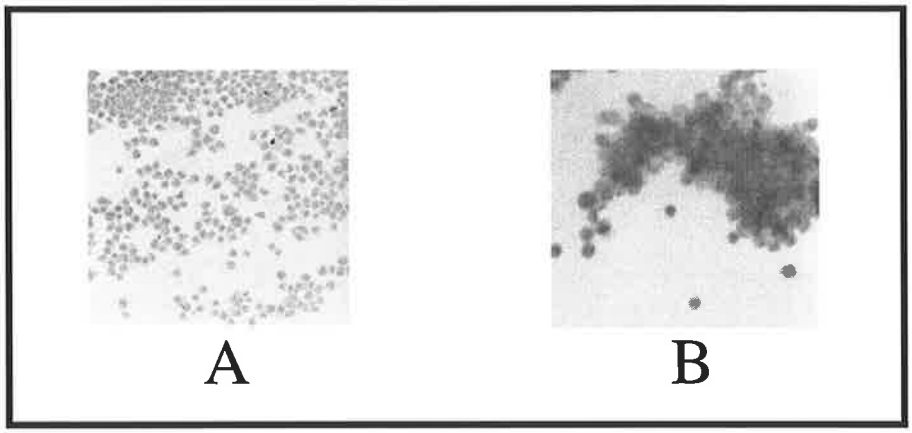
[a]All bacterial strains can be found in Table 2.3
[b]Invasion percentages were calculated by dividing the number of bacteria at time 0 after gentamicin treatment the number of bacteria initially added x 100
[c]Growth Index (GI) was calculated by the number of bacteria at timepoint 24 h divided by the number of bacteria at time 0 h.

As expected, the $PhoP^c$ (RMA1090) strain gave a lower invasion level than the *S. typhi* Ty2 and RMA1030 strains, (Table 4.3). The GI at 24 h (Table 4.3) for *S. typhi* Ty2 showed similar growth to that in HeLa cells (Table 4.1). There was a significant difference in growth at 24 h between Ty2 and RMA1030 (Figure 4.2) ($P< 0.3$ at 24 h, students T-test), however the difference was judged to be insufficient for an assay to screen *S. typhi pqa/pqr* mutants for an intracellular growth defect.

4.4 U937 cells and PMA-U937 cells

U937 cells normally grow as a cell suspension but can be differentiated by treatment with 6 ng/ml of phorbol myristate acetate (PMA) for 72 h to form macrophage-like cells, with many of the characteristics of macrophages (Section 4.1). U937 and PMA-U937 cells display different morphologies when stained with Diff-Quick (Section 2.12.3). The PMA-U937 cells adhere to glass after the 72 h differentiation and were directly fixed and stained on a cover slip (Figure 4.3 A) However the U937 cells are non-adhesive and required centrifugation onto a glass slide prior to fixeing and staining (Figure 4.3 B). These results are identical to those reported previously (Hone *et al.*, 1992).

Figure 4.3: Microscopic comparison of U937 and PMA-differentiated U937 cells



This Figure shows U937 and PMA-U937 cells stained with Diff-Quick (Section 2.12.3)
(A) Undifferentiated U937 cells stained with Diff-Quik
(B) U937 cells differentiated with 6 ng/ml of PMA for 72 h (PMA-U937 cells) stained with Diff-Quik.

4.5 PMA-U937 cell assay

4.5.1 Initial assessment of sampling timepoints for the PMA-U937 cell assay

The PMA-U937 tissue culture assay was based on the assay by Hone *et al.*, 1992 and is described in Section 2.12.4.2.

Initially, the *S. typhi* bacteria were added to the PMA-U937 cells at a ratio of 10:1 (bacteria:cells) and the growth/survival of the bacteria at 0, 6, 24, 36 and 48 h was determined. The growth curve for the *S. typhi* bacteria was found to plateau after 36 h and to decrease by 48 h (Table 4.4) with the clearest difference in growth between the *S. typhi* wildtype and PhoP$^-$ bacteria being at 24 h.

Table 4.4: Growth Index (GI) of *S. typhi* Ty2 and RMA1030 (PhoP-) in PMA-U937 cells at different timepoints

	GI[b]				
Stain[a]	0 (h)	6 (h)	24 (h)	36 (h)	48 (h)
Ty2	1	4	21	20	14
RMA1030 (PhoP$^-$)	1	2	3	9	6

[a]All bacterial strains can be found in Table 2.3
[b]Growth Index (GI) was calculated by the number of bacteria at timepoint 0, 6, 24, 36 and 48 h respectively divided by the number of bacteria at time 0 h.

By visual inspection, the majority of the infected cells remained adherent to the bottom of the 24 well trays at the 24 h timepoint and most of these (> 95%) excluded trypan blue indicating that they were still viable. However at times greater than 24 h approximately 20-30% stained with trypan blue suggesting some cell death was occuring. Consequently the maximum time limit of the assay was set at 24 h. Timepoints at 6 and 18 h were chosen as the middle sampling points for the PMA-U937 assay. The presence of PMA in the media for the duration of the bacterial infection/growth assay was assessed and it was found to have no effect on the *Salmonella* growth (data not shown). This indicated that the U937 cells were are fully differentiated by PMA by 72 h. Consequently in future experiments, PMA was only used for the 72 h differentiation period and was not added for the remainder of the assay.

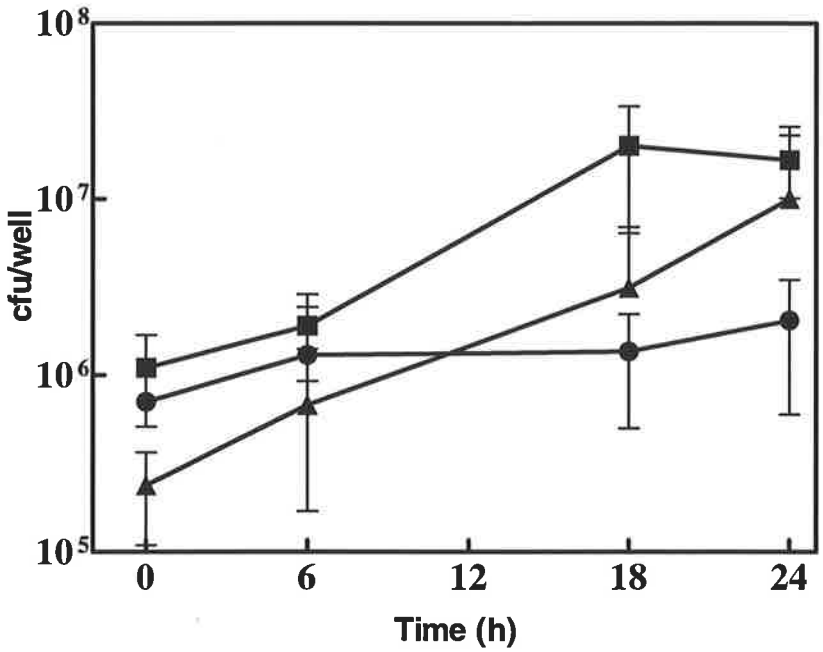
4.5.2 Invasion and growth of *S. typhi* PhoP$^-$, PhoP$^+$, PhoP^c and *E. coli* K12 strains in PMA-U937 cells

S. typhi Ty2 and isogenic PhoP$^-$ (RMA1030) and PhoP^c (RMA1090) strains were assessed for their ability to invade and grow in PMA-U937 cells (Figure 4.4, Table 4.5). The *S. typhi* PhoP$^-$ mutants displayed invasion comparable to that of the wildtype Ty2 strain, however an approximate 10-fold decrease in invasion for the PhoP^c strain was observed (Figure 4.4, Table 4.5). This was consistant with the HeLa cell results (Section 4.2) and *S. typhimurium* PhoP^c data from Behlau & Miller, (1993). The net growth index (GI) at 24 h for

Ty2 in the PMA-U937 cells was 25 (Table 4.5), and the PhoP^c (RMA1090) strain had a GI of 42 (Table 4.5), a possible explanation for this net increase in growth of *S. typhi* PhoP^c strain compared to Ty2 is discussed later in Chapter 7. The *E. coli* K12 strain (DH5) was also assayed (Table 4.4) and the bacteria were added at a 100:1 (bacteria:cells) ratio as this strain is non-invasive. As can be seen in Table 4.4, this strain had a very low level of invasion (discussed in section 4.7) and had a decrease in growth (GI = 0.5) at 24 h indicating that the PMA-U937 cells were able to kill the *E. coli* strain.

The PhoP$^-$ strain (RMA1030) displayed limited intracellular net growth (GI = 2, Figure 4.4, Table 4.5) when compared to the wildtype strain. The difference in net growth between Ty2 and RMA1030 was significant ($P<0.01$ at 24 h, students T-test), indicating that the assay was suitable to assay the *S. typhi pqa/pqr* mutants (Section 3.2).

Figure 4.4: Net growth of *S. typhi* strains in U937-PMA cells



This figure shows the net growth characteristics of *S. typhi* Ty2 (■), RMA1030 (PhoP$^-$) (●) and RMA1090 (PhoP^c) (▲) strains after invasion of U937-PMA cells. *S. typhi* strains were added at a ratio of 10:1 (bacteria:cells) and each point represents the arithmetic mean of 4 assays expressed as viability of cfu/well ± SD at a given time in hours.

Table 4.5: Invasion and Growth Index (GI) of *S. typhi* and *E. coli* strains in PMA-U937 cells

Bacterial strains[a]	Invasion[b]	GI[c]
Ty2 (PhoP$^+$)	6.3 ± 2.5	15
RMA1030 (PhoP$^-$)	4.3 ± 1.3	2
RMA1090 (PhoP^c)	0.9 ± 0.69	42
DH5	0.25 ± 0.09	0.5

[a]All bacterial strains can be found in Table 2.1 and Table 2.3
[b]Invasion percentages were calculated by dividing the number of bacteria at time 0 after gentamicin treatment the number of bacteria initially added x 100
[c]Growth Index (GI) was calculated by the number of bacteria at timepoint 24 h divided by the number of bacteria at time 0 h.

During this study a problem was encountered that was similar to one previously described by Abshire *et al.*, (1993A). As the experiments were done over a long time period (two years), it was necessary to establish multiple cultures from frozen stocks, rather than keeping the U937 cells in continuous culture over this period. The different sub-cultures of U937 cells gave slightly different results. Though growth curves for *S. typhi* strains were similar, and the *S. typhi* Ty2 wildtype strain always showed statistically significant greater growth than the *S. typhi* PhoP$^-$ null strain, different amounts of invasion were noted which affected the overall GI. Overall, the GI for *S. typhi* was shown to range from 6-30 in different assays performed at different times.

4.5.3 Complemented RMA1030 PhoP$^-$ strains in PMA-U937 cells

As complementation of the growth/survival defect of *S. typhimurium* PhoP$^-$ strains in macrophages has not previously been shown, it was of interest to determine whether the growth defect of the *S. typhi* PhoP$^-$ mutant in PMA-U937 cells could be complemented with cloned *S. typhimurium phoP/Q* genes. Initially complementation was attempted with the *S. typhimurium phoP/Q* genes cloned into pSUP203, a medium copy number vector (pRMSB1). The growth of *S. typhi* Ty2, RMA1030 and RMA1170 (RMA1030 + pRMSB1) in the PMA-

U937 assay was assessed. The GI at 24 h (Table 4.6-Assay I) indicated that the plasmid did not complement the PhoP null mutation in PMA-U937 cells, although pRMSB1 had previously been shown to restore non-specific acid phosphatase activity to RMA1170 on X-pho plates (ie: gave blue colonies) (Section 3.4). As *phoP/Q* are regulators, it was thought that the higher copy number of PhoP/Q in the complemented strain may be affecting bacterial growth. This had been implied previously by the observation that the *S. typhimurium phoP/Q* genes cloned in the high copy number plasmid (pBluescript KS^+) could not be maintained in *S. typhi* $PhoP^-$ bacterial cells (Section 3.4). Therefore RMA1164 (Ty2 + pSUP203), RMA1166 (Ty2 + pRMSB1 (*phoP/Q* in pSUP203)), RMA1168 (RMA1030 + pSUP203) and RMA1170 (RMA1030 + pRMSB1 (*phoP/Q* in pSUP203)) (Table 4.6-Assay II) were assayed for growth in PMA-U937 cells.

Table 4.6: Growth Index (GI) of *S. typhi* Ty2, $PhoP^-$ and complemented $PhoP^-$ strains in PMA-U937 cells

Assay I[c]	
Bacterial strains[a]	GI[b]
Ty2 ($PhoP^+$)	23
RMA1030 ($PhoP^-$)	3
RMA1170 ($PhoP^-$ + pRMSB1)	3
Assay II[c]	
Bacterial strains[a]	GI[b]
RMA1164 (Ty2 + pSUP203)	9
RMA1166 (Ty2 + pRMSB1)	1
RMA1168 ($PhoP^-$ + pSUP203)	1
RMA1170 ($PhoP^-$ + pRMSB1)	1
Assay III[d]	
Bacterial strains[a]	GI[b]
Ty2 ($PhoP^+$)	6
RMA1030 ($PhoP^-$)	1
RMA1325 ($PhoP^-$ + pCL1921)	1
RMA1327 ($PhoP^-$ + pRMSB34)	6

[a]All bacterial strains can be found in Table 2.3
[b]Growth Index (GI) was calculated by the number of bacteria at timepoint 24 h divided by the number of bacteria at time 0 h.
[c]Medium copy number plasmids were used in these assays
[d]Low copy number plasmids were used in this assay

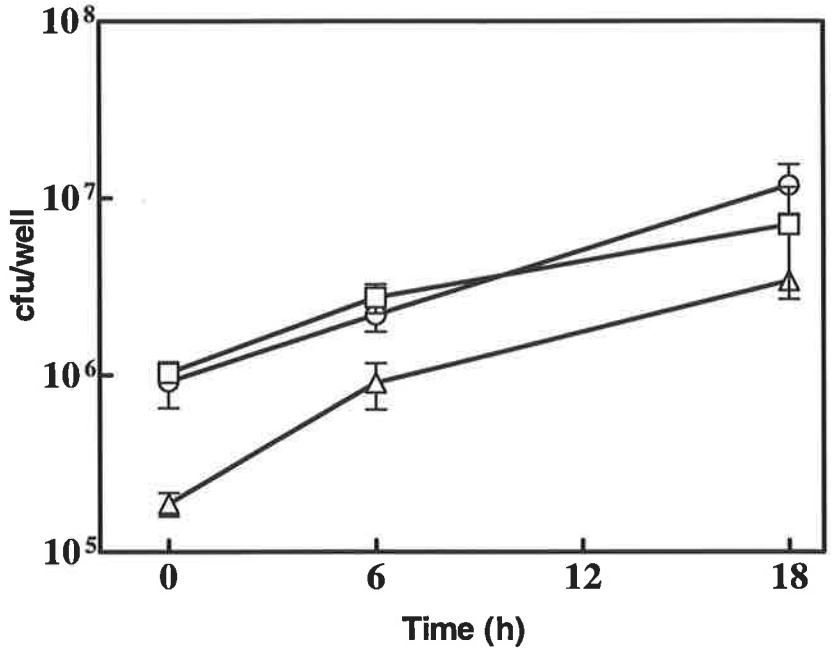
As can been seen from the results in Table 4.6-Assay II, a higher copy number of the *phoP/Q* genes due to pRMSB1 clearly affects the growth of the wildtype *S. typhi* Ty2 bacteria in the PMA-U937 cell assay. Finally, the *S. typhi* RMA1030 (PhoP$^-$) strain complemented with pRMSB34 (*phoP/Q* cloned into the low copy number vector pCL192) was assayed in the PMA-U937 cell assay. The results in Table 4.6-Assay III conclusively show that the growth defect for the RMA1030 strain is due to the *phoP* mutation as it can be complemented by the *S. typhimurium phoP/Q* genes.

4.5.4 Invasion and net growth of *S. typhimurium* strains in PMA-U937 cells.

As the *S. typhi* PhoP$^-$ mutant displayed reduced net growth in the PMA-U937 cell line compared to the wildtype Ty2 strain, it was proposed that the *S. typhimurium* PhoP$^-$ mutant would show a similar defect in net growth compared to the parent C5 strain. Therefore the *S. typhimurium* C5 and isogenic PhoP$^-$ (RMA1010) and PhoP^c (RMA1024) mutants ability to invade and grow within PMA-U937 cells was investigated (Figure 4.5, Table 4.7). However, since *S. typhimurium* bacterial infection of the PMA-U937 cells resulted in significant cell death by 24 h (as indicated by lack of trypan blue exclusion, data not shown), the assay was taken to 18 h rather than 24 h as used for *S. typhi*.

The *S. typhimurium* strains were observed to have a greatly increased level of invasion (approximately 10-20 fold) compared to *S. typhi* strains (Table 4.7); this has been noted before for HeLa cell invasion (Mills & Finlay, 1994). Therefore the bacterial:PMA-U937 cell ratio for *S. typhimurium* was adjusted to 1:1 instead of 10:1 as used for *S. typhi* so the numbers of bacteria invading the PMA-U937 cells were similar for both species.

Figure 4.5: Net growth of *S. typhimurium* strains in PMA-U937 cells



This figure shows the net growth characteristics of *S. typhimurium* C5 ( ), RMA1010 (PhoP$^-$) ( ) and RMA1024 (PhoP^c) (Δ) strains after invasion of U937-PMA cells. *S. typhimurium* strains were added at a ratio of 1:1 (bacteria:cells) and each point represents the arithmetic mean of 4 assays expressed as viability of cfu/well ± SD at a given time in hours.

Table 4.7: Invasion and Growth Index (GI) of *S. typhimurium* strains in PMA-U937 cells

Bacterial strains[a]	Invasion[b]	GI[c]
C5 (PhoP$^+$)	95.7 ± 4.7	7
RMA1010 (PhoP$^-$)	82.7 ± 4.8	32
RMA1024 (PhoP^c)	14.5 ± 2.6	10

[a]All bacterial strains can be found in Table 2.2
[b]Invasion percentages were calculated by dividing the number of bacteria at time 0 after gentamicin treatment the number of bacteria initially added x 100
[c]Growth Index (GI) was calculated by the number of bacteria at timepoint 18 h divided by the number of bacteria at time 0 h.

As expected the *S. typhimurium* PhoP^c (RMA1024) mutant displayed reduced invasion compared to the wildtype and PhoP$^-$ derivative (Table 4.7) (Behlau & Miller, 1993). The *S. typhimurium* PhoP$^-$ mutant had no net growth limitations compared to the *S. typhimurium* wildtype strain (Figure 4.6) and actually gave a higher G.I. than wildtype (Table 4.7). Hence,

the net growth restriction caused by the PMA-U937 cells for the *S. typhi* PhoP$^-$ mutation in *S. typhi* appeared to be specific for *S. typhi* (discussed in Chapter 7).

4.6 Invasion and growth of *S. typhi pqa/pqr* mutants in PMA-U937 cells

4.6.1 Initial screen for invasion and growth of the *S. typhi pqa/pqr*::Mu*dJ* insertion mutants in PMA-U937 cells

As the difference between *S. typhi* Ty2 and RMA1030 (PhoP$^-$) was shown to be significantly different in Section 4.5.2, the invasion and growth capacities of the five *S. typhi pqa/pqr*::Mu*dJ* insertion mutants within PMA-U937 cells was investigated. Initially, the five mutant strains (RMA2310 (*pqaA*::Mu*dJ*), RMA2312 (*pqaD*::Mu*dJ*), RMA2316 (*pqrB*::Mu*dJ*), RMA2326 (*pqaB*::Mu*dJ*) and RMA1180 (*pqrA*::Mu*dJ*)), were tested at 0 and 24 h and the invasion and net growth can be seem in Table 4.8. None of the mutants appeared to be defective in invasion and two mutants (RMA2310 (*pqaA*) and RMA2326 (*pqaB*)) gave a lower GI than *S. typhi* Ty2.

Table 4.8: Invasion and net growth of *S. typhi pqa/pqr*::Mu*dJ* mutants in PMA-U937 cells

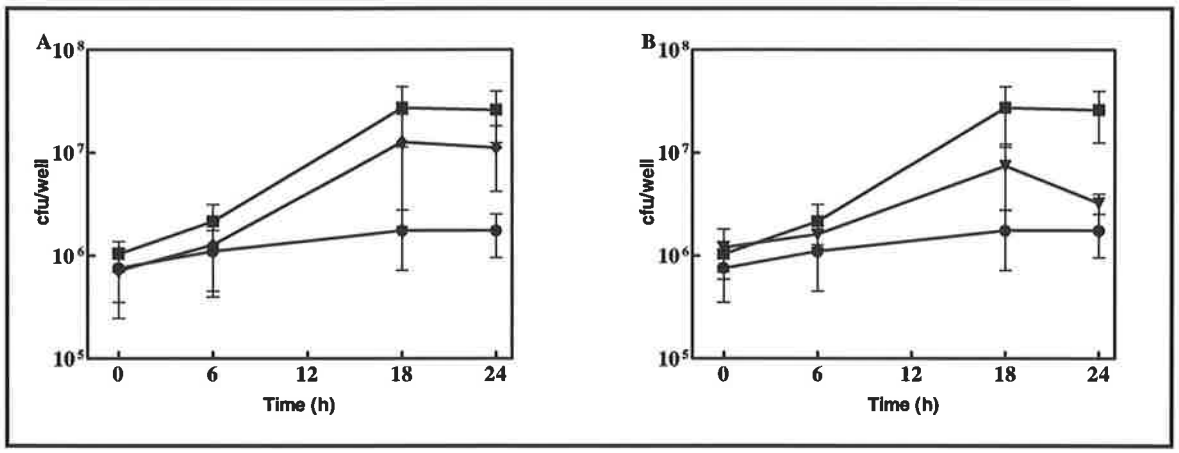
Bacterial strains[a]	Invasion[b]	GI[c]
Ty2 (PhoP$^+$)	9	9
RMA1030 (PhoP$^-$)	6	2
RMA2310 (*pqaA*::Mu*dJ*	5	4
RMA2312 (*pqaD*::Mu*dJ*)	7	14
RMA2316 (*pqrB*::Mu*dJ*)	7	23
RMA2326 (*pqaB*::Mu*dJ*)	5	2
RMA1180 (*pqrA*::Mu*dJ*)	7	12

[a]All bacterial strains can be found in Table 2.3
[b]Invasion percentages were calculated by dividing the number of bacteria at time 0 after gentamicin treatment the number of bacteria initially added x 100
[c]Growth Index (GI) was calculated by the number of bacteria at timepoint 24 h divided by the number of bacteria at time 0 h.

4.6.2 Further testing of RMA2310 (*pqaA*) and RMA2326 (*pqaB*) for growth in PMA-U937 cells

The results for *S. typhi pqaA* (RMA2310) and *pqaB* (RMA2326) Mu*dJ* insertion mutants GI from Table 4.8 warranted re-assessment in PMA-U937 cells and these mutants were assayed at 0, 6, 18 and 24 h. The *S. typhi pqa*A (RMA2310) strain showed a slight decrease in intracellular net growth compared to *S. typhi* Ty2 (GI of 16) (Figure 4.6 A) however this was not significant by the students T-test as P=0.06, therefore P>0.05, and the growth difference is not significant. The *S. typhi pqaB::*Mu*dJ* (RMA2326) strain showed a decreased intracellular net growth compared to *S. typhi* Ty2 (Figure 4.6 B) (GI of 3) which was significant (P=0.02, therefore P<0.05).

Figure 4.6: Net growth of *S. typhi* Ty2, PhoP⁻ (RMA1030), *pqa*A::Mu*dJ* (RMA2310), and *pqa*B::Mu*dJ* (RMA2326)strains in PMA-U937 cells.



(A): This figure shows the net growth characteristics of *S. typhi* Ty2 (■), RMA1030 (PhoP⁻) (●), and RMA2310 (*pqa*A::Mu*dJ*) (◆), strains after invasion of PMA- U937 cells.
(B): This figure shows the net growth characteristics of *S. typhi* Ty2 (■), RMA1030 (PhoP⁻) (●), and RMA2326 (*pqaB*::Mu*dJ*) (▼), strains after invasion of PMA-U937 cells.
S. typhi strains were added at a ratio of 10:1 (bacteria:cells).
Each point represents the arithmetic mean of 4 assays expressed as viability of cfu/well± SD at a given time in hours.
Growth Index (GI) was calculated by the number of bacteria at timepoint 24 h divided by the number of bacteria at time 0 h.

Table 4.9: Invasion and net growth of of *S. typhi* Ty2, PhoP⁻ (RMA1030), *pqaA*::Mu*dJ* (RMA2310), and *pqaB*::Mu*dJ* (RMA2326) strains in PMA-U937 cells.

Bacterial strains[a]	Invasion[b]	GI[c]
Ty2 (PhoP⁺)	5.5 ± 1.3	25
RMA1030 (PhoP⁻)	2.8 ± 0.7	2
RMA2310 (*pqaA*::Mu*dJ*)	6.8 ± 2.5	16
RMA2326 (*pqaB*::Mu*dJ*)	4.9 ± 2.4	3

[a]All bacterial strains can be found in Table 2.3
[b]Invasion percentages were calculated by dividing the number of bacteria at time 0 after gentamicin treatment the number of bacteria initially added x 100
[c]Growth Index (GI) was calculated by the number of bacteria at timepoint 24 h divided by the number of bacteria at time 0 h.

The *S. typhi pqaB*::Mu*dJ* insertion mutant was also assayed for growth in HeLa cells to ensure that the mutation did not cause a non-specific intracellular growth defect. The *pqaB*::Mu*dJ* mutant gave a similar level of growth compared to the *S. typhi* Ty2 strain (data not shown), indicating that the growth defect was specific to macrophage-like PMA-U937 cells. Further analysis of the *S. typhi pqaB*::Mu*dJ* insertion mutant and testing a defined *S. typhi pqaB* mutant for growth in PMA-U937 cells is discussed in Section 6.10.3.

4.7 LDH Assay

The PMA-U937 assay strongly suggested differences between *S. typhi* Ty2, PhoP⁻ and *pqaB*::Mu*dJ* mutant strains with respect to the bacterial GI. However, it was possible that the differences in the *Salmonella* cfu/well at the timepoints (particularly 0 and 24 h) for the PMA-U937 cell assay were actually due to the bacteria having a cytotoxic effect on the infected macrophage-like cells. This would result in lower cfu/well counts independent of net growth differences between the *Salmonella* strains. As mentioned previously, the majority of the infected cells excluded trypan blue at 24 h indicating that they were viable. As a further test, lactate dehydrogenase (LDH) (a stable cytosolic enzyme which is released during cell lysis) levels were assayed and used to evaluate the amount of PMA-U937 cells remaining viable

over the 24 h assay. The amount of LDH contained within the PMA-U937 during infection of various *Salmonella* strains (Table 4.11). The data showed a 25-35 % variation in the number of PMA-U937 cells per well before bacterial infection (Table 4.11), which is consistent with data for other adherent cells lines (Monack *et al.*, 1996). This variation difference in the number of PMA-U937 cells may explain the large error bars seen in Figures 4.4, 4.5 and 4.6. Both *S. typhi* Ty2 and PhoP$^-$ (RMA1030) strains caused some cytolysis of the U937 cells during invasion but at 24 h, the damage was comparable, indicating that the difference in G.I. (Table 4.4) between the two strains was not due to differences in PMA-U937 cytolysis by the bacteria. Both the *S. typhi* PhoP^c (RMA1090) and *pqaB*::Mu*dJ* (RMA2326) strains also caused some cytolysis of PMA-U937 cells, albeit less than wildtype. PMA-U937 cells infected with *S. typhimurium* strains were also tested for release of LDH and at 18 h the bacteria displayed an amount of PMA-U937 cytolysis comparable to that of the *S. typhi* Ty2 strain at 24 h (data not shown).

Table 4.11: Lactate Dehydrogenase (LDH) assay for *S. typhi* infection of PMA-U937 cells

Strain[a]	LDH Units/Well (0 h)[b]	Fold decrease[c] (0 h)	LDH Units/Well (24 h)	Fold decrease[c] (24 h)
Uninfected	$4.03 \times 10^{-3} \pm 1.47 \times 10^{-3}$	1.00	$4.50 \times 10^{-3} \pm 9.22 \times 10^{-4}$	0.87
Ty2 (PhoP$^+$)	$2.03 \times 10^{-3} \pm 9.25 \times 10^{-4}$	0.50	$1.09 \times 10^{-3} \pm 9.47 \times 10^{-4}$	0.27
RMA1030 (PhoP$^-$)	$1.96 \times 10^{-3} \pm 1.15 \times 10^{-3}$	0.49	$1.16 \times 10^{-3} \pm 9.55 \times 10^{-4}$	0.29
RMA1090 (PhoP^c)	$2.98 \times 10^{-3} \pm 1.55 \times 10^{-3}$	0.74	$2.41 \times 10^{-3} \pm 1.25 \times 10^{-3}$	0.60
RMA2326 (*pqaB*::Mu*dJ*)	$2.92 \times 10^{-3} \pm 1.27 \times 10^{-3}$	0.72	$2.30 \times 10^{-3} \pm 1.50 \times 10^{-3}$	0.57

[a]All bacterial strains are described in Table 2.3.
[b]LDH units/well are presented as the mean of 4 assays ± SD.
[c]Fold decrease is the decrease in LDH units/well compared to uninfected cells at time 0

4.8 Summary

In this chapter, a tissue culture model (PMA-differentiated U937 cells) was used to study the invasion and intracellular net growth of *S. typhi* Ty2, PhoP$^-$, PhoP^c , *pqa/pqr* mutants and *E. coli* K12. The invasion and net growth of *S. typhimurium* C5, PhoP$^-$ and PhoP^c derivatives

in these cells was also investigated. The *S. typhi* strains mentioned above were also assayed in an epithelial (HeLa) cell line to test for general defects in intracellular net growth.

The *S. typhi* PhoP^c strain was found to be defective in invasion in both epithelial (HeLa) and macrophage-like (PMA-U937) cell lines compared to PhoP$^-$ and PhoP$^+$ strains (Section 4.2; Section 4.5.2). No invasion defects were noted for any of the five *S. typhi pqa/pqr*::Mu*dJ* insertion mutants. The *S. typhi* PhoP$^-$ strain was found to be growth-deficient in the PMA-U937 cells compared to the wildtype strain and this mutation could be complemented by a low-copy number plasmid containing the *S. typhimurium* phoP/Q genes (Section 4.5.2; Section 4.5.3). One of the *S. typhi pqa/pqr*::Mu*dJ* insertion mutants (*pqaB*::Mu*dJ*) showed a significant defect in intracellular growth compared to the wildtype *S. typhi* Ty2 strain (Section 4.6) and this is discussed further in Chapter 6.

The *S. typhimurium* PhoP$^-$, PhoP$^+$ and PhoP^c strains showed no defects in invasion or growth in the PMA-U937 assay (Section 4.5.4). These results and implications for *S. typhi phoP/Q* gene regulation are discussed further in Chapter 7.

Chapter 5

Identification and analysis of the *S. typhi pqaA* gene

5.1 Introduction

Antimicrobial peptides (AP) are part of the host innate immune response, and the number and diversity of AP's is extremely large with over 40 having been identified to date (Ganz & Lehrer, 1995). Defensins, melittin, magainins and cecropins belong to the same family of antimicrobial peptides. They are short cationic oligopeptides which adopt α-helical structures in hydrophobic environments, have been shown to form channels in artificial membranes (Christensen *et al.*, 1988; Duclohier *et al.*, 1989; Kagan *et al.*, 1990; Tosteson & Tosteson, 1984) and are toxic for a variety of target cells, including Gram-negative bacteria (Boman, 1991; Boman *et al.*, 1991). The cationic peptides must cross the OM of Gram-negative bacteria to reach the cytoplasmic membrane target, and being cationic they are expected to bind to the negatively charged LPS on the bacterial surface; this surface interaction has been demonstrated for magainin (Rana *et al.*, 1991).

Melittin, the principal toxic component of bee venom, consists of 26 amino acids, and has been shown to interact strongly with and disrupt lipid bilayer membranes (Sessa *et al.*, 1969). Melittin has been shown to act on virus membranes and cause lysis in a similar manner to complement (Esser *et al.*, 1979). No *S. typhimurium phoP/Q* regulated gene has been shown to be involved with melittin resistance thus far.

The *S. typhi pqaA*::Mu*d*J fusion was found to be strongly upregulated by PhoP/Q (β-galactosidase activity was increased 10-fold in the *pqaA*::Mu*d*J PhoP^c strain (RMA2510) compared to the same mutation in the PhoP$^-$ chromosomal background (RMA1110) (Section 3.7.4). The *S. typhi pqaA*::Mu*d*J mutant (RMA2310) was also found to be sensitive to the

antimicrobial peptide melittin (Baker *et al.*, 1997) but not the antimicrobial peptides protamine (Section 3.6.4) or magainin II (Daniels, 1994). The *S. typhimurium* PhoP$^-$ mutant has been shown to be extremely sensitive to antimicrobial peptides (Fields *et al.*, 1989; Groisman *et al.*, 1992B; Miller *et al.*, 1990; Porter *et al.*, 1997; Selsted *et al.*, 1992).

During the inital project, 130 bp of the *S. typhi pqaA* gene was isolated (Baker, 1993) (discussed in Section 5.2) and was found to have no homology to any other protein in the databases by BlastN/X/P 2.0 analysis (Altschul *et al.*, 1997), indicating the identification of a novel *Salmonella* gene. Therefore I felt this locus was worth further investigation and this chapter deals with the isolation and analysis of the entire *S. typhi pqaA* gene, the *pqaA* encoded protein and of the DNA adjacent to the *pqaA* locus.

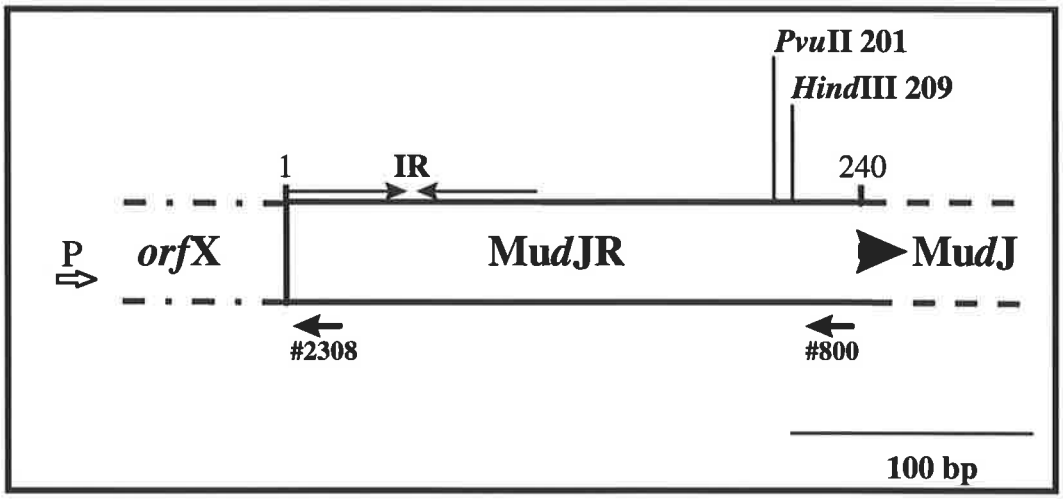
5.2 Isolation of sequence from the *pqaA*::Mu*dJ* RHS flanking DNA

This section contains work from a previous project (Baker, 1993) and was not performed as thesis work but is included to assist clarity and understanding of Section 5.3. The original isolation of a 130 bp fragment from the *S. typhi pqaA*::Mu*dJ* insertion mutant (RMA2310) was accomplished using a "PCR walking" technique (Willoughby *et al.*, 1991). This method involved a Ligatable primer (LP #801, Table 2.5) and a specific primer based on the RHS of the Mu*dJ* transposon (#800) (Table 2.5) (Mu*dJ* RHS, Figure 5.1).

The *S. typhi* RMA2310 (*pqaA*::Mu*dJ*) chromosome was fully digested with *Bgl*II, *Bam*HI and partially digested with *Sau*3A, ligated to double stranded LP and used as a template for "PCR walking" PCR (Willoughby *et al.*, 1991). A 600 bp fragment was obtained from the partial *Sau*3A digest ligation PCR mixture and digested with *Hind*III (a *Hind*III site is located at the end of the LP and within the Mu*dJ* fragment (Figure 5.1), cloned into pBluescript KS$^+$ to produce plasmid pRMSB5 and sequenced with M13 forward and reverse primers with dye

terminator sequencing (Baker, 1993). The pRMSB5 insert as depicted in Figure 5.2 contains 132 bp of the putative "*pqaA*" sequence flanked by the LP and Mu*dJ* RS transposon sequences (Baker, 1993).

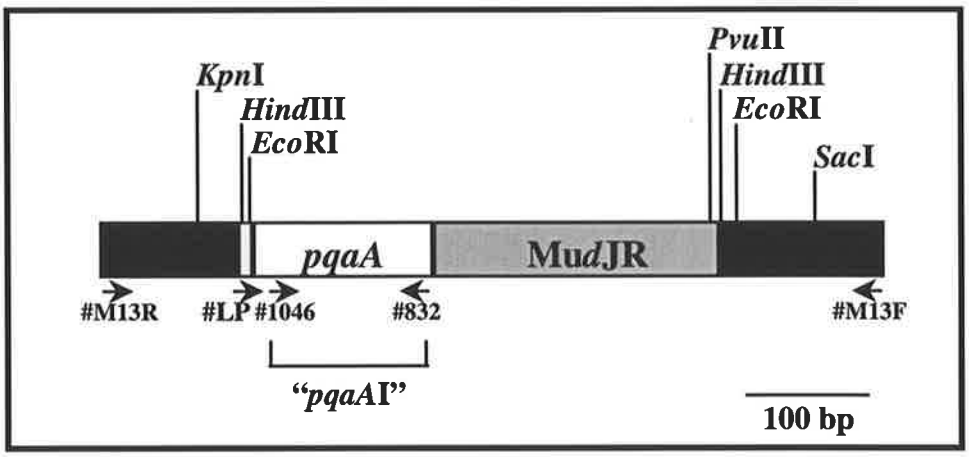
Figure 5.1: Schematic diagram of the RHS of Mu*dJ*



This schematic diagram of the first 240 nts of the RHS of Mu*dJ* is labelled 1-240 with 1 being the first nucleotide of the Mud*J*R transposon located next to the chromosomal insertion. This figure is modified from MuR sequence given in Metcalf *et al.*, (1990) (gbD: M33723). The transposon is represented in the orientation as inserted into an transcriptionally active ORF (represented by *orfX* with the direction of the transcriptional activity represented by the promoter (P) and an open arrow). Continuation into the Mu*dJ* transposon towards the Mu*dJ* LHS is represented by a solid arrowhead (➨). Restriction enzyme sites *Pvu*II and *Hind*III and their locations are marked as is the location of the 48 bp inverted repeat (IR, →←). The position of the oligonucleotides #800 and #2308 and direction of extention (←) are also labelled.

The partial "*pqaA*" sequence obtained showed no similarity to any sequence from the databases available at NCBI by BlastN/X/P 2.0 analysis (Altschul *et al.*, 1997). Although the Mu*dJ*R homology was strong enough to indicate that the correct *pqaA*-Mu*dJ* fusion fragment had been isolated, the DNA homology of the Mu*dJ*R sequence to MuR (Metcalf *et al.*, 1990) by FASTA (in DNASIS), analysis was only 89.9% over 210 bp (Figure 5.3).

Figure 5.2: Schematic diagram of pRMSB5 insert



This schematic diagram of the HindIII insert of pRMSB5 shows the *pqaA*-Mu*d*J (*pqaA* - unshaded box, Mu*d*JR - grey box) PCR fragment inserted between HindIII sites in pBluescript KS^+ (black boxes). The position of oligonucleotides #LP, #M13F, #M13R, #832 and #1046 and direction of extension (indicated by arrows →) are shown as well as the PCR product "*pqaA*I" produced with oligonucleotides #1046 and #832.

Figure 5.3: Homology between pRMSB5 cloned Mu*d*JR sequence and MuR

89.2% identity in 213 bp overlap

```
                    10         20         30         40         50
pRMSB5MudJR.SEQ  TGAAGCGGCGCACGAAAAGCGCAAAACTTTTAAAGGATAATGCCGAAAC-TAAGGTTTTC
                 :::::::::::::::::: ::: :::   ::  : :::::   :::::: ::: ::::::
MUR.SEQ          TGAAGCGGCGCACGAAAAACGCGAAAGCGTTTCACGATAAATGCGAAACGTAAAGTTTTC
                         10         20         30         40         50         60

                 60         70         80         90        100        110
pRMSB5MudJR.SEQ  GCATTTATCGTG-AACGCTTTCG-GTTTTTCGTGCGAGTGCTCATTAAGGAAAGGGACAA
                 :::::::::::: :::::::::: X:::::::::::     :::::::::::::: ::::
MUR.SEQ          GCATTTATCGTGAAACGCTTTCGCGTTTTTCGTGCGCCGCTTCATTAAGGAAAGGAACAA
                         70         80         90        100        110        120

                 120        130        140        150        160        170
pRMSB5MudJR.SEQ  TGACAACATTACTTAACCCCTATTTTGGTGAGTTTGGCGGCATGTACGTGCCAGAAATCC
                 :::::::::::::::::::::::::::::::::::::::::::::::::::::: ::::::
MUR.SEQ          TGACAACATTACTTAACCCCTATTTTGGTGAGTTTGGCGGCATGTACGTGCCACAAATCC
                         130        140        150        160        170        180

                 180        190        200        210
pRMSB5MudJR.SEQ  TGATGCCTGCTCTGCGCCAGCTGGGAGAAGCTT
                 :::::::::::::::::::::::: :::::::X
MUR.SEQ          TGATGCCTGCTCTGCGCCAGCTGGAAGAAGCTT
                         190        200        210
```

This figure compares the similarity between the cloned Mu*d*JR sequence contained in pRMSB5 with MuR (Metcalf *et al.*, 1990) (gbD: M33723). Sequences are represented in the same orientation as Figure 5.1. The 48 bp inverted repeat contained within MuR is underlined and the *Hind*III site at the end of the cloned fragment is represented in bold.

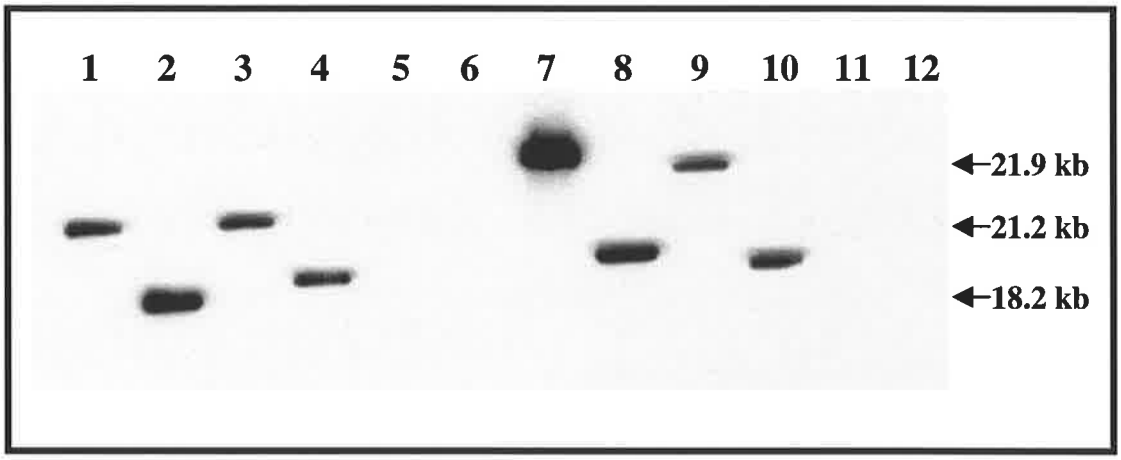
Sequence comparison by DNASIS of the cloned Mu*d*JR sequence and MuR (Metcalf *et al.*, 1990), shows that most of the mismatches seen are in the 48 bp inverted repeat region (IR)

located within the Mu*d*JR (Figure 5.1, Figure 5.3). It is likely that the IR in the cloned Mu*d*J-*pqaA* fragment is disrupted due to nt changes in the original PCR as it has previously been reported to be a difficult region to PCR (Metcalf *et al.*, 1990). In this project it was relatively easy to amplify the pRMSB5 insert by PCR with #M13F and #M13R primers ("*pqaA*-Mu*d*JR") with 55°C annealing, 72°C extension temperatures but no PCR product was ever obtained by PCR (using the specific #1046 oligonucleotide designed to *pqaA* and oligonucleotide #800) using a chromosomal template, and similar PCR conditions (data not shown).

5.3 Southern analysis of *Salmonella*, *E. coli* and *Shigella* DNA with the *pqaA* probe.

As the 600 nt pRMSB5 insert obtained for the *pqaA*::Mu*d*J had no homology to anything in the database for the 132 nt *pqaA* sequence and the homology to MuR (Metcalf *et al.*, 1990) obtained for the Mu*d*JR fragment had many mismatches, it was essential to determine that the unique 132 nt fragment sequence did come from the *pqaA* gene. Therefore two oligonucleotides were designed (#832 and #1046 (Figure 5.2; Table 2.5)), to give a product size of 110 nt (named "*pqaA*I") in a PCR reaction with a *S. typhi* Ty2 chromosomal DNA template. This product was labelled with DIG-11-dUTP in a DIG-PCR labelling reaction (Section 2.7.1.2) and used in Southern hybridisation to probe *S. typhi* Ty2, RMA1110 (*pqaA*::Mu*d*J, PhoP$^-$) and RMA2310 (*pqaA*::Mu*d*J), *S. typhimurium* LT2, *Shigella flexneri* and *E. coli* K12 chromosomal DNA. It was expected that by digesting with the same restriction enzyme, the Mu*d*J insertion should affect the size of the band obtained for *pqaA* in the RMA2310 strain compared to the wildtype parental *S. typhi* Ty2 strain. The results of this Southern are shown in Figure 5.4 and Table 5.1.

Figure 5.4: Southern hybridisation analysis of *Salmonella*, *E. coli* and *Shigella* chromosomal DNA with the "*pqaA*I" DIG labelled probe



Southern blot analysis of *Salmonella*, *E. coli* and *Shigella* *Bgl*II and *Sal*I digested chromosomal DNA probed with the DIG-labelled "*pqaA*I" PCR product (Figure 5.2). The approximate size of bands are shown in kilobases (kb) and were calculated by comparison with *Bgl*II digested λ marker DNA (Section 2.5.2.2). The order of the lane loading is labelled numerically at the top of the figure and the sample in the lanes are; Lane 1: *S. typhi* RMA1110 (*pqaA*::Mu*dJ*) [*Bgl*II], Lane 2: *S. typhi* Ty2 [*Bgl*II] , Lane 3: *S. typhi* RMA2310 (*pqaA*::Mu*dJ*) [*Bgl*II], Lane 4: *S. typhimurium* EX730 [*Bgl*II] , Lane 5: *S. flexneri* PE577 [*Bgl*II] , Lane 6: *E. coli* DH5 [*Bgl*II] , Lane 7: *S. typhi* RMA1110 (*pqaA*::Mu*dJ*) [*Sal*I], Lane 8: *S. typhi* Ty2 [*Sal*I] , Lane 9: *S. typhi* RMA2310 (*pqaA*::Mu*dJ*) [*Sal*I] , Lane 10: *S. typhimurium* EX730 [*Sal*I] , Lane 11: *S. flexneri* PE577 [*Sal*I] , Lane 12: *E. coli* DH5 [*Sal*I]

Table 5.1: Band sizes from the Southern hybridisation analysis of *Salmonella*, *E. coli* and *Shigella* chromosomal DNA with the "*pqaA*I" DIG labelled probe

Figure 5.4 Lane No's	Restriction Enzyme	RMA1110	*S. typhi* Ty2	RMA2310	EX730	PE577	DH5
1-6	*Bgl*II	21.2	18.2	21.2	19.3	-	-
7-12	*Sal*I	21.9	20.0	21.9	19.8	-	-

This table gives the band sizes obtained from the Southern blot analysis of *Salmonella*, *E. coli* and *Shigella* *Bgl*II and *Sal*I digested chromosomal DNA probed with the DIG-labelled "*pqaA*I" PCR product (Figure 5.4). The approximate size of bands are shown in kilobases (kb) and the sizes of the bands detected were calculated in kb by comparison with *Bgl*II digested λ markers (Section 2.5.2.2).

A shift in band size (3.0 kb for *Bgl*II digests and 1.9 kb for the *Sal*I digest) was seen for the strains with a *pqaA*::Mu*dJ* insertion compared to wildtype *S. typhi* Ty2 chromosomal DNA, verifying that the unique sequence obtained from the *pqaA*-Mu*dJR* fusion did belong to

the *S. typhi pqaA* gene. The *S. typhimurium* LT2 strain gave similar unique bands compared to *S. typhi* chromosomal DNA (Figure 5.4, Table 5.1). However, even after extensive exposure, no bands could be seen for the *Shigella* and *E. coli* chromosomal DNA, the Southern hybridisation was repeated with chromosomal DNA from a greater number of *Shigella* and *E. coli* strains (at low stringency) and the same result was obtained, confirming that the *pqaA* gene is likely to be *Salmonella* specific (data not shown).

5.4 Isolation of further *S. typhi* Ty2 *pqaA* gene sequence

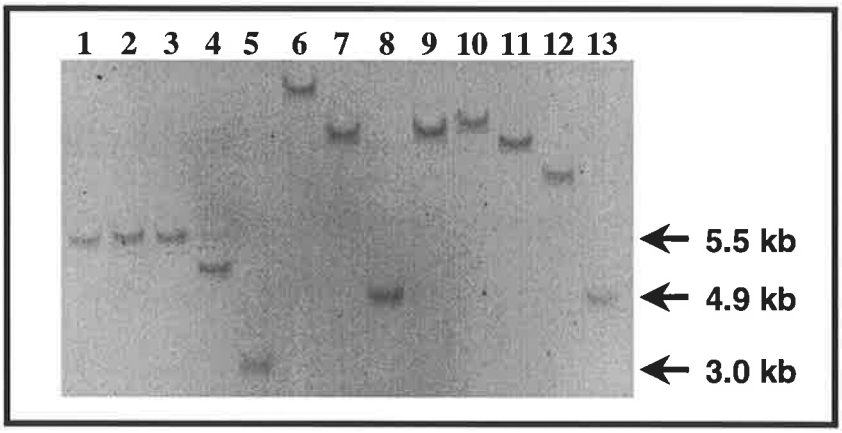
5.4.1 Probing of *pqaA*::Mu*dJ* left flanking DNA for Inverse PCR

The DIG-labelled "Mu*dJL*" probe used in Chapter 3 for the LHS of Mu*dJ* was used to probe RMA2310 chromosomal DNA digested with *Dra*I, *Nhe*I, *Mlu*I and *Hind*III to determine if a suitable sized fragment could be isolated by inverse PCR. Unfortunately all the bands obtained were greater than 7 kb in size (data not shown) and therefore unsuitable for an inverse PCR reaction (Section 2.5.3.3).

5.4.2 Southern analysis of wildtype *S. typhi* Ty2 DNA

As the *Salmonella pqaA* gene appeared to be specific to *Salmonella,* it was judged that *pqaA* gene sequence cloned into a plasmid would be simple to screen in *E. coli* due to a low background. Therefore a "Shotgun cloning" approach based on a plasmid bank of *S. typhi* DNA was undertaken. The *S. typhi* Ty2 chromosomal DNA (doubly digested with restriction enzymes) was probed with the "*pqaA*I" probe (Section 5.3) and the results are shown in Figure 5.5 and Table 5.2.

Figure 5.5: Southern hybridisation analysis of *S. typhi* Ty2 chromosomal DNA with the "*pqaA*I" DIG labelled probe



Southern blot analysis of *S. typhi* Ty2 "double-digested" chromosomal DNA probed with the DIG-labelled "*pqaA*I" PCR product (Figure 5.1)(Section 2.7). The approximate size of bands are shown in kilobases (kb) and were calculated by comparison with *Eco*RI digested SPP1 DNA markers (Section 2.5.2.2). The order of the lane loading is labelled numerically at the top of the figure and the sample in the lanes are; Lane 1: *S. typhi* Ty2 [*Hind*III/*Pst*I], Lane 2: *S. typhi* Ty2 [*Hind*III/*Eco*RI], Lane 3: *S. typhi* Ty2 [*Hind*III/*Bam*HI], Lane 4: *S. typhi* Ty2 [*Hind*III/*Sal*I], Lane 5: *S. typhi* Ty2 [*Hind*III/*Eco*RV], Lane 6: *S. typhi* Ty2 [*Bam*HI/*Pst*I], Lane 7: *S. typhi* Ty2 [*Bam*HI/*Sal*I], Lane 8: *S. typhi* Ty2 [*Sal*I/*Eco*RV], Lane 9: *S. typhi* Ty2 [*Sal*I/*Pst*I], Lane 10: *S. typhi* Ty2 [*Bam*HI/*Eco*RI], Lane 11: *S. typhi* Ty2 [*Pst*I/*Eco*RI], Lane 12: *S. typhi* Ty2 [*Sal*I/*Eco*RI], Lane 13: *S. typhi* Ty2 [*Eco*RI/*Eco*RV]

Table 5.2: Southern hybridisation analysis of *S. typhi* Ty2 chromosomal DNA with "*pqaA*I"

Figure 5.4: Lane No's	Restriction Enzymes	*S. typhi* Ty2
Lane 1	*Hind*III/*Pst*I	5.5
Lane 2	*Hind*III/*Eco*RI	5.5
Lane 3	*Hind*III/*Bam*HI	5.7
Lane 4	*Hind*III/*Sal*I	4.7
Lane5	*Hind*III/*Eco*RV	3.0
Lane 6	*Bam*HI/*Pst*I	>8.5
Lane 7	*Bam*HI/*Sal*I	>8.5
Lane 8	*Sal*I/*Eco*RV	4.9
Lane 9	*Sal*I/*Pst*I	>8.5
Lane 10	*Bam*HI/*Eco*RI	>8.5
Lane 11	*Pst*I/*Eco*RI	>8.5
Lane 12	*Sal*I/*Eco*RI	>8.5
Lane 13	*Eco*RI/*Eco*RV	4.9

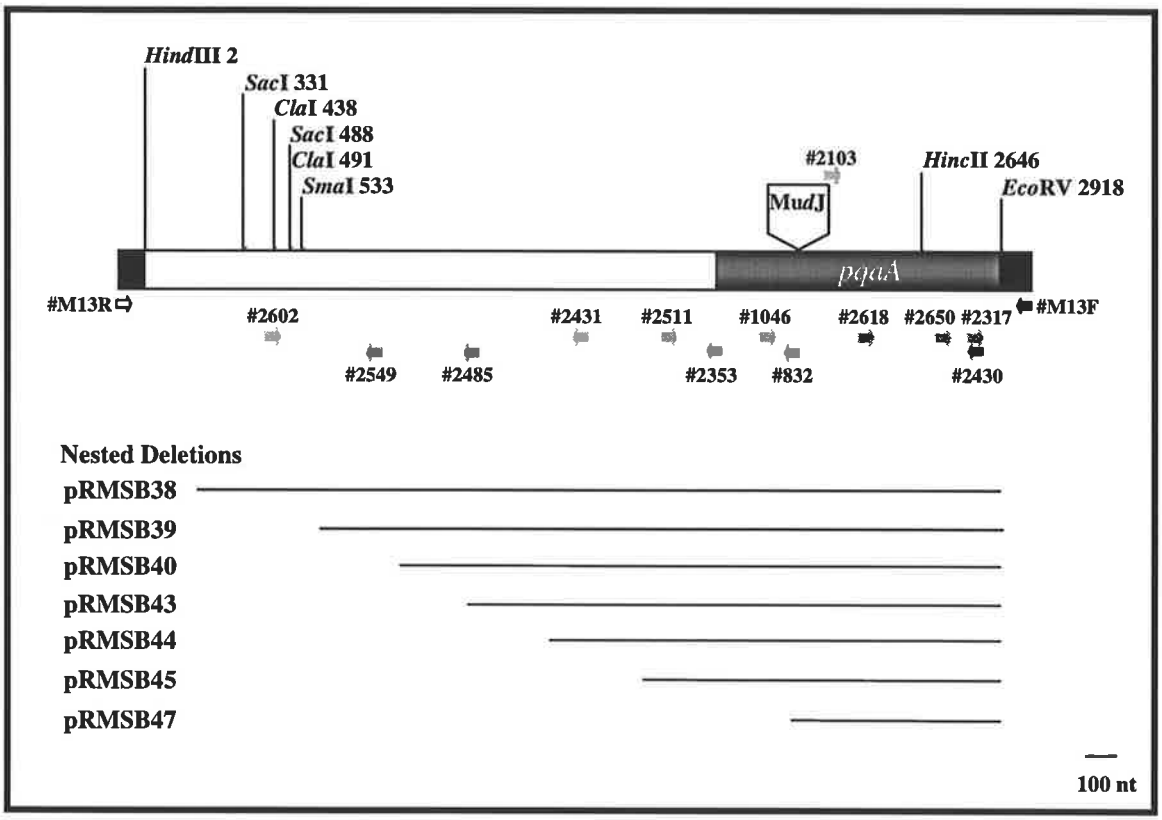
This table gives the band sizes obtained from the Southern blot analysis of double-digested *S. typhi* Ty2 chromosomal DNA probed with the DIG-labelled "*pqaA*I" PCR product (Figure 5.4). The approximate size of bands are shown in kilobases (kb) and the sizes of the bands detected were calculated in kb by comparison with *Eco*RI digested SPP1 markers (Section 2.5.2.2).

The results showed that the smallest band obtained (thus potentially easiest to clone) was for the *Hind*III/*Eco*RV digest which gave a band of ~3kb (Figure 5.5). Therefore ~10 μg of *S. typhi* Ty2 chromosomal DNA was digested with *Hind*III and *Eco*RV and electrophoresed on a 1% TAE gel. The chromosomal DNA between between *Eco*RI digested SPP1 marker bands 5 and 6 (between 3.59 and 2.81 kb) was isolated as described in Section 2.5.2.5. This chromosomal DNA was ligated to similarly digested pBluescript KS^+, transformed into competent DH5α cells and transformants were plated onto LA containing Amp plates.

Approximately 400 white colonies were then screened by spotting miniprep DNA onto nylon membrane (DNA was made by using the short microtitre tray miniprep method, (Section 2.5.1.3) and probing with the "*pqaA*I" probe. One clone was obtained and the plasmid was named pRMSB19 (in RMA1121) (Figure 5.6).

The *Hind*III/*Eco*RV insert from pRMSB19 was then sequenced using a combination of #M13F and #M13R oligonucleotides, pRMSB19 nested deletions (Section 2.5.2.6), and with oligonucleotides designed from the newly determined sequence (Table 2.5) (Figure 5.6). Sequencing and analysis (translation of the sequence using DNASIS) of the *Hind*III/*Eco*RV fragment revealed that the putative 5' end of the *S. typhi pqaA* gene had been obtained (Figure 5.6), however the 3' end of the *pqaA* gene still needed to be isolated.

Figure 5.6: Schematic diagram of the pRMSB19 *Hind*III/*Eco*RV insert and sequencing strategy



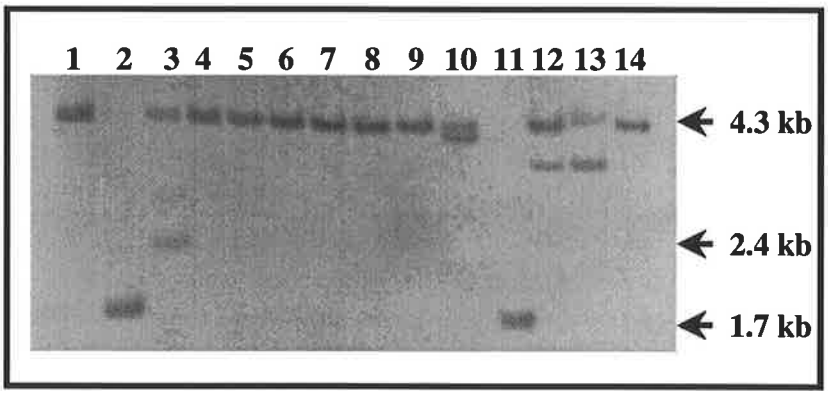
This schematic restriction enzyme map of the *S. typhi pqaA* *Hind*III/*Eco*RV insert in pRMSB19 shows 2.92 kb containing the *pqaA* gene (indicated by a dark grey rectangle) and surrounding sequence flanked by the pBluescriptKS$^+$ multiple cloning site (indicated by black boxes). Restriction enzymes and their positions are marked. The oligonucleotides #M13F (black arrow), #M13R (open arrow) (Table 2.5) and oligonucleotides designed for sequencing (light and dark grey solid arrows) and their relative positions are marked as well as the locations for the nested deletion derivatives of pRMSB19.

5.4.3 Use of SSP-PCR and IPCR to obtain the 3' end of the *S. typhi pqaA* gene

To obtain the remainder of the *S. typhi pqaA* gene, it was decided to use the SSP-PCR method (Section 2.5.3.4). Therefore, a further Southern was performed with *S. typhi* RMA2310 (*pqaA*::Mu*dJ*) chromosomal DNA (digested with *Hind*III as the primary enzyme and *Eco*RV etc as secondary enzymes), probed with the DIG-labelled "Mu*d*JL" primer (Section 3.8.1). The results can be seen in Figure 5.7 and Table 5.3 and it can be seen that the *Hind*III digest gave a fragment size of 4.3 kb, and in most of the double digests the second

enzyme did not restrict this fragment. It was also noted that in some of the digests where the second enzyme did cut the *Hind*III fragment, it did not cut completely, giving two bands; one of which was the same size as the 4.3 kb *Hind*III fragment, the other smaller band was therefore presumed to be a DNA fragment cut with both enzymes. Six of the double digestions gave fragments smaller than 4 kb and therefore potentially suitable for SSP-PCR and it was decided to use three of these; *Hind*III/*Hinc*II ~1.7 kb, *Hind*III/*Cla*I ~3.9 kb and *Hind*III/*Sal*I ~3.4 kb.

Figure 5.7: Southern hybridisation analysis of *S. typhi* RMA2310 chromosomal DNA with the "Mu*d*JL" DIG-labelled PCR probe.



Southern blot analysis of *S. typhi* RMA2310 (*pqaA*::Mu*dJ*) "double-digested" chromosomal DNA probed with the DIG-labelled "Mu*d*JL" PCR product (Figure 3.8.1). The approximate size of bands are shown in kilobases (kb) and were calculated by comparison with *Eco*RI digested SPP1 DNA markers (Section 2.5.2.2). Only bands detected above 1.5 kb are shown. The order of the lane loading is labelled numerically at the top of the figure and the sample in the lanes are; Lane 1: *S. typhi* RMA2310 (*pqaA*::Mu*dJ*) [*Hind*III], Lane 2: *S. typhi* RMA2310 [*Hind*III/*Eco*RV], Lane 3: *S. typhi* RMA2310 [*Hind*III/*Eco*RI], Lane 4: *S. typhi* RMA2310 [*Hind*III/*Sma*I], Lane 5: *S. typhi* RMA2310 [*Hind*III/*Pst*I], Lane 6: *S. typhi* RMA2310 [*Hind*III/*Xba*I], Lane 7: *S. typhi* RMA2310 [*Hind*III/*Sac*I], Lane 8: *S. typhi* RMA2310 [*Hind*III/*Not*I], Lane 9: *S. typhi* RMA2310 [*Hind*III/*Bam*HI], Lane 10: *S. typhi* RMA2310 [*Hind*III/*Cla*I], Lane 11: *S. typhi* RMA2310 [*Hind*III/*Hinc*II], Lane 12: *S. typhi* RMA2310 [*Hind*III/*Kpn*I], Lane 13: *S. typhi* RMA2310 [*Hind*III/*Sal*I], Lane 13: *S. typhi* RMA2310 [*Hind*III/*Xho*I]

Table 5.3: Southern hybridisation analysis of *S. typhi* RMA2310 chromosomal DNA with "Mu*d*JL" DIG-labelled PCR probe

Figure 5.5: Lane No's	Restriction Enzymes	RMA2310 (*pqaA*::Mu*d*J)
Lane 1	*Hind*III	4.3
Lane 2	*Hind*III/*Eco*RV	1.8
Lane 3	*Hind*III/*Eco*RI	4.3, 2.4
Lane 4	*Hind*III/*Sma*I	4.3
Lane 5	*Hind*III/*Pst*I	4.3
Lane 6	*Hind*III/*Xba*I	4.3
Lane 7	*Hind*III/*Sac*I	4.3
Lane 8	*Hind*III/*Not*I	4.3
Lane 9	*Hind*III/*Bam*HI	4.3
Lane 10	*Hind*III/*Cla*I	4.3, 3.9
Lane 11	*Hind*III/*Hinc*II	1.7
Lane 12	*Hind*III/*Kpn*I	4.3, 3.5
Lane 13	*Hind*III/*Sal*I	4.3, 3.4
Lane 14	*Hind*III/*Xho*I	4.3

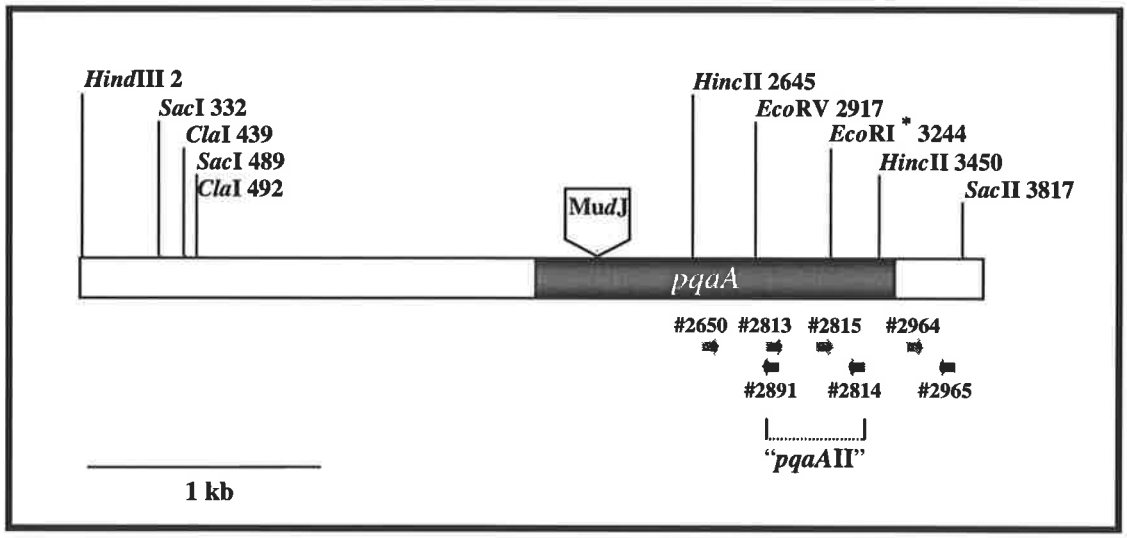
This table gives the band sizes obtained from the Southern blot analysis of double-digested *S. typhi* RMA2310 (*pqaA*::Mu*d*J) chromosomal DNA probed with the DIG-labelled "Mu*d*JL" PCR product (Figure 5.5)(Section 2.7). The approximate size of bands are shown in kilobases (kb) and the sizes of the bands detected were calculated in kb by comparison with *Eco*RI digested SPP1 markers (Section 2.5.2.2).

Therefore RMA2310 chromosomal DNA was digested with these enzymes and ligated to similarly digested pBluescript KS^+ plasmid and the ligation mixture was used as the SSP-PCR template (Section 2.5.3.4). PCR reactions were then performed using the Mu*d*JL oligonucleotide #2103 (Figure 3.5) and #M13F or #M13R primers respectively. Unfortunately, none of the PCR reactions gave a band of the anticipated size, so the oligonucleotide #2650 (Table 2.5) based on the *S. typhi pqaA* sequence (located ~260 nt before the *Eco*RV site, Figure 5.6) was used instead of oligonucleotide #2103. The SSP-PCR reaction was then repeated with *Hind*III/*Cla*I and *Hind*III/*Sal*I templates and oligonucleotides #2650 and #M13F or #M13R as appropriate, and again no correct sized products were obtained.

A *Sac*I restriction enzyme site lies on the chromosome just before the *S. typhi pqaA* gene (Figure 5.6) and the *Hind*III/*Eco*RI digest gave a fragment size of 2.4 kb when probed with the

"Mu*dJL*" probe (Figure 5.7, Table 5.3), indicating that an *Eco*RI site is likely to be 3' to the *Eco*RV site in *pqaA*. Therefore, *S. typhi* Ty2 chromosomal DNA was digested with *SacI/Eco*RI, ligated to similarly digested pBluescriptKS$^+$, and the ligation mixture used for a SSP-PCR reaction with #2650 and #M13R to give an expected PCR product size of ~600 nt. The SSP-PCR reaction with the *SacI/Eco*RI template gave a band of the correct size which was purified and directly sequenced with oligonucleotides #2650 and #M13R. (It was later noted when sequencing over the *Eco*RI site region that the enzyme had cut the *S. typhi* chromosomal DNA by star activity on a sequence of GATTTC, rather than the correct site of GAATTC) (Figure 5.8).

Figure 5.8: *S. typhi pqaA* and surrounding DNA sequencing map

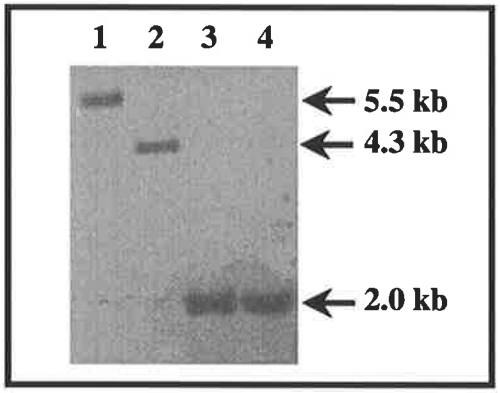


This schematic restriction enzyme map of the *S. typhi pqaA* and surrounding sequence shows 3.9 kb containing the *pqaA* gene (indicated by a dark grey rectangle) and surrounding sequence. Restriction enzymes and their positions are marked, including the *Eco*RI site which is represented by *Eco*RI* as it represents an *Eco*RI site only restricted by star activity. Oligonucleotides designed for sequencing (light and dark grey solid arrows) and their relative positions are marked as well as the PCR product "*pqa*AII" produced with oligonucleotides #2813 and #2814.

The direct sequencing of the *SacI/Eco*RI SSP-PCR product revealed that not all of the *pqaA* ORF had been isolated (Figure 5.8) so oligonucleotides #2813 and #2814 (Table 2.5) were designed to *S. typhi pqaA* sequence downstream of the *Eco*RV fragment to create a

second *S. typhi pqaA* probe ("*pqaA*II") (Figure 5.8). Another Southern hybridisation analysis was then performed using DIG-labelled "*pqaA*II" PCR probe, this time using *Eco*RV and double digests with *Eco*RV and a variety of enzymes (Figure 5.9).

Figure 5.9: Southern hybridisation analysis of *S. typhi* Ty2 chromosomal DNA with the "*pqaA*II" DIG labelled probe



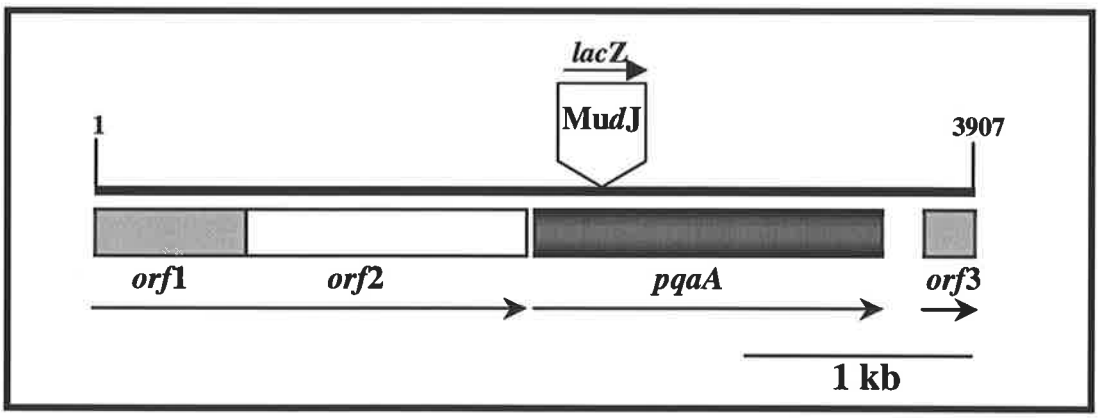
Southern blot analysis of *S. typhi* single and "double-digested" chromosomal DNA probed with the DIG-labelled "*pqaA*II" PCR product (Figure 5.8)(Section 2.7). The approximate size of bands are shown in kilobases (kb) and were calculated by comparison with the *Eco*RI digested SPP1 DNA markers (Section 2.5.2.2). The order of the lane loading is labelled numerically at the top of the figure and the sample in the lanes are; Lane 1: *S. typhi* Ty2 [*Hind*III], Lane 2: *S. typhi* RMA2310 (*pqaA*::Mud*J*) [*Hind*III], Lane 3: *S. typhi* Ty2 [*Eco*RV], Lane 4: *S. typhi* Ty2 [*Eco*RV/ *Hind*III]

The results showed that an *Eco*RV fragment of 2.0 kb was detected. Therefore *S. typhi* Ty2 chromosomal DNA was digested with *Eco*RV, diluted and religated and used as the template for an inverse PCR reaction with oligonucleotides #2981 and #2815 (Figure 5.9; Table 2.5; Section 2.5.3.3) designed to the *S. typhi pqaA* sequence: The resulting IPCR produced a correctly sized product of 2.0 kb which was purified and directly sequenced with oligonucleotides (Figure 5.7) and dye terminator sequencing (Section 2.5.2.7.1). The DNA sequencing of the fragment showed that the sequence of the entire *S. typhi pqaA* gene had been isolated, and double stranded sequencing of the *S. typhi pqaA* region was continued for 309 nts after the end of the *pqaA* gene. The analysis of the *pqaA* gene and surrounding DNA sequence are presented below in Section 5.5.

5.5 The *S. typhi pqaA* gene and surrounding sequence

Sequencing of the *S. typhi pqaA* gene and adjacent DNA (3907 nt) revealed 2 partial ORFs and two full ORFs (homologies discussed below) (Figure 5.10 and Figure 5.11). The location and several properties, including G+C% content of each of the ORF's designated ORF1, ORF2, PqaA and ORF3, are summarised in Table 5.4. Analysis of the DNA sequence for transcription and translation details, and significant similarities with other known proteins, revealed by comparison with sequence databases (BlastN/X 2.0; Altschul *et al.*, 1997)) are described below for each of the ORF's. The ORF1, ORF2 and ORF3 will be discussed in Sections 5.5.1, 5.5.2 and 5.5.3, respectively with more detailed analysis of the *S. typhi pqaA* gene presented in Section 5.6. Localisation of the *S. typhi pqaA* gene on the *S. typhimurium* chromosome will be discussed in Section 5.7.

Figure 5.10: Schematic map of the *S. typhi pqaA* gene and adjacent DNA



This schematic representation of the *S. typhi pqaA* gene and adjacent DNA shows two full open reading frames present in the total 3907 nts of DNA, *orf2* and *pqaA* shown as open and dark grey shaded boxes respectively. The Mud*J* insertion between nucleotides 2230 and 2231 is shown as well as the direction of transcription for lacZ (→). Two partial ORF were also found to be present encoded by *orf1* and *orf3*, represented by light grey boxes. The direction of transcription for each *orf* is indicated by an arrow (→) below the *orf*'s.

Figure 5.11: Sequence outline of the *S. typhi pqaA* gene and surrounding DNA

```
          10        20        30        40        50        60
5'AAGCTTTGTCAGAAAGAAATTAGGCGTCAGCTTCCCCGGCCGTACGTTGGGAATCCATGC
ORF1 S  F  V  R  K  K  L  G  V  S  F  P  G  R  T  L  G  I  H  A
```

```
        70         80         90        100        110        120
GCTTGTGGCTGACGCTTCACTTACCAGGGGATGGAAAATGGCAGCCTCGCTACGGGGCGC
 L  V  A  D  A  S  L  T  R  G  W  K  M  A  A  S  L  R  G  A

       130        140        150        160        170        180
AGGAGAAGAGCTTCTCGATAGCTATGAACAGGAACGCCGCCCGGTAGCCGAATCACTGCT
 G  E  E  L  L  D  S  Y  E  Q  E  R  R  P  V  A  E  S  L  L

       190        200        210        220        230        240
CCACCTTTCTACCCGATTACTTGACTCTCAAAAACAGGGTGGCATCAAACGAGAACGCGA
 H  L  S  T  R  L  L  D  S  Q  K  Q  G  G  I  K  R  E  R  D

       250        260        270        280        290        300
CGTTCAACAACTTGACATCCAGTATACCAACTCCCCCTTAGCCCACACGTTGCCCGAACG
 V  Q  Q  L  D  I  Q  Y  T  N  S  P  L  A  H  T  L  P  E  R

       310        320        330        340        350        360
TCAGCATGGGTTACAAGCGGGAGAAAGAGCTCCGGATGCTCCTCTTTTGGGCGCCGGCGG
 Q  H  G  L  Q  A  G  E  R  A  P  D  A  P  L  L  G  A  G  G

       370        380        390        400        410        420
CCAATCATTACGATTATTTCAGTTACTCCAGGGTCCGGACTGGAATCTGTTGGCCTACGA
 Q  S  L  R  L  F  Q  L  L  Q  G  P  D  W  N  L  L  A  Y  E

       430        440        450        460        470        480
AACCCACGGTAAGGTCATCGATGCGCGGCGAGTCTGCGTATTCATCACATTGGCGAGCAG
 T  H  G  K  V  I  D  A  R  R  V  C  V  F  I  T  L  A  S  R

       490        500        510        520        530        540
GACGAGCTCATCGATACCCTGGGGCACTTCCGGGAGTCCTATCATCTGGCCCGGTCAATG
 T  S  S  S  I  P  W  G  T  S  G  S  P  I  I  W  P  G  Q  C

       550        560        570        580        590        600
CGTGCTGATCCGACCCGATGGCTATGTCGGCGCATTCTTTCACGGCAAACAGAGCAACGA
 V  L  I  R  P  D  G  Y  V  G  A  F  F  H  G  K  Q  S  N  D

       610        620        630        640        650        660
TATTGAAAATTATCTCTCTCGTTTTGCCATAGGGATTAAAGATGAATACTAATGTCTATG
 I  E  N  Y  L  S  R  F  A  I  G  I  K  D  E  Y  *
                                  ORF2 M  N  T  N  V  Y  E

       670        680        690        700        710        720
AGAACACCGACAGCGAAACTATCACCCCGCTCAACAAGCGGCGTATTTTGCCTGTTTTCC
  N  T  D  S  E  T  I  T  P  L  N  K  R  R  I  L  P  V  F  L

       730        740        750        760        770        780
TGCTTGTCGGCCTTTACGCCGCCAGTACAGCGGCTGTGATGTCGGTACTGCCTTTTTATA
  L  V  G  L  Y  A  A  S  T  A  A  V  M  S  V  L  P  F  Y  I

       790        800        810        820        830        840
TCCGCGAGATGGGCGGTTCGCCGCTTATCATTGGAATCATCATCGCCACTGAGGCTTTTA
  R  E  M  G  G  S  P  L  I  I  G  I  I  I  A  T  E  A  F  S

       850        860        870        880        890        900
GCCAATTTTGTGCAGCGCCCCTGATTGGCCACCTTTCCGATCGCGTTGGCCGCAAGCGAA
  Q  F  C  A  A  P  L  I  G  H  L  S  D  R  V  G  R  K  R  I

       910        920        930        940        950        960
TACTGATTGTCACGCTGGCTATTGCGGCGATAAGTTTACTATTACTCGCCAACGCGCAAT
  L  I  V  T  L  A  I  A  A  I  S  L  L  L  L  A  N  A  Q  C

       970        980        990       1000       1010       1020
GTATCCTGTTTATCCTGCTCGCCCGCACGCTTTTTGGCATTAGCGCCGGGAATTTGTCAG
  I  L  F  I  L  L  A  R  T  L  F  G  I  S  A  G  N  L  S  A

      1030       1040       1050       1060       1070       1080
CCGCCGCAGCCTATATTGCCGATTGTACGCACGTCAGAAATCGGCGCCAGGCAATCGGTA
  A  A  A  Y  I  A  D  C  T  H  V  R  N  R  R  Q  A  I  G  I
```

```
     1090       1100       1110       1120       1130       1140
TCCTCACAGGCTGCATTGGTTTAGGCGGTATTGTCGGGGCAGGCGTTTCCGGGTGGCTAT
  L  T  G  C  I  G  L  G  G  I  V  G  A  G  V  S  G  W  L  S

     1150       1160       1170       1180       1190       1200
CGCGTATCAGTCTGGGTGCGCCGATCTACGCCGCCTTTATACTTGTCCTTGGGGCTGCCC
  R  I  S  L  G  A  P  I  Y  A  A  F  I  L  V  L  G  A  A  L

     1210       1220       1230       1240       1250       1260
TGGTCGCGATTTGGGGGTTAAAAGACCCTTCCACAACATCACGTACCACAGATAAAATAG
  V  A  I  W  G  L  K  D  P  S  T  T  S  R  T  T  D  K  I  A

     1270       1280       1290       1300       1310       1320
CGTCGTTCTCTGCCCGCGCTATTTTAAAGATGCCAGTCCTTCGCGTCTTAATCATCGTAA
  S  F  S  A  R  A  I  L  K  M  P  V  L  R  V  L  I  I  V  M

     1330       1340       1350       1360       1370       1380
TGCTTTGTCATTTCTTCGCCTATGGCATGTACTCTTCACAATTACCTGTTTTTCTTTCTG
  L  C  H  F  F  A  Y  G  M  Y  S  S  Q  L  P  V  F  L  S  D

     1390       1400       1410       1420       1430       1440
ACACCTTCATCTGGAATGGGCTTCCCTTTGGGCCAAAAGCGTTAAGCTATCTGTTAATGG
  T  F  I  W  N  G  L  P  F  G  P  K  A  L  S  Y  L  L  M  A

     1450       1460       1470       1480       1490       1500
CGGACGGGGTTATTAATATTTTCGTTCAGCTATTTCTGTTAGGTTGGGTGAGCCAATATT
  D  G  V  I  N  I  F  V  Q  L  F  L  L  G  W  V  S  Q  Y  F

     1510       1520       1530       1540       1550       1560
TTTCGGAGCGAAAGCTAATTATCCTCATCTTCGCCCTTCTTTGTACTGGATTTCTCACTG
  S  E  R  K  L  I  I  L  I  F  A  L  L  C  T  G  F  L  T  A

     1570       1580       1590       1600       1610       1620
CGGGTATCGCCACGACCATACCAGTGCTTATTTTTGCTATCGTTTGTATTAGCATCGCTG
  G  I  A  T  T  I  P  V  L  I  F  A  I  V  C  I  S  I  A  D

     1630       1640       1650       1660       1670       1680
ATGCGCTAGCCAAACCCACTTATCTTGCCGCCTTGTCCGTCCATGTATCGCCTGCCCGAC
  A  L  A  K  P  T  Y  L  A  A  L  S  V  H  V  S  P  A  R  Q

     1690       1700       1710       1720       1730       1740
AAGGTATCGTCATCGGAACGGCGCAGGCATTAATCGCAATCGCTGATTTTATATCCCCCG
  G  I  V  I  G  T  A  Q  A  L  I  A  I  A  D  F  I  S  P  V

     1750       1760       1770       1780       1790       1800
TATTGGGCGGATTTGTCCTGGGTTATGCCCTGTATGGCGTCTGGATCGGTATAGCTATCT
  L  G  G  F  V  L  G  Y  A  L  Y  G  V  W  I  G  I  A  I  S

     1810       1820       1830       1840       1850       1860
CTGTCGCCATTATTGGTCTGGTGACGGCAATGATTTACCTTTCAAAAAGTTCACCGCTAA
  V  A  I  I  G  L  V  T  A  M  I  Y  L  S  K  S  S  P  L  I

     1870       1880       1890       1900       1910       1920
TAGCGAAACCAGAAACAGAATAATGTGACAACGTCACAAGCATTATTACTCCCTTCTTCC
  A  K  P  E  T  E  *

     1930       1940       1950       1960       1970       1980
CTTATTTATCAAAAGTATTGTACTTATATTGAATGTTTAAGAGGTATTTATGGAAATTAT
                                 PqaA M  F  K  R  Y  L  W  K  L  C

     1990       2000       2010       2020       2030       2040
GCTGGTTGGCATTTGCCCTTGTAAAGCGCGGGGAAAGCATGAAAAAAATATATCTCGTTG
  W  L  A  F  A  L  V  K  R  G  E  S  M  K  K  I  Y  L  V  V

     2050       2060       2070       2080       2090       2100
TTATCGTACTGTTTTTTATTTCTACAAAAGTTTATACACTACTTCATAACAACATCTTCT
  I  V  L  F  F  I  S  T  K  V  Y  T  L  L  H  N  N  I  F  F
```

```
      2110       2120       2130       2140       2150       2160
TTTGTCGAAACAGCCCGGAGTGTGATTTGTCTCATGTTCTCCCCGACTACCGGGAGCAGA
  C  R  N  S  P  E  C  D  L  S  H  V  L  P  D  Y  R  E  Q  I

      2170       2180       2190       2200       2210       2220
TTTCAGGCACACCGCTAAAATATACCTTAATTAGTACAGCTCCATTAGCACAGGTGGTAG
  S  G  T  P  L  K  Y  T  L  I  S  T  A  P  L  A  Q  V  V  V

      2230       2240       2250       2260       2270       2280
TCAGACACTATGAATTATTATCGCAACACTGGTCGCCAGATGACATGGTAACCCCTGCGC
  R  H  Y  E  L  L  S  Q  H  W  S  P  D  D  M  V  T  P  A  Q

      2290       2300       2310       2320       2330       2340
AATGGCGACATAACGTTGATATTTATATCCCTGAAACGGCAAAAGAGCACCATGCTTTGG
  W  R  H  N  V  D  I  Y  I  P  E  T  A  K  E  H  H  A  L  V

      2350       2360       2370       2380       2390       2400
TGGTTGTAAACAACGGAATTAATTATGAAAAAGGCATACAAATCCCCAGTAAACCCGTTG
  V  V  N  N  G  I  N  Y  E  K  G  I  Q  I  P  S  K  P  V  D

      2410       2420       2430       2440       2450       2460
ATTTTACTCAGCAAACACTGGCAAGCATCGCCCGTGATACGAATACCATCGTAATATCAG
  F  T  Q  Q  T  L  A  S  I  A  R  D  T  N  T  I  V  I  S  V

      2470       2480       2490       2500       2510       2520
TGAGTGATATACCTAACCAATATTTAACCTTTCAGGATGATAAAAAACCACTGAAAGAGG
  S  D  I  P  N  Q  Y  L  T  F  Q  D  D  K  K  P  L  K  E  D

      2530       2540       2550       2560       2570       2580
ACGAGAGCGTTTCCCGAAGCTGGGCATTATTTATGGAAGCGCCGGAGCAACGTGAGTTAA
  E  S  V  S  R  S  W  A  L  F  M  E  A  P  E  Q  R  E  L  M

      2590       2600       2610       2620       2630       2640
TGCCTTTAAATATTCCTATGGTCACTGCCATATCGCAAGCGATGAGATTAGCAAAAAAGG
  P  L  N  I  P  M  V  T  A  I  S  Q  A  M  R  L  A  K  K  E

      2650       2660       2670       2680       2690       2700
AGTTAACGCAATGGAATATAAATTCCTTTATCATTACAGGTATTTCAAAGCGTGGGTGGA
  L  T  Q  W  N  I  N  S  F  I  I  T  G  I  S  K  R  G  W  T

      2710       2720       2730       2740       2750       2760
CCACCTGGTTGTCAGCTATTGCCGATCCTGATGTTGAAGCAATTGTTCCCTTCGCCATTG
  T  W  L  S  A  I  A  D  P  D  V  E  A  I  V  P  F  A  I  D

      2770       2780       2790       2800       2810       2820
ATCTACTTGATATTGATGCTTCACTTGAGCATATTTATCAATCATACGGTGGAAATTGGC
  L  L  D  I  D  A  S  L  E  H  I  Y  Q  S  Y  G  G  N  W  P

      2830       2840       2850       2860       2870       2880
CCATTACATTCTACCCCTACTACCAGCAAGGCATAGATGAGAAAATAAAATCCCCCACAT
  I  T  F  Y  P  Y  Y  Q  Q  G  I  D  E  K  I  K  S  P  T  F

      2890       2900       2910       2920       2930       2940
TTACCCAGCTAAGGCAAATAATAGATCCGTTAAGATATCTCAATACTATTTACCAACCCC
  T  Q  L  R  Q  I  I  D  P  L  R  Y  L  N  T  I  Y  Q  P  R

      2950       2960       2970       2980       2990       3000
GCCTTGCAATACCGAAATATATTATTAATGCAAGTGGCGATGATTTCTTCGTACCAGACA
  L  A  I  P  K  Y  I  I  N  A  S  G  D  D  F  F  V  P  D  N

      3010       3020       3030       3040       3050       3060
ATACTCGCTTTTATTATAGCAAACTTCCTGGCGTAAAATCATTACGTATAGTGCCTAATA
  T  R  F  Y  Y  S  K  L  P  G  V  K  S  L  R  I  V  P  N  M

      3070       3080       3090       3100       3110       3120
TGAACCACTACTCAATTAACCAGTTCGCTGAAGGAAGCCTCGTTCCATTTATTAACCGGT
  N  H  Y  S  I  N  Q  F  A  E  G  S  L  V  P  F  I  N  R  F
```

```
      3130       3140       3150       3160       3170       3180
TTCAAAGTAAAAAAACATTACCGCAGTTGATCGGCCTTATTCACCACCATCTTCTTACGG
  Q  S  K  K  T  L  P  Q  L  I  G  L  I  H  H  H  L  L  T  V

      3190       3200       3210       3220       3230       3240
TCTATCTCTCAGAAGCGCCGGTAAAAGTCGTTCGCTGGACAGCCAACAATCCAAATGCGC
  Y  L  S  E  A  P  V  K  V  V  R  W  T  A  N  N  P  N  A  R

      3250       3260       3270       3280       3290       3300
GTGATTTCCGTTATGCCTGTGGCATCCGCTATCAGCCGCTTACAATAGATATTCCCGCCA
  D  F  R  Y  A  C  G  I  R  Y  Q  P  L  T  I  D  I  P  A  N

      3310       3320       3330       3340       3350       3360
ACAATAAGATCAGTATTACGTTAAATGAACCGAAGACAGGTTGGGAAGCTACCTATATCG
  N  K  I  S  I  T  L  N  E  P  K  T  G  W  E  A  T  Y  I  E

      3370       3380       3390       3400       3410       3420
AAGCCACTTTTAATGATGGCTATGTCGCCACGAGTCAGGTTTATATTACGCCCGATGAAA
  A  T  F  N  D  G  Y  V  A  T  S  Q  V  Y  I  T  P  D  E  K

      3430       3440       3450       3460       3470       3480
AATACCCACAGACAGCACCACCTTCCGTTAACGCCGCATGTCAAACGTTACCGGGGCGTG
  Y  P  Q  T  A  P  P  S  V  N  A  A  C  Q  T  L  P  G  R  G

      3490       3500       3510       3520       3530       3540
GATTAGGGGAAAACGATAGCCCCGATTGAATTTTCAAGTAATTGTCTGATTTTAAATAAT
  L  G  E  N  D  S  P  D  *

      3550       3560       3570       3580       3590       3600
TTTATCCATCACGCTTGCGATGGATAAAAATCCTCTGCGATAAATTTTTGTTATATTGAT

      3610       3620       3630       3640       3650       3660
CACGTTCTAACCACAAGATAGGTGATTTGAAAATAACCTTAAAATATTGTGGTCAGGCCG

      3670       3680       3690       3700       3710       3720
CTAGACCACAATGAGAGGATTAATCATGAAAAGAAAAACAATGGGATGGCTTATCGTTTT
     ORF3 M  R  G  L  I  M  K  R  K  T  M  G  W  L  I  V  F

      3730       3740       3750       3760       3770       3780
TCTTCTATTTATAGTTTACATGCTCAACTATATGGATCGTTCAGCATTGTCGATAACTGC
 L  L  F  I  V  Y  M  L  N  Y  M  D  R  S  A  L  S  I  T  A

      3790       3800       3810       3820       3830       3840
CCCCTTGATCGAAAAGGAGTTTAGGATTAACGCCGCGGAGATGGGAATGATCTTCAGCGC
 P  L  I  E  K  E  F  R  I  N  A  A  E  M  G  M  I  F  S  A

      3850       3860       3870       3880       3890       3900
ATTTTTTATCGGATACGCCCTGTTTAATTTTATTGATGGCTGGGCCAGCGATAAAGTTGG
 F  F  I  G  Y  A  L  F  N  F  I  D  G  W  A  S  D  K  V  G


GCCAAAA 3'
 P  K
```

The figure shows nt sequence of the *S. typhi pqaA* region (gbD: AF188291). The nucleotide sequence is numbered in accordance with Genbank and is shown from nt 1 to nt 3907. The amino acid translations are represented by single letter code below the first nt of each codon. The potential initiation codons are bolded and potential ribosome binding sites (RBS) are underlined. Three putative promoters were found and the –10 boxes are highlighted and sequence of the –10 and –35 regions are given below. A putative terminator sequence after the end of *pqaA* is highlighted in white text.

Promoter	Location	Sequence
*pqaA*p$_1$	1905-1928	TTATTActcccttcttcccctTATTTA
*pqaA*p$_2$	1970-1995	ATGGAAattatgctggttggCATTTG
*orf3*p$_1$	3626-3648	TTTGAAaataaccttaaAATATT

The putative promoter regions are listed above and refer to the sequence given in Figure 5.11. The highlighted capital letters refer to those bases that are identical to the consensus sequence for the –35 (TTGACA) and –10 (TATAAT) regions, the capital bases not highlighted do not match the consensus. The lower case letters indicate the sequence between the –35 and –10 boxes.

Table 5.4: General analysis of the *S. typhi pqaA* gene and surrounding sequence

ORF	G+C content[a]	Location in sequence	Predicted MW (kDa)	#aa	Hydrophobicity analysis[b]
ORF1	53.1%	2-652	-	217	-0.31
ORF2	49.7%	642-1883	43.9	414	1.02
PqaA	41.9%	1953-3509	59.3	519	-0.21
ORF3	40.1%	3671-3907	-	79	0.61

[a]Percent guanine plus cytosine (G+C) of coding region.
[b]According to Kyte & Doolitttle (1982), as implemented in PROSIS

5.5.1 Analysis of the partial ORF1 in the *S. typhi pqaA* region

As only the carboxy-terminal end of the open reading frame was identified for ORF1, no promoter or RBS could be found. A terminator codon (TAA) was found at position 650 (Figure 5.11). Analysis of the partial ORF1 protein upstream of the *S. typhi pqaA* gene by BlastN/X/P 2.0 (Altschul *et al.*, 1997) revealed no similarity at the DNA level but BlastX analysis revealed amino acid sequence homology to the *Streptomyces aereofaciens* tetracycline 6-hydroxylase protein (Chl-2) (470 aa) which is responsible for the chlorination of tetracycline (Dairi *et al.*, 1995) and an oxygenase homolog (LanE) (491 aa) in *Streptomyces cyanogenus* which is potentially involved in landomycin biosynthesis (Westrich *et al.*, 1999). The homologies of ORF1 to these *Streptomyces* proteins are outlined in Table 5.5.

Table 5.5: Homology table for ORF1

	% Identity[a]		
	STPORF1	SAChl-2	SCLanB
STPORF1	100	25.5 (184)	29.1 (189)
SAChl-2		100	39.6 (189)
SCLanB			100

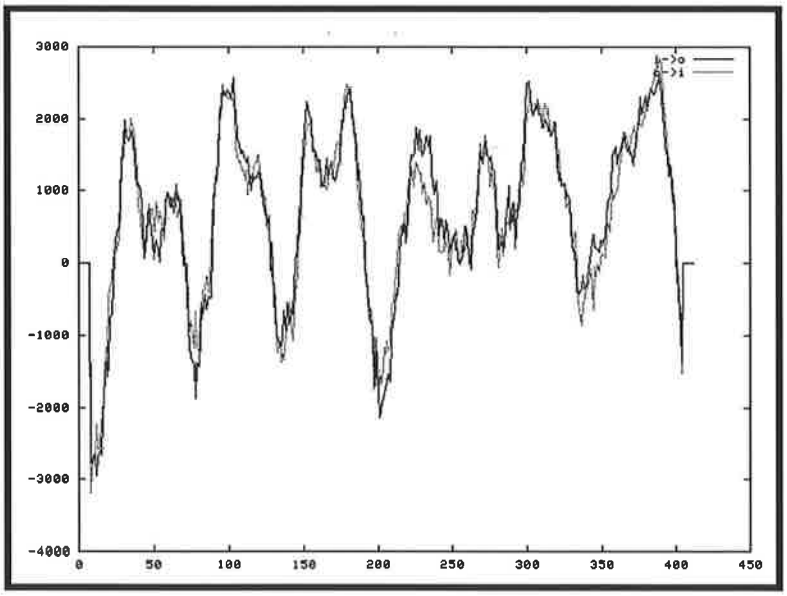
[a]This table shows the percentage of identical amino acids determined with FASTA as implemented in PROSIS. Numbers in the brackets indicate the number of amino acids over which the % identity occurs.

STPORF1 *S. typhi* ORF1 in PqaA region
SAChl-2 *S. aerofaciens* Chl-2 (gbP: JC4098)
SCLanB *S. cyanogenus* LanB (gbP: AAD13534)

5.5.2 Analysis of ORF2 in the *S. typhi pqaA* region

Analysis of the sequence for *orf2* gave a potential methionine initiation codon at position 642. This initiation codon overlaps with the termination codon of the proceeding *orf1* indicating that they may be translationally coupled. A putative RBS can be found upstream of the *orf2* methionine start codon and a stop codon (TAA) can be found at position 1881, a putative promoter sequence (-10 and –35 regions) could not be found. Analysis of the complete ORF2 encoded directly upstream of the *S. typhi* PqaA protein by hydropathy plot and transmembrane analysis indicated that it was likely to contain many transmembrane regions (Figure 5.12).

Figure 5.12: Hydropathy plot and transmembrane analysis of *S. typhi* ORF2



Using the computer program TMpredict (Hofmann & Stoffel, 1993) a model for the topology of ORF2 was proposed. The suggested transmembrane domains are shown in the table below with two potential models described. The corresponding hydropathy plot shows that ORF2 has at least 9 potential transmembrane domains. Positive numbers on the Y-axis indicate hydrophobic regions. The positions of every 50th amino acid is marked on the X-axis.

Model 1 (strongly preferred)					Model 2 (alternative model)				
TM#	Start	End	Length	Orientation	TM#	Start	End	Length	Orientation
1	22	47	26	o-i	1	22	40	19	i-o
2	50	76	27	i-o	2	50	70	21	o-i
3	87	105	19	o-i	3	87	114	28	i-o
4	144	162	19	i-o	4	147	167	21	o-i
5	169	190	22	o-i	5	169	193	25	i-o
6	217	238	22	i-o	6	219	237	19	o-i
7	260	283	24	o-i	7	260	284	25	i-o
8	292	310	19	i-o	8	292	320	29	o-i
9	380	399	20	o-i	9	380	400	21	i-o

Analysis of the *orf2* DNA by BlastN/X 2.0 (Altschul *et al.*, 1997) revealed no significant similarity at the DNA level, however analysis using BlastX found that ORF2 had homology with many proteins contained within the proton-dependant multidrug efflux systems proteins, commonly known as the Major Facilitator Superfamily (MFS). In particular, the ORF2 protein had homology with 12-TMS (transmembrane) multidrug efflux proteins; these proteins have a secondary stucture of 12 putative TMSs, with the amino- and carboxy-terminal and a large central domain located in the cytoplasm (Bolhuis *et al.*, 1997). Only 9 transmembrane domains were predicted by TMpredict computer analysis, indicating that there

are likely to be other transmembrane regions in ORF2 currently unidentified. The 12-TMS family are involved with drug efflux systems (Paulsen *et al.*, 1996) which catalyze drug:H^+ antiport systems and the export of a large range of drugs including tetracycline, fluoroquinolones, tetraphenylphosphonium compounds, chloramphenicol and puromycin (Paulsen *et al.*, 1996).

ORF2 displayed similarity to the *Enterococcus hirae* NapC protein (400 aa) (a tentative tetracylin efflux protein) (Solioz & Strausak, 1997), the *Vibrio anguillarum* TetA(G) (393 aa) (a class G tetracycline resistance determinant) (Zhao & Aoki, 1992), *E. coli* TN1721 transposon TETA(A) (399 aa), a tetracycline resistance determinant (Allmeier *et al.*, 1992; Waters *et al.*, 1983) and the *Salmonella ordonez* TETA(D) protein (394 aa), a class D tetracycline resistance gene (Allard *et al.*, 1993). Other homologs included the *Staphylococcus aereus* NorA protein (388 aa) which mediates active efflux of fluoroquinolones (Kaatz *et al.*, 1993); *Bacillus subtilis* BMR1 (389 aa), an energy-dependant efflux pump responsible for decreased drug accumulation in multi-drug resistant cells (Ahmed *et al.*, 1994; Neyfakh *et al.*, 1991)) and *Bacillus subtilis* BMR2 (400 aa), a second energy-dependant efflux pump responsible for decreased drug accumulation in multi-drug resistant cells (Ahmed *et al.*, 1995). The % identity with these proteins is listed in Table 5.6.

Multiple alignment and sequence analysis of the 12-TMS families has shown that sequence similarity between these proteins tends to be greater in the N-terminal half and conserved motifs can be seen in both the N-terminal and C-terminal parts of the proteins (Paulsen & Skurray, 1993). It has been hypothesised that the C-terminal regions of the MFS transporters are involved with substrate specificity and the N-terminal regions are primarily involved in the transport energy process.

Table 5.6: Homology table for ORF2

	% Identity[a]							
	STPORF2	EHNapC	VATETA(G)	ECTETA(A)	SOTETA(D)	SANorA	BSBMR1	BSBMR2
STPPORF2	100	24.8 (379)	28.0 (389)	26.6 (369)	25.3 (396)	23.5 (383)	23.0 (382)	24.8 (399)
EHNapC		100	26.3 (365)	31.0 (364)	26.8 (380)	24.3 (395)	24.9 (381)	26.6 (399)
VATETA(G)			100	62.0 (382)	52.6 (382)	22.6 (359)	21.2 (359)	22.5 (356)
ECTETA(A)				100	48.7 (380)	25.2 (365)	24.4 (360)	26.2 (362)
SOTETA(D)					100	24.9 (385)	23.0 (356)	25.1 (358)
SANorA						100	45.8 (378)	38.5 (382)
BSBMR1							100	52.6 (386)
BSBMR2								100

[a]. This table shows the percentage of identical amino acids determined with FASTA as implemented in PROSIS. Numbers in the brackets indicate the number of amino acids over which the % identity occurs.

STP0RF2	*S. typhi* ORF2 in the PqaA region
EHNapC	*Enterococcus hirae* NapC (gbP: CAA04021)
VATETA(G)	*Vibrio anguillarum* TETA(G) (gbP: P51563)
ECTETA(A)	*E. coli*, Tn1721 TETA(G) (gbP: P02982)
SOTETA(D)	*S. ordonez* TETA(D) (gbP: P33733)
SANorA	*Staphyloccoccus aureus* NorA (gbP: AAA16158)
BSBMR1	*Bacillus subtilus* BMR1 (gbP: P33449)
BSBMR2	Bacillus subtilus BMR2 (gbP: P39843)

A number of highly conserved motifs have been found (Paulsen & Skurray, 1993); Motif A, located in the cytoplasmic loop between TMS2 and TMS3, has been suggested to either act as a cytoplasmic gate which controls passage of the substrate to and from the cytoplasm (Yamaguchi *et al.*, 1990; Yamaguchi *et al.*, 1992). Alternatively, it may be involved in promoting global conformational changes in the protein that enable the substrate to translocate across the membrane (Yamaguchi *et al.*, 1992). Motif B is located within TMS 4 of the proteins and has been proposed to be involved with energy coupling (Paulsen & Skurray, 1993). Motif C has been suggested to be required for linking proton translocation to antiport but not symport of a substrate (Griffith *et al.*, 1992; Paulsen and Skurray, 1993). Motif D2 is located within TMS1 but has not yet been assigned a role (Paulsen and Skurray, 1993). Motif G is conserved only in the 12-TMS family of proteins and probably corresponds to a C-terminal duplication of motif C (Paulsen & Skurray, 1993) however its role has not yet been defined. These motifs can all be seen in the clustal alignment for the *S. typhi* ORF2 and the homologous proteins in Figure 5.13.

Figure 5.13: Alignment of *S. typhi* ORF2 with homologous proteins

```
                                                                        aa
STPORF2     MNTNVYENTDSETITPLNKRRILPVFLLVGLYAASTAAVMSVLPFYIREMGGSP----LI    56
EHNapC      ------------MNTTINKHALVFGFTSVFLIGLGLTIVNPVIPFMVEQYTKNTQQQATT    48
VATETA(G    --------------MRSS---AIIALLIVGLDAMGLGLIMPVLPTLLRELVPAEQV-AGH    42
ECTETA(G)   --------------MKPNRP-LIVILSTVALDAVGIGLIMPVLPGLLRDLVHSNDV-TAH    44
SOTETA(D)   ----------------MNKP-AVIALVITLLDAMGIGLIMPVLPSLLREYLPEADV-ANH    42
SANorA      --------------MNKQ---ILVLYFNIFLIFLGIGLVIPVLPVYLKDLGLTG----SD    39
BSBMR1      --------------MEKKNITLTILLTNLFIAFLGIGLVIPVTPTIMNELHLSG----TA    42
BSBMR2      ----------MKKSINEQKTIFIILLSNIFVAFLGIGLIIPVMPSFMKIMHLSG----ST    46
                                          .       .  * *  .
                                        lgxxxxxPvxP
                                          Motif D2

STPORF2     IGIIIATEAFSQFCAAPLIGHLSDRVGRKRILIVTLAIAAISLLLLANAQCILFILLART   116
EHNapC      VTLLSAIYAFSMFLAAPMLGALSDRFGRKIILISSLFGSAIGYYLFGFGGALWILFLGRI   108
VATETA(G    YGALLSLYALMQVVFAPMLGQLSDSYGRRPVLLASLAGAAVDYTIMASAPVLWVLYIGRL   102
ECTETA(G)   YGILLALYALMQFACAPVLGALSDRFGRRPVLLVSLAGAAVDYAIMATAPFLWVLYIGRI   104
SOTETA(D)   YGILLALYAVMQVCFAPLLGRWSDKLGRRPVLLLSLAGAAFDYTLLALSNVLWMLYLGRI   102
SANorA      LGLLVAAFALSQMIISPFGGTLADKLGKKLIICIGLILFSVSEFMFAIGQNFLILMLSRV    99
BSBMR1      VGYMVACFAITQLIVSPIAGRWVDRFGRKIMIVIGLLFFSVSEFLFGIGKTVEMLFISRM   102
BSBMR2      MGYLVAAFAISQLITSPFAGRWVDRFGRKKMIILGLLIFSLSELIFGLGTHVSIFYFSRI   106
               . .  *      .*  *   *  *.. ..   *   .    .            *
                                GxLaDrxGrkxxl                   lxxxRx
                                  Motif A                        MotifB
```

```
STPORF2     LFGISA-GNLSAAAAYIADCTHVRNRRQAIGILTGCIGLGGIVGAGVSGWLSRISLGAPI   175
EHNapC      IEGLTG-GEISAILAYFADLTPIESRTKYFGWISATVGIGTAAGPLIGGFLAQYGPAIPL   167
VATETA(G)   VSGVTG-ATGAVAASTIADSTGEGSRARWFGYMGACYGAGMIAGPALGGMLGGISAHAPF   161
ECTETA(G)   VAGITG-ATGAVAGAYIADITDGDERARHFGFMSACFGFGMVAGPVLGGLMGGFSPHAPF   163
SOTETA(D)   ISGITG-ATGAVAASVVADSTAVSERTAWFGRLGAAFGAGLIAGPAIGGLAGDISPHLPF   161
SANorA      IGGMSAGMVMPGVTGLIADISPSHQKAKNFGYMSAIINSGFILGPGIGGFMAEVSHRMPF   159
BSBMR1      LGGISAPFIMPGVTAFIADITTIKTRPKALGYMSAAISTGFIIGPGIGGFLAEVHSRLPF   162
BSBMR2      LGGVSAAFIMPAVTAYVADITTLKERSKAMGYVSAAISTGFIIGPGAGGFIAGFGIRMPF   166
            . *..            ** .   .   * .     *   *   *         *
            xxqGxgaa                              gxxxGPxxGGxl
            Motif B                                  Motif C

STPORF2     YAAFILVLGAALVAIWGLKDPSTT-SRTTDKIAS-------FSARAILKMPVLRVLIIVM   228
EHNapC      YVASFLSLSNAVYGYFFMPESLTKRERTRNLSLQQ--INPFKQLQLVFTFRSVKWLLITG   225
VATETA(G)   IAAALLNGFAFLLACIFLKETHHS-HGGTGKPVR---IKPFVLLRLDDALRGLGALFAVF   218
ECTETA(G)   FAAAALNGLNFLTGCFLLPESHK----GERRPLRREALNPLASFRWARGMTVVAALMAVF   217
SOTETA(D)   VIAAILNACTFLMVFFIFKPAVQT-E---EKPADE--KQESAGISFITLLKPLALLLFVF   215
SANorA      YFAGALGILAFIMSIVLIHDPKKV-STNGFQKLEP-------QLLTKINWKVFITPVILT   211
BSBMR1      FFAAAFALLAAILSILTLREPERN-PENQEIKGQK-------TGFKRIFAPMYFIAFLII   214
BSBMR2      FFASAIALIAAVTSVFILKESLSI-EERHQLSSHTK-ESNFIKDLKRSIHPVYFIAFIIV   224
              *       .

STPORF2     LCHFFAYGMYSSQLPVFLSDTFIWNGLPFGPKALSYLLMADGVINIFVQLFLLGWVSQYF   288
EHNapC      FLIWLPNGSFQAIFAQFSIDTFHLS-----PIIIGFTFSLIGIMDIFAQLLIMPILLKFW   280
VATETA(G)   FIIQLIGQVPAALWVIYGEDRFQWN-----TATVGLSLAAFGATHAIFQAFVTGPLSSRL   273
ECTETA(G)   FIMQLVGQVPAALWVIFGEDRFHWD-----ATTIGISLAAFGILHSLAQAMITGPVAARL   272
SOTETA(D)   FTAQLIGQIPATVWVLFTESRFAWD-----SAAVGFSLAGLGAMHALFQAVVAGALAKRL   270
SANorA      LVLSFGLSAFETLYSLYTADKVNYS-----PKDISIAITGGGIFGALFQIYFFDKFMKYF   266
BSBMR1      LISSFGLASFESLFALFVDHKFGFT-----ASDIAIMITGGAIVGAITQVVLFDRFTRWF   269
BSBMR2      FVMAFGLSAYETVFSLFSDHKFGFT-----PKDIAAIITISSIVAVVIQVLLFGKLVNKL   279
                      .   .               .                 *

STPORF2     SERKLIILIFALLCTGFLTAGIATTIPVL---IFAIVCISIADALAKPTYLAALSVHVSP   345
EHNapC      RENQIITMGITSEMIGYSVIILSAFYGSIPCFIIGMVFFGLGDAIFSPSYNGLISTYASK   340
VATETA(G)   GERRTLLFGMAADGTGFVLLAFATQGWMV---FPILLLLAAG-GVGMPALQAMLSNNVSS   329
ECTETA(G)   GERRALMLGMIADGTGYILLAFATRGWMA---FPIMVLLASG-GIGMPALQAMLSRQVDE   328
SOTETA(D)   SEKTIIFAGFIADATAFLLMSAITSGWMV---YPVLILLAGG-GIALPALQGIISAGASA   326
SANorA      SELTFIAWSLIYSVIVLVLLVIADGYWTI---MVISFVVFIGFDMIRPAITNYFSNIAG-   322
BSBMR1      GEIHLIRYSLILSTSLVFLLTTVHSYVAI---LLVTVTVFVGFDLMRPAVTTYLSKIAG-   325
BSBMR2      GEKRMIQLCLITGAILAFVSTVMSGFLTV---LLVTCFIFLAFDLLRPALTAHLSNMAG-   335
             *   .                                        .  *.     *

STPORF2     ARQGIVIGTAQALIAIADFISPVLGGFVLGYALYGV-WIGIAISVAIIGLVTAMIYLSKS   404
EHNapC      EDQGKIQGASQSIQALARVIGPMIGGQLYANFHHTM---PFIIGFILLGLATFIVKPKVK   397
VATETA(G)   NKQGALQGTLTSLTNLSSIAGPLGFTALYSATAGAWNGWVWIVGAILYLICLPILRRPFA   388
ECTETA(G)   ERQGQLQGSLAALTSLTSIVGPLLFTAIYAASITTWNGWAWIAGAALYLLCLPALRRGLW   390
SOTETA(D)   ANQGKLQGVLVSLTNLTGVAGPLLFAFIFSQTQQSADGTVWLIGTALYGLLLAICLLIRK   386
SANorA      DRQGFAGGLNSTFTSMGNFIGPLIAGALFDVHIEAP--IYMAIGVSLAGVVIVLIEKQHR   380
BSBMR1      NEQGFAGGMNSMFTSIGNVFGPIIGGMLFDIDVNYP--FYFATVTLAIGIALTIAWKAPA   383
BSBMR2      NQQGFVAGMNSTYTSLGNIFGPALGGILFDLNIHYP--FLFAGFVMIVGLGLTMVWKEKK   393
              **   *       .     *      .                    .
                                 GxxxGPL
                                 Motif G

STPORF2     SPLIAKPETE   414
EHNapC      RSP-------   400
VATETA(G)   TSLVI-----   393
ECTETA(G)   SGAGQRADR-   399
SOTETA(D)   PAPVAATC--   394
SANorA      AKLKQQDL--   388
BSBMR1      HLKAST----   389
BSBMR2      NDAAALN---   400
```

Alignment of the amino acid sequences of all of the above as determined using the default settings of the program CLUSTAL (Higgins & Sharp, 1988). *, identical residues, ., similar residues. The consensus sequences of the motifs are displayed as follows: x, any amino acid; capital letters, amino acid occurrence frequency in the displayed sequence is greater than 70%; lowercase letters, frequency of occurrence is greater than 40%. Motifs A, B, C, D2, and G, correspond to the motifs previously described by Paulsen & Skurray (1993).

5.5.3 Analysis of the partial ORF3 in the *S. typhi pqaA* region

Analysis of the sequence of the *S. typhi pqaA* locus *orf3*, gave a potential initiation methionine codon at nt 3671 (Figure 5.11). A putative RBS and –10, -35 promoter regions were also found (Figure 5.11). As only the partial 5' end of the ORF3 was sequenced, no termination codon was found. Analysis of the partial ORF3 protein downstream of the *S.typhi pqaA* gene by BlastN/X 2.0 analysis (Altschul *et al.*, 1997) revealed no significant similarity to anything at the DNA level, but BlastX analysis revealed similarity to members of the pthalate permease family. Members of this family which gave strong homology to ORF3 were *Bacillus subtilis* YjmG (a hexuronate transporter-like protein) (422 aa) (Rivolta *et al.*, 1998), *Pseudomonas putida* PcaK (a 4-hydroxybenzoate transporter) (448 aa) (Harwood *et al.*, 1994), *E. coli* DGOT (a D-galactonate transporter) (445 aa) and (Burland *et al.*, 1993), *Pseudomonas putida* PSEPU (a probable glucarate transporter) (456 aa) (Burlingame *et al.*, 1998). The sequence identity between ORF3 and these proteins are outlined in Table 5.7.

Table 5.7: Homology table for ORF3 in *S. typhi pqaA* region

	% Identity[a]				
	STPORF3	BSYjmG	PPPcaK	ECGOT	PPGUDT
STPORF3	100	52.3 (65)	39.7 (73)	39.4 (66)	31.3 (67)
BSYjmG		100	32.6 (144)	25.5 (416)	25.2 (254)
PPPcaK			100	37.8 (434)	25.5 (141)
ECGOT				100	18.0 (316)
PPGUDT					100

[a]This table shows the percentage of identical amino acids determined with FASTA as implemented in PROSIS. Numbers in the brackets indicate the number of amino acids over which the % identity occurs.

STPORF3 *S. typhi* ORF3 in the PqaA region
ECDGOT *E. coli* DGOT (gbP: P31457)
PPPcaK *Pseudomonas putida* PcaK (gbP: AAA85137)
BSYjmG *Bacillus subtilis* YjmG (gbP: AAC46332)
PPGUDT *Pseudomonas putida* GUDT (gbP: P42205)

5.6 Analysis of the *S. typhi pqaA* gene

5.6.1 Homology analysis of *S. typhi* PqaA protein

Analysis of the *S. typhi pqaA* gene sequence indicated that there were two potential initiation methionine codons at 1953 and 2019 (Figure 5.11). Potential RBSs can be found upstream of each potential start codon and the putative promoter sequences *pqaA*p_1 and *pqaA*p_2 were found in front of the each of methionine codons respectively (Figure 5.11). Neither the RBSs or potential promoters highly correspond to the consensus sequences for the bacterial RBS, -10 and –35 regions. This infers that *pqaA* is strongly regulated by a regulator protein such as PhoP and therefore is potentially expressed only when needed. Comparison of the potential *pqaA* promoter regions using BlastN and DNASIS with promoter regions of known *phoP/Q* regulated genes detected no homologous regions (data not shown). A termination codon (TGA) was found at nt 3507 and a putative weak terminator stem/loop structure (energy = -8.3) was found after the termination codon (white text, Figure 5.11).

Analysis of the *S. typhi pqaA* protein by BlastN/X/P 2.0 (Altschul *et al.*, 1997), revealed no significant similarity to anything in the databases at the DNA level and BlastX/P analysis revealed similarity to a 422 aa protein encoded by *Thermotoga maritima* (TM0945) which has no known function. Comparison of PqaA to TM0945 by FASTA analysis as implemented in PROSIS is shown in Figure 5.14.

Figure 5.14: Homology analysis of *S. typhi* PqaA and *T. maritima* TM0945

26.4% identity in 368 aa overlap

```
        50        60        70        80        90       100
PQAA     FCRNSPECDLSHVLPDYREQISGTPLKYTLISTAPLAQVVVRHYELLSQHWSPDDMVTPA

TM0945   MKRFLAILLILPVMVLAIHPLDLLVRARGNPVYETIIATTTQDGDEIYILKSYGMNWQNI
                10        20        30        40        50        60
      110       120       130       140       150       160
PQAA     QWRHNVDIYIPETAK-EHHALVVVNNGINYEKGIQIPSKPVDFTQQTLASIARDTNTIVI
         :: :.:.: .:.. . ...:.  ...: . :.. .  .. .. ....:  .:.. ..  :
TM0945   QWFHRVGIILPSNLNYKDRAFFFITGGSRKEENERYYDSFLEDVKENL-WVAKEFEAPFI
                70        80        90       100       110
       170       180       190       200       210       220
PQAA     SVSDIPNQYLTFQDDKKPLKEDESVSRSWALFMEAPEQRELMPLNIPMVTAISQAMRLAK
          :.:.:::  .:      :.:::. .. .. .:.:.:.   ..:: .::. .. .::  :.
TM0945   VVGDVPNQ-PIF-----GLREDALIAETFKMFLENPD--PFLPLLVPMTYGVIKAMDTAQ
       120       130             140       150         160       170
        230         240       250       260       270       280
PQAA     KELTQW--NINSFIITGISKRGWTTWLSAIADPDVEAIVPFAIDLLDIDASLEHIYQSYG
         . :..   . ..:...: ::::::::.:.:: :: : ::.:.. : :.:.: : :  ..:
TM0945   DFLEKKGVEIKGFMVSGASKRGWTTYLTAIFDPRVFAIAPMVYDNLNIEAQLLHQ-KEYY
              180       190       200       210       220       230
          290       300       310       320       330       340
PQAA     GNWPITFYPYYQQGIDEKIKSPTFTQLRQIIDPLRYLNTIYQPRLAIPKYIINASGDDFF
         :... ..  : ..:. : ...   ..: .:.:: :     . ::..:: .. ...:...
TM0945   GTYSEKLRDYQERGLFEILENDLGKRLLEIVDP--Y---AMRLRLSLPKILVLGTNDEYW
               240       250       260             270       280
          350       360       370       380       390       400
PQAA     VPDNTRFYYSKLPGVKSLRIVPNMNHYSINQFAEGSLVPFINRFQSKKTLPQLIGLIHHH
         . :....: ..::: . :   :: .... ..  :    .. . :.   .::..  . ..
TM0945   TVDSANLYVDDLPGETFLFYSPN-DPHNLKNVKE-IIETLSSFFKMYPKLPKVEFFYRDG
           290       300         310         320       330       340
          410       420       430       440       450       460
PQAA     LLTVYLSEAPVKVVRWTANNPNARDFRYACGIRYQPLTIDIPANNKISITLNEPKTGWEA
         . :    . :..  : : ...:::: :  .. .. ... .... :... ..:..  .:
TM0945   KIFVERIPEIVDAELWFA-RSKSRDFRKA--VWLRR-GVEETDDSLIGVPPEKPEGFHQA
             350       360         370         380       390
                470       480
PQAA.AMI TYIEATFNDGYVATSQVYITPDE
          .. .:..
TM0945   YFLRVTLEINGLRMKLCSKMMVE
       400       410       420
```

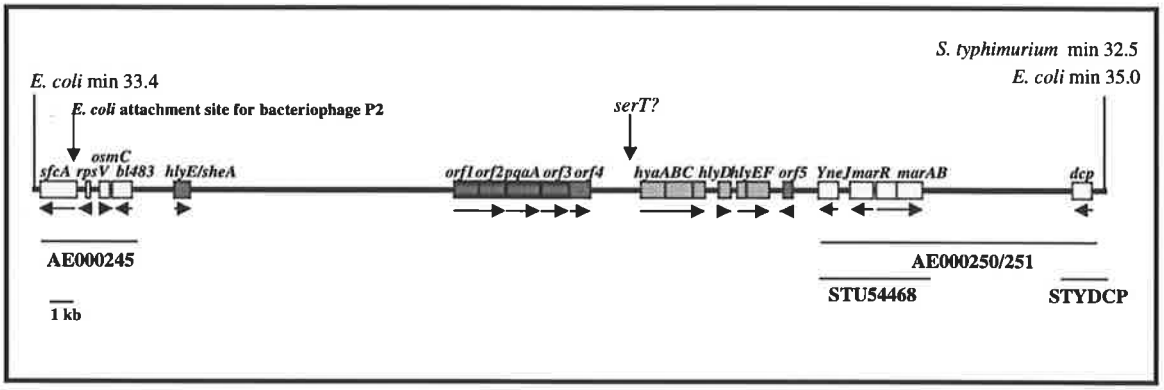
This figure shows the similarity of the *S. typhi* PqaA protein to *Thermotoga maritima* TM0945 protein using FASTA as implemented in PROSIS.

5.7 Location of the *pqaA* locus

The *S. typhi pqaA* gene and adjacent ORFs (*orf1*, *orf2* and *orf3*) were found to have no significant similarity to known *E. coli* or *Salmonella* genes. The *S. typhi* chromosome is currently being sequenced by the Sanger Centre (Sanger Centre, 1999) and contigs are available for analysis. The *S. typhi pqaA* gene and adjacent DNA was used in a BlastN 2.0 search (Altschul *et al.*, 1997) against the Sanger database and these sequences were found to be located in the middle of a 300 kb contig [Contig 345, 1999]. The sequence was

downloaded and 2 kb sections were analysed extending outwards from the *pqaA* region using BlastN/X 2.0 homology searches until nt sequences with ≥ 80% homology to *E. coli* DNA were found. The contig was not analysed for ORFs as the sequence still has numerous errors in it and only ORFs which gave > 60% amino acid homology to other proteins by BlastX are shown in Figure 5.15.

Figure 5.15: Schematic diagram of the *S. typhi pqaA* gene and surrounding DNA



This figure shows the schematic diagram of 51 kb of DNA obtained from the *S. typhi* Sanger database sequencing project [contig 345, 1999] containing the *pqaA* gene. The Genbank accession numbers of *E. coli* DNA which showed >80% homology to this region (gbD: AE000245, AE000250/251) and *S. typhimurium* DNA showing >95% homology (gbD: STU54468, STYDCP) are indicated by lines. ORFs which showed >60% homology to known ORFs are labelled and direction of gene transcription indicated with arrows (→). An attachment site for the *E. coli* bacteriophage P2 is labelled with a downward arrow (↓) as well as the location that *serT* is normally found (just upstream of *hyaA*) in *E. coli* (gbD: AE000199).

The *S. typhi pqaA* gene is located in the middle of a 30.4 kb DNA fragment which is bordered by DNA with > 80% homology to *E. coli* Genbank sequences AE000245 (located at 33.4 min on the *E. coli* chromosome) and AE000250/251 (located at 34.8-35.0 min on the *E. coli* chromsome) (Blattner *et al.*, 1997) (Figure 5.15). The DNA and protein similarities of the *S. typhi pqaA* region encoded ORFs (Figure 5.15) detected by BlastN/X analysis are given below starting from the LHS of Figure 5.15 at SfcA and finishing with Dcp, are listed below with the BlastN/X sequence identity in brackets.

At the start of the *S. typhi pqaA* region four ORFs were found to have strong similarity with proteins encoded in the *E. coli* AE000245 sequence; the first ORF had strong similarity with SfcA (88%) (an enzyme involved with central intermediary metabolism and gluconeogeneis); the second with RpsV (87%) (30S ribosomal subunit protein S22); the third with OsmC (92%) (an osmotically inducible protein), and the fourth with a hypothetical protein Bl483 (88%). The BlastN homology over this sequence gave 85% identity at the DNA level and then the similarity ended after BL483. Upstream from this region HlyA/ClyA (86%) was found, which encodes a "silent haemolysin" in *E. coli* (encoded within gbD: AE000216, at 26.5 min). HlyA/ClyA has been shown to be upregulated by the *S. typhimurium* SlyA regulator (Ludwig *et al.*, 1999). The *S. typhi pqaA* locus Orf1, Orf2, PqaA and Orf3 showed 99% homology at the amino acid level (less than 100% due to errors in the *S. typhi* contig sequence). The Orf3 protein is followed by Orf4 with 60 % identity to a hypothetical *Haemophilus influenzae* zinc-type alcohol dehydrogenase (gbD: AAC21731) and a *Bacillus subtililis* zinc-containing alcohol dehydrogenase (gbP: AF015825). The next group of proteins with homology was the *E. coli* Hya operon encoding the [NiFe] hydrogenase 1, involved in aerobic respiration (gbD: AE000199, at min 22.2-22.3) (Menon *et al.*, 1990; Menon *et al.*, 1991). The *pqaA* region encoded ORFs had the indicated homology with the Hya proteins as follows: HyaA (72 %), HyaB (62 %), HyaC (64 %), HyaD (53 %), HyaE (36%) and HyaF (30%). The next protein, Orf5 is likely to be an outer membrane protein as it had 72 % identity to the porin OmpC from Klebsiella pneumoniae (gbD: CAA83913), 73 % identity to the porin OmpN from *E. coli* (gbD: AF035618), 70 % identity to the *E. coli* porin OmpC (gbD: AE000310), 71 % identity to the *S. typhi* porin OmpC (gbD: AAA27169) and 70 % identity to the porin OmpC from *S. typhimurium* (gbD: AF039309). Following this region, the next region had high similarity to the *mar* locus region in *E. coli* (gbD: AE000250) (Cohen *et al.*, 1993), and *S. typhimurium* (STU54468) (Sulavik *et al.*, 1997), which is involved in multiple antibiotic resistance in both strains. The nt sequence identity to the *mar*

locus of *S. typhimurium* was 97 %, and 85% to that of *E. coli*. The *E. coli* homology continued at this level over gbD: AE000250 and then into gbD: AE000251 which contains many hypothetical proteins and the protein Dcp (dipeptidyl carboxypeptidase II) (Henrich *et al.*, 1990). The Dcp protein has also been cloned and the location mapped on the *S. typhimurium* chromosome (gbD: STYDCP) (Miller & Hamilton, 1992). The *E. coli dcp* gene is located at 35. 0 min and the *S. typhimurium dcp* gene located at 32.5 min therefore identifying the end of the *S. typhi pqaA* gene region to be located at 32.5 min on the *S. typhimurium* chromosome. DNA homology (85 % identity) was also found to an *E. coli* attachment site for P2 bacteriophage at the end of *sfcA* (Figure 5.15). In the *E. coli* Hya operon (AE000199), the gene encoding the tRNA-Ser, *serT* is located 400 nt upstream from *hyaA*, however although the entire 51 kb *S. typhi pqaA* locus was searched using DNASIS for similarity to *serT*, no homologous region was found. The DNA between Bl483 and *hlyE/clyA*, *orf4* and hyaA, *hyaF* and *orf5*, and *orf5* and *yneJ* (Figure 5.?) was also searched extensively using DNASIS and BlastN for any repeat regions, other bacteriophage genes and transposon elements, however none were found (data not shown). The potential of the 30.4 kb *pqaA* region to be a pathogenicity island will be discussed in Chapter 7.

5.8 Further analysis of *S. typhi pqaA*

5.8.1 Regulation of the *S. typhi pqaA* gene

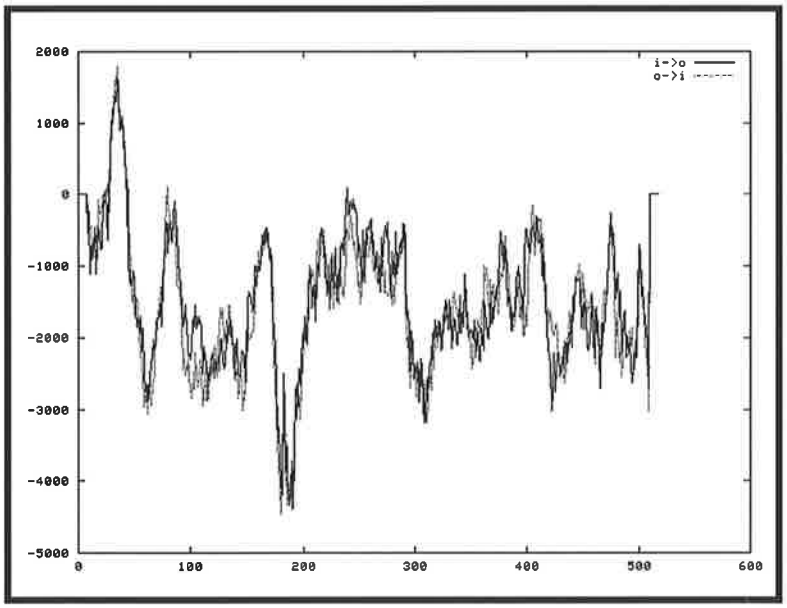
The *S. typhi pqaA* gene has been shown to be strongly regulated by *phoP/Q*, however to test whether it was also regulated by PmrA/B, a *pmrA*-null mutation (*pmrA*::Tn*10*d) from the *S. typhimurium* strain JSG421 was transduced by P22 phage (Section 2.6.3.2) into *S. typhi* PhoP^c *pqaA*::Mu*d*J (RMA2510) to give RMA1338. The PhoP^c *pqaA*::Mu*d*J (RMA2510) and PhoP^c *pqaA*::Mu*d*J, *pmrA*::Tn*10*d strains (RMA1338) were compared on LA containing X-gal

plates for LacZ activity, however no difference was seen between the two strains, indicating that the *S. typhi pqaA* gene is not regulated by the PmrA/B operon.

5.8.2 Initial structural analysis of the *S. typhi* PqaA protein

As the homology analysis of the *S. typhi* PqaA protein by BlastX/P 2.0 (Altschul *et al.*, 1997) gave no results to indicate a function for the protein, a hydrophobicity analysis of the protein was performed which revealed that the hydrophobicity was –0.21 (indicating that it was a highly hydrophilic protein). Further analysis by TMpredict (Hofmann & Stoffel, 1993) suggested that there was a transmembrane region near the start of the protein (Figure 5.16).

Figure 5.16: Hydrophobicity and transmembrane analysis of the *S. typhi* PqaA protein



Using the computer program TMpredict (Hofmann & Stoffel, 1993) a model for the topology of PqaA was proposed. The suggested transmembrane domains are shown in the table below with two potential models described. The corresponding hydropathy plot shows that ORF2 has at least 1 potential transmembrane domain. Positive numbers on the Y-axis indicate hydrophobic regions. The positions of every 100th amino acid is marked on the X-axis.

Model 1 (strongly preferred)					Model 2 (alternative model)				
TM#	Start	End	Length	Orientation	TM#	Start	End	Length	Orientation
1	26	44	19	o-i	1	26	44	19	i-o

Analysis of the hydrophobic region at the N-terminal of the *S. typhi* PqaA protein (Figure 5.16), by SignalP V1.1 (http://www.cbs.dtu.dk/services/SignalP/sp_characteristics.html), indicated that although the N-terminal part of the PqaA protein contained a potential signal sequence in the hydrophobic section, it was unlikely to be cleaved and the protein was likely to be a cytoplasmic membrane protein (Nielsen *et al.*, 1997). Therefore this gave an indication of the *S. typhi* PqaA protein's structure: the N-terminal portion was likely to be a potential membrane anchor in the cytoplasmic membrane with the hydrophilic C-terminal part of the protein in the cytoplasm (although it could not be ruled out at this stage that it might be located in the periplasm.

As the only protein with similarity to the *S. typhi* PqaA protein has only been identified from sequence analysis, an attempt was made to overexpress the PqaA protein to verify that the coding region did encode a protein of the expected size.

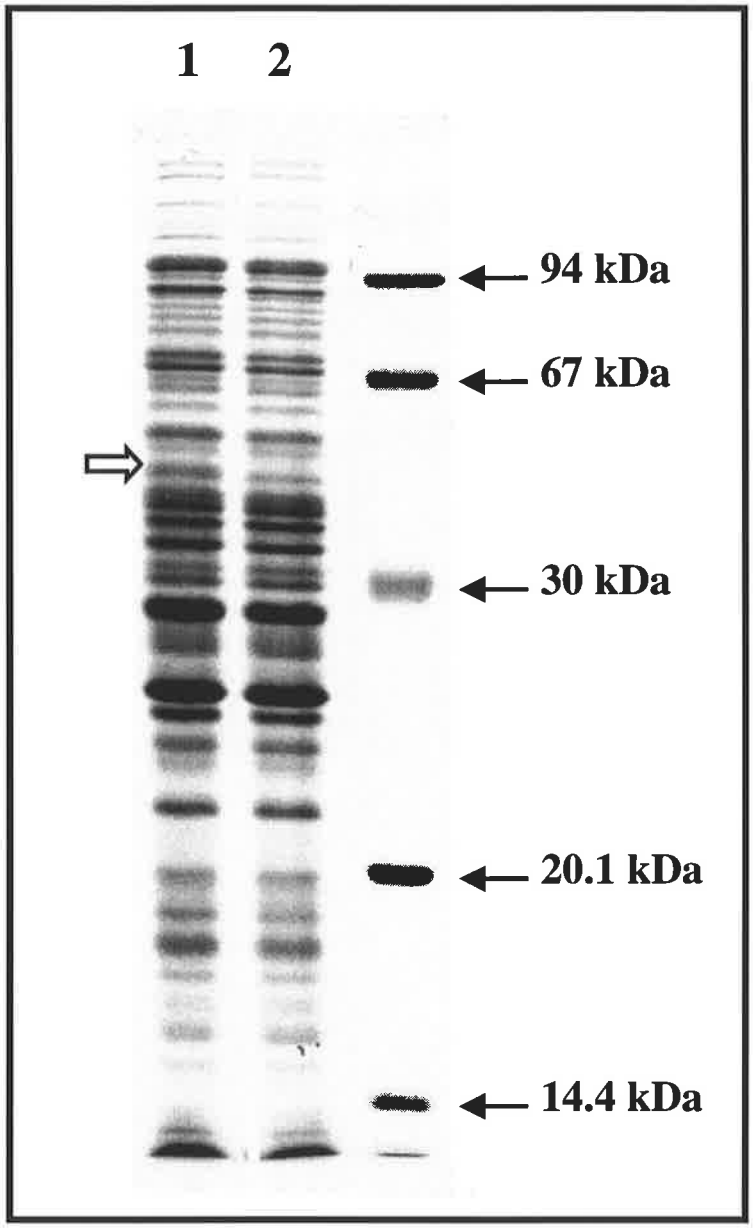
5.8.3 Overexpression of the *S. typhi* PqaA protein

The *S. typhi pqaA* gene encodes a protein with a predicted MW of 59.3 kDa. O verexpression studies were initially undertaken using the pET11 based vector pET11BYZ (Vindurampulle, 1994) which carries a strong RBS. An oligonucleotide was designed to the *S. typhi pqaA* sequence; #2986 (Table 2.5) which provided a *Nde*I site at the start of the *pqaA* gene such that the gene would be cloned with the first methionine at the optimal position for the RBS in pET11BYZ. An *S. typhi* Ty2 chromosomal template was used for a PCR with oligonucleotides #2986 and #2987 (located after the 3' end of the *pqaA* gene, Table 2.5) to produce a predicted 1.62 kb product. The PCR was performed, and a band of the correct size was produced, digested with *Nde*I and *Bam*HI and ligated to similarly digested pET11BYZ. The ligation was then transformed into DH5α competent cells and Amp^R transformants screened for the correct insert. A plasmid was produced (pRMSB82) (in RMA1362) which

was then transformed into E2096 which is a heat inducible overexpression strain containing pGP1-2 encoding T7 RNA polymerase (Tabor & Richardson, 1985) to produce RMA1376. The colonies produced were grown at 30°C but were very small, even after 48 h of growth, indicating that the PqaA protein may be slightly toxic for growth. RMA1376 was cultured overnight in LB at 30°C, then subcultured for 3 h at 30°C but only produced a culture with an OD_{600} of ~0.4 which is about half the OD normally expected. After being shifted to 42°C for 10 min with the addition of 1 mM IPTG, then culturing for 2 h at 37°C in LB with aeration, the total cell proteins were analysed by electrophoresis on a 10% SDS-polyacrylamide gel. Although this experiment was repeated several times, no expression of an ~ 59 kDa protein was seen.

A second overexpression experiment used the salt-induced T7 RNA polymerase host strain GJ1158. This strain can be cultured at 37°C and is induced by the addition of 0.6 M NaCl (Bhandari & Gowrishankar, 1997). Therefore the pRMSB82 plasmid was transformed into GJ1158 to create RMA1369. The strains containing the pRMSB82 plasmid grew significantly better at 37°C than the E2096 derivatives at 30°C and the growth in liquid media attained an OD_{600} of 0.6 after 2 h of subculturing and salt-induced overexpression was then performed (Section 2.8.2). The whole cell samples were electrophoresed on a 10% SDS-polyacrylamide gel and the overexpressed ~ 59kDa protein can be seen in Figure 5.17.

Figure 5.17: Overexpression of the *S. typhi* PqaA protein



This figure shows the overexpression of the *S. typhi* PqaA protein. The strains were grown in LB and protein was induced using the T7 promoter/RNA polymerase and NaCl system (Bhandari & Gowrishankar, 1997) and electrophoresed on an SDS-15% polyacrylamide gel and stained with Coomassie Blue (Section 2.8.2). Protein band sizes were calculated in kDa compared to Pharmacia LMW markers (Section 2.8.1) and are indicated on the side of the Figure with solid arrows (➨). The overexpressed 59.3 kDa PqaA protein is indicated by an open arrow (⇛). Lanes contain; Lane 1: RMA1369 (pRMSB82 in GJ1158), Lane 2: RMA1368 (pET11BYZ in GJ1158)

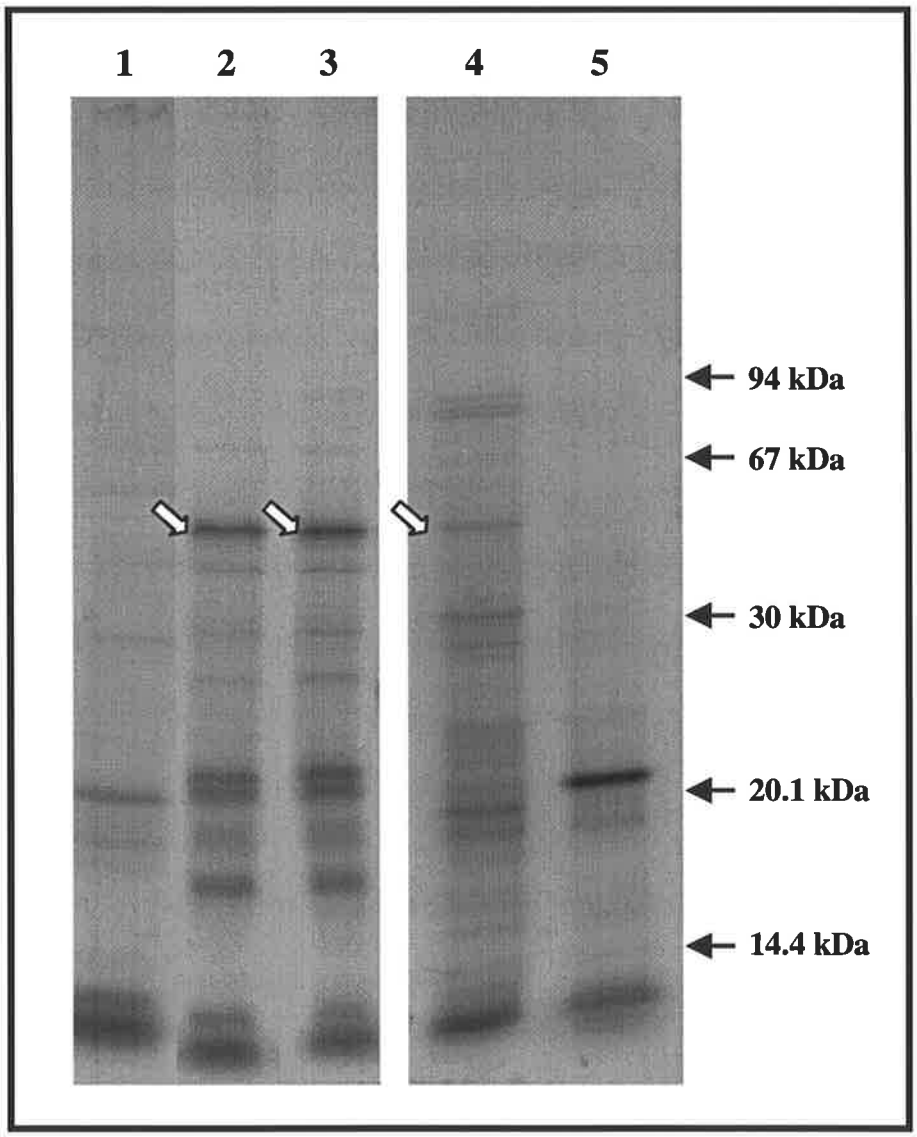
Unfortunately, the overexpression of *S. typhi* PqaA from strain RMA1369 could not be repeated, indicating that the clone may be unstable. As pET11BYZ is a medium copy number plasmid it was decided to clone the *pqaA* gene into the low copy number based plasmid

pWSK29 (Wang & Kushner, 1991) with the ORF in the T7 orientation. An oligonucleotide was designed with a *Hind*III site incorporated into it: (#2989; Table 2.5) and used to perform a PCR reaction with #2987 (Table 5.2) using *S. typhi* Ty2 chromosomal DNA as a template. A 1.74 kb PCR product was produced, digested with *Hind*III and *Bam*HI and ligated to similarly digested pWSK29. The ligation mixture was then transformed into DH5a and Amp^R transformants were selected on LA containing Amp. The resultant plasmids were named pRMSB84 and pRMSB85 (in RMA1364 and RMA1365, respectively). These plasmids were transformed into E2096 (to produce strains RMA1373 and RMA1374 respectively) and GJ1158 (to produce strains RMA1392 and RMA1393 respectively). After "normal overexpression" (both temperature and salt-induced) was tried with these low copy number clones and no overexpressed ~59 kDa protein was produced (data not shown), it was decided to use L-[S^{35}] methionine labelling to detect the *S. typhi* PqaA protein.

5.8.4 L-[S^{35}]-methionine labelling of the *S. typhi pqaA* gene product

L-[S^{35}] methionine overexpression with the E2096 strains carrying the pRMSB82 pRMSB84, and pRMSB85 clones was performed (Section 2.8.3) and the results can be seen in Figure 5.18. The results from Figures 5.18 show that the *S. typhi pqaA* gene does encode a 59.3 kDa protein as predicted from its sequence. Cell fractionation was not undertaken to localize PqaA however due to the limited amount of labelled protein produced.

Figure 5.18: L-[S^{35}] methionine labelling of protein produced from the cloned *S. typhi pqaA* gene

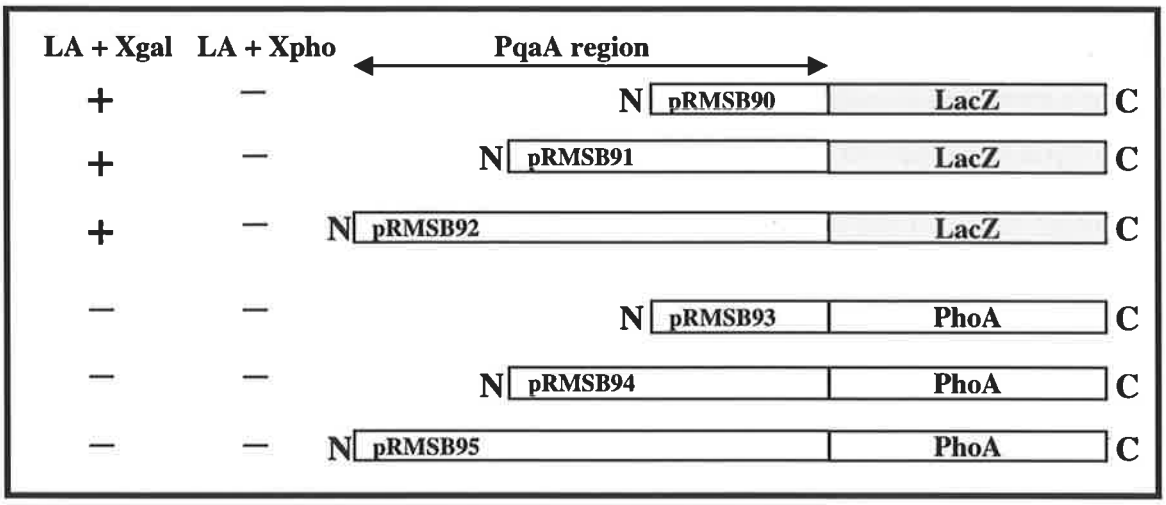


This figure shows the detection of production of the *S. typhi* PqaA protein by L-[S^{35}] methionine labelling (Section 2.8.3). The 59.3 kDa PqaA protein is indicated by an open arrow (⇛) and protein bands were calculated by comparison with Pharmacia LMW markers Section 2.8.1). The lanes are numbered at the top of the figure and contents of the lanes are listed below; Lane1: RMA1381 (pWSK29 in E2096), Lane2: RMA1373 (pRMSB84 in E2096), Lane3: RMA1374 (pRMSB85 in E2096), Lane4: RMA1376 (pRMSB82 in E2096), Lane5; RMA1377 (pET11bYZ in E2096)

5.8.5 Further structural analysis of the *S. typhi* PqaA protein

Due to the difficulty of overexpressing the *S. typhi* PqaA protein, another approach to determine the subcellular location of the hydrophilic part of the PqaA protein was undertaken. Computer analysis predicted that the majority of the *S. typhi* PqaA protein was located in the cytoplasm. To test this, PqaA-LacZ (β-galactosidase) and PqaA-PhoA (alkaline phosphatase) fusion proteins were made using the vectors pRMCD70 (LacZ) and pRMCD28 (PhoA) (Daniels *et al.*, 1998). Three different PCR products were produced with the following oligonucleotides: #2988 (located before the promoter region of *pqaA*) and #3067, #3068 and #3069 (Table 2.5) respectively, located at three different points in the C-terminal portion of the protein. Three PCR reactions were performed using *S. typhi* Ty2 chromosomal DNA as a template and oligonucleotides #2988-#3067, #2988-#3068, #2988-#3069 to produce PCR products of 0.63 kb, 1.15 kb and 1.70 kb respectively. The PCR products were digested with *Xba*I and *Eco*RI and ligated to similarly digested pRMCD70 and pRMCD28, transformed into DH5α and Amp^R transformants were selected. The resultant plasmids were named pRMSB90, pRMB91, and pRMSB92 (in RMA1324, RMA1347 and RMA1349, respectively) to produce PqaA-LacZ fusions, and pRMSB93, pRMSB94 and pRMSB95 (in RMA1403, RMA1405 and RMA1406, respectively) to produce the PqaA-PhoA fusions. All six strains were streaked out onto LA containing Amp and X-gal and LA containing Amp and X-pho and screened at the single colony level (Figure 5.19). The β-galactosidase fusions (from pRMSB90, pRMSB91 pRMSB92) were the only strains to produce blue colonies on selective media (LA containing Amp and X-gal), indicating that the hydrophilic portion of the *S. typhi* PqaA protein is located in the cytoplasm.

Figure 5.19: Schematic diagram of PqaA-LacZ and -PhoA fusions



This figure shows the results from plating strains contain the *pqaA*-LacZ fusions (from pRMSB90, pRMSB91, pRMSB92) and *pqaA*-PhoA fusions (from pRMSB93, pRMSB94 and pRMSB95) onto LA containing X-gal and LA containing X-pho. Open boxes indicate the PqaA protein, and LacZ and PhoA fusion boxes are labelled.
N: amino terminal, C: carboxy terminal
+ = Blue colonies and - = white colonies, scored at the single colony level.

5.8.6 Analysis of the rare codon usage of the *S. typhi* PqaA protein

It was interesting that it was so difficult to overexpress the *S. typhi* PqaA protein as overall it is a hydrophilic protein and doesn't contain many transmembrane regions (which usually are the reasons for difficulty in obtaining overexpression of a protein). Therefore it was decided to analyse the codon usage for *pqaA*. It has previously been proposed (Grosjean & Fiers, 1982) that efficiency of translation is affected by the codon usage in mRNA, and that rare or minor codons are rarely encoded in efficiently expressed genes and are considered to be modulating codons. It has also been suggested that the presence of these modulating codons within the first 25 amino acids results in stalling of the ribosome, leading to weakly expressed proteins (Chen & Inouye, 1990). The first 25 codons of the *S. typhi pqaA* gene were analysed (Figure 5.20) and it was found that two modulating codons lay after each methionine at the start of the protein (as it is not known which Met is likely to be the start codon). The *S. typhi* PqaA gene was also analysed for total codon usage using DNASIS and

the results can be seen in Table 5.8.

Figure 5.20: Analysis of the *S. typhi* PqaA first 25 amino acids

```
       1953        1962        1971        1980        1989        1998
TTA TAT TGA ATG TTT AAG AGG TAT TTA TGG AAA TTA TGC TGG TTG GCA TTT GCC
            Met Phe Lys Arg Tyr Leu Trp Lys Leu Cys Trp Leu Ala Phe Ala

       2007        2016        2025        2034        2043        2052
CTT GTA AAG CGC GGG GAA AGC ATG AAA AAA ATA TAT CTC GTT GTT ATC GTA CTG
Leu Val Lys Arg Gly Glu Ser Met Lys Lys Ile Tyr Leu Val Val Ile Val Leu

       2061        2070        2079        2088        2097        2106
TTT TTT ATT TCT ACA AAA GTT TAT ACA CTA CTT CAT AAC AAC ATC TTC TTT TGT
Phe Phe Ile Ser Thr Lys Val Tyr Thr Leu Leu His Asn Asn Ile Phe Phe Cys
```

This figure uses sequence based on Figure 5.11 and the two potential start (methionine) codons are bolded and the "modulating codons (AGG (arginine), GGG (glycine), ATA (isoleucine) and CTA (leucine) are shadowed.

Table 5.8: *S. typhi pqaA* codon usage

CODON	COUNT	%AGE	CODON	COUNT	%AGE	CODON	COUNT	%AGE	CODON	COUNT	%AGE
TTT-Phe	14	2.7%	TCT-Ser	2	0.4%	TAT-Tyr	19	3.7%	TGT-Cys	4	0.8%
TTC-Phe	7	1.4%	TCC-Ser	4	0.8%	TAC-Tyr	8	1.5%	TGC-Cys	1	0.2%
TTA-Leu	18	3.5%	TCA-Ser	9	1.7%	TAA-***	0	0.0%	TGA-***	0	0.0%
TTG-Leu	5	1.0%	TCG-Ser	3	0.6%	TAG-***	0	0.0%	TGG-Trp	11	2.1%
CTT-Leu	10	1.9%	CCT-Pro	9	1.7%	CAT-His	6	1.2%	CGT-Arg	7	1.4%
CTC-Leu	5	1.0%	CCC-Pro	11	2.1%	CAC-His	6	1.2%	CGC-Arg	5	1.0%
CTA-Leu	**4**	**0.8%**	CCA-Pro	8	1.5%	CAA-Gln	14	2.7%	**CGA-Arg**	**3**	**0.6%**
CTG-Leu	3	0.6%	CCG-Pro	10	1.9%	CAG-Gln	11	2.1%	**CGG-Arg**	**2**	**0.4%**
ATT-Ile	23	4.4%	ACT-Thr	5	1.0%	AAT-Asn	15	2.9%	AGT-Ser	7	1.4%
ATC-Ile	11	2.1%	ACC-Thr	8	1.5%	AAC-Asn	14	2.7%	AGC-Ser	8	1.5%
ATA-Ile	**13**	**2.5%**	ACA-Thr	13	2.5%	AAA-Lys	21	4.1%	**AGA-Arg**	**3**	**0.6%**
ATG-Met	8	1.5%	ACG-Thr	8	1.5%	AAG-Lys	6	1.2%	**AGG-Arg**	**2**	**0.4%**
GTT-Val	14	2.7%	GCT-Ala	6	1.2%	GAT-Asp	23	4.4%	GGT-Gly	3	0.6%
GTC-Val	5	1.0%	GCC-Ala	11	2.1%	GAC-Asp	4	0.8%	GGC-Gly	8	1.5%
GTA-Val	9	1.7%	GCA-Ala	11	2.1%	GAA-Glu	13	2.5%	**GGA-Gly**	**4**	**0.8%**
GTG-Val	4	0.8%	GCG-Ala	5	1.0%	GAG-Glu	10	1.9%	**GGG-Gly**	**4**	**0.8%**

This table shows the codon usage of the *S. typhi pqaA* gene (Figure 5.11: 1953-3509, 518 aa). The rare codons (defined as rare or modulator codons by Grosjean & Fiers (1982) are CUA (leucine), AUA (isoleucine), AGA/AGG/CGA/CGG (arginine) and GGA/GGG (glycine) and are outlined in bold.

A total 6.9% of the codons are rare or modulating codons (Table 5.8) which is sufficient to potentially inhibit translation (Grosjean & Fiers, 1982), combined with the modulating codons seen within the first 25 codons of the *S. typhi pqaA* gene provides a probable cause for the difficulties observed in obtaining overexpression of the PqaA protein.

5.8.7 Functional analysis of the *S. typhi* PqaA protein

As the low copy number *pqaA* pWSK29 clones (pRMSB84 and pRMSB85) had been shown by L-[S^{35}] protein labelling to produce the 59 kDa PqaA protein, it was felt that the pRMSB84 and pRMSB85 plasmids could be used to complement the *S. typhi pqaA*::Mu*dJ* (RMA2310) mutant in melittin sensitivity assays (as the *S. typhi pqaA*::Mu*dJ* insertion mutant has previously been shown to be sensitive to melittin) (Baker *et al.*, 1997). However, during the melittin sensitivity assay analysis, it was found that the *S. typhi pqaA*::Mu*dJ* (RMA2310) mutant was no longer sensitive to melittin compared to the *S. typhi* Ty2 parental strain (Table 5.9). The *S. typhi* $PhoP^c$ mutant (RMA1090) was also tested and it was found to have increased resistance to melittin compared to the *S. typhi* Ty2 strain (Table 5.9), correlating with previous results for polymyxin B assays (Section 3.6.5).

Table 5.11: *S. typhi* strains and melittin resistance

S. typhi Strain	Melittin resistance
Ty2	47.1 ± 5.9
RMA1030 ($PhoP^c$)	0.0 ± 0.0
RMA1090 ($PhoP^c$)	78.2 ± 4.4
RMA2310 (*pqaA*::Mu*dJ*)	50.0 ± 2.3

This table shows the percentage survival of survival of *S. typhi* strains after exposure for 1 hour to 7.5 μg/ml of melittin at 37°C (Section 2.11.2). The mean value and standard deviation were calculated from two repeated assays.

5.8.8 Deletion/Insertion mutagenesis of *pqaA*

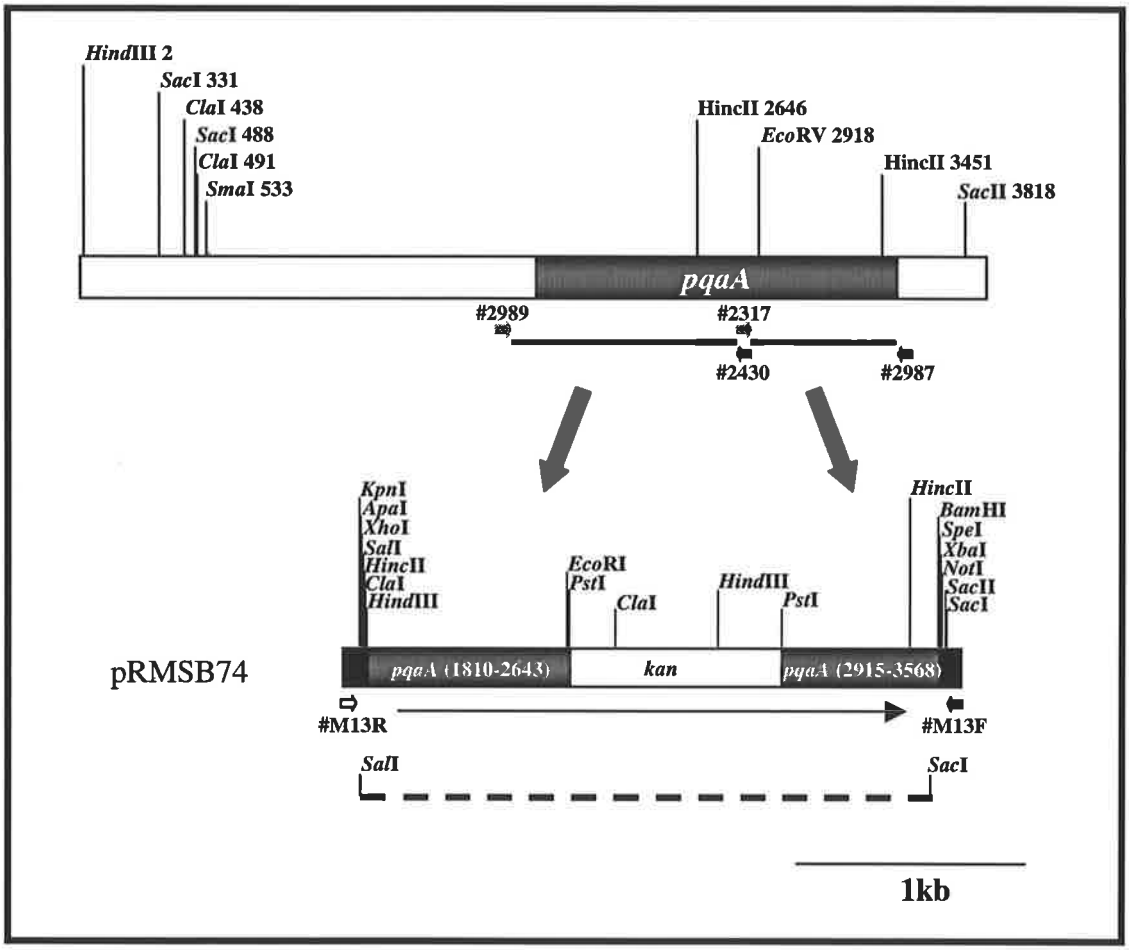
As the *S. typhi pqaA*::Mu*dJ* insertion mutant appeared to have lost the sensitivity to melittin phenotype, I wanted to construct a defined mutant in *pqaA* to test whether the mutation causing sensitivity had been lost during the studies or whether an error had occurred during the original melittin assays.

Due to the lack of restriction enzymes site contained within the *S. typhi pqaA* gene, the construct was built using PCR and oligonucleotides containing restriction enzyme sites (Figure 5.21; Table 2.5). A PCR reaction using the *S. typhi* Ty2 chromosomal DNA as a template and oligonucleotides #2987 (*pqaA*: 3569-3546, Figure 5.11) (includes a *Bam*HI site) and #2317 (*pqaA*: 2835-2852, Figure 5.11) (located 3' to the *Hinc*II site in *pqaA*) (Figure 5.21). The 0.73 kb PCR product was purified, digested with *Bam*HI and *Hinc*II, and ligated to *Bam*HI and *Sma*I digested pBC. The ligation mixture was transformed into DH5α and transformants were selected on LA containing Cml, IPTG and X-gal for blue white colour selection. The resultant plasmid was named pRMSB71 (in RMA1351). A second PCR was then set up with *S. typhi* Ty2 chromosomal DNA and oligonucleotides #2989 (*pqaA*: 1806-1828) (contains a *Hind*III site) and #2430 (*pqaA*: 2861-2843)) and the 1.1 kb product was purified, digested with *Hind*III and *Hinc*II, and ligated to *Hind*III and *Eco*RV digested pRMSB71. The ligation mixture was transformed into DH5α, transformants were selected on LA containing Cml, screened for the appropriate second insert and the resultant plasmid was named pRMSB73 (in RMA1353).

The *kan* cartridge used for the mutagenesis was the non-polar *kan* cartridge (*aphT*) from pSB315 which has been modified from the *aph* (aminoglycoside 3'phosphotransferase) gene encoding Kan resistance, devoid of its transcription terminator (Galán *et al.*, 1992), therefore allowing disruption of the gene it is inserted in but permitting downstream genes to be expressed as normal. For clarity, the *aphT* gene shall be referred to as *kan* from now on. The *kan* cartridge was obtained by *Pst*I digestion of pSB315, and the *Pst*I digested *kan* cartridge was ligated into *Pst*I digested pRMSB73, transformed into DH5α and transformants were selected on LA containing Cml and Kan. The plasmids obtained were screened for the correct orientation of the *kan* cartridge (which contains a *Cla*I site near the beginning) and a correctly orientated *kan* insert was obtained, and the plasmid was named pRMSB74 (in RMA1354). It was noted that although the pRMSB74 plasmid contained the *S. typhi pqaA* gene which has no

homolog in *E. coli*, with a deletion of 272 bp and a Kan cartridge insert, the colonies still grew poorly, indicating that the *pqaA* gene may be toxic when cloned into high copy number vectors such as pBC.

Figure 5.21: Outline of construction of *S. typhi pqaA*::Kan insert for mutagenesis



This figure shows the outline of the construction of pRMSB74 and the schematic restriction enzyme map of the *S. typhi pqaA*::Kan insert in pRMSB74 shows the two cloned sections of *pqaA* (1810-2643 and 2915-3568 corresponding to Figure 5.11) (dark grey reactangle) with the kan cartridge from pSB315 cloned into the PstI site between them (outlined rectangle). The *pqaA*::Kan insert is flanked by the pBC MCS (black boxes)..The direction of transcription of *pqaA* and Kan is shown by a line arrow (→). Positions of the restriction enzymes and the oligonucleotides #M13F (black arrow), #M13R (open arrow) (Table 2.5) and their positions are marked. The *SacI*/*SalI* fragment from pRMSB74 which was cloned into the suicide vector pCACTUS is indicated by a dashed line.

The insert from pRMSB74 was then digested with *Sac*I and *Sal*I and ligated to a similarly digested pCACTUS, temperature sensitive suicide plasmid (which also contains a *sacB* gene),

and the ligation mixture was transformed into DH5α and selected on LA containing Cml and Kan, at 30°C. After screening for the correct insert, the resulting plasmid was named pRMSB88 (in RMA1332), pRMSB88 was then electroporated into *S. typhi* Ty2 creating strain RMA1395 , selected on LA containing Cml and Kan, and grown at 30°C. For the actual mutagenesis, RMA1395 was grown overnight at 30°C and then subcultured 1/20 for 2 h at 37°C and plated out on LA containing sucrose (as the *sacB* gene is toxic to the host bacteria when expressed in the presence of sucrose) and Cml, at 37°C to select for insertion mutants and loss of the suicide plasmid. However although this was repeated many times, and the plasmid was even retransformed into *S. typhi* Ty2 (creating RMA1396), the mutagenesis process was unsuccessful. The pRMSB74 *pqaA*::Kan clone was sequenced at both ends and appeared to be correct. More *pqaA* DNA may have been needed in the clone (possibly at least 1 kb each side of the *kan* cartridge rather than just 0.65 and 0.83 kb as were contained in pRMSB88), however time constraints did not allow this exercise to be repeated.

5.9 Summary

In this chapter, *S. typhi pqaA* gene was fully sequenced as well as approximately 1.5 kb of adjacent DNA (Section 5.4). The *pqaA* gene was shown to be *Salmonella* specific (Section 5.3) and potentially located on a previously undescribed putative *Salmonella* pathogenicity island (Section 5.7) (discussed further in Chapter 7).

The *S. typhi pqaA* gene was confirmed to encode a 59.3 kDa protein by overexpression analysis and the protein has an N-terminal transmembrane region which is likely to be located in the cytoplasmic membrane with the C-terminal highly hydrophilic portion located in the cytoplasm (Section 5.8.2; Section 5.8.3; Section 5.8.2; Section 5.8.5). The protein is highly regulated by the PhoP/Q operon and is likely to also be regulated at the translational level by

containing 6.9% rare amino acids, including at least two in the first 25 amino acids, therefore making it difficult to easily overexpress (Section 5.8.6). Unfortunately upon retesting in this study, the *S. typhi pqaA*::Mu*dJ* mutants had lost the sensitivity to melittin, and although studies with a defined mutant were proposed, the mutant was unable to be constructed to retest for melittin resistance (Section 5.8.7; Section 5.8.8).

Upstream of the *S. typhi pqaA* gene are two ORFs, the first of which has homology to tetracycline-6 hydroxylase and an oxygenase enzymes, and may be in an operon with ORF2. ORF2 encodes a protein which has high homology to the MFS superfamily, specifically the 12-TMS proteins which are involved in multi-drug efflux systems. ORF2 had many of the motifs which characterise this family and had approximately the same homology (around 25%) to all of the seven 12-TMS proteins discussed in this chapter. It is unlikely to encode a tetracycline resistance protein as all the TetA homologs had higher homology to each other (~50%) than to the ORF2. Due to time constraints the protein was unable to be studied further but it would be interesting to test mutants in this ORF2 against a variety of drugs to see if any sensitivity could be determined. The regulation of this ORF2 is also not known and testing whether it could be PhoP/Q regulated would also worth-while for future studies as NorA has recently been shown to be regulated by a two component regulatory system (Fournier *et al.*, 2000).

The partial ORF3 downstream of the PqaA protein had homology to members of a permease family which are involved in transport of substances such as hexuronate, glucarate and 4- hydroxybenzoate). Further discussion of these results will be continued in Chapter 7.

Chapter 6

Identification and analysis of the *S. typhi pqaB* gene

6.1 Introduction

Initial analysis of the *S. typhi* mutant with the *pqaB*::Mu*dJ* fusion showed that it was strongly upregulated by *phoP/Q* (β-galactosidase activity was increased 15-fold in the *pqaB*::Mu*dJ* PhoP^c strain (RMA2526) compared to the same mutation in the PhoP$^-$ chromosomal background) (Section 3.7.4). Phenotypic analysis of the *S. typhi* Ty2 *pqaB*::Mu*dJ* mutant indicated that it was sensitive to the antimicrobial peptide melittin (Baker *et al.*, 1997) and had reduced net growth in PMA-U937 cells compared to *S. typhi* Ty2 (Section 4.6.2).

The *S. typhimurium* PhoP$^-$ mutant has been previously shown to be extremely sensitive to antimicrobial peptides, including melittin (Groisman *et al.*, 1992B) although no *S. typhimurium phoP/Q* regulated genes characterised so far have been shown to be involved with melittin resistance. During the course of this study, it was reported that the *S. typhimurium* PhoP/Q regulates genes encoding the PrmA, PmrB (PmrA/B) two-component regulatory system. This system in turn regulates genes including *pmrE/ugd* (Groisman *et al.*, 1997; Gunn & Miller, 1996) and the recently identified *pmrF* operon which is involved with PmB resistance and lipid A modifications (Gunn *et al.*, 1998B; Guo *et al.*, 1997). PmrA/B regulated genes have been shown to affect modification of the LPS core and lipid A regions with ethanolamine and the addition of 4-amino-arabinose (4AA) to the 4' phosphate of lipid A (Gunn *et al.*, 1998B). The *pmrF* locus was predicted to contain an operon encoding seven proteins involved with the 4-amino-arabinose modification of lipid A (Gunn *et al.*, 1998B).

In this chapter the *S. typhi* PhoP/Q regulated *pqaB*::Mu*d*J fusion was identified by cloning and sequence analysis as being almost identical to the fifth gene in the *S. typhimurium* PmrA/B regulated *pmrF* operon.

6.2 Identification of the *S. typhi pqaB* mutant

6.2.1 Isolation of *S. typhi pqaB* sequence from the LHS of the Mu*d*J transposon insertion

As the *S. typhi pqaB*::Mu*d*J fusion mutant had previously been shown to exhibit multiple phenotypes including melittin sensitivity, (Baker *et al.*, 1997) and reduced net growth in PMA-U937 cells (Section 4.6.2), the *S. typhi pqaB* gene was further characterised. An initial attempt to clone the *pqaB* gene was based on the Mu*d*J left inverse PCR (IPCR) method used previously (Section 3.8.1). The "Mu*d*JL" probe (Section 3.8.1.1) was used for Southern analysis of *Dra*I, *Hind*III and *Mlu*I digested RMA2326 (*pqaB*::Mu*d*J) chromosomal DNA. The digested RMA2326 DNA was electrophoresed on a 0.7 % (w/v) TAE agarose gel, transferred to nylon and probed with the "Mu*d*JL" probe (Section 2.7). The results of this Southern hybridisation analysis can be seen in Table 6.1 along with the corresponding predicted IPCR band size.

Table 6.1: Southern hybridisation analysis of *S. typhi* RMA2326 (*pqaB*::Mu*d*J chromosomal DNA with the "Mu*d*J-L probe"

Restriction Enzyme	Southern Band Size (kb)	Predicted IPCR Product Size (kb)
*Dra*I	2.9	2.3
*Hind*III	2.8	2.2
*Mlu*I	1.25	0.65

Chromosomal DNA from RMA2326 (*pqaB*::Mu*d*J) was digested with the indicated enzymes and probed by Southern analysis with the "Mu*d*J-L" probe (Section 3.8.1.1; Section 2.7). The sizes of the bands detected were calculated in kb by comparison with *Eco*RI digested SPP1 markers (Section 2.5.2.2). The predicted inverse PCR product size is ~600 bp less than the Southern hybridisation band.

All three digests gave suitably sized bands (under 3 kb) for use in IPCR, and it was decided to use *Hind*III and *Mlu*I. Therefore, RMA2326 chromosomal DNA was digested with *Hind*III and *Mlu*I, religated and used as a template for IPCR with #2103 and #2104 oligonucleotides (Table 2.5). The *Mlu*I IPCR produced a product of the expected size (0.65 kb, Table 6.1) (data not shown) which was ligated into pGEMT, transformed into DH5α, and Amp^R transformants were selected and screened. The resulting plasmid (pRMSB21) (in strain RMA1262) was sequenced with the #2103 oligonucleotide in a dye-terminator sequencing reaction. The expected sequence for Mu*dJ* was obtained (Figure 6.1a) and the flanking DNA sequence adjacent to the Mu*dJ* transposon had homology to the *S. typhimurium hisD* gene (Figure 6.1 b).

Figure 6.1a: BlastN analysis of pRMSB21 - Mu*dJ* LHS homology

```
pRMSB21: 348 TGTATTGATTCACTTGAAGTACGAAAAAAACCGGGAGGACATTGGATTATTCGGGATCTG 289
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MudJL:     1 TGTATTGATTCACTTGAAGTACGAAAAAAACCGGGAGGACATTGGATTATTCGGGATCTG 60

pRMSB21: 288 ATGGGATTAGATTTGGTGGGGCTTGCAAGCCTGTAGTGCAAATTTTAGTCGTTAATCAAT 229
             |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
MudJL:    61 ATGGGATTAGATTTGGTGGGGCTTGCAAGCCTGTAGTGCAAATTTTAGTCCTTAATCAAT 120

pRMSB21: 228 GAAACGCGAAAGATAGTAAAAAATTGCTTTTGTTTCATTGAAAATACGAAAAACAAAAAC 169
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MudJL:   121 GAAACGCGAAAGATAGTAAAAAATTGCTTTTGTTTCATTGAAAATACGAAAAACAAAAAC 180

pRMSB21: 168 ACTGCAAATCATTTCAATAACAGCTTCAAAAAACGTTCAA 129
             |||||||||||||||||||||||||||||||||||||
MudJL:   181 ACTGCAAATCATTTCAATAACAGCTTCAAAAAACGTTCAA 220
```

The sequence obtained from the pRMSB21 plasmid with dye-terminator sequencing was analysed by the BlastN 2.0 homology program (Altschul *et al.*, 1997) and the first part of the sequence (1-129 nt) showed homology to the Mu*dJ* LHS (gbD: M64097)

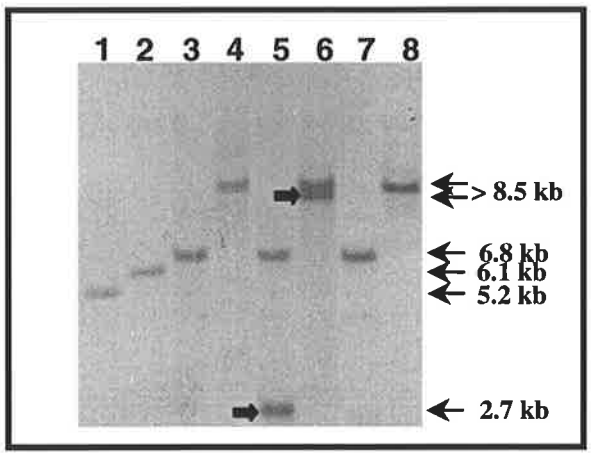
Figure 6.1b: BlastN analysis of pRMSB21 - *hisD* homology

```
pRMSB21: 349 ACCCCTGAAGAGATCGCCGCCGCCGGCGCGCGTCTGAGCGACGAATTAAAACAGGCGATG 408
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HisD:   2110 ACCCCTGAAGAGATCGCCGCCGCCGGCGCGCGTCTGAGCGACGAATTAAAACAGGCGATG 2169

pRMSB21: 409 ACCGCTGCCGTCAAAAATATTGGAA 433
             |||||||||||||||||||| ||
HisD:   2170 ACCGCTGCCGTCAAAAATATTGAAA 2194
```

The sequence obtained from the pRMSB21 plasmid with dye-terminator sequencing was analysed by the BlastN 2.0 homology program (Altschul *et al.*, 1997) and the latter part of the sequence (349-433 nt) showed homology to the *S. typhimurium hisD* (gbD: X13464)

The high similarity of the *S. typhi* Mu*dJ* LHS flanking DNA to the *S. typhimurium hisD* gene was surprising as all the *S. typhi pqa/pqr*::Mu*dJ* mutants had previously been tested for auxotrophic growth on minimal media and none had shown any growth limitations (Daniels, 1994). Therefore it seemed unlikely that the *S. typhi* Mu*dJ* insertion for *pqaB* was in the *S. typhi hisD* gene. As the Mu*dJ* transposon used for the mutagenesis was originally contained in the *S. typhimurium* TT10288 strain in the *hisD* gene (Hughes & Roth, 1988), it seemed likely that a duplication event had occurred during the transposition process. To investigate this, a PCR-DIG-labelled probe ("*hisD*") was constructed using oligonucleotides #2275 and #2276 (Table 2.5) designed to a region just outside of the *S. typhimurium hisD* gene (gbD: X13464) and using *S. typhimurium* C5 chromosomal DNA as a template (Section 2.7.1.2). *S. typhi* Ty2, RMA2310 (*pqaA*::Mu*dJ*) and RMA2326 (*pqaB*::Mu*dJ*) chromosomal DNA was digested with *Hind*III and *Pst*I, electrophoresed on a 0.7% (w/v) TAE gel, transferred to nylon and probed with the "*hisD*" probe (Section 2.7)(Figure 6.2).

Figure 6.2: Southern hybridisation analysis of *E. coli* and *Salmonella* chromosomal DNA with the "*hisD*" probe



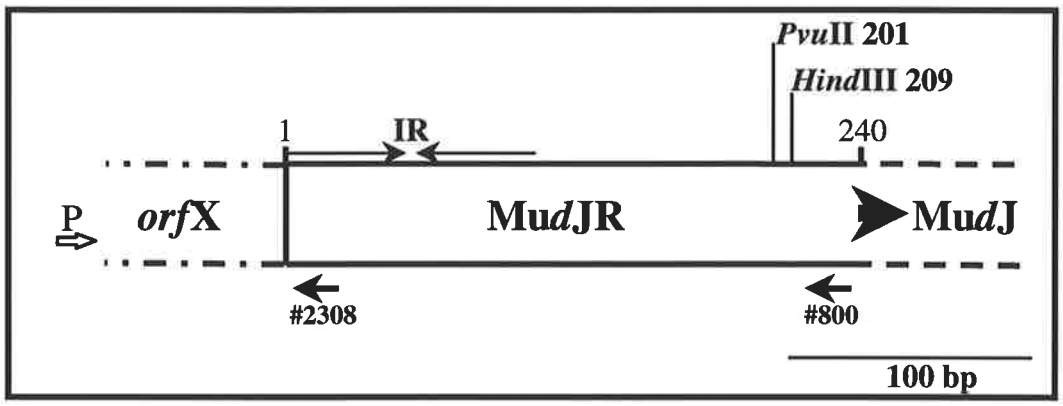
Chromosomal DNA from *E. coli* DH5, *S. typhimurium* LT2 P9003 *S. typhi* Ty2, RMA2310 (*pqaA*::Mu*dJ*) and RMA2326 (*pqaB*::Mu*dJ*) was digested with *Hind*III and *Pst*I and probed with the DIG-labelled "*hisD*" PCR product (Section 2.7). The size of the bands are indicated and were calculated by comparison with *Eco*RI digested SPP1 marker DNA (Section 2.5.2.2). The extra bands detected in RMA2326 are indicated by solid arrows (→). The order of the lane loading is labelled numerically at the top of the figure and the sample in the lanes are; Lane 1: *E. coli* DH5 [*Hind*III], Lane 2: *S. typhimurium* P9003 [*Hind*III], Lane 3: *S. typhi* Ty2 [*Hind*III], Lane 4: *S. typhi* Ty2 [*PstI*I], Lane 5: *S. typhi* RMA2326 (*pqaB*::Mu*dJ*) [*Hind*III], Lane 6: *S. typhi* RMA2326 [*PstI*I], Lane 7: *S. typhi* RMA2310 (*pqaB*::Mu*dJ*) [*Hind*III], Lane8: *S. typhi* RMA2310 [*Hind*III].

Figure 6.2 shows clearly that two bands were detected for the *S. typhi pqaB*::Mu*d*J chromosomal DNA *Hind*III digest, and a doublet can be seen for the *Pst*I digest compared to the single bands seen for the *S. typhi* and RMA2310 (*pqaA*::Mu*d*J) digests respectively. The extra *hisD* band in RMA2326 indicated that a duplication of the *hisD* gene had occurred on the LHS of the Mu*d*J transposon during the mutagenesis transduction. Consequently, the flanking DNA from the RHS of the *pqaB*::Mu*d*J insertion needed to be obtained to identify the *S. typhi pqaB* gene.

6.2.2 Isolation of *S. typhi pqaB* sequence from the RHS of the Mu*d*J transposon insertion

To identify the *S. typhi pqaB* gene sequence, DNA adjacent to the RHS of the Mu*d*J transposon insertion needed to be obtained. As the RHS of the Mu*d*J transposon contains a 48 nt inverted repeat (Figure 6.3) it was considered too difficult to use oligonucleotides designed for an IPCR to isolate *pqaB* DNA from this end.

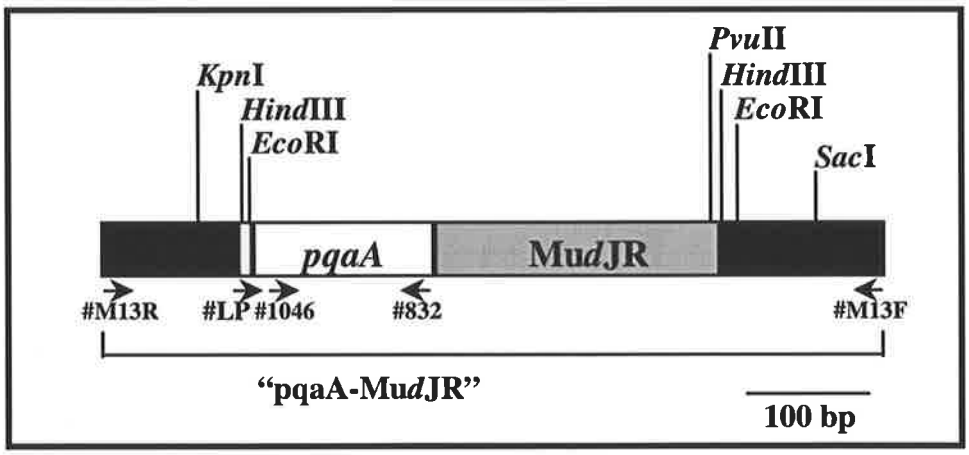
Figure 6.3: Schematic diagram of the RHS of Mu*d*J



Schematic diagram of the first 240 nts of the RHS of Mu*d*J, labelled 1-240 with 1 being the first nucleotide of the Mud*J*R transposon located next to the chromosomal insertion. This figure is modified from MuR sequence given in Metcalf *et al.*, (1990) (gbD: M33723). The transposon is represented in the orientation as inserted into an transcriptionally active ORF (represented by *orfX* with the direction of the transcriptional activity represented by the promoter (P) and an open arrow). Continuation into the Mu*d*J transposon towards the Mu*d*J LHS is represented by a solid arrowhead (➨). Restriction enzyme sites *Pvu*II and *Hind*III and their locations are marked as well as the location of the 48 bp inverted repeat (IR, →←). The position of the oligonucleotides #800 and #2308 and direction of extention (←) are also labelled.

However, part of the *pqaA* gene sequence was originally isolated from the RHS of Mu*dJ* with a "walking PCR" method (Section 5.1). The 130 bp of *pqaA* sequence isolated, along with adjacent DNA from the Mu*dJ* RHS was cloned into a pBluescript KS^+ plasmid, pRMSB5 (Figure 6.4) (Baker, 1993).

Figure 6.4: Schematic diagram of pRMSB5 insert



This schematic diagram of the HindIII insert of pRMSB5 shows the *pqaA*-Mu*dJ* (*pqaA* - unshaded box, Mu*dJ*R - grey box) PCR fragment inserted between HindIII sites in pBluescript KS^+ (black boxes). The position of oligonucleotides #LP, #M13F, #M13R, #832 and #1046 and direction of extension (indicated by arrows →) are shown as well as the PCR product "*pqaA*-Mu*dJ*R" produced with oligonucleotides #M13F and #M13R.

The #M13F and #M13R oligonucleotides (Table 2.5) were used to PCR DIG-11-dUTP label the insert from the pRMSB5 plasmid for use as probe ("*pqaA*-Mu*dJ*R" (Figure 6.4; Section 2.7.1.2)). This probe was designed such that the *pqaA* component of the probe would hybridise to a single band containing the *pqaA* gene at the same position in both *S. typhi* Ty2 and RMA2326 (*pqaB*::Mu*dJ*) and to a different sized band in RMA2310 (*pqaA*::Mu*dJ*) (due to the *pqaA*::Mu*dJ* insertion). Sufficient Mu*dJ* RHS DNA would be also be labelled allowing hybridisation to the Mu*dJ* transposon fragment in RMA2310 (at the same position as *pqaA*) and to different position in RMA2326, indicating the location of the *pqaB* gene. A Southern hybridisation was performed using *S. typhi* Ty2, RMA2310 and RMA2326 chromosomal DNA digested with *Hind*III and *Pvu*II (as both these enzymes are located within the RHS of

Mu*dJ*, Figure 6.3), electrophoresed on a 0.7% TAE gel, then transferred to nylon and probed with the "*pqaA*-Mu*dJR*" probe (Section 2.7). The results can be seen in Table 6.2.

Table 6.2: Southern hybridisation analysis of *S. typhi* Ty2, RMA2310 (*pqaA*::Mu*dJ*) and RMA2326 (*pqaB*::Mu*dJ*) chromosomal DNA with the "*pqaA*-Mu*dJR*" probe

	Strains			
Enzymes	*S. typhi* Ty2 (*pqaA* band) (kb)	RMA2310 (*pqaA* band) (kb)	RMA2326 (*pqaA* band) (kb)	RMA2326 (additional band) (kb)
*Hind*III	5.5	2.50	5.4	2.3
*Pvu*II	>8.5	2.55	>8.5	2.2

Chromosomal DNA was digested with the indicated enzymes and probed by Southern analysis with the "*pqaA*-Mu*dJR*" probe (Figure 6.3; Section 2.7). The sizes of the bands detected were calculated in kb by comparison with *Eco*RI digested SPP1 markers (Section 2.5.2.2).

A single band was seen for the *S. typhi* Ty2 and RMA2310 (*pqaA*::Mu*dJ*) chromosomal DNA. These bands were different sizes due to the *pqaA*::Mu*dJ* insertion within RMA2310, signifying that it was the *pqaA* gene being detected. RMA2326 (*pqaB*::Mu*dJ*) had bands of an identical size to the *S. typhi* Ty2 parental strain, due to detection of the *pqaA* gene, however an additional band was seen for the RMA2326 chromosomal DNA: *Hind*III (2.3 kb) and *Pvu*II (2.2 kb) (Table 6.2), indicating the position and size of the *S. typhi pqaB* gene.

Due to the difficulty of performing an IPCR with the RHS of Mu*dJ*, it was decided to use the SSP-PCR method (Section 2.5.3.4) to identify the *pqaB* gene. Southern hybridisation using the "*pqaA*-Mu*dJR*" probe was performed with *S. typhi* Ty2 and RMA2326 chromosomal DNA digested with *Hind*III and various enzymes (Table 6.3) whose sites are located in the pBluescript KS^+ multiple cloning site. The unique bands seen for RMA2326 compared to *S. typhi* Ty2 for this Southern hybridisation are listed in Table 6.3.

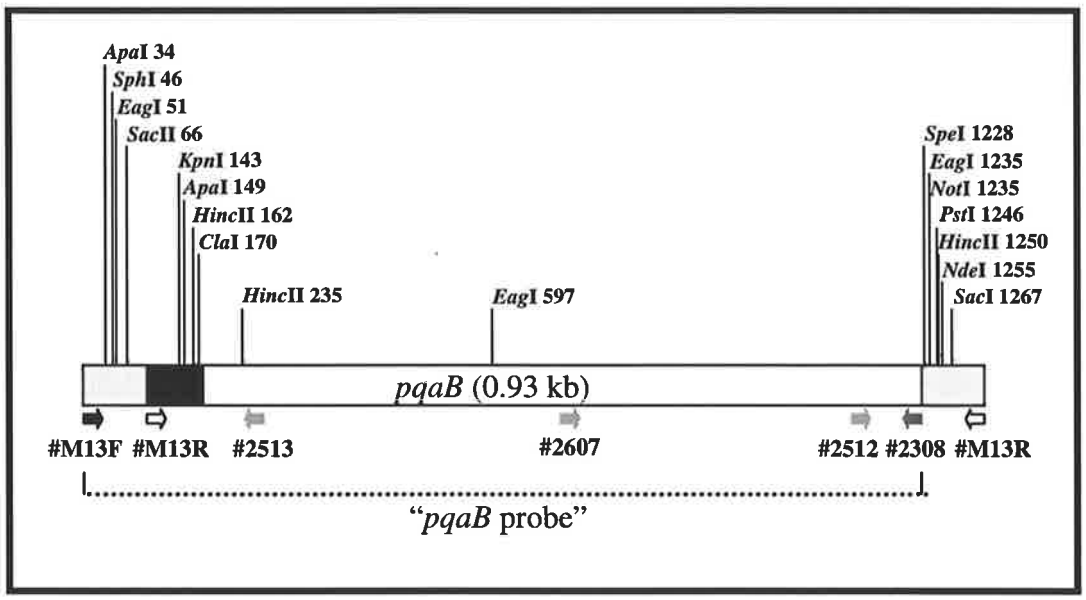
Table 6.3: Southern hybridisation analysis of unique bands for RMA2326 (*pqaB*::Mu*dJ*) chromosomal DNA with the "Mu*dJ*-R-*pqaA*" probe.

Restriction enzymes	RMA2326 (*pqaB*::Mu*dJ*) (unique band size kb)
*Hind*III/*Cla*I	0.93
*Hind*III/*Eco*RV	1.65
*Hind*III/*Hinc*II	0.93
*Hind*III/*Kpn*I	2.23
*Hind*III/*Pst*I	2.23
*Hind*III/*Xba*I	2.23

Chromosomal DNA from *S. typhi* Ty2 and RMA2326 (*pqaB*::Mu*dJ*) was digested with the indicated enzymes and probed by Southern hybridisation analysis with the "*pqaA*-Mu*dJ*R" probe (Section 2.7). Only unique bands detected in RMA2326 compared to *S. typhi* Ty2 chromosomal DNA are reported above. The sizes of the bands detected were calculated in kb by comparison with *Eco*RI digested SPP1 markers (Section 2.5.2.2).

As the *Hind*III digest from Table 6.3 gave a band size of 2.23 kb with the "Mu*dJ*-R-*pqaA*" probe, it was unlikely that secondary enzymes in the *Hind*III/*Pst*I, *Hind*III/*Kpn*I and *Hind*III/*Xba*I double digests were cutting the *Hind*III fragment. Therefore *Hind*III/*Eco*RV (1.65 kb), *Hind*III/*Hinc*II (0.93 kb) and *Hind*III/*Cla*I (0.93 kb) enzyme combinations were selected for the chromosomal digests of *S. typhi* RMA2326 (*pqaB*::Mu*dJ*) DNA. RMA2326 chromosomal DNA was digested with the above enzymes, ligated to similarly digested pBluescript KS^+ plasmid DNA and used as a template for SSP-PCR with oligonucleotide #2308 which primes out of the end of the RHS of the Mu*dJ* transposon (Figure 6.3) and #M13F or #M13R oligonucleotides (Table 2.5), depending on the orientation of the cloned RMA2326 fragment (Section 2.5.3.4). A product of the expected size (0.93 kb) for the *Hind*III/*Cla*I SSP-PCR reaction was obtained, ligated to pGEMT, transformed into DH5α and plated out onto selective NA containing Amp plates. The resultant plasmid was named pRMSB50 (Figure 6.5) (in RMA1292) and the insert was sequenced with #2308, #M13F and #2607 oligonucleotides with dye-terminator sequencing (Section 2.5.2.7.1). Analysis with BlastX 2.0 analysis (Altschul *et al.*, 1997) gave ~ 70 % identity to an ORF encoded in *E. coli* upstream of the *E. coli pmrD* gene (gbD: AE000315) (Blattner *et al.*, 1997).

Figure 6.5: Restriction enzyme map of the *pqaB HindIII/ClaI* SSP-PCR insert in pRMSB50



The schematic restriction enzyme map of the *S. typhi pqaB HindIII/ClaI* SSP-PCR insert in pRMSB50 showing the 0.93 kb partial *pqaB* gene as an outlined rectangle, flanked by part of pBluescript KS^+ MCS on the left, (black box), with the pGEMT multiple cloning site (light grey box). Restriction enzymes and their positions are marked. The oligonucleotides (Table 2.5), #M13F (black arrow), #M13R (open arrow) (Table 2.5), #2308 (dark grey arrow) (from the end of Mu*d*JR, Figure 6.3), #2312 and #2313 (light grey arrows) (designed for IPCR with *pqaB*), #2607 (light grey arrow) (designed for sequencing) and their relative positions are also marked. The "*pqaB*" probe position is outlined with a dashed line.

A probe for the *S. typhi pqaB* fragment ("*pqaB*") was derived by PCR-DIG-11-dUTP (Section 2.7.1.2) labelling the pRMSB50 insert with oligonucleotides #2308 and #M13F. This was then used to probe a number of single digests of the *S. typhi* Ty2 chromosomal DNA to identify a larger *pqaB* fragment which could then be isolated by IPCR. The results of this Southern hybridisation are listed in Table 6.4.

From Table 6.4 it can be seen that the largest band detected under 3 kb in size was obtained for the *Eco*RV fragment (~2.9 kb). Therefore, *S. typhi* Ty2 chromosomal DNA was digested with *Eco*RV and used as a template for IPCR (Section 2.5.3.3) with two oligonucleotides designed 100 nt in from the end of the known sequence (#2512 and #2513) (Figure 6.5; Table 2.5).

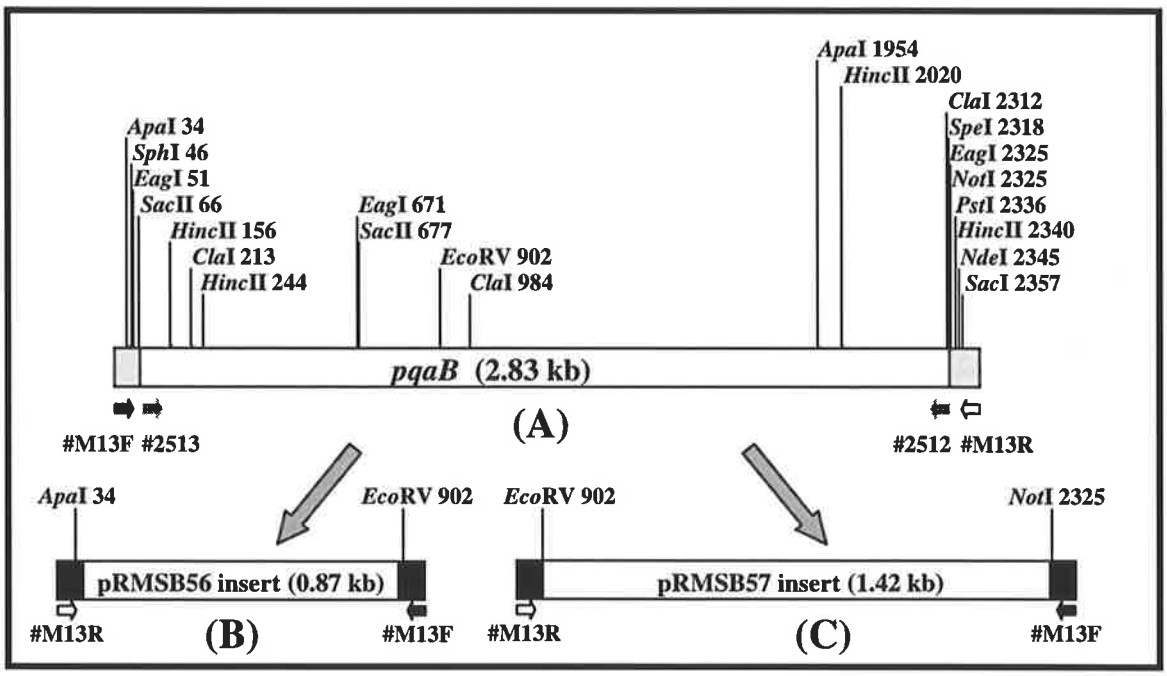
Table 6.4: Southern hybridisation analysis of *S. typhi* Ty2 chromosomal DNA with the "*pqaB*" fragment probe

Restriction enzyme	*S. typhi* Ty2 (Band Size kb)
*Bam*HI	>8.5
*Cla*I	2.7
*Eco*RV	2.9
*Hind*III	>8.5
*Pst*I	>8.5

Chromosomal DNA from *S. typhi* Ty2 was digested with the indicated enzymes and probed by Southern analysis with the "*pqaB*" probe (Section 2.7). The sizes of the bands detected were calculated in kb by comparison with *Eco*RI digested SPP1 markers (Section 2.5.2.2).

A DNA band was produced from the IPCR reaction of the correct size (2.9 kb), ligated to pGEMT, transformed into DH5α and Amp^R transformants were selected on NA containing Amp. The resulting plasmid was named pRMSB53 (in RMA1295) and the *Eco*RV IPCR fragment is represented in a schematic diagram in Figure 6.6. To aid sequencing of the 2.9 kb *Eco*RV IPCR fragment, the pRMSB53 plasmid was digested with *Apa*I and *Eco*RV and the resulting 0.87 kb *ApaI/Eco*RV fragment ligated to similarly digested pBC, transformed into DH5α and Cml^R transformants were selected on NA containing Cml. The resultant plasmid was named pRMSB56. The pRMSB53 plasmid was also digested with *Not*I and *Eco*RV and the resulting 1.46 kb *NotI/Eco*RV fragment ligated to similarly digested pBC, transformed into DH5α and Cml^R transformants were selected on NA containing Cml. The plasmid obtained was named pRMSB57 (Figure 6.6).

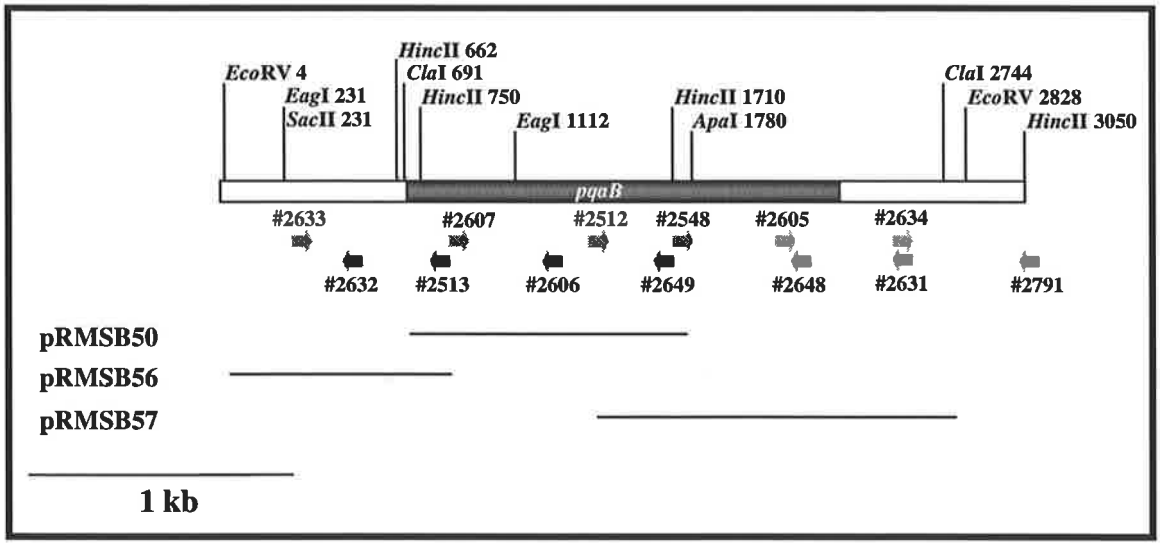
Figure 6.6: Restriction enzyme map of the *pqaB Eco*RV IPCR insert in pRMSB53 and subclones



(A): The schematic restriction enzyme map of the *S. typhi pqaB Eco*RV IPCR insert in pRMSB53 shows 2.83 kb of insert containing the *pqaB* gene as an outlined rectangle, flanked by the pGEMT multiple cloning site (light grey boxes). Positions of the restriction enzymes and their positions are marked. The oligonucleotides #M13F (black arrow), #M13R (open arrow) (Table 2.5) and #2512 and #2513 (pale grey arrows) (Figure 6.5) and their relative positions are also marked.
(B): The schematic restriction enzyme map of the *S. typhi pqaB ApaI/Eco*RV insert from pRMSB53 in pRMSB56 shows 0.87 kb of insert containing the *pqaB* gene as an outlined rectangle, flanked by the pBC multiple cloning site (black boxes). Positions of the relevant restriction enzymes and their positions are marked. The oligonucleotides #M13F (black arrow) and #M13R (open arrow) and their relative positions are also marked.
(C): The schematic restriction enzyme map of the *S. typhi pqaB NotI/Eco*RV insert from pRMSB53 in pRMSB57 shows 1.42 kb of insert containing the *pqaB* gene as an outlined rectangle, flanked by the pBC multiple cloning site (black boxes). Positions of the relevant restriction enzymes and their positions are marked. The oligonucleotides #M13F (black arrow) and #M13R (open arrow) and their relative positions are also marked.

The pRMSB50, pRMSB53, pRMSB56 and pRMSB57 inserts were then subjected to dye-terminator sequencing with #M13F, #M13R and various oligonucleotides designed for this purpose (Figure 6.7) (Table 2.5; Section 2.5.2.7.2). Double stranded sequencing of the *Eco*RV IPCR DNA fragment confirmed that the entire *S. typhi pqaB* gene had been isolated and details about the *pqaB* gene and surrounding DNA are presented below in Section 6.3.

Figure 6.7: Restriction enzyme map of the *S. typhi pqaB* gene and adjacent sequence



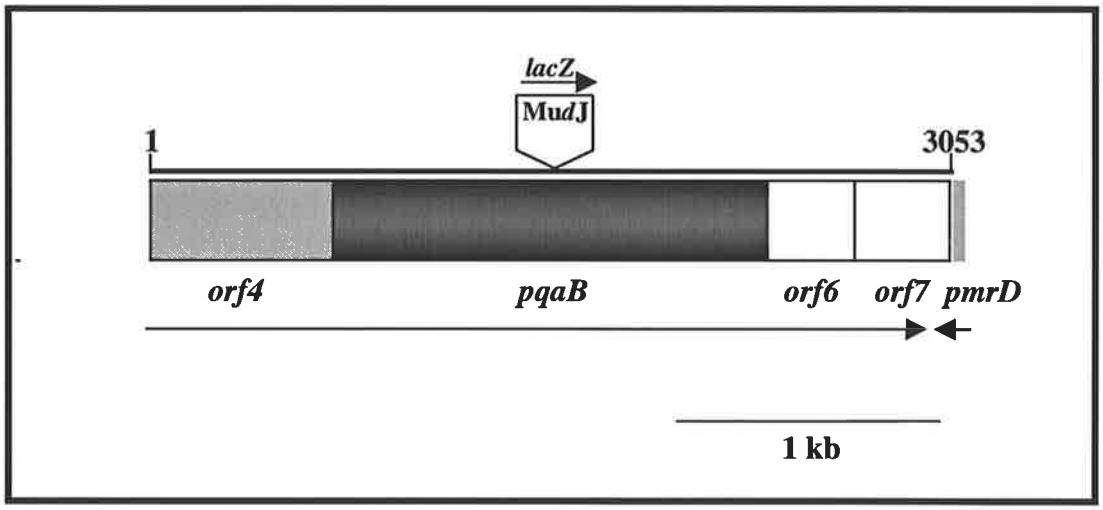
This schematic diagram shows the sequencing outline for the *S. typhi pqaB* gene (indicated by a dark grey rectangle) and surrounding sequence. Restriction enzymes and their positions are marked. Oligonucleotides designed for sequencing (light and dark grey solid arrows) and their relative positions are marked as well as the locations for the inserts of pRMSB50 (Figure 6.5), pRMSB56 and pRMSB57 (Figure 6.6).

6.3 Analysis of the *S. typhi pqaB Eco*RV fragment and surrounding DNA

The sequence of the *S. typhi pqaB Eco*RV DNA fragment (2830 nts) was analysed using BlastN/X 2.0 (Altschul *et al.*, 1997) and *pqaB* was found to have ~70% similarity to an *E. coli* K-12 ORF (gbD: AE00315, *orf550*) and ~ 98% homology with the fifth ORF (*orf5*) of the *S. typhimurium* PmrA/B regulated *pmrF* operon (gbD: AF036677; Gunn *et al.*, 1998B) at the aa level. The sequence adjacent to the *S. typhi pqaB* gene within the *Eco*RV fragment showed strong similarity to the *S. typhimurium pmrF* operon *orf4*, *orf6* and *orf7* genes. Therefore the *orf*s adjacent to the *S. typhi pqaB* fragment were given the same names as the equivalent *orf*s in *S. typhimurium* (Figures 6.8 and 6.9). The Mu*dJ* insertion was found to be between nucleotides 1528 and 1529 of the *Eco*RV fragment (Figure 6.8), corresponding to nucleotides 847 and 848 of the *pqaB* gene. Due to the high level of homology between the *S. typhi* and *S. typhimurium pmrF* and *pqaB* loci, an oligonucleotide based on the sequence of the *S. typhimurium pmrD* gene (#2971, Figure 6.7) (Gunn *et al.*, 1998B) was designed in order to

obtain the full *S. typhi orf7* sequence (sequence included in Figures 6.8 and 6.9). The location and several properties of each of the *S. typhi pqaB* operon ORFs (ORF4, PqaB, ORF6 and ORF7) and the properties of the seven ORFs in the *S. typhimurium pmrF* operon are summarised in Table 6.5. The similarity between *E. coli* (gbD: AE000315), *S. typhimurium pmrF* operon (gbD: AF036677) and the *S. typhi pqaB Eco*RV fragment (gbD: AF0718082) regions are shown in Figure 6.10. Analysis of each of the seven proteins encoded by the *S. typhimurium pmrF* operon and the homologous proteins in the *S. typhi pqaB* operon will be discussed in Section 6.4.

Figure 6.8: The *S. typhi pqaB* operon and adjacent DNA



This figure shows the *S. typhi pqaB* gene and adjacent DNA (3053 nt)(gbD: AF0718082). The full *S. typhi pqaB* and *orf6, orf7* genes are represented by dark grey and open boxes respectively and the partial *orf4* and *pmrD* genes are represented by light grey boxes. The Mu*dJ* transposon insertion into the *S. typhi pqaB* gene at 847 nt into the *pqaB* gene is shown. The direction of transcription for each ORF is indicated by an arrow (→) below the ORF's.

Figure 6.9: Sequence and translation of the *S. typhi* Ty2 *pqaB* gene and adjacent DNA

```
            10         20         30         40         50         60
   5'GATATCCTGCTGGCCGGCACCGCCTGGCCGGGAAAAAATATCGGCAACGCTAATGCCGGG
ORF4 D  I  L  L  A  G  T  A  W  P  G  K  N  I  G  N  A  N  A  G

            70         80         90        100        110        120
   ATTATTCGTGAAACGGCAACATACCATGAGACGGGACTACATGCCTGGGATCACCATGCG
   I  I  R  E  T  A  T  Y  H  E  T  G  L  H  A  W  D  H  H  A

           130        140        150        160        170        180
   TGGCAGACTCACAGCGGCCACTGGAGTATCCGCCAGCTTGAAGAAGATATTGCGCGCGGC
   W  Q  T  H  S  G  H  W  S  I  R  Q  L  E  E  D  I  A  R  G

           190        200        210        220                   240
   ATAACGGCGCTTGAGGCCATTATCGGAAAACCCGTCACCTGCTCAGCCGCGGCCGGCTGG
   I  T  A  L  E  A  I  I  G  K  P  V  T  C  S  A  A  A  G  W

           250        260        270        280        290        300
   CGAGCGGATGGGCGCGTTGTTCGTGCGAAAGAACCGTTCAACCTGAGGTATAACAGCGAT
   R  A  D  G  R  V  V  R  A  K  E  P  F  N  L  R  Y  N  S  D

           310        320        330        340        350        360
   TGCCGTGGAACCACCTTATTCCGTCCGCTACTGATGCCGGGCCAGACAGGGACGCCGCAA
   C  R  G  T  T  L  F  R  P  L  L  M  P  G  Q  T  G  T  P  Q

           370        380        390        400        410        420
   ATCCCGGTCACCCTACCCACGTGGGATGAGGTTATCGGCCCCGCCGTTCAGGCGCAGTCG
   I  P  V  T  L  P  T  W  D  E  V  I  G  P  A  V  Q  A  Q  S

           430        440        450        460        470        480
   TTCAATACCTGGATTATTTCCCGTATGTTGCAGGACAAAGGCACGCCGGTATATACCATC
   F  N  T  W  I  I  S  R  M  L  Q  D  K  G  T  P  V  Y  T  I

          490        500        510        520        530        540
   CATGCGGAAGTCGAAGGTATTGTCCATCAGCCGCTGTTTGAAGATTTGCTCGTTCGTGCA
   H  A  E  V  E  G  I  V  H  Q  P  L  F  E  D  L  L  V  R  A

           550        560        570        580        590        600
   CGCGATGCGGGGATCACCTTTTGTCCTCTGGGCGAACTGCTGCCGGCATCGCCTGAGTCT
   R  D  A  G  I  T  F  C  P  L  G  E  L  L  P  A  S  P  E  S

           610        620        630        640        650        660
   CTGCCGTTGGGGCAAATTGTACGTGGACACATCCCTGGCCGTGAAGGTTGGCTGGGGTGT
   L  P  L  G  Q  I  V  R  G  H  I  P  G  R  E  G  W  L  G  C

           670        680        690        700        710        720
   CAACAGGCAGTGAGCGCCTCATGATGAAATCGATACGCTATTATCTGGCTTTCGCCGCGT
   Q  Q  A  V  S  A  S  *
                     PqaB  M  M  K  S  I  R  Y  Y  L  A  F  A  A  F

           730        740                   760        770        780
   TTATCGCGCTCTACTATGTTATTCCTGTCAACAGCCGCCTGCTCTGGCAGCCGGATGAAA
     I  A  L  Y  Y  V  I  P  V  N  S  R  L  L  W  Q  P  D  E  T

           790        800        810        820        830        840
   CGCGCTATGCCGAAATAAGTCGGGAGATGCTGGCGTCTGGCGACTGGATCGTGCCGCATT
     R  Y  A  E  I  S  R  E  M  L  A  S  G  D  W  I  V  P  H  F
```

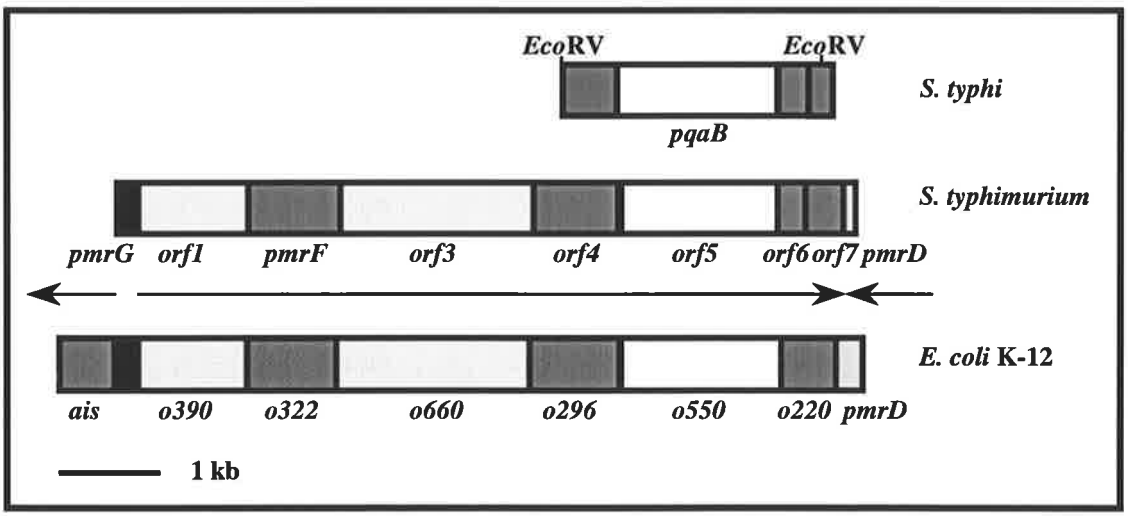
```
      850       860       870       880       890       900
TTCTGGGATTACGTTATTTTGAAAAACCGATAGCCGGTTACTGGATAAACAGTCTGGGTC
  L   G   L   R   Y   F   E   K   P   I   A   G   Y   W   I   N   S   L   G   Q

      910       920       930       940       950       960
AGTGGCTTTTTGGCGCTACCAATTTTGGCGTGCGGGCCGGCGCTATTTTAACGACATTGC
  W   L   F   G   A   T   N   F   G   V   R   A   G   A   I   L   T   T   L   L

      970       980       990      1000      1010      1020
TGGCCGCAGCGCTGGTGGCGTGGCTGACGTTCCGCTTATGGCGGGATAAACGTACCGCGT
  A   A   A   L   V   A   W   L   T   F   R   L   W   R   D   K   R   T   A   L

     1030      1040      1050      1060      1070      1080
TGCTCGCCTCCGTGATCTTTCTCTCCCTCTTTGCCGTTTACAGTATCGGGACGTATGCGG
  L   A   S   V   I   F   L   S   L   F   A   V   Y   S   I   G   T   Y   A   V

     1090      1100      1110      1120      1130      1140
TACTCGACCCGATGATCGCGCTTTGGCTTACGGCCGGGATGTGTTGTTTCTGGCAGGGGA
  L   D   P   M   I   A   L   W   L   T   A   G   M   C   C   F   W   Q   G   M

     1150      1160      1170      1180      1190      1200
TGCAGGCCACGACCCGAACGGGCAAGATTGGGATGTTTTTGCTACTGGGCGCAACCTGCG
  Q   A   T   T   R   T   G   K   I   G   M   F   L   L   L   G   A   T   C   G

     1210      1220      1230      1240      1250      1260
GTCTGGGTGTATTAACTAAAGGGTTCCTCGCCCTGGCCGTACCGGTGGTGAGCGTGCTGC
  L   G   V   L   T   K   G   F   L   A   L   A   V   P   V   V   S   V   L   P

     1270      1280      1290      1300      1310      1320
CGTGGGTTATTGTTCAGAAACGCTGGAAGGACTTTTTGCTTTACGGCTGGCTGGCGGTCT
  W   V   I   V   Q   K   R   W   K   D   F   L   L   Y   G   W   L   A   V   L

     1330      1340      1350      1360      1370      1380
TGAGCTGCTTTGTGGTTGTCCTTCCCTGGGCGATCGCCATTGCGCGACGCGAAGCCGATT
  S   C   F   V   V   V   L   P   W   A   I   A   I   A   R   R   E   A   D   F

     1390      1400      1410      1420      1430      1440
TCTGGCATTACTTTTTCTGGGTGGAGCATATCCAGCGATTCGCCATGAGCGATGCCCAGC
  W   H   Y   F   F   W   V   E   H   I   Q   R   F   A   M   S   D   A   Q   H

     1450      1460      1470      1480      1490      1500
ATAAAGCCCCTTTCTGGTACTATCTGCCGGTGCTGCTCGCGGGAAGTTTACCGTGGCTGG
  K   A   P   F   W   Y   Y   L   P   V   L   L   A   G   S   L   P   W   L   G

     1510      1520      1530      1540      1550      1560
GGTTACTGCCTGGCGCGCTTAAACTGGGCTGGCGCGAGCGAAACGGCGCATTCTATTTGC
  L   L   P   G   A   L   K   L   G   W   R   E   R   N   G   A   F   Y   L   L

     1570      1580      1590      1600      1610      1620
TCGGATGGACGATCATGCCGCTTCTCTTTTTCAGCATTGCGAAAGGGAAACTGCCCACCT
  G   W   T   I   M   P   L   L   F   F   S   I   A   K   G   K   L   P   T   Y

     1630      1640      1650      1660      1670      1680
ATGTTCTTTCCTGTTTCGCACCGATAGCGATACTCATGGCGCGCTTCGTCCTGCATAACG
  V   L   S   C   F   A   P   I   A   I   L   M   A   R   F   V   L   H   N   V

     1690      1700                1720      1730      1740
TAAAAGAAGGCGTCGCCGCGCTACGTGTCAACGGCGGGATCAACCTGGCGTTCGGGATAA
  K   E   G   V   A   A   L   R   V   N   G   G   I   N   L   A   F   G   I   I
```

```
      1750       1760       1770                  1790       1800
TCGGGATCGTCGCGGCGTTTGTCGTCTCCTCATGGGGCCCGCTGAAATCGCCGGTGTGGA
  G  I  V  A  A  F  V  V  S  S  W  G  P  L  K  S  P  V  W  T

      1810       1820       1830       1840       1850       1860
CGCATATCGAAACCTATAAAGTGTTTTGCGTCTGGGGCGTCTTTACTGTTTGGGCGTTTG
  H  I  E  T  Y  K  V  F  C  V  W  G  V  F  T  V  W  A  F  V

      1870       1880       1890       1900       1910       1920
TGGGCTGGTATAGCCTCTGTCATAGCCCAAAATACCTATTACCCGCGTTTTGTCCGCTGG
  G  W  Y  S  L  C  H  S  P  K  Y  L  L  P  A  F  C  P  L  G

      1930       1940       1950       1960       1970       1980
GACTGGCGCTGCTGTTCGGCTTTTCCGTCCCCGACAGGGTGATGGAGTCCAAACAGCCTC
  L  A  L  L  F  G  F  S  V  P  D  R  V  M  E  S  K  Q  P  Q

      1990       2000       2010       2020       2030       2040
AGTTTTTTGTCGAAATGACCCAGGCGCCTTTGGCATCAAGCCGCTATATTCTTGCCGACA
  F  F  V  E  M  T  Q  A  P  L  A  S  S  R  Y  I  L  A  D  S

      2050       2060       2070       2080       2090       2100
GCGTGGGCGTCGCCGCCGGGCTGGCCTGGAGTCTGAAACGAGACGATATTATGCTCTACG
  V  G  V  A  A  G  L  A  W  S  L  K  R  D  D  I  M  L  Y  G

      2110       2120       2130       2140       2150       2160
GACACGCGGGCGAACTCAGGTATGGTCTTAGCTATCCTGATGTACAGAATAAATTCGTTA
  H  A  G  E  L  R  Y  G  L  S  Y  P  D  V  Q  N  K  F  V  K

      2170       2180       2190       2200       2210       2220
AAGCCGACGATTTTAACGCCTGGCTCAACCAACATCGTCAGGAGGGCATTATTACGCTAG
  A  D  D  F  N  A  W  L  N  Q  H  R  Q  E  G  I  I  T  L  V

      2230       2240       2250       2260       2270       2280
TACTTTCAATAGACAAAGACGAAGACATCAGCGCACTTTCCCTTCCCCCTGCGGACAATG
  L  S  I  D  K  D  E  D  I  S  A  L  S  L  P  P  A  D  N  V

      2290       2300       2310       2320       2330       2340
TTGATTATCAGGGGCGTCTGGTGTTAATTCAGTATCGGCCTAAATGATCGGCATCGTTCT
  D  Y  Q  G  R  L  V  L  I  Q  Y  R  P  K  *
                                      ORF6  M  I  G  I  V  L

      2350       2360       2370       2380       2390       2400
GGTGCTTGCCAGTCTGCTCAGCGTTGGCGGTCAGCTCTGCCAGAAGCAAGCGACACGGCC
 V  L  A  S  L  L  S  V  G  G  Q  L  C  Q  K  Q  A  T  R  P

      2410       2420       2430       2440       2450       2460
ATTGACGACGGGCGGACGCCGCCGTCACTTGATGCTGTGGCTGGGCCTGGCGCTGATATG
 L  T  T  G  G  R  R  R  H  L  M  L  W  L  G  L  A  L  I  C

      2470       2480       2490       2500       2510       2520
CATGGGTGCAGCGATGGTGCTGTGGCTGCTGGTGCTACAAACCCTGCCGGTGGGAATTGC
 M  G  A  A  M  V  L  W  L  L  V  L  Q  T  L  P  V  G  I  A

      2530       2540       2550       2560       2570       2580
TTACCCGATGTTGAGTCTGAATTTTGTCTGGGTCACGCTGGCGGCCTGGAAAATCTGGCA
 Y  P  M  L  S  L  N  F  V  W  V  T  L  A  A  W  K  I  W  H

      2590       2600       2610       2620       2630       2640
TGAACAGGTTCTGCCCCGCCACTGGTTGGGCGTTGCATTAATTATCAGCGGCATTATCAT
 E  Q  V  L  P  R  H  W  L  G  V  A  L  I  I  S  G  I  I  I
```

```
      2650       2660       2670       2680       2690       2700
TCTGGGGAGTGCGGCATAATGGCGTAATGTGGGGACTGATAAGCGTTGCGATTGCCCCC
 L  G  S  A  A  *
            ORF7  M  G  V  M  W  G  L  I  S  V  A  I  A  P

      2710       2720       2730       2740       2750       2760
CTCGCCCAGTTGAGTTTAGGATTCGCCATGATGCGACTGCCATCGATAGCGCATCCACTG
L  A  Q  L  S  L  G  F  A  M  M  R  L  P  S  I  A  H  P  L

      2770       2780       2790       2800       2810       2820
GCGTTTATTTCCGGGCTGGGCGCATTTAATGCCGCGACCCTGGCGCTATTCGCCGGTTTG
A  F  I  S  G  L  G  A  F  N  A  A  T  L  A  L  F  A  G  L

                 2840       2850       2860       2870       2880
GCGGGATATCTGGTTTCCGTCTTCTGCTGGCAGAAAACATTACACATGCTCGCGCTCAGC
A  G  Y  L  V  S  V  F  C  W  Q  K  T  L  H  M  L  A  L  S

      2890       2900       2910       2920       2930       2940
AAAGCCTATGCGCTGCTGAGTCTCAGTTATGTGCTTGTCTGGGTAGCCTCAATGTTGTTA
K  A  Y  A  L  L  S  L  S  Y  V  L  V  W  V  A  S  M  L  L

      2950       2960       2970       2980       2990       3000
CCCGGTTTGCAAGGCGCTTTCAGCCTAAAAGCGATGCTCGGCGTATTGTGCATTATGGCG
P  G  L  Q  G  A  F  S  L  K  A  M  L  G  V  L  C  I  M  A

      3010       3020       3030       3040       3050
GGGGTAATGCTGATTTTTCTGCCCGCCAGATCATGATGGCTTGCGCGTCAACC 3'
G  V  M  L  I  F  L  P  A  R  S  *
```

This figure shows the sequence analysis of the *S. typhi pqaB* region of DNA and adjacent genes (gbD: AF0718082). The nucleotides sequence is numbered in accordance with Genbank and is shown from nt 1 to nt 3053. The amino acid translations are represented by single letter code below the first nt of each codon. The potential start (ATG) codon is bolded and the potential ribosome binding sites (RBS) are underlined

Figure 6.10: *S. typhi, S. typhimurium* and *E. coli* regions containing *pqaB* and *pqaB* homologs



This figure shows the location of the sequenced *S. typhi Eco*RV fragment (gbD: AF0718082) containing the *S.typhi pqaB* gene and surrounding genes. The *S. typhimurium pmrF* operon (gbD: AF036677) and homologous *E. coli* (gbD: AE000315) region are also shown. Relevant restriction sites are also indicated and arrows (→) indicate direction of transcription.

Table 6.5: Analysis of the *S. typhi pqaB* gene, surrounding sequence, and the *S. typhimurium pmrF* operon

S. typhi pqaB operon (Figure 6.6)					
ORF	G+C content[a]	Location in sequence	Predicted MW (kDa)	#aa	Hydrophobicity analysis[b]
ORF4	58.0%	1-684	-	228	-0.15
PqaB	54.3%	681-2327	61.7	550	0.53
ORF6	58.0%	2324-2659	12.1	112	1.11
ORF7	54.0%	2659-3036	13.2	126	1.38

S. typhimurium pmrF operon (AFO36677; (Gunn, *et al.*, 1998B))					
ORF	G+C content[a]	Location in sequence	Predicted MW (kDa)	#aa	Hydrophobicity analysis[b]
ORF1	56.5%	548-1702	41.8	385	0.00
PmrF	53.7%	1708-2688	36.5	327	0.15
ORF3	54.1%	2688-4667	73.6	660	-0.13
ORF4	57.1%	4667-5563	33.0	299	-0.16
ORF5	54.1%	5563-7206	61.8	548	0.54
ORF6	60.7%	7206-7538	12.0	111	1.09
ORF7	53.9%	7541-7978	15.4	146	1.25

[a]Percent guanine plus cytosine (G + C) of coding region.
[b]According to (Kyte & Doolittle, 1982), as implemented in PROSIS

6.4 Analysis of the *S. typhimurium pmrF* operon and the homologous proteins in the *S. typhi pqaB* operon.

The *S. typhimurium pmrF* operon was recently identified as being both PhoP/Q and PmrA/B regulated and highly likely to encode the enzymes required for the biosynthesis of 4AA, a structural modification of the lipid A region of LPS (Gunn *et al.*, 1998B). To help analyse the enzymes encoded by the *Salmonellae pmrF/pqaB* operon, homology analysis with databases using BlastN/X/P 2.0 (Altschul *et al.*, 1997) was undertaken and hydropathy plot analysis of *S. typhimurium* and *S. typhi pmrF/pqaB* operon encoded ORFs were performed and are shown when appropriate.

6.4.1 Analysis of ORF1 of the *S. typhimurium pmrF* operon

Analysis of the *S. typhimurium pmrF* operon ORF1 by BlastN/X/P 2.0 (Altschul *et al.*, 1997) revealed similarity to several putative amino transferases at the amino acid level, such as the *Steptomyces griseus* aminotransferase StrS (378 aa) which is involved with the N-methyl-L-glucosamine pathway (Distler, 1988) and the *Streptomyces tendae* L-Lysin 2-aminotransferase NikC (426 aa), which is involved in nikkomycin D biosynthesis (Bruntner & Bormann, 1998). Other putative amino transferases include the *Brucella melitensis* perosamine synthetase Per (367 aa) which potentially catalyses the conversion of GDP-4-keto-6-deoxymannose to 4-NH_2-4, 6-dideoxymannose (perosamine) (Godfroid *et al.*, 1998) and the *E. coli* O157 perosamine synthetase Per (366 aa) which potentially encodes the acetyl transferase to make GDP-*N*-acetylperosamine (Wang & Reeves, 1998). The homologies of ORF1 to the above mentioned proteins and to the *E. coli* K12 homolog (O390) are shown in Table 6.6.

Table 6.6: Homology table for *S. typhimurium prmF* operon ORF1

	% Identity[a]					
	STYMORF1	BMPer	ECORF390	ECO157Per	SGStrS	STNDNikC
STMORF1	100	35.7 (311)	82.1 (385)	34.3 (364)	34.4 (381)	39.2 (199)
BMPer		100	35.7 (311)	52.6 (361)	37.0 (349)	33.5 (209)
ECORF390			100	33.6 (360)	33.8 (382)	40.7 (199)
ECO157Per				100	32.6 (362)	38.0 (200)
SGStrS					100	41.5 (207)
STNDNikC						100

[a]This table shows the percentage of identical amino acids determined with FASTA as implemented in PROSIS. Numbers in the brackets indicate the number of amino acids over which the % identity occurs.

STMORF1 *S. typhimurium* ORF1 in PmrF operon
BMPer *Brucella melitensis* Per (gbP: AAC98613)
ECORF390 *E. coli* K12 ORF390 (gbP: P77690)
ECO157 *E. coli* O157 Per (gbP: AAC32343)
SGStrS *Streptomyces griseus* StrS (gbP: CAA68523)
STNDNikC *Streptomyces tendae* NikC (gbP: CAA75797)

6.4.2 Analysis of PmrF of the *S. typhimurium pmrF* operon

Analysis of the *S. typhimurium pmrF* operon PmrF by BlastX/P 2.0 (Altschul *et al.*, 1997) revealed similarity to proteins involved in the glucosylation of *S. flexneri* O-antigen such as the bacteriophage SfII bactoprenol glucosyl transferase Bgt (309 aa) (Mavris *et al.*, 1997) and bacteriophage SfX bactoprenol glucose transferase GtrB (305 aa) which catalyse the formation of bactoprenol-linked glucose from UDP-glucose to bactoprenol (Guan & Verma, 1999; Mavris *et al.*, 1997). The homologies of PmrF to these *S. flexneri* bacteriophage proteins and *E. coli* ORF322 are shown in Table 6.7 and the hydropathy plot analysis showing similar hydrophobic regions can be seen in Figure 6.11.

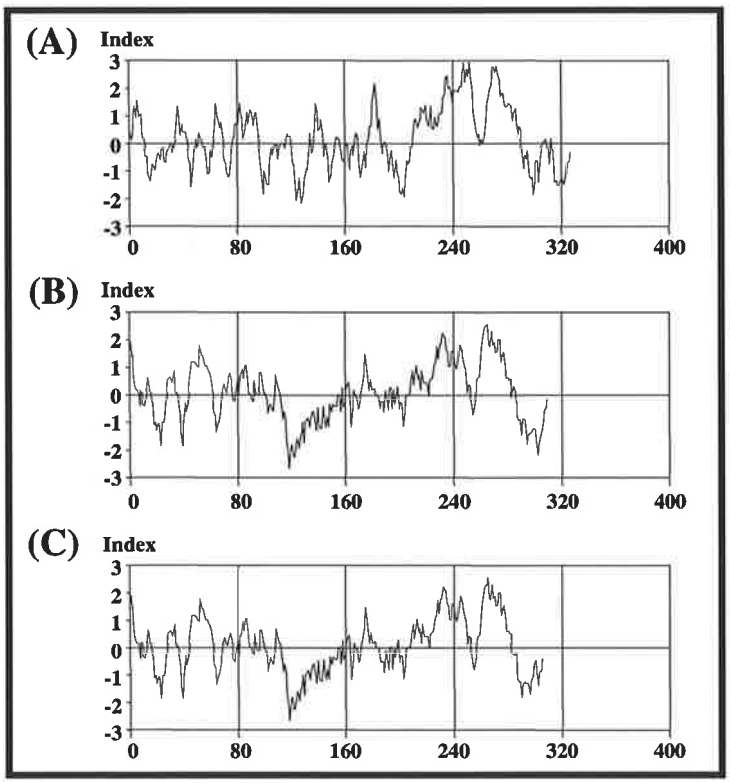
Table 6.7: Homology table for *S. typhimurium prmF* operon PmrF

	% Identity[a]			
	STYMPmrF	SFIIBgt	SFXGtrB	ECORF322
STMPmrF	100	32.7 (306)	32.5 (305)	87.7 (318)
SFIIBgt		100	95.7 (302)	30.9 (311)
SFXGtrB			100	31.6 (304)
ECORF322				100

[a]This table shows the percentage of identical amino acids determined with FASTA as implemented in PROSIS. Numbers in the brackets indicate the number of amino acids over which the % identity occurs.

STMPmrF	*S. typhimurium* PmrF in PmrF operon
SFIIBgt	Bacteriophage SfII Bgt (gbP: AAC39272)
SFXGtrB	Bacteriophage SfX GtrB (gbP: AAF22454)
ECORF322	*E. coli* K12 ORF322 (gbP: P77757)

Figure 6.11: Hydropathy plot analysis of *S. typhimurium* PmrF, Bacteriophage SfII Bgt and Bacteriphage SfX GtrB



This figure shows the hydropathy plot analysis of: (A) *S. typhimurium* PmrF protein, (B) Bacteriophage SfII Bgt protein and (C) Bacteriophage SfX GtrB protein. The hydropathy plots were generated by the method of Kyte & Doolittle in Prosis. Positive numbers on the Y-axis indicate hydrophobic regions. The position of every 80th amino acid is marked on the X-axis.

6.4.3 Analysis of ORF3 of the *S. typhimurium* pmrF operon

The *S. typhimurium orf3* in the *pmrF* operon encodes a large protein (660 aa) which when analysed by BlastX/P 2.0 (Altschul *et al.*, 1997) showed similarity to two types of proteins. The amino terminal half of ORF3 showed homology to putative formyl methyl transferase proteins including the *Bacillus subtilis* methionyl-tRNA formyltransferase Fmet (317 aa) and the *Pseudomonas aeruginosa* methionyl-tRNA formyltransferase Fmet (314 aa) which modify the free amino group of the aminoacyl moiety of methionyl-tRNA (Mazel *et al.*, 1997), and the *Brucella melitensis* formyl transferase WbkC (259 aa) which catalyses the conversion of GDP-4-NH2-4, 6-dideoxymannose to GDP-4-formamido-4, 6-dideoxymannose (Godfroid *et al.*, 1998). The carboxy terminal half of ORF3 showed homology to putative

oxidoreductases including the *Pseudomonas aeruginosa* oxidoreductase Rmd (284 aa) which converts GDP-4-keto-6-deoxy-mannose to GDP-D-rhamnose (Rocchetta *et al.*, 1998), the *Sulfolobus solfataricus* ORFC39_019 (310 aa) and a *Streptomyces fradiae* ORF333 (333 aa) dTDPglucose 4, 6-dehydratases (Charlebois *et al.*, 1999; Merson-Davies & Cundliffe, 1994). These putative oxidoreductases possess an amino acid "fingerprint" GxxGxxG which shows involvement with NAD as a cofactor for the formation of a ketose intermediate during enzymatic function (Frey, 1987). Homologies of ORF3 to the formyl-transfer proteins and the *E. coli* K12 homolog (ORF660) are shown in Table 6.8, homologies to the oxido-reductases are shown in Table 6.9.

Table 6.8: Homology analysis of *S. typhimurium pmrF* operon ORF3

	% Identity[a]				
	STYMORF 3	ECORF66 0	BMWbk C	BSFMT	PAFMT
STMORF1	100	79.4 (660)	37.9 (153)	28.0 (311)	24.2 (297)
ECORF66 0		100	29.2 (257)	29.3 (311)	26.1 (295)
BMWbkC			100	24.2 (165)	28.9 (159)
BSFMT				100	42.1 (311)
PAFMT					100

[a]This table shows the percentage of identical amino acids determined with FASTA as implemented in PROSIS. Numbers in the brackets indicate the number of amino acids over which the % identity occurs.

STMORF3	*S. typhimurium* ORF1 in the *pmrF* operon
ECORF660	*E. coli* K12 ORF660 (gbP: P77398)
BMWbkC	*Brucella melitensis* WbkC (gbP: AAC98617)
BSFMT	*Bacillus subtilis* FMT (gbP: P94463)
PAFMT	*Pseudomonas* FMT (gbP: O85732)

Table 6.9: Homologies of *S. typhimurium pmrF* operon ORF3

	% Identity[a]				
	STYMORF3	ECORF660	PARmd	SFORF333	SSORFC39
STMORF1	100	79.4 (660)	24.6 (228)	26.4 (280)	26.8 (261)
ECORF660		100	24.6 (228)	26.4 (280)	26.8 (261)
PARmd			100	26.3 (251)	28.1 (249)
SFORF333				100	31.6 (323)
SSORFC39					100

[a]This table shows the percentage of identical amino acids determined with FASTA as implemented in PROSIS. Numbers in the brackets indicate the number of amino acids over which the % identity occurs.

STMORF3	*S. typhimurium* ORF1 in PmrF operon
ECORF660	*E. coli* K12 ORF660 (gbP: P77398)
PARmd	*Pseudomonas aeruginosa* Rmd (gbP: AAC72282)
SFORF333	*Streptomyces fradiae* ORF333 (gbP: S49054)
SSORFC39	*Sulfolobus solfataricus* ORF-c39_019 (gbP: CAB57495)

6.4.4 Analysis of ORF4 in the *S. typhimurium pmrF* operon

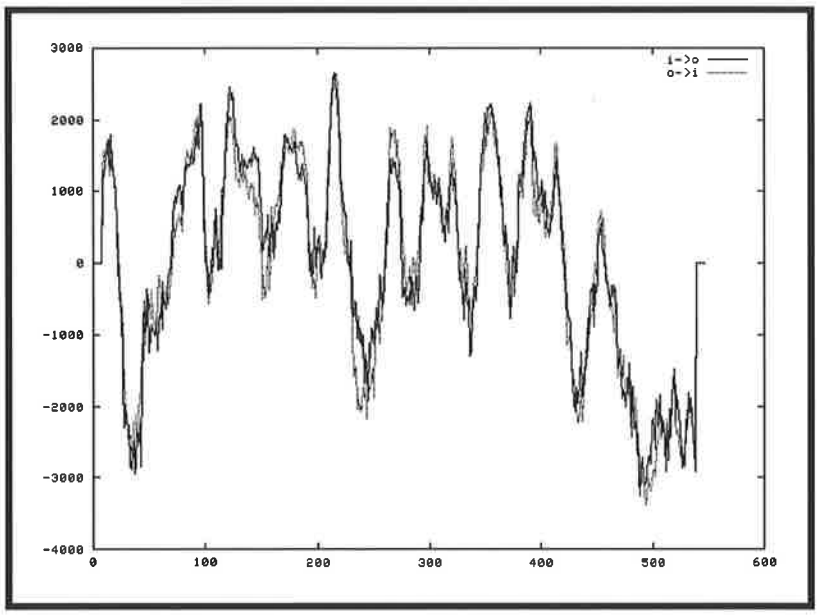
The *S. typhimurium pmrF* operon ORF4 was analysed by BlastX/P 2.0 (Altschul *et al.*, 1997) and only showed similarity to the partial *S. typhi pqaB* operon ORF4 (99.1% over 227 aa) and *E. coli* ORF296 homolog (73.2% over 298aa) (gbD: AE000315) and nothing else either at the nucleotide or amino acid level. The *S. typhi pqaB* partial ORF4 also demonstrated homology to the *E. coli* ORF4 (68.6% over 226 aa). Hydrophobicity analysis of the *S. typhimurium* ORF4 suggested that it was a highly hydrophilic protein (-0.16) (Table 6.5).

6.4.5 *S. typhimurium* pmrF operon ORF5/*S. typhi pqaB* operon PqaB

Analysis of the *S. typhimurium pmrF* operon ORF5 and the *S. typhi* PqaB protein with BlastN/X/P 2.0 (Altschul *et al.*, 1997) gave no significant similarity to anything in the database at the nucleotide or protein level except to the corresponding *E. coli* K12 ORF550

(gbD: AE000315). The *S. typhimurium* ORF5 homology to PqaB was 98.2% (over 548 aa) and the *E. coli* K12 ORF550 was 71.3% (over 550aa). *S. typhi* PqaB had 71.0% (over 550 aa) homology to the *E. coli* K12 ORF550 protein. As the *S. typhimurium* and *S. typhi* ORF5 and PqaB proteins were so similar, only PqaB was analysed by hydropathy plot and transmembrane analysis. The results (Figure 6.12), indicated that it was likely to contain many transmembrane regions.

Figure 6.12: Hydrophobicity plot analaysis of the *S. typhi* PqaB protein



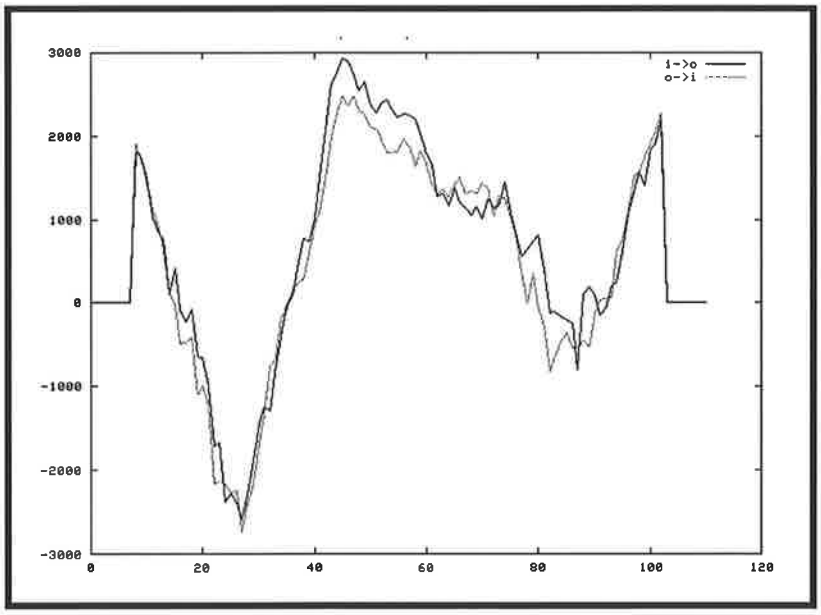
Using the computer program TMpredict (Hofmann & Stoffel, 1993), a model for the topology of PqaB was proposed. The suggested transmembrane domains are shown in the table below with two potential models described. The corresponding hydropathy plot shows that ORF2 has at least 12-13 potential membrane domains. Positive numbers on the Y-axis indicate hydrophobic regions. The position of every 100th amino acid is marked on the X-axis.

Model 1 (strongly preferred)					Model 2 (alternative model)				
TM#	Start	End	Length	Orientation	TM#	Start	End	Length	Orientation
1	5	23	19	o-i	1	7	25	19	i-o
2	89	107	19	i-o	2	86	104	19	o-i
3	112	135	24	o-i	3	113	135	23	i-o
4	135	154	20	i-o	4	163	198	36	o-i
5	163	198	36	o-i	5	205	228	24	i-o
6	205	228	24	i-o	6	257	280	24	o-i
7	257	280	24	o-i	7	290	307	18	i-o
8	290	307	18	i-o	8	311	331	21	o-i
9	311	331	21	o-i	9	347	365	19	i-o
10	347	365	19	i-o	10	381	399	19	o-i
11	381	399	19	o-i	11	406	424	19	i-o
12	406	424	19	i-o	12	447	465	19	o-i
13	447	465	19	o-i					

6.4.6 Analysis of ORF6 of the *S. typhimurium pmrF* operon and *S. typhi pqaB* operon

Analysis of the *S. typhimurium pmrF* operon ORF6 and the *S. typhi pqaB* operon ORF6 by BlastN/X/P 2.0 (Altschul *et al.*, 1997) gave no significant similarity to any sequence at both the nucleotide and protein level apart from each other (protein identity: 97.3% over 111 aa). Very little similarity to the potential corresponding ORF220 in the *E. coli* K12 genome (gbD: AE000315) was observed as the *S. typhimurium* homology was 26.3% identity over 38 aa and the *S. typhi* 24.1 % identity over 58 amino acids. Analysis of the *S. typhi* ORF6 by hydropathy plot and transmembrane analysis indicated that it was highly hydrophobic and likely to contain 3-4 transmembrane regions (Figure 6.13).

Figure 6.13: Hydropathy plot and transmembrane analysis of *S. typhi* ORF6



Using the computer program TMpredict (Hofmann & Stoffel, 1993) a model for the topology of ORF6 was proposed. The suggested transmembrane domains are shown in the table below with two potential models described. The corresponding hydropathy plot shows that ORF2 has at least 9 potential transmembrane domains. Positive numbers on the Y-axis indicate hydrophobic regions. The positions of every 20th amino acid is marked on the X-axis.

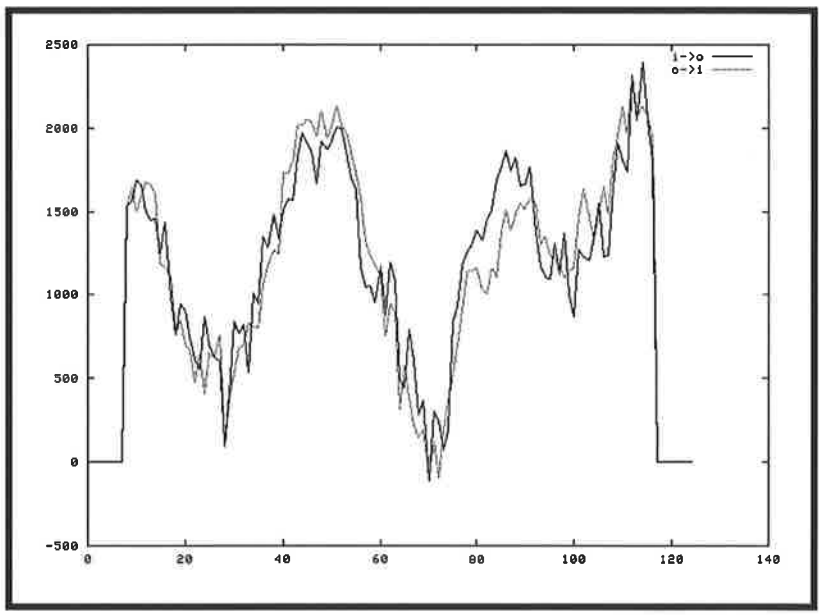
Model 1 (strongly preferred)				
TM#	Start	End	Length	Orientation
1	1	19	19	i-o
2	36	55	20	o-i
3	64	85	22	i-o
4	94	111	18	o-i

Model 2 (alternative model)				
TM#	Start	End	Length	Orientation
1	1	19	19	o-i
2	36	58	23	i-o
3	94	111	18	o-i

6.4.7 Analysis of ORF7 of the *S. typhimurium pmrF* operon and *S. typhi pqaB* operon

Analysis of the *S. typhimurium* pmrF operon ORF7 and the *S. typhi pqaB* operon ORF7 by BlastN/X/P 2.0 (Altschul *et al.*, 1997) revealed no significant similarity at the nucleotide or amino acid level apart from each other (97.6% identity over 125 aa) and the potential corresponding ORF6 in the *E. coli* K12 chromosome (gbP: AE000315): *S. typhimurium* homology (67.5% identity over 123 aa) and *S. typhi* (67.5 % identity over 123 amino acids. Analysis of the *S. typhi* ORF7 by hydropathy plot and transmembrane analysis indicated that it was highly hydrophobic and likely to contain 4 transmembrane regions (Figure 6.14)

Figure 6.14: Hydropathy plot and transmembrane analysis of *S. typhi* ORF7



Using the computer program TMpredict (Hofmann & Stoffel, 1993) a model for the topology of ORF7 was proposed. The suggested transmembrane domains are shown in the table below with two potential models described. The corresponding hydropathy plot shows that ORF2 has at least 9 potential transmembrane domains. Positive numbers on the Y-axis indicate hydrophobic regions. The positions of every 20th amino acid is marked on the X-axis.

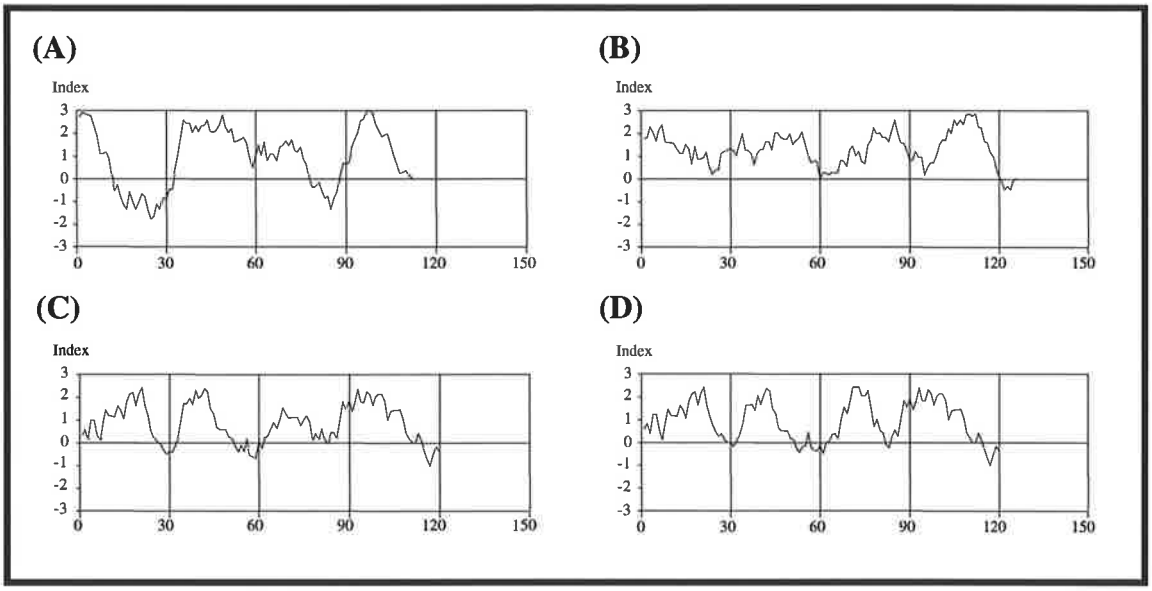
Model 1 (strongly preferred)				
TM#	Start	End	Length	Orientation
1	1	22	22	i-o
2	34	64	31	o-i
3	76	97	22	i-o
4	105	121	17	o-i

Model 2 (alternative model)				
TM#	Start	End	Length	Orientation
1	1	24	24	o-i
2	42	64	23	i-o
3	105	121	17	o-i

6.4.8 Putative role for ORF6 and ORF7

Although no homologies were detected for ORF6 and ORF7, they were found to be highly hydrophobic proteins with 3-4 predicted membrane regions (with 4 transmembrane regions being the preferred model)(Section 6.4.6; Section 6.4.7). Consequently, these proteins have structural homology with the small hydrophobic proteins of Bacteriophages SFII and SfX which have 4 proposed transmembrane regions, indicating that they are likely to be integral membrane proteins (Figure 6.15).

Figure 6.15: Hydropathy plots of ORF6, 7 and Gtr and Orf2



This figure shows the hydropathy plot analysis of: (A) *S. typhi* ORF6 protein, (B) *S. typhi* ORF7 protein, (C) Bacteriophage SfX GtrA protein and (D) Bacteriophage SfII ORF2 protein. The hydropathy plots were generated by the method of Kyte & Doolittle in Prosis. Positive numbers on the Y-axis indicate hydrophobic regions. The position of every 30th amino acid is marked on the X-axis.

The GtrA and Orf2 proteins have been shown to be similar to other proteins encoded within LPS, cell-wall teichoic acid and sugar biosynthesis loci in a variety of organisms such as RfbI (ORF10x1) in *S. flexneri*, GtcA in *Listeria monocytogenes* and Orf15x3 in *Vibrio anguillarum* (Allison & Verma, 2000; Guan *et al.*, 1999; Mavris *et al.*, 1997). It has been hypothesised by Guan *et al.*, (1999) that the SfX GtrA is the flippase for the UndP-glucose

precursor and proposed that GTR multimers could act together to transport the glucose precursor and/or act with other LPS transport genes such as RfbX. ORF6 and ORF7 (particularly ORF7) have structural homology to GtrA and ORF2 (Figure 6.15) and therefore it is possible that ORF6 and ORF7 may play a role in transfer of the Bactoprenol-4AA-lipid A across the membrane.

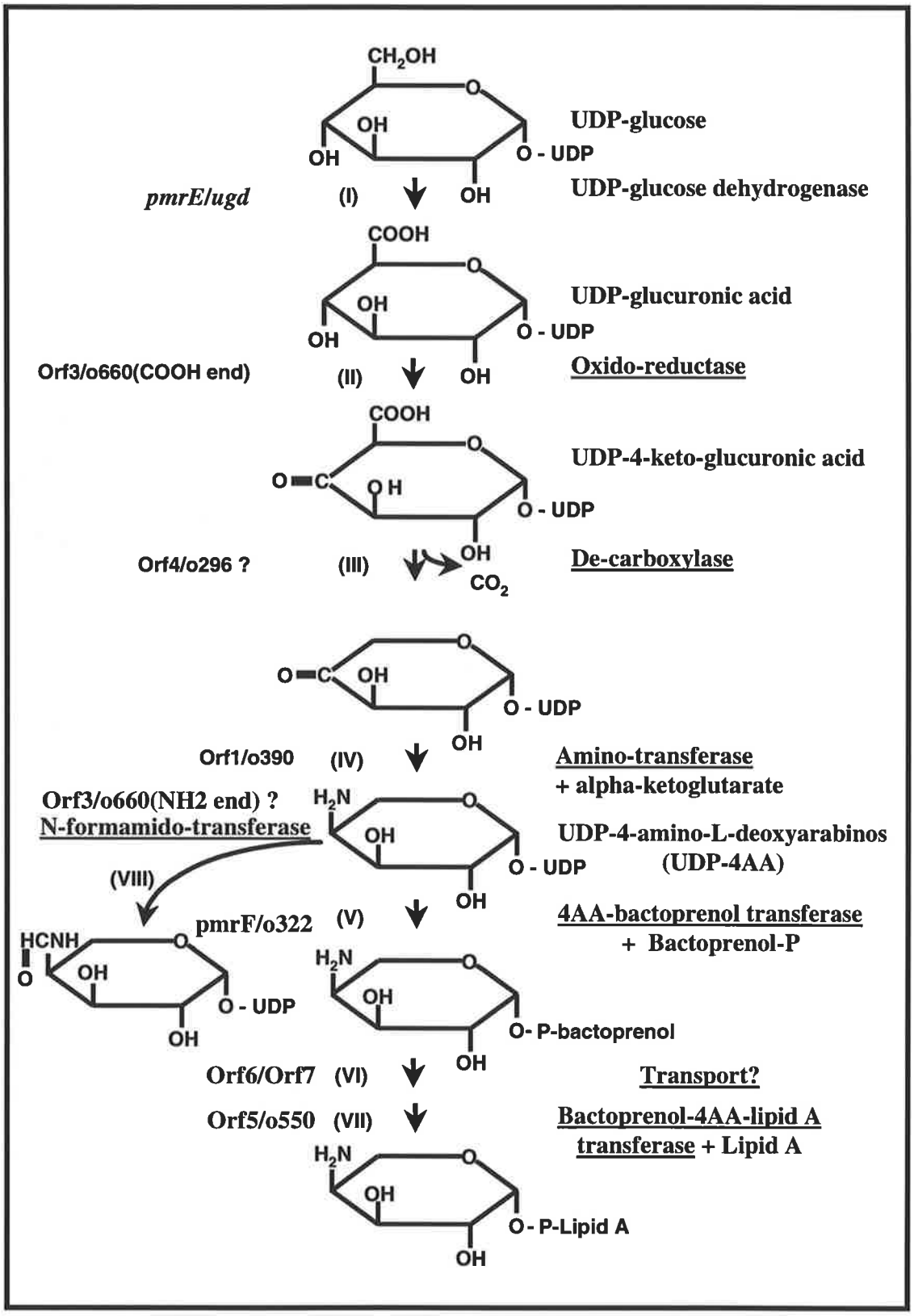
6.5 Putative biosynthesis pathway for 4-aminoarabinose

It was reported by Gunn *et al.*, (1998B) that the *pmrF* mutation was in the second ORF of the *S. typhimurium pmrF* operon and this study showed that the *S. typhi pqaB*::Mu*dJ* transposon insertion causes a mutation in ORF 5 of this operon. To obtain an understanding of the possible role of the *pmrF* and ORF 5/*pqaB* gene products in 4AA-lipid A modification, the previous section described the analysis of the ORFs in the operons by hydropathy plot and BlastX 2.0 analysis (Altschul *et al.*, 1997) (Section 6.4). These analyses allow a speculative, putative pathway for biosynthesis of 4AA and the modification of lipid A by 4AA to be proposed (Figure 6.16).

The *de novo* biosynthesis of C5 monosaccharides has been extensively studied in plants (Feingold & Barbar, 1990; Tenhaken & Thulka, 1996). UDP-glucuronic acid is the key precursor, and is obtained from the metabolic pool by the action of UDP-glucose dehydrogenase on UDP-glucose (Figure 6.16 I). UDP-glucose dehydrogenase is encoded by the remotely located *pmrE (ugd) gene* ((Groisman *et al.*, 1997; Gunn *et al.*, 1998B; Morona *et al.*, 1995; Valdivia & Falkow, 1996). The enzymes encoded by the *pmrF/pqaB* operon are then predicted to act on UDP-glucuronic acid. Initially, an oxido-reductase encoded by the carboxy terminal half of ORF 3 (Section 6.4.3) converts UDP-glucuronic to UDP-4-keto-glucuronic acid (Figure 6.16, II). The next step is the removal of the carboxylic group in a decarboxylation reaction (Figure 6.16, III). The nature of the enzymes which perform this step are poorly characterised and no sequences have been reported. The only ORF which

could potentially be assigned as encoding this function is ORF 4 which encodes a hydrophilic protein (Section 6.4.4; Figure 6.16, IV). The de-carboxylation reaction results in a C5 keto intermediate which could be stereo-specifically reduced at the C4 position by an amino-transferase encoded by ORF 1 (Section 6.4.1; Figure 6.16, IV) resulting in UDP-4AA. The following two steps result in lipid A modification by 4AA. The PmrF protein has both amino acid sequence and hydropathy profile similarity with glucosyl transferases (Section 6.4.2). In reactions analogous to those recently described for the glucosylation of *Shigella flexneri* O-antigen (Mavris *et al.*, 1997; Guan *et al.*, 1999), 4AA is transferred to bactoprenol to form 4AA-P-bactoprenol by the *pmrF* encoded glycosyl transferase (Figure 6.16, V). From this intermediate, 4AA is transferred to a free phosphate on lipid A by a specific transferase encoded by ORF 5 (Figure 6.16, VII). The 550 aa ORF 5 protein is highly hydrophobic with multiple potential membrane spanning regions and little similarity to other proteins (Section 6.4.5). In this regard, it is similar to the specific glucosyl transferases (GtrX, GtrI, GtrII, GtrV), which modify the *S. flexneri* O-antigen (Bastin *et al.*, 1997; Guan *et al.*, 1999; Huan *et al.*, 1997A; Huan *et al.*, 1997B; Mavris *et al.*, 1997; Verma *et al.*, 1993). The remaining unassigned ORFs 6 and 7 (ORF 6 in the *E. coli* K-12 operon) encode small, highly hydrophobic proteins with 4 putative transmembrane regions and structural similarity to S. flexneri bacteriophage SfX GtrA and bacteriophage SfII ORF2 (Section 5.4.8). GtrA and ORF2 have been proposed to play a role in transport in sugar biosynthesis pathways (Guan *et al.*, 1999), indicating a potentially similar role for ORF6 and ORF7 in the 4AA pathway (Figure 5.16, VI). An interesting aside is that the amino terminal half of ORF3 protein has approximately 28% identity with proteins able to formylate a variety of molecules (Section 6.4.3). Thus, it can be speculated that the amino group of 4AA can be formylated by this domain of ORF3 (Figure 6.16, VIII). This type of modification has been reported for the 4-amino sugar component (perosamine) of the O-antigen of *Brucella* sp. (Moreno *et al.*, 1987). The presence of this modification on 4-AA has not been reported.

Figure 6.16: Putative pathway for the biosynthesis of 4AA and modification of lipid A.



The predicted functions of individual *pmrF* operon gene products are described in the text (Section 6.4; Section 6.5) and the genes and ORFs described here correspond to Figure 6.10.

6.6 PmrA-PmrB regulation of the *S. typhi pqaB* gene.

The *S. typhimurium pmrF* operon has been shown to be strongly regulated by PmrA/B (Gunn *et al.*, 1998B). To test whether the *S. typhi pqaB* gene is also regulated by PmrA/B, a *pmrA*-null mutation (*pmrA*::Tn*10*d) from the *S. typhimurium* strain JSG421 was transduced by P22 phage (Section 2.6.3.2) into the *S. typhi pqaB*::Mu*dJ*, PhoP$^+$ (RMA2326) and *pqaB*::Mu*dJ*, PhoP^c (RMA2356) strains to create RMA1307 and RMA1309 respectively. These strains produced white colonies on LA containing X-gal plates compared to intense blue colonies of their *pmrA*$^+$ parent strains. This indicated that the *S. typhi pqaB*::Mu*dJ* fusion was strongly regulated by the PmrA/B operon. β-galactosidase assays were performed on overnight cultures grown in LB (Section 2.10); the results obtained for each *S. typhi* strain are presented in Table 6.10. These results showed that *pqaB* expression was PmrA/B dependent, as the β-galactosidase activity of *S. typhi* RMA1307 and RMA1309 strains were greatly down-regulated (66- and 125-fold respectively) compared to their *pmrA*$^+$ parental strains.

Table 6.10: β-galactosidase assay showing PmrA-PmrB regulation of the *S. typhi pqaB* gene

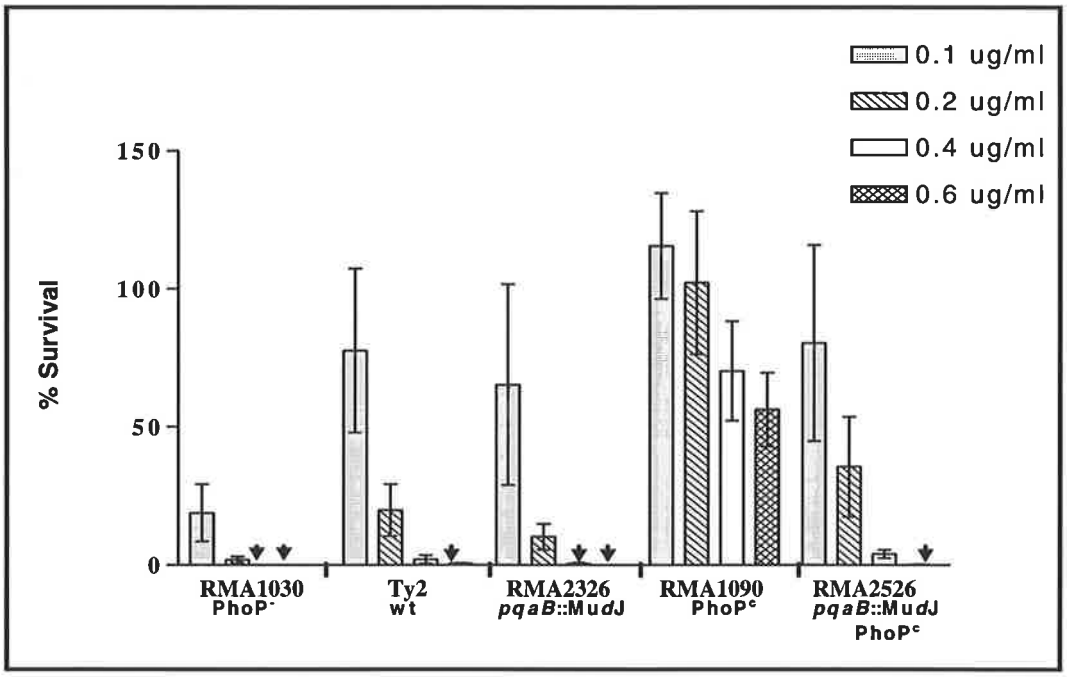
S. typhi Strains[a]		β-galactosidase units[b]
RMA1126	(PhoP$^-$, *pqaB*::Mu*dJ*)	17 ± 1
RMA2326	(PhoP$^+$, *pqaB*::Mu*dJ*)	395 ± 1
RMA2526	(PhoP^c, *pqaB*::Mu*dJ*)	996 ± 35
RMA1307	(PhoP$^+$, *pqaB*::Mu*dJ*, *pmrA*::Tn*10)*	6 ± 1
RMA1309	(PhoP^c, *pqaB*::Mu*dJ*, *pmrA*::Tn*10)*	8 ± 4

[a]All bacterial strains are described in Table 2.3
[b]β-galactosidase units are presented as the mean of 2 duplicate assays± SD
β-galactosidase assays were performed on overnight cultures gown in LB as described in Section 2.10.

6.7 Polymyxin B sensitivity of *S. typhi pqaB* mutant.

Since the *S. typhimurium pmrF* mutant is sensitive to PmB compared to the wildtype strain (Gunn *et al.*, 1998B), the sensitivity of the *S. typhi pqaB* mutant to this antimicrobial agent was tested. In preliminary experiments it was found that *S. typhi* Ty2 bacteria were approximately 10-fold more sensitive to PmB than the *S. typhimurium* C5 strains (Section 3.6.5). The survival of *S. typhi* Ty2, RMA1030 (PhoP$^-$), RMA1090 (PhoP^c), RMA2326 (*pqaB*::Mu*dJ*), RMA2526 (*pqaB*::Mu*dJ*, PhoP^c), RMA1307 (*pqaB, pmrA::*Tn*10)* and RMA1309 (*pqaB, pmrA::*Tn*10,* PhoP^c) strains were assayed with four concentrations of PmB (0.6, 0.4, 0.2 and 0.1 μg ml^{-1}) and the results are shown in Figure 6.17.

Figure 6.17: Polymyxin B resistance of *S. typhi* strains



This figure shows the percentage survival of *S. typhi* RMA1030 (PhoP$^-$), Ty2, CDM326 (*pqaB*::Mu*dJ*), RMA1090 (PhoP^c) and RMA2526 (*pqaB*::Mu*dJ*, PhoP^c) strains after exposure for 1 hour to 4 concentrations (0.1, 0.2, 0.4 and 0.6 μg ml^{-1}) of polymyxin B at 37°C (Section 2.11.1). Each bar represents the arithmetic mean of 3 assays expressed as percentage survival ± SD. Arrows (↓) indicate survival ≤ 1%.

Little difference in survival was observed between the *S. typhi* Ty2 wildtype and RMA1030 (PhoP$^-$) strains, however RMA1090 (PhoP^c) showed increased resistance to PmB (discussed in Chapter 7). This correlates well with previous *S. typhimurium* data (Groisman *et*

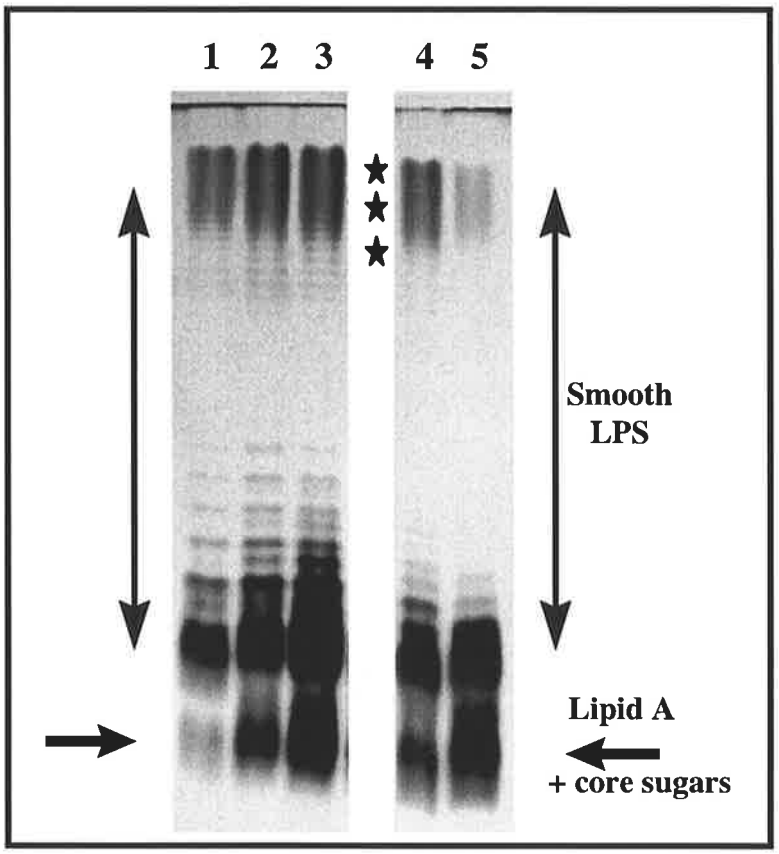
al., 1997; Gunn *et al.*, 1998B). Little or no difference was seen between *S. typhi* Ty2 and the *pqaB*::Mu*d*J insertion mutant (RMA2326) but a large difference was observed between the RMA2526 (*pqaB*::Mu*d*J, PhoP^c) and RMA1090 (PhoP^c) strains. These data indicate that the putative 4AA modifications to the LPS due to this operon affects PmB resistance in both *S. typhi* and *S. typhimurium.*

6.8 Comparison between *S. typhi S. typhi pqaB*::Mu*d*J LPS.

Since the *S. typhimurium pmrF* operon affects lipid A modification by 4AA and both *pqaB* in *S. typhi* and *pmrF* in *S. typhimurium* affect PmB resistance comparisons by gel analysis of the LPS produced by *S. typhi* wildtype, PhoP$^-$ and PhoP^c strains, and the *S. typhi* strains with the *pqaB* mutation in a PhoP$^+$ and PhoP^c background were performed (Figure 6.18).

A subtle difference between the LPS of the *S. typhi pqaB* mutants (RMA2326 and RMA2356) and their isogenic PhoP$^+$ (Ty2) and PhoP^c (RMA1090) counterparts could be seen, indicated by the lack of discrete LPS bands in the high molecular range (marked by *** in Figure 6.18). The lack of discrete bands was more apparent with RMA2526, which also seemed to produce LPS with a reduced number of O-antigen chains, this result was reproduced on three separate occasions (data not shown).

Figure 6.18: Comparison of *S. typhi* and *pqaB* mutant lipopolysaccharides (LPS)



Samples (representing 10^8 cells) were prepared from 18 h cultures and electrophoresed on an SDS-20% polyacrylamide gel, then subjected to silver staining to detect LPS (Section 2.9). The strains in each lane are; Lane 1: *S. typhi* RMA1030 ($PhoP^-$), Lane 2: *S. typhi* Ty2 ($PhoP^+$), Lane 3: *S. typhi* RMA1090 ($PhoP^c$), Lane 4: *S. typhi* RMA2326 (*pqaB*::Mu*d*J, $PhoP^+$), Lane 5: *S. typhi* RMA2526 (*pqaB*::Mu*d*J, $PhoP^c$)
***, indicate that the LPS chains lack discrete banding. Smooth LPS: O-antigen chains; LipidA + core sugar molecules are indicated by downward arrows.

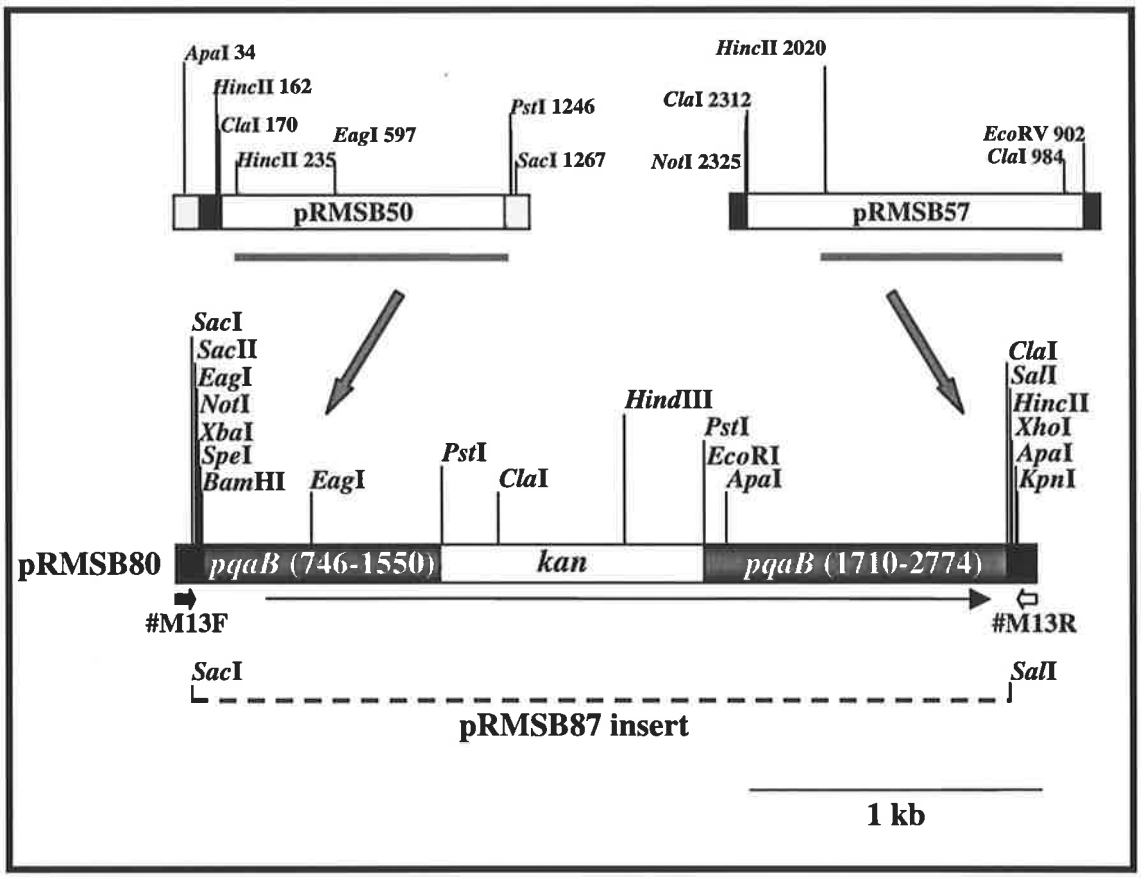
6.9 Construction of the defined *pqaB*::Kan mutant

A defined mutation in the *S. typhi pqaB* gene was needed to confirm the phenotypes seen for the *pqaB*::Mu*d*J mutant and to test whether they were due to the *pqaB* gene alone or due to a polar affect on the downstream genes (ORF6 and ORF7). Consequently, a defined mutant with the non-polar Kan cartridge (*aphT*) from pSB315 was constructed. This *aphT* cartridge has been modified from the *aph* (aminoglycoside 3'-phosphotransferase) gene encoding Kan resistance and is devoid of its transcription terminator (Galán *et al.*, 1992), therefore allowing disruption of the gene it is inserted in but allowing downstream genes (in this case ORF6 and

ORF7) to be expressed as normal. For clarity, the *aphT* cartridge will be referred to as *kan* from now on in the text. To create the insertion mutation (Figure 6.19), the *Hinc*II/*Pst*I fragment from pRMSB50 (Figure 6.5) was cloned into *Sma*I/*Pst*I digested pBC, transformed into DH5α and transformants were selected with blue/white colour selection and Cml^R creating pRMSB76 (in RMA1356). The *Hinc*II/*Cla*I fragment from pRMSB57 (Figure 6.6) was then ligated into *Eco*RV/*Cla*I digested pRMSB76, transformed into DH5α and transformants were screened for the double insert and Cml^R. The resulting plasmid was named pRMSB79 (in RMA1359). The pSB315 plasmid was then digested with *Pst*I (to obtain the *kan* cartridge) and the *kan* cartridge was then ligated to *Pst*I digested pRMSB79, the ligation mixture was transformed into DH5α and Cml/Kan^R transformants were selected. Since a *Cla*I site is contained within the *kan* cartridge near the start, digestion with *Cla*I was performed to ensure the correct orientation of the *kan* cartridge was obtained, and the resultant plasmid was named pRMSB80 (in RMA1360) (Figure 6.19).

The pRMSB80 plasmid was then digested with *Sac*I/*Sal*I, the digest was electrophoresed on a 0.8% TAE gel and the *Sac*I/*Sal*I *pqaB*::Kan insert was gel extracted (Section 2.5.2.5) and ligated to *Sac*I/*Sal*I digested pCACTUS, a temperature sensitive suicide plasmid. The ligation mixture was transformed into DH5α, plated out on LA containing Cml and Kan at 30°C and the resultant Cml/Kan^R plasmid containing the *pqaB*::Kan insert was named pRMSB87 (in RMA1331).

Figure 6.19: Restriction enzyme map of pRMSB80 insert



The schematic restriction enzyme map of the *S. typhi pqaB*::Kan insert in pRMSB80 shows the two cloned sections of *pqaB* (746-1550 and 1710-2774 corresponding to Figures 6.5 and 6.6) (dark grey reactangle) with the Kan^R cartridge cloned into the *Pst*I site between them (outlined rectangle). The *pqaB*::Kan insert is flanked by the pBC MCS (outlined in horizontal lines)..The direction of transcription of *pqaB* and Kan is shown by a line arrow (→). Positions of the restriction enzymes and the oligonucleotides #M13F (black arrow), #M13R (open arrow) (Table 2.5) and their positions are marked. The *SacI*/*SalI* fragment from pRMSB80 which was cloned into the suicide vector pCACTUS is indicated by a dashed line.

The plasmid pRMSB87 was then electroporated into *S. typhi* Ty2 and the transformants plated out on LA containing Cml and Kan at 30°C. The resultant *S. typhi* strain (RMA1383) containing pRMSB87 was grown at 30°C overnight in LB, subcultured 1/20 at 37°C in LB (to prevent plasmid replication) and plated out on LA containing Kan and 0.6% sucrose (as pCACTUS contains a *sacB* gene which is lethal when expressed whilst bacteria are grown on media containing sucrose) to ensure only the *pqaB*::Kan insert had crossed over into the chromosome. Two *S. typhi* $PhoP^+$ *pqaB*::Kan strains were obtained which were named RMA1399 and RMA1400. In addition two *S. typhi* $PhoP^c$ *pqaB*::Kan mutants were obtained

in the same manner from RMA1090 and these was named RMA1401 and RMA1402. These strains were all determined to be Kan^R and Cml^S, indicating that only the *pqaB*::Kan insert had crossed over into the chromosome and the remainder of the pRMSB80 plasmid lost. PCR was performed with #2607 and #2648 (Table 2.5) and demonstrated that the *pqaB* gene had increased in size by approximately 1 kb (correlating with the size of the *kan* cartridge) as seen by gel motility (data not shown).

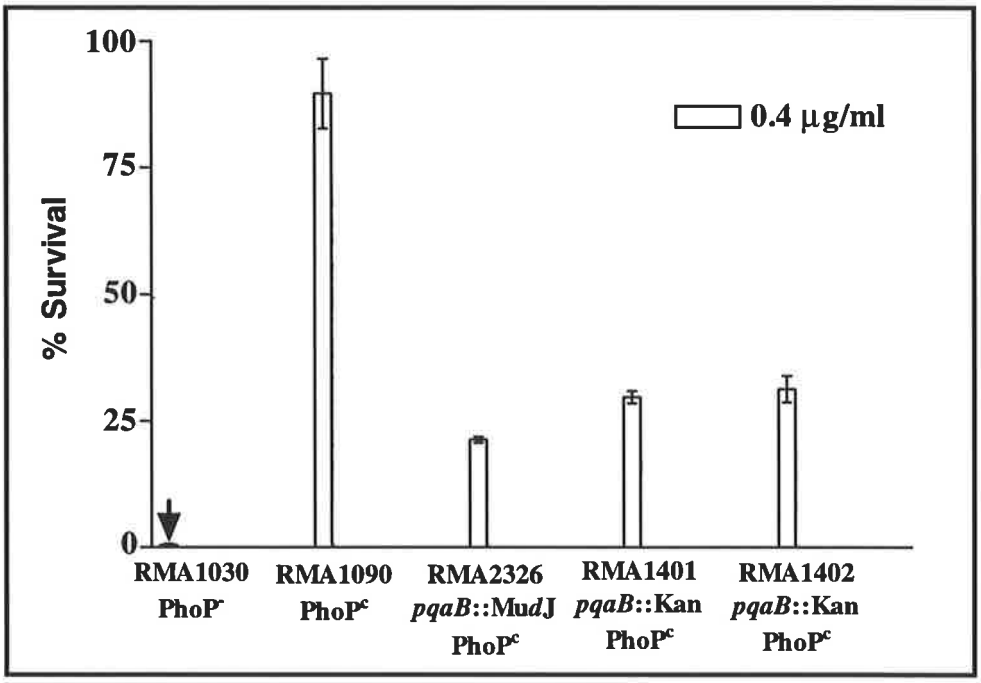
6.10 Phenotypes of the defined *S. typhi pqaB*::Kan mutants

6.10.1 PmB resistance of the defined *pqaB*::Kan mutant

As the RMA2326 *pqaB*::Mu*dJ* mutant had previously been shown to have a greatly diminished resistance to PmB when compared to the *S. typhi* RMA1090 $PhoP^c$ parental strain and limited difference was noted with the $PhoP^+$ backgrounds, it was decided to test the defined *pqaB*::Kan mutants in the $PhoP^c$ backgrounds. As 0.4 μg/ml PmB gave almost ~100% survival for the RMA1090 constitutive strain, and the RMA2326 *pqaB*::Mu*dJ* mutant had greatly decreased survival (Section 6.7), this was the concentration of PmB used to test the defined *pqaB*::Kan mutant.

The survival of *S. typhi* RMA1030 ($PhoP^-$), RMA1090 ($PhoP^c$), RMA2526 (*pqaB*::Mu*dJ*, $PhoP^c$), RMA1401 (*pqaB*::Kan, $PhoP^c$) and RMA1402 (*pqaB*::Kan, $PhoP^c$) were tested against 0.4 μg/ml of PmB (Section 2.11.2) and the results are shown in Figure 6.20. The results show that the defined *pqaB*::Kan mutant in the $PhoP^c$ background is slightly more resistant to 0.4 mg/ml PmB than the *S. typhi pqaB*::Mu*dJ*, $PhoP^c$ mutant but is clearly much more sensitive than the parental *S. typhi* RMA1090 $PhoP^c$ mutant strain.

Figure 6.20: PmB resistance of *S. typhi* strains and the defined *pqaB*::kan mutant



This figure shows the percentage survival of survival of *S. typhi* RMA1030 (PhoP$^-$), RMA1090 (PhoP^c), RMA2526 (*pqaB*::Mu*dJ*, PhoP^c), RMA1401 (*pqaB*::Kan, PhoP^c) and RMA1402 (*pqaB*::Kan, PhoP^c) strains after exposure for 1 hour to 0.4 µg/ml of polymyxin B at 37°C (Section 2.11.1). Each bar represents the arithmetic mean of 2 assays expressed as percentage survival ± SD. Arrows (↓) indicate survival ≤ 1%.

6.10.2 Melittin resistance of the defined mutant

The *S. typhi pqaB*::Mu*dJ* mutant (RMA2326) has previously been shown to be more sensitive to the antimicrobial peptide melittin than the wildtype *S. typhi* Ty2 strain. To confirm that the defined *S. typhi pqaB*::Kan insertion mutants were also sensitive. *S. typhi* Ty2 (PhoP$^+$), RMA1030 (PhoP$^-$), RMA2326 (*pqaB*::Mu*dJ*), RMA1399 (*pqaB*::Kan) and RMA1400 (*pqaB*::Kan) were tested against 7.5 µg/ml melittin (Section 2.11.2) and the results are shown in Table 6.11. By the students T-test, the *S. typhi pqaB* mutants all showed a P value of less than 0.05 indicating that they had significantly reduced survival compared to the wildtype strain and therefore the *S. typhi pqaB* gene is likely to be involved with melittin resistance.

Table 6.11: *S. typhi* and melittin survival

Strain	% Survival	P value
Ty 2 ($PhoP^+$)	47.1 ± 5.9	
RMA1030 ($PhoP^-$)	0.0 ± 0.0	
RMA2326 (*pqaB*::Mu*dJ*, $PhoP^+$)	14.5 ± 0.6	0.03
RMA1399 (*pqaB*::Kan, $PhoP^+$)	16.7 ± 0.3	0.04
RMA1400 (*pqaB*::Kan, $PhoP^+$)	14.0 ± 2.0	0.03

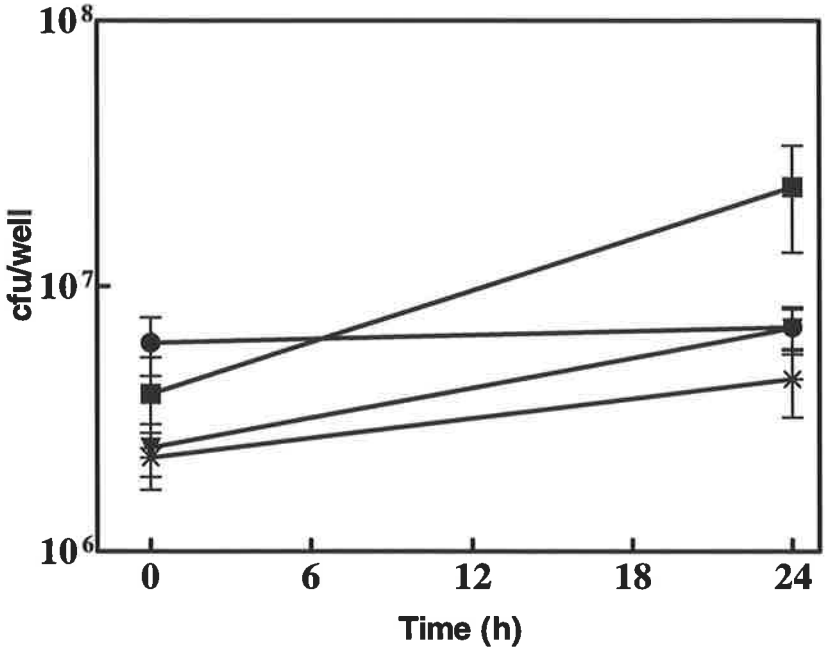
This table shows the percentage survival of survival of *S. typhi* strains after exposure for 1 hour to 7.5 µg/ml of melittin at 37°C (Section 2.11.2). The mean value and standard deviation were calculated from a mean of 2 assays.

6.10.3 Intracellular growth of defined *S. typhi pqaB* mutant in PMA-U937 cells

The *S. typhi pqaB*::Mu*dJ* insertion mutant showed a growth defect in PMA-U937 cells (Section 4.5.4). As the mutation in *S. typhi pqaB*::Mu*dJ* insertion mutant is due to a large transposon mutation (~ 10 kb), to establish whether the growth defect in PMA-U937 cells was due to the *pqaB* mutation or whether the downstream *orf6* and *orf7* genes were also involved, the defined non-polar *pqaB* mutant RMA1399 was tested in the PMA-U937 assay at 0 and 24 h (Figure 6.21, Table 6.12; Section 2.12.4.2).

The results showed that the defined mutant (*pqaB*::Kan) was significantly defective in intracellular survival (GI of 3) compared to the *S. typhi* wildtype strain (GI of 6) ($P = 0.03$ at 24 h, therefore $P<0.05$) and had a similar growth restriction compared to the *S. typhi pqaB*::Mu*dJ* insertion mutant (GI = 2), indicating that the *S. typhi pqaB* mutation is involved with growth in PMA-U937 cells. Neither of the *S. typhi pqaB* mutants were found to be as defective in net growth as the *S. typhi* $PhoP^-$ (RMA1030) mutant (GI = 1), as expected from Section 4.6.2.

Figure 6.21: Net growth of *S. typhi* Ty2, PhoP⁻ and *pqaB* mutant strains in PMA-U937 cells



This figure shows the net growth characteristics of *S. typhi* Ty2 (■), RMA1030 (PhoP⁻) (●), RMA2326 (*pqaB*::Mu*dJ*) (▼)and RMA1399 (*pqaB*::Kan) (✶)strains after invasion of PMA-differentiated U937 cells. *S. typhi* strains were added at a ratio of 10:1 (bacteria:cells).
Each point represents the arithmetic mean of 3 assays expressed as viability of cfu/well ± SD at a given time in hours.
Growth Index (GI) was calculated by the number of bacteria at timepoint 24 h divided by the number of bacteria at time 0 h.

Table 6.12: Invasion and net growth of of *S. typhi* Ty2, PhoP⁻ (RMA1030), *pqa*A::Mu*dJ* (RMA2310), and *pqaB*::Mu*dJ* (RMA2326) strains in PMA-U937 cells.

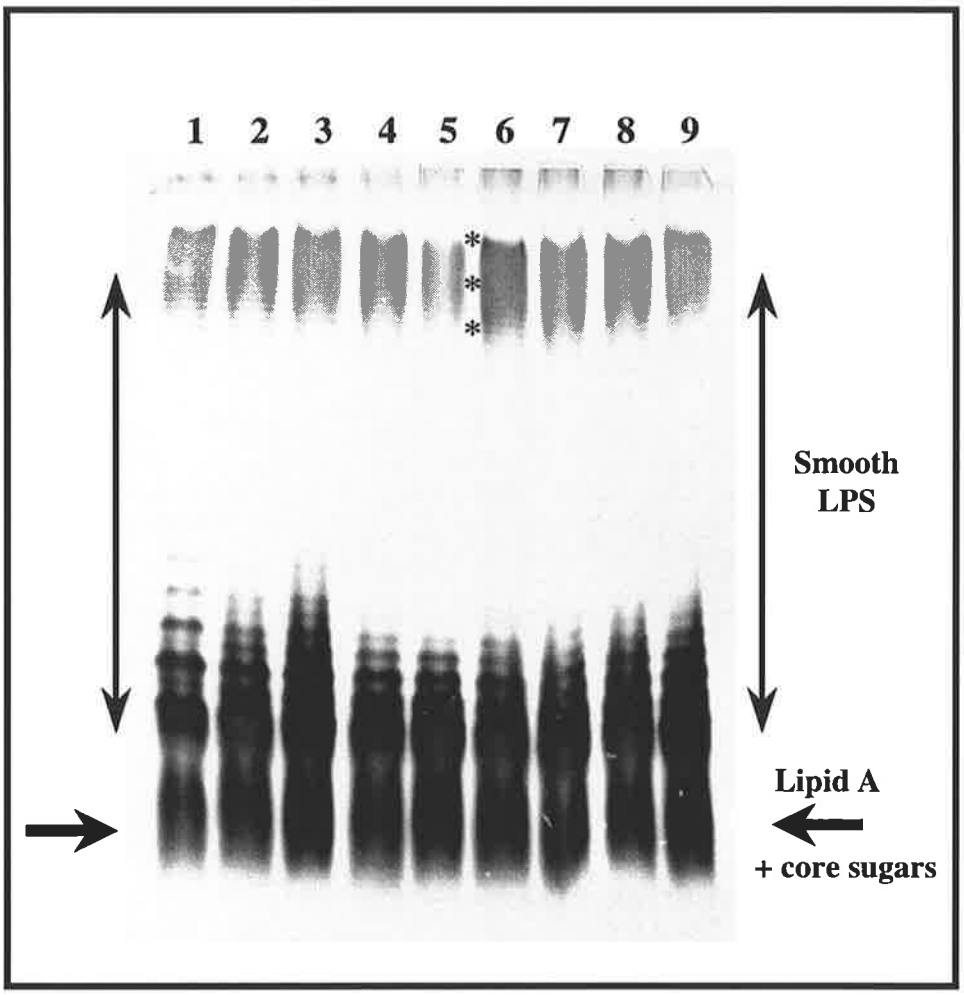
Bacterial strains[a]	Invasion[b]	GI[c]
Ty2 (PhoP⁺)	12.6 ± 2.5	6
RMA1030 (PhoP⁻)	13.0 ± 3.6	1
RMA2326 (*pqaB*::Mu*dJ*)	23.4 ± 9.5	2
RMA1399 (*pqaB*::Kan)	21.2 ± 7.6	3

[a]All bacterial strains can be found in Table 2.3
[b]Invasion percentages were calculated by dividing the number of bacteria at time 0 after gentamicin treatment the number of bacteria initially added x 100
[c]Growth Index (GI) was calculated by the number of bacteria at timepoint 24 h divided by the number of bacteria at time 0 h.

6.10.4 The defined *S. typhi pqaB*::Kan mutants and LPS

A subtle difference was previously noted between the LPS of the *S. typhi pqaB*::Mu*d*J mutants and their isogenic PhoP$^+$ and PhoP^c counterparts (Section 6.8) as a lack of discrete LPS bands in the high molecular range. Therefore LPS from the *S. typhi* defined *pqaB*::Kan mutants in PhoP$^+$ and PhoP^c backgrounds were compared to both the parental strains and *pqaB*::Mu*d*J mutants (Figure 6.22). It can be noted that the discrete LPS bands were apparent in the LPS of the *S. typhi pqaB*::Kan mutants compared to the *S. typhi pqaB*::Mu*d*J mutants, indicating that the *S. typhi pqaB* operon ORF6 and ORF7 may be involved in the discrete banding.

Figure 6.22: Comparison between *S. typhi* and *S. typhi pqaB* mutants and LPS



Samples (representing 10^8 cells) were prepared from 18 h cultures and electrophoresed on an SDS-20% polyacrylamide gel, then subjected to silver staining to detect LPS (Section 2.9). The strains in each lane are; Lane 1: *S. typhi* RMA1030 (PhoP$^-$), Lane 2: *S. typhi* Ty2 (PhoP$^+$), Lane 3: *S. typhi* RMA1090 (PhoP^c), Lane 4: *S. typhi* RMA2326 (*pqaB*::Mu*d*J, PhoP$^+$), Lane 5: *S. typhi* RMA2526 (*pqaB*::Mu*d*J, PhoP^c), Lane 6: *S. typhi* RMA1399 (*pqaB*::Kan, PhoP$^+$), Lane 7: *S. typhi* RMA1400 (*pqaB*::Kan, PhoP$^+$), Lane 8: *S. typhi* RMA1400 (*pqaB*::Kan, PhoP^c), Lane 9: *S. typhi* RMA1400 (*pqaB*::Kan, PhoP^c) ***, indicate that the LPS chains lack discrete banding.
Smooth LPS: O-antigen chains; LipidA + core sugar molecules are indicated by arrows

6.11 Complementation of *S. typhi pqaB* mutants

In addition to testing defined *S. typhi pqaB* mutants for polymyxin B and melittin sensitivity, growth in PMA-U937 cells and LPS analysis, oligonucleotides were designed (#3001, #3002, #3003 and #3004; Table 2.5) to PCR amplify and clone the *S. typhi pqaB*, *orf6* and *orf7* genes from *S. typhi* Ty2 for complementation of the *S. typhi pqaB*::Mu*dJ* insertion mutant. Although this part of the study was undertaken, it was found that the *S. typhi pqaB*, *orf6* and *orf7* genes could not be cloned and maintained in DH5α cells (possibly due to the high hydrophobicity of encoded proteins). However the results for the defined mutation in *pqaB*, clearly showed that the *S. typhi pqaB* gene was involved in polymyxin B and melittin sensitivity and growth in PMA-U937 cells; and I therefore judged that complementing the *pqaB*::Mu*dJ* mutation to confirm the phenotypes mentioned above was not needed.

6.12 Summary

The *S. typhi pqaB* gene was identified, cloned and sequenced along with adjacent DNA and was found to have high homology (~98 %) to ORF5 of the *S. typhimurium pmrF* operon (Section 6.2; Section 6.3). The *S. typhimurium pmrF* operon has been identified as being involved with 4AA lipid A modification and PmB resistance and a putative pathway for the biosynthesis of 4AA was outlined; this suggested that *pqaB/orf5* encodes a bactoprenol-4AA-lipid A transferase (Section 6.5).

The *S. typhi pqaB* gene was found to be strongly regulated by the PmrA/B operon, involved in PmB and melittin resistance and growth in PMA-U937 cells (Section 6.6; Section 6.10.2; Section 6.10.3). LPS analysis showed a lack of discrete high molecular range bands in *S. typhi pqaB*::Mu*dJ* mutants, and the LPS of these strains appeared to have fewer O-antigen chains (Section 6.8). This phenotype was not observed for the defined *pqaB*::Kan muant (Section 6.10.4), indicating that the small, hydrophobic ORF6 and ORF7 proteins may be

involved with LPS O-antigen polymerisation or other modifications and this will be discussed further in Chapter 7.

Chapter 7

Discussion

7.1 Introduction

S. typhi is the causative agent of typhoid fever and is host specific for humans. Although the actual mechanisms involved in host specificity have not yet been thoroughly characterised, host specificity in chickens and mice has been shown to primarily involve the reticuloendothelial system where *Salmonella* host-specific serotypes were shown to be unable to survive in the visceral organs (particularly the spleen and liver) (Barrow *et al.*, 1994). The spleen and liver contain macrophages which have been proposed to be a "safe-haven" for *Salmonella*, providing a niche in which they are able to survive and multiply whilst being protected from the immune system (Dunlap *et al.*, 1991; Edwards *et al.*, 2000; Nnalue *et al.*, 1992). The *Salmonella* PhoP/Q global regulator may be potentially involved in host specificity as it has been shown to be involved with macrophage survival (Fields *et al.*, 1989; Miller *et al.*, 1989). Mutations in the *S. typhi phoP* gene have been shown to abrogate *S. typhi* virulence in humans, indicating that important virulence loci are controlled by PhoP/Q in *Salmonella typhi* (Hohmann *et al.*, 1996A).

Little was known about the *S. typhi* PhoP/Q operon and PhoP/Q regulated genes before this study commenced, and my initial aims included investigation of the PhoP/Q regulon in both *S. typhi* and *S. typhimurium*, noting differences in PhoP/Q regulated genes between the strains, and characterising a number of previously identified PhoP/Q regulated genes of *S. typhi*.

7.2 Comparison of *S. typhi* and *S. typhimurium phoP/Q* regulated genes

Isogenic strains of *S. typhimurium* C5 PhoP$^-$, PhoP^c and *S. typhi* Ty2 PhoP$^-$ mutants had been constructed previously (Baker *et al.*, 1997) and an *S. typhi* PhoP^c mutant was constructed during this work (Section 3.3). These strains were analysed for differences in *S. typhi* and *S. typhimurium phoP/Q* regulated genes as judged by: whole cell protein analysis, analysis of known *phoP/Q* regulated gene activity (*phoN*), magnesium dependent growth requirements, antimicrobial peptide sensitivity, and analysis of LPS (Chapter 3).

While differentiating PhoP$^-$, PhoP$^+$ and PhoP^c phenotypes according to the level of *phoP/Q* regulated non-specific acid phosphatase (PhoN) activity, it became apparent that the PhoN activity levels between the two strains was different (Section 3.6.3); *S. typhi* had lower levels of non-specific acid phosphatase than *S. typhimurium*. The difference in *phoN* regulation between *S. typhi* and *S. typhimurium* may either reflect lower intrinsic activity of the *S. typhi* PhoN protein, or PhoP/Q may not up-regulate *phoN* to the same degree in *S. typhi* as *S. typhimurium*. PhoN is not apparently involved in *Salmonella* pathogenesis (Fields *et al.*, 1989) and may have been acquired by *Salmonella* species by horizontal transfer prior to the diversification of the present day *Salmonellae* (Groisman *et al.*, 1992A). I speculate that the genes may have accumulated DNA sequence changes due to low selective pressure and analysis of the DNA sequences of the *S. typhimurium* and *S. typhi phoN* genes would allow this proposal to be tested.

The whole cell proteins produced by *S. typhi*, *S. typhimurium*, and isogenic PhoP$^-$ and PhoP^c derivatives were compared during this study (Section 3.6.1). Whereas *S. typhimurium* Pag and Prg proteins were readily detected, *S. typhi* Pag and Prg proteins were fewer in number and the Pag and Prg proteins detected were different in apparent molecular mass when *S. typhi* and *S. typhimurium* were compared. The lower numbers of PhoP/Q regulated proteins

for *S. typhi* may indicate that there either are fewer *S. typhi* PhoPQ regulated proteins than for *S. typhimurium,* or that their expression is lower and hence regulated differently. The latter proposal is consistent with the lower level of *phoN* expression seen for *S. typhi* compared with *S. typhimurium.*

Previously, *S. typhimurium* C5 and *S. typhi* Ty2 PhoP$^-$ strains (RMA1010 and RMA1030, respectively) have been complemented with plasmids containing *phoP*$^+$ and *phoP*^{c} from *S. typhimurium* (Baker, 1993). These complemented strains gave blue colonies on LA containing X-pho (RMA1030 itself gave white colonies), which demonstrated that the PhoP$^-$ mutation in RMA1030 and RMA1010 could be complemented by the cloned *phoP*$^+$ and *phoP*^{c} genes of *S. typhimurium.* Furthermore, the greater intensity of blue colonies for both *phoP*$^+$ and *phoP*^{c} complemented *phoP*$^-$ strains compared to strains with chromosomal *phoP*$^+$and *phoP*^{c} alleles, suggested that an overexpression of PhoP/Q was occurring in the plasmid complemented strains (Section 3.4; Section 3.6.3). The results that were obtained for the complementation of the *S. typhi* PhoP$^-$ *pqa/pqr*::Mu*dJ* insertion mutants for the analysis of their LacZ activity on solidified media (Section 3.6.3) also suggests that multiple copies of *phoP/Q* can "overregulate" *phoP* regulated genes, i.e: increased up or down regulation compared to the single copy chromosomal *phoPQ* locus. The *phoP/Q* operon has been shown to be transcriptionally autoregulated (Soncini *et al.*, 1995), and it would appear that multiple copies of *phoP/Q*$^+$ or *phoP/Q*^{c} alleles lead to increased levels of expression/repression under the environmental conditions used.

This scenario appears to have acted to the original study's advantage when using the screening method devised for isolation of PhoP/Q regulated genes in *S. typhi* (Section 1.14; Baker, 1993), allowing isolation of mutants such as RMA1112 (*pqaD*::Mu*dJ*) and RMA1116 (*pqrB*::Mu*dJ*) whose fusions appear to be regulated at a low level by the chromosomally encoded *phoP/Q* locus (Section 3.7.4).

It has been reported by Garcia Vescovi *et al.*, (1996) that PhoP/Q was responsive to intracellular Mg^{2+} and Ca^{2+} *in vitro*. *S. typhi* PhoP$^+$, PhoP$^-$ and PhoP^c strains were tested on N-buffer agarose plates containing various amounts of Mg^{2+} (Section 3.5). The results showed a greater requirement for Mg^{2+} by *S. typhi* PhoP$^-$ compared to wildtype which is consistent with the data presented for *S. typhimurium* (Garcia Vescovi *et al.,* 1996), indicating that the *S. typhi* PhoP/Q is likely to respond to similar environmental signals as *S. typhimurium* (e.g. extracellular Mg^{2+} levels).

The increased sensitivity of *S. typhimurium* PhoP$^-$ mutants to antimicrobial peptides was originally demonstrated by Fields *et al.*, (1989), and *S. typhi* PhoP$^-$ strains were tested for this phenotype. The results obtained indicated that the *S. typhi* RMA1030 (PhoP$^-$) was sensitive to the antimicrobial agents protamine and polymyxin B (Section 3.6.4; Section 3.6.5) compared to *S. typhi* Ty2 (PhoP$^+$). Moreover, the increased sensitivity to antimicrobial peptides of *S. typhi* compared to *S. typhimurium* shown in this study and previous work in our laboratory has not previously been reported (Daniels, 1994; Section 3.6.4; Section 3.6.5).

A possible reason for the differences in antimicrobial agent sensitivity between *S. typhi* and *S. typhimurium* was suggested when the LPS of *S. typhi* Ty2, PhoP$^-$ and PhoP^c, and *S. typhimurium* C5 and PhoP$^-$, PhoP^c derivatives were examined by SDS-PAGE gel (Section 3.6.2). Previous chemical analysis had indicated that *S. typhimurium* PhoP^c LPS had shorter O antigen chains (Guo *et al.*, 1997), and a difference was expected to be seen between the LPS of the *S. typhimurium* wildtype and its PhoP^c derivative. As can be seen in Figure 3.3, there was a remarkable difference between *S. typhimurium* C5 and PhoP^c LPS, with much shorter O antigen chains being evident for the PhoP^c strain. These results correlate with the report from Guo *et al.*, (1997) but conflict with previous studies (Miller & Mekalanos, 1990), which reported that that the PhoP^c mutation did not affect LPS. The differences seen for *S. typhimurium* wildtype and PhoP^c strains is in direct contrast to the *S. typhi* Ty2, PhoP$^-$ and PhoP^c strains for which no difference in O-antigen chain length could be detected (Figure 3.3).

The LPS of both *S. typhi* Ty2 and *S. typhimurium* C5 strains showed no reproducible differences compared to their PhoP$^-$ derivatives; this was previously reported for *S. typhimurium* wildtype and PhoP$^-$ strains (Galán & Curtiss, 1989B; Miller & Mekalanos, 1990). Interestingly, the modal chain length of the LPS for *S. typhi* is much longer than that of the LPS of *S. typhimurium* suggesting that they may have different *wzz* genes.

7.3 Identification of *S.typhi pqa/pqr*::Mu*d*J insertion mutants

During this study, the identities of the *S. typhi pqaA*::Mu*d*J, *pqaB*::Mu*d*J, *pqaD*::Mu*d*J, *pqrA*::Mu*d*J and *pqrB*::Mu*d*J were found. The characterisation of the *S. typhi pqaA* and *pqaB* genes will be discussed below in Sections 7.4 and 7.6.

The sequence identification of the *S. typhi pqaD*, *pqrA* and *pqrB* genes gave some interesting insights into the nature of the genes. The *S. typhi pqaD* gene has been identified as having very high sequence homology to the *E. coli deoA* gene (Section 3.8.1.2) which encodes thymidine phosphorylase and is involved in the pyrimidine salvage pathway in *E. coli* and *S. typhimurium*. The pathway helps to assimilate exogenous free bases and nucleosides; the nucleosides are usually metabolised to free bases before being used for nucleotide synthesis to enable the exogenous nucleosides and their pentose moities to be available as a source of carbon and energy. Finally, they also help to recover free bases and nucleosides produced from intracellular nucleotide turnover as some ribonucleotides are degraded during normal growth and the recovery of these free bases and nucleosides requires salvage enzymes (Cohen & Wolfenden, 1971). The regulation of the *deo* operon is complex and involves CytR, DeoR and the cAMP-CRP complex, and now it also appears to be upregulated by PhoP/Q (Section 3.7.3; Section 3.7.4). It is possible that this pathway may be upregulated in stressful situations (eg. poor growth or intra-cellular conditions) to help bacterial survival.

The *S. typhi pqrA* gene was shown to have strong homology to the *E. coli gcvA* gene which encodes an autoregulated LysR type regulator (Wilson & Stauffer, 1994). GcvA regulates the glycine-cleavage enzyme system (*gcv* operon) which catalyses the cleavage of glycine, generating CO_2, NH_3 and a one-carbon unit which can be used for cellular methylation reactions (Wilson & Stauffer, 1994; Wilson *et al.*, 1993). The *E. coli gcv* operon is also regulated by leucine-responsive regulatory (Lrp) proteins: GcvR and PurR. Together with GcvA they activate the glycine cleavage system in the presence of glycine and repress in the presence of purines (Jourdan & Stauffer, 1998; Wonderling & Stauffer, 1999). The *S. typhi* PhoP/Q system represses the *pqrA/gcvA* gene (Section 3.7.3; Section 3.7.4). Recently it has been shown that the DNA adenine methylase deficient (*dam*) mutants are defective in invasion of, and cytotoxicity for, M-cells (Garcia-del Portillo *et al.*, 1999), and *dam* methylation of genes has previously been shown to be involved in regulation of virulence genes (Heithoff *et al.*, 1999B). It may be possible that control of the *gcv* operon is involved in indirect regulation of these genes by altering the amount of one-carbon units available for methylation reactions.

The *S. typhi pqrB* gene has been shown to have homology to the *E. coli rsd* gene which has been shown to be upregulated at the beginning of stationary phase, and the Rsd protein has been shown to specifically bind to the σ^{70} subunit of RNA polymerase (Jishage & Ishihama, 1998; Jishage & Ishihama, 1999). Most of the exponentially expressed genes in *E. coli* have promoters which require the σ^{70} subunit to be transcribed. Many stationary phase genes require the σ^{38} subunit for transcription, production of which increases during the stationary phase of growth (Nickerson & Curtiss, 1997). The level of σ^{70} tends to remain constant at all growth phases, even if the transcription of genes under the control of σ^{70} is decreased (Jishage & Ishihama, 1999). The Rsd protein has been proposed to play a role in controlling the σ^{70} function in stationary phase growth by binding to the σ^{70} subunit and inhibiting binding to the

core RNA polymerase enzyme (Jishage & Ishihama, 1998; Jishage & Ishihama, 1999). The *S. typhi pqrB*::Mu*dJ* insertion was originally isolated as a *pqa* gene on McConkey agar plates (Baker, 1993), although it appears as a *pqr* gene based on analysis of liquid grown cultures and of cells grown on LA + X-gal plates (Section 3.6.4; Section 3.6.5). This indicates that environmental growth conditions may affect the PhoP/Q regulation of either *pqrB/rsd* or another regulator responsive to these conditions may be involved. MacConkey agar contains bile salts and it has previously been reported that bile is able to dramatically regulate the *S. typhimurium phoP* repressed genes *prgC* and *prgH* independently of PhoP/Q (Van Velkinburgh & Gunn, 1999). It is possible that some *Salmonella* PhoP/Q regulated proteins are expressed in stationary phase, although none have been identified so far, and that the Rsd protein plays a role in their regulation, or that the Rsd protein is involved in the downregulation of *Salmonella* PhoP/Q regulated proteins expressed in exponential growth phase by inhibiting transcription.

The *S. typhi pqrA* and *pqrB* genes both showed strong homology to *E. coli* regulatory genes. This was interesting as although *Salmonella* PhoP/Q operon has been shown to regulate many genes, the transcriptional regulator protein PhoP does not bind near the promoter sequences of any *phoP/Q* regulated genes so far isolated (Dr John S. Gunn, Department of Microbiology, University of Texas Health Science Centre at San Antonio, personal communication). It may be possible that PhoP acts directly on the promoter region of other regulators which then up/down-regulate the "PhoP/Q regulated genes". This scenario is known to occur as *S. typhimurium* PhoP/Q has been shown to regulate another two-component regulatory system, PmrA/B (Gunn & Miller, 1996).

Analysis of the promoter regions from the *E. coli deoA* and *gcvA*, and *S. typhimurium rsd* genes did not show any homologous DNA regions comparable to known phoP/Q regulated genes (Section 3.10). Two REP (repetitive extragenic palindromic) sequences were found in front of the *E. coli* deoA gene (Section 3.8.1.2). These sequences have been reported to be

involved in the expression of the *deo* operon (Valentin-Hansen *et al.*, 1982) and REP sequences are located in front of many genes throughout the *E. coli* chromosome. As yet none of the other genes located downstream from REP sequences have been shown to be PhoP/Q regulated but it would be interesting to analyse these genes for PhoP/Q regulation as this could potentially be another way of PhoP/Q regulating many genes, including so called "house-keeping" genes.

Unfortunately, due to time constraints, no further analysis and characterisation of the *S. typhi pqaD/deoA*, *pqrA/gcvA* and *pqrB/rsd* genes were undertaken, however if time had been available further study, analysis of the regulatory genes *pqrA* and *pqrB* may have proved useful to understanding of how PhoP/Q regulates genes in *Salmonella*. Clearly though, I have found evidence for a cascade of regulators being involved in the control of PhoP/Q regulated genes which correlates with the identification of the PmrA/B two-component regulatory system as this has been shown to regulate its own subset of PhoP/Q regulated genes.

7.4 Identification of the *S. typhi pqaA* gene, localisation on the *Salmonella* chromosome and analysis of surrounding DNA

The *S. typhi pqaA*::Mu*dJ* insertion mutant was originally characterised as a novel *Salmonella* gene (Baker, 1993), affecting sensitivity antimicrobial peptide melittin (Baker *et al.*, 1997). The *pqaA* gene and 1.5 kb of adjacent DNA was fully sequenced (Section 5.4).

The *pqaA* gene was shown to be *Salmonella* specific, has a lower G+C content (41.9%) than the average *Salmonella* chromosome (52%) and was surrounded by genes not contained in *E. coli* indicating that it may be located on a previously unidentified *Salmonella* pathogenicity island. Analysis of 51 kb of DNA from the *S. typhi* sequencing project (Sanger database (Sanger Centre, 1999)) showed that the *S. typhi pqaA* gene was located on a 30.4 kb

DNA region which was flanked by DNA with significant similarity to the *E. coli* K-12 genome sections: Genbank accession numbers AE000245 (located at 33.4 minutes on the *E. coli* chromsome) and AE000250/AE000251 (located at 35.0 minutes on the *E. coli* chromosome) (Figure 5.15). In the *S. typhi pqaA* DNA region with strong similarity to the *E. coli* AE000245 section, a bacteriophage attachment site with 85% identity at the DNA level to the attachment site for bacteriophage P2 was found (Figure 5.15). Further downstream from the *S. typhi pqaA* locus, *orf*s were found that had similarity to the *E. coli* K12 *hyaA* operon (gbD: AE000199, which normally is located at 22.2-22.3 min on the *E. coli* chromosome), adjacent to the *E. coli serT* gene (tRNA-Ser). However, no homology to the *serT* gene in the 51 kb of *S. typhi* DNA was detected. Downstream from the *pqaA* gene was the *mar* locus, involved in multiple antibiotic resistance (Cohen *et al.*, 1993; Sulavik *et al.*, 1997), and following this the *dcp* gene (which encodes dipeptidyl carboxypeptidase). The *dcp* gene has been isolated and sequenced in *S. tyhimurium*, and is located at 32.5 minutes on the *S. typhimurium* chromosome (Miller & Hamilton, 1992), therefore providing a location for the 30.4 kb region containing the *pqaA* gene on the *Salmonella* chromosome (Figure 5.15). The *pqaA* region of DNA was also found to encode the *S. typhimurium* SlyA regulated "silent haemolysin" *hlyE/clyA* gene in *E. coli* K-12 (Ludwig *et al.*, 1999), however as it is not known whether *slyA* is present in *S. typhi*, it is not known whether this haemolysin would be expressed. As to whether this region is a pathogenicity island, the rules for defining a pathogenicity island have been defined (Hacker *et al.*, 1997) and are as follows: carriage of (often many) virulence genes; the region must have different G+C content in comparison to DNA of the host bacteria; occupation of a large chromosomal region (often > 30 kb); presence of distinct genetic units, often flanked by direct repeats; association with tRNA genes and/or insertion sequence (IS) elements at their boundaries; presence of (often cryptic) 'mobility' genes (IS elements, integrases, transposases, origins of plasmid replication); and instability. Therefore, it is possible that the *S. typhi pqaA* may be located on a new *Salmonella*

pathogenicity island as the *orf2*, *pqaA* and *orf3* had lower G+C% than the normal *Salmonella* percentage (Table 5.5), a bacteriophage attachment site was found, the presence of the *hya* operon and loss of *serT* indicates that the region may have had previous insertions in the past, and at least one known virulence gene (the haemolysin *hlyE/clyA*) was found in this region. As the *S. typhi pqaA* gene encodes a novel protein, it is yet not known whether it is involved in virulence. No insertion sequences or direct repeats were found but the sequence of this region is still under construction and contains errors (Sanger Centre, 1999), so further investigation will have to wait until the sequence is completed. In conclusion, I propose this region to be a potential pathogenicity island in *S. typhi*.

Further analysis of *pqaA* confirmed that the *S. typhi pqaA* gene encoded a 59.3 kDa protein by overexpression analysis, and the protein has an N-terminal transmembrane region which is likely to be located in the cytoplasmic membrane with the C-terminal, highly hydrophilic portion located in the cytoplasm (Sections 5.8.3; Section 5.8.4; Section 5.8.5) The *S. typhi pqaA* gene is highly regulated by the PhoP/Q operon and is likely to also be regulated at the translational level by containing 6.9% rare amino acids, including at least two in the first 25 amino acids, therefore accounting for difficulty in overexpression of the protein (Section 5.8.6). Unfortunately for reasons unknown, upon retesting, the *S. typhi pqaA*::Mu*dJ* mutant had lost the sensitivity to melittin, and although studies with a defined mutant were proposed, the mutant was unable to be constructed in time to retest for melittin resistance.

Upstream of the *S. typhi pqaA* gene are two ORFs, the first of which has homology to tetracycline-6 hydroxylase and an oxygenase enzymes and may be encoded in an operon with ORF2 (Section 5.5.1). ORF2 encodes a protein which has high homology to the MFS superfamily, specifically the 12-TMS proteins which are involved in multi-drug efflux systems (e.g. NorA). ORF2 had many of the motifs which characterise this family and had approximately the same homology (around 25%) to all of the seven 12-TMS proteins discussed in this chapter (Section 6.5.2). It is unlikely to encode a tetracycline resistance

protein as all the TetA homologs had higher homology to each other (~50%) than to the ORF2. The protein was unable to be studied further but it would be interesting to test mutants in this ORF2 against a variety of drugs to see if any sensitivity could be determined. The regulation of this ORF2 is also not known and testing whether it could be PhoP/Q regulated would also worth-while in future studies as NorA has recently been shown to be regulated by a two component regulatory system (Fournier *et al.*, 2000).

The partial ORF3 downstream of the PqaA protein had homology to members of a pthalate permease family which are involved in transport of substances such as hexuronate, glucarate and 4- hydroxybenzoate) (Section 5.5.3). ORF3 appeared to have its own promoter and was located well downstream of a *pqaA* terminator so they are unlikely to be transcriptionally and translationally coupled. However it would still be worthwhile to test if this gene is PhoP/Q regulated as well.

7.5 Tissue Culture studies

In this study, a tissue culture model (PMA-differentiated U937 cells) was used to study the invasion and intracellular net growth of *S. typhi* Ty2, PhoP$^-$, PhoP^c, *pqa/pqr* mutants and *E. coli* K12. The invasion and net growth of *S. typhimurium* C5, PhoP$^-$ and PhoP^c derivatives in these cells was also investigated. The *S. typhi* strains mentioned above were also assayed in an epithelial cell line (HeLa) to test for general defects in intracellular net growth.

The differences in levels of invasion noted between *S. typhi* bacteria in U937 cells (Section 4.3.2) and PMA-U937 cells (4.5.2) indicated that the PMA-U937 cells are more phagocytic than the undifferentiated monocytic U937 cells as expected for macrophage-like cells. Although differentiation of the U937 cells causes the cells to become adhesive and stick down onto the plastic trays, the invasion level was higher for the PMA-U937 cells than was seen with the HeLa cell invasion (Section 4.2) (also an adhesive cell line). These higher

levels suggest that the higher bacterial uptake rates are due to a combination of invasion by the *S. typhi* bacteria (supported by the low level of uptake seen for the non-invasive *E. coli* K12 strain (Section 4.5.2) and phagocytosis by the PMA-U937 cells.

The U937-PMA cell assay was able to differentiate between *S. typhi* Ty2 and its PhoP$^-$ derivative (Section 4.5.2) indicating that it was a suitable assay to test the *S. typhi pqa/pqr* mutants. The *S. typhi* PhoP$^-$ (RMA1030) strain could also be complemented (Section 4.5.3) by *S. typhimurium phoP/Q* genes for the growth defect seen in PMA-U937 cells demonstrating that PhoP/Q regulation of the *S. typhi pqa/pqr* genes is involved with *S. typhi* growth within PMA-U937 cells. In the HeLa cell assay (Section 4.2), the *S. typhi* PhoP$^-$ and PhoP^c mutants did not show a net growth defect compared to wildtype *S. typhi* Ty2, indicating that any net growth defects in the PMA-U937 cell assay were specific to the macrophage-like cells. The assay also appeared to be specific for *S. typhi* strains as there was no intracellular net growth defect for the *S. typhimurium* PhoP$^-$ (RMA1010) strain which was comparable to that of wildtype *S. typhimurium* C5 (Section 4.5.4). Possible reasons for this will be discussed below. The data for the *S. typhi* PhoP^c mutant does not correlate with published observations with a *S. typhimurium* PhoP^c mutant which was found to be defective in intracellular net growth within mouse peritoneal macrophages (Miller & Mekalanos, 1990). The observed difference may be due to the use of a different macrophage-like cell line (human) for our experiments compared to the mouse macrophage cell line used by Miller & Mekalanos (1990).

No growth restriction for the *S. typhimurium* C5, PhoP$^-$ (RMA1010) or the PhoP^c (RMA1024) mutants in the PMA-U937 cells, comparable to that of *S. typhimurium* C5 (Section 4.5.4) was observed. This result does not agree with a recent report by Schwan *et al.*, (2000) which found that *S. typhimurium* had a 2-3 fold growth defect compared to *S. typhi* Ty2 in PMA-differentiated U937 cells. However the disparity in these results may be due to the experimental conditions used. The assay used by Schwan *et al.*, (2000) only differentiated

the U937 cells with PMA for 12-16 h (compared to 72 h in the assay used in this study), and therefore the cells are not likely to be fully differentiated compared to the PMA-U937 cells used in this study; this may be one reason why different results were obtained.

Initial results indicated that none of the *S. typhi pqa/pqr*::Mu*d*J mutants were deficient in invasion of PMA-U937 cells (Section 4.6.1), however both the *S. typhi pqa*B::Mu*d*J (RMA2326) and *pqaA*::Mu*d*J (RMA2310) appeared to have a defect in net growth compared to the wildtype *S. typhi* Ty2 strain. Further analysis showed that the RMA2310 (*pqaA*::Mu*d*J) mutant was slightly (but not significantly as P = 0.06) affected for intracellular growth in PMA-U937 cells, however the *S. typhi pqaB* (RMA2326) mutant had a significantly lower intracellular net growth level at 24 h compared to *S. typhi* Ty2 in PMA-U937 cells (P = 0.02) (Section 4.6.2). The net growth of the *S. typhi pqaB*::Mu*d*J mutant was not affected compared to the wildtype strain in HeLa cells, indicating that the intracellular net growth defect was specific to the macrophage-like PMA-U937 cell line. Strain RMA2326 (*pqaB*::Mu*d*J) was found to be defective in net growth in the PMA-U937 cells during the last 6 h of infection but overall was not as deficient in intracellular net growth as the *S. typhi* PhoP$^-$ mutant (RMA1030) (Section 4.6.2). This indicates that other *pqa* genes are involved with *S. typhi* survival in PMA-U937 cells, which was supported by the fact that the *S. typhi* PhoP^c mutant strain was not at all deficient in net intracellular growth compared to the parental *S. typhi* Ty2 strain. The *S. typhi* defined *pqaB* mutant was also shown to be involved in growth in PMA-U937 macrophage-like cells (Section 6.10.3), confirming that this gene has an effect on the growth of *S. typhi* in PMA-U937 cells.

As the *S. typhi* strains do not appear to be cytotoxic for the monocyte-derived macrophages over the 24 h assay from the LDH data (Section 4.7), infection of the PMA-U937 cells could be used to test when *pqa/pqr* genes are switched on/off after invasion. By using a reporter system, such as β-galactosidase, green fluorescent protein (GFP) or

chloramphenicol transacetylase assays, a particular gene could be studied to determine whether it is switched on or off early or late during infection of the macrophage-like cells.

7.6 Characterisation of the *S. typhi pqaB* gene

The *S. typhi pqaB* gene was identified, cloned and sequenced along with adjacent genes, and was initially found to have ~ 70 % homology to ORF550 in the *E. coli* K-12 database (gbD: AE000315). During this study, the *S.typhimurium pmrF* operon was characterised and *pqaB* was shown to have high (~98%) homology to ORF5 of the *S. typhimurium pmrF* operon (Section 6.3). The *S. typhimurium pmrF* operon has been identified as being involved with 4AA lipid A modification and PmB resistance (Gunn *et al.*, 1998B). A putative pathway for the biosynthesis of 4AA based on sequence alignments and predicted biochemistry is described in Section 6.5 and indicates that *pqaB*/ORF5 encodes a bactoprenol-4AA-lipid A transferase.

The *pmrF* operon was found to be strongly regulated by the PmrA-PmrB operon (Gunn *et al.*, 1998B). A *pmrA*::Tn*10*d mutation was transduced into the *S. typhi pqaB*::Mu*d*J mutant and expression of the *S. typhi pqaB*::Mu*d*J fusion was strongly reduced (Section 6.6). Hence *pqaB*/ORF5 is also regulated by PmrA-PmrB, as expected from its location in the *pmrF* operon.

As the *S. typhimurium pmrF* operon is involved with PmB resistance, the *S. typhi* strains were also tested for PmB resistance. The *pqaB*::Mu*d*J, PhoP^{c} (RMA2526) strain was more sensitive to PmB than RMA1090 (PhoP^{c}), as was the defined *pqaB*::Kan mutant, indicating that the *S. typhi pqaB* gene is also involved with PmB resistance (Section 6.7; Section 6.10.1). The sensitivity of *pmrF* and *pqaB* mutants to PmB is consistent with these genes affecting the same pathway for LPS modifications (ORF's 1 to 7, putative functions outlined in Section 6.5).

The *S. typhi pqaB*::Mu*d*J mutation has also been shown to be involved with melittin resistance and the *S. typhi pqaB*::Kan defined mutant was also shown to be sensitive to melittin compared to the *S. typhi* Ty2 wildtype strain (Section 6.10.2), indicating that the 4AA modifications of LPS may also be involved with *S. typhi* melittin resistance as well as PmB resistance.

It has been suggested that since LPS is negatively charged and most antimicrobial peptides (including PMB and melittin) are cationic at physiological pH, mutations which alter the phosphate content or LPS substitutions may change the negative charge of the LPS and therefore affect the ability of the cationic peptides to bind to the LPS (Groisman, 1994). An increase in both ethanolamine and 4AA substitution results in a lower surface negative charge of the lipid A, and inner core parts of the LPS, and therefore give a greater resistance to cationic peptides (Helander *et al.*, 1994). As both *S. typhi* and *S. typhimurium* PhoP^c mutants have their *pqa/pag* genes constitutively up-regulated, this would explain why they have a higher resistance to antimicrobial agents such as PmB and melittin as seen for the *S. typhi* PhoP^c (RMA1090) strain compared to the wildtype strain (Section 6.7, Section 5.8.7), and PmB resistance for *S. typhimurium* (Gunn *et al.*, 1998B).

The *S. typhi* pqaB::Mu*d*J mutation was also found to subtly affect LPS, seen by the lack of discrete banding of high molecular range LPS which was the only difference seen between the *S. typhi pqaB* mutants (RMA2326 and RMA2526) and their wildtype (Ty2) and PhoP^c (RMA1090) counterparts (Section 6.8). The LPS of strain RMA2526 (*pqaB*::Mu*d*J, PhoP^c) also appeared to have fewer O antigen chains which was not seen for the defined (non-polar) *pqaB*::Kan, PhoP^c mutant (Section 6.10.4). Therefore it is likely that the Mu*d*J insertion in RMA2526 is polar and may affect expression of ORF6 and ORF7. ORF6 and ORF7 are small, highly hydrophobic proteins with 3-4 potential transmembrane regions (Section 6.4.6; Section 6.4.7), indicating that they may be integral membrane proteins and these results (Section 6.10.4), indicate that they may be involved in LPS O antigen synthesis (Section

6.4.8). As complementation of the *pqaB*::Mu*dJ* mutant could not be achieved (Section 6.11), a defined polar mutation in *pqaB* would be useful to define the role that ORF6 and ORF7 may play in LPS O antigen synthesis. In conclusion, the *S. typhi pqaB* mutant studies indicated that 4AA modification of lipid A is needed for intracellular growth in PMA-U937 cells and antimicrobial peptide resistance.

7.7 Conclusions

This study has shown significant differences between *S. typhi* and *S. typhimurium* PhoP/Q regulated genes based on PhoN activity and comparison of whole cell proteins. Five *S. typhi* phoP/Q regulated genes were identified, and two potential regulators involved in the *S. typhi phoP/Q* regulatory cascade were identified.

The most significant results however were the differences between *S. typhi* and *S. typhimurium* LPS, and differences in antimicrobial peptide sensitivity, indicating differences in lipid A modifications. The *pqaB* gene was identified as being identical to the *orf5* in the *S. typhimurium pmrF* operon which is involved in 4AA modification of lipid A. The higher level of resistance of *S. typhimurium* to antimicrobial peptides may be due to an increased amount of LPS/lipid A modifications for *S. typhimurium* compared to *S. typhi*; e.g. modifications such as the 4AA and ethanolamine may be regulated/added to give increased modifications in *S. typhimurium* compared to *S. typhi.* This would correlate with the lower *phoN* expression and fewer *phoP/Q* regulated proteins seen by whole cell protein analysis. Alternatively, undetected *pag/pqa* genes in either *S. typhimurium* or *S. typhi* may give different modifications for *S. typhimurium* LPS compared to *S. typhi.* Either of these proposals would result in *S. typhi* LPS having a relatively higher net negative charge than *S. typhimurium*, which correlates with the fact that while the *S. typhi pqaB* mutant demonstrated sensitivity to melittin, an *S. typhimurium pmrF* mutant (which also affects 4AA modification

of LPS) was shown not to affect sensitivity to melittin (Dr. John S. Gunn, Department of Microbiology, University of Texas Health Science Centre at San Antonio, personal communication). The U937-PMA assay used in this study may be sensitive enough to detect these differences in *S. typhi* strains but not *S. typhimurium*, explaining why, unlike the *S. typhi* PhoP$^-$ mutant, the *S. typhimurium* PhoP$^-$ mutant was not limited in net growth in the PMA-U937 cells compared to the wild-type strain. Given the importance of LPS in *Salmonella* virulence, the differences noted in this study may impact on the relative virulence of *Salmonella* species in different hosts. As outlined in Section 1.8.9 and Section 1.13, lipid A modifications have been shown to potentially affect the hosts immune response to *Salmonella* infections. The human Tlr4 complex expressed on macrophages has been shown to be non-responsive to tetra-acyl lipid A whereas murine Tlr4 will respond to full and deacylated lipid A (Golenbock *et al.*, 1991; Poltorak *et al.*, 2000). This could mean that *S. typhi* is host-adapted to human LPS receptors and modifies its LPS according, and therefore may be able to influence and/or escape from host immune responses in humans but not mice or other "non-specific" host species. The particular lipid A modifications that *S. typhi* has could be very specific for human cells and analysis of structural modifications of LPS isolated from *S. typhi* Ty2, PhoP$^-$, PhoP^c and *pqaB*::Mu*dJ* derivatives by mass spectrometry to confirm and extend the known differences between *S. typhi* and *S. typhimurium* LPS may be useful in helping to define mechanisms for host specificity.

Bibliography

Abigail, A. S. & Dixie, D. W. (1994). *Salmonella* Infections. In *Bacterial Pathogenesis: A molecular approach*, pp. 229-243: ASM Press.

Abshire, K. A. & Heffron, F. (1993A). Growth rate paradox of *Salmonella typhimurium* within host macrophages. *J Bacteriol* **175**, 3744-3748.

Abshire, K. Z. & Heffron, F. (1993B). Analysis of proteins synthesized by *Salmonella typhimurium* during growth within a host macrophage. *J Bacteriol* **175**, 3734-3743.

Achtman, M., Manning, P. A., Edelbluth, C. & Herrlich, P. (1979). Export without proteolytic processing of inner and outer membrane proteins encoded by F sex factor *tra* cistrons in Escherichia coli minicells. *Proc Natl Acad Sci USA* **76**, 4837-4841.

Adams, P., Fowler, R., Howell, G., Kinsella, N., Skipp, P., Coote, P. & O'Conner, C-D. (1999). Defining protease specificity with proteomics: A protease with a dibasic amino acid recognition motif is regulated by a two-component signal transduction system in *Salmonella*. *Electrophoresis* **20**, 2241-2247.

Ahmed, M., Borsch, C. M., Taylor, S. S., Vazquez-Laslop, N. & Neyfakh, A. A. (1994). A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates. *J Biol Chem* **269**, 28506-28513.

Ahmed, M., Lyass, L., Markham, P. N., Taylor, S. S., Vazquez-Laslop, N. & Neyfakh, A. A. (1995). Two highly similar multidrug transporters of *Bacillus subtilis* whose expression is differentially regulated. *J Bacteriol* **177**, 3904-3910.

Ahmer, B. M. M., van Reeuwijk, J., Watson, P. R., Wallis, T. S. & Heffron, F. (1999). *Salmonella* SirA is a global regulator of genes mediating enteropathogenesis. *Mol Microbiol* **31**, 971-982.

Allard, J. D., Gibson, M. L., Vu, L. H., Nguyen, T. T. & Bertrand, K. P. (1993). Nucleotide sequence of class D tetracycline resistance genes from *Salmonella ordonez*. *Mol Gen Genet* **237**, 301-305.

Allison, G. E. & Verma, N. K. (2000). Serotype-converting bacteriophages and O-antigen modification in *Shigella flexneri*. *TIM* **8**, 17-23.

Allmeier, H., Cresnar, B., Greck, M. & Schmitt, R. (1992). Complete nucleotide sequence of Tn1721: gene organization and a novel gene product with features of a chemotaxis protein. *Gene* **111**, 11-20.

Alpuche Aranda, C. M., Swanson, J. A., Loomis, W. P. & Miller, S. I. (1992). *Salmonella typhimurium* activates virulence gene transcription within acidified macrophage phagosomes. *Proc Natl Acad Sci USA* **89**, 10079-10083.

Alpuche Aranda, C. M., Racoosin, E. L., Swanson, J. A. & Miller, S. I. (1994). *Salmonella* stimulate macrophage macropinocytosis and persist within spacious phagosomes. *J Exp Med* **179**, 601-608.

Alpuche Aranda, C. M., Berthiaume, E. P., Mock, B., Swanson, J. A. & Miller, S. I. (1995). Spacious phagosome formation within mouse macrophages correlates with *Salmonella* serotype pathogenicity and host susceptibility. *Infect Immun* **63**, 4456-4462.

Altmeyer, R. M., McNern, J. K., Bossio, J. C., Rosenshine, I., Finlay, B. B. & Galán, J. E. (1993). Cloning and molecular characterization of a gene involved in *Salmonella* adherance and invasion of cultured epithelial cells. *Mol Microbiol* **7**, 89-98.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389-3402.

Anderson, D. M. & Schneewind, O. (1997). A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. *Science* **278**, 1140-1143.

Anderson, M. S., Bulawa, C. E. & Raetz, C. R. P. (1985). The biosynthesis of gram negative endotoxin: formation of lipid A precursors from UDP-GluNAc in extracts of *Escherichia coli*. *J Biol Chem* **260**, 15536-15541.

Arricau, N., Hermant, D., Waxin, H., Ecobichon, C., Duffey, P. S. & Popoff, M. Y. (1998). The RcsB-RcsC regulatory system of *Salmonella typhi* differentially modulates the expression of invasion proteins, flagellin and Vi Antigen in response to osmolarity. *Mol Microbiol* **29**, 835-850.

Ascensao, J. L. & Mickman, J. K. (1984). Differentiation of human leukemic cell lines (HL60, U937) towards macrophages is accompanied by production of colony-stimulating activity (CSA). *Exp Hematol* **12**, 177-182.

Aspedon, A. & Groisman, E. A. (1996). The antibacterial action of protamine: evidence for disruption of cytoplasmic membrane energization in *Salmonella typhimurium*. *Microbiol* **142**, 3389-3397.

Bahrmand, A. R. & Velayati, A. A. (1997). Antimicrobial resistance pattern and plasmid profile of *Salmonella typhi* isolated from an outbreak in Tehran province. *Scan J Infect Dis* **29**, 265-269.

Bajaj, V., Hwang, C. & Lee, C. A. (1995). HilA is a novel OmpR/ToxR family member that activates the expression of *Salmonella typhimurium* invasion genes. *Mol Microbiol* **18**, 715-727.

Bajaj, V., Lucas, R. L., Hwang, C. & Lee, C. A. (1996). Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. *Mol Microbiol* **22**, 703-714.

Baker, S. J. (1993). Isolation and characterisation of PhoP regulated genes in *Salmonella typhi*. *Department of Microbiology and Immunology*. Adelaide: University of Adelaide, B. Sc (Hons) Thesis..

Baker, S. J., Daniels, C. & Morona, R. (1997). PhoP/Q regulated genes in *Salmonella typhi*: identification of melittin sensitive mutants. *Microb Pathog* **22**, 165-179.

Barrow, P. A., Huggins, M. B. & Lovell, M. A. (1994). Host specificity of *Salmonell*a infection in chickens and mice is expressed *in vivo* primarily at the level of the reticuloendothelial system. *Infect Immun* **62**, 4602-4610.

Bastin, D. A., Lord, A. & Verma, N. K. (1997). Cloning and analysis of the glucosyl transferase gene encoding type I antigen in *Shigella flexneri*. *FEMS Microbiol Lett* **156**, 133-139.

Bäumler, A. J., Kusters, J. G., Stojiljkovic, I. & Heffron, F. (1994). *Salmonella typhimurium* loci involved in survival within macrophages. *Infect Immun* **62**, 1623-1630.

Bäumler, A. J. & Heffron, F. (1995). Identification and sequence analysis of *IpfABCDE*, a putative fimbrial operon of *Salmonella typhimurium*. *J Bact* **177**, 2087-2097.

Bäumler, A. J., Tsolis, R. M. & Heffron, F. (1996). The *lpf* fimbrial operon mediates adhesion of *Salmonella typhimurium* to murine Peyer's patches. *Proc Natl Acad Sci USA* **93**, 279-283.

Bäumler, A. J. (1997). The record of horizontal gene transfer in *Salmonella*. *TIM* **5**, 318-322.

Bäumler, A. J., Tsolis, R. M., Ficht, T. A. & Adams, L. G. (1998). Evolution of host adaptation in *Salmonella enterica*. *Infect Immun* **66**, 4579-4587.

Beaman, L. & Beaman, B. L. (1984). Macrophages and phagocytic killing. *Ann Rev Microbiol* **38**, 27-48.

Bearson, S. M. D., Benjamin, W. H. J., Swords, W. E. & Foster, J. W. (1996). Acid shock indcution of RpoS is mediated by the mouse virulence gene *mviA* of *Salmonella typhimurium*. *J Bacteriol* **178**, 2572-2579.

Bearson, S., Bearson, B. & Foster, J. W. (1997). Acid stress responses in enterobacteria. *FEMS Microbiol Lett* **147**, 173-180.

Bearson, B. L., Wilson, L. & Foster, J. W. (1998). A low pH-inducible, PhoPQ-dependent acid tolerance response protects *Salmonella typhimurium* against inorganic acid stress. *J Bacteriol* **180**, 2409-2417.

Behlau, I. & Miller, S. I. (1993). A PhoP-repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. *J Bacteriol* **175**, 4475-4484.

Belden, W. J. & Miller, S. I. (1994). Further characterization of the PhoP regulon: identification of new PhoP-activated virulence loci. *Infect Immun* **62**, 5095-5101.

Bennett, I. L. & Hook, E. W. (1959). Infectious diseases (some aspects of salmonellosis). *Ann Rev Med* **10**, 1.

Beutler, B. (2000). Tlr4: central component of the sole mammalian LPS sensor. *Cur Opin Immunol* **12**, 20-26.

Bhandari, P. & Gowrishankar, J. (1997). An *Escherichia coli* host strain useful for efficient overproduction of cloned gene products with NaCl as the inducer. *J Bacteriol* **179**, 4403-4406.

Bignon, C., Daniel, N. & Dijiane, J. (1993). β-galactosidase and chloramphenicol acetyl transfer assays in 96-well plates. *Biotechniques* **15**, 243-245.

Blanc Potard, A. B. & Groisman, E. A. (1997). The *Salmonella selC* locus contains a pathogenicity island mediating intramacrophage survival. *EMBO J* **16**, 5376-5385.

Blaser, M. J. & Newman, L. S. (1982). A review of human *Salmonellosis*. I. Infective dose. *Rev Infect Dis* **4**, 1096-1106.

Blattner, F. R., Plunkett, G. I., Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B. & Shao, Y. (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* **277**, 1453-1474.

Bolhuis, H., van Veen, H. W., Poolman, B., Driessen, A. J. M. & Konings, W. N. (1997). Mechanisms of multidrug transporters. *FEMS Microbiol Rev* **21**, 55-84.

Boman, H. G. (1995). Peptide antibiotics and their role in innate immunity. *Ann Rev Immunol* **70**, 1514-1518.

Brown, M. C. M., Weston, A., Saunders, J. R. & Humphreys, G. O. (1979). Transformation of *E. coli* C600 by plasmid DNA at different phases of growth. *FEMS Microbiol Lett* **5**, 219-222.

Brozek, K. A. & Raetz, C. R. H. (1990). Biosyntheis of lipid A in *Escherichia coli* acyl carrier protein-dependent incorporation of laurate and myristate. *J Biol Chem* **265**, 15410-15417.

Bruntner, C. & Bormann, C. (1998). The *Streptomyces tendae* Tu901 L-lysine 2-aminotransferase catalyzes the intitial reaction in nikkomycin D biosynthesis. *Eur J Biochem* **254**, 347-355.

Buchmeier, N. A. & Heffron, F. (1989). Intracellular survival of wild-type *Salmonella typhimurium* and macrophage-sensitive mutants in diverse populations of macrophages. *Infect Immun* **57**, 1-7.

Buchmeier, N. A. & Heffron, F. (1990). Induction of *Salmonella* stress proteins upon infection of macrophages. *Science* **248**, 730-732.

Buchmeier, N. A. & Heffron, F. (1991). Inhibition of macrophage phagosome-lysosome fusion by *Salmonella typhimurium*. *Infect Immun* **59**, 2232-2238.

Buchmeier, N. A., Lipps, C. J., So, M. Y. H. & Heffron, F. (1993). Recombination-deficient mutants of *Salmonella typhimurium* are avirulent and sensitive to the oxidative burst of macrophages. *Mol Microbiol* **7**, 933-936.

Burland, V., Plunkett, G. I., Daniels, D. L. & Blattner, F. R. (1993). DNA sequence and analysis of 136 kilobases of the *Escherichia coli* genome: organizational symmetry around the origin of replication. *Genomics* **16**, 551-561.

Burland, V., Plunkett, G. r., Sofia, H. J., Daniels, D. L. & Blattner, F. R. (1995). Analysis of the *Escherichia coli* genome VI: DNA sequence of the region from 92.8 through 100 minutes. *Nucleic Acids Res* **23**, 2105-2119.

Burlingame, R. P., Lauer, G. D., Platz, J. G., Rudd, E. A., Ally, A., Ally, D. & Backman, K. C. (1998). *Pseudomonas putida*: putative glucarate transporter,. *Genbank, direct submission* .

Burns-Keliher, L., Nickerson, C. A., Morrow, B. J. & Curtiss, R. (1998). Cell-specific proteins synthesized by *Salmonella typhimurium*. *Infect Immun* **66**, 856-861.

Cárdenas, L. & Clements, J. (1992). Oral immunization using live attenuated *Salmonella* spp. as carriers of foreign antigens. *Clin Microbiol Rev* **5**, 328-342.

Carlomagno, M. S., Chiariotti, L., Alifamo, P., Nappo, A. G. & Bruni, C. B. (1988). Structure and function of the *Salmonella typhimurium* and *Escherichia coli* K-12 histidine operons. *J Mol Biol* **203**, 585-606.

Casadaban, M. J. & Cohen, S. N. (1979). Lactose genes fused to exogenous promoters in one step using Mu-*lac* bacteriophage: *in vivo* probe for transcriptional control sequence. *Proc Natl Acad Sci USA* **76**, 4530-4533.

Castilho, B. A., Olfson, P. & Casadaban, M. J. (1984). Plasmid insertion mutagenesis and *lac* gene fusion with mini-Mu bacteriphage transposons. *J Bacteriol* **158**, 488-495.

Charlebois, R. L., Singh, R. K., Chan-Weiher, C. C. Y., Allard, G., Chow, C., Confalonieri, F., Curtis, B., Duguet, M., Erauso, G., Faguy, D., Gaasterland, T., Garrett, R. A., Gordon, P., Jeffries, A. C., Kozera, C., Kushwaha, N., Lafleur, E., Medina, N., Peng, X., Penny, S. L., She, Q., St Jean, A., van der oost, J., Young, F., Zivanovic, Y., Doolittle, W. F., Ragan, M. A. & Sensen, C. W. (1999). Gene content and organization of a 281-kbp contig from the genome of the extremely thermophilic archaeon, *Sulfolobus solfataricus* P2. *Genbank, direct submission* .

Château, M.-T. & Caravano, R. (1993). Infection of differentiated U937 cells by *Salmonella typhimurium*: Absence of correlation between oxidative burst and antimicrobial defence. *FEMS Immun Med Microbiol* **7**, 111-118.

Chen, G-F. T. & Inouye, M. (1990). Suppression of the negative effect of minor arginine codons on gene supression; preferential usage of minor codons within the first 25 codons of the *Escherichia coli* genes. *Nucleic Acids Res* **18**, 1465-1473.

Chen, L. M., Kaniga, K. & Galán, J. E. (1996). *Salmonella* Spp are cytotoxic for cultured macrophages. *Mol Microbiol* **21**, 1101-1115.

Cherrington, C. A., Hinton, M., Mead, G. C. & Chopra, I. (1991). Organic acids: chemistry, antibacterial activity, and practical applications. *Adv Microb Physiol* **32**, 87-108.

Christensen, B., Fink, J., Merrifield, R. B. & Mauzerall, D. (1988). Channel-forming properties in cecropins and related model compounds incorporated into planar lipid membranes. *Proc Natl Acad Sci USA* **85**, 5072-5076.

Cianciotto, N. P., Eisenstein, B. I., Mody, C. H., Toews, G. B. & Engleberg, N. C. (1989). A *Legionella pneumophila* gene encoding a species-specific surface protein potentiates initiation of intracellular infection. *Infect Immun* **57**, 1255-1262.

Cirillo, D. M., Valdivia, R. H., Monack, D. M. & Falkow, S. (1998). Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. *Mol Microbiol* **30**, 175-188.

Clark, M. A., Jepson, M. A., Simmons, N. L. & Hirst, B. H. (1994). Preferential interaction of *Salmonella typhimurium* with mouse Peyer's patch M cells.*Res Microbiol* **145**, 543-552.

Clark, M. A., Reed, K. A., Lodge, J., Stephen, J., Hirst, B. H. & Jepson, M. A. (1996). Invasion of murine intestinal M Cells by *Salmonella typhimurium inv* mutants severely deficient for invasion of cultured cells. *Infect Immun* **64**, 4363-4368.

Clark, M. A., Hirst, B. H. & Jepson, M. A. (1998). Inoculum composition and *Salmonella* Pathogenicity Island 1 regulate M-Cell invasion and epithelial destruction by *Salmonella typhimurium*. *Infect Immun* **66**, 724-731.

Clemens, J. D. & El-Morshidy, S. (1984). Construction of a potential live oral bivalent vaccine for typhoid fever and cholera-*Escherichia coli*-related diarrheas. *Infect Immun* **46**, 564-569.

Clementz, T., Bednarski, J. J. & Raetz, C. R. H. (1996). Function of the *htrB* high temperature requirement of *Escherichia coli* in the acylation of lipid A: HtrB catalyzed incorporation of laurate. *J Biol Chem* **271**, 12095-12102.

Clementz, T., Zhou, Z. & Raetz, C. R. H. (1997). Function of the *Escherichia coli msbB* gene, a multicopy suppressor of *htrB* knockouts, in the acylation of lipidA. *J Biol Chem* **272**, 10353-10360.

Cohen, R. M. & Wolfenden, R. (1971). Cytidine deaminase from *Esherichia coli*. *J Biol Chem* **246**, 7561-7565.

Cohen, S. P., Levy, S. B., Foulds, J. & Rosner, J. L. (1993). Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. *J Bacteriol* **175**, 7856-7862.

Collazo, C. M., Zierler, M. K. & Galán, J. E. (1995). Functional analysis of the *Salmonella typhimurium* invasion genes *invI* and *invJ* and identification of a target of the protein secretion apparatus encoded in the *inv* locus. *Mol Microbiol* **15**, 25-38.

Collazo, C. M. & Galán, J. E. (1996). Requirement for exported proteins in secretion through the invasion-associated type-III system of *Salmonella typhimurium*. *Infect Immun* **64**, 3524-3531.

Collazo, C. M. & Galán, J. E. (1997). The invasion-associated type-III protein secretion system in *Salmonella* - a review. *Gene* **192**, 51-59.

Collinson, S. K., Emody, L., Muller, K.-H., Trust, T. J. & Kay, W. W. (1996). The location of four fimbrin-encoding genes, *agfA*, *fimA*, *sefA* and *sefD*, on the *Salmonella enteriditis* and/or *S. typhimurium Xba*I-*Bln*I genomic restriction maps. *J Bacteriol* **173**, 4773-4781.

Conlan, J. W. (1996). Neutrophils prevent extracellular colonization of the liver microvasculature by *Salmonella typhimurium*. *Infect Immun* **64**, 1043-1047.

Conlan, J. W. (1997). Critical roles of neutrophils in host defense against experimental systemic infections of mice by *Listeria monocytogenes*, *Salmonella typhimurium*, and *Yersinia enterocolitica*. *Infect Immun* **65**, 630-635.

Cotter, P. A. & Miller, J. F. (1998). *In vivo* and *ex vivo* regulation of bacterial virulence gene expression. *Curr Opin Microbiol* **1**, 17-26.

Coughlin, R. T., Tonsager, S. & McGroarty, E. J. (1983). Quantitation of metal cation bound to membranes and extracted lipopolysaccharide of *Eschrichia coli*. *Biochem* **22**, 2002-2007.

Couper, M. R. (1997). Strategies for the rational use of antimicrobials. *Clin Infect Dis* **24**, S1154-156.

Crago, A. M. & Koronakis, V. (1998). *Salmonella* InvG forms a ring-like multimer that requires the InvH lipoprotein for outer membrane localization. *Mol Microbiol* **30**, 47-56.

Dairi, T., Nakano, T., Aisaka, K., Katsumata, R. & Hasegawa, M. (1995). Cloning and nucleotide sequence of the gene responsible for chlorination of tetracycline. *BioSci Biotechnol Biochem* **59**, 1099-1106.

Daniels, C. (1994). Characterisation and cloning of PhoP regulated genes in *Salmonella typhi*. *Department of Microbiology and Immunology*. Adelaide: University of Adelaide B.Sc. (Hons) Thesis.

Daniels, C., Vindurampulle, V. & Morona, R. (1998). Overexpression and topology of the *Shigella flexneri* O-antigen polymerase (Rfc/Wzy). *Mol Microbiol* **28**, 1211-1222.

Daniels, J. J. D., Autenrieth, I. B., Ludwig, A. & Goebel, W. (1996). The Gene *slyA* of *Salmonella typhimurium* is required for destruction of M Cells and intracellular survival but not for invasion or colonization of the murine small intestine. *Infect Immun* **64**, 5075-5084.

Darlison, M. G. & Guest, J. R. (1984). Nucleotide sequence encoding the iron-sulphur protein subunit of the succinate dehydrogenase of *Escherichia coli*. *Biochem* **223**, 507-517.

Darveau, R. P. (1998). Lipid A diversity and the innate host response to bacterial infection. *Curr Opin Microbiol* **1**, 36-42.

Darwin, K. H. & Miller, V. I. (1999). Molecular basis of the interaction of *Salmonella* with the intestinal mucosa. *Clin Microbiol Rev* **12**, 405-428.

Distler, J. (1988). Putative aminotransferase in *Steptomyces griseus*. *Genbank: Direct submission* .

Dorsa, W. J. (1997). New and established carcass decontamination procedures commonly used in the beef-processing industry. *J Food Protect* **60**, 1146-1151.

Dougan, G. (1994). The molecular basis for the virulence of bacterial pathogens: implications for oral vaccine development. *Microbiol* **140**, 215-224.

Dragunsky, E. M., Wooden, C. R., Vargo, S. A. & Levenbook, I. S. (1989). *Salmonella typhi* vaccine strain in vitro; low infectivity in human cell line U937. *J Biol Standard* **17**, 353-360.

Dragunsky, E. M., Rivera, E., Hochstein, H. D. & Levenbook, I. S. (1990). *In vitro* characterization of *Salmonella typhi* mutant strains for live oral vaccines. *Vaccine* **8**, 263-268.

Duclohier, H., Molle, G. & Spach, G. (1989). Antimicrobial peptide magainin I from *Xenopus* skin forms anion-permeable channels in planar lipid bilayers. *Biophys J* **56**, 1017-1021.

Dunlap, N. E., Benjamin, W. H., McCall, R. D. J., Tilden, A. B. & Briles, D. E. (1991). A 'safe-site' for *Salmonella typhimurium* is within splenic cells during the early phase of infection in mice. *Microb Pathog* **10**, 297-310.

Dunstan, S. J., Simmons, C. P. & Strugnell, R. A. (1999). Use of in vivo-regulated promoters to deliver antigens from attenuated *Salmonella enterica* var. Typhimuirum. *Infect Immun* **67**, 5133-5141.

DuPont, H. L. & Steele, J. H. (1987). Use of antimicrobial agents in animal feeds: implications for human health. *Rev Infect Dis* **9**, 447-460.

Earampamoorthy, S. & Koff, R. S. (1975). Health hazards of bivalve-mollusk ingestion. *Ann Intern Med* **83**, 107-110.

Edsall, G., Gaines, S., M., L., Tigertt, W. D., Sprinz, H., Trapani, R. J., Mandel, A. D. & Benenson, A. S. (1960). Studies on infection and immunity in experimental typhoid fever. I. Typhoid fever in chimpanzees orally infected with *Salmonella typhosa*. *J Exp Med* **112**, 143-166.

Edwards, R. A., Schifferli, D. M. & Maloy, S. R. (2000). A role for *Salmonella* fimbriae in intraperitoneal infections. *Proc Natl Acad Sci USA* **97**, 1258-1262.

Eichelberg, K., Ginocchio, C. C. & Galán, J. E. (1994). Molecular and functional characterization of the *Salmonella typhimurium* invasion genes *invB* and *invC* - homology of InvC to the F_0f_1 ATPase family of proteins. *J Bacteriol* **176**, 4501-4510.

Eichelberg, K., Hardt, W. D. & Galán, J. E. (1999). Characterization of SprA, an AraC-like transcriptional regulator encoded within the *Salmonella typhimurium* Pathogenicity Island 1. *Mol Microbiol* **33**, 139-152.

Emmerth, M., Goebel, W., Miller, S. I. & Hueck, C. J. (1999). Genomic subtraction identifies *Salmonella typhimurium* prophages, F-related plasmid sequences, and a novel fimbrial operon, *stf*, which are absent in *Salmonella typhi*. *J Bact* **181**, 5652-5661.

Esser, A. F., Bartholomew, R. M., Jensen, F. C. & Müller-Eberhard, H. J. (1979). Dissambly of viral membranes by complement independent of channel formation. *Proc Natl Acad Sci USA* **76**, 5843-5847.

Fang, F. C., Krause, M., Roudier, C., Fierer, J. & Guiney, D. G. (1991). Growth regulation of a *Salmonella* plasmid gene essential for virulence. *J Bacteriol* **173**, 6783-6789.

Fang, F. C., Libby, S. J., Buchmeier, N. A., Loewen, P. C., Switala, J., Harwood, J. & Guiney, D. G. (1992). The alternative sigma factor *katF* (*rpoS*) regulates *Salmonella* virulence. *Proc Natl Acad Sci USA* **89**, 11978-11982.

Farmer, J. J. I., McWhorter, A. C. & Brenner, D. J. (1984). The *Salmonella-Arizona* group of Enterobacteriaceae: nomenclature, classification, and reporting. *Clin Microbiolog Newsletter* **6**, 63-66.

Farmer, J. J. I., Davis, B. R. & Hickman-Brenner, F. W. (1985). Biochemical identification of new species and biogroups of Enterbacteriaceae isolated from clinical specimens. *J Clin Microbiol* **21**, 46-76.

Fath, M. J. & Kolter, R. (1993). ABC transporters: bacterial exporters. *Microbiol Rev* **57**, 995-1017.

Feingold, D. S. & Barbar, G. A. (1990). Nucleotide sugars. In *Carbohydrates*, pp. 39-78. Edited by P. M. Dey. London: Academic Press.

Fields, P. I., Sqanson, R. V., Haidaris, C. G. & Heffron, F. (1986A). Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci USA* **83**, 5189-5193.

Fields, P. I., Haidaris, C. G., Swanson, R. V., Parsons, R. L. & Heffron, F. (1986B). Virulence determinants of *Salmonella* required for survival in macrophages. *Vaccines* **86**, 205-211.

Fields, P. I., Groisman, E. A. & Heffron, F. (1989). A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. *Science* **243**, 1059-1062.

Fierer, J., Eckmann, L., Fang, F., Pfeifer, C., Finlay, B. B. & Guiney, D. (1993). Expression of the *Salmonella* virulence plasmid gene *spvB* in cultured macrophages and non-phagocytic cells. *Infect Immun* **61**, 5231-5236.

Finlay, B. B. & Falkow, S. (1997). Common themes in microbial pathogenicity revisited. *Microbiol Mol Biol* **61**, 136-169.

Folkesson, A., Advani, A., Sukupolvi, S., Pfeifer, J. D., Normark, S. & Lofdahl, S. (1999). Multiple insertions of fimbrial operons correlate with the evolution of *Salmonella* serovars responsible for human disease. *Mol Microbiol* **33**, 612-622.

Formal, S. B., Baron, L. S., Kopecko, D. J., Washington, O., Powell, C. & Life, C. A. (1981). Construction of a potential bivalent vaccine strain: introduction of *Shigella sonnei* form I antigen genes into the *galE Salmonella typhi* Ty21a typhoid vaccine strain. *Infect Immun* **34**, 746-750.

Foster, J. W. & Hall, H. K. (1990). Adaptive acidification tolerance response of *Salmonella typhimurium*. *J Bacteriol* **172**, 771-778.

Foster, J. W. (1991). *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. *J Bacteriol* **173**, 6896-6902.

Foster, J. W. & Hall, H. K. (1992). Effect of *Salmonella typhimurium* ferric uptake regulator (*fur*) mutations on iron- and pH-regulated protein synthesis. *J Bacteriol* **174**, 4317-4323.

Fournier, B., Aras, R. & Hooper, D. C. (2000). Expression of the multidrug resistance transporter NorA from *Staphylococcus aureus* is modified by a two-component regulatory system. *J Bacteriol* **182**, 664-671.

Franzon, V. L. & Manning, P. A. (1986). Molecular cloning and expression in *Escherichia coli* K-12 of the gene for a hemagglutinin from *Vibrio cholerae*. *Infect Immun* **52**, 279-284.

Frey, P. A. (1987). Complex pyridine nucleotide-dependent transformation. In *Pyridine nucleotide coenzymes: chemical, biochemical, and medical aspects*, pp. 461-511. Edited by D. Dolphin, R. Poulson & O. Avramovic. New York: John Wiley & Sons.

Friedrich, M. J., Kinsey, N. E., Vila, J. & Kadner, R. J. (1993). Nucleotide sequence of a 13.9 kb segment of the 90 kb virulence plasmid of *Salmonella typhimurium*: the presence of fimbrial biosynthetic genes. *Mol Microbiol* **8**, 543-558.

Fu, Y. X. & Galán, J. E. (1998). Identification of a specific chaperone for SptP, a substrate of the centisome 63 Type III secretion system of *Salmonella typhimurium*. *J Bacteriol* **180**, 3393-3399.

Fu, Y. X. & Galán, J. E. (1999). A *Salmonella* protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. *Nature* **401**, 293-297.

Galán, J. E. & Curtiss, I. R. (1989A). Cloning and molecular characterization of genes who products allow *Salmonella typhimurium* to penetrate tissue culture cells. *Proc Natl Acad Sci USA* **86**, 6383-6387.

Galán, J. E. & Curtiss, R. D. (1989B). Virulence and vaccine potential of *phoP* mutants of *Salmonella typhimurium*. *Microb Pathog* **6**, 433-443.

Galán, J. E., Ginocchio, C. & Costeas, P. (1992). Molecular and functional characterization of the *Salmonella typhimurium* invasion gene *invA*: homology of InvA to members of a new protein family. *J Bacteriol* **17**, 4338-4349.

Galán, J. E. (1994). *Salmonella* entry into mammalian cells: different yet converging signal transduction pathways. *TCB* **4**, 196-199.

Galán, J. E. (1996). Molecular genetic bases of *Salmonella* entry into host cells. *Mol Microbiol* **20**, 263-271.

Galán, J. E. (1999). Interaction of *Salmonella* with host cells through the centisome 63 type III secretion system. *Curr Opin Microbiol* **2**, 46-50.

Galishoff, S. (1983). Triumph and Failure: The American response to the urban water supply problem, 1860-1923. In *Pollution and reform in American cities, 1870-1930*, pp. 43-44. Edited by M. V. Melosi. Austin, Texas.

Galyov, E. E., Wood, M. W., Rosqvist, R., Mullan, P. B., Watson, P. R., Hedges, S. & Wallis, T. S. (1997). A secreted effector proein of *Salmonella dublin* is translocated into eukaryotic cells and mediates inflammation and fluid secretion in infected ileal mucosa. *Mol Microbiol* **25**, 903-912.

Ganz, T. & Lehrer, R. I. (1995). Defensins. *Pharmacol Therapeut* **66**, 191-205.

Garcia Vescovi, E., Soncini, F. C. & Groisman, E. A. (1994). The role of the PhoP/PhoQ regulon in *Salmonella* virulence. *Res Microbiol* **145**, 473-480.

Garcia Vescovi, E., Ayala, Y. M., Di Cera, E. & Groisman, E. A. (1997). Characterization of the bacterial sensor protein PhoQ. Evidence for distinct binding sites for Mg2+ and Ca2+. *J Biol Chem* **272**, 1440-1443.

Garcia Vescovi, E., Soncini, F. C. & Groisman, E. A. (1996). Mg^{2+} as an extracellular signal: environmental regulation of *Salmonella* virulence. *Cell* **84**, 165-174.

Garcia-del Portillo, F., Pucciarelli, M. G. & Casadesus, J. (1999). DNA adenine methylase mutants of *Salmonella typhimurium* show defects in protein secretion, cell invasion and M cell cytotoxicity. *Proc Natl Acad Sci USA* **96**, 11578-11583.

Germainier, R. & Fürer, E. (1975). Isolation and characterization of *galE* Mutant Ty 21a of *Salmonella typhi*: A candidate strain for a live, oral typhoid vaccine. *J Infect Dis* **131**, 553-558.

Germanier, R. (1984). Typhoid fever. In *Bacterial Vaccines*, pp. 137-165: Academic Press.

Gianella, R. A., Broitman, S. A. & Zamcheck, N. (1972). Gastric acid barrier to ingested microorganisms in man: studies *in vivo* and *in vitro*. *Gut* **13**, 251-256.

Ginannella, R. A., Rout, W. R. & Formal, S. B. (1975). Pathogenesis of *Salmonella*-mediated intestinal fluid secretion: activation of adenylate cyclase and inhibition by indomethacin. *Gastroenterol* **69**, 1238-1245.

Ginocchio, C. & Galán, J. E. (1995). Functional conservation among members of the *Salmonella typhimurium* InvA family of proteins. *Infect Immun* **63**, 729-732.

Ginocchio, C. & Galán, J. E. (1995). Functional conservation among members of the *Salmonella typhimurium* InvA family of proteins. *Infect Immun* **63**, 729-732.

Godfroid, F., Taminiau, B., Danese, I., Denoel, P., Tibor, A., Weynants, V., Cloeckaert, A., Godfroid, J. & J-J., L. (1998). Identification of the persamine synthetase gene of *Brucella melitensis* 16M and involvement of lipopolysaccharide O side chain in *Brucella* survival in mice and in macrophages. *Infect Immun* **66**, 5485-5493.

Golenbock, D. T., Hampton, R. Y., Qureshi, N., Takayama, K. & Raetz, C. R. (1991). Lipid A-like molecules that antagonize the effects of endotoxins on human monocytes. *J Biol Chem* **266**, 19490-19498.

Griffith, J. K., Baker, M. E., Rouch, D. A., Page, M. G. P., Skurray, R. A., Paulsen, I. T., Chater, K. F., Baldwin, S. A. & Henderson, P. J. F. (1992). Membrane transport proteins: implications of sequence comparisons. *Curr Opin Cell Biol* **4**, 684-695.

Groisman, E. A., Chiao, E., Lipps, C. J. & Heffron, F. (1989). *Salmonella typhimurium phoP* virulence gene is a transcriptional regulator. *Proc Natl Acad Sci U S A* **86**, 7077-7081.

Groisman, E. A. & Saier, M. H., Jr. (1990). *Salmonella* virulence: new clues to intramacrophage survival. *TIB* **15**, 30-33.

Groisman, E. A., Saier, M. H., Jr. & Ochman, H. (1992A). Horizontal transfer of a phosphatase gene as evidence for mosaic structure of the *Salmonella* genome. *EMBO J* **11**, 1309-1316.

Groisman, E. A., Parra Lopez, C., Salcedo, M., Lipps, C. J. & Heffron, F. (1992B). Resistance to host antimicrobial peptides is necessary for *Salmonella* virulence. *Proc Natl Acad Sci U S A* **89**, 11939-11943.

Groisman, E. A., Heffron, F. & Solomon, F. (1992C). Molecular genetic analysis of the *Escherichia coli phoP* locus. *J Bacteriol* **174**, 486-491.

Groisman, E. A. & Ochman, H. (1993). Cognate gene clusters govern invasion of host epithelial cells by *Salmonella typhimurium* and *Shigella flexneri*. *Embo J* **12**, 3779-3787.

Groisman, E. A. (1994). How bacteria resist killing by host-defense peptides. *TIM* **15**, 30-33.

Groisman, E. A. & Ochman, H. (1997). Pathogenicity islands-bacterial evolultion in quantum leaps. *Cell* **87**, 791-794.

Groisman, E. A., Kayser, J. & Soncini, F. C. (1997). Regulation of polymyxin resistance and adaptation to low-Mg^{2+} environments. *J Bacteriol* **179**, 7040-7045.

Grosjean, H. & Fiers, W. (1982). Preferential codon usage in prokaryotic genes: the optimal codon-anticodon interaction energy and the selective codon usage in efficiently expressed genes. *Gene* **18**, 199-209.

Guan, G., Bastin, D. A. & Verma, N. K. (1999). Functional analysis of the O antigen glucosylation gene cluster of *Shigella flexneri* bacteriophage SfX. *Microbiol* **145**, 1263-1273.

Gulig, P. A. (1990). Virulence plamids of *Salmonella typhimurium* and other Salmonellae. *Microb Pathog* **8**, 3-11.

Gulig, P. A., Danbara, H., Guiney, D. G., Lax, A. J. & Norel, F. (1993). Molecular analysis of the *spv* virulence genes of the *Salmonella* virulence plasmids. *Mol Microb* **7**, 825-830.

Gunn, J. S., Alpuche Aranda, C. M., Loomis, W. P., Belden, W. J. & Miller, S. I. (1995). Characterization of the *Salmonella typhimurium pagC/pagD* chromosomal region. *J Bacteriol* **177**, 5040-5047.

Gunn, J. S. & Miller, S. I. (1996). PhoP-PhoQ activates transcription of *pmrAB*, encoding a two-component regulatory system involved in *Salmonella* typhimurium antimicrobial peptide resistance. *J Bacteriol* **178**, 6857-64.

Gunn, J. S., Hohmann, E. L. & Miller, S. I. (1996). Transcriptional regulation of *Salmonella* virulence: a PhoQ periplasmic domain mutation results in increased net phosphotransfer to PhoP. *J Bacteriol* **178**, 6369-6373.

Gunn, J. S., Belden, W. J. & Miller, S. I. (1998A). Identification of PhoP-PhoQ activated genes within a duplicated region of the *Salmonella typhimurium* chromosome. *Microb Pathog* **25**, 77-90.

Gunn, J. S., Lim, K. B., Krueger, J., Kim, K., Guo, L., Hackett, M. & Miller, S. I. (1998B). PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Mol Microbiol* **27**, 1171-1182.

Guo, L., Lim, K. B., Gunn, J. S., Bainbridge, B., Darveau, R. P., Hackett, M. & Miller, S. I. (1997). Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*. *Science* **276**, 250-253.

Guo, L., Lim, K. B., Poduje, C. M., Daniel, M., Gunn, J. S., Hackett, M. & Miller, S. I. (1998). Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. *Cell* **95**, 189-198.

Hacker, J., Blum-Oehler, G., Mühldorfer, I. & Tschäpe, H. (1997). Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Mol Microbiol* **23**, 1089-1097.

Hackett, J. (1990). *Salmonella*-based vaccines. *Vaccine* **8**, 5-11.

Hakura, A., Morimoto, K., Sofuni, T. & Nohmi, T. (1991). Cloning characterization of the *Salmonella typhimurium ada* gene, which encodes O^6-methylguianine-DNA methyltransferase. *J Bacteriol* **173**, 3663-3672.

Hall, H. K. & Foster, J. W. (1996). The role of Fur in the acid tolerance response of *Salmonella typhimurium* is physiologically and genetically separablefrom its role in iron acquisition. *J Bacteriol* **178**, 5683-5691.

Hammer-Jespersen, K. (1983). Nucleoside catabolism. In *Metabolism of nucleotides, nucleosides and nucleobases in microorganisms*, pp. 203-258. Edited by A. Munch-Petersen. London: Academic Press Inc Ltd.

Hampton, M. D., Ward, L. R., Rowe, B. & Threlfall, E. J. (1998). Molecular fingerprinting of multidrug-resistant *Salmonella enterica* serotype Typhi. *Emerg Infect Dis* **4**, 317-320.

Harding, C. V. & Geuze, H. J. (1993). Antigen processing and intracellular traffic of antigens and MHC molecules. *Curr Opin Cell Biol* **5**, 596-605.

Hardt, W. D., Urlaub, H. & Galán, J. E. (1998A). A substrate of the centisome 63 type III protein secretion system of *Salmonella typhimurium* is encoded by a cryptic bacteriophage. *Proc Natl Acad Sci USA* **95**, 2574-2579.

Hardt, W. D., Chen, L. M., Schuebel, K. E., Bustelo, X. R. & Galán, J. E. (1998B). *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell* **93**, 815-826.

Harwood, C. S., Nichols, N. N., Kim, M. K., Ditty, J. L. & Parales, R. E. (1994). Identification of the *pcaRKF* gene cluster from *Pseudomonas putida*: involvement in chemotaxis, biodegradation, and transport fo 4-hydroxybenzoate. *J Bacteriol* **176**, 6479-6488.

Hashimoto, Y., Li, N., Yokoyama, H. & Ezaki, T. (1993). Complete nucleotide sequence and molecular characterization of ViaB region encoding Vi antigen in *Salmonella typhi*. *J Bacteriol* **175**, 4456-4465.

Hass, R., Bartels, H., Topley, N., Hadam, M., Köhler, L., Goppelt-Strübe, M. & Resch, K. (1989). TPA-induced differentiation and adhesion of U937 cells: Changes in ultrastructure, cytoskeletal organization and expression of cell surface antigens. *Eur J Cell Biol* **48**, 282-293.

Hatha, A. A. M. (1997). Prevalence of *Salmonella* in fish and crustaceans from markets in Coimbatore, South India. *Food Microbiol* **14**, 111-116.

Hayward, R. D. & Koronakis, V. (1999). Direct nucleation and bundling of actin by the SipC protein of invasive *Salmonella*. *EMBO J* **18**, 4926-4934.

Heffernan, E. J., Harwood, J., Fierer, J. & Guiney, D. (1992). The *Salmonella typhimurium* virulence plasmid complement resistance gene *rck* is homologous to a family of virulence-related outer membrane protein genes, including *pagC* and *ail*. *J Bacteriol* **174**, 84-91.

Heithoff, D. M., Conner, C. P. & Mahan, M. J. (1997). Bacterial infection as assessed by *in vivo* gene expression. *Proc Natl Acad Sci USA* **94**, 934-939.

Heithoff, D. M., Conner, C. P., Hentschel, U., Govantes, F., Hanna, P. C. & Mahan, M. J. (1999A). Coordinate intracellular expression of *Salmonella* genes induced during infection. *J Bacteriol* **181**, 799-807.

Heithoff, D. M., Sinsheimer, R. L., Low, D. A. & Mahan, M. J. (1999B). An essential role for DNA adenine methylation in bacterial virulence. *Science* **284**, 967-970.

Helander, I. M., Kilpelainen, I. & Vaara, M. (1994). Increased substitution of phosphate groups in lipopolysaccharides and lipid A of the polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium* - a ^{31}P-NMR study. *Mol Microbiol* **11**, 481-487.

Henrich, B., Monnerjahn, U. & Plapp, R. (1990). *dcp* gene of *Escherichia coli*: cloning, sequencing, transcript mapping, and characterization of the gene product. *J Bacteriol* **175**, 7290-7300.

Hensel, M., Shea, J. E., Baumler, A. J., Gleeson, C., Blattner, F. & Holden, D. W. (1997). Analysis of the boundaries of *Salmonella* Pathogenicity Island 2 and the corresponding chromosomal region of *Escherichia coli* K-12. *J Bacteriol* **179**, 1105-1111.

Hensel, M., Shea, J. E., Waterman, S. R., Mundy, R., Nikolaus, T., Banks, G., Vazqueztorres, A., Gleeson, C., Fang, F. C. & Holden, D. W. (1998). Genes encoding putative effector proteins of the Type III secretion system of *Salmonella* Pathogenicity Island 2 are required for bacterial virulence and proliferation in macrophages. *Mol Microbiol* **30**, 163-174.

Hermant, D., Menard, R., Arricau, N., Parsot, C. & Popoff, M. Y. (1995). Functional conservation of the *Salmonella* and *Shigella* effectors of entry into epithelial cells. *Mol Microbiol* **17**, 781-789.

Herrero, M., De Lorenzo, V. & Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J Bacteriol* **172**, 6557-6567.

Hersh, D., Monack, D. M., Smith, M. R., Ghori, N., Falkow, S. & Zychlinsky, A. (1999). The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. *Proc Natl Acad Sci USA* **96**, 2396-2401.

Higgins, I. M. & Sharp, P. M. (1988). CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene* **73**, 237-244.

Hilbert, F., del Portillo, F. G. & Groisman, E. A. (1999). A periplasmic D-alanyl-D-alanine dipeptidase in the gram-negative bacterium *Salmonella enterica*. *J Bacteriol* **181**, 2158-2165.

Hitchcock, P. J. & Brown, T. M. (1983). Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J Bacteriol* **54**, 269-277.

Hmiel, S. P., Snavely, M. D., Florer, J. B., Maguire, M. E. & Miller, C. G. (1989). Magnesium transport in *Salmonella typhimurium*: genetic characterisation and cloning of three magnesium transport loci. *J Bacteriol* **171**, 4742-4751.

Hofmann, K. & Stoffel, W. (1993). TMBASE - A database of membrane spanning protein segments (http://www.ch.embnet.org/cgi-bin/TMPRED_form_parse). *Biol Chem Hoppe-Seyler* **374**, 166.

Hohmann, E. L., Oletta, C. A., Killeen, K. P. & Miller, S. I. (1996A). PhoP/PhoQ-deleted *Salmonella typhi* (Ty800) is a safe and immunogenic single-dose typhoid fever vaccine in volunteers. *J Infect Dis* **173**, 1408-1414.

Hohmann, E. L., Oletta, C. A. & Miller, S. I. (1996B). Evaluation of a PhoP/PhoQ-deleted, *aroA*-deleted live oral *Salmonella typhi* vaccine strain in human volunteers. *Vaccine* **14**, 19-24.

Hone, D. M., Attridge, S. R., Forrest, B., Morona, R., Daniels, D., LaBrooy, J. T., Bartholomeusz, R. C., Shearman, D. J. & Hackett, J. (1988). A *galE* via (Vi antigen-negative) mutant of *Salmonella typhi* Ty2 retains virulence in humans. *Infect Immun* **56**, 1326-33.

Hone, D. M., Tacket, C. O., Harris, A. M., Kay, B., Losonsky, G. & Levine, M. M. (1992). Evaluation in volunteers of a candidate live oral attenuated *Salmonella typhi* vector vaccine. *J Clin Invest* **90**, 412-20.

Hone, D. M., Tacket, C. O., Levine, M. M., Hasday, J. & Harris, A. M. (1993). Induction of tumor necrosis factor secretion from U937 macrophage-like cells after infection with *Salmonella typhi*. *Vaccine* **93**, 43-47.

Hook, E. W. (1961). Salmonellosis: certain factors influencing the interaction of *Salmonella* and the human host. In *Bull NY Acad Med*, pp. 499.

Hook, E. W. (1990). 199. *Salmonella* species (including typhoid fever). In *In Principles and practice of infectious diseases*, pp. 1700-1716. Edited by Mardell et al. New York: Churchill Livingston.

Hopkins, S. (1997). Dendritic cells of the murine Peyer's patches colocalize with *Salmonella typhimurium* avirulent mutants in the subepithelial dome. *Adv Exp med Biol* **417**, 105-109.

Hornick, R. B., Greisman, S. E. & Wooward, T. E. (1970). Typhoid fever: pathogenesis and immunologic control. *New Eng J Med* **283**, 686-739.

Houng, H. H., Noon, K. F., Ou, J. T. & Baron, L. S. (1992). Expression of Vi antigen in *Escherichia coli* K-12: characterization of ViaB from *Citrobacter freundii* and identity of ViaA with RcsB. *J Bacteriol* **174**, 5910-5915.

Huan, P. T., Bastin, D. A., Whittle, B. L., Lindberg, A. A. & Verma, N. K. (1997A). Molecular characterization of the genes involved in O-antigen modification, attachment, integration and excision in *Shigella flexneri* bacteriophage SfV. *Gene* **195**, 217-227.

Huan, P. T., Whittle, B. L., Bastin, D. A., Lindberg, A. A. & Verma, N. K. (1997B). *Shigella flexneri* type-specific antigen V: cloning, sequencing and characterization of the glucosyl transferase gene of temperate bacteriophage SfV. *Gene* **195**, 207-216.

Huang, X.-Z., Tall, B., Schwan, W. R. & Kopecko, D. J. (1998). Physical limitations on *Salmonella typhi* entry into cultured human intestinal epithelial cells. *Infect Immun* **66**, 2928-2937.

Hueck, C. J., Hantman, M. J., Bajaj, V., Johnston, C., Lee, C. A. & Miller, S. I. (1995). *Salmonella typhimurium* secreted invasion determinants are homologous to *Shigella* Ipa proteins. *Mol Microbiol* **18**, 479-490.

Hueck, C. J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* **62**, 379-433.

Hughes, K. T. & Roth, J. R. (1988). Transitory cis complementation: A method for providing transposition functions to defective transposons. *Genetics* **119**, 9-12.

Ishibashi, Y. & Arai, T. (1990). Specific inhibition of phagosome-lysosome fusion in murine macrophages mediated by *Salmonella typhimurium* infection. *FEMS Microbiol Immunol* **64**, 35-44.

Ishibashi, Y. & Arai, T. (1996). A possible mechanism for host-specific pathogenesis of *Salmonella* serovars. *Microb Pathog* **21**, 435-446.

Islam, A., Butler, T., Kabir, I. & Alam, N. H. (1993). Treatment of typhoid fever with ceftriaxone for 5 days or chloramphenicol for 14 days: a randomized clinical trial. *Antimicrob Agents Chemo* **37**, 1572-1575.

Jensen, V. B., Harty, J. T. & Jones, B. D. (1998). Interactions of the invasive pathogens Salmonella typhimurium, Listeria monocytogenes, and Shigella flexneri with M cells and murine peyer's patches. *Infect Immun* **66**, 3758-3766.

Jiang, X.-M., Neal, B., Santiago, F., Lee, S. J., Romana, L. K. & Reeves, P. R. (1991). Structure and sequence of the rfb (O antigen) gene cluster of *Salmonella* serovar typhimurium (strain LT2). *Mol Microbiol* **5**, 695-713.

Jishage, M. & Ishihama, A. (1998). A stationary phase protein in *Escherichia coli* with binding activity to the major sigma subunit of RNA polymerase. *Proc Natl Acad Sci USA* **95**, 4953-4958.

Jishage, M. & Ishihama, A. (1999). Transcriptional organization and *in vivo* role of the *Escherichia coli rsd* gene, encoding the regulator of RNA polymerase sigma D. *J Bacteriol* **181**, 3768-3776.

Johnson, E. M., Krauskopf, B. & Baron, L. S. (1965). Genetic mapping of Vi and somatic antigenic determinants in *Salmonella*. *J Bacteriol* **90**, 302-308.

Johnston, C., Pegues, D. A., Hueck, C. J., Lee, A. & Miller, S. I. (1996). Transcriptional activation of *Salmonella typhimurium* invasion genes by a member of the phosphorylated response-regulator superfamily. *Mol Microbiol* **22**, 715-727.

Jones, B. D. & Falkow, S. (1994). Identification and characterization of a *Salmonella typhimurium* oxygen-regulated gene required for bacterial internalization. *Infect Immun* **62**, 3745-3752.

Jones, B. D., Ghori, N. & Falkow, S. (1994). *Salmonella typhimurium* intitiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *J Exp Med* **180**, 15-23.

Jones, B. D. (1996). Salmonellosis: Host immune responses and bacterial virulence determinants. *Ann Rev Immun* **14**, 533-561.

Jones, B. D., Nichols, W. A., Gibson, B. W., Sunshine, M. G. & Apicella, M. A. (1997). Study of the role of the *htrB* Gene in *Salmonella typhimurium* virulence. *Infect Immun* **65**, 4778-4783.

Jourdan, A. D. & Stauffer, G. V. (1998). Mutational analysis of the transcriptional regulator GcvA: amino acids important for activation, repression and DNA binding. *J Bacteriol* **180**, 4865-4871.

Kaatz, G. W., Seo, S. M. & Ruble, C. A. (1993). Efflux-mediated fluoroquinolone resistance in *Staphylococcus aureus*. *Antimicrob Agents Chemother* **37**, 1086-1094.

Kagan, B. L., Selsted, M. E., Ganz, T. & Lehrer, R. I. (1990). Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. *Proc Natl Acad Sci USA* **87**, 210-214.

Kaniga, K., Bossio, J. C. & Galán, J. E. (1994). The *Salmonella typhimurium* invasion genes *invF* and *invG* encode homologues of the AraC and PulD family of proteins. *Mol Microbiol* **13**, 555-568.

Kaniga, K., Trollinger, D. & Galán, J. E. (1995A). Identification of two targets of the Type III protein secretion system encoded by the *inv* and *spa* loci of *Salmonella typhimurium* that have homology to the *Shigella* IpaD and IpaA proteins. *J Bacteriol* **177**, 7078-7085.

Kaniga, K., Tucker, S., Trollinger, D. & Galán, J. E. (1995B). Homologs of the *Shigella* IpaB and IpaC invasins are required for *Salmonella typhimurium* entry into cultured epithelial cells. *J Bacteriol* **177**, 3965-3971.

Karmaker, S., Biswas, D., Shaikh, N. M., Chatterjee, S. K., Kataria, V. K. & Kumar, R. (1991). Role of a large plasmid of *Salmonella typhi* encoding multiple drug resistance. *J Med Microbiol* **34**, 149-151.

Kasahara, M., Nakata, A. & Shinagawa, H. (1991). Molecular analysis of the *Salmonella typhimurium phoN* gene, which encodes nonspecific acid phosphatase. *J Bacteriol* **173**, 6760-6765.

Kasahara, M., Nakata, A. & Shinagawa, H. (1992). Molecular analysis of the *Escherichia coli phoP-phoQ* operon. *J Bacteriol* **174**, 492-498.

Kauffmann, F. (1954). *Enterobacteriaceae*, 2nd edn. Copenhagen: Munksgaard.

Keitel, W. A., Bond, N. L., Zahradnick, J. M., Cramton, T. A. & Robbins, J. B. (1994). Clinical and serological responses following primary and booster immunization with *Salmonella typhi* Vi capsular polysaccharide vaccines. *Vaccine* **12**, 195-199.

Khan, S. A., Everest, P., Servos, S., Foxwell, N., Zahringer, U., Brade, H., Rietschel, E. T., Dougan, G., Charles, I. G. & Maskell, D. J. (1998). A lethal role for lipid A in *Salmonella* infections. *Mol Microbiol* , 571-579.

Kier, L. D., Weppelman, R. & Ames, B. N. (1977). Regulation of two phosphatases and a cyclic phosphodiesterase of *Salmonella typhimurium*. *J Bacteriol* **130**, 420-428.

Kier, L. D., Weppelman, R. M. & Ames, B. N. (1979). Regulation of nonspecific acid phosphatase in *Salmonella*: *phoN* and *phoP* genes. *J Bacteriol* **138**, 155-161.

Klein, J. B., Schepers, T. M., Dean, W. L., Sonnenfeld, G. & McLeish, K. R. (1990). Role of intracellular calcium concentration and protein kinase C activation in IFN-gamma stimulation of U937 cells. *J Immunol* **144**, 4305-4311.

Klena, J. D., Ashford, R. S. & Schnaitman, C. A. (1992). Role of *Escherichia coli* K-12 *rfa* genes and the *rfp* gene of *Shigella dysenteriae* 1 in the generation of lipopolysaccharide core heterogeneity and attachment of O antigen. *J Bacteriol* **174**, 7297-7303.

Kohbata, S., Yokoyama, H. & Yabuuchi, E. (1986). Cytopathogenic effect of Salmonella typhi GIFU 10007 on M cells of murine ilial Peyer's patches in ligated ileal loops: An ultrastructural study. *Microbiol Immun* **30**, 1225-1237.

Kossaczka, Z., Bystricky, S., Bryla, D. A., Shiloach, J., Robbins, J. B. & Szu, S. C. (1997). Synthesis and immunological properties of Vi and Di-*O*-acetyl pectin protein conjugates with adipic acid dihydrazide as the linker. *Infect Immun* **65**, 2088-2093.

Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Laratejero, M., Sukhan, A., Galán, J. E. & Aizawa, S. (1998). Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science* **280**, 602-605.

Kwon, Y. M. & Ricke, S. C. (1998). Induction of acid resistance of *Salmonella typhimurium* by exposure to short-chain fatty acids. *App Env Microbiol* **64**, 3458-3463.

Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**, 105-132.

Lacraz, S., Dayer, J.-M., Nicod, L. & Welgus, H. G. (1994). 1,25-Dihydroxyvitamin D_3 dissociates production of interstitial collagnase and 92-kDa gelatinase in human mononuclear phagocytes. *J Biol Chem* **269**, 6485-6490.

Lan, R. & Reeves, P. R. (1996). Gene transfer is a major factor in bacterial evolution. *Mol Biol Evol* **13**, 47-55.

Landick, R. & Oxender, D. L. (1985). The complete nucleotide sequences of the *Escherichia coli* LIV-BP and LS-BP genes. Implications for the mechanism of high-affinity branched-chain amino acid transport. *J Biol Chem* **260**, 8257-8261.

Landy, M. (1954). Studies on Vi antigen. VI. Immunization of human beings with purified Vi antigen. *Am J Hyg* **60**, 52-62.

Lange, R. & Hengge-Aronis, R. (1991). Identification of a central regulator of stationary phase gene expression *Escherichia coli*. *Mol Micro* **5**, 49-59.

Latreille, P. (2000). The sequence of *Salmonella typhimurium* fragment STMF1 (I-Ceu-F). In *The Salmonella typhimurium genome sequencing project*. St Louis: Washington University Genome Sequencing Center.

Le Minor, L., Veron, M. & Popoff, M. Y. (1982). Taxonomie des *Salmonella*. *Ann Microbiol* **133B**, 223-243.

Le Minor, L. & Popoff, M. Y. (1987). Designation of *Salmonella enterica* sp. nov.. nom. rev.. as the type and only species of the genus *Salmonella*. *J Int Syst Bactiol* **37**, 465-468.

Le Minor, L. & Popoff, M. Y. (1988). *Antigenic formulas of the Salmonella serovars*, 5th edn. Paris, France: WHO Collaborative Cebtre for reference and research on *Salmonella*, Institute Pasteur.

Leclerc, G. J., Tartera, C. & Metcalf, E. S. (1998). Environmental regulation of *Salmonella typhi* invasion-defective muatants. *Infect Immun* **66**, 682-691.

Lee, C. A., Jones, B. D. & Falkow, S. (1992). The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. *Proc Natl Acad Sci USA* **87**, 4304-4308.

Lee, I. S., Slonczewski, J. L. & Foster, J. W. (1994). A low-pH-inducible, stationary-phase acid tolerance response in *Salmonella typhimurium*. *J Bacteriol* **176**, 1422-1426.

Lee, I. S., Lin, S. J., Hall, H. K., Bearson, B. & Foster, J. W. (1995). The stationary-phase sigma factor σ^{38} (RpoS) is required for a sustained acid tolerance response in virulent *Salmonella typhimurium*. *Mol Microbiol* **17**, 155-167.

Lehrer, R. I., Lichtenstein, A. K. & Ganz, T. (1993). Defensins: Antimicrobial and cytotoxic peptides of mammalian cells. *Ann Rev Immunol* **11**, 105-128.

Lerner, C. G. & Inouye, M. (1990). Low copy number plasmids for regulated low-level expression of cloned genes in *Escherichia coli* with blue/white insert screening capability. *Nucleic Acids Res* **18**, 46.

Lesse, A. J., Campagnari, A. A., Bittner, W. E. & Apicella, M. A. (1990). Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis. *J Immunol Methods* **126**, 109-117.

Levine, M. M., Ferreccio, C., Abrego, P., Martin, O. S., Ortiz, E. & Cryz, S. (1999). Duration of efficacy of Ty21a, attenuated *Salmonella typh*i live oral vaccine. *Vaccine* **17**, 22-27.

Li, J., Ochman, H., Groisman, E. A., Boyd, E. F., Solomon, F., Nelson, K. & Selander, R. K. (1995). Relationship between evolutionary rate and cellular location among the Inv/Spa invasion proteins of *Salmonella enterica*. *Proc Natl Acad Sci USA* **92**, 7252-7256.

Libby, S. J., Goebel, W., Ludwig, A., Buchmeier, N., Bowe, N., Fang, F. C., Guiney, D. G., Songer, J. G. & Heffron, F. (1994). A cytolysin encoded by *Salmonella* is required for survival within macrophages. *Proc Natl Acad Sci USA* **91**, 489-493.

Libby, S. J., Goebel, W., Muir, S., Songer, G. & Heffron, F. (1990). Cloning and characterization of a cytotoxin gene from *Salmonella typhimurium*. *Res Microbiol* **141**, 775-783.

Lin, J., Lee, I. S., Frey, J., Slonczewski, J. L. & Foster, J. W. (1995). Comparitive analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. *J Bacteriol* **177**, 4097-4103.

Liu, S. L., Ezaki, T., Miura, H., Matsui, K. & Yabuuchi, E. (1988). Intact motility as a *Salmonella typhi* invasion-related factor. *Infect Immun* **56**, 1967-1973.

Ludwig, A., Bauer, S., Benz, R., Bergmann, B. & Goebel, W. (1999). Analysis of the SlyA-controlled expression, subcellular localization and pore-forming activity of a 34 kDa haemolysin (ClyA) from Escherichia coli K-12. *Mol Microb* **31**, 557-567.

Lugtenberg, B., Meijers, J., Peters, R., van der Hoek, P. & van Alphen, L. (1975). Electrophoretic resolution of the major outer membrane protein of *Escherichia coli* into four bands. *FEBS Lett* **58**, 254-258.

Lundberg, U., Vinatzer, U., Berdnik, D., von Gabain, A. & Baccarini, M. (1999). Growth phase-regulated induction of *Salmonella*-induced macrophage apoptosis correlates with transient expression of SPI-1 genes. *J Bacteriol* **181**, 3433-3437.

Lynn, W. A. & Golenbock, D. T. (1992). Lipopolysaccharide antagonists. *Immunol Today* **13**, 271-276.

MacFarlane, A. S., Schwacha, M. G. & Eisenstein, T. K. (1999A). In vivo blockage of nitric oxide with aminoguanidine inhibits immunosuppression induced by an attenuated strain of *Salmonella typhimurium*, potentiates *Salmonella* infection, and inhibits macrophage and polymorphonuclear leukocyte influx into the spleen. *Infect Immun* **67**, 891-898.

Macfarlane, E. L. A., Kwasnicka, A., Ochs, M. M. & Hancock, R. E. W. (1999B). PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. *Mol Microbiol* **34**, 305-316.

Makela, P. H., Sarvas, M., Calcagno, S. & Lounatmaa, K. (1978). Isolation and characterization of polymyxin-resistant mutants of *Salmonella*. *FEMS Microbiol* **3**, 323-326.

Makino, K., Shinagawa, H., Amemura, M. & Nakata, A. (1986A). Nucleotide sequence of the *phoB* gene, the positive regulatory gene for the phosphate regulon of *Escherichia coli* K-12. *J Mol Biol* **190**, 37-44.

Makino, K., Shinagawa, H., Amemura, M. & Nakata, A. (1986B). Nucleotide sequence of the *phoR* gene, a regulatory gene for the phosphate regulon of *Escherichia coli* K-12. *J Mol Biol* **192**, 549-556.

Mavris, M., Manning, P. A. & Morona, R. (1997). Mechanism of bacteriophage SfII-mediated serotype conversion in *Shigella flexneri*. *Mol Microbiol* **26**, 939-950.

Mazel, D., Coic, E., Blanchard, S., Saurin, W. & Marliere, P. (1997). A survey of polypeptide deformylase function throughout the eubacterial lineage. *J Mol Biol* **266**, 939-949.

McCarthy, M. P. (1987). Typhoid and the politics of public health in nineteenth-century Philadelphia. In *Memoirs of the American Philosophical Society*, pp. 1-10. Philadelphia: American Philosophical Society.

McCormick, B. A., Colgan, S. P., Delparcher, C., Miller, S. I. & Madara, J. L. (1993). *Salmonella typhimurium* attachment to human intestinal epithelial monolayers - transcellular signalling to subepithelial neutrophils. *J Cell Biol* **123**, 895-907.

McCormick, B. A., Miller, S. I., Carnes, D. & Madara, J. L. (1995). Transepithelial signaling to neutrophils by *Salmonellae* - a novel virulence mechanism for gastroenteritis. *Infect Immun* **63**, 2302-2309.

McCormick, B. A., Parkos, C. A., Colgan, S. P., Carnes, D. K. & Madara, J. L. (1998). Apical secretion of a pathogen-elicited epithelial chemoattractant (PEEC) activity in response to surface colonization of intestinal epithelia by *Salmonella typhimurium*. *J Immunol* **160**, 455-456.

Menon, N. K., Robbins, J., Peck, J. H. D., Chatelus, C. Y., Choi, S. & Przybyla, A. E. (1990). Cloning and sequencing of a putative *Escherichia coli* (NiFe) hydrogenase-1 operon containing 6 open reading frames. *J Bacteriol* **172**, 1969-1977.

Menon, N. K., Robbins, J., Wendt, J. C., Shanmuam, K. T. & Przybyla, A. E. (1991). Mutational analysis and characterization of the *E. coli hya* operon which encodes [NiFe] hydrogenase 1. *J Bacteriol* **173**, 4851-4861.

Merson-Davies, L. A. & Cundliffe, E. (1994). Analysis of five tylosin biosynthetic genes from the tyllBA region of the *Streptomyces fradiae* genome. *Mol Microbiol* **13**, 349-355.

Metcalf, W. W., Steed, P. M. & Wanner, B. L. (1990). Identification of phosphate starvation-inducible genes in *Escherichia coli* K-12 by DNA sequence analysis of *psi*::*lacZ*(Mu d1) transcriptional fusions. *J Bacteriol* **172**, 3191-3200.

Miller, C. G. & Hamilton, S. (1992). Cloning and nucleotide sequence of the *Salmonella typhimurium dcp* gene encoding dipeptidyl carboxypeptidase. *J Bacteriol* **174**, 1626-1630.

Miller, J. H. (1972). Experiments in molecular genetics. In *Cold Spring Harbor*. New York: Cold Spring Harbor Laboratory Press.

Miller, L. J., Schwarting, R. & Springer, T. A. (1986). Regulated expression of the Mac-1, LFA-1, p150,95 glycoprotein family during leukocyte differentiation. *J Immunol* **137**, 2891-2990.

Miller, S. I., Kukral, A. M. & Mekalanos, J. J. (1989). A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. *Proc Natl Acad Sci U S A* **86**, 5054-5058.

Miller, S. I. & Mekalanos, J. J. (1990). Constitutive expression of the *phoP* regulon attenuates *Salmonella virulence* and survival within macrophages. *J Bacteriol* **172**, 2485-2490.

Miller, S. I., Pulkkinen, W. S., Selsted, M. E. & Mekalanos, J. J. (1990). Characterization of defensin resistance phenotypes associated with mutations in the *phoP* virulence regulon of *Salmonella typhimurium*. *Infect Immun* **58**, 3706-3710.

Miller, S. I. (1991). PhoP/PhoQ: macrophage-specific modulators of *Salmonella* virulence? *Mol Microbiol* **5**, 2073-2078.

Miller, S. I., Loomis, W. P., Alpuche Aranda, C., Behlau, I. & Hohmann, E. (1993). The PhoP virulence regulon and live oral *Salmonella* vaccines. *Vaccine* **11**, 122-125.

Miller, V. L., Beer, K. B., Loomis, W. P., Olson, J. A. & Miller, S. I. (1992). An unusual *pagC*::Tn*phoA* mutation leads to an invasion and virulence-defective phenotype in *Salmonellae*. *Infect Immun* **60**, 3763-3770.

Mills, D. M., Bajaj, V. & Lee, C. A. (1995). A 40 kb chromosomal fragment encoding *Salmonella typhimurium* invasion genes is absent from the corresponding region of the *Escherichia coli* K-12 chromosome. *Mol Microbiol* **15**, 749-759.

Mills, S. D. & Finlay, B. B. (1994). Comparison of *Salmonella typhi* and *Salmonella typhimurium* invasion, intracellular growth and localization in cultured human epithelial cells. *Microb Pathog* **17**, 409-423.

Mills, S. D. & Finlay, B. B. (1998). Isolation and characterization of *Salmonella typhimurium* and *Yersinia pseudotuberculosis*-containing phagosomes from infected mouse macrophages - *Y. Pseudotuberculosis* traffics to terminal lysosomes where they are degraded. *Eur J Cell Biol* **77**, 35-47.

Mills, S. D., Ruschkowski, S. R., Stein, M. A. & Finlay, B. B. (1998). Trafficking of porin-deficient *Salmonella typhimurium* mutants inside HeLa cells: OmpR and EnvZ mutants are defective for the formation of *Salmonella*-induced filaments. *Infect Immun* **66**, 1806-1811.

Minta, J. O. & Pambrun, L. (1985). In vitro induction of cytologic and functional differentiation of the immature human monocyte-like cell line U-937 with phorbol myristate acetate. *Am J Pathol* **119**, 111-126.

Mirold, S., Rabsch, W., Rohde, M., Stender, S., Tschape, H., Russmann, H., Igwe, E. & Hardt, W. D. (1999). Isolation of a temperate bacteriophage encoding the Type III effector protein SopE from an epidemic *Salmonella typhimurium* strain. *Proc Natl Acad Sci USA* **96**, 9845-9850.

Mizuno, T., Wurtzel, E. T. & Inouye, M. (1982). Osmoregulation of gene expresion. - II. DNA sequence of the *envZ* gene of the *ompB* operon of *Escherichia coli* and characterization of its gene product. *J Biol Chem* **257**, 13692-13698.

Monack, D. M., Raupach, B., Hromockyj, A. E. & Falkow, S. (1996). *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. *Proc Natl Acad Sci USA* **93**, 9833-9838.

Moncrief, M. B. C. & Maguire, M. E. (1998). Magnesium and the role of *mgtC* in growth of *Salmonella typhimurium*. *Infect Immun* **66**, 3802-3809.

Moreno, E., Mayer, H. & Moriyon, I. (1987). Characterization of a native polysaccharide hapten from *Brucella melitensis*. *Infect Immun* **55**, 2850-2853.

Morona, R., van den Bosch, L. & Manning, P. A. (1995). Molecular, genetic and topological characterization of O-antigen chain length regulation in *Shigella flexneri*. *J Bacteriol* **177**, 1059-1068.

Munford, R. S. & Hall, C. L. (1986). Detoxification of bacterial lipopolysaccharides (endotoxin) by a human neutrophil enzyme. *Science* **1986**, 1035-1063.

Murphy, C. K. & Beckwith, J. (1996). Export of proteins to the cell envelope in *Escherichia coli*. In *Escherichia coli and Salmonellae: cellular and molecular biology*, pp. 967-978. Edited by F. C. Neidhardt, I. Curtiss, R., J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, M. Riley, W. S. Reznikoff, M. Schaechter & H. E. Umbarger. Washington, D. C.: ASM Press.

Murray, B. E., Mathewson, J. J., DuPont, H. L., Ericsson, C. D. & Reves, R. R. (1990). Emergence of resistant fecal *Escherichia coli* in travelers not taking prophylactic antimicrobial agents. *Antimicrob Agents Chemo* **34**, 515-518.

Nambu, M., Morita, M., Watanabe, H., Ulenoyama, Y., Kim, K. M., Tanaka, M., Iwai, Y., Kimata, H., Mayumi, M. & Mikawa, H. (1989). Regulation of Fc gamma receptor expression and phagocytosis of a human monoblast cell line U937. Participation of cAMP and protein kinase C in the effects of IFN-gamma and phorbol ester. *J Immunol* **143**, 4158-4165.

Neyfakh, A. A., Bidnenko, V. E. & Chen, L. B. (1991). Efflux-mediated multidrug resistance in *Bacillus subtilis*: similarities and dissimilarities with the mammalian system. *Proc Natl Acad Sci USA* **88**, 4781-4785.

Nickerson, C. A. & Curtiss, R. (1997). Role of sigma factor RpoS in initial stages of *Salmonella typhimurium* infection. *Infect Immun* **65**, 1814-1823.

Nielsen, H., Engelbrecht, J., Brunak, S. & von Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering* **10**, 1-6.

Nikaido, H. (1976). Outer membrane of *Salmonella typhimurium*: transmembrane diffusion of some hydrophobic substances. *Biochem Biophys Acta* **433**, 118-132.

Nnalue, N. A., Shnyra, A., Hultenby, K. & Lindberg, A. A. (1992). *Salmonella choleraesuis* and *Salmonella typhimurium* associated with liver cells after intravenous innoculation of rats are localized mainly in Kupffer cells and multiply intracellularly. *Infect Immun* **60**, 2758-2768.

Nummila, K., Kipelainen, I., Zahringer, U., Vaara, M. & Helander, I. M. (1995). Lipopolysaccharides of polymyxin B-resistant mutants of *Escherichia coli* are extensively substituted by 2-aminoethyl pyrophosphate and contain aminoarabinose in lipid A. *Mol Microbiol* **16**, 271-278.

Ochman, H., Gerber, A. S. & Hartl, D. L. (1988). Genetic applications of an inverse polymerase chain reaction. *Genetics* **120**, 621-623.

Ochman, H. & Groisman, E. A. (1996). Distribution of pathogenicity islands in *Salmonella spp. Infect Immun* **64**, 5410-2.

Ochman, H., Soncini, F. C., Solomon, F. & Groisman, E. A. (1996). Identification of a pathogenicity island required for *Salmonella* survival in host cells. *Proc Natl Acad Sci USA* **93**, 7800-7804.

Oh, Y. K., Alpuche Aranda, C., Berthiaume, E., Jinks, T., Miller, S. I. & Swanson, J. A. (1996). Rapid and complete fusion of macrophage lysosomes with phagosomes containing *Salmonella typhimurium. Infect Immun* **64**, 3877-3883.

Okeke, I. N., Lamikanra, A. & Edelman, R. (1999). Socioeconomic and behavioral factors leading to aquired bacterial resistance to antibiotics in developing countries. *Emerg Infect Dis* **5**, 18-27.

Old, D. C. (1990). *Salmonella*. In *Topley and Wilson's principles of bacteriology, virology and immunity*, pp. 469-493. Edited by D. BI. London: Arnold.

Olsen, E. R., Dunyak, D. S., Jurss, L. M. & Poorman, R. A. (1991). Identification and characterisation of *dppA*, an *Escherichia coli* gene encoding a periplasmic dipeptide transport protein. *J Bacteriol* **173**, 234-244.

Pang, T., Bhutta, Z. A., Finlay, B. B. & Altwegg, M. (1995). Typhoid fever and other salmonellosis: a continuing challenge. *TIM* **3**, 253-255.

Parra Lopez, C., Baer, M. T. & Groisman, E. A. (1993). Molecular genetic analysis of a locus required for resistance to antimicrobial peptides in *Salmonella typhimurium*. *Embo J* **12**, 4053-62.

Paulsen, I. T. & Skurray, R. A. (1993). Topological, structure and evolution of two families of proteins involved in antibiotic and antiseptic resistance in eukaryotes and prokaryotes-an analysis. *Gene* **124**, 1-11.

Paulsen, I. T., Brown, M. H. & Skurray, R. (1996). Proton-dependent multidrug efflux systems. *Microbiolog Rev* **60**, 575-608.

Pearlman, E., Jiwa, A. H., Engleberg, N. C. & Eisenstein, B. I. (1988). Growth of *Legionella pneumophila* in a human macrophage-like (U937) cell line. *Microbial Pathog* **5**, 37-95.

Pedrinaci, S., Huelin, C., Patarroya, M., Ruiz Cabello, F. & Garrido, F. (1989). Studies on CD11a and CD18 molecules with two new monoclonal antibodies: differential myelomonocytic antigen expression of PMA treated HL60 and U937 cell lines. *Hybridoma* **8**, 13-23.

Pegues, D. A., Hantman, M. J., Behlau, I. & Miller, S. I. (1995). PhoP/PhoQ transcriptional repression of *Salmonella typhimurium* invasion genes: evidence for a role in protein secretion. *Mol Microbiol* **17**, 169-81.

Penheiter, K. L., Mathur, N., Giles, D., Fahlen, T. & Jones, B. D. (1997). Non-invasive *Salmonella typhimurium* mutants are avirulent because of an inability to enter and destroy M cells of ilieal Peyer's patches. *Mol Microbiol* **24**, 697-709.

Peterson, W. L., Mackowiak, P. A., Barnett, C. C., Marling-Cason, M. & Haley, M. L. (1989). The human gastric bactericidal barrier: mechanisms of action, relative antibacterial activity, and dietary influences. *J Infect Dis* **159**, 979-983.

Pickard, D., Li, J., Roberts, M., Maskell, D., Hone, D., Levine, M., Dougan, G. & Chatfield, S. (1994). Characterization of defined *ompR* mutants of *Salmonella typhi*: *ompR* is involved in the regulation of Vi polysaccharide expression. *Infect Immun* **62**, 3984-3993.

Pier, G. B., Grout, M., Zaidi, T., Meluleni, G., Mueschenborn, S. S., Banting, G., Ratcliff, R., Evans, M. J. & Colledge, W. H. (1998). *Salmonella typhi* uses CFTR to enter intestinal epithelial cells. *Nature* **393**, 79-82.

Pohlman, T. H., Munford, R. S. & Harlan, J. M. (1987). Deacylated lipopolysaccharide inhibits neutrophil adherence to endothelium induced by lipopolysaccharide *in vitro*. *J Exp Med* **165**, 1393-1402.

Poltorak, A., Ricciardi-Castagnoli, P., Citterio, S. & Beutler, B. (2000). Physical contact between lipopolysaccharide and Toll-like receptor 4 revealed by genetic complementation. *Proc Natl Acad Sci USA* **97**, 2163-2167.

Porter, E. M., Vandam, E., Valore, E. V. & Ganz, T. (1997). Broad-spectrum antimicrobial activity of human intestinal defensin 5. *Infect Immun* **65**, 2396-2401.

Prasad, R., Chopra, A. K., Chary, P. & Peterson, J. W. (1992). Expression and characterization of the cloned *Salmonella typhimurium* enterotoxin. *Microb Pathog* **13**, 109-121.

Priess, H., Brauer, B., Schmidt, C. & Kamp, D. (1987). Sequence of the left end of Mu. In *Phage Mu*, pp. 277-296. Edited by N. Symonds, A. Toussaint, P. Van de Putte & M. M. Howe. New York: Cold Spring Harbor Laboratory.

Prince, R. W., Xu, Y., Libby, S. J. & Fang, F. C. (1994). Cloning and sequencing of the gene encoding the RpoS (KatF) σ factor from *Salmonella typhimurium* 14028s. *Biochem Biophys Acta* **1219**, 198-200.

Pugin, J., Schurer, M., C. C., Leturcq, D., Moriarty, A., Ulevitch, R. J. & Tobias, P. S. (1993). Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. *Proc Natl Acad Sci USA* **90**, 2744-2748.

Pugsley, A. P. (1993). The complete general secretory pathway in gram-negative bacteria. *Microbiol Rev* **57**, 50-108.

Pulkkinen, W. S. & Miller, S. I. (1991). A *Salmonella typhimurium* virulence protein is similar to a *Yersinia enterocolitica* invasion protein and a bacteriophage lambda outer membrane protein. *J Bacteriol* **173**, 86-93.

Qi, S.-Y., Li, Y., Szyroki, A., Giles, I. G., Moir, A. & O'Conner, C. D. (1995). *Samonella typhimurium* responses to a bactericidal protein from human neutrophils. *Mol Microbiol* **17**, 523-531.

Radzun, H. J., Parwaresch, M. R., Sundstrom, C., Nilsson, K. & Eissner, M. (1983). Monocytic origin of the human hematopoitic cell line U-937 and its convertibility to macrophages evidenced by isoenzyme mapping. *Int J Cancer* **31**, 181-186.

Raetz, C. R. (1996). Bacterial lipopolysaccharides: a remarkable family of bioactive macroamphiphiles. In *Escherichia coli and Salmonella. Cellular and Microbiology*, pp. 1035-1063. Edited by F. C. Neidhardt, R. I. Curtiss, J. L. Ingraham, E. C. C. Lin & K. B. Low. Washington: ASM Press.

Rakeman, J. L., Bonifield, H. R. & Miller, S. I. (1999). A HilA-independent pathway to *Salmonella typhimurium* invasion gene transcription. *J Bacteriol* **181**, 3096-3104.

Ralph, P., Moore, M. A. & Nillson, K. (1976). Lysozyme syntheis by established human and murine hystiocytic lymphoma cell lines. *J Exp Med* **143**, 1528-1533.

Rana, F. R., Macias, E. A., Sultany, C. M., Modzrakowski, M. C. & Blazyk, J. (1991). Interactions between magainin 2 and *Salmonella typhimurium* outer membranes: effect of lipopolysaccharide structure. *Biochem* **30**, 5858-5866.

Rathman, M., Barker, L. P. & Falkow, S. (1997). The unique trafficking pattern of *Salmonella typhimurium*-containing phagosomes in murine macrophages is independent of the mechanism of bacterial entry. *Infect Immun* **65**, 1475-1485.

Reeves, M. W., Evins, G. M., Heiba, B. D., Plikaytis, B. D. & Farmer III, J. J. (1989). Clonal nature of *Salmonella typhi* and its gentic relatedness to other *Salmonellae* as shown by multilocus enzyme electrophoresis, and proposal of *Salmonella bongori* comb. nov. *J Clin Microbiol* **27**, 313-320.

Reinhart, R. A. (1988). Magnesium metabolism: a review with special refererence to intracellular content and serum levels. *Arch Intern Med* **148**, 2415-2420.

Rhen, M., Riikonen, P. & Taira, S. (1993). Transcriptional regulation of *Salmonella* enterica virulence plasmid genes in cultured macrophages. *Mol Microbiol* **10**, 45-56.

Rivolta, C., Soldo, B., Lazarevic, V., Joris, b., Mauel, C. & Karamata, D. (1998). A 35.7 kb DNA fragment from the *Bacillus subtilis* chromosome containing a putative 12.3 kb operon involved in hexuronate catabolism and a perfectly symmetrical hypothetical catabolite-responsive element. *Microbiol* **144**, 877-884.

Robbe-Saule, V., Coynault, C. & Norel, F. (1995). The live oral typhoid vaccine Ty21a is a *rpoS* mutant and susceptible to various environmental stresses. *FEMS Microbiol Lett* **126**, 171-176.

Robbins, J. D. & Robbins, J. B. (1984). Reexamination of the protective role of the capsular polysaccharide (Vi antigen) *Salmonella typhi*. *J Infect Dis* **150**, 436-449.

Rocchetta, H. L., Pacan, J. C. & Lam, J. S. (1998). Synthesis of the A-band polysaccharide sugar D-rhamnose requires Rmd and WbpW: identification of multiple AlgA homologues, WbpW and ORF488, in *Pseudomonas aeruginosa*. *Mol Microbiol* **29**, 1419-1434.

Roland, K. L., Martin, L. E., Esther, C. R. & Spitznagel, J. K. (1993). Spontaneous *pmrA* mutants of *Salmonella typhimurium* LT2 define a new two-component regulatory system with a possible role in virulence. *J Bacteriol* **175**, 4154-64.

Roland, K. L., Esther, C. R. & Spitznagel, J. K. (1994). Isolation and characterization of a gene, *pmrD*, from *Salmonella typhimurium* that confers resistance to polymyxin when expressed in multiple copies. *J Bacteriol* **176**, 3589-3597.

Roudier, C., Krause, M., Fierer, J. & Guiney, D. G. (1990). Correlation between the presence of sequences homologous to the *vir* region of the *Salmonella dublin* plasmid pSDL2 and the virulence of twenty-two Salmonella serotypes in mice. *Infect Immun* **58**, 1180-1185.

Rowe, B., Ward, L. R. & Threlfall, E. J. (1991). Treatment of multiresistant typhoid fever. *Lancet* **337**, 1422.

Royer-Pokora, B., Kunkel, L. M., Monaco, A. P., Goff, S. C., Newburger, P. E., Baehner, R. L., Cole, F. S., Curnutte, J. T. & Orkin, S. H. (1986). Cloning the gene for an inherited human disorder-chronic granulomatous disease - on the basis of its chromosomal nature. *Nature (London)* **322**, 32-38.

Rubin, R. H. & Weinstein, L. (1977). *Salmonellosis: Microbiologic, pathologic, and clinical features.* New York: Stratton Intercontinental Medical Book Corporation.

Ryan, C. a., Hargrett-Bean, N. T. & Blake, P. A. (1989). *Salmonella typhi* infections in the United States, 1975-1984: increasing role of foreign travel. *Rev Infect Dis* **11**, 1-8.

Saiki, R. K. & Gelfand, D. H. (1989). Introducing AmpliTaq DNA polymerase. *Amplification* **1**, 4-6.

Salmond, P. C. & Reeves, P. J. (1993). Membrane traffic wardens and protein secretion in Gram-negative bacteria. *TIB* **18**, 7-12.

Sanderson, K. F. & Roth, J. R. (1988). Linkage map of Salmonella typhimurium, edition VII. *Microbiol Rev* **52**, 485-532.

Sanderson, K. E., Hessel, A. & Rudd, K. E. (1995). Genetic map of *Salmonella typhimurium*, Edition VIII. *Microbiol Rev* **59**, 241-303.

Sanger Centre (1999). *Salmonella typhi* sequencing project. *EMBL/Genbank* **Web address: ftp://ftp.sanger.ac.uk/pub/pathogens/st**.

Schmidt, G., Jann, B. & Jann, K. (1969). Immunochemistry of R lipopolysaccharide biosynthesis in enteric bacteria. *Microbiol Rev* **57**, 655-682.

Schnaitman, C. A. & Klena, J. D. (1993). Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiol Rev* **57**, 655-682.

Schneider, K. & Beck, C. F. (1986). Promoter-probe vectors for the analysis of divergently arranged promoters. *Gene* **42**, 37-48.

Schwan, W. R., Huang, X.-Z., Hu, L. & Kopecko, D. J. (2000). Differential bacterial survival, replication, and apoptosis-inducing ability of *Salmonella* serovars within human and murine macrophages. *Infect Immun* **68**, 1005-1013.

Scott, M. G., Yan, H. & Hancock, R. E. W. (1999). Biological properties of structurally related α-helical cationic antimicrobial peptides. *Infect Immun* **67**, 2005-2009.

Segal, A. W. & Abo, A. (1993). The biochemical basis of the NADPH oxidase of phagocytes. *Trends Biochem Sci* **18**, 43-47.

Selsted, M. E., Miller, S. I., Henschen, A. H. & Ouellette, A. J. (1992). Enteric defensins: antibiotic peptide components of intestinal host defense. *J Cell Biol* **118**, 929-36.

Sessa, G., Freer, J. H., Colacicco, G. & Weissman, G. (1969). *J Biol Chem* **244**, 3575-3582.

Shafer, W. M., Martin, L. E. & Spitnagel, J. K. (1984). Cationic antimicrobial proteins isolated from human neutrophil granulocytes in the presence of diisopropyl fluorophosphate. *Infect Immun* **45**, 29-35.

Shea, J. E., Hensel, M., Gleeson, C. & Holden, D. W. (1996). Identification of a virulence locus encoding a second Type III secretion system in Salmonella typhimurium. *Proc Natl Acad Sci USA* **93**, 2593-2597.

Shyamala, V. & Ames, G. F. (1993). Single specific primer-polymerase chain reaction (SSP-PCR) and genome walking. In *PCR Protocols: Current methods and applications*, pp. 339-348. Edited by B. A. White. Totowa: Humana Press.

Siebers, A. & Finlay, B. B. (1996). M cells and the pathogenesis of mucosal and systemic infections. *TIM* **4**, 22-29.

Simon, R., Priefer, U. & Pühler, A. (1983). A broad host range mobilisation system for *in vivo* genetic engineering: Transposon mutagenesis in gram negative bacteria. *Biotech* **1**, 784-791.

Sirard, J.-C., Niedergang, F. & Kraehenbuhl, J.-P. (1999). Live attenuated *Salmonella*: a paradigm of mucosal vaccines. *Immunolog Rev* **171**, 5-26.

Sitrin, R. G., Pan, P. M., Srikanth, S. & Todd III, R. F. (1998). Fibrinogen activates NF-kB transcription factors in mononuclear phagocytes. *J Immunol* **161**, 1462-1470.

Smith, R. L., Banks, J. L., Snavely, M. D. & Maguire, M. E. (1993A). Sequence and topology of the CorA magnesium transport systems of *Salmonella typhimurium* and *Escherichia coli* . Identification of a new class of transport protein. *J Biol Chem* **268**, 14071-14080.

Smith, R. L., Tao, T. & Maguire, M. E. (1993B). Membrane topology of a P-Type ATPase - the MgtB magnesium transport protein of *Salmonella typhimurium*. *J Biol Chem* **268**, 22469-22479.

Snavely, M. D., Gravina, S. A., Cheung, T.-B. T., Miller, C. G. & Maguire, M. E. (1991). Magnesium transport in *Salmonella typhimurium*: regulation of *mgtA* and *mgtB* expression. *J Biol Chem* **266**, 824-829.

Solioz, M. & Strausak, D. (1997). A putative tetracyclin efflux system of *Entrococcus hirae*. *Genbank - direct submission* .

Soncini, F. C., Vescovi, E. G. & Groisman, E. A. (1995). Transcriptional autoregulation of the *Salmonella typhimurium phoPQ* operon. *J Bacteriol* **177**, 4364-4371.

Soncini, F. C. & Groisman, E. A. (1996). Two-component regulatory systems can interact to process multiple environmental signals. *J Bacteriol* **178**, 6796-6801.

Soncini, F. C., Garcia Vescovi, E., Solomon, F. & Groisman, E. A. (1996). Molecular basis of the magnesium deprivation response in *Salmonella typhimurium*: identification of PhoP-regulated genes. *J Bacteriol* **178**, 5092-5099.

Spitznagel, J. K. (1990). Antibiotic proteins of human neutrophils. *J Clin Invest* **86**, 1381-1386.

Storm, D. R., Rosenthal, K. S. & Sqanson, P. E. (1977). Polymyxin and related peptide antibiotics. *Ann Rev Biochem* **46**, 723-763.

Stumpe, S., Schmid, R., Stephens, D., Georgiou, G. & Bakker, E. P. (1998). Identification of OmpT as the protease that hydrolyzes the antimicrobial peptide protamine before it enters growing cells of *Escherichia coli*. *J Bacteriol* **180**, 4002-4006.

Sukupolvi, S. & Vaara, M. (1989). *Salmonella typhimurium* and *Escherichia coli* mutants with increased outer membrane permeability to hydrophobic compounds. *Biochem Biophys Acta* **988**, 377-387.

Sukupolvi, S., Lorenz, G., Gordon, J. I., Bian, Z., Pfeifer, J. D., Normark, S. J. & Rhen, M. (1997). Expression of thin aggregative fimbriae promotes interaction of *Salmonella typhimurium* SR-11 with mouse small intestinal cells. *Infect Immun* **65**, 5320-5325.

Sulavik, M. C., Dazer, M. & Miller, P. F. (1997). The *Salmonella typhimuirum mar* locus: molecular and genetic analyses and assessment of its role in virulence. *J Bacteriol* **179**, 1857-1866.

Sundström, C. & Nilsson, K. (1976). Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int J Cancer* **17**, 565-577.

Tabor, S. & Richardson, C. C. (1985). A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci USA* **82**, 1074-1076.

Tacket, C. O., Forrest, B., Morona, R., Attridge, S. R., LaBrooy, J., Tall, B. D., Reymann, M., Rowley, D. & Levine, M. M. (1990). Safety, immunogenicity, and efficacy against cholera challenge in humans of a typhoid-cholera hybrid vaccine derived from *Salmonella typhi* Ty21a. *Infect Immun* **58**, 1620-1627.

Tacket, C. O., Hone, D. M., Curtiss, R. d., Kelly, S. M., Losonsky, G., Guers, L., Harris, A. M., Edelman, R. & Levine, M. M. (1992). Comparison of the safety and immunogenicity of Δ*aroC* Δ*aroD* and Δ*cya* Δ*crp* *Salmonella typhi* strains in adult volunteers. *Infect Immun* **60**, 536-541.

Takada, H. & Kotani, S. (1992). Structure-function relations of lipid A. In *Bacterial endotoxic lipopolysaccharides, Molecular biochemistry and cellular biology*, pp. 107-130. Edited by D. C. Morrison & J. L. Ryan. Boca Raton: CRC Press, Inc.

Takeuchi, A. (1967). Electron microscope studies of experimental *Salmonella* infection. *Am J Pathol* **50**, 109-119.

Tao, T., Grulich, P. F., Kucharski, L. M., Smith, R. L. & Maguire, M. E. (1998). Magnesium transport in *Salmonella Typhimurium* - biphasic magnesium and time dependence of the transcription of the *mgtA* and *mgtB* loci. *Microbiol* **144**, 655-664.

Tartera, C. & Metcalf, E. S. (1993). Osmolarity and growth phase overlap in regulation of *Salmonella typhi* adherance to and invasion of human epithelial cells. *Infect Immun* **61**, 3084-3089.

Tenhaken, R. & Thulka, O. (1996). Cloning of an enzyme that synthesizes a key nuclotide-sugar precursor of hemicellulose biosynthesis from soybean: UDP-glucose dehydrogenase. *Plant Physiol* **112**, 1127-1134.

Tokada, H. & Kotani, S. (1992). Bacteial endotoxic lippolysaccharides. In *Molecular Biochemistry and Cellular Biology*, pp. 107-130. Edited by D. C. Morrison & J. L. Ryan. Boca Raton, FL.: CRC Press.

Tosteson, M. T. & Tosteson, D. C. (1984). Activation and inactivation of melittin channels. *Biophys J* **45**, 112-114.

Tramont, E. C., Chung, S., Berman, S., Keren, D., Kapfer, C. & Formal, S. B. (1984). Safety and antigenicity for typhoid-*Shigella sonnei* vaccine (strain 5076-1C). *J Infect Dis* **50**, 98-110.

Tsai, C. M. & Frasch, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* **119**, 115-119.

Tsutsumi, A., Kubo, M., Fujii, H., Freire-Moar, J., Turck, C. W. & Ransom, J. T. (1993). Regulation of protein kinase C isoform proteins in phorbol ester-stimulated Jurkat T lymphoma cells. *J Immunol* **150**, 1746-1752.

Uchiya, K., Barbieri, M. A., Funato, K., Shah, A. H., Stahl, P. D. & Groisman, E. A. (1999). A *Salmonella* virulence protein that inhibits cellular trafficking. *EMBO J* **18**, 3924-3933.

Unanue, E. R. (1984). Antigen-presenting function of the macrophage. *Ann Rev Immunol* **2**, 395-428.

Vaara, M. (1981A). Effect of ionic strength on polymyxin resistance of *pmrA* mutants of *Salmonella*. *FEMS Microbiol Lett* **11**, 321-326.

Vaara, M. (1981B). Increased outer membrane resistance to ethylenediamine-tetraacetate and cations in novel lipid A mutants. *J Bacteriol* **148**, 426-434.

Vaara, M., Vaara, T., Jensen, M., Helander, I., Nurminen, M., Rietschel, E. T. & Makela, P. H. (1981). Characterization of the lipopolysaccharide from the polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium*. *FEBS Lett* **129**, 145-149.

Vaara, M. & Vaara, T. (1983). Polycations sensitise enteric bactria to antibiotics. *Antimicrob Agents Chemo* **24**, 114-122.

Vaara, M. (1992). Agents that increase the permeability of the outer membrane. *Microbiol Rev* **56**, 395-411.

Valdivia, R. H. & Falkow, S. (1996). Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. *Mol Microbiol* **22**, 367-378.

Valentin-Hansen, P., Aiba, H. & Schuemperli, D. (1982). The structure of tandem regulatory regions in the *deo* operon of *Escherichia coli* K12. *EMBO J* **1**, 317-322.

Van Velkinburgh, J. C. & Gunn, J. S. (1999). PhoP-PhoQ-regulated loci are required for enhanced bile resistance in *Salmonella* spp. *Infect Immun* **67**, 1614-1622.

Verma, N. K., Verma, D. J., Huan, P. T. & Lindberg, A. A. (1993). Cloning and sequencing of the glucosyl transferase-encoding gene from converting bacteriophage X (SFX) of *Shigella flexneri*. *Gene* **129**, 99-101.

Virlogeux, I., Waxin, H., Ecobichon, C., Lee, J. O. & Popoff, M. Y. (1996). Characterization of the *rcsA* and *rcsB* genes from *Salmonella typhi: rcsB* through *tviA* is involved in regulation of Vi antigen synthesis. *J Bacteriol* **178**, 1691-1698.

Vladoianu, I.-R., Chang, H. R. & Pechere, J.-C. (1990). Expression of host resistance to *Salmonella typhi* and *Salmonella typhimurium*: bacterial survival within macrophages of murine and human origin. *Microbial Pathog* **8**, 83-90.

Wallis, T. S., Paulin, S. M., Plested, J. S., Watson, P. R. & Jones, P. W. (1995). The *Salmonella dublin* virulence plasmid mediates systemic but not enteric phases of salmonellosis in cattle. *Infect Immun* **63**, 2755-2761.

Wang, L. & Reeves, P. R. (1998). Organization of *Escherichia coli* O157 O-antigen gene cluster and identification of its specific genes. *Infect Immun* **66**, 3545-3551.

Wang, R. F. & Kushner, S. R. (1991). Construction of verstile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* **100**, 195-199.

Waterman, S. R. & Small, P. L. C. (1998). Acid-sensitive enteric pathogens are protected from killing under extemely acidic conditions of pH 2.5 when they are inoculated onto certain solid food sources. *App Env Microbiol* **64**, 3882-3886.

Waters, S. H., Rogowsky, P., Grinsted, J., Altenbuchner, J. & Schmitt, R. (1983). The tetracycline resistance determinants of RP1 and Tn1721: nucleotide sequence analysis. *Nucleic Acids Res* **11**, 6089-6105.

Watson, P. R., Paulin, S. M., Bland, A. P., Libby, S. J., Jones, P. W. & Wallis, T. S. (1999). Differential regulation of enteric and systemic salmonellosis by *slyA*. *Infect Immun* **67**, 4950-4954.

Way, J. S., Josephson, K. L., Pillai, S. D., Abbaszadegan, M., Gerba, C. P. & Pepper, I. L. (1993). Specific detection of *Salmonella* spp. by multiplex polymerase chain reaction. *Appl Environ Microbiol* **59**, 1473-1479.

Ways, D. K., Posekany, K., de Vente, J., Garris, T., Chen, J., Hooker, J., Qin, W., Cook, P., Fletcher, D. & Parker, P. (1994). Transforming growth factor-β potentiates vitamin D3-induced terminal monocytic differentiation of human leukemic cell lines. *Cell Growth Differ* **5**, 1195-1201.

Westrich, L., Domann, S., Faust, B., Bedford, D., Hopwood, D. A. & Bechthold, A. (1999). Cloning and characterisation of a gene cluster from *Streptomyces cyanogenus* S136 probably involved in landomycin biosynthesis. *FEMS Microbiol Lett* **170**, 381-387.

Whitfield, C. (1995). Biosynthesis of lipopolysaccharide O antigens. *TIM* **3**, 178-185.

Whittington, V. (1988). Two Fevers - Gold and Typhoid. In *A social history of Western Australia during the decade 1891-1900 under the particular influence of these and other related articles - people in events*. Bently, WA.

Wick, M. J., Harding, C. V., Normark, S. J. & Pfeifer, J. D. (1994). Parameters that influence the efficiency of processing antigenic epitopes expressed in *Salmonella typhimurium*. *Infect Immun* **62**, 4542-4548.

Wick, M. J., Harding, C. V., Twesten, N. J., Normark, S. J. & Pfeifer, J. D. (1995). The *phoP* locus influences processing and presentation of *Salmonella typhimurium* antigens by activated macrophages. *Mol Microbiol* **16**, 465-476.

Willoughby, J. J., Russell, W. C., Thirkell, D. & Burdon, M. G. (1991). Isolation and detection of urease genes in *Ureaplasma urealyticum*. *Infect Immun* **59**, 2463-2469.

Wilmes-Riesenberg, M. R., Foster, J. W. & Curtiss, R. (1997). An altered *rpoS* allele contributes to the avirulence of *Salmonella typhimurium* LT2. *Infect Immun* **65**, 203-210.

Wilson, R. L., Stauffer, L. T. & Stauffer, G. V. (1993). Roles of the GcvA and PurR proteins in negative regulation of the *Escherichia coli* glycine cleavage enzyme system. *J Bacteriol* **175**, 5129-5134.

Wilson, R. L. & Stauffer, G. V. (1994). DNA sequence and characterization of GcvA, a LysR family regulatory protein for the *Escherichia coli* glycine cleavage enzyme system. *J Bacteriol* **176**, 2862-2868.

Wonderling, L. D. & Stauffer, G. V. (1999). The cyclic AMP receptor protein is dependent on GcvA for regulation of the gcv operon. *J Bacteriol* **181**, 1912-1919.

Wong, K. K., McClelland, M., Stillwell, L. C., Sisk, E. C., Thurston, S. J. & Saffer, J. D. (1998). Identification and sequence analysis of a 27-kilobase chromosomal fragment containing a *Salmonella* Pathogenicity Island located at 92 minutes on the chromosome map of *Salmonella eterica* serovar Typhimurium LT2. *Infect Immun* **66**, 3365-3371.

Wood, M. W., Jones, M. A., Watson, P. R., Hedges, S., Wallis, T. S. & Galyov, E. E. (1998). Identification of a pathogenicity island required for *Salmonella* enteropathogenicity. *Mol Microbiol* **29**, 883-891.

Woolfson, A., Huebner, R., Wasas, A., Chola, S., Godfrey-Faussett, P. & Klugman, K. (1997). Nasopharyngeal carriage of community-aquired, antibiotic-resistant *Streptococcus pneumoniae* in a Zambian paediatric population. *Bull WHO* **75**, 453-462.

Wosten, M. & Groisman, E. A. (1999). Molecular characterization of the PmrA regulon. *J Biol Chem* **274**, 27185-27190.

Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevich, R. J. & Mathison, J. C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **249**, 1431-1433.

Yamaguchi, A., Ono, N., Akasaka, T., Noumi, T. & Sawai, T. (1990). Metal-tetracycline/H^+ antiporter of *Escherichia coli* encoded by a transposon, Tn*10*. The role of the conservered dipeptide, Ser^{65}-Asp^{66}, in tetracycline transport. *J Biol Chem* **265**, 15525-15530.

Yamaguchi, A., Someya, Y. & Sawai, T. (1992). Metal-tetracycline/H+ antiporter of Escherichia coli encoded by transposon Tn10. The role of a conserved sequence motif, GXXXXRXGRR, in a putative cytoplasmic loop between helices 2 and 3. *J Biol Chem* **267**, 19155-19162.

Zhao, J. & Aoki, T. (1992). Nucleotide sequence analysis of the class G tetracycline resistance determinant from *Vibrio anguillarum*. *Microbiol Immunol* **36**, 1051-1060.

Zhou, D. G., Mooseker, M. S. & Galán, J. E. (1999A). An invasion-associated *Salmonella* protein modulates the actin-bundling activity of plastin. *Proc Natl Acad Sci USA.* **96**, 10176-10181.

Zhou, D., Mooseker, M. S. & Galán, J. E. (1999B). Role of the *S. typhimurium* actin-binding protein SipA in bacterial internalization. *Science* **283**, 2092-2095.

Corrigenda

Abstract

General comments:
The abstract cannot be shortened as it contains detailed relevant information reflecting the nature of the results obtained.
Second page, The Mu*d*J transposon is discussed in detail in the introduction of Chapter 3.

First page, Lines 8-9. "A large number of *pags/prg*s involved with virulence have been identified in *Salmonella*, e.g. for survival in macrophages, invasion of mammalian cells, protein secretion, and for antimicrobial peptide resistance."

First page, Lines 16-17. The PhoP/Q regulation mentioned refers to the effect of *phoP* and *phoQ* chromosomal mutations and not the inducing conditions for the wildtype *phoP/Q* genes.

First page, Lines 16-17. "Comparison of *S. typhi* and *S .typhimurium* wildtype, *phoP12*, ($PhoP^-$) (PhoP null mutations in which *pqr/prg* genes are up-regulated and *pqa/pag*s are down-regulated) and *phoP24* ($PhoP^c$) (PhoP constitutive mutations in which *pqa/pag* genes are upregulated and *pqr/prg*s are downregulated)"

First page, Five lines up. "..strains showed differences in both the number and apparent molecular masses of PhoP/Q regulated proteins,.."

First page, Two lines up. "..encoding a non-specific acid phosphatase was noted, with lower.."

Third page, Line 6 and elsewhere. L-[S^{35}]-methionine is to be replaced with L-[^{35}S]-methionine.

Third page, line 6-8. "The *S. typhi pqaA* gene encodes a 518 aa (59.3 kDa) protein which was confirmed by overexpression and L-[^{35}S]-methionine labelling experiments, and had no homology to other bacterial genes when compared to the sequence databases with Blast 2.0 N/X/P programs."

Third page, 8-10 lines up "It was found that the *S. typhi* $PhoP^-$ mutant was growth restricted in the phorbol myristate acetate (PMA) differentiated U937 (PMA-U937) cells; this growth defect was able to be complemented by a low copy number plasmid carrying the *S. typhi*miurum *phoP/Q* genes."

Chapter 1

Section 1.8.5 *Salmonella* and transepithelial signalling of polymorphonuclear cells (PMS).

Page 1, 5 lines up. .."(Le Minor & Popoff, 1987).

Page 5, Line 1. "Other patients may carry bacteria in their gall bladders for years..".

Page 5, 8 lines up. "..late 1800s, early 1900s..".

Page 6, 3 lines up. "..contracted the deadly fever and returned to hospital care in the major cities..".

Page 9, Line 8. "..(previously the "gold standard" agent (Islam *et al.*, 1993))..".

Page 9, Line 9. "..R plasmids: large transferable, conjugative plasmids..".

Page 14, 2 lines up. "..or moderate (pH 4.4) acid could enable..".

Page 15, lines 3-5. "The systems are classified as either σ^{38}-dependent or –independent (σ^{38} is an alternative sigma factor encoded by *rpoS*.."

Page 15, 9 lines up. "..ATR response including the major iron regulatory protein Fur..".

Page 16, 11 lines up. "..*S. typhi* do not contain the *pef*, *lpf* or *stf* operons and accordingly do not produce either PEF or LPF fimbriae."

Page 14, Line 9 and Page 26, Line 11. PMN's should be replaced with PMNs.

Page 28, Line 15. "The *Salmonella* induced "non-degradative" phagosomes..".

Page 32, Lines 14-15. "..via the *S. typhimurium* SapABCDE (ATP-binding cassette) locus (Parra Lopez *et al.*, 1993).".

Page 33, Figure 1.2 The square in the O-antigen repeat unit represents an different sugar in the repeat unit.

Page 33, last line "..according to the Kaufmann-White scheme..".

Page 38, 7 lines up. "..single amino acid substitution ($Thr^{48}\rightarrow Ile$) in the periplasmic domain of PhoQ (Gunn *et al.*, 1996; Garcia Vescovi *et al.*, 1997).".

Page 40, Table 1.1b. In addition to those proteins listed, it should be noted that other PhoP regulated proteins (e.g. TraT) have also been identified (Valdivia & Falkow, 1997).

Page 41, Line 5. "These periplasmic phosphatases..".

Page 46, Line 12. "..cell invasion protein of *Yersinia enterocolitica*..".

Page 46, Line 19. "..macrophages, however the actual function of *pagC* during survival in macrophages by *Salmonellae* has not been defined."

Page 47, Lines 16-17. "..a product of the *Yersinia* virulence plasmid..".

Page 47, Lines 20-21. "..by Southern hybridisation analysis (Gunn *et al.*, 1998A)."

Pages 48-49, Section 1.9.7.4 and Page 64-68, Section 1.13. SP's should read SPs.

Page 55, 3 lines up. "..and by doing so to change PhoQ conformation..".

Page 66, Line 11. ""It has been found that the host specificity of *Salmonella* infections.."

Page 67, 6 lines up. "..differently to deacetylated forms of Lipid A..".

Chapter 2

Page 77, Line 8. dNTP's should be replaced with dNTPs.

Page 80, Line 13. RNASE A should be replaced with RnaseA.

Page 81, Lines 3-4. "Plasmid, restriction enzyme and PCR DNA fragments..".

Pages 81, Line 4 & Page 82 Line 6. Quiagen should be replaced with Qiagen.

Page 81, 4 lines up. "..treated with exonuclease III, then incubated with klenow and S1-nuclease enzymes.."

Page 91, Section 2.6.2.1. Super-competent cells give an average of 10^7 transformants per µg of DNA.

Page 93, Section 2.6.3.1 P22 phage propagation

Page 93, 9 lines up. "..0.1 ml of 1 M $MgSO_4$ and 0.01 ml of 1 M $CaCl_2$ were added..".

Page 94, Line 7. "..were prepared according to the manufacturer's protocol."

Page 96, Line 7. "..Whatman blotting paper..".

Page 98, 11 lines up. "..Coomasie Brilliant Blue G250 (dissolved in 5% (v/v) perchloric acid)."

Pages 99 and 100, Section 2.8 "0.4 M (isopropyl-β-D-thiogalactopyranoside (IPTG) should be changed to "0.4 mM (isopropyl-β-D-thiogalactopyranoside (IPTG)" throughout this entire section.

Page 100, 11 lines up. "..to induce the pGP1-2 λ_{PL} promoter..".

Page 100, 6 lines up. "..and resuspended in 120 µl of 1 x SDS sample buffer (Section 2.8.1)."

Page 103, Line 6, "Section 2.11.1 Polymyxin B (PmB) assays".

Page 103, 5 lines up "Bacteria, grown for 16 h in LB were subcultured..".

Page 105, Line 10. "..the PMA-U937 cells stain with a dark blue nucleus..".

Page 106, 6-9 lines up. "After 1 ml of bacteria (*S. typhi* 10^7 bacteria/ml, *S. typhimurium* 10^6 bacteria/ml) were added to the HeLa or PMA-U937 cells in the 24 multi-well plates (*S. typhi* strains were added at a ratio of 10:1 and *S. typhimurium* strains at 1:1 (bacteria:tissue culture cells)), invasion was allowed to occur for 2 h, the supernatant was removed and the cells washed once with 1 ml of PBS, and the appropriate media containing 0.2 mg/ml gentamicin (Gibco BRL) was added for 1 h."

Page 107, 5 lines up. "..according to the manufacturer's instructions..".

Chapter 3

Page 109, Section 3.1 LHS stands for Left Hand Side and RHS stands for Right Hand Side.

Page 113, 5-6 lines up. Pag's should read Pags and Prg's should read Prgs.

Page 114 and Figure 3.2, Section 3.6.1. The growth media used for this assay was LB which contains mM amounts of Mg^{2+}.

Page 115, 6 lines up. "..a larger modal chain length than the LPS of *S. typhimurium* C5."

Page 118, 5 lines up. "..strains were tested for sensitivity to protamine..".

Page 119, 7 lines up. "..strains against various concentrations..".

Page 123, Table 3.6 Fifth column heading (second row) should read $PhoP^-$ + pRMSB1.

Page 129 and onwards. The term homology is used in Chapter 3 as it was felt that the genes discussed were likely to be related by the evolutionary process of divergence from a common ancestor.

Page 135, 6 lines up. "..and probed with the PCR DIG-dUTP labelled *gcvA* probe.."

Page 136, 3 lines up. ""..Mu*d*J-left" probe and was known to cut at a site just over 1 kb into..".

Page 141, Figure 3.14. Although the band in Lane 1 is faint it is visible, and the position is also indicated by an arrow.

Page 143, Lines 9-10. "The whole cell proteins produced by *S. typhi*, *S. typhimurium* and respective $PhoP^-$ and $PhoP^c$ derivatives were compared by 1-D SDS-polyacrylamide gel electrophoresis, and it was found that *S. typhi* Pag and Prg proteins were fewer in number and had different apparent molecular masses than *S. typhimurium* Pag and Prg proteins (Section 3.6.1).

Chapter 4

Page 144, Line 8. "..decreased survival in macrophage cell lines and decreased invasion of other mammalian cells, a monocyte-derived human cell line..".

Page 146, 13 lines up. "..bacterial strain's ability..".

Page 148, Section 4.3.1 "..cells were aliquoted into 1 ml volumes..".

Page 150, Line 4 should read "There was a significant difference in growth at 24 h between Ty2 and RMA1030 (Figure 4.2) ($P<0.03$ at 24 h, students T-test),

Page 150, 2 lines up. "..prior to fixing and staining (Figure 4.3 B)."

Page 151, Figure 4.3. The photograph was taken at the same resolution (10 x objective) for both U937 and PMA-U937 cells, therefore it was felt a scale bar was not needed.

Page 152, 11 lines up. "This indicated that the U937 cells were fully differentiated by PMA by 72 h."

Page 156, 10-11 lines up. "Therefore the ability of the *S. typhimurium* C5 and isogenic PhoP$^-$ (RMA1010) and PhoP^c (RMA1024) mutants, to invade and grow within PMA-U937 cells was investigated (Figure 4.5, Table 4.7).

Page 157, Figure 4.5 figure legend should read
This figure shows the net growth characteristics of *S. typhimurium* C5 (□), RMA1010 (PhoP$^-$) (○) and RMA1024 (PhoP^c) (Δ) strains after invasion of U937-PMA cells. *S. typhimurium* strains were added at a ratio of 1:1 (bacteria:cells) and each point represents the arithmetic mean of 4 assays expressed as viability of cfu/well ± SD at a given time in hours.

Page 159-160, Section 4.6.2. References to Figures 4.6A and 4.6B in the text should be followed by a reference to Table 4.9.

Page 161, lines 1-2. "The amount of LDH contained within the PMA-U937 cells during infection with various *Salmonella* strains (Table 4.11) was measured".

Page 163, Line 5. AP's should read APs.

Chapter 5

Page 185, Line 6-7. "..(a tentative tetracycline efflux protein)..".

Page 186, Table 5.6 legend "..*Bacillus subtilis*.."

Page 190, Line 7. "Neither the RBSs nor the potential promoters highly correspond..".

Page 194, Line 13. Figure 5.? should be read as Figure 5.15.

Page 193, Line 14. "..*Bacillus subtilis* zinc-containing alcohol dehydrogenase..".

Page 196, 11 lines up "Overexpression studies were initially undertaken using the pET11 based vector pET11BYZ (Vindurampulle, 1994) which carries a strong RBS."

Page 197, Line 9. "..no expression of a ~ 59 kDa protein was seen..".

Page 204, Section 5.8.7 Table 5.11 should be labelled Table 5.9 and RMA1030 ($PhoP^-$).

Page 205, Section 5.8.8 pBC is pBC-KS^+ (Table 2.4).

Page 206, Figure 5.21 legend, Line 3 "..(dark grey rectangle)..".

Chapter 6

Page 225, Figure 6.9 legend. "The potential start (ATG) codon is shown in bold..".

Page 240 (Figure 6.17) & Page 246 (Figure 6.20). For ease of comparison between strains, I chose to calculate and present % survival rather than log cfu.

Page 245, 11 lines up. "..and only a limited difference was noted with the $PhoP^+$ backgrounds,..".

Page 249, Line 8. "..indicating that the *S. typhi pqaB* operon ORF6 and/or ORF7 may be..".

Page 250, 2 lines up. "..the defined *pqaB*::Kan mutant..".

Page 150, 1 lines up. "..indicating that the small, hydrophobic ORF6 and/or ORF7 may be.."

Chapter 7

Page 257, Line 7. "..in the presence of glycine and repress it in..".

Page 260, Line 13. ""..isolated and sequenced in *S. typhimurium*..".

Page 268, Line 9. "..to potentially affect the host's immune response..".

Biobliography

Throughout the text, these references should be changed.

Abigail & Dixie (1994) should be Salyers and Whitt (1994).

Abshire & Heffron should be Abshire & Neidhardt.

Blanc Potard & Groisman (1997) should read Blanc-Potard & Groisman (1997).

Maniatis (1982) should be Sambrook *et al.*, (1989).

Abshire, K. A. & Neidhardt F. C. (1993A). Growth rate paradox of *Salmonella typhimurium* within host macrophages. *J Bacteriol* **175**, 3744-3748.

Abshire, K. Z. & Neidhardt F. C. (1993B). Analysis of proteins synthesized by *Salmonella typhimurium* during growth within a host macrophage. *J Bacteriol* **175**, 3734-3743.

Blanc-Potard, A. B. & Groisman, E. A. (1997). The *Salmonella selC* locus contains a pathogenicity island mediating intramacrophage survival. *EMBO J* **16,** 5376-5385.

Salyers, A. & Whitt, D. (1994). *Salmonella* Infections. In *Bacterial Pathogenesis: A molecular approach*, pp. 229-243: ASM Press.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). Molecular cloning: A laboratory manual, vol. 1, 2 & 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

These references have had spelling errors modified

Adams, P., Fowler, R., Howell, G., Kinsella, N., Skipp, P., Coote, P. & O'Connor, C. D. (1999). Defining protease specificity with proteomics: A protease with a dibasic amino acid recognition motif is regulated by a two-component signal transduction system in *Salmonella*. *Electrophoresis* **20**, 2241-2247.

Bearson, S. M. D., Benjamin, W. H. J., Swords, W. E. & Foster, J. W. (1996). Acid shock induction of RpoS is mediated by the mouse virulence gene *mviA* of *Salmonella typhimurium*. *J Bacteriol* **178**, 2572-2579.

Brozek, K. A. & Raetz, C. R. H. (1990). Biosynthesis of lipid A in *Escherichia coli* acyl carrier protein-dependent incorporation of laurate and myristate. *J Biol Chem* **265**, 15410-15417.

Cohen, R. M. & Wolfenden, R. (1971). Cytidine deaminase from *Escherichia coli*. *J Biol Chem* **246**, 7561-7565.

Esser, A. F., Bartholomew, R. M., Jensen, F. C. & Müller-Eberhard, H. J. (1979). Disassembly of viral membranes by complement independent of channel formation. *Proc Natl Acad Sci USA* **76**, 5843-5847.

Fields, P. I., Swanson, R. V., Haidaris, C. G. & Heffron, F. (1986A). Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci USA* **83**, 5189-5193.

Galyov, E. E., Wood, M. W., Rosqvist, R., Mullan, P. B., Watson, P. R., Hedges, S. & Wallis, T. S. (1997). A secreted effector protein of *Salmonella dublin* is translocated into eukaryotic cells and mediates inflammation and fluid secretion in infected ileal mucosa. *Mol Microbiol* **25**, 903-912.

Giannella, R. A., Broitman, S. A. & Zamcheck, N. (1972). Gastric acid barrier to ingested microorganisms in man: studies *in vivo* and *in vitro*. *Gut* **13**, 251-256.

Giannella, R. A., Rout, W. R. & Formal, S. B. (1975). Pathogenesis of *Salmonella*-mediated intestinal fluid secretion: activation of adenylate cyclase and inhibition by indomethacin. *Gastroenterol* **69**, 1238-1245.

Hall, H. K. & Foster, J. W. (1996). The role of Fur in the acid tolerance response of *Salmonella typhimurium* is physiologically and genetically separable from its role in iron acquisition. *J Bacteriol* **178**, 5683-5691.

Lacraz, S., Dayer, J.-M., Nicod, L. & Welgus, H. G. (1994). 1,25-Dihydroxyvitamin D_3 dissociates production of interstitial collagenase and 92-kDa gelatinase in human mononuclear phagocytes. *J Biol Chem* **269**, 6485-6490.

Le Minor, L. & Popoff, M. Y. (1988). *Antigenic formulas of the Salmonella serovars*, 5th edn. Paris, France: WHO Collaborative Centre for reference and research on *Salmonella*, Institute Pasteur.

Leclerc, G. J., Tartera, C. & Metcalf, E. S. (1998). Environmental regulation of *Salmonella typhi* invasion-defective mutants. *Infect Immun* **66**, 682-691.

Qi, S.-Y., Li, Y., Szyroki, A., Giles, I. G., Moir, A. & O'Connor, C. D. (1995). *Salmonella typhimurium* responses to a bactericidal protein from human neutrophils. *Mol Microbiol* **17**, 523-531.

Salmond, G. P. C. & Reeves, P. J. (1993). Membrane traffic wardens and protein secretion in Gram-negative bacteria. *TIB* **18**, 7-12.

Sessa, G., Freer, J. H., Colacicco, G. & Weissman, G. (1969). Interaction of a lytic polypeptide, melittin, with lipid membrane systems. *J Biol Chem* **244**, 3575-3582.

Tokada, H. & Kotani, S. (1992). Bacterial endotoxic lippolysaccharides. In *Molecular Biochemistry and Cellular Biology*, pp. 107-130. Edited by D. C. Morrison & J. L. Ryan. Boca Raton, FL.: CRC Press.

These references should be added to the bibliography

Mahan, M. J., Slauch, J. M., Hanna, P. C., Camilli, A., Tobias, J. W., Waldor, M. K. & Mekalanos, J. J. (1993A). Selection for bacterial genes that are specifically induced in host tissues - the hunt for virulence factors. *Infect Agent Dis* **2**, 263-268.

Mahan, M. J., Slauch, J. M. & Mekalanos, J. J. (1993B). Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* **259**, 686-688.

Valdivia, R. H., & Falkow, S. (1997). Fluorescence-based isolation of bacterial genes expressed within host cells. *Science* **277,** 2007-2011.