



Molecular Characterisation of the Haemolysin (HlyA) and the Region Downstream of *hlyA* in *Vibrio cholerae* O1

Monica A. Ogierman, B.Sc.(Hons.) (Adelaide)

Department of Microbiology and Immunology

Microbial Pathogenesis Unit

The University of Adelaide,

Adelaide, 5005

AUSTRALIA

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Abstract

The haemolysin (HlyA) of El Tor *Vibrio cholerae* O1 is a potent toxin with both enterotoxic and cytolytic activities and is considered an alternate diarrhoeogenic factor to the cholera toxin, for El Tor and non-O1 strains. Although originally termed "haemolysin" because of its ability to lyse erythrocytes, it is also capable of lysing a variety of mammalian cells in culture. Osmotic protection and artificial lipid bilayer assays show that HlyA forms moderately anion selective pores with a single-channel conductance of 350pS in 1M KCl and an estimated diameter of <1.6 nm. HlyA is predicted to consist of amphipathic β -sheets which may be involved in pore formation.

Stable C-terminal truncations of HlyA were constructed in order to define structural domains that relate to function. Deletions at the C-terminus of pro-HlyA exposed a proteolytically sensitive site approximately 15 kDa from the C-terminus. Deletions of only 12 amino acids expose this site and cleavage occurs at this site efficiently in both *E. coli* and *V. cholerae*. Cleavage at the proteolytically sensitive site, together with the cleavage at the N-terminus that is normally required for activation, produces a 50 kDa derivative of HlyA. This central 50 kDa region of pro-HlyA was able to form pores in sheep erythrocytes and cause lysis, and defines a minimum haemolytic domain, characterised thus far. HlyA is homologous to the VAH1, ASH1 and AHH1 haemolysins of *V. anguillarum*, *A. salmonicida* and *A. hydrophila*, respectively, with a high degree of homology within the 50 kDa region of HlyA. The positions of the cysteine residues are conserved within these haemolysins, and are all contained within the 50 kDa domain, which may reflect conservation of secondary structure. The region Gln-609 to Thr-598 located at the C-terminus of 50 kDa HlyA (15 kDa from the C-terminus of pro-HlyA) is essential for the production of secreted, active haemolysin. The C-terminal 15 kDa of HlyA is not essential for secretion, pore-formation (cytolytic activity) or cleavage and so its role remains unknown.

HlyA is secreted as an 80 kDa pro-toxin that requires the removal of the N-terminal 15 kDa pro-region to release the mature 65 kDa protein. The soluble haemagglutinin / protease (SHA) produced by *V. cholerae* was identified as at least one factor that is involved

with the activation of HlyA. SHA is known to activate other virulence determinants of *V. cholerae*, including the cholera enterotoxin. Activation of HlyA was shown not to occur simultaneously with its secretion, as activity continues to increase in cell-free culture supernatants.

The region downstream of *hlyAB* was sequenced and characterised and a triacylglyceride-lipase operon (*lipAB*) encoding a 33 kDa lipase (LipA), and a 32 kDa accessory protein (LipB), was identified. LipA was previously known as HlyC, however as the amino acid sequence is highly homologous to the triacylglyceride-specific lipase of *Pseudomonas* spp, *hlyC* was re-named *lipA*. LipA contains the highly conserved pentapeptide and catalytic triad amino acid regions of the catalytic site of other lipases. LipB is homologous to the accessory-lipase proteins (lipase-specific foldase) required by *Pseudomonas* and various other bacterial species for the production of mature active lipase, and consistent with this, both *lipA* and *lipB* are required to restore a lipase-deficient *lipA* null mutant of *V. cholerae*. The intergenic stop codon for *lipA* overlaps the ribosome binding site for *lipB*, and a stem-loop resembling a rho-independent terminator is present immediately downstream from *lipB*, suggesting *lipA* and *lipB* form a lipase operon in *V. cholerae*.

A putative metalloprotease gene (*prtV*) was identified downstream of *lipAB*, but is transcribed in the opposite direction, and is predicted to share the same putative transcriptional terminator with *lipAB*. PrtV is 102 kDa and is highly homologous to the immune-inhibitor A (InA) metalloprotease of *Bacillus thuringiensis* and zinc-binding and catalytic domains conserved among many metalloproteases is present in PrtV.

The genetic organization of this region suggests it is possibly part of a pathogenicity island, encoding products capable of damaging host cells, or involved in nutrient acquisition by *V. cholerae*. However, neither *lipA* nor *prtV* null mutants were attenuated in the infant mouse model, nor did they exhibit reduced colonization potential when compared with wild-type in competition experiments.

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Monica A. Ogierman



I dedicate this thesis to my parents,

Margaret and Raymond
Ogierman



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LIST OF ABBREVIATIONS

A₆₀₀: absorbance at 600nm

A: adenine

aa: amino acid

ACP: acyl carrier protein

Ap: ampicillin

ATP: adenosine 5'-triphosphate

bp: base pair

BSA: bovine serum albumin

CAT: chloramphenicol acetyl-transferase

C: cytosine

Cm: chloramphenicol

CTP: cytosine 5'-triphosphate

cyto: cytoplasm

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleoside triphosphate

ddNTP: dideoxyribonucleoside triphosphate

DTT: dithiothreitol

EDTA: ethylene-diamine-tetra-acetic acid

EtBr: ethidium bromide

G: guanine

GTP: guanosine 5'-triphosphate

HlyA: haemolysin

IM: inner membrane

IPTG: isopropyl- β -D-thiogalactopyranoside

kb: kilobase pairs

kDa: kilodalton

Km: kanamycin
LB: luria broth
LPS: lipopolysaccharide
MFRHA: mannose-fucose resistant haemagglutinin
mRNA: messenger ribonucleic acid
NA: nutrient agar
NB: nutrient broth
nt: nucleotide
O-Ag: O-antigen
OD: optical density
oligo: oligonucleotide
OM: outer membrane
ORF: open reading frame
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PEG: polyethylene glycol-6000
P: periplasm
R: resistant
RBC: red blood cells
RBS: ribosome binding site
Rif: rifampicin
RNA: ribonucleic acid
RNase: ribonuclease
rpm: revolutions per minute
S: sensitive
SD: Shine-Dalgarno
SDS: sodium dodecyl sulphate
SHA: soluble haemagglutinin / protease

Sm: streptomycin

sRBC: sheep red blood cells

T: thymine

Tc: tetracycline

TEMED: N,N,N',N'-tetramethyl-ethylene-diamine

Tn: transposon

Tris: Tris-(hydroxymethyl)-aminomethane

TTP: thymidine 5'-triphosphate

U: uracil

UV: ultraviolet

v/v: volume per volume

WC: whole cells

w/v: weight per volume

X-gal: 5-bromo-4-chloro-3-indolyl- β -galactopyranoside

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Chapter 1

INTRODUCTION

1.1 The causative agent of cholera

Over a century ago Robert Koch first saw a "comma shaped organism" under the microscope, isolated from the gut of an infected cholera victim which he subsequently identified as the causative agent of the disease cholera (Koch, 1884). The organism responsible for cholera is *Vibrio cholerae*, a Gram-negative rod with a single polar flagellum. *V. cholerae* is divided into serogroups O1 and non-O1, based on the O-antigen of the lipopolysaccharide (LPS). While more than 155 serogroups have been identified, it was believed that only strains belonging to O1 were associated with epidemic and pandemic cholera until 1992, with the emergence of *V. cholerae* O139. With the exception of O139, the non-O1 serogroups are associated with sporadic cases of gastroenteritis and occasionally with extraintestinal infections, and are not associated with epidemic spread (Morris, 1990).

Strains of the *V. cholerae* O1 serogroup are further divided into biotypes, El Tor and classical, and are distinguished on the basis of a number of characteristics. Originally, haemolytic phenotype was used to distinguish members of these biotypes, as it was generally considered that El Tor isolates were haemolytic for sheep erythrocytes, while classical strains were not. However, this proved unreliable due to the subsequent variability of the haemolytic status of El Tor strains (discussed further in Section 1.5.1). The most reliable method to date used to distinguish biotype is the resistance of El Tor *V. cholerae* (and susceptibility of classical strains) to Mukerjee's type IV vibriophage (Murkerjee, 1963).

Since the turn of the 19th century, classical isolates of *V. cholerae* have been responsible for six cholera pandemics (Blake, 1994). It was generally believed that strains of the El Tor biotype were unable to cause cholera, until the advent of the seventh cholera pandemic, originating in South-East Asia in 1962, where the causative agent was El Tor *V. cholerae* O1. In 1992, an epidemic of cholera began in India and Bangladesh which quickly

and efficiently spread to neighbouring countries (overviewed by Blake, 1994). The causative agent was a novel serotype O139, which was able to evade previously acquired immunity to O1 cholera and was not detected using the standard O1 antigen test (Hall *et al.*, 1994). It was initially believed that this would be the beginning of the eighth pandemic (Swerdlow and Ries, 1993), however, O139 vibrios have not been predominant in recent years.

The *V. cholerae* serotype O139 is closely related to the El Tor strains of *V. cholerae* O1. Both possess highly homologous virulence genes and the regulation of virulence expression is indistinguishable (Calia *et al.*, 1994; Hall *et al.*, 1994; Johnson *et al.*, 1994). O139 isolates do however differ from O1 in the LPS structure, as O139 does not produce O-Ag (Manning *et al.*, 1994). In addition, O139 isolates are distinct in their ability to produce a polysaccharide capsule layer which is thought to increase resistance to killing by serum (Johnson *et al.*, 1993; Weintraub *et al.*, 1994) and are more effective in the spread and proliferation of the organism within the environment (Islam *et al.*, 1993).

1.2 The disease process

There are sequential and distinct stages involved in a cholera infection. The organism is transmitted via the faecal-oral route and the disease process is initiated by the ingestion of contaminated food or water. Alternatively, incompletely cooked contaminated seafood may also be a source of infection. The acidic environment of the stomach provides a partial barrier against infection by *V. cholerae*, as demonstrated by a human volunteer study where the numbers of vibrio organisms required to produce infection decreased 10^4 fold when stomach acidity was reduced using sodium bicarbonate (Hornick *et al.*, 1971). However, if sufficient numbers of bacteria do survive the acidic conditions of the stomach, the organism proceeds to the small intestine where it must adhere to, and colonise in order to establish a successful infection.

Flagellar motility and chemotaxis are required to facilitate vibrios in arriving at and penetrating the intestinal mucosa (Guentzel and Berry, 1975; Freter *et al.*, 1981). However, the relationship between motility and virulence in *V. cholerae* is not clearly defined. Previous

studies found that non-motile mutants are less virulent than wildtype in animal models, and exhibit reduced adsorption (Guentzel and Berry 1975; Jones and Freter, 1976). Subsequently, the motility status of the cell was shown to have no effect on colonization or adherence (Attridge and Rowley, 1983; Teppema *et al.*, 1987), and non-motile mutants were found to be fully virulent in the infant mouse model (Freter *et al.*, 1981; Richardson, 1991). More recently, Gardel and Mekalanos (1996) have clearly shown an association between motility phenotype and virulence factor expression. While hyperswarming mutants are defective in the production of two primary virulence factors, the cholera toxin (CT) and the TCP pili, nonmotile mutants displayed increased expression of these factors. Chemotactic vibrios show greater ability to penetrate the mucus gel of the gut mucosa during infection of mice, compared to non-chemotactic vibrios (Freter and O'Brien, 1981; Freter *et al.*, 1981). Homologues of the well characterised chemotactic transducer proteins in *E. coli* involved in sensing environmental stimuli and signalling the flagellar motor, have been identified in *V. cholerae* (Everiss *et al.*, 1994; Harkey *et al.*, 1994).

The next stage towards colonisation requires the adhesion of the organism to the small-intestinal mucosa, where it must avoid being flushed away by the peristaltic motion of the gut. *V. cholerae* utilises a combination of secreted colonization modifiers and cell-associated adherence factors (reviewed in Manning, 1994). The colonization modifiers are generally hydrolytic enzymes and include DNases and proteases. Two DNases are produced by *V. cholerae* and it is postulated that they reduce the viscosity of the epithelial mucus layer, which is rich in DNA, and the hydrolysis of the DNA may also provide nutrients for the invading organism (Focareta and Manning, 1987; 1991). The soluble haemagglutinin/protease (SHA/protease) produced by *V. cholerae* is a zinc-dependent metalloprotease related to the *Pseudomonas aeruginosa* elastase (Häse and Finkelstein, 1990; 1991) and has mucinase activity which may also assist with the penetration of the organism into the mucous layer (Finkelstein *et al.*, 1983).

The major adherence factor for *V. cholerae* O1 is the filamentous pilus TCP (toxin co-regulated pilus) (reviewed recently in Manning, 1997). TCP is essential for colonization and virulence in both the infant mouse model (Taylor *et al.*, 1987; Sharma *et al.*, 1989; Voss

et al., 1996) and in human volunteer studies (Herrington *et al.*, 1988). The major subunit of the pilus, TcpA, is encoded within the large *tcp* operon consisting of 15 genes which encode proteins involved in pilus assembly and transport (Kaufman *et al.*, 1993; Ogierman *et al.*, 1993). The C-terminal coding domain of *tcpA* differs between El Tor and classical isolates, which may account for the differences in both antigenic epitopes and protection between the two biotypes (Jonson *et al.*, 1991a and b; Sun *et al.*, 1991; Attridge *et al.*, 1993). Expression of TCP in the two biotypes requires different cultural conditions (Jonson *et al.*, 1991a and b; Voss and Attridge, 1993), which may be attributed to differences in the major regulatory regions of *tcp* (Thomas *et al.*, 1995; Ogierman *et al.*, 1996).

Immediately downstream of the *tcp* operon lies the *acf* locus which encodes outer membrane proteins involved in colonization (Peterson and Mekalanos, 1988; Hughes *et al.*, 1995). The colonization defect of mutants in *acf* are not as severe as mutants in *tcp* in infant mice, and therefore the *acf* locus is proposed to provide an accessory colonization factor to TCP (Peterson and Mekalanos, 1988). The *tcp-acf* gene cluster is part of a pathogenicity island of *V. cholerae* O1 and O139 and resembles that of an integrated phage (Kovach *et al.*, 1996). Several haemagglutinins (HA) produced by *V. cholerae* have been implicated as adherence factors. Haemagglutinins are molecules that are able to mediate agglutination of erythrocytes and this ability has been associated with the ability to adhere to the microvilli of brush border membranes (Jones *et al.*, 1976). However, a clear association between the haemagglutinins of *V. cholerae* and virulence is yet to be made.

There is also evidence to suggest that the LPS of *V. cholerae* may play a role in adherence to the gut epithelium (Freter and Jones, 1976; Chitnis *et al.*, 1982; Attridge and Rowley, 1983). However, Iredell *et al.* (1997) have recently shown that *V. cholerae rfb* mutants, that are defective in O-antigen biosynthesis, are also unable to assemble TCP on the cell surface. The TcpA pilin subunit accumulated in the periplasm and the outer membrane of these mutants (Iredell and Manning, 1997). A plasmid encoding the *rfb* region was able to restore the production of both O-antigen and TCP, which suggests that functional TCP assembly depends on an intact LPS structure (Iredell *et al.*, 1997).

Following successful adherence to the mucosa of the small intestine, *V. cholerae* O1 then secretes an array of toxins during an infection. The most significant is the cholera toxin (CT) and non-O1 strains can produce a toxin that is identical (Yamamoto *et al.*, 1983a) or similar (Yamamoto *et al.*, 1983b) to *V. cholerae* O1 CT (CT and other toxins will be discussed further in Sections 1.3 and 1.4). The action of CT is primarily responsible for the copious volumes of diarrhoea, which is the major and most devastating symptom of cholera disease (Levine *et al.*, 1983). CT acts on mucosal cells causing fluid and ion loss from tissues, leading to hypotension and electrolyte imbalance (Spangler, 1992). A person with full blown cholera can lose up to 20 litres of water a day, which can rapidly lead to death if left untreated. Presumably, the purpose of CT (and possibly other toxins) is to provide nutrients which enable the organism to survive and multiply to astonishing levels. This was clearly demonstrated in a study conducted by Levine and co-workers (1988a), where human volunteers were fed ca. 10^6 CFU of the El Tor strain N16961 and were subsequently found to excrete ca. 3×10^7 vibrios/g of stool, in an average volume of 4.2 litres. This represents an average increase of organisms of approximately 10^5 -fold.

It is proposed that *V. cholerae* utilizes the SHA/protease as a "detachase", enabling the exit and release of the organism from the human gut into the environment, aiding in the spread of the organism (Finkelstein *et al.*, 1992). Once released into the environment, vibrios can survive for long periods in estuarine and fresh water, adapting a dormant "viable but non-culturable state" (Colwell *et al.*, 1985) which can transform into a culturable state when the environmental conditions are favorable (Huq *et al.*, 1990).

The expression of genes involved with the virulence, colonization and survival of *V. cholerae* in the host are co-ordinately regulated by a complex cascade involving the ToxR/ToxS/ToxT system (DiRita, 1992). ToxR is a transcriptional activator that transduces a wide variety of environmental signals including pH, osmolarity and temperature, into changes of gene expression (reviewed in Skorupski and Taylor, 1997). The cellular global regulator cAMP-CRP also influences the expression of the ToxR regulon (Skorupski and Taylor, 1997). The cascade begins with ToxR, which directly activates transcription of genes encoding CT, the outer membrane protein OmpU, and ToxT. ToxT then

transcriptionally activates other virulence genes such as *tcp*, *acf* and the *ctx* genes which encode the cholera toxin (DiRita *et al.*, 1991; Skorupski and Taylor, 1997).

ToxR spans the inner membrane and has a cytoplasmic domain involved in DNA binding and transcriptional activation, and a periplasmic domain which interacts with the ToxS protein (Miller *et al.*, 1987 and 1989; DiRita and Mekalanos, 1991). *toxS* is co-transcribed with *toxR* and its product is proposed to stabilize the formation of a ToxR dimer (Miller *et al.*, 1989; DiRita and Mekalanos, 1991). ToxT is a member of the AraC family of transcriptional activators and the *toxT* gene is located within the *tcp* cluster (Higgins *et al.*, 1992; Ogierman and Manning, 1992). Recent studies on gene expression in *V. cholerae* have shown that bile drastically reduces transcription of *ctx* and *tcp*, while motility is significantly increased (Gupta and Chowdhury, 1997). TcpH is another regulator which couples environmental signals to the expression of the ToxR regulon, by influencing expression of ToxT, and consequently all *toxT* regulated genes (Carroll *et al.*, 1997).

1.3 The cholera enterotoxin (CT)

1.3.1 An overview of CT

It is without doubt that the cholera enterotoxin is the primary virulence factor of *V. cholerae*, responsible for the voluminous diarrhoea characteristic of a cholera infection. Extensive studies in both human volunteers and animal models have shown that strains devoid of CT do not produce full blown cholera. As little as 5µg of pure cholera toxin can produce between 1-6 litres of diarrhoea in human volunteers, demonstrating its potency (Levine *et al.*, 1983). CT has been the object of intense investigation at the biochemical and genetic level, and is consequently one of the best understood bacterial toxins to date. The structure and biological activity of CT closely resembles the heat-labile enterotoxin (LT) of *E. coli*, whose crystal structure has been elucidated (Sixma *et al.*, 1991; Spangler, 1992).

CT is a member of the A-B subunit toxins, where the A fragment has catalytic ADP-ribosyltransferase activity, while the B fragment is required for binding to host cells and translocation into the cytosol (Gill and King, 1975). Each CT molecule consists of a doughnut-shaped ring of five identical B subunits, attached to a single A subunit (Sixma *et*

al., 1991). While the genes encoding the A subunit (*ctxA*) and the B subunit (*ctxB*) overlap, they are not translationally coupled. The *ctxB* cistron is however, translated at a higher efficiency than that of *ctxA*, resulting in the 1:5 stoichiometry (Mekalanos *et al.*, 1983). The A subunit is activated upon cleavage into the subdomains A1 and A2, which remain linked by a single disulphide bond (Gill and Rappaport, 1979). The proteolytic processing of the A subunit can be mediated by a variety of enzymes found in the gut, including trypsin (Gill and Rappaport, 1979). The SHA/protease produced by *V. cholerae* is also able to nick and thereby activate the A subunit of CT (Booth *et al.*, 1983; Finkelstein *et al.*, 1983). However, despite its ability to cleave the CT, the SHA/protease is not considered a virulence determinant as mutants in the gene encoding the SHA/protease (*hap*) were found to be fully virulent using an infant rabbit model (Finkelstein *et al.*, 1992).

Disulphide bond formation is crucial for CT biogenesis and the enzyme responsible is the DsbA homologue, TcpG (Peek and Taylor, 1992; Yu *et al.*, 1992). The B subunit of CT degrades in the periplasm of *tcpG* mutants and consequently the CT holotoxin fails to assemble (Peek and Taylor, 1992; Yu *et al.*, 1992). In *E. coli*, DsbA functions as a disulphide bond isomerase that is crucial for the correct folding of secreted proteins (Bardwell *et al.*, 1991). TcpG of *V. cholerae* has 40% sequence identity with DsbA and the crystal structure of DsbA (Martin *et al.*, 1993) resembles that of TcpG (Hu *et al.*, 1997). TcpG is also required for the functional maturation of the TCP pilus. *tcpG* mutants still express TCP on their surface, however the pili no longer function to promote colonization (Peek and Taylor, 1992).

The receptor for the active cholera toxin is the GM₁ ganglioside of the host enterocyte of the gut. The neuraminidase (NANase) produced by *V. cholerae* converts higher order gangliosides to GM₁ (Holmgren *et al.*, 1975), thereby increasing the number of receptors available for CT. However, while several studies show that NANase plays a role in the binding and uptake of CT, it is not considered a primary virulence factor (Haksar *et al.*, 1974; Galen *et al.*, 1992).

Active CT attaches to the receptor via the B subunit and the A₁ subunit must then penetrate the host cell membrane, which requires reduction of the disulphide bond linking

the A₁ and A₂ subunits (Tomasi and Montecucco, 1981). The exact mechanism by which A₁ enters the cell is unclear, however evidence suggests that it may do so via receptor-mediated endocytosis (Janicot *et al.*, 1991; Lencer *et al.*, 1992). Once inside the host cell, the enzymatic A₁ subunit triggers a metabolic cascade, disrupting many crucial cellular functions. The A₁ subunit ADP-ribosylates the host cell G_s protein (a GTP-hydrolysing protein) which in turn regulates the activity of adenylate cyclase. Adenylate cyclase mediates the conversion of ATP to cyclic AMP (cAMP), a crucial cellular messenger. High levels of cAMP leads to an increase in secretion of Cl⁻ ions from crypt cells, and a decrease in adsorption of Na⁺ ions by villus cells, resulting in a net movement of electrolytes into the gut lumen, followed by loss of fluid (Field, 1971). Despite the massive loss of fluid however, CT does not disrupt adsorption (Drasar and Barrow, 1985) and so replacement of lost fluid and salts (rehydration therapy) is an effective means of treatment for those afflicted with cholera.

The extracellular secretion of the cholera toxin requires the products of the *eps* locus, as mutations in *eps* abolish secretion and lead to the accumulation of assembled cholera toxin in the periplasm, and in addition affects the secretion of SHA/protease and a chitinase (Overbye *et al.*, 1993; Sandkvist *et al.*, 1993; Sandkvist *et al.*, 1995). The Eps components are homologous to proteins of the general secretory pathway (Pugsley *et al.*, 1990), which are essential for secretion in other organisms including *Aeromonas hydrophila*, *Klebsiella oxytoca* and *Pseudomonas aeruginosa*.

1.3.2 Phage encoded CT

A major discovery in cholera research was recently unveiled by Waldor and Mekalanos (1996). The genes encoding the CT (and flanking DNA) are encoded by a filamentous phage, CTXΦ, which is related to the M13 coliphage. The CTXΦ can replicate as a plasmid or integrate into a specific site in the chromosome of *V. cholerae* to form stable lysogens. The receptor for CTXΦ is the TCP pilus involved in intestinal colonisation, and transduction of the phage was shown to occur *in vivo*, within the gastrointestinal tract. This provided a mechanism by which non-toxigenic, avirulent strains of *V. cholerae* could be

converted to toxigenic virulent strains. Waldor and Mekalanos (1996) conclude that the emergence of pathogenic *V. cholerae* is a result of horizontal gene transfer, that may depend on *in vivo* expression of TCP. The implication is that any *V. cholerae* strain expressing TCP may potentially be a target for the CTX Φ and therefore acquire virulence, a factor which must be now considered when developing cholera vaccines.

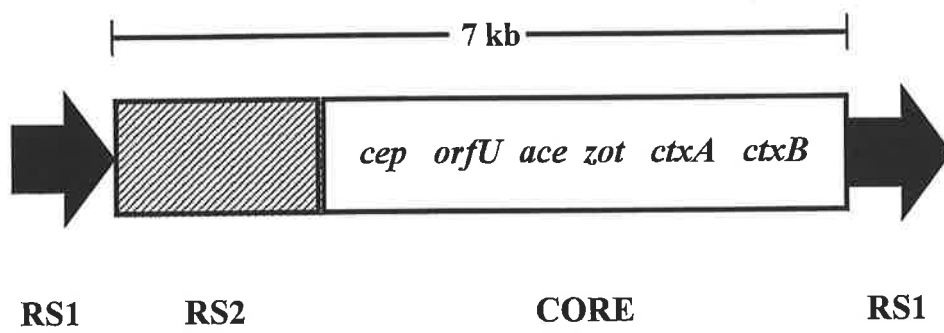
The genes encoding CT (*ctxAB*) lie in a segment of DNA termed the "core" region and 5' to the core lies RS2 (Figure 1.1). The core and RS2 make up the CTX Φ genome and this region is flanked by two or more copies of a repeated sequence RS1. The core and RS sequences are collectively known as the CT genetic element, and this element has the structure of a compound transposon (Pearson *et al.*, 1993). A comparison of toxinogenic and non-toxinogenic *V. cholerae* strains reveal that the CT element is absent in non-toxinogenic strains (Mekalanos, 1983; Miller and Mekalanos, 1984). Recombination between the RS1 elements can result in tandem duplication and amplification of the core region, as well as deletion of the core region (Goldberg and Mekalanos, 1986). In some El Tor strains of *V. cholerae*, the CT element is tandemly duplicated and can undergo amplification upon growth in the rabbit intestine, while classical strains have two copies of the element, which are separated on the chromosome (Mekalanos, 1983 and 1985). The RS1 sequences encode a site-specific recombination system, and it was demonstrated that a suicide vector carrying RS1 was able to integrate into an 18 base pair target site (*attRS1*), located on the chromosome of non-toxicogenic *V. cholerae* strains. This site-specific recombination was *recA* independent (Pearson *et al.*, 1993). The nucleotide sequence of the RS1 and RS2 genetic elements are almost identical, and genes within these regions encode products required for integration, replication and regulatory functions of CTX Φ (Waldor *et al.*, 1997).

In addition to the *ctx* genes, the core region carries *cep*, *orfU*, *ace* and *zot* (Fasano *et al.*, 1991; Pearson *et al.*, 1993; Trucksis *et al.*, 1993) and these genes are proposed to represent the CTX Φ genome (Waldor and Mekalanos, 1996). The genomic organization of CTX Φ is conserved with those of other filamentous phages, including M13 (Waldor and Mekalanos, 1996). In addition, the products of *zot* and *orfU* have been shown to be

Figure 1.1 Structure of the CT element of *V. cholerae*.

The CT element consists of the core region, the RS2 and RS1 elements. The core region and RS2 are encoded by the bacteriophage, CTX Φ (Waldor and Mekalanos, 1996).

CTX Φ GENOME



required for the morphogenesis of CTX Φ (Waldor and Mekalanos, 1996). The *zot* gene product is homologous to gene I, which is found in filamentous phage of *E. coli* and *Pseudomonas* and *Xanthomonas* (Koonin, 1992). The product of gene I is required for phage assembly (Russel, 1995).

Previously the *cep*, *ace* and *zot* genes of the core region of the *V. cholerae* chromosome were proposed to encode potential virulence factors, and thus this region was designated the "virulence cassette" (Trucksis *et al.*, 1993). However, the recent findings of Waldor and Mekalanos (1996) cast doubt on the proposed roles of these gene products. As the *cep* gene product showed homology to the *fxp*-encoded flexible pilin subunit of *Aeromonas hydrophila* and strains with deletions in *cep* were defective for intestinal colonization, the gene was known as the core-encoded pilin or *cep* (Pearson *et al.*, 1993). However, the amino acid products of both *cep* and *fxp* are closely related to the M13 virion capsid protein, and it is suggested that *cep* encodes the virion capsid of CTX Φ (Waldor and Mekalanos, 1996). These authors suggest therefore that Fxp and Cep are phage particles and not pili, as was originally suggested (Ho *et al.*, 1990; Pearson *et al.*, 1993).

The *zonula occludens* toxin (ZOT) was originally reported by Fasano *et al.* (1991) as a toxin that increases the permeability of the small intestinal mucosa, by affecting the structure of the intercellular tight junctions (*zonula occludens*). ZOT was identified by measuring the activity of culture supernatants from CT-negative and CT-positive strains of *V. cholerae* using Ussing chambers. Trucksis *et al.* (1993) proposed that the *ace* gene product, which increased the potential difference across epithelial membranes and induced fluid secretion in ligated rabbit ileal loops, functions as an accessory cholera enterotoxin. However, based on the conservation of the genetic organization of filamentous phage such as CTX Φ , the *ace* gene product is now believed to participate in the receptor binding of CTX Φ (Waldor and Mekalanos, 1996).

1.4 Other toxins of *Vibrio cholerae*

It is well established that cholera toxin is responsible for diarrhoea, the major symptom of cholera. However, candidate vaccine strains of both El Tor (Kaper *et al.*,

1984b) and classical (Kaper *et al.*, 1984a) *V. cholerae* devoid of cholera toxin still produced residual diarrhoea in 50% of human volunteers (Levine *et al.*, 1988a). In addition, many recipients experienced other adverse reactions such as headache, fever, nausea, intestinal cramps and vomiting. The results of these trials suggested the existence of additional enterotoxins(s) in *V. cholerae* O1, that are distinct from CT (Levine *et al.*, 1988a). Most non-O1 non-O139 vibrios do not produce the cholera-like toxin, yet in many instances the non-O1 non-O139 clinical cases are virtually indistinguishable from cholera cases (Ramamurthy *et al.*, 1993; Mukhopadhyay *et al.*, 1995) and thus it was hypothesised that additional enterotoxin(s) also exist in these serogroups. It has also been suggested, however, that colonization of the small intestine by *V. cholerae* may be sufficient to produce reactogenicity. The search for additional diarrhoeagenic factors has revealed a variety of toxins produced by both O1 and non-O1 strains of *V. cholerae*. Zot and Ace have been described earlier, and the haemolysin and other toxins will be discussed further in the following sections.

1.4.1 Shiga-like toxin

Some strains of both *V. cholerae* O1 and *V. cholerae* non-O1 produce a protein that is cytotoxic to HeLa cells. Antibody specific for the shiga toxin of *Shigella dysenteriae* was able to neutralize this cytotoxicity, and hence the toxin was considered to be "shiga-like" (O'Brien *et al.*, 1984; Gyobu *et al.*, 1991). The shiga-like toxin was initially believed to be the alternate diarrhoeagenic factor of *V. cholerae*, however a non-O1 strain producing this toxin failed to produce diarrhoea in human volunteers (Morris *et al.*, 1990). The gene encoding the shiga-like toxin has yet to be cloned and analysed.

1.4.2 Heat-Stable toxin (NAG-ST)

Several strains of *V. cholerae* non-O1 produce a 17 amino acid heat-stable enterotoxin (NAG-ST) that is similar to the heat-stable toxin of enterotoxigenic strains of *E. coli* and *Yersinia enterocolitica* (Honda *et al.*, 1985; Takeo *et al.*, 1985; Arita *et al.*, 1986; Ogawa *et al.*, 1990; Arita *et al.*, 1991). Interestingly, non-O1 strains appear to produce

either the cholera toxin or NAG-ST, but not both (Morris, 1994). A human volunteer study undertaken by Morris *et al.* (1990) demonstrated that a NAG-ST-producing and colonising strain of *V. cholerae* non-O1 was able to cause diarrhoea of similar severity to that of cholera. However, the frequency of clinical isolates of *V. cholerae* non-O1 that produce NAG-ST is low, which indicates that there is an as yet undefined additional factor(s) involved with the diarrhoeagenicity of non-O1 vibrios (Morris, 1994). The NAG-ST is generally not produced by the O1 serogroup, and one study has revealed that only 1 out of 197 O1 isolates produced this toxin and was also positive for the cholera toxin gene (Takeda *et al.*, 1991).

1.4.3 Thermostable direct haemolysin (TDH)

Vibrio parahaemolyticus is a major cause of diarrhoea and gastroenteritis associated with the consumption of seafood (Blake, 1980). The agent responsible for the enterotoxicity of this species is the thermostable-direct haemolysin (TDH) (Nishibuchi *et al.*, 1992). TDH is a heat-stable dimer composed of two identical subunits of 165 amino acids (Honda and Iida, 1993). Purified TDH is both cardiotoxic and rapidly lethal for mice (Honda *et al.*, 1976), produces a positive response in the rabbit ileal loop assay and is also able to cause diarrhoea in monkeys (Zen-Yoji *et al.*, 1974). Non-O1 *V. cholerae* produce a toxin related to the TDH (Honda *et al.*, 1985; Gyobu *et al.*, 1991) and genes homologous to TDH-encoding genes have been found on a plasmid in some non-O1 strains (Honda *et al.*, 1986). It was previously believed that TDH-encoding genes were not present in *V. cholerae* O1 (Terai *et al.*, 1991), however an open reading frame homologous to the TDH gene of *V. parahaemolyticus* was recently identified in the *V. cholerae* O1 classical strain Z17561 (A. Fallarino, personal communication).

TDH acts by forming pores in the host cell membranes, causing colloidal osmotic haemolysis (Honda *et al.*, 1992). *V. parahaemolyticus* is classified into two groups, according to the ability of the organism to cause TDH-mediated haemolysis on special blood agar (Wagatsuma medium). Haemolytic activity on this agar is called the Kanagawa phenomenon (Takeda, 1983). A positive Kanagawa phenomenon is closely associated with

pathogenicity for humans, as most clinical isolates are Kanagawa positive, while nearly all environmental isolates are Kanagawa negative (Miyamoto *et al.*, 1969; Takeda, 1983).

1.4.4 The non-membrane-damaging cytotoxin (NMDCY)

NMDCY is a 35 kDa heat labile, trypsin-sensitive toxin that is produced by some clinical strains of *V. cholerae* non-O1 (Saha *et al.*, 1996). NMDCY elicits the cell rounding of Chinese hamster ovary cells (CHO) and HeLa cells, without causing damage to the membrane (hence its designated name) and purified toxin causes fluid accumulation in the rabbit ileal loop assay (Saha *et al.*, 1996). The morphological changes elicited by NMDCY closely resemble those of the non-membrane damaging cytotoxic enterotoxins of *Clostridium difficile* and *Bacteroides fragilis* (Saha *et al.*, 1996). The authors note that the production of the El Tor haemolysin from these strains masks the activity of NMDCY, and so NMDCY was isolated under conditions which suppressed the production of the haemolysin (Saha *et al.*, 1996). The toxin was originally isolated from a clinical strain of *V. cholerae* O26, however, by using specific monoclonal antibodies, it was established that NMDCY production is widespread among strains of *V. cholerae*, including toxigenic and non-toxigenic *V. cholerae* O1 and environmental non-O1 non-O139 isolates. NMDCY was also detected among other enteric bacteria, including clinical isolates of *V. parahaemolyticus*, *Aeromonas* spp. and *Shigella* spp. (Saha and Nair, 1997). Despite the cytotoxic and enterotoxic activities of NMDCY, it has yet to be determined whether this toxin contributes to the diarrhoea observed with clinical isolates of non-O1 and non-O139 *V. cholerae*.

1.5 The *V. cholerae* O1 Haemolysin (HlyA)

1.5.1 The haemolytic status of *V. cholerae* O1 strains

The *hlyA* gene is a highly conserved genetic element among the virulence genes in *V. cholerae*, and has been detected in both O1 and non-O1 *V. cholerae*. (Goldberg and Murphy, 1984; Manning *et al.*, 1984; Brown and Manning, 1985; Manning *et al.*, 1988; Morris, 1990). The presence of the *hlyA* gene, however, does not always reflect the haemolytic status of the cell. Traditionally, El Tor strains were considered haemolytic and

classical strains were considered non-haemolytic and this distinction was originally used to differentiate between the biotypes (Pollitzer, 1959). However, due to the variability of El Tor strains, haemolytic (Hly) phenotype is no longer solely used to determine biotype. The majority of non-O1 strains of *V. cholerae* are haemolytic (Sakazaki *et al.*, 1967).

Since 1961, weakly haemolytic variants of El Tor strains were identified and by the mid 1960s and 1970s, most El Tor strains were considered non-haemolytic (de Moor, 1963; Gallut, 1974). However in more recent years, the proportion of haemolytic El Tor isolates associated with disease has increased (Barrett and Blake, 1981; Iwanga *et al.*, 1982; Kaper *et al.*, 1982). In addition, Goldberg and Murphy (1984) have reported variation of haemolytic phenotype within individual isolates of the El Tor strain RV79, which is phenotypically non-haemolytic, but can convert to a haemolytic phenotype at moderate frequencies. The nucleotide sequence of *hlyA* and the regulatory region for both haemolytic and non-haemolytic El Tor RV79 is identical and the levels of *hlyA*-specific mRNA in recombinant *E. coli* are comparable (Rader and Murphy, 1988). The reason for the non-haemolytic status of some El Tor strains is not known.

As classical strains are unable to lyse sheep erythrocytes, they were regarded as non-haemolytic (Pollitzer, 1959), but have since been shown to lyse both chicken and rabbit erythrocytes (Richardson *et al.*, 1986). The Hly⁻ phenotype of classical strains for sheep erythrocytes is due to a defect in the structural gene, *hlyA*. The *hlyA* gene has an 11 bp deletion which generates an early stop codon and premature termination of *hlyA* translation produces a truncated HlyA protein of 27 kDa, designated HlyA* (Alm *et al.*, 1988; Rader and Murphy, 1988). This defect in the *hlyA* gene is conserved in all classical strains tested (Alm and Manning, 1990b). There is an inverted repeat around the deleted region which can potentially form a stem loop structure, and it has been suggested that this loop structure may have facilitated the generation of the deletion, in a past replication event (Alm *et al.*, 1988). The regulatory region of *hlyA* is identical in both biotypes, although less mRNA specific for *hlyA** is detected in recombinant *E. coli* compared to that for *hlyA*. This difference may be attributed to greater degradation of classical *hlyA*-specific mRNA (Rader and Murphy, 1988).

A second haemolysin, unrelated to the El Tor haemolysin was isolated from classical strains, however the significance of this second haemolysin is not known at this stage (Richardson *et al.*, 1986). A novel haemolysin gene *hlx* was recently isolated from the El Tor strain N86, the product of which was haemolytic when expressed in *E. coli* (Nagamune *et al.*, 1995). However, the haemolytic activity and ability to cause fluid accumulation in ligated ileal loops of a *hlx* null mutant of *V. cholerae* did not differ from that of wildtype. The *hlx* gene is unrelated to *hlyA* and shows no homology to any other toxin genes (Nagamune *et al.*, 1995).

1.5.2 Activities of El Tor HlyA and classical HlyA*

The El Tor haemolysin (HlyA) has cytotoxic, haemolytic (cytolytic) and enterotoxin activities. It has also been demonstrated to be cardiotoxic and rapidly lethal for mice (Honda and Finkelstein, 1979). The HlyA toxin is referred to as a 'haemolysin', based on its ability to lyse eukaryotic erythrocytes from a variety of species including rabbit, goat, mouse, sheep, chicken, and humans (Honda and Finkelstein, 1979). However, the cytolytic and cytotoxic capabilities of HlyA extend to cultured nucleated cell types including Y-1 adrenal cells (Honda and Finkelstein, 1979), HEp-2 cells (Alm *et al.*, 1991), Vero cells (Hall and Drasar, 1990) and intestinal Henle 407 cells (Zitser *et al.*, 1997). Marked intestinal epithelial damage attributed to HlyA was observed in the intestines of infected mice, where the El Tor strain O17 displayed significant sloughing off of the epithelium when compared to the isogenic *hlyA::Km^R* mutant (Alm *et al.*, 1991). HlyA is a potent toxin, as the binding of less than 10^4 molecules per cell *in vitro* is lethal for cultured cell lines (Zitser *et al.*, 1997). The haemolysin is capable of inducing fluid accumulation in ligated ileal segments of adult rabbits, and the accumulated fluid is bloody, viscous and mucoid (Alm *et al.*, 1991).

The classical product HlyA* is a truncated form of the El Tor haemolysin and while it retains the cytotoxic and enterotoxin activities associated with HlyA (Alm *et al.*, 1991), there is contradictory evidence as to whether it retains the cytolytic activity. HlyA* was unable to cause any significant lysis in a cytolytic assay using a mouse antigen-presenting tumour cell line internally labelled with ^{51}Cr (Alm *et al.*, 1991). However, Richardson *et*

al. (1986) showed that Hly* was able to lyse rabbit (but not sheep) erythrocytes, when expressed in *E. coli*. It is possible that HlyA* differs from HlyA in host cell specificity.

Non-O1 *V. cholerae* strains produce a haemolysin that is biologically, physiochemically and immunologically indistinguishable from the El Tor haemolysin, and is often referred to as the "El Tor-like haemolysin" (Yamamoto *et al.*, 1986). Purified non-O1 haemolysin is rapidly lethal for mice, increases vascular permeability of rabbit skin and is able to lyse erythrocytes from a variety of species (Yamamoto *et al.*, 1984) and in addition is also cytotoxic for adrenal Y-1 cell lines and Chinese hamster ovary cells (McCardell *et al.*, 1985; Spira *et al.*, 1986). Ichinose *et al.* (1987) demonstrated that the non-O1 haemolysin causes fluid accumulation in the ileal loop model, and analogous to the El Tor HlyA, the fluid accumulation attributed to non-O1 HlyA was invariably mucoid and bloodied and was accompanied by a histological change in the intestinal mucosa.

1.5.3 HlyA maturation

HlyA was originally purified by Honda and Finkelstein (1979) and based on gel filtration on Sephadex columns, its molecular size was predicted to be 20 kDa. However, the migration of HlyA, as with many other cytotoxins, was probably retarded due to the interaction of HlyA with the Sephadex gel. The haemolysin is actually synthesized as an 82 kDa pre-pro-protein which is processed to an 80 kDa pro-form following the removal of the signal sequence during secretion (Manning *et al.*, 1984; Goldberg and Murphy, 1985; Alm *et al.*, 1988; Hall and Drasar, 1990; Yamamoto *et al.*, 1990a). The inactive 80 kDa pro-haemolysin is secreted into the extracellular medium where it is cleaved to produce the haemolytically active 65 kDa form (Alm *et al.*, 1988; Hall and Drasar, 1990; Yamamoto *et al.*, 1990a; Nagamune *et al.*, 1996). Haemolysins produced from a variety of bacterial pathogens are initially produced as inactive precursors, which may serve to protect the bacteria from the toxic effects of the proteins. Amino-terminal sequencing analysis of the 65 kDa form of HlyA has established that 15 kDa are removed from the N-terminus of pro-HlyA during maturation, however, there is conflict over the exact site of cleavage (Hall and Drasar, 1990; Yamamoto *et al.*, 1990a; Nagamune *et al.*, 1996). The processing of HlyA is

explored and discussed further in Chapter 4. Nagamune *et al.* (1997) have recently shown that the N-terminal 15 kDa pro-region acts as an intramolecular chaperone required for the correct folding of the mature haemolysin, and the sequence of the pro-region is similar to that of the molecular chaperone Hsp90.

1.5.4 HlyA Export

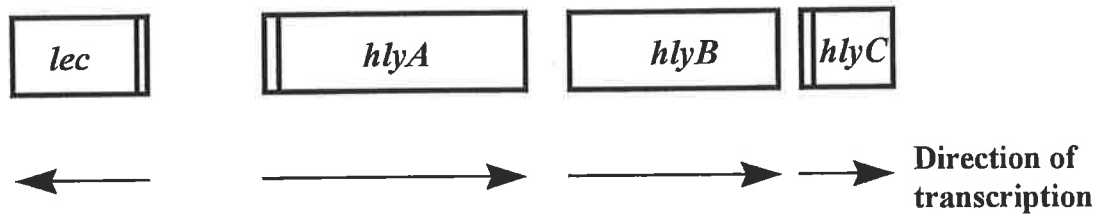
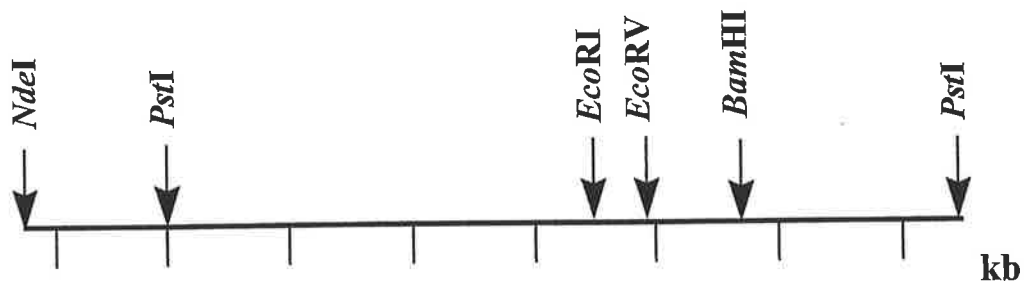
The transport of HlyA across the inner membrane presumably occurs via the Sec-dependent system (Pugsley *et al.*, 1990), with the concomitant removal of the N-terminal 25 amino acid signal peptide (Hall and Drasar, 1990; Yamamoto *et al.*, 1990a). HlyA resides transiently in the periplasm, prior to secretion into the extracellular medium. Neither the mechanism whereby HlyA is translocated from the periplasm across the outer membrane, nor the secretion signal within HlyA, is known at this stage. When HlyA is expressed in *E. coli*, it remains trapped in the periplasm and is not secreted, however *tol* mutants that leak periplasmic proteins are able to mimic the secretion of HlyA into the extracellular media (Mercurio and Manning, 1985). While the *eps* locus of *V. cholerae* is required for the secretion of the cholera toxin, SHA/protease and the chitinase (Overbye *et al.*, 1993; Sandkvist *et al.*, 1993), it is not known whether it is required for the secretion of HlyA.

1.5.5 Genetic organization of the *hly* locus

The genetic organisation of the *hly* locus is shown in Figure 1.2. The structural gene *hlyA* encoding the haemolysin was initially localised on a 3.5 kb *Pst*I/*Eco*RI fragment by transposon insertion and deletion analysis (Manning *et al.*, 1984). *hlyA* from both El Tor and classical biotypes have since been cloned (Manning *et al.*, 1984; Goldberg and Murphy, 1984 and 1985; Richardson *et al.*, 1986) and sequenced (Alm *et al.*, 1988; Rader and Murphy, 1988; Yamamoto *et al.*, 1990a). A potential promoter for *hlyA* was identified by primer extension analysis and transcription initiates 430 nt upstream of the translational start site (Williams and Manning, 1991). This unusually long untranslated region is seen with both El Tor and classical strains, but its role is not known (Williams and Manning, 1991).

Figure 1.2 The *hly* locus of El Tor *V. cholerae* O1.

Genes of the *hly* locus are labelled A, B and C. The lecithinase gene, *lec*, is divergently transcribed to *hlyA*. The upper line shows relevant restriction enzyme sites of this region.



1 kb



The *E. coli* α -haemolysin operon also has a large untranslated 5'-region similar in length to that of *V. cholerae hlyA* (Welch and Pellet, 1988), however the genes are unrelated.

The lecithinase (phospholipase) gene *lec* lies upstream of, but is transcribed divergently to, the *hlyA* gene (Fiore *et al.*, 1997). The 45 kDa gene product Lec, was shown to degrade the phospholipid lecithin (phosphatidylcholine) of egg yolk agar, and a mutation in *lec* abolished this activity (Fiore *et al.*, 1997). While phospholipases have been implicated in the pathogenic process of bacterial disease, studies examining the effect of a *lec* mutation exclude this gene as a virulence determinant of *V. cholerae*. Lec does not contribute to the morphological changes of cultured intestinal epithelial cells incurred by the exposure to *V. cholerae* culture supernatants. Examination of the mutant in the *in vivo* ligated intestinal loop demonstrated that it does not cause fluid accumulation. *lec* exhibits homology to other *Vibrionaceae* phospholipases that are able to lyse erythrocytes, including the thermolabile haemolysin from *V. parahaemolyticus* and the glycerophospholipid-cholesterol acyltransferases from *Aeromonas salmonicida* and *A. hydrophila* (Fiore *et al.*, 1997).

Downstream of *hlyA* lies the *hlyB* gene and there is a putative rho-independent terminator of moderate strength in the *hlyA-hlyB* intergenic region (Alm *et al.*, 1988). Northern blot analysis show that transcription of *hlyB* can occur from the upstream *hlyA* promoter, which suggests there is transcriptional readthrough of the putative terminator (Williams and Manning, 1991). However, this readthrough occurs at low levels and is only seen when *hlyU* is present in multicopies (Williams and Manning, 1991). *hlyB* is predicted to encode a 60.3 kDa protein based on sequence analysis and cell fractionation, however, HlyB migrates aberrantly at 71 kDa on an SDS-polyacrylamide gel due to the unusually highly charged C-terminus of the protein (Alm and Manning, 1990a). HlyB has previously been implicated in the secretion of the haemolysin, as HlyA remains trapped in the periplasm of *hlyB* mutants and is released only in stationary phase, presumably due to cell lysis (Alm and Manning, 1990a). However, HlyB shares significant homology with the methyl-accepting chemotaxis proteins (MCPs) of *E. coli*, which are involved in environmental sensing and signal transduction and also shares sequence similarity with TcpI and AcfB of

V. cholerae (Manning, 1994; Everiss *et al.*, 1994). MCPs are integral inner membrane proteins with distinct periplasmic ligand-binding and cytoplasmic signalling domains (Boyd *et al.*, 1983). The most significant homology between HlyB and the MCPs lies within the C-terminal half, particularly in the region referred to as the 'highly conserved domain' of MCPs (Alley *et al.*, 1992).

A potential rho-independent terminator lies downstream of *hlyB* and this is followed by the *hlyC* gene, the product of which is proposed to encode an 18.3 kDa protein which shows homology to the triacyl-glyceride lipases of *Pseudomonas* spp. (Manning, 1994; Casanova and Peterson, 1995). The characterisation of the genetic locus downstream of *hlyAB* has been modified and extended and is discussed further in Chapter 6.

1.5.6 Regulation of *hlyA* expression

1.5.6.1 HlyU

HlyU is a small protein (11.9 kDa) which transcriptionally upregulates the expression of *hlyA*. The *hlyU* locus was first identified using *hlyA-cat* transcriptional fusions to screen a genomic library of *V. cholerae* O17 in *E. coli* (Williams and Manning, 1991). The amino acid sequence of HlyU suggests that it belongs to the family of small regulatory proteins, including NolR (*Rhizobium meliloti*), SmtB (*Synechococcus*) and the ArsR repressors (*E. coli*, *S. aureus* and *S. xylosus*) (Williams *et al.*, 1993). A putative helix-turn-helix motif characteristic of a DNA binding domain lies within HlyU (Williams *et al.*, 1993) and initial experiments confirm the DNA binding ability of HlyU. Gel-retardation assays show that HlyU-enriched extracts isolated from both *E. coli* and *V. cholerae* are able to specifically bind a 124-bp fragment incorporating the -59 to -183 upstream region of the *hlyA* mRNA start site (Coates, 1995). Mutants in *hlyU* are significantly less haemolytic than wildtype, although HlyA can still accumulate in stationary phase, which demonstrates that HlyU is not essential for the production of HlyA, despite its ability to up-regulate the *hlyA* gene (Williams *et al.*, 1993).

A *V. cholerae hlyU* mutant is attenuated, displaying a 100-fold increase in LD₅₀ when compared with wild type in the infant mouse model (Williams *et al.*, 1993). It is

possible that HlyU may control other, unidentified proteins required for full virulence, although it is not known whether the decrease in virulence of a *hlyU* mutant is due only to a reduction of *hlyA* expression, as the LD₅₀ values for a *hlyA* mutant are comparable with those of a *hlyU* mutant (Williams *et al.*, 1993). A comparison of the colonisation abilities of the *hlyU* mutant with wildtype show a slight reduction in colonisation ability with the mutant, which suggests that HlyU may control a colonisation factor(s) (Williams *et al.*, 1993).

The Hcp protein (for Haemolysin co-regulated protein) is also regulated by HlyU and was first identified by comparing the protein profile of O17 and its *hlyU* mutant (Williams *et al.*, 1993). Two genes, *hcpA* and *hcpB* have been identified and sequenced and the products HcpA and HcpB, are identical (Williams *et al.*, 1996). Southern blot analysis shows that *hcpA* and *hcpB* are present in all *V. cholerae* strains examined, however, not all strains produce the Hcp protein (Williams *et al.*, 1996). Both *hcpA* and *hcpB* express proteins which migrate as 28 kDa on SDS-PAGE, however the coding capacity of each gene is only 19 kDa. Williams *et al.* (1996) have suggested that Hcp runs aberrantly during polyacrylamide gel electrophoresis due to post-translational modification of two cysteine residues.

The mechanism of upregulation by HlyU appears to be different for HcpAB and HlyA. While *hlyA* and *hcp* are co-regulated by HlyU, the promoter regions do not share any sequence homology and therefore do not have a consensus binding sequence for HlyU. While consensus -10 and -35 sequences have been identified in the promoter of *hlyA*, the *hcp* promoter contains consensus sequences for σ^{54} DNA binding proteins and the integration host factor (Williams and Manning, 1991; Williams *et al.*, 1996). HlyU is able to activate the *hlyA* promoter, but not the *hcp* promoters in *E. coli*, which suggests that HlyU requires an intermediate regulator in order to activate *hcp* (Williams *et al.*, 1996). In support of the existence of an intermediate protein, *E. coli* derived protein extracts enriched in HlyU were unable to bind the promoter regions of *hcp*, while extracts obtained from *V. cholerae* demonstrated weak DNA binding abilities (Coates, 1995). HlyU may therefore be

part of a multi-component regulatory cascade, which is a common mechanism utilised by pathogenic bacteria for the regulation of virulence gene expression (Miller *et al.*, 1989).

Hcp does not appear to contribute to the pathogenesis of *V. cholerae*, as mutants defective in both *hcp* genes remain fully virulent in the infant mouse model (Williams *et al.*, 1996). To date, Hcp is the only protein secreted from *V. cholerae* which does not use an N-terminal signal sequence. Amino-terminal analysis has revealed that only the N-terminal methionine of Hcp is processed during secretion (Williams *et al.*, 1996).

1.5.6.2 The role of Iron

In addition to HlyU regulation, haemolysin production is also regulated by the levels of available iron in the media. Low levels of iron increase the production of HlyA, while high iron levels repress its production (Stoebner and Payne, 1988). The synthesis of haemolysins from *Serratia marcescens* (Poole and Braun, 1988) and a nephropathogenic *E. coli* (Waalwijk *et al.*, 1983) are also regulated by iron. It is well recognised that iron acquisition by bacterial pathogens is associated with virulence. In *V. anguillarum*, there is a clear correlation between virulence and the iron-sequestering system, as loss of the plasmid-encoding iron-uptake genes or mutations within the genes, renders the bacteria avirulent (Crosa *et al.*, 1977; Crosa *et al.*, 1980; Singer *et al.*, 1991). Because of the involvement of iron in HlyA synthesis, it is proposed that one role of the El Tor haemolysin may be to increase the levels of available iron during an infection. In the mammalian host, iron is limited as it is associated with host iron-binding proteins, such as ferritin or lactoferrin. HlyA is able to lyse epithelial cells and erythrocytes which may function to release intracellular haem compounds and provide a source of iron. Bloody fluid accumulation and tissue damage in ligated ileal loops have been attributed to the O1 (Alm *et al.*, 1991; Zitzer *et al.*, 1997) and non-O1 El Tor haemolysin (Ichinose *et al.*, 1987).

Approximately 100 nts upstream of the *hlyA* transcriptional start site lies a putative Fur binding site (Alm, 1989; Williams and Manning, 1991). In *E. coli*, the Fur protein represses transcription of iron-regulated genes in the presence of sufficient iron, by binding to the highly conserved consensus sequence (Fur box) located in the promoter of these genes

(reviewed in Silver and Walderhaug, 1992). The Fur box is a 19 bp consensus sequence with dyad symmetry and may be present twice within a promoter, as primary and secondary sites (de Lorenzo *et al.*, 1987; Wee *et al.*, 1988). Interactions of the *E. coli* Fur with DNA of the promoter regions of the siderophore aerobactin and haemolysin operons have been directly visualised using atomic force microscopy and Dark-field electron microscopy (Le Cam *et al.*, 1994). *V. cholerae* has a *fur* locus analogous to that of *E. coli*, and *E. coli fur* is able to complement *V. cholerae* Fur mutants, and vice versa (Stoebner and Payne, 1988; Litwin *et al.*, 1992). Fur homologues have been identified in other Gram negative pathogens, including *V. vulnificus* (Litwin and Calderwood, 1993), *V. anguillarum* (Tolmasky *et al.*, 1994), *Salmonella typhimurium* (Foster and Hall, 1992), *Yersinia pestis* (Staggs and Perry, 1991) and *Pseudomonas aeruginosa* (Prince *et al.*, 1993). There is also evidence for an additional iron-responsive regulatory system in *V. cholerae*, as proteins have been identified that are regulated by iron in a Fur-independent manner (Litwin and Calderwood, 1994).

Iron-limitation in *V. cholerae* results in the co-ordinate expression of other genes, including virulence factors and genes involved in iron uptake. The production of the siderophore vibriobactin, which binds ferric iron (Sigel and Payne, 1982) and other outer membrane proteins, is controlled by the availability of iron. Iron-regulated outer membrane proteins of *V. cholerae* include IrgA, of unknown function but which is associated with virulence (Goldberg *et al.*, 1990) and ViuA, which is the receptor for vibriobactin (Butterton *et al.*, 1992; Stoebner *et al.*, 1992). Upstream of the *irgA* and *viuA* genes are nucleotide sequences homologous to the *E. coli* Fur box (Goldberg *et al.*, 1991; Butterton *et al.*, 1992) and Fur has been shown to repress both genes (Litwin and Calderwood, 1994). 2-D gel electrophoresis comparing protein extracts from a *V. cholerae fur* mutant with wildtype have shown that Fur represses the expression of at least 22 other proteins (Litwin and Calderwood, 1994).

In addition to the siderophore-mediated iron-acquisition system, *V. cholerae* can acquire iron from mammalian iron compounds such as haem or haemoglobin (Stoebner and Payne, 1988). The haem or haemoglobin-utilization system has been characterised and

involves at least the inner membrane HutB protein and the outer membrane HutA protein (Henderson and Payne, 1993). HutA is homologous to TonB-dependent receptors (Goldberg *et al.*, 1992; Henderson and Payne, 1994a) and is transcriptionally regulated by iron in a Fur-dependent manner (Henderson and Payne, 1994a). Together, the HutA and HutB system can transport the entire haem molecule into the cell (Henderson and Payne, 1994a). Henderson and Payne (1994b) have analysed single and double mutants of the vibriobactin and haem utilization systems in the infant mouse model and have shown that disruption of either system reduces the ability of *V. cholerae* to cause disease, especially when small inocula are used.

1.5.6.3 Other factors involved in *hlyA* expression ?

While the ToxR protein transcriptionally activates the cholera toxin and Tcp genes (Miller and Mekalanos, 1984; Taylor *et al.*, 1987), examination of a *toxR::km^R* mutant *in vitro* has excluded its involvement in *hlyA* activation (Williams and Manning, 1991). The *hlyR* locus, closely linked to, but distinct from *toxR*, was previously proposed to be an activator of *hlyA* expression (von Mechow *et al.*, 1985), however, as a Hly⁻ *hlyR* strain can be restored to Hly⁺ status by transduction with a *hlyA* transducing phage, the role of *hlyR* is unclear (Varcoe, 1991). Post-transcriptional control mechanisms may also be involved in *hlyA* regulation, since non-haemolytic El Tor strains produce identical levels of *hlyA*-specific mRNA as haemolytic strains (Rader and Murphy, 1988).

1.5.7 The role of HlyA in disease

There is sufficient evidence to implicate the El Tor haemolysin as a potent toxin. However, despite the extensive studies investigating the biological activities of HlyA, its role in the pathogenesis of *V. cholerae* remains unclear. Honda and Finkelstein (1979) first questioned the relevance of HlyA in virulence, as haemolytic phenotype does not always correlate with disease. However, HlyA has been implicated as a virulence determinant for the El Tor biotype in the infant mouse model. Mouse studies comparing the virulence of

hlyA strains with wildtype show a 50-fold increase in LD₅₀ for the *hlyA* mutant of El Tor *V. cholerae*, strain O17 (Alm *et al.*, 1991; Williams *et al.*, 1993).

As discussed earlier, HlyA is capable of inducing fluid accumulation in rabbit ileal loops, and so is deemed enterotoxic. However, a clear association between the haemolysin and the production of diarrhoea in infected individuals, is yet to be made. Kaper *et al.* (1988) constructed derivatives of the candidate vaccine strains JBK70 and CVD101, which carry a 400bp *HpaI* deletion in the *hlyA* gene. These strains still produced diarrhoea in 33% of the recipients (Levine *et al.*, 1988a) and so it was concluded that *hlyA* was not responsible for the residual diarrhoea seen with volunteers receiving JBK70 and CVD101. However, Alm *et al.* (1991) suggest that the enterotoxic domain of HlyA was not inactivated in these strains. The 27 kDa HlyA* produced by classical *V. cholerae* was inferred to be enterotoxic (Alm *et al.*, 1991), and as this truncated protein corresponds to the N-terminus of the 80 kDa El Tor HlyA, the *HpaI* deletion removed only 2.5 kDa of the 27 kDa HlyA.

CVD103 is a cholera toxin A⁻B⁺ derivative of the classical strain 569B, and volunteers receiving this strain developed mild diarrhoea (Levine *et al.*, 1988b). However, when this strain was genetically tagged by inserting a mercury-resistance (Hg^R) cartridge into *hlyA* and administered to human volunteers, the side effect of diarrhoea was eliminated. A similar vaccine strain CVD110 was constructed in an El Tor *V. cholerae* strain, which is devoid of cholera toxin, zonula occludens toxin, accessory cholera enterotoxin and HlyA, but was still reactogenic and elicited an unacceptable level of diarrhoea in human volunteers (Tacket *et al.*, 1993).

Despite the cytotoxic/enterotoxic activities of the non-O1 haemolysin, it is not certain whether the toxin is responsible for the pathogenic mechanism of the gastroenteritis of *V. cholerae* non-O1 isolates. The symptoms of non-O1 induced gastroenteritis include abdominal cramps, fever, and mucoid bloody diarrhoea (Blake, 1980; Hughes *et al.*, 1978). While haemolysin is produced by most non-O1 clinical isolates (McIntyre *et al.*, 1965), not all haemolytic non-O1 vibrios cause disease, and so a direct link between the toxin and disease cannot be made. A recent study by Singh *et al.* (1996) suggests that the enterotoxicity of non-O1 vibrios is independent of haemolytic phenotype. A colonising,

haemolytic non-O1 strain fed to human volunteers did not cause disease (Morris *et al.*, 1990).

While these vaccine trials do not implicate HlyA or the toxins described earlier (section 1.4) as primary virulence factors, they do not exclude any involvement of the toxins in the pathogenesis of cholera. These toxins may be secondary to the cholera toxin, contributing to diarrhoeagenicity in strains devoid of CT or when conditions are not optimal for cholera toxin production. By comparing *V. cholerae* strains O17 and 569B with their respective *hlyA::Km^R* mutants, Alm *et al.* (1991) observed a delay in fluid accumulation in rabbit ileal loops and a delay in the onset of deaths among infected infant mice with the mutants, which suggests that HlyA may act early in an infection, prior to action of the cholera toxin. In addition to contributing to diarrhoeagenicity, these toxins may be involved in nutrient acquisition for the bacteria. The search for the elusive secretogenic factor(s) of *V. cholerae* O1 continues.

1.6 Other Pore-forming toxins

Pore-forming toxins produced by bacterial pathogens are often associated with the virulence of the organism. Many of these toxins have common requirements: they must be expressed at the appropriate time, be secreted extracellularly, activated, bind to and inserted into the host cell membrane in order to exert their lethal effect. Different toxins have employed various mechanisms for these processes and the following section examines the strategies of representatives of different types of toxins, produced by Gram negative and Gram positive bacteria. The way in which these water-soluble toxins insert into the hydrophobic domain of the host cell membrane is discussed further in Chapter 3.

1.6.1 The *Escherichia coli* alpha-haemolysin: An RTX toxin

The alpha-haemolysin (HlyA) of *E. coli* is a virulence factor associated with urinary tract and other extra-intestinal infections (Welch *et al.*, 1981). The haemolysin is the prototype for the RTX family of pore-forming toxins which are potent cytotoxins of various

nucleated cell types and are calcium dependent. There is a high degree of sequence homology among the RTX toxins and the activation and secretion of these toxins are thought to be mechanistically analogous. The designation RTX (repeats in the toxin) refers to the presence of a tandemly repeated nine amino acid sequence present in all toxin members (Welch, 1991). Other Gram negative pathogens producing RTX toxins include *Proteus mirabilis* and *P. vulgaris*, *Morganella morganii*, and various *Actinobacillus* strains. The *Pasteurella haemolytica* leukotoxin and the *Bordetella pertussis* adenylate cyclase/haemolysin are also RTX toxins (the RTX toxins were recently reviewed in Welch *et al.*, 1995).

The *E. coli* alpha-haemolysin is synthesised as a 110 kDa inactive pro-toxin that requires HlyC-dependent acylation for activation. It is secreted via the HlyB/HlyD transport complex and the genetically unlinked TolC outer membrane protein (Goebel and Hedgpeth, 1982; Härtlein *et al.*, 1983; Wagner *et al.*, 1983; Wandersman and Delepelaire, 1990).

1.6.1.1 Pore formation and activities of *E. coli* HlyA

E. coli HlyA lyses erythrocytes and is cytotoxic for a wide variety of nucleated host cell types, including leukocytes, renal tubular epithelial cells and endothelial cells. Pore formation by HlyA has been demonstrated in erythrocytes, artificial lipid membranes and more recently in human macrophages. However, there is conflict as to whether a toxin monomer or oligomer is responsible for the pore (Bhakdi *et al.*, 1986; Menestrina *et al.*, 1987; Menestrina, 1988; Benz *et al.*, 1989 and 1992; Menestrina *et al.*, 1996). Previous predictions for the pore-diameter have been made, but there is some dispute over the size (Bhakdi *et al.*, 1986). Moayeri and Welch (1994) have shown that the HlyA pore can actually increase in diameter over time, ranging from <0.6 to >1.2 nm. This increase in pore diameter is temperature dependant and does not appear to be the result of additional toxins inserting into the membrane. Two distinct conformational states of HlyA associated with erythrocytes and liposomes have been observed, a reversible pre-lytic state and an irreversible lytic state (Bakas *et al.*, 1996; Moayeri and Welch, 1997). Toxin associated with erythrocytes at 0 to 20°C is defined as the pre-lytic state, as there is a lag period prior to

lysis following a shift to 23°C which is in contrast to the immediate lysis observed following the removal of an osmotic protectant from erythrocytes exposed to haemolysin.

In addition to causing cell death by osmotic lysis, sub-lytic levels of the *E. coli* alpha-haemolysin can disrupt the normal cellular function of eukaryotic target cells. HlyA is able to induce the synthesis and release of inflammatory mediators from granulocytes, mast cells and macrophages (König *et al.*, 1986; König *et al.*, 1994) while human neutrophils exposed to sub-lytic levels of HlyA are subsequently unable to bind chemotactic factors (Welch *et al.*, 1986). Recently, Walev *et al.* (1996) demonstrated that pore-formation by *E. coli* HlyA induced rapid and massive shedding of the interleukin 6 receptor (IL-6R) and the LPS receptor CD14. Cleaved soluble IL-6R was then capable of binding its ligand and inducing an IL-6-specific signal in cells that lacked IL-6R. This 'trans-signaling' is thought to widen the spectrum of responsive cells and offers a possible explanation as to how pore-forming toxins can promote inflammatory responses and have long-range effects within the host. The authors suggest that the insertion of the toxin into the host cell membrane causes membrane-located metallo-proteinases to come into contact with these receptors, thereby cleaving and releasing IL-6R and CD14.

1.6.1.2 Activation of *E. coli* HlyA

HlyC directs the cytoplasmic modification of inactive pro-HlyA to the active form (Goebel and Hedgpeth, 1982; Nicaud *et al.*, 1985; Hardie *et al.*, 1991) by mediating the transfer of a fatty acyl group from an acyl carrier protein to pro-HlyA (Issartel *et al.*, 1991). It has been shown both *in vivo* and *in vitro* that two internal lysine residues (Lys-564 and 690) of HlyA are acylated, and that these modifications must occur for wild type activity (Stanley *et al.*, 1994; Ludwig *et al.*, 1996). Two domains (FAI and FAII) within the protoxin interact independently with HlyC, and these regions span the target lysine residues (Stanley *et al.*, 1996). FAI was shown to have a greater affinity for HlyC compared with FAII and so consequently this region is acylated at a higher frequency (Stanley *et al.*, 1996). The *B. pertussis* adenylate cyclase toxin (CyaA) is modified in an analogous manner to the *E. coli* haemolysin, however only a single lysine residue is modified (Hackett *et al.*, 1994).

While the fatty acid acylation of pro-HlyA is required for the production of a haemolytically active protein, the precise role of the acylation is not known. It was previously believed that acylation of the haemolysin was required for the efficient binding to erythrocyte membranes (Oropeza-Wekerle *et al.*, 1989; Boehm *et al.*, 1990b). However, more recent studies have shown that non-acylated forms of HlyA are able to bind to erythrocytes at wild-type levels and conclude that binding is independent of the HlyC-mediated modification (Bauer and Welch, 1996; Moayeri and Welch, 1997). In addition, pro-HlyA is able to form pores in planar lipid bilayers, demonstrating that acylation is not an essential requirement for pore formation (Ludwig *et al.*, 1996). It has been suggested that the acylation of HlyA and indeed other RTX toxins, may confer host and cell-type specificity. Mutants with substitutions at the acylation sites of the *E. coli* HlyA have been shown to be altered in their target-cell specificity (Pellet and Welch, 1996). Monoclonal antibody accessibility studies have shown the conformation of the N-terminus of the non-acylated pro-HlyA associated with the membrane differs to that of acylated HlyA, which suggests that acylation may alter conformation (Moayeri and Welch, 1997).

The binding of Ca^{2+} to the peptide repeats is required for haemolytic activity, and was previously believed to be required for the adsorption of HlyA to erythrocyte membranes (Ludwig *et al.*, 1988; Boehm *et al.*, 1990a and 1990b). However, recent binding assay studies suggest that the binding of HlyA to target cells does not require the glycine-rich repeat region and show that binding is both Ca^{2+} and temperature independent (Bauer and Welch, 1996). Dobereiner *et al.* (1996) have also shown that HlyA has channel forming activities in artificial membranes, in the absence of Ca^{2+} .

1.6.1.3 Secretion of *E. coli* HlyA

The HlyB protein of the secretion apparatus of *E. coli* belongs to the well-characterised ATP-binding cassette (ABC) superfamily of transporters, found in both eukaryotes and prokaryotes, and is involved in the transport of a variety of substrates (Higgins, 1992). Secretion systems similar to that of *E. coli* found in other Gram negative pathogens are used to transport not only other RTX toxins, but also proteases of *Erwinia*

chrysanthemi, and the metalloproteases of *S. marcescens* and *P. aeruginosa* (Welch, 1991; Hughes *et al.*, 1992; Coote, 1992; Ghigo and Wandersman, 1992). There are numerous examples where ABC translocators can be exchanged within Gram negative pathogens, albeit with differing degrees of efficiency.

The *E. coli* haemolysin is secreted in one step through both membranes, directly from the cytoplasm to the extracellular medium, without any detectable periplasmic intermediates (Felmlee and Welch, 1988; Koronakis *et al.*, 1989b). HlyA lacks an N-terminal signal sequence (Goebel and Hedgpeth, 1982, Felmlee *et al.*, 1985) and is secreted in a sec-independent manner (Gentschev *et al.*, 1990; Holland *et al.*, 1990).

Secretion of HlyA in *E. coli* requires the trans-envelope complex formed by the inner membrane proteins HlyB and HlyD and the outer membrane protein TolC (Wagner *et al.*, 1983; Mackman *et al.*, 1986; Gray *et al.*, 1989; Wandersman and Delepelaire, 1990; Wang *et al.*, 1991). HlyB is an ABC transporter protein that functions as a dimer with fused membrane domains and cytoplasmic ATP binding domains (Hyde *et al.*, 1990; Wang *et al.*, 1991; Gentschev and Goebel, 1992, Koronakis *et al.*, 1993). The hydrolysis of ATP bound to the cytoplasmic domain is an absolute requirement for protein translocation by HlyB (Koronakis *et al.*, 1991; Koronakis *et al.*, 1995; Sheps *et al.*, 1995). HlyD is an accessory protein that functions in conjunction with HlyB and is a member of the membrane fusion protein family (MFP) (Dinh *et al.*, 1994). The third component of the transenvelope complex, TolC, interacts with the inner membrane components of the complex to enable protein export, as its presence is required for protein export out of both intact and sphaeroplasted cells (Koronakis *et al.*, 1997). TolC functions as a trimer (Koronakis *et al.*, 1997), which is consistent with the previous findings of Benz *et al.* (1993), that oligomeric but not monomeric TolC is able to form ion-permeable channels. Each monomer of TolC consists of a membrane domain, and a periplasmic domain which is proposed to form the bridge to the inner membrane transport complex (Koronakis *et al.*, 1997).

The C-terminus of HlyA contains the specific sequence required for its extracellular secretion and was determined by extensive studies involving deletion analysis, localised mutagenesis and fusions of the C-terminus to heterologous proteins (Gray *et al.*, 1986;

Mackman *et al.*, 1986; Koronakis *et al.*, 1989b; Hess *et al.*, 1990; Stanley *et al.*, 1991; Kenny *et al.*, 1991; Jarchau *et al.*, 1994; Zhang *et al.*, 1995). C-terminal secretion signals are found in a variety of other bacterial toxins and the *E. coli* haemolysin transport machinery is able to transport heterologous toxins. While this suggests there is a specific recognition feature, no consensus signal sequence has been confirmed at either the primary or secondary level. The C-terminus of HlyA shares very little sequence homology with the secretion signal of other family members, and often it is the C-terminus that is the most divergent part of the protein. Various secondary structures have been assigned to the secretion signal of HlyA and there are similarities in the basic features with other proteins of this family. However, there is conflicting evidence for the importance of these structures. It has also been proposed that dispersed residues within the C-terminus, which are unlinked to any obvious structural feature, act co-operatively in order to be recognised by the transport machinery (Stanley *et al.*, 1991; Kenny *et al.*, 1992; Chervaux and Holland, 1996).

1.6.1.4 Genetic organisation and regulation of the *hlyCABD* operon

The *hlyCABD* operon of *E. coli* encodes the genes involved in the synthesis (*hlyA*), acylation (*hlyC*) and transport (*hlyBD*) of the haemolysin. A Rho-independent transcriptional terminator is situated between *hlyA* and *hlyB* of the *hlyCABD* operon such that transcription of the operon produces a major *hlyCA* transcript and following read-through of the terminator, a minor *hlyCABD* transcript (Koronakis *et al.*, 1988a; Welch and Pellet, 1988). As expression of HlyBD requires read-through of the terminator, both proteins are present in low levels in the cell (Koronakis *et al.*, 1988b and 1989a). The haemolysin operon is either chromosomally encoded within pathogenicity islands of *E. coli* human uropathogenic isolates, or found on large transmissible plasmids in animal isolates (Müller *et al.*, 1983; Welch *et al.*, 1983; Blum *et al.*, 1994 and 1995; Swenson *et al.*, 1996). The coding region of the operons from chromosomal or plasmid origin have 97% sequence identity, while the untranslated regions and regions 5' to promoters are heterologous (Knapp *et al.*, 1985; Hess *et al.*, 1986; Welch and Pellet, 1988).

An upstream sequence *hlyR*, enhances the expression of the plasmid-encoded haemolysin. Clones encoding *hlyCABD* but not *hlyR* are poorly haemolytic (Goebel and Hedgpeth, 1982) and sequential 5' removal of the *hlyR* sequence gradually decreases the level of haemolytic activity (Vogel *et al.*, 1988). *hlyR* functions only in *cis* and is proposed to antiterminate transcription at the *hlyA-hlyB* terminator (Koronakis *et al.*, 1988a and 1989a). The haemolysin is subject to regulation by environmental factors such as osmolarity, temperature and anaerobic conditions (Mouriño *et al.*, 1994) and analysis of mutants show that *hlyR* is essential for HlyA expression under high-salt (osmolarity) conditions (Carmona *et al.*, 1993). A transcriptional silencer, *hlyM*, lies downstream of the *hly* promoter within the *hlyC* gene, with no internal promoter. Deletion of *hlyM* increases the activity of *hlyA::galK* fusions and *hlyM* only functions in *cis* (Jubete *et al.*, 1995).

The expression of the *E. coli* haemolysin is a complex process involving the chromosomally encoded genes *hha* and *rfaH*. The Hha protein is 8.6 kDa (Nieto *et al.*, 1991) and is involved in the repression of plasmid-encoded haemolysin in high-salt media (Carmona *et al.*, 1993). A mutation in the *hha* locus increases the rate of transcription of plasmid-encoded *hlyCA* (Nieto *et al.*, 1991) via alterations in DNA topology. Hha is proposed to be a histone-like protein (Carmona *et al.*, 1993) and is homologous to YmoA of *Yersinia enterocolitica*, which has been shown to be a temperature-dependant modulator of virulence factor expression (De la Cruz *et al.*, 1992; Cornelis *et al.*, 1991). Both Hha and YmoA are considered members of a class of modulators of gene expression in enterobacteria (De la Cruz *et al.*, 1992; Milkuskis and Cornelis, 1994). A *hha* chromosomal mutation overcomes the silencing imposed by *hlyM* and so it is believed that Hha interacts with the *hlyM* region in order to silence the *hly* promoter, however *hlyR* is able to overcome this modulating effect, allowing a higher level of transcription (Jubete *et al.*, 1995). *hha* mutants show an increase in haemolytic activity, even in *hlyR*⁻ strains (Godessart *et al.*, 1988; Nieto *et al.*, 1991). In addition to the haemolysin, Hha also modulates the expression of the Vir adhesin of human and bovine *E. coli* isolates (Mouriño *et al.*, 1996).

The *rfaH* product is a *trans*-acting factor that affects the expression of both plasmid- and chromosomally-encoded haemolysin in *E. coli*. Recent work has shown that RfaH

enhances the elongation of the *hlyCA* transcript which therefore allows full expression of the *hlyCABD* operon (Bailey *et al.*, 1996; Leeds and Welch, 1997). RfaH is homologous to the essential prokaryotic transcription-terminator cofactor NusG, and it is proposed that RfaH regulates transcript elongation in an analogous way to NusG (Bailey *et al.*, 1996; Nieto *et al.*, 1996). A *cis*-acting regulatory sequence known as *ops* (operon polarity suppressor) has been identified upstream of the *hly* operon which functions together with RfaH to enhance transcription of the *hly* operon (Bailey *et al.*, 1996; Nieto *et al.*, 1996; Leeds and Welch, 1997). Deletions within the *ops* element reduce *hly* transcript elongation, in a similar manner to that seen with *rfaH* null mutants (Bailey *et al.*, 1996; Nieto *et al.*, 1996; Leeds and Welch, 1997). Cell extracts from a *rfaH* null mutant showed reduced ability to elongate transcription of the *hly* operon compared with wild-type extract and the addition of purified RfaH was able to overcome this effect (Bailey *et al.*, 1996). This conserved sequence is widespread as it is present in many Gram-negative species. It is located in all RfaH-regulated operons, especially those involved with bacterial virulence and fertility (Nieto *et al.*, 1996). *ops* is also part of the previously noted JUMPStart element located in the upstream region of several RfaH-enhanced polysaccharide gene clusters (Hobbs and Reeves, 1994).

A strong correlation between the LPS and haemolytic status of a cell has previously been established in other systems (Camprubi *et al.*, 1990). Both RfaH and RfaC are proposed to indirectly enhance the haemolytic activity of *E. coli* due to their effect on the LPS status (Wandersman and Letoffe, 1993; Leeds and Welch, 1996; Bauer and Welch, 1997). These proteins participate in LPS biosynthesis and mutations in either gene produce a deep rough phenotype. Extracellular *E. coli* haemolysin rapidly decays and is inactivated due to aggregation in an *rfaC* mutant and it is proposed that the haemolysin must interact with LPS to form a stable active conformation (Bauer and Welch, 1997). An association of purified haemolysin with an LPS structure has previously been demonstrated (Bohach and Snyder, 1986; Ostolaza *et al.*, 1991).

1.6.2 The *Serratia marcescens* haemolysin

The *Serratia marcescens* haemolysin produces only small zones of lysis on blood agar due to the degradation and aggregation of the haemolysin in the culture medium (Schiebel *et al.*, 1989). When this toxin was initially characterised, it represented a novel type of haemolysin as its secretion and activation system was unique among other described systems (Braun *et al.*, 1992). However, the *Serratia* haemolysin and activation/secretion proteins are now the prototype of a family whose members include the haemolysins of *Proteus mirabilis* and *P. vulgaris* (Uphoff and Welch, 1990), *Haemophilus ducreyi* (Palmer and Munson, 1995), *Edwardsiella tarda* (Hirono *et al.*, 1997) and the filamentous hemagglutinin of *Bordetella pertussis* (Locht *et al.*, 1993). This type of haemolysin is produced by nearly all clinical strains of *S. marcescens* and *Serratia liquefaciens* as well as other *Serratia* species (Ruan and Braun, 1990).

The haemolytic activity of *Serratia* requires the product of two genes, *shlA* and *shlB*, which encode polypeptides ShlA (165 kDa) and ShlB (62 kDa), respectively (Braun *et al.*, 1987; Poole *et al.*, 1988). Both ShlA and ShlB contain typical N-terminal signal sequences which are removed during export across the cytoplasmic membrane (Poole *et al.*, 1988). ShlA is secreted and is the haemolysin proper while ShlB remains in the outer membrane and is required for both the activation and secretion of ShlA (Braun *et al.*, 1987; Poole *et al.*, 1988; Schiebel *et al.*, 1989; Schiebel and Braun, 1989). Recombinant ShlA expressed in the absence of ShlB in *E. coli* is inactive (ShlA*) and remains in the periplasm (Schiebel *et al.*, 1989). ShlA* can be activated in two ways; by the addition of cell lysates containing ShlB as well as partially purified ShlB. ShlA remains active after the removal of ShlB by column chromatography, suggesting that this activation is irreversible (Ondraczek *et al.*, 1992). The modification by ShlB is required for ShlA to associate with erythrocyte membranes (Schiebel and Braun, 1989).

Functional domains have been assigned to the primary structure of the ShlA haemolysin. The carboxy-terminus of ShlA is the pore-forming domain while the region adjacent to this is involved in membrane binding. Haemolytic activity of ShlA is gradually

reduced by progressive deletion of the C-terminus, and further deletions produce a protein that is able to associate with erythrocyte membranes, but is no longer haemolytic (Poole *et al.*, 1988). The *in vivo* activation and secretion of the *Serratia* haemolysin appears to be tightly coupled (Schönherr *et al.*, 1993), and domains for both functions are located at the N-terminus of the protein. The secretion signal lies within the N-terminal 238 residues of ShlA and the region modified by ShlB is present in the first 149 residues of ShlA (Schiebel *et al.*, 1989; Ondraczek *et al.*, 1992; Schönherr *et al.*, 1993). The N-terminal fragment of ShlA (ShlA') is also able to complement inactive ShlA*, but only when synthesized in the presence of ShlB. In contrast to the stable modification of ShlA* by ShlB, the activation of ShlA* by ShlA' is reversible, as removal of ShlA' renders ShlA* inactive (Ondraczek *et al.*, 1992). A 15 kDa tryptic fragment derived from the N-terminal 149 residues of ShlA is able to convert ShlA* to the active form, and hence the activation site has been assigned to this domain (Schönherr *et al.*, 1993). Point mutations within this N-terminal region have identified two asparagine residues, found in two short sequence motifs (ANPN), that are involved in secretion and activation (Schönherr *et al.*, 1993). Interestingly, this motif has been found in other proteins such as the haemolysins of *P. mirabilis* and *H. ducreyi*, respectively, the FHA protein of *Bordetella pertussis*, and the high-molecular weight proteins and haem/haemopexin binding protein of *Haemophilus influenza* (Uphoff and Welch, 1990; Barenkamp and Leininger, 1992; Palmer and Munson, 1995).

It was previously believed that ShlB covalently modified ShlA*. Hertle and Braun (1996) have recently shown that this is not the case. The phospholipid phosphatidylethanolamine (PE) and its precursor phosphatidyl-serine (PS) are required for the *in vitro* activation of ShlA, in the presence of ShlB. While crude or partially purified ShlB extracts can activate crude ShlA*, highly purified ShlB is unable to activate highly purified ShlA* and the activity can only be restored by the addition of PE or PS. In addition, ShlA can be inactivated by digestion with phospholipase A2, which digests PE. Kinetic studies show that PE acts as a cofactor and not as substrate in the enzymic reaction. The authors propose that ShlB acts as a chaperone and that PE is required to stabilize ShlA*, such that it is recognised by ShlB for secretion and activation (Hertle and Braun, 1996).

The *Serratia* haemolysin is capable of forming channels in both erythrocyte and artificial membranes (Braun *et al.*, 1987; Schiebel and Braun, 1989; Schönherr *et al.*, 1994). However it is not certain whether ShlA requires oligomerisation to form a pore in the host cell membrane. Schiebel and Braun (1989) originally proposed that a single ShlA molecule forms a channel, as the rate of ShlA mediated lysis was greater at 4°C than 20°C, and oligomerisation is usually inhibited at low temperatures. However, more recent evidence suggests that ShlA monomers oligomerise in order to form the anion selective channels (Schönherr *et al.*, 1993; Schönherr *et al.*, 1994). Super-haemolytic mutants derived by single amino acid replacements have identified residues within ShlA that reduce inactivation by aggregation. These mutants also differ from wild type in that they are non-haemolytic at 4°C, due to an inability to adsorb and insert into the erythrocyte membrane (Hilger and Braun, 1995).

The *S. marcescens* haemolysin stimulates the release of the inflammatory mediators histamine and leukotrienes (König *et al.*, 1987) and may also play a role in iron acquisition (Pool and Braun, 1988). The production of ShlA is regulated by iron at the transcriptional level by a Fur-type repressor. A potential Fur-binding consensus sequence has been identified upstream of the *shlAB* operon (Poole and Braun, 1988).

ShlA and ShlB of *Serratia* have 47% and 55% identity with the homologues HpmA and HpmB, respectively, of *P. mirabilis* and *P. vulgaris* (Uphoff and Welch, 1990). HpmA* can be activated *in vitro* by ShlB and complemented by an ShlA fragment, and ShlA* can be activated by HpmB and complemented by an HpmA fragment (Ondraczek *et al.*, 1992). The amino acid sequence of the haemolysin gene cluster *hhdA* and *hhdB* of *Haemophilus ducreyi* also share homology to ShlA and ShlB (Palmer and Munson, 1995). FhaC, essential for the production of the filamentous haemagglutinin (FHA) in *Bordetella pertussis*, is homologous to ShlB and HpmB and the N-terminus of FHA displays homology to ShlA and HpmA (Willems *et al.*, 1994). However, despite this homology, FhaC and HpmB fail to transcomplement, as FhaC cannot secrete and activate HpmA and HpmB cannot secrete and activate FHA (Jacob-Dubuisson *et al.*, 1997).

1.6.3 The *Aeromonas aerolysin*

The virulence of the opportunistic human pathogen *A. hydrophila* has been shown to be associated with the production of aerolysin (haemolysin) (Chakraborty *et al.*, 1987) which is a potent channel-forming cytotoxin secreted by many *Aeromonas* spp. (Daily *et al.*, 1981; Burke *et al.*, 1982). Aerolysin is synthesized as a 54 kDa pre-pro-toxin with a typical N-terminal signal sequence that is processed during translocation across the inner membrane (Husslein *et al.*, 1988). The inactive pro-aerolysin folds and dimerizes in the periplasm, prior to being released from the cell (Hardie *et al.*, 1995). Pro-toxin binds to the host cell receptor which effectively concentrates the toxin on the cell surface, however it is unable to oligomerise as it requires the proteolytic cleavage of approximately 40 amino acids from the C-terminus for activation (Howard and Buckley, 1985; van der Groot *et al.*, 1992). Removal of the C-terminal peptide exposes a hydrophobic patch on the toxin surface, which may drive the oligomerisation and insertion of aerolysin into the host membrane (van der Groot *et al.*, 1994a). The aerolysin dimers must disassociate prior to oligomerisation (van der Groot *et al.*, 1993a). The cleavage site is located on a flexible loop of aerolysin and is processed by several mammalian proteases including trypsin and chymotrypsin, as well as several metalloproteases produced by *A. hydrophila* (Garland and Buckley 1988; van der Groot *et al.*, 1992).

The transmembrane protein glycophorin has been isolated from murine erythrocytes and identified as the aerolysin receptor (Howard and Buckley, 1982; Gruber *et al.*, 1994). More recently, Nelson *et al.* (1997) have shown that the major glycoprotein Thy-1 of T-lymphocytes, functions as a high affinity receptors for the aerolysin.

Aerolysin oligomerises into heptameric structures on the cell surface prior to inserting into the host cell membrane, which then form amphipathic β -barrels (Garland and Buckley, 1988; Wilmsen *et al.*, 1992; van der Groot *et al.*, 1993b; Parker *et al.*, 1994; Moniatte *et al.*, 1996). The aerolysin pores are approximately 1 nm in diameter (van der Groot *et al.*, 1994b). The *S. aureus* α -toxin (Gouaux *et al.*, 1994) and the anthrax toxin (Milne *et al.*, 1994) also form oligomers with 7-fold symmetry.

The aerolysin shares many properties with the outer membrane porins of Gram negative bacteria and the *Staphylococcus aureus* α -toxin; the proteins are hydrophilic with no hydrophobic stretches in the primary structure, have extensive β -sheet secondary structure, and oligomerise to form stable channels with similar properties (reviewed in Parker *et al.*, 1996). In addition, the oligomerisation of aerolysin and α -toxin is dependant on certain histidine residues and can be blocked by zinc. Despite these similarities, the primary structures of the aerolysin and the α -toxin are virtually unrelated, with only a small region of homology (Howard *et al.*, 1987). This region of homology is also present in the *P. aeruginosa* haemolysin (Hayashi *et al.*, 1989) and the oxygen-labile perfringolysin-O toxin (Parker *et al.*, 1996) and these toxins share similar properties with aerolysin and α -toxin. The primary structure of the aerolysin shows significant homology along the entire length to the *Clostridium septicum* α -toxin, even though these toxins are phylogenetically different (Ballard *et al.*, 1995). The activation and mechanism of action of aerolysin resembles that of the *C. septicum* α -toxin (Ballard *et al.*, 1993).

The extracellular secretion of aerolysin across the outer membrane requires the products of the *exe* genes (Jiang and Howard, 1992; Howard *et al.*, 1993; Jahagirdar and Howard, 1994). In addition, a type IV leader peptidase gene *tapD*, is required for the secretion of aerolysin in *A. hydrophila* (Pepe *et al.*, 1996). *tapD* is located in the cluster of genes involved in the biogenesis of *P. aeruginosa* type IV pili and presumably TapD processes the Exe preproteins in addition to its role in pilus biogenesis (Pepe *et al.*, 1996). The signal for extracellular secretion within the aerolysin molecule itself is not presently known, as it may only be present in the tertiary structure of the protein, however, mutations in the central serine / threonine rich region do inhibit secretion (Wong and Buckley, 1991).

1.6.4 Pore-forming toxins of Gram positive bacteria

A number of Gram positive pathogens are capable of producing pore-forming toxins that contribute to the pathological effects of the organism. These include the *Staphylococcus aureus* α -toxin (Bhakdi and Tranum-Jensen, 1991), the thiol-activated toxins and the *Clostridium septicum* α -toxin (Ballard *et al.*, 1992). Secretion of toxins from Gram positive

bacteria is a much simpler process than that in Gram negative bacteria, as there is no outer membrane to cross.

The *S. aureus* α -haemolysin (α -HL) is considered an important virulence factor which contributes to tissue damage and is lethal in mouse models (Patel *et al.*, 1987; Bhakdi and Trantum-Jensen, 1991; Callegan *et al.*, 1994; O'Callaghan *et al.*, 1997). α -HL is secreted as a water-soluble monomer that binds to eukaryotic cells and assembles on the surface into heptameric pores (Gouaux *et al.*, 1994; Song *et al.*, 1996). Assembly of a non-lytic pre-pore precedes membrane permeabilization, following conformational change (Walker *et al.*, 1992; 1993) and a 15 residue sequence lining the pore has been identified (Valeva *et al.*, 1996). The α -HL monomer is able to bind to human platelets, rabbit erythrocytes, monocytes, lymphocytes and endothelial cells (Bhakdi and Trantum-Jensen, 1991). The assembled pore of α -HL has secondary and tertiary structural features similar to those of the aerolysin, as both toxins assemble into heptameric oligomers with long β -strands that form β -sheets and there is very little α -helical structure (Bhakdi and Trantum-Jensen, 1991; Parker *et al.*, 1994).

More than 20 members of the family of thiol-activated cytolytins have been characterised thus far, both at the biochemical and molecular level (for a recent review, refer to Tweten, 1995). These cytolytins share common biochemical properties, as they lose activity upon exposure to oxygen, which can be restored by the addition of sulfhydryl compounds and they show immunological cross-reactivity and are homologous at the amino acid level (Alouf and Geoffroy, 1991; Braun and Focareta, 1991). The most thoroughly characterised thiol-activated toxins include listeriolysin-O of *Listeria monocytogenes*, streptolysin-O of *Streptococcus pyogenes*, pneumolysin of *Streptococcus pneumoniae* and the perfringolysin-O (theta toxin) of *Clostridium perfringens*. All these toxins are considered significant virulence factors of these pathogens. *L. monocytogenes* is the only thiol-activated cytolytin producer that is an intracellular pathogen and it is the listeriolysin O which mediates the escape from the phagosome into the cytosol of the infected cell (Gaillard *et al.*, 1987; Bielecki *et al.*, 1990; Kuhn *et al.*, 1990). The mode of action of these toxins is also similar, as they utilise cholesterol as the membrane receptor and then oligomerise and

insert into the host cell membrane. Cholesterol concentrates the toxin on the cell surface thereby promoting oligomerisation. It has been shown that cholesterol has multiple roles in the formation of perfringolysin-O channels. In addition to functioning as the receptor, cholesterol is involved in the targeting, oligomerisation, membrane insertion and stabilization of the membrane pore (Rossjohn *et al.*, 1997). Rossjohn *et al* (1997) have elucidated the crystal structure of perfringolysin-O and have shown the molecule consists predominantly of β -sheets. Thiol-activated oligomers form similar characteristic arc- and ring-shaped structures, as visualised by negative stain electron microscopy (examples include: Hebert *et al.*, 1992; Sekiya *et al.*, 1993; Morgan *et al.*, 1994).

The *C. septicum* α -toxin is a β -barrel pore-forming toxin. It is produced as an inactive toxin that requires proteolytic cleavage of the C-terminus for activation, and the toxin shares mechanistic and sequence similarity to aerolysin (Ballard *et al.*, 1993). The C-terminal propeptide, that is cleaved during maturation, was recently shown to function as an intramolecular chaperone (Sellman and Tweten, 1997).

1.7 Aims of this Thesis

The El Tor haemolysin is a potent toxin, capable of both cytolytic and enterotoxic activities and is an additional enterotoxigenic substance that exists in *V. cholerae*. While the haemolysin itself is not an essential virulence determinant, it is believed to contribute to the pathogenicity of the organism. Although much is known about cytolysins of other systems, the *V. cholerae* haemolysin does not fit into one of the previously described classes of cytolysins and so this study will potentially reveal novel features with regards to its maturation and activity.

Specific aims are to establish whether HlyA is a pore-forming toxin and if so, determine some of the physical properties of the pore. Mutagenesis of the HlyA protein will be performed in order to define functional domains, involved in pore-formation, activation and secretion. A further aim is to determine whether any factor(s) produced by *V. cholerae* are involved with the activation of HlyA.

Additional gene products are often required for the production of mature haemolysins in other systems and these genes are usually encoded in close proximity to the structural gene. It is not known whether additional genes maximize the activity of the El Tor haemolysin. The region flanking *hlyA* is also of interest as it encodes other potential virulence determinants. Therefore, an additional aim is to investigate the region downstream of *hlyA* to determine whether any auxiliary *hlyA* genes lie in this region, and to determine if there are any previously uncharacterised genes that contribute to the pathogenicity of *V. cholerae*.

Chapter 2

MATERIALS AND METHODS

2.1 Bacterial strains and plasmids

The *Vibrio cholerae* and *Escherichia coli* strains used are listed in Tables 2.1 and 2.2 respectively. Plasmids used in this study are listed in Table 2.3.

2.2 Maintenance of bacterial strains

Strains in routine use were maintained at -70°C as a suspension of bacteria in glycerol (32% v/v) and Bacto-peptone (0.6% w/v, Difco). Fresh cultures from glycerols were prepared by streaking a loopful of the glycerol suspension onto a nutrient agar plate (with antibiotic where appropriate) followed by incubation overnight at 37°C.

2.3 Growth media

The following nutrient media were used for bacterial cultivation. Nutrient broth (NB) (Oxoid), prepared at double strength (16 g/l) with added sodium chloride (NaCl) (5 g/l) and Luria broth (LB) were the general growth medium for *E. coli* K-12 and *V. cholerae* strains, respectively. LB is composed of Bacto-tryptone (10 g/l) (Difco), Bacto-yeast extract (5 g/l) (Difco) and NaCl (5 g/l). LB agar with 10% (w/v) sucrose (but no added NaCl) was prepared by the addition of an appropriate volume of a filter-sterilised solution of sucrose (50% w/v). NA is nutrient agar composed of Lab-Lemco powder (Oxoid) (10 g/l), peptone (Oxoid) (10 g/l), NaCl (5 g/l) and Agar (Media Makers) (15 g/l). Brain heart infusion (BHI) broth is composed of BHI powder (37 g/l) (Difco-Bacto), cysteine (0.5 g/l) and Hemin solution (1 ml/l). M13 Minimal medium was prepared as described by Miller (1972) and supplemented prior to use with MgSO₄, glucose and thiamine-HCl to final concentrations of 0.2 mg/ml, 0.5% (w/v) and 50 µg/ml, respectively. Terrific broth (Sambrook *et al.*, 1989) consisted of 1.2% (w/v) Bacto-tryptone (Difco), 2.4% (w/v) Bacto-yeast extract (Difco),

Table 2.1 *V. cholerae* strains used in this study

Strain	Description	Reference/Source
O17	El Tor, Ogawa, Sm ^R	K. Bhaskaran
V945	$\Delta hlyA$ O17	This study
V1218	Km ^R :: <i>lipA</i> O17	This study
V1219	Km ^R :: <i>priV</i> O17	A. Fallarino

Table 2.2 *E. coli* strains used in this study

Strain	Description	Reference/Source
DH5	F ⁻ , <i>endA-1</i> , <i>recA-1</i> , <i>hsdR-17</i> (r _K ⁻ m _K ⁺), <i>deoR</i> , <i>thi-1</i> , <i>supE-44</i> , <i>gyrA-96</i> , <i>relA-1</i> , λ ⁻	B. R. L. (Gibco)
DH5α	F ⁻ , φ80 <i>lacZ</i> ΔM15, Δ(<i>lacZYA</i> -B.R.L. <i>argF</i>)U169, <i>endA-1</i> , <i>recA-1</i> , <i>hsdR-17</i> (r _K ⁻ m _K ⁺), <i>deoR</i> , <i>thi-1</i> , <i>supE-44</i> , <i>gyrA-96</i> , <i>relA-1</i> , λ ⁻	J. Pohlner ^a

^a Max Planck-Institut für Biologie, Tübingen, FRG.

Table 2.3 Plasmids used in this study

Plasmid	Description	Reference or source
pUC21	Ap ^R	Vieira and Messing (1991)
pBluescript S/K	Ap ^R	Stratagene
pGP1-2	Km ^R	Tabor and Richardson (1987)
pCACTUS	Temperature sensitive suicide vector contains <i>sacB</i> gene for positive selection of recombinants. Tc ^R	C. Clark ^a
pGEM-T	Ap ^R	Promega
pBR325	Ap ^R Cm ^R	Prentki <i>et al</i> (1981)
pPM431	Tc ^R	Manning <i>et al</i> (1984)
pPM3039	pSUP301 carrying <i>hlyU</i> on a 2.7kb <i>Pst</i> I fragment. Km ^R	Williams and Manning (1991)
pPM2181	pACYC184 carrying Km ^R cartridge on 1.2 kb <i>Hinc</i> II fragment	U. Strocher ^b

^{a / b} University of Adelaide, Adelaide, South Australia.

0.4% glycerol, 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4 . Blood agar plates contain 5% sheep red blood cells (sRBCs). Incubations were at 37°C unless otherwise specified.

Antibiotics were added to broth and solid media at the following final concentrations: ampicillin (Ap; Sigma), 100 µg/ml; chloramphenicol (Cm; Calbiochem, prepared in 100% ethanol), 25 µg/ml; kanamycin sulphate (Km; Sigma), 25 µg/ml; rifampicin (Rif; Sigma), 200 µg/ml; 40 µg/ml; streptomycin (Sm; Sigma), 50 µg/ml; tetracycline (Tc; Calbiochem, prepared in 50% ethanol), 8 µg/ml for *E. coli* and 4 µg/ml for *V. cholerae* strains.

2.4 Animals

Swiss infant mice weighing between 2.4-2.7g were used for *in vivo* studies of *V. cholerae* O1 strains. Antisera were raised in adult New Zealand white rabbits.

2.5 Chemicals and reagents

Chemicals were Analar grade. Phenol, polyethylene glycol-6000 (PEG), sodium dodecyl sulphate (SDS) and sucrose were from BDH Chemicals. Tris was Trisma base from Boehringer Mannheim. Caesium chloride (Cabot) was technical grade. Ethylene-diamine-tetra-acetic-acid, disodium salt (EDTA) was Analar analytical grade from Ajax Chemicals. Antibiotics were purchased from Sigma (ampicillin, kanamycin sulphate, rifampicin, gentamycin), and Calbiochem (tetracycline, chloramphenicol). All other antimicrobial agents (dyes, detergents and antibiotics) were purchased from Sigma Chemical Co., BDH Chemicals Ltd., Glaxo, or Calbiochem. The following electrophoresis grade reagents were obtained from the sources indicated: acrylamide and ammonium persulphate (Bio-Rad), ultra pure N,N'-methylene bis-acrylamide and urea (BRL).

The four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP) were obtained from Boehringer-Mannheim. Adenosine-5'-triphosphate, sodium salt (ATP), herring sperm DNA and dithiothreitol (DTT) were obtained from Sigma. X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside) and IPTG (isopropyl-β-D-thiogalacto-pyranoside) were purchased from Boehringer-Mannheim. [^{35}S]-Methionine (1,270 Ci/mmol), were purchased from Amersham. Digoxigenin (DIG) DNA labeling and

detection kits were purchased from Boehringer-Mannheim. Detergent Tween 20 was purchased from Sigma.

Sterile, deionised and filtered water was used to prepare all buffers and reagents for DNA manipulation, otherwise reagents and buffers were prepared with deionised water.

2.6 Enzymes and immunoconjugates

Lysozyme was obtained from Sigma. Pronase and proteinase K were from Boehringer-Mannheim. All restriction endonucleases were purchased from either Boehringer-Mannheim, New England Biolabs, Pharmacia or Amersham and used according to the suppliers instructions. Other DNA modifying enzymes were purchased from the following suppliers: New England Biolabs (T4 DNA ligase), Amersham (T4 DNA polymerase, T4 DNA ligase) and Boehringer-Mannheim (DNA polymerase I, Klenow fragment of DNA polymerase I, and molecular biology grade alkaline phosphatase). Taq polymerase (Ampli Taq) was purchased from Perkin Elmer Cetus Corp. Sequencing kits using either dye-labelled primer or dye-labelled terminators were purchased from Applied Biosystems. Double-stranded Nested Deletion kits were purchased from Pharmacia.

Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Kirkegaard and Perry Laboratories Inc. Anti-digoxigenin-POD (Fab fragments) was obtained from Boehringer Mannheim.

2.7 Cell Fractionation

The cell fractionation procedure was a modification of that described by Osborn *et al.* (1972). Cells were grown in NB to mid-exponential phase at 37°C (50 ml, OD₆₅₀ of 0.6). 1 ml of culture was centrifuged (15K, 1 min) and the pellet resuspended in 100 µl 1x SDS sample buffer (whole cell fractions). The remaining cells were pelleted in a Sorvall SS-34 rotor, (10,000 rpm, 10 min, 4°C) and resuspended in 1 ml of 20% (w/v) sucrose, 30 mM Tris-HCl pH 8.1, transferred to SM-24 tubes and chilled on ice. Cells were converted to sphaeroplasts with 0.1 ml of 1 mg/ml lysozyme in 0.1 M EDTA pH 7.3 for 30 min on ice. Cells were centrifuged as above and the supernatant collected (periplasmic fraction). The

cell pellet was frozen in an ethanol dry ice bath for 30 min, thawed and dispersed vigorously in 3 ml 3 mM EDTA, pH 7.3. Cells were lysed by 60 x 1 sec pulses with a Branson Ultrasonifier. Unlysed cells and large cell debris were removed by low speed centrifugation (7,000 rpm, 5 min, 4°C). The supernatant containing the membranes and the cytoplasm was centrifuged at 35,000 rpm in a 80Ti rotor for 60 min at 4°C in a Beckman L8-80 ultracentrifuge. The supernatant (cytoplasmic fraction) was collected and the membrane pellet was resuspended in 1 ml 25 mM Tris-HCl (pH 7.5) 50 mM MgCl₂. Triton X-100 was added at a final concentration of 2% to 500 µl of the membrane suspension and kept on ice for 30 min, with intermittent vortexing. Separation of the inner and outer membrane fractions was achieved by centrifugation (35,000 rpm, 60 min). The triton soluble fraction was isolated (inner membrane fraction) and the triton insoluble fraction (the pellet) was resuspended in 500 µl of 25 mM Tris-HCl (pH 7.5) 50 mM MgCl₂ (outer membrane fraction). Fractions were mixed with an equal volume of 2 x SDS sample buffer and subjected to SDS-PAGE analysis.

2.8 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western transfer

2.8.1 SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 15% polyacrylamide gels (unless specified otherwise) using a modification of the procedure of Lugtenberg *et al.* (1975) as described previously by Achtman *et al.* (1978). Samples were heated at 100°C for 3 min in SDS sample buffer (0.25 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 15 (w/v) bromophenol blue) prior to loading. Gels were electrophoresed at 100 V for 5 h. Proteins were stained with gentle agitation overnight at room temperature in 0.06% (w/v) Coomassie Brilliant Blue G250 (dissolved in 5% (v/v) perchloric acid). Destaining was accomplished with several changes of 5% (v/v) acetic acid, with gentle agitation for 24 h. 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 Western transfer and detection

The procedure used was a modification of that described by Towbin *et al.* (1979). Samples were subjected to SDS-PAGE and transferred to nitrocellulose (Schleicher and Schuell) at 200 mA for 2 h in a Trans-Blot Cell (Biorad). The transfer buffer used was 25 mM Tris-HCl pH 8.3, 192 mM glycine and 5% (v/v) methanol. After transfer, the nitrocellulose sheet was incubated for 30 min in 5% (w/v) skim milk powder in TTBS (0.05% (v/v) Tween 20, 20 mM Tris-HCl, 0.9% (w/v) NaCl) to block non-specific protein binding sites. Primary antiserum was diluted in TTBS, 0.02% (w/v) skim milk powder and incubated with gentle agitation at room temperature for 2-16 h. Unbound antibody was removed by washing the nitrocellulose sheet three times for 10 min in TTBS with shaking. The filter was then incubated for 2-16 h with either goat anti-rabbit IgG or goat anti-mouse IgG conjugated with horseradish peroxidase (Nordic Immunology). The secondary antibody was diluted in TTBS at 1/5000 for colour detection and at 1/30,000 for ECL (enhanced chemiluminescence) detection. The filter was then washed four times (5 min intervals) with TTBS, followed by two 5 min washes in TBS (20 mM Tris-HCl, 0.9% (w/v) NaCl). The colour detection method utilised peroxidase substrate (9.9 mg 4-chloro-1-naphthol dissolved in 3.3 ml -20°C methanol and added to 16.5 ml TBS containing 15 µl hydrogen peroxide). Filters were incubated in peroxide substrate for 10-15 min with shaking, as described by Hawkes *et al.* (1982). The ECL detection reagent was prepared according to the manufacturer's directions (Amersham): the filter was incubated in the reagent for 1 min, drained, covered with clear plastic and exposed to X-ray film (Kodak X-Omat) for 5 min.

HlyA specific antibodies were used at the following concentrations: rabbit α -dHly, raised against SDS-PAGE purified 80 kDa HlyA (this work) was used at 1/5,000. Rabbit α -65, raised against purified 65 kDa product was generously provided by K. Yamamoto (Yamamoto *et al.*, 1990a). This antiserum was used at 1/50,000. Mouse monoclonal antibody mA6, raised towards gel-purified 80 kDa HlyA (Sheilds, 1991) was used at 1/1000.

2.9 DNA extraction procedures

2.9.1 Plasmid extraction procedures

Plasmid DNA was isolated by one of the three following procedures:

Method 1: Qiagen-tip 20 (Qiagen) was used to purify ca. 9-20 µg DNA, according to the manufacture's instructions.

Method 2: Large scale plasmid purification was performed by the three step alkali lysis method (Garger *et al.*, 1983). Cells from a one litre culture were harvested (6,000 rpm, 15 min, 4°C, GS-3, Sorvall) and resuspended in 24 ml of solution 1 (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA). Freshly prepared lysozyme (4 ml of 20 mg/ml in Solution 1) was mixed with the cell suspension and incubated at room temperature for 10 min. Addition of 55 ml of Solution 2 (0.2 M NaOH, 1% (w/v) SDS), followed by a 5 min incubation on ice resulted in total lysis of the cells. Protein, chromosomal DNA and high molecular weight RNA were precipitated by the addition of 28 ml Solution 3 (3 M potassium acetate, 2 M acetic acid, pH 4.8) with incubation on ice for 15 min. The precipitate was then removed by centrifugation (8,000 rpm, 20 min, 4°C, GSA, Sorvall). The supernatant was extracted with an equal volume of a TE saturated phenol, chloroform, isoamyl alcohol mixture (25:24:1). Plasmid DNA from the aqueous phase was precipitated with 0.6 volumes of 100% (v/v) propan-2-ol at room temperature for 10 min and collected by centrifugation (10,000 rpm at 4°C, 35 min, GSA, Sorvall). After washing in 70% (v/v) ethanol, the pellet was dried *in vacuo* and resuspended in 4.8 ml TE. Plasmid DNA was further purified from contaminating protein, chromosomal DNA and RNA by centrifugation on a two step CsCl ethidium bromide gradient according to Garger *et al.* (1983). The DNA band was removed by side puncture of the tube with a 19 gauge needle attached to a 1 ml syringe. The ethidium bromide was extracted using isoamyl alcohol. CsCl was then removed by dialysis overnight against three changes of 5 litres 1x TE at 4°C. DNA was stored at 4°C.

Method 3: Small scale plasmid purification was performed by the three step alkali lysis method using a modification of Garger *et al.* (1983). Overnight bacterial cultures (1.5 ml) were transferred to a microfuge tube, harvested by centrifugation (45 sec, Eppendorf), and resuspended in 0.1 ml of Solution 1 (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA). The addition of 0.2 ml of solution 2 (0.2 M NaOH, 1% (w/v) SDS) followed by a 5 min incubation on ice resulted in cell lysis. After the addition of 0.15 ml of solution 3 (60 ml of 5 M potassium acetate, pH 4.8, 11.5 ml of glacial acetic acid and 28.5 ml of H₂O) and a 5 min incubation on ice, protein, chromosomal DNA and high molecular weight RNA were collected by centrifugation (90 sec, Eppendorf). The supernatant was transferred to a fresh tube and extracted with TE-equilibrated phenol. Plasmid DNA was precipitated by the addition of 2 volumes of 100% ethanol and a 30 min incubation at -70°C. The DNA was collected by centrifugation (15 min, Eppendorf), washed with 70% (v/v) ethanol and dried *in vacuo*. The pellet was resuspended in 40 µl of 1x TE.

2.9.2 Preparation of *V. cholerae* genomic DNA

Genomic DNA from *V. cholerae* was prepared according to Manning *et al.* (1986). Cells from a 20 ml shaken overnight culture were pelleted in a bench centrifuge for 10 min and washed once with TES buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 50 mM NaCl). The pellet was then resuspended in 2 ml of 25% (w/v) sucrose, 50 mM Tris-HCl, pH 8.0 and 1 ml of lysozyme (10 µg/ml in 0.25 mM EDTA, pH 8.0) was added and the mixture incubated on ice for 20 min. TE buffer (0.75 ml) and 0.25 ml of lysis solution (5% (w/v) sarkosyl, 50 mM Tris-HCl, pH 8.0, 0.25 mM EDTA, pH 8.0) were added, together with 2 mg solid pronase. The mixture was gently mixed, and incubated at 56°C for 60 min. This was followed by three extractions with 4 ml TE-saturated phenol and two extractions with 4 ml diethyl-ether. The genomic DNA was precipitated with four volumes of ice cold 100% ethanol, spooled on heat-sealed pasteur pipette, washed in 70% ethanol and dissolved in 1 ml of TE.

2.10 Analysis and manipulation of DNA

2.10.1 DNA quantitation

The concentration of DNA in solutions was determined by measurement of absorption at 260 nm and assuming an A_{260} of 1.0 is equal to 50 μg DNA/ml (Miller, 1972).

2.10.2 Restriction endonuclease digestion of DNA

Most cleavage reactions were done using the restriction enzyme buffer SPK (10x: 200 mM Tris-HCl pH 7.5, 50 mM MgCl_2 , 5 mM dithiothreitol, 1 mM EDTA, 500 mM KCl and 50% glycerol). Otherwise, digests were carried out using NE buffers from New England Biolabs as described by the manufacturer. 0.1-0.5 μg of DNA were incubated with 2 units of each restriction enzyme in a final volume of 20 μl , at 37°C, for 1-2 h. The reactions were terminated by heating at 65°C for 10 min. Prior to loading onto a gel, a one tenth volume of tracking dye (15% (w/v) Ficoll, 0.1% (w/v) bromophenol blue, 0.1 $\mu\text{g}/\text{ml}$ RNase A) was added.

2.10.3 Calculation of restriction fragment size

The sizes of restriction enzyme fragments were calculated by comparing their relative mobility with that of *EcoRI* digested *Bacillus subtilis* bacteriophage SPP1 DNA. The sizes (kilobases, kb) used were: 8.5; 7.35; 6.10; 4.84; 3.59; 2.81; 1.95; 1.86; 1.51; 1.39; 1.16; 0.98; 0.72; 0.48; 0.36; 0.09 (Bresatec).

2.10.4 Analytical and preparative separation of restriction fragments

Electrophoresis of digested DNA was carried out at room temperature on horizontal, 0.6%, 0.8%, 1%, or 1.2% (w/v) agarose gels (Seakem HGT). Gels were run at 100V in TBE buffer (67 mM Tris base, 22 mM boric acid and 2 mM EDTA, final pH 8.8). After electrophoresis the gels were stained in distilled water containing 2 $\mu\text{g}/\text{ml}$ ethidium bromide.

DNA bands were visualized by trans-illumination with UV light and photographed using thermal paper (K65HM) and a Mitsubishi Video imaging system (Tracktel).

When extracting DNA from an agarose gel, the gel was electrophoresed at 100V in TAE buffer, (40 mM Tris acetate and 2 mM EDTA) and the required DNA band was excised and the gel slice placed inside dialysis tubing with ca. 500 μ l of sterile TAE. The gel slice was then positioned in an electrophoretic tank filled with 0.5 x TAE buffer and electrophoresed for 1-2 h. The DNA was then precipitated with two volumes of ethanol and one tenth volume of 3 M sodium acetate, pH 5.0 for ca. 2 h at -20°C . The precipitated DNA was collected by centrifugation (15 min, Heraeus Biofuge 15), washed once with 1 ml 70% (v/v) ethanol, dried *in vacuo* and dissolved in TE buffer.

Alternatively DNA was extracted from a gel slice using GENE-Clean DNA extraction kit (Bio 101) according to the manufacturer's instructions.

2.10.5 Dephosphorylation of DNA using alkaline phosphatase

Restriction enzyme-digested DNA was treated with alkaline phosphatase by the following method. 0.1-0.5 μ g of digested plasmid DNA was incubated with 1 unit of alkaline phosphatase (Calf intestinal: CIP), for 15 min at 37°C . The reaction was terminated by the addition of EDTA, pH 8.0 to a final concentration of 3 mM followed by heating at 65°C for 10 min. The reaction mix was then extracted with TE-saturated phenol and DNA was precipitated and collected as described in section 2.10.4.

2.10.6 End-filling with Klenow fragment

Protruding DNA ends created by cleavage with restriction endonucleases were end-filled using the Klenow fragment of *E. coli* DNA polymerase I. Typically, 1 μ g of digested DNA or purified DNA fragments, 2 μ l of 10 x Klenow buffer (0.1 M Tris-HCl, 0.1 M MgCl_2), 1 μ l of each dNTP (2 mM) and 1 unit of Klenow fragment were mixed in a final volume of 20 μ l and incubated for 5-10 min. Polymerase activity was stopped by heating at 65°C for 10 min.

2.10.7 *In vitro* cloning

DNA to be subcloned was combined with appropriately cleaved vector DNA, at a ratio of insert to vector of ca. 3:1, then ligated with T4 DNA ligase (2u) in a volume of 50 μ l and a final buffer concentration of 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM dithiothreitol (DTT), 0.6 mM ATP for 16 hr at 4°C. The ligated DNA was then used directly for transformation of *E. coli* strains.

2.11 DNA sequencing

2.11.1 Dye-primer sequencing

Dye-primer sequencing reactions were carried out using the PRISMTM Ready reaction Dye primer cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. DNA template concentration was ca. 100 ng/ μ l.

The following reagents were aliquoted into four 0.5 ml centrifuge tubes:

Reagent:	A	C	G	T
d/ddNTP mix	4 μ l	4 μ l	8 μ l	8 μ l
DNA template	1 μ l	1 μ l	2 μ l	2 μ l

Each reaction was overlayed with 20 μ l of light mineral. Tubes were placed in a Cetus-Perkin Elmer model thermal cycler (preheated to 95°C) and cycled as follows

Rapid thermal ramp to 95°C

95°C for 30 sec

Rapid thermal ramp to 55°C

55°C for 30 sec

Rapid thermal ramp to 70°C

70°C for 60 sec

15 cycles total

followed by

Rapid thermal ramp to 95°C

95°C for 30 sec

Rapid thermal ramp to 70°C

70°C for 60 sec

15 cycles total

Rapid thermal ramp to 4°C and hold

Samples were combined in 80 µl of 95% (v/v) ethanol with 3 µl of 3M sodium acetate and precipitated on ice for 10-15 min. DNA was pelleted at 13,000 rpm for 15 min (Eppendorf). Samples were dried *in vacuo* and stored at -20°C until needed. Prior to loading onto the sequencing gel the samples were resuspended in 5 µl deionized formamide/50 mM EDTA (pH 8.0) 5:1 (v/v) and heated to 95°C for 2 min.

2.11.2 Dye-terminator sequencing

Sequencing reactions were carried out on 1 µg of double-stranded plasmid DNA using the protocol provided by Applied Biosystems. The following reagents were added to the DNA as a reaction premix: 5x TACS buffer (400 mM Tris-HCl, 10 mM MgCl₂, 100 mM (NH₄)₂SO₄ pH9.0), dNTP mix (750 µM dITP, 150 µM dATP, 150 µM dTTP, 150 µM dCTP), dye deoxy A,C,G and T terminator and diluted amplitaq. The volume was made up to 20 µl with water and the mixture overlaid with oil prior to thermal cycling. The following cycle protocol was used.

Rapid thermal ramp to 96°C

96°C for 30 sec

Rapid thermal ramp to 50°C

50°C for 30 sec

Rapid thermal ramp to 60°C

60°C for 4 min

25 cycles total

Once the reaction cycles were completed, the reaction volumes were adjusted to 100 μ l. The aqueous phase was transferred to a sterile microfuge tube and extracted twice with 120 μ l of phenol:chloroform:water (7:1:2, v/v). The DNA was precipitated with 300 μ l of 95% (v/v) ethanol and 10 μ l of 3M sodium acetate at -20°C for 16-18 hrs. DNA was pelleted by centrifugation at 13,000 rpm for 15 min. Samples were dried *in vacuo* and stored at -20°C until needed. Prior to loading onto the sequencing gel the samples were resuspended in 5 μ l deionized formamide/50 mM EDTA (pH 8.0) 5/1 (v/v) and heated to 95°C for 2 min.

2.11.3 Analysis of DNA sequences

Raw sequencing data from the 373A automated sequencer were analysed using the Applied biosystems Seq Ed program version 6.0. Sequencing data were analysed using the following computer programs: LKB DNA and protein analysis programs, DNASIS and PROSIS (Hitachi Software). NCBI (BLASTX) (Gish and States, 1993) was used for protein/nucleotide Data-base homology searches.

2.12 Bacterial transformation, electroporation and conjugation

2.12.1 Bacterial transformation

Transformation was performed essentially according to the method described by Brown *et al.* (1979). *E. coli* K-12 strains were made competent for transformation with plasmid DNA as follows: an overnight shaken culture (in NB) was diluted 1:20 into NB and incubated with shaking until the culture reached an A_{600} of 0.6 (ca. 4×10^8 cells/ml). The cells were chilled on ice for 20 min, pelleted at 4°C in a bench centrifuge, resuspended in 0.5 volume of ice-cold 100 mM MgCl_2 , centrifuged again and resuspended in 0.1 volume of cold 100 mM CaCl_2 . The cells were allowed to stand for 60 min on ice before addition of DNA. Competent cells (0.2 ml) were then mixed with DNA (volume made to 100 μ l with 1x TE buffer and left on ice for a further 30 min. The cell/DNA mixture was heated at 37°C for 5 min and then 3 ml NB was added followed by incubation with shaking at 37°C for 1-2 h. 100 μ l aliquots of the culture were then plated onto selection plates directly or concentrated by centrifugation prior to plating.

2.12.2 Electroporation of *Vibrio cholerae*

Electroporation of plasmid DNA was performed essentially as described by Stoeber and Payne (1988). Cells were grown in LB to an A_{650} of 0.5. They were then washed in sucrose electroporation buffer (272 mM sucrose, 7 mM sodium phosphate buffer, pH 7.4, 1 mM $MgCl_2$) and resuspended in ice-cold sucrose buffer at 1/10 the original volume. 5 μ g plasmid DNA was added to 100 μ l of cell suspension, placed in an ice cold sterile *E. coli* PulserTM cuvette (2 mm electrode gap, BIO-RAD) and left on ice for 30 min. Electroporation conditions were 2000 V at 25 μ F capacitance and 200 ohms (Gene Pulser BIO-RAD). The cells were returned to ice for 30 min, then diluted in LB (3 ml) and incubated at 37°C for 1 h, and plated out on appropriate media.

2.12.3 Bacterial conjugation

Overnight NB or LB cultures were diluted at 1:20 and grown to early exponential phase with slow agitation. Donor and recipient bacteria were mixed at a ratio of 1:10 and the cells pelleted by centrifugation (3000 rpm, 10 min, bench centrifuge). The pellet was gently resuspended in 200 μ l of broth and spread onto a cellulose acetate membrane filter (0.45 mm, type HA, Millipore Corp.) on a NA plate. This plate was incubated for 3 hrs at 37°C. The cells were then resuspended in 10 ml NB and samples plated onto selective agar.

2.13 Synthesis of oligodeoxynucleotides

Oligodeoxynucleotides (oligos) were synthesized using reagents purchased from Applied Biosystems or Ajax Chemicals (acetonitrile). Synthesis was performed on an Applied Biosystems 381A DNA synthesizer in the trityl-off mode and butanol extracted prior to use. Oligos used are listed in table 2.4.

2.14 Polymerase chain reaction (PCR)

The procedure used was essentially as described by Delidow *et al.* (1996). PCR amplification was performed in a 50 μ l reaction volume containing PCR buffer (1.5 mM

Table 2.4 Oligonucleotides used in this study

Oligonucleotide	Sequence ^a
# 173	5' CACGGCATTCCCACAATCA 3'
# 1007	5' CAATACGTGCGATAGCCA 3'
# 1009	5' CACACGCAAACCTCAAGGA 3'
# 1019	5' CCTTTGAAGTAGTATGGC 3'
# 2048	5' CAAGT <u>ACGTAC</u> GCCGATTCTGGGTT 3'
# 2113	5' TTAT <u>CCCGGG</u> TGTTTAATGCGCAAGA 3'
# 2114	5' ACAC <u>CCCGGG</u> GATAAGCGGTGATGGTAC 3'
# 2208	5' TGTTCGCACAATATGTGC 3'
# 2209	5' TGGATTTGGGCACCT 3'
# 2217	5' GGATTAACCATGAGCAGC 3'
# 2316	5 TGGATTAATACAGCGTGG 3'

^a Nucleotides that deviate from the normal sequence are underlined

MgCl₂, 10 mM Tris pH8.4, 50 mM KCl), 1.5 U of Taq polymerase, 20 pM of primer and 100 ng of *V. cholerae* chromosomal DNA. The dNTPs were used at a final concentration of 2 μ M. The thermocycler (Perkin Elmer Cetus) was programmed to incubate samples at 95°C for 5 min and then to carry out 25 cycles consisting of 95°C for 30 sec., 62°C for 30 sec., 72°C for 1 min followed by a final extension at 72°C for 5 min (approximately 1 min/kb DNA). 10 μ l of this reaction product was analysed on a 0.8% agarose gel, the remaining product was purified and used for cloning. PCR products were ligated directly into pGEM-TTM (PROMEGA) and transformed into DH5 α .

2.15 Preparation of DNA Probes

DNA plasmid probes and PCR fragment probes were labelled with digoxigenin-11-dUTP (DIG-11-dUTP; Boehringer Mannheim) according to the manufacturer's protocol. DNA fragment probes were labelled using random-primed labelling. The DNA (10 ng-3 μ g) was denatured at 95°C for 10 min, then chilled on ice for 3 min before the addition of 2 μ l Hexanucleotide mix, 2 μ l dNTP labelling mix and 2 U Klenow fragment. The reaction volume was made up to 20 μ l with sterile Milli-Q water, and incubated at 37°C for 20 hr. The reaction was stopped by the addition of 2 μ l of 0.2 M EDTA, pH 8.0. DNA was precipitated by the addition of 2.5 μ l 4 M LiCl and 75 μ l of pre-chilled 100% ethanol. After a minimum of 2 h at -20°C, DNA was collected by centrifugation at 12,000g for 10 min. The DNA pellet was washed with 50 μ l 70% ethanol (-20°C), dried *in vacuo* and resuspended in 20 μ l 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

2.16 Southern transfer and hybridisation

Transfer of DNA from agarose gels to nylon (Hybond-N⁺, Amersham) was performed as described by Southern (1975) and modified by Sambrook *et al.* (1989). Prior to hybridization with labelled probe, filters were incubated for 4 h at 42°C in a pre-hybridization solution containing 5 x SSC (0.15 M NaCl, 0.015 M sodium citrate), 50 mM sodium phosphate buffer, pH 6.4, 50% formamide, 5x Denhardt's reagent (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% Fraction V BSA) and 0.1 mg/ml single stranded herring

sperm DNA (Sigma) (Sambrook *et al.*, 1989). Pre-hybridization fluid was discarded and replaced with fresh hybridization. Denatured probe was added and hybridization allowed to occur for 16-24 h at 42°C. Filters were washed with 2x SSC (2x 5 min), 0.1% SDS at RT, then with 0.2x SSC, 0.1% SDS (2x 15min) at 65°C. Filters were then incubated in blocking reagent (5% Skim milk in buffer 1 [0.1 M Tris-HCl, 0.15 M NaCl pH 7.5]) for 1 h before being incubated with anti-digoxigenin-POD Fab fragments (1/10,000 dilution in Buffer 1, Boehringer Mannheim) for at least 30 min. Unbound antibody-conjugate was removed by washing the filters in Buffer 1 (4x 5min) and then PBS (2x 5min). Detection of target DNA was performed by using either ECL (Amersham or Boehringer Mannheim), as described previously for Western blot detection or by colour detection. For colour detection, filters were incubated with α -Dig-AP Fab fragments (1/5000 dilution in Buffer 1) for at least 30 min. The membranes were equilibrated for 2 min with buffer 3 (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M MgCl₂, pH 9.5) prior to the addition of the substrate (prepared according to manufacturer's instructions). The filters were incubated in substrate solution at room temperature, in the dark without shaking (between 5 min to 1 h). The reaction was terminated by washing the membranes for 5min with Buffer 4 (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0) and then air dried.

2.17 DNA colony blot and hybridisation

This procedure is a modification of the method described by Grunstein and Hagness (1975). Bacterial colonies were transferred onto a nitrocellulose filter (Schleicher and Schuell) by overlaying the colonies on an agar plate, after overnight incubation. The filter was gently lifted off the plate and transferred colony-side up to Whatmann 3mm paper soaked with lysis solution (10% SDS). After 5 min at RT, the filter was transferred to 3 mm paper soaked with denaturation solution (0.5 M NaOH, 1.5 M NaCl) for a further 5 min. Following denaturation the filter is transferred to 3 mm paper soaked with neutralisation solution (1.5 M NaCl, 0.5 M Tris-HCl pH 8.0) for 5 min, then air-dried before being baked at 80°C *in vacuo* for 60-90 min. Cell debris was removed from the filter by placing it in

0.1% SDS and gently scrubbing the surface. Prehybridisation, hybridisation and detection were performed as described above for Southern transfers.

2.18 T7 RNA Polymerase Expression System

This procedure is a modification of the method described by Tabor and Richardson, (1987). Proteins induced by this method were either labelled with [^{35}S]-methionine or left unlabelled. For labelled proteins, the protocol is as follows: The plasmid pGP1-2 carries the T7 RNA polymerase under the control of the lambda P_L promoter. This plasmid was transformed into *E. coli* strains containing a plasmid with the specific gene of interest under control of the T7 RNA polymerase promoter. A 10 ml Terrific Broth broth with ampicillin and kanamycin was inoculated with a single colony and shaken at 30°C overnight. The culture was subcultured 1:20 and incubated with constant shaking at 30°C. When the cultures reached an A_{600} of 0.6, the 0.4 ml of the cell suspension were centrifuged (15K, 1 min) and washed (3 x) in minimal media to remove all available methionine. The cells were resuspended in 1 ml methionine assay media (Difco-Bacto) and incubated at 30°C for 60 min. The cells were then incubated at 42°C for 20 min to induce the pGP1-2 P_L promoter by the inactivation of cI_{ts} , allowing expression from the λP_L promoter. Rifampicin was added to a final concentration of 200 $\mu\text{g/ml}$ to inactivate the *E. coli* RNA polymerase and incubation was continued at 42°C for a further 10 minutes. Cultures were then incubated at 37°C for 1 hr with shaking to ensure the breakdown of non-specific mRNA. Samples were pulsed with 10 μCi of [^{35}S]-methionine for 5 min at 37°C. The cells were centrifuged (15K, 1 min) and resuspended in 120 μl of 1x SDS sample buffer. 50 μl of sample was boiled for 5 min and loaded onto SDS-PAGE gels for analysis. Gels were subsequently stained with Coomassie G250 and dried on Whatman 3MM chromatography paper at 60°C for 2 hr on a Bio-Rad gel drier. [^{35}S]-methionine autoradiography was performed at room temperature for 1-5 days using Kodak XR-100 film.

For induced unlabelled proteins: The cells were subcultured from an overnight culture as described above, and grown with shaking at 30°C to A_{600} of 0.9. The cultures

were shifted to 42°C for 30 min, followed by a 1 h incubation at 37°C, with shaking. Whole cell fractions were isolated, subjected to SDS-PAGE and western immunoblotting.

2.19 Preparation of rabbit antisera

Antiserum was raised against gel-isolated, partially purified protein. The rabbit was immunized without adjuvant by subcutaneous injection of the gel homogenates on day 1, day 10 and day 17. The rabbits were exsanguinated by cardiac puncture under anaesthesia 10 days after the last immunization and the serum stored at -20°C. A working stock of the antiserum was kept at 4°C with 0.05% (w/v) azide.

2.20 *In vivo* assays for virulence using the infant mouse cholera model

2.20.1 LD₅₀ studies

The virulence of a given strain was assessed by determining the 48 h LD₅₀ value (Attridge and Rowley, 1983). The LD₅₀ is the number of organisms capable of killing 50% of the infant mice within 48 h. 3-4 day old infant mice used in these studies were removed from their mothers about 5-6 hr before use, to permit emptying of stomach contents. Unless stated otherwise, the challenge strain was grown on nutrient agar with appropriate antibiotic selection where necessary at 37°C. Serial dilutions of each strain were made in peptone saline (0.1% (w/v) solution of protease-peptone [Difco] in saline). Each dilution was then administered orally to groups of 5-6 infant mice (0.1 ml dose per mouse delivered via using a smooth-tipped hypodermic needle (22 g)). The mice were kept at 25°C for 48 h after which time the number of survivors in each group was noted. Using these data, a plot of cumulative percentage mortality versus log₁₀ challenge dose (Reed and Muench, 1938) was plotted and the 48 h LD₅₀ dose determined by interpolation.

2.20.2 Competition experiments

Competition experiments were performed by feeding the infant mice a mixed inoculum comprising approximately equal numbers of parent and mutant organisms: the dose of each strain equal to ca. 10 LD₅₀ of the parent strain (Attridge, 1979). The mice

were maintained at 25°C for 22-24 h before being sacrificed and the small intestine removed; this was then homogenised in 2 ml saline using a homogeniser (Ultraturrax). To determine the ratio of parent and mutant organisms present at the time of sacrifice, dilutions of each gut homogenate were spread onto NA and selective agar. From the number of colonies present on these plates the ratio of parent to mutant was calculated.

2.21 Preparation of protein for amino-terminal analysis

An overnight culture was subcultured 1/20 into 500 ml of Terrific broth and protein expression was induced using the T7 RNA polymerase system (section 2.18). A 10 ml periplasmic fraction was isolated (section 2.7) and this fraction was concentrated to a volume of 1 ml using a centrifugal filter device (Ultrafree^R-15, Millipore). An equal volume of SDS-loading buffer was added to a 100 µl aliquot and the sample electrophoresed in SDS on a 20% polyacrylamide gel and stained with Coomassie Brilliant Blue. The desired protein was excised from the gel and the acrylamide band containing protein was subjected to an additional electrophoresis on 20% PAGE gel. The protein was transferred to PVDF-membrane according to the manufacturers' instructions (Bio-Rad) and stained with 0.025% Coomassie Blue R-250 dissolved in 40% methanol (then destained with 50% methanol), to visualise the protein. The protein on the filter was then subjected to amino-terminal sequencing, by Dr. J. Walsh of Macquarie University, Sydney NSW.

2.22 Haemolysis assays

2.22.1 Haemolysis assays and blood plates

Liquid haemolysis assays were performed using either *V. cholerae* culture supernatant or *E. coli* periplasmic fractions. *V. cholerae* supernatants were filtered through a 0.22 µm Millipore filter prior to use. An aliquot of the sample to be examined was mixed with an equal volume of a 5% suspension of washed sheep red blood cells and incubated at 37°C. Aliquots were taken at various time points and pelleted in a microcentrifuge for 45 sec. Haemolytic activity (haemoglobin release) was assessed by measurement of A₄₁₄.

Haemolysis was expressed as a percentage of total lysis as determined by lysis of sRBC with 2% SDS.

2.22.2 Inhibition of Haemolysis

HlyA was titrated to determine the concentration required to produce 50% haemolysis at 37°C after 2 h. This HlyA dilution was pre-incubated with diluted antibody for 1 h at 37°C, prior to adding sRBC. Dilutions of antisera used were as follows: α -65 1/3000 and mA6 1/500. The SHA specific antiserum (R946) was a kind gift from Professor J. Holmgren (University of Göteborg, Sweden) and was used at 1/ 1000 dilution.

2.22.3 Osmotic protection assay

Assay mixtures consist of a 5% sRBC suspension resuspended in either saline or 30 mM carbohydrate. An equal volume of HlyA-containing supernatant from *V. cholerae* was added to the sRBC suspension and the percentage of total haemolysis was determined at various time intervals. Haemolysis was measured as the increase in OD₄₁₄ in the supernatant of the sRBC/haemolysin suspension. After 120 min, an aliquot was washed (3x) with saline (to remove any free HlyA and carbohydrate) and finally resuspended in saline. The suspension was incubated at 37°C for 10 min after which the percentage of total haemolysis was determined.

2.22.4 Reproducibility of experimental data

The data reported are means for three or more samples or are the representative data for experiments that were conducted three or more times with essentially identical results.

2.22.5 Association of HlyA with sheep erythrocyte membrane

A 2.5 ml aliquot of supernatant containing HlyA was added to an equal volume of a 10% suspension of sRBC. 1.5 ml aliquots were taken at various time points and microcentrifuged at 15K rpm for 5 min. The supernatant was carefully removed with a pasteur pipette and the pellet was washed (3x) with saline, and (3x) with Na₂HPO₄ (to

hypertonicity lyse the erythrocytes), centrifuging for 5 min at 15K rpm after each wash. The final centrifugation was for 15 min at 15K rpm to pellet all sRBC membrane. The pellet was resuspended in 300 μ l 1x SDS sample buffer, boiled for 5 min and 80 μ l aliquots subjected to SDS-PAGE and immunoblotting.

2.23 Black lipid bilayer assay

2.23.1 Preparation of protein

An overnight culture of *V. cholerae* was subcultured at a 1/50 dilution into 20 ml BHI supplemented with 3% glycerol and grown at 37°C with vigorous shaking for 4 h. Culture supernatants were collected after centrifugation at 15K for 20 min. 1M Tris-HCl (pH 8.0) was added to the cell-free supernatant at a 1/20 dilution of the volume. HlyA was precipitated by the addition of Ammonium sulphate at 60% saturation (381.5 grams / litre supernatant), with stirring on ice. Precipitated protein was isolated by centrifugation (16K for 45 min at 4°C) and the pellet was resuspended in 100 μ l TCU buffer (20 mM Tris, 150 mM NaCl, 6 M urea, pH 7.0). An equal volume of glycerol was then added. The preparation was stored at -20°C until required for analysis in the black lipid bilayer experiments.

2.23.2 Black lipid bilayer assay

The method used for the black lipid experiment is described in detail by Benz *et al* (1978). The apparatus consisted of a Teflon cell with two water-filled compartments connected by a partition with a small circular hole with a surface area of 0.5 mm². A black lipid bilayer membrane was constructed by painting the lipid diphytanoyl phosphatidylcholine (1% (wt/vol) solution in *n*-decane) across the hole. Bilayer formation was indicated when the membrane was black in reflected light. The small circular hole was pretreated with a chloroform-lipid mix. After the membrane turned black, 50 ng/ml of HlyA was added to the aqueous phase in both chambers, with stirring to allow equilibration. The aqueous phase was unbuffered 1M KCl (pH 6.0). The current through the bilayer membrane was measured with two calomel electrodes connected in series with a voltage

source and a current amplifier. The amplified signal was monitored with a storage oscilloscope and recorded on a strip chart.

Chapter 3

The pore-forming abilities of the *V. cholerae* O1 El Tor haemolysin (HlyA)

3.1 Introduction

Cytolysins often contribute to the pathogenicity of bacteria, and in some cases, act solely to cause the manifestations of the disease. Many were initially classified by their ability to lyse erythrocytes, and hence were referred to as "haemolysins". This classification, however, is often misleading, as many haemolysins are capable of lysing a range of nucleated cell types. Cell lysis can be induced by a detergent-like action or enzymatic activity, such as with mellitin or phospholipases, respectively. However, many cytolysins are pore-forming toxins, that lyse cells by inserting into the host cell membrane to form defined channels. The pore acts as a molecular sieve, allowing the leakage of K^+ from the cell, but initially retaining larger cytoplasmic components. The intracellular osmotic pressure becomes greater than that in the medium, the cell swells and bursts. It is noteworthy, however, that pore-forming toxins can also exert non-lytic inhibitory activities against host cells. For example, exposure to sub-lytic levels of the *Escherichia coli* α -haemolysin can cause the release of leukotrienes from human granulocytes (Scheffer *et al.*, 1985) and human neutrophils no longer bind chemotactic factors (Welch *et al.*, 1986).

A large family of cytolytic toxins known as the RTX group are produced by Gram-negative bacteria. Members are calcium-dependent and have a common structural feature of a tandem array of a nine amino acid repeat (RTX for repeats in toxin) (Felmlee and Welch, 1988). These toxins are produced by genera of the *Enterobacteriaceae* and *Pasteurellaceae*, and the best characterised is the *E. coli* α -haemolysin. While all RTX members are cytotoxic for a variety of nucleated cells, the individual toxins often differ in cell- and host-specificity (Welch, 1991). The mechanisms for activation, secretion and mode of action of RTX toxins are similar, and these toxins are encoded by a common set of related genes organised in an operon (Braun *et al.*, 1993).

Cytolysins which differ from the RTX toxins include the *Serratia marcescens* haemolysin, the *Aeromonas* aerolysin and haemolysins from *Vibrionaceae* (Braun and Focareta, 1991). Pore-forming toxins are also produced by Gram-positive bacteria, and include the thiol-activated Listeriolysin O, Streptolysin O and Perfringolysin of *Listeria monocytogenes*, *Streptococcus pyogenes* and *Clostridium perfringens*, respectively. A well characterised pore-former of Gram positive bacteria is the *Staphylococcus aureus* α -toxin.

A paradoxical property of pore-forming toxins is they can exist in both water-soluble and membrane-associated forms. That is, a molecule with a hydrophilic surface must insert into the hydrophobic membrane lipid-bilayer. Conformational changes in the protein can accommodate this process, which serve to expose previously hidden hydrophobic and amphipathic regions. Colicin A is a water-soluble toxin that effectively turns "inside-out" on insertion into the membrane (Parker *et al.*, 1989; Benedetti *et al.*, 1992). Other pore-forming toxins oligomerise in order to form channels. Both aerolysin and α -toxin monomers must oligomerise in order to form heptameric ring-structured channels (Bhakdi *et al.*, 1981; Wilmsen *et al.*, 1992; Gouaux *et al.*, 1994)

While it was known that the El Tor haemolysin (HlyA) of *Vibrio cholerae* is a potent toxin, with both enterotoxic and cytotoxic / cytolytic activities (Honda and Finkelstein, 1979; Alm *et al.*, 1991; Ichinose *et al.*, 1987), very little was known about its mode of action at the commencement of this study. Although HlyA is lytic for a diverse range of erythrocytes, it is capable of lysing a variety of mammalian cells in culture (Honda and Finkelstein, 1979; Alm *et al.*, 1991).

This chapter describes the characterisation of HlyA as a member of the pore-forming toxins in order to gain an insight into the mechanism by which the toxin exerts host-cell damage.

3.2 Results

3.2.1. Osmotic protection

It is known that cytolytic activities of pore-forming toxins can be blocked by molecules sufficiently large enough to act as osmotic protectants (Welch, 1991). In order to

assess whether HlyA of *V. cholerae* strain O17 is a pore-forming toxin, osmotic protection assays were undertaken.

A suspension of sheep erythrocytes was exposed to supernatants containing HlyA, in the presence or absence of various carbohydrates (sucrose, mannose, maltose, raffinose, dextrin 20 and dextran 4) at 30 mM. The protective effects of the carbohydrates were then assessed in a liquid haemolysis assay, with aliquots taken for measurement at 30 minute intervals for 120 minutes (Figure 3.1 panel A). While the haemolytic activity of HlyA was virtually unaffected by the presence of sucrose, mannose, maltose and raffinose (haemolytic activity is slightly reduced in the presence of carbohydrates, however the difference is not significant), the samples containing dextrin 20 and dextran 4 displayed only background levels of haemolysis (<10%) (Figure 3.1A). Supernatants of the isogenic strain *V. cholerae* O17 $\Delta hlyA$ (V945) (described further in section 4.2.1) that are devoid of HlyA, also produced only background levels of haemolysis (not shown).

In order to ensure that the protective carbohydrates inhibit haemolysis by osmotic protection, and not by preventing pore-formation, an aliquot of the sheep erythrocyte suspension was taken at 120 minutes (indicated with an arrow in Figure 3.1A) and washed several times to remove carbohydrate and any unassociated HlyA, and resuspended in phosphate buffered saline. Following the removal of the osmotic protectant, the sample lysed immediately and showed haemolytic activity comparable with that seen with HlyA not exposed to protective carbohydrate (not shown). sRBC that were not exposed to either HlyA or carbohydrate, remained intact throughout this procedure. The immediate lysis suggests that HlyA had formed pores in the sheep erythrocytes during the course of the assay, but lysis was prevented due to the osmotic stabilization provided by either dextrin 20 or dextran 4.

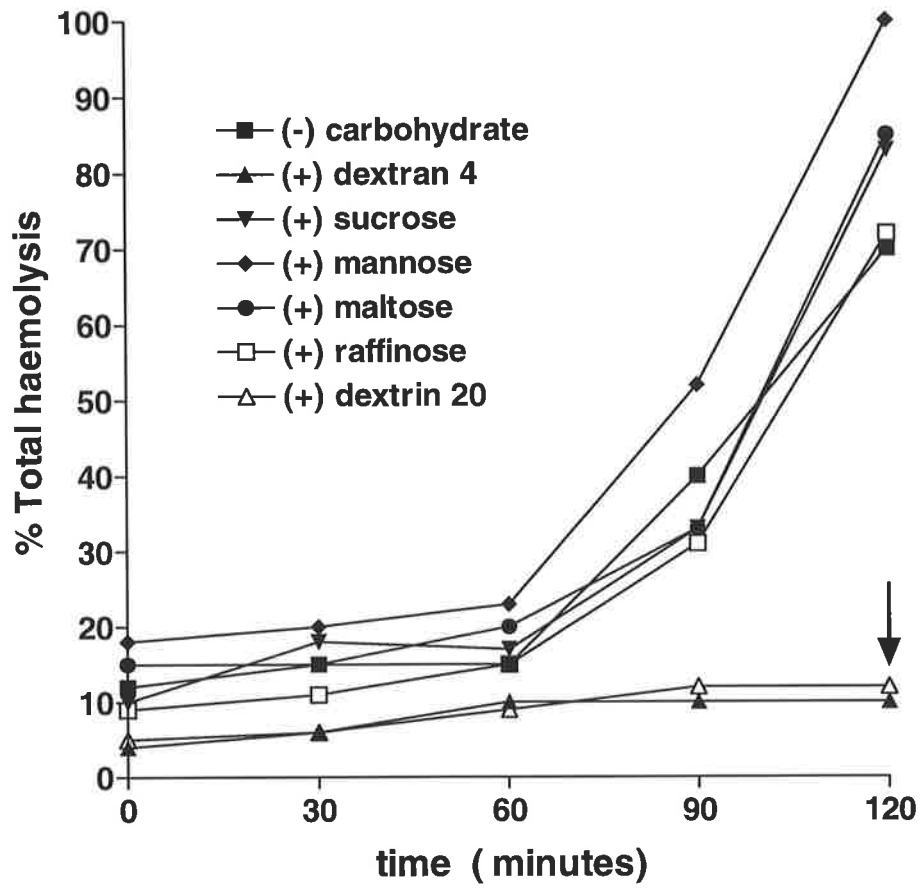
The level of HlyA associated with sheep erythrocyte membranes in the presence or absence of dextrin 20 was also examined. Aliquots of the sRBC suspension were taken at 0, 60 and 120 minutes from the assay described in Figure 3.1A. The sRBC suspension was washed to remove both dextrin 20 and any unassociated HlyA. The sRBCs were then hypertonically lysed and the total erythrocyte membrane collected after centrifugation. The

Figure 3.1 Osmotic protection of HlyA.

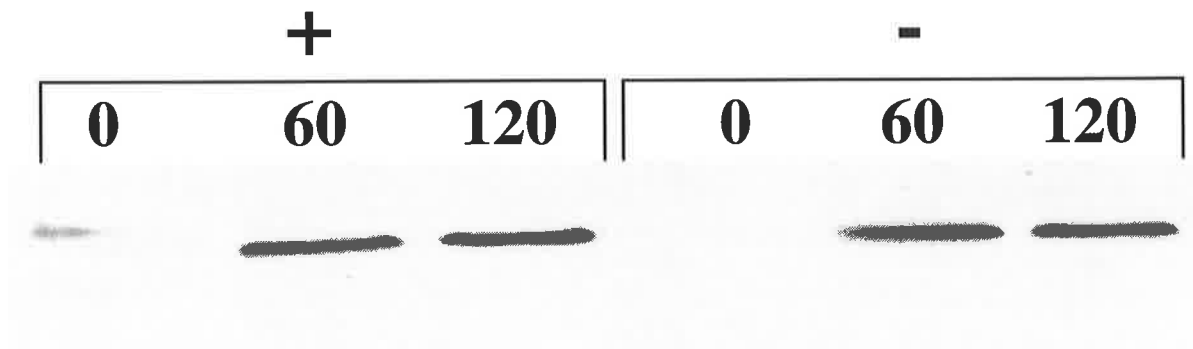
Panel A. Osmotic protection of sRBCs exposed to HlyA by dextrin 20 and dextran 4. Time dependant haemolysis of sRBCs exposed to HlyA was determined in the presence or absence of various carbohydrates (30 mM sucrose, mannose, maltose, raffinose, dextrin 20 and dextran 4) at 37°C and was expressed as % total lysis of sRBCs. At 120 minutes (indicated by the arrow) the suspension containing dextrin 20 and dextran 4 were washed to remove carbohydrate and any free HlyA. The cells were re-suspended in saline and reincubated at 37°C for a further 5 minutes, after which time the samples reached maximal haemolytic activity.

Panel B. Western immunoblot of HlyA associated with sRBC membrane in the presence or absence of dextrin 20. Aliquots were taken at 0, 60 and 120 minutes from the assay described in panel A, and were washed with saline to remove dextrin 20 and any unassociated HlyA. The remaining sRBCs were hypertonically lysed and the erythrocyte membranes were collected by centrifugation and resuspended in 300 µl 1 X SDS-sample buffer. An aliquot of each sample was subjected to SDS-PAGE and western immunoblotting using the anti-HlyA antiserum α -65.

A



B



sRBC membranes were electrophoresed on an SDS polyacrylamide gel, followed by western immunoblot analysis using α -65 antiserum for the detection of HlyA. This antiserum was raised towards purified native 65 kDa HlyA (Yamamoto *et al.*, 1990a). At 60 and 120 minutes (Fig. 3.1 panel A) the 65 kDa form of HlyA was associated with sRBC membranes in both the presence and absence of dextrin 20, (Figure 3.1, panel B) which demonstrates that the carbohydrate does not inhibit the association of HlyA with sRBC. These data collectively suggest that dextrin 20 (and others) acts as an osmotic protectant when inhibiting HlyA-mediated lysis and lysis must therefore be the result of HlyA mediated pore formation in the erythrocyte membrane.

The mean hydrated diameters of mannose, sucrose, maltose, raffinose, dextrin 20 and dextran 4 are 0.72, 0.9, 0.96, 1.1, 1.6 and 3.5 nm, respectively. Dextrin 20 and dextran 4 were all able to prevent HlyA-induced lysis, however, carbohydrates with molecular weights less than that of dextrin 20, were unable to do so. Assuming pores formed by HlyA act as a molecular sieve, then the pore diameter is estimated to be less than 1.6 nm in diameter (slightly less than the size of dextrin 20).

3.2.2 Black lipid bilayer analysis

The channel-forming ability of HlyA was subsequently analysed in artificial/black lipid bilayers. These experiments were performed at the University of Würzburg, Germany, in the laboratory of Professor R. Benz. Proteins examined by this method are added to an aqueous salt solution bathing a planar bilayer. Individual pores form in the membrane in a time-dependant manner, which is measured by a step-wise increase in conductance (current divided by applied voltage) between two electrodes placed on either side of the membrane. Amplification of the current allows measurement of a single channel, formed either by a protein monomer or oligomer.

3.2.2.1 Isolation of HlyA

Analysis of the pore-forming ability of HlyA in artificial membranes, initially required the isolation of the HlyA protein. An overnight culture of *V. cholerae* strain O17

was sub-cultured in BHI broth supplemented with 3% glycerol and grown at 37°C for four hours. Culture supernatants were collected and HlyA was precipitated by the addition of solid ammonium sulphate to 60% saturation. This preparation of HlyA was highly unstable, as activity was lost after short term storage at either 4°C or -20°C, and the addition of 6M urea did not enhance stability significantly. This loss of activity may be attributed to non-specific aggregation of HlyA monomers, as a result of the increased concentration of protein (A. Ludwig, personal communication). Precipitated HlyA remained stable only after the addition of 50% glycerol prior to storage at -20°C.

3.2.2.2 Single-channel analysis of HlyA

Ammonium sulphate precipitated HlyA was added to one of the two aqueous compartments containing 1M KCl, five minutes after the artificial membrane was in the black (bilayer) state. The insertion of HlyA into the membrane occurred after an additional five minutes. Figure 3.2 shows a single-channel recording (which is representative of ten such recordings) of a lipid bilayer (diphytanoyl phosphatidylcholine in *n*-decane) in the presence of HlyA and a single pore created by HlyA is indicated (arrow A). Current fluctuations were observed (Fig. 3.2, arrow B) which is indicative of the opening and closing of pores in the membrane. Channels in the bilayer may either be formed by a rigid structure, such as the detergent resistant hexamers of the Staphylococcal α -toxin (Ikigai and Nakae, 1985) or by a structure which opens and closes as with the *E. coli* α -haemolysin (Benz *et al.*, 1989). The average life-time of a channel formed by HlyA is estimated to be approximately one minute (Figure 3.2).

The single-channel recordings suggest that the ion-permeable channel of HlyA has a conductance of 350 pS in 1M KCl, however, a small fraction of 150 pS channels were also recorded. A histogram of the conductance fluctuations is shown in Figure 3.3 and incorporates 400 conductance steps. Haemolysins from the El Tor *V. cholerae* strains JBK70 and KM169 also exhibit a small fraction of 150 pS channels, which suggests the minor 150 pS channel is present in all haemolysins of *Vibrio cholerae* El Tor tested so far (Menzl *et al.*, 1996; R. Benz, personal communication).

Figure 3. 2 Black lipid bilayer exposed to HlyA.

Single-channel recording of a diphytanoyl phosphatidylcholine/*n*-decane membranes in the presence of 50 ng/ml El Tor haemolysin from *V. cholerae* O17. The aqueous phase contained 1M KCl (pH 6). The applied membrane potential was 20 mV at 20°C. Arrow A indicates the formation of a single pore, and arrow B indicates a fluctuation in conductance.

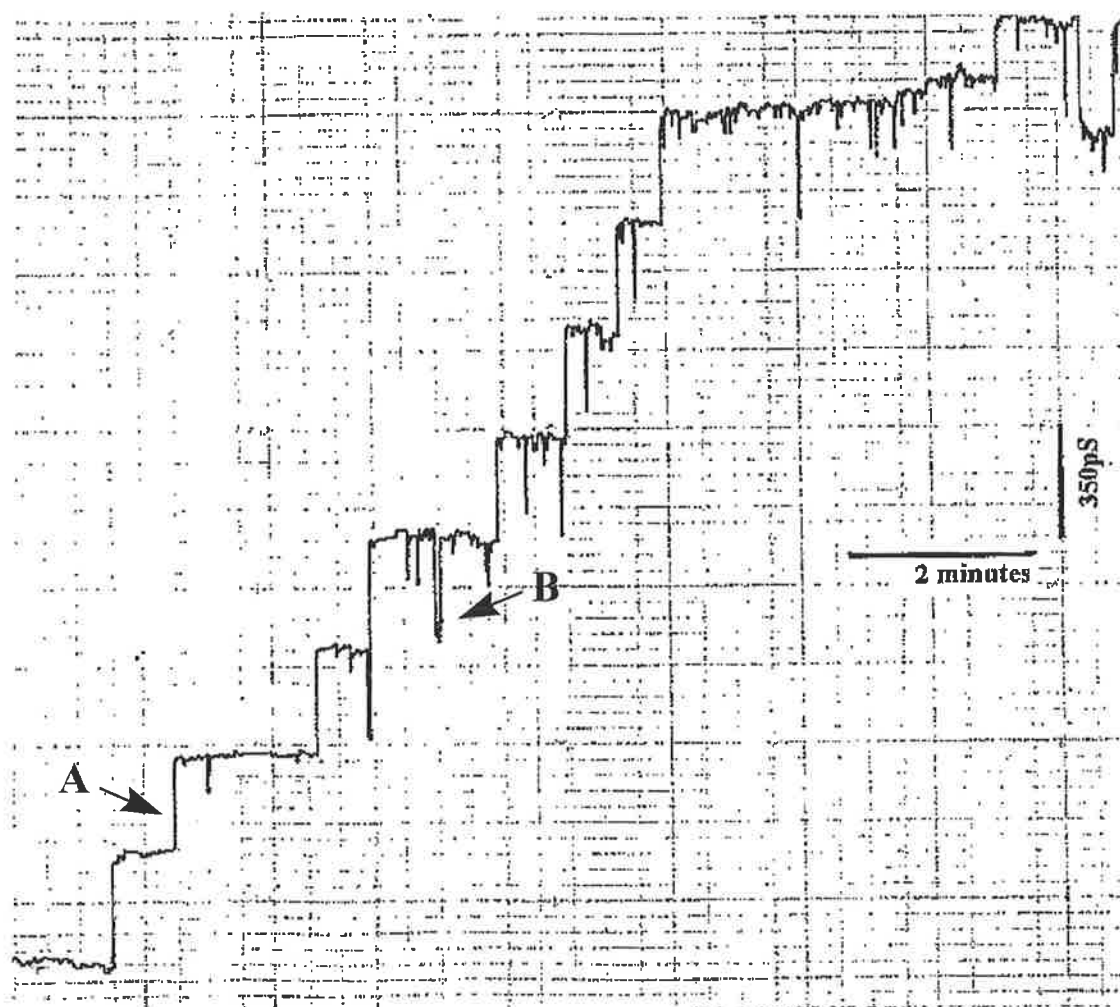
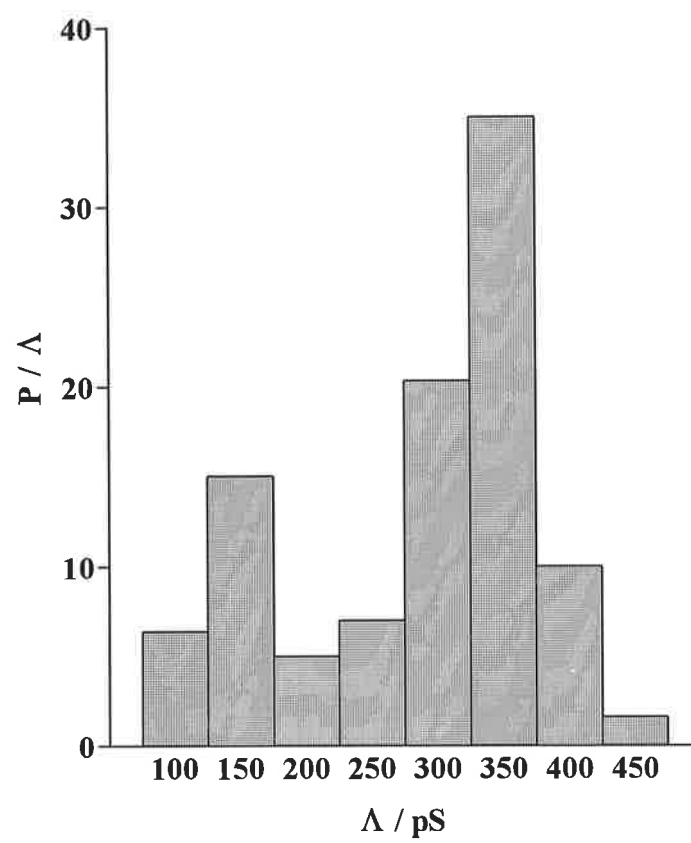


Figure 3.3 Summary of conductance fluctuations of Bilayer recordings.

Histogram of the conductance fluctuations observed with membranes formed from diphytanoyl phosphatidylcholine/*n*-decane in the presence of HlyA. The average single-channel conductance (Λ) was 150 pS for the left-hand maximum and 350 pS for the right-hand maximum. The total number of conductance fluctuations was 400 events. The aqueous phase contained 1M KCl and 50 ng/ml HlyA. The voltage applied to the membrane was 20 mV at 20°C. P is probability.



Precipitated supernatants isolated from an isogenic strain, *V. cholerae* O17 Δ hlyA (V945) were used to ensure there were no artefacts in the system. There was no increase of conductance with these supernatant fractions, despite being added to the aqueous phase in higher concentrations. This demonstrates that the single-channel recordings of Figure 3.2 were specific for HlyA.

The effect of different salt solutions on the single-channel conductivity of HlyA channels was examined and is shown in Table 3.1. Potassium (KCl) was replaced with Lithium (LiCl), and this had only a minor influence on the conductivity of the channel (conductivity decreased from 350 pS to 275 pS). However, the change in conductivity was more noticeable when the anion was changed. When the chloride ion (KCl) was replaced with acetate (KCH_3COO), conductivity dropped to 130 pS. This suggests that the haemolysin channel may be anion selective. However, despite the possible anion selectivity, there was a 1:1 ratio between conductance and KCl concentration (Figure 3.4), which is consistent with the absence of a binding site (Chakraborty *et al.*, 1990).

3.2.3 Prediction of pore-forming structures of HlyA

The only two protein structures capable of traversing the membrane are α -helices and β -strands (Jähnig., 1990) which require a minimum of twenty and ten amino acids, respectively (Lear *et al.*, 1988; Oiki *et al.*, 1988). The surface of the membrane spanning region must be hydrophobic, and contain sufficient residues to traverse the membrane. However, the requirements for pore-forming domains are somewhat different. Part of the protein is embedded into the lipid membrane while other parts are exposed to the aqueous filled channel. The region facing the membrane must be hydrophobic, while the region facing the interior of the channel, must be hydrophilic. Such structures are termed amphipathic, and can consist of either α -helices or β -strands. To form a pore with β -sheets, hydrogen bonds are formed between adjacent strands, and to form a β -barrel, it is estimated that a minimum of eight strands are required (Lesk *et al.*, 1989).

Unfortunately, there is no reliable consensus sequence that can be applied when looking for pore-forming structures within a protein. Many amino acid substitutions are

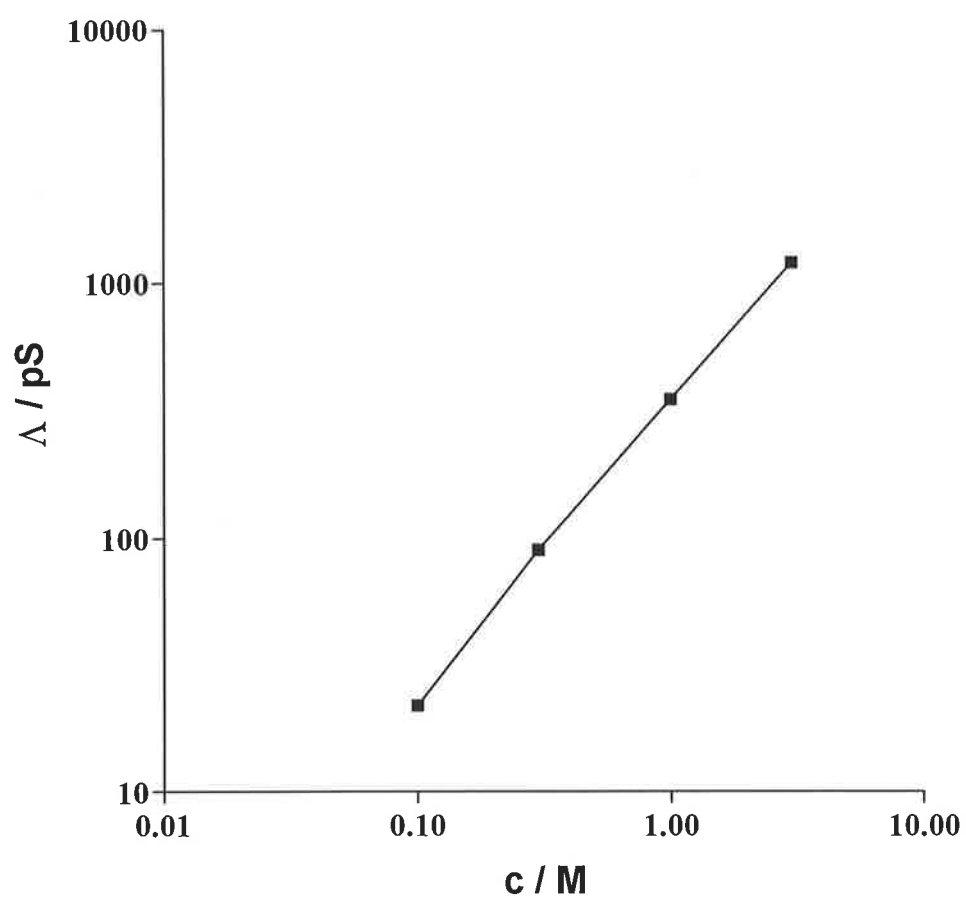
Table 3.1

Average single-channel conductance of the El Tor haemolysin of *Vibrio cholerae* O17 in different salt solutions. Concentration is given in M.

Salt	Molarity	Average single-channel conductance (pS)
KCl	0.1	22
	0.3	90
	1.0	350
	3.0	1200
KCH ₃ COO	1.0	130
LiCl	1.0	275

Figure 3.4 HlyA has a linear relationship with KCl concentration.

The average single channel conductance (Λ) of the HlyA channel as a function of the KCl concentration (M) in the aqueous phase. The membranes were formed from diphytanoyl phosphatidylcholine/*n*-decane. The points represent the means of at least 100 single events. The voltage applied to the membrane was 20 mV at 20°C.



permitted in an amphipathic structure, provided the amphipathic nature is conserved (Hancock and Brown, 1992). In the β -sheet structure, alternate amino acids are exposed to the aqueous channel and lipid membrane interior, respectively. Thus, if the arrangement of alternating polar and non-polar amino acids is conserved, many amino acid substitutions are permitted (Hancock and Brown, 1992). Bacterial porins consist of predominantly β -sheet structures, which form β -barrels (Nau and Konisky, 1989; Siehnel *et al.*, 1990). However, alignment of the primary sequence of bacterial porins did not provide an adequate consensus for the pore-forming domains, even though the structural and physical features were conserved (Siehnel *et al.*, 1990; Jeanteur *et al.*, 1991).

In order to predict any membrane-spanning amphipathic structures that may be involved with the pore-formation of HlyA, a FORTRAN computer program called AMPHI (Jähnig, 1990) was applied to the HlyA sequence. This method is based on the well-known hydrophobicity analysis of Kyte and Doolittle (1982), where each amino acid is allocated a hydrophobicity index, h . When the hydrophobicity of a protein is plotted as an average over seven neighbouring residues $H_7(i)$, this allows the prediction of membrane-spanning helices. However, this approach is limited as it does not distinguish between hydrophobic or amphipathic helices. The AMPHI program utilises equations specifically designed to predict amphipathic α -helices and β -strands (Jähnig, 1990). To predict an amphipathic α -helix, the average $H_\alpha(i)$ over the ten residues on one side of a predicted membrane-spanning α -helix is plotted, and an amphipathic α -helix is predicted when $H_\alpha(i)$ varies within a range of 3.6 residues (the number of residues per turn in an α -helix), over a region of twenty residues (sufficient to span the membrane). (ie: $H_\alpha(i) \geq 1.6$ and $H_\alpha(i+2) \leq 0.8$) In order to predict amphipathic β -strands, the structure must be at least ten residues to span the membrane. The average $H_\beta(i)$ of the five amino acid residues on one side of a predicted membrane-spanning β -strand is plotted, and an amphipathic β -strand is predicted if $H_\beta(i)$ varies with a period of two residues (that is, $H_\beta(i) \geq 1.6$ and $H_\beta(i+1) \leq 0.4$).

No amphipathic α -helices were predicted for HlyA, however, four amphipathic β -strands were predicted using the AMPHI program. Table 3.2 illustrates the $H_\beta(i)$ and $H_\beta(i+1)$ values of the amino acids within the predicted β -strands of HlyA. It should be

Table 3.2 Potential amphipathic β -sheets in HlyA^{a/b}

β -sheet 1			β -sheet 2		
position	aa	$H\beta(i) / H\beta(i+1)$	position	aa	$H\beta(i) / H\beta(i+1)$
166	H	-0.48	314	E	-0.16
167	V	1.84	315	L	1.92
168	A	0.06	316	G	-1.08
169	F	2.82	317	V	2.92
170	Y	-1.4	318	T	-1.7
171	I	2.24	319	G	3.2
172	S	-0.4	320	G	-1.16
173	V	2.3	321	V	2.36
174	N	-0.92	322	E	-1.78

β -sheet 3			β -sheet 4		
position	aa	$H\beta(i) / H\beta(i+1)$	position	aa	$H\beta(i) / H\beta(i+1)$
426	F	[1.08]	634	Y	-2.84
427	I	-0.82	635	V	1.68
428	I	2	636	R	-1.78
429	D	-1.36	637	A	3.22
430	S	3.04	638	G	-1.58
431	S	-1.56	639	A	2.82
432	V	2.16	640	A	-0.56
433	N	-1.56	641	I	1.9
434	I	[1.0]	642	D	0.18
435	R	-1.56	643	A	1.9
			644	L	0.1

^aThe position of the amino acids (aa) within HlyA are indicated.

^bThe $H\beta(i)$ and $H\beta(i+1)$ were obtained using the FORTRAN program AMPHI (Jähnig *et al.*, 1989).

The $H\beta(i)$ values that are not optimal are bracketed.

noted, however, that the $H\beta(i)$ values of two amino acids (Phe-426 and Ile-434) located at either end of β -sheet 3, are not optimal and so the true amphipathic β -sheet may only contain seven residues (Table 3.2). However, it is noteworthy that two synthetic 8 and 10 amino acid peptides were able to form pores in an artificial lipid bilayer and the peptide of 10 amino acids was haemolytic for human red blood cells (Oropeza-Wekerle *et al.*, 1992). These peptides were derived from predicted α -helical structures of the *E. coli* α -haemolysin. Clearly the lengths of these peptides are not sufficient to span the lipid bilayer, and the authors suggest that the peptides must insert efficiently into the membrane and be capable of intermolecular aggregation in the membrane.

Jähnig (1990) states that amphipathic β -strands can only be predicted when eight are present, as at least eight must be present to form a β -barrel (Lesk *et al.*, 1989). However, this requirement could be satisfied with the oligomerisation of monomers in the membrane. For example, eight monomers, each with only one β -strand, can form a barrel provided that hydrogen bonds can form between neighbouring β -sheets (Ojcius and Young, 1991). Thus, if the prediction of four amphipathic β -sheets in HlyA is correct, this implies that HlyA must oligomerise to form a β -barrel structure.

Even though HlyA is overall a hydrophilic protein, when the amino acid sequence was also subjected to 'Predictprotein' computer algorithms (Rost, 1996) to predict any hydrophobic structures capable of spanning the membrane, no hydrophobic β -sheets were predicted. In addition only one α -helix at position 637-659 was predicted using the PHDhtm program (Rost *et al.*, 1995). Since one α -helix was predicted, and although this helix had the minimum level of hydrophobicity required to insert into a lipid membrane, it is unlikely that this structure plays a role in pore-formation, although it may have a role in initial membrane interactions.

3.2.4 A comparison of HlyA with other haemolysins

It was of interest to determine whether the *V. cholerae* El Tor haemolysin was homologous to other bacterial haemolysins. A search of the database showed the amino acid sequence of HlyA is 57.4%, 44.7%, 44.8% homologous to the *Vibrio anguillarum* VAH1

haemolysin (Hirono *et al.*, 1996), the *Aeromonas salmonicida* 17-2 ASH4 haemolysin (Hirono and Aoki, 1993) and the *Aeromonas hydrophila* ATCC7966 AHH1 haemolysin (Hirono and Aoki, 1991), respectively (Table 3.3). This is in agreement with the findings of Hirono and co-workers (1996). While the *V. anguillarum* VAH1 gene is predicted to encode a 751 amino acid protein similar in size to the 82 kDa pre-pro-HlyA of *V. cholerae*, the haemolysin genes of *A. salmonicida* ASH4 and *A. hydrophila* are predicted to encode 63.4 kDa and 63.66 kDa proteins, respectively.

The positions of six cysteine residues were conserved in all four haemolysins, located at positions 182, 200, 497, 511, 537 and 549 of HlyA (however, it should be noted that the cysteine residue at position 511 in HlyA was not present in AHH1). The regions of homology between the sequences and conserved cysteine residues are highlighted in Figure 3.5. The amino acid sequence of the regions containing the potential amphipathic β -sheets of HlyA (section 3.2.3) was also compared with the amino acid sequence in the other haemolysins (Figure 3.5). The regions of HlyA containing β -sheets one, two and three, were homologous with regions in VAH1, ASH4 and AHH4, however, the most highly conserved β -sheets were two and three. The region containing β -sheet four was highly conserved in VAH4.

While the *Vibrio vulnificus* cytotoxin has only two small distinct regions (I and II) of homology to HlyA, the organization of these regions and positions of conserved cysteine residues suggest interspecies relatedness (Yamamoto *et al.*, 1990b). Regions I and II are identical in the *hlyA* gene sequences of *V. cholerae* El Tor strains O17, N86 and RC79 (Yamamoto *et al.*, 1990b).

3.3 Summary and Conclusion

The data presented here show that HlyA of *Vibrio cholerae* O1 is a member of the pore-forming toxins. The channel formed by HlyA had a single-channel conductance of 350 pS in 1M KCl and appears to be moderately anion selective. Zero-current membrane potential studies on the haemolysin of the *V. cholerae* El Tor strain JBK70 have confirmed the anion selectivity (Menzl *et al.*, 1996). Using osmotic protection assays, the diameter of

Table 3.3Comparison of *V. cholerae* HlyA with other haemolysins / cytolytins^a

	<i>V. cholerae</i> ^b HlyA	<i>V. anguillarum</i> ^c VAH1	<i>A. salmonicida</i> ^d ASH4	<i>A. hydrophila</i> ^e AHH1	<i>V. vulnificus</i> ^f Vvh
<i>V. cholerae</i> HlyA	100 [741]	57.4 [728]	44.7 [584]	44.8 [582]	28.5 [400]
<i>V. anguillarum</i> VAH1		100 [751]	43 [549]	45.7 [543]	25.8 [411]
<i>A. salmonicida</i> ASH4			100 [578]	82.4 [578]	24.2 [401]
<i>A. hydrophila</i> AHH1				100 [577]	24.4 [406]
<i>V. vulnificus</i> Vvh					100 [471]

^a Numbers, % identity determined using FASTA as implemented by PROSIS Version 7 (LKB-Hitachi Software).
Numbers in brackets, number of aa over which the indicated identity occurs.

^b Accession No. X16945

^c Accession No. 1911832

^d Accession No. S26577

^e Accession No. A61372

^f Accession No. P19247

Figure 3.5 A comparison of HlyA with various other haemolysins

The amino acid sequence of the *V. cholerae* El Tor HlyA is highly homologous to the haemolysins VAH1 (*V. anguillarum*), ASH4 (*A. salmonicida*) and AHH1 (*A. hydrophila*). The conserved cysteine residues and the amphiphilic β -sheets predicted for HlyA (section 3.2.3) are highlighted. The alignment of the amino acid sequences were performed using Clustal V (Higgins and Sharp, 1988 and 1989). Identical residues are indicated by asterisks, conserved residues with similar properties are indicated by dots. Gaps (dashes) have been introduced to optimize alignment. The number of the last amino acid in each row is indicated for each protein.

β -sheet 2

HlyA	F P V D N I N E K F E R K E V S G F	E L G V T G G V E	V S G D G P K A K L E A R A S Y T Q S R W L T	345
VAH1	F P R E N L N A E Y E V K E V S G F	Q L G V S G G V E	V S A D G P K G K L E A N A S Y N Q S R W L T	342
ASH4	L P T N - L N S K Y E H R R I S G F	E V G V T G G V E	V N K D G P K A S W R R R P S S A S S V T M P	309
AHH1	L P T N - L N S K Y E H R E I S G F	E V G V T G G V E	V N K D G P R P S W R R R P S S A S S A S S P	309
	* * . . *	* * . * * * *	* * * . * *	

HlyA	Y N T Q D Y R I E R N A K N A Q A V S F T W N R Q Q Y -	A T A E S L L N R S T D A L W V N T Y P V	393
VAH1	F K T H D Y R V E R S T N N A Q N V S F K W N R S Q Y -	A T A E S M M N R S Y D A I W V N T Y P V	390
ASH4	- T T P R I I G S N A R P E C P E G E F Q L G A G S I R D G G V T A L L Q D R H -	R L G N G V R C	356
AHH1	- T T P R I T G L N A P A E R P E G E F Q L G A R S I R D R R V P A L L Q D G H -	V W A W - L R R	355
	*	.	

β -sheet 3

HlyA	D V N R I S P L S Y A S F V P K M D V I Y K A S A T E T G S T D	F I I D S S V N I R	P I Y N G A Y K	443
VAH1	D T N R I N P I G Y A S F V P K M D V I Y K A A P N V T G T T E	F T I D S S V N I R	P I Y N G A Y K	440
ASH4	G S Q P H P A A E L Q G L R A E S G C H L Q G G A D E T G S T E	F K I D S S V N I R	P I Y T G I Y K	406
AHH1	G S Q P H P A A Q L Q G V C A E S D V I Y K A A P D E T G S T E	F K I D S S V N I R	P I Y T G I Y K	405
		* * . *	* * * * * * * * * * * * *	

HlyA	H Y Y V V G A H Q S Y H G F E D T P - R R R R I T K S A S F T V D W D H P V F T G G R P V N L Q L A S	492	
VAH1	H Y Y V V G G H Q S Y H G L K N T T - R R R R V S K V T S F T V D W E H P V F T G G R P V N L Q L G S	489	
ASH4	H Y Y V V G G H V S F Q G F E D V D K R R R V T A S T S F K V D W N H P V F T G G R P V N L Q L G G	456	
AHH1	H Y Y V V G A H V S F Q G F E D T D K R R R V A A S T S F K V D W N H P V F T G G R P V N L Q L G G	455	
	* * * * * . * * . . * . .	* * * * * . * * * * * * * * * * * * * . .	

HlyA	F N N R	C	I Q V D A Q G R L A A N T	C	D S Q Q S A Q S F I Y D Q L G R Y V S A S N T K L	C	L D G B A	542
VAH1	F N N R	C	V D V A D N G V L S A S S	C	D E N Q N S Q S F I Y D Q L G R Y V S A N D I H Q	C	L D G Q S	539
ASH4	F D N R	C	L S A G A E H G L S A V T	C	D E T S A A Q S F I Y D Q Y G R Y V S A L D T R R	C	L D G N N	506
AHH1	F D N R	C	L S A D A N H G L S A V T	C	D E T S A A Q S S I Y D E Y G R Y V S A Q D T L R	C	L D G N N	505
	* . * * *	.	* . *	.	* * * * *	.	* * * * *	

HlyA	L D A L Q P	C	N Q N L T Q R W E W R K G T D E L T N V Y S G E S L G H D K Q T G E L G L Y A S S N D	592				
VAH1	L G Q L Q P	C	S S S L V Q R W E W I E N S D K L S N V F S H E L L G H D K T S G E L G L Y A D G N D	589				
ASH4	L G Q L Q S	C	R L S L G Q R W E W K A D S D A L S N L S A H Q L L G H N K Q T G E L A L Y D E N G N	556				
AHH1	L G Q L Q S	C	S L S L G Q R W E W K A D S D A L S N L S A H Q L L V H D K Q S G A L G L Y D E N G N	555				
	* . * * *	*	* * * * *	.	* * . * . . .	* * . * . * * .	.	

β -sheet 4[illegible][illegible][illegible][illegible]

the HlyA channel was estimated to be <1.6 nm. This is in full agreement with the findings of Ikigai *et al.* (1996) who estimated pore size by both osmotic protection and electron microscopy. Menzl *et al.* (1996) estimate a minimum diameter of 0.7 nm for HlyA using artificial lipid membranes, however the authors concede that this is an estimate only due to the limitations of this assay when determining the diameter of smaller channels, such as HlyA.

The amino acid sequence of HlyA was analysed for potential pore-forming structures using the AMPHI (Jähnig, 1990) computer program. Four potential amphipathic β -sheets were predicted for HlyA and as at least eight β -sheets are required to form a β -barrel (Lesk *et al.*, 1989), this suggests that HlyA must oligomerise in order to form a channel. It has since been demonstrated that HlyA monomers do oligomerise on the membranes of rabbit erythrocyte ghosts, liposomes (Ikigai *et al.*, 1996) and human intestinal cells (Zitzer *et al.*, 1997). No amphipathic α -helices were predicted for HlyA.

A search of the data based revealed high homology between HlyA and the VAH1, ASH4 and AHH1 haemolysins of *Vibrio anguillarum*, *Aeromonas salmonicida*, and *Aeromonas hydrophila*, respectively. β -sheet structures one, two and three predicted for HlyA were conserved with all four haemolysins, while β -sheet four was conserved in VAH1. Six cysteine residues were also conserved which raises the possibility that secondary structure of the proteins is also conserved.

Chapter 4

Analysis of C-terminal truncations of the *V. cholerae* O1 El Tor haemolysin (HlyA)

4.1 Introduction

Many toxins are first translated as inactive precursor or "pro-" toxins, which require proteolytic cleavage to convert them into the mature, active form. For example, the *Aeromonas* cytotoxin requires the removal of 25 amino acids from the C-terminus outside the cell, by a protease produced by *Aeromonas* (Howard *et al.*, 1987; Husslein *et al.*, 1988; Buckley, 1992). Similarly, the pro-cytotoxin of the opportunistic pathogen *Pseudomonas aeruginosa* is converted to the active form by the removal of 20 amino acids from the C-terminus (Hayashi *et al.*, 1989). Diphtheria toxin requires proteolytic cleavage of a precursor to generate two polypeptides A and B, which remain joined by a single disulphide bond. Maturation of the *Vibrio cholerae* El Tor haemolysin (HlyA) requires the removal of an N-terminal 15 kDa peptide from the precursor to become active (Hall and Drasar, 1990; Yamamoto *et al.*, 1990a; Nagamune *et al.*, 1996). HlyA is initially synthesised as an 82kDa pre-pro-toxin in the cytoplasm and after removal of the signal peptide is secreted as an inactive 80 kDa pro-toxin (Alm *et al.*, 1988; Yamamoto *et al.*, 1990a; Nagamune *et al.*, 1996). Cleavage of the pro-form is then required to release the active 65 kDa toxin (Yamamoto *et al.*, 1990a; Nagamune *et al.*, 1996).

The cleavage site of pro-HlyA was initially localised to the N-terminus by amino-terminal sequencing of the purified 65 kDa product, however there is conflict over the exact site of processing (Hall and Drasar, 1990; Yamamoto *et al.*, 1990a; Nagamune *et al.*, 1996). At the commencement of this work, little was known about the actual cleavage event and the factor(s) involved. It was postulated that HlyA may be autoproteolytic, as degradation and specific cleavage products were observed during the purification of 80 kDa pro-HlyA, and cleavage was not inhibited by the protease inhibitor phenyl-methyl-sulphonyl fluoride (PMSF) (Alm *et al.*, 1988; Alm, 1989). It was also suggested that cleavage may be

attributed to the soluble-haemagglutinin-protease (SHA-protease) (Alm *et al.*, 1988; Hall and Drasar, 1990; Yamamoto *et al.*, 1990a), which is able to cleave several factors associated with virulence, including cholera toxin, fibronectin, mucin and lactoferrin (Finkelstein *et al.*, 1983; Booth *et al.*, 1984).

As discussed in section 1.5, HlyA is a potent bifunctional toxin with both enterotoxic and cytolytic activities and is considered an additional diarrhoeagenic factor to the cholera toxin, for El Tor and non-O1 strains (Ichinose *et al.*, 1987; Alm *et al.*, 1991). The cytolytically active domain of HlyA was localised to a 65 kDa cleavage product (Yamamoto *et al.*, 1986). Using animal models, enterotoxicity was localised to the N-terminus of HlyA for both classical HlyA* and El Tor HlyA and it was proposed that enterotoxicity lies in the N-terminal 15 kDa cleavage product of HlyA (Alm *et al.*, 1991). Classical strains produce only a truncated version of the haemolysin, HlyA*, which is essentially the N-terminal 27 kDa of the El Tor haemolysin (Alm *et al.*, 1991; Rader and Murphy, 1988).

The various domains of HlyA relating to specific functions were unknown at the commencement of this study. In other systems, domains involved in membrane-binding, pore-formation, activation and secretion have been well defined, enabling a better understanding of the role in the pathogenesis. Examples include the *E. coli* α -haemolysin and the *S. marcescens* haemolysin, and are reviewed in sections 1.6.1 and 1.6.2.

The aim of this chapter was to identify and localise structure / function domains of the El Tor haemolysin (HlyA), involved with pore-formation, cleavage and secretion.

4.2 Results

4.2.1 Construction of a $\Delta hlyA$ mutant in *V. cholerae*

4.2.1.1 Creating a deletion in the *hlyA* gene

To analyse various constructs encoding mutated HlyA proteins in *V. cholerae* and eliminate any possibility of recombination to restore an intact *hlyA* gene, a chromosomal deletion of *hlyA* in *V. cholerae* strain O17 was constructed (V945). The source of *hlyA* was pPM431 (Manning *et al.*, 1984), which carries the *hlyA*, *hlyB* and *hlyC* genes on the 6.4 kb *Pst*I fragment (Alm and Manning, 1990a) shown in Figure 1.2. A 3.5 kb *Pst*I / *Eco*RI

fragment carrying *hlyA* was isolated from pPM431 and cloned into the *Pst*I and *Eco*RI restriction sites of pBluescript SKTM to generate plasmid pPM4343 (Figure 4.1).

pPM4343 was then digested with *Nru*I and *Aat*II, to delete a 2057 bp fragment containing most of *hlyA*. The remaining *hlyA* would encode only 9 and 46 amino acids of the N- and C-terminus, respectively. The *Nru*I and *Aat*II sites lie at nts 1170 and 3227 respectively, of the 3.5 kb *Pst*I / *Eco*RI fragment containing *hlyA* (shown in Figure 1.2). The DNA was end-filled, religated and transformed into DH5 and clones were initially screened for a haemolytic negative phenotype by patching colonies onto blood agar. Hly⁻ cells were then confirmed to carry plasmids which had lost the 2 kb *Nru*I / *Aat*II fragment. To promote efficient homologous recombination of the *hlyA* deletion with the *V. cholerae* chromosome, approximately 300-400 bp of DNA should be present on either side of the deletion. To ensure adequate homologous DNA in this region, the *Pst*I / *Eco*RI fragment with the *Nru*I / *Aat*II deletion was ligated together with a 2.7 kb *Pst*I / *Eco*RI fragment encoding *hlyB*, which lies downstream of *hlyA*, and were shot-gun cloned into the *Pst*I site of pBR325 (Figure 4.2). pBR325 was chosen as pBR322-based plasmids are gradually lost from *V. cholerae* if there is no antibiotic selection for the plasmid (U. H. Stroehrer, personal communication). Cm^R, Ap^S, Tet^S and haemolytic negative transformants were isolated and the resulting construct was designated pPM4348 (Figure 4.2).

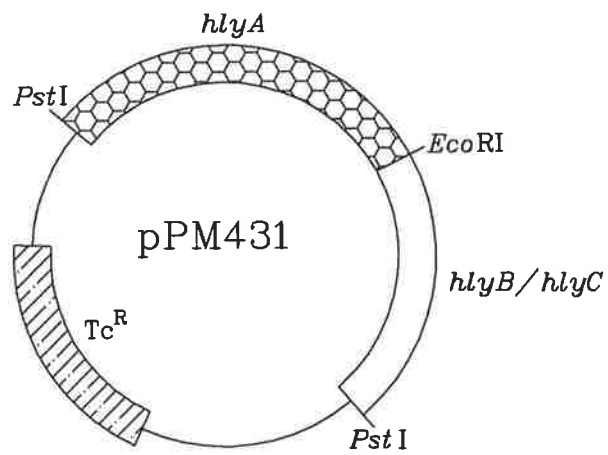
4.2.1.2 Construction of *V. cholerae* O17 Δ *hlyA* (V945)

In order to obtain a *V. cholerae* strain with a chromosomal deletion in *hlyA*, pPM4348 was electroporated into O17. An overnight culture of this strain was then subcultured 1/20 in the presence of antibiotic (Cm) and grown to late exponential phase. It was again subcultured using a 1/20 dilution, with no antibiotic selection (ie: no selection for the plasmid), and this was repeated four times. Cells in which homologous recombination had occurred between the chromosome and the mutated *hlyA* gene on the plasmid (containing the deletion) were identified by their non-haemolytic phenotype on blood agar.

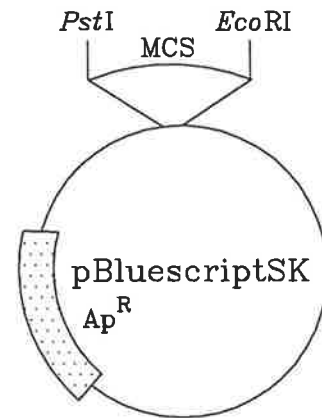
Southern hybridization was then used to confirm the isolation of a Δ *hlyA* strain of O17 (Figure 4.3A). The 3.5 kb *Pst*I / *Eco*RI fragment of pPM4343 carrying *hlyA* and

Figure 4.1 Cloning *hlyA* into pBluescript SK.

The 3.5 kb *Pst*I / *Eco*RI fragment of pPM431 (Manning *et al.*,1984) carrying *hlyA* was isolated and ligated into the *Pst*I / *Eco*RI restriction sites of pBluescript SK to generate pPM4343.



Isolate the 3.5 kb
*Pst*I/*Eco*RI
fragment



Digest with
*Pst*I/*Eco*RI

Mix and Ligate

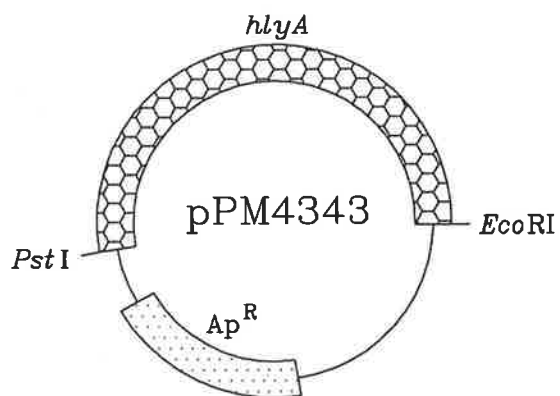
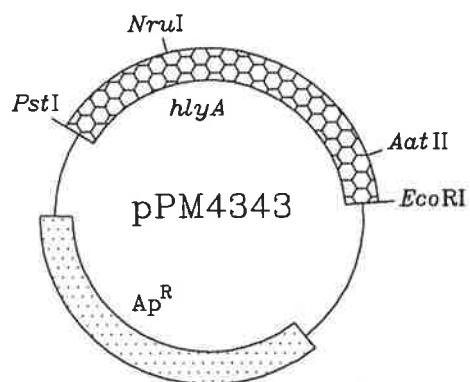
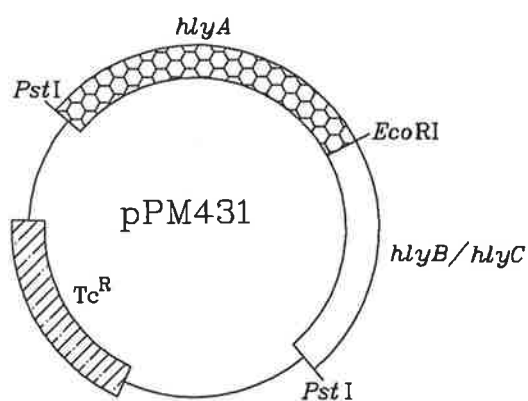
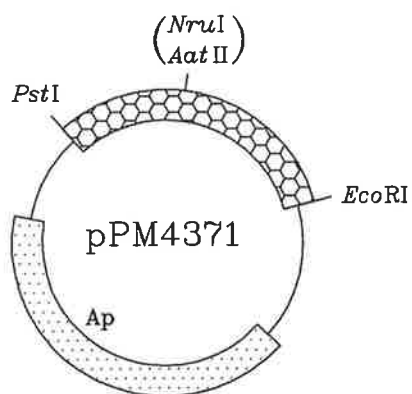


Figure 4.2 Generation of pPM4348 which carries a large deletion in *hlyA*.

pPM4343 was digested with *NruI* / *AatII*, end-filled and re-ligated to remove the intervening 2 kb that encodes most of HlyA, to generate pPM4371. To ensure efficient homologous recombination with genomic DNA at a later stage, additional DNA normally found downstream of *hlyA* was cloned adjacent to the deletion in *hlyA*. ($\Delta hlyA$). The *PstI* / *EcoRI* of pPM4371 was isolated and mixed together with both the 2.9 kb *EcoRI* / *PstI* isolated from pPM431 and pBR325 linearized with *PstI*. The resulting construct, pPM4348 carries $\Delta hlyA$, *hlyB* and *hlyC*.



Digest with *NruI/AatII*
End-fill, dilute and religate



Isolate *PstI/EcoRI*
fragment

Isolate *PstI/EcoRI*
fragment

Mix and ligate

Digest with *PstI*
alkaline phosphatase

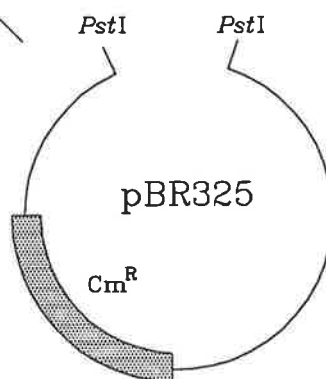
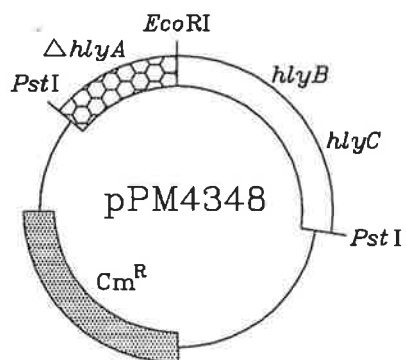
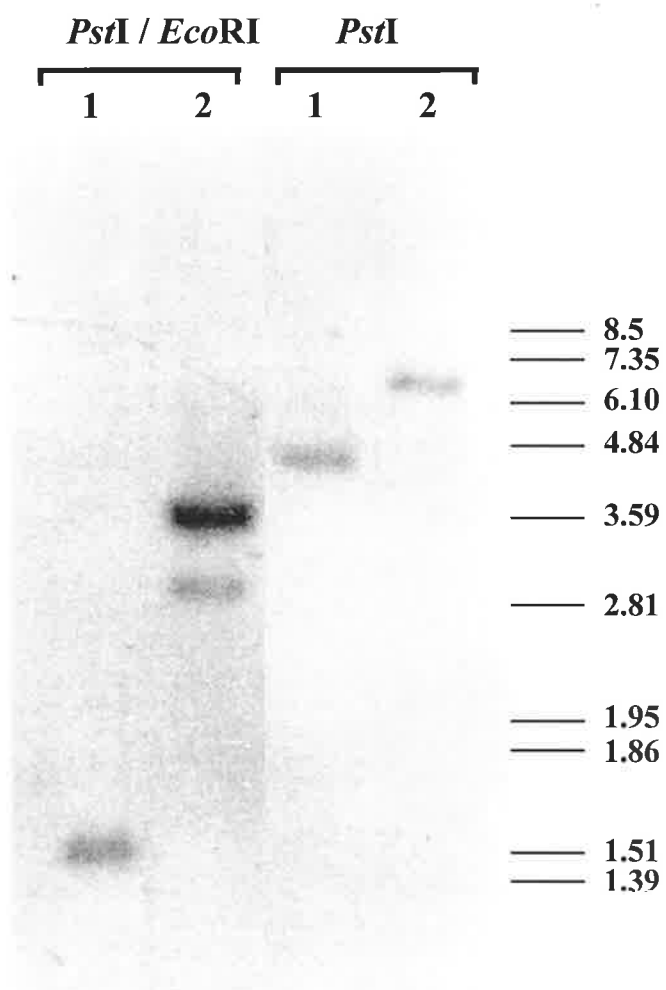


Figure 4.3 Confirmation of the $\Delta hlyA$ mutant (V945) of *V. cholerae*.

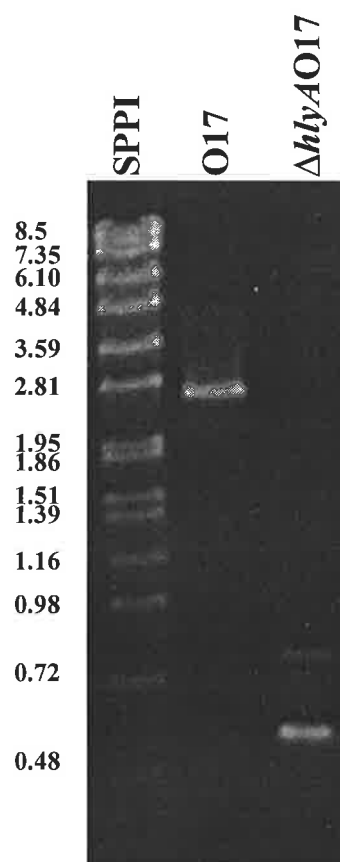
Panel A: A Southern blot of chromosomal DNA of *V. cholerae* O17 and O17 $\Delta hlyA$ digested with either *Pst*I / *Eco*RI or *Pst*I restriction endonucleases (as indicated). The probe used was a DIG-labelled 3.5 kb *Pst*I/*Eco*RI fragment of pPM4343 carrying *hlyA*. Lane 1: $\Delta hlyA$ O17 and 2: O17. A decrease of approximately 2 kb is observed with restriction fragments generated from O17 $\Delta hlyA$, compared to that obtained from O17 DNA. The position of the SPP1 size markers are indicated to the right of the Figure, and are given in kb.

Panel B: PCR amplification of the *hlyA* region in *V. cholerae* O17 and $\Delta hlyA$ O17 using oligonucleotides #1009 and #1019. Fragments of approximately 2.59 kb and 0.54 kb were generated from O17 and O17 $\Delta hlyA$, respectively. DNA fragments were electrophoresed on a 1% agarose gel. The SPP1 size markers are indicated, and are given in kb.

A



B



flanking DNA was isolated, DIG-labelled and used as the probe. Genomic DNA was extracted from both O17 and potential O17 Δ *hlyA* cells, and digested with *Pst*I / *Eco*RI and *Pst*I. Since *hlyA* lies on a 6.4 kb *Pst*I fragment and a 3.5 kb *Pst*I / *Eco*RI fragment, the strain containing a 2 kb deletion across *hlyA* should generate a 4.4 kb *Pst*I and 1.5 kb *Pst*I / *Eco*RI fragment, and Fig 4.3 shows this is the case, confirming the isolation of *V. cholerae* O17 Δ *hlyA*.

PCR amplification of the chromosome spanning the *hlyA* region was also used to confirm the mutation. Amplification with oligonucleotides #1009 and #1019 generated a 2.59 kb fragment from O17 and a 0.54 kb fragment the O17 Δ *hlyA* *V. cholerae* (Figure 4.3B).

4.2.2 Production and analysis of C-terminal truncations of HlyA in *E. coli*

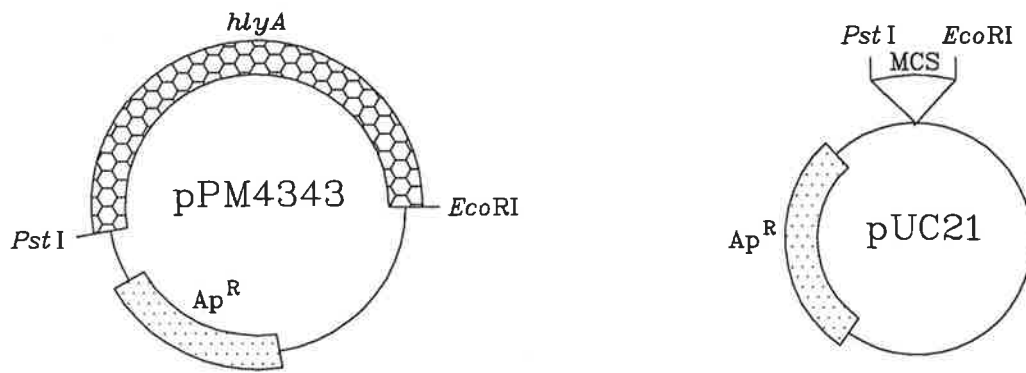
According to previous reports, the 15 and 65 kDa products originate from the N- and C-terminus of pro-HlyA, respectively (Hall and Drasar, 1990; Yamamoto *et al.*, 1990a; Nagamune *et al.*, 1996) and so it was considered to be important to express the N-terminal 15 kDa of HlyA in order to establish a possible role for this product. In addition, to define the minimum region of the 65 kDa HlyA required for haemolytic activity, the strategy chosen was to obtain a series of C-terminally-truncated HlyA proteins. Stable truncated derivatives of HlyA could also be used to define regions required for secretion and regions that are recognised by various anti-HlyA antibodies. In summary, this strategy was chosen to define structure / function domains of pro-HlyA.

4.2.2.1 Sequential Deletions at the 3' end of *hlyA*

The 3.5 kb *Pst*I / *Eco*RI fragment encoding *hlyA* was recloned from pPM4343 into the *Pst*I / *Eco*RI sites of the lower copy number vector pUC21, as the high copy plasmid pPM4343 was unstable in both *E. coli* and *V. cholerae*. The resulting clone pPM2684 (Figure 4.4), is oriented such that *hlyA* is under the control of the bacteriophage T7 promoter. This plasmid was then digested with *Kpn*I and *Eco*RI and one end of the linearized fragment was sequentially digested with exonuclease III using the Erase-a-base

Figure 4.4 Construction of pPM2684 and generation of 3' deletions.

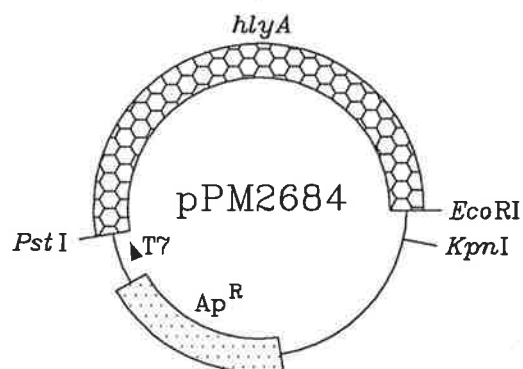
The 3.5 kb *Pst*I / *Eco*RI fragment of pPM4343 was isolated and ligated into the *Pst*I / *Eco*RI of the MCS of pUC21. The resulting construct was designated pPM2684, and carries *hlyA* behind the bacteriophage T7 promoter (Tabor and Richardson, 1987). To obtain 3' nested deletions in *hlyA*, pPM2684 was restricted with *Eco*RI and *Kpn*I. The DNA end created by *Eco*RI was then sequentially deleted by digesting with exonuclease III. The end of the DNA created by *Kpn*I is not susceptible to exonuclease III digestion.



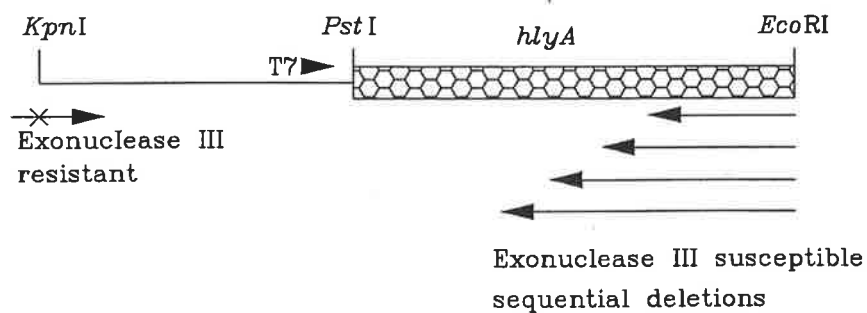
Isolate the 3.5 kb
*Pst*I/*Eco*RI
fragment

Digest with
*Pst*I/*Eco*RI

Mix and Ligate



Digest with
*Kpn*I/*Eco*RI



system (Promega) (Figure 4.4). DNA restricted with *Kpn*I is resistant to exonuclease III digestion, however DNA generated by digestion with *Eco*RI, is susceptible to exonuclease III. Thus the 3' end of the *hlyA* gene was sequentially deleted, the products religated and the end-points of the deletions were determined by DNA sequencing using Dye-labelled reverse primer. The exonuclease III-generated derivatives of pPM2684 are summarized in Figure 4.5.

4.2.2.2 Expression of C-terminal truncations of HlyA in *E. coli*

The constructs generated by exonuclease III digestion were then used to generate C-terminally truncated forms of HlyA, using the T7 promoter / RNA polymerase system of Tabor and Richardson (1987). The expression of *hlyA* encoded by pPM2684 and its derivatives is under the control of the T7 RNA polymerase and promoter. Whole cell fractions of *E. coli* containing proteins produced after the induction of the T7 polymerase were electrophoresed on a SDS-PAGE gel followed by staining with 0.4% Coomassie Brilliant blue. A decrease in the size of 80 kDa pro-HlyA as a result of C-terminal truncations is illustrated in Figure 4.6A. All the truncated proteins are visible on the gel except for the protein encoded by pPM2700 which could only be detected when labelled with [³⁵S]-methionine followed by autoradiography (not shown), which suggests it is unstable. pPM2700 encodes the N-terminal 15 kDa pro-region of HlyA, and still includes the proposed cleavage sites (Hall and Drasar, 1990; Yamamoto *et al.*, 1990a; Nagamune *et al.*, 1996).

4.2.2.3 Reactivity of C-terminal truncations to specific antibodies

The reactivity of HlyA and the C-terminally deleted derivatives to polyclonal antisera and the cleavage of C-terminally truncated forms of HlyA was examined by western immunoblot analysis. The gels used for the western immunoblots were produced using identical conditions to those of Figure 4.6A. Antiserum α -65 was raised towards purified 65 kDa product (Yamamoto *et al.*, 1986) and as previously demonstrated by Alm *et al.* (1988) recognises both full-length 80 kDa and cleaved 65 kDa haemolysin. In addition, α -

Figure 4.5 A summary of exonuclease III derived C-terminal truncations of HlyA.

Plasmid pPM2684 contains the intact *hlyA* gene cloned in vector pUC21 and the deletions are derivatives of pPM2684. Relevant regions of HlyA and the end-points of the deletions are shown in amino acids. The specific N-terminal cleavage site is indicated with an arrow.

Cleavage site



pro-HlyA

NH₂



COOH

pPM2684	741
pPM2685	729
pPM2686	682
pPM2687	653
pPM2688	639
pPM2689	623
pPM2691	609
pPM2692	583
pPM2693	533
pPM2696	473
pPM2697	429
pPM2699	249
pPM2700	179

Figure 4.6 Expression of C-terminally truncated HlyA from *E. coli* and their reactivity with anti-HlyA antiserum.

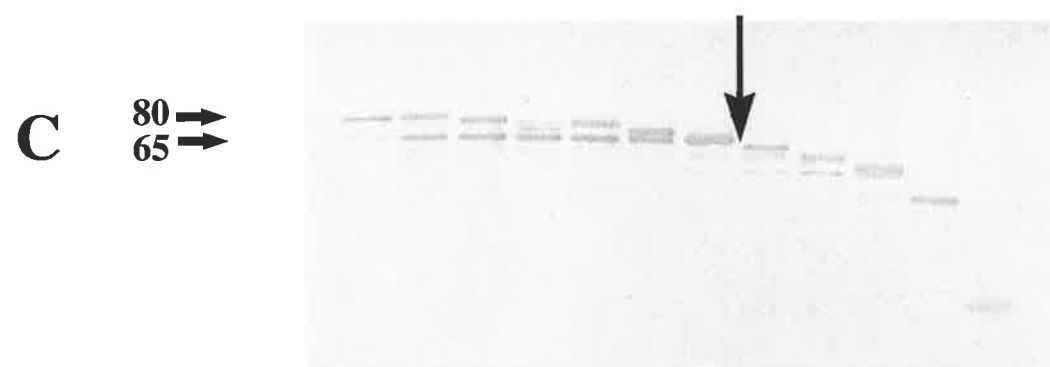
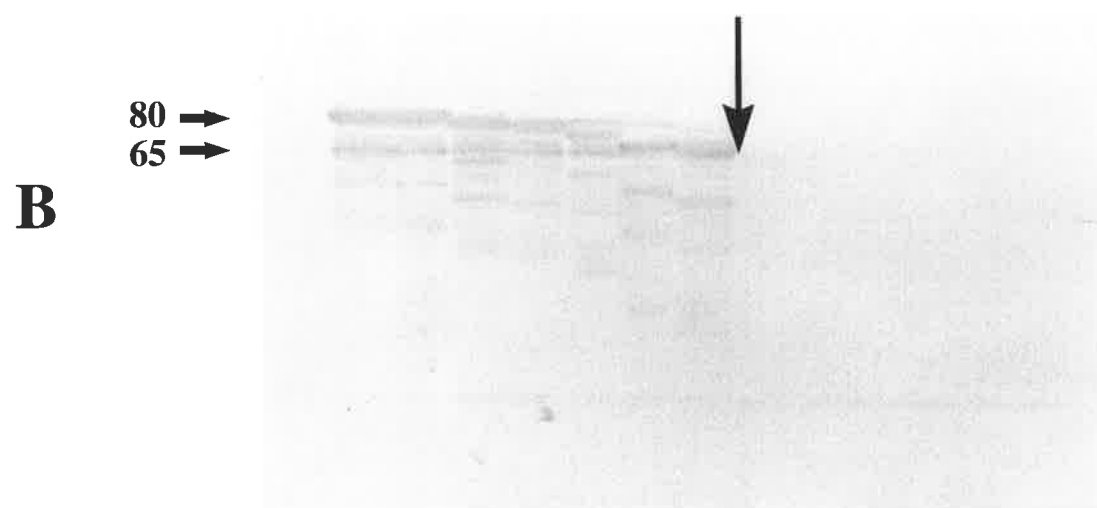
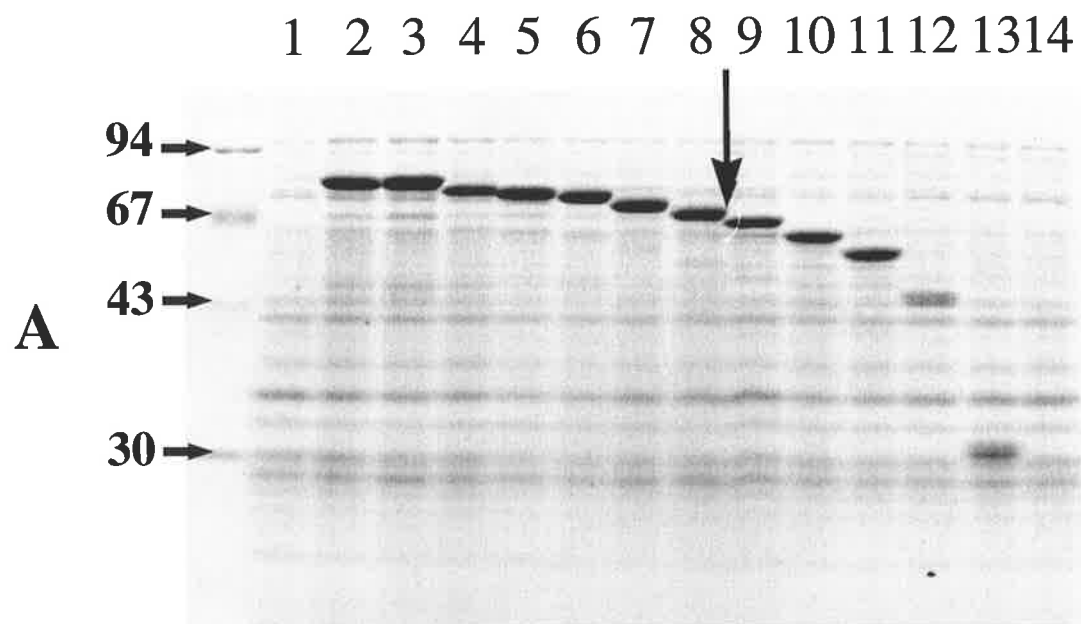
Expression of the C-terminal truncations of HlyA was induced using the T7 RNA polymerase / promoter system of Tabor and Richardson, 1987. After induction, whole cell fractions were subjected to SDS-PAGE and either stained with Coomassie Brilliant blue or subjected to western immunoblot using specific anti-HlyA antisera. Lanes 1-14 are pUC21, pPM2684, 2685, 2686, 2687, 2688, 2689, 2691, 2692, 2693, 2696, 2697, 2699 and pPM2700.

Panel A: Coomassie stained gel. HlyA and all truncated HlyA are visible except for the smallest truncated HlyA, encoded on pPM2700. Molecular masses are shown and are given in kDa.

Panel B: α -65 used in this immunoblot was raised to purified 65kDa HlyA (Yamamoto *et al*, 1986). The end-point of recognition is indicated by a vertical arrow.

Panel C: α -dHlyA used in this immunoblot was raised against SDS-PAGE purified denatured 80kDa HlyA, and all the truncated HlyA are recognised (except for pPM2700-encoded protein).

The end-point for the presence of the 65* kDa band is indicated with a vertical arrow in panels B and C and corresponds to pPM2691.



65 recognises C-terminal truncated HlyA derivatives which are 65 kDa or greater (Figure 4.6B; lanes 3-8). Removal of more than 15 kDa from the C-terminus resulted in loss of recognition by α -65 (Figure 4.6B; lanes 9-13: the endpoint of recognition is indicated by the arrow).

For further analysis of the C-terminal truncations of HlyA, it was essential to have a specific antiserum which recognises all of the truncated HlyA shown in Figure 4.6A. *V. cholerae* containing pPM3039 was chosen as the source of HlyA. pPM3039 encodes HlyU, which transcriptionally up-regulates *hlyA* such that the level of HlyA production is higher than that of the parental O17 strain (Williams and Manning, 1991). 500 mls of the culture supernatant was TCA precipitated on ice for 30 mins to concentrate the HlyA protein. However, the culture supernatant of O17 [pPM3039] also contains the 28 kDa Hcp protein, which is co-ordinately up-regulated with HlyA (Williams *et al.*, 1996). It was therefore necessary to purify the TCA-precipitated HlyA by SDS-PAGE. The polyacrylamide gel was stained with Coomassie blue and denatured 80 kDa HlyA was excised and homogenized. The homogenized gel slice was then resuspended in incomplete Freund's adjuvant and used as the immunogen for the antiserum. This antiserum was designated α -dHlyA (anti-denatured HlyA). A western-immunoblot using this antiserum to whole cell fractions of the T7 induced C-terminal truncations is shown in Figure 4.6C. All the C-terminal truncations of HlyA were detected except for pPM2700 which was also not identified by Coomassie staining of the gel (Fig 4.6A)

The monoclonal antibody A6 (mA6) raised towards purified 80 kDa HlyA (Sheilds, 1991) was also examined for reactivity to HlyA and while it recognised native 80 kDa HlyA, it did not recognise any of the HlyA C-terminal deletions (not shown). Recognition of various anti-HlyA antibodies to HlyA and its deleted derivatives is summarised in Figure 4.7.

Plasmid pPM2684 encodes native HlyA and produces both the 80 and the 65 kDa HlyA (Figure 4.6B and C, lane 2). As the mature 65 kDa product is derived from the C-terminus of pro-HlyA, we expected a decrease in the 65 kDa product as a result of the sequential C-terminal deletions. However, in lanes 3-8 (Fig 4.6B and C), a 65 kDa protein

Figure 4.7 A summary of the phenotypes of the C-terminal truncations (Δ C) of HlyA.

The haemolytic activity, reactivity with specific antibodies and ability to produce a 65* kDa cleavage product of the various C-terminal deletions of HlyA are shown. Up to 15 kDa can be removed from the C-terminus of HlyA before haemolytic activity, ability to cleave to a 65* kDa product, and recognition by α -65 antiserum, is abolished. This end-point lies between proteins encoded by pPM2691 and pPM2692. Plasmid pPM2684 encodes native pro-HlyA. The end-points are shown in amino acids. The specific cleavage site is indicated with an arrow.

pro-HlyA		Cleavage site		$\Delta C < 15$		Haemolytic ^a	Reactivity to antibody ^b			Cleavage to
	15kDa	65kDa				activity	a	b	c	65*kDa
pPM2684				741	++++	+	+	+		+
pPM2685				729	++	+	+	-		+
pPM2686				682	++	+	+	-		+
pPM2687				653	++	+	+	-		+
pPM2688				639	++	+	+	-		+
pPM2689				623	++	+	+	-		+
pPM2691				609	++	+	+	-		+
pPM2692				583	-	-	+	-		
pPM2693				533	-	-	+	-		
pPM2696				473	-	-	+	-		
pPM2697				429	-	-	+	-		
pPM2699				249	-	-	+	-		
pPM2700				179	-	-	-	-		

^a Measured on 5% Blood agar

^b Reactivity to antibodies: α -65 (a); α -dHly (b); mA6 (c)

was also detected for the C-terminally truncated HlyA which is contradictory to the previously reported cleavage sites (Hall and Drasar, 1990; Yamamoto *et al.*, 1990; Nagamune *et al.*, 1996). Approximately 15 kDa could be removed from the C-terminus before the ability to produce a 65 kDa product was lost.

This raised the possibility that cleavage may actually occur at the C- and not the N-terminus of pro-HlyA, such that mature 65 kDa HlyA would be N-terminally derived (Figure 4.8, hypothesis I). The N-terminal cleavage site was previously determined by amino-terminal sequencing of purified 65 kDa HlyA. However, both 80 and 65 kDa HlyA are unstable and readily degrade during purification procedures (Yamamoto *et al.* 1986; Alm *et al.*, 1988; Nagamune *et al.*, 1996; Alm, 1989), so it is possible that a breakdown product(s) of the 65 kDa was sequenced instead of the 65 kDa itself. This would require a proteolytically sensitive site approximately 15 kDa from the N-terminus, which cleaves during purification of the 65 kDa.

Alternatively, there may be a proteolytically sensitive site 15 kDa from the C-terminus of pro-HlyA, that is exposed as result of the C-terminal deletions (Figure 4.8, hypothesis II). Cleavage at this site would produce an atypical, 65 kDa breakdown product, prior to the normal cleavage at the N-terminus of pro-HlyA.

As the origin of the 65 kDa product of the C-terminal truncations is uncertain, it will be referred to as 65* kDa. The ability of HlyA and its deleted derivatives to cleave to the 65* kDa product is summarised in Figure 4.7.

4.2.2.4 The end-point of haemolytic activity of C-terminal truncations of HlyA

The haemolytic activity of HlyA and the C-terminal truncations was assessed in both *E. coli* and *V. cholerae*. Plasmid DNA encoding the C-terminal deletions of HlyA was transformed into DH5 α or electroporated into V945 and the resulting colonies patched in triplicate on 5% blood agar and incubated overnight. Even though HlyA remains in the periplasm of *E. coli* and is not secreted, a small zone of haemolysis on blood agar is still visible due to the release of periplasmic contents upon cell death (Mercurio and Manning,

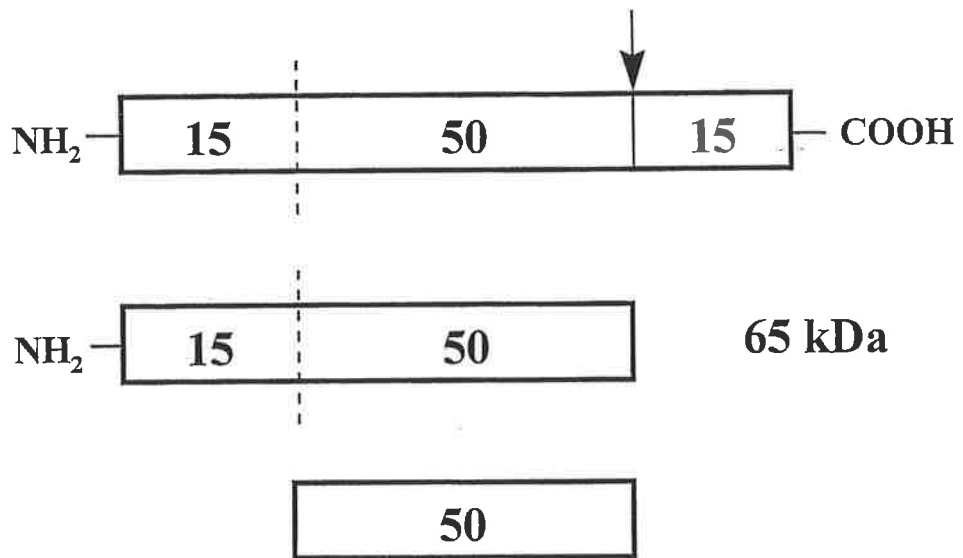
Figure 4.8 Two hypotheses generated from section 4.2.2

Up to 15kDa can be removed from the C-terminus of pro-HlyA before haemolytic activity, and the ability to produce a 65* kDa cleavage product, is abolished. Two hypotheses have been proposed and are outlined below. Pro-HlyA is illustrated at the top of the Figure, and the sizes of various domains are given in kDa. The specific cleavage site required for HlyA activation is indicated by a solid arrow and the proteolytically sensitive site is shown with a dotted line.

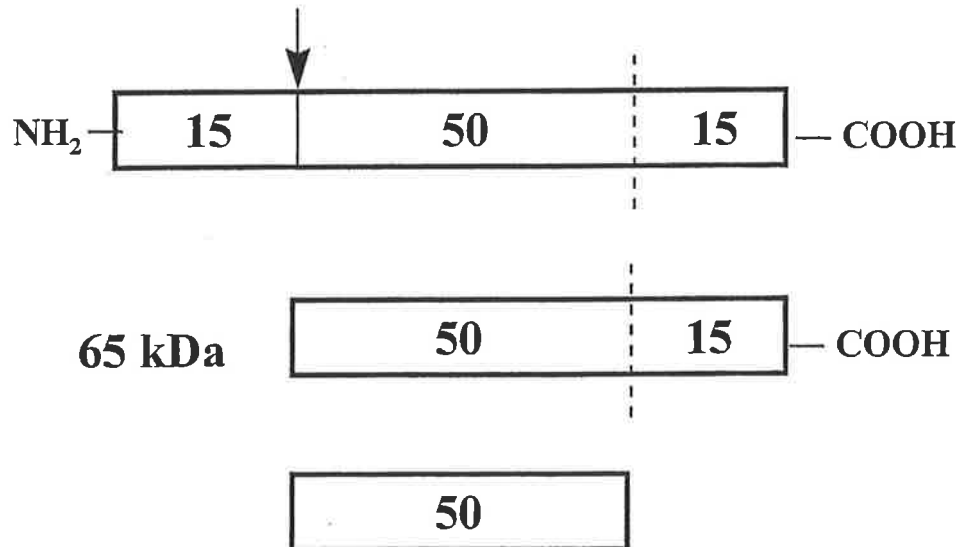
Hypothesis I: Cleavage occurs 15 kDa from the C-terminus of pro-HlyA to generate a N-terminally derived haemolytic 65 kDa product. This 65 kDa HlyA has a proteolytically sensitive site 15 kDa from the N-terminus, which cleaves during protein purification. Cleavage at this site would generate a 50 kDa product, and would give rise to the N-terminal sequence data obtained for purified 65 kDa (Hall and Drasar, 1990; Yamamoto *et al.*, 1990; Nagamune *et al.*, 1996).

Hypothesis II: Cleavage occurs 15 kDa from the N-terminus of pro-HlyA to generate a C-terminally derived haemolytic 65 kDa product, which has N-terminal amino acid sequence consistent with data reported by Hall and Drasar (1990), Yamamoto *et al.* (1990) and Nagamune *et al.* (1996). However, a proteolytically sensitive site 15 kDa from the C-terminus can be exposed as a result of deletions into the C-terminus. Cleavage at this site would generate an atypical 65* kDa product prior to cleavage at the specific N-terminal site. Cleavage of the 65* kDa at the specific N-terminal site would generate a 50 kDa product.

I



II



1985). The endpoint of haemolytic activity for the C-terminal truncations expressed in either *E. coli* or *V. cholerae* is the same and is summarised in Figure 4.7. Approximately 15 kDa can be removed from the C-terminus of pro-HlyA before haemolytic activity is lost.

15 kDa can be removed from the C-terminus before haemolytic activity and the ability to produce a 65* kDa product is lost. This supports the hypothesis (Figure 4.8, hypothesis I) that the active 65 kDa domain of HlyA may be derived from the N-terminus of pro-HlyA. This possibility is explored further in the following sections.

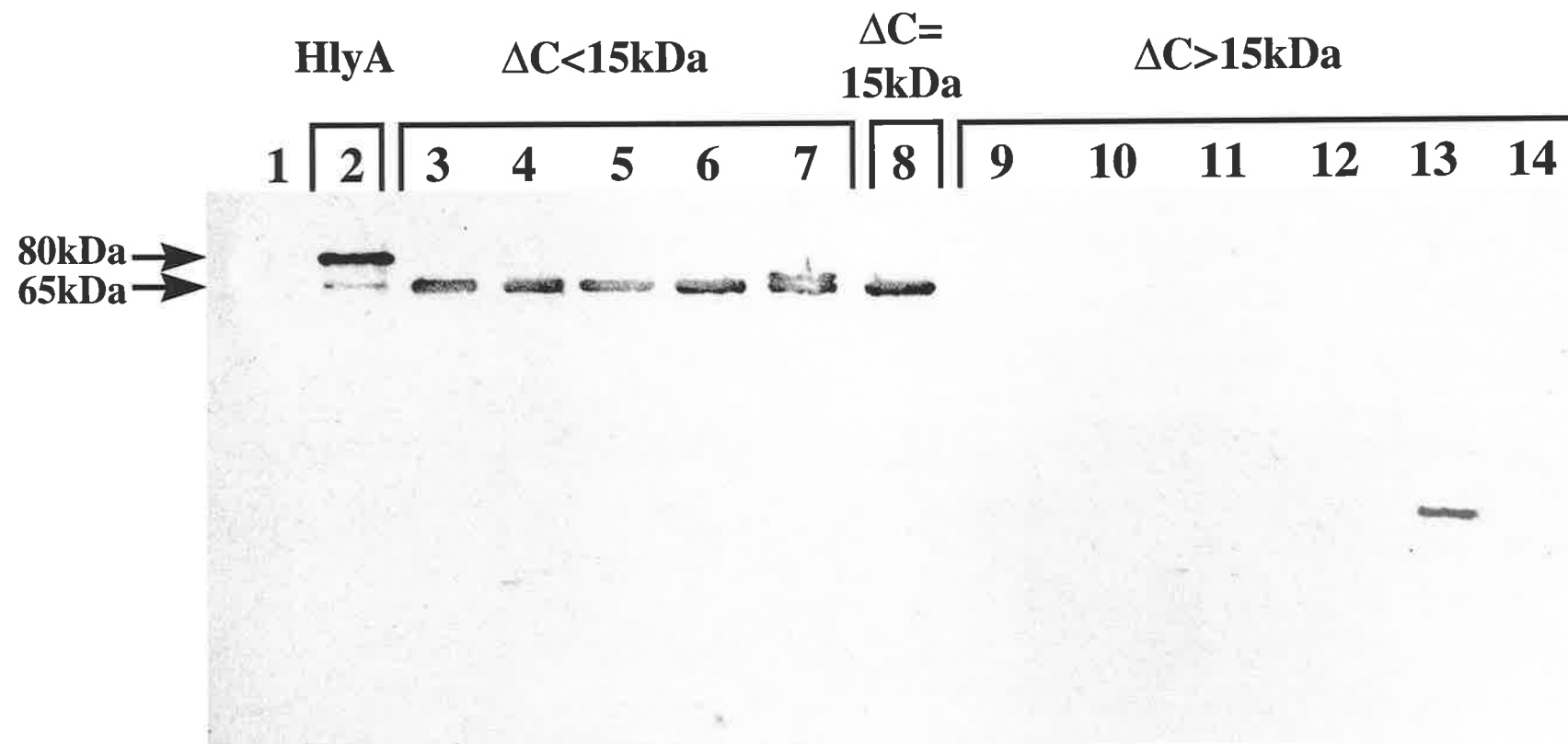
4.2.2.5 Analysis of native and C-terminally truncated HlyA from the *E. coli* periplasm.

It was known from previous work (Mercurio and Manning, 1985) that HlyA is periplasmically trapped, when expressed in *E. coli*. Therefore, to examine and compare both haemolytic activity and cleavage of HlyA and its truncated derivatives, periplasmic fractions were isolated and characterised.

Periplasmic fractions of the T7 induced cultures producing either HlyA or its C-terminal truncations were subjected to SDS-PAGE followed by western immunoblotting using α -dHlyA. Only native HlyA and derivatives with less than 15 kDa removed from the C-terminus were detected in the periplasmic fraction (Figure 4.9, lanes 2-8). HlyA in which greater than 15 kDa had been deleted were not detected, with the exception of the truncated protein produced by pPM2699 (lane 13). As with the whole cell fractions, native HlyA encoded by pPM2684 is present predominantly as the pro-80 kDa form with only some 65 kDa, in the periplasmic fractions. However, the C-terminal truncations are present only as the cleaved 65* kDa form. The pro-forms of these truncated HlyA were present in the whole cell fractions (Figure 4.6B and C, lanes 3-8), but were not detected in periplasmic fractions (Figure 4.9, lanes 3-8) (with the exception of pPM2689, lane 7). It appears that deletions of the C-terminus of up to 15 kDa destabilise the pro-form in the *E. coli* periplasm, and these pro-forms consequently cleave to the seemingly more stable 65* kDa product.

Figure 4.9 Western blot analysis of *E. coli* periplasmic fractions containing C-terminally truncated HlyA (Δ C).

Periplasmic fractions of T7 induced cultures were isolated, subjected to SDS-PAGE and western immunoblotting using α -dHlyA antiserum. Only C-terminal truncations (Δ C) of up to and including 15kDa are detected, the last to be detected encoded by pPM2691. The exception is pPM2699 which is also detected in the periplasm (lane 13). Native HlyA is present predominantly as the 80kDa form, while the C-terminal truncations have all cleaved to a 65* kDa form. Lanes 1-14 are pUC21, pPM2684, 2685, 2686, 2687, 2688, 2689, 2691, 2692, 2693, 2696, 2697, 2699 and pPM2700



4.2.2.6 Cellular location of C-terminally truncated HlyA in *E. coli*

Pro-haemolysin with greater than 15 kDa removed from the C-terminus was detected in whole cells, but not periplasmic fractions, of *E. coli*. To determine the cellular location(s) of truncated HlyA, cell fractionations of *E. coli* were performed. *E. coli* strains containing HlyA and a selection of truncated HlyA were induced using the T7 expression system described earlier. Various cellular fractions were isolated and electrophoresed on a 15% polyacrylamide gel, followed by western immunoblotting using α -dHlyA. The truncated HlyA proteins chosen contained the smallest deletion (pPM2685), the last deletion detected in the periplasm (pPM2691) and the first deletion not detected in the periplasm (pPM2692). Plasmids pPM2691 and pPM2692 also define the endpoints summarised in Figure 4.7.

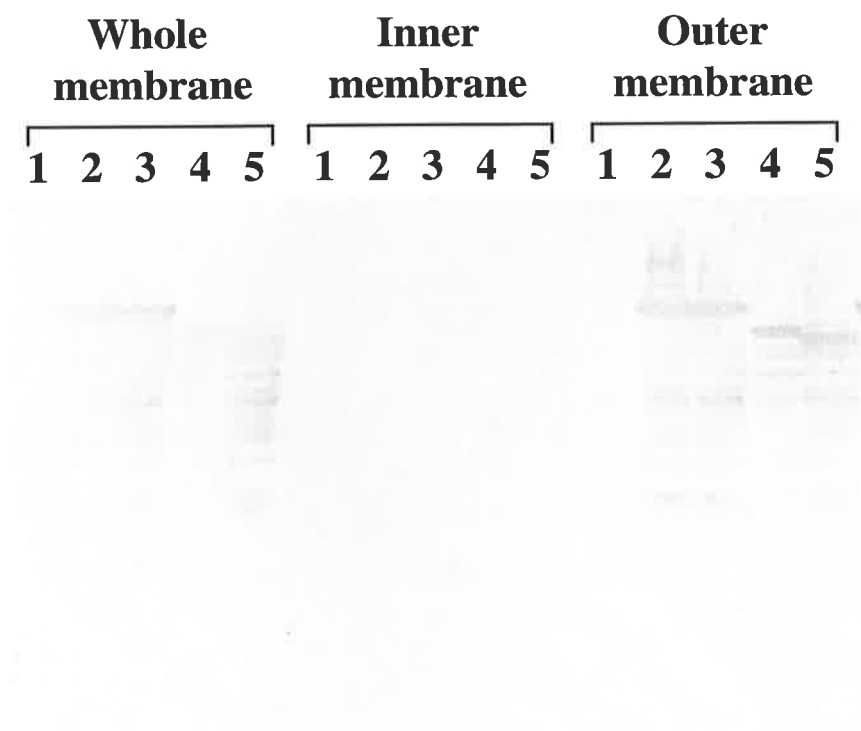
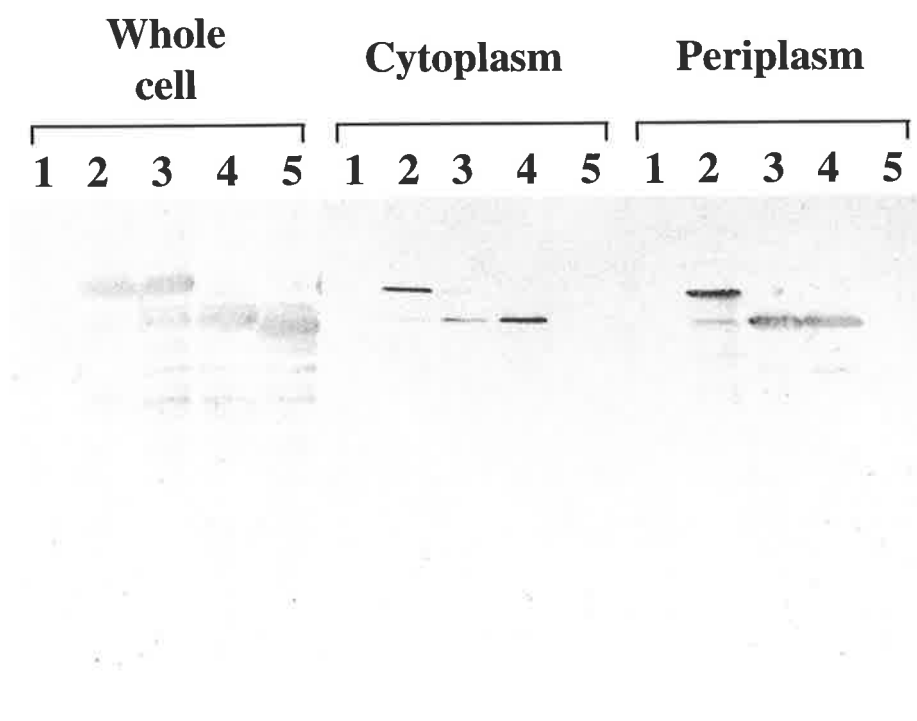
Native HlyA (pPM2684) and the truncated form with less than 15 kDa removed from the C-terminus (pPM2685 and pPM2691) were detected in all fractions, with the exception of the inner membrane (Figure 4.10). The majority of protein was localised to the outer membrane (Figure 4.10, lanes 2-4). As the haemolysin has a typical signal sequence (Alm *et al.*, 1988; Rader and Murphy, 1988) it is expected to reach the inner membrane, however, as *E. coli* lacks the transport machinery required for HlyA secretion, it is perhaps not surprising to find HlyA trapped in OM. Truncated HlyA with greater than 15 kDa removed from the C-terminus (pPM2692) was located only in the outer membrane (Figure 4.10, lane 5). This indicates that this truncation of HlyA is unstable in both the cytoplasm and periplasm of *E. coli*, (and is presumably degraded by proteases present in these compartments), but is stable in the more protective environment of the outer membrane. However, there is still considerable degradation of HlyA and all the truncated derivatives located in the outer membrane (Figure 4.10).

4.2.3 An assay for HlyA activation

While the haemolysin remains trapped in the outer membrane when expressed in *E. coli*, some protein is detected in the periplasm (4.2.2.6). Similarly, the 65* kDa product of the C-terminal truncations of HlyA are also present in periplasmic fractions of *E. coli*. Thus,

Figure 4.10: Cellular localization of HlyA and C-terminally truncated forms of HlyA in *E. coli*.

Expression was induced using the T7 promoter/RNA polymerase system (Tabor and Richardson, 1987). The cells were fractionated and analysed in SDS on a polyacrylamide gel followed by western immunoblotting using α -dHlyA antiserum. Lanes 1-5: pUC21, pPM2684, pPM2685, pPM2691, pPM2692.



it is possible to compare the relative haemolytic activities of native HlyA and the 65* kDa product of truncated HlyA, to help elucidate the origin of the 65* kDa protein. Additionally, as HlyA is present predominantly in the unprocessed 80 kDa form in the *E. coli* periplasm, it should be possible to determine whether factors produced from *V. cholerae* are involved with HlyA activation. This section describes the construction of an *E. coli* based haemolysis assay.

4.2.3.1 Activation of HlyA in the extracellular media

The haemolytic activity of *V. cholerae* O17 expressing HlyA increases with cell growth (Figure 4.11A). Presumably the increase in activity can be partially attributed to an increase of HlyA synthesis as the number of HlyA-producing cells increase and possibly increased expression in late exponential phase. However, it is also possible that activation of HlyA occurs and is rate limiting. Activation could occur either by autoproteolysis or by a factor encoded by *V. cholerae*. *V. cholerae* O17 expressing HlyA was grown to an A₆₀₀ of 0.53 at 37°C (highlighted Figure 4.11A) and the supernatant collected and filtered. The cell-free supernatants were then incubated at 37°C for a total of six hours, with aliquots taken at hourly intervals and the haemolytic activity determined by adding an equal volume of 5% sRBC followed by a further one hour incubation. Haemolytic activity was observed to increase for the first three hours, followed by a decline (Fig 4.11B). While this demonstrates that secretion and activation are separate events, it does not distinguish between activation due to autoproteolysis or activation by factors secreted by *V. cholerae*. The decline in activity is presumably due to post-activation degradation of HlyA.

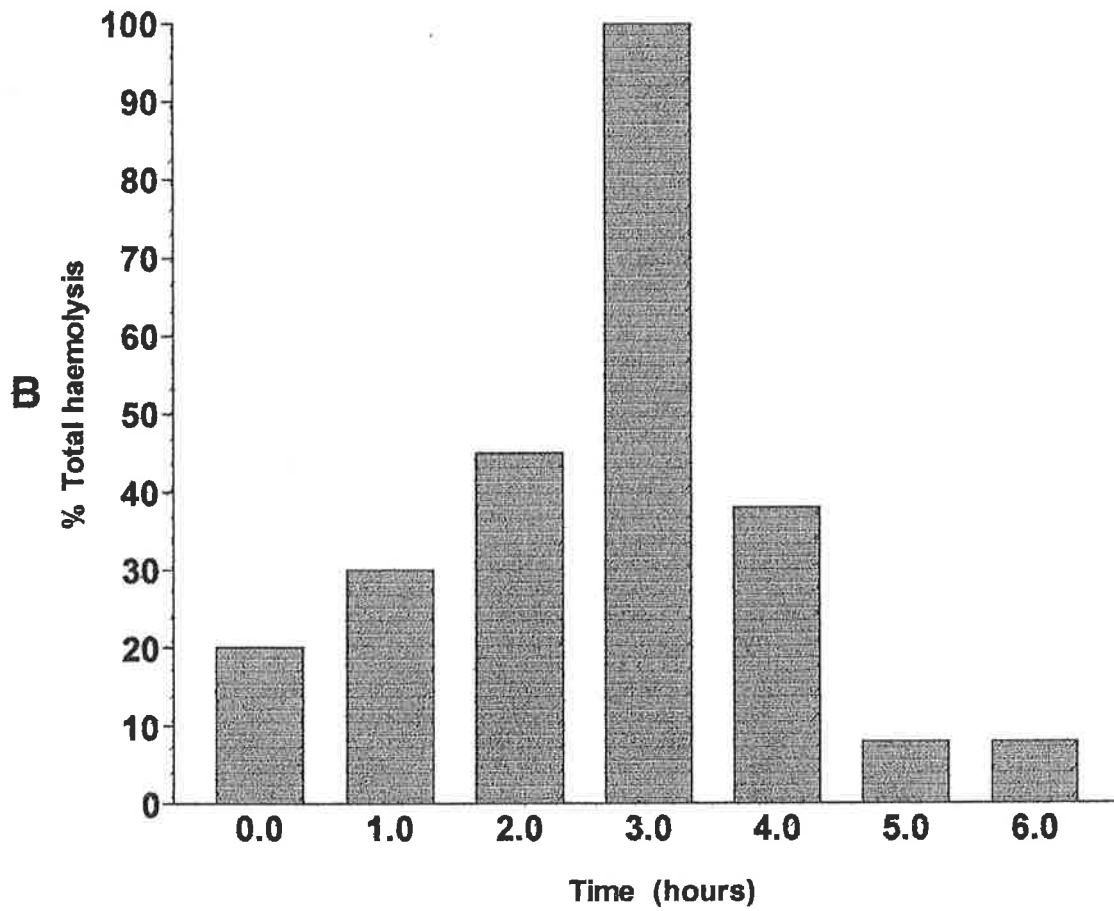
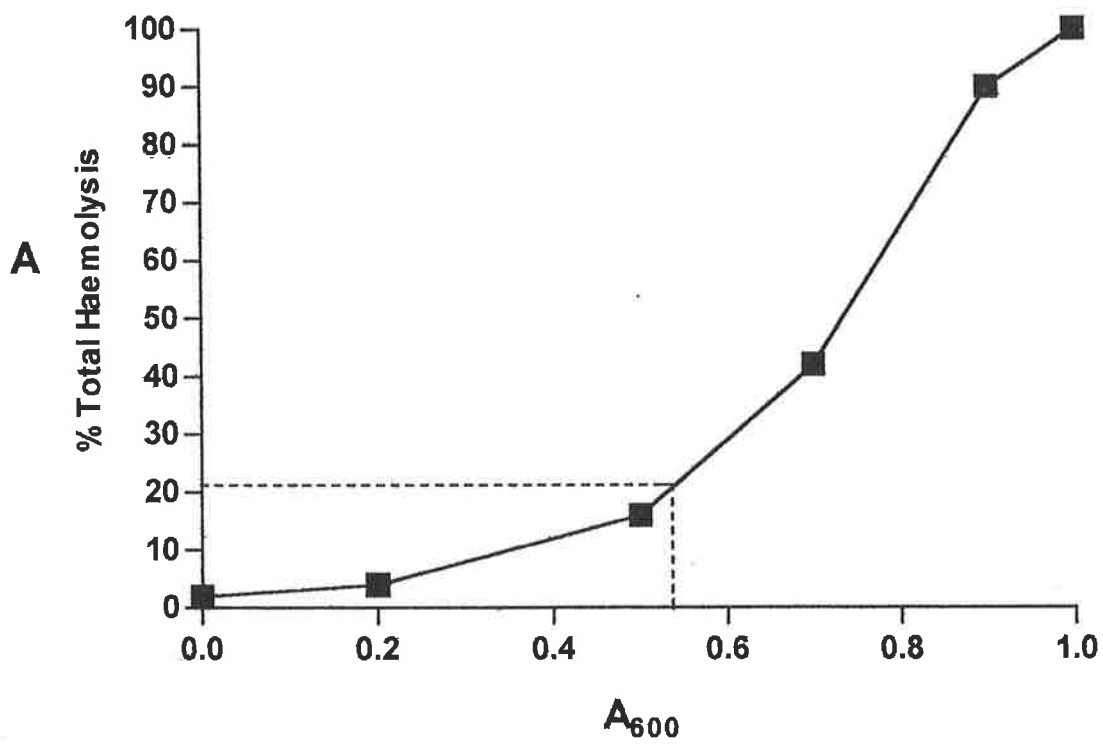
4.2.3.2 Activation of *E. coli* periplasmic HlyA by V945 supernatants

An assay using HlyA isolated from the *E. coli* periplasm was developed in order to determine whether factors encoded by *V. cholerae* are involved with the activation of HlyA. HlyA encoded by pPM2684 is present predominantly as 80 kDa when isolated from the *E. coli* periplasm (Figure 4.9). *E. coli* periplasmic fractions containing HlyA were pre-incubated with an equal volume of *V. cholerae* Δ hlyA (V945) supernatant for one hour at

Figure 4.11 Cell-free activation of HlyA in *V. cholerae* culture supernatants.

Panel A: *V. cholerae* O17 was subcultured at 37°C and supernatant fractions were collected at various A₆₀₀ and the haemolytic activity measured.

Panel B: The cell-free supernatant fraction collected at A₆₀₀ of 0.53 (indicated with a dotted line in panel A) was incubated for up to six hours at 37°C with aliquots removed at various times and mixed with a suspension of sRBC. Each mixture was incubated at 37°C for one hour, after which time the haemolytic activity was determined.



37°C, after which the haemolytic activity was determined and compared with and without pre-exposure to V945 supernatant (Figure 4.12A). The periplasmic fractions containing HlyA showed an increase in haemolytic activity after exposure. *E. coli* supernatants were unable to activate the periplasmic fractions (data not shown) suggesting the existence of other factor(s) produced by *V. cholerae* which are involved with the activation of the haemolysin.

4.2.3.3 Inhibition of the activation of HlyA by anti-SHA antiserum

HlyA isolated from the *E. coli* periplasm has been shown to be activated by a factor(s) encoded by *V. cholerae* V945 (Figure 4.12A). One possible candidate is the soluble haemagglutinin / protease (SHA) which has been shown to nick the A subunit of CT thereby activating it, as well as being able to cleave other proteins such as fibronectin, mucin and lactoferrin (Finkelstein *et al.*, 1983; Booth *et al.*, 1984). Antiserum directed to the SHA (R946) was used to determine if it could inhibit the activation of HlyA seen in Figure 4.12A. The assay of Figure 4.12A was modified slightly such that the *E. coli* periplasmic fractions containing HlyA were diluted to give virtually no haemolysis (0-20%) over two hours, but maximal activation after exposure to V945 supernatant. When V945 supernatants were pre-incubated with R946 the activation was completely abrogated (Fig 4.12B), suggesting that SHA is capable of activating HlyA. This experiment was also performed using non-specific antiserum, and this antiserum was unable to duplicate the effects of R946, thereby acting as a negative control.

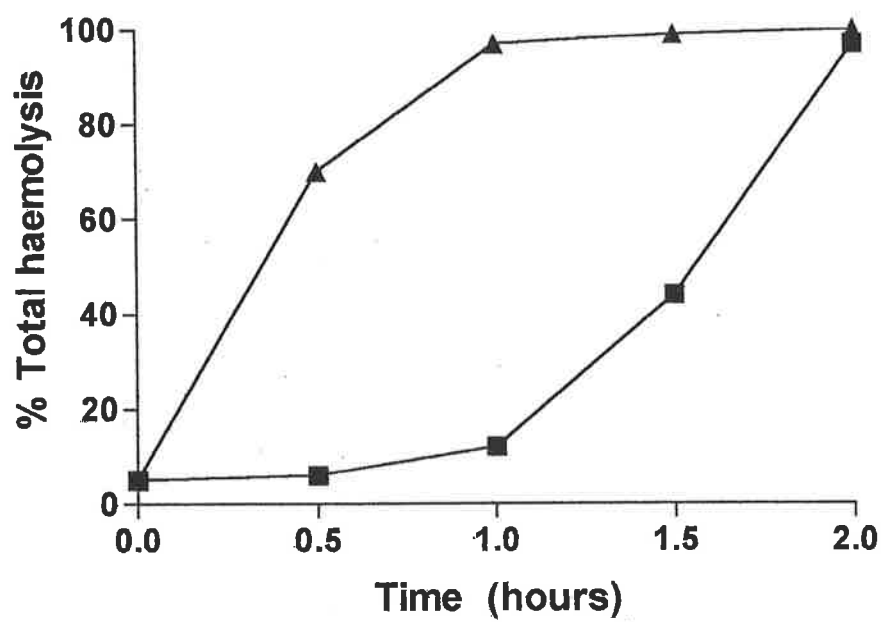
The SHA is a metallo-protease, and so presumably the activation of HlyA (Figure 4.12A) is a result of protein cleavage. However, western immunoblots of HlyA before and after exposure to V945 supernatants did not show a significant conversion of the 80 kDa to the 65 kDa (data not shown), suggesting that this system is not sensitive enough. Subsequently, however, Nagamune *et al.* (1996) have been able to demonstrate this conversion using pro-HlyA and SHA/protease purified by column chromatography.

Figure 4.12 Activation of HlyA by V945 supernatants and inhibition by α -SHA / protease (R946).

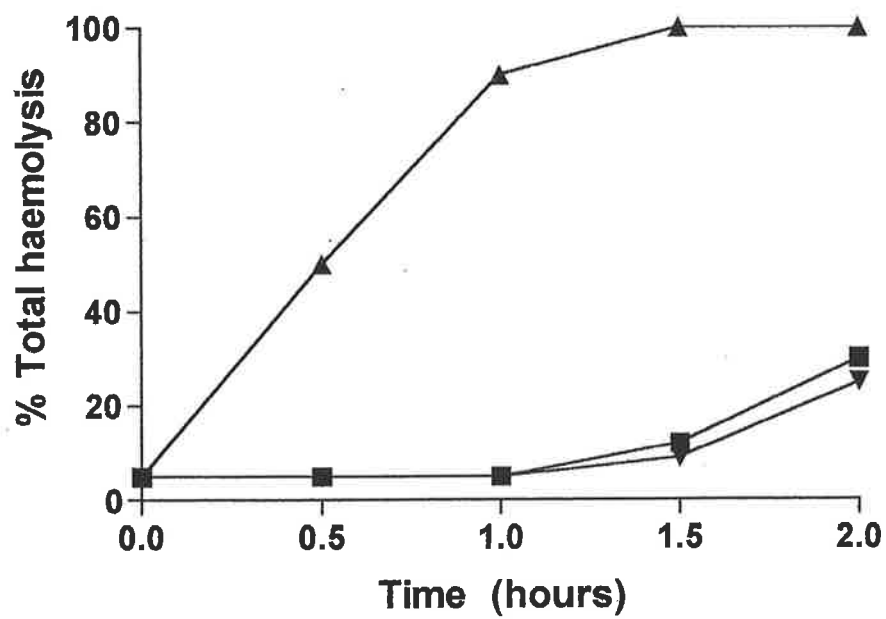
Panel A: Activation of HlyA extracted from *E. coli* periplasms, by V945 culture supernatant. Periplasmic fractions of *E. coli* expressing HlyA (pPM2684) were pre-incubated with either V945 culture supernatant or broth for one hour at 37°C. After the addition of an equal volume of 5% sRBCs, the haemolytic activity was measured over two hours. Activity was greatly increased when the periplasmic fractions were pre-incubated with V945 supernatant prior to the addition of sRBCs, with the exception of the two hour sample which was comparable to the control.

Panel B: Inhibition of HlyA activation by anti-soluble haemagglutinin-protease antiserum (R946). The periplasmic fractions containing HlyA (described in panel A) were diluted to give virtually no haemolysis, but maximal activation by V945 supernatant, after two hours. When V945 supernatants were pre-incubated for 1hr at 37°C with antiserum specific for the SHA (R946), the ability to increase the haemolytic activity of the periplasmic fractions was completely lost.

A



B



- ▲— pPM2684+S/N
- pPM2684+broth
- ▼— pPM2684+S/N+R946

4.2.4 Haemolytic activity of the 65* kDa product

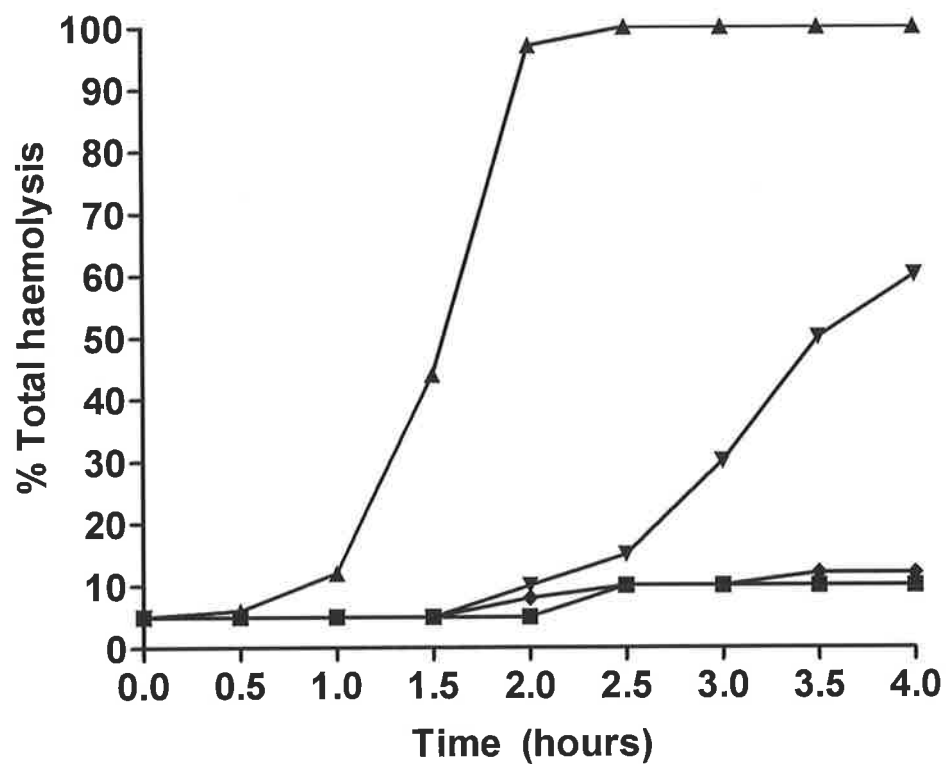
Further analysis of the 65* kDa product of C-terminally truncated pro-HlyA was required to determine whether it represents native 65 kDa HlyA, or an atypical breakdown product. The haemolytic activity of the *E. coli* periplasmic fractions containing HlyA encoded by pPM2684 (Figure 4.9, lane 2) and the 65* kDa derivative encoded by pPM2691 (Figure 4.9 lane 8) were measured in a liquid haemolysis assay. Native HlyA displayed maximum lytic activity after two hours, in contrast to the 65* kDa derivative which remained virtually non-haemolytic (Figure 4.13). These assays show clearly that the 65* kDa protein is not the normal active cleavage product of HlyA.

These data support the theory (Figure 4.8, hypothesis II), that the 65* kDa product is an atypical breakdown product of the C-terminal deletions of HlyA, which have not processed at the specific N-terminal cleavage site. It was therefore necessary to determine whether the 65* kDa could be activated by V945 supernatants (containing SHA) in the same way as native HlyA, using the system established in section 4.2.3.

The periplasmic fractions containing the 65* kDa product (encoded by pPM2691) were pre-incubated with either an equal volume of V945 supernatant or nutrient broth for one hour at 37°C, after which the haemolytic activity was determined. The fraction showed an increase in haemolytic activity after exposure to V945 supernatant and pre-incubation of the V945 supernatants with the anti-SHA antiserum R946, inhibited this activation (Figure 4.13). The activation of the 65* kDa protein by the SHA protease suggests that an additional cleavage was required for activation, presumably in the same manner that native 80 kDa HlyA was activated, which suggests that the 65* kDa requires N-terminal cleavage for activation (consistent with the second hypothesis presented in Figure 4.8). However, as with native HlyA (section 4.2.3.3), western immunoblotting of the 65* kDa product before and after exposure to V945 supernatants failed to show cleavage of the protein. Periplasmic fractions containing the C-terminal truncations of HlyA encoded by pPM2685 through to pPM2689 were also examined, and gave similar results to that obtained for the pPM2691-encoded protein.

Figure 4.13 Activation of the 65* kDa derivative of HlyA encoded by pPM2691 by SHA.

The haemolytic activity of periplasmic fractions of *E. coli* expressing HlyA (pPM2684) or the 65* kDa (pPM2691) derivative was measured over four hours. HlyA produced maximum lysis after two hours. While the 65* kDa derivative was not active after four hours, activity increased when the periplasmic fractions were pre-incubated with V945 supernatants, prior to the addition of sRBCs. This activation was inhibited when the V945 supernatants were pre-incubated for 1hr at 37°C with antiserum R946. R946 is anti-soluble haemagglutinin-protease antiserum.



- ▲— pPM2684 + broth
- ▼— pPM2691 + V945 S/N
- ◆— pPM2691 + broth
- pPM2691 + V945 S/N + R946

While the *E. coli* periplasmic fractions containing the 65* kDa derivative of HlyA were not haemolytic in this assay, a zone of lysis was produced when the colonies were patched onto blood agar (as discussed earlier in section 4.2.2.4 and summarised in Figure 4.7). This is probably a result of the differing lengths of incubation times (approximately 20 hrs for incubation on blood agar compared to 4 hrs for the liquid haemolysis assay), which would allow for N-terminal cleavage.

4.2.5 Analysis of C-terminally truncated HlyA in *V. cholerae*

Analysis of the C-terminal truncations of HlyA in *E. coli* is limited, as *E. coli* is unable to support the secretion of HlyA. Also, cleavage of HlyA may differ in *E. coli* from its usual host, *V. cholerae*. Therefore, a study in V945 ($\Delta hlyA$ O17 *V. cholerae*) was undertaken to examine secretion, cleavage and activation of native HlyA and its C-terminally truncated derivatives.

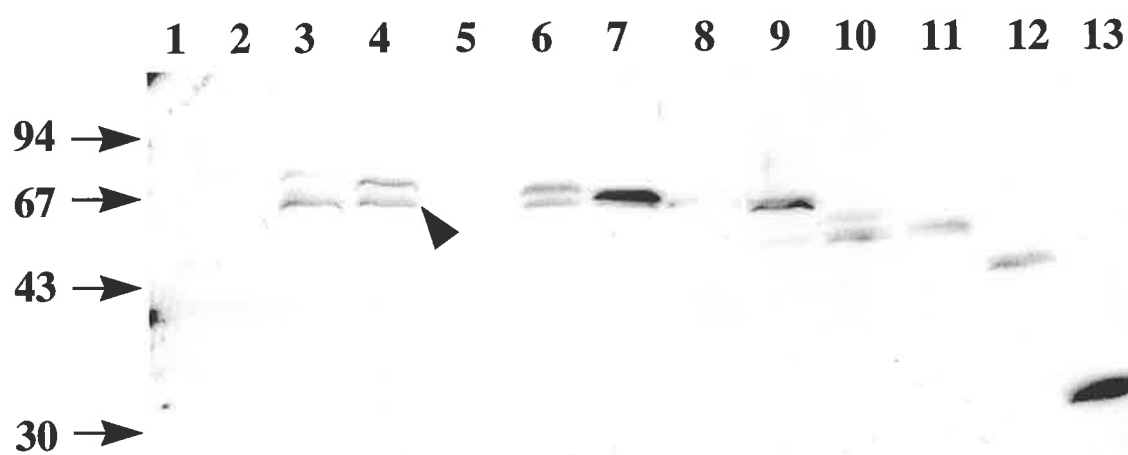
4.2.5.1 Detection of C-terminally truncated HlyA in *V. cholerae*

To determine whether pPM2684 and its derivatives are expressed in *V. cholerae*, whole cell fractions of V945 strains expressing HlyA and the C-terminal deletions were collected at $A_{600}=0.8$ and these fractions were subjected to SDS-PAGE and western immunoblotting. α -dHlyA was used for detection, as it recognises all of the C-terminally truncated HlyA derivatives (section 4.2.2.3). The western immunoblot of V945 whole cell fractions (Figure 4.14A) was similar to *E. coli* whole cell fractions expressing HlyA C-terminal truncations (Figure 4.6C), however less protein was detected from V945 presumably because protein expression was not induced. A decrease in the size of pro-HlyA was observed with the sequential C-terminal deletions, and deletions of up to 15 kDa produced a 65* kDa protein, indicated with a triangle in Figure 4.14A (lanes 3-8, however, no protein was detected in lane 5). It should be noted that a HlyA product of approximately 50 kDa was detected in lanes 9 and 10.

Figure 4.14 C-terminally truncated HlyA expressed in *V. cholerae* V945

Whole cell (panel A) and supernatant (panel B) fractions of V945 cultures expressing HlyA and its truncated derivatives were collected at A₆₀₀ of 0.8. Supernatant fractions were precipitated with TCA. The fractions were electrophoresed on a polyacrylamide gel followed by western immunoblot analysis, using α -dHlyA. The 65* kDa derivative of HlyA is indicated with an arrowhead). Molecular mass is shown and given in kDa. Lanes 1-13 are pUC21, pPM2684, 2685, 2686, 2687, 2688, 2689, 2691, 2692, 2693, 2696, 2697 and 2699.

A



B



4.2.5.2 Secretion of C-terminally truncated HlyA

To determine whether the C-terminal truncations of HlyA are secreted when expressed in V945, culture supernatants were collected at $A_{600}=0.9$ and precipitated with TCA. A western immunoblot using α -dHlyA of the supernatant fractions revealed that native HlyA expressed from pPM2684 was secreted, as were C-terminal truncations of up to 15 kDa (Figure 4.14B, lanes 2-8). C-terminal deletions of HlyA greater than 15 kDa were not detected in the supernatant (Figure 4.14B, lanes 9-12), with the exception of the protein encoded by pPM2699 (lane 13). This establishes that the C-terminal 15 kDa of pro-HlyA is not required for secretion.

The HlyA truncates precipitated with TCA have been processed to unusual sizes, of approximately 70 kDa, 75 kDa and 50 kDa (Figure 4.14B). While TCA precipitation was useful in establishing which of the HlyA truncations are secreted, it is clearly not suitable for analysing the normal processing of HlyA. In addition, the protein bands on the gel in Figure 4.14B are not quantitative, as the levels of protein have exceeded the linear range of the autoradiographic film.

Therefore, the examination of unprecipitated HlyA (pPM2684) and a truncated derivative (pPM2691) from V945 supernatants ($A_{600}=0.9$) was undertaken via western immunoblotting (Figure 4.15). α -65 antiserum was chosen as it is more sensitive than α -dHlyA and able to detect unprecipitated HlyA. Both unprocessed (80 kDa) and processed (65 kDa) forms of HlyA were detected, with greater amounts of the 65 kDa product (Figure 4.15). The 65* kDa product encoded by pPM2691 was also detected in the supernatant fractions, however, a 50 kDa cleavage product was also detected (Figure 4.15), which supports hypothesis II (Figure 4.8). Native HlyA was present in greater amounts than truncated HlyA encoded by pPM2691.

4.2.5.3 Cellular localisation of truncated HlyA in *V. cholerae*

C-terminal truncations greater than 15 kDa of HlyA are not detected in supernatant fractions when expressed in V945. These derivatives of HlyA are therefore either not

Figure 4.15 Cleavage products of HlyA (pPM2684) and the 65* kDa HlyA (pPM2691) in *V. cholerae* supernatants.

Western immunoblot analysis using α -65 antiserum, of V945 culture supernatants containing HlyA (pPM2684) and its truncated 65* kDa derivative (pPM2691), collected at A₆₀₀ of 0.8. Both the 80 kDa and 65 kDa forms are seen with native HlyA. A 50 kDa breakdown product of the 65* kDa derivative of HlyA is indicated.

pUC21

pPM2684

pPM2691



← **80 kDa**

← **65 kDa**

← **50 kDa**

secreted, or are secreted but rapidly degraded in the extracellular medium. To distinguish between the two possibilities, cellular fractions of V945 expressing truncated HlyA (not detected in the supernatant, pPM2692), were compared to that of native HlyA (pPM2684) in a western immunoblot. Whole cell fractions revealed more cell associated truncated HlyA (pPM2692) than native HlyA (Figure 4.16). Truncated HlyA was localised to the outer membrane fraction (Figure 4.15), while the majority of native HlyA was detected in the supernatant with some protein present in the outer membrane (Figure 4.16). It appears that the removal of more than 15kDa from the C-terminus of HlyA disrupts secretion, such that the protein remains cell-associated.

4.2.5.4 Haemolytic activity of C-terminally truncated HlyA in *V. cholerae*

V945 strains expressing native and truncated HlyA were subcultured and grown at 37°C and supernatants were collected at various stages of cell growth and the haemolytic activity measured in a liquid haemolysis assay. The growth curve was similar for all constructs examined (not shown). Although the haemolytic activity of the supernatants containing either intact HlyA or truncated HlyA increased with cell growth, the HlyA-truncations exhibited a significant "lag" in activity represented as a "shift" in the curve (Figure 4.17). As shown previously in Figure 4.15, V945 [pPM2684] supernatants have greater levels of HlyA than the C-terminal truncation (pPM2691), which would account for the lag in haemolytic activity. While all C-terminal truncations of less than 15 kDa (pPM2685-pPM2689, pPM2691) were assayed, only pPM2691 and pPM2686 are shown in figure 4.17, as the others lie within that range. As noted earlier, C-terminal truncations of pro-HlyA greater than 15 kDa are not active and are therefore excluded from Figure 4.17.

4.2.6 A minimum haemolytic domain of 50 kDa

Pro-haemolysin cleaves to a 65* kDa product when up to 15 kDa is removed from the C-terminus. The data presented thus far support the hypothesis (Figure 4.8, II) that these truncated forms of pro-HlyA cleave atypically at a proteolytically sensitive site 15kDa from the C-terminus, and this cleavage gives rise to the 65* kDa product. If this is the case,

Figure 4.16 Localisation of HlyA with greater than 15 kDa removed from the C-terminus (pPM2692) in *V. cholerae* V945.

V945 strains expressing native HlyA (pPM2684) and a C-terminally truncated derivative (pPM2692) were subcultured to an $A_{600}=0.8$. The cells were fractionated (as indicated) and analysed in SDS on a polyacrylamide gel followed by western immunoblotting using α -dHlyA antiserum. Supernatants were precipitated with TCA.

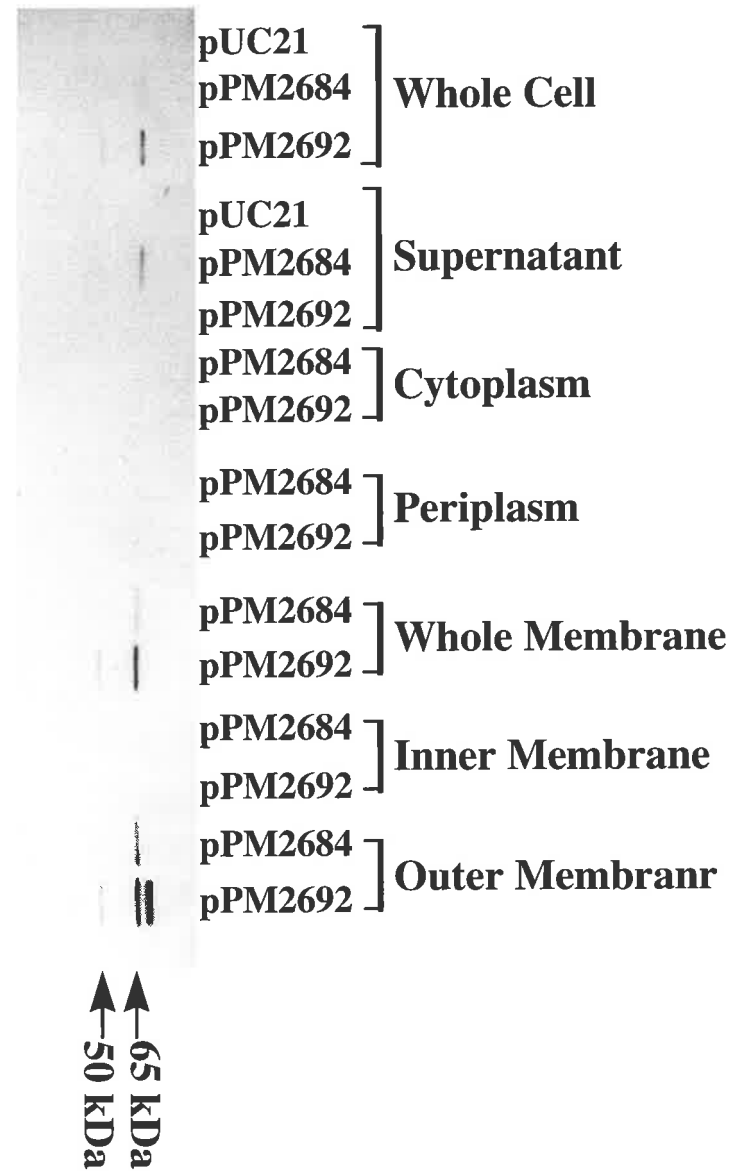
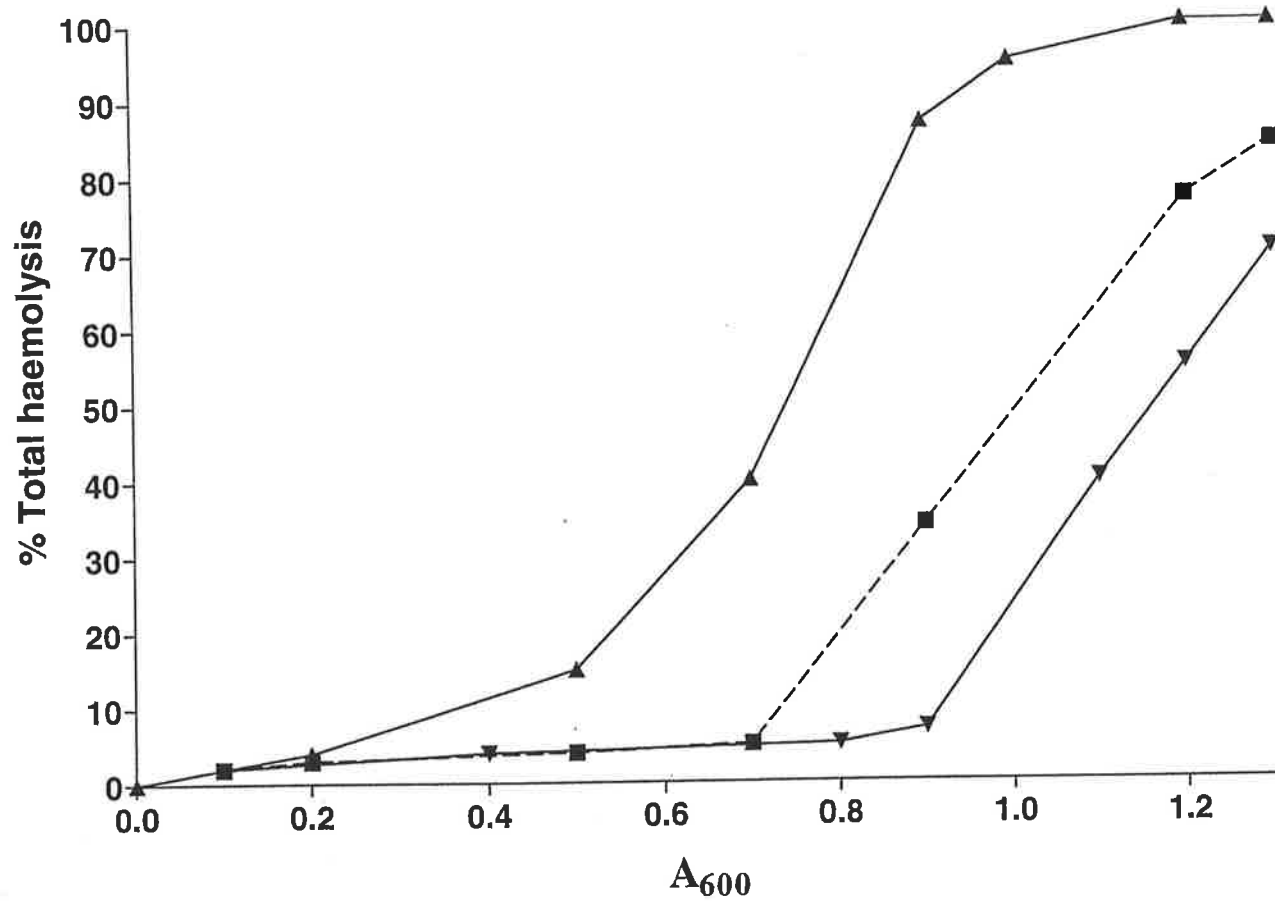


Figure 4.17 Haemolytic activity of native and C-terminally truncated HlyA isolated from *V. cholerae* supernatants.

Culture supernatants were collected at various stages of cell growth (A₆₀₀) of V945 expressing either HlyA (pPM2684) or the C-terminal truncations of pro-HlyA. The haemolytic activity of the supernatants were determined and expressed as % total lysis. C-terminal truncations of up to 15kDa (pPM2685 - pPM2689, and pPM2691) exhibit a lag in haemolytic activity when compared with native HlyA. Only the data for pPM2691 and pPM2686 is shown, others fall within that range. C-terminal truncations greater than 15kDa were non-haemolytic (<10%) and are therefore not shown on the graph.



—▲— pPM2684

-■- pPM2691

—▼— pPM2686

then the 65* kDa derivative of HlyA has not cleaved at the specific N-terminal site, and consequently the haemolytic domain of the 65* kDa would be expected to be 50 kDa. The 50 kDa would be derived from the central region of pro-HlyA, with approximately 15 kDa removed from both the N- and C- terminus.

4.2.6.1 Membrane associated form of 65* kDa HlyA is 50 kDa.

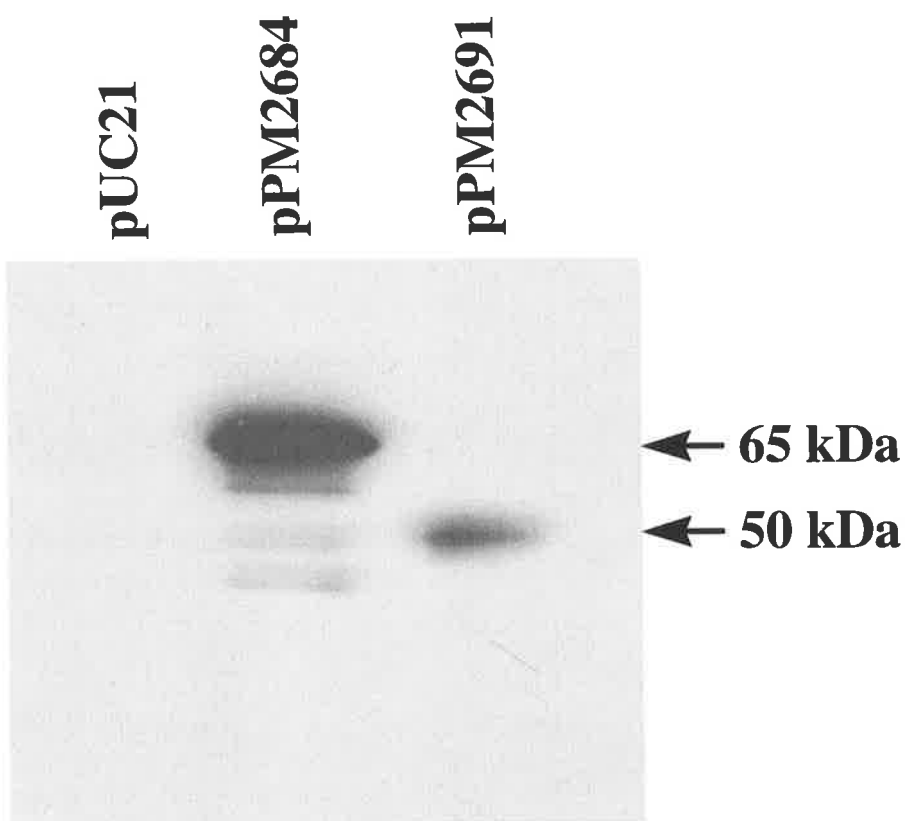
A 50 kDa breakdown product of 65* kDa HlyA was observed previously in V945 supernatants (Figure 4.15). In order to determine if the haemolytic domain of the 65* kDa is 50 kDa, supernatants of V945 expressing either native HlyA, or the 65* kDa derivative (encoded by pPM2684 and pPM2691, respectively) were collected at $A_{600}=0.9$ and filtered. The supernatants were incubated with a 5% suspension of sRBC for two hours at 37°C. sRBC membranes were then collected, electrophoresed on SDS-PAGE gels and subjected to western immunoblotting using α -65 for detection. As expected, the membrane-associated form of native 80 kDa HlyA was 65 kDa, but the membrane-associated form of the 65* kDa was 50 kDa (shown in Figure 4.18), which also confirms hypothesis II (Figure 4.8). There is considerably less membrane associated 50 kDa compared with native 65 kDa, which is to be expected as native HlyA is present in higher concentrations than the 65* kDa, in the supernatant fractions (Figure 4.15).

4.2.6.2 Osmotic protection of the 50 kDa HlyA

As mentioned in Chapter 3, lysis of erythrocytes exposed to pore-forming toxins can be prevented by the presence of carbohydrates, provided the diameter of the carbohydrate is larger than that of the pore. As discussed in section 3.2.1, both dextran 4 and dextrin 20 were able to prevent El Tor HlyA mediated lysis of sheep erythrocytes. This assay was adopted to establish that the 50 kDa derivative of HlyA also forms pores, of approximately the same diameter, in sRBC membranes. The C-terminally truncated HlyA encoded by pPM2691 is 65* kDa, which gives rise to the 50 kDa product that associates with erythrocyte membranes. V945 strains expressing native HlyA (pPM2684) and C-terminally truncated HlyA (pPM2691) were grown at 37°C to $A_{600}=0.9$, after which supernatant

Figure 4.18. A 50 kDa derivative of HlyA associates with sheep erythrocyte membranes.

V945 culture supernatants from cells expressing either the cloning vector (pUC21), HlyA (pPM2684) or the 65* kDa derivative (pPM2691) were exposed to an equal volume of 5% sRBCs for 2hrs at 37°C. sRBC membranes were isolated, subjected to SDS-PAGE followed by western immunoblotting using α -65. The active membrane-associated forms of native HlyA and the 65* kDa derivative are 65kDa and 50 kDa, respectively. The 50 kDa represents the minimum region of HlyA required for haemolytic activity.



fractions were collected. sRBC suspensions were exposed to an equal volume of these fractions, in the presence or absence of 30 mM raffinose, dextran 4 or dextrin 20. Haemolytic activity was determined at 0 and 120 minutes. Lysis induced by both HlyA and the 50 kDa fragment was prevented by the addition of either dextran 4 or dextrin 20, but not by raffinose (data not shown). This experiment demonstrates that the 50 kDa derivative of HlyA forms pores in the erythrocyte membranes, of similar size to native HlyA and in addition, demonstrates that the C-terminal 15 kDa of pro-HlyA is not essential for pore-formation.

The region encoding the C-terminal 15 kDa of pro-HlyA has been deleted in pPM2691, which encodes the 65* kDa. Since specific cleavage occurs 15 kDa from the N-terminus (Hall and Drasar, 1990; Yamamoto *et al.*, 1990a; Nagamune *et al.*, 1996), this 50 kDa haemolytic HlyA must be derived from the central region of pro-HlyA. This 50 kDa region represents the minimum domain required for haemolytic activity characterised thus far.

4.3 Summary and conclusions

A $\Delta hlyA$ *V. cholerae* O17 strain (V945) was constructed which enabled the analysis of various mutant HlyA derivatives without the possibility of recombination with wild type *hlyA*. In addition, *V. cholerae* culture supernatants could be isolated that are devoid of haemolysin, but obtained from cultures grown under identical conditions to those used to produce active HlyA.

The 80 kDa pro-haemolysin is inactive until cleaved to the 65 kDa form (Nagamune *et al.*, 1996) and correlating with this, it has been shown that activation occurs predominantly after secretion into the *V. cholerae* supernatants. This suggested that factor(s) secreted from *V. cholerae* may be involved with HlyA activation. Thus an *E. coli*-based assay was established which identified the SHA/protease as a factor involved with HlyA activation. The involvement of SHA/protease in HlyA activation is supported by Nagamune *et al.* (1996) who demonstrated that the SHA / protease is able to convert inactive 80kDa HlyA to the active 65kDa.

In order to define functional domains within the El Tor pro-haemolysin, a series of stable C-terminal truncations were constructed. An antiserum, designated α -dHlyA, that recognises all these truncations was generated using denatured 80 kDa HlyA as an immunogen. The specific cleavage required for HlyA activation occurs 15 kDa from the N-terminus, such that the mature 65 kDa HlyA is derived from the C-terminus. However, western immunoblotting using specific anti-HlyA antisera showed that the C-terminal truncations of HlyA, of up to 15 kDa, were able to be cleaved to a 65 kDa product. This 65 kDa product was designated 65* kDa. In addition, up to 15 kDa could be removed from the C-terminus of the pro-HlyA before haemolytic activity in both *E. coli* and *V. cholerae* was lost. There were two possible explanations for these findings.

Firstly, it was possible that the specific cleavage of pro-HlyA required for activation, may actually occur 15 kDa from the C- and not the N-terminus. An alternative explanation was that a second proteolytically sensitive site exists approximately 15 kDa from the C-terminus of pro-HlyA. Further investigations revealed the latter hypothesis to be the case, and the following model is proposed: Deletions at the C-terminus of pro-HlyA expose a proteolytically sensitive region, 15 kDa from the C-terminus (Figure 4.19B and 4.19C). Deletions of as little as 12 amino acids are able to expose this site. Cleavage then occurs efficiently at this region in both *E. coli* and *V. cholerae* to produce an atypical 65* kDa breakdown product. This 65* kDa product still contains the N-terminal 15 kDa pro-region and is not active (Figure 4.19D). Exposure of the 65* kDa to proteases such as SHA cleaves the protein to an active 50 kDa form (Figure 4.19E). This cleavage and activation presumably occurs by the specific N-terminal cleavage normally required for HlyA activation (Nagamune *et al.*, 1996). This 50 kDa derivative of HlyA is haemolytic and forms pores of approximately equal diameter to those formed by native HlyA (Chapter 3). The 50 kDa protein, derived from the central region of pro-HlyA, therefore represents a minimum haemolytic domain of the El Tor haemolysin characterised thus far.

The experiments described here show that the C-terminal 15 kDa region of pro-HlyA is not essential for secretion, pore-formation (haemolytic activity) or cleavage and its role

Figure 4.19 Schematic representation of the proteolytic processing of HlyA.

(A) Pro-HlyA, with the 15 kDa and 65 kDa domains indicated. The specific cleavage site of HlyA is indicated by a vertical arrow. (B) Pro-HlyA can also be divided into the central 50 kDa region and the N- and C-terminal 15kDa regions. (C) C-terminal deletions (ΔC) expose a proteolytically sensitive site 15kDa from the C-terminus (indicated by a black triangle). (D) Pro-HlyA then cleaves atypically at this site to produce a 65* kDa derivative, which has not cleaved at the specific N-terminal site. This 65* kDa is non-haemolytic and is not the normal active 65kDa HlyA. (E) Cleavage at the specific N-terminal site by SHA (and other proteases?) releases a haemolytic 50 kDa product. This 50 kDa is a derivative of the normal 65kDa HlyA, with 15kDa removed from the C-terminus.

therefore, remains unknown. It is possible that the C-terminal 15 kDa enhances protein stability, as less protein is detected in *V. cholerae* supernatants when this region is removed.

Chapter 5

Analysis of the 50 kDa derivative of the *V. cholerae* O1 El Tor haemolysin (HlyA)

5.1 Introduction

The functional domains of various cytolysins have been well characterised. This has enabled a more complete understanding of how these toxins are produced, and how they exert their toxic effects. While it is known that the haemolysin (HlyA) of *Vibrio cholerae* contributes to virulence in El Tor and non-O1 strains (Ichinose *et al.*, 1987; Alm *et al.*, 1991; Williams *et al.*, 1993), the mechanism by which it does so is poorly understood. Very little is known about the functional domains within the HlyA protein. Functional information of a toxin can be obtained by analysis of mutant proteins, firstly by the construction of general mutations and is usually followed by more specific mutagenesis.

Primary structure analysis may reveal features that relate to function which allow the construction of a basic model. For example, the structural features of the pore formed by the *S. aureus* α -toxin were predicted by analysis of its primary sequence (Menestrina *et al.*, 1992). A model for the pore-forming structure of the *E. coli* α -haemolysin was proposed using a combination of structural predictions and site-specific deletions (Ludwig *et al.*, 1991). This later identified two regions of only 8 and 10 amino acids which are independently capable of mediating cell lysis and are able to form pores in an artificial lipid bilayer (Oropeza-Wekerle *et al.*, 1992). Sequence comparison of proteins can identify homologous regions which may represent functional domains. Point mutations initially identified two asparagine residues involved in the activation and secretion of the *Serratia* haemolysin (ShlA) and sequence comparison revealed that these residues are part of a short sequence motif (ANPN), present in other proteins that are secreted in a similar manner to ShlA (Barenkamp and Leininger, 1992; Schönher *et al.*, 1993; Palmer and Munson, 1995).

Protein fragments derived from deletions or chemical cleavage, and complementation of these fragments often provide vital information about functional domains. C-terminal

truncations of ShlA produced by *Serratia marcescens* showed the N-terminus was required for secretion (Ondraczek *et al.*, 1992) and functional complementation using a 15 kDa N-terminal tryptic fragment of ShlA showed the activation site of the haemolysin was located in that N-terminal fragment (Schönherr *et al.*, 1993).

Chemical modification or site-directed mutagenesis can define specific residues crucial for certain functions. Point mutations within ShlA identified two amino acids (Asn-69 and Asn-109) which are essential for the active protein (Schönherr *et al.*, 1993), while other single amino acid replacements showed increased activity when compared to wild-type ShlA, presumably by increasing the stability of the mutant ShlA (Hilger and Braun, 1995). Transfer of the *Aeromonas aerolysin* across the outer membrane was inhibited by changing a single tryptophane near the middle of the protein (Wong and Buckley, 1991). Chemical modification of four histidine residues of the *Staphylococcus aureus* α -toxin identified residues required for specific receptor binding and oligomerisation (Menestrina *et al.*, 1992).

Mapping antigenic regions of haemolysins recognised by antibodies which inhibit activity is also a useful technique in identifying functional domains (and may provide suitable non-functional peptides or toxoids for protective immunisation). Protective epitopes of the *E. coli* α -haemolysin were identified by determining the antibody binding to cyanogen bromide fragments (Ji and O'Hanley, 1990).

C-terminal deletions show that the C-terminal 15 kDa of pro-HlyA is neither essential for secretion nor pore-formation (Chapter 4). In addition, these deletions have defined a 50 kDa region of HlyA that is haemolytically active. This 50 kDa protein is derived from the central region of pro-HlyA, after the removal of approximately 15 kDa from both the N- and C-termini. Deletions at the C-terminus possibly expose a proteolytically sensitive site which results in the removal of 15 kDa from the C-terminus of pro-HlyA. This C-terminal cleavage, in conjunction with the specific cleavage that occurs at the N-terminus (Nagamune *et al.*, 1996), produces the 50 kDa HlyA (Figure 4.19). The C-terminal end of the 50 kDa derivative of pro-HlyA appears to be critical for activity and secretion (Chapter 4).

This chapter localises regions essential for activity and secretion within the 65 kDa HlyA and examines the cleavage that leads to the production of the 50 kDa derivative of

HlyA. The C-terminal 15 kDa of HlyA is expressed independently of the rest of the protein and used for epitope mapping. Anti-HlyA antibodies that inhibit haemolysis are identified and the epitopes which they recognise are localised.

5.2 Results

5.2.1 Localising the C-terminal end of 50 kDa HlyA

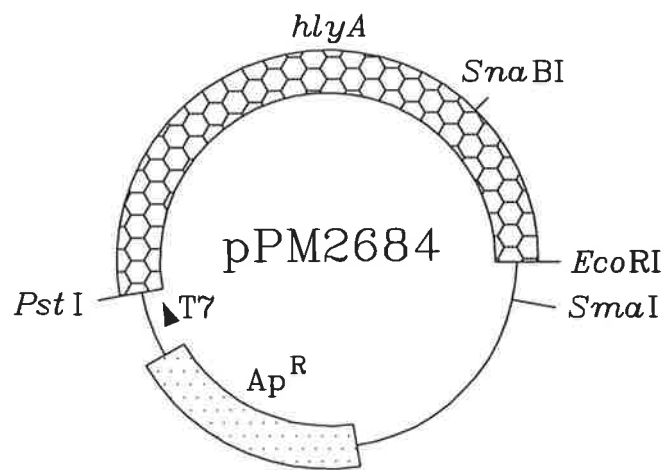
Deletions of the C-terminus of pro-HlyA ranging from only 12 amino acids to 15 kDa are still haemolytic, while deletions greater than 15 kDa abolish activity. pPM2691 encodes the last C-terminally truncated HlyA that still retains haemolytic activity. A further deletion of 27 amino acids (encoded by pPM2692) results in the loss of haemolytic activity and secretion from *V. cholerae*. The haemolytic endpoint defines the C-terminal end of the 50 kDa derivative of HlyA and the proposed proteolytically sensitive site of pro-HlyA was also localised to this C-terminal region (Figure 4.7). Clearly, this is an important region of HlyA.

To further localise the minimum C-terminal region required for haemolytic activity, a *Sna*BI restriction site which lies between the two end points of the deletions in pPM2691 and pPM2692 was utilised to construct a truncation at this site (Figure 5.1). Plasmid pPM2684 (encoding *hlyA*) was digested with *Sna*BI (site at nt 2932 in the 3.5 kb *Pst*I / *Eco*RI fragment) and *Sma*I (lies downstream in the polylinker) and the intervening 0.6 kb fragment deleted. The resulting construct was designated pPM4342, and the end-point of the deletion is shown in Figure 5.2. The truncated HlyA protein produced by pPM4342 (not shown) was non-haemolytic, suggesting that this deletion removed a vital part of the 50 kDa encoding region. This localised the C-terminal end of the 50 kDa HlyA derivative to the 12 amino acids Thr-598 to Gln-609 (Figure 5.2).

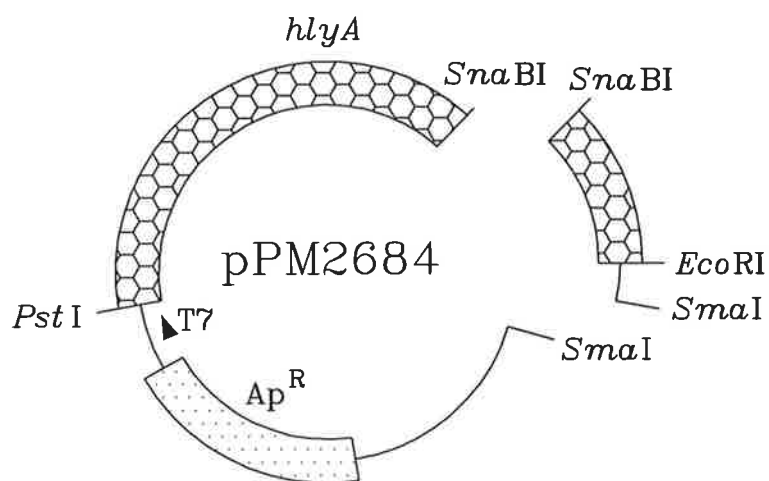
The haemolytic endpoint was more precisely located by site-directed mutagenesis of this region. A *Sma*I restriction enzyme site (*Sma*I*) was introduced between the endpoints of pPM4342 and pPM2691 to generate pPM4352 (Figures 5.2 and 5.3). This initially required two separate PCR reactions, both utilising pPM4343 as the DNA template. The first PCR reaction using oligonucleotide #2114 and M13 reverse primer amplified a 2.9 kb

Figure 5.1 Construction of pPM4342.

Plasmid pPM2684 harbouring *hlyA* was digested with *Sna*BI and *Sma*I restriction enzymes, diluted and re-ligated to remove the intervening DNA. The resultant construct, pPM4342 encodes C-terminally truncated HlyA.



Digest with
*Sna*BI/*Sma*I



Dilute and religate

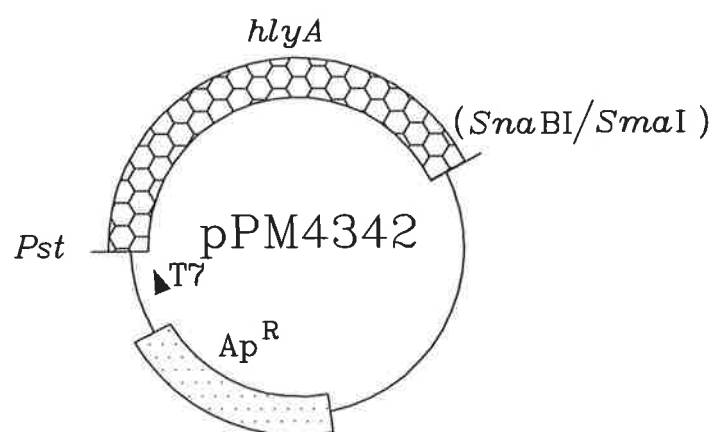
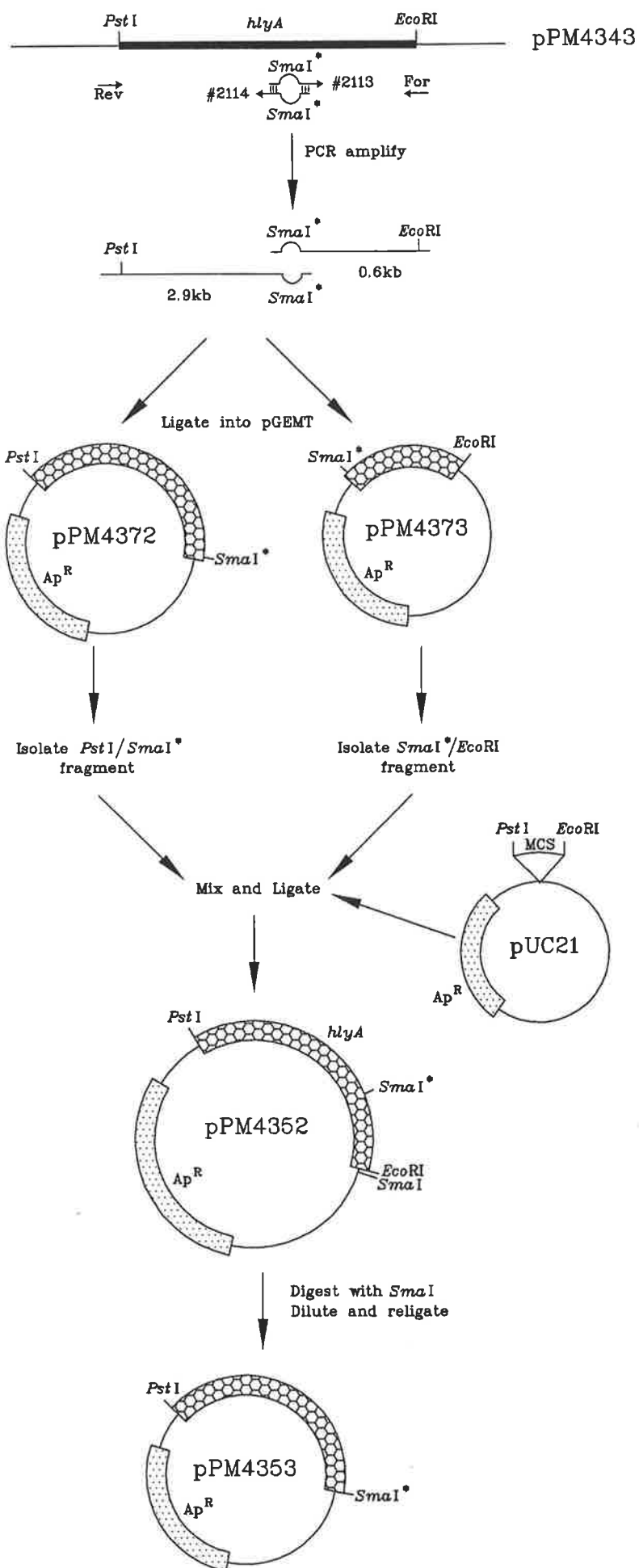


Figure 5.2 Localisation of the minimal C-terminal region of HlyA required for haemolytic activity and secretion.

Approximately 15 kDa can be removed from the C-terminus of pro-Hly before haemolytic activity is lost. The region where activity is lost corresponds to the proposed proteolytically sensitive site of HlyA (Chapter 4) and is indicated by an arrow. The amino acid sequence of this region is compared to the amino acid sequence and associated phenotypes of various mutations within this region. Plasmid pPM2684 encodes native HlyA. pPM2691, pPM4353, pPM4342 and pPM2692 are C-terminal truncations of HlyA. pPM4351 encodes HlyA with an in-frame deletion of 15 amino acids. Altered amino acids are shown in bold. The amino acids underlined are required for protein stability and secretion and the boxed amino acids are essential for haemolytic activity.

Figure 5.3 Introduction of a *Sma*I site in *hlyA* (pPM4352) and a *Sma*I cut-down (pPM4353).

Two PCR products were generated in two separate reactions, using pPM4343 as the DNA template. The first product generated by PCR amplification of a 2.9 kb region using oligonucleotides M13 Reverse (Rev) and #2114 was ligated into pGEM-T to generate pPM4372. The second product generated by PCR amplification of a 0.6 kb region using oligonucleotides M13 forward (-21) (For) and # 2113 was also ligated into pGEM-T to generate pPM4373. Oligonucleotides # 2113 and # 2114 are partially complementary and both deviate from the normal sequence as a *Sma*I restriction site (*Sma*I*) has been incorporated into them. The 2.9 kb *Pst*I / *Sma*I* fragment of pPM4372 and the 0.6 kb fragment of pPM4373, were isolated and ligated into pUC21 restricted with *Pst*I / *Eco*RI. The resulting construct pPM4352, carries *hlyA* with the newly incorporated *Sma*I* restriction site. pPM4352 was then digested with *Sma*I and re-ligated to remove the intervening DNA and generate pPM4353.



region and the fragment generated was ligated into pGEM-T and designated pPM4372. The second PCR reaction amplified a 0.6 kb region using oligonucleotide #2113 and M13(-21) and this fragment was also ligated into pGEM-T and designated pPM4373. Oligonucleotides #2113 and #2114 are partially complementary and contain several mismatches which incorporate a *SmaI* restriction site (*SmaI**). The 2.9 kb product of pPM4372 is flanked by *PstI* and *SmaI** restriction sites, and the 0.6 kb fragment of pPM4373 is flanked by *SmaI** and *EcoRI* (Figure 5.3). These restriction sites were used to isolate the fragments which were then ligated together using the common *SmaI** site introduced by PCR, into the *PstI* / *EcoRI* sites of pUC21. This regenerated the 3.5 kb fragment encoding *hlyA* present in pPM2684, but the fragment now incorporates a *SmaI** site (Figures 5.2 and 5.3). This construct was designated pPM4352, and although HlyA encoded on this plasmid has two amino acid changes, Thr to Pro (603) and Asp to Gly (604) in the region of interest (Figure 5.3), they did not affect haemolytic activity (not shown). This alteration was confirmed by sequencing across the region using oligonucleotide #1007.

The *SmaI** site introduced in pPM4352 and the *SmaI* site downstream in the vector polylinker were utilised to create a deletion in HlyA, from amino acid 604 onwards, and the resulting construct was designated pPM4353 (Figures 5.2 and 5.3). The C-terminally truncated HlyA encoded by pPM4353 was weakly haemolytic on blood agar when expressed in *V. cholerae* V945. Culture supernatants of this strain were not haemolytic in liquid haemolysis assays (not shown), so it is possible that truncated HlyA encoded by pPM4353 is not secreted. It should be noted that V945 expressing pPM4353 exhibited poor growth on solid media, which suggests that the product of this construct may be detrimental to the cell, or the construct itself may be unstable.

To determine if C-terminally truncated HlyA encoded on pPM4353 is secreted, supernatant and other cellular fractions were isolated from V945 expressing this protein. These fractions, as well as those isolated from V945 expressing native HlyA from pPM2684, were electrophoresed on a 15% polyacrylamide gel, followed by western immunoblotting using α -dHly antiserum for detection (Figure 5.4). Truncated HlyA (pPM4353) was not detected in the culture supernatant, but was present in the outer

Figure 5.4 Location of C-terminally truncated HlyA encoded by pPM4353 in V945.

V945 derivatives expressing native HlyA (pPM2684) and a C-terminally truncated form (pPM4353) were subcultured to an $A_{600}=0.8$. The cells were fractionated (as indicated) and analysed in SDS on a 15% polyacrylamide gel followed by western immunoblotting using α -dHlyA antiserum. Supernatants were precipitated with TCA.



pUC21	]	whole cell
pPM2684		
pPM4353		
pUC21	]	supernatant
pPM2684		
pPM4353		
pPM2684	]	cytoplasm
pPM4353		
pPM2684	]	periplasm
pPM4353		
pPM2684	]	whole membrane
pPM4353		
pPM2684	]	inner membrane
pPM4353		
pPM2684	]	outer membrane
pPM4353		

membrane (and whole membrane) fraction. This suggests that the protein is not secreted and remains cell-associated. However, we cannot exclude the possibility that some protein is exported but rapidly degraded. It should be noted that the protein encoded by pPM4353 is running higher than 65 kDa, as the C-terminus is fused to several vector-encoded amino acids. Native HlyA was present in both the supernatant and outer membrane of V945.

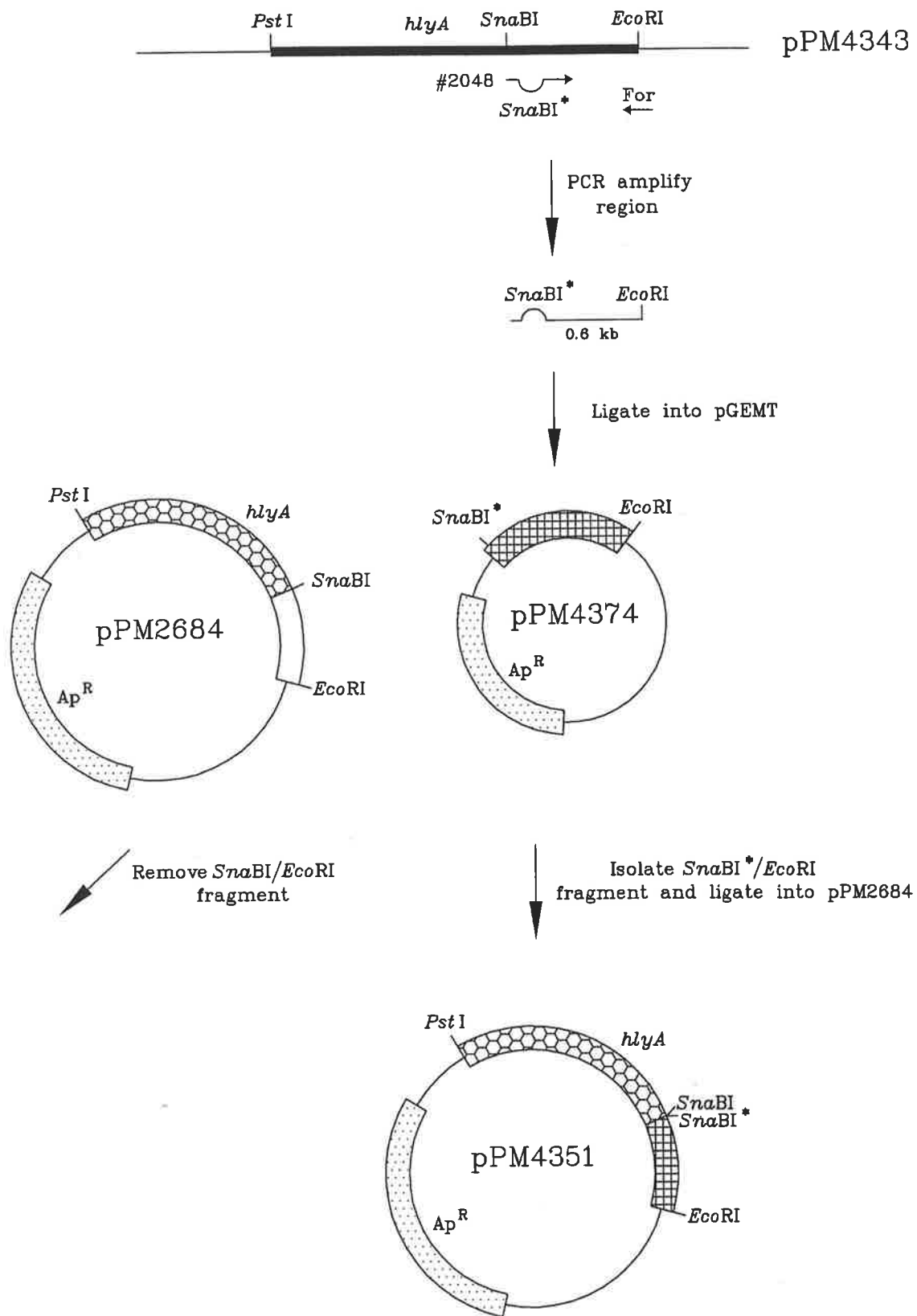
When approximately 15 kDa are removed from the C-terminus of pro-HlyA, the truncated protein (pPM2691), is still haemolytic and secreted. The C-terminal amino acid of this protein is Gln-609 (Figure 5.2). In plasmid pPM4353, a further seven amino acids Thr-603 to Gln-609 are removed from the C-terminus and the protein is weakly haemolytic, but is not secreted. This suggests that amino acids Thr-603 to Gln-609 (underlined in Figure 5.2) are essential for secretion. When an additional five amino acids, Thr-598 to Tyr-602, are removed from the C-terminus (pPM4342), haemolytic activity is completely abolished. These five amino acids (highlighted in Figure 5.2) are therefore critical for haemolytic activity. This has localised the minimum C-terminal region of HlyA required for haemolytic activity to within five amino acids.

5.2.2 Removing critical amino acids of HlyA

The region Thr-598 to Gln-609 lies approximately 15 kDa from the C-terminus of pro-HlyA and is proposed to be critical for the production of active, secreted haemolysin (section 5.2.1). In order to confirm this, an in-frame deletion of amino acids Thr-598 to Ser-612 was constructed and designated pPM4351 (Figure 5.5). The region of the protein removed from HlyA is shown in Figure 5.2. The deletion was achieved utilising the *Sna*BI site in pPM2684 (marking the endpoint of pPM4342) and incorporating another *Sna*BI downstream by PCR mutagenesis (at the endpoint of pPM2691) (Figure 5.2). Mutagenic oligonucleotide #2048 contains several mis-matches which incorporate a *Sna*BI site (*Sna*BI*), and was used together with M13(-21) primer to PCR amplify a 0.6 kb region of *hlyA*, using pPM4343 as the template. This fragment was ligated into pGEM-T to generate pPM4374. The 0.6 kb *Sna*BI / *Eco*RI fragment of pPM2684 (encoding native *hlyA*) was

Figure 5.5 Construction of an in-frame deletion of essential amino acids in pro-HlyA (pPM4351).

A 0.6 kb region of *hlyA* was PCR amplified with oligonucleotides M13 forward (-21) (For) and #2048 using pPM4343 as the template. Oligonucleotide #2048 contains several mismatches which incorporate a *Sna*BI site (*Sna*BI*) site. This new *Sna*BI* site is situated 15 amino acids downstream from a *Sna*BI site present in *hlyA*. The amplified product was ligated into pGEM-T to generate pPM4374. A 0.6 kb *Sna*BI / *Eco*RI fragment was removed from pPM2684 (which encodes *hlyA*) and replaced with the 0.6 kb *Sna*BI* / *Eco*RI fragment of pPM4374 to generate pPM4351. pPM4351 encodes modified HlyA which has an in-frame deletion of the 15 amino acids Thr-598 to Ser-612 which lie 15 kDa from the C-terminus. These amino acids are crucial for haemolytic activity and secretion.



replaced with the *Sna*BI* / *Eco*RI fragment of pPM4374, such that the intervening nucleotides encoding 15 amino acids, were removed to produce pPM4351 (Figure 5.2 and 5.5). These 15 amino acids include those proposed to be required for activity and secretion (section 5.2.1). The mutagenesis was confirmed by DNA sequencing using oligonucleotide #1007.

In order to ensure pPM4351 produces stable protein and also to examine the cleavage of the protein, pPM4351 was expressed in *E. coli* using the T7 RNA polymerase induction system as described earlier. Western immunoblot analysis of whole cell fractions revealed that this modified 80kDa HlyA is able to cleave to the 65 kDa product (Figure 5.6, panel A). Cell fractionations localised the majority of modified HlyA to the whole membrane fraction, and the level of protein was comparable to that of native HlyA (Figure 5.6, panel B). A 15 kDa cleavage product of pPM4351 was observed in the whole membrane fraction. The levels of modified HlyA compared to native HlyA in the periplasm were greatly reduced. This suggests that modified HlyA is unstable in the periplasm and is degraded more readily than unmodified HlyA.

Both *E. coli* and V945 expressing modified HlyA were non-haemolytic and modified HlyA was not secreted in V945 (data not shown). As the amino acid region Thr-598 to Ser-612 has been removed in the deletion of pPM4351, these data support the previous findings (section 5.2.1) that the two regions, Thr-598 to Tyr-602 and Thr-603 to Gln-609 are essential for haemolytic activity and secretion, respectively.

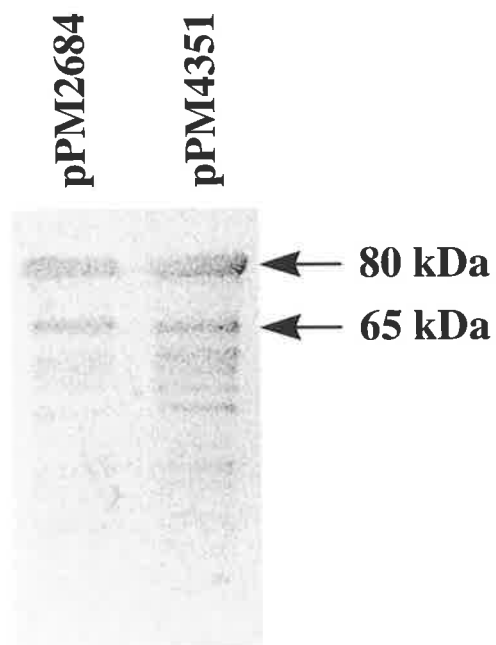
5.2.3 A comparison of El Tor HlyA with other haemolysins

As discussed in Chapter 3, the *V. cholerae* El Tor haemolysin shares homology with the VAH1, ASH4 and AHH1 haemolysins of *V. anguillarum*, *A. salmonicida* and *A. hydrophila*, respectively. In Chapter 4, the central 50 kDa region of pro-HlyA was identified as a minimum domain required for haemolytic activity. It was proposed to determine whether this 50 kDa region represents a conserved cytolytic domain and so a comparison of this region was made with the corresponding regions of VAH1, ASH4 and AHH1 (Figure 5.7). The N-terminal end of the 50 kDa derivative of pro-HlyA is defined by

Figure 5.6 Analysis of modified HlyA encoded by pPM4351 in *E. coli*.

Expression of native HlyA (pPM2684) and modified HlyA (pPM4351) were induced using the T7 promoter/RNA polymerase system (Tabor and Richardson, 1987). The cells were fractionated and analysed in SDS on a 15% polyacrylamide gel followed by western immunoblotting using α -dHlyA antiserum. The 80 kDa and 65 kDa forms of native and modified HlyA present in whole cell fractions are indicated (panel A). Modified HlyA was present in the periplasm, however, in reduced amounts compared with native HlyA. A 15 kDa cleavage product of modified HlyA was detected in whole membrane fractions (panel B).

A



B

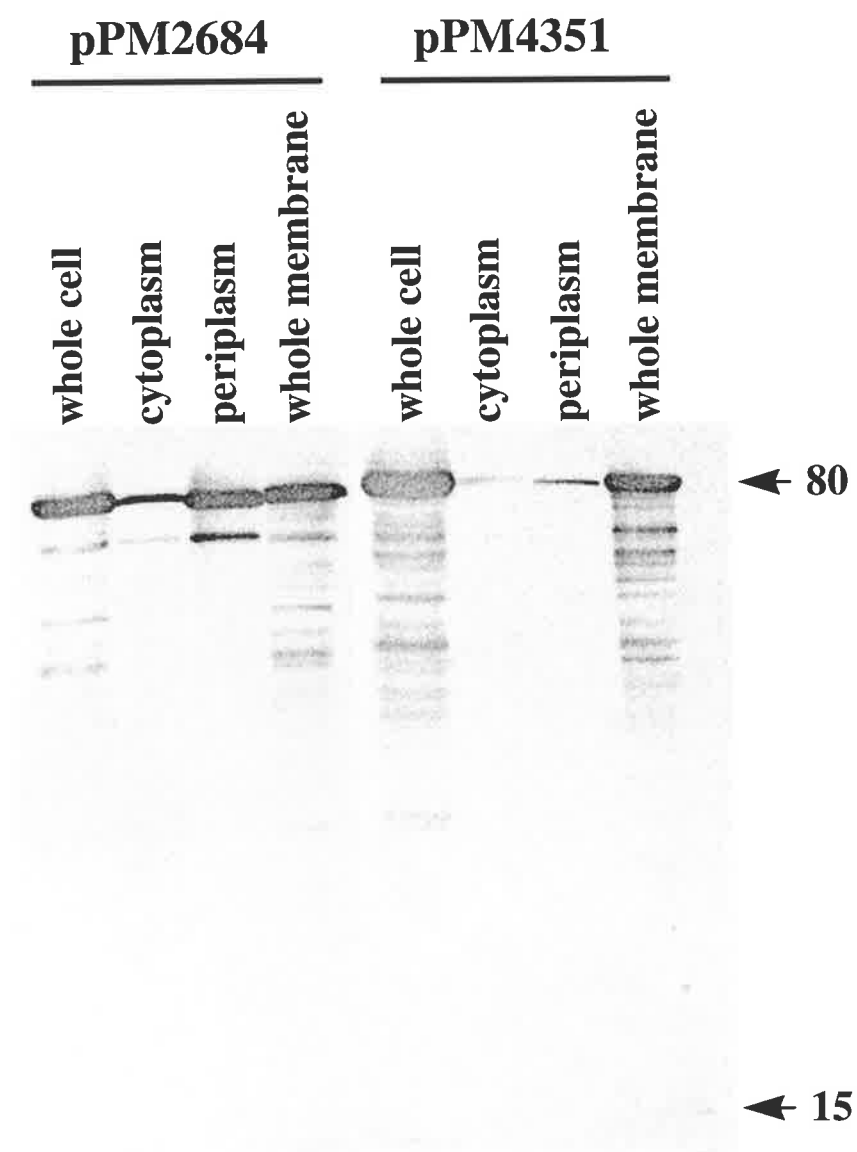


Figure 5.7 Comparison of the 50 kDa derivative of HlyA (Vc50) with various other haemolysins.

The central 50 kDa region of *V. cholerae* HlyA was aligned with the most homologous regions of the haemolysins VAH1 (*V. anguillarum*; Va50), ASH4 (*A. salmonicida*; As50) and AHH1 (*A. hydrophila*; Ah50). The conserved cysteine residues are highlighted. The alignment of the amino acid sequences was performed using Clustal V (Higgins and Sharp, 1988 and 1989). Identical residues are indicated by asterisks, conserved residues with similar properties are indicated by dots. Gaps (dashes) have been introduced to optimize alignment. The number of the last amino acid in each row is indicated for each protein.

Vc50 NSETNTLPHVAFYISVNRAISDEE**C**TFNNSWLWKNEKGSRP**F****C**KDANISL 207
Va50 GSETNSLPHVAFYLVNKRPNNTDYE**C**TFNNSHLW--DRGTRV**F****C**DYPNISL 206
As50 ATAEAGLPRGAFYLLKLNKISVAE**C**TFPRSR**TW**--DRGDRL**F****C**DSPNISL 172
Ah50 PAEEAGLPHVAFYLVNVRKISDAE**C**TFPRSR**TW**--SRGDRL**F****C**DSPNISL 172

. ** . *** . . * . ***** * * . * * ** . ****

Vc50 IYRVNLERSLQYGVGSATPDAKIVRISLDDDDSTGAGIHLNDQLGYRQFG 257
Va50 IYRVNLERSLQYGTALQHRTE**N**C-RISLDDDDTTGAGI-ISM**T**LDYRQSG 254
As50 VYRVNLERSLQFGNTGSATPDAKIVRISLDDDESAGGGIQLNEDLTWSENI 222
Ah50 VYRVNLERSLQFGNTASATPDAKIVRISLDEESAGAGIQLNEDLSWSENI 222

. ***** . . ***** . . . * . * . *

Vc50 ASYTTLDAYFREWSTDAIAQDYRFVFNASNNKAQILKTFPVDNINEKFER 307
Va50 ADYVTLDAYFRDWSTDAIAQDYQFVFNSSNNKAQILKTFPRENLNAEYEV 304
As50 ADYLLLDGWARDYATDAIAQDYRFTIDASNTKAAVLKSLPTN-LNSKYEH 271
Ah50 ADYLLLDGWARDYATDAIAQDYRFTTEASNTKAAVLKSLPTN-LNSKYEH 271

* * ** . * . ***** . * . . ** ** . ** . * . * *

Vc50 KEVSGFELGVTGGVEVSGDGPKAKLEARASYTQSRWLTYNTQDYRIERNA 357
Va50 KEVSGFQLGVSGGVEVSADGPKGKLEANASYNQSRWLTFKTHDYRVERST 354
As50 RRISGFEGVGTGGVEVNKDGPKASWRRRPSSASSVTMP-TTPRIIGSNAR 320
Ah50 REISGFEGVGTGGVEVNKDGP RPSPWRRRPSSASSASSP-TTPRITGLNAP 320

. . *** . ** . ***** . *** . . * * *

Vc50 KNAQAVSFTWNRQQY--ATAESLLNRSTDALWVNTYPVDVNRISPLSYAS 405
Va50 NNAQNVSFKNRNSQY--ATAESMMNRSYDAIWVNTYPVDTNRINPIGYAS 402
As50 PECPEGEFQLGAGSIRDGGVTALLQDRH--RLGNGVRCGSQPHPAELQG 368
Ah50 AERPEGEFQLGARSIRDRRVPALLQDGH--VWAW-LRRGSQPHPAQLQG 367

. *

Vc50 FVPKMDVIYKASATETGSTDFIIDSSVNIRPIYNGAYKHYYVVGHAHQS**Y**H 455
Va50 FVPKMDVIYKAAPNVTGTTEFTIDSSVNIRPIYNGAYKHYYVVG**G**HQS**Y**H 452
As50 LRAESGCHLQGGADETGSTEFKIDSSVNIRPIYTG**I**YKHYYVVG**G**HVS**F**Q 418
Ah50 VCAESDVIYKAAPDETGSTEFKIDSSVNIRPIYTG**I**YKHYYVVG**A**HVS**F**Q 417

. ** . * ***** * ***** . * *

Vc50 GFEDTP-RRRITKSASF**T**VDWDHPVFTGGRPVNLQLASFNNR**C**IQVDAQG 503
Va50 GLKNTT-RRRVSKVTS**F**VDWEHPVFTGGRPVNLQLGSFNNR**C**VDVADNG 501
As50 GFEDVDKRRRV**T**ASTSF**K**VDWNHPVFTGGRPVNLQLGGFDNR**C**LSAGAEH 468
Ah50 GFEDTDKRRRV**A**ASTSF**K**VDWNHPVFTGGRPVNLQLGGFDNR**C**LSADANH 467

* . . *** . . ** *** ***** . * ***

Vc50 RLAANT**C**DSQQAQSFYIDQLGRYVSASNTKL**C**LDGEALDALQP**C**NQNLT 553
Va50 VLSASS**C**DENQNSQSFYIDQLGRYVSANDIHQ**C**LDGQSLGQLQP**C**SSSLV 551
As50 GLSAVT**C**DETSAAQSFYIDQYGRYVSALDTRR**C**LDGNNLGQLQS**C**RLSLG 518
Ah50 GLSAVTFDETSAAQSSYID**E**YGRYVSAQDTLR**C**LDGNNLGQLQS**C**LSLG 517

* . * . * . ** *** ***** ***** * * * *

Vc50 QRWEWRKGTDELTVYSGESLGHDKQTGELGLYASSNDA--VSLRTITAY 601
Va50 QRWEWIENSDKLSNVFSHELLGHDKTSGELGLYADGNDQ--VSVRTITAY 599
As50 QRWEWKADSDALS**N**LSAHQLLGHNKQTGELALYDENGNPADVSVRTLTSY 568
Ah50 QRWEWKADSDALS**N**LSAHQLLVHDKQSGALGLYDENGNPQNVSVRTLTSY 567

***** . * * . * . * * * * * . * * * * *

the N-terminal cleavage site between Ala-157 and Asn-158 (Nagamune *et al.*, 1996), while the C-terminal end of the 50 kDa was localised to Tyr-602 in section 5.2.1. The *V. anguillarum* VAH1 precursor is a similar size to the HlyA precursor, and so the central 50 kDa region of VAH1 was used in the comparison. The *Aeromonas* haemolysins ASH4 and AHH1 both lack the C-terminal 15 kDa present in the *V. cholerae* pro-HlyA and so only the N-terminal 15 kDa was removed in the comparison. The 50 kDa region of HlyA was 70%, 50.8% and 51.3% identical to the corresponding regions of VAH1, ASH4 and AHH1, respectively, and there was 85% homology between these regions in ASH4 and AHH1 (Table 5.1). Homology between these haemolysins was greatest between the 50 kDa regions aligned in Figure 5.8, compared to analysis of the entire protein (section 3.2.4). The cysteine residues conserved within these four haemolysins (section 3.2.4) are all located within the 50 kDa domain.

The *Vibrio vulnificus* cytolyisin VvhA shares two regions of homology with El Tor HlyA and there is conservation of the location of cysteine residues (Yamamoto *et al.*, 1990b). The homologous regions and conserved cysteine residues are located within the 50 kDa domain of HlyA. That is, the 56 kDa cytolyisin of *V. vulnificus* lacks both the N- and C-terminal 15 kDa regions present in El Tor HlyA, even though both cytolyisins have virtually identical biological characteristics. It is worth noting that Iwanaga and Ichinose (1991) have characterised an aberrant haemolysin produced by a *V. cholerae* non-O1 strain NO37, that is antigenically very similar to HlyA, but is only 48 kDa. This haemolysin also lacks the N- and C-terminal 15 kDa present in HlyA.

A schematic representation of alignments of the various haemolysins to HlyA with respect to homologous regions and positions of conserved cysteine residues more clearly shows the potential significance of the central 50 kDa region of pro-HlyA (Figure 5.8). The secondary structure of the 50 kDa region may be conserved among HlyA, VAH1, ASH4, AHH1 and VvhA haemolysins, as the location of cysteine residues are conserved within this region. It is worth noting that pre-treating HlyA with the reducing agent dithiothreitol (DTT) inhibits haemolytic activity (data not shown), which infers that disulphide bond formation in HlyA is critical for active protein.

Table 5.1

Comparison of the central 50 kDa region of *V. cholerae* HlyA with other haemolysins/cytolysins^a

	Vc50 ^b	Va50 ^c	As50 ^d	Ah50 ^e
Vc50	100 [445]	70 [444]	50.8 [449]	51.3 [448]
Va50		100 [441]	47.3 [446]	50.1 [445]
As50			100 [444]	85.1 [444]
Ah50				100 [443]

^a Numbers, % identity determined using FASTA as implemented by PROSIS Version 7 (LKB-Hitachi Software).

Numbers in brackets, number of aa over which the indicated identity occurs.

^b Vc50: central 50 kDa region of *V. cholerae* HlyA

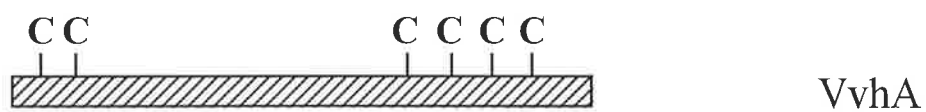
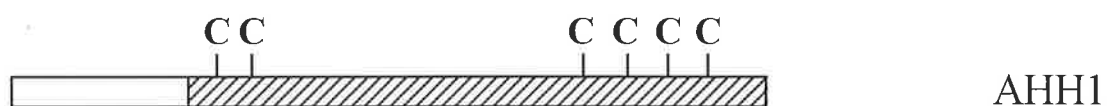
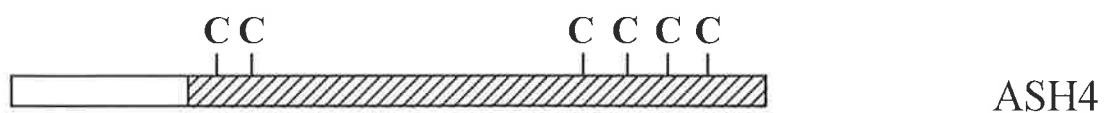
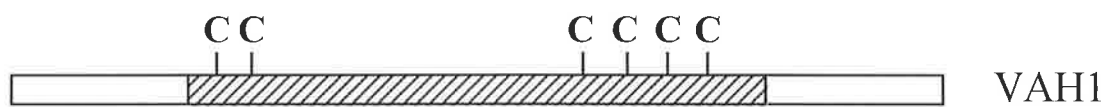
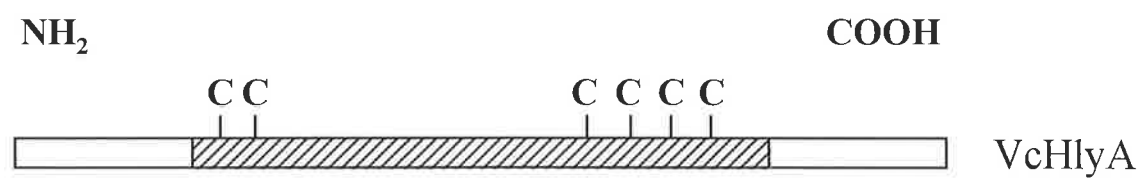
^c Va50: central 50 kDa region of *V. anguillarum* VAH1

^d As50: C-terminal 50 kDa region of *A. salmonicida* ASH4

^e Ah50: C-terminal 50 kDa region of *A. hydrophila* AHH1

Figure 5.8 Schematic alignment of HlyA (VcHlyA) with various other haemolysins.

The pro-forms of various haemolysins were aligned according to regions of homology and conserved cysteine residues (C). The haemolysins examined were as follows: HlyA (*V. cholerae*), VAH1 (*V. anguillarum*), ASH4 (*A. salmonicida*), AHH1 (*A. hydrophila*), VvhA (*V. vulnificus*) and VcNO37 (*V. cholerae* NO37). The hatched region (approximately 50 kDa), contains the highest degree of homology between HlyA and the other haemolysins as well as all the conserved cysteine residues. This 50 kDa region may represent a conserved domain critical for haemolytic activity.



5.2.4 Expression of the C-terminal 15kDa of pro-HlyA

This section describes the expression of the C-terminal region of pro-HlyA. The reason for this was two-fold. Firstly, stable expression of this region could help localise region(s) recognised by specific anti-HlyA antisera (discussed further in section 5.2.5). Previously, the stable expression of the N-terminus of HlyA was achieved which allowed initial localisation of antibody recognition (Chapter 4). Cyanogen bromide cleavage of a protein is often used to localise epitopes, however, this method was unsuccessful for HlyA as the protein completely degraded during the process (data not shown).

An additional reason for expressing the C-terminal 15 kDa of pro-HlyA, is that its role remains unclear. It is not essential for either pore-formation or secretion (Chapter 4) and so the expression of this region could be useful in future experiments to help elucidate a role.

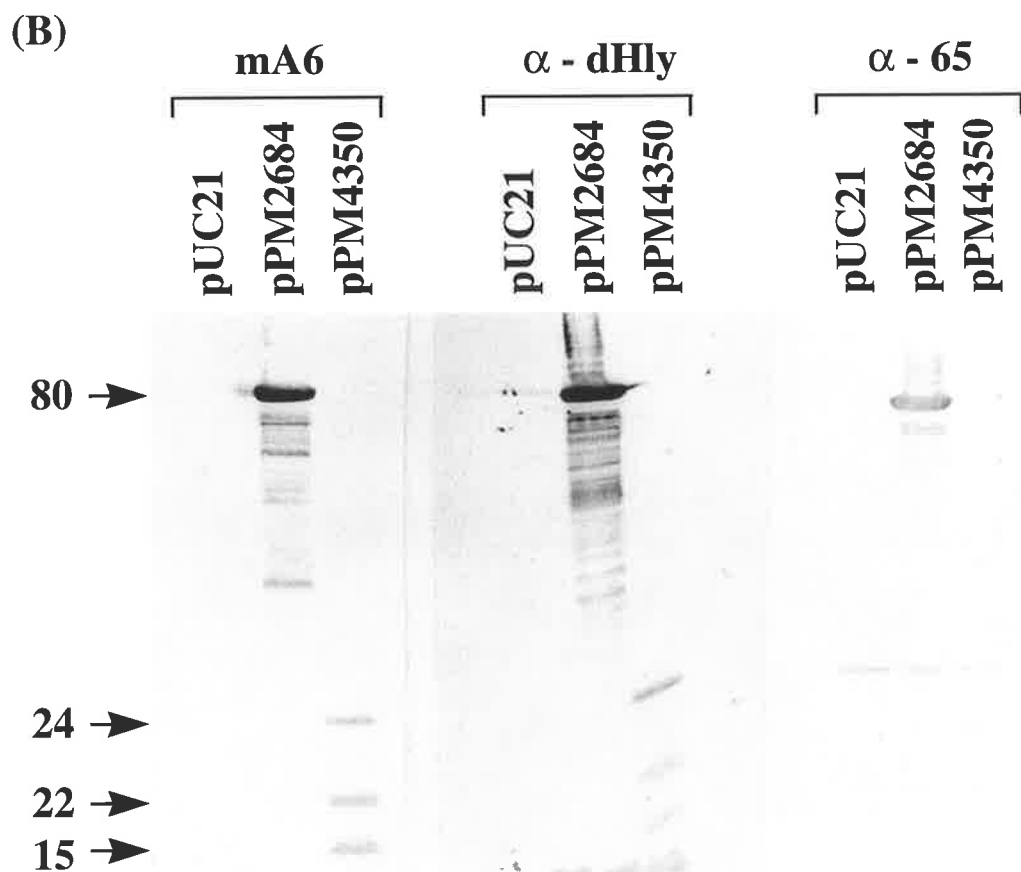
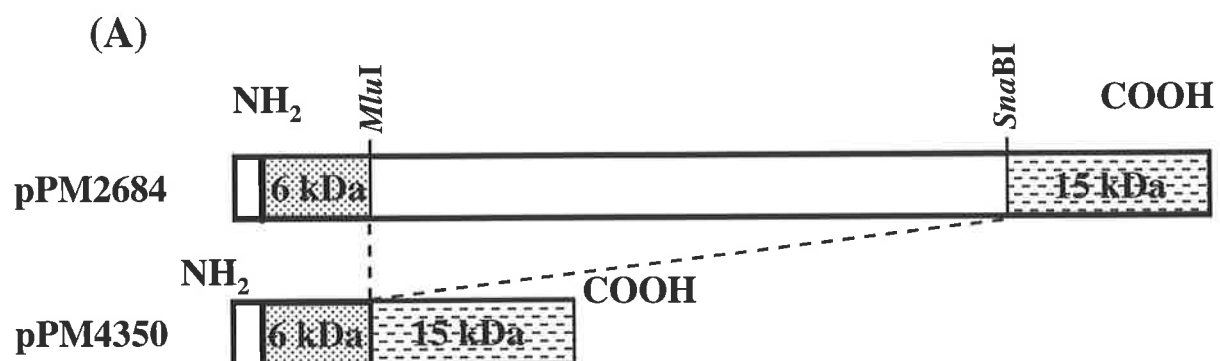
5.2.4.1 Construction and expression of pPM4350

In order to express the C-terminal 15kDa of pro-HlyA, an in-frame deletion within *hlyA* was constructed which removed most of the region encoding the 65kDa domain of HlyA. The construction of pPM4350 is shown schematically in Figure 5.9 (panel A). Plasmid pPM2684 was digested with *MluI* / *SnaBI*, end-filled and re-ligated to remove the intervening DNA to generate pPM4350. pPM4350 encodes a 24 kDa protein, with the N-terminal 5.8 kDa fused to the C-terminal 15 kDa of HlyA. The N-terminal cleavage site required for HlyA maturation (Yamamoto *et al*, 1990a; Hall and Drasar, 1990; Nagamune *et al*, 1996) is not present in the internal deletion of HlyA encoded by pPM4350.

Expression of truncated and native HlyA encoded by pPM4350 and pPM2684, respectively, was achieved using the T7 RNA polymerase system (Tabor and Richardson, 1987). Whole cell fractions were subjected to SDS-PAGE and western immunoblotting using α -dHly, mA6 and α -65 antibodies for detection (Figure 5.9, panel B). Three protein bands approximately, 24 kDa, 22 kDa and 15 kDa were produced from pPM4350. These proteins were detected by both α -dHly and mA6, but not by α -65 (Figure 5.9). The 24

Figure 5.9 Expressing the C-terminal 15 kDa of pro-HlyA from pPM4350.

Schematic representation of the construction of pPM4350, which encodes an in-frame internal deletion in HlyA. The *hlyA* gene of pPM2684 was digested with *Mlu*I and *Sna*BI and re-ligated to remove the intervening DNA (panel A). Expression of native HlyA (pPM2684) and internally truncated HlyA (pPM4350) in *E. coli* was achieved using the T7 system (Tabor and Richardson, 1987). Whole cell fractions of induced strains were subjected to SDS-PAGE and western immunoblotting (panel B). Proteins were detected with either mA6, α -dHly or α -65 antibodies, as indicated. Three protein bands, approximately 24 kDa, 22 kDa and 15 kDa encoded by pPM4350, were detected by mA6 and α -dHly, but not by α -65. The 24 kDa protein corresponds to the truncated HlyA protein encoded by pPM4350, removal of the signal sequence produces the 22 kDa protein and cleavage of this protein generates the 15 kDa product.



kDa protein corresponds to the pPM4350-encoded HlyA deletion and removal of the 25 amino acid signal peptide during protein export from the cytoplasm to the periplasm, would generate the 22 kDa protein. The 15 kDa protein is proposed to be a C-terminal derivative of the 22 kDa protein encoded by pPM4350, as the 22 kDa protein contains the proteolytically sensitive site that is normally located 15 kDa from the C-terminus of pro-HlyA (Chapter 4). Two other bands were detected by α -65 in all three lanes (pUC21, pPM2684 and pPM4350) and are therefore likely to be background only. It should be noted that while the normal N-terminal 15kDa cleavage product of pro-HlyA encoded by pPM2684 was not detected in the gel of Figure 5.9, it has previously been seen on other gels (data not shown; Alm *et al.*, 1988). It appears that the truncated protein encoded by pPM4350 cleaves more readily than the full length protein encoded by pPM2684.

5.2.4.2 N-terminal sequence analysis of the 15kDa product of pPM4350

The amino-terminus of the 15kDa product of pPM4350 was sequenced to confirm its origin and attempt to locate the exact position of the C-terminal proteolytically sensitive site of HlyA. Expression of pPM4350 (and pUC21 as a negative control) was induced using the T7 RNA polymerase system described earlier. Periplasmic fractions of the induced cultures were isolated and concentrated using a centrifugal filter device (Ultrafree^R-15, Millipore). The samples were subjected to SDS-PAGE and the 15 kDa cleavage product of pPM4350 was excised from the Coomassie stained polyacrylamide gel shown in Figure 5.10. To further purify the 15 kDa protein, the excised band was re-electrophoresed on an another polyacrylamide gel. The protein was then transferred from the gel onto a PVDF filter and subjected to amino-terminal sequencing.

The N-terminal amino acids obtained for the 15kDa cleavage product encoded by pPM4350 were Ile-Asn-Gln-Gln-Lys-Arg-Gly-Thr-Ile-Thr. According to the amino acid sequence of HlyA (Yamamoto *et al.*, 1990a), the 10 N-terminal amino acids of the 15 kDa are a hybrid containing amino acids that span the point of fusion of the deleted region in pPM4350 (illustrated in Figure 5.11). The first 6 amino acids (Ile-73 to Arg-78) are from the N-terminus and the seventh amino acid Gly, encoded by GGT, is created as a result of

Figure 5.10 Isolation of the 15 kDa product of pPM4350.

A Coomassie stained 15% SDS polyacrylamide gel of periplasmic fractions of *E. coli* strains carrying either pPM4350 or pUC21 whose expression was induced using the T7 RNA polymerase / promoter system (Tabor and Richardson, 1987). pPM4350 encodes internally truncated HlyA which cleaves to a 15 kDa product (shown with an arrow). This 15 kDa protein was excised from the gel, transferred to a filter and subjected to N-terminal amino acid analysis. The standard molecular mass markers are shown (in kDa) to the left of the panel.

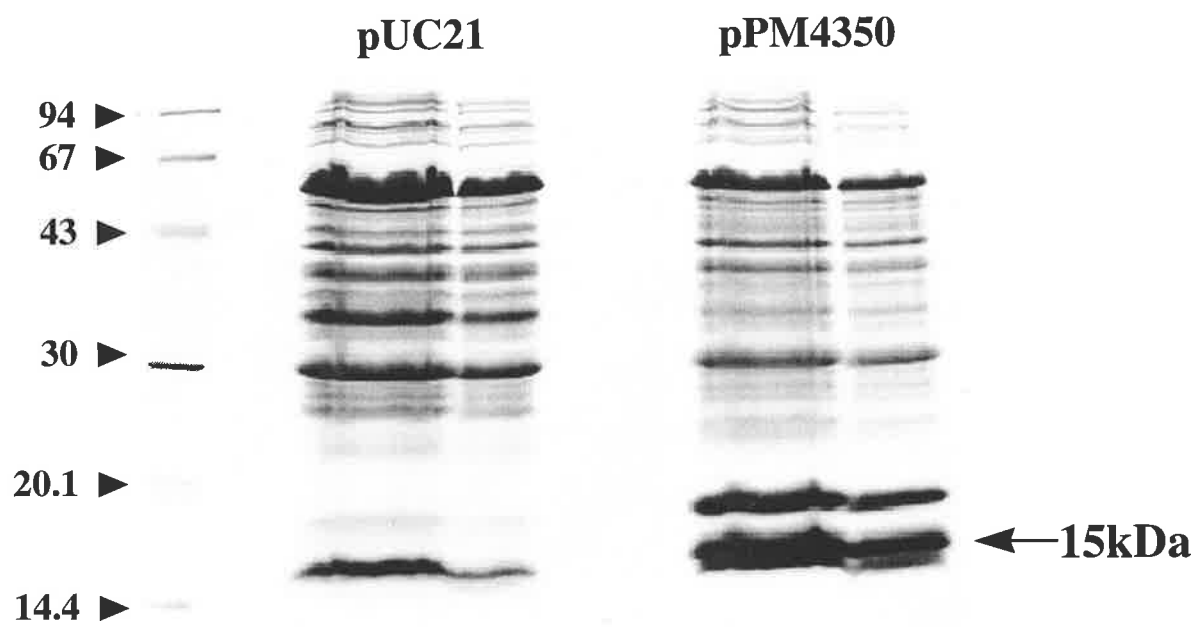
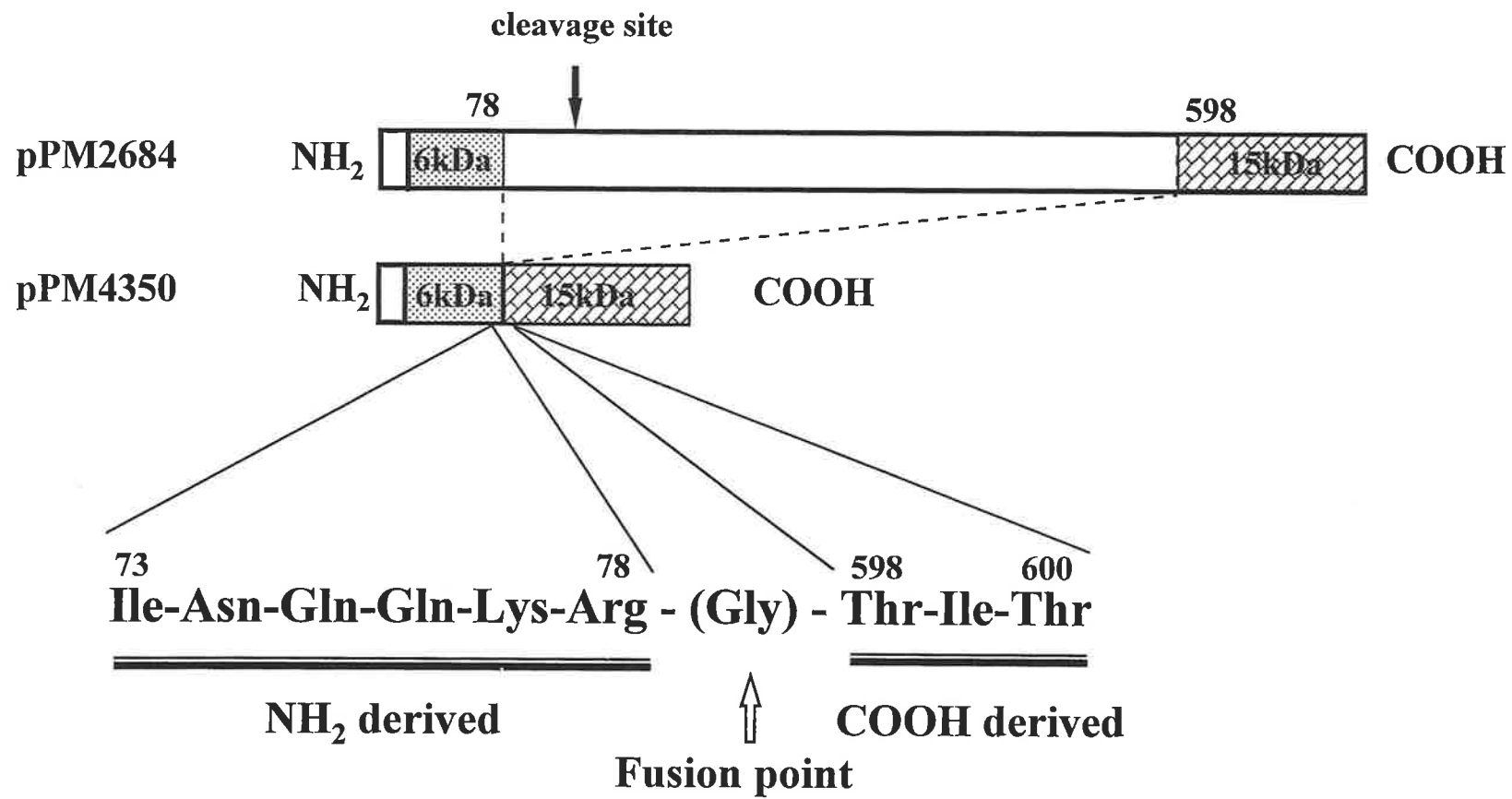


Figure 5.11 N-terminal analysis of the 15 kDa cleavage product of pPM4350 encoded truncated HlyA.

A schematic representation of the ten amino acids obtained from the N-terminal analysis of the 15kDa cleavage product of truncated HlyA encoded by pPM4350. The first six amino acids (Ile-73 to Arg-78) are derived from the N-terminus of HlyA and the last three (Thr-598 to Thr-600) are derived from the C-terminus. The seventh amino acid (Gly) was created at the point of fusion between N- and C-terminal HlyA. Amino acids are given in universal three letter codes.



the ligation between the end-filled *Mlu*I and the blunt-end *Sna*BI sites used to construct the deletion. The final three amino acids (Thr-598 to Thr-600) are from the C-terminus of HlyA. Therefore, with the exception of the first seven amino acids, the rest of the 15 kDa protein must be derived from the C-terminus of pro-HlyA.

N-terminal sequence analysis has confirmed that the 15kDa protein is a cleavage product derived from the C-terminus of the 22 kDa protein encoded by pPM4350, and apart from the first seven amino acids, the product corresponds to the C-terminal 15 kDa region of pro-HlyA. Cleavage of the HlyA derivative encoded by pPM4350 has occurred in the vicinity of the region predicted to contain the proteolytically sensitive site (Chapter 4). According to N-terminal analysis, cleavage has occurred approximately 6 kDa from the N-terminus of pro-HlyA (Figure 5.11), between the Val-72-Ile-73 residues. However, as proteolytically sensitive sites are usually located in exposed parts of the protein that are accessible to proteases, it is possible that the exposed region was maintained in the internally truncated HlyA of pPM4350.

5.2.5 Characterisation of anti-HlyA antibodies

5.2.5.1 Inhibition of haemolysis by α -65 and mA6

HlyA specific polyclonal antiserum α -65 and a monoclonal antibody mA6 were tested for their ability to neutralize the haemolytic activity of *V. cholerae* O17 culture supernatants in a liquid haemolysis assay. Supernatants containing HlyA were pre-incubated with serial dilutions of antibody for one hour at 37°C, after which sRBCs were added and incubated for a further two hours. Both α -65 and mA6 were able to inhibit haemolytic activity, with a neutralisation titre of 1/3000 and 1/1000, respectively (not shown). The antiserum α -dHly was also examined, but did not inhibit haemolysis.

It was then determined whether the inhibition of haemolysis by α -65 or mA6 was due to a decrease in the amount of HlyA associated with the sRBC membranes. Serial two-fold dilutions of HlyA were pre-incubated at 37°C for one hour, either in the presence (+) or absence of antibody (-). sRBC suspensions were added to each of these dilutions, and incubated at 37°C for two hours, after which haemolytic activity was determined (Figure

5.12). SRBC membranes were collected from the assay corresponding to the level of HlyA that gave approximately 50% lysis in the absence of antibody, but total inhibition in the presence of antibody (indicated by a dotted line in Figure 5.12). These samples were electrophoresed on SDS-PAGE gels and subjected to western immunoblotting using α -65 antiserum for detection (Figure 5.12). It can be seen that HlyA protein has associated with the sRBC membranes during the haemolysis assay, both in the presence (+) or absence (-) of antibody, but there is a reduction of sRBC-associated HlyA after exposure to either α -65 or mA6 (Figure 5.12).

5.2.5.2 Localising the regions recognised by the antibodies

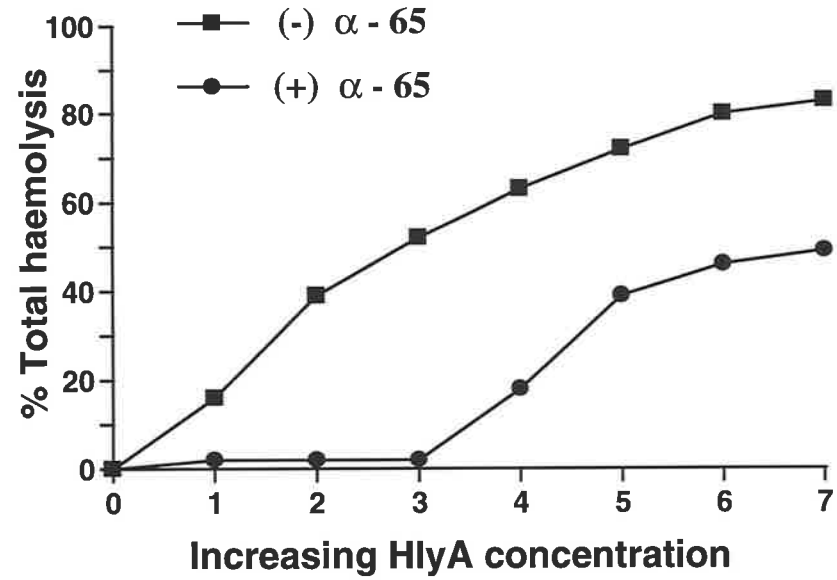
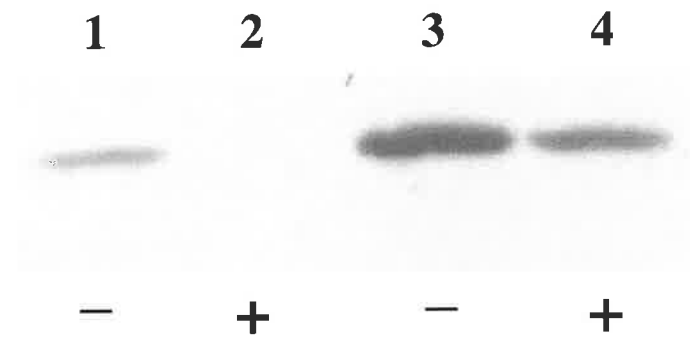
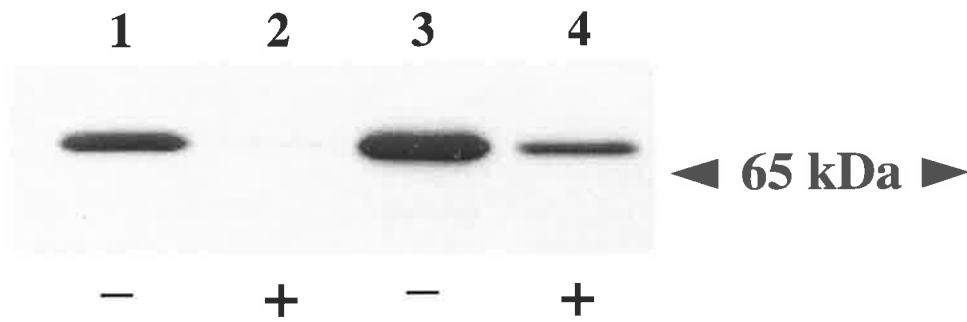
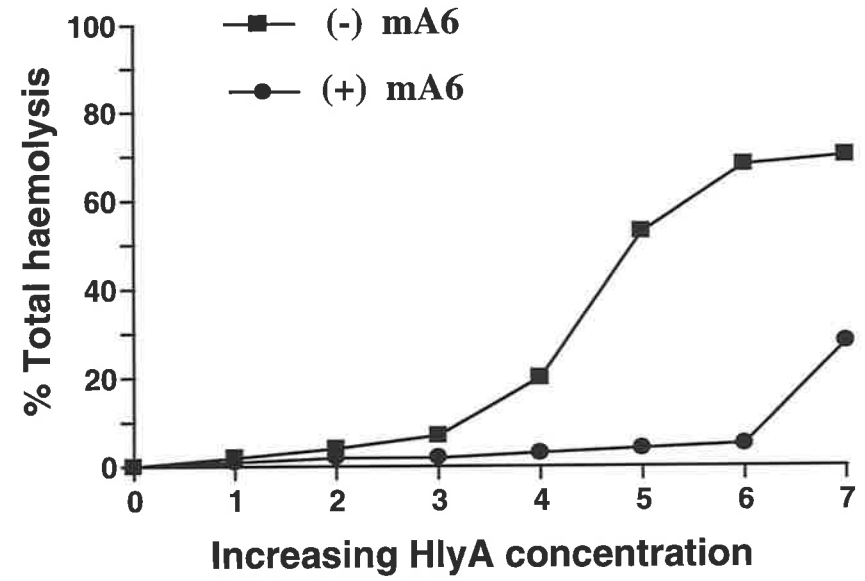
As the HlyA-specific antibodies α -65 and mA6 inhibit haemolysis, it was of interest to determine what regions of pro-HlyA they recognise. This section summarizes data previously obtained (Chapters 4 and 5) and also incorporates new data which help define HlyA regions recognised by α -65 and mA6.

α -65 recognises both pro-80 kDa HlyA and mature 65kDa and in addition, recognises the 50 kDa derived from the central region of pro-HlyA (discussed in Chapter 4). As shown previously (section 4.2.2.3), α -65 recognises C-terminal truncations of pro-HlyA of up to 15kDa and removal of more than 15kDa abolished recognition. α -65 was able to detect the C-terminal truncation encoded by pPM2691, but was unable to recognise the protein when a further 27 amino acids were removed from the C-terminus (pPM2692). Thus the C-terminal cut-off point for recognition lies between the end-points encoded by pPM2691 and pPM2692.

Additional mutants of HlyA have been constructed which may further localise the region(s) recognised by α -65. These mutants are encoded by pPM4353, pPM4342 and pPM4351 and details of their construction are described in sections 5.2.1 and 5.2.2. Expression of constructs encoding either native HlyA (pPM2684) or mutations of HlyA (pPM2685, pPM2691, pPM2692 [section 4.2.2]; pPM4342, pPM4353 [section 5.2.1] and pPM4351 [section 5.2.2]), were induced using the T7 system (Tabor and Richardson, 1987) and whole cell fractions were collected. These fractions were electrophoresed on

Figure 5.12 Inhibition of haemolysis by α -65 and mA6 antibodies.

Dilutions of HlyA were pre-incubated for 1 hr at 37°C in the presence (+) or absence (-) of antibody prior to the addition of an equal volume of a 5% sRBC suspension. Haemolytic activity was determined for each HlyA dilution after two hours and expressed as % total lysis. Haemolytic activity increases with increasing HlyA concentration and this is inhibited by the presence of either α -65 (panel A) or mA6 (panel B) as determined by a shift in the curve. Below the graphs are western immunoblots of HlyA associated with sRBC membranes taken at 0 hrs (lanes 1 and 2) and 2 hrs (lanes 3 and 4) after the addition of sRBCs, from the assay described above. Aliquots were taken from the dilution of culture supernatant that gave 50% lysis in the absence (-) of antibody, but total inhibition in the presence (+) of antibody (indicated by a dotted line). SRBC membranes were isolated and subjected to western immunoblotting, using α -65 for detection.

A**B**

duplicate polyacrylamide gels and the proteins were detected with either α -dHly or α -65 antisera in a western Immunoblot (Figure 5.13). α -dHly was used as a positive control, to ensure that all the HlyA mutants were expressed (Figure 5.13). The only mutant of HlyA not recognised by α -65 was the C-terminal truncation encoded by pPM2692 (Figure 5.13). It should be noted that the protein encoded by pPM4351 is showing reduced recognition by α -65 compared to α -dHly. It is possible that the deletion of pPM4351 has removed epitope(s) recognised by α -65. Figure 5.14 summarizes recognition by α -65.

While α -65 recognised C-terminally truncated HlyA encoded by pPM2691, pPM4353 and pPM4342, it was unable to do so when amino acids Leu-584 to Arg-597 were deleted from pPM4342, as in construct pPM2692 (Figure 5.14). This suggests that these amino acids (highlighted in Figure 5.14) are required for recognition by α -65 and the epitope recognised by α -65 either contains these residues or the removal of these residues significantly change the folding so that the epitope is no longer formed. HlyA encoded by pPM4351 carries an in-frame deletion of the region Thr-598 to Ser-612, but still contains the region from Leu-584 to Arg-597 (Figure 5.14) and this altered HlyA was recognised by α -65 (Figure 5.14). α -65 antiserum does not recognise HlyA with the large internal truncation, encoded by pPM4350, or the C-terminal 15kDa cleavage product of this protein (section 5.2.4.1), which represents the 15 kDa C-terminus of pro-HlyA. Truncated HlyA encoded by pPM4350 does not contain the Leu-584 to Arg-597 region (Figure 5.14), and since α -65 does not recognise this protein, this supports the notion that these amino acids are critical for recognition.

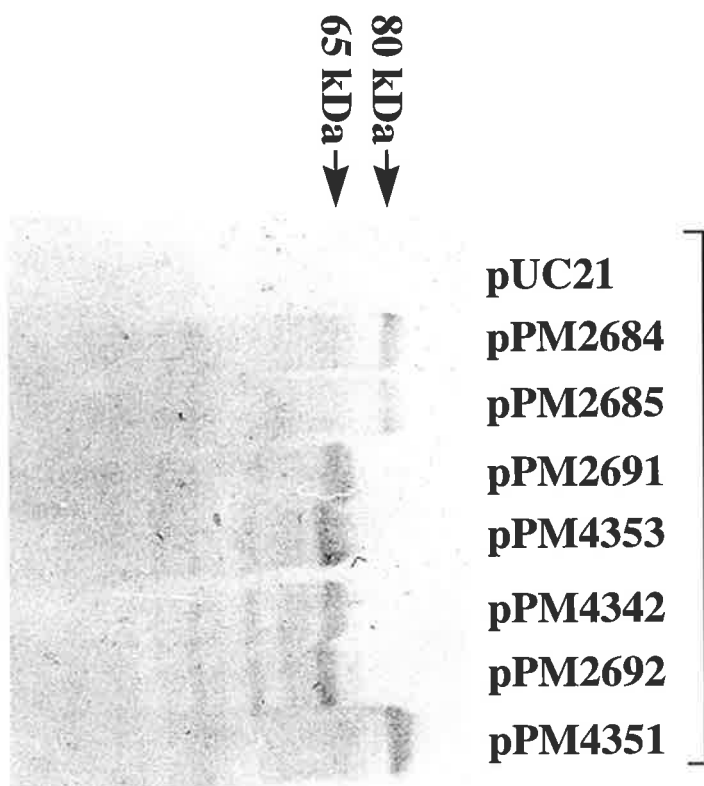
The monoclonal antibody mA6 recognises both 80 kDa and 65 kDa HlyA, and in addition recognises the C-terminal 15 kDa of pro-HlyA (section 5.2.4). However, mA6 was unable to recognise C-terminal truncations of pro-HlyA, and recognition is lost with deletions of only 12 amino acids (section 4.2.2.3). These data collectively show that amino acids 730 to 741 are critical for recognition by mA6.

Immunoblotting of α -65 and mA6 to HlyA on SDS polyacrylamide gels should represent recognition of predominantly linear epitopes. However, the possibility cannot be excluded that there is some re-folding of protein during western immunoblotting, in which

Figure 5.13 Recognition of native and mutated HlyA by α -65 and α -dHly antisera.

The expression of various constructs encoding native or mutated HlyA was induced using the T7 RNA polymerase/promoter system (Tabor and Richardson, 1987). The vector pUC21 was included as a negative control. Whole cell fractions were electrophoresed in SDS on duplicate 15% polyacrylamide gels, followed by western immunoblotting using either α -dHly or α -65 antisera. α -dHly recognised all HlyA derivatives examined. α -65 recognised all HlyA derivatives examined except for the C-terminally truncated HlyA encoded by pPM2691. This protein lacks the Leu-584 to Arg-597 region of HlyA predicted to be recognised by α -65.

α - dHly



α - 65

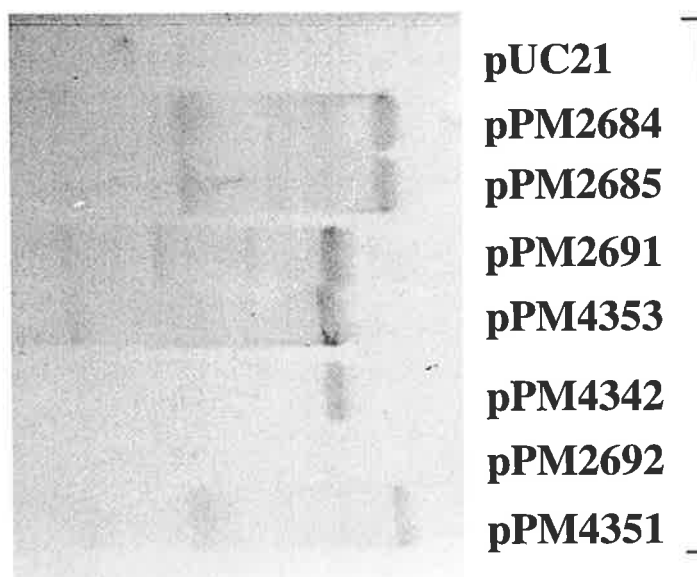
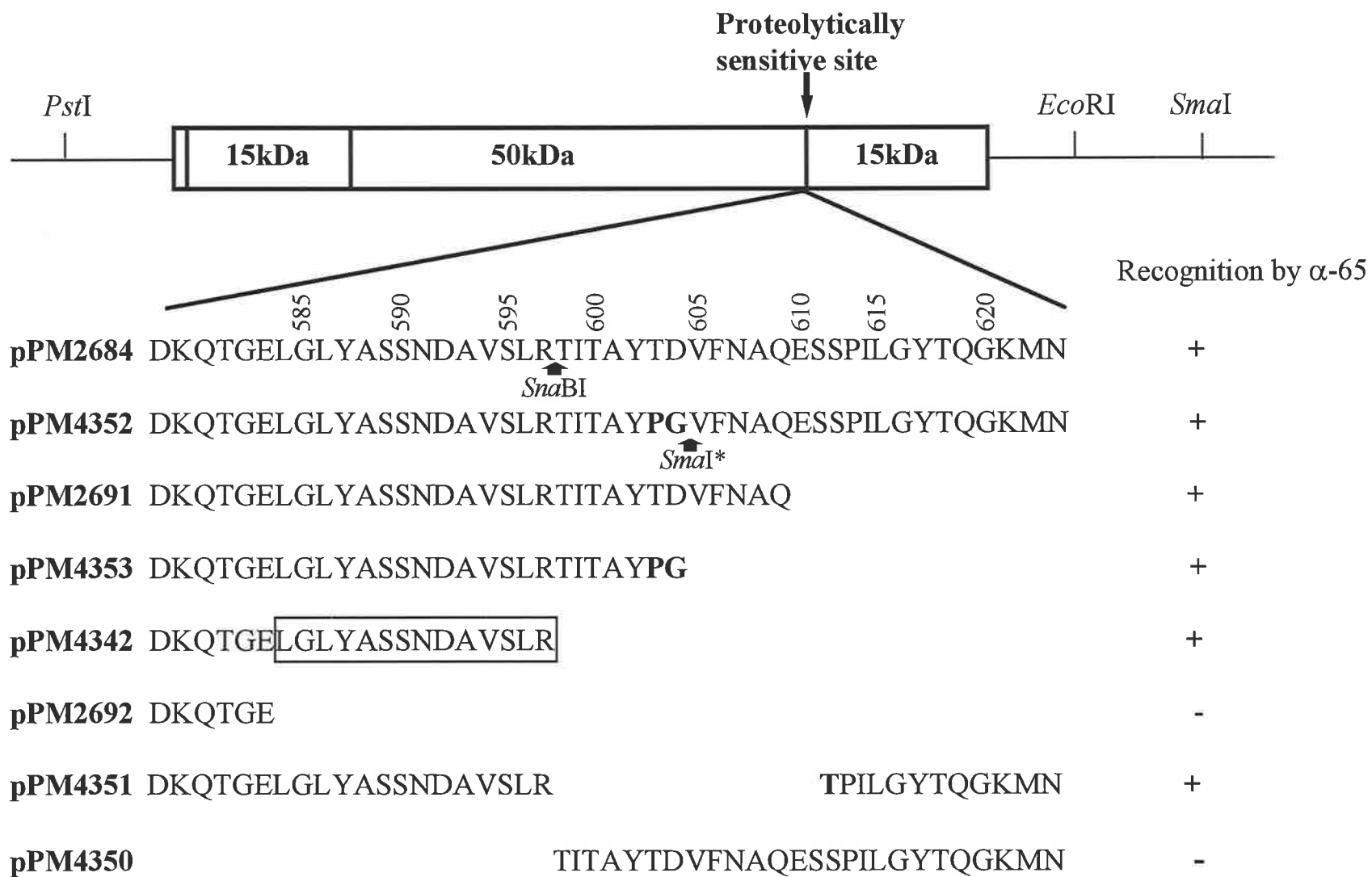


Figure 5.14 Epitope mapping of α -65 to HlyA.

The amino acid sequence of the region 15 kDa from the C-terminus in native and mutated HlyA is compared, and the recognition (+) or loss of recognition (-) by α -65 to these proteins is indicated. Plasmid pPM2684 encodes native HlyA and the amino acids altered in other constructs are shown in bold. The boxed amino acid region is proposed to be required by α -65 for recognition of HlyA.



case, antibodies may be recognising conformational epitopes. An overall summary of the regions within HlyA that are recognised by the α -65 and mA6 antibodies is shown in Figure 5.15.

5.3 Summary and Conclusions

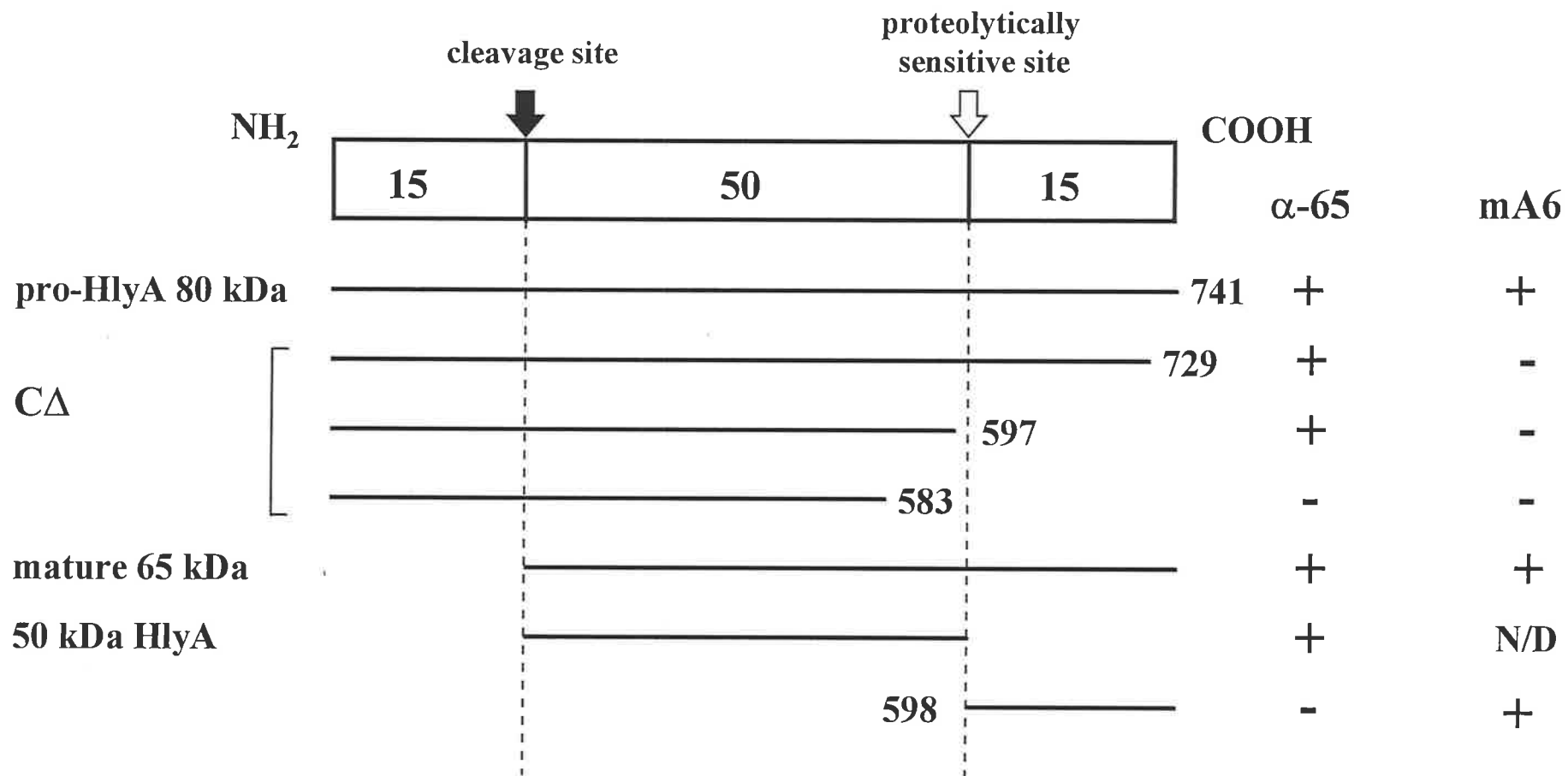
The central 50 kDa region of pro-HlyA was identified as a minimum domain required for haemolytic activity (Chapter 4). Deletions from the C-terminus of up to 15 kDa can be tolerated before activity and secretion ability is lost. Further removal of the C-terminus of pro-HlyA identified two significant regions at the C-terminus of the 50 kDa. The first region, Thr-603 to Gln-609, was essential for the secretion (stability) of HlyA. The second region, Thr-598 to Tyr-602, was crucial for haemolytic activity. These findings were supported by the in-frame deletion of pro-HlyA (pPM4351) which only removed amino acids Thr-598 to Ser-612. This deletion abolished activity and the ability of HlyA to be secreted.

The 50 kDa derivative of HlyA is highly homologous to the VAH1, ASH1 and AHH1 haemolysins of *V. anguillarum*, *A. salmonicida* and *A. hydrophila*, respectively. Based on regions of homology, the VvhA haemolysin of *V. vulnificus* can also be aligned with this 50 kDa region. The relative location of six cysteine residues is conserved within the HlyA, VAH1, ASH1, AHH1 and VvhA haemolysins (Chapter 3). These cysteine residues are all contained within the 50 kDa domain, which may reflect conservation of secondary structure. The conservation of the amino acid sequence, and of the arrangement of cysteine residues within the 50 kDa region, suggests that this may represent a domain essential for cytolytic activity. The *Aeromonas* haemolysins ASH1 and AHH1 do not contain the C-terminal 15 kDa region present in the *V. cholerae* pro-HlyA, and the VvhA cytotoxin lacks both the N- and C-terminal 15 kDa regions.

An internally truncated HlyA (encoded by pPM4350) with 6 kDa of the N-terminus fused to 15 kDa of the C-terminus cleaved to a 15kDa product. The protein encoded by pPM4350 did not contain the N-terminal cleavage site (Hall and Drasar, 1990; Yamamoto *et al.*, 1990a; Nagamune *et al.*, 1996). Amino-terminal sequence analysis confirmed that the

Figure 5.15 A summary of the regions within HlyA recognised by α -65 and mA6 antibodies.

α -65 recognises 80 kDa pro-HlyA, 65 kDa mature HlyA and the 50 kDa derivative of pro-HlyA, but does not recognise the C-terminal 15 kDa of pro-HlyA. This antiserum recognises C-terminal deletions (C- Δ) in pro-HlyA of up to 15 kDa, however, deletions greater than 15 kDa are no longer recognised. mA6 recognises both 80 kDa and 65 kDa HlyA as well as the C-terminal 15 kDa of pro-HlyA, but does not recognise any of the C-terminal deletions of pro-Hly described in Chapter 4. (N/D, not done).



15kDa product of pPM4350 was derived from the C-terminus, and therefore represents the C-terminal 15 kDa of pro-HlyA. While cleavage in this instance occurred in close proximity to our predicted cleavage site, it had actually occurred within the N-terminal region of pro-HlyA, at the junction / fusion point of the internally truncated HlyA. Proteolytic processing sites are generally in surface loops, or in relatively flexible interdomain segments. This structure is probably maintained in the protein produced by pPM4350 allowing access to proteolytic enzymes.

Anti-HlyA antibodies α -65 and mA6 both inhibit haemolysis. The exact means by which they do so is unknown at this stage, however less HlyA is associated with sheep erythrocyte membranes in the presence of these antibodies. The amino acid regions Leu-584 to Arg-597 and Asp-730 to Asn-741 are required for recognition by α -65 and mA6, respectively. These antibodies therefore recognise either a linear epitope contained within the amino acid regions, or a conformational epitope that requires these amino acids for the correct conformation.

Chapter 6

Characterisation of the region downstream of the *hlyA* locus of *V. cholerae* O1

6.1 Introduction

The *hlyA* locus is closely linked to a number of other potential virulence-associated determinants. A lecithinase / phospholipase gene *lecA*, is divergently transcribed to *hlyA* (Fiore *et al.*, 1997) and downstream of *hlyA* lies *hlyB*, whose gene product is highly homologous to methyl-accepting-chemotactic proteins of *E. coli* which are involved in signalling the flagellar motor in response to environmental stimuli (Everiss *et al.*, 1994). A gene located downstream of *hlyB* designated *hlyC* was also identified (Alm and Manning, 1990a) and shown to be transcriptionally induced during infection in an infant mouse model of cholera, suggesting it plays a role during infection of the host (Camilli and Mekalanos, 1995). Based on the previously noted homology of HlyC with lipases of other bacterial species, such as the *Pseudomonas aeruginosa* LipA protein (Manning, 1994; Casanova and Peterson, 1995; Camilli and Mekalanos, 1995), it is proposed herein to rename *hlyC* to *lipA*.

This chapter describes the examination of the nucleotide sequence and genetic organization of the region downstream of *hlyAB*. Specific chromosomal mutations in *lipA* and a previously uncharacterised *orf* (*pvtV*) which lie downstream of *hlyAB*, were constructed and the possible involvement of these *orfs* in the haemolytic activity and pathogenicity of *V. cholerae* O1 were evaluated.

6.2 Results

6.2.1 Expressing LipA

The *V. cholerae* O1 *hlyA* and *hlyB* genes have previously been characterised and are encoded on a 6.4 kb-*Pst*I fragment of pPM431 (Manning *et al.*, 1984; Alm *et al.*, 1988; Radar and Murphy, 1988; Alm and Manning, 1990a; Yamamoto *et al.*, 1990a). An additional gene, *lipA* (previously designated *hlyC*) was identified downstream of *hlyAB* and

predicted to encode a 18.3 kDa cell-associated protein with no signal peptide (Alm and Manning, 1990a; Casanova and Peterson, 1995). This *orf* was predicted to be a triacylglyceride-specific lipase based on similarity to other bacterial lipases (Manning, 1994; Camilli and Mekalanos, 1995; Casanova and Peterson, 1995).

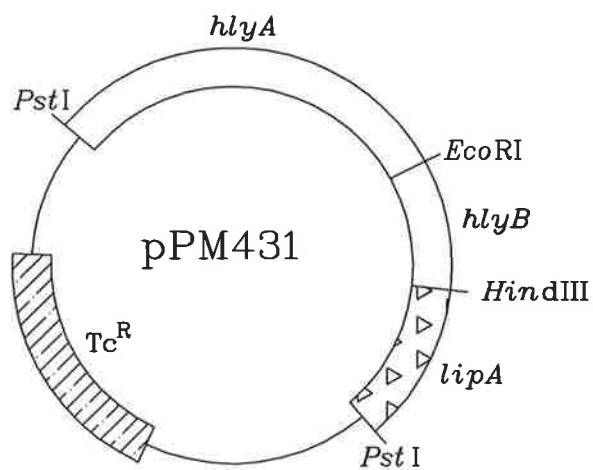
Due to the close proximity of *lipA* to *hlyA*, it was of interest to determine whether the product of *lipA* is involved in haemolytic activity and ultimately investigate its role in the virulence of *V. cholerae*. To facilitate the characterisation of this protein, the *lipA* gene was cloned behind an inducible promoter. This was achieved by sub-cloning the terminal 1.19 kb *HindIII* / *PstI* fragment of the 6.4 kb *PstI* fragment of pPM431 (Manning *et al.*, 1984), into the *HindIII* / *PstI* sites of the pBluescript SKTM polylinker, to generate pPM2656 (Figure 6.1). Expression of *lipA* from pPM2656 was achieved using the T7 RNA polymerase / promoter system of Tabor and Richardson (1987). Induced protein was labelled with [³⁵S]-methionine in the presence of rifampicin and whole cell samples were subjected to SDS-PAGE, stained with Coomassie blue and the gel dried and subjected to autoradiography. A 29 kDa protein produced by pPM2656 was detected (Figure 6.2), which disagrees with the previously reported size of 18 kDa (Alm and Manning, 1990a; Casanova and Peterson, 1995) and can only be explained as the result of read-through of *lipA* into the vector.

6.2.2 Re-sequencing *lipA*

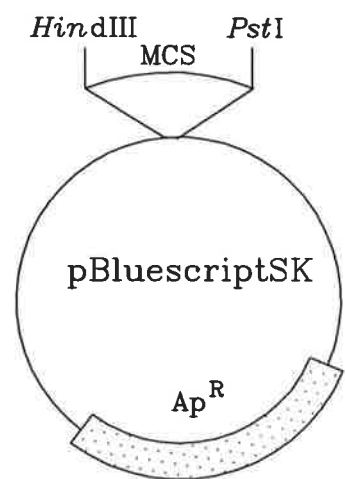
The expression of *lipA* from pPM2656 produced a 29 kDa protein, while earlier reports predicted an 18 kDa protein. To further examine this inconsistency, *lipA* was re-sequenced from pPM2656 (Figure 6.1), by extension from the forward and reverse primer binding sites present in pPM2656. Several differences were detected from previous reports (Alm and Manning, 1990a; Casanova and Peterson, 1995). A (CG) to (GC) transition (Arg to Ala) was found at nts 5724 / 5725 of the 6.4 kb *PstI* fragment of pPM431 (Figure 6.1), and following position 6078 bp, a "C" was inserted leading to a shift in the reading frame. This opens the reading frame to the end of the fragment with no detectable stop codon, and thus pPM431 does not contain the complete *lipA* gene, as reported previously.

Figure 6.1 Cloning *lipA* behind the T7 promoter.

A 1.19kb *HindIII* / *PstI* fragment carrying *lipA* was isolated from pPM431 (Manning *et al.*, 1984) and subsequently ligated between the *HindIII* / *PstI* restriction endonuclease sites of pBluescript SK. The resultant plasmid was designated pPM2656, where *lipA* is orientated behind the inducible T7 promoter.



Isolate the 1.19 kb
PstI/*HindIII*
fragment



Digest with
HindIII/*PstI*

Mix and Ligate

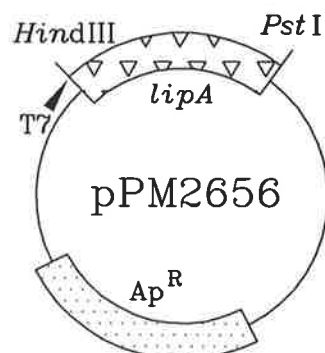


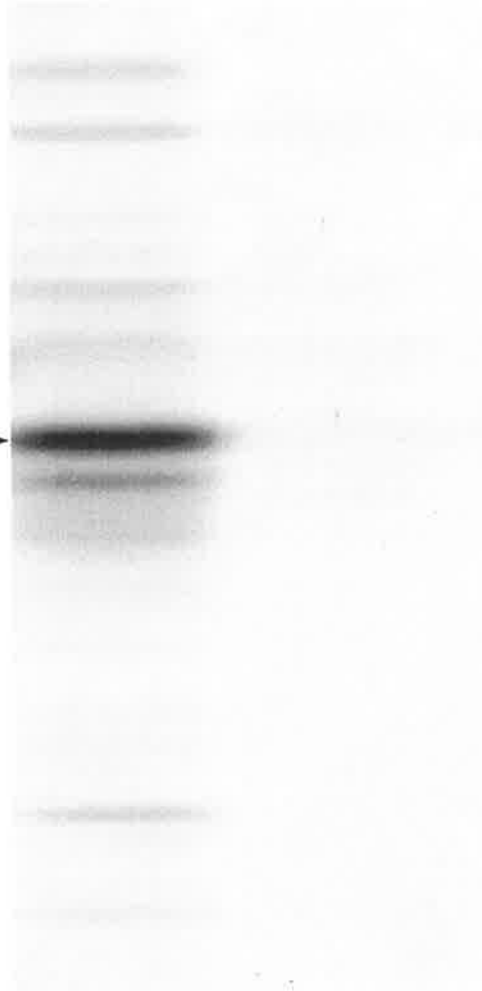
Figure 6.2 Expression of truncated LipA.

E. coli strains carrying either pPM2656 or pBluescript SK were induced using the T7 polymerase / promoter system of Tabor and Richardson (1987). Proteins were intrinsically labelled with [^{35}S]-methionine and whole cell fractions were electrophoresed in SDS on a 15% polyacrylamide gel. The gel was dried and subjected to autoradiography. Truncated LipA (29 kDa) is indicated by an arrow.

29kDa →

pPM2656

pBluescript SK



6.2.3 Isolation of the downstream region of *hlyAB*

As the *hly* locus contains several potential virulence-associated determinants, it was of interest to explore the region downstream of *hlyAB* and this section describes the isolation and characterisation of this region.

6.2.3.1 Mapping restriction sites downstream of *hlyAB*

In order to fully characterise *lipA* the complete gene must be obtained and as described in section 6.2.2, pPM431 contains only a partial copy of the gene. Thus, it was necessary to map restriction sites downstream of the 6.4 kb *Pst*I fragment of pPM431 in the chromosome of *V. cholerae* O1. Chromosomal DNA was restricted with various enzymes for seven hours at 37°C followed by electrophoresis on a TAE agarose gel. The DNA was subjected to Southern hybridization (Figure 6.3, panel A) using a 0.7 kb DIG-labelled PCR product as a probe. The probe was generated by PCR amplification of the region between nts 5830-6446 using oligonucleotide #173 and M13 reverse primer, and pPM2656 as the template. Using the known restriction sites of the 6.4 kb *Pst*I fragment of pPM431 (Alm and Manning, 1990a; Yamamoto *et al.*, 1990a) and the size of the various restriction fragments detected in the Southern blot, a putative restriction map of the region downstream of the *Pst*I site was generated (Figure 6.3, panel B).

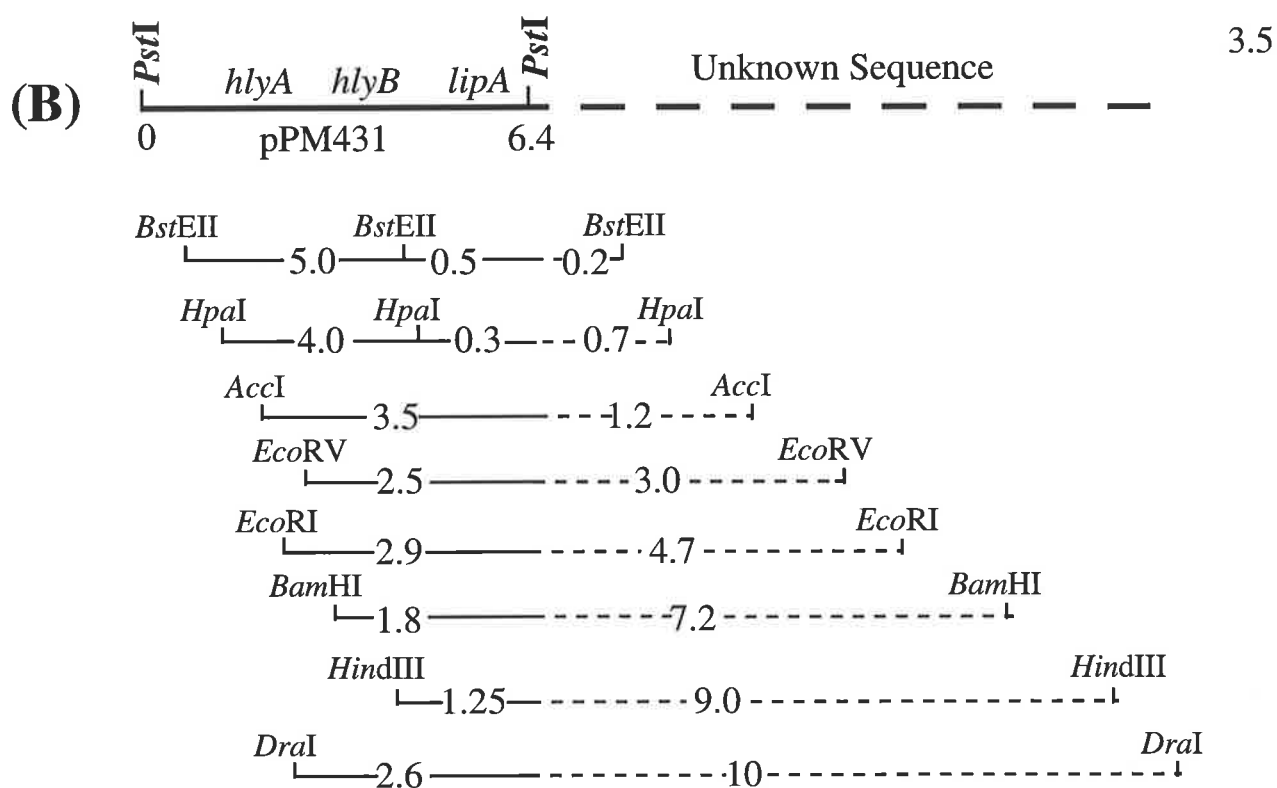
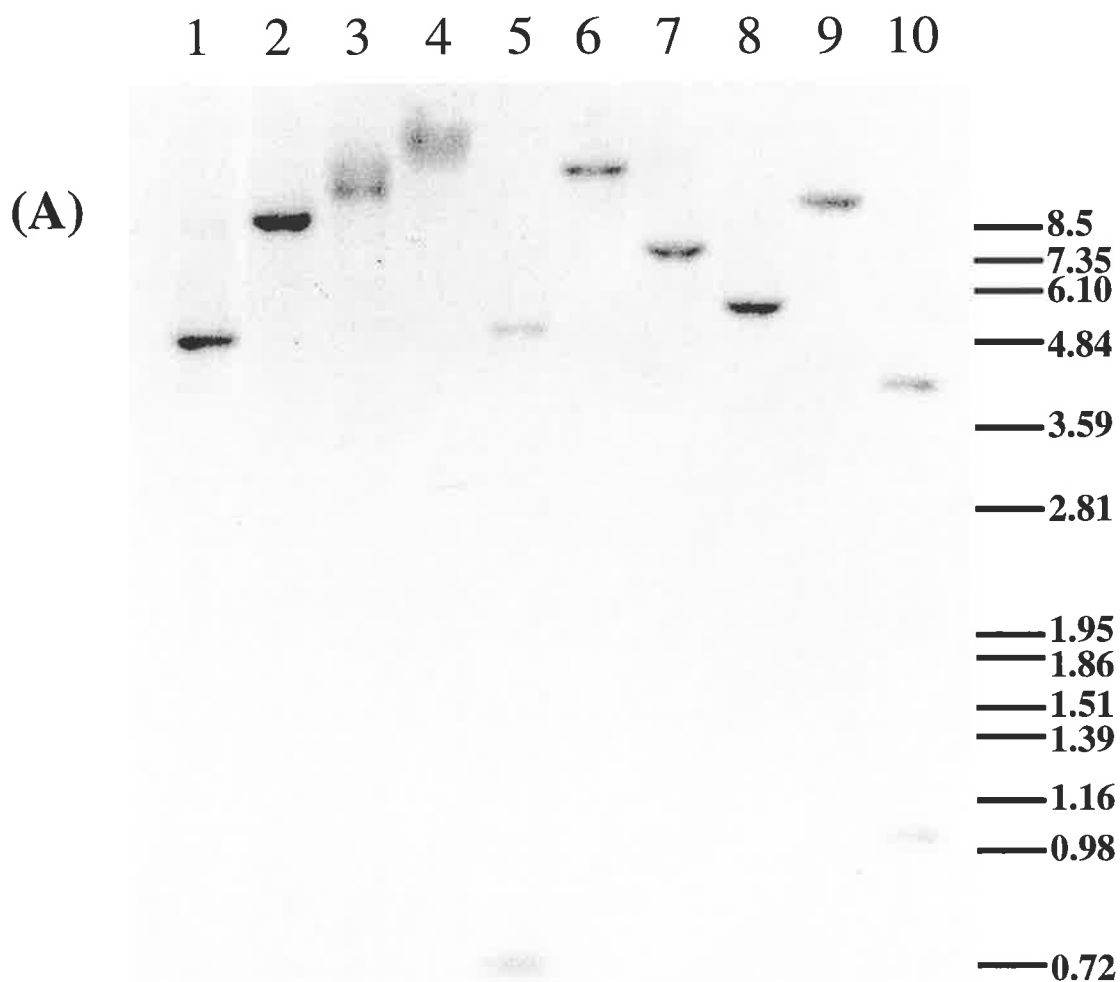
6.2.3.2 Construction of Sub-genomic libraries of *V. cholerae* O1

According to the Southern blot analysis of Figure 6.3, *Eco*RV and *Eco*RI restriction sites lie 3 kb and 4.7 kb downstream of the 6.4 kb *Pst*I site, respectively. These restriction sites were utilised to construct two sub-genomic chromosomal libraries of *V. cholerae*. Initially, a sub-genomic library was generated by digesting chromosomal DNA with *Eco*RV at 37°C for seven hours, followed by electrophoresis in a TAE agarose gel. Based on the restriction map in Figure 6.3, *Eco*RV fragments of approximately 5-6 kb were isolated and purified from the gel, and subsequently ligated into *Eco*RV restricted pBluescript SKTM. The ligated DNA was transformed into *E. coli* DH5 α and the library generated was screened

Figure 6.3 Mapping of restriction sites downstream of *hlyAB*.

Panel A shows a Southern blot of chromosomal DNA from *V. cholerae* O17 digested with various restriction enzymes, and probed with a DIG-labelled fragment derived from the PCR amplification of the region from nts 5830 to 6446. Lanes 1-10: *AccI*; *BamHI*; *BstXI*; *BglII*; *BstEII*; *DraII*; *EcoRI*; *EcoRV*; *HindIII*; *HpaI*. The *EcoRI*-restricted SPP1 size markers are shown in kb to the right of lane 10.

Panel B shows a putative restriction enzyme map of the chromosomal region downstream of *hlyAB*, and is derived from data obtained from the Southern blot in panel A. The 6.4 kb *PstI* fragment carrying *hlyAB* and partial *lipA*, has previously been cloned and sequenced (Manning *et al.*, 1984; Alm *et al.*, 1988; Alm and Manning, 1990a) and this DNA is indicated with a solid line. The sequence downstream from this region is unknown and is indicated with a dotted line. Restriction sites, both known and predicted, are indicated.



in a DNA colony blot using a DIG-labelled PCR probe described earlier in section 6.2.3.1. As a result, pPM4355 was isolated and this construct encodes complete *lipA* on a 5.6 kb *EcoRV* fragment (Figure 6.4).

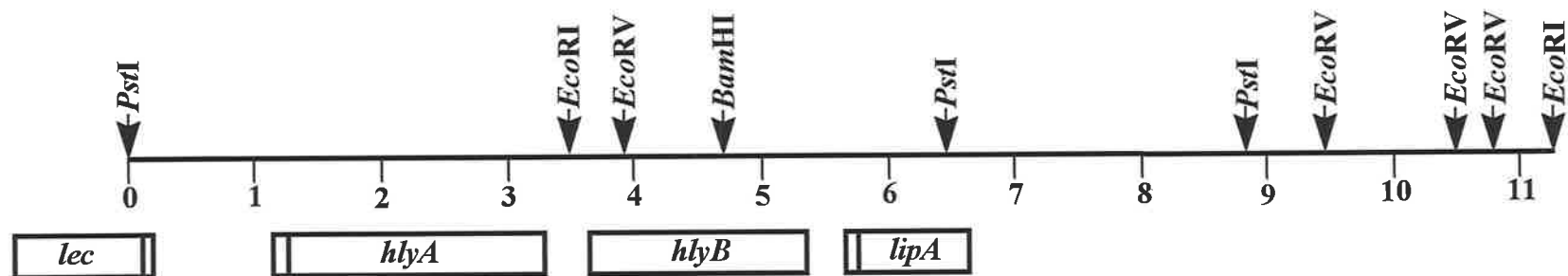
Following the sequencing of the insert of pPM4355 (discussed later in Section 6.2.4), an additional chromosomal library was constructed which enabled the isolation of DNA further downstream. This library was generated in the same way as the *EcoRV* library, with the exception that the chromosomal DNA was restricted with *Bam*HI / *Eco*RI and fragments of approximately 6-7 kb were isolated and ligated into pGEM3Zf. The DNA library was screened using a DIG-labelled PCR product of the region that lies between 6863-7510 nts (Figure 6.4). The region was amplified with oligonucleotides #2208 and #2209, using pPM4355 as the template. The sub-genomic clone pPM5105 was isolated from the DNA library and incorporates DNA downstream of *hlyAB* on a 6.4 kb *Bam*HI / *Eco*RI fragment (Figure 6.4).

6.2.3.3 Subcloning the downstream region of *hlyAB*

To obtain the complete *lipA* gene and determine what lies downstream, the 4.7 kb region 3' to the 6.4 kb *Pst*I site (Figure 6.4) was sequenced. This region is present on both pPM4355 and pPM5105, and so appropriate subclones (represented schematically in Figure 6.5) were generated as follows: The 2.5 kb and 0.6 kb *Pst*I fragments of pPM4355 (sites lie at nts 6446 and 9884, and downstream in the polylinker of pPM4355) were isolated and ligated into pBluescript KS which had been previously linearized with *Pst*I and treated with alkaline phosphatase. The constructs with the 2.5 kb or 0.6 kb *Pst*I fragments were designated pPM4358 and pPM4359, respectively. Various deletions within the 2.5 kb *Pst*I fragment of pPM4358 were constructed as follows; pPM4358 was restricted with enzymes *Hpa*I / *Hind*III (*Hpa*I site lies at nt 7179 and *Hind*III downstream in the polylinker) and religated to remove the intervening 1.8 kb of DNA, to generate pPM4360. The first 200 bp of the insert of pPM4360 was removed using the *Bam*HI (site lies upstream in vector polylinker) and *Bst*EII (lies at nt 6640) restriction sites, which generated plasmid pPM4361. pPM4363 was constructed by digesting pPM4358 with *Hpa*I / *Bam*HI (*Hpa*I site lies at nt

Figure 6.4 Sub-genomic cloning of the region downstream of *hlyAB*.

A 5.6 kb *EcoRV* and a 6.4 kb *Bam*HI / *Eco*RI fragment carrying *lipA* was isolated from a sub-genomic chromosomal library of *V. cholerae* O1 strain O17. The *EcoRV* fragment was ligated into pBluescript SK to generate pPM4355. The *Bam*HI / *Eco*RI fragment was ligated into pGEM3Zf to generate pPM5105. pPM431 has been described elsewhere (Manning *et al.*, 1984).



pPM431



pPM4355



pPM5105

7179 and *Bam*HI lies upstream in the polylinker) followed by re-ligation to remove the intervening 0.7 kb. pPM4362 was generated by restricting pPM4358 with *Acc*I (sites lie 7686 and downstream in the vector polylinker) and re-ligating to remove the intervening 1.3 kb. The 1.3 kb *Acc*I fragment of pPM4358 was also isolated and ligated into pBluescript KS which had been linearised with *Acc*I and treated with alkaline phosphatase. This generated plasmid pPM4364.

To ensure unambiguous sequence data, the 1.3 kb insert of pPM4363 was further sub-cloned, as follows: The 0.9 kb and 0.27 kb *Bst*XI fragments of pPM4363 (generated by sites at nts 7796, 8746 and downstream in the vector polylinker) were isolated and end-filled, and ligated separately into the end-filled *Bst*XI site of pBluescript KS. It was necessary to end-fill the *Bst*XI restricted DNA, as there is ambiguity in the recognition sequence for *Bst*XI, such that sticky-ends of DNA created by *Bst*XI are not necessarily compatible. The 0.9 kb and 0.27 kb *Bst*XI fragments that were successfully ligated into pBluescript KS were designated pPM4366 and pPM4365, respectively. The 0.9 kb insert of pPM4366 was further subcloned, to generate pPM4367 and pPM4368. pPM4367 was achieved by restricting pPM4366 with *Mlu*I (site lies at nt 8282) and *Sac*I (site lies downstream, in the vector polylinker) and re-ligating to remove the intervening 0.4 kb of DNA. Similarly, pPM4368 was generated by restricting pPM4366 with *Mlu*I and *Xba*I (*Xba*I lies upstream in the vector polylinker).

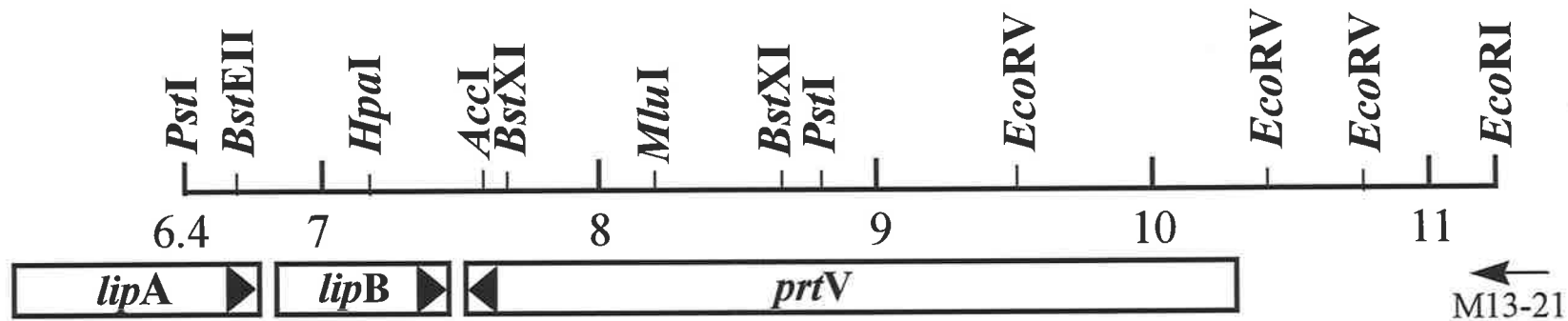
Two *Eco*RV fragments of approximately 0.9 kb and 0.4 kb were isolated from pPM5105 and ligated separately into pBluescriptKS-Cm, to generate pPM5106 and pPM5107, respectively (Figure 6.5) (*Eco*RV sites lie at nts 9580, 10462 and 10837).

6.2.4 Nucleotide sequence analysis of region downstream of *hlyAB*

It has previously been reported that *lipA* encodes an 18.3 kDa protein (Alm and Manning, 1990a; Casanova and Peterson, 1995), however, sequencing 3' to the 6.4 kb *Pst*I site revealed a continuation of the open reading frame for *lipA* to 933 bp which is now predicted to encode a 33 kDa protein (Figure 6.5 and 6.6).

Figure 6.5 Strategy used for sequencing the 4.7 kb *Pst*I / *Eco*RI region downstream of *hlyAB*.

The number scale indicates the position in kb on the sequence and the location of relevant restriction sites are indicated. The various sub-clones used for sequencing the region downstream of the 6.4 kb *Pst*I site of pPM431 (Manning *et al.*, 1984) are shown as lines below the map, and are annotated with the subclone name. Sequencing was achieved using either dye-labelled primers (-21 M13 universal primer or M13 reverse primer), or dye-labelled dideoxyterminators with synthetic oligonucleotides #2217 and #2316, as indicated. The region sequenced covered part of *lipA* and the entire *lipB* and *priV*.



pPM4359
 pPM5106
 pPM5107
 pPM4358
 pPM4360
 pPM4361
 pPM4362
 pPM4363
 pPM4364
 pPM4365
 pPM4366
 pPM4367
 pPM4368

Figure 6.6 Nucleotide sequence of the *V.cholerae* O1 region containing *lipA*, *lipB* (*orf1*) and *prtV* (*orf2*).

The nucleotide sequence starts from nucleotide position 5461 as it is a continuation of that shown in Figure 6.4, and continues to position 11116, the last 6 nts corresponding to an *EcoRI* site. ORFs are indicated by the single letter code, below the nucleotide sequence with the stop codons indicated by asterisks. The first and last nt or amino acid of each line is numbered. The position of potential ribosome binding site (rbs) sequences are indicated in boldface and putative -35 and -10 promoter sequences are indicated. The positions of relevant restriction enzyme cleavage sites are also indicated. Initiation codons are underlined and LipA is proposed to initiate with leucine. The initiation codon previously predicted for *lipA* (Alm and Manning 1990a; Casanova and Peterson, 1995) at nt 5578-5580 is underlined. *PrtV* (ORF2) is in the opposite polarity to the nucleotide sequence and the position of the N- and C-terminal ends are shown. The putative translation initiation and termination sites of *prtV* are indicated by complementary strand sequence. Potential signal cleavage sites of LipA and *PrtV* are indicated by a vertical arrow. The proposed bi-directional terminator is marked with inverted arrows which correspond to a proposed stem-loop structure. The two direct repeats containing two copies of a putative rbs of *prtV* are indicated with arrows in the same direction. Sequence from nucleotides 5461-6446 was originally reported by Alm and Manning (1990a), however some corrections are reported here. These sequence data have been submitted to the EMBL/GenBank Data libraries under the accession number X16945.

TTGACA TATAAT
 5461 GTTAATGAAATGTTATTAATTTATGTTTTGTTTCACATTTTGACCTTTTGAGATTTCGGAATCCGCCACTTCTCTTAGCTTAATTAACGGTAAGAGGTCTGACTTCTACCGAGTAATG 5580
 -35 -10 +1
 5581 rbs lipA
 5' ACCCTGTTTTTCAGTTAATGAGACTCACGCCACTTCGTGTGGTTCCAAGACTGAAGACAAGAACCAGAGAAGGAATATCCCTTGAATAAAATCATTATATTAATCGCTCTCTCACTGTTTTTCT 5700
 L N K I I I L I A L S L F S 14
 5701 TCCTCAATCTGGGCCGGAACCAAGTGTCTCACGCGTTATCGCAACAAGGCTATACCCAAACCGTTATCCAATTGTCTTAGTTTCATGGCCTATTTGGCTTCGACACCTTGGCGGGGATGGAT 5820
 15 S S I W A G T S A H A L S Q Q G Y T Q T R Y P I V L V H G L F G F D T L A G M D 54
 ↑
 5821 TACTTTCACGGCATTCCACAATCACTGACCCGAGATGGCGCTCAAGTGTATGTGGCACAGGTTTCAGCGACCAACAGCTCCGAGCGCCGAGGTGAGCAGTTATTGGCTCAGGTGGAATCG 5940
 55 Y F H G I P Q S L T R D G A Q V Y V A Q V S A T N S S E R R G E Q L L A Q V E S 94
 BstEII
 5941 CTACTGGCGGTAACCGGAGCGAAAAAGTGAACCTTGATTGGGCATAGTCATGGTGGCCCCACCATTGCTATGTGGCATCAGTGCGTCCGGATTTAGTCGCCTCAGTGACCAGTATTGGC 6060
 95 L L A V T G A K K V N L I G H S H G G P T I R Y V A S V R P D L V A S V T S I G 134
 6061 GGGGTGCATAAAGGCTCTGCCGTTGCCGACTTAGTGCGTGGTGTGATTCCATCGGGTTCGGTGAGTGAGCAGGTAGCGGTTGGGTTAACCCAAAGGCTTGGTGGCTTTGATTGATCTGCTC 6180
 135 G V H K G S A V A D L V R G V I P S G S V S E Q V A V G L T Q G L V A L I D L L 174
 6181 TCTGGCGGAAAGCGCATCCACAAGATCCCCTTGCTTCGCTGGCGGCGCTCACCACCGAAGGATCGCTGAAATTCAATCAGTATTATCCTGAAGGTGTTCCACATCAGCGTGTGGAGAG 6300
 175 S G G K A H P Q D P L A S L A A L T T E G S L K F N Q Y Y P E G V P T S A C G E 214
 6301 GGGGCGTACCAAGTGAATGGGTACGTTACTACTCATGGAGTGGCGCTGCGACCGTCACCAATATTCTTGATCCAAGCGATGTGGCGATGGGGTTGATTGGGTGGTCTTTAATGAGCCG 6420
 215 G A Y Q V N G V R Y Y S W S G A A T V T N I L D P S D V A M G L I G L V F N E P 254
 PstI
 6421 AATGATGGCTTGGTCGCAACCTGCAGTACTCATCTTGGCAAAGTGATCCGTGATGACTACCGGATGAACCATTTGGATGAGATCAACGGTCTGCTTGGTATTCACTCCCTGTTTGAAACC 6540
 255 N D G L V A T C S T H L G K V I R D D Y R M N H L D E I N G L L G I H S L F E T 294
 rbs lipB BstEII
 6541 GATCCGGTAACGTTATATCGCCAGCATGCGAACCGTCTCAAACAAGCTGGATTGTAGGAGCTTGCCATGAAAAAATCGCATGGAGTTTGGGGATTTTGGTGACCATAGGGGCACTCTGT 6660
 295 D P V T L Y R Q H A N R L K Q A G L * M K K I A W S L G I L V T I G A L C 18
 6661 GCGATTGTTTGGCCATCTTGGTATCCCTCTCGGCCCTTTAGTGACCACGCCATCGCAGCGGACATACAAGCAGATCAATCGAGTCCGCGCATCTCTTGGAATATTTTCTCTCAGGCTTG 6780
 19 A I V W P S W Y P S R P L V T T P S Q A D I Q A D Q S S P R D L L E Y F L S G L 58
 6781 GGTGAAACGCTCTTTACCCGTGATTTCAGCAGCAAGTGCAGCGTTATGAGCAGGAAAAACCAAGGCTTGCTCATGATAGCAGTCTGTTTTGCACAATATGTGCAGTATAAAGCGGCGTTATCT 6900
 59 G E T S L P V I Q Q Q V Q R Y E Q E N Q G L L I D S S L F A Q Y V Q Y K A A L S 98
 6901 GAGCTGACGCTGCCGCAAGCCAGCGGGGGGCTTTTCGACCCAAGAGTGGTGGCAATTGCACCAGAGCTTGTTAGATCTGCAAGCTCGTTATTTTTCTGCTGAGCAGCAAGCGCTGTTTGCA 7020
 99 E L T L P Q A S G G L S T Q E W W Q L H Q S L L D L Q A R Y F S A E Q Q A L F A 138

7021 GAGGAGAATCGGCTGCGCGAATTGGCGATCGAAAAGCGCCGAATTTATGAGCAGTATGGGCAAAGTGAAGAGGCGCAGCGAGCTTGGCAAAGCGCTGTTGCTCGACCAGCCCGATTTTATT 7140
139 E E N R L R E L A I E K R R I Y E Q Y G Q S E E A Q R A W Q A L L L D Q P D F I 178
7141 CAGCGTAGTGAGGCGACAGCGCAGCTATTGCCGCGAGTTAACTCAAGCGGGGAGGGGGATACGAGCAACGCTATTTGGCTCGCGTTGCCTTGGTGGGTGAGCAAGGGGCGCAGCGTTTA 7260
179 Q R S E A T A Q L L P Q L T Q A G Q G D T Q Q R Y L A R V A L V G E Q G A Q R L 218
7261 GCGGAGCTCGATGATTCCCGTGCGACGTTTGAACAGCAGTTTCAGGACTATTATCAAGCGCGGGCCGCTATATTAGTTTCGCAACGAGCTCTCGGCCAGTGAGCAGCAAACCCAAATTCAG 7380
219 A E L D D S R A T F E Q Q F Q D Y Y Q A R A A I L V R N E L S A S E Q Q T Q I Q 258
7381 CAGTTACGAGAGCAGCACCTTTGCGCCTGAGCAGTGGCGCGTATTGATGCGTTAGAGCGGCTGAAAGATAACGAGAATAAGGCTTTTCGCATTGGCATGAGCCTTAAAAAAGGGGCACC 7500
259 Q L R E Q H F A P E Q W R R I D A L E R L K D N G E * 284
7501 CGAAGGTGCCCAAATCCATGGAAGGAGAAGGAAGATTACAGTTTGACTTTGATGGTGATGGTGGTGACTGCGCCGCGGTATCCATCACTTTGAGCTGCACATCATGGTGCCCATAGCT 7620
← AAT
967 COOH- * L K V K I T I T T V A G R D D M V K L Q V D H H G Y S 933
BstEII
7621 TGGGAAAATCGCAGTAAACATACGACCCCGCTTAATTTTACCATTTCGGCAGTGTCCATTTCGGTGTGACAAATGCGGCCATCACTGTTCGGTGTGCTGGTTGACCACATGGTGACCCAGCGACC 7740
892 P F I A T F M R G R K I K G N P L T W E T D V I R G D S D T S T S W M T V W R G 893
7741 TAAATGGATATAGTTGGCACTGGCTTGTGGTAACGCATTTCGGTGTGTCCACTTTAATGGTTTGTGATGAGTATCGCTATCGCCTTTGTTCATCCGTCACCGTTAAAGTGACAGAGTAAC 7860
852 L H I Y N A S A Q P L A N P T D V K I T Q Q H T D S D G K D D T V T L T V S Y S 853
7861 GCCTGCTTTGGTATATGACCAAGTTGGAGCGGCTTCGGTACTGGTTTGACCGTTACCAAAATCCACAAATAACTGACGATATTGCCATCGCTATCACTACTGGTGTTTTGTGACATCAC 7980
812 G A K T Y S W T P A A E T S T Q G N G F D W L Y S V I N G D S D S S T N Q S M V 813
7981 AGAGAGCCCTCGACTTTTCAGTTCAAAGCGAGCCACAGGCGGATATTTCCGTTGGTGACTTTTCGACAAGCGAACAACACCGTACTCATTCTCTTTACTTTGTGAGAGCAGGTTCGATCTT 8100
772 S L G E V K L E F R A V P A I N E T T V K S L R V V G Y E N E K S Q S L L D I K 773
8101 CAAACCAAATGGCATCACTTTGCGGCCAGAATCTGGAGCTTGAGGCGAGCTGTAATCTTGATCGTCCGAGAACGAGGCATTTCGCGGTTAAGTTCATATCTTCCAGCGTATTGCCATCAGC 8220
732 L G F P M V K R G S D P A Q P S S Y D Q D D S F S A N A T L N M D E L T N G D A 693
8221 CGTGACCAGTTTGAGCGGCGCTTGATCAAACAGTGAGAAGGTTCATCAGCACCTTGGAACGCGTTTGTGCCACTTCCCCTGTTTTTGACCAGACCAAGGCATTTTGGTCGGCATCGAC 8340
692 T V L K L P A Q D F L S F T A D R V Q F R T Q A V E G T K S W V L A N Q D A D V 653
8341 ACGGCCTAGCCAGCCTTCAACCGGATGTTTGCCAACCCAGTTATCCGCGTAAGATTTCGTCCACATACCACACCGAGCAAGCCCGGCTCGAATGACATCAGTTGCCCGAAGCGTTTCAAATT 8460
652 R G L W G E G P H K G V W N D A Y S E D V Y W V L L G P E F S M L Q G F R K L N 613
8461 GGCTAAGCCTTGGTCAACGTCATTATGGCTGCGCCATTGCAGCAAGTAATAGTGATTGGCTTCGTGGAAGCCACCGTTTTCGGTGAAACCTTGGAACGCAAAGCTGGATGTGCCTTCTGC 8580
612 A L G Q D V D N H S R W Q L L Y Y H N A E H F G G N K T F G Q F A F S S T G E A 573
8581 GTTATCGATCAAGGTCTGATTGCCATCCACCTCAAGACGTAAGTCATCGACATACAGACCTTCCATGGCCAGACCGCCATCCGTCAGTAATCAAATGCCAGTTCAACGGTTTGGCCTGC 8700
572 N D I L T Q N G D V E L R L D D V Y L G E M A L G G D T L Y D F A L E V T Q G A 533
8701 CCAAGCAGAGAGATCAAATTGCGCATCTACCCAGCCACCATCAGATTGGCCTGAGATGGCAGGTACTAAACCTGATTTAAACGATCATCCATCGTCGTGATATTACCGGCAATCGGTTT 8820
532 W A S L D F Q A D V W G G D S Q G S I A P V L G S K F P D D M T T I N G A I P K 493

8821 GCCGTTAAATCAGCACACGCGCGTAGTCGTAATCTTTTTCATCTCTGGAACACAGCGCTTTAAAGCGCAACGTTGGCTTGGCTGCCTGCTGGGATCGTCAATTGGACGCGCTCATTCGGGTTTTTCAG 8940
492 G N I L V R A Y D Y D K E I Q F W A K F R L T A Q S G A P I T L P R S M R N K L 453
PstI
8941 ATCATCGCCACGGTTCGAGTAGAAGGAGAACTCACCTTCTGCAGGCTTAATGCCTTCAACCCGTTTCATCGGCAGAGTCACCTTACCACATGTTTCGGGCGTGAGTTATCTGTGGTTTGGAA 9060
452 D D G R N S Y F S F E G E A P K I G E V R K M P L T V K V M N P R S N D T T Q F 413
9061 TAGCGTAACACGCGCGGTTTGGCTTCTAACTCATTAAATCGAAAGCTGCTCATGGTTAATCCAGCGTCCGCCAATCGAATTTTGTAAAGAACTGCTTAGCCCATGAACTGAAAGCCGTGGG 9180
412 L T V V R P K A E L E N I S L Q E H N I W R G G I S N Q L F Q K A W S S F A T P 373
9181 CTGTGTACCGCCAATTTTGGCCGCCAGCTGCCAGATGACATGATTGACCAATAAGAGACGGGCTCTCCCGTACCTGTGTACTGGGTGTCATATTCATCGGGCAGACCTAAATCGTGACC 9300
372 Q T G G I K G A W S G S S M I S W Y S V P E G T G T Y Q T D Y E D P L G L D H G 333
9301 ATATTCTGTGGGCACACACGCGCGCAGCCGCATCAATCGGTTGAATGGTGTAAATCAAAGGCAGCGAATTGGCCATTGAAGCGTCCAGGAACGTTGCTTTTCGTGCCTTCAAGAACATGGTA 9420
332 Y E H A C V G A A A D I P Q I T Y D F A A F Q G N F R G P V N S K T G E L V H Y 293
9421 ACGGCCGAGGTTAAACCGGTGTGACCAATCGCATCCGCGCCCAACACACCGCCACCCGCTTCTTACCCACAGAGGCATGGAATCATCAAGTGATCTATCAGCCCATCTGGCTCACG 9540
292 R G L N F R H S W I A D A G L V G G G A E E G V S A H F I M L H D I V G D P E R 253
EcoRV
9541 GAAATTACCGTTACCGTTGTAGTCATAGCGATCTTCGATATCGTAATCGGCAAGGTTAATGTTTGGATCGCGCGCAAGTTGATCCAGTGCTTCGCGAACCAGCTCCCAGCATTTCATATC 9660
252 F N G N G N Y D Y R D E I D Y D A L N I N P D R A L Q D L A E R V L E R A N M D 213
9661 ATTGTTGGTACCGGAGAGTTGCCACCGTAATAAGCCGATTTTTTTGAGGCACGATACCATCCTGCTGCTTGGCGGAGACACTGTAGCTGTTGCCAGATTCACTCTCGTAATATTGACG 9780
212 N N T G P S N G G Y Y A A N K S A R Y W G A A Q G S V S Y S N G S E S E Y Y Q R 173
9781 CATTGAGATAAAGTTTTTACCCTTTGGACCGGTATAGCCTTTGTCCGAGAACAGCAAATCTTGGTAGTGAGAAGGCTCATAACGATCGTAGAGCATCTCAGTATGCTCTTTCGTGAGCG 9900
172 M S I F N E G N P G T Y G K D S F L L D Q Y H S P E Y R D Y L M E T H E K T L R 133
9901 GTTATCATCCCAAGGGAGATCGGGGAAATCGATCAATAGCGCGAGCACTTTGTCCGTGCGCTTTTGAACCAACATCTAAAGCAAAAACACGCGCCTTGTGCGGGCCTTTTGTCTATCGAT10020
132 N D D W P L D P F D I L L A L V K D T R K Q G V D L A F V R A K H P G K Q K D I 93
10021CACTTTAAGAATTTTCGCACGCTGCTCGAGCGCTTTCTTACCAAATTGCGCATCACCTTTGAATCCGAGCGAATTTTCTCCTCCAGATAACGTTCTAGCGCAATACGTTTATCAACGTC10140
92 V K L I K A R Q E L A K K G F Q A D G K F G S R I K E E L Y R E L A I R K D V D 53
10141AGAGGCATCGGCAGGAATTTGTCCGTTGCGGACTAACATTTCAATTAAATTATCTCATTCACCACGCGCTAAATCAATGGGTGTTTGAGCGTATAATCCACTGCTGAAAAAATGCGCTAT10260
52 S A D A P I Q G T R V L M E I L K D E N V V G L D I P T Q A Y L G S S F F S A I 13
prtV ↑
10261GGCGGCAGCTAATAGCGTTTTTTTGATCGTTTTTCATTTTATTTCCCTTAATATTTCCCTTATTTAAGGTGTGAAAAGGCCAGCCGAAAATAACAATCTTCGGTTAACCAGATTGGGTGATGG10380
GTAAATAAAGGAATTATAAAGGAATAAAT
← ←
12 A A A L L T K K I T K M -NH₂ rbs rbs 1

EcoRV

10381CTATGGCGACTCTGGATTAATACAGCGTGGCTTTAATTGAGGGTTTTCACCTGAGGAAGAGGATTCGCTATCAGCTTTGATATCGTTATTCTTATTATTAAACCTTTGCTGTAAGTAGAG10500

10501GGATAATGCTTTTTTGTGCTGCTCATAAGAAAGCCCTTCGCAGATAATGCCTTGTTCAATCAAGGCACTAACTAGCTTTTCATCATTAGGCTGAGCATTGGCAAATAATAAGGTGGAATA10620

G TATTAT
+1 -10

10621GCAGATTACCCATAATCCAAATAGGGTGCGCATAGTACATATCCATATGTAAAGTATTTGTAACAATTTATGGCTGGCGATAATTCATAGCAAGCCTGAATTATTCATTGTTTGGCATAG10740

ATGGGT
-35

EcoRV

10741CATCACATTTGCAACACATTGAGTAAACAAATCATCAATCGAATAAATGACTAATAAGACAATTAAGTATGCACAATATCGACATGGATAAATGATATCTATGTCGATATAAAACATCGT10860

10861AATTGTTAGTTAGGTCGTAAATAAAGCAATTAGAGCACATAAATAATCAGTGAAATTAAACATGCTATCAATATATGCGAAATCCCATCACTCGATTGACGATAAAATAAAGGGAAA10980

10981TAATTTGATCTTGCTTCATTGATTTTGTTCGATTTGACGCTGTCGTGGTGTGAGCGGTTTTGGTACACTCTATCCCTTTCCCTACTTGACGCCGAGCGGTGTTGATCGGTCAAAAGAG11100

EcoRI

11101GGGATAGGGCGACGAATCGAGAGCGGATTGAAGCCTTATACTGCAAGCGTTGGCTAGAATTC 3' 11116

It was also reported that translation of *lipA* initiates at the AUG codon corresponding to nt 5578-5580 of the *Pst*I fragment, and that LipA does not possess an amino terminal signal sequence (Alm and Manning 1990a; Casanova and Peterson, 1995). However, it is predicted that the codon UUG, which normally encodes for leucine, but can act as an alternate initiation codon (Gold and Stormo, 1987) and lies 27 codons downstream of the previously reported start site, is the translational initiation site for *lipA* (Figure 6.6). Initiation of translation from UUG would generate an excellent amino terminal signal sequence according to the constraints of von Heijne (1985). Cleavage of the signal sequence is predicted to occur between residues Ala-25 and Leu-26, with an Ala residue at -1 and -3, and the helix-breaking residue, Gly at -6 (Figure 6.6). While AUG is used 91% as the preferred initiation codon of prokaryotes (Gold and Stormo, 1987), this does not necessarily infer that *lipA* will be poorly transcribed, as other factors such as a good ribosome binding site (AGGA) with optimal spacing (7 bases from the initiation codon of *lipA*) may compensate for the non-optimal initiation codon (Kozak, 1983).

Beyond the termination codon of *lipA* are two additional open reading frames, *orf1* and *orf2* which has been subsequently designated *lipB* and *pvtV*, respectively (section 6.2.5). *lipB* initiates 9 bp downstream of the stop codon of the preceding *lipA* and its potential ribosome binding site (RBS), AGGAG, overlaps the stop codon of *lipA*, suggesting that they are translationally coupled. *pvtV* lies downstream of *lipA* and *lipB* but is transcribed in the opposite direction and is preceded by two copies of a putative ribosome binding site, present on a 10 bp tandem repeat sequence, TAAGGAAATA (the RBS is underlined) (Figure 6.6). Multiple ribosome binding sites have previously been reported for the chloramphenicol acetyltransferase gene, which is preceded by three functional ribosome binding sites (Ambulos *et al.*, 1986). A typical signal sequence is present at the N-terminus of *PrtV* (Figure 6.6), which conforms with the constraints of von Heijne (1985). There is a charged residue (Lys) at the extreme N-terminus, followed by a hydrophobic stretch. Cleavage of the signal sequence is predicted to occur between residues Ala-23 and Gln-24, with a -1 of Ala, and the helix-breaking residue, Gly, at -4.

A stem-loop structure resembling a *rho*-independent terminator was found in the intergenic region between *lipB* and *prtV*, and probably functions as a bidirectional terminator (Figure 6.7). The free energy of the structure ($\Delta G = -22.4 \text{ kCal mol}^{-1}$) indicates that it would be highly favourable (Tinoco *et al.*, 1973), and multiple A residues are present 5' and 3' to the stem-loop.

Primer extension analysis has been used to map the 5' end of the *lipA* mRNA and localised a potential σ^{70} consensus promoter at appropriate spacing from the 5' end at nt 5544, with the (-35) sequence TTGAGA spaced 17 nts upstream of (-19) sequence TCTCTT, as shown in Figure 6.6 (S. Williams, personal communication). Potential -10 and -35 sequences upstream from the *prtV* ATG codon (Figure 6.6) were identified by primer extension analysis of *prtV* mRNA (S. Williams, personal communication).

6.2.5 Homology of *V. cholerae* LipA, LipB and PrtV with other proteins

The predicted protein sequences obtained were used to search the NCBI database for similar entries, using BLAST. Significant matches were obtained for *lipA*, *lipB* and *prtV* and are discussed in detail in the following sections.

6.2.5.1 *lipA* is homologous to the lipases of *Pseudomonas* spp.

A search of the database with the 33 kDa LipA of *V. cholerae* shows strong homology with the triacylglyceride-specific lipase genes of *Pseudomonas* spp. (Table 6.1). The highest degree of similarity was observed with *P. aeruginosa* (59%) (Chihara-Siomi *et al.*, 1992; Wohlfarth *et al.*, 1992), while others included the lipase genes of *P. fragi* (47%) (Kugimiya *et al.*, 1986; Aoyama *et al.*, 1988), *P. glumae* (43%) (Frenken *et al.*, 1992), *P. cepacia* (43%) (Jorgensen *et al.*, 1991) and *Acinetobacter calcoaceticus* (45%) (Kok *et al.*, 1995a), all of which share homologies in significant regions.

Sequence alignment studies reveal lipases and lipoprotein lipases have a consensus pentapeptide Gly-X₁-Ser-X₂-Gly (where X₁ is His in prokaryotic lipases), similar to the one common to serine proteases (Antonian., 1988; Chihara-Siomi *et al.*, 1992; Derewenda and Sharp, 1993). Crystallization and 3-D structure analysis of eukaryotic lipases has revealed a

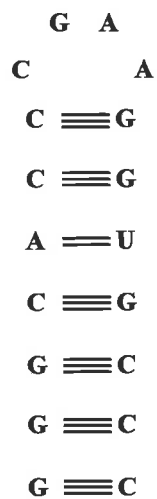
Figure 6.7 A potential bidirectional terminator downstream of *lipAB*.

The stem-loop structure ($\Delta G = -22.4 \text{ kCal mol}^{-1}$) in the intergenic region of *lipB* and *pvtV* is proposed to be a bi-directional terminator. Arrows indicate the direction of transcription of the flanking genes. The complementary strand is only shown for *pvtV*.

lipB
(*orf1*)

GAUAACGGAGAAUAAGGCUUUUCGCAUUGGCAUGAGCCUUAAAAAAG

Asp Asn Gly Glu ***



$$\Delta G = -22.4 \text{ kcal mol}^{-1}$$

priV
(*orf2*)

AAAUCCAUGGAAGGAGAAGGAAGAAUUACAGUUUGACUUU

*** Leu Lys Val Lys



Table 6.1Comparison of *V.cholerae* LipA with other lipases^a

	<i>V.cholerae</i> ^b	<i>P.aeruginosa</i> ^c	<i>P. fragi</i> ^d	<i>A.calcoaceticus</i> ^e	<i>P.glumae</i> ^f	<i>P.cepacia</i> ^g
<i>V.cholerae</i>	100 [312]	59.1 [308]	47.3 [262]	45.2 [301]	42.8 [228]	42.6 [216]
<i>P. aeruginosa</i>		100 [311]	45 [262]	51.6 [275]	39.2 [237]	37.7 [231]
<i>P. fragi</i>			100 [277]	39 [272]	42.9 [182]	43.4 [182]
<i>A. calcoaceticus</i>				100 [323]	36.2 [240]	37.5 [216]
<i>P. glumae</i>					100 [358]	78 [364]
<i>P. cepacia</i>						100 [364]

^aNumbers, % identity determined using FASTA as implemented by PROSIS Version 7 (LKB-Hitachi software).
Numbers in brackets, number of aa over which the indicated identity occurs.

^bAccession No. X16945

^cAccession No. JT0958

^dAccession No. P08658

^eAccession No. X80800

^fAccession No. Q05489

^gAccession No. A16316

catalytic triad of residues Ser-Asp-His (Brady *et al.*, 1990; Winkler *et al.*, 1990) which is also conserved among *Pseudomonas* lipases (Chihara-Siomi *et al.*, 1992; Jaeger *et al.*, 1993). The serine residue is common to both the pentapeptide and the catalytic triad. The sequence alignment of *V. cholerae* LipA with the lipase of *P. aeruginosa* showed that both the pentapeptide Gly-His-Ser-His-Gly (108-112) and catalytic triad Ser (110) Asp (256) His (278) regions are conserved (Figure 6.8). The homology also included the signal peptide sequence which supports the prediction for the alternative initiation codon (UUG) for *lipA* (section 6.2.4).

The *P. aeruginosa* lipase has a single functional disulphide bond connecting residues Cys(183) and Cys(235) (Jaeger *et al.*, 1993), and the presence and the position of the Cys residues are also conserved in *P. cepacia* (Jorgensen *et al.*, 1991), *P. glumae* (Frenken *et al.*, 1992), as well as the *V. cholerae* lipase shown here, with Cys(212) and Cys(262) highlighted in Figure 6.8.

6.2.5.2 *lipB* is homologous to accessory-lipases

The amino acid sequence of *orf2* was 29% and 25% homologous to accessory-lipase proteins LipB of *A. calcoaceticus* (Kok *et al.*, 1995b) and LipH of *P. aeruginosa* (Chihara-Siomi *et al.*, 1992; Wohlfarth *et al.*, 1992), respectively (Table 6.2). Based on this homology, *orf2* was designated *lipB*. A hydropathy plot of the derived amino acid sequence of *V. cholerae* LipB (data not shown) reveals a distinct hydrophobic region at the N-terminus, between the residues 4 to 28, and is highlighted in the alignment with the *P. aeruginosa* LipH (Figure 6.9). This is indicative of membrane-associated domains, as the hydrophobic region would be able to span the membrane bilayer. Indeed, Frenken *et al.* (1993a) showed LipB of *P. glumae* is anchored in the inner membrane by an N-terminal hydrophobic transmembrane helix domain located at the N-terminus, while the rest of the protein is periplasmically located.

Most lipases from *Pseudomonas* spp. require a downstream accessory gene for activity, and has been reported for *P. aeruginosa* PAO1 and TE3285, *P. cepacia* DSM3959, *P. glumae* and *P. sp* 109 (Wohlfarth *et al.*, 1992; Chihara-Siomi *et al.*, 1992;

Figure 6.8 Comparison of the *V. cholerae* O1 (Vc) and *P. aeruginosa* (Pa) lipase (LipA) proteins.

LipA of *P. aeruginosa* is a triacylglyceride-specific lipase (Chihara-Siomi *et al.*, 1992; Wohlfarth *et al.*, 1992). The pentapeptide consensus motif (GX₁SX₂G) found in the active site of this family of lipases is highlighted. Squares indicate the conserved residues (Ser, Asp, His) of the catalytic triad. The cysteine residues conserved among *Pseudomonas* spp. and present in *V. cholerae* LipA are indicated by closed circles. The homologous proteins were initially found using BLAST e-mail searches of the databases at NCBI. The alignment of the amino acid sequences was performed using Clustal V (Higgins and Sharp, 1988 and 1989). Identical residues are indicated by asterisks (*), conserved residues with similar properties are indicated by dots (•). Gaps (dashes) have been introduced by the program to optimize the alignment.

VcLipA MNK I I I L I A L S L F S S S I W A G T S A H A L S Q Q G - Y T Q T R Y P I V L V H G L F G F D T 49
PaLipA M K K K S - L L P L G L - - A I G L A S L A A S P L I Q A S T Y T Q T K Y P I V L A H G M L G F D N 47
* . * * . . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . *

VcLipA L A G M D Y F H G I P Q S L T R D G A Q V Y V A Q V S A T N S S E R R G E Q L L A Q V E S L L A V T 99
PaLipA I L G V D Y W F G I P S A L R R D G A Q V Y V T E V S Q L D T S E V R G E Q L L Q Q V E E I V A L S 97
. * . * * * * * . * * * * * * * * . . * * . . * * * * * * * * * * . . * . .

VcLipA G A K K V N L I G H S H G G P T I R Y V A S V R P D L V A S V T S I G G V H K G S A V A D L V R G V 149
PaLipA G Q P K V N L I G H S H G G P T I R Y V A A V R P D L I A S A T S V G A P H K G S D T A D F L R Q - 146
* *

VcLipA I P S G S V S E Q V A V G L T Q G L V A L I D L L S G G K A H P Q D P L A S L A A L T T E G S L K F 199
PaLipA I P P G S A G E A I L S G L V N S L G A L I S F L S S G S T G T Q N S L G S L E S L N S E G A A R F 196
* * . * * . * . * * . . * * * * . * * . * . * . * . * . * . * . * . * . * . *

VcLipA N Q Y Y P E G V P T S A C G E G A Y Q V N G V R Y Y S W S G A A T V T N I L D P S D V A M G L I G L 249
PaLipA N A K Y P H G V P T S A C G E G A Y K V N G V S Y Y S W S G S S P L T N F L D P S D A F L G A S S L 246
* * * . * . * * . * * * * * * * * . *

VcLipA V F N E - - P N D G L V A T C S T H L G K V I R D D Y R M N H L D E I N G L L G I H S L F E T D P V 297
PaLipA T F K N G T A N D G L V G T C S S H L G M V I R D N Y R M N H L D E V N Q V F G L T S L F E T S P V 296
* . . . * * * * * . * * * . *

VcLipA T L Y R Q H A N R L K Q A G L 312
PaLipA S V Y R Q H A N R L K N A S L 311
. . * * * * * * * * * . * . *

Table 6.2Comparison of *V.cholerae* LipB with other accesory proteins^a

	<i>V.cholerae</i> ^b	<i>A.calcoaceticus</i> ^c	<i>P.aeruginosa</i> ^d
<i>V.cholerae</i>	100 [284]	29.3 [249]	25.4 [248]
<i>A.calcoaceticus</i>		100 [343]	33 [285]
<i>P.aeruginosa</i>			100 [282]

^aNumbers, % identity determined using FASTA as implemented by PROSIS Version 7 (LKB-Hitachi Software). Numbers in brackets, number of aa over which the indicated identity occurs.

^b Accession No. X16945

^c Accession No. X80800

^d Accession No. Q01725

Figure 6.9 A comparison of *V. cholerae* O1 LipB (ORF1) with *P. aeruginosa* LipH.

LipH of *P. aeruginosa* is an accessory-lipase protein, required for the production of active triacylglyceride-specific lipase (Jaeger *et al.*, 1993; Oshima-Hirayama *et al.*, 1993). Hydrophobic residues at the *V. cholerae* LipB N-terminus proposed to be an anchoring domain, are indicated as closed circles. The homologous proteins were initially found using BLAST e-mail searches of the databases at NCBI. The alignment of the amino acid sequences were performed using Clustal V (Higgins and Sharp, 1988 and 1989). Identical residues are indicated by asterisks (*), conserved residues with similar properties are indicated by dots (·). Gaps (dashes) have been introduced by the program to optimize the alignment.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50			
VcLipB	M	K	K	I	A	W	S	L	G	I	L	V	T	I	G	A	L	C	A	I	V	W	P	S	W	Y	P	S	R	P	L	V	T	T	P	S	Q	A	D	I	Q	A	D	Q	S	S	P	R	D	L	50		
PaLipH	M	P	-	-	-	-	-	-	-	-	A	K	V	A	P	L	P	T	S	F	R	G	T	S	V	D	G	S	F	S	V	D	A	S	G	N	L	L	I	T	R	D	I	-	-	-	R	N	L	38			
	*															*															*							*		*					*		*						
VcLipB	L	E	Y	F	L	S	G	L	G	E	T	S	L	P	V	I	Q	Q	Q	V	Q	R	Y	-	-	-	E	Q	E	N	Q	G	L	L	I	D	S	S	L	F	A	Q	Y	V	Q	Y	K	A	A	L	97		
PaLipH	F	D	Y	F	L	S	A	V	G	E	E	P	L	Q	Q	S	L	D	R	L	R	A	Y	I	A	A	E	L	Q	E	P	A	R	G	Q	A	L	A	L	M	Q	Q	Y	I	D	Y	K	K	E	L	88		
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VcLipB	S	E	L	T	-	-	L	P	Q	A	S	G	L	S	T	Q	E	W	W	Q	L	H	Q	S	L	L	D	L	Q	A	R	Y	F	S	A	E	Q	Q	-	A	L	F	A	E	E	N	R	L	R	144			
PaLipH	V	L	L	E	R	D	L	P	R	L	A	D	-	-	-	L	D	A	L	R	Q	R	E	A	A	V	K	A	L	R	A	R	I	F	S	N	E	A	H	V	A	F	F	A	D	E	E	T	Y	N	135		
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VcLipB	E	L	A	I	E	K	R	R	I	Y	E	Q	Y	G	Q	S	E	E	A	-	-	-	-	-	Q	R	A	W	Q	A	L	L	L	D	Q	P	D	F	I	-	-	Q	R	S	E	A	T	A	Q	L	187		
PaLipH	Q	F	T	L	E	R	L	A	I	R	Q	D	G	K	L	S	A	E	E	K	A	A	A	I	D	R	L	R	A	S	L	P	E	D	Q	Q	E	S	V	L	P	Q	L	Q	S	E	L	Q	Q	Q	185		
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VcLipB	L	P	Q	L	T	Q	A	G	Q	G	D	T	Q	Q	R	Y	L	A	R	V	A	L	V	G	E	Q	G	A	Q	R	L	A	E	L	D	D	S	R	A	T	F	E	Q	Q	F	Q	D	Y	Y	Q	237		
PaLipH	T	A	A	L	Q	A	A	G	A	G	P	E	A	I	R	Q	M	-	R	Q	Q	L	V	G	A	E	A	T	T	R	L	E	Q	L	D	R	Q	R	S	A	W	K	G	R	L	D	D	Y	F	A	234		
	.																																																				

Jorgensen *et al.*, 1991; Frenken *et al.*, 1993a; Ihara *et al.*, 1992). However, this accessory protein was not detected in all *Pseudomonas* strains examined (Tan and Miller, 1992; Johnson *et al.*, 1993), which is perhaps not surprising considering the diversity of *Pseudomonas* spp.

6.2.5.3 *prtV* is homologous to metalloproteases

The amino acid sequence of *orf2* located downstream of *lipAB*, was found to be 40.9% homologous to the secreted neutral metalloprotease, immune inhibitor A (InA) of *Bacillus thuringiensis* var. *alesti* (Table 6.3). Consequently, *orf2* was named *prtV* (protease of *Vibrio cholerae*). The alignment of the amino acid sequence of PrtV and InA is shown in Figure 6.10. The InA protease has been shown to specifically cleave antibacterial proteins produced by the insect host (Dalhammar and Steiner, 1984) and has been implicated as a virulence determinant of *B. thuringiensis* (Lovgren *et al.*, 1990).

The primary sequence of InA has the zinc-binding and catalytic active site residues present in various other metalloproteases, including thermolysin (Lovgren *et al.*, 1990), and these are also present in PrtV. The significant residues are highlighted in panel A of Figure 6.11. The zinc-binding signature pattern present in PrtV is sufficient to detect members of the zinc-metalloproteinase superfamily of proteins (Jongeneel *et al.*, 1989; Murphy *et al.*, 1991).

PrtV also shares homology with a different group of proteases, including *Achromobacter* protease I (API), *Xanthomonas* carboxyl proteinase (XCP) and *Clostridium histolyticum* collagenase (ColH) (Table 6.3). While the overall homology among these proteins is limited, there is a small highly conserved region present (Figure 6.11, panel B), the function of which is currently unknown. This region was not conserved in *B. thuringiensis* InA.

A summary of the extended *hly* locus obtained by data presented thus far, is presented schematically in Figure 6.12.

Table 6.3Comparison of *V. cholerae* PrtV with other proteases^a

	<i>V. cholerae</i> ^b	<i>B. thuringiensis</i> ^c	<i>A. Lyticus</i> ^d	<i>Xanthomonas</i> spp ^e	<i>C. histolyticum</i> ^f
<i>V. cholerae</i>	100 [919]	40.9 [651]	45 [82]	40.2 [87]	36.8 [152]
<i>B. thuringiensis</i>		100 [687]	16.5 [97]	24.6 [57]	17.7 [96]
<i>A. Lyticus</i>			100 [653]	51.6 [157]	33.7 [89]
<i>Xanthomonas</i> spp				100 [798]	26.8 [97]
<i>C. histolyticum</i>					100 [898]

^aNumbers, % identity determined using FASTA as implemented by PROSIS Version 7 (LKB-Hitachi Software).

Numbers in brackets, number of aa over which the indicated identity occurs.

^bPrtV, Accession No. X16945^cInA, Accession No. p23382^dAPI, Accession No. sp15636^eXCP, Accession No. D83740^fColH, Accession No. D29981

Figure 6.10 Comparison of *V. cholerae* O1 PrtV (ORF2) with *B. thuringiensis* InA.

Immune inhibitor A (InA) of *B. thuringiensis* is a secreted metallo-protease which cleaves antibacterial proteins produced by the insect host (Dalhammar and Steiner, 1984). The homologous proteins were initially found using BLAST e-mail searches of the databases at NCBI. The alignment of the amino acid sequences was performed using Clustal V (Higgins and Sharp, 1988 and 1989). Identical residues are indicated by asterisks (*), conserved residues with similar properties are indicated by dots (·). Gaps (dashes) have been introduced by the program to optimize the alignment.

PrtV	M K T I K K T L L A A A I A S F F S S G L Y A Q T P I D L G V V N E D K L I E M L V R T G Q I P A D	50
InA	M K D A K A D - T K E K L N Q - - - P A T G T P A A	22
	* * * * *	
PrtV	A S D V D K R I A L E R Y L E E K I R S G F K G D A Q F G K K A L E Q R A K I L K V I D K Q K G P H	100
InA	T G P V - K G G L N G K V P - - - - - - - - - - - - - - T S P A	39
	* * * * *	
PrtV	K A R V F A L D V G Q K R T D K V L A L L I D F P D L P W D D N R L T K E H T E M L Y D R Y E P S H	150
InA	K Q K A Y N - - - G D V R K D K V L V L L V E Y A D F - - K H N N I D K E P G Y M Y S E D F N K E H	84
	* * * * *	
PrtV	Y Q D L L F S D K G Y T G P N G E N F I S M R Q Y Y E S E S G N S Y S V S G Q A A G W Y R A S K N A	200
InA	Y E K M L Y G D E P F A L E D G S K I E T F K Q Y Y E E Q S G G S Y T V D G T V T K W L T V P G K A	134
	* * * * *	
PrtV	A Y Y G G N S P G T N N D M N A - - - R E L V R E A L D Q L A R D P N I N L A D Y D I E D R Y D Y N	247
InA	A D Y G A D A A T G H D N K G P K G P R D L V K D A L K A - A V D S G L D L S Q F D Q F D Q Y D V N	183
	* * * * *	
PrtV	G N G N F R E P D G V I D H L M I F H A S V G E E A G G G V L G A D A I W S H R F N L G R Y H - V L	296
InA	G D G N Q N Q P D G L I D H L M I I H A G V G Q E A G G G K L G D D A I W S H R W T V G P K P F A I	233
	* * * * *	
PrtV	E G T K S N V P G R F N G Q F A A F D Y T I Q P I D A A A G V C A H E Y G H D L G L P D E Y D T Q Y	346
InA	E G T Q A K V P Y W - G G K M A A F D Y T I E P E D G A V G V N A H E Y G H D L G L P D E Y D T D Y	282
	* * * * *	
PrtV	T G T G E P V S Y W S I M S S G S W A G K I G G T Q P T A F S S W A K Q F L Q N S I G G R W I N H E	396
InA	T G H G E P I Q A W S V M S G G T W A G K I A G T T P T S F S P Q N K E F F Q K T I G G N W A N I V	332
	* * * * *	
PrtV	Q L S I N E L E A K P R V V T L F Q - - T T D N S R P N M V K V T L P M K R V E G I K P A E G E F S	444
InA	E V D Y E K L N K G I G L A T Y L D Q S V T K S N R P G M I R V N L P D K D V K T I R P A F G K Q Y	382
	* * * * *	

[illegible]

Figure 6.11 A comparison of conserved domains in PrtV with other proteases.

Panel A A comparison of the region around the known active sites and Zinc binding domains (indicated with an "A" and "Z", respectively) of *B. thermoproteolyticus* thermolysin, with the predicted ones of *B. thuringiensis* InA, *P. aeruginosa* elastase and *V. cholerae* PrtV.

Panel B A comparison of a region in PrtV with *Achromobacter* protease I (API), *Xanthomonas* carboxyl proteinase (XCP) and *C. histolyticum* collagenase (ColH). The function of this highly conserved region is currently unknown, and is the only significant region of homology among these proteins. This region was not conserved with *B. thuringiensis* InA.

The number of the first and last amino acid for each protein is indicated. Homologous proteins were initially found using BLAST e-mail searches of the databases at NCBI. The alignment of the amino acid sequences was performed using Clustal V (Higgins and Sharp, 1988 and 1989). Identical residues are indicated by asterisks, conserved residues with similar properties are indicated by dots. Gaps (dashes) have been introduced to optimise alignment.

A

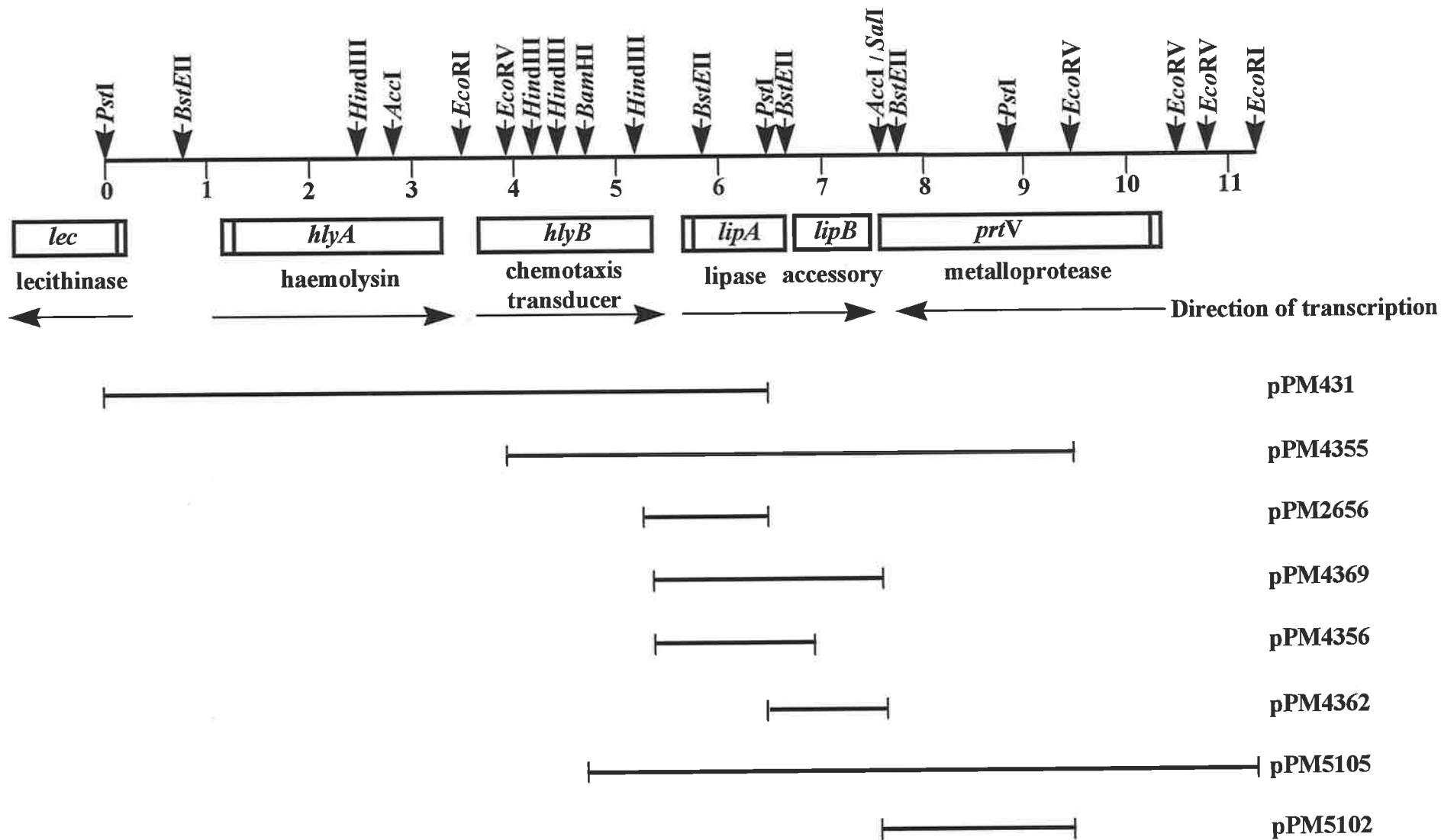
PrtV	315	D Y T I Q P I D A A A G V C A H E Y G H D L G L P D E - - - Y D T Q Y T G T G E P V S Y W S I M S S	361
InA	251	D Y T I E P E D G A V G V N A H E Y G H D L G L P D E - - - Y D T D Y T G H G E P I Q A W S V M S G	297
Thermolysin	127	G Q T F I P L S G G I D V V A H E L T H A V T D Y T A G L I Y Q N E S G A I N E A I - - - S D I F G	173
Elastase	126	A T V F Y P L V S - L D V A A H E V S H G F T E Q N S G L I Y R G Q S G G M N E A F - - - S D H A G	171
		. * . * * * *	
		Z A Z	A

B

PrtV	778	T S S D S D G N I V S Y L W D F G N G Q T S T E A A P T W S Y T K A G S Y S V T L T V T D D K G D S	827
API	495	S S T D S D G S I A S R S W N F G D G S T S T A T N P S K T Y A A A G T Y T V T L T V T D N G G A T	544
XCP	657	S S T D S D G S I A S H A W T F G D G S T S T A T S P S H T Y S A A G T Y S V A E T V T D N A G A T	706
ColH	750	G S F D P D G K I V S Y E W D F G D G N K S N E E N P E H S Y D K V G T Y T V K L K V T D D K G E S	799
		* * . * * * * * * * . * * * * . * . * * * . * .	

Figure 6.12 Genetic organisation of the *hly* and *lip* locus, and the *prtV* gene of *V. cholerae* O1.

Genes of the *hly* locus are labelled A and B, and genes of the *lip* locus are labelled A (formerly *hlyC*) and B. The metallo-protease gene *prtV* is transcribed in the opposite direction. The upper line shows relevant restriction enzyme sites of this region. Plasmid pPM431 has been described elsewhere (Manning *et al.*, 1984). Plasmids pPM4355, pPM2656, pPM4369, pPM4356, pPM4362, and pPM5105 used in this chapter are shown. The orientation of inserts are such that the T7 promoter is to the left of each insert (except for pPM5105, where the T7 promoter is to the right of the insert).



6.2.6 Expression of LipA and PrtV

The *lipA*, *lipB* and *prtV* genes of *V. cholerae* potentially encode 33, 32.6 and 102 kDa proteins, respectively. To confirm this, plasmids with various combinations of the genes were constructed and are shown schematically in Figure 6.12.

6.2.6.1 Cloning *lipA* and *lipB* behind the inducible T7 promoter

The strategies undertaken for the cloning of *lipA*, *lipB* and *lipAB* are as follows: a 1.3 kb fragment encoding only LipA, was generated by PCR amplification of the region between nts 5426 and 6704 using pPM4355 as the template and was ligated into pGEM-T to generate pPM4356 (Figure 6.13). pPM4358 (section 6.2.3.3) was digested with *AccI* (sites lie at nt 7686 and downstream in the vector polylinker) such that the intervening 1.3 kb of DNA was removed. The resulting construct was designated pPM4362 and encodes only LipB (Figure 6.13). Construct pPM4369, carrying both *lipA* and *lipB* was generated by combining the inserts of pPM4356 (*lipA*) and pPM4362 (*lipB*). An *ApaI* / *PstI* digest of pPM4356 was used to liberate the 1.3 kb insert carrying *lipA* (*ApaI* site lies in the polylinker, and *PstI* at nt 6446) and the fragment was ligated together with the 1.2 kb *PstI* / *AccI* fragment isolated from pPM4362, using the common *PstI* site, and this fragment ligated into the *ApaI* / *AccI* sites of pBluescript SKTM (Figure 6.13). The clones pPM4355, pPM4369, pPM4356 and pPM4362 (Figure 6.12) are orientated such that *lipA* and *lipB* are under the control of the T7 promoter.

6.2.6.2 Detection of LipA and PrtV

Protein expression was achieved using the T7 promoter / RNA polymerase system of Tabor and Richardson (1987), as described earlier (section 6.2.1). Two proteins migrating at 33 kDa and 30 kDa were produced by strains carrying pPM4356, pPM4369 and pPM4355 (Figure 6.14, panel A), all of which carry *lipA*. As pPM4356 encodes only LipA, and LipA has a predicted molecular mass of 33 kDa, and 30 kDa after the removal of the signal sequence, it can be concluded that the two observed bands (indicated by arrows A and B) represent unprocessed and processed LipA, respectively. Truncated LipA of 29 kDa was

Figure 6.13 Construction of plasmids encoding LipA, LipB and LipAB.

Oligonucleotides #2160 and #2158 were used to PCR amplify a region carrying the entire *lipA* gene, using pPM4355 as a template. The PCR product carrying *lipA* was ligated into pGEM-T, to generate pPM4356. The plasmid pPM4362 carrying only *lipB*, was constructed by digesting pPM4358 (Figure 6.5) with *AccI* and re-ligating such that the intervening 1.3 kb fragment is removed. pPM4369 contains both *lipA* and *lipB* and was generated by ligating the 1 kb *ApaI* / *PstI* fragment of pPM4356 together with the 1.2 kb *PstI* / *AccI* insert of pPM4362 into the *ApaI* / *AccI* sites of pBluescript SK.

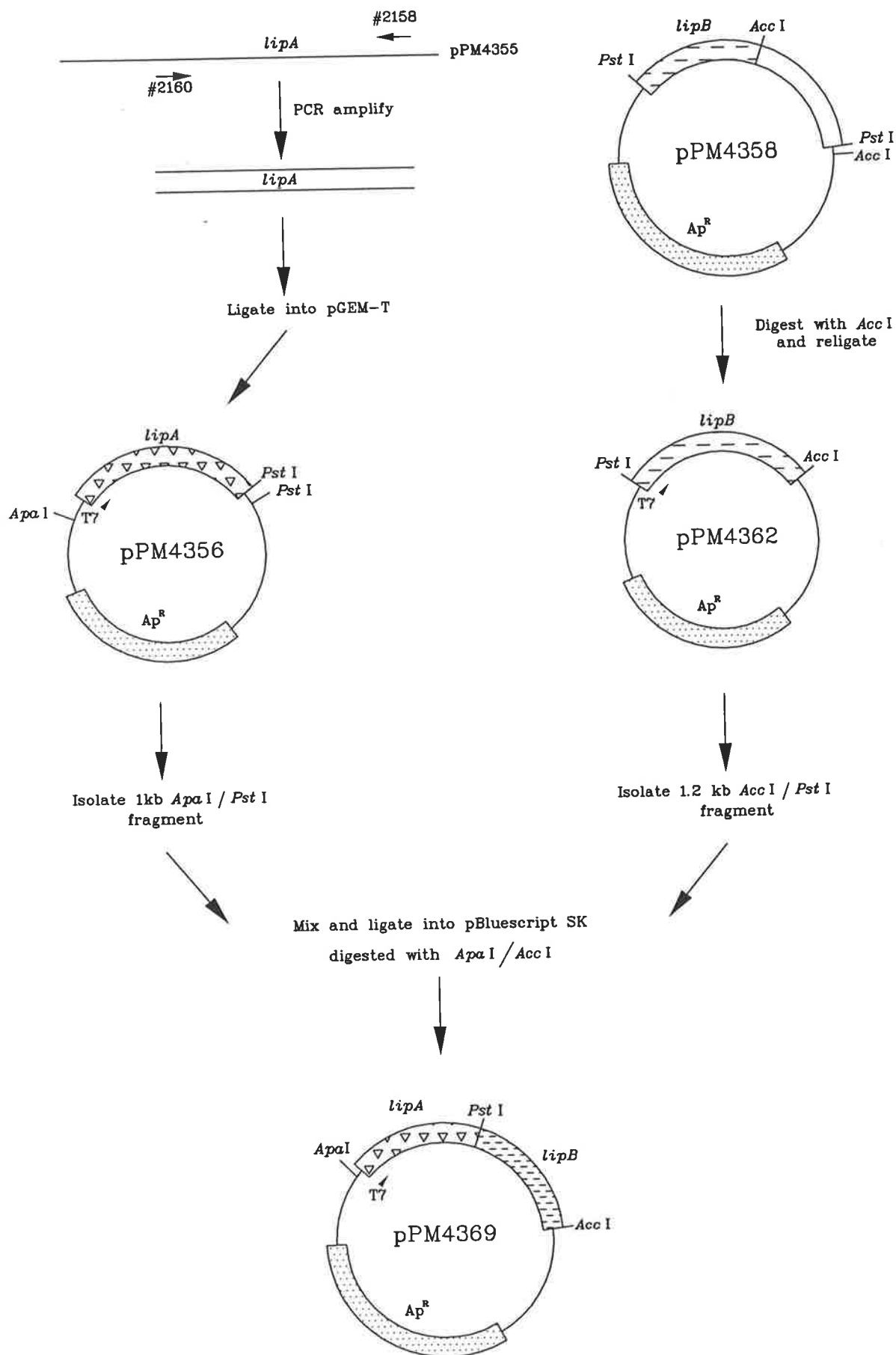
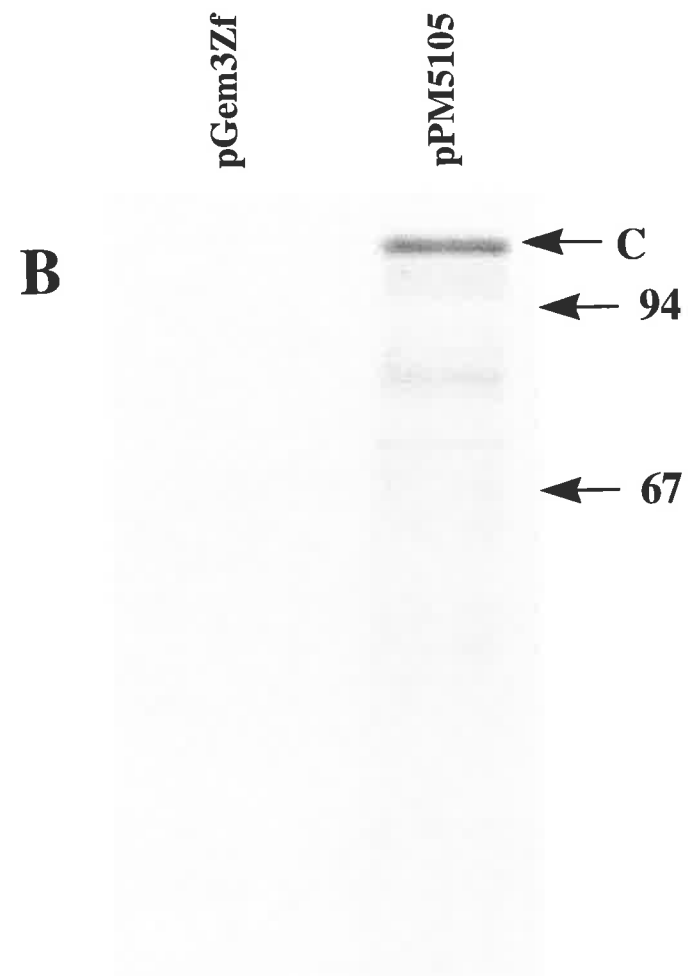
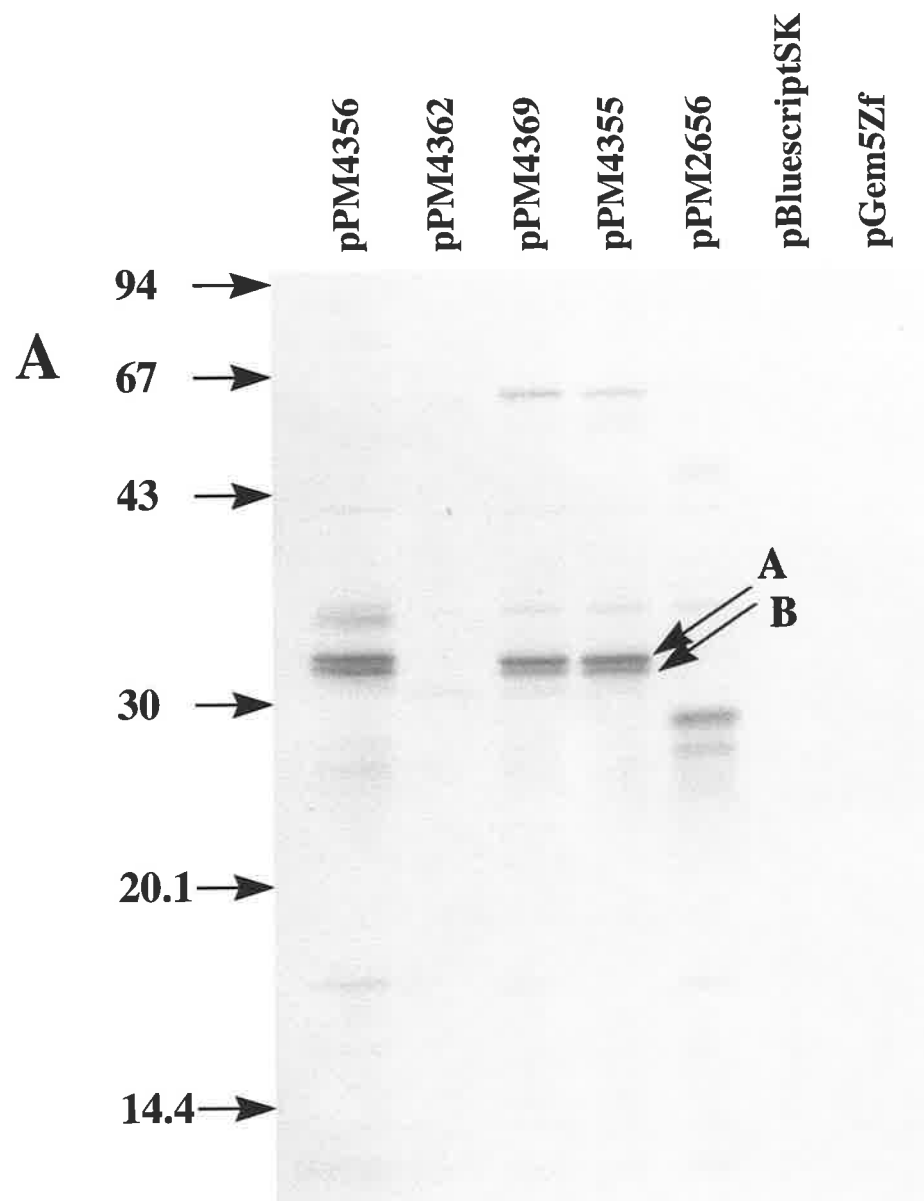


Figure 6.14 Synthesis of LipA and PrtV.

Expression of LipA and PrtV was achieved using the T7 polymerase / promoter system of Tabor and Richardson (1987). Proteins were intrinsically labelled with [^{35}S]-methionine and whole cell fractions subjected to SDS-15% PAGE followed by autoradiography of the dried gel. The plasmids used are described in Figure 6.12. The molecular mass standards are shown in kilodaltons. **Panel A:** Unprocessed and processed LipA are indicated by arrows A and B, respectively. LipB was not detected and is discussed further in the text. **Panel B:** PrtV is indicated by arrow C.



produced by incomplete *lipA* encoded by pPM2656 (Figure 6.14, panel A), as discussed earlier (Section 6.2.1).

It was not possible to detect a band correlating with the predicted molecular mass, 32.6 kDa, of LipB (Figure 6.14, panel A). LipB has only one methionine residue, which limits labelling with [^{35}S]-methionine and hence detection by autoradiography. Furthermore, the initiation methionine may be processed as observed with other proteins (Ben-Bassat *et al.*, 1987). pPM5105 (Figure 6.12) produced a protein of approximately 102 kDa (Figure 6.14, panel B) which is consistent with the coding capacity of the *prtV* gene.

6.2.7 Construction of a *lipA* mutant of *V. cholerae* O17

6.2.7.1 Introduction of a Km^{R} cartridge into *lipA*

To investigate the role of *lipA*, a specific mutation was constructed in *V. cholerae* O17 using the suicide vector pCACTUS to introduce a Km^{R} cartridge into the gene. pCACTUS has a temperature sensitive replicon and encodes Cm^{R} . The replicon is inoperative at 42°C, but supports plasmid replication at 30°C. It also encodes the *SacB* gene from *Bacillus subtilis*, which produces a toxic product when bacteria are grown in the presence of sucrose (Kaniga *et al.*, 1991).

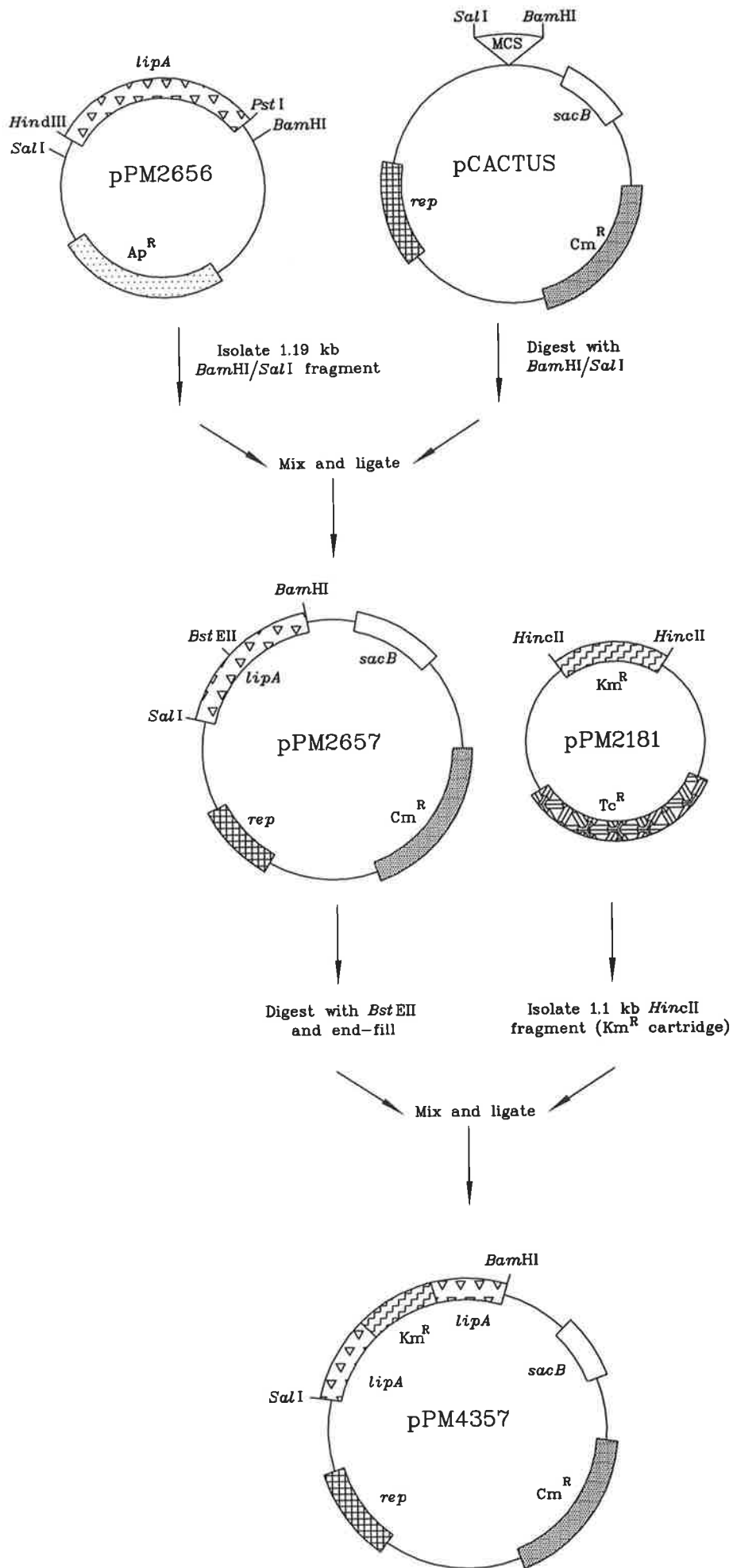
A 1.19 kb *SalI* / *Bam*HI fragment carrying most of *lipA* was isolated from pPM2656 and ligated into the *SalI* / *Bam*HI sites of pCACTUS (Figure 6.15). The DNA was transformed into DH5 α , and transformants were selected at 30°C in the presence of Cm . The resulting construct was designated pPM2657 (Figure 6.15). A 1.2 kb *Hinc*II Km^{R} cartridge isolated from pPM2181 (Stroeher, 1992) was then inserted into the end-filled *Bst*EII restriction site site of pPM2657, 981bp downstream of the *lipA* start codon. pCACTUS carrying *lipA::Km^R* was isolated from *E. coli* after growth at 30°C, and was designated pPM4357 (Figure 6.15).

6.2.7.2 Construction of a *lipA::Km^R* strain of O17 (V1218)

The pCACTUS-based construct pPM4357, carrying *lipA::Km^R*, was electroporated into *V. cholerae* O17 and grown at 30°C in the presence of Km and Cm . Aliquots of an

Figure 6.15 Construction of *lipA*::Km^R in the suicide vector pCACTUS.

A 1.19 kb *Sal*I / *Bam*HI fragment carrying partial *lipA* was isolated from pPM2656 (Figure 6.1) and ligated into the temperature sensitive suicide vector pCACTUS, to generate pPM2657. A *Hinc*II Km^R cartridge isolated from pPM2181 (Stroeher, 1992) was subsequently inserted into the end-filled *Bst*EII restriction site of pPM2657, which lies in *lipA*. The construct generated was pPM4357.



overnight culture of this strain were plated on Km-containing BHI media and grown overnight at 42°C (growth of the strain at the non-permissive temperature of 42°C in the presence of Km selects for strains in which recombination with host DNA has occurred, since the plasmid pCACTUS cannot replicate at this temperature). The resulting colonies were then inoculated into Luria broth in the presence of Km and grown overnight at 30°C to allow for the resolution of co-integrates of plasmid and host chromosomal DNA. Cells which did not resolve the cointegrate were selected against by growth in the presence of sucrose at 30°C. Selection for Km^R Cm^S sucrose^R colonies at 37°C ensured that a double cross-over recombination event had occurred in *V. cholerae*, such that the intact copy of *lipA* had been replaced with the inactive copy, and that the plasmid pCACTUS was no longer present.

To confirm the presence of a *lipA* mutation in the chromosome of *V. cholerae* O17, genomic DNA was extracted and digested with *Bst*XI and examined by Southern hybridization, using DIG-labelled pPM2656 as probe. *lipA* lies on a 2.0 kb *Bst*XI fragment, as detected in O17 and pPM431 (Figure 6.16). An additional larger fragment was detected for pPM431, which represents homology between the vectors: pBluescript of the probe and pBR322 of pPM431. Digestion of *lipA*::Km^R mutants with *Bst*XI resulted in an increase of 1.2 kb, to produce a 3.2 kb fragment (Figure 6.16). This confirms the isolation of a *lipA*::Km^R strain of *V. cholerae* O17, which was subsequently designated V1218.

6.2.8 Analysis of *lipA* and *prtV* mutants of *V. cholerae* O1

6.2.8.1 The effect of a *lipA* mutation on lipase activity

As *lipA* and *lipB* of *V. cholerae* are predicted to encode a lipase and accessory lipase based on sequence homology, the effect of the *lipA* mutation on lipase activity was examined. Agar plates containing emulsified tributyrin were inoculated with various strains and incubated at 37°C for 24 hours. While the parental *V. cholerae* O17 strain displayed a zone of clearing consistent with lipase activity, the isogenic *lipA* mutant V1218 was unable to do so (Figure 6.17) which is in agreement with the previous findings of Camilli and Mekalanos (1995).

Figure 6.16 Confirmation of the *lipA::Km^R* mutant (V1218) of *V. cholerae*.

A Southern blot of chromosomal DNA of *V. cholerae* O17 and putative O17 *lipA::Km^R* mutants digested with the restriction endonuclease *Bst*XI and probed with DIG-labelled pPM2656 and DIG-labelled molecular size marker SPP1 (the size markers are shown in kb). The 2 kb or the 3.1 kb *Bst*XI fragments harbouring either *lipA* or *lipA::Km^R*, respectively, are indicated. *V. cholerae* O17 and pPM431 were included as controls. Lanes 1-15 are putative *lipA::Km^R* mutants, however, lane 8 is still wild-type. The *V. cholerae* strain from which DNA in lane 12 was derived was confirmed to be a *lipA::Km^R* mutant and designated V1218.

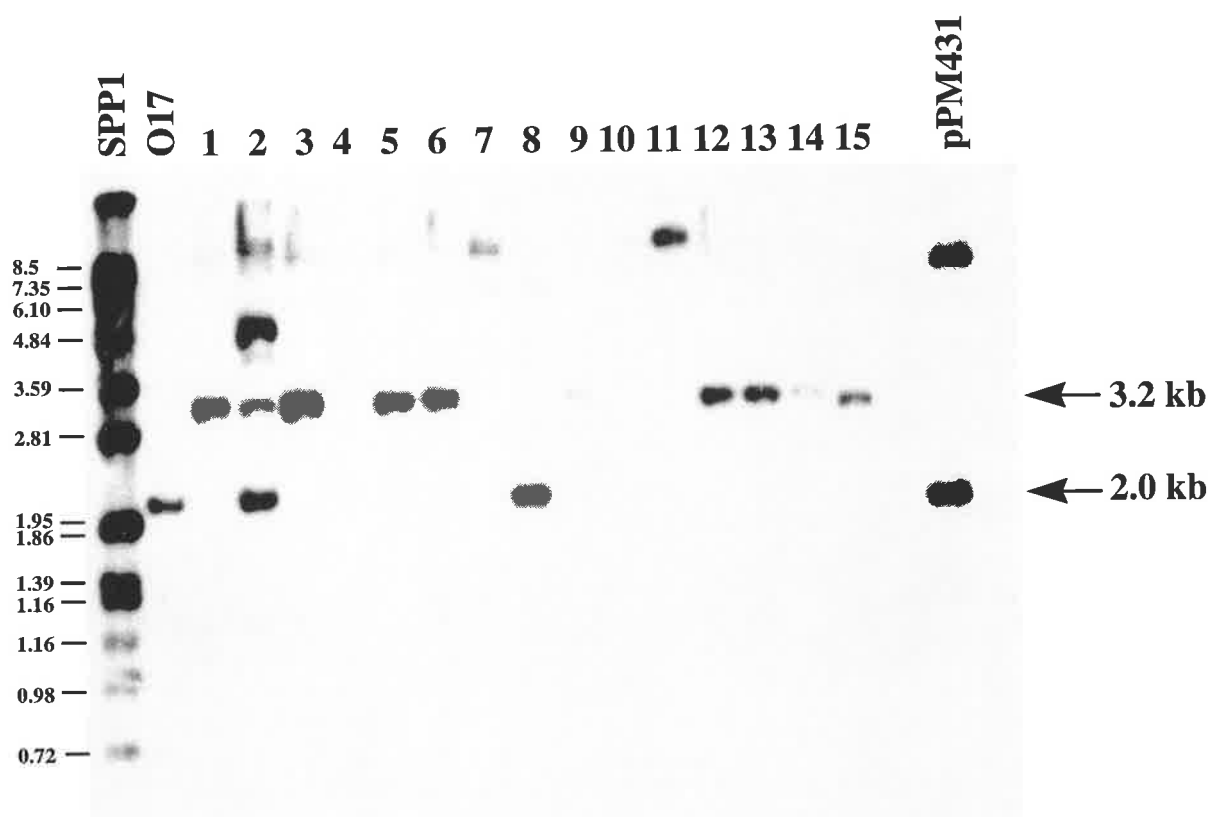
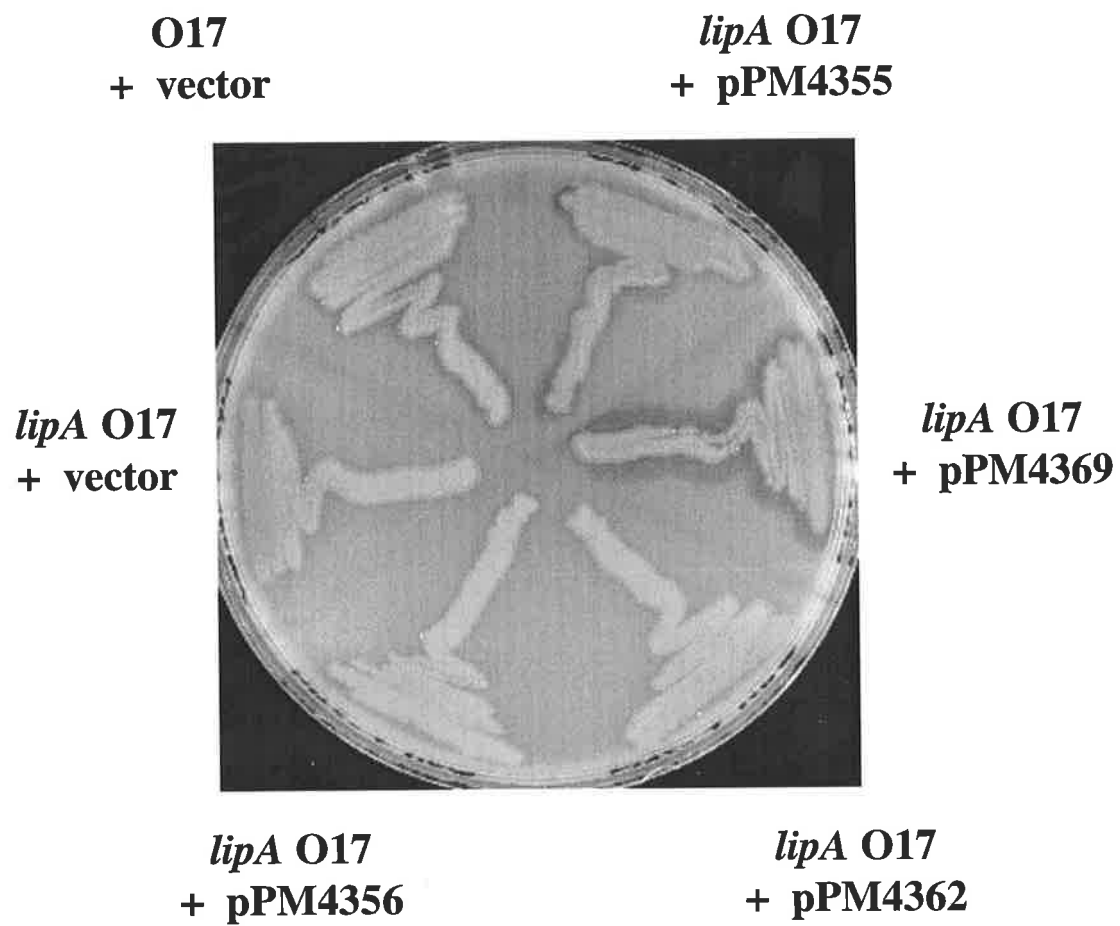


Figure 6.17 Analysis of lipase activity of the *lipA* mutant V1218 and complementation of the mutation.

A comparison of the lipase activity of *V. cholerae* O1 strain O17 and its isogenic *lipA* mutant (V1218) containing various plasmids (Figure 6.12), as indicated by a zone of clearing on LB agar containing emulsified tributyrin after an incubation period of 24 hrs. Below the agar plate is a summary table of the results. *lipA* O17 is the *lipA* mutant of *V. cholerae* O1 strain O17.



Strain	Lipase Activity
O17 + pBluescript	+
<i>lipA</i> O17 + pBluescript	—
<i>lipA</i> O17 + <i>lipA lipB</i> (pPM4355; pPM4369)	+
<i>lipA</i> O17 + <i>lipA</i> (pPM4356)	—
<i>lipA</i> O17 + <i>lipB</i> (pPM4362)	—

In order to complement the LipA defect and define the corresponding region required to do so, a variety of plasmid constructs (Figure 6.12) were electroporated into V1218. Plasmid pPM4355 containing the 5.6 kb *EcoRV* fragment carrying *lipA*, *lipB* and additional downstream DNA, was able to restore the lipase activity of V1218, confirming the isolation of the lipase operon of *V. cholerae* (Figure 6.17). Similarly, plasmid pPM4369 carrying only *lipA* and *lipB* was also able to restore activity. However, the independent expression of either *lipA* or *lipB* in strains carrying pPM4356 or pPM4362, respectively, did not restore lipase activity (Figure 6.17). It can therefore be concluded, that *V. cholerae* requires the product of both the *lipA* and *lipB* genes for lipase activity. These data are consistent with the findings of other bacterial species, such as *Pseudomonas*, as described earlier.

6.2.8.2 The effect of *lipA* and *prtV* mutations on haemolytic activity

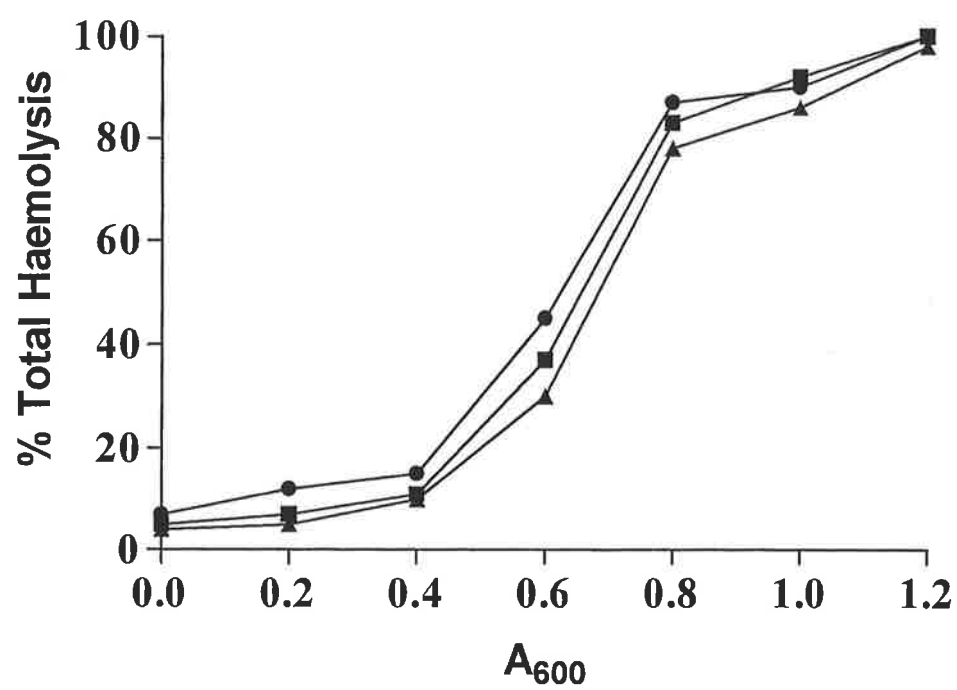
The *lipA* (V1218) and *prtV* (V1219) mutants of *V. cholerae* were assayed in order to determine the effect of these mutations on haemolytic activity. V1219 was constructed by inserting a Km^R cartridge into the *PstI* site of *prtV*, which was then recombined into the *V. cholerae* O17 chromosome (A. Fallarino, personal communication). Culture supernatants of the parental strain O17 and mutated strains V1218 and V1219 were collected at various stages of cell growth, filtered and the haemolytic activity measured in a liquid haemolysis assay. The haemolytic activity of *V. cholerae* was unaffected by a mutation in either *lipA* or *prtV* (Figure 6.18).

6.2.8.3 The effect of *lipA* and *prtV* mutations on virulence

The *in vivo* consequences of introducing various mutations into the O17 strain were assessed using the infant mouse cholera model. The virulence of parent and isogenic mutant was compared by performing simultaneous (48-hr) LD₅₀ titrations. The values obtained show that neither V1218 (LD₅₀ of 5.6×10^3 compared with 4.4×10^4 for O17) nor V1219 (LD₅₀ of 8.8×10^3 compared with 9.2×10^3 for O17) are attenuated.

Figure 6.18 A comparison of haemolytic activity of *V. cholerae* O17 with *lipA* O17 (V1218) and *pvtV* O17 (V1219).

Culture supernatant was collected at various stages of growth (A_{600}) and incubated with a 5% sRBC suspension at 37°C for 2 hrs. The haemolytic activity was then measured and expressed as % total lysis. Neither V1218 nor V1219 differed in haemolytic activity compared to the wild type strain O17.



—■— O17
—▲— V1218
—●— V1219

In addition, competition experiments (Attridge *et al.*, 1996) were performed to compare the colonisation potential of a mutant strain with wild-type O17. These involve the administration of a mixed inoculum of the two strains, with intestinal contents harvested 24 hours later. Any significant change in the ratio of mutant:parent bacteria signifies a difference in colonisation potential. For both mutants, the output ratios (ORs) of mutant:parent bacteria recovered at 24 hrs were not significantly different from the ratio present in the challenge inoculum. For V1218, the mutant:parent input ratio 2.3, and the median OR was 1.2 (n=7); for V1219 the input ratio was 1.0, and the median OR was 2.6 (n=6).

6.3 Summary and conclusions

The region downstream of *hlyAB* in *V. cholerae* O1 was investigated. Sub-genomic DNA libraries of *V. cholerae* O1 strain O17 were constructed, from which plasmids pPM4355 and pPM5105 carrying the region downstream of *hlyAB* were isolated. Sequence analysis of this region has revealed three *orfs* designated *lipA*, *lipB* and *ptrV* which potentially encode 33 kDa, 32.6 kDa and 102 kDa proteins, respectively, and have been confirmed for *lipA* and *prrV*. A summary of the genetic organisation of the extended *hlyAB* locus is shown in Figure 6.12. The *lipA* and *lipB* genes are predicted to be translationally coupled, as the stop codon for *lipA* overlaps the ribosome binding site for *lipB*. Downstream of *lipAB* lies *ptrV*, which is transcribed in the opposite direction. A potential *rho*-independent terminator is present in the intergenic region between *lipAB* and *ptrV* and may function as a bi-directional terminator.

The *lipA* gene was previously known as *hlyC*, and was reported to encode an 18.3 kDa protein with no potential signal sequence (Manning *et al.*, 1984; Alm and Manning, 1990a; Casanova and Peterson, 1995). However, by re-sequencing this gene and over-expressing its protein product, it was shown to encode a 33 kDa protein, with a potential signal peptide at the amino terminus that conforms to the constraints of von Heijne (1985). The 6.4 kb *Pst*I fragment of pPM431 (Manning *et al.*, 1984) therefore carries an incomplete *lipA* gene. Both unprocessed (33 kDa) and processed (30 kDa) forms of the LipA protein

were detected on an SDS polyacrylamide gel. LipA has an atypical start codon of UUG, but a good match to the consensus RBS and is translated sufficiently to be detected in T7 over-expression analysis.

A comparison of the predicted amino acid sequences of LipA, LipB and PrtV in the PIR and SWISS-PROT databases revealed significant homologies. The 33 kDa LipA protein of *V. cholerae* is highly homologous to the triacylglyceride-specific lipases of several *Pseudomonas* spp. (*P. aeruginosa*, *P. fragi*, *P. glumae* and *P. cepacia*) and *Acinetobacter calcoaceticus*, and all share sequence homologies in significant regions. The consensus pentapeptide Gly-X₁-Ser-X₂-Gly (where X₁ is His in prokaryotic lipases) is conserved among neutral lipases and is also present in LipA of *V. cholerae*. LipA also contains the three highly conserved amino acids Ser-Asp-His, known as the three dimensional catalytic triad. The serine residue of the triad is the same as that of the conserved pentapeptide.

Immediately downstream of the *V. cholerae* *lipA* gene lies *lipB*. The amino acid sequence of LipB is homologous to the accessory-lipase of *A. calcoaceticus* and *P. aeruginosa*, which have been shown to be required for the production of active lipases. Consistent with this, the *lipA::Km^R* mutant of *V. cholerae* (V1218) could only be complemented by *lipA* in conjunction with *lipB*, but not by *lipA* alone, which demonstrates that both LipA and LipB are essential for lipase activity of *V. cholerae*. The LipB protein was not detected due to a lack of methionine residues which are required for detection in the T7 RNA polymerase / promoter system used here. However, based on these expression studies, it is presumed that LipB is expressed.

The *prtV* gene lies downstream of *lipAB* and the product of this gene is predicted to encode a metallo-protease. PrtV is homologous to the InA metalloprotease of *Bacillus thuringiensis* and zinc-binding and catalytic active site residues conserved among metalloproteases are present in PrtV. The presence of the Zinc-binding domain is sufficient to detect members of the zinc-metalloproteinase superfamily of proteins. *B. thuringiensis* is an insect pathogen and InA is thought to contribute to the pathogenesis of the bacteria. PrtV shares a small, highly conserved region of homology with a variety of other proteases,

including *Achromobacter* protease I (API), *Xanthomonas* carboxyl proteinases (XCP) and *C. histolyticum* collagenase (ColH). The function of this conserved region is unknown.

As *lipA* and *prtV* are in close proximity to *hlyA*, their possible involvement in the haemolytic activity of *V. cholerae* was examined. Null mutants in either the *lipA* (V1218) or *prtV* gene (V1219) were still haemolytic, which eliminates any essential role of these genes in activity. Both V1218 and V1219 were unaffected for the LD50 and colonisation ability in the infant mouse cholera model. But, as the products of both genes are likely to be secreted, the proteins produced by the wild-type vibrios may compensate the mutant strains in the provision of lipase or protease function in the competition experiments. It is possible that the LipAB and PrtV proteins are involved in the acquisition of nutrients, which appears to be a general theme of the genes encoded within this region of the *V. cholerae* chromosome (Figure 6.12).

Chapter 7

DISCUSSION

7.1 Introduction

The El Tor haemolysin (HlyA) of *V. cholerae* O1 is a potent toxin that is cytotoxic/cytolytic for a variety of cell types. In addition, it is enterotoxic and is considered to be an additional diarrhoeagenic factor to the cholera toxin in El Tor strains. Its involvement in pathogenesis was realised when El Tor mutants devoid of HlyA were shown to be significantly attenuated for virulence (Alm *et al.*, 1991). HlyA is secreted as an inactive 80 kDa pro-toxin that requires the removal of 15 kDa to release the mature, active 65 kDa form.

At the commencement of these studies, very little was known about the mode of action of HlyA and the functional domains within the protein. The studies undertaken here investigate these aspects and in addition explore the region of the *V. cholerae* chromosome downstream of the *hlyAB* locus.

7.2 HlyA is a pore-forming toxin

A combination of osmotic protection and artificial lipid membrane studies have established that the El Tor haemolysin exerts its cytolytic effect via pore formation. HlyA pores have a single channel conductance of 350 pS in 1M KCl and the effects of different salt solutions on the channel conductivity suggests that it is moderately anion selective. The pore diameter of HlyA is estimated to be less than 1.6 nm, as sheep erythrocytes were protected from HlyA-mediated lysis by the inclusion of carbohydrates with a diameter greater than 1.6 nm. Moayeri and Welch (1994) have shown that the diameter of the *E. coli* α -haemolysin pore actually increases in a temperature dependent manner over time and therefore estimates of pore diameter from osmotic protection experiments are dependent on the concentrations of toxin and osmotic protectant, assay time and incubation temperature. Consequently, the authors caution the use of osmotic protection when estimating pore diameter. However, while the conditions used here in the osmotic protection studies for

HlyA differ to those of Ikigai *et al.* (1996), they have estimated the diameter to be in the range of 1.2 and 1.6 nm, and confirmed it morphologically by electron microscopy.

Despite the common requirements of pore-forming toxins and bacterial porins to insert into the membrane and form channels, there is no reliable consensus sequence for pore-forming domains (Siehnel *et al.*, 1990; Jeanteur *et al.*, 1991; Hancock and Brown, 1992). This is perhaps highlighted by the findings that even though the channels formed by HlyA have similar properties to those of the *Aeromonas aerolysin* and the *Staphylococcus aureus* α -toxin (Chakraborty *et al.*, 1990; Wilmsen *et al.*, 1990; Menzl *et al.*, 1996), the toxins share virtually no sequence homology. Apart from the hydrophobic N-terminal signal sequence, HlyA is essentially hydrophilic with no obvious hydrophobic structures capable of spanning a lipid membrane. Thus, the FORTRAN program AMPHI (Jähnig, 1990) was employed to predict any amphipathic structures, which may be involved in channel formation. While no amphipathic α -helices were predicted, HlyA was predicted to contain four amphipathic β -sheets. However, at least eight β -sheets are required to form β -barrel (Lesk *et al.*, 1989), which suggests that HlyA must oligomerise in order to form a pore. Recent findings have indeed demonstrated that HlyA monomers oligomerise on the membranes of rabbit erythrocyte ghosts and liposomes (Ikigai *et al.*, 1996) and cultured intestinal cells (Zitzer *et al.*, 1997).

A detailed crystallographic analysis of purified HlyA together with electron microscopy analysis of the membrane-bound state may confirm its structure and determine the subunit stoichiometry required for channel formation. In addition, various amino acids within HlyA could be labelled with polarity-sensitive dyes, which indicate the movement of labelled residues into the membrane during pore-formation (Valeva *et al.*, 1995). It is also not known whether HlyA has a pre-lytic membrane-associated conformation. The *E. coli* haemolysin has two distinct cell-associated conformations, such that binding and lysis are separable events (Bauer and Welch, 1996; Moayeri and Welch, 1997). The topology of membrane-associated El Tor haemolysin at a low temperature (0-2°C) would need to be compared to that at a higher temperature (23-37°C), by determining the surface accessibility of the protein to specific antibodies and proteases. Collectively, these studies may unravel

the mechanism of how the water-soluble HlyA molecule inserts into the hydrophobic environment of the membrane.

While the mode of cytolytic action of the haemolysin has been elucidated, it is yet to be determined how HlyA exerts its enterotoxic activities. It is conceivable that the mode of action for both activities is the same: that is, via pore-formation. It is well documented that sub-lytic levels of the *E. coli* haemolysin disrupt a range of normal cellular functions, which produce various pathophysiological effects (Section 1.6.1.1). Perhaps the insertion of HlyA into intestinal epithelial cells at sub-lytic levels interferes with the normal flow of fluid across the membranes, leading to a net loss of fluid into the gut.

7.3 Cleavage and activation of HlyA

Many toxins are not active when first translated and require further processing to convert into the mature active form, and the nature of activation varies depending on the toxin. The *E. coli* haemolysin is covalently modified by a gene encoded within the same operon (Hardie *et al.*, 1991; Issartel *et al.*, 1991). In contrast, other cytolsins require proteolytic cleavage for activation, such as the α -toxin of *Clostridium septicum* (Ballard *et al.*, 1993), the haemolysin of *Pseudomonas aeruginosa* (Hayashi *et al.*, 1989) and the *Aeromonas aerolysin* (Howard and Buckley, 1985).

It was of interest to examine the requirements for HlyA maturation. HlyA is secreted as an 80 kDa precursor which requires the removal of 15 kDa to release the active 65 kDa haemolysin (Nagamune *et al.*, 1996) and correlating with this, it has been shown that activation occurs predominantly after secretion. Purified 80 kDa HlyA is able to cleave into 65 and 15 kDa products, which led to the suggestion that HlyA is autoproteolytic (Alm, 1989; Alm *et al.*, 1988). However, HlyA isolated from the *E. coli* periplasm was activated by the addition of *V. cholerae* culture supernatants devoid of HlyA, implying that other factor(s) are involved with activation. Specific anti-SHA/protease antiserum was able to inhibit this activation, demonstrating the involvement of SHA/protease in the activation of HlyA. This did not exclude the possibility that additional factors exist which may also activate HlyA, as is the case with the activation of the cholera enterotoxin (CT). While the

SHA/protease has been shown to proteolytically nick and therefore activate the A subunit of CT (Booth *et al.*, 1984), null mutants devoid of the SHA/protease gene (*hap*) were still fully virulent in infant rabbits (Finkelstein *et al.*, 1992), thus demonstrating that the role of SHA/protease could easily be replaced by other proteases, possibly those in the gut. Indeed, Nagamune *et al.* (1996) have recently shown that a variety of enzymes including SHA/protease, trypsin, α -chymotrypsin, subtilisin BPN', papain and thermolysin can activate HlyA purified from *E. coli* via N-terminal cleavage.

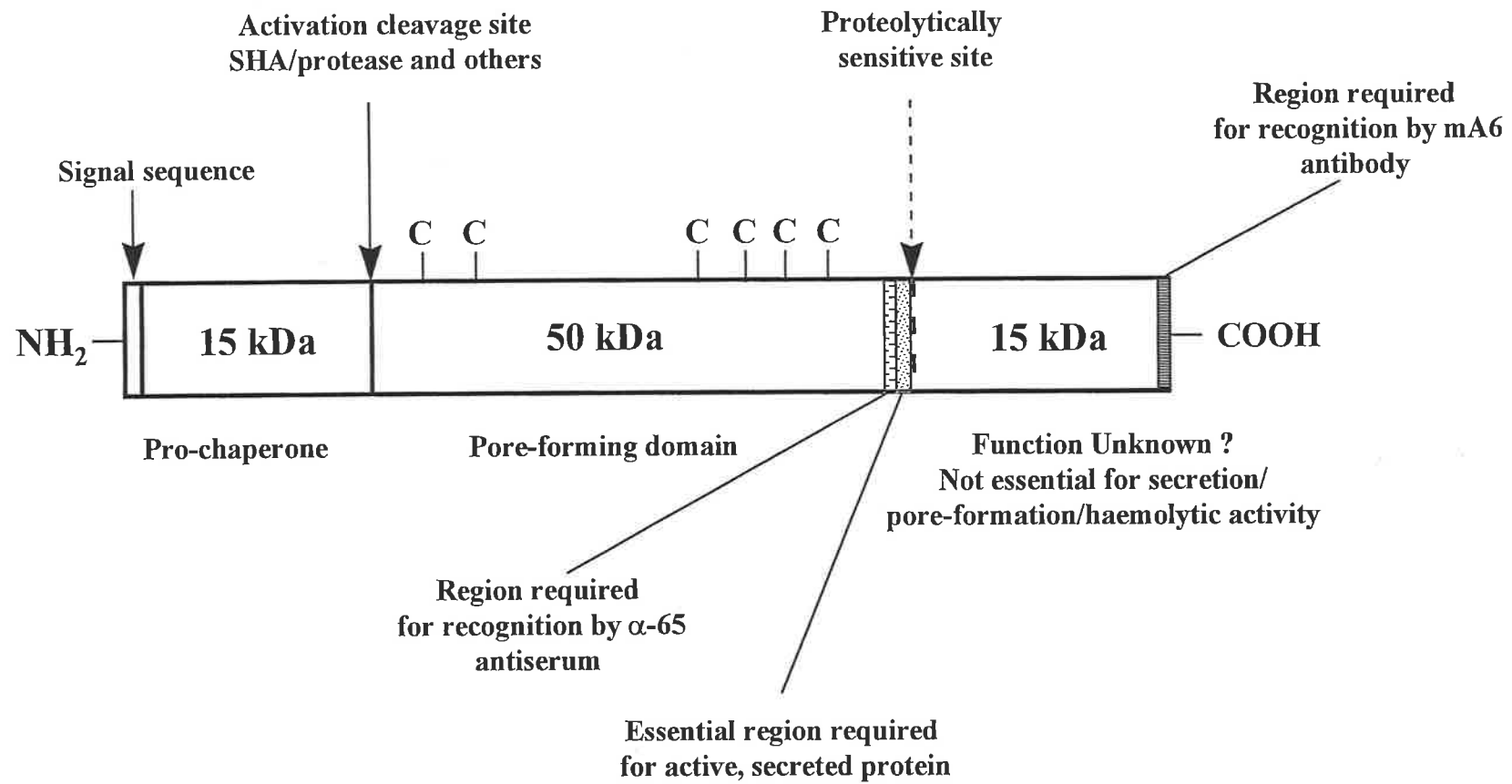
7.4 A minimum haemolytic domain of HlyA

One approach to define functional domains involved in membrane binding, pore-formation, maturation and extracellular secretion of toxins is to examine the effects of sequentially removing the C-terminus of the protein. This approach localised the pore-forming and membrane binding domains of the *Serratia* haemolysin (Poole *et al.*, 1988). Thus in order to help identify and localise functional domains within the *V. cholerae* HlyA, a range of stable sequential C-terminal deletions were produced. To analyse these deletions and various other mutated derivatives of HlyA, a Δ *hlyA* derivative of *V. cholerae* strain O17 (V945) was constructed and in addition a polyclonal antiserum which recognises the C-terminal truncations was generated.

C-terminal deletions of 80 kDa pro-HlyA expose a proteolytically sensitive site approximately 15 kDa from the C-terminus (Figure 7.1). Deletions ranging from only 12 amino acids to 15 kDa expose this site and cleavage occurs efficiently at this site in both *V. cholerae* and *E. coli*. The removal of the C-terminal 15 kDa from pro-HlyA produces a 50 kDa protein, following the removal of the N-terminal pro-region that is normally required for HlyA activation (Nagamune *et al.*, 1996). The 50 kDa is therefore derived from the central region of pro-HlyA, excluding both the N- and C-terminal 15 kDa regions (Figure 7.1). This 50 kDa derivative was identified as a minimum active domain of HlyA characterised thus far, capable of lysis via pore-formation in erythrocyte membranes. Osmotic protection studies demonstrate that the pore diameter is comparable to those formed by native HlyA,

Figure 7.1 A summary of the model proposed for the HlyA protein.

HlyA is a pore-forming toxin (Chapter 3), and the 82 kDa precursor form of the toxin is shown. The amino-terminal signal sequence is removed during secretion, and N-terminal cleavage is required for the activation of pro-HlyA, (Hall and Drasar, 1990; Yamamoto *et al.*, 1990), and the SHA/protease is at least one protease involved with its activation (Nagamune *et al.*, 1996; Section 4.2.3). The N-terminal 15 kDa pro-region functions as an intra-molecular chaperone (Nagamune *et al.*, 1997). A proteolytically sensitive site lies at the C-terminus, and is exposed as a result of C-terminal deletions in pro-HlyA (Section 4.2.2). Cleavage at both the N- and C-terminus produces a 50 kDa derivative of HlyA, and this derivative is able to form pores in erythrocyte membranes and is haemolytically active, and represents a minimum haemolytic domain, defined thus far (Section 4.2.6). As all the cysteine residues of HlyA are contained within the 50 kDa region, and this region is present in the VAH1, ASH4, AHH1 haemolysins of *V. anguillarum*, *A. salmonicida* and *A. hydrophila*, respectively, the 50 kDa may represent a conserved domain (Section 5.2.3). A region essential for the production of active, secreted HlyA lies at the C-terminus of the 50 kDa region (Section 5.2.2). Regions essential for the recognition of α -65 and mA6 antibodies are highlighted (Section 5.2.5). The role of the C-terminal 15 kDa region of pro-HlyA is unknown, but it is not essential for secretion, pore-formation or haemolytic activity (Chapters 4 and 5).



however further analysis in artificial lipid membranes is needed to determine whether other structural features of the pore are conserved.

The central 50 kDa region of pro-HlyA may represent a conserved domain critical for cytolytic activity. This region is highly homologous to the haemolysins VAH1, ASH4 and AHH1 of *Vibrio anguillarum* (Hirono *et al.*, 1996), *Aeromonas salmonicida* (Hirono and Aoki, 1993) and *Aeromonas hydrophila* (Hirono and Aoki, 1991), respectively. In addition, the *Vibrio vulnificus* cytotoxin VvhA shares regions of homology with HlyA (Yamamoto *et al.*, 1990b) within the 50 kDa region of HlyA. The location of cysteine residues is conserved among these cytotoxins which suggests that the tertiary structure may also be conserved, as the disulphide bonds that form between cysteine residues are often crucial for the folding and stability of the protein. The El Tor haemolysin has seven cysteine residues. The first of these is Cys-7, which is located within the signal sequence and is therefore removed during the translocation of HlyA from the cytoplasm to the periplasm (Hall and Drasar, 1990; Yamamoto *et al.*, 1990a). The other six cysteine residues (Cys-182, 200, 497, 511, 537 and 549), located in the mature 65 kDa HlyA are all contained within the 50 kDa derivative of HlyA (Figure 7.1). While these six cysteine residues are conserved among the VAH1, ASH4, AHH1 and VvhA haemolysins, site-directed mutagenesis altering individual cysteine residues would be required to determine which residues are required for active protein.

It appears that the breakdown of the 80 kDa HlyA to the 50 kDa derivative may also occur as a result of purification procedures. It was demonstrated that TCA precipitation of HlyA produces a 50 kDa product and Ikigai *et al.* (1996) reported the presence of a 55 kDa derivative of HlyA during immunoblotting of toxin oligomers. A 52 kDa derivative of HlyA that was haemolytic and able to form channels in the lipid bilayer membranes was observed during the SDS-PAGE purification of HlyA (Menzl *et al.* 1996). Perhaps purification of HlyA leads to degradation of the C-terminus in a manner analogous to the C-terminal deletions constructed *in vitro* (Chapter 4), and this results in the exposure and cleavage of the proteolytically sensitive site. The haemolysin is secreted into the human gut during

infection where it would be exposed to a multitude of proteolytic enzymes, and so it is conceivable that degradation to the 50 kDa may also occur *in vivo*.

A region crucial for the production of secreted active haemolysin was identified 15 kDa from the C-terminus of native 65 kDa HlyA (Figure 7.1). HlyA which has had 15 kDa removed from the C-terminus (up to residue Gln-609) is still able to be secreted and is haemolytically active. A further deletion of the C-terminus up to residue Tyr-602 retains some activity but is unstable, however a further deletion up to residue Arg-597 abolishes haemolytic activity. This was confirmed by an in-frame deletion of pro-HlyA which removed residues Thr-598 to Ser-612, leading to a form which is no longer active or secreted. It is possible that this region maintains a conformation of HlyA that is crucial for secretion and activity.

Two regions within HlyA, (Leu-584 to Arg-597) and (Asp-730 to Asn-741) were required for the recognition by the polyclonal antiserum α -65 and the monoclonal antibody mA6, respectively (Figure 7.1). However, it is not known whether these antibodies recognise a linear epitope, or a conformational epitope that requires these amino acids for correct conformation. A synthetic peptide incorporating these amino acids could be used to distinguish between these two possibilities. As both α -65 and mA6 inhibit haemolytic activity *in vitro*, identifying epitopes recognised by these antibodies may identify a functional domain(s). A decrease of membrane-associated HlyA in the presence of either antibody is observed, however, it is not known at what stage the maturation process is inhibited. These antibodies may interfere with N-terminal cleavage, and therefore the rate of conversion of 80 kDa HlyA to 65 kDa HlyA in the presence and absence of these antibodies needs to be determined.

While the cytolytic domain was localised to the central 50 kDa region of pro-HlyA, the region responsible for enterotoxicity is yet to be defined. Alm *et al.* (1991) proposed that enterotoxicity lies within the N-terminal 15 kDa region, however, this seems unlikely as Nagamune *et al.* (1997) have shown that the N-terminal pro-region functions as an intramolecular chaperone, that is essential for the correct folding of the haemolysin. Assessment

of the C-terminal truncations of HlyA and the 50 kDa derivative of HlyA in the rabbit ileal loop assay or Ussing chambers may help localise the enterotoxic domain.

7.5 Role of the C-terminus of HlyA?

The N-terminal 15 kDa of pro-HlyA is essential for the production of active haemolysin, however the role of the C-terminal 15 kDa remains unclear (Figure 7.1). The C-terminal region is not essential for haemolytic activity, secretion, or the N-terminal cleavage, however, the possibility that it contributes in some way to these processes cannot be excluded. A comparison between native HlyA and the C-terminal deletion lacking this region (pPM2691) of the specific activity, rate of secretion and rate of cleavage is required to determine this. It is interesting to note that the cytotoxin VvhA of *V. vulnificus* lacks both the N-terminal 15 kDa chaperone region and the C-terminal 15 kDa regions of the El Tor HlyA, even though both VvhA and HlyA have virtually similar biological characteristics. The *Aeromonas* haemolysins ASH1 and AHH1 which are homologous to HlyA also do not contain a homologue of the C-terminal region.

7.6 The secretion signal of HlyA

The amino-terminal signal peptide of HlyA (Figure 7.1) directs translocation across the cytoplasmic membrane (Hall and Drasar, 1990; Yamamoto *et al.*, 1990a) presumably via the Sec-dependent pathway (Pugsley *et al.*, 1990). HlyA is then transported across the outer membrane into the extracellular medium, via an unknown mechanism. The extracellular secretion signal within HlyA is also unknown, however it was demonstrated that the C-terminal 15 kDa region is not required for secretion. The secretion signal therefore lies within the N-terminal 65 kDa of the 80 kDa form of HlyA. While HlyA derivatives with greater than 15 kDa removed from the C-terminus were unable to fold correctly and remained in the cytoplasm, the C-terminal deletion (pPM2699) encoding only the N-terminal 27 kDa of HlyA was an exception, as it is secreted when expressed in *V. cholerae*. It is possible therefore, that the extracellular secretion signal is located within this N-terminal region of HlyA. Fusions of C-terminally truncated HlyA with a heterologous protein may

enhance the stability of the truncations, and thus may be secreted. Random chemical mutagenesis of the N-terminal region of HlyA may also help determine whether this region is required for secretion.

Alternatively, the extracellular transport signal may not be a single linear sequence, but may comprise several different regions generated only in the tertiary structure, as is the case for the pullulanase (PulA) of *Klebsiella oxytoca*. Minor sequence changes were found not to affect the secretion of PulA (Sauvonnnet *et al.*, 1995), and subsequently two non-adjacent sequences were identified as being essential for extracellular secretion (Sauvonnnet and Pugsley, 1996). Random deletions of HlyA within a HlyA- β -lactamase gene fusion may assist in the identification of secretion domain(s). Secretion of the altered HlyA- β -lactamase fusions can be determined by using nitrocefin-containing agar. Nitrocefin is an indicator of β -lactamase activity, and colonies that secrete active β -lactamase fusions produce a red halo (Sauvonnnet and Pugsley, 1996).

7.7 Downstream of *hlyA*: a lipase operon and a putative metalloprotease

7.7.1 *lipAB* and *prtV*

Sequence analysis of the region downstream of the *hly* locus has revealed three ORFs and the corresponding genes were named *lipA*, *lipB* and *prtV*. The triacylglycerol lipase operon of *V. cholerae* comprises of *lipA* and *lipB*, and expression studies in a *lipA*::Km^R mutant of *V. cholerae* (V1218) indicate that the product of both genes are essential for lipase activity. LipA and LipB are highly homologous to the lipase and accessory-lipase, respectively, of *Pseudomonas* spp. and *Acinetobacter calcoaceticus*. LipA contains the highly conserved consensus pentapeptide (Gly-X₁-Ser-X₂-Gly) and the three dimensional catalytic triad (Ser-Asp-His), found in almost all lipases. Accessory genes with homology to *lipB* have been shown to encode lipase-specific foldases, essential for the production of active lipase (Jorgensen *et al.*, 1991; Chihara-Siomi *et al.*, 1992; Wohlfarth *et al.*, 1992; Frenken *et al.*, 1993a and 1993b), and based on this homology and the expression

studies in V1218, it is predicted that LipB functions in an analogous manner. While LipA of *P. aeruginosa* requires LipB for activation, there is no sequence similarity between the LipB protein and any known chaperone, suggesting that the mechanism of activation by LipB is different from that of chaperones (Oshima-Hirayama *et al.*, 1993).

Primer extension analysis has located a potential start site for *lipA* transcription and a potential σ^{70} consensus promoter sequence has been identified (S. Williams, personal communication). As the stop codon for *lipA* overlaps the ribosome binding site for *lipB*, these genes are predicted to be translationally coupled. Primer extension analysis of the *lipB* transcript would determine whether *lipB* is expressed from a mono-cistronic message, indicative of an internal promoter, or whether it is expressed from a poly-cistronic message carrying both *lipA* and *lipB*.

The *lipA* gene, previously known as *hlyC*, was reported to encode an 18.3 kDa protein with no potential signal sequence (Alm and Manning, 1990a; Camilli and Mekalanos, 1995; Casanova and Peterson, 1995). However, it was established that it actually encodes a 33 kDa protein, with a potential signal peptide at the amino terminus. While both unprocessed (33 kDa) and processed (30 kDa) forms of the LipA protein were detected on a SDS-polyacrylamide gel, N-terminal sequence analysis of both forms would confirm the translational start site, and establish whether the N-terminus has been processed during translocation across the inner membrane, indicative of a signal peptide. LipB was not detected in the T7 RNA polymerase/promoter expression system, due to a lack of methionine residues available for labelling. To overcome this limitation and confirm the size of the LipB, amino acids within the protein could be radiolabelled with [^{14}C]-protein hydrolysate.

The regulation of the *lipAB* operon remains unknown. *lipA* expression is not regulated (*in vitro*) by iron levels, microaerophilic conditions or by conditions that affect the expression of ToxR regulated genes (Camilli and Mekalanos, 1995). The ToxRST system regulates several significant virulence determinants of *V. cholerae*, including the cholera enterotoxin and toxin co-regulated pilus (Peterson and Mekalanos, 1988). Camilli and Mekalanos (1995) have raised the possibility that *lipA* is regulated by HlyU, however there is no evidence to support this (S. Williams, personal communication). It is possible,

therefore, that the *lipAB* operon is regulated by a novel system in *V. cholerae*. To identify regulatory elements involved with lipase production, fusions of the *lipA* promoter with a reporter gene, such as chloramphenicol-transacetylase, could be used to screen a *V. cholerae* plasmid library in *E. coli*. Alternatively, random transposon mutagenesis of the *V. cholerae* chromosome could be screened for loss of lipase activity on emulsified tributyrin agar.

Lipases are extremely versatile enzymes, capable of catalysing a variety of reactions, including the hydrolysis of the ester linkage in fats and trans-esterification reactions. The action of lipases is unique, as they are able to hydrolyse fats at the water-lipid interface and can reverse the reaction in non-aqueous media (Ghosh *et al.*, 1996; Bosley, 1997). Consequently, lipases are significant biocatalysts in a wide variety of biotechnological applications, used in the pharmaceutical industry and as additives in detergents for the domestic market (Ghosh *et al.*, 1996; Bosley, 1997). Extensive research has established that fungi and bacteria provide the best tools for commercial production (Bosley, 1997). For example, the lipase of *P. glumae* PG1 was previously patented and used in detergents. The Danish company "Novo Nordisk" has expressed the *lipase* gene of the *Humicola* spp. fungus in a fermenter utilising *Aspergillus* spp. and the product, *Lipolase*TM, is now widely used in commercial washing powders. The potential use of the *V. cholerae* lipase in industry is yet to be explored. The enzyme efficiency would need to be determined, and compared to lipases already commercially available. Lipase activity can be measured by the amount of lipase required to catalyse the hydrolysis of esters to butyric acid (Chihara-Siomi *et al.*, 1992), and varying conditions such as pH, temperature and time, substrate specificity and sensitivity to inhibitors will determine its spectrum of activity.

Downstream of *lipAB* lies *prtV*, which is predicted to encode a metalloprotease based on homology with the metalloprotease InA, of *Bacillus thuringiensis*. *prtV* is transcribed in the opposite direction to *lipAB*, and a rho-independent terminator present in the intergenic region between *lipAB* and *prtV* is proposed to function as a bi-directional terminator of transcription. PrtV contains functionally important zinc-binding and catalytic active site residues conserved among metalloproteases, including thermolysin (Jongeneel *et al.*, 1989; Lovgren *et al.*, 1990; Murphy *et al.*, 1991).

7.7.2 Role of LipAB and PrtV in disease ?

Since mutants of either LipA or PrtV retain full virulence, it is clear that neither protein provides an indispensable function in the *V. cholerae* O17 strain. Competition experiments between the *lipA* and *prtV* mutants against the parent strain showed no difference in the ability to colonise the small intestine. However, as both LipA and PrtV are presumed to be secreted, the wild-type vibrios may compensate the mutant strains in the provision of lipase or protease function. The possibility, therefore, that either LipA or PrtV play a role in colonisation cannot be ruled out. It is conceivable that these proteins are involved with the attachment or detachment of the organism to the intestinal epithelium. Either protein may assist in the degradation of the mucous layer of the small intestine, allowing a more close association with the epithelial cells. The PrtV metalloprotease may function in an analogous manner to the SHA/protease, which degrades several putative receptors of the adhesins of *V. cholerae* (Finkelstein *et al.*, 1992). Adherence assays examining the ability of *lipA* and *prtV* mutants to attach and detach from cultured human intestinal cells would be required to address these issues. Analysis of lipase/protease production of *V. cholerae* from patient and environmental isolates may provide a correlation between production of these proteins, and the ability to cause disease or survive in the environment.

Pancreatic lipases are active in the small intestine, working in conjunction with Bile to emulsify fat globules in foods, and so it is possible that LipA will also function in the small intestine; the site of a *V. cholerae* infection. Camilli and Mekalanos (1995) have shown that *lipA* is transcriptionally induced during infection in the infant mouse cholera model, which suggests that LipA may in fact play a role during infection of the host. Perhaps LipA acts in conjunction with HlyA. The formation of HlyA-induced pores in host cell membranes may create sites where the membrane lipids are accessible to degradation by the lipase. There is evidence that both the phospholipase C and listeriolysin O of *Listeria monocytogenes* function together in the vacuole escape process that is required for a successful infection by *L. monocytogenes* (Andrews and Portnoy, 1994).

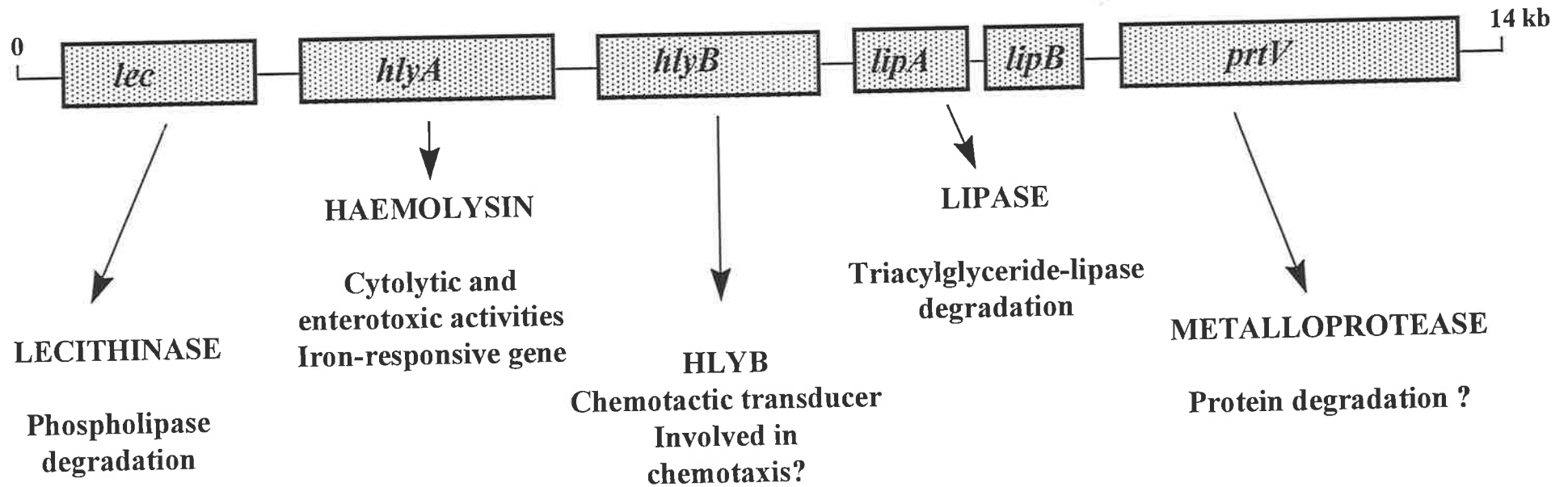
HlyA requires proteolytic cleavage for activation and the possible involvement of PrtV in this process was investigated. Null mutants of *prtV* were fully haemolytic in a liquid haemolysis assay. However, the endogenous enzyme SHA/protease is also capable of processing HlyA (Section 7.3), and so a conclusion cannot be made at this stage. Direct evidence could be obtained from the *in vitro* processing and activation of precursor HlyA by purified PrtV. In addition, a double mutant eliminating both PrtV and SHA/protease in *V. cholerae* could be examined for haemolytic activity. If the mutant retains haemolytic activity, then this suggests that there are additional endogenous protease(s), involved in the proteolytic activation of HlyA.

7.7.3 The *hly* locus: A pathogenicity island?

Linked to the *hly* locus of *V. cholerae* are a number of potential virulence-associated determinants including a haemolysin, lecithinase and methyl-accepting chemotactic protein (section 1.5.5) (Figure 7.2). The clustering of virulence genes raises the possibility that this locus is part of a pathogenicity island (PI). PI are thought to facilitate the spread of virulence genes among the bacterial population, via the excision and acquisition of large genomic fragments (reviewed recently in Hacker *et al.*, 1997). The haemolysin gene cluster of uropathogenic strains of *E. coli* is located on a PI and is associated with genes involved in uropathogenicity, such as those associated with pyelonephritis associated pili production (Blum *et al.*, 1994 and 1995; Swenson *et al.*, 1996). A PI has been identified in strains of *V. cholerae* O1 and O139, comprising of the *tcp* and *acf* gene clusters, an integrase gene, *int*, and the regulatory gene *toxT* (Kovach *et al.*, 1996). All are encoded by a putative integrated phage (Waldor and Mekalanos, 1996).

Initial examination of the *hly* locus of *V. cholerae*, however, suggests that it does not fulfil all the criteria of a PI. PI often differ in %G+C content, in comparison to the rest of the chromosome. The %G+C content of the *hly* locus is 46.6% which falls well within the average range of *V. cholerae* DNA. The boundary regions of PI have a vital role in the integration/excision of DNA and consequently PI are flanked by either direct repeats, insertion sequence elements or tRNA loci. These features are not present within the

Figure 7.2 Schematic representation of the genes of the *hly* locus and their associated activities.



sequenced region of the *hly* locus and flanking genes. However, PI are large elements ranging between 30-200 kb and the *hly* locus characterised thus far, is only 11 kb and so it is possible that the boundaries are not contained within the sequenced region.

It is possible that the *hly* locus of *V. cholerae* is involved in the acquisition of nutrients, which appears to be a general theme of the genes encoded within this region (Figure 7.2). The HlyA toxin is capable of host tissue damage which would release a variety of cellular components including iron, membrane lipids and proteins. The enzymes capable of degrading these substances (Lec, LipA and PrtV) are encoded in the vicinity of *hlyA*. The level of HlyA production is responsive to the levels of available iron (Stoebner and Payne, 1988), an essential requirement for bacterial survival. A chemotactic transducer HlyB, also encoded in this locus, could monitor the nutrient status of the surrounding environment, and signal the bacteria to move accordingly. All these factors may contribute to bacterial growth and survival during an infection of the host.

It is of interest therefore, to extend the sequence of this region in order to determine what lies beyond these characterised genes. Currently, the *Vibrio cholerae* genome of the El Tor strain N16961 is being sequenced by TIGR (The Institute for Genomic Research). As a similar locus exists in this strain, data generated from this project may uncover the boundaries characteristic of a PI.

7.8 Concluding Remarks

The El Tor haemolysin (HlyA) is a potent toxin that forms pores in eukaryotic membranes, leading to colloidal cell lysis. This places HlyA within the ever-increasing family of pore-forming toxins, that incorporates members of the well-characterised *E. coli*-, *Serratia*- and *Aerolysin*-type haemolysins, as well as those produced by Gram-positive pathogens. However, the process leading to the production of mature, functionally active HlyA, appears to be unique, and this aspect alone prompts further investigation into the El Tor haemolysin.

This thesis has provided a number of insights into the structure/function relationships within HlyA. However, it is clear that there are unresolved questions, with respect to the C-

terminal 15 kDa domain, and the signals required for secretion and membrane insertion/pore-formation. Some of the constructs generated here may be invaluable to the crystallographer in determining the three-dimensional structure of the protein and of the pore. The genetic organisation of the region flanking the *hly* locus has a number of interesting features, suggestive of a pathogenicity island, and future analysis of the region may provide insights into the evolution/acquisition of these interesting traits.

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