Serotype conversion in *Vibrio cholerae* O1

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A thesis submitted for the degree of Doctor of Philosophy.

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Dedicated to my beautiful

and loving

wife Diana

The time will come when diligent research over long periods will bring to light things which now lie hidden. A single lifetime, even though entirely devoted to the sky, would not be enough for the investigation of so vast a subject... And so this knowledge will be unfolded only through long successive ages. There will now come a time when our decendants will be amazed that we did not know things that are so plain to them... Many discoveries are reserved for ages still to come, when memory of us will have been effaced. Our universe is a sorry little affair unless it has in it something for every age to investigate... Nature does not reveal her mysteries once and for all.

Seneca, Natural Questions,

Book 7, first century

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I thank my family for their support and encouragement throughout the many years of study.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. The author consents to the thesis being made available for photocopying and loan, if applicable and if accepted for the award of the degree.

Uwe Horst Ströher

LIST OF ABBREVIATIONS

A: adenine

aa: amino acid

ACP: acyl carrier protein

Ap: ampicillin

ATP: adenosine 5'-triphosphate

BHI: brain heart infusion

bp: base pair

BSA: bovine serum albumin

CAT: chloramphenicol acetyltransferase

C: cytosine

Cm: chloramphenicol

CTP:cytosine 5'-triphosphate

cyto: cytoplasm

DNA: deoxyribonucleic acid

DNase: deoxyribonuclease

dNTP: deoxyribonucleoside triphosphate

ddNTP: dideoxyribonucleoside triphosphate

DOC: deoxycholate

DTT: dithiothreitol

EDTA: ethylene-diamine-tetra-acetic acid

EtBr: ethidium bromide

G: guanine

Gm: gentamycin

GTP:guanine 5'-triphosphate

HAI: haemagglutination inhibition assay

Hly: haemolysin

IM: inner membrane

IPTG: isopropyl-b-D-thiogalactopyranoside

kb: kilobase pairs

kDa: kilodalton

KDO: keto-3-deoxy-D-mannonse-octulosonic acid

Km: kanamycin

LB: Luria broth

LOS: lipooligosaccharide

LPS: lipopolysaccharide

MFRHA: mannose-fucose resistant haemagglutinin

mRNA: messenger ribonucleic acid

NA: nutrient agar

NB: nutrient broth

NMR: nuclear magnetic resonnance

nt: nucleotide

OD: optical density

oligo: oligodeoxynucleotides

om: outer membrane

ORF: open reading frame

OS: oligosaccharide

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PEG: polyethylene glycol-6000

peri: periplasm

R: resistant

RBC: red blood cells

RBS: ribosome binding site

RF: replicative form

Rif: rifampicin

RNA: ribonucleic acid

RNase: ribonuclease

rpm: revolutions per minute

S: sensitive

SD: Shine-Dalgarno

SDS: sodium dodecyl sulphate

Sm: streptomycin

Sp: spectinomycin

SRBC: sheep red blood cell

T: thymine

Tc: tetracycline

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

Tn: transposon

Tris: Tris (hydroxymethyl) aminomethane

TTP:thymine 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-D-galacto-pyranoside

Abstract

Vibrio cholerae O1 the aetiological agent of cholera in man, exists as two biotypes, Classical and El Tor and two distinct serotypes, Inaba and Ogawa. A third rare and unstable serotype Hikojima is as yet ill understood. The three serotypes are distingished on the basis of antigens on their lipopolysaccharide. Inaba strains express the A and C antigens, Ogawa strains A, B and a small amount of C, whereas Hikojima strains express all three antigens. The serotype of a given strain is not fixed in that the strains can undergo spontaneous serotype conversion from Ogawa to Inaba and vice versa. This phenomenon was first observed in 1918 and since that time much research has gone into identifying the antigens and the molecular basis of the switch. This thesis has sought to elucidate the mechanism by which serotype conversion takes place.

The *rfbT* gene has been shown to be responsible for the expression of the B antigen and has been cloned from a number of Inaba and Ogawa strains. Analysis of the nucleotide sequence has revealed that Inaba strains appear to have a defect in this gene. This results in a truncated protein which is non-functional. In Ogawa strains the gene is highly conserved. The *rfbT* gene is found within a 20kb *Sac*I fragment which encodes O-antigen biosynthesis in *V. cholerae* O1. *RfbT* has its own promoter and this fact together with its low G-C content of 32% makes it distinct from the rest of the *rfb* operon. Inactivation of *rfbT* by insertion of an antibiotic marker, results in the loss of the B antigen from Ogawa strains.

The isogenic Ogawa-Inaba strains were used to asses the role of serotype in pathogenesis. The B antigen does not appear to affect the pathogenesis *per se* although it probably plays a role in avioding the host immmune response. Other mutantions affecting O-antigen biosynthesis have a marked effect on the virulence by attenuating the strain. This appears to be due to the loss of motility and the toxin co-regulated pilus.

The RfbT protein molecular weight is 32kDa in Ogawa strains. The protein has been localized using specific antiserum to the inner membrane of *V. cholerae* . This is in general agreement with the model for O-antigen and LPS biosynthesis which is though to occur at the

inner membrane or periplasmic space. The proposed topology of RfbT places most of the protein in the periplasmic space.

The expression of the B and C antigens are interdependant in that the B antigen is either a modified form of C or is masking C. The analysis of these antigens using serotype converted strains and NMR has not as yet revealed their structure.

Contents

CHAPTER 1

Introduction

1.1 Lipopolysaccharide

The lipopolysaccharide (LPS) of Gram negative bacteria is the most abundant molecule on the cell surface, where it provides a protective barrier to hydrophobic agents and detergents (Osborn, 1979). The best characterised and most extensively studied LPS is that of *Salmonella typhimurium* and studies on this organism together with *Escherichia coli* has provided a model for LPS biosynthesis.

The chemical structure of LPS has been determined and shown to consist of three distinct regions; lipid A, the core oligosaccharide and the O-antigen (Fig. 1.1) (Lüderitz *et al.*, 1968). The lipid A forms part of the lipid bilayer of the outer membrane, the core is subsequently attached to the lipid A via an acid labile linkage with keto-3-deoxy-D-mannose-octulosonic acid (KDO) (Schmidt *et al.*, 1969).

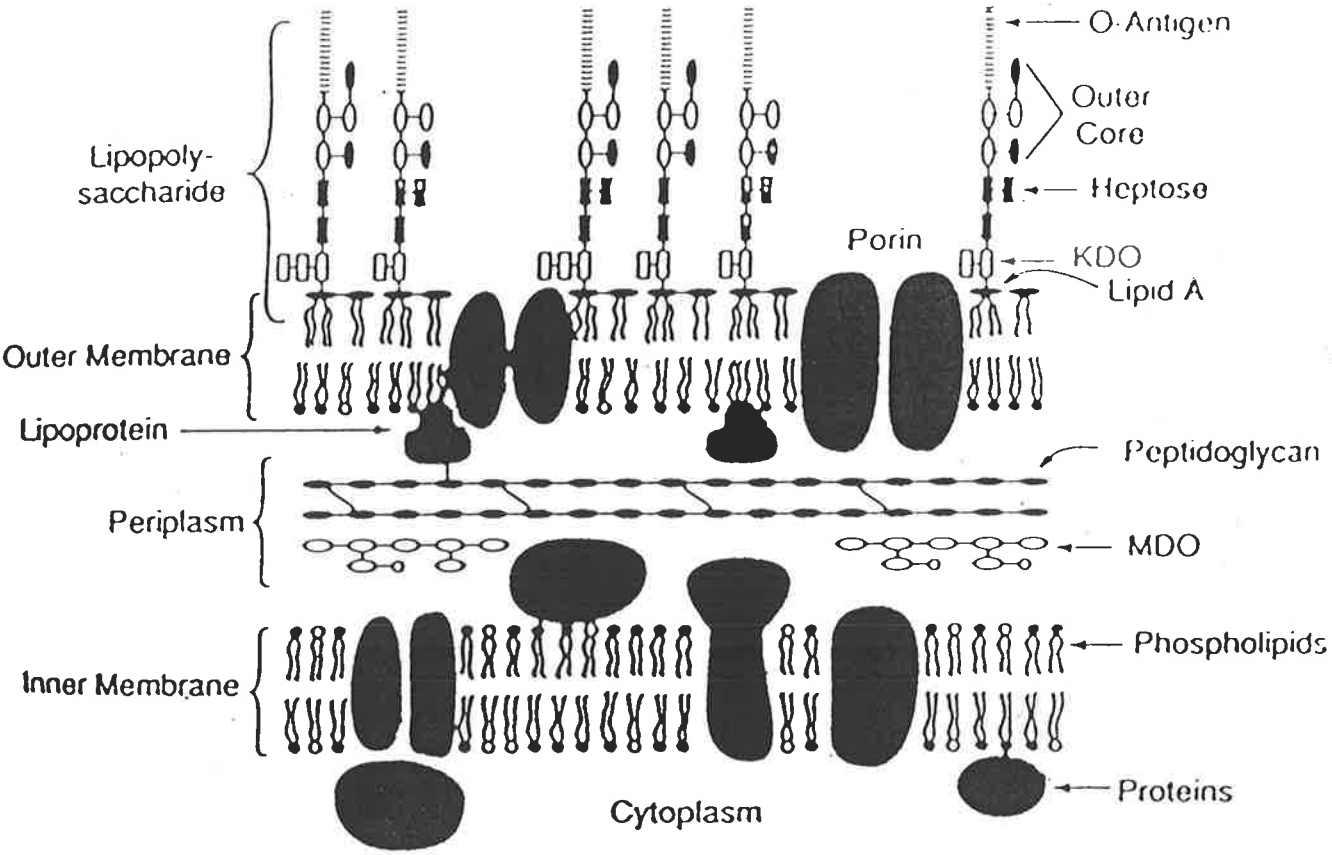
The outermost region is the O-antigen which is linked to the core and carries the serological O-specificity of Gram-negative bacteria (Lüderitz *et al.*, 1971; Jann and Westphal, 1975). The O-antigen usually consists of a polysaccharide or monosaccharide polymer of variable length.

1.1.1 Lipid A

Unlike the rest of the LPS molecule (core and O-antigen), assembly of lipid A not only involves the biosynthesis of sugars, namely glucosamine, but also the linkage of fatty acids.

Figure 1.1: Schematic representation of the *Salmonella typhimurium* envelope.

The envelope of *S. typhimurium* is divided into three distinct domains; Inner or cytoplasmic membrane, peptidoglycan and the outer membrane. The LPS makes up part of the outer membrane; i.e. the fatty acids from the lipid A make up the outer leaflet of the lipid bilayer. The LPS is further divided into the core oligosaccharide and the O-antigen.
Ovals and rectangles represent sugar residues within the LPS. The KDO is the linking sugar between the core and the lipid A. MDO are membrane derived oligosaccharides.
Figure is reproduced from Raetz (1990).


O-Antigen
Outer Core
Heptose
KDO
Lipid A
Porin
Lipopoly-saccharide
Outer Membrane
Lipoprotein
Peptidoglycan
Periplasm
MDO
Phospholipids
Inner Membrane
Proteins
Cytoplasm

The lipid A of *E. coli* and *S. typhimurium* consists of a β-1-6 linked disaccharide of glucosamine, which is acylated with R-3-hydroxymyristate at position 2,3,2' and 3'and is phosphorylated at position 1 and 4'. The 2,3 R-3-hydroxymyristate is further esterified with laurate and myristate (Fig. 1.2; Takayama *et al.*, 1983).

The lipid A molecule is linked to the core oligosaccharide via KDO by an α 2-6' linkage (Strain *et al.*, 1983a, 1983b).

The biosynthesis of lipid A has only been recently elucidated. This has been due to a number of factors, which include the difficulty in obtaining mutants defective in lipid A biosynthesis and a lack of understanding of the sites of acylation.

Initial work was performed using temperature sensitive mutants defective in KDO synthesis; this leads to the accumulation of a lipid A precursor that is not fully acylated, and is called lipid IV_A (Galanos and Lüderitz, 1975; Rick and Osborn, 1977; Rick *et al.*, 1977; Rick and Young, 1982). The starting product of lipid A is UDP-acetyl-glucosamine (Anderson *et al.*, 1985). This product is acylated with R-3-hydroxymyristate at position 2 and 3; this requires the enzyme UDP-N-acetyl glucosamine acyltransferase (*lpxA*) and an acyl carrier protein (ACP) carrying R-3-hydroxymyristoyl (Crowell *et al.*, 1986; Anderson and Raetz, 1987; Coleman and Raetz, 1988; Anderson *et al.*, 1988). The next significant step is the formation of lipid IV_A by the formation of tetra-acyl-disaccharide-1-phosphate (Radika and Raetz, 1988) from uridine-di-phosphate-2,3,-diacyl-glucosamine (UDP-2,3-diacyl-GluN). This is carried out by the enzyme lipid A disaccharide synthase (Ray *et al.*, 1984; Crowell *et al.*, 1986; Crowell *et al.*, 1987) encoded by *lpxB*. The final stage to lipid IV_A is phosphorylation of the 4' end of the glucosamine molecule by 4' kinase (Ray and Raetz, 1987).

Without KDO the lipid IV_A cannot be acylated to form lipid A. Thus, temperature sensitive KDO synthesis mutants such as *kdsA* and *kdsB* accumulate the underacylated disaccharide-bis-phosphate precursor of lipid A (Rick and Osborn, 1977).

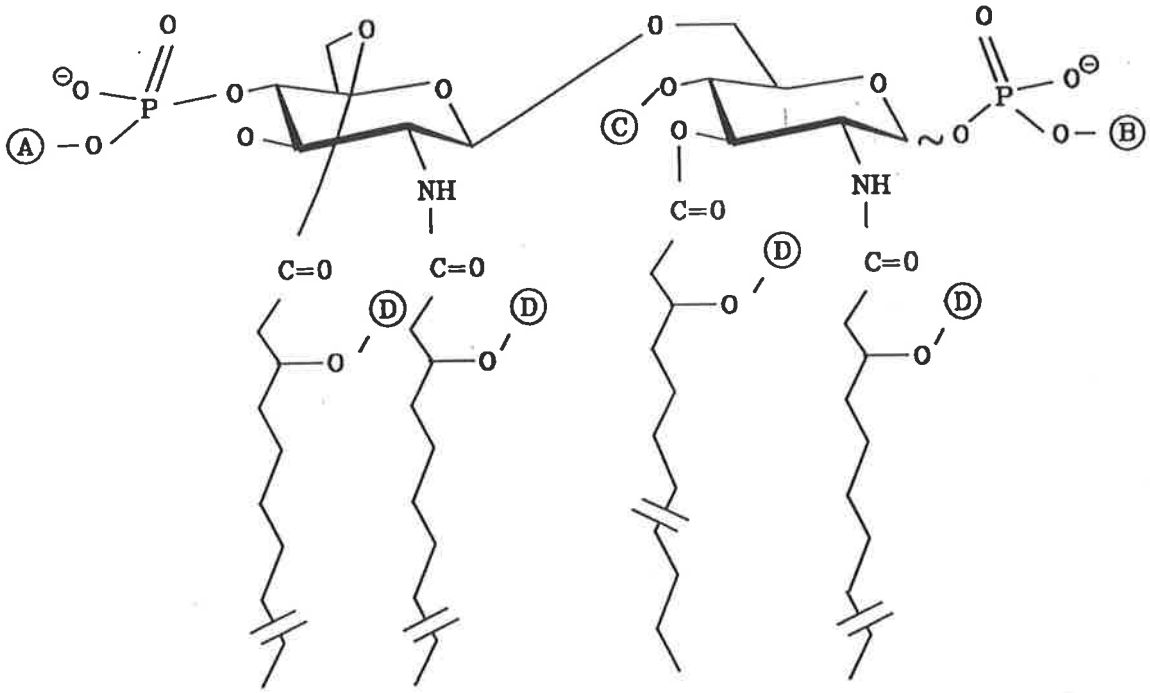
The next step is the addition of two KDO units to the lipid IV_A. This is done by KDO transferase encoded by *kdtA* (Clementz and Raetz, 1988). This product KDO2-IV_A is

Figure 1.2: Structure of lipid A.

The structure of lipid A shown is similar in many organisms. The circled letters indicate points of structural variation between the lipid A of different bacteria. The major points of variation between species are as follows:

1. the presence and nature of residues attached at the A and B positions to the backbone phosphate groups
2. differing substitutions of the glucosaminyl-saccharide present at position C
3. the type and chain length of fatty acids
4. 3-O-acetylation of the 3-hydroxy fatty acids at position D

Diagram was taken from Rietschel *et al.* (1983).


O
⊖O
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A
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C=O
D
O
NH
C=O
D
O
O
P
O⊖
O
B

then acylated with laurate and myristate due to the utilization of the appropriate ACPs and two as yet unidentified novel acyltransferases. This structure of lipid A with 2 KDOs is the minimal LPS required for cell viability. Any further reduction in the LPS results in a non-viable organism either due to membrane instability or the accumulation of toxic intermediates.

1.1.2 LPS Core region

The LPS core oligosaccharide of *S. typhimurium* and *E. coli* can be divided into the inner and outer core regions (see Fig. 1.3).

The inner core consists of the KDO linked to lipid A and the two heptose units. The outer core contains the N-acetylglucosamine, glucose and galactose sugars.

The genes required for core biosynthesis have been cloned (Creeger and Rothfield, 1979; Creeger *et al.*, 1979; Coleman, 1983; Kadan *et al.*, 1985; Austin *et al.*, 1990; Pradel and Schnaitman, 1991). Although initial knowledge concerning LPS core structure has come from mutants with incomplete LPS core structures (Hancock and Reeves, 1976) more recent data have been obtained from the cloned *rfa* genes.

Some of the core mutants that have been defined have the following sugar defects:-

Ra: Complete core

Rb1: Lacking N-acetylglucosamine

Rb2: Lacking glucose II

Rc: Lacking galactose I

Rd1: Lacking glucose I

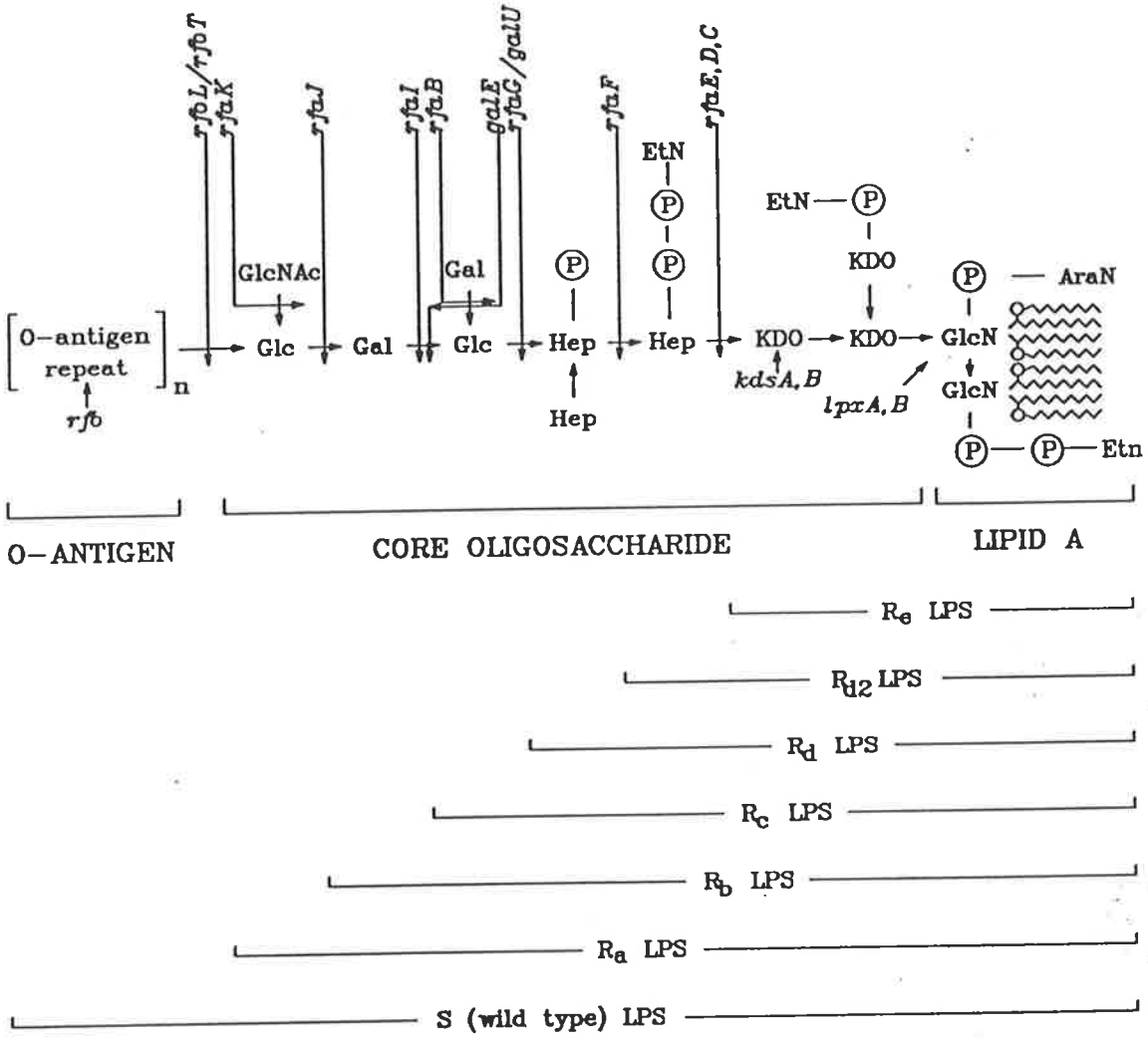
Rd2: Lacking heptose II

Re: Lacking heptose I

Any mutation affecting the main oligosaccharide chain, i.e. the core backbone sugars will prevent the addition of all units distal to it, including the O-antigen. An organism is

Figure 1.3: Definition of chemotype variants and genetic loci involved in the biosynthesis of the *S. typhimurium* LPS molecule.

Schematic diagram of the smooth LPS of *S. typhimurium.* Key: Glc, D-glucose; Gal, D-galactose; GlcN, D-glucosamine; GlcNAc, N-acetyl-D-glucosamine; Hep, L-glycero-D-mannose-heptose; KDO, 2-keto-3-deoxy-D-manno-octulosonic acid; AraN, 4-amino-L-arabinose; P, phosphate; EtN, ethanolamine; , hydroxy and nonhydroxy fatty acids; Ra to Re indicate incomplete R form lipopolysaccharides. The arrows with the *rfa*, *rfb*, *lpxA,B*, *kdsA,B* and *gal* genes show the location of the enzymatic steps encoded by these genes. Diagram adapted from Lüderitz *et al*, (1971); Hitchcock *et al*, (1986); Raetz (1990).


rfbL/rfbT
rfaK
rfaJ
rfaI
rfaB
galE
rfaG/galU
rfaF
rfaE,D,C
EtN
GlcNAc
Gal
Hep
KDO
AraN
O-antigen repeat
n
rfb
Glc
Gal
Glc
Hep
Hep
KDO
KDO
GlcN
GlcN
Etn
kdsA,B
lpxA,B
O-ANTIGEN
CORE OLIGOSACCHARIDE
LIPID A
Re LPS
Rd2 LPS
Rd1 LPS
Rc LPS
Rb LPS
Ra LPS
S (wild type) LPS

considered to be rough if it lacks either the O-antigen or any of the LPS core sugars. A smooth organism contains complete lipopolysaccharide. Thus, one can obtain mutants with differing levels of roughness (Fig. 1.3). Many mutants have been selected by phage resistance since a number of bacteriophage use the core as their site of adsorption (Hellerquist and Lindberg, 1971, Wilkinson *et al.*, 1972). The type of mutants and the genes affected vary between *E. coli* and *S. typhimurium* due to a difference(s) in the backbone of the core.

The structure of the *S. typhimurium* core oligosaccharide is:

GlcII-GalI-GlcI-HepII-HepI-KDO

The structure of the *E. coli* core oligosaccharide is:

GlcIII-GlcII-GlcI-HepII-HepI-KDO

(Creeger and Rothfield, 1979; Rick, 1987)

Thus, not all the genes found in *Salmonella* are able to be complemented by genes from *E. coli* although analogous genes have been proposed such as *rfaM* and *rfaN* in *E. coli* for *rfaI* and *rfaJ* in *Salmonella* (Austin *et al.*, 1990).

The genes involved in core biosynthesis can be classified into three distinct groupings: firstly, those genes involved in the synthesis of specific sugar nucleotides; secondly, genes encoding the transferases; and thirdly, the regulatory genes *rfaH* in *Salmonella* and *sfrB* in *E. coli* K-12 (Wilkinson and Stocker, 1968; Lindberg and Hellerquist, 1980; Kuo and Stocker, 1972; Beutin *et al.*, 1981).

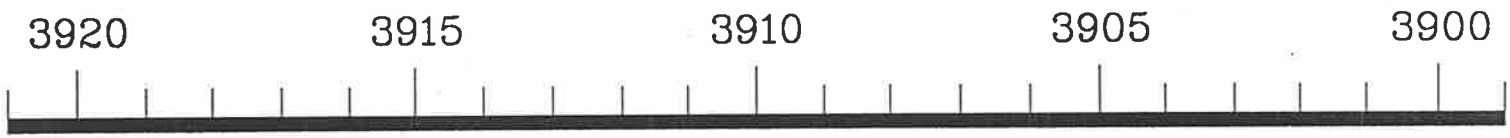
There are also a number of other mutants not classified as *rfa*. These mutants lack some of the core sugars whose biosynthesis is not encoded by *rfa* genes. For example, *galE* mutants are unable to synthesize UDP-galactose (Nikaido, 1962). In *S. typhimurium galU* mutants lack UDP-glucose pyrophosphorylase and are unable to synthesize UDP-glucose and are thus like *rfaG* mutants (Nakae and Nikaido, 1971).

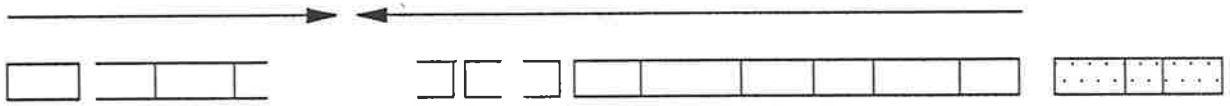
The genes for core biosynthesis appear to be clustered in the *rfa* locus (Fig. 1.4; Mäkelä and Stocker, 1984). The genes *rfaK*, *rfaJ*, *rfaI* and *rfaB* are located in the main *rfa*

Figure 1.4: Physical map of *rfa* loci of *E. coli* and *S. typhimurium.*

Physical maps of the *rfa* loci of *E. coli* K-12 and *S. typhimurium* were derived from Schnaitman *et al.* (1991). The loci are approximately 18kb in length. The scale indicates distance in kilobase coordinates on the *E. coli* K-12 restriction map of Kohara *et al.* (1987). The genes which have been completely sequenced are indicated by complete boxes. Open boxes indicate open reading frames which terminate where the sequence is not known. The bracketed genes have only been localized on restriction fragments and have not been sequenced. The direction of transcription is shown by the arrows, which appears to be the same for both organisms.

E. coli and S. typhimurium rfa operons





E. coli

rfa D F G L (K) Z Y J I B 38k P G Q kdtA fpg

S. typhimurium

rfa D F G L K Z Y J I (B) (G)

Table 1.1: *rfa* genes for *Salmonella* unless otherwise specified.

rfaL	links O-polysaccharide to core (Cynkin and Osborn, 1968)
rfaK	N-acetylglucosamine transferase (Hellerquist and Lindberg, 1971)
rfaJ	Glucosyltransferase II (Kadan *et al.*, 1985)
rfaI	Galactosyltransferase I (Kadan *et al.*, 1985)
rfaB	Galactosyltransferase II (Kadan *et al.*, 1985)
rfaG	Glucosyltransferase I (Kadan *et al.*, 1985)
rfaH	Positive regulator for *rfa* genes (Lindberg and Hellerquist, 1971)
sfrB (*E.coli*)	Positive regulator for *rfa* genes (Beutin *et al.*,1981)
rfaM (*E.coli*)	Glucosyltransferase II (Austin et al., 1990)
rfaN (*E. coli*)	Glucosyltransferase III (Austin et al., 1990)
rfaD	ADP-1-glycero-D-mannoheptose-6-epimerase (Coleman, 1983)
rfaF	Heptosyltransferase II (Mäkelä and Stocker, 1984)
rfaE	Heptosyltransferase I/Synthesis of Heptosyl donor (Mäkelä and Stocker, 1984)
rfaC	Heptosyltransferase I/Synthesis of Heptosyl donor (Mäkelä and Stocker, 1984)

cluster and are organised into an operon (Austin *et al.*, 1990). The position of other *rfa* genes is less well understood although it appears as if *rfaG* is also in the cluster containing *rfaK*, *rfaJ*, *rfaI* and *rfaB*. More recently the transcriptional organization of this region has been studied using *lacZ* and *cat* transcriptional fusions. These studies have identified a promoter region upstream of *rfaG*, *rfaB*, *rfaI* and *rfaJ* (Brazas *et al.*, 1991). The other *rfa* genes are in the same region of the chromosome but there is some uncertainty as to the distance between these genes since not all of the *rfa* genes have been cloned as a single functional unit (Kadan *et al.*, 1985; Austin *et al.*, 1990).

The proposed order and locations of the promoters of the *rfa* genes can be seen in Figure 1.4. These genes were located by the use of transposon insertions and complementation analysis (Austin *et al.*, 1990).

The other *rfa* genes *rfaA*, *rfa C*, *rfaD*, *rfaE* and *rfaF* are involved in heptose biosynthesis for the inner core. The best studied gene in this cluster is *rfaD*, encoding ADP-1-glycero-D-mannoheptose epimerase, which has been cloned, sequenced and had its promoter identified (Coleman, 1983; Pegues *et al.*, 1990).

The inner core can be considered to contain the KDO sugar residues. These sugars are essential for cell viability: KDO is produced from arabinose-5-phosphate and involves two enzymes. The first enzyme is KdsA which is a KDO-8-phosphate synthetase, the second enzyme KdsB is the KDO-CMP synthetase (Rick and Osborn, 1977; Sanderson and Roth, 1988; Woisetschläger and Högenauer, 1987). This KDO deravitive is then linked to lipid IV_A via KdtA. Subsequent assembly of the core occurs via the sequential addition of high energy UDP-sugar precursors to the growing backbone by the sugar transferases.

1.1.3 O-polysaccharide: Genetics and sugar biosynthesis

The O-antigen of the lipopolysaccharide is highly polymorphic, not only between, but within species. In *Salmonella* the O-polysaccharide was studied using anti-sera and found to

show great structural variation (Kaufmann, 1966; Lüderitz *et al.*, 1968), it is due to this that the O-polysaccharide is referred to as the O-antigen or the heat stable somatic antigen.

The O-antigen consists of a polymer of a variable number of identical oligosaccharide units. This structure is usually a heteropolymer consisting of a few monosaccharides. In *Salmonella typhimurium*, the O-antigens consist of a repeat unit of four hexoses (abequose, mannose, rhamnose and galactose). This structure is assembled from the nucleotide sugars CDP-abequose, GDP-mannose, dTDP-L-rhamnose and UDP-galactose on the undecaprenol phosphate intermediate. The biochemical pathway for the sugars has been elucidated and the *rfb* genes involved have been assigned various functions (Fig. 1.5). The sugars are synthesised prior to polymerization and modification of the O-antigen units (Jann and Jann, 1984; Mäkelä and Stocker, 1984). Any defect in O-antigen biosynthesis or assembly will lead to a rough phenotype, i.e. complete core but no O-antigen, which is in contrast to variable length LPS core mutants. The genes required for O-antigen biosynthesis are termed the *rfb* genes; these genes are found in a cluster in *Salmonella typhimurium* LT2 and other enterobacteriaceae at 42 mins on the genetic map (Sanderson and Roth, 1988).

It has been known for many years that the *rfb* region not only contains the genes that determine the synthesis of specific monosaccharides but also those that determine correct assembly into the LPS O-antigen. This was shown by genetic crosses between *Salmonellae* of different O groups (Mäkelä, 1965, 1966; Kishi and Iseki, 1973a, 1973b). The first genetic-biosynthetic approach was carried out by Nikaido *et al.* (1967) and Levinthal and Nikaido (1969). These studies were the first in which a genetic analysis was directly linked to the biosynthesis of O-antigen. Using deletions they proposed a gene order for the *rfb* cluster. More recently, the *rfb* cluster from *Salmonella typhimurium* strain LT2 has been cloned and sequenced (Jiang *et al.*, 1991). The proposed map of the *rfb* operon of *Salmonella typhimurium* strain LT2 is shown in figure 1.6. Table 1.2 shows the *rfb* genes associated with the sugar biosynthesis and the known transferase genes.

Nucleotide sequence analysis of the *rfb* region from various *Salmonella* serovars, namely A, B and D, has allowed the genetic basis of serology to be studied (Verma and

Figure 1.5: Biosynthetic pathway for the sugars in the *Salmonella typhimurium* O-antigen.

The biosynthetic pathways for the sugars in the *S. typhimurium* O-antigen have been known for a number of years. The sugars are all derived from glucose which is modified by either the *rfb* gene products, or in the case of galactose by genes outside of *rfb*. The possible site of action of the various gene products is indicated.

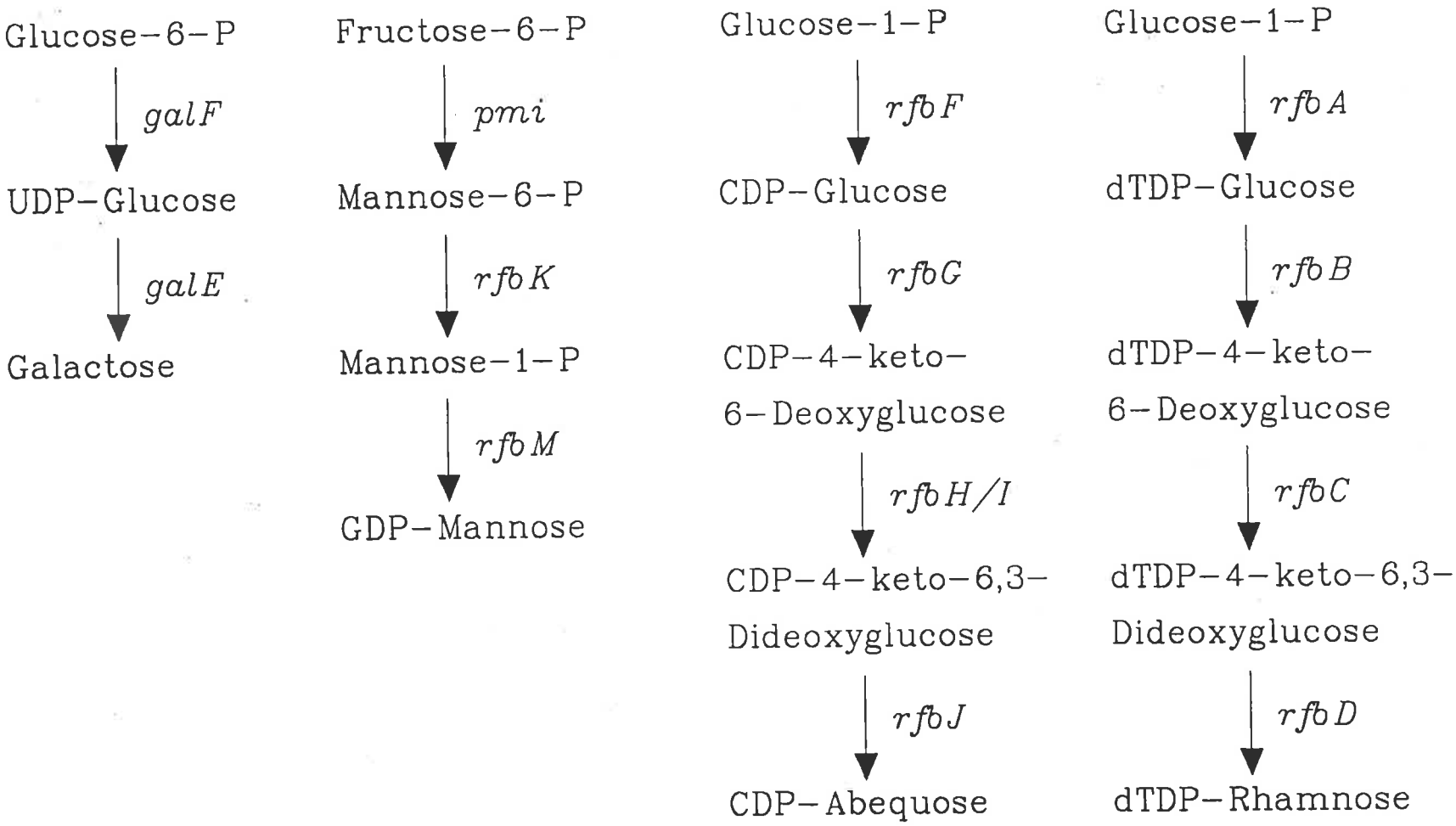

Glucose-6-P
galF
UDP-Glucose
galE
Galactose
Fructose-6-P
pmi
Mannose-6-P
rfbK
Mannose-1-P
rfbM
GDP-Mannose
Glucose-1-P
rfbF
CDP-Glucose
rfbG
CDP-4-keto-
6-Deoxyglucose
rfbH/I
CDP-4-keto-6,3-
Dideoxyglucose
rfbJ
CDP-Abequose
Glucose-1-P
rfbA
dTDP-Glucose
rfbB
dTDP-4-keto-
6-Deoxyglucose
rfbC
dTDP-4-keto-6,3-
Dideoxyglucose
rfbD
dTDP-Rhamnose

Figure 1.6: Physical map of the *rfb* operon of *Salmonella typhimurium* LT2.

The *rfb* locus of *S.typhimurium* is divided into five distinct domains.

1. Biosynthesis genes for rhamnose
2. Biosynthesis genes for abequose
3. Region containing genes for the transferases
4. Biosynthesis genes for mannose
5. Region containing the gene for the mannose transferase.

ORF = open reading frames: These ORF have as yet no assigned genes for which a functional assay exists.

The whole of the *rfb* operon from *S.typhimurium* has been sequenced (Jiang *et al.*, 1991). The genes marked are those for which precise functions are known and there exist functional assays.

Salmonella typhimurium rfb Operon

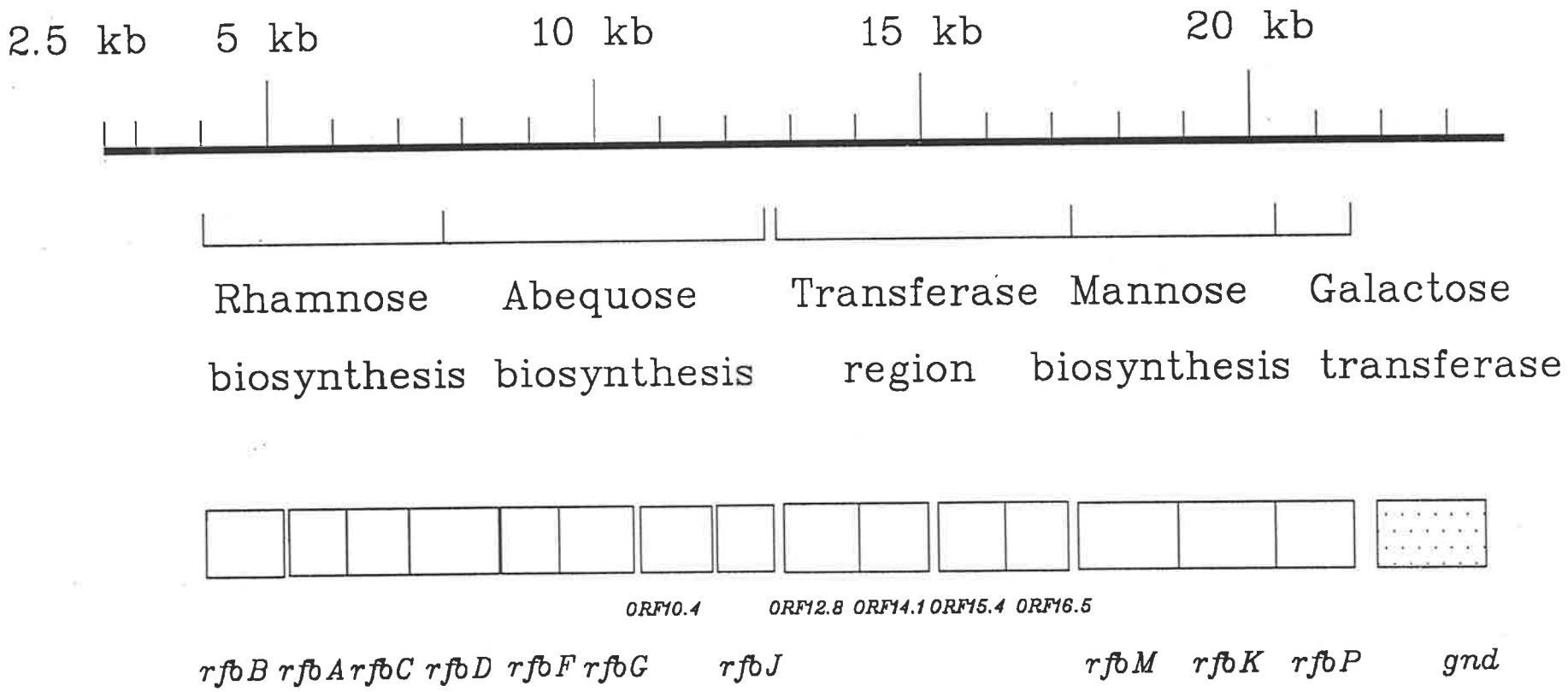


Table 1.2 Table of *rfb* genes *S. typhimurium* and their protein products and functions

The genes for the LPS biosynthesis are clustered in an operon. For many of the products of the *rfb* operon an assay system exists for function. This has allowed a putative biochemical pathway for O-antigen biosynthesis to be determined.

rfbA: Glucose-1-phosphate thymidyltransferase

rfbB: dTDP-D-glucose 4,6-dehydrogenase

rfbC: dTDP-6-deoxy-D-glucose-3,5 epimerase

rfbD: dTDP-6-deoxy-L-mannose dehydrogenase

rfbF: Glucose-1-phosphate cytidyltransferase

rfbG: CDP-glucose-4,6 dehydrogenase

rfbH: abequose synthesis

rfbI: abequose synthesis

rfbJ: abequose synthetase

rfbK: (mannose-6-phosphate) phosphomannomutase

rfbL: phosphomannomutase (outside of rfb)

rfbM: Mannose-1-phosphate guanylyltransferase

rfbN: Rhamnose transfrease

rfbP: Galactosyl-P-P-undercaprenol synthetase (galactose transferase

Reeves, 1989, Liu *et al.*, 1991). *Salmonella* groups A, B and D have paratose, abequose and tyvelose respectively as the immunodominant sugar in their O-antigens, which are otherwise identical. The gene *rfbJ* from group B strain encodes abequose synthetase (Wyk and Reeves, 1989); two other genes *rfbS* and *rfbE* are involved in the unique sugar biosynthesis of serovars *t*yphi (group A) and *paratyphi* (group D), respectively. The gene *rfbS* encodes paratose synthetase, whereas *rfbE* encodes CDP-tyvelose epimerase i.e. which converts CDP-paratose to CDP-tyvelose (Verma *et al.*,1988, 1989; Liu *et al.*, 1991).

Not all the genes involved in O-antigen biosynthesis are clustered in the *rfb* operon. The genes that encode the synthesis of UDP-galactose, which is used in both the core and the O-antigen, lie outside of the *rfb* operon, although *galF* probably lies close to the *rfb* cluster (Nakae and Nikaido, 1971a, 1971b; Nakae, 1971; Liu *et al.*, 1991).

The gene required for the conversion of fructose-1-phosphate to mannose-6-phosphate (*pmi*) also lies outside of the *rfb* region (Collins and Hackett, 1991a).

Although the *rfb* gene cluster has been sequenced and thoroughly studied by the use of deletions, point mutations and complementation analysis, the exact position of all of the *rfb* genes within the operon is still not totally clear (Jiang *et al.*, 1991).

Deletions arising from the rhamnose end of the *rfb* operon are unstable and accumulate secondary mutantions in order to relieve the toxic accumulation of unfinished O-units, which prevent the reuse of the undecaprenol lipid carrier (Mäkelä *et al.*, 1976). Deletions starting from the histidine end of the operon do not have these problems since incomplete O-units linked to undecaprenol do not occur; this is because RfbP, which is encoded by the last gene, is required to transfer the UDP-galactose to undecaprenol phosphate (Juasa *et al.*, 1969). Consequently this has allowed some mapping of the genes within the *rfb* operon such as those for rhamnose biosynthesis. The intermediate formed in rhamnose biosynthesis, dTDP-4-keto-6-deoxyglucose, is also used for the synthesis of thomasamine seen in the enterobacterial common antigen ECA (Jann and Jann, 1967; Mäkelä and Mayer, 1976; Mäkelä *et al.*, 1976). Deletions in the rhamnose biosynthesis pathway were found to have secondary mutations in *rfe-rff* (ECA cluster) (Mäkelä *et al.*, 1976),

presumably because incomplete ECA would saturate the undecaprenol phosphate carrier with incomplete oligosaccharide units similar to those described for incomplete O-antigen assembly.

1.1.4 O-antigen assembly

The linkage and polymerization of O-antigen repeat units can be divided into three steps -

1. the O-antigen oligosaccharide is synthesized on the antigen carrier lipid ACL (undecaprenol phosphate) or bactoprenol,
2. the oligosaccharide is polymerized into the O-antigen chain, and
3. polymerized O-antigen oligosaccharide chains are translocated to lipid A-core.

1.1.5 O-repeat unit biosynthesis

The initial and only reversible step in biosynthesis of the O-antigen of *Salmonella* O serogroups A, D, E, (Robbins and Wright, 1971; Wright and Kanegasaki, 1971) and C2, C3 (Shibaev *et al.*, 1979) is the transfer of galactose to undecaprenol phosphate. This generates a diphosphate linkage by the transfer of galactose-phosphate from UDP-galactose (Osborn and Tze-Yuen, 1968; Wright *et al.*, 1967; Robbins and Wright, 1971). The remaining sugars TDP-rhamnose, GDP-mannose and CDP-abequose are then sequentially transferred to the undecaprenol diphosphate-galactose intermediate. Each of these steps is mediated by a specific transferase, all of which are membrane associated (Osborn *et al.*, 1972a, 1972b).

1.1.6 Polymerization of O-antigen repeat units

For polymerization of O-antigen repeat units to occur, the complete O units are required. Thus, the repeat units are polymerised as a long chain still attached to the lipid carrier (Weiner *et al.*, 1965; Robbins *et al.*, 1966). This mechanism has once again been most widely studied in *Salmonella.* The type of linkage formed by the polymerase contributes to the structure and hence antigenicity of the O-antigen (Staub and Bagdian, 1966; Kanegasaki and Wright, 1970). The polymerization occurs at the reducing end which, in the case of *Salmonella typhimurium*, means a glycosidic linkage is formed between the reducing terminal galactose of the growing chain and the non-reducing mannose of the O-repeat unit. This releases the undecaprenol diphosphate, which is subsequently dephosphorylated to undecaprenol-phosphate to be recycled. The enzymes involved in this polymerization fractionate with the cytoplasmic membrane.

In *S. typhimurium*, the *rfc* gene encodes a protein responsible for this polymerization; strains that lack or are mutant for *rfc* have only a single O unit in their LPS (Naide *et al.*, 1965; Sanderson and Hurley, 1987). The *rfc* gene has been cloned from *S. typhimurium* and sequenced (Collins and Hackett, 1991b). Other bacterial species such as *Shigella flexneri* also appear to have an *rfc* polymerase activity (Simmons and Romanowska, 1987; Mavris, M. and Morona, R., personal communication). This gene maps within the *rfb* locus for *Sh. flexneri* and *S. typhimurium* C1 but not for *S. typhimurium* of group B where it maps between *gal* at 18 min and *trp* at 34 min (Mäkelä, 1966).

The O-antigen polymerization of other *Salmonella* groups (C and L) and in *E.coli* O8 and O9 does not occur via a *rfc*-dependent polymerization (Schmidt *et al.*, 1976; Jann *et al.*, 1979; Mäkelä and Stocker, 1984). The polymerization is *rfe* dependent although some steps are analogous to the previously described polymerization. The *rfe-rff* gene clusters have recently been cloned, and using such clones it has conclusively been shown that *E. coli* O9 uses *rfe* for the D-mannan polymerisation (Ohta *et al.*, 1991). The O-antigen of *E.coli* O9 consists of a pentasaccharide repeat unit containing only mannose, i.e. a single sugar (Prehm

et al., 1975). The initial step is the linkage of a glucose residue to undecaprenol, similar to the galactose in *Salmonella* group B and E. Subsequent linkage does not appear to involve polymerization of undecaprenol linked repeats but is a sequential transfer of mannose to the non-reducing terminus of the chain (Jann *et al.*, 1982). The O-antigen of *E.coli* O9 is subsequently linked to the core-LPS via the terminal reducing glucose, since the core lacks one glucose residue which is provided by the O-antigen (Weisgerber *et al.*, 1984).

1.1.7 O-polysaccharide translocation to lipid A-core

The transfer of the O-polysaccharide to the core probably occurs at the periplasmic face of the cytoplasmic membrane, but the exact method of translocation of the complete LPS molecule to the cell surface remains unknown (Robbins and Wright, 1971; Osborn, 1979; Mulford and Osborn, 1983). It has recently been shown that the translocation of the O-polysaccharide to the lipid A-core requires the proton motive force, since energy uncouplers block the final step in LPS assembly (McGrath and Osborn, 1991; Marino *et al.*, 1991).

For the attachment of O-polysaccharide, the core must be complete otherwise the phenotype will be rough, although the O-polysaccharide can be detected as lipid linked hapten (Beckmann *et al.*, 1964; Lindberg *et al.*, 1972). Unfinished O-units are unable to be attached to the core *in vivo* (Yuasa *et al.*, 1969) whereas incomplete O-polysaccharide can be attached *in vitro* (Nikaido and Nikaido, 1965; Shibaev *et al.*, 1979).

The O-antigen chains attached to the core are not of a homogeneous length, nor is all of the core substituted with O-antigen, thus, there must be some regulation of core substitution which balances polymerization and translocation (Goldman and Levie, 1980; Tsai and Frasch, 1982; Hitchcock and Brown, 1983). The translocation of the O-units is carried out by a linkage between the non-reducing sugar chain of the core and the reducing sugar of the O-antigen chain. This step releases the undecaprenol attached to the O-antigen chain, thus, allowing recycling of the lipid carrier. The O-antigen ligase(s) which is involved

has not been identified. In *S. typhimurium* two genes are involved, *rfaL* and *rfbT*, and mutants in either have the same rough phenotype characterised by complete core and O-polysaccharide linked to the lipid carrier.

1.1.8 LPS export

The mechanism of LPS export is poorly understood. The substrates needed for LPS biosynthesis such as the nucleotide sugars, ATP and R-3-hydroxymyristoyl carrier proteins are found or function on the inner surface of the cytoplasmic membrane. It is known that once LPS is exported, it cannot move back from the outer membrane which is in contrast to glycerophospholipids (Osborn *et al.*, 1972a, 1972b; Jones and Osborn, 1977). It has been proposed that LPS export occurs via zones of adhesion between the inner and outer membranes, at either the Bayer's junctions or the periseptal annuli (Muehlradt *et al.*, 1974; Bayer, 1975; Rothfield *et al.*, 1987), and that the LPS then diffuses across the cell surface.

The mechanism for exporting the LPS across these zones of adhesion or across the membranes is not known, although a number of systems have been proposed (Osborn, 1979; Raetz, 1990). These include:

1. the Flip-Flop of O-antigen, core-lipid A or complete LPS across the membranes,
2. budding of vesicles containing LPS, and
3. the continuity between the inner and outer membranes involving a filter for LPS.

So far, there is very little direct evidence for any of these systems. Although the energy coupling of O-antigen translocation reported by Osborn and co-workers (1991) suggests a system of high energy dependent translocators or that even a "flippase" may exist.

1.2 O-antigen variation in Gram negative bacteria

A number of distinct systems for O-antigen variation have been identified in Gram negative bacteria. Four organisms of particular interest are *Shigella flexneri*, *Salmonella typhmurium*, *Neisseria gonorrhoeae* and *Haemophilus influenzae*. This is not only because they are major disease causing organisms but they also display novel systems for O-antigen variation.

Sh. flexneri and *S. typhimurium* use bacteriophage which lysogenise the cell and subsequently alter their serotype, by modification of the O-antigen (Matsui, 1958; Iseki and Hamano, 1959; Okada *et al.*,1960; Giammanco, 1968) .

N. gonorrhoeae displays serotypic variation by employing a system of transcriptional attenuation (Stein and Petricoin, 1990).

H. influenzae lipooligosaccharide (LOS) expression uses a tetramer of the nucleotides CAAT as a translational switch (Cope *et al.*,1991).

These mechanisms are discussed in more detail in the following sections. The system employed by *V. cholerae* is unknown, but may be by one of the above mechanisms or by a novel process.

1.2.1 *Shigella flexneri* lipopolysaccharide modification

Within *Shigella flexneri* there are 12 distinct serotypes and these arise due to substitution of the O-antigen backbone (Fig. 1.7) (Ewing and Carpenter, 1966). A thirteenth serotype type 6 exists, which is atypical and contains a different core. Its core is the same as that of *E. coli* R1 whereas the other serogroups have the core of *E. coli* R3 (Katzenellenbogen and Romanowska, 1980; Simmons 1983a; 1983b). There is still some doubt as to the exact sugar composition of type 6 O-antigen (it may be isolate dependent) but what is known is that it varies markedly from those of the other O-serogroups of *Sh. flexneri* (Katzenellenbogen *et*

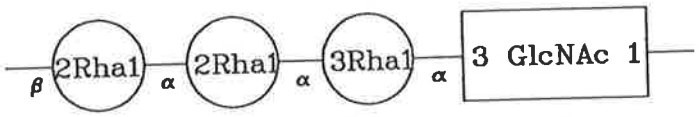
Figure 1.7: *Shigella flexneri* O-antigen variation.

Sh. flexneri exists as a number of serotypes called: 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b, 5a, 5b, X, Y and type 6. Chemical and physical studies and monoclonal antibodies have allowed the chemical structure associated with the type antigens (I, II, IV, V, VI) and group antigens (3,4; 7,8; 6) to be determined.

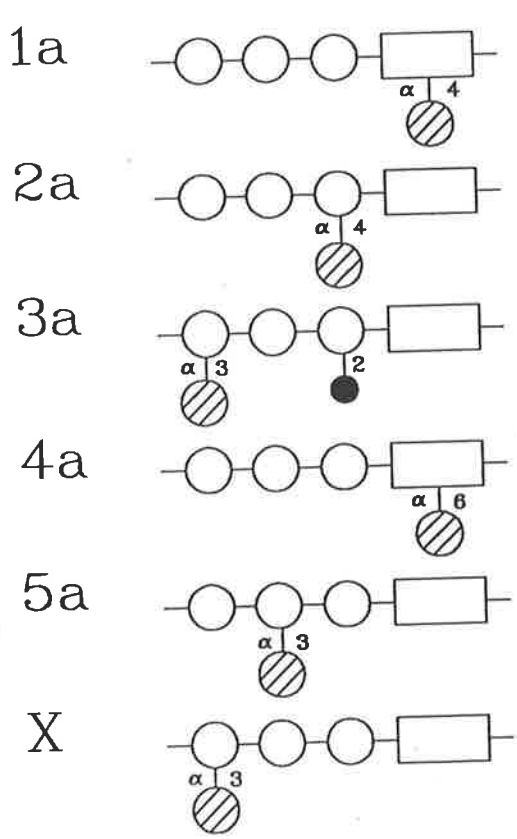
The structures of the O-antigen are shown which also shows the antigens exhibited by each serotype. All the serotypes are related to the basic O-antigen structure (Y serotype) which is composed of a tetrasaccharide having the sugars:

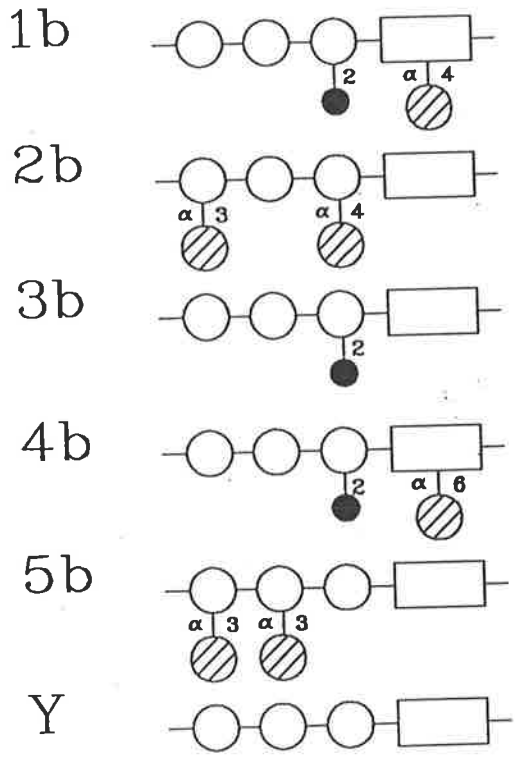
RhamnoseI-RhamnoseII-RhamnoseIII-N-acetyl glucosamine.

Substitution with either glucose or acetyl groups at various positions alters the group or type antigen.



glucosyl ● acetyl





al., 1976; Dmitrier *et al.*, 1979). Genetic studies also clearly demonstrate that the *rfb* region of type 6 strains is unrelated to those of all the other serotypes which are essentially identical (Macpherson *et al.*, 1991; Cheah *et al,*. 1991)

The O-antigen backbone of *Sh. flexneri* type Y consists of rhamnose and N-acetylglucosamine. This is the basic structure of the Y serotype O-antigen (Lindberg *et al.*, 1973; Kenne *et al.*, 1977). All other serotypes are derived from Y by either glycosylation or O-acetylation or both. This modification of the lipopolysaccharide can occur at the N-acetylglucosamine or on the rhamnose units. The rhamnose III is the only rhamnose sugar to be acetylated. Rhamnose III, along with the other rhamnose units can be glycosylated. The N-Acetyl-glucosamine can be glycosylated but it cannot be acetylated (Fig. 1.7)

The immuno-dominance of the O-antigen is best illustrated in *Sh. flexneri* since immunity to one serotype is not cross protective for any of the other serotypes (Lindberg *et al.*, 1991).

The genes encoding the proteins involved in O-antigen biosynthesis are in a cluster termed *rfb*, similar to *Salmonella*, *E. coli* and *V. cholerae*, but the genes involved in modification are unlinked to *rfb* and are in fact phage encoded. These bacteriophage are temperate and, upon lysogenization alter the O-antigen and thus, the serotype of the strain. Each phage carries its own specific modifying enzyme which is stably expressed as a consequence of the phage integrating into the chromosome at their corresponding attachment site (Luria and Burrows, 1957; Timakov *et al.*, 1970; Petrovskaya and Licheva, 1982). This makes the strain resistant to super-infection with the same phage.

The modifications from the basic Y serotype to the other serotypes may require a single phage if it is a single modification, such as Y to 3b requiring phage Sf6 (Clark *et al.*, 1991). Alternatively, it may require several phages to modify serotype Y to X and then to 2b, with phage f-7,8 and f-II sequentially (Giammanco, 1968; Lindberg *et al.*, 1978; Gemski *et al.*, 1975; Petrovskaya and Licheva, 1982; Clark *et al.*, 1991).

Thus, the phage encode either an O-acetylase (Sf6) or a glycosylase (f-7,8) which modifies the O-antigen. The serotype conversion seen in *Sh. flexneri* is similar to the effect

some bacteriophage, such as ε15, f27 and P22, have in *Salmonella* (Kanegasaki and Wright, 1973; Lindberg *et al.*, 1978). The serotype variation and conversion in *Sh. flexneri* is therefore completely bacteriophage mediated, the chromosomal loci for LPS biosynthesis providing only the basic Y O-antigen structure (Clark *et al.*, 1991; Macpherson *et al.*, 1991).

1.2.2 *Salmonella typhimurium* lipopolysaccharide modification

As with *Sh. flexneri, S. typhimurium* lipopolysaccharide can be modified by two means: O-acetylation and glycosylation. This may occur by either chromosomally or bacteriophage encoded genes (Iseki and Kashiwagi, 1955; Mäkelä, 1966; Mäkelä and Mäkelä, 1966; Haigwara *et al.*, 1966).

The bacteriophage mediated modification alters the lipopolysaccharide in such a way as to make the strain resistant to further infection (i.e. similar to *Sh. flexneri*) with the same or closely related bacteriophage. This modification always occurs at one or the other of the monosaccharides that are hydrolysed by the phage upon adsorption (Lindberg, 1977). Thus, the phage g_{341} O-acetylates the galactose in the O-antigen repeat of *S. typhimurium*, whereas the phage P22 glycosylates this sugar (Young *et al.*, 1964; Haigwara *et al.*, 1966, Wright, 1971). The modification occurs only if the phage has integrated into the host chromosome and the strain become lysogenic. This system is similar to that described for *Sh. flexneri* lipopolysaccharide modification.

There are two possible modifications that can occur by chromosomally encoded genes, acetylation and glycosylation. The O-acetylation is mediated by *oafA* (Mäkelä, 1966; Johnson *et al.*, 1966; Kishi and Iseki, 1973b). This modification occurs at either the abequose, rhamnose or glucose sugars in the O-antigen depending on the serogroup of *S. typhimurium* (Hellerquist *et al,*. 1970, 1972). Glycosylation is mediated by three chromosomally encoded genes, *oafB*, *oafE* and *oafC* (Stocker *et al.*, 1960; Wright and

Barzilai, 1971; Kishi and Iseki, 1974). Glycoslation occurs at the galactose residue and requires the various *oaf* genes in different serogroups.

1.2.3 Lipooligosaccharide

Most Gram negative bacteria contain a LPS of similar structure, made up of Lipid A, core and O-antigen, but several organisms display a different outer cell surface. *Haemophilus influenzae*, *Bordetella* and *Neisseria* do not have defined O-antigen, but instead possess an antigenic oligosaccharide structure. This oligosaccharide is an extension of the basic core structure and consequently it has been referred to as LOS (lipooligosaccharide) (Hitchcock *et al.*, 1986).

The LOS is similar to LPS in its biological activity in that it protects the cell from hydrophobic agents and is endotoxic. Furthermore it has also been shown to play a major role in virulence determination (Zwahlen *et al.*, 1986; Syrogiannopoulos *et al.*, 1988). Organisms such as *Haemophilus ducreyi* and *H. influenzae* have reduced virulence upon loss of the outer portion of the LOS. In contrast, changes in the antigens of the LOS help these organisms evade the host immune response (Kimura and Hansen, 1986; Odumeru *et al.*, 1987; Weiser *et al.*, 1989a).

The LOS structure is unknown but it has been proposed to contain lipid A and then a core oligosaccharide of three domains. The basal oligosaccharide and terminal lactoside domains appear to be conserved. The third domain called the elongation segment is responsible for the variability of the LOS (Griffiss *et al.*, 1988).

1.2.4 *Haemophilus influenzae* lipooligosaccharide variation

H. influenzae is an encapsulated Gram negative bacterium, which is a major cause of septicaemia and meningitis. This organism also possesses a LOS which can undergo spontaneous antigenic variation. Recently, some of the genes for the biosynthesis of the LOS have been cloned and expressed in *E. coli* (Spinola *et al.*, 1990; Cope *et al.*,1991).

The molecular basis of antigenic phase variation in *H. influenzae* LOS has been extensively studied. There appear to be three distinct levels of oligosaccharide (OS) expression (++++), (+) and (-); thus the switching is not simply an on/off variation. The genetic loci *lic*-1 is necessary for the expression of at least two distinct LOS structures, and was the first region to be cloned. Other loci *lic*-2 and *lic*-3 have been cloned and these also play a role in the expression of other LOS variants (Cope *et al.*, 1991; Maskell *et al.*, 1991). *H. influenzae* has a number of other loci which are involved in LOS biosynthesis but not antigenic variation (Zwahlen *et al.*, 1985; 1986).

Sequence analysis of these *lic* regions has revealed the mechanism responsible for antigenic variation, and it is the same for all three loci. The system involves a translational switch using the tetramer CAAT which determines if genes within the loci are in or out of the translational reading frame. The number of tetramers is highly variable, and up to 30 tetramers have been found (Weiser *et al.*, 1989a, 1989b). The heterogeneity seen of (-), (+) or (++++) can not be explained by the in or out of frame switch alone. The number of repeats play a significant role, in that either none, one or two ATG start codons are in frame (Fig. 1.8). The reason why a minor difference between the 5' nucleotide sequence and/or the N-terminus of the protein alters expression between (+) and (++++) is not understood. This system with differing levels of expression is the result of alterations to the expression of *lic*-1. The other two loci, *lic*-2 and *lic*-3 do not seem to be involved (Weiser *et al.*, 1989; Maskell *et al.*, 1991).

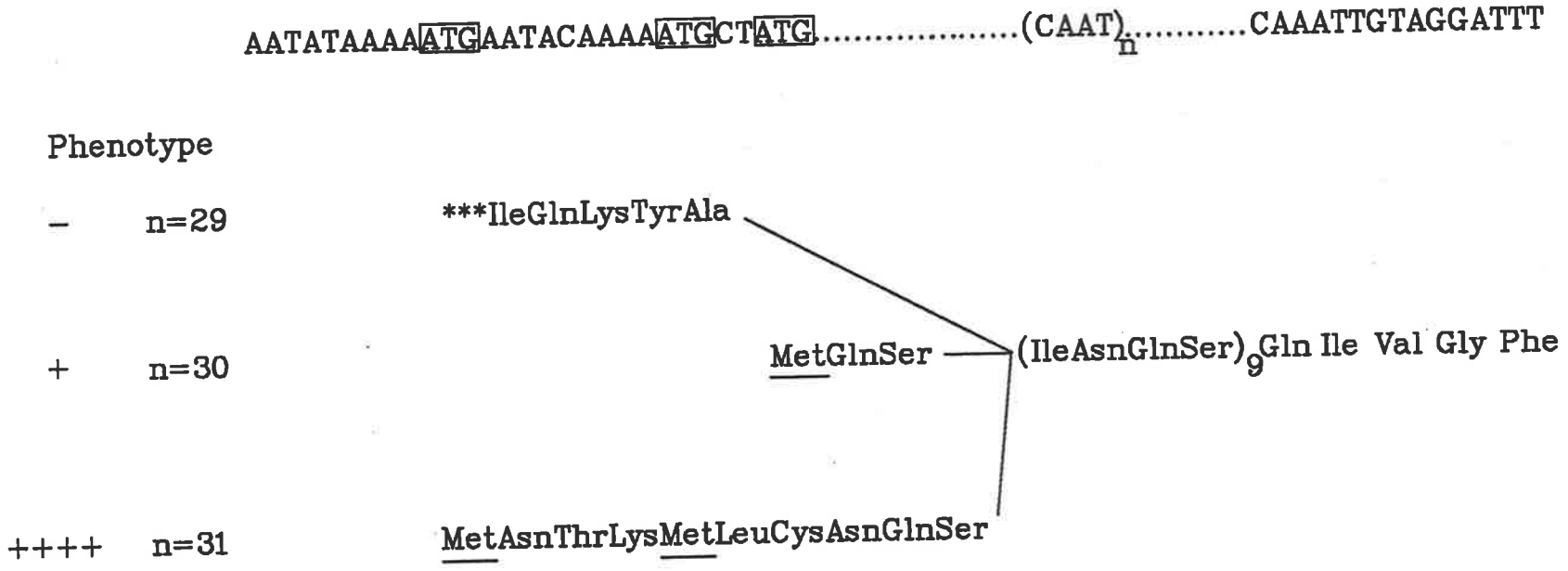
It is interesting to note that a similar translational switch occurs in the expression of the opacity proteins of *N. gonorrhoeae* and *N. meningiditis*. In these cases switching

Figure 1.8: Proposed mechanism of *Haemophilus influenzae* LOS phase variation.

Phase variation in *Haemophilus influenzae* LPS expressed by *lic*-1 is determined by a translational switch. The nucleotide sequence of the 5' end of *licA* is shown with the possible ATG start codons boxed. Variation in the number of CAAT repeats (n) shifts the reading frame of *licA*, altering the translational phase upstream of the repeats. The three possible amino-terminal translational products (n= 29-31) are shown below the sequence. Depending upon the number of CAAT repeats there is either none, one or two initiation codons in frame causing variable expression of *licA*. The three levels of *licA* expression corresponding to the translational phases determine the epitope -, +, ++++.

Diagram from Weiser *et al.*(1989b).

Mechanism of Haemophilus LOS Phase Variation



involves pentamers of CTCTT. This is only an on/off switch like in *lic*-2 and *lic*-3 (Stern *et al.*,1986; Stern and Meyer, 1987). The similarity between *Haemophilus* and *Neisseria* species goes further in that they also share common virulence factors (Koomey and Falkow, 1984).

1.2.5 *Neisseria gonorrhoeae* lipooligosaccharide variation

The mechanism employed by *N. gonorrhoeae* for LOS variation has only recently been elucidated. The lipooligosaccharide on the surface of the organism can vary in molecular weight from 3150 to 7100 . This variation in the molecular weight is due to a difference in the oligosaccharide, since the number of sugars and their chemical composition alters (Griffiss *et al.*, 1987, 1988).

A region involved in LOS biosynthesis has been cloned and sequenced. This locus contains two genetic units *lsi*-1 and *lsi*-3. The protein encoded by *lsi*-3 is involved in the polymerization of the oligosaccharide (OS) while the secondary structure involving the mRNA prior to *lsi*-1 regulates the expression of this protein. The secondary structure upstream from *lsi*-3 regulates the gross level of the transcribed message. Efficient transcription of a complete message only occurs if the small peptide *lsi*-1 is transcribed, preventing the formation of a Rho-independent terminator (Stein and Petricion, 1990).

The small Lsi1 peptide contains a number of codons that are rarely used by *N. gonorrhoeae* and thus the abundance of the rare tRNA's is critical to the translational efficiency. In a rich environment the level of the rare tRNA is elevated compared to the level in an environment where the cell is stressed. In a nutritionally stressed environment this would result in a decreased level of translation of *lsi*-1 which in turn causes an increase in the amount of message that is terminated at the stem loop of the mRNA leading to reduction in translation of *lsi*-3. The amount and molecular size of the OS will be regulated by the amount of the *lsi*-3 gene product (Stein and Petricoin, 1990).

There may be other systems operating in *Neisseria* that have not as yet been found. The mechanism by which the sugar composition itself varies is unknown.

1.3 *Vibrio cholerae*

V. cholerae is divided into more than 30 O-serotypic groups (Shimada and Sakazaki, 1977). Only *V. cholerae* of the O1 serotype is associated with cholera in man, the other serotypes are usually referred to as non-O1 or non-cholera *vibrios*. The O1 serotype exists as two biotypes; classical and El Tor, these are distinguished by a number of characteristics. The two biotypes were originally differentiated on the ability of El Tor strains to produce a soluble haemolysin for sheep erythrocytes, however marked variability is seen in the haemolytic phenotype. Strains of the El Tor biotype can agglutinate chicken erythrocytes whereas classical strains cannot. There are, however, a number of exceptions (Gan and Tjia, 1963; Roy *et al.*, 1965; Rizvi *et al.*, 1965; Gangarosa *et al.*, 1967; Pesigan *et al.*, 1967). The most accurate and reliable test is the sensitivity of all Classical *V. cholerae* to Mukerjee's group IV vibriophage to which El Tor strains are resistant (Mukerjee and Roy 1961; Monsur *et al.*, 1965). Furthermore the two biotypes can be distinguished by their polymyxin B sensitivity.

The classical biotype is thought to have been responsible for the first six of the seven great cholera pandemics which have occured since the early 19th Century. Prior to the seventh pandemic the El Tor biotype was thought not to cause cholera since it was only associated with mild diarrhoea. However, in 1962 with the outbreak of the seventh pandemic originating in South-East Asia the disease caused by El Tor strains was defined as true cholera. Thus, it was clear that both *V. cholerae* biotypes are pathogenic for man.

V. cholerae O1 of both biotypes can be further subdivided into three serotypes depending on the O-antigens on the lipopolysaccharide. These serotypes are designated Inaba, Ogawa and Hikojima, which all share a common antigen refered to as the A antigen. There are two specific antigens, B and C, which are expressed to varying degrees on the

different serotypes. Inaba strains express only C and A, while Ogawa strains express all three antigens A, B and C, although C is present in a much reduced amount compared to Inaba (Burrows *et al.*, 1946a, 1946b; Sakazaki and Tamura, 1971; Redmond *et al.*, 1973; Redmond, 1979). Redmond (personal communication) showed by absorption of an Ogawa anti-serum with Inaba cells that 5-10% of activity against Ogawa cells remained, indicating an Ogawa specific antigen termed B antigen. The third serotype termed Hikojima is extremely rare and unstable and expresses all three antigens in high amounts (Nobechi, 1923). The exact nature of Hikojima strains is unknown although it has been proposed that such strains may be segregating diploids (Bhaskaran and Sinha, 1971) or strains undergoing high frequency serotype conversion.

1.3.1 *Vibrio cholerae* Lipopolysaccharide

The LPS of *V. cholerae* has been extensively studied by a number of groups, particularly at the chemical level. The structure of a lipid A has been determined but there are still many questions unanswered about the structures of the core and O-antigen (Raziuddin, 1977; Broady *et al.*, 1981).

There are significant differences in the *V. cholerae* LPS compared to other Gram negative bacteria such as *Salmonella* and *E. coli*. Nevertheless the general structure of the *V. cholerae* LPS consists of lipid A, core oligosaccharide and O-antigen regions.

1.3.2 *Vibrio cholerae* Lipid A

The lipid A component of *V. cholerae* LPS consists of a β-1,6-linked D-glucosamine oligosaccharide which is substituted with a phosphate group ester bound to the non-reducing glucosamine residue and a pyrophosphorylethanolamine (PP-Etm) linked to C-1 of the

reducing glucosamine residue (Fig. 1.9) (Raziuddin, 1977; Broady *et al.*, 1981). This represents the backbone structure of the lipid A:

P-GlcN(β 1-6)GluN-1-PP-Etm.

The glucosamine residues are further substituted at their hydroxyl and amino groups by a number of fatty acids (Armstrong and Redmond, 1973). Three of these fatty acids, tetradecasoic, hexadecanoic and 3-hydroxydodecanoic acid are involved in ester linkages whereas 3 hydroxytetradecanoic acid (3 hydroxymyristic acid) is an amide linkage. The fatty acids found in the lipid A occur in both Ogawa and Inaba serotypes as well as in non-O1 *Vibrio* strains (Broady *et al.*, 1981). The exact position of the ester linked fatty acids is not known but *V. cholerae* lipid A is similar to most lipid A species studied, although it differs from that of *Salmonella* and other species in containing D-3-hydroxydodecanoic acid which is 3-O-acylated by D-3-hydroxydodecanoic acid.

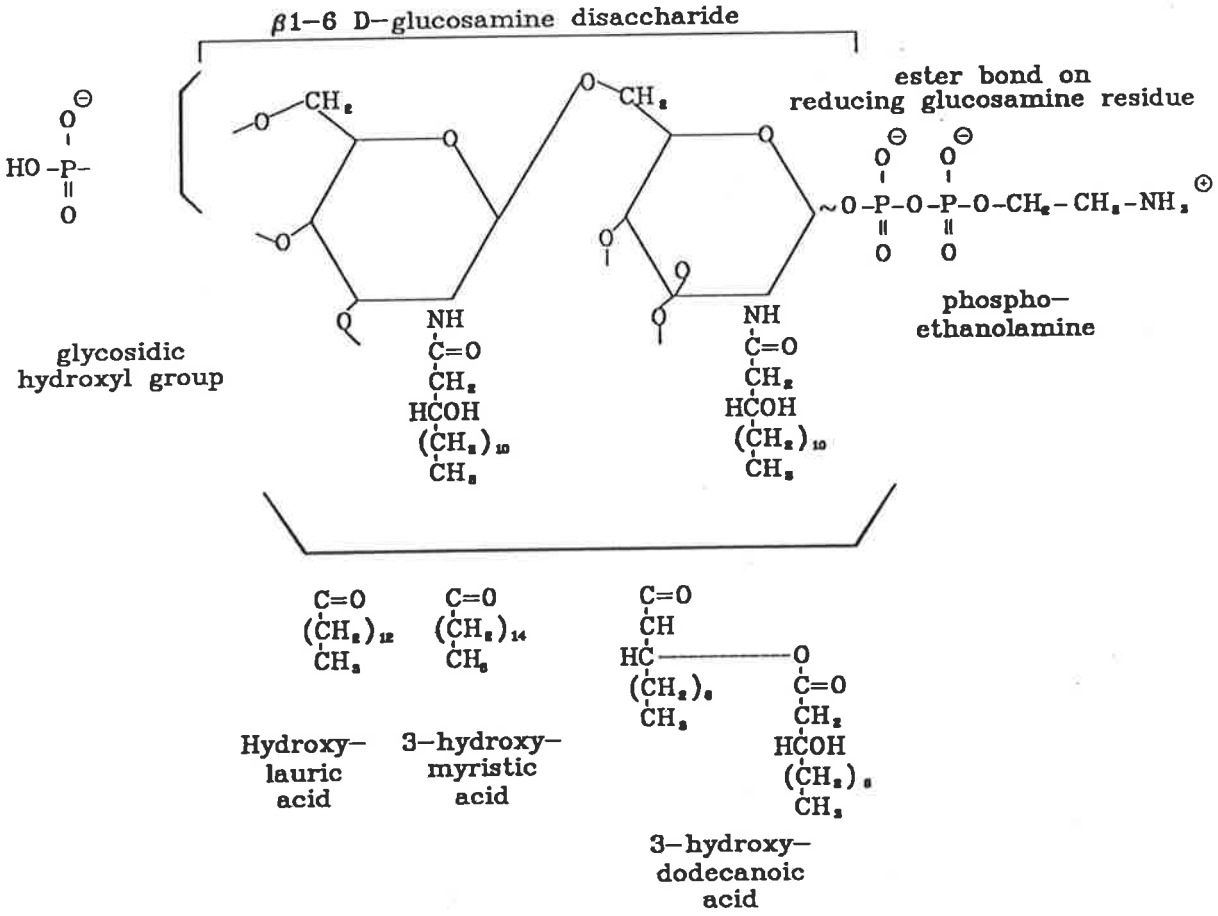
As in *Salmonella*, the lipid A is linked to the KDO, although the existence of KDO has been a point of controversy (Brade, 1985).

1.3.3 *Vibrio cholerae* Core sugars

The core oligosaccharide of *V. cholerae* contains glucose, heptose, fructose and ethanolamine phosphate, and the presence of 2-keto-deoxyoctonic acid (KDO) has only recently been reported (Brade, 1985). Initial investigations did not detected the presence of KDO in the core of *V. cholerae* (Hisatsune *et al.*, 1983). This can be explained since the KDO can only be released from the LPS by harsh hydrolytic conditions, unlike other species such as *Salmonella* where the KDO is more readily released. At least 2 KDO sugar units can be detected, a KDO-5-phosphate and KDO-7-O-8-phosphate (Brade 1985; Redmond, personal communication). This finding supports the studies which have shown KDO or KDO-like material in other genera of the Vibrionacaeae family (Hisatsune *et al.*, 1981; Banoub *et al.*, 1985).

Figure 1.9: Chemical structure of the lipid A of *V. cholerae*.

The backbone of the lipid A is a β-1,6-D-glucosamine disaccharide (A), the glycosidic phosphate group is substituted with phosphorylethanolamine. The *V. cholerae* lipid A differs from other species in containing D-3-hydroxydodecanoic acid 3-O-acylated by D-3-hydroxydodecanoic (B) and is therefore highly saturated with fatty acids. The lipid A of *V. cholerae* O1 exhibits both distinct and common features compared to other species of Gram-negative bacteria. Diagram is modified from Broady *et al.* (1981)


β1–6 D–glucosamine disaccharide
ester bond on reducing glucosamine residue
glycosidic hydroxyl group
phospho–ethanolamine
Hydroxy–lauric acid
3–hydroxy–myristic acid
3–hydroxy–dodecanoic acid

The remaining sugars found in the core region of the LPS form a backbone substituted at various points by sugars such as fructose, heptose, ethanolamine and N-acetyl glucosamine. It has been reported that the D-fructose was acid labile and may be the linking sugar between the core oligosaccharide and the lipid A (Jann *et al.*, 1973).

More recent data have shown that the fructose can be destroyed by periodate oxidation and therefore cannot be the linkage sugar. Since fructose is released by mild acid hydrolysis it seems likely that it branches off the core oligosaccharide backbone (Kaca *et al.*, 1986; Redmond, personal communication). The proposed structure for the core oligosaccharide is shown in Figure 1.10

1.3.4 *Vibrio cholerae* O-polysaccharide

The O-polysaccharide of *V. cholerae* has been examined by both chemical and physical means, including nuclear magnetic resonance spectroscopy (NMR).

The O-polysaccharide and core oligosaccharide can be separated from the rest of the LPS molecule by mild acid hydrolysis. The lipid A fraction is chloroform soluble whereas the polysaccharide fraction is water soluble. The polysaccharide moieties can then be separated on a Sephadex G50 column yielding two polysaccharide fractions of 10,000 MW and 1,000 MW (Kenne *et al.*, 1979). The 1,000 MW fraction can be refractionated to give a 1,000 MW fraction corresponding to the core oligosaccharide and a fraction of D-fructose (Gustafsson and Holme, 1985).

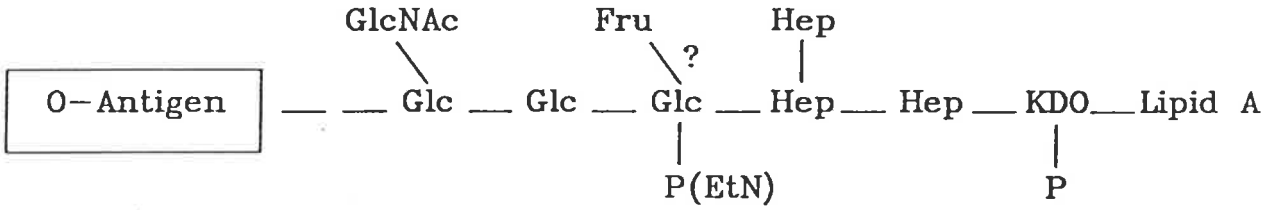
The sugars present in the O-saccharide of *V.cholerae* O1 appear to be identical regardless of serotype (Raziuddin, 1980). The most common sugars found in the O-polysaccharide are -

1. Perosamine, and
2. Quinovosamine

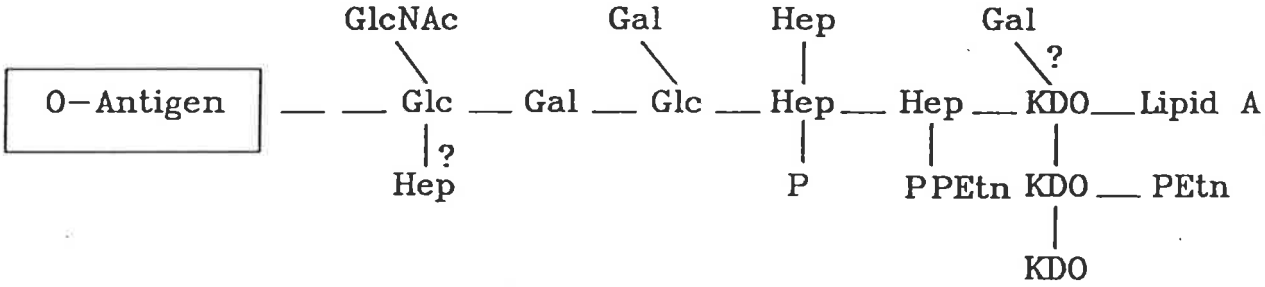
Figure 1.10: Proposed structure of the *Vibrio cholerae* O1 core

Comparison between the putative *V. cholerae* O1 core and the *E. coli* K-12 core show a number of similarities in that the backbone of the core consists of five monosaccharide units linked to KDO-lipid A. The backbone of *V. cholerae* O1 contains glucose (Glc) and heptose (Hep) whereas the *E. coli* K-12 backbone contains glucose, heptose and galactose (Gal). The type of substitution of the backbone and the sugars involved vary between the two species. Both cores are substituted by N-acetyl glucosamine (GlcNAc), heptose and phosphoethanolamine (P(EtN)). *V. cholerae* O1 core contains fructose (Fru) which is absent in *E. coli* K-12 which instead contains galactose. The number and type of linkages involving KDO also vary between the species. The structure of the *V. cholerae* O1 core is that proposed by N. Parker, M. Batley, J. Redmond and P.A. Manning (Manusript in preparation)

Vibrio cholerae



Escherichia coli K–12



Earlier reports suggested that the O-antigen also contained other sugars such as heptose, fructose, glucosamine and ethanolamine (Raziuddin, 1980; Majumder and Mukherjee, 1983) but these sugars now appear to be exclusively present in the core. This is based on the fact that the sugars of the core oligosaccharide are located at the reducing end of the perosamine (Hisatsune *et al.*, 1989). Furthermore rough strains lacking O-antigen still contain sugars such as glucose, fructose, heptose and glucosamine (Hisatsune and Kondo, 1980).

Perosamine is the major sugar in the O-antigen (Jann *et al.*, 1973; Redmond, 1975), and forms a polymer of approximately 17-18 units which has been shown by NMR spectroscopy to exist as a homopolymer. Since perosamine is substituted with tetronic acid, this would give rise to a 10,000 MW polysaccharide upon acid hydrolysis of LPS. If acid hydrolysis gives rise to unbranched perosamine, approximately sixty units would be required to form a 10,000 MW fraction. The perosamine residues have been shown to be α-(1-2) linked (Redmond, 1979; Kenne *et al.*, 1979). The perosamine backbone is substituted at various positions with other sugars known to be associated with the O-antigen. Some data sugests that the sugar D-glycero-L-manno-heptose is present at the non-reducing end of the polysaccharide polymer in Inaba strains (Sen *et al.*, 1979). Another sugar present in Inaba strains is D-glycero-L-glucoheptose, whereas in Ogawa strains these sugars have been proposed to exist as the optical isomers L-glycero-D-mannoheptose and L-glycero-D-glucoheptose (Guhathakurta *et al.*, 1986). These proposed differences in the sugar comosition between Inaba and Ogawa strains cannot be explained from the sequence of the *rfb* region in *V. cholerae* (Manuscript in prep.). Furthermore since the two serotypes can be expressed in *E. coli* by the introduction of the *V. cholerae rfb* operon it seems unlikely that these sugars play a role in the B and C antigen determination (Manning *et al.*, 1986). The perosamine is not only substituted with sugars but also acylated with 3-deoxy-L-glycero-tetronic acid (Redmond, 1979; Kenne *et al.*, 1982). This substitution is common to both Inaba and Ogawa and it has been proposed that it is the A antigen common to both serotypes (Kenne *et al.*, 1982). The perosamine can be further substituted at the C-3 position whereas the tetronate can be substituted at C-2 (Guhathakurta *et al.*, 1986) (Fig. 1.11).

Figure 1.11: Structural formulae for amino sugars in *V. cholerae* LPS.

A. Perosamine-4-NH_2-4,6-dideoxy-mannose

B. Quinovosamine-2-NH_2-2,6-dideoxy-D-glucose

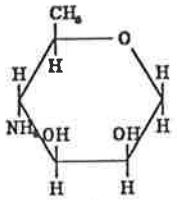
C. 4-NH_2-4-deoxy-L-arabinose

D. Repeat unit of *V. cholerae* O-antigen, comprising a (1→2) linked 4-amino-4,6-dideoxy-a-D-manno-pyranosol residue. The amino groups are acylated with 3-deoxy-L-tetronic acid.

The figure is based on Kenne *et al.* (1982).

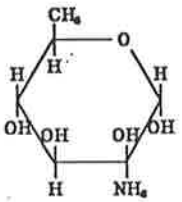
A

PEROSAMINE
4–NH_2–4,6–DIDEOXY–MANNOSE



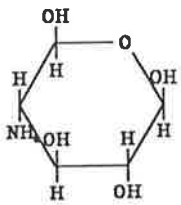
B

QUINOVOSAMINE
2–NH–2,6–DIDEOXY–D–GLUCOSE



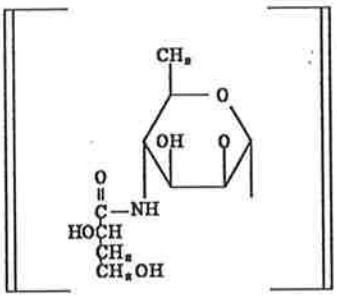
C

4–NH_2–4–DEOXY–L–ARABINOSE



D

L–GLYCEROL TETRONIC ACID
AND PEROSAMINE



The unique sugar quinovosamine has as yet not been localised, although its ratio to perosamine suggests it may be a "capping sugar", either on the distal or proximal end of the O-antigen (Jann *et al.*, 1973; J.W. Redmond, personal communication).

The sugar 4-NH_2-4-deoxy-L-arabinose has so far only been found in the Ogawa serotype (Fig. 1.11). The original observation by Redmond (1978) has been both confirmed and disputed by a number of other workers, thus the existence of this sugar is still in doubt (Redmond, 1978; Hisatsune *et al.*, 1978; Kabir, 1982). This may be due to the property of amino sugars, in that they are only stable in acid conditions and require modified thin layer chromatography stains for visualization (Redmond, 1978). The 4-NH_2-4-deoxy-L-arabinose of *Salmonella* is released under mild acid hydrolysis whereas the *V. cholerae* sugar is only released under very harsh hydrolytic conditions. Although antibody absorptions have proved negative using anti-B monoclonal antibody, the possibility that 4-NH_2-4-deoxy-L-arabinose is involved in the "B" antigen cannot be ruled out (J.W. Redmond, unpublished data).

1.3.5 *Vibrio cholerae* O1 Sugar analysis and antigenicity

A number of studies have been carried out to correlate the various O-antigen polysaccharides with particular antigenic specificities (Sen *et al.*, 1980; Majumdar *et al.*, 1983; Guhathakurta *et al.*, 1986). These oligosaccharides have been generated by partial hydrolysis of either O-antigen or O-antigen-core oligosaccharide from Ogawa and Inaba strains. It has been shown that these oligosaccahrides and monosaccharides can inhibit agglutination of *V. cholerae* cells using antiserum to *V. cholerae* LPS. The data indicate that the glucuronic acid followed by glucosamine are the most effective inhibitors and the immmunodominat sugars, regardless of serotype. The glucosamine is present in the core of *V. cholerae* O1, but as yet no glucoronic acid has been found in the LPS of *V. cholerae* O1. It is surprising to find that a sugar found exclusively in the core should be immunodominant, and the claim of the immunodominance of glucuronic acid must be looked at critically. It must be pointed out that in all cases, the oligosaccharides were better inhibitors than any of

the constituent monosaccharides. The contribution of the L and D heptoses unique to each serogroup may play a role in the "B" and "C" antigens, although there is no evidence to support this possibility (Guhathakurta *et al.*, 1986).

Work with monoclonal antibodies to LPS has shown that the A antigen is present as multiple determinants, i.e. there is more than one antigenic site per lipopolysaccharide molecule. In contrast, the B and C antigens appear to be single determinants. The low epitope density on the lipopolysaccharide molecule has hampered qualitative identification of the antigens. These data may explain why it has not been possible to precisely define the three antigenic determinants (Gustafsson and Holme, 1985).

1.3.6. Genetics of *Vibrio cholerae* O-antigen biosynthesis

The genes involved in O-antigen biosynthesis in *V. cholerae* strains 569B (Inaba) and O17 (Ogawa) have been cloned and expressed in *E. coli* K-12 (Manning *et al.*, 1986; Ward *et al.*, 1987). More recently, the locus associated with O-antigen biosynthesis has been mapped near *ilv* and *arg* on the *V. cholerae* chromosome (Ward and Manning, 1989) and corresponding to the locus associated with serotype specificity of the O-antigen, *oag*, described by Bhaskaran (1960).

Using the cloned O-antigen biosynthesis genes Morona *et al.* (1991) have obtained evidence supporting the proposed existence of glucose in the core oligosaccharide of *V. cholerae*. If the genes for O-antigen biosynthesis of *V. cholerae* are introduced into *E. coli* strains lacking glucose in their core, the production of *V. cholerae* O-antigen attached to the *E. coli* core is eliminated. This is consistent with the reported presence of glucose in the *V. cholerae* LPS-core-oligosaccharide.

1.3.7 Non-O1 and non-cholera *Vibrio* LPS

The LPS of non-O1 and non-cholera *Vibrios* is of interest since these organisms can cause disease in man and animals, and studies of their structure, biosynthesis and composition may complement studies on *V. cholerae* O1 LPS.

Many studies have been carried out on the non-O1 and non-cholera *Vibrios* with respect to their LPS composition. Initial reports indicated that the presence of perosamine and quinovosamine is characteristic of the O1 group (Hisatsune *et al.*, 1983). However, more recent findings have shown that quinovosamine is found in *V. proteus* and *V. metschnikovii* (Kondo *et al.*, 1988) but not in *V. parahaemolyticus* or *V. fluvialis* (Nazarenko *et al.*, 1988; Kondo *et al.*, 1989).

The LPS core of O1 and non-O1 groups is serologically identical using antiserum to rough strains, i.e. strains lacking the O-antigen. This would indicate that all *V. cholerae* have the same or very similar core and that the serogroups are identified solely on the basis of their O-antigen (Shimada and Sakazaki, 1973; Donovan and Furniss, 1982).

The non-O1 *Vibrios* have O-antigens of a highly variable nature. Unlike the O1 group, where the O-antigen consists of a monosaccharide (perosamine) which is polymerized and substituted, the non-O1 groups may have tetrasaccharide repeat units, such as O:21 which has a rhamnose, heptose, N-acetyl-galactose, N-acetyl-glucose repeat (Ansari *et al.*,1986). Other non-O1 serotypes are similar to O1 and have a monosaccharide polymer which is highly branched (Sen and Mukherjee, 1978; Ansari *et al.*, 1986).

The non-cholera *Vibrios* also have a different core structures containing acids related to KDO, such as 3-deoxyhetulosonaric and 2-octulosonic acid (Kondo *et al.*, 1989). The strains of *V. parahaemolyticus* group O7 and O12 contain the acid 3-deoxy-D-threo-hexulosonic acid instead of KDO whereas other strains contain as yet unidentified KDO-like substances (Kondo *et al.*, 1988, 1989). The structure of the core of *V. ordalii* has been elucidated and consists of heptose-heptose-glucose branched with heptose and glucose (Banoub and Hodder, 1985).

The other sugars found in the non-cholera *Vibrios* are the same as those commonly found in the LPS of other Gram negative organisms such as *Salmonella* and *E. coli* (Hisatsune *et al.*, 1980). Investigations have determined the structure of the O-antigen of one of the non-cholera *Vibrios*, that of *V. fluvialis*. This has a backbone of L-rhamnose which is substituted with N-acetyl-glucosamine (Nazarenko *et al.*, 1988).

1.4 *Vibrio cholerae* Serotype conversion

In *V. cholerae* O1 two serotypes were initially described (Kabeshima, 1918). These are now designated Inaba and Ogawa but were originally called J and F forms, respectively. A third serotype called Hikojima was subsequently described which carried all three antigens, A, B and C (Nobechi, 1923; Gardner and Venkatram, 1935).

V. cholerae O1 strains are not fixed, but can undergo serotype conversion. This occurs at a low frequency of approximately 10^{-5} for the Ogawa to Inaba conversion (Bhaskaran and Gorrill, 1957). This phenomenon has been known since 1918 although as yet no mechanism has been proposed. The first report by Kabeshima (1918) of serotype-conversion occurred as a result of exposure of the organism during growth to antiserum directed against its serotype (Ogawa). Since these original studies were carried out prior to a firm definition of Inaba and Ogawa, the experiments have been modified and repeated. In 1947, Shrivastava and White reported the isolation of Inaba strains from Ogawa cells grown in the presence of anti-Ogawa serum. The converse experiment of growing Inaba cells in anti-Inaba serum only gave rise to rough strains. Thus, it appeared that only the serotype-conversion from Ogawa to Inaba could be observed.

Until 1966, serotype conversion had only been reported *in vitro*. All previous reports of *in vivo* serotype conversion were dismissed as multiple infections of patients or patients being reinfected with another serotype. In 1967 Gangarosa *et al.* reported that after eight days of infection with a *V. cholerae* biotype El Tor serotype Ogawa strain, Inaba organisms

were found in the stool of a patient. This was followed by a relapse, indicating that the organism had multiplied in the intestine, and suggesting that a change in serotype may have arisen and enabled the host immune response to be evaded. In all, the patient excreted all three serotypes within ten days of contracting cholera. What is uncertain is whether the patient received more than one serotype at the time of initial infection, although the authors believed this was unlikely (Gangarosa *et al.*, 1967). It should be noted that all putative Hikojima strains isolated from this patient were typed as Inaba. This is suggestive of the highly unstable nature of the Hikojima serotype, since the other serotypes were stable when retested.

Serotype switching from Inaba to Ogawa was first reported in 1966 (Sheehy *et al.*, 1966). In a laboratory which exclusively used Inaba serotype strains a worker acquired an infection, and after three days began to excrete Ogawa strains. The reason this switch had not been reported earlier may be due to a much lower conversion rate from Inaba to Ogawa than the reverse switch. Thus, in humans where the *V. cholerae* multiply to large numbers and presumably a selection via the immune response can occur, it is possible to obtain Inaba to Ogawa serotype convertants.

Studies using germ free mice confirmed the ability of *V. cholerae* to undergo serotype conversion (Sack and Miller, 1969). In particular this demonstrated the ability of *V. cholerae* to change serotype not only from Inaba to Ogawa and vice versa but also the change from smooth to rough strains lacking O-antigen and then back to smooth strains. Thus, it appears as though *V. cholerae* can change serotypes and also the presence or absence of O-antigen. The immune response of the mouse host appeared to place selective pressure on seroconversion since;

1. if antibody to one serotype was present, the change to another serotype was accelerated, and
2. if immunosuppressive drugs such as cyclophosphamide, which suppresses antibody formation were given, the serotype conversion was absent or significantly reduced.

These data indicate that serotype conversion is selected for by the presence of specific antibodies (Sack and Miller, 1969). These observations correlate well with earlier studies where cells were grown in the presence of specific antibodies, which allowed selection for serotype conversion (Shrivastara and White, 1947; Nobechi and Nakano, 1967).

The question of *in vitro* Inaba to Ogawa serotype conversion is still unanswered. Sakazaki and Tamura (1971) attempted to show this change *in vitro*. They looked at thirteen strains, six Ogawa, six Inaba and one Hikojima strain. The bacteria were grown in the presence of monospecific antibodies to either Inaba or Ogawa. Of the Ogawa strains tested, all showed a number of cells changing to either Inaba, Hikojima or rough strains. The Inaba strains showed no seroconversion although some rough cells were isolated. Thus, it seems likely that either the rate of Inaba to Ogawa conversion is extremely low *in vitro* (Sakazaki and Tamura, 1971) or only some Inaba strains can seroconvert to Ogawa.

The significance of serotype conversion to the spread and persistence of cholera epidemics has not been demonstrated. Data to support the notion that serotype conversion is important comes from observations of the current epidemic in South America. Extensive biochemical analyses and ribo-probing has shown that the epidemic strain, an El Tor Inaba is distinctive. However, identical strains of the Ogawa serotype have now begun to appear (Salazar-Lindo *et al.*, 1991). This suggests that the epidemic strain has undergone a serotype conversion.

Several reports have suggested that serotype conversion may be mediated by a lysogenic conversion of *V. cholerae* by the bacteriophage CP-T1 (Ogg *et al.*, 1978, 1979). It has been proposed that CP-T1 may becomes lysogenic upon infection in a manner similar to phages which infect *Sh. flexneri* and *S. typhimurium* (section 1.2.1 and 1.2.2). Like the phages which modify the LPS of *Sh. flexneri* and *S. typhimurium*, phage CP-T1 has been shown to use the O-antigen of the lipopolysaccharide as its receptor and can infect both Inaba and Ogawa strains (Guidolin and Manning, 1985). The proposed CP-T1 lysogens which are resistant to phage killing are then presumed to be seroconverted cells (Barksdale and Arden, 1974; Ogg *et al.*, 1978, 1979). This would be similar to the mechanism in *Salmonella* and

Shigella. This seems unlikely to be the reason for serotpye-conversion in *V. cholerae* since CP-T1 has been shown not to form lysogens (Guidolin and Manning, 1985). It would appear to be more likely that any serotype converted strains detected were either spontaneous Ogawa to Inaba converts or may be due to the ability of CP-T1 to mediate generalized transduction (Ogg *et al.*, 1981). In this case it is possible that the phage transduce the serotype from one strain to another although at a low frequency. Furthermore any rough strains detected are likely to be spontaneous CP-T1 resistant mutants.

Using clones of the genes for *V. cholerae* O-antigen biosynthesis, it has been possible to express the Inaba and Ogawa serotypes in *E. coli* K-12 (Manning *et al.*, 1986). No readily detectable differences have been identified within the *rfb* regions suggesting that the genetic event associated with serotype conversion is subtle (Ward *et al.*, 1987).

Thus, although serotype conversion in *V .cholerae* has been known for over 70 years, the molecular basis for the conversion has not been identified. Extensive chemical analysis of *V. cholerae* LPS has not yet allowed the identification of the three antigens involved in serotyping Inaba, Ogawa and "Hikojima" strains.

1.5 Aims of this thesis

V. cholerae O1 is divided into three distinct serotypes, Inaba, Ogawa and a third rare and unstable serotype Hikojima. The serotype of a strain is fixed and can undergo serotype conversion. *V. cholerae* O1 was first reported to serotype convert in 1918. Since then the mechanism and basis for the serotype change has been the subject of much research.

The aims of this study are to:

1. To identify the differences between Inaba and Ogawa serotypes at both the DNA and protein level and to propose a model for the molecular mechanism behind serotype-conversion.

2. To examine the role of serotype-conversion in virulence of *V. cholerae* O1 in an animal model.

3. To identify the three antigens, A,B, and C, which are responsible for serotyping of *V. cholerae* O1.

CHAPTER 2

MATERIALS AND METHODS

2.1 Growth media

The following nutrient media were used for bacterial cultivation. Nutrient broth (NB) (Difco), prepared at double strength (16 g/l) with added sodium chloride (NaCl) (5 g/l) or Luria broth (LB), were the general growth medium for both *V. cholerae* and *E. coli* K-12 strains. Luria broth (LB) is composed of bacto-tryptone (10 g/l) (Difco), bacto-yeast (5 g/l) (Difco) and NaCl (5 g/l). 2xYT medium was prepared as described by Miller (1972). Minimal medium (M13 minimal media) was prepared as described by Miller (1972) and supplemented prior to use with $MgSO_4$, glucose and thiamine-HCl to concentrations of 0.2 mg/ml, 0.5%(w/v) and 50µg/ml, respectively. Terrific broth was prepared as described by Maniatis *et al.* (1989).

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). Soft agar contains equal volumes of NB and NA. H agar consisted of bacto-tryptone (16 g/l) (Difco), NaCl (8 g/l) and bacto-agar (12 g/l) (Difco). H top agar was identical to H agar but contained only 8 g/l bacto-agar.

Antibiotics were added to broth and solid media at the following final concentrations: ampicillin (Ap), 25 µg/ml; chloramphenicol (Cm), 25 µg/ml; kanamycin (Km), 25 µg/ml; rifampicin (Rif), 200 µg/ml; gentamycin (Gm), 40 µg/ml; tetracycline (Tc), 12 µg/ml for *E. coli* and 4 µg/ml for *V. cholerae* strains.

Incubations were at 37°C unless otherwise specified. Liquid cultures, were normally grown in 20 ml McCartney bottles.

2.2 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 anti-microbial 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) and their corresponding dideoxy-ribonucleotide triphosphate homologues (ddATP, ddCTP, ddGTP and ddTTP), 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.

D-threo(dichloroacetyl-1-^{14}C) was obtained from Amersham.

M13 sequencing primer and α[^{32}P]-dCTP, at a specific activity of 1,700 Ci/mMole were obtained from BRESATEC (Adelaide). The -35 sequencing primer was obtained from New England Biolabs. [^{35}S]-Methionine (1,270 Ci/mmole), and [^{35}S]-dATP (>1000 Ci/mmole) were purchased from Amersham. Sequenase^{TS} was purchased from I.U.B. Digoxigenin (DIG) DNA labeling and detection kits were purchased from Boehringer-Mannheim.

Detergents DOC, NonidetP40 were from BDH, Sarkosyl from Geigy, Brij35 from ICN and Tween20 from Sigma.

2.3 Enzymes

DeoxyribonucleaseI (DNaseI) and lysozyme were obtained from Sigma. Pronase and proteinaseK 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 polymeraseI, Klenow fragment of DNA polymeraseI, 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.

2.4 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. Oligos were routinely of a purity that no further purification was required.

2.5 Maintenance of bacterial strains

For long term storage, all strains were maintained as lyophilized cultures, stored *in vacuo* in sealed glass ampoules. When required, an ampoule was opened and its contents suspended in several drops of the appropriate sterile broth. Half the contents were then transferred to a 10 ml bottle of NB and incubated with shaking overnight at the appropriate temperature. The other half was streaked onto two nutrient agar plates and incubated overnight at the appropriate growth temperature. Antibiotics were added to the media when appropriate. If the colony form was uniform, single colonies were selected and picked off plates for subsequent storage or use. Short-term storage of strains in routine use was as a suspension of freshly grown bacteria in glycerol (32%v/v) and peptone (0.6%w/v) at -70^{o}C. Fresh cultures from glycerols were prepared by streaking a loopful of the glycerol suspension onto a nutrient agar plate (with or without antibiotic as appropriate) followed by incubation overnight just prior to use.

Bacterial strains were prepared for long-term storage by suspension of several loopfulls in a small volume of sterile skimmed milk. Approximately 0.2 ml aliquots of this thick bacterial suspension were dispensed into sterile 0.25x4 inch freeze drying ampoules and the end of each ampoule was plugged with cotton wool. The samples were then lyophilized in a freeze drier. After the vacuum was released, the cotton wool plugs were pushed well down the ampoule and a constriction was made just above the level of the plug. The ampoules were evacuated to a partial pressure of 30 microns and then sealed at the constriction without releasing the vacuum. Finally the ampoules were labelled and stored at 4^{o}C.

2.6 Bacterial strains and plasmids

The *Vibrio cholerae* strains used are listed in Table 2.1. Strains of the El Tor biotype were distinguished from the classical biotype by resistance to the antibiotic polymyxin B (50 units/ml) and sensitivity to biotype specific typing phages. Table 2.2 describes the *Escherichia coli* K-12 strains used in this study.

The plasmid cloning vectors which were used in this study are listed in Table 2.3.

2.7 Transformation procedure

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_{650} OD of 0.6 (4x 10^8 cells/ml). The cells were chilled on ice for 20 min, pelleted at 4°C in a bench centrifuge, resuspended in half volume of cold 100mM $MgCl_2$, centrifuged again and resuspended in a tenth volume of cold 100 mM $CaCl_2$. The cells were allowed to stand for 60 min on ice before addition of DNA. Competent cells (0.2ml) were then mixed with DNA (volume made to 100ml with 1x TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and left on ice for a further 30 min. The cell/DNA mixture was heated at 42°C for 2 min and then 3ml NB was added followed by incubation with shaking at 37°C for 1-2 hr. The culture was then plated onto selection plates directly or concentrated by centrifugation and plated. Cells with sterile buffer were included as a control.

Table 2.1 *V. cholerae* strains

Strains	Biotype/ Serotype	Genotype/ Phenotype	Source
O17	El Tor, Ogawa	Sm^R	K. Bhaskaran
569B	Classical, Inaba	Sm^R, motile	K. Bhaskaran
CA401	Classical, Inaba		C. Parker
NSW14	El Tor, Ogawa		P. Desmarchelier
NSW10	El Tor, Ogawa		P. Desmarchelier
NSW4	El Tor, Ogawa		P. Desmarchelier
NSW1	El Tor, Ogawa		P. Desmarchelier
1074-78*	El Tor, Ogawa		J. Kaper
1196-78*	El Tor, Ogawa		J. Kaper
M791	El Tor, Ogawa	RV79 *arg*::*VcA1*	J. Mekalanos
H-1	El Tor, Ogawa		S.C. Pal
AA13993	El Tor, Inaba		B. Kay
Z17561	Classical, Inaba		B. Kay
64	El Tor, Ogawa		S.C. Pal
CA411	Classical, Ogawa		J. Berry
BM69	El Tor, Inaba		S.C. Pal
8233	El Tor, Inaba		J. Berry
AA14073	El Tor, Ogawa		B. Kay
358	El Tor, Inaba		S.C. Pal
1621	El Tor, Ogawa		J.E. Ogg
C5	El Tor, Ogawa		P. Guinee
433	Hikojima		T. Holme
1602	El Tor, Hikojima		T. Holme
1603	El Tor, Hikojima		T. Holme

C31	El Tor, Ogawa		P. Guinee
V90	El Tor, Inaba		S.C. Pal
GN9006	El Tor, Inaba	*pyrA*-201::Tn*5*	B. Green
KUKI	Classical, Inaba		D. Rowley
B149	El Tor, Inaba		P. Desmarchelier
CD17	El Tor, Inaba	Type IV^R	D. Rowley
RJ234	El Tor, Ogawa	RV79::Tn*1*-24	R.K. Holmes
O162	Classical, Ogawa		D. Rowley
V663	Classical, Inaba	569B::Tn*5*	H. Ward
V665	Classical, Inaba	569B::Tn*5*	H. Ward
V667	Classical, Inaba	569B::Tn*5*	H. Ward
V671	Classical, Inaba	569B::Tn*5*	H. Ward

* These strains are non-toxigenic enviromental isolates

Table 2.2 *Escherichia coli* K-12 strains

Strain	Genotype/Phenotype	Source
DH1	F^-, *gyrA*-96, *recA*-1 *relA*-1, *endA*-1, *thi*-1, *hsdR*-1,*supE*-44, λ^-	B. Bachman
DS410	F^-, *minA*, *minB*, *rpsL*	D. Sherratt
S17-1	RP4-2-Tc::Mu-Km::Tn7/ *pro*, *hsdR*	U. Priefer
SM10	RP4-2-Tc::Mu, *thi*, *thr*, *leu*, *supE*	U. Priefer
JM101	F'[*traD*-36, *proA*,*B*, $lacI^q$ *lacZ*, ΔM15], *supE*, *thi*-1, Δ[*lac-proA*,*B*]	A Sivaprasad
DH5	F^-, *endA-1*, *recA-1*, *hsdR-17*(r_K^- m_k^+), *deoR*, *thi-1*, *supE*-44, *gyrA*-96, *relA-1*, λ^-	B.R.L.
DH5α	F^-, ϕ80 *lacZ* ΔM15, Δ(*lacZYA-argF*)U169, *endA-1*, *recA-1*, *hsdR-17* r_K^- m_k^+), *deoR*, *thi-1*, *supE*-44, *gyrA*-96, *relA-1*,λ^-	B.R.L.

Table 2.3: Plasmids and cloning vehicles

Plasmid/ Phage	Antibiotic marker	Reference
pME305	Tc	Rella *et al.* (1985)
pPH1JI	Gm, Sp, Sm	Beringer *et al.* (1978)
pRK290	Tc	Ditta *et al.* (1980)
pSUP201-1	Ap, Cm	Simon *et al.* (1983)
J225	Ap, Km	J. Hackett (unpublished)
pHC79	Tc, Ap	Hohn (1980)
pUC18	Ap	Messing & Vieira (1982)
pBluescript(K/S)	Ap	Stratagene
pGP1-2	Km	Tabor & Richardson (1985)
pEVX20	Tc	Morona *et al.* (1991)
pRMB1	Tc	Morona *et al.* (1991)
pTTQ181	Ap	M. Stark (1987)
pPM3024	Ap	Williams and Manning (1991)
pPM3035	Ap	Williams and Manning (1991)
M13mp18		Messing & Vieira (1982)
M13mp19		Messing & Vieira (1982)

2.8 Electroporation of *Vibrio cholerae*

Electroporation of plasmid DNA was performed essentially as described by Stoebner and Payne (1988). Cells were grown in LB to an A_{650} of 0.5. They were then washed in sucrose electroporation buffer (272mM sucrose, 7mM sodium phosphate buffer, pH 7.4, 1mM $MgCl_2$) and resuspended in ice-cold sucrose buffer at 1/10 the original volume. 5µg plasmid DNA was added to 100 µl of cell suspension, placed in a cuvette and left on ice for 30 min. Electroporation conditions were 2000 V at 25 µF capacitance and 200 ohms. The cells were returned to ice for 30 min, then diluted in LB (3ml) and incubated at 37°C for 45 min, and plated out on appropriate media.

2.9 Bacterial conjugation

Overnight broth cultures grown in NB or LB were diluted 1:20 and grown to early exponential phase with slow agitation. Donor and recipient bacteria were mixed at a ratio of 1:1 and the cells pelleted by centrifugation (5000 rpm, 5 min, bench centrifuge). The pellet was gently resuspended in 200 µl of broth and spread onto a cellulose acetate membrane filter (0.45 µm, type HA, Millipore Corp.) on a NA plate. This plate was incubated for 6-16 hr at 37°C. The cells were then resuspended in 10 ml NB and samples plated onto selective agar and incubated overnight at 37°C.

2.10 Plasmid DNA extraction procedures

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

Method 1: Triton X-100 cleared lysates were prepared from 10 ml overnight cultures by a modification of the procedure of Kahn *et al.* (1979). Cells were resuspended in 0.4 ml 25% (w/v) sucrose in 50 mM Tris-HCl, pH 8.0. Lysozyme (50 µl, 10 mg/ml freshly prepared in H_2O) and 50µl of 0.25M EDTA, pH 8.0 were added to cells in Eppendorf tubes and left to stand on ice for 15 min. 0.5 ml TET buffer (50mM Tris-HCl, 66mM EDTA, pH 8.0, 0.4% Triton X-100) was added followed by a brief mixing by inversion of the tubes. The chromosomal DNA was then pelleted by centrifugation (20 min, 4°C, Eppendorf). The supernatant was extracted twice with TE saturated phenol (pH 7.5) and twice with diethyl-ether. Plasmid DNA was precipitated by the addition of 0.6 weight of propan-2-ol and allowed to stand at -70°C for 30 min. The precipitate was collected (10 min, Eppendorf), washed once with 1 ml 70% (v/v) ethanol, dried *in vacuo* and resuspended in 50 µl 1X TE buffer.

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 (50mM glucose, 25mM Tris-HCl, pH 8.0, 10mM 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 (0.2M NaOH, 1% (w/v) SDS), followed by a 5 min incubation on ice resulted in total lysis of the cells. After the addition of 28 ml solution 3 (60 ml 5M potassium acetate, pH 4.8, to which was added 11.5 ml glacial acetic acid and 28.5 ml of H_2O) and incubation on ice for 15 min, protein, chromosomal DNA and high molecular weight RNA were removed by centrifugation (8,000 rpm, 20 min, 4°C, GSA, Sorvall). The supernatant was then 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

weight 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 4.8ml TE. Plasmid DNA was 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 (50mM glucose, 25mM Tris-HCl, pH 8.0, 10mM EDTA). The addition of 0.2 ml of solution 2 (0.2M NaOH, 1% (w/v) SDS) followed by a 5 min incubation on ice resulted in cell lysis. After the addition of 0.15ml of solution 3 (60ml of 5M potassium acetate, pH 4.8, 11.5ml of glacial acetic acid and 28.5ml of H_2O) 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 once with TE-equilibrated phenol and once with diethyl ether. Plasmid DNA was precipitated by the addition of 2 volumes of 100% ethanol and a 2 min incubation at room temperature. 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.

2.11 Analysis and manipulation of DNA

2.11.1 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).

2.11.2 Restriction endonuclease digestion of DNA

Most cleavage reactions were done using the restriction enzyme buffer SPK (10x: 200 mM Tris-HCl pH 7.5, 50mM $MgCl_2$, 5mM dithiothreitol, 1mM EDTA, 500mM KCl and 50% glycerol). The remaining restriction digests were carried out using EB buffer (10mM Tris-HCl, pH7.5, 6mM $MgCl_2$, 1mM DTT) as a basis, with either the addition of NaCl or KCl as described by the manufacturers. 0.1-0.5 µg of DNA or purified restriction fragments were incubated with 2 units of each restriction enzyme in a final volume of 20 µl, at 37°C, for 1-2 hr. 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 µg/ml RNase A) was added.

2.11.3 Calculation of restriction fragment size

The sizes of restriction enzyme fragments were calculated by comparing their relative mobility with that of *Eco*RI digested *Bacillus subtilis* bacteriophage SPP1 DNA. The calculated sizes of the SPP1 *Eco*RI standard fragments used differ from those published (Ratcliff *et al.*, 1979) and were calculated with the program DNAFRAG (Rood and

Gawthorne, 1984) using bacteriophage lambda and plasmid pBR322 as standards. The sizes (kilobases, kb) used were: 8.37; 7.2; 6.05; 4.9; 3.55; 2.68; 1.73; 1.61; 1.29; 1.19; .99; .86; .63; .48; .38; (Franzon and Manning, 1986).

2.11.4 Analytical and preparative separation of restriction fragments

Electrophoresis of digested DNA was carried out at room temperature on horizontal, 0.6%, 0.8% or 1% (w/v) agarose gels (Seakem HGT), 13 cm long, 13 cm wide and 0.7 cm thick. Gels were run at 100V for 4-5 hr in either 1x TBE buffer (67mM Tris base, 22mM boric acid and 2mM EDTA, final pH 8.8), or 1x TAE buffer (40mM Tris acetate and 2mM EDTA). After electrophoresis the gels were stained in distilled water containing 2 μg/ml ethidium bromide. DNA bands were visualized by trans-illumination with UV light and photographed using either Polaroid 667 positive film or 665 negative film.

For preparative gels Sea Plaque (Seakem) low-gelling-temperature agarose at a concentration of 0.6% (w/v) was used for separation of restriction fragments, which were recovered by the following methods:

Method 1: DNA bands were excised and the agarose melted at 65°C. Five volumes of 20mM Tris-HCl, 1mM EDTA, pH 8.0 buffer were added and the agarose extracted with phenol:water (1:1) and then phenol:chloroform (1:1). Residual phenol was removed with chloroform and the DNA precipitated with two volumes of ethanol and one tenth volume of 3M sodium acetate, pH 5.0. DNA was collected by centrifugation (15 min, Eppendorf), washed once with 70% (v/v) ethanol and dried *in vacuo* before being resuspended in 1x TE buffer.

Method 2: After electrophoresis the required DNA bands were excised and then placed inside dialysis tubing. This was then positioned in an electrophoretic tank

filled with 0.5x TAE buffer. A current was applied causing the DNA to move out of the gel and into the buffer contained in the dialysis tubing. The DNA was then extracted with an equal volume of TE saturated phenol and precipitated with two volumes of ethanol and one tenth volume of 3M sodium acetate, pH 5.0.

2.11.5 Dephosphorylation of DNA using alkaline phosphatase

Restriction enzyme digested DNA was treated with alkaline phosphatase by the following method. 0.1-0.5 µg of digested plasmid DNA was incubated with 1 unit of alkaline phosphatase (Calf intestinal: CIP), for 30 min at 37°C. The reaction was terminated by the addition of EDTA, pH 8.0 to a final concentration of 3mM followed by heating at 65°C for 10 min. The reaction mix was then extracted twice with hot (56°C) TE saturated phenol and twice with diethyl ether. DNA was precipitated overnight at -20°C with two volumes of ethanol and 1/10 volume of 3M sodium acetate pH 8.0. The precipitate was collected by centrifugation (15 min, Eppendorf), washed once with 1 ml 70% (v/v) ethanol, dried *in vacuo* and dissolved in 1x TE buffer.

2.11.6 End-filling with Klenow fragment

Protruding ends created by cleavage with restriction endonucleases were filled in using the Klenow fragment of *E. coli* DNA polymerase I. Typically, 1µg of digested DNA or purified DNA fragments, 2µl of 10 x nick-translation buffer (Maniatis *et al.*, 1982), 1µl of each dNTP (2mM) and 1 unit of Klenow fragment were mixed in a final volume of 20µl and incubated for 30min. Samples were phenol/chloroform extracted twice in a total volume of

100µl and precipitated in 2 volumes of 100% ethanol and 1/10 volume of 3M sodium acetate for 30min in a dry ice/ethanol bath. Pellets were dried *in vacuo* and resuspended in a total volume of 20µl of 1 x TE.

2.11.7 Kinasing

Oligonucleotide DNA primers were kinased using $\gamma[^{32}P]$-dATP. The reaction mix consisted of 10 mM DTT, 1 µl 10x kinase buffer, 3 units of polynucleotide kinase, 10 µl of $\gamma[^{32}P]$ and 60 µg of primer. This reaction mix was made up to 10 µl in water and incubated at 37°C for 30 minutes. The 10x kinase buffer consisted of 500 mM Tris pH 7.4 and 100 mM $MgCl_2$.

2.11.8 Labelling of Double Stranded DNA Probes

Purified DNA fragments were labelled with $\alpha[^{32}P]$-dCTP using a Hexaprime DNA Labelling kit (Bresatec).

The DNA (50-100ng) was denatured by heating at 95°C for 5 min in 0.1mM EDTA pH8.0 and chilled on ice. Ethanolic $\alpha[^{32}P]$-dCTP (50µCi) was dried down and resuspended with the denatured DNA and 12µl of a hexanucleotide/nucleotide/buffer cocktail in a final volume of 24µl. Klenow enzyme was added and the reaction was incubated for 20 min at 40°C. The reaction was terminated by heating at 65°C for 10 min. To remove unincorporated nucleotides from the labelled DNA the reaction was made up to 50µl and precipitated for 10 min on ice with 1/2 volume of 7.5M ammonium acetate, 3 volumes of 100% ethanol and 1µl of glycogen. After centrifugation (20 min, Eppendorf 5414) the pellet was washed in 70%(v/v) ethanol and spun briefly before resuspending in water. The probe was heated at 100°C for 5 min before addition to prehybridized filter.

2.11.9 Nick Translation

Nick translation reactions with DNA polymeraseI were modified from Maniatis *et al.* (1982) and carried out as follows: 25μCi of α[^{32}P]-dCTP (1,700 Ci/mmole in ethanol) was dried *in vacuo* in an Eppendorf tube, resuspended with 80 μl of H_2O, 10 μl of 10x nick translation buffer (500mM Tris-HCl, pH 7.2, 100mM $MgCl_2$, 1mM DTT, 500 μg/ml BSA) and 1 μl each of 2mM dATP, dGTP and dTTP. DNA (1 μg) and DNase (10 mg/ml; 1 μl) was added and incubated at 37°C for 10 minutes. DNA polymeraseI (5 units) was added to the mix and allowed to incubate at 16°C for 2 hr. α[^{32}P]-labelled DNA was separated from unincorporated label by centrifugation through a mini-column of Sepharose CL-6B.

2.11.10 Dot Blots with oligonucleotide probes

Strains to be tested were grown up overnight on agar plates. These colonies were transferred to nitrocellulose discs (Schleicher and Schuell) and the colonies lysed by the following method. The nitrocellulose disc was placed colonies up, on Whatman 3MM paper soaked in 0.5M NaOH and allowed to stand for 5 to 10 minutes. The discs were then subsequently treated for 5 min incubations by placing them sequentially on Whatman 3MM paper soaked in the following solutions: 0.1M NaOH, 1.5M NaCl; 1M Tris-HCl pH 7.5 (x2); 0.5M Tris-HCl pH 7.5, 1.5M NaCl. The discs were then air dried, followed by baking under vacuum between Whatman 3MM sheets for 2 h at 80°C. All bacterial debris was removed by a 2 hr wash at 65°C in 3x SSC (20x SSC is 0.34M NaCl, 75mM sodium citrate, pH 7.0) plus 0.2% SDS. Prior to hybridization, nitrocellulose discs were incubated for 4-5 hr at 42°C in 15 ml of pre-hybridization solution (containing 56.25 ml 4M NaCl, 22.5ml 1M Tris-HCl pH 7.6, 9 ml 0.25M EDTA, 25 ml 50x Denhardt's reagent (0.1%(w/v) Ficoll, 0.1%(w/v) polyvinylpyrrolidone, 0.1%(w/v) fraction V BSA), 2.5 ml denatured Herring Sperm DNA

made to a final volume of 250 ml and a final concentration of 0.2% SDS). Pre-hybridization fluid was discarded and replaced with fresh hybridization buffer (as for pre-hybridization but with the exclusion of Herring Sperm DNA). The kinased oligonucleotide was added, and hybridization allowed to occur for 16-24 hr at 42°C. Filters were removed and washed twice for 30 min in 6x SSC. After air drying (15-20 min at room temperature), the discs were placed for autoradiography at -70°C with intensifying screens.

2.11.11 Southern transfer and hybridization

Bi-directional transfers of DNA from agarose gels to nitrocellulose paper (Schleicher and Schuell) were performed as described by Southern (1975) and modified by Maniatis *et al.* (1982).

Prior to hybridization with radio-labelled probe, filters were incubated for 4 hr at 44°C in a pre-hybridization solution containing 50% (v/v) formamide, 50mM sodium phosphate buffer, pH 6.4, 5xSSC 7.0), 5x Denhardt's reagent and 83µg/ml single stranded herring sperm DNA (Sigma) (Maniatis *et al.*, 1982). Pre-hybridization fluid was discarded and replaced with fresh hybridization buffer (as for pre-hybridization solution, with the exclusion of herring sperm DNA). Denatured probe was added and hybridization allowed to occur for 16-24 hr at 44°C.

Filters were washed twice with shaking at 37°C for 30 min in 2xSSC, containing 0.1% (w/v) SDS. This was followed by two further washes in 0.1xSSC plus 0.1% (w/v) SDS at 65°C. After drying in the air (15min, room temperature), the filters were covered in plastic wrap and placed on film for autoradiography at -70°C with intensifying screens.

2.11.12 *In vitro* cloning

DNA to be subcloned (200 vg) was cleaved in either single or double restriction enzyme digests. This was combined with 20 ng of similarly cleaved vector DNA, then ligated with 2 units of T4 DNA ligase in a volume of 50 µl in a final buffer concentration of 20mM Tris-HCl, pH 7.5, 10mM $MgCl_2$, 10mM dithiothreitol (DTT), 0.6mM ATP for 16 hr at 4°C. The ligated DNA was then used directly for transformation of *E. coli* strains. Transformants were screened for insertional inactivation of the appropriate drug resistance gene (eg. Ap or Tc), wherever possible, prior to plasmid DNA isolation.

2.12 Preparation of *V. cholerae* or *E. coli* genomic DNA

Genomic DNA from either *V. cholerae* or *E. coli* 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 (50mM Tris-HCl, pH 8.0, 5mM EDTA, 50mM NaCl). The pellet was then resuspended in 2ml of 25% (w/v) sucrose, 50mM Tris-HCl, pH 8.0 and 1ml of lysozyme (10µg/ml in 0.25mM EDTA, pH 8.0) was added and the mixture incubated on ice for 20min. TE buffer (0.75ml) and 0.25ml of lysis solution (5%(w/v) sarkosyl, 50mM Tris-HCl, pH 8.0, 0.25mM EDTA, pH 8.0) were added, together with 2mg solid pronase. The mixture was gently vortexed, transferred to a 50 ml Ehrlenmeyer flask and incubated at 56°C for 60 min. This was followed by three extractions with TE-saturated phenol and two extractions with diethyl-ether. The genomic DNA was precipitated with four volumes of 100% ethanol and resuspended in 1ml of TE.

2.13 Polymerase Chain Reaction Protocol (PCR)

The procedure used was a modification of Saiki and Gelfand (1984). PCR amplification was performed in a 50 µl reaction volume containing PCR buffer (1.5mM $MgCl_2$, 10mM Tris pH8.4, 50mM KCl), 1.5U of Taq polymerase (Cetus), 20pM of primer and 100ng of *V. cholerae* chromosomal DNA. The dNTPs were used at a concentration of 2µM. 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. 10µl of this reaction product was analysed on a 0.8% agarose gel, the remaining product was purified and used for cloning.

2.14 M13 cloning and sequencing procedures

2.14.1 Preparation of M13 replicative form (RF) DNA

Fresh 2xTY broth (10 ml) was inoculated with 10 µl of an overnight culture of JM101 (in M13 minimal medium). A single plaque of M13mp18 or M13mp19 picked from an H agar plate with a sterile toothpick was added to this bottle. The culture was grown at 37°C with vigorous shaking for 6 hr. Bacterial cells were removed by centrifugation (5,000 rpm, 10 min, bench centrifuge) and the supernatant added to 1 litre of NB containing 10 ml of a shaken overnight culture of JM101. Following incubation for 14 hr at 37°C with shaking, replicative form (RF) DNA was prepared as described previously for plasmid DNA purification (section 2.10).

2.14.2 Cloning with M13mp18 and M13mp19

The M13 vectors, M13mp18 and M13mp19 (Messing and Vieira, 1982; Vieira and Messing, 1982) were used for selective cloning of restriction enzyme generated DNA fragments. Stocks of M13 vectors cleaved with various enzyme combinations and treated with alkaline phosphatase were stored at 4°C. Plasmid DNA was cut with the appropriate enzyme combinations for subcloning into the M13 vectors. The ligation conditions used for blunt ends and cohesive ends were identical. The reaction mixtures consisted of the DNA to be cloned (100 ng) and the DNA vector (20 ng) in a final volume of 10 µl of ligation buffer. Ligation with T4 DNA ligase was carried out overnight at 4°C.

2.14.3 Transfection of JM101

The *E. coli* strain JM101 was made competent for transformation/transfection as described in section 2.7. Competent cells (0.2 ml) were added directly to the ligation mixes and incubated on ice for 30 min. This was followed by a 2 min heat shock at 42°C. Cells were then transferred to sterile test tubes to which was added a mixture of JM101 indicator cells (200 µl), 100mM IPTG (40 µl) and 2% (w/v) X-gal in N, N'-dimethyl formamide (40 µl) and finally 4 ml of H top agar. The mixture was poured as an overlay onto an H agar plate and incubated overnight at 37°C.

2.14.4 Screening M13 vectors for inserts

White plaques were picked from X-gal/IPTG plates with sterile toothpicks and added to 1 ml 2xTY broth in microfuge tubes containing a 1:100 dilution of an overnight culture of

JM101. These tubes were incubated for 5 hr at 37°C. The cells were pelleted by centrifugation (30 sec, Eppendorf). RF DNA, suitable for restriction analysis, was prepared by the miniprep method. After restriction enzyme digestion, DNA was examined on 1% (w/v) agarose gels.

2.14.5 Purification of single stranded template DNA

M13 RF DNA containing appropriate inserts was reintroduced into JM101 and single white plaques from this transfection picked with sterile toothpicks to inoculate 2 ml of 2xTY broth containing 20 µl of an overnight culture of JM101. After vigorous shaking at 37°C for 6 hr, the culture was transferred to Eppendorf tubes and centrifuged for 10 min. The supernatant was transferred to a clean tube and recentrifuged for 5 min. Three methods of lysing phage and collecting single stranded phage were employed. They are as follows:

Method 1: A 1 ml aliquot of the supernatant from each tube was withdrawn and mixed in a fresh tube with 0.27 ml of 20% (w/v) polyethylene glycol (PEG), 2.5M NaCl. These tubes were then incubated at room temperature for 15 min. The phage were pelleted by centrifugation for 10 min in an Eppendorf centrifuge and the supernatant discarded. Following another short spin (10 sec), the remainder of the PEG/NaCl supernatant was removed with a drawn out Pasteur pipette. The pellets were resuspended in 0.2 ml of 1xTE buffer. Redistilled TE saturated phenol (0.1 ml) was then added to the phage suspension and the tubes were briefly vortexed. After standing for 15 min at room temperature, the tubes were centrifuged for 2 min and 0.15 ml of the top phase transferred to clean tubes. To the aqueous phase 6 µl of 3M sodium acetate pH 5.0 and 400 µl absolute ethanol was added. Single stranded DNA was precipitated at -20°C overnight, followed by centrifugation for 15 min in

an Eppendorf centrifuge. DNA pellets were washed once with 1 ml 70% (v/v) ethanol followed by centrifugation. After drying *in vacuo* the pellets were resuspended in 25 µl 1x TE buffer and stored at -20°C until required.

Method 2: This method is the same as method 1 to precipitate the phage, which was then resuspended in 300 µl of TE buffer (110mM Tris HCl, 0.1mM EDTA, pH8.0). 300 µl of TE-saturated phenol was added and the mix was vortexed sporadically for 10 min. The tubes were centrifuged for 3 min, and then the extraction was repeated with chloroform:isoamyl alcohol (24:1). The liberated single stranded DNA was precipitated by the addition of 1/10 volume 5M $NaClO_4$ and 1 vol. of isopropanol. Single stranded DNA was precipitated at -20°C overnight, followed by centrifugation for 15 min in an Eppendorf centrifuge. DNA pellets were washed once with 1 ml 70% (v/v) ethanol followed by centrifugation. After drying *in vacuo* the pellets were resuspended in 50 µl 1x TE buffer and stored at -20°C until required.

Method 3: 1 ml of the supernatant was added to a microfuge tube containing 250 µl of a 20% PEG/3.5M ammonium acetate solution. This was vortexed and incubated on ice for 30 min. The phage were collected by centrifugation (15 min, Eppendorf) and all the supernatant carefully removed. The pellet was dissolved in 100 µl 1x TE buffer. 50 µl redistilled TE saturated phenol was added and the tube vortexed for 2 min followed by incubation at room temperature for 5 min. 50 µl chloroform was then added, vortexed for 2 min, spun in a microfuge for 5 min and the upper aqueous phase was transferred to a fresh tube. The phenol/chloroform phase was extracted with 100 µl 1x TE buffer, spun as before and the aqueous phases combined. The combined aqueous phases were then extracted with an equal volume of phenol/chloroform (three more times). This was then extracted with chloroform and 250 µl of the supernatant transferred to a microfuge tube

containing 125 µl 7.5M ammonium acetate. After addition of 0.75 ml 95% (v/v) ethanol the tubes were stored overnight at -20°C. The DNA was collected by centrifugation (15 min, Eppendorf), and the pellet washed twice with 95% (v/v) ethanol. The pellet was dried *in vacuo* before resuspending the DNA in 20 µl distilled water.

2.14.6 Dideoxy sequencing protocol with Klenow fragment

The method was based on that described by Sanger *et al.* (1977, 1980). Stock solutions of the four dNTPS and ddNTPs were 10 mM in 1x TE buffer and stored frozen at -20°C. Working stocks of the dNTPs were made by diluting to 0.5mM with 1x TE. Working stocks of the ddNTPs were diluted to the following concentrations in 1x TE: ddATP (0.1mM), ddCTP (0.1mM), ddGTP (0.3mM) and ddTTP (0.5mM). The deoxynucleotide mixes (A, C, G, T) were made for each of the four sequencing reactions, with $\alpha[^{32}P]$-dCTP, as follows:

Components	Mixes			
	A°(µl)	C°(µl)	G°(µl)	T°(µl)
0.5 mM dATP	4	40	40	40
0.5 mM dCTP	--	5	--	--
0.5 mM dGTP	40	40	6	40
0.5 mM dTTP	40	40	40	6
10x TE buffer	10	10	10	10

Mixes of N° and working solutions of ddNTPs were made by the addition of the following combination of components:

Components	Mixes			
	A°+ddA	C°+ddC	G°+ddG	T°+ddT
N°	7 µl	7 µl	7 µl	7 µl
ddNTP	14 µl	14 µl	14 µl	14 µl

The mixes were stored at -20°C until required for later use in sequencing reactions. The annealing of synthetic primer to template was achieved by incubating 6 µl template, 1 µl M13 primer, 1 µl 10x TM buffer (100mM Tris-HCl, pH 8.0, 50mM $MgCl_2$) and 2 µl water. The mixture was heated at 65°C for 60 min and then allowed to cool at room temperature. Rows of four microfuge tubes (one tube for each sequencing reaction) were prepared containing 2 µl of annealed DNA. 5 µCi of $\alpha[^{32}P]$-dCTP was dispensed into each of four tubes marked A, C, G and T and dried *in vacuo*. The solution of appropriate N°/ddN mix was used to resuspend the dried label. The N°/ddN label mix (2 µl) was aliquoted into each of four tubes (one for each sequencing reaction) containing 2 µl of the dried annealed DNA. To the side of each tube was added .25 units of Klenow fragment. These components were simultaneously brought together by a brief spin in an Eppendorf centrifuge and the reaction mixes incubated at 37°C for 15 min. Chase solution (2 µl), consisting of 0.25mM of each dNTP and 0.025 units/ml Klenow, was added to the side of each tube and the chase reaction started by another brief spin. After 15 min at 37°C, 4 µl formamide dye mix (95% (w/v) formamide, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue, 10mM EDTA pH 8.0) was added to stop the reaction. Reaction mixes were heated in a 100°C heating block for 2.5 min and immediately 0.5-1.0 µl loaded onto 6% polyacrylamide denaturing gels (see below). Remaining reaction mixes were stored at -20°C and, for re-running, these samples were boiled for 60 sec immediately prior to loading.

2.14.7 Dideoxy sequencing protocol with Sequenase^{TM}

The dideoxy chain termination procedure of Sanger *et al.* (1977) was modified to encompass the use of Sequenase^{TM} (modified T7 DNA polymerase) in place of Klenow (Tabor and Richardson, 1987). All reagents were stored at -20^{o}C. Two types of labelling and termination mixes were used, namely the dGTP mixes and the dITP mixes. The contents of the dGTP mixes are as follows :

Labelling Mix (dGTP):	7.5 mM dGTP, dCTP and dTTP
ddG Termination Mix (dGTP):	80 mM dNTP, 8 mM ddGTP, 50 mM NaCl
ddA Termination Mix (dGTP):	80 mM dNTP, 8 mM ddATP, 50 mM NaCl
ddC Termination Mix (dGTP):	80 mM dNTP, 8 mM ddCTP, 50 mM NaCl
ddT Termination Mix (dGTP):	80 mM dNTP, 8 mM ddTTP, 50 mM NaCl

The dITP mixes were used to reduce gel artifacts due to secondary structures in DNA synthesized in the sequencing reaction (Barnes *et al.*, 1983; Gough and Murray, 1983). The dITP mixes were as follows :

Labelling Mix (dITP):	15 mM dITP, 7.5 mM dCTP, 7.05 mM dTTP
ddG Termination Mix (dITP):	160 mM dITP, 80 mM dATP, dCTP dTTP, 1.6 mM ddGTP, 50 mM NaCl
ddA Termination Mix (dITP):	160 mM dITP, 80 mM dATP, dCTP dTTP, 8 mM ddATP, 50 mM NaCl
ddC Termination Mix (dITP):	160 mM dITP, 80 mM dATP, dCTP dTTP, 8 mM ddCTP, 50 mM NaCl
ddT Termination Mix (dITP):	160 mM dITP, 80 mM dATP, dCTP dTTP, 8 mM ddTTP, 50 mM NaCl

Normally the labelling mix was diluted 1:5 with water to obtain the working concentration, however, to read long sequences in a single reaction, a dilution of 1:2 was used. The synthetic primer was annealed to the template by incubating 7 µl template (5-10 nM), 1 µl primer (500 nM) and 2 µl 5x Sequenase buffer (200mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 250mM NaCl). The mixture was heated in a metal block at 65°C for 3 minutes and then the block containing the tubes was allowed to cool to room temperature. To the annealed mixture, 2 µl of the appropriately diluted labelling mix, 1 µl DTT (0.1 M), 0.5 µl $\alpha[^{35}S]$-dATP (1000 Ci/mmol) and 2 µl of diluted Sequenase^{TM} (1:8 dilution in 1x TE buffer) was added, spun, mixed, and then incubated for 5 minutes at room temperature. 3.5 µl of this mix was then aliquoted into four microfuge tubes, prewarmed to 37°C, labelled A, C, G and T, each containing 2.5 µl of the corresponding termination mix, then spun briefly to start the termination reaction. After 5 minutes at 37°C, 4 µl Stop solution (95% (v/v) formamide, 20mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v)xylene cyanol) was added to each of the reactions. Reaction mixes were heated to 100°C for 2 min and immediately 1.2 µl loaded onto the sequencing gel. For re-running, these samples were kept at -20°C for up to 2 weeks and heated to 100°C for 3 min prior to loading.

2.14.8 Sequencing using dye-labelled primers

Sequencing reactions were carried out on 1mg of double stranded plasmid DNA using the protocol provided by Applied Biosystems.

In dye-labelled primer sequencing the DNA was split into four tubes labelled A, C, G and T containing 160ng, 160ng, 320ng and 320ng of DNA respectively. To each tube the following reagents were added: Dye primer 0.4pmol, 5x cycle sequencing buffer (400mM

Tris-HCl, pH8.9, 100mM $(NH_4)_2SO_4$ pH9.0, 25mM $MgCl_2$), d/ddNTP mixes and diluted Taq polymerase .5units (1 in 8).

d/ddA mix 1.5mM ddATP, 62.5µM dATP, 250µMdCTP,375µM c^7dGTP, 250µM dTTP)

d/ddC mix 0.75mM ddCTP, 250µM dATP, 62.5µMdCTP, 375µM c^7dGTP, 250µM dTTP)

d/ddG mix .125mM ddGTP, 250µM dATP, 250µMdCTP, 94µM c^7dGTP, 250µM dTTP)

d/ddT mix 1.25mM ddTTP, 250µM dATP, 250µMdCTP, 375µM c^7dGTP, 62.5µM dTTP)

Reagent	A	C	G	T
Dye-primer	1µl	1µl	2µl	2µl
d/dNTP mix	1µl	1µl	2µl	2µl
5x Cycle buffer	1µl	1µl	2µl	2µl
DNA template	1µl	1µl	2µl	2µl
Diluted Taq	1µl	1µl	2µl	2µl
Total Vol.	5µl	5µl	10µl	10µl

Each reaction was overlayed with 20µl of light mineral oil and spun.

Tubes were placed in a Cetus-Perkin Elmer model thermal cycler and cycled as follows

Rapid thermal ramp to 95°C

95°C for 30sec

Rapid thermal ramp to 55°C

55°C for 30sec

Rapid thermal ramp to 70°C

70°C for 60sec

15 cycles total

followed by

Rapid thermal ramp to 95°C

95°C for 30sec

Rapid thermal ramp to 70°C

70°C for 60sec

15 cycles total

Rapid thermal ramp to 4°C and hold

Samples were combined in 80µl of 95% (v/v) ethanol with 3µl of 3M sodium acetate and precipitated on ice. DNA was pelleted at 13,000 rpm for 15 min (Eppendorf 5414). 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/50mM EDTA (pH 8.0) 5:1 (v/v) and heated to 95°C for 2min.

2.14.9 Sequencing with dye-labelled terminators

Sequencing reactions were carried out on 1µg 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 (400mM Tris-HCl, 10mM $MgCl_2$, 100mM $(NH_4)_2SO_4$ pH9.0), dNTP mix (750µM dITP, 150µM dATP, 150µM dTTP, 150µM dCTP) , dye deoxy A,C,G and T terminator and diluted amplitaq. The volume was made up to 20µl with water. The mixture was overlayed with oil prior to thermal cycling. The following cycle protocol was used.

Rapid thermal ramp to 98°C

98°C for 30sec

Rapid thermal ramp to 50°C

50°C for 30sec

Rapid thermal ramp to 60°C

60°C for 4min

25 cycles total

Following cycling the reaction volumes were adjusted to 100µl and subsequently extracted twice with 120µl of phenol:chloroform:water (7:1:2, v/v). The DNA was precipitated with 300µl of 95% (v/v) ethanol and 10µl of 3M sodium acetate. DNA was pelleted at 13,000 rpm for 15 min (Eppendorf 5414). 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/50mM EDTA (pH 8.0) 5/1 (v/v) and heated to 95°C for 2min.

2.14.10 DNA sequencing gels

Polyacrylamide gels for DNA sequencing were prepared using glass plates 33 x 39.4 cm and 33 x 42 cm. Spacers and combs were high density polystyrene (0.25 mm thick). The gel mix contained 70 ml acrylamide stock [5.7% (w/v) acrylamide, 0.3% (w/v) bis-acrylamide, 8M urea in 1x TBE buffer (89mM Tris base, 89mM boric acid, 2.5mM EDTA, pH 8.3)], plus 420 µl 25% ammonium persulphate and 110 µl TEMED (N,N,N',N'-tetramethyl-ethylene-diamine, Sigma). After thorough mixing the gel mix was poured into a clean gel sandwich and the comb inserted. Polymerization took place for 60 min, with the gel in a horizontal position. The gel was mounted onto the sequencing apparatus. Gels were pre-electrophoresed at 700 V for 30 min. After the samples had been loaded the gel was electrophoresed at a constant voltage (700 V) for 15 min, and then increased to 1200 V (33 mA). After 4 hr the samples were reloaded into a second set of wells on the same gel. The gel was further electrophoresed, initially at 700 V, then 1200 V for 2.5 hr by which time the bromophenol blue dye front from the second loading, had reached the bottom of the gel. Plates were separated and tissue paper was used along the borders of the gel to hold it to the plate during the fixation procedure which involved slowly washing the gel using 2 litres of 10% (v/v) acetic acid, 20% (v/v) ethanol applied using a 60 ml syringe. The gel was then dried at 100°C for 20 min. Plastic wrap was used to cover the gel before placing on film for

autoradiography. Autoradiography was performed at room temperature, without the use of intensifying screens, for 16-24 hr.

Sequencing gels for the Applied Biosystems 373A automated sequencer were essentially the same as above. The gel was clamped into the sequencer and was pre-electrophoresed for 10 min at 1000 V. Samples were boiled for 2 min prior to loading and run for 14hr at a constant current of 40mA. Data were collected for a period of 13hr, analysed and displayed on screen of the Macintosh IICX as chromatograms.

2.14.11 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.

2.15 Analysis and manipulation of RNA

2.15.1 RNA preparation

RNA was prepared by a modification of the method described by Aiba *et al.* (1981). Overnight (NB) cultures of either *V. cholerae* or *E. coli* harbouring a plasmid of interest were subcultured 1:10 and grown to $OD_{650} = 1$. Five ml of culture was centrifuged and the pellet was resuspended in 0.5 ml of solution A (0.02mM Na acetate pH 5.5, 0.5% SDS, 1mM EDTA). This was extracted 3 to 4 times with hot (65°C) phenol (equilibrated with a solution containing; 0.02 mM Na acetate, 0.02mM KCl, 0.01mM $MgCl_2$ at pH 5.2). The aqueous

solution was then precipitated with two volumes of 100% ethanol and one tenth volume of 3M Na acetate pH 5.2. To remove contaminating DNA the precipitate obtained was resuspended in H_2O and incubated at 37°C for 10-15 min with DNase buffer (10x: 200mM Tris-HCl pH 7.6, 50mM $MgCl_2$) and 1 µl of DNAse enzyme (10 u/µl, BRESATEC). This was re-extracted with phenol, the RNA precipitated, dried *in vacuo* and resuspended in water.

2.15.2 Quantitation of RNA

The concentration of RNA in solutions was determined by measurement of absorption at 260nm and assuming an A_{260} of 1.0 is equal to 40µg RNA/ml (Miller, 1972).

2.15.3 Northern Transfer

Glyoxalation of RNA, electrophoresis and Northern transfer procedures were essentially as described by Thomas (1980).

Denaturation of RNA: The RNA samples were denatured by incubation of RNA (30µg) with 1M deionised glyoxal and 10mM sodium phosphate buffer pH 6.5 at 50°C for 60 min. Reactions were chilled on ice and 5 µl of sample buffer containing 50%(v/v) glycerol, 10mM sodium phosphate buffer pH 6.5 and bromophenol blue was added.

Electrophoresis: The samples were electrophoresed on a horizontal 1.2% agarose gel in 10mM sodium phosphate pH 6.5, 0.1mM EDTA. The electrophoresis apparatus used was the HE 100 SuperSub (Hoefer) which ensured adequate recirculation of buffer. Maintenance of a constant pH is required for the glyoxal adduct to remain associated with the RNA

Transfer: Transfer was set up according to Thomas (1980) in 20xSSC (3M NaCl, 0.3M trisodium citrate). A charged 0.45µm nylon membrane was used (BioTrace. Gelman Sciences). After 17 hr transfer the nylon membrane was air dried and placed RNA side down on a UV transilluminator (254nm) for 2 min to fix the RNA to the membrane.

Removal of Glyoxal: To remove the glyoxal adduct the filter was treated with 20mM Tris pH8.0 at 100°C and allowed to cool to room temperature.

Prehybridization: Filters were prehybridized for 7 hr at 42°C in buffer containing 50%(v/v) formamide, 50mM sodium phosphate buffer pH 6.5, 5xSSC , 5x Denhardts reagent and 250µg/ml single stranded herring sperm DNA.

Hybridization and Washing: Denatured probe was added to prehybridized filters and incubated at 42°C for 24 hr. Filters were washed twice for 5 min at room temperature in 2xSSC, SDS 0.1%(w/v). This was followed by two washes for 15 min at 65°C in 0.1xSSC, SDS 0.1%(w/v). After air drying the filters were covered in plastic wrap and placed on film for autoradiography at -70°C with intensifying screens.

2.15.4 Primer Extension

Synthetic 18mer oligo. primers were radioactively labelled with $\gamma[^{32}P]$-ATP at the 5' end by T4 polynucleotide kinase as described in section 2.11.7. The kinasing reaction consisted of 60 ng of primer, 20µCi $\gamma[^{32}P]$-ATP, kinase buffer (50mM Tris pH7.4, 10mM $MgCl_2$, 5mM DTT) and 2U T4 polynucleotide kinase (Boehringer) in a volume of 10 µl and was incubated at 37°C for 30 min. The reaction mixture was made up to 80 µl and precipitated with 2 volumes of ethanol, 1/10 volume of 3M NaAc and 20 µg glycogen

overnight at -20°C. Labelled primers were centrifuged for 15 min, washed in 70% ethanol, dried and resuspended in H_2O.

The kinased primer (6ng) and RNA (30µg) were precipitated together at -20°C with 3 volumes of ethanol and 1/20 volume of 4M NaCl. After centrifugation, pellets were washed in 70%(v/v) ethanol and dried *in vacuo*.

Primers were hybridised to RNA by resuspending pellets in 10µl hybridisation mix (10mM Tris pH 8.3, 200mM KCl), heating at 80°C for 3 min and incubation at 42°C for 60-90 min.

Extension of annealed primers was achieved by the addition of 24µl extension mix (10mM Tris pH 8.3, 14mM $MgCl_2$, 14mM DTT, 2mM each dNTPs) and 10U AMV reverse transcriptase (Pharmacia), followed by a 60 min incubation at 42°C.

Reactions were treated with DNase free RNaseA, phenol/chloroform extracted and precipitated overnight with 3 volumes ethanol and 1/10 volume 3M Na acetate pH5.5 at -20°C. Pellets were recovered, washed in 70% ethanol and dried before resuspension in 5 µl H_2O and 5 µl formamide loading buffer. Samples were boiled at 100°C for 3 min prior to loading on 6% polyacrylamide gels.

Dideoxy sequencing reactions were performed using a T7 DNA polymerase sequencing system as described by the manufacturers (Promega). Reactions were performed using 60ng of the above primers and 4µg of double stranded DNA template.

2.16 Assay for Chloramphenicol Acetyltransferase (CAT)

The cell lysates for this assay were prepared essentially as described by Gross and Rappuoli (1988). The assay was performed as described by Gorman *et al.* (1982).

Cultures to be assayed were grown to equivalent optical densities in CFB or AKI media. Optical densities were adjusted to A_{600} of 0.8 and 1.5ml of cells were collected. After centrifugation (2 min, Eppendorf 5414) cell pellets were resuspended in 300µl of

0.25M Tris pH7.8. Cells were lysed by sonication for 60 sec on ice with a Branson sonicator. Cell debris was removed by centrifugation at 4°C (15 min, Eppendorf 5414). The supernatants were collected and heated at 65°C for 10 min. The lysates were centrifuged (5min, Eppendorf 5414) and the supernatants retained. These were snap frozen in a dry ice/ethanol bath and stored at -20°C. Assays were performed using 15µl of a 1/10 dilution of cell lysate in a final volume of 170µl containing 0.25M Tris pH7.8, 0.125µCi[^{14}C]-chloramphenicol and 0.5mM acetyl Co-A. Incubation was at 37°C for 30 min. Reactions were extracted with ethyl acetate, vortexed and centrifuged (5 min, Eppendorf 5414). The organic phase was removed and dried down in a Speedvac evaporator for 35 min. The samples were resuspended in 10µl of ethyl acetate and spotted in 2.5µl aliquots onto silica gel thin layer chromatography (TLC) plates. The chromatography tank was equilibrated for 1hr with 100ml of 95:5 (v/v) chloroform:methanol. Thin layer chromotography was run in this solvent until the solvent front was 1cm from the top of the TLC sheet. TLC sheets were air dried and exposed to X-ray film for 24 hr at room temperature.

2.17 Protein analysis

2.17.1 Minicell procedures

Plasmid encoded proteins were analysed in minicells using the E. coli strain DS410. Minicells were purified and the plasmid-encoded proteins labelled with [^{35}S]-methionine as described by Kennedy *et al.* (1977) and modified by Achtman *et al.* (1979). Cells were initially collected by centifugation (Sorval rotor GSA, 7,000 rpm, 10 min) Minicell purification involved separation of minicells from whole cells (500 ml overnight culture in LB) by centrifugation (Sorval rotor HB4, 6,000 rpm, 20 min) through two successive 20% sucrose gradients in 1x buffered saline gelatin ((BSG) .85%(w/v) NaCl, 0.03%(w/v) KH_2PO_4, 0.06%(w/v) Na_2HPO_4, 0.1%(w/v) gelatine) . The minicells were removed by puncturing the centrifuge tube and withdrawing the cells into a 10ml syringe. The minicells

were subesquently washed in minimal media. The optical density of the cells was adjusted to an A650 of 0.6. Minicells were then preincubating in minimal medium to degrade long lived mRNAs corresponding to chromosomally encoded genes, then pulse labelled with 12.5 μCi [^{35}S]-methionine in the presence of methionine assay medium (Difco) for 60 min. Following incubation with [^{35}S]-methionine cells were washed and resuspended in 1ml of nutrient broth and incubated at 37oC for 5 min. Minicells were subsequently solubilized by heating at 100^{o}C in 100 μl of 1x sample buffer (Lugtenberg *et al.*, 1975) and analysed by SDS-PAGE.

2.17.2 SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on either 11-20% polyacrylamide gradients (for proteins) or straight 20% polyacrylamide gels (for lipopolysaccharides) using a modification of the procedure of Lugtenberg *et al.* (1975) as described previously by Achtman *et al.* (1978). Samples were heated at 100^{o}C for 3 min in SDS sample buffer (.25mM 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 generally electrophoresed at 100 V for 5 hr (11-20% gradient gels) or 10mA constant current for 16 hr (20% PAGE gels). 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 hr. 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).

2.17.3 Induction of Proteins Using IPTG

An overnight culture was diluted 1/10, with appropriate antibiotic, and incubated with shaking for 1 1/2hr at 37°C. IPTG was added to 1/2 of the culture at a final concentration of 1mM and incubation continued for 3 hr at 37°C. 1 ml sample of culture was placed in a microcentrifuge and cells were collected by centrifugation and resuspended in 50 µl of 1x SDS smaple buffer. 10 µl of sample was boiled and loaded onto SDS-PAGE gels for analysis.

2.17.4 T7 RNA Polymerase Expression System

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 LB broth with ampicillin and kanamycin was inoculated with a single colony and shaken at 30°C overnight. The culture was subcultured 1:10 and incubated with constant shaking at 30°C. When an A_{590} O.D. of 0.6 was reached, the cells were incubated at 42°C for 20 minutes to induce the pGP1-2 P_L promoter by the inactivation of cI_{ts}, allowing expression from the λpL promoter. Rifampicin was added to a final concentration of 200 µg/ml to inactivate the *E. coli* RNA polymerase and incubation was continued at 42°C for a further 20 minutes. The culture was then left for at least 2 hr shaking at 37°C. 1 ml of culture was transferred to microfuge tubes, spun to pellet the cells and resuspended in 100 µl of 1x SDS sample buffer. 10 µl of sample was boiled for 2 min and loaded onto SDS-PAGE gels for analysis. Gels were subsequently stained with Coomassie G250.

2.17.5 Cell Fractionation

The cell fractionation procedure was a modification of that described by Osborn *et al.* (1972a). Cells were grown in BHI to mid-exponential phase at 37°C (50 ml, OD_{650} of 0.6). 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, 30mM 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 0.1M 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 3mM EDTA, pH 7.3. Cells were lysed by 60 x 1 sec bursts with a Branson Ultrasonifier. Unlysed cells and large cell debris were removed by low speed centrifugation (5,000 rpm, 5 min, 4°C). The supernatant containing the membranes and the cytoplasm was centrifuged at 35,000 rpm in a 50Ti 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 25% sucrose, 10mM Tris-HCl pH 7.8, 1mM EDTA. The separation of the outer and inner membrane fractions was performed by using a step gradient between 55% and 30% sucrose. The membrane bands were collected, pelleted by centrifugation and suspended in 0.1 ml of 10mM sodium phosphate buffer pH 6.8. Samples were mixed with SDS sample buffer and subjected to SDS-PAGE analysis.

2.17.6 Autoradiography

SDS-PAGE gels were dried on Whatman 3MM chromatography paper at 60°C for 2 hr on a Bio-Rad gel drier. [^{35}S]-methionine and [^{35}S]-dATP autoradiography was performed at room temperature for 1-7 days without intensifying screens using Kodak XR-100 film. For autoradiography with [^{32}P] labelled DNA, the gels were exposed to film for 6-72 hr at -70°C, using intensifying screens.

2.17.7 Western Transfer and Protein Blotting

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 hr in a Trans-Blot Cell (Biorad). The transfer buffer used was 25mM Tris-HCl pH 8.3, 192mM glycine and 5%(v/v) methanol. For LPS transfer the buffer contained 20%(v/v) methanol and the transfer time was 1hr at 500mA. 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, 20mM Tris-HCl, 0.9%(w/v) NaCl) to block non-specific protein binding sites. The primary antiserum was diluted 1/100 in TTBS, 0.02%(w/v) skim milk powder and incubated with gentle agitation at room temperature for 2-16 hr. The nonspecific antibody was removed by washing the nitrocellulose sheet three times for 10 min in TTBS with shaking. Bound antibody was detected using an anti-antibody coupled with horseradish peroxidase and peroxidase substrate. This was accomplished by incubating the filter for 2-16 hr (gentle agitation) with goat anti-rabbit IgG coupled with horseradish peroxidase (Nordic Immunology) at a dilution of 1/5,000 in TTBS. The filter was then washed four times (5 min intervals) with TTBS, followed by two 5 min washes in TBS (20mM Tris-HCl, 0.9%(w/v) NaCl). The antigen-antibody complexes were then visualized using peroxidase substrate (9.9mg 4-chloro-1-napthol dissolved in 3.3 ml -20^{o}C methanol added to 16.5 ml TBS containing 15 µl hydrogen peroxide) which was added and allowed to incubate for 10-15 min with shaking, as described by Hawkes *et al.* (1982).

2.17.8 Transfer for N-terminal sequencing

Samples were run in SDS on 15% polyacrylamide gel and stained with Coomassie blue G250, the protein of interest was excised and rerun to remove any contaminants. Samples were subsequently transfered to polyvinylidene difluride (PVDF) (BioRad) at 200

mA for 2 hr in a Trans-Blot Cell (Biorad). The transfer buffer used was 25mM Tris-HCl pH 8.3, 192mM glycine and 10%(v/v) methanol. Proteins were stained for 10 min in 0.025%(w/v) Coomassie blue R-250 in 40%(v/v) methanol. The membrane was destained in 50%(v/v) methanol and subsequently air dried. The membrane was send to A. Gooley (Macquarie University Sydney) to be analysed on a 470A Applied Biosystems Protein Sequencer.

2.18 Preparation of rabbit antisera

Adult rabbits were obtained from the Central Animal House of the University of Adelaide for the production of antisera to partially purfied protein by subcutaneous immunization. The rabbit was immunized without adjuvant by subcutaneous injection of the gel homogenates on day 1, day 10 and day 17. The rabbits were bled by cardiac puncture under anesthesia 10 days after the last immunization.

2.19 Lipopolysaccharide procedures

2.19.1 Preparation of whole cell lysates (WCL)

Whole cell lysates (WCL) were prepared by the method of Hitchcock and Brown (1983). Cells were grown overnight in NB and 1.5 ml was spun down in an Eppendorf centrifuge for 5 min. The pellets were solubilized in 50 µl of lysing buffer containing 2%(w/v) SDS, 4%(v/v) β-mercaptoethanol, 10%(v/v) glycerol, 1M Tris-HCl pH 6.8, and 0.1%(w/v) bromophenol blue. Lysates were heated at 100°C for 10 min. 2.5 µg of

Proteinase K solubilized in 10 µl of lysing buffer was added to each sample and incubated at 60°C for 2-4 hr. Samples were stored at -20°C.

2.19.2 LPS silver staining

Silver staining of LPS in polyacrylamide gels was performed using the method of Tsai and Frasch (1982). The following procedure was used:

i) fixation overnight in 40%(v/v) ethanol, 10%(v/v) acetic acid;

ii) oxidation for 5 min with 0.7%(w/v) periodic acid in 40%(v/v) ethanol, 10%(v/v) acetic acid;

iii) 4 washes with water at 30 min each;

iv) staining for 10 min, in a solution containing 28 ml 0.1N NaOH, 2 ml concentrated NH_4OH and 5 ml 20%(w/v) $AgNO_3$ in a total volume of 150 ml;

v) developing in a solution of 50 mg citric acid and 0.5 ml formaldehyde in 1 litre. The citric acid was dissolved in water and heated to 37°C and formaldehyde added just before use. Distilled, deionized water which had been passed through a series of Millipore filters and had a conductivity of not more than 18 mega ohms/cm was used to rinse all glassware and in the preparation of solutions.

2.19.3 Isolation of LPS from *V. cholerae*

LPS was isolated from several strains during the course of these studies for sensitization of red blood cells (RBCs) for haemagglutination inhibition assay (HIA). LPS was extracted from cells with hot 90%(w/v) phenol/water using the procedure of Westphal and Jann (1965). A 100ml bacterial saline suspension (20mg/ml dry weight) was heated to

68°C. An equal volume of 90%(v/v) phenol/water (prewarmed to 68°C) was mixed into the cell suspension and stirred continuously at 68°C for 30 min The mixture was allowed to cool and the resulting phases separated by centrifugation in glass buckets (1500rpm, 20min, Coolspin MSE). The aqueous phase was collected and stored at 4°C. The phenol phase was re-extracted with half the original volume of prewarmed water (68°C) for 30 min with stirring, and the phases again separated by centrifugation. The aqueous phases were combined and dialysed against 5 liters of water, overnight at 4°C. Insoluble material was pelleted by centrifugation (500g for 5 min at 4°C in an SS34 rotor, Sorvall). The LPS was precipitated from the supernatant with 5-6 volumes of cold 100% ethanol containing 250mg of sodium acetate powder. The precipitate was collected by centrifugation (4000g for 30min at 4°C in a GSA rotor, Sorvall) and resuspended in 25ml of distilled water containing 5mM $MgCl_2$. A small quantity (approximately 1mg) of DNAse and RNAse I was added, and incubated for 60 min at room temperature. This was followed by the addition of pronase (1-2 mg) and further incubation for 30 min The mixture was again centrifuged at low speed (300g for 10 min at 4°C in an SS34 rotor, Sorvall), before deposition of LPS by ultracentrifugation (100,000g for 2 hr at 4°C; 60Ti rotor in a Beckman L8-80). Each pellet was resuspended in 5ml distilled water, of which 0.25ml was used for dry weight determination and 0.1ml for the estimation of protein by Lowry's method (Lowry *et al.*, 1951) using BSA as a standard. All preparations were stored at 4°C.

2.20 Haemagglutination inhibition assay (HIA)

LPS was alkali treated prior to use by overnight incubation of LPS at room temperature in the presence of .25M NaOH, the LPS was subsequently neutralized to pH 7.0 by the addition of HCl. A haemagglutination assay was performed by adding 25 µl of sheep red blood cells (SRBC), sensitized with alkali treated, purified LPS, to 25 µl of 2-fold

dilutions of the appropriate antiserum. *V. cholerae* Ogawa LPS was obtained from S. Attridge. The monoclonal antibodies α-A (20B) and α-C (13B) were as described in Ward *et al.* (1987). Monoclonal antibody α-B (VCO8) was obtained from Wellcome Diagnostics, Kent U.K. Trays were incubated at 37°C for 60 min and the haemagglutination end point determined. Four haemagglutinating units of antibody were used in the HIA.

The HIA was performed as follows. The antigen being tested (LPS) was diluted out serially in 25 µl volumes. 25 µl of anti-serum (4 haemagglutinating units, as described above), was then added to each well. The trays were incubated at 37°C for 60 min and then an equal volume, i.e. 50 µl, of sensitized SRBC was added to each well. The trays were incubated for a further 60 min at 37°C and the end points determined.

2.21 Electronmicroscopy

2.21.1 Preparation of protein A-gold

Colloidal gold (10nm-15nm diameter) was treated with dextran, activated with glutaraldehyde and then coupled to protein (Pharmacia), according to the method of Hicks and Molday (1984).

2.21.2 Immuno-gold electron microscopy

Immuno-gold labelling was performed using an adaption of the method from Levine *et al.* (1984). Plastic coated grids were placed face down on 40µl of a washed bacterial suspension. Excess liquid was removed and the grid was placed face down on a drop of anti-serum for 15 min After thorough washing, the grids were placed on drops of Protein A-gold for 15 min After further washing, the grids were examined with a JEOL JEM 1005 electron

microscope, using an accelerating voltage of 60 kv. All incubation and wash solutions consisted of PBS, pH 7.2 and 1%(w/v) of BSA.

Cells were initially resuspended in PBS with 1mM $MgCl_2$ and all subsequent wash solutions included 1%(w/v) of BSA.

2.22 *In vivo* assay for virulence using the infant mouse cholera model

The infant mouse cholera model was first described by Ujiiye *et al.* (1968) and subsequently extended by Chaicumpa and Rowley (1972).

3-4 day old infant mice used in these studies were removed from their parents 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. Organisms were either simply diluted or spun down and resuspended in peptone saline (0.1%(w/v) solution of protease-peptone [Difco] in saline). After appropriate dilution, 0.1 ml aliquots were administered orally to infant mice using a smooth-tipped hypodermic needle (22g). After challenge, the mice were not returned to their mothers but were kept in tissue-lined plastic containers in a laboratory incubator at 25°C.

Forty-eight hours after challenge, the survival of mice within each group was noted and plotted.

2.23 *V. cholerae* motility assay

Motility was tested by swarming of the bacteria in soft agar and is based on a modification by S. Attridge (PhD Thesis, University of Adelaide, 1979) of the sloppy agar

overlay method devised by Stocker (1949). A fresh culture of a test organism was diluted and plated onto NA such that 100-200 colonies would develop per plate. Following overnight incubation, each plate was overlaid with 5ml of 0.3% soft agar, allowed to set at room temperature and incubated at 37^{o}C for 2-3hr. Colonies which comprise motile bacteria develop a halo as the organisms swim in the soft agar overlay.

Chapter3

Characterization of the locus associated with serotype conversion in *V. cholerae*

3.1 Introduction

The species *Vibrio cholerae* is divided into several serogroups on the basis of their O-antigens (Sakazaki and Tamura, 1971). The disease, Asiatic cholera, is caused only by strains belonging to serogroup O1. The O1 serogroup is divided into two major serological types, Ogawa and Inaba. Strains of the Ogawa group express three antigens, A, B and C, whereas Inaba strains express only A and C antigen. It is on the basis of these antigens that the serotype is determined (Redmond *et al.*, 1973; Burrows *et al.*, 1946b; Sakazaki and Tamura, 1971).

The genes determining the biosynthesis of the O-antigen from strains O17 (Ogawa) and 569B (Inaba) have been cloned and expressed in *E. coli* K-12 (Manning *et al.*, 1986). Preliminary analysis suggested that the function(s) associated with serotype determination mapped within a 20kb *Sac*I (*Sst*I) fragment. This fragment has been completely sequenced from O17 (pRMB1) and 569B (pEVX20)(Fig. 3.1) It was therefore necessary to subclone this region in order to subject the contained genes to detailed genetic analyses which may aid in understanding the mechanism of serotype specificity and conversion. This chapter describes the characterization of the serotype locus and identifies the gene necessary for interconversion between the Inaba and Ogawa serotypes.

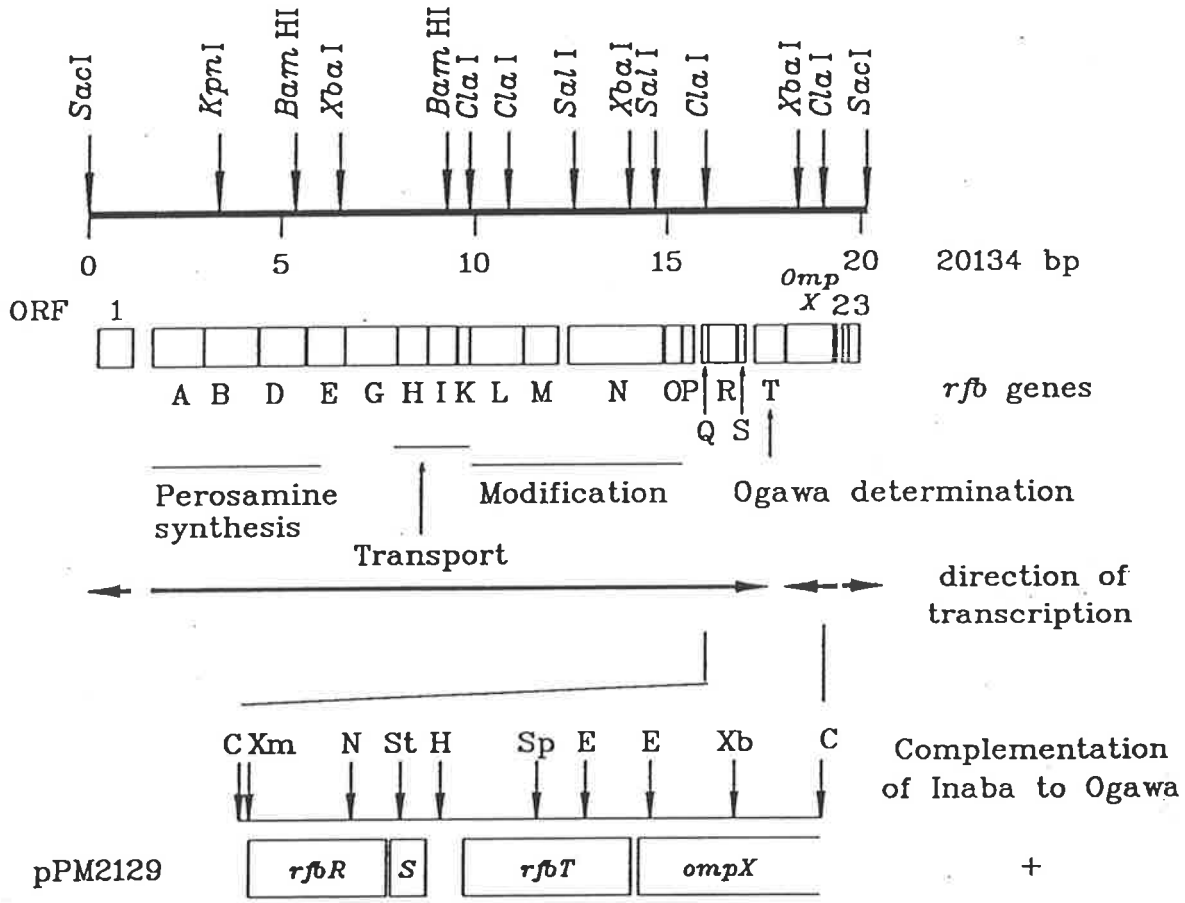

SacI
KpnI
BamHI
XbaI
BamHI
ClaI
ClaI
SalI
XbaI
SalI
ClaI
XbaI
ClaI
SacI
0
5
10
15
20
20134 bp
ORF 1
Omp X 23
A B D E G H I K L M N OP R T
Q S
rfb genes
Perosamine synthesis
Transport
Modification
Ogawa determination
direction of transcription
C Xm N St H Sp E E Xb C
Complementation of Inaba to Ogawa
pPM2129
rfbR
S
rfbT
ompX
+

Figure 3.1: Genetic organization of the 20 kb *Sac*I fragment encoding O-antigen biosynthesis in *V.cholerae* O1.

The upper line shows a restriction map of the *Sac*I fragment containing the genes involved in O-antigen biosynthesis (Ward and Manning, 1989). The lower part shows the positions within the coding region of the various *rfb* genes. The regions containing the genes for perosamine biosynthesis, transport and modification have been identified by homology to genes from other species. Arrows indicate the direction of transcription.
Plasmid pPM2129, containing the 3kb *Cla*I fragment in pUC18, has been shown by slide agglutination with typing sera to convert 569B to its Ogawa form.

3.2 Results

3.2.1. The 3kb *Cla*I fragment from *V. cholerae* O17 cosmid pRMB1 can convert Inaba to Ogawa

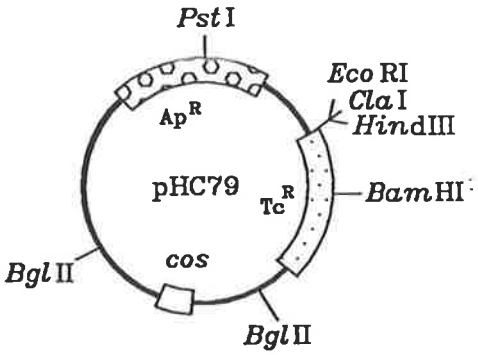
Genetic manipulation in *V. cholerae* has been hindered due to the extra-cellular deoxyribonucleases which make transformation difficult (Focareta and Manning, 1987; 1991). The most efficient method for introducing cloned genes back into *V. cholerae* is by conjugal mobilization. The vectors described by Simon *et al.* (1983) are readily suitable for this purpose. A similar mobilizable plasmid, pPM2101, has been constructed and used for the reintroduction of cosmid clones into *V. cholerae* (Fig. 3.2) (Sharma *et al.*, 1989).

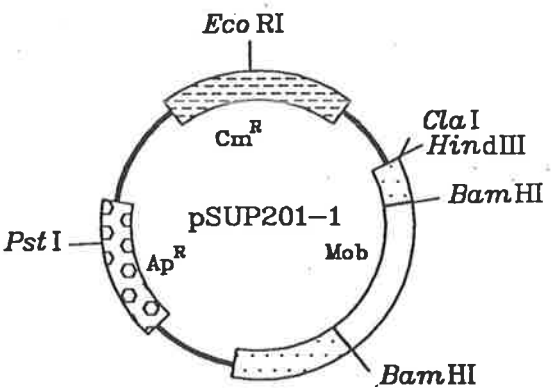
The 3kb *Cla*I fragments from pEVX20 (Inaba) and pRMB1 (Ogawa) were cloned into the *Cla*I site of pPM2101 to generate pPM2123 and pPM2122, respectively (Fig. 3.3). Plasmids pPM2101, pPM2122 and pPM2123 were introduced into the *V. cholerae* strains O17(Og), 569B(In), BM69(In) and CA411(Og) and the serotypes of the strains were determined by slide agglutination. Only the introduction of pPM2122 into 569B and BM69 was able to alter the serotype, suggesting that the Ogawa form is dominant. This is supported by the finding that pPM2123 in O17 does not alter the serotype even though the plasmid copy number gives a gene dosage of approximately 30(Inaba): 1(Ogawa). This agrees with the data presented by Morona *et al.* (1990). Thus, the 3kb *Cla*I fragment contained within pPM2122 can mediate the conversion of 569B from the Inaba serotype to the Ogawa form.

To facilitate further studies and to permit construction of deletions within the 3kb *Cla*I region, the fragment was cloned from pRMB1 into the *Acc*I site of pUC18. This plasmid was designated pPM2129.

Figure 3.2: Construction of pPM2101.

The cosmid cloning vector pHC79 was cleaved with *Eco*RI and the protruding ends filled using Klenow fragment of DNA polymerase I. Plasmid pSUP201-1 was cleaved with *Bam*HI, the fragment containing the RP4 mobilization region (Mob) was isolated and the protuding ends filled. The cleaved pHC79 and Mob fragment were ligated and the ligation transformed into strain S17-1 selecting for Tc^R. The correct constructs were selected by their ability to be mobilized into strain SM10 selecting for Tc^R (pPM2101) and Km^R (counterselection for S17-1) and confirmed by restriction analysis (Sharma *et al.*, 1989).





Cleave with *Eco* RI
End fill with Klenow fragment

Cleave with *Bam*HI
End fill with Klenow fragment

Ligate

Transform into strain S17–1 and select for Tc^R

Select for mobilization into strain SM10 screening for Tc^R and Km^R

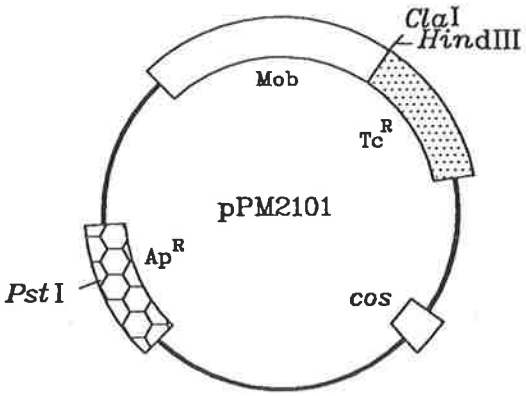
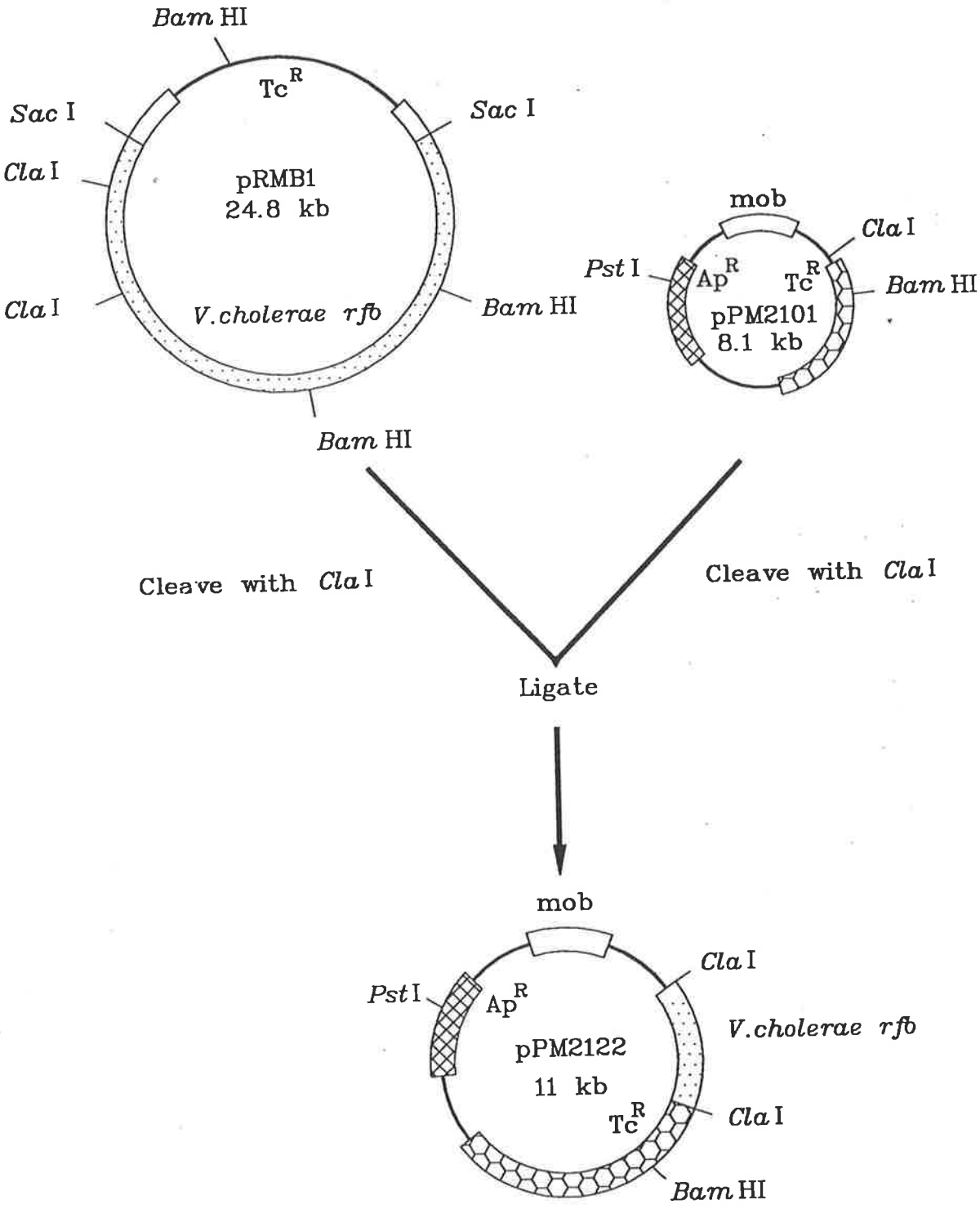


Figure 3.3: Construction of pPM2122 and pPM2123

The plasmids pEVX20 and pRMB1 contain the 20kb *Sac*I fragment encoding biosynthesis of the Inaba and Ogawa O-antigens of *V. cholerae* O1, respectively. The constructions of pPM2122 and pPM2123 were identical except that pPM2123 was generated using pEVX20. pRMB1 DNA was cleaved with *Cla*I and the fragments were ligated to the mobilizable vector pPM2101 which had also been cleaved with *Cla*I. The ligation was transformed into the strain DH5. Clones were selected by Ap^R and screened for Tc^S due to insertional inactivation of the *tet* resistance gene. The plasmid generated was designated pPM2122 (Ogawa) from pRMB1 and pPM2123 (Inaba) from pEVX20.


Bam HI
Tc R
Sac I
Sac I
Cla I
pRMB1
24.8 kb
Cla I
V.cholerae rfb
Bam HI
Bam HI
mob
Cla I
Pst I
Ap R
Tc R
Bam HI
pPM2101
8.1 kb
Cleave with Cla I
Cleave with Cla I
Ligate
mob
Cla I
Pst I
Ap R
V.cholerae rfb
pPM2122
11 kb
Tc R
Cla I
Bam HI

3.2.2 Inaba and Ogawa strains appear to have no conserved alterations within the 3kb *Cla*I region.

The sequence of the 3kb *Cla*I fragment from pRMB1 is discussed in chapter 4 and is shown in Fig. 4.2 . The sequence variations between pRMB1 and pEVX20 across this region are shown in Table 3.1.

The most obvious difference is an 8bp deletion (TAAAAGGC) from base 16,374-16,382 in the *rfbR* gene of pEVX20 (Inaba). To determine if this difference is conserved in other strains across the Inaba and Ogawa serotypes, a 16nt synthetic oligodeoxynucleotide (oligo) 5'GATGTAAAAGCCTGCT 3' spanning the deletion was used. The oligo was radioactively labelled and used to probe genomic DNA from 26 *V. cholerae* O1 strains (Fig. 3.4). The results show that not all Inaba strains have this 8bp deletion and also that this deletion can occur in Ogawa strains, as observed with strain RJ234, although, this change appears to be rare in Ogawa strains. This result suggests that *rfbR* is not the serotype converting gene, since the 8bp deletion leads to a frameshift and a truncated RfbR protein. Thus, the other regions of sequence divergence in the 3kb *Cla*I fragment needed to be analysed.

Comparison of the sequence of the *Cla*I fragment of pEVX20 and pRMB1 revealed a variation which could be investigated by Southern analysis. A base change at nt 17,672 resulted in a *Dra*I site in the *rfbT* gene from pEVX20. Plasmids pEVX20, pRMB1, pPM2122 and pPM2123 were analysed by *Dra*I restriction endonuclease digestion (Fig. 3.5). The *Dra*I restriction patterns seen with pRMB1 and pPM2122 as well as with pEVX20 and pPM2123 corresponds to the sequence data. Plasmid pPM2129 was used as a radioactively labelled probe to screen chromosomal DNA of 26 *V. cholerae* O1 strains digested with *Dra*I (Fig. 3.6 A and B). The Southern hybridization analysis revealed that all *V. cholerae* strains examined contain the 3kb *Cla*I region, but that the *Dra*I polymorphism determined by sequence analysis is not conserved within the Inaba and Ogawa serogroups. Furthermore, no

Table 3.1 Sequence variation within the 3kb *Cla*I fragment between Ogawa strain O17 and Inaba Strain 569B

There are three sequence differences within the 3kb *Cla*I fragment. The first of these is an 8bp deletion in the Inaba strain leading to a truncated protein for RfbR instead of a 26kDa protein. The second change leads to an amino acid substitution from glutamine in Ogawa, to a lysine in Inaba in RfbT and also generates a *Dra*I site in 569B (underlined). The third base substitution leads to a TGA stop codon in the *rfbT* gene of the Inaba strain 569B instead of a glycine (Ogawa). This gives rise to a 27kDa truncated protein instead of the 32kDa protein in Ogawa strain O17. The first two of these changes have been probed, using an oligo. and Southern hybridization, respectively.

Table 3.1: Sequence variation across the 3kb *Cla*I fragment

```
CTA CTA GAT GTA AAA GGC TGC TTG ATT ACG ATT GAT    Ogawa
Leu Leu Asp Val Lys Gly Cys Leu Ile Thr Ile Asp
          16374
Leu Leu Asp G.....lyL euA spT yrA sp* **
CTA CTA GAT G            TGC TTG ATT ACG ATT GAT    Inaba

TCA TTA TAT TTT CAA AAA AAT ACA    Ogawa
Ser Leu Tyr Phe Gln Lys Asn Asp
             17672
Ser Leu Tyr Phe Lys Lys Asn Asp
TCA TTA TAT TTT AAA AAA AAT ACA    Inaba
           --- ---

GAA AAG CAT AAT GGA ATT GAA TAT    Ogawa
Glu Lys His Asn Gly Ile Glu Tyr
             17840
Glu Lys His Asn ***
GAA AAG CAT AAT TGA ATT GAA TAT    Inaba
```

Figure 3.4: Oligonucleotide serotyping of *V. cholerae* O1

Genomic DNA from 26 *V. cholerae* O1 strains was blotted onto nitrocellulose. Plasmids pEVX20, pRMB1, pPM2122 and pPM2123 were used as controls. The DNA was probed with the end labelled oligonucleotide #286 (5'GATGTAAAAGGCTGCT3') which spans the 8bp deletion in the *rfb*R gene of Inaba strain 569B. After probing the filter was subjected to autoradiography. Strains and their respective serotypes are shown above the autoradiograph. Inaba (I), Ogawa (O) and Hikojima (H).

1	C5(O)	8233(I)	1602(H)	1603(H)	433(H)	BM69(I)
2	358(I)	64(O)	H-1(O)	AA14073(O)	AA13993(I)	Z17561(I)
3	RJ234(O)	CD17(I)	M791(O)	B149(I)	NSW14(O)	NSW10(O)
4	NSW4(O)	NSW1(O)	GN9006(I)	1621(O)	Kuki(I)	569B(I)
5	CA401(I)	017(O)				
6			pEVX20(I)	pRMB1(O)	pPM2122(O)	pPM2123(I)

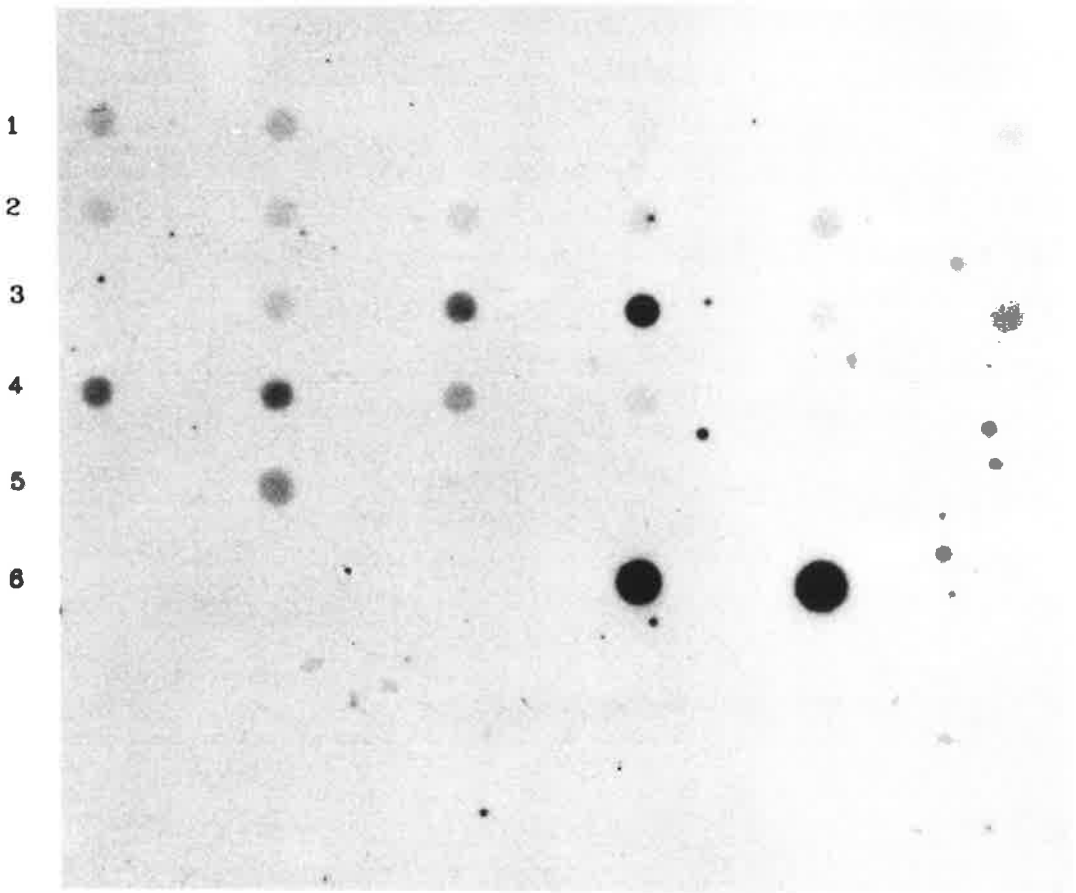


Figure 3.5: Analysis of the *rfbT* gene from strains O17 (Ogawa) and 569B (Inaba) by restriction endonuclease digestion.

The 24.8kb plasmids pEVX20 and pRMB1 were cleaved with the restriction endonuclease *Dra*I. Plasmids pPM2122 and pPM2123 were included to show that the *Dra*I polymorphism was contained within the 3kb *Cla*I fragment. The fragments of interest are marked by arrows. In pPM2123 there is a new band at ~500bp the smaller 300bp fragment is not visible on the gel. SPP1 phage DNA digested with *Eco*RI was used as a size marker. SPP1 EcoRI restriction fragment sizes in kb; 8.37, 7.2, 6.05, 4.9, 3.55, 2.63, 1.73, 1.61, 1.29, 1.19, .99, .86, .63, .48, .38 .

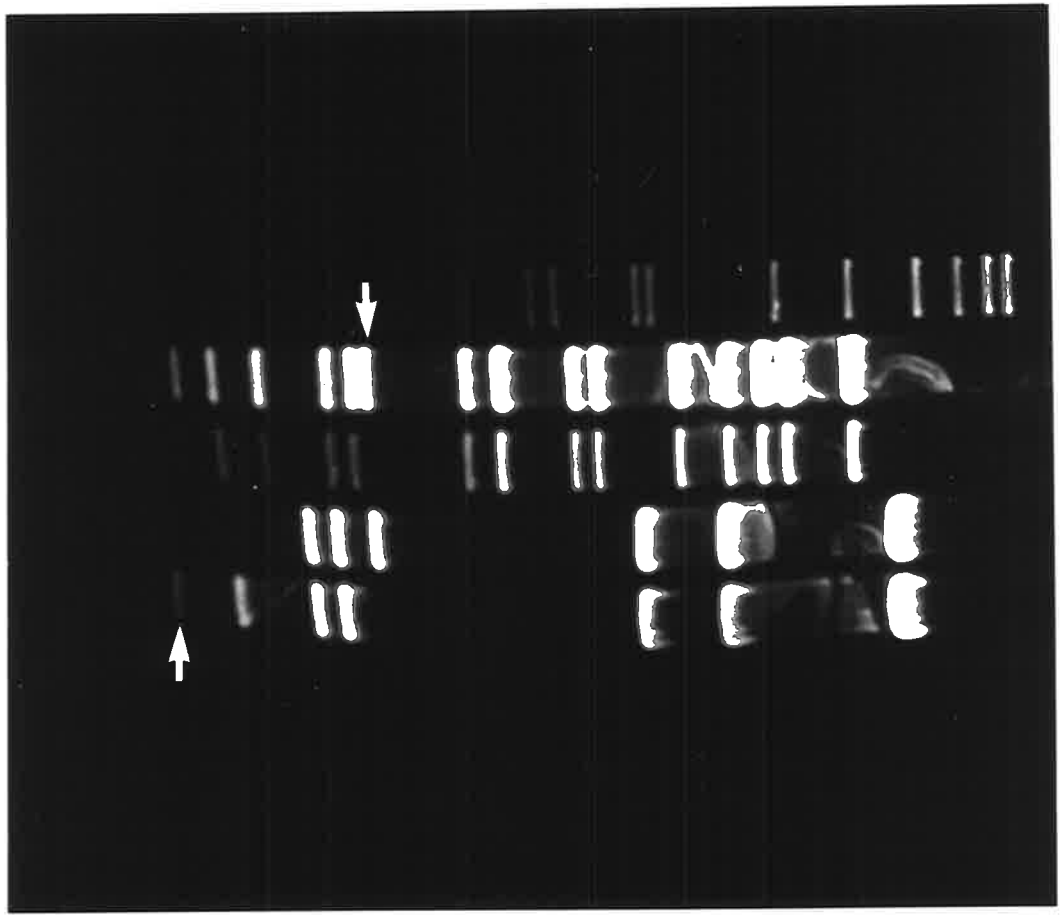
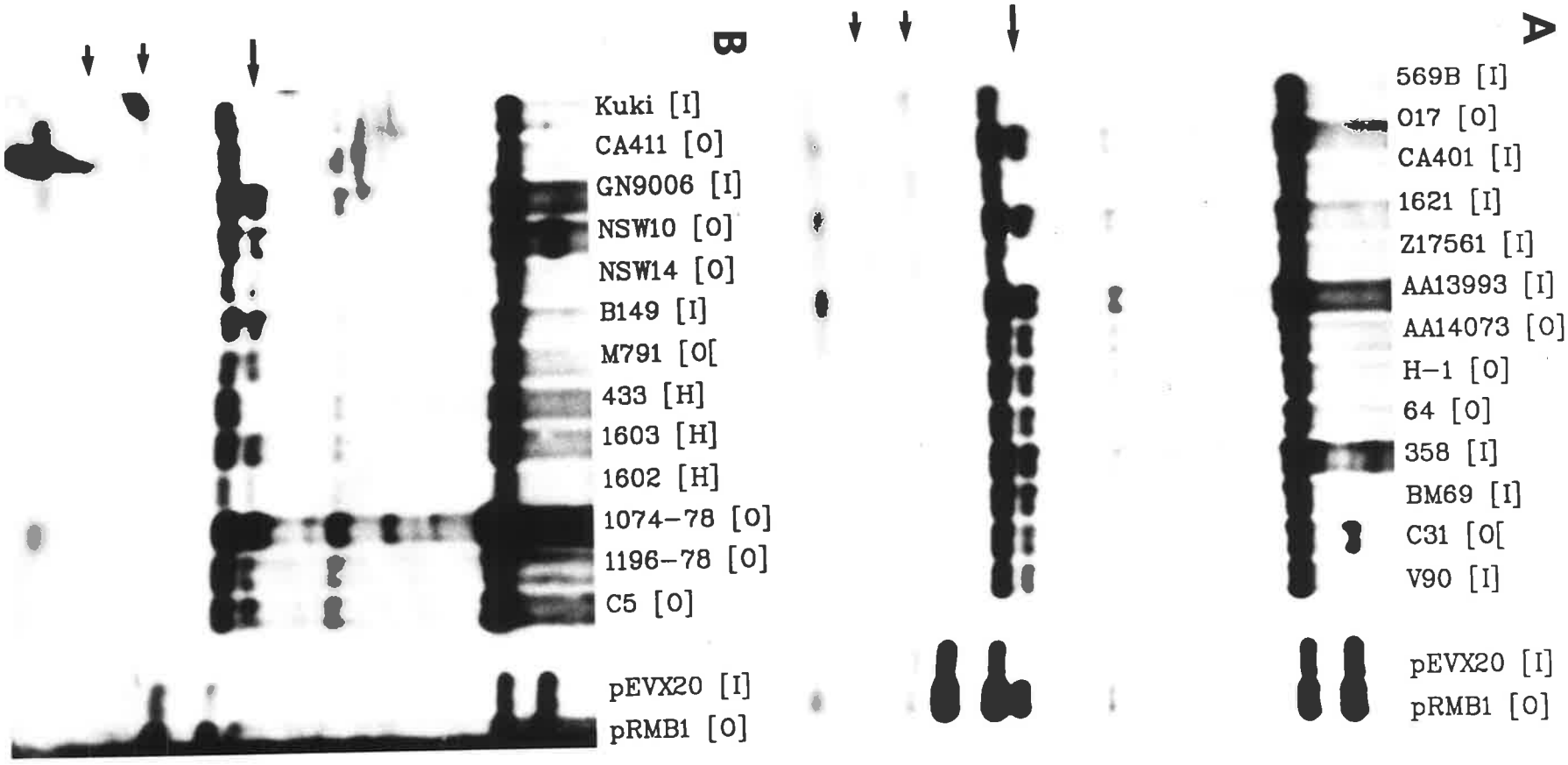

SPP1
pRMB1
pEVX20
pPM2122
pPM2123

Figure 3.6: Southern analysis of chromosomal DNA isolated from Inaba and Ogawa serotypes of *V. cholerae* O1.

Genomic DNA of 26 *V. cholerae* O1 strains was digested with the restriction endonuclease *Dra*I and electrophoresed on a 1% agarose gel. The plasmids pRMB1 and pEVX20 were used as positive and negative controls. After transfer to nitrocellulose the filter was probed with $\alpha[^{32}P]$-dCTP nick translated pPM2122, washed and subjected to autoradiography. The serotype of each strain is given in brackets; as either Inaba (I), Ogawa (O) or Hikojima (H). The 860bp DNA fragment of interest is identified by an arrow. The two smaller fragments of 560bp and 300bp are also indicated.

Panel A contains those strains that have been used throughout this study.

Panel B includes a number of environmental isolates as well as Hikojima strains.


B
Kuki [I]
CA411 [O]
GN9006 [I]
NSW10 [O]
NSW14 [O]
B149 [I]
M791 [O[
433 [H]
1603 [H]
1602 [H]
1074-78 [O]
1196-78 [O]
C5 [O]
pEVX20 [I]
pRMB1 [O]
A
569B [I]
017 [O]
CA401 [I]
1621 [I]
Z17561 [I]
AA13993 [I]
AA14073 [O]
H-1 [O]
64 [O]
358 [I]
BM69 [I]
C31 [O[
V90 [I]
pEVX20 [I]
pRMB1 [O]

other changes in the *Dra*I fragments in either number or length were detected. Thus, it was not possible to identify any conserved differences between Inaba and Ogawa strains, which could be responsible for serotype.

The third sequence divergence between pEVX20 and pRMB1 is a point mutation at position 17,840. This base substitution leads to a stop codon in *rfbT* in the Inaba strain 569B leading to a truncated protein of 27kDa instead of the predicted RfbT of 32kDa. This change is not easily detected by probing and requires the cloning of *rfbT* genes from a number of strains (see Chapter 4).

3.2.3 *RfbT* is the gene required for serotype conversion

Since no conserved change appeared in either *rfbR* or *rfbT* it was decided to firstly determine exactly which gene was involved in serotype specificity by site-directed mutagenesis of each of the open reading frames within the 3kb *Cla*I region.

Plasmid pPM2129 was used as the basis for a series of restriction endonuclease generated deletions and for the insertion of a kanamycin resistance (Km^R) cartridge to determine which of the four genes within the 3kb *Cla*I fragment are required for serotype specificity. The relative position of the 3kb *Cla*I fragment within the 20kb *Sac*I O-antigen region is shown in Fig. 3.1. Each gene within the 3kb *Cla*I fragment of pPM2129 was either completely or partially removed by deletion (Fig. 3.7). Plasmid pPM2132 is a *Hinc*II deletion of pPM2129 which completely removes *rfbT* and *ompX*. This clone is no longer able to convert strain 569B (Inaba) to Ogawa when introduced by electroporation. *RfbT* is required for this conversion since the entire *ompX* gene is not present in pPM2129. Several other deletions, pPM2134, pPM2133 and pPM2135 have *rfbR* and *rfbS* either completely or partially removed. Most of *ompX* is also deleted in pPM2135. These plasmids are still able to convert 569B from Inaba to Ogawa. That is except when *rfbT* is removed, these plasmids

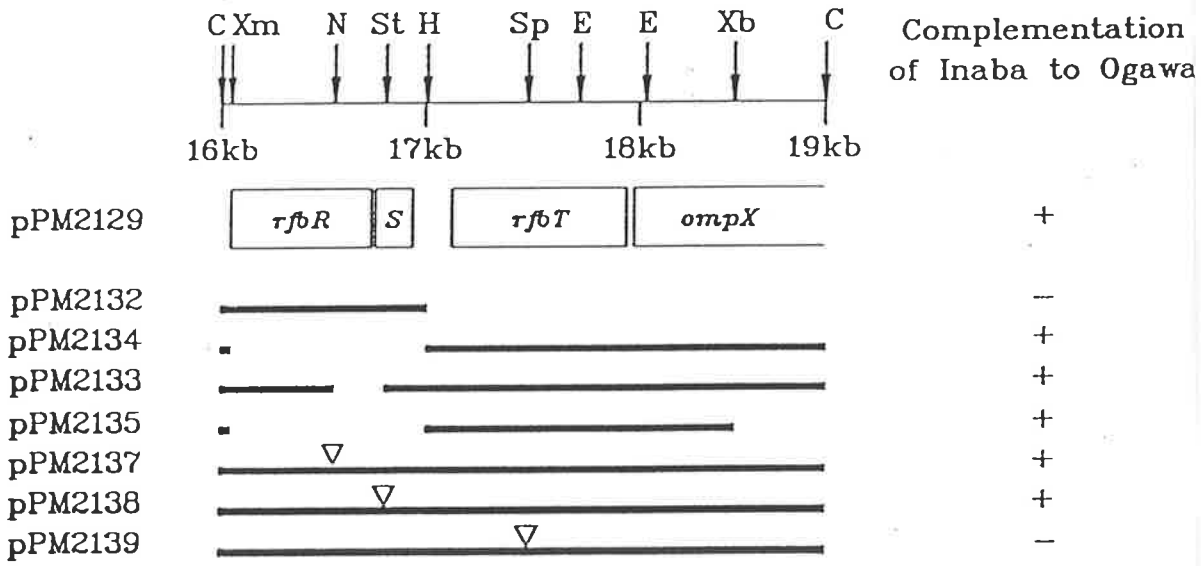

C Xm N St H Sp E E Xb C
Complementation of Inaba to Ogawa
16kb
17kb
18kb
19kb
pPM2129
rfbR
S
rfbT
ompX
+
pPM2132
–
pPM2134
+
pPM2133
+
pPM2135
+
pPM2137
+
pPM2138
+
pPM2139
–

Figure 3.7: Localization of the gene encoding synthesis of the Ogawa specific B antigen

A physical map of pPM2129 and the relevant restriction sites is shown at the top. Plasmid pPM2129 containing the 3kb *Cla*I fragment from pRMB1 was cloned into pUC18. Plasmid pPM2132 was obtained by a deletion from the *Hinc*II site through to a *Hinc*II site in the vector leaving *rfbR* and *rfbS* intact but removing *rfbT*. Plasmid pPM2133 and pPM2134 represent *Nru*I/*Stu*I and *Hinc*II/*Xmn*I deletions, respectively. These plasmids interrupt or remove *rfbR* and *rfbS*, but leave *rfbT* and the C-terminal coding region of *ompX*. Plasmid pPM2135 is a deletion clearly showing that only *rfbT* is required for serotype specificity. Plasmids pPM2137, pPM2138 and pPM2139 contain the Km^R cartridge inserted into the *Nru*I, *Stu*I and *Spe*I sites of pPM2129, respectively. Insertions are indicated by the triangles. The ability of the various plasmids to mediate conversion from Inaba strain 569B to Ogawa is indicated and was determined by slide agglutination using typing sera.

C, *Cla*I; E, *Eco*RI; H, *Hinc*II; N, *Nru*I; Sp, *Spe*I; St, *Stu*I; Xb, *Xba*I; Xm, *Xmn*I.

are able to convert 569B, from expressing A and C antigens to expressing the A, B and C antigens. Thus, it seems most likely that *rfbT* is the only gene required for the Ogawa serotype specificity. To confirm this, a number of insertion mutants (pPM2137, pPM2138 and pPM2139) were constructed (Fig 3.7). The insertions clearly showed that none of the other genes played a role in serotype specificity and that only the insertion in *rfbT* had any effect on the ability of the plasmid to mediate serotype conversion.

The deletions have been constructed in such a way that a promoter in front of *rfbR* could still lead to transcription of *rfbT*. However, the Km^R insertions in *rfbR* and *rfbS* indicate that *rfbR* and *rfbS* are not required for serotype specificity and that *rfbT* probably has its own promoter since the Km^R cartridge is expected to be polar.

3.3 Summary and Conclusion

During the course of this study, the region necessary for serotype specificity could be further localized (Morona *et al.*, 1990) and it was anticipated to lie within a 3kb *Cla*I fragment which contains three complete ORF designated *rfbR, rfbS* and *rfbT* and a partial ORF called *ompX* (see Chapter 4). By deletion and insertion mutagenesis it was possible to show that *rfbR* and *rfbS* as well as *ompX* are not required for the determination of serotype specificity. Indeed, the data imply that *rfbR* due to the 8 bp deletion is not required for O-antigen biosynthesis.

Thus, it has been shown conclusively that the *rfbT* gene is the only gene needed for serotype conversion from the Inaba to Ogawa form and that, in merodiploids, the Ogawa form is dominant. Therefore, the *rfbT* gene product is responsible for synthesis of the B antigen seen in Ogawa strains, but appears to play no role in the expression of the O-antigen in Inaba strains. This is further discussed in Chapter 5. A number of other *V. cholerae* O1 strains of the Inaba, Ogawa and Hikojima serotypes have been analysed in order to detect a

possible conserved difference between strains of the various serotypes. These studies have not revealed any conserved change either in *rfbR* or in *rfbT*. The change leading to a stop codon and premature termination of translation in *rfbT* of 569B is consistent with the notion that Inaba strains are defective in *rfbT*, and this is examined in Chapter 4.

Chapter 4

Genetic Characterization of the *rfbT* Gene from Inaba and Ogawa Strains of *Vibrio cholerae* O1

4.1 Introduction

It had not been possible to show a conserved DNA change between Inaba and Ogawa strains by dot blot and Southern hybridization. However the *rfbT* gene of the Inaba strain 569B has an amber mutation leading to a truncated protein of 27kDa instead of a 32kDa protein in Ogawa strain O17. Consequently it was decided to look at a number of Inaba and Ogawa strains more closely and to examine the degree of variability of the region surrounding and within the *rfbT* gene.

The advent of PCR (Mullis *et al.*, 1986; Saiki and Gelfand, 1989) and the cloning and sequencing of PCR products has provided a useful tool to look at a number of strains without having to make extensive cosmid or plasmid banks and subsequently isolate the cloned genes. Thus, the minor change leading to a stop codon in *rfbT* in strain 569B can be readily examined in other Inaba strains. In this chapter, the nucleotide sequence of *rfbT* is analysed and the sequence of *rfbT* from 7 strains determined. A promoter in front of *rfbT* is identified by primer extension and confirmed by cloning into the promoter detection vector pPM3024. Based on the sequence data a mechanism is proposed for serotype conversion from Inaba to Ogawa and vice versa.

4.2 Results

4.2.1 Nucleotide sequence determination of *rfbT* from O17 and 569B

The nucleotide sequence of *rfbT* was determined as part of the sequencing of the 20kb *Sac*I fragment which encodes O-antigen biosynthesis in *V. cholerae* O1. The sequencing strategy of the 3kb *Cla*I fragment encoding *rfbR*, *rfbS*, *rfbT* and part of *ompX* is shown in Fig. 4.1, the sequence of this region is shown in Fig. 4.2.

4.2.1.1. Nucleotide sequence analysis of *rfbT*

The *rfb* region of *V. cholerae* O1 is an operon of approximately 18kb, and the ORF's have been defined by the order in which they are transcribed within this operon. Analysis of the nucleotide sequence of the 3kb *Cla*I fragment from strain O17 has revealed three ORF's called *rfbR*, *rfbS* and *rfbT*. The ORF's of *rfbR* and *rfbS* could encode proteins of 7kDa and 27kDa respectively. The ORF for *rfbT* has two possible initiation codons (ATG-met) at nucleotide positions 17148 and 17190. The correct initiation codon was determined by N-terminal analysis which is discussed in Chapter 6. The ORF codes for 286 amino acids which when translated would give rise to a protein of 32kDa. The 32kDa protein correlates well with a protein seen on Coomassie blue stained SDS-PAGE as described in Chapter 6.

No polarity was observed with plasmids pPM2137 and pPM2138 (sections 3.2.1 and 3.2.2) which contain a Km^R cartridge upstream of *rfbT*. This seems to indicate the presence of a promoter in front of *rfbT*. Analysis of *E.coli* promoters has defined two consensus hexamer regions (Pribnow, 1975, 1976; Schaller *et al.*, 1975; Takanami *et al.*, 1976; Seeburg *et al.*, 1977; Hawley and McClure, 1983). The "Pribnow box" is a sequence located 10nt

Figure 4.1: Strategy used for dideoxy sequencing of the 3kb *Cla*I fragment.

The 3kb *Cla*I fragment was cloned into M13 in two orientations. The fragment was sequenced using -40 universal primer and synthetic oligodeoxynucleotides. The synthetic oligo allowed the sequencing of both the Inaba and Ogawa clones. The numbers below the arrows refer to the oligonucleotide in our laboratory collection. The -40* arrow is sequence derived from a subclone fused to the *V. cholerae ompV* gene.

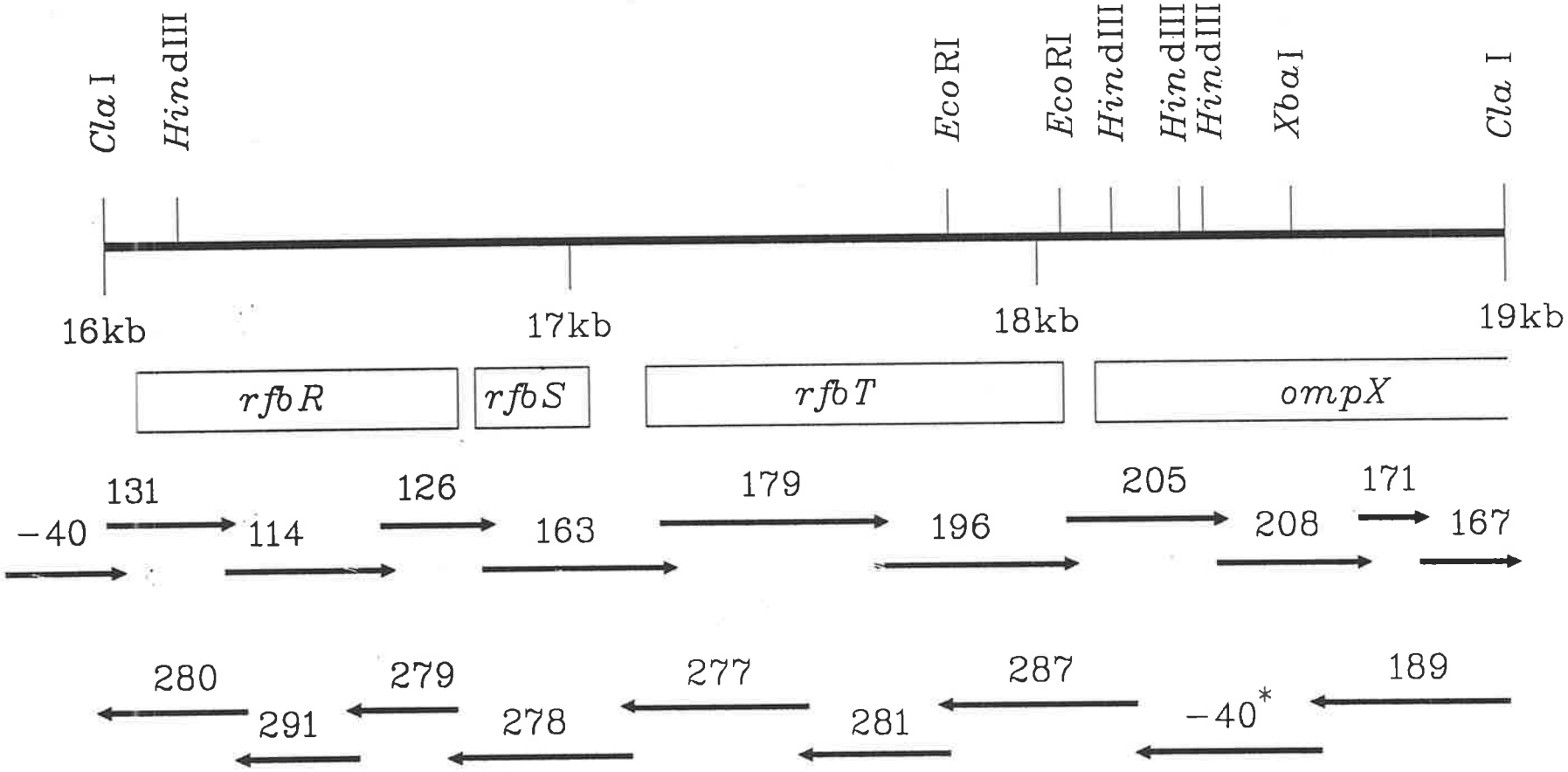

ClaI
HindIII
EcoRI
EcoRI
HindIII
HindIII
HindIII
XbaI
ClaI
16kb
17kb
18kb
19kb
rfbR
rfbS
rfbT
ompX
131
126
179
205
171
−40
114
163
196
208
167
280
279
277
287
189
291
278
281
−40*

Figure 4.2: Nucleotide sequence of the 3kb *Cla*I fragment of the O-antigen operon *V.cholerae* O17.

The sequence of the 3kb *Cla*I fragment is shown. This region contains three complete genes *rfbR*, *rfbS* and *rfbT* and part of *ompX*, which is transcribed in the opposite orientation to the *rfb* genes and has not been shown on the sequence. The nucleotides are numbered relative to the 20 kb sequence of the *Sac*I (*Sst*I) fragment from O17. The amino acids are numbered relative to the start of each protein. The * represent the stop codons

A more detailed diagram of the upstream region of *rfbT* can be found in figure 4.3.

```
    16038     16048     16058     16068     16078     16088     16098     16108     16118     16128     16138     16148
5'ATCGATTTTGGTCATGCTCGCTTAGATTTCCTTAAACGATATGGTCACTTTTAAGCTGGAATGCCTTCTGCGGATACGCTTTCTCGTGTGATGGGTATGATTAATCCTGTAGCTTTGCAA
  I  D  F  G  H  A  R  L  D  F  L  K  R  Y  G  H  F  *  rfbR M  P  S  A  D  T  L  S  R  V  M  G  M  I  N  P  V  A  L  Q
                                                          1                                                            20

    16158     16168     16178     16188     16198     16208     16218     16228     16238     16248     16258     16268
AGAAGCTTCATTACCTGGATGAAGGACTGCCATACACTAACGGATGGTGAAGTTATTGCCATCGACGGTGAAACATTACGCGGCTCTTATGACCGCTCGAAAGGCAAAGGAACAATCCAC
R  S  F  I  T  W  M  K  D  C  H  T  L  T  D  G  E  V  I  A  I  D  G  E  T  L  R  G  S  Y  D  R  S  K  G  K  G  T  I  H
                                                                                                                        60
21

    16278     16288     16298     16308     16318     16328     16338     16348     16358     16368     16378     16388
ATGGTGAACGCTCTTGCTACAGCAAATGGAATGAGCATTGGGCAACTGAAGGTTGATTCTAAGAGCAACGAGATTACCGCGATCCCCAAGCTACTTGACTTACTAGATGTAAAAGGCTGC
M  V  N  A  L  A  T  A  N  G  M  S  I  G  Q  L  K  V  D  S  K  S  N  E  I  T  A  I  P  K  L  L  D  L  L  D  V  K  G  C
                                                                                                                       100
61

    16398     16408     16418     16428     16438     16448     16458     16468     16478     16488     16498     16508
TTGATTACGATTGATGCAATGGGCTGCCAAAAGAAAATAGCACAGAAAATTCTTGATAAAGAAGCGGATTATTTATTGGCGGTCAAAGGTAATCAGGGAATGCTTGAGCAAGCCTTTGAT
L  I  T  I  D  A  M  G  C  Q  K  K  I  A  Q  K  I  L  D  K  E  A  D  Y  L  L  A  V  K  G  N  Q  G  M  L  E  Q  A  F  D
                                                                                                                       140
101

    16518     16528     16538     16548     16558     16568     16578     16588     16598     16608     16618     16628
GATTATCTTCGAATGGACATGCTTCACGACTTCGACGGTAGTTCTTATAGTACACAAGAAAAAAGTCACGGAAGAATAGAAACGAGAGTGGCTTTAGTGAATCGCGATTTGTCGGTTTTG
D  Y  L  R  M  D  M  L  H  D  F  D  G  S  S  Y  S  T  Q  E  K  S  H  G  R  I  E  T  R  V  A  L  V  N  R  D  L  S  V  L
                                                                                                                       180
141

    16638     16648     16658     16668     16678     16688     16698     16708     16718     16728     16738     16748
GGTGATATTGAACATGAATGGCCTGAGCTTAAATCAATGGGCACCGTGGCTTCAATTCGACAAGAATCGGCAGTCGCAACAGAGCAAGATGTGAGTATTCGTTACTACATATGCTCTAAA
G  D  I  E  H  E  W  P  E  L  K  S  M  G  T  V  A  S  I  R  Q  E  S  A  V  A  T  E  Q  D  V  S  I  R  Y  Y  I  C  S  K
                                                                                                                       220
181

    16758     16768     16778     16788     16798     16808     16818     16828     16838     16848     16858     16868
GAATTGGAAGCTCAAACGTTACTTGAAGCGACTTGTTCTCACTAGGGGGTAGAGGTCATGGATTGGTCACTTGATACCGCATTTTGTGAGGACAATTCACGCATTAGAGCGGACGATCGA
E  L  E  A  Q  T  L  L  E  A  T  C  S  H  *        rfbS  M  D  W  S  L  D  T  A  F  C  E  D  N  S  R  I  R  A  D  D  R
221                                  234               1                                                                21

    16878     16888     16898     16908     16918     16928     16938     16948     16958     16968     16978     16988
GCAGAGGCCTTTGCAAGGATCAGGCAGATATGTTTGAACCTATTAAAGAGCGAACCCACGTTTAAAGGTGGTATCAAACGTAAACGGATGAACTGCGCAATGAACGAAAAGTACCTAAGT
A  E  A  F  A  R  I  R  Q  I  C  L  N  L  L  K  S  E  P  T  F  K  G  G  I  K  R  K  R  M  N  C  A  M  N  E  K  Y  L  S
                                                                                                                        61
22
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    16998     17008     17018     17028     17038     17048     17058     17068     17078     17088     17098     17108
AAGGTTCTTGAAAGCCTTACGTGACGGTGATGTTCATGCGGTTTCCGTGCTAAAGGCTTTGGATGAAATTCCTTCTAAATGTCAATAAAATGGCAAATCCATTATTGGTCAACAATGCCC
K  V  L  E  S  L  T  *     *
62                  68

    17118     17128     17138     17148     17158     17168     17178     17188     17198     17208     17218     17228
TTTCAGGTCCTCAAACCTGCATCTGCAAGTTGATTCTGTATGTTATTTTTTACGCTAATATTATTTAAAATTGAGGTAGTAATGAAACATCTAATAAAAAACTATGTACAAAAATTAATT
                                                                                 rfbT M  K  H  L  I  K  N  Y  V  Q  K  L  I
                                                                                      1                                    13

    17238     17248     17258     17268     17278     17288     17298     17308     17318     17328     17338     17348
AAAACAGAGCTTGATGCTATTCAGTCAAAGTCTGTTCATGATAATCGAAACTTCATTTACAATGGAGAGTTTTTAATTCTTGAAAGCGAATTTGGATTGCATTGTTTTCCCAGAGTGCAG
K  T  E  L  D  A  I  Q  S  K  S  V  H  D  N  R  N  F  I  Y  N  G  E  F  L  I  L  E  S  E  F  G  L  H  C  F  P  R  V  Q
14                                                                                                                    53

    17358     17368     17378     17388     17398     17408     17418     17428     17438     17448     17458     17468
TTGAACCATGCTTTAAGCTACAAAAACCCAAACTTTGATTTAGGTATGCGTCACTGGATTGTTAATCATTGTAAGCATGACACCACTTATATTGATATCGGTGCAAACGTTGGAACTTTC
L  N  H  A  L  S  Y  K  N  P  N  F  D  L  G  M  R  H  W  I  V  N  H  C  K  H  D  T  T  Y  I  D  I  G  A  N  V  G  T  F
54                                                                                                                    93

    17478     17488     17498     17508     17518     17528     17538     17548     17558     17568     17578     17588
TGTGGAATCGCTGCTCGTCATATTACACAAGGAAAAATTATAGCGATAGAACCACTCACAGAAATGGAAAATAGTATTAGGATGAATGTTCAATTAAATAATCCACTAGTTGAGTTTCAT
C  G  I  A  A  R  H  I  T  Q  G  K  I  I  A  I  E  P  L  T  E  M  E  N  S  I  R  M  N  V  Q  L  N  N  P  L  V  E  F  H
94                                                                                                                    133

    17598     17608     17618     17628     17638     17648     17658     17668     17678     17688     17698     17708
CATTTTGGCTGTGCAATAGGTGAGAATGAAGGGGAAAATATTTTCGAAGTTTATGAGTTTGATAATAGGGTGTCATCATTATATTTTCAAAAAAATACAGACATAGCAGATAAGGTTAAA
H  F  G  C  A  I  G  E  N  E  G  E  N  I  F  E  V  Y  E  F  D  N  R  V  S  S  L  Y  F  Q  K  N  T  D  I  A  D  K  V  K
134                                                                                                                   173

    17718     17728     17738     17748     17758     17768     17778     17788     17798     17808     17818     17828
AATAGCCAAGTTCTGGTTAGAAAGTTAAGTAGTTTAGATATATCGCCTACTAACTCTGTAGTTATAAAAATTGATGCTGAAGGCGCAGAAATAGAGATATTAAACCAGATTTACGAATTC
N  S  Q  V  L  V  R  K  L  S  S  L  D  I  S  P  T  N  S  V  V  I  K  I  D  A  E  G  A  E  I  E  I  L  N  Q  I  Y  E  F
174                                                                                                                   213

    17838     17848     17858     17868     17878     17888     17898     17908     17918     17928     17938     17948
ACAGAAAAGCATAATGGAATTGAATATTATATTTGCTTTGAATTTGCAATGGGTCATATACAGAGGTCTAATAGAACTTTTGATGAGATTTTTAACATAATAAACTCAAAATTCGGAAGT
T  E  K  H  N  G  I  E  Y  Y  I  C  F  E  F  A  M  G  H  I  Q  R  S  N  R  T  F  D  E  I  F  N  I  I  N  S  K  F  G  S
214                                                                                                                   253
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    17958     17968     17978     17988     17998     18008     18018     18028     18038     18048     18058     18068
AAGGCATATTTTATTCATCCATTATCATCCGCTGAACATCCTGAGTTTAATAAAGCAACGCAGGATATTAATGGGAATATCTGTTTTAAATATGTATCATAAAATAATTTAATATATTCC
K  A  Y  F  I  H  P  L  S  S  A  E  H  P  E  F  N  K  A  T  Q  D  I  N  G  N  I  C  F  K  Y  V  S  *
254                                                                                                   286

    18078     18088     18098     18108     18118     18128     18138     18148     18158     18168     18178     18188
GTATGTCATTGCAAGTTCAACAGACATTTCCGAAGAGTTCACTATACAGTTTAGTATAGCTTTGTGCATAGCGATGTGCTGTGAATTCCTGTTCAAAACGTTTGCGAGCATTAATACCTA

    18198     18208     18218     18228     18238     18248     18258     18268     18278     18288     18298.    18308
ATTTTTCACAAAGTTTTGTATCATGTTCTATTTTTAACATTGCATCGGAAAAGCTTTGACTGTCAGCTGGCGGAACAACTAACCCTGTTTCGCCATTGATGTTCACATAAGATGAACCTG

    18318     18328     18338     18348     18358     18368     18378     18388     18398     18408     18418     18428
TGCCAATATCACTACTAATAATGGCTTTACAGTACATTTGTGCTTCGATAAGTGATATACCAAAAGCTTCTGAGCGTAGGTGAGATGGGAAAACAAAAGCTTTAGAAAGTAAATGTAATA

    18438     18448     18458     18468     18478     18488     18498     18508     18518     18528     18538     18548
TGACTTTGTCTTCCTCAGAAATGAATCCTACTAGCTTAACGTTTTCAAGATTGTGTTTGGCAATATAAGACTCTAGTTTTACTCTTTCCGGACCGTCACCAGCAATTATAACAGGTAGCT

    18558     18568     18578     18588     18598     18608     18618     18628     18638     18648     18658     18668
GGTTGATTTTTGCTGCTTCAAGTAAGAAATCTAGACCCTTGTAGTACCGTAATACGCCAACAAATAAGAAAAAACCTTCCCCTACTTTTTCTCGCCATTTATTAATATTATCGTTTGATG

    18678     18688     18698     18708     18718     18728     18738     18748     18758     18768     18778     18788
GGATAGGATAAGTATTCTCATCAACGGCAAGTGGAATTATTTTTACCTTATTTTTATGCCTTAACAAATTCTCGCTTGTGTGTGCATATTGTGGTGATGTTGCCACAAGGATATTTGCTT

    18798     18808     18818     18828     18838     18848     18858     18868     18878     18888     18898     18908
TATCGGAATGATACGTTACAATACTTGGATTCGATGAGCCAAATAAAGAGAGAAAGTCTCGATTTAAAAAATGACTCTCAAGTGGTTGATATAATTTCTTTAAGCACTTTTGACGAATAA

    18918     18928     18938     18948     18958     18968     18978     18988     18998     19008
CGGTTGGCCATGGATAATGGTAATGGATTATGTCAGCCCATTTTGAAAGTTTCTTGAATTGTCTAATTAGCTTCCACGAGAATCCATTTGAAGAGATTTCAATCGAT 3'
```

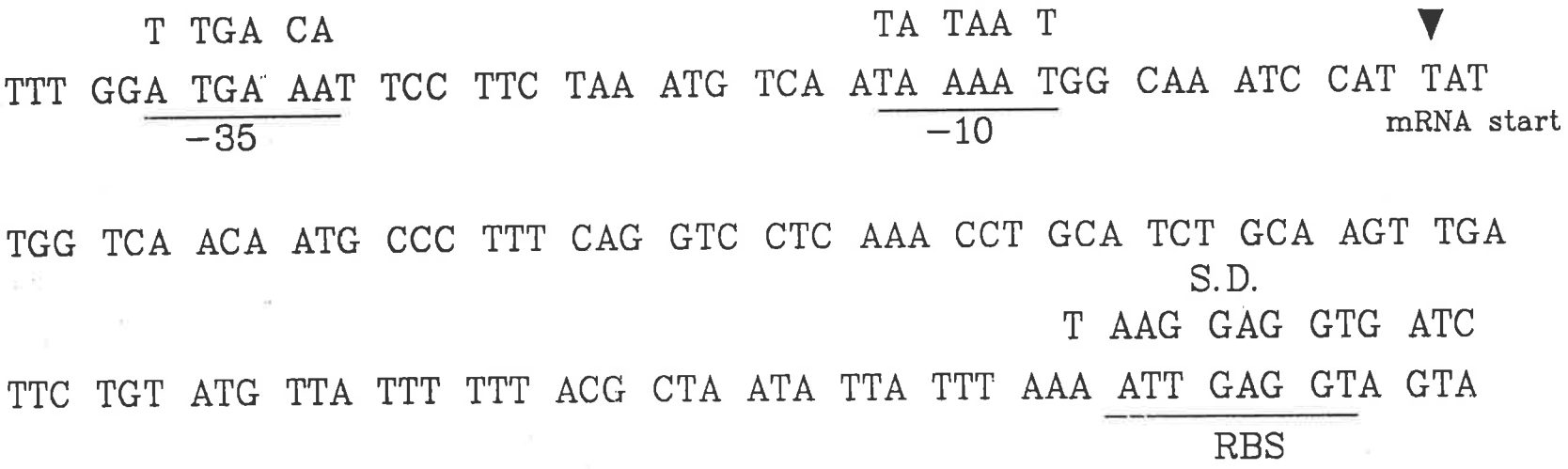
upstream from the messenger RNA start. The consensus sequence is T_{80} A_{95} T_{45} A_{60} A_{50} T_{96}, where the subscript represents the precentage occurrence of the base most frequently found in that position (Siebenlist *et al.*, 1980; Hawley and McClure, 1983). The other site is termed the "-35 region" and is 35 bases upstream of the transcriptional start (Pribnow, 1979; Hawley and McClure, 1983; Studnicka, 1987). The -35 region has been implicated as the initial recognition site for RNA polymerase. There is also a consensus sequence for this region: T_{82} T_{84} G_{98} A_{65} C_{54} A_{45} (Hawley and McClure, 1983). The role of the individual bases within the hexamers has been closely analysed by site directed mutagenesis. Changes within this sequence have marked effects by decreasing/increasing the promoter activity (Rosenberg and Court, 1979; Hawley and McClure, 1983).

A third region has been identified by Gentz and Bujard (1985). This consists of a hexamer of "A"'s centered around the -43 position and may play an additional role in promoter activity. The use of primer extension analysis on *rfbT* (section 4.2.4) has enabled the previously discussed promoter sequences to be localized. A promoter was detected 5' to the *rfbT* ORF that shows homology with both the "Pribnow box" (-10 region) and the "-35 region". A possible "-10 region" is located between nt 17074 and 17079 and reads (**TAAAAT**) (Fig. 4.3). The sequence shows remarkable homology to the consensus sequence and differs only at the central T which shows the greatest variability. This region is in fact at -15 which is not optimal. The reasons for this may be that the spacing in *V. cholerae* shows greater variability. The primer extension analysis (section 4.2.4) differs for the actual mRNA start between *V. cholerae* and *E. coli* it is thus possible that some processing of the 5' end of the mRNA has occurred. The corresponding -35 region located between nt 17051 and 17056 (Fig 4.3) is **ATGAAA** where 4 of the 6 bases are identical to the *E. coli* consensus sequence. The change from a C to an A at position 17055 is not unusual since the C is present in only 54% of *E. coli* promoters, but the T to A change at nt 17051 may have an effect on promoter efficiency. Both the *hly*A and MFRHA promoters

Figure 4.3: Upstream region of *rfbT*.

The 144 nt upstream of the translational start are shown. The *E. coli* consensus promoter sequences for the "Pribnow box" and the "-35 region" are shown above the putative *rfbT* promoter. The Shine-Dalgarno (S.D.) sequence and corresponding ribosome binding site (RBS) are also indicated. The 5' end of the mRNA was determined by primer extension on total RNA preparations from *V. cholerae* and *E. coli* harbouring the cloned *rfbT* gene and is indicated by an arrow.

The first few amino acids of RfbT are shown and are based on N-terminal protein sequence analysis (Chapter 6).


T TGA CA
TA TAA T
TTT GGA TGA AAT TCC TTC TAA ATG TCA ATA AAA TGG CAA ATC CAT TAT
−35
−10
mRNA start
TGG TCA ACA ATG CCC TTT CAG GTC CTC AAA CCT GCA TCT GCA AGT TGA
S.D.
T AAG GAG GTG ATC
TTC TGT ATG TTA TTT TTT ACG CTA ATA TTA TTT AAA ATT GAG GTA GTA
RBS
ATG AAA CAT CTA ATA AAA AAC TAT GTA CAA AAA TTA ATT AAA ACA GAG
Met Lys His Leu Ile Lys Asn Tyr Val Gln Lys Leu Ile Lys Thr Glu

from *V. cholerae* show similar sequence divergence (Williams and Manning , 1991; A. Barker personal communication).

The spacing between the -10 and -35 regions appears to play an important role for promoter strength. The limits of the spacing are 15 to 21 nt, with maximal strength at 17±1 nt (Rosenberg and Court, 1979; Harley and McClure, 1983). The 17 nt spacing between -10 and -35 regions seen in the *rfbT* promoter is optimal for the *E. coli* consensus promoter region. The region upstream of *rfbT* shows no homology to the -43 hexamer, but this is not unusual and has been seen with other *V. cholerae* promoters such as the *hlyA* (Williams and Manning, 1991).

For efficient initiation of translation of the mRNA a ribosome binding site (RBS) is required which is usually 5-8 nt from the initiation codon (ATG). The ribosome binding site displays homology with the free 3' end of the 16S rRNA, and is often referred to as a Shine-Dalgarno sequence (Shine and Dalgarno, 1974). The Shine-Dalgarno sequence is 5'AAGGAGGU 3' and any mutations in this region drastically reduce the level of translational initiation (Gold *et al*, 1981; Kozak, 1983). The optimal spacing of the RBS from the start codon is 7 nt and spacings of less than 5 and greater than 9 are rare. Reducing this spacing results in less efficient translation (Gold *et al*, 1981; Kozak, 1983). The RBS upstream of *rfbT* is 5'AUU**GAGGU** 3' which shows strong homology to the Shine-Dalgarno (5'AAGGAGGU 3') consensus sequence although the spacing is only 4 nt from the AUG start which could result in less than optimal translation. The RBS for *rfbT* shows the same spacing and homology as that for *hlyA* (Williams and Manning, 1991).

4.2.1.2. Transcriptional Terminators

Analysis of the sequence downstream of *rfbT* has not identified a Rho-independent terminator. There appears to be no region after the stop codon which can form the secondary

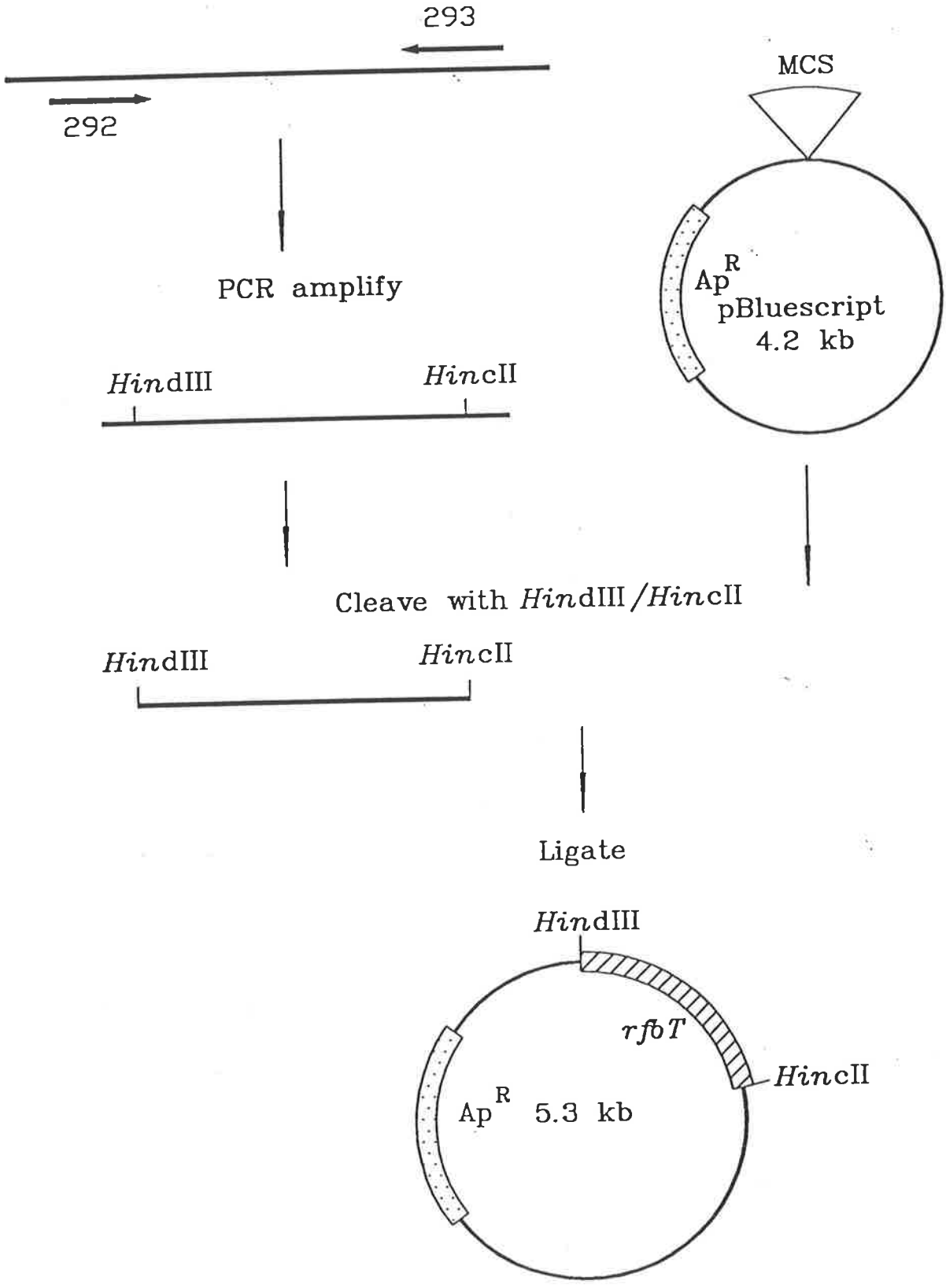
structure characteristic of Rho-independent terminators (Rosenberg and Court, 1979). The characteristics of a Rho-dependent terminator are less well defined although Rho-dependent transcriptional termination elements have been defined in a number of systems (Court *et al.*, 1980; Stanssens *et al.*, 1986; Stewart *et al.*, 1986). The most common feature of Rho dependent termination is a C over G rich region upstream of the 3' endpoint of the mRNA (Alifano *et al.*, 1991). Since the 3' end of the mRNA is not known, the possibility exists that *rfbT* has a Rho dependent terminator. It is unlikely that the mRNA of *rfbT* continues far into *ompX* since it is transcribed from the opposite strand.

4.2.2. Cloning of other *rfbT* genes

With the availability of the sequence for the *rfbT* gene it was possible to synthesize two synthetic oligodeoxynucleotide primers, #292 (5' CCAAACACAATCTTGAAA 3')and #293 (5' TTTGCTGACAATATGTGG 3') which could be used to PCR amplify this region from *V. cholerae* genomic DNA. These primers bind to base pair position 18488 and 15977 respectively within the 20kb sequenced *Sac*I fragment. It was decided to use strains of both El Tor and classical biotypes and both Inaba and Ogawa serotypes for the amplification and cloning. The region amplified was larger than the structural gene and this allowed the PCR product to be cleaved internally with two restriction endonucleases and the cloning of a *Hin*cII/*Hin*dIII 1.15kb fragment into pBluescript K/S (Fig 4.4). This approach was taken instead of direct cloning of the PCR fragments which seemed to have a very low efficiency.

Figure 4.4: Cloning of various *rfbT* genes from Inaba and Ogawa strains.

Two oligonucleotides #292 and #293 were employed to PCR amplify a region of DNA from a number of strains. The PCR product was cleaved with *Hinc*II/*Hind*III and ligated to *Hinc*II/*Hind*III cleaved pBluescript K/S. The ligation was transformed into *E. coli* strain DH5 and potential clones screened by cleavage with *Hinc*II/*Hind*III. Two clones from each PCR amplification were used for sequencing.


293
292
PCR amplify
MCS
Ap R
pBluescript
4.2 kb
HindIII
HincII
Cleave with HindIII/HincII
HindIII
HincII
Ligate
HindIII
rfbT
HincII
Ap R
5.3 kb

4.2.3. Nucleotide sequence determination and analysis of various *rfbT* genes

A number of clones from each PCR reaction were sequenced using forward and reverse dye-labelled M13 primers on double stranded plasmid DNA. *Eco*RI deletions were generated and this allowed the sequence data to be extended further using M13 primers. Those regions not covered by the M13 primers were sequenced using two synthetic oligo/primers and dye-labelled terminators. All sequencing was carried out on a 373A Applied Biosystems DNA sequencer. Fig. 4.5 shows the sequencing strategy employed. Two independent clones were sequenced from each strain to rule out any changes which may have arisen from the lack of fidelity of Taq polymerase in the PCR amplification. This was done to ensure that any sequence divergence seen between strains was not a PCR artefact.

Analysis of the sequence of the *rfbT* gene a number of strains has revealed several differences between the Ogawa and Inaba serotypes (Table 4.1). The *Dra*I polymorphism previously investigated (section 3.2.2) by Southern analysis is not conserved within serotypes. The same base involved in the *Dra*I polymorphism i.e. nt 17672 changes from C to T in O17 to 569B also varies in CA401 (Inaba) and Z17561 (Inaba) in that the C is deleted. It is unusual to find two different changes in the same base. This may indicate that this region is prone to sequence variation. As predicted, all Inaba strains have an alteration in which the *rfbT* reading frame is prematurely terminated, leading to a truncated RfbT protein (Table 4.1). This implies that even a small truncation at the carboxy terminus of the RfbT protein destroys its activity.

One of the original variations seen between O17 and 569B was a G to T base substitution leading to a stop codon and a truncated RfbT protein in 569B. This stop codon is not conserved in the different Inaba strains, suggesting that the Inaba serotype appears to be derived from random mutations in the Ogawa *rfbT* gene. Two of the strains sequenced, CA401 and Z17561 have exactly the same sequence variations and this may indicate that these two strains are closely related although, their dates of isolation are well separated.

Figure 4.5: Sequencing strategy used for PCR products.

The relative position of the *Hin*cII/*Hin*dIII fragment within the 20kb *Sac*I region is shown on the top line. Cloned PCR products were sequenced using either dye-labelled primers (ie. -21 universal primer or reverse primer), or dye-labelled dideoxyterminators with synthetic oligonucleotides #196 and #277. The PCR clones were cleaved with *Eco*RI to facilitate sequencing with dye primers. The region sequenced covered the whole of *rfbT* including the 5' mRNA. Although both strands were not entirely sequenced the data were unambiguous.

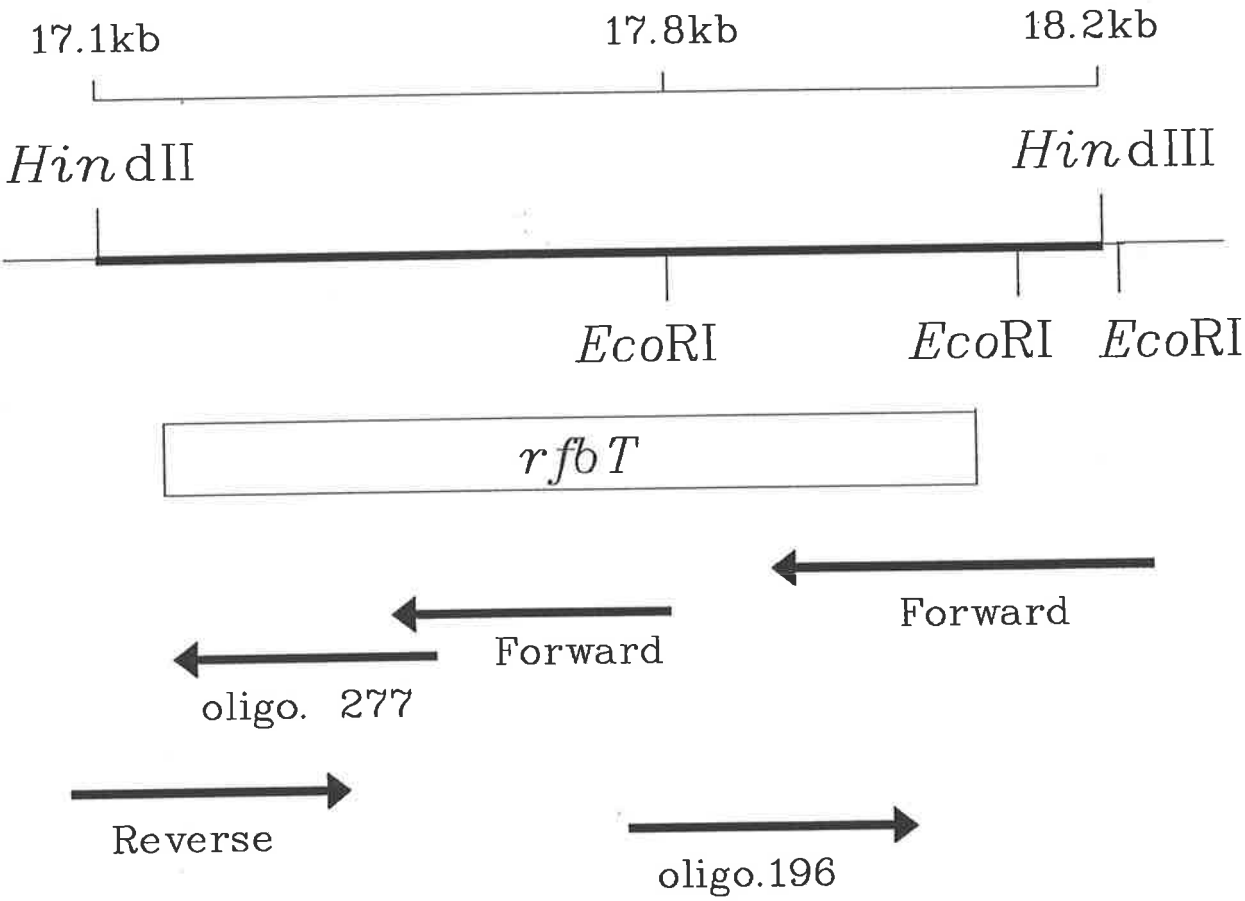

17.1kb
17.8kb
18.2kb
HindII
HindIII
EcoRI
EcoRI
EcoRI
rfbT
Forward
Forward
oligo. 277
Reverse
oligo.196

Table 4.1: Nucleotide sequence changes in the *rfb* region of different *Vibrio cholerae* O1 strains relative to strain O17 (Ogawa).

Strain (biotype/ serotype)[b]	Changes	Consequence
569B (C/I)	G_{6654} to A	no effect, lies in an intergenic region
	Del(T_{16378} to C_{16385})	frameshift in RfbR, gives a truncated protein
	C_{17672} to A	RfbT-Q_{163} to K_{163}
	G_{17844} to T	RfbT-G_{219} to stop
	C_{18086} to G	
	T_{19631} to G	ORF2-Y_{47} to D_{47}
H-1 (E/O)	A_{17729} to G	no change to RfbT-R_{180}
64 (C/O)	no changes	
BM69 (E/I)	C inserted after T_{17976}	frameshift: RfbT-S_{263}AE to F_{263}Rstop
CA401 (C/I)	G_{17327} to T	RfbT-L_{46} to W_{46}
	$TACA_{17495}$ to ACAC	RfbT-T_{102} to H_{102}
	C_{17672}deleted	frameshift: RfbT-Q_{163}KNTDI to K_{163}IQTstop
Z17561 (C/I)	G_{17327} to T	RfbT-L_{46} to W_{46}
	$TACA_{17495}$ to ACAC	RfbT-T_{102} to H_{102}
	C_{17672} deleted	frameshift: RfbT-Q_{163}KNTDI to K_{163}IQTstop

b The biotypes and serotypes are abbreviated : C, classical; E, El Tor; I, Inaba; O, Ogawa.

Other strains have a random scattering of changes and there is no correlation between the biotypes i.e. El Tor and classical strains.

All of the Ogawa strains sequenced, O17, H-1 and 65, have an ORF corresponding to a protein of 32kDa. The only sequence variation seen between Ogawa strains was in H-1 in where there was a conservative base substitution, giving rise to the same amino acid. It thus appears that the Ogawa *rfbT* gene is highly conserved.

4.2.4. Promoter identification and localization

Since plasmids pPM2127 and pPM2128 are unable to mediate serotype conversion and all deletions and insertions in *rfbR* and *rfbS* have no effect on *rfbT* expression this suggested the presence of a promoter immediately prior to *rfbT*. Isolated total RNA was used in a Northern blot using pPM2129 as a probe but no message for either *rfbR*, *rfbS* or *rfbT* could be detected. Thus, it appears that there is little message for *rfbR*, *rfbS* and *rfbT*. This is not surprising due to the lack of an identifiable -43 sequence and the less that optimal position of the -10 region. Therefore primer extension analysis was performed. This is a more sensitive method of detecting mRNA, and it identifies the 5' end of the message. The extension was performed on total RNA isolated from *V.cholerae* strains and *E.coli* strains with or without the *rfbT* clone using oligo #381 (5' AGACTTTGACTGAATAGC 3') which binds at base pair position 17261 (Fig 4.6). Both O17 and 569B produce mRNA for *rfbT* as does the *E.coli* strain carrying the *rfbT* clone, implying that the promoter in front of *rfbT* is recognized in both *V. cholerae* and *E.coli*. There is a difference seen in the extension product in *E. coil* compared to *V. cholerae*. In *E. coli* the messenger RNA is one base longer. This difference does not markedly affect the actual spacing between the transriptional start point in relationship to the putative promoter. No extension product was detected in *E.coli* from pPM2134 (*Hinc*II/*Xmn*I cutdown Fig. 3.7), which does not contain the putative promoter.

Figure 4.6: Primer extension analysis.

Total cellular RNA was isolated from the various strains shown. Primer extensions were performed by using oligonucleotide #381 hybridized to the RNA and extended with AMV reverse transcriptase. The position of the major extension product is indicated by the arrow. The corresponding sequencing ladder was obtained using the same oligonucleotide as a primer with pPM2129 as template.

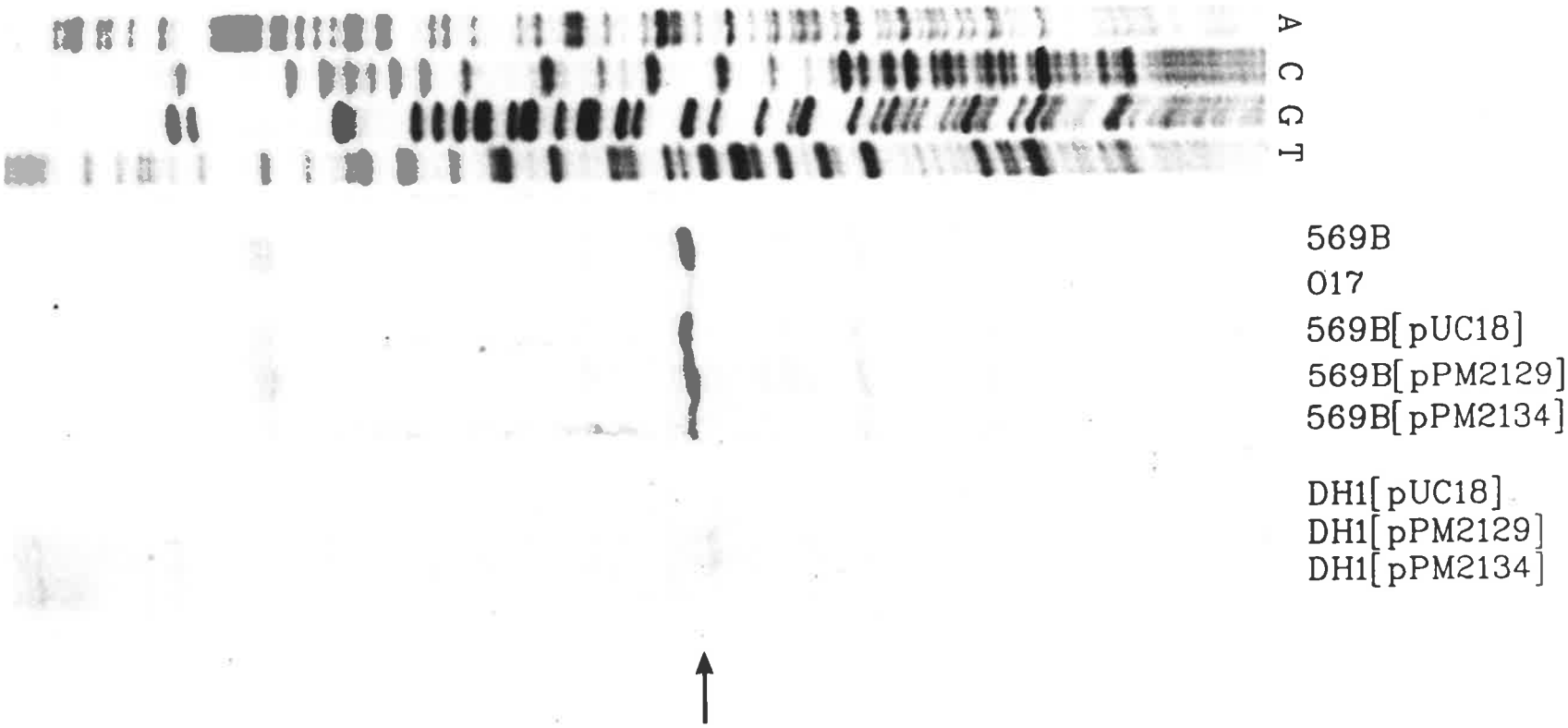
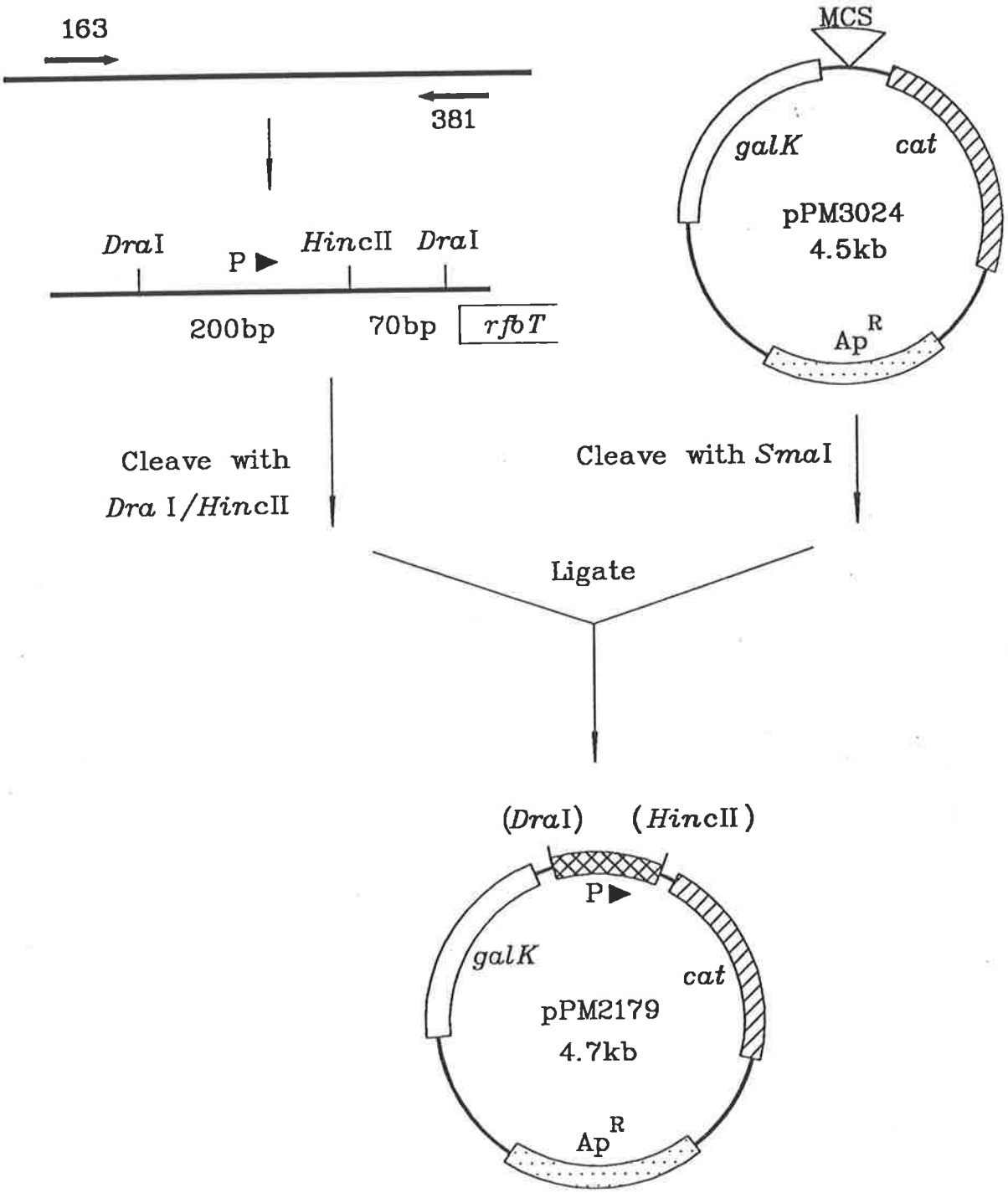

A C G T
569B
017
569B[pUC18]
569B[pPM2129]
569B[pPM2134]
DH1[pUC18]
DH1[pPM2129]
DH1[pPM2134]

Figure 4.7: Cloning of the *rfbT* promoter into pPM3024.

Using two oligonucleotides, #381, #163, and PCR amplification an approximately 300bp fragment is generated. The PCR product was cleaved with *Dra*I and *Hinc*II to generate two fragments of 70bp and 200bp. The 200bp fragment containing the *rfbT* promoter was cloned into the promoter detection vector pPM3024 via the *Sma*I site. The resultant plasmids were designated pPM2179 and pPM2180 which are both in the same orientation.


163
381
DraI
P
HincII
DraI
200bp
70bp
rfbT
MCS
galK
cat
pPM3024
4.5kb
Ap R
Cleave with
Dra I/HincII
Cleave with SmaI
Ligate
(DraI)
(HincII)
P
galK
cat
pPM2179
4.7kb
Ap R

This plasmid, however is still able to mediate serotype conversion. This might be explainedif the *rfbT* gene was being transcribed at a very low level from a vector promoter sequence. Since no extension product was detected of a differing size, this would point to the putative vector promoter to be distal form the insert. This result may also be explained if there are very low levels of mRNA from this new promoter region.

The primer extension provides the precise 5' end of the mRNA for *rfbT* and it is thus possible to identify the putative promoter sequences as discussed in Section 4.2.1.1.

To confirm the primer extension result and to rule out the possibility that the message detected came from processing of a larger mRNA, a small 200bp *DraI*/*Hinc*II fragment was cloned into the promoter detection vector pPM3024 (Williams and Manning, 1991)(Fig 4.7). This vector can be used in a chloramphenicol acetyl transferase (CAT) assay in *V. cholerae*. The clones, pPM2179 and pPM2180, confirm the presence of a promoter within this region and shows that it is active in both *V. cholerae* and *E. coli* (Fig 4.8). The relative strength of the promoter can be gauged by comparison to the positive control vector pPM3035 which contains the promoter for the kanamycin resistance cartridge, this promoter was chosen since it is active in *V. cholerae*; in *E. coli* the *rfbT* promoter appears to be weaker than the promoter in pPM3035 although in *V. cholerae* the promoters appear of approximately equal strength.

4.2.5. RNA Secondary Structure

The start of the *rfbT* mRNA is 99 nt prior to the start of translation, leaving a long 5' untranslated tail (Fig. 4.9). A potential secondary structure of a 114 nt region, from the mRNA start to just inside *rfbT* is represented in Fig. 4.9 and has a free energy of -27.6 kcal/mol. It is interesting to note that the AUG start codon and the ribosome binding site (Shine Dalgarno) (AUUGAGG) are contained within this proposed secondary structure of the mRNA. Thus, it could be necessary to eliminate the secondary structure prior to translation.

Figure 4.8: Promoter detection by chloramphenicol transacetylase (CAT) assay.

CAT was assayed by thin layer chromatography of *V. cholerae* strain O17 and *E. coli* DH5 cell lysates containing either: -ve control plasmid, pPM3024; +ve control plasmid, pPM3035; the *rfbT* promoter clones plasmids pPM2179 or pPM2180. O = origin; U = unmodified chloramphenicol; M = monoacetylated chloramphenicol.

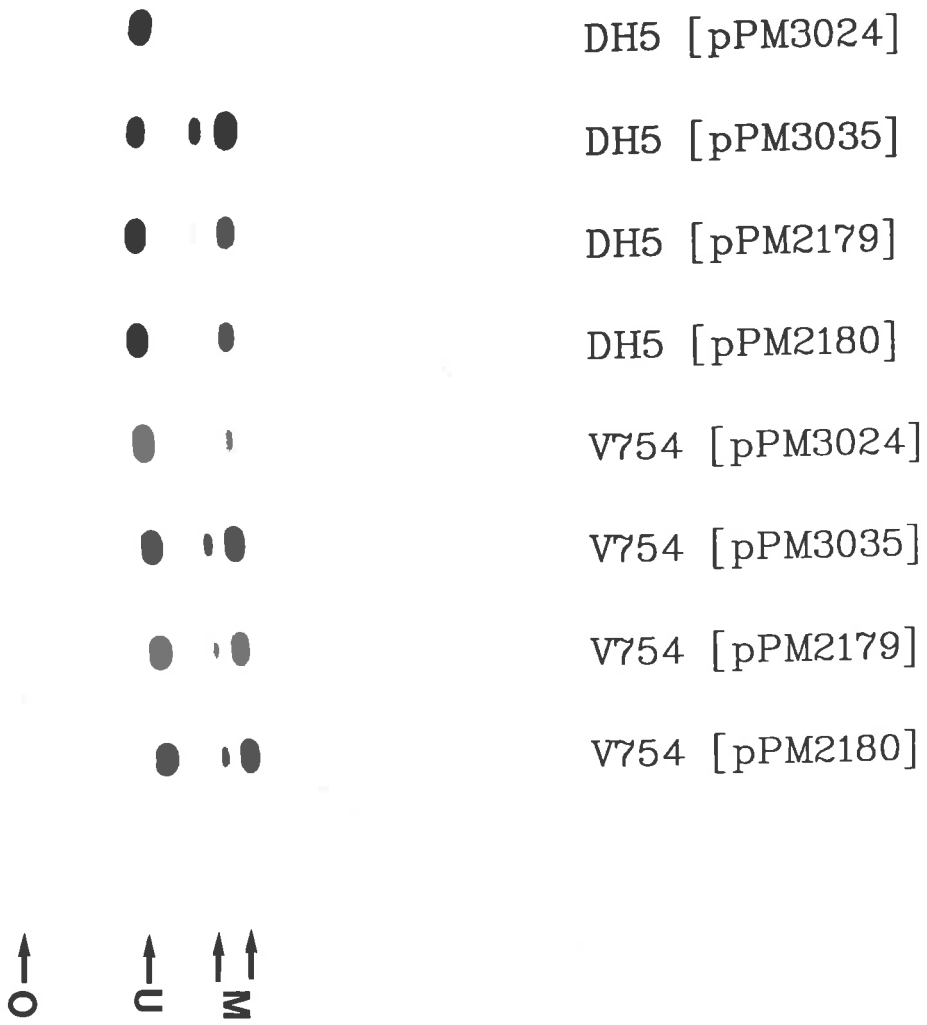
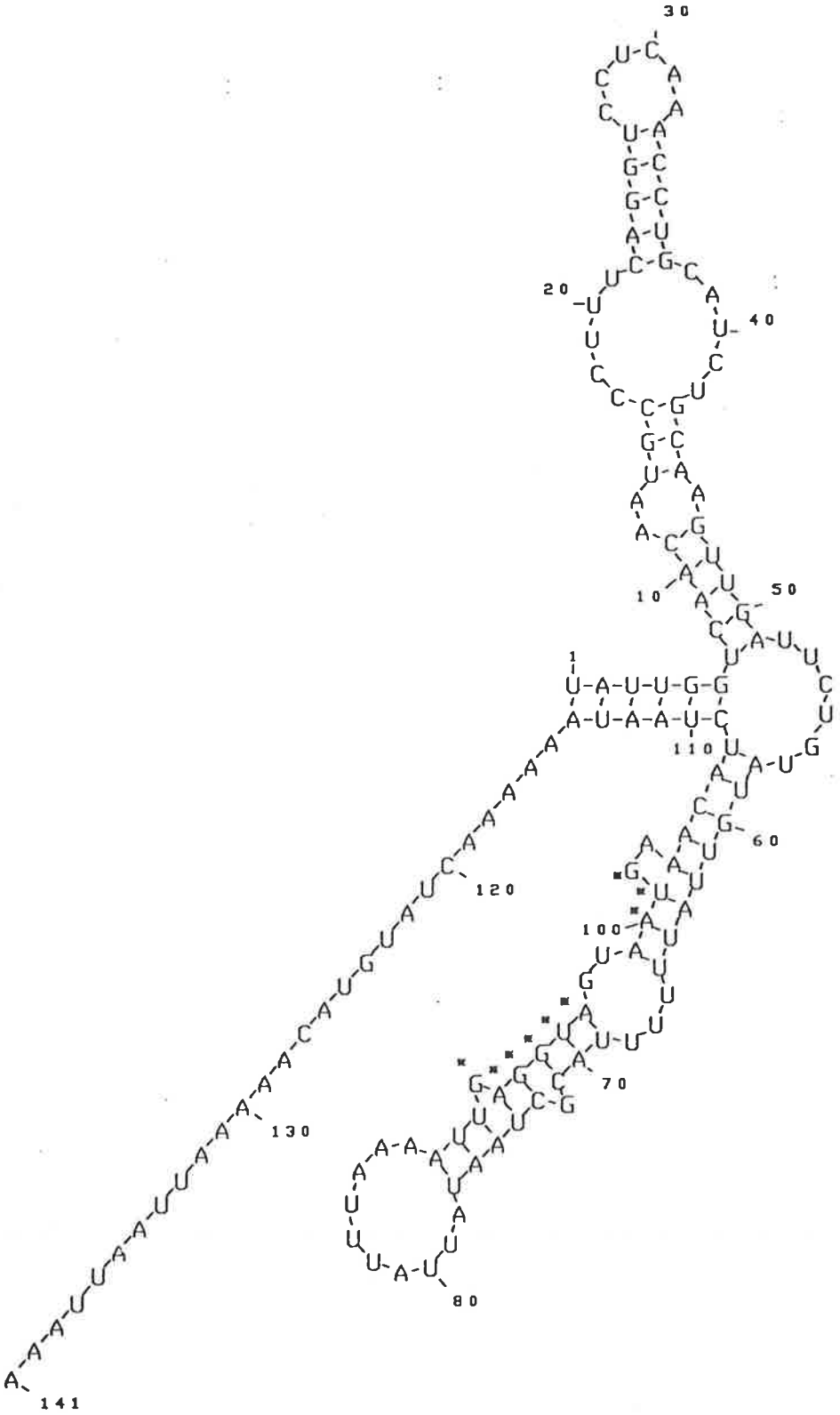

DH5 [pPM3024]
DH5 [pPM3035]
DH5 [pPM2179]
DH5 [pPM2180]
V754 [pPM3024]
V754 [pPM3035]
V754 [pPM2179]
V754 [pPM2180]
O
C
M

Figure 4.9: Predicted mRNA secondary structure of the 5' end of the *rfbT* mRNA

The 5' untranslated mRNA of *rfbT* is 99 nt in length. The ribosome binding site (RBS); GAGGUA and the AUG start/initiation codon are both indicated by asterisks (*). Both of these regions are involved in the proposed secondary structure of the mRNA . The free energy of this structure is -27.6 kcal/mol and was determined using DNASIS computer software (Salser, 1977).

RNA Secondary Structure

Free Energy −27.6kCal/Mol



The suggested secondary structure would reduce the level of translation and thus regulate the amount of RfbT produced, since the involvement of the RBS in secondary structure represents a major factor in ribosome accessability and thus translation efficiency (McCarthy *et al.*, 1985).

4.3 Summary and Conclusions

Using PCR clones of the *rfbT* gene it has been possible to show that Inaba strains appear to have random mutations within the *rfbT* gene leading to a non-functional truncated RfbT protein. There appears to be no correlation between the strains regardless of serotype or biotype. It was surprising to find that nt 17672 shows great variability although these changes do not always lead to a truncated product. Furthermore CA401 and Z17561 show the same sequence variations which is surprising since these strains have markedly different isolation dates. The *rfbT* gene is conserved in Ogawa strains and the only sequence difference detected led to a conserved amino acid substitution.

Sequence analysis has revealed a region in front of *rfbT* which shows strong homology with the *E. coli* σ^{70} consensus promoters. Primer extension using RNA from *V. cholerae* and *E. coli* has allowed the 5' end of the message to be mapped and the *rfbT* promoter to be identified. The presence of the promoter was confirmed by cloning the region prior to the mRNA start of *rfbT* into the promoter detection vector pPM3024. The vector pPM3035 with has the Km^R cartridge promoter cloned into it was used as a positive control promoter. The *rfbT* promoter clones, pPM2179 and pPM2180 which are both in the same orientation, appear to be weaker in activity in *E. coli* than the positive control pPM3035, although they have similar strength in *V. cholerae*. The *rfbT* mRNA has a long 5' tail which has the potential to form a strong secondary structure and which may have a marked influence on the translation of the *rfbT* message. The poor spacing of the -10 region

combined with the lack of a "-43 sequence" in the *rfbT* promoter suggests that it is not a strong promoter, which together with the weak activity detected in the CAT assays, may explain the inability to detect mRNA on a Northern blot. The spacing of the -10 region probably plays the greatest role in this low activity since the *hlyA* promoter is strongly transcribed and it also lacks an identifiable -43 sequence. Footprinting of the DNA using RNA polymerase can be used to confirm the presence of a promoter in front of *rfbT*. To determine if the message is degraded at the 5' end a phosphate exchange reaction could be done to determine if the terminal phosphate is stil present (Alifano *et al.*, 1992). The stability of the message is unknown and this also plays an important role in the ability to detect message in a Northern blot.

Chapter 5

Analysis of the expression of the A, B and C antigens of the O-antigen on *V. cholerae* O1 and *rfb* mutants

5.1 Introduction

The A, B and C antigens associated with the O1 serotype have been localized to the O-antigen of the LPS; but the exact chemical composition of these antigens is unknown. The expression of the three antigens is highly variable, in that Inaba strains express high levels of A and C antigen whereas Ogawa strains express A and B but express very little C. There also appears to be variation in the amount of B antigen expressed in different Ogawa strains (Sakazaki and Tamura, 1971). Using serotype converted strains, it should be possible to look at the expression of the B antigen on the cell surface This was done using two approaches; haemagglutination inhibition and direct immunogold electronmicroscopy. Both of these methods rely on the use of monoclonal antibodies which recognize the three antigens A, B and C. Furthermore using these approaches it should be possible to answer the question of whether or not the B antigen is a modified form of the C antigen or if the B antigen is independent of C.

Chemical fractionation of the LPS molecule and haemagglutination inhibition assays should help to more clearly define the epitopes presented by the three antigens, and may help in identifying the antigens themselves.

The use of western blots, also provides a means of looking at the expression of the B antigen and determining its association with a (definite) fraction of the LPS molecule.

This chapter also analyses the importance of LPS and serotype on virulence of *V. cholerae* O1. This was done using a mutant in *rfbT* which converts an Ogawa strain to the

Inaba form. The use of transposon mutants within the *rfb* operon were used to examine the role the LPS plays in virulence and the expression of various surface components.

5.2 Results

5.2.1 Construction of an *rfbT* mutant

5.2.1.1 Introduction of a Km^R cartridge into *rfbT*

The nucleotide sequence of the *rfbT* gene has been determined and, by complementation and mutation analysis, it has been shown to be required for the conversion of an Inaba strain expressing the A and C antigens to the Ogawa serotype, expressing A, B and C. However, it has not been shown that *rfbT* is necessary in an Ogawa strain for expression of the B antigen. Therefore, the Km^R cartridge from plasmid J225 was cloned into the *Spe*I site in *rfbT* to generate an *rfbT* mutant. Fig. 5.1 shows the stepwise construction of the plasmid pPM2139 containing the mutant *rfbT* gene.

5.2.1.2 Subcloning into plasmid pRK290

Plasmid pRK290 was developed by Ditta *et al.* (1980) and can be used as an efficient cloning and mobilizing vector, in a wide variety of Gram-negative bacteria. This plasmid contains two unique cloning sites, *Eco*RI and *Bgl*II, encodes Tc^R, and can be mobilized from *E. coli* K-12 strains SM10 or S17-1 which have the RP4 transfer genes integrated into the chromosome (Simon *et al.*, 1983). This facilitates the conjugation of pRK290 into *V. cholerae* in the same way as pPM2101 (see Section 3.2.1). The 4.1kb *Pst*I/*Bam*HI fragment

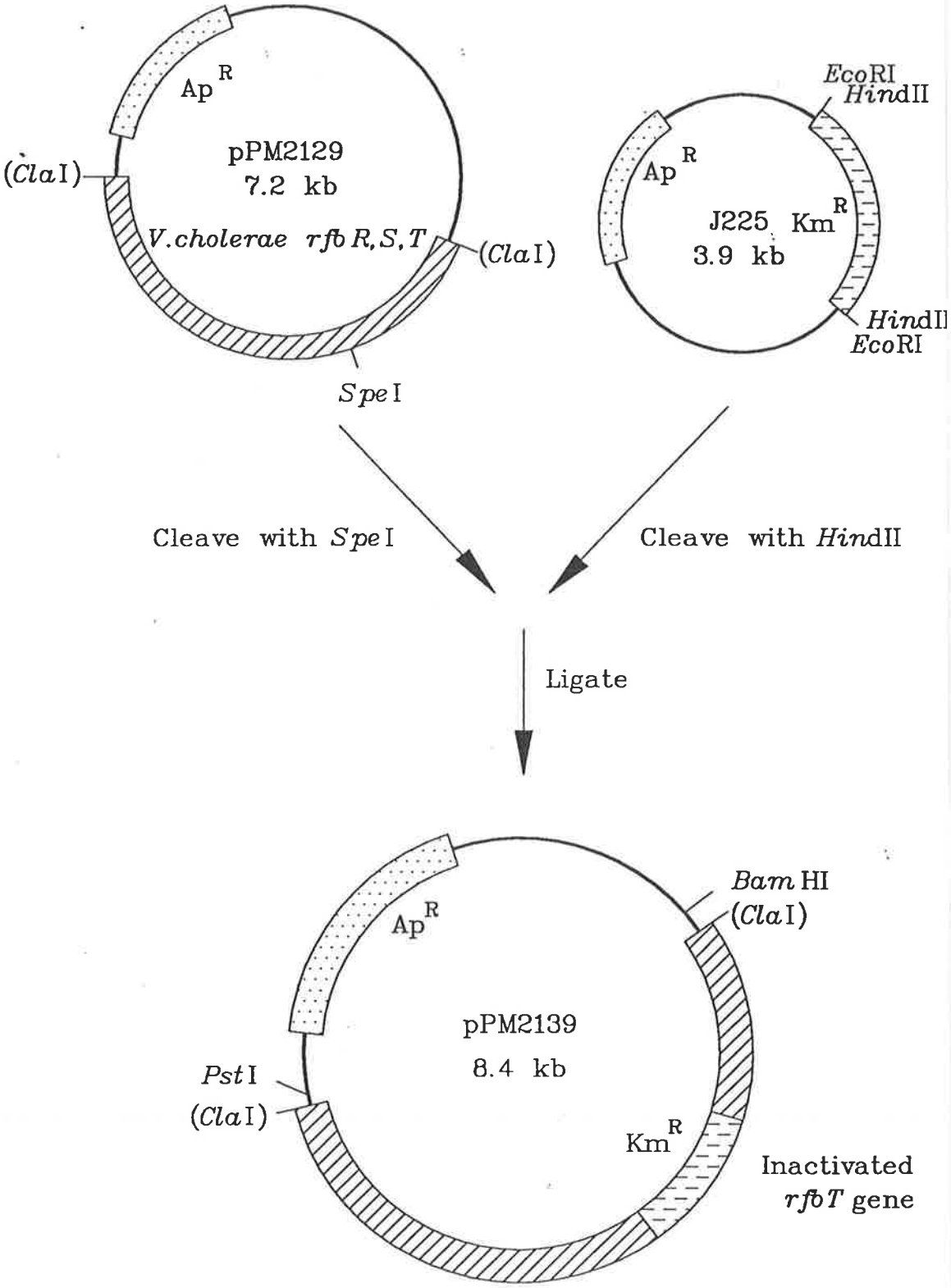

Ap R
pPM2129
7.2 kb
(ClaI)
V.cholerae rfb R,S,T
(ClaI)
SpeI
EcoRI
HindII
Ap R
J225 Km R
3.9 kb
HindII
EcoRI
Cleave with SpeI
Cleave with HindII
Ligate
Ap R
BamHI
(ClaI)
pPM2139
8.4 kb
PstI
(ClaI)
Km R
Inactivated
rfbT gene

Figure 5.1: Construction of plasmid pPM2139 containing an *rfbT*::Km^R mutation.

Plasmid pPM2129 was cleaved with *Spe*I to create a blunt end site into which the Km^R cartridge was cloned. Plasmid J225 was cleaved with *Hin*dII, allowing the purification of the Km^R cartridge from the Ap^R vector. Purified Km^R cartridge was ligated with *Spe*I cleaved pPM2129 to generate plasmid pPM2139.

from pPM2139 was cloned into the *Bgl*II site of pRK290 (Fig. 5.2). The resultant plasmid designated pPM2142 was selected using both Tc^R and Km^R.

5.2.1.3 Construction of *rfbT*::Km^R strains of *V. cholerae*

Plasmid pPM2142 was introduced into strain O17 by conjugation from S17-1 and transconjugants were selected as Tc^R, Km^R and Sm^R. Plasmid pPH1JI (Beringer *et al.*, 1978) was then introduced into these transconjugants by selection for Gm^R. Since both pRK290 and pPH1JI belong to the IncP incompatability group, these plasmids are unable to co-exist within the same cell. Therefore by selecting for Km^R Gm^R, and screening for Tc^S, cells can be isolated in which a reciprocal recombination event has occurred and pPM2142 has been expelled from the cell. Such a cell would now have the intact *rfbT* gene replaced with the defective *rfbT*::Km^R gene (Fig. 5.3). Cells with the Km^R Gm^R phenotype no longer behave like Ogawa cells in that they do not agglutinate with anti-Ogawa typing sera, indicating the loss of the B antigen (Table 5.1).

In order to assess the effects of the *rfbT* mutation on virulence it was necessary to remove the plasmid pPH1JI from the mutant strains, since it has been observed that this plasmid attenuates *V. cholerae* strains in an unknown manner (Alm *et al.*, 1991). The possibility that the introduction of pPH1JI alters the serotype of the strain was ruled out by slide agglutination. The removal of pPH1JI was achieved by incompatability, as it was for pPM2142 (section 5.2.6.2)(Fig. 5.3). In this case the IncP plasmid pME305 was introduced via conjugation. This plasmid has a temperature sensitive replicon (Rella *et al.*, 1985) and can only replicate at the permissive temperature of 30°C. Thus, once pPH1JI was displaced the strain could be grown at 42°C to cure it of pME305. This yielded strain V874 (Fig. 5.3), which could be complemented by the reintroduction of pPM2129, thus restoring the expression of the B antigen.

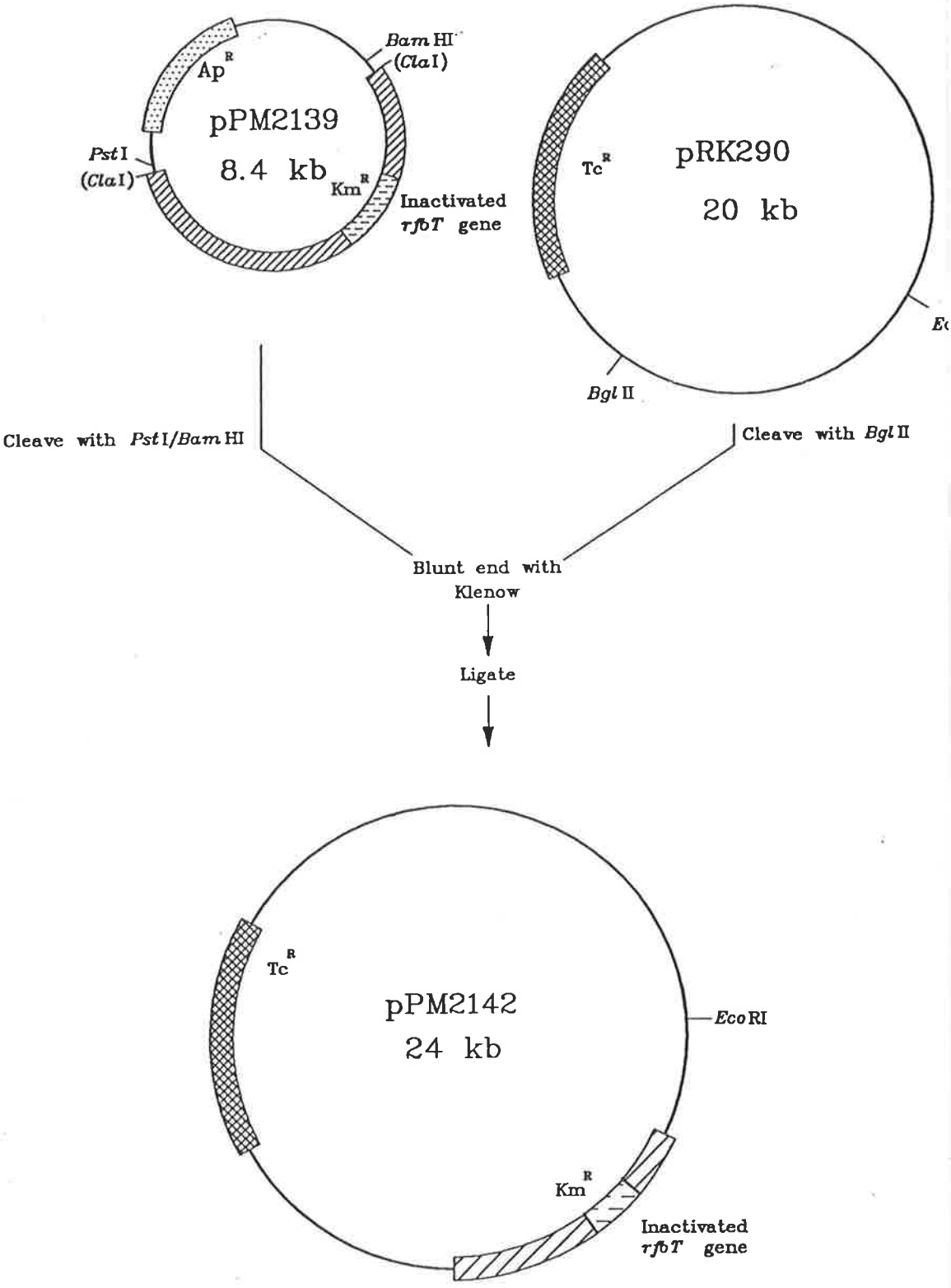

Bam HI
(Cla I)
Ap R
pPM2139
8.4 kb
Pst I
(Cla I)
Km R
Inactivated
rfbT gene
Tc R
pRK290
20 kb
Bgl II
Cleave with Pst I/Bam HI
Cleave with Bgl II
Blunt end with
Klenow
Ligate
Tc R
pPM2142
24 kb
Eco RI
Km R
Inactivated
rfbT gene

Figure 5.2: Construction of plasmid pPM2142.

Plasmid pPM2139 was cleaved with *Pst*I and *Bam*HI to generate a 4.1kb fragment containing the mutant *rfbT*::Km^R gene. The protruding ends were blunt ended with Klenow fragment of DNA polymerase I. The vector, pRK290, was cleaved with *Bgl*II and the protruding ends filled. The cleaved pRK290 and pPM2139 DNAs were ligated and transformed into strain S17-1 selecting for Tc^R and Km^R. The resultant plasmid was designated pPM2142.

Figure 5.3: Introduction of a *rfbT*::Km^R mutation into the chromosome of *V. cholerae*.

Plasmid pPM2142 (Tc^R, Km^R) was mobilized from *E. coli* strain S17-1 into *V. cholerae* O17. The resultant transconjugants were Sm^R, Tc^R and Km^R. Recombination between the chromosome and pPM2142 was allowed to occur. Plasmid pPH1JI (Gm^R), which also belongs to the IncP incompatability group, was introduced via conjugation into the *V. cholerae* strain containing pPM2142. Sm^R, Km^R, Gm^R colonies were then selected and screened for Tc^S . The *V. cholerae* strains in which the *rfbT*::Km^R mutation had recombined into the chromosome were isolated and analysed further using Southern hybridization (Fig. 5.4).

Plasmid pME305, a temperature sensitive replicon derivative of RP4 and incompatible with pPH1JI, was introduced into the *V. cholerae rfbT*::Km^R mutant strain by conjugation. The transconjugants were selected by Km^R, Tc^R and screened for Gm^S to ensure the loss of pPH1JI . The strains were then grown at 42°C to cure plasmid pME305 and to produce isogenic plasmid-free strains except for the *rfbT*::Km^R mutation. This strain was designated V874.

In the diagram B = *Bgl*II, S = *Spe*I and C = *Cla*I.

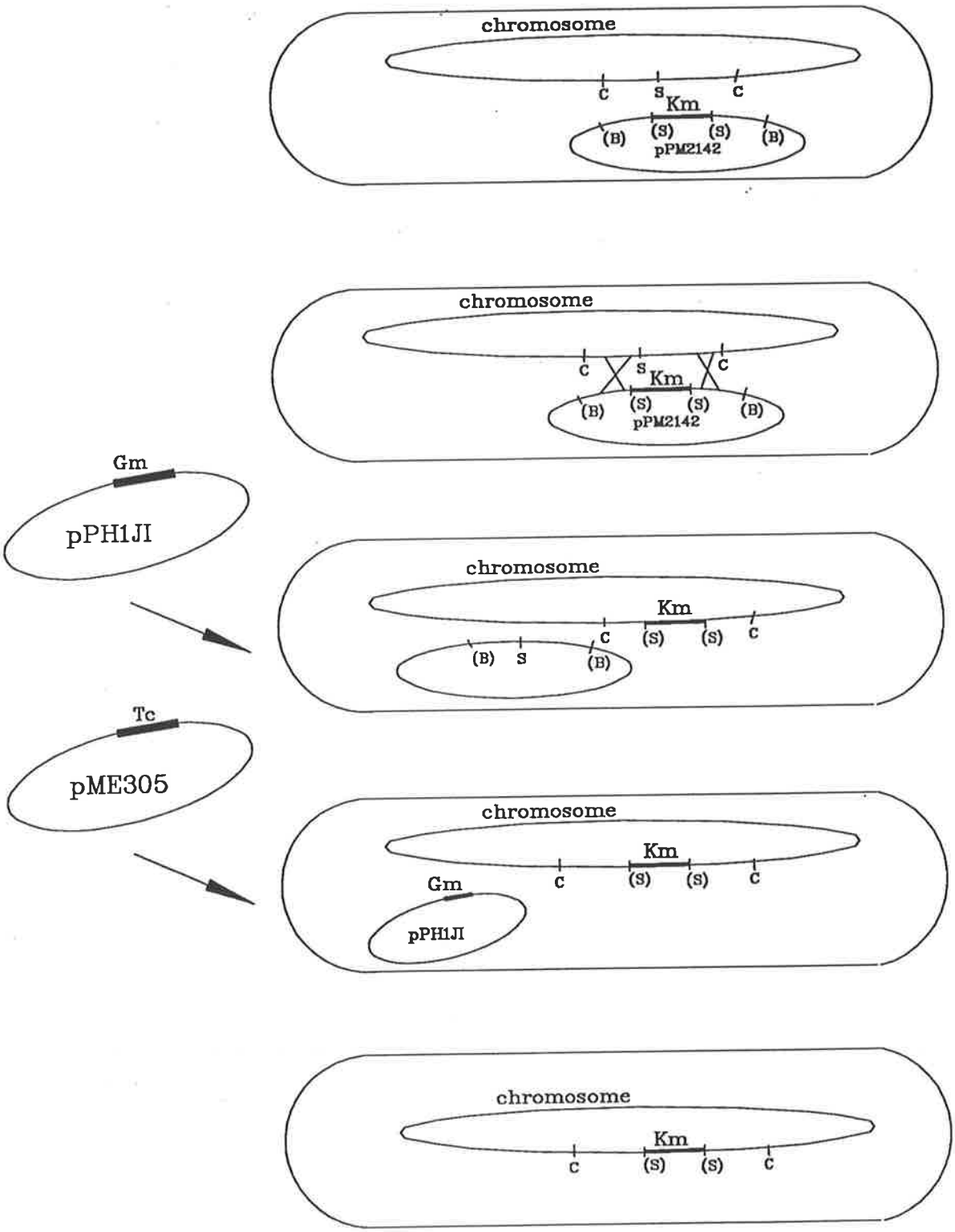

chromosome
C
S
C
Km
(B)
(S)
(S)
(B)
pPM2142
chromosome
C
S
Km
C
(B)
(S)
(S)
(B)
pPM2142
Gm
pPH1JI
chromosome
Km
C
(S)
(S)
C
(B)
S
(B)
Tc
pME305
chromosome
Km
C
(S)
(S)
C
Gm
pPH1JI
chromosome
Km
C
(S)
(S)
C

Table 5.1: Slide agglutination of serotype converted strains.

The strains were agglutinated with either anti Inaba or anti Ogawa typing sera (Wellcome). All strains were grown overnight on antibiotic containing nutrient agar plates, resuspended in saline and then agglutinated.

The plasmids used are pPM2101, pPM2122 and pPM2123 which are: -ve control, *rfbT* from Ogawa strain O17 and *rfbT* from Inaba strain 569B repectively.

Slide Agglutination		
Strain/ plasmid	anti-Inaba	anti-Ogawa
O17	-	+
O17 [pPM2101]	-	+
O17 [pPM2122]	-	+
O17 [pPM2123]	-	+
569B	+	-
569B [pPM2101]	+	-
569B [pPM2122]	-	+
569B [pPM2123]	+	-
BM69	+	-
BM69 [pPM2101]	+	-
BM69 [pPM2122]	-	+
BM69 [pPM2123]	+	-
CA411	-	+
CA411 [pPM2101]	-	+
CA411 [pPM2122]	-	+
CA411 [pPM2123]	-	+
V874	+	-
V874 [pPM2122]	-	+

To confirm that the *rfbT*::Km^R mutation had been recombined into the chromosome of *V. cholerae*, genomic DNA of Gm^R, Km^R, Tc^S cells was extracted and digested with the restriction endonuclease *Eco*RI. An oligo which binds within the *rfbT* gene (#196 :5' TTTCGAAGTTTATGAGTT 3') was labelled with digoxigenin-11-dUTP using terminal transferase and used as a probe in Southern hybridization. Since the Km^R cartridge contains no sites for *Eco*RI, the fragment expected in the *V.*
cholerae chromosome with the recombined insertion should be 1.1kb larger i.e. an increase in fragment size from 5.4 kb to 6.5 kb. This was confirmed by the Southern hybridization (Fig. 5.4)

5.2.2 Immunogold Electron-microscopic Studies on Serotype Converted Strains

Using monoclonal antibodies and immunogold electronmicroscopy it is possible to directly quantitate the B and C antigens on the cell surface. Prior to immunogold and HIA studies all strains containing pPM2101, pPM2122 and pPM2123 were tested by slide agglutination using typing sera (Table 5.1). The Inaba strains 569B and BM69 altered in their agglutination pattern only when carrying pPM2122. All other plasmids had no effect on the serotyping of Inaba or Ogawa strains. The O17 *rfbT* mutant (V874) could only be agglutinated with anti-Inaba sera, but the introduction of plasmid pPM2122 complemented the *rfbT* mutant which led to the same agglutination pattern as the O17 parent (Table 5.1).

The monoclonal antibodies, H8 and 13B, corresponding to anti-B and anti-C respectively, were used on 17 strains (see Table 5.2) for the quantitation of the relative levels of the various antigens using immunogold electronmicroscopy. The number of gold particles in a given area were counted on four different cells and the average calculated (Table 5.2). In 569B although the level of B expressed is significantly increased when pPM2122 is present the level of C expressed on the cell surface does not alter significantly when taking the

Figure 5.4: Southern hybridization analysis of *rfbT*::Km^R mutant, V874.

Genomic DNA of *V. cholerae* strains O17, 569B and V874 was digested with the restriction endonuclease *Eco*RI and electrophoresed on a 0.8% agarose gel. After transfer to nitrocellulose, the filter was probed with an digoxigenin-11-d-UTP 3' end labelled oligonucleotide #196 (5'TTTCGAAGTTTATGAGTT3'). The 5.4 kb *Eco*RI fragment harbouring the *rfbT* locus present in O17 and 569B is absent in V874. A new 6.5 kb *Eco*RI fragment containing the Km^R cartridge was detected. The change in fragment size is indicated by arrows. SPP1 phage DNA cleaved with *Eco*RI was used as a size standard on the ethidium bromide stained agarose gel (not shown). Size of SPP1 *Eco*RI fragments in kb; 8.37, 7.2, 6.05, 4.9, 3.55, 2.63, 1.73, 1.61, 1.29. 1.19, .99, .86, .63, .48, .38 .

O17
569B
V874
6.5 kb
5.4 kb

Table 5.2: Immunogold electronmicroscopic quantitation of B and C antigens by Immunogold electronmicroscopy.

Quantitation of the B and C antigens was undertaken using monoclonal antibodies which recognize the B and C antigens. The number of gold particles bound were counted in a given area on the cell surface and are given in the table. This was done on four individual cells per group and the average taken after subtraction of the background. All strains were grown overnight on antibiotic containing nutrient agar plates. The same antibodies were used for the HIA assays and in the determination of antigens on degraded LPS. Strains O17 and CA401 are Ogawa, 569B and BM69 are Inaba.

Immunogold Electron Microscopy		
Strain/ plasmid	anti-B	anti-C
O17	54.5 ± 13	12.2 ± 9
O17 [pPM2101]	57.0 ± 10.5	14.0 ± 7.5
O17 [pPM2122]	63.0 ± 8.7	15.7 ± 8
O17 [pPM2123]	43.3 ± 13.7	11.2 ± 6.4
569B	2.0 ± 1	84.5 ± 18
569B [pPM2101]	1.5 ± 2	97.3 ± 20.7
569B [pPM2122]	35 ± 5.8	66.0 ± 16.3
569B [pPM2123]	1.0 ± 1.5	64.5 ± 8.1
BM69	3.0 ± 2	64.3 ± 11.3
BM69 [pPM2101]	3.7 ± 2.5	63.5 ± 12.4
BM69 [pPM2122]	136 ± 29.3	73.5 ± 14
BM69 [pPM2123]	2.9 ± 3.4	75.0 ± 11.8
CA401	104 ± 19.1	6.0 ± 4
CA401 [pPM2101]	135 ± 25.9	10.0 ± 6.5
CA401 [pPM2122]	170 ± 56.6	12.0 ± 2
CA401 [pPM2123]	86.5 ± 14.4	8.0 ± 9
V874	5.0 ± 3.7	95.0 ± 20.3

standard deviation into account i.e. does not appear to be a simple conversion from C to B. The same result is seen with BM69. In the serotype converted strains the level of the C antigen does not vary significantly regardless of whether the Inaba or Ogawa *rfbT* gene is introduced. However in the case of the *rfbT* mutant (V874) the level of antigen C is increased in the absence of the B antigen compared to the O17 parent which shows low levels of C antigen. A possible reason for the apparent lack of variability of the C antigen expression upon introduction of the cloned *rfbT* gene may have to do with the plasmid instability. An experiment determining plasmid stability of pPM2122 has shown that after overnight incubation 40% of the cells have lost the plasmid and subsequently only 60% of the cells are actively producing the B antigen. This is confirmed by the presence of a few cells which appear to lack the B antigen even if they are supposed to carry pPM2122.

The *rfbT* mutant represents a true serotype converted strain since the mutation acts like the wild type *rfbT* gene from Inaba strains. In the mutant there is only a single copy per cell integrated into the chromosome and is thus, under the same control as in an Inaba strain. The mutant represents a serotype converted strain not from Inaba to Ogawa but from Ogawa to Inaba and allows a more accurate analysis of antigen expression and suggests that the B and C antigens may not be independent. Thus the ideal serotype converted strain would contain a chromosomally integrated *rfbT* gene from O17 in 569B to convert from Inaba to Ogawa.

Figure 5.5 shows a number of serotype converted strains probed with monoclonal antibodies to the B and C antigens.

5.2.3 Haemagglutination Inhibition Assay using LPS from Serotype Converted Strains

LPS was extracted from derivatives of four strains, either the wild type strain, or harbouring one of the plasmids pPM2101, pPM2122 or pPM2123. The LPS was used in

Figure 5.5a: Immunogold electronmicrographs.

Electronmicrographs showing binding of monoclonal antibodies to the B and C antigen of *V. cholerae* LPS. Antibodies were used at 1 in 50 dilution, the gold particles were 10nm in diameter. The black bars represents 330nm.

A. 569B α-C

B. 569B[pPM2101] α-C

C. 569B[pPM2122] α-C

D. 569B[pPM2123] α-C

E. 569B α-B

F. 569B[pPM2101] α-B

G. 569B[pPM2122] α-B

H. 569B[pPM2123] α-B

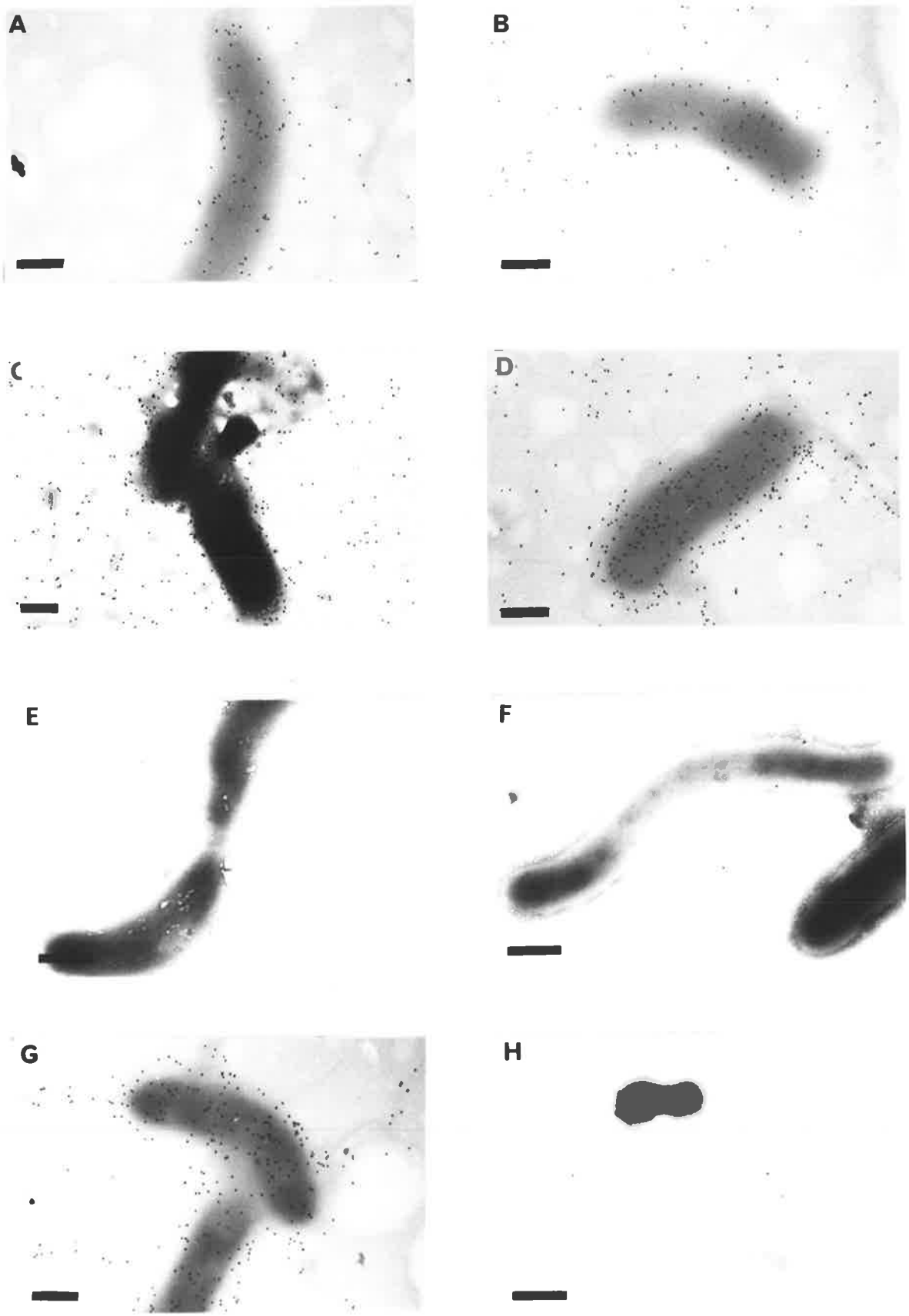

A
B
C
D
E
F
G
H

Figure 5.5b: Immunogold electronmicrographs.

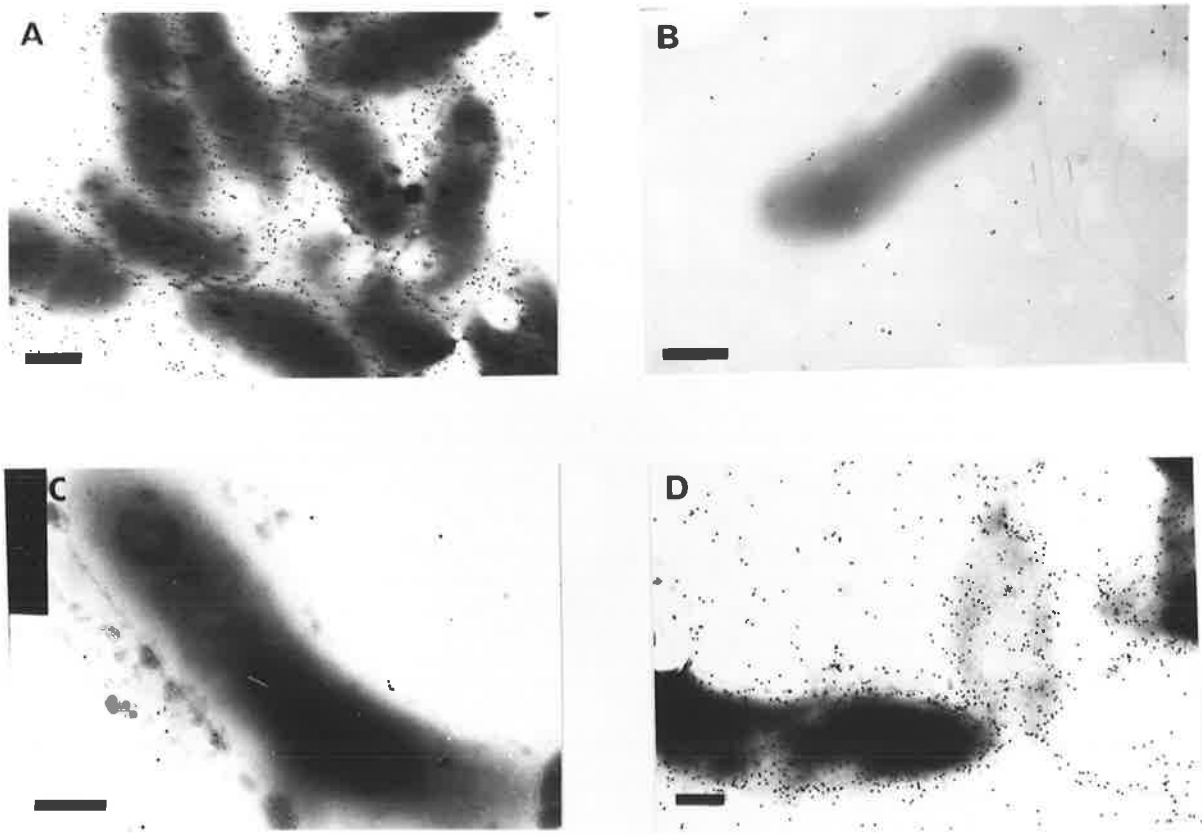
Electronmicrographs showing binding of monoclonal antibodies to the B and C antigens of *V. cholerae* LPS. Antibodies were used at 1 in 50 dilution. The gold particles were 10nm in diameter. The black bars represents 330nm.

A. O17 α-B

B. O17 α-C

C. V874 α-B

D. V874 α-C


A
B
C
D

haemagglutination inhibition assays (HIA) to determine the minimal inhibitory concentration of LPS for a given monoclonal antibody. Three monoclonal antibodies 20B, H8 and 13B, were used and recognize the antigens A, B and C, respectively.

These results indicate that the A antigen is invariable and completely independent of the B and C antigens. The C antigen also appears not to be affected by the introduction of either pPM2101, pPM2122 or pPM2123. The expression of the C antigen on Ogawa strains is approximately one-fifth of the expression on Inaba strains by immunogold, this result is not confirmed by HIA where there appears to be only a two fold difference. This may be due to several factors such as the inaccuracy of HIA using two-fold dilutions and also that, in the HIA, purified LPS was used and not whole cells.. The level of the B antigen is invariable in Ogawa strains and is dependent upon the introduction of pPM2122 into Inaba strains. The results are summarized in Table 5.3 and closely resemble those of the immunogold electronmicroscopy. The antisera were diluted two-fold across the haemagglutination trays. The strains used O17, CA411, 569B and BM69, were two Ogawa and two Inaba isolates. These results do not give a clear idea of whether or not the C antigen is converted to B. This is possibly due to a number of factors such as the dilution factor and that the plasmid pPM2122 is unstable in *V. cholerae* (section 5.2.2). Thus, the lower levels of C antigen on Ogawa cells does not appear to be due to conversion to B. From the HIA the expression of C remains unchanged indicating that B is independent of C.

5.2.4 SDS-Page Analysis of Serotype Converted Strains

The LPS from the four different sets of strains was subjected to SDS-PAGE and subsequently visualised using the silver staining procedure (Fig 5.6). The gel used was a 7.5-25% hyperbolic gradient gel with twice the normal concentration of SDS. It was found that this concentration of SDS gives better resolution for *V. cholerae* LPS molecules with O-

Table 5.3: Haemagglutination inhibitions for quantitation of antigens.

Strain [b]	Plasmid	Haemagglutination inhibition[a] with Monoclonal antibody		
		α-A	α-B	α-C
569B (I)	no plasmid	0.156	-[c]	0.078
	pPM2101	0.156	-	0.078
	pPM2122	0.078	0.018	0.078
	pPM2123	0.078	-	0.078
CA411 (O)	no plasmid	0.039	0.009	0.039
	pPM2101	0.039	0.005	0.039
	pPM2122	0.039	0.009	0.039
	pPM2123	0.078	0.005	0.039
O17 (O)	no plasmid	0.039	0.018	0.078
	pPM2101	0.156	0.018	0.078
	pPM2122	0.078	0.018	0.078
	pPM2123	0.156	0.009	0.078
BM69 (I)	no plasmid	0.078	-	0.078
	pPM2101	0.156	-	0.078
	pPM2122	0.078	0.039	0.078
	pPM2123	0.156	-	0.078

HIA was carried out using two fold serial dilution of the purified LPS antigens and monoclonal antibodies to the A,B and C antigens.

a Haemagglutination inhibition is measured as the lowest amount of LPS (μg) required to inhibit the agglutination of sheep erythrocytes coated with O162 (Ogawa) LPS. LPS used was purified from 100ml cultures

b The serotype of the strains are indicated; I, Inaba; O, Ogawa.

c -, indicates no inhibition was observed implying the absence of the antigen corresponding to the particular antibody.

Figure 5.6: Silver stain of serotype-converted strains.

SDS-PAGE analysis of whole cell lysates from O17, 569B, BM69 and CA411 containing either no plasmid, pPM2101, pPM2122 or pPM2123 and strain V874. The 7.5-20% gel was silver stained to show the LPS. Cell lysates were prepared from 1ml cultures containing $2x10^9$ cells/ml. Cells were treated with Proteinase K in 100µl of lysing buffer. 5µl samples were loaded.

O-antigen +
LipidA/Core
569B
569B [pPM2101]
569B [pPM2123]
569B [pPM2122]
CA411
CA411 [pPM2101]
CA411 [pPM2123]
CA411 [pPM2122]
017
017 [pPM2101]
017 [pPM2123]
017 [pPM2122]
V874
BM69
BM69 [pPM2101]
BM69 [pPM2123]
BM69 [pPM2122]

antigen attached. No detectable difference in the banding pattern of the complete LPS molecules (O-antigen plus core plus lipid A) was detected in the strains regardless of serotype.

The association of the B antigen with the LPS was demonstrated using SDS-PAGE on a 15% polyacrylamide gel and western blotting (Fig 5.7). A polyclonal antiserum raised to O17 and extensively absorbed with 569B to remove any antibodies to the C and A antigens was used in the western blot. All the Ogawa strains (O17 and CA411) and the Inaba strains which had been converted to Ogawa (569B[pPM2122]and BM69[pPM2122]) expressed the B antigen on their O-antigen. The *rfbT* mutant, V874, shows no B antigen associated with any portion of the LPS. This confirms the slide agglutination and immunogold electronmicroscopic results section 5.2.2 and 5.2.3 (Table 5.1, 5.2). No B antigen could be detected associated with the core-lipid A region of the LPS. The lower levels of staining of the serotype converted strains is possibly due in part to plasmid instability in that not all of the cells contain pPM2122 (section 5.2.1), and that in the HIA and immunogold labelling monoclonal antiserum was used and not the absorbed polyclonal sera. The B antigen is thus diluted on the cell surface after each cell division in those cells not harbouring the plasmid.

5.2.5 Expression of the Antigens A, B and C on Chemically Degraded LPS

Acetone dried cells were prepared from five strains (O17, 569B, BM69[pPM2101], BM69[pPM2122] and BM69[pPM2123]) and were sent to Dr. J. Redmond (Macquarie University, Sydney). The LPS was extracted and subjected to various chemical treatments. Samples of this degraded LPS were then analysed by haemagglutination inhibition for the presence of the three antigens, A, B and C, this was done so that any reactive material could be further analysed by Nuclear Magnetic Resonance (NMR) spectroscopy.

Figure 5.7: Western blot analysis of serotype converted *V. cholerae* strains.

Panel A:

Cell lysates of *V. cholerae* strains O17, 569B, BM69 and CA411 containing either no plasmid, pPM2101,pPM2122 or pPM2123 were run in SDS on a 15% polyacrylamide gel and transferred via electroblotting to nitrocellulose. Using a serum raised to O17 and absorbed with 569B. The B antigen can be detected exclusively in the O-antigen fraction of the LPS.

Panel B:

SDS-PAGE analysis of whole cell lysates from O17, 569B, BM69 and CA411 containing either no plasmid, pPM2101, pPM2122 or pPM2123 and strain V874. The 15% polyacrylamide gel was silver stained to show the LPS. Cell lysates were prepared from 1 ml cultures containing $2x10^9$ cells/ml. Cells were treated with proteinase K in 100µl of lysing buffer. 5µl samples were loaded.

LipidA/Core
O-antigen +
LipidA/Core
BM69
BM69 [pPM2101]
BM69 [pPM2123]
BM69 [pPM2122]
017
017 [pPM2101]
017 [pPM2123]
017 [pPM2122]
CA411
CA411 [pPM2101]
CA411 [pPM2123]
CA411 [pPM2122]
569B
569B [pPM2101]
569B [pPM2123]
569B [pPM2122]
V874

LipidA/Core
O-antigen +
LipidA/Core
BM69
BM69 [pPM2101]
BM69 [pPM2123]
BM69 [pPM2122]
017
017 [pPM2101]
017 [pPM2123]
017 [pPM2122]
CA411
CA411 [pPM2101]
CA411 [pPM2123]
CA411 [pPM2122]
V874
569B
569B [pPM2101]
569B [pPM2123]
569B [pPM2122]

Of the samples tested, only the hydrazine treated reacetylated LPS showed any inhibition indicating the presence of the A, B and C antigens (Table 5.4) although the levels of the three antigens was reduced by 1000 fold. The alternative treatment, hydrazine treated, reacetylated, 1% formic acid for 1 hr at 100^{o}C appeared to destroy the C and B antigens, although it left a small amount of the A antigen intact. Thus, it apppears that the A antigen is more stable than either B or C. The level of the A antigen is very low compared to the phenol water extracted LPS. The A antigen inhibits to 3mg whereas the control LPS inhibits to 0.03mg. Other treatments such as periodate oxidation, and acid and base hydrolysis also destroy the antigens. This would indicate that the antigens are labile and are readily lost during treatment of the LPS. Therefore less aggresive degradation protocols are required to be able to identify the three antigens such as bacteriophage degradation of the LPS.

5.2.6 Virulence of *V. cholerae rfb* mutants

5.2.6.1 Virulence of *V. cholerae* mutants lacking the O-antigen

A number of CP-T1 phage-resistant transposon-insertion mutants of strain 569B have been isolated (Table 5.5) (Ward and Manning, 1989). These mutants are rough i.e. lack the O-antigen, which is not surprising since CP-T1 uses the O-antigen as its receptor (Guidolin and Manning, 1986). These mutants have transposons inserted within the genes for perosamine biosynthesis and are thus defined *rfb* mutants.

The effect of a loss of the O-antigen on the virulence of *V. cholerae* was examined in the infant mouse cholera model. The mutant strains were markedly attenuated, with mice readily surviving doses approximately 50 to 100 fold higher than the LD_{50} of the smooth control strains (Table 5.5). The loss of the O-antigen also affects two surface associated functions involved in virulence; adherence and motility (Freter and O'Brien, 1981; Attridge and Rowley 1983; Taylor *et al.*, 1987; Sharma *et al.*, 1989). Electronmicroscopy was used to

Table 5.4: Haemagglutination inhibition assays on chemically degraded LPS.

The LPS used was water/phenol extracted from acetone dried cells and then chemically degraded by hydrazine. Initial concentration of LPS was 200mg/ml. The monoclonal antibodies used recognize the three antigens A, B and C. The concentrations given in the Table are final levels of inhibition by LPS in mg/ml. The control LPS O162 and 569B is from water/phenol extracted cells without hydrazine treatment. N/D indicates no detectable inhibition at 200mg/ml. Monoclonal antibodies used were 20B αA, H8 αB and 13B αC.

HAI assay of hydrazine treated reacetylated LPS			
Strain/ LPS	anti-A	anti-B	anti-C
O17	1.50	2.30	18.75
569B	0.35	N/D	4.70
BM69 [pPM2101]	0.75	N/D	9.40
BM69 [pPM2122]	0.35	2.30	2.30
BM69 [pPM2123]	0.75	N/D	18.75
O162 LPS	5×10^{-4}	1×10^{-3}	1.7×10^{-2}
569B LPS	1×10^{-3}	N/D	7.5×10^{-4}

Table 5.5: Virulence of 569B and its *rfb* mutants in the infant mouse cholera model.

Strain	Mutation	Survival[a]	
		Dose	
		10^7	10^8
569B	*rfb*$^+$	1	0
V661	*rfb*-2	8	3
V663	*rfb*-6	7	3
V665	*rfb*-7	8	8
V669	*rfb*-9	8	8
V671	*rfb*-10	8	7

Survival[a] is measured as the number of mice from a group of eight that were still alive at 48hr after receiving the challenge dose

One experiment (of three), in which some of the mice died from a challenge with the *rfb* mutants is shown. The LD_{50} of 569B for infant mice was $5x10^5$ bacteria. The results were consistent across all three experiments.

examine both toxin co-regulated pilus (TCP) and the flagella. Since these two structures are known to be responsible for adherence and motility respectively. No TCP was detected on the surface of cells of strain 569B mutants grown in CFA broth. Examination of the flagella revealed both sheathed and what appeared to be unsheathed flagella; no other differences could be detected between rough and smooth strains. Motility studies using soft agar overlay revealed that the O-antigen mutants showed reduced zones of swarming (Fig 5.8), indicative of reduced motility, i.e. flagellar function.

Analysis of the cell envelope of the *rfb* mutants by SDS-PAGE revealed an altered protein profile in which one protein reduced and another markedly increased. This is consistent amongst the mutants (Fig 5.9). An altered protein profile has been observed in other bacteria with LPS defects. One possible explanation is that there is a co-requirement for intact LPS for the proteins to be correctly incorporated into the outer membrane (Hancock and Reeves, 1975; Ames *et al.*, 1974; Lugtenberg *et al.*, 1976).

Thus, *V. cholerae* strains lacking O-antigen do not appear to be able to assemble pili (TCP) on the cell surface, furthermore, a large amount of the major structural subunit, TcpA, accumulates in the periplasm of these mutants (data not shown). It is thus reasonable to infer that complete LPS is required for fully functional flagella and the assembly of TCP.

5.2.6.2 Effect of the *rfbT*::Km^R mutation on virulence in the infant mouse cholera model

The *V. cholerae* strain O17 and its *rfbT* mutant (V874) were assessed for virulence in the infant mouse cholera model. The infant mice were innoculated intra-gastrically with various doses of bacteria and were subsequently checked at regular intervals over a period of 48 hr for their state of health and survival (Fig. 5.10). Two strains of mice were used due to a change in the availability of LacA mice. The Swiss mouse strain used was extensively tested and gave the same virulence pattern in the infant mouse cholera model. *V. cholerae* O17 and

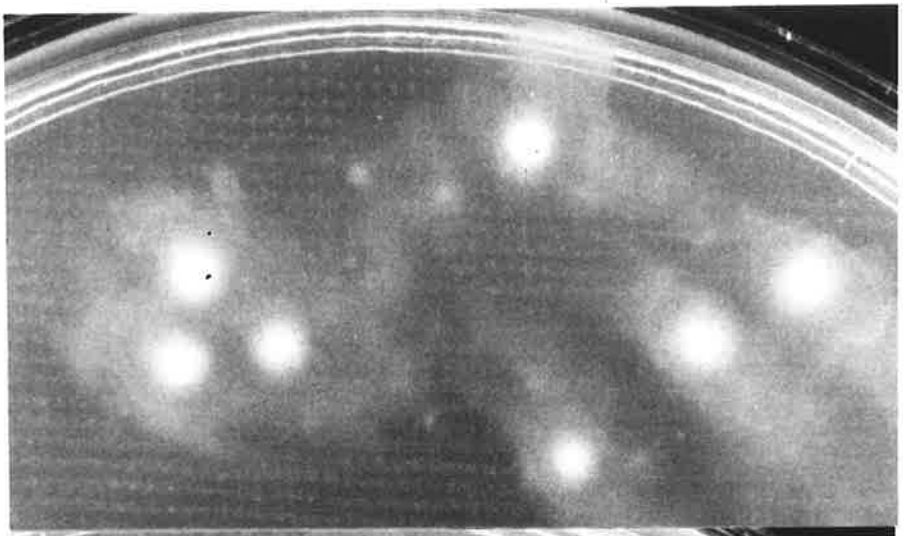
Figure 5.8: Motility of *V. cholerae rfb* mutants in soft agar.

Strains 569B and V663 (569B *rfb*-6) were plated out at a density to give 100-300 colonies per plate. The colonies were overlaid with 0.3% nutrient agar and incubated for 2-3 hr at 37°C. Motility is detected by the diffuse zone around the colonies.

A. 569B

B. V663

A



B

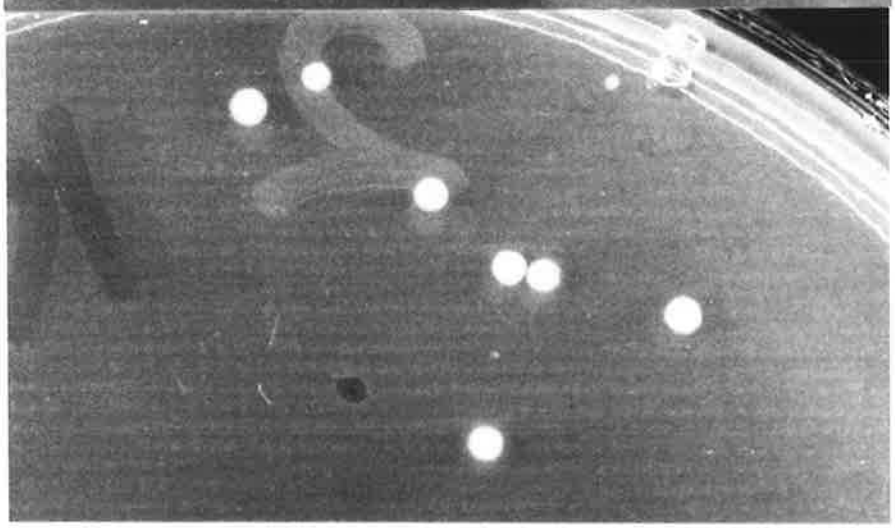
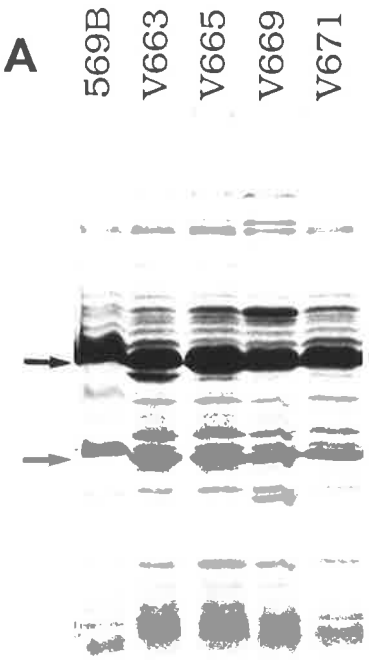


Figure 5.9: Analysis of the cell envelope of *V. cholerae rfb* mutants.

Cell envelopes of strain 569B and its *rfb* mutants were run in SDS on a 15% polyacrylamide gel. Panel A shows the protein profiles after staining with Coomassie blue G-250. A number of the major membrane proteins alter in their expression, these are indicated by the arrows. Panel B is SDS-PAGE analysis of whole cell lysates from 569B and the *rfb* mutants. The mutants lack the O-antigen/lipidA-core region. The 15% polyacrylamide gel was silver stained to detect the LPS. Cell lysates were prepared from 1ml cultures containing $2x10^9$ cells/ml. Cells were treated with proteinase K in 100µl of lysing buffer. 5µl samples were loaded.


A
569B
V663
V665
V669
V671


B
569B
V663
V665
V669
V671

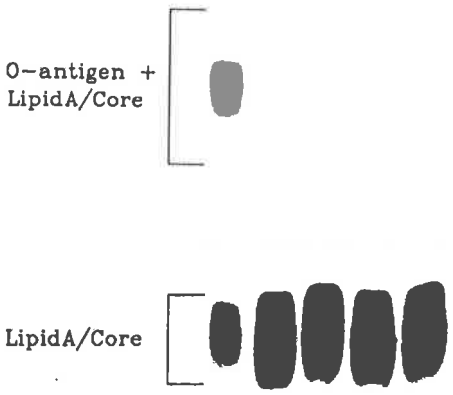

O-antigen +
LipidA/Core
LipidA/Core

Figure 5.10: Effect of the *rfbT*::Km^R mutation on virulence in the infant mouse cholera model.

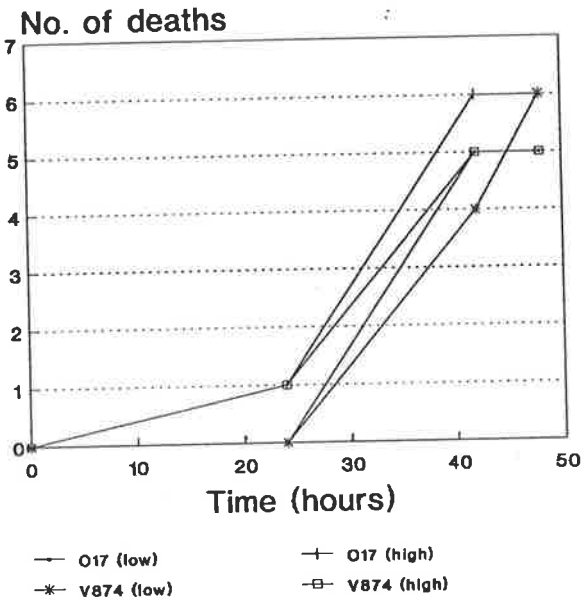
The *V. cholerae* strain O17 and its mutant V874 were administered intragastrically to infant mice. The number of deaths at each time point was recorded. Six mice were used for each strain.

Panel A is for LacA mice given either low ($1x10^6$) or high ($1x10^7$) doses of organisms.

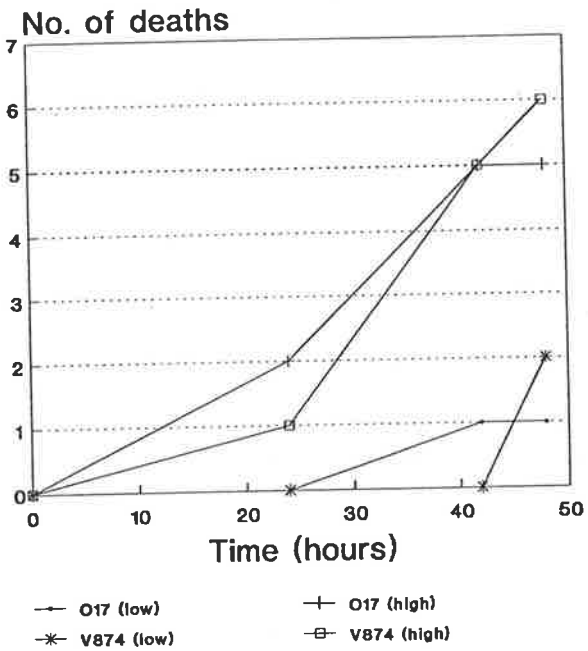
Panel B is for Swiss mice given either low ($1x10^4$) or high ($1x10^5$) doses of organisms.

The LD50 for strain O17 was $1x10^5$ in LacA and $5x10^4$ in Swiss mice.

LacA


No. of deaths
7
6
5
4
3
2
1
0
0
10
20
30
40
50
Time (hours)
O17 (low)
V874 (low)
O17 (high)
V874 (high)

Swiss


No. of deaths
7
6
5
4
3
2
1
0
0
10
20
30
40
50
Time (hours)
O17 (low)
V874 (low)
O17 (high)
V874 (high)

V874 showed no significant difference in either the rate of death of the infant mice or the overall death rate after 48 hr. Since O17 and V874 can be considered as isogenic Ogawa and Inaba strains, respectively, this indicates that the serotype i.e. the expression of the B antigen on the lipopolysaccharide does not affect its virulence. This is the first time isogenic strains have been used in a study of the virulence of Ogawa versus Inaba. This result however, does not rule out the possibility that a change in serotype is important for evading the immune response of the host.

5.3 Summary and conclusions

Inaba strains, such as 569B and BM69, are readily serotype-converted by the introduction of the *rfbT* gene from an Ogawa strain. Using NMR and HIA on chemically modified LPS and silver staining to check for any alteration in the mobility of the LPS it was hoped that the B antigen may be structurally or chemically identified. This has not been possible since the three antigens A,B and C appear to be very labile and are destroyed by most chemical treatments. Using HIA assays and immunogold electronmicroscopy it has been shown that the three antigens are surface-associated and that the expression of these antigens appears to be independent. However expression of the C antigen on the *rfbT* mutant, V874, increased markedly compared with the O17 parent. These data suggest that the expression of B and C are linked, and that C is converted to B or masked by the B antigen. The serotype-converted strains 569B shows lower expression of the B antigen compared to wild type Ogawa strains in both HIA, western blots and IEM. This is probably due to plasmid loss. The serotype converted BM69 only shows lower levels of the B antigen in the western blot. The strains for the silverstaining and western blot were grown in broth and not on plates which may contribute to plasmid loss.

Virulence studies using the infant mouse cholera model, showed that the presence of the B antigen is unimportant for pathogenesis *per se*. Its involvement in avoiding the

immune response of the host, however, cannot be ruled out from these experiments. Other mutants in the *rfb* operon which affect O-antigen biosynthesis have a marked effect on the organism by reducing its virulence. This may be due in part to the reduced motility and the lack of TCP on *rfb* mutants since both are known virulence factors. Furthermore rough strains lacking the O-antigen are more susceptible to non-specific host defense mechanisms such as bile salts and pH.

Chapter 6

Characterization of the RfbT protein

6.1 Introduction

The genetic locus determining the Ogawa serotype specificity in *V. cholerae* has been cloned and sequenced. An open reading frame encoding the RfbT protein of approximately 32-34kDa has been shown to be responsible for this activity.

Over-expression and purification of the protein would allow specific antiserum to be raised and facilitate N-terminal amino acid sequencing. This is important since it was not known which of the two possible ATG start codons represents the actual start.

The cellular compartmentalization of proteins involved in O-antigen biosynthesis in *V. cholerae* has not been determined. Thus, the localization of RfbT could indicate where the O-antigen is modified to produce the B antigen, and perhaps shed light on the pathway by which O-antigen and LPS are exported. Localization can be achieved with the use of a specific antiserum to RfbT and by cell fractionation. This chapter looks at the overexpression of the RfbT protein. The protein is partially purified for N-terminus sequencing and the raising of specific antibodies. The localization of RfbT in both *E. coli* and *V. cholerae* is under taken. In *E. coli* the protein is localized to a cellular compartment by labelling RfbT with [^{35}S]-methionine. In *V. cholerae* the protein was localized by the use of specific antibodies to RfbT.

6.2 Results

6.2.1 Expression of RfbT in minicells

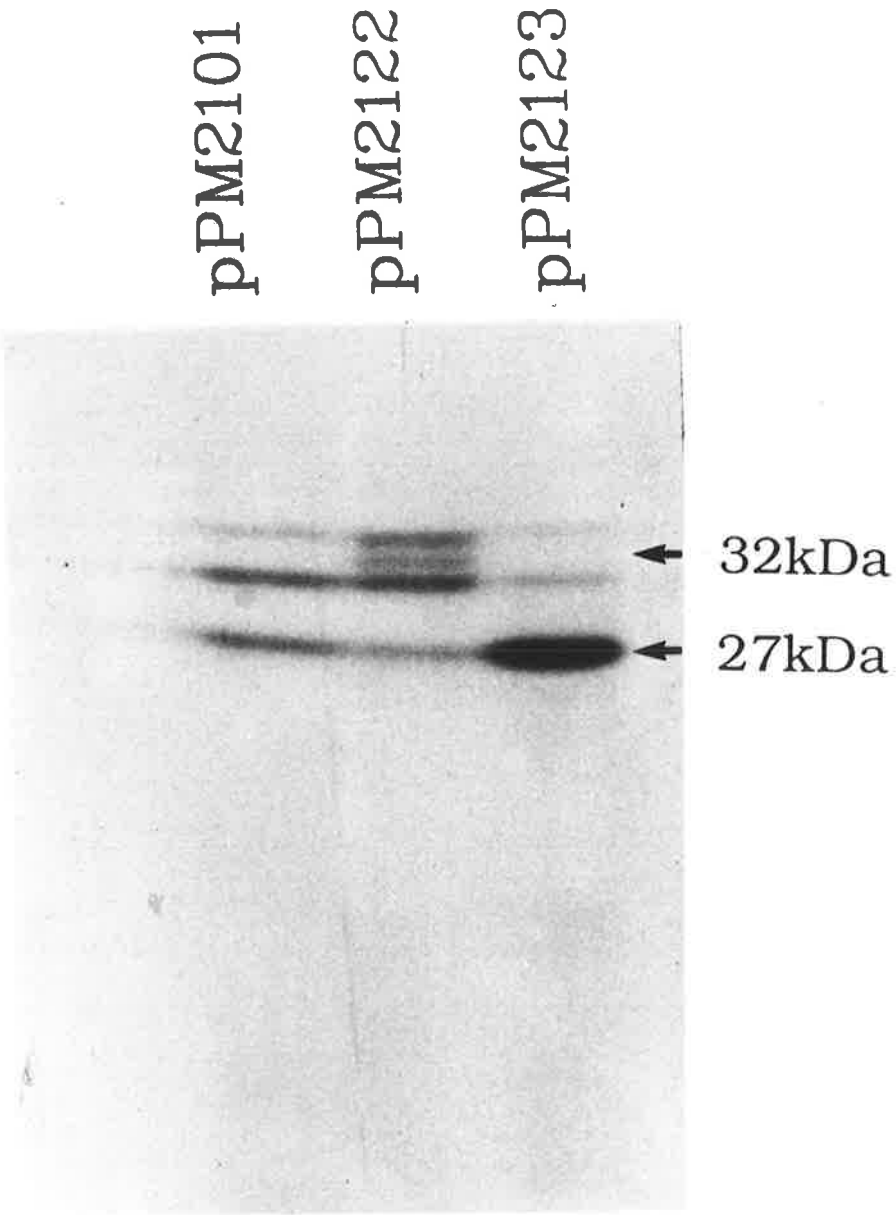
Plasmids pPM2101, pPM2122 and pPM2123 were transformed into the *E. coli* K-12 minicell strain DS410. Plasmid pPM2122 contains three complete open reading frames corresponding to the *V. cholerae* RfbR, RfbS and RfbT proteins. The RfbT protein of pPM2123 is predicted to have a size of 27kDa compared to the 32kDa protein encoded on pPM2122. From the minicell data (Fig. 6.1) a protein corresponding to approximately 32kDa is encoded by plasmid pPM2122. There appears to be a product of 27kDa in pPM2123 which is obscured by the products of the ampicillin resistance gene, *bla*, which comigrate in the region of 25-30kDa (Fig. 6.1). In no case could candidates for RfbR and RfbS be detected: the reasons for this is that *rfbT* has its own promoter while *rfbR* and *rfbS* do not. Furthermore, *rfbR* and *rfbS* do not have ribosome binding sites since they are translationally coupled to the rest of the *rfb* operon.

6.2.2 Expression of RfbT from Ogawa in pTTQ181

Since the expression of RfbT in the minicell strain was low it was decided to attempt to over-express the protein in a different system. The 3kb *Cla*I fragment was cloned from pRMB1 into the AccI site of the expression vector pTTQ181 (Stark, 1987), and was designated pPM2126. Plasmid pTTQ181 is pUC based and contains the P_{tac} promoter which is inducible with IPTG. This system should be expected to allow overexpression of the genes encoded on pPM2126. Cells harbouring the induced clone were fractionated and the fractions run in SDS on a 15% polyacrylamide gel which was stained with Coomassie blue G-250. There was no observable difference between the control pTTQ181 and the induced pPM2126 clone. This result indicates that the RfbT protein is not overexpressed to a readily

Figure 6.1: Expression of plasmid-encoded proteins in the minicell producing strain DS410.

Plasmid-encoded proteins were analysed using the *E. coli* minicell producing strain DS410. Minicells harbouring the plasmids pPM2101, pPM2122 and pPM2123 were purified on sucrose gradients, labelled with [^{35}S]-methionine and solubilized in SDS sample buffer. The plasmid-encoded proteins were visualized by autoradiography after electrophoretic separation in SDS on a 15% polyacrylamide gel. The RfbT proteins from O17 (pPM2122) and 569B (pPM2123) are indicated by arrows.


pPM2101
pPM2122
pPM2123
32kDa
27kDa

detectable level in this system. It was thus decided to use an alternate expression system based on the T7 promoter/RNA polymerase system of Tabor and Richardson (1985)

6.2.3 Overexpression and purification of RfbT

6.2.3.1 Overexpression of RfbT Ogawa using Bluescript K/S

The 3kb *Cla*I fragment was cloned into the plasmid pBluescript K/s (expression system) in both orientations. This plasmid can be used in conjunction with plasmids such as pGP1-2 which contain the T7 RNA polymerase under the control of the λP_L regulatable promoter. This system is designed to allow very high levels of expression of genes cloned downstream of the T7 promoter (Tabor and Richardson, 1985). In one orientation in Bluescript K/S the genes are under the control of the T7 promoter, (pPM2127), in the other orientation (pPM2128) they are inverted relative to the T7 promoter and thus can not be overexpressed from the T7 promoter (Fig. 6.2), although they are now under the control of the T3 promoter.

The T7 expression system uses two steps for over-expression, the initial step is a temperature shift from 30^{o}C to 42^{o}C. This is necessary since the T7 RNA polymerase is repressed by λcI857 at 30^{o}C but is induced by a temperature shift to 42^{o}C. The second step is the addition of rifampicin which inhibits the *E. coli* RNA polymerases but allows the T7 RNA polymerase to function. Since the genes cloned into pPM2127 are under the control of the T7 promoter, this should lead to high level expression of RfbR, RfbS and RfbT. Cycloserine was used to kill any cells that had acquired spontaneous Rif^R. This system gave only low levels of RfbT expression, but was a significant improvement on the pTTQ181 expression because a protein corresponding to 32kDa could be visualized on a SDS-PAGE gel (Fig. 6.3 and Fig 6.4).

Figure 6.2: Construction of plasmids pPM2127 and pPM2128.

Plasmid pRMB1 and pBluescript K/S were digested with the restriction endonucleases *Cla*I and *Acc*I, respectively. The 3kb *Cla*I fragment from pRMB1 containing the *rfbR*, *S*, and *T* genes and *ompX'* was cloned into pBluescript in both orientations, thus placing the genes either under the control of the T7 or T3 promoters. These plasmids were designated pPM2127 and pPM2128, respectively.

ompX' is truncated *ompX*

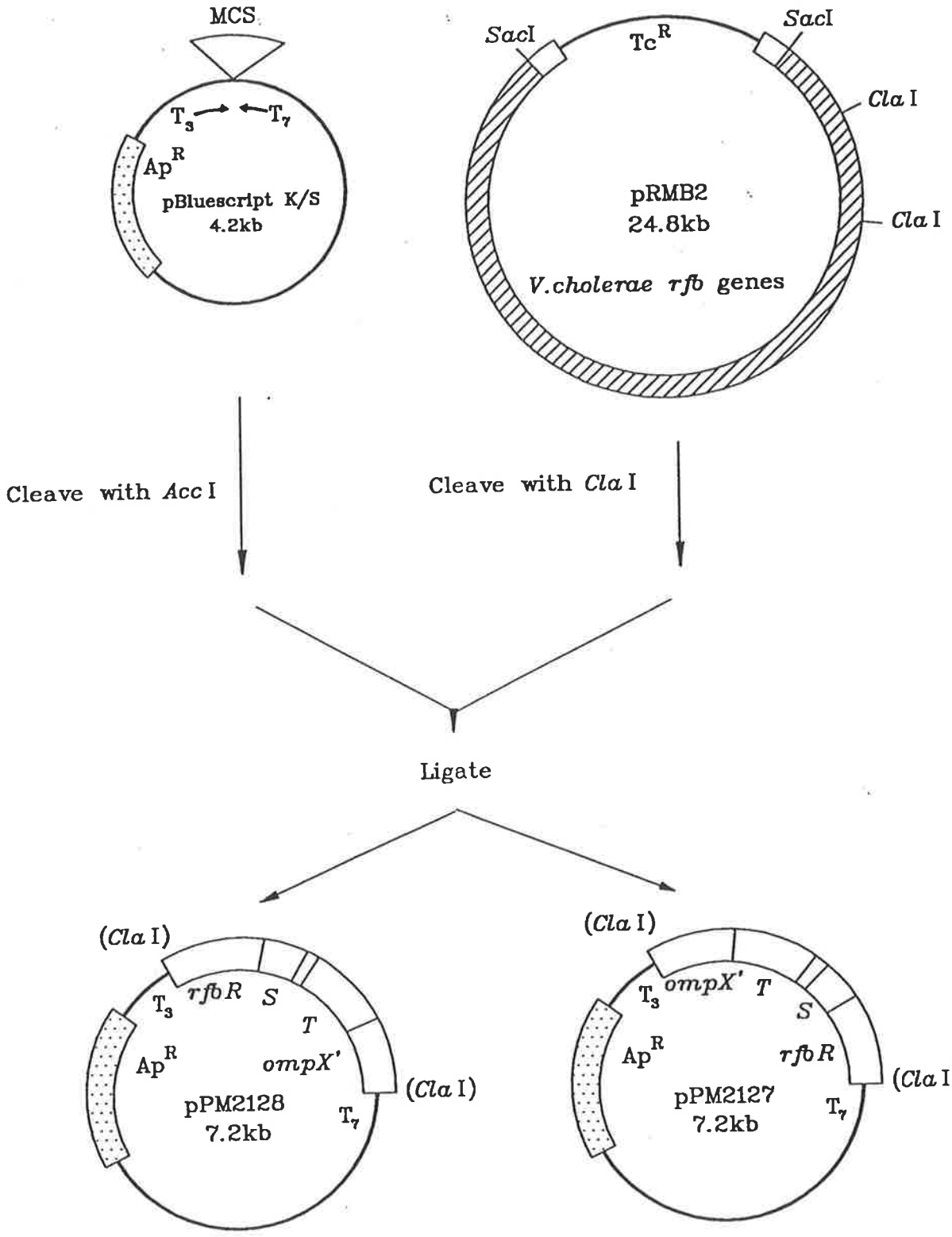

MCS
T3
T7
ApR
pBluescript K/S
4.2kb
SacI
TcR
SacI
ClaI
pRMB2
24.8kb
V.cholerae rfb genes
ClaI
Cleave with AccI
Cleave with ClaI
Ligate
(ClaI)
T3
rfbR
S
T
ApR
ompX'
pPM2128
7.2kb
T7
(ClaI)
(ClaI)
T3
ompX'
T
S
ApR
rfbR
pPM2127
7.2kb
T7
(ClaI)

Figure 6.3: Purification of the RfbT protein using the T7 promoter/RNA polymerase system in the presence of cycloserine.

DH5 cells containing pGP1-2 and pPM2127 were induced by a temperature shift from 30^{o}C to 42^{o}C and the addition of rifampicin. Cycloserine was added to kill any spontaneous rifampicin resistant cells. Extractions with 5% DOC were performed on whole membranes after disrupting the cells in a French Pressure Cell. Two sequential extractions were performed on the membrane material (1^{o} and 2^{o}) and the insoluble material removed by centrifugation at 35,000rpm for 1 hr (2^{o} pellet). The material was separated by electrophoresis in SDS on a 15% polyacrylamide gel and stained with Coomassie blue G250.

Panel A: extractions were carried out in the presence of 10mM $MgCl_2$.

Panel B: extractions were carried out in the absence of 10mM $MgCl_2$

Material was either boiled in SDS loading buffer or heated to 37^{0}C prior to being run on SDS-PAGE.

The molecular weight markers (M.W.M.) were; 94, 67, 43, 30, 20.1 and 14.4.

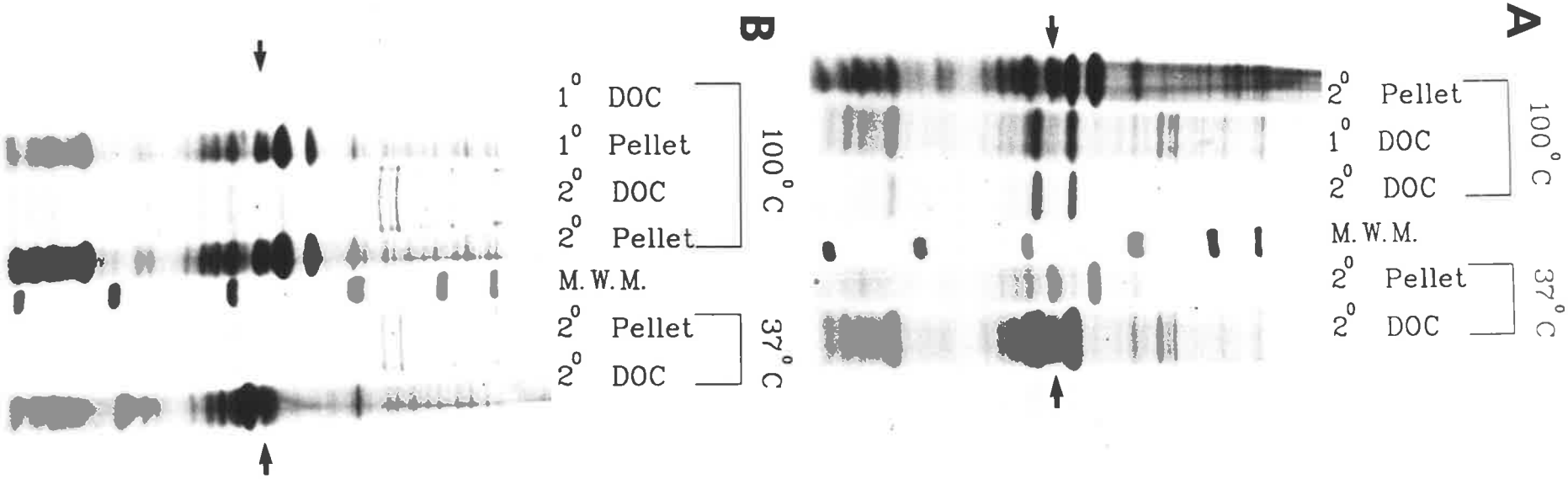

B
1° DOC
1° Pellet
2° DOC
2° Pellet
100° C
M. W. M.
2° Pellet
2° DOC
37° C
A
2° Pellet
1° DOC
2° DOC
100° C
M. W. M.
2° Pellet
2° DOC
37° C

Figure 6.4: Purification of the RfbT protein using the T7 promoter/RNA polymerases system.

DH5 cells containing pGP1-2 and either pPM2127 or pBluescript (pB/S) were induced by a temperature shift from 30°C to 42°C and by the addition of rifampicin. Whole cells were broken with a French Pressure Cell and the whole membrane fraction collected by ultracentrifugation (35,000 rpm for 1 hr) and subjected to various extractions.

pB/S represents DH5 cells containing pBluescript K/S and, pPM2127 is the clone in which *rfbT* is under the control of the phage T7 promoter.

The tracks correspond to:

1: Whole membrane fraction

2: Soluble fraction after extraction with 5% Sarkosyl in the presence of 10mM $MgCl_2$

3: Soluble fraction after 1st extraction with 5% DOC in the presence of 10mM $MgCl_2$

4: Soluble fraction after 2nd extraction with 5% DOC in the presence of 10mM $MgCl_2$

5: Insoluble fraction after extraction with 5% Sarkosyl and 5% DOC.

6: Soluble fraction after extraction of 5 with 4M Urea

7: Soluble fraction after extraction of 5 with 8M Urea

8: Insoluble residue after detergent and urea extractions

The samples were electrophoresed in SDS on a 15% polyacrylamide Gel and stained with Coomassie blue G250.

Panel A: Samples were boiled in SDS prior to loading on the gel

Panel B: Samples were heated to 37°C in SDS prior to loading on the gel

The position of the RfbT protein is indicated by the large arrow. The *E. coli* OmpA protein and porins are indicated by the triangle and diamond respectively.

The molecular weight markers (M.W.M.) were; 94, 67, 43, 30, 20.1 and 14.4.

A
1
2
3
4
5
6
7
8
pB/S
pPM2127
pB/S
pPM2127
pB/S
pPM2127
pB/S
pPM2127
pB/S
pPM2127
pB/S
pPM2127
pB/S
pPM2127
M.W.M.

B
1
2
3
4
5
6
7
8
pB/S
pPM2127
pB/S
pPM2127
pB/S
pPM2127
pB/S
pPM2127
pB/S
pPM2127
pB/S
pPM2127
pB/S
pPM2127
M.W.M.

The expression of the protein could be further upregulated by omitting the cycloserine. With this system, it was possible to partially purify the RfbT protein (Fig. 6.4). This material was subsequently used for N-terminal analysis (Section 6.2.6) and the raising of antibodies specific to RfbT (Section 6.2.5). The reasons for the difference in the levels of expression between the presence and absence of cycloserine is not known. Since the action of cycloserine is to stop peptidoglycan biosynthesis and this ties up the undecaprenol lipid carrier this could indirectly affect proteins involved in LPS biosynthesis. The constructs used for the T7 over-expression lead to a long 5' mRNA tail of *V. cholerae* origin, it may be at the translational level that the lower production of RfbT occurs by some unknown feedback system i.e. if there is no or little undecaprenol lipid carrier LPS production may be down-regulated thus affecting the production of RfbT. Since both O-antigen and peptidoglycan biosynthesis use the same undecaprenol lipid carrier.

6.2.3.2 Purification of RfbT from a low level producer

Several attempts were made to purify the RfbT protein from the low level producer (DH5 [pPM2127/pGP1-2] with cycloserine). Upon cell fractionation the protein appeared to be associated with the membrane fraction. Thus, it was decided to try to extract the protein from the membrane by the use of various salts at differing concentrations ranging from 20mM to 500mM (Table 6.1). None of the salts appeared to have any effect on the solubilization of the protein. Other extractions were performed using a number of detergents with or without the addition of magnesium chloride (Table 6.1). The procedure which gave maximum purity of RfbT involved two extractions with deoxycholate (DOC) in the presence of 10mM $MgCl_2$ at 37^{o}C. This removed a number of contaminating proteins and left most of RfbT associated with the insoluble fraction (Fig. 6.3).

The partially purified RfbT protein was run on SDS-PAGE and the band corresponding to 32kDa RfbT protein was excised from the stained gel. The gel slice

Table 6.1: Extraction procedures with salts and detergents:

Salt	**Conc^n**
LiCl	10mM ±10mM $MgCl_2$
LiCl	50mM ±10mM $MgCl_2$
LiCl	100mM ±10mM $MgCl_2$
LiCl	500mM ±10mM $MgCl_2$
NaCl	10mM ±10mM $MgCl_2$
NaCl	50mM ±10mM $MgCl_2$
NaCl	100mM ±10mM $MgCl_2$
NaCl	500mM ±10mM $MgCl_2$
Detergent	**Conc^n**
Tween20	1% ±10mM $MgCl_2$
Sarkosyl	1% ±10mM $MgCl_2$
NonidetP40	1% ±10mM $MgCl_2$
BRIJ35	1% ±10mM $MgCl_2$
DOC	1% ±10mM $MgCl_2$

Extractions were carried out on membrane material using either LiCl or NaCl salts at concentrations ranging from 10mM to 500mM with or without the addition of $MgCl_2$.

Other extractions were performed using various detergents such as Tween20, Sarkosyl, NonidetP40, Brij35 and DOC with or without the addition of $MgCl_2$.

All extractions were carried out for 30min at room temparture with intermittent vortexing.

containing the RfbT protein was homogenized with Span oil and used for the primary immunization of a rabbit to raise a specific antiserum to RfbT.

6.2.3.3 Purification of RfbT from a high level producer

Cells producing high levels of RfbT (section 6.2.3.1) were fractionated and the fractions run in SDS on a 15% polyacrylamide gel. As shown earlier section (6.2.3.2) the RfbT protein appears to be associated with the membrane. A series of solubilizations were carried out using either 5% Sarkosyl and 5% DOC in the presence of magnesium chloride. It was decided to use the higher levels of detergent to try to solubilize the protein out of the membrane fraction. Further extractions with either 4M or 8M urea were performed on the detergent insoluble material (Fig. 6.4). The major contaminating protein appears to be OmpA because it alters its mobility from 28kDa to approximately 43kDa with respect to RfbT if the samples are solubilized at either 37^{o}C or 100^{o}C. Other contaminants migrating in the proximity of RfbT are the *E. coli* porins OmpF and OmpC. The porins only enter the gel if the samples are boiled prior to loading, otherwise the porins exist as trimers which do not enter the gel effectively. The RfbT protein is the major protein in the 4M or 8M urea fractions, however some of the RfbT protein remains insoluble. This may be due to the formation of intracellular inclusion bodies and not due to insufficient urea or detergent. The formation of inclusion bodies is not unusual for proteins which show high levels of expression.

The RfbT protein migrates at approximately 32kDa regardless of whether the samples were boiled or heated to 37^{o}C prior to loading on the gel. This would indicate that RfbT probably does not form dimers or trimers like porins. This size corresponds to that predicted for RfbT from the nucleotide sequence.

The purified RfbT from the 4M and 8M urea fractions was run in SDS on a 15% polyacrylamide gel and the protein band excised. This material, which was considerably

purer than the material from the low level producer, was homogenized, and used to boost the rabbit that had previously been immunized with the partially purified RfbT from the low level producer.

6.2.4 N-terminal amino acid sequence analysis of RfbT

From the nucleotide sequence analyses two possible start codons within the same ORF could give rise to proteins of similar molecular size, 34kDa compared to 32kDa. The 32kDa protein has a better ribosome binding site than the larger 34kDa protein. Consequently, it was decided to determine the N-terminal amino acid sequence of RfbT in order to confirm the identity of the protein and to confirm which of the two possible inframe ATG start codons gave rise to the RfbT protein.

The 4M urea soluble fraction of RfbT was mixed with SDS sample buffer and run in SDS on a 15% polyacrylamide gel. The RfbT protein was excised from the gel and reelectrophoresed. This material was subsequently transferred via electro-blotting to PVDF protein sequencing membrane, and the N-terminal sequence was determined on an Applied Biosystems 470 peptide sequencer (by A.Gooley, Macquarie University, Sydney).

The N-terminal sequence for the 32kDa RfbT protein is shown below and demonstrates that the second ATG is the actual start of translation of the protein. This result more closely approximates the size predicted from SDS-PAGE gel and fits the better ribosome binding site than if the first ATG codon was used.

N-terminal sequence: **Met Lys His Leu Ile Lys Asn Trp Val Glu Lys Leu Ile Lys**

6.2.5 Cellular location of RfbT in *E. coli*

DH5 cells containing pGP1-2 and either pBluescript K/S, pPM2127 or pPM2128 were labelled with ^{35}[S]-methionine using the T7 polymerase expression system (Tabor and

Richardson, 1985). The cells were subsequently fractionated into cytoplasm, periplasm, inner and outer membranes. The protein fractions were run in SDS on a 15% polyacrylamide gel and visualized by autoradiography. The RfbT protein can be detected in the whole cells and inner membrane fractions, although there is some protein seen in the other fractions which may be due to contaminating inner membrane material (Fig. 6.5). The periplasmic fraction also contains a significant amount of RfbT. This could be due to a number of factors, such as small membrane vesicles formed upon sphaeroplasting which can only be removed from the periplasmic fraction by high speed ultracentrifugation. One other possibility is that RfbT is on the periplasmic surface of the inner membrane and subsequently is solubilized to some extent upon the formation of sphaeroplasts. The protein material seen in the pPM2127 track may either be breakdown products of RfbT or RfbR and RfbS.

6.2.6 Stability of labelled RfbT in *E. coli*

The T7 promoter/RNA polymerase expression system (Tabor and Richardson, 1985) was used to specifically label proteins under the control of the T7 promoter. This system has the advantage over minicells that there are no *bla* gene products to interfere with the analysis of the proteins. The proteins were labelled with ^{35}[S]-methionine and subsequently chased for various lengths of time by the addition of cold methionine. The samples were run in SDS on a 15% polyacrylamide gel and the proteins visualized by autoradiography (Fig. 6.6). None of the time points from 1min to 60 min show any degradation of the labelled RfbT. This would indicate that RfbT has a long half life and that it is stable in *E. coli* K-12. Candidates for the other two proteins, RfbR and RfbS, could be visualized, although a longer exposure of the autoradiograph does reveal these bands more clearly, it also reveals a number of other bands, some of which correspond to proteins seen in the control tracks and possible breakdown products. The poor expression of RfbR and RfbS is probably due to the lack of any identifiable ribosome binding site since the *rfbR* and *rfbS* genes are translationally

Figure 6.5: Cellular localization of the RfbT protein in *E. coli* using [^{35}S]-methionine labelled proteins.

High level expression was induced using the T7 transcription system (Tabor and Richardson, 1985) and the translated proteins were labelled with [^{35}S]-methionine. *E. coli* DH5 cells harbouring pGP1-2 and either plasmids pBluescript K/S, (pB/S),pPM2127 and pPM2128 were used. The cells were fractionated and analysed in SDS on a 15% polyacrylamide gel followed by autoradiography. RfbT could be detected in the whole cell, periplasmic and inner membrane fractions. The small amount of RfbT seen in the other fractions is likely to be due to membrane contamination. The labelled RfbT protein is approximately 32kDa and is indicated by an arrow.

W.C. is whole cells

Cyto. is cytoplasmic fraction

Peri. is periplasmic fraction

I.M. is inner membrane fraction

O.M. is outer membrane fraction

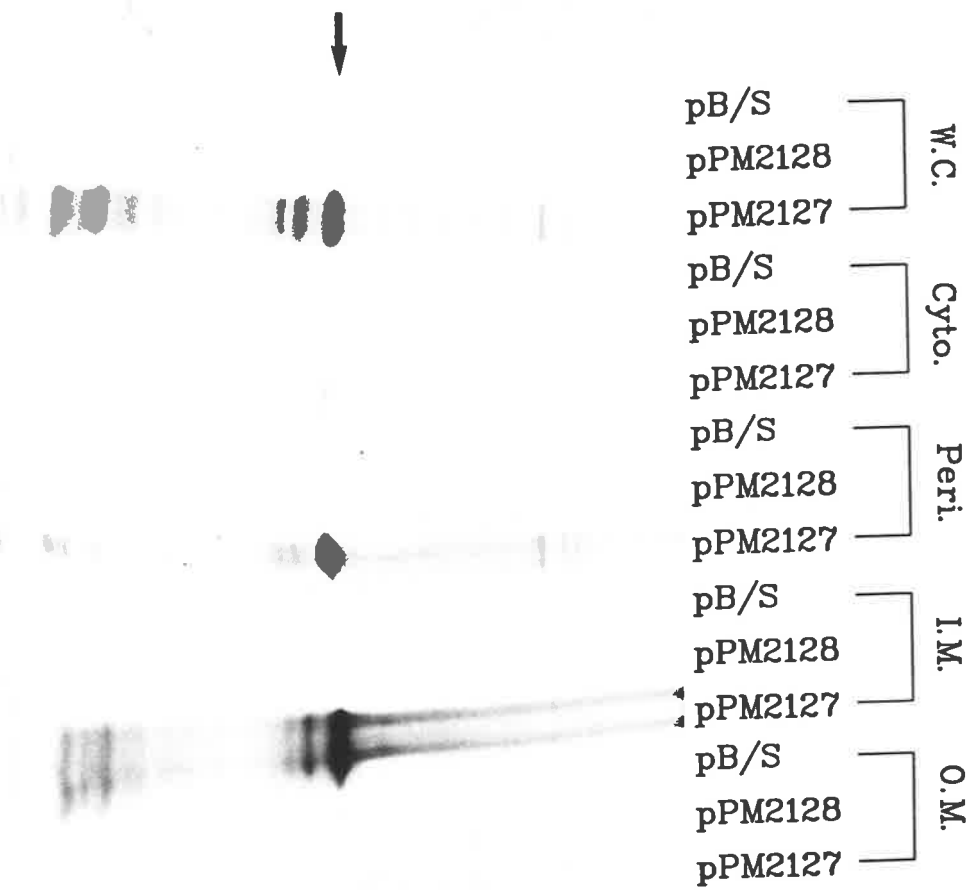

pB/S
pPM2128
pPM2127
W.C.
pB/S
pPM2128
pPM2127
Cyto.
pB/S
pPM2128
pPM2127
Peri.
pB/S
pPM2128
pPM2127
I.M.
pB/S
pPM2128
pPM2127
O.M.

Figure 6.6: Stability of labelled RfbT in *E. coli*.

Proteins expressed from the T7 promoter were labelled for 2min with [^{35}S]-methionine in *E. coli* DH5 cells harbouring pGP1-2 and either pBluescript K/S (pB/S), pPM2128 or pPM2127. The labelling was followed by a chase of cold methionine and samples were taken at 1 min, 5 min and 60 min after addition of the cold methionine. The proteins were analysed in SDS on a 15% polyacrylamide gel followed by autoradiography. The arrow indicates the position of the RfbT protein. The other two smaller arrows indicate the possible protein products of *rfbR* and *rfbS*. The triangle and diamond indicate RfbR and RfbS respectively.

pB/S
pPM2128
pPM2127
T1
pB/S
pPM2128
pPM2127
T5
pB/S
pPM2128
pPM2127
T60

coupled to the rest of the *rfb* operon. The levels of the three proteins detected does not correspond to the number of methionine residues, since, RfbT has only 5 residues whereas RfbR and RfbS have 10 and 3 residues respectively. Mutants in RfbR and RfbS would help in the identification of these proteins in the T7 polymerase system. The RfbR protein has an expected molecular size of 27kDa and is probably the second band under RfbT, RfbS is only 7kDa and thus migrates well below the other two proteins. The only unique band in this region is indicated at the bottom of the gel (Fig 6.6).

6.2.7 Detection of RfbT in *V. cholerae*

The antiserum used for detection was raised against partially purified RfbT. This serum was absorbed 5 times overnight with the *rfbT* mutant strain (V874) to remove any non-specific antibodies. Whole cell lysates were solubilized, and run on SDS-PAGE and and probed with antiserum after transfer to a nitrocellulose filter (Fig. 6.7). In strain O17 the serum reacts most strongly with a 32kDa protein and with a 27kDa protein in 569B (Fig. 6.7). These sizes correspond to those expected from the sequence data of the RfbT protein in O17 and 569B.

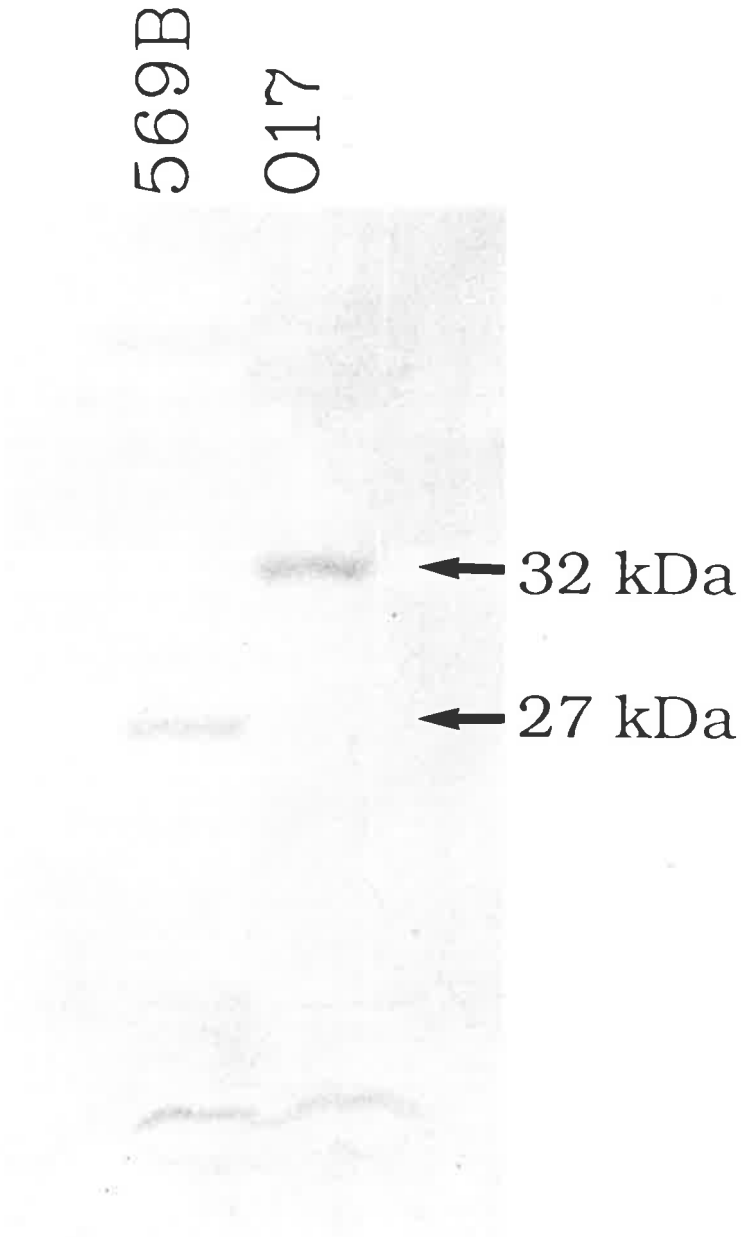
6.2.8 Cellular localisation of RfbT in *V. cholerae*

Results obtained from cell fractionation experiments in which membrane proteins have been overexpressed may not necessarily reflect their true cellular location (Tommassen, 1986). This may well be the case with the *E. coli* fractionations to localize RfbT. In this case the protein was over-expressed using the T7 promoter/RNA polymerase system.

Since the RfbT protein could be readily detected in whole cells of *V. cholerae* using the antiserum to RfbT, cell fractionations of *V. cholerae* were undertaken. The cells were

Figure 6.7: Detection of RfbT in *V. cholerae* O1 strains O17 and 569B.

Whole cells of *V. cholerae* strains O17 and 569B were analysed on a 15% polyacrylamide gel followed by transfer to nitrocellulose. Using antiserum raised to gel-purified RfbT protein, RfbT can be visualised on a Western blot. O17 and 569B have proteins of differing sizes as predicted from the nucleotide sequence. The O17 RfbT is 32 kDa whereas the 569B protein is 27 kDa. The proteins are identified by arrows.


569B
017
32 kDa
27 kDa

fractionated and the fractions run on SDS- PAGE, the proteins transferred to nitrocellulose and probed with the antiserum. In both O17 and 569B, the RfbT protein was found exclusively in the inner membrane fraction (Fig. 6.8). These data confirm those obtained previously (Section 6.2.5) and suggest that RfbT is localized and acts at the inner membrane. This supports the model that LPS is assembled and modified at or within the inner membrane prior to export to the outer membrane (Robbins and Wright, 1971; Osborne, 1979; Mulford and Osborne, 1983;) . Since the truncated RfbT product of 569B is also found in the same cellular location, it seems likely that the domain(s) of the protein which interact with the membrane and are responsible for its location must be within the first 27kDa of the protein. No corresponding protein was detected in fractions of the *rfbT* mutant, V784.

6.2.9 Computer analysis of the RfbT protein

6.2.9.1. Codon Usage and amino acid composition

A summary of the codon usage within the coding region of the *rfbT* mRNA is shown in Table 6.2. Table 6.2 shows the *rfbT* codon usage compared to other sequenced *V. cholerae* genes. Many of the genes sequenced from *V. cholerae* are involved in virulence. These genes tend to have a G+C content of approximately 40% (P. Manning, personal communication) compared to the overall G+C content of *V. cholerae* of 48% and may apply a bias to the Table. The codon usage of *rfbT* is also compared to the other *rfb* genes of *V. cholerae* (Table 6.3).

Since the G+C content of *rfbT* is very low (31.7%) compared to the rest of the *rfb* operon (39.1 %) and *V. cholerae* in general this leads to a predominance of amino acids such as phenylalanine (6.9%), isoleucine (11.1%), asparagine (9.4%) and lysine (6.4%), when compared to *V. cholerae* in general. Codons which use predominantly G and C are reduced in RfbT such as leucine (6.2%), serine (6.1%), alanine (4.8%) and arginine (3.0%) when

Figure 6.8: Cellular localization of RfbT in *V. cholerae* using antisera.

Whole cells (W.C.) of *V. cholerae* O17, 569B and V874 were fractionated into cytoplasm (cyto), periplasm (peri), inner (I.M.) and outer membrane (O.M.). The fractions were solubilized in SDS and the proteins separated on a 15% polyacrylamide gel and transferred to nitrocellulose. Antiserum raised to purified RfbT was used in a Western blot to detect the protein. The only fractions to contain RfbT were whole cells and inner membrane. V874 (an rfbT null mutant) did not react with the antiserum. The RfbT proteins are indicated by arrows. The molecular weight markers (M.W.M.) were; 94, 67, 43, 30, 20.1 and 14.4.

W.C. is whole cells

Cyto. is cytoplasmic fraction

Peri. is periplasmic fraction

I.M. is inner membrane fraction

O.M. is outer membrane fraction

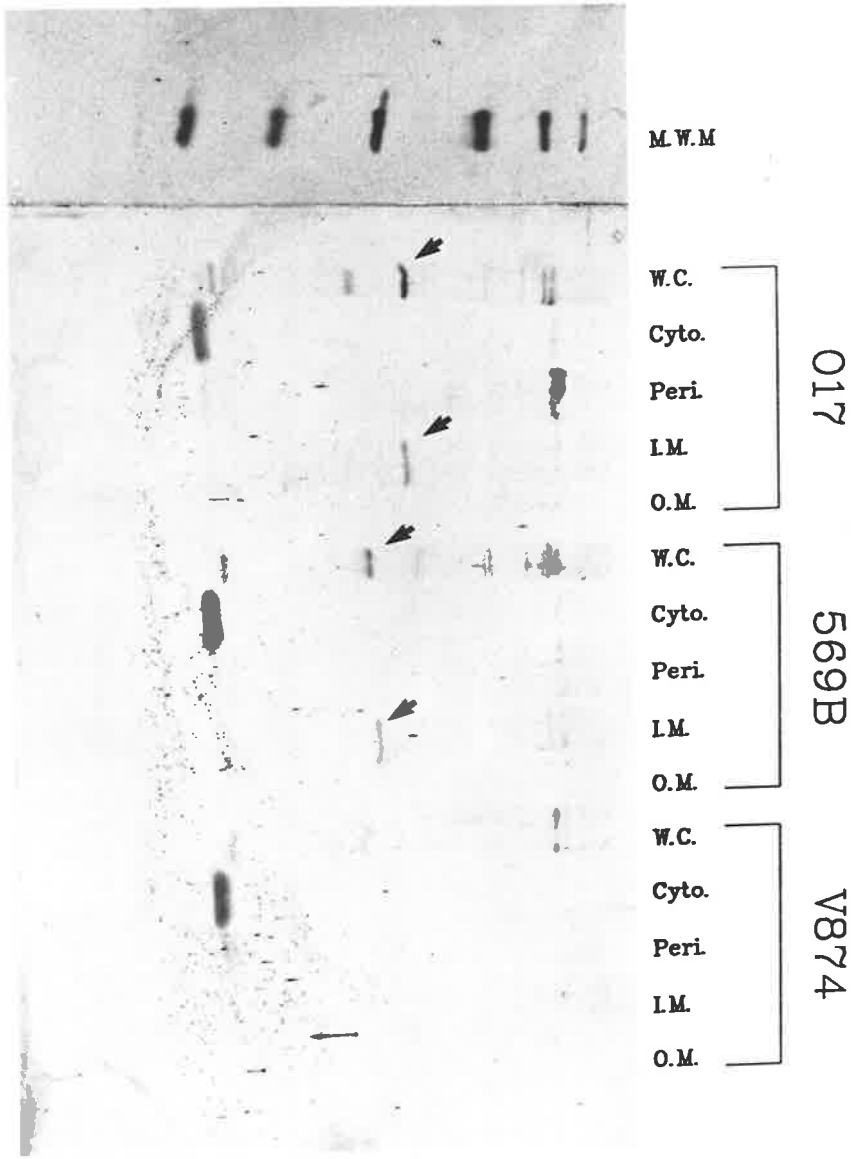

M.W.M
W.C.
Cyto.
Peri.
I.M.
O.M.
O17
W.C.
Cyto.
Peri.
I.M.
O.M.
569B
W.C.
Cyto.
Peri.
I.M.
O.M.
V874

Table 6.2: Codon usage of *rfbT* compared to *V. cholerae* O1, the *rfb* operon of *V. cholerae* and to *E. coli* high and low expressed genes.

Codon	aa	Percent in *V.cholerae*	Percent in *rfb*	Percent in *rfbT*	Percent in *E. coli* (high)	Percent in *E.coli* (low)
UUU	Phe	70.6	73.9	75.0	18.3	64.9
UUC	Phe	29.4	26.1	25.0	81.7	35.1
UUA	Leu	28.2	31.4	55.5	1.4	16.4
UUG	Leu	18.9	20.9	11.1	1.5	15.3
CUU	Leu	17.9	22.5	11.1	3.5	12.0
CUC	Leu	9.7	4.3	5.5	4.8	10.8
CUA	Leu	12.6	14.5	11.1	0.6	3.5
CUG	Leu	12.6	6.4	5.5	88.2	41.9
AUU	Ile	50.7	52.8	53.1	18.6	49.5
AUC	Ile	28.4	21.4	9.4	81.2	39.6
AUA	Ile	20.9	25.8	37.5	0.2	10.9
AUG	Met	100.0	100.0	100.0	100.0	100.0
GUU	Val	34.5	39.0	66.6	50.6	26.0
GUC	Val	15.7	10.3	0.0	6.8	22.8
GUA	Val	25.1	34.2	20.0	26.2	15.7
GUG	Val	24.7	16.5	13.3	16.4	35.5
UCU	Ser	20.9	30.5	20.0	38.8	14.0
UCC	Ser	7.7	6.9	6.6	34.4	12.8
UCA	Ser	21.6	21.1	40.0	2.1	13.4
UCG	Ser	11.9	9.3	6.6	3.6	16.2
AGU	Ser	22.1	18.4	26.6	1.6	16.1
AGC	Ser	15.9	13.8	20.0	19.4	27.4
CCU	Pro	30.5	32.3	28.6	6.1	16.0
CCC	Pro	13.1	11.1	14.3	1.0	15.5
CCA	Pro	36.0	42.4	57.1	11.9	23.5
CCG	Pro	20.5	14.1	0.0	81.0	45.0
ACU	Thr	26.6	36.0	36.4	40.3	37.8
ACC	Thr	25.7	16.4	9.1	52.2	21.0
ACA	Thr	27.3	29.3	45.4	3.0	37.0
ACG	Thr	20.4	18.2	9.1	4.5	26.2
GCU	Ala	31.7	36.7	42.9	38.8	15.3
GCC	Ala	16.2	13.2	0.0	8.8	28.4
GCA	Ala	29.8	33.2	50.0	26.6	22.3
GCG	Ala	22.4	16.9	7.1	25.8	34.0
UAU	Tyr	66.4	66.7	72.7	24.5	65.2
UAC	Tyr	33.6	33.3	27.3	75.5	34.8
CAU	His	65.7	64.3	92.9	18.4	60.0
CAC	His	34.3	35.7	7.1	81.6	40.0
CAA	Gln	65.7	66.7	50.0	10.7	38.9
CAG	Gln	34.3	33.3	50.0	89.3	61.1
AAU	Asn	62.3	63.4	63.0	5.5	53.6
AAC	Asn	37.7	36.6	37.0	94.5	46.4
AAA	Lys	71.6	66.3	66.7	79.0	72.5
AAG	Lys	28.4	33.7	33.3	21.0	27.5
GAU	Asp	77.0	78.0	83.3	34.2	68.3
GAC	Asp	23.0	22.0	16.7	65.8	31.7
GAA	Glu	64.5	67.2	65.2	79.7	65.7
GAG	Glu	35.5	32.8	34.8	21.3	34.3
UGU	Cys	61.2	64.7	83.3	39.6	45.3
UGC	Cys	38.8	35.3	16.7	60.4	54.7
UGG	Trp	100.0	100.0	100.0	100.0	100.0
CGU	Arg	28.1	24.7	22.2	74.7	31.8
CGC	Arg	20.5	14.6	0.0	25.0	40.3
CGA	Arg	15.6	16.9	11.1	0.0	9.6
CGG	Arg	6.7	4.1	0.0	0.2	10.8
AGA	Arg	21.4	27.0	33.3	0.0	4.6
AGG	Arg	7.6	12.8	33.3	0.0	2.9
GGU	Gly	42.5	43.0	23.5	58.4	35.5
GGC	Gly	25.1	19.8	11.8	40.0	33.2
GGA	Gly	17.9	23.0	41.2	0.5	14.6
GGG	Gly	14.6	14.2	11.8	1.1	16.7
UAA	Stop	45.6	41.2	100.0	95.8	51.1
UAG	Stop	27.2	29.4	0.0	4.2	4.2
UGA	Stop	27.2	29.4	0.0	0.0	44.7

This codon usage table was complied from 72 sequenced *V. cholerae* O1 genes (P. Manning manuscript in preparation). The *rfb* operon contains 17 of these genes and has a G+C content of 38%. In most cases the genes for the *rfb* operon fall between the general usage of codons for *V. cholerae* and *rfbT*. The codon usage is also given for high and low expressed genes of *E. coli* (Anderson and Kurland, 1990).

compared to *V. cholerae* (Table 6.3). However other amino acids such as glycine and proline which also use a predominance of G and C do not show such a marked shift, although in all amino acids there is a trend towards using the codons with the lower G+C content. For amino acids such as proline the CCG codon is not used, nor is the GCC codon used for alanine or the CGC and CGG codons for arginine. These codons are normally used at 20.5%, 16.2%, 20.5% and 6.7% respectively. The shift of RfbT from the *V. cholerae* codon usage is less pronounced if RfbT is compared to the rest of the *rfb* operon (Table 6.2). This is due to the *rfb* operon having a low G+C content of 39.1%. Comparison of the *rfbT* codon usage with that of *E. coli* (Andersson and Kurland, 1990) clearly shows that *rfbT* fits the *V. cholerae* codon usage Table more closely, since there is a strong bias to the codons with a high G+C content in *E. coli* for leucine, isoleucine, proline, glutamine, asparagine and cysteine.

It is not possible to predict if the expression of RfbT is influenced to any degree by its codon usage (Gouy and Gautier, 1982; Robinson *et al.*, 1984). Other factors are also likely to influence the expression of *rfbT*. The ribosome binding site infront of *rfbT* is not ideal, and the possible secondary structure of the mRNA prior to rfbT and the less than optimal spacing of the -10 region prior to the transcriptional start point may also play a role in determining the level of *rfbT* expression.

6.2.9.2 Hydropathy plots of RfbR , RfbS and RfbT

Plots of the hydrophobic nature of the RfbR, RfbS and RfbT proteins were made using the algorithm of Kyte and Doolittle (1982) (Fig. 6.9). The plot for RfbR suggests that overall the protein is hydrophilic with a mean hydropathic value of -0.21. There is only one broad hydrophobic domain between residues 80 and 110. However this region is interspersed with a number of charged residues suggesting that there are no transmembrane domains. Thus, RfbR is likely to be a soluble protein.

Table 6.3: Relative percentage of amino acids in proteins in *V. cholerae*, the *rfb* operon and *rfbT*

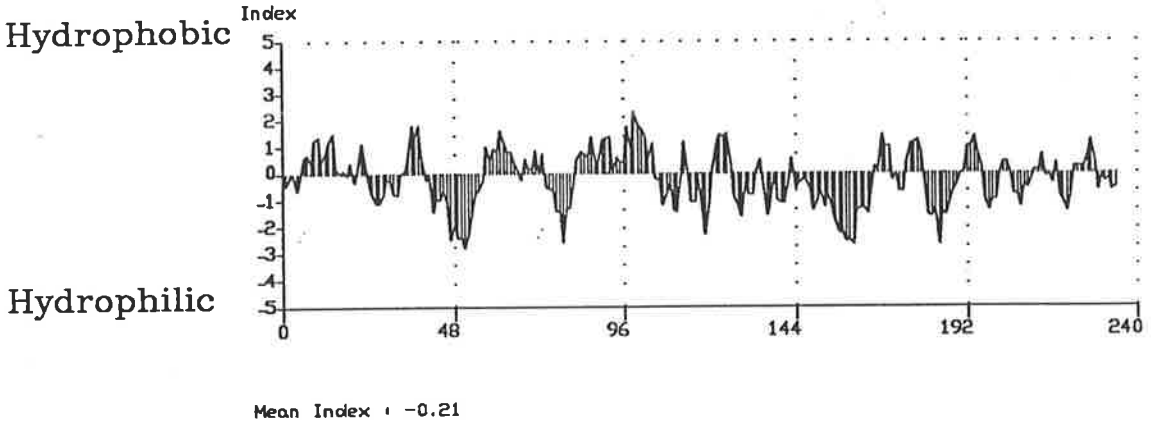
aa	% of aa V. cholerae	% of aa in *rfb*	% of aa in *rfbT*
Phe	4.7	6.6	6.9
Leu	9.3	4.7	6.2
Ile	6.9	9.3	11.1
Met	2.3	7.8	1.7
Val	6.8	2.4	5.2
Ser	8.3	7.6	6.1
Pro	3.4	7.8	2.4
Thr	5.4	3.8	3.7
Ala	7.0	4.3	4.8
Tyr	3.8	6.6	3.8
His	2.3	3.6	4.8
Gln	4.1	2.7	3.4
Asn	5.4	3.1	9.4
Lys	5.9	4.7	6.3
Asp	5.4	6.4	4.2
Glu	6.0	5.3	8.0
Cys	1.2	6.8	2.0
Trp	1.1	1.3	0.3
Arg	4.4	1.2	3.0
Gly	6.2	4.2	5.2

The table is constructed from 71 sequenced *V. cholerae* O1 genes. The *rfb* operon contains 17 ORFs from which the relative percentage of amino acids was constructed.

Figure 6.9: Hydropathy plot of the RfbR, RfbS and RfbT proteins.

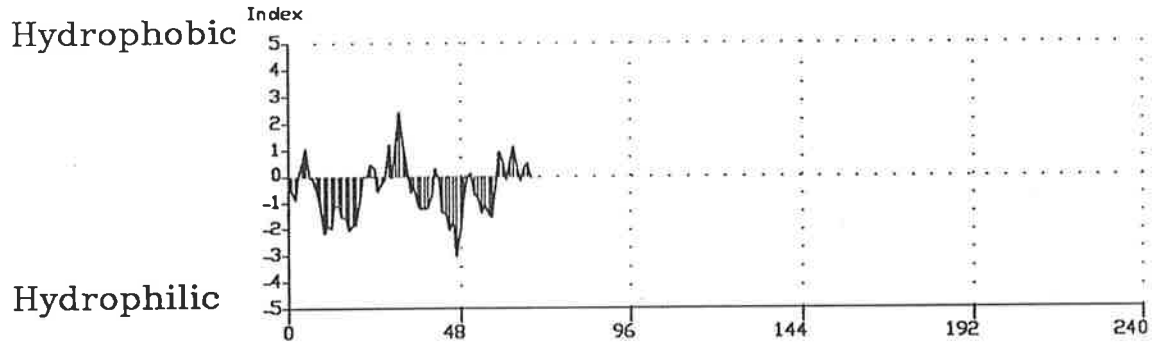
The amino acid sequences of RfbR, RfbS and RfbT proteins were analysed according to Kyte and Doolittle (1982) using a window of six amino acids.

RfbR



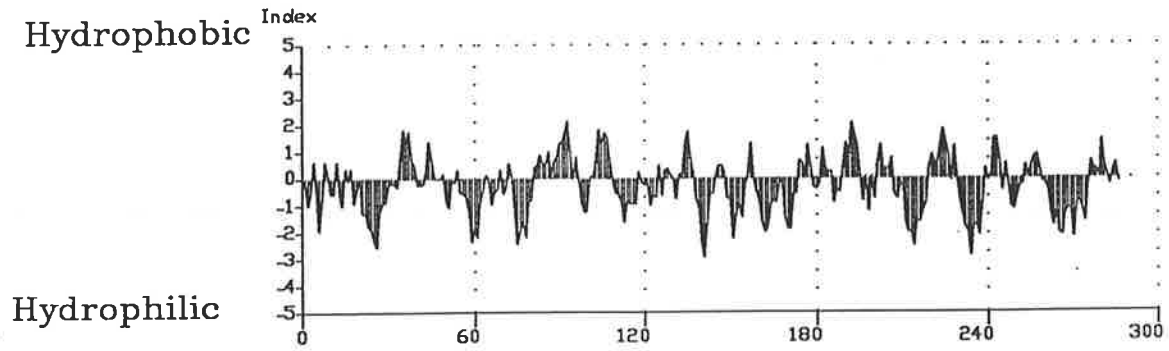
Mean Index = -0.21

RfbS



Mean Index = -0.56

RfbT



Mean Index = -0.28

The plot for RfbS has a hydropathic index of -0.56 which also indicates that the overall nature of this protein is soluble. Since neither RfbR or RfbS have a recognizable signal sequence these proteins are likely to be cytoplasmic.

The plot for RfbT shows this protein to be hydrophilic with a mean hydropathic value of -0.28. RfbT has a number of hydrophobic domains although only the domains between amino acids 86 to 98 and 121 to 130, are large enough to traverse the membrane and thus act as membrane anchors. The other hydrophobic domains all contain charged residues and are thus unlikely to be trans-membrane. Consistent with its inner membrane location, RfbT does not have an N-terminal signal sequence.

6.2.9.3 Secondary protein structure of RfbR, RfbS and RfbT

The secondary structures of RfbR, RfbS and RfbT were predicted using Chou, Fasman and Rose analysis (Fig. 6.10, 6.11 and 6.12) (Rose 1978; Chou and Fasman, 1974a; 1974b; 1978).

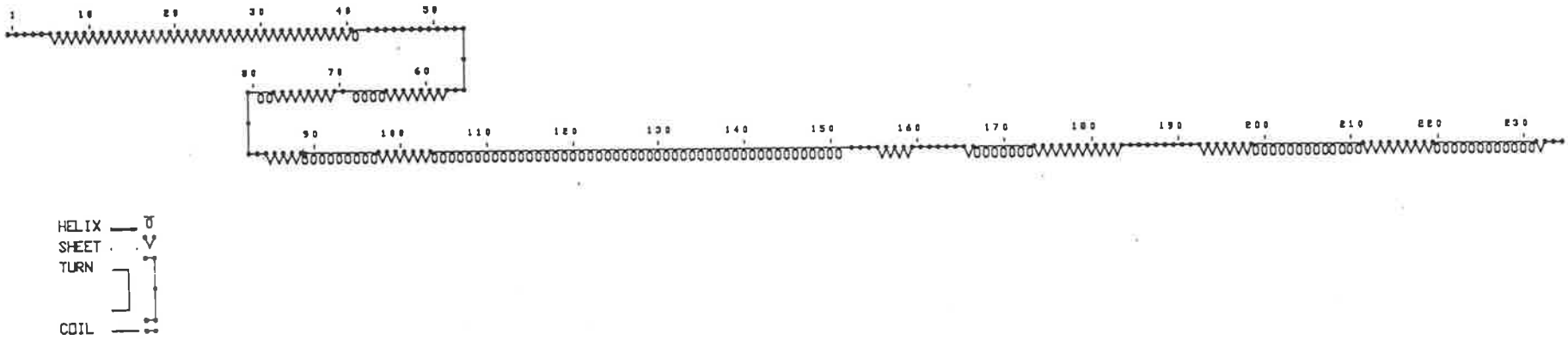
Neither RfbR nor RfbS have any domains, such as possible DNA binding domains, which give a clue to their function. RfbR does, however, have large stretches of either beta-sheet or alpha-helix. There appear to be no transmembrane domains since any hydrophobic stretches are interspersed with charged amino acid residues. Furthermore the only region which is hydrophobic has both alpha-helix and beta-sheet.

RfbT appears to cross the membrane twice. The number of amino acids required to cross the membrane varies depending upon the secondary structure of the region (Engelman and Zaccai, 1980; Engelman *et al.*, 1980; Paul and Rosenbush, 1984). Regions of beta-sheet require approximately nine residues to traverse the membrane, whereas alpha-helical regions require more residues. The region from amino acids 80 through to 95 is in a region containing beta-sheet and thus there are enough residues to traverse the membrane. The second region predicted to be involved in membrane anchoring is also in a section of beta sheet from residue 118 to 139, the transmembrane region is from residue 121 to 130.

Figure 6.10: Predicted secondary structure of the RfbR protein.

The amino acid sequence of the RfbR protein was subjected to analysis using the algorithm of Chou, Fasman and Rose (1978). The protein is 234 amino acids in length.

RfbR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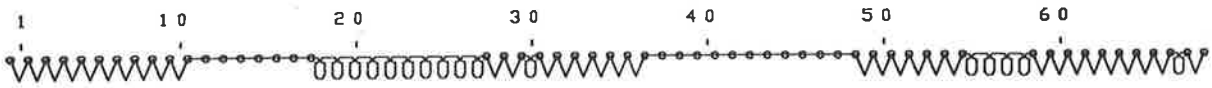
200
210
220
230
HELIX
SHEET
TURN
COIL

Figure 6.11: Predicted secondary structure of the RfbS protein.

The amino acid sequence of the RfbS protein was subjected to analysis using the algorithm of Chou, Fasman and Rose (1978). The protein is 68 amino acids in length.

RfbS



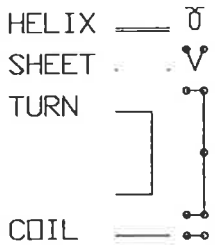
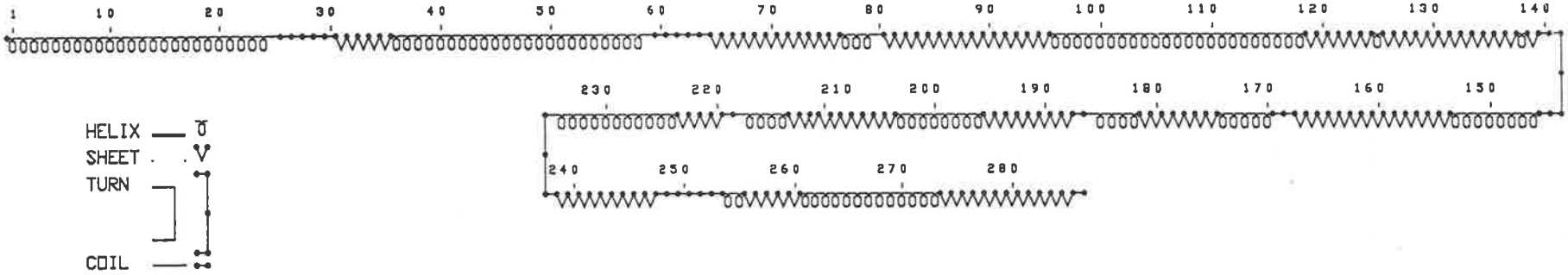


Figure 6.12: Predicted secondary structure of the RfbT protein.

The amino acid sequence of the RfbT protein was subjected to analysis using the algorithm of Chou, Fasman and Rose (1978). The protein is 286 amino acids in length.

RfbT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230
220
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200
190
180
170
160
150
240
250
260
270
280
HELIX
SHEET
TURN
COIL

6.3 Summary and conclusions

The RfbT proteins in *V. cholerae* O1 strains O17 and 569B are 32kDa and 27kDa, respectively, as visualized in *E. coli* minicells. This result was confirmed by the use of specific RfbT antibodies and Western blotting in *V. cholerae* and coincides with the sizes predicted from the nucleotide sequence.

Since the RfbT protein could not be detected by Coomassie blue staining in wild type *V. cholerae* attempts were made to over-express it. The difficulties observed when trying to over-express RfbT suggest that RfbT is not translated efficiently. This is possibly due to the long 5' mRNA leader (see chapter 4), and the poor ribosome binding site prior to *rfbT*, and would appear to play a role in regulating expression of the protein. However, using the T7 promoter/polymerase system, high level expression of RfbT was achieved and this allowed the N-terminal amino acid sequence to be determined. This has confirmed the second of the two possible ATG initiation codons as the correct start. The production of a specific antiserum to RfbT made it possible to determine that the protein is localized in the inner membrane as was predicted from computer analysis of the amino acid sequence. This is the probable site of O-antigen modification i.e. the addition of the B antigen to the LPS.

The labelling of the RfbT protein in *E. coli* K-12 minicells and pulse chase experiments indicates that RfbT is extremely stable. Together with the low level of RfbT protein in *V. cholerae* O1 wild type, this tends to suggest that RfbT is only required in catalytic amounts.

Chapter7

Discussion

7.1 Introduction

V. cholerae O1 can be subdivided into two biotypes, classical and El Tor (Feeley, 1965; Sen, 1969), both of which can cause cholera in humans. These biotypes can be further subdivided into two common serotypes, Inaba and Ogawa, and a third rare serotype, Hikojima.

The serotypes are differentiated by three antigens expressed on the O-antigen of the lipopolysaccharide: Inaba strains express A and C antigens whereas Ogawa strains express A, B and some C antigen. Hikojima strains are unstable but appear to express high levels of all three antigens (Sakazaki and Tamura, 1971). The LPS of *V. cholerae* has been extensively studied and although the three antigenic determinants were described many years ago, the chemical and genetic basis for these determinants is still unknown.

The expression of the B and C antigens is not fixed. Thus, an Ogawa strain can lose the ability to produce the B antigen, and so become Inaba and an Inaba strain can gain this ability, to become Ogawa. However, it is clear that this interconversion between the serotypes is not equal, and the latter is said to occur at a much reduced frequency (Shrivastava and White, 1947; Bhaskaran and Gorrill, 1957; Sheehy *et al.*, 1966; Nobechi and Nakano, 1967). Neither the reason for this switching nor the molecular mechanism involved is understood. This thesis has thought to investigate these various problems.

7.2 Results

7.2.1 The *rfbT* locus of *V. cholerae* O1 and serotype conversion

This laboratory has cloned and sequenced a 20kb *Sac*I restriction fragment from *V. cholerae* strains O17 (Ogawa) and 569B (Inaba) (Manuscripts in prep.). This region encodes the genes, *rfb*, necessary for O-antigen biosynthesis and assembly in *V. cholerae* (Manning *et al.*, 1986; Ward *et al.*, 1987). Analysis of this sequence has allowed several interesting regions to be identified by homology to other known sequenced genes. Thus, it has been possible to predict the genes encoding: perosamine biosynthesis, tetronate biosynthesis and export of the LPS or O-antigen.

The region of greatest divergence between the sequences from O17 (Ogawa) and 569B (Inaba) is in a 3kb *Cla*I fragment. This region was of prime interest, since it was shown to encode the functions required for the expression of the Ogawa serotype, i.e. the B antigenic determinant.

The DNA encoding the structural gene for the *V. cholerae* O1 RfbT protein and its surrounding genes was cloned from strains O17 and 569B into *E. coli* K-12 to generate plasmids pPM2122 and pPM2123, respectively. These plasmids contain the 3kb *Cla*I region which shows the greatest sequence divergence between Inaba and Ogawa. It is this region which is involved in determination of the serotype of *V. cholerae* O1 as either Inaba or Ogawa. The 3kb *Cla*I fragment contains three complete genes *rfbR*, *rfbS* and *rfbT*. Using specific restriction endonuclease generated deletions and insertion mutants, it has been conclusively shown that *rfbT* is the only gene required to convert an Inaba strain expressing A and C antigens to the Ogawa form expressing A, B and C. The product of this gene can convert strains of both the classical (569B) and El Tor (BM69) biotypes from Inaba to Ogawa.

Nucleotide sequence analysis revealed three differences between Inaba (569B) and Ogawa (O17) within the 3kb *Cla*I region. The most striking difference is an 8bp deletion in *rfbR* of 569B. Using an oligo probe spanning this region a number of Ogawa and Inaba strains were probed to establish whether this change is conserved between serotypes. This revealed that not all Inaba strains have the deletion suggesting that the deletion is not a conserved difference between Inaba and Ogawa. Since this deletion, which generates a frameshift within *rfbR*, is present in both Inaba and some Ogawa strains (RJ234) it seems unlikely that *rfbR* plays an essential role in O-antigen biosynthesis.

The second sequence alteration is a polymorphism in the number of *Dra*I sites within the *rfbT* gene in 569B and O17. This arises due to a base substitution (C to A) leading to an amino acid replacement (Glutamine to Lysine). This could be a significant alteration, due to the substitution of a neutral amino acid with a positively charged amino acid. This has been shown to have significant effects in other proteins (Wissmann *et al.*, 1991). *V. cholerae* strains of various serotypes and biotypes were screened by Southern analysis, using pPM2123 as a probe. This has shown that the *Dra*I restriction site polymorphism is also not a conserved variation across serotypes.

The third sequence divergence is a G to T base substitution, which leads to a TGA stop codon in *rfbT* of 569B, resulting in a truncated protein of approximately 27kDa instead of the functional 32kDa protein. PCR was used to amplify the region of interest from a number of Inaba and Ogawa strains. These regions containing the *rfbT* gene were subsequently cloned and sequenced. Sequence analysis of these clones has revealed that Inaba strains appear to have random scattering of changes in *rfbT*, leading to truncated RfbT proteins ranging in size from 17kDa to 30kDa. This implies that even small deletions in the carboxy-terminus of the RfbT protein render it non-functional. The RfbT protein in different Ogawa strains is highly conserved and any sequence divergence leads to a conserved amino acid substitution. In the case of strain H-1, there is a base change from A to G which leads to a change in the codon used but not the amino acid due to the third base wobble.

These data imply that Inaba strains arise from mutations in *rfbT* resulting in a non-functional product. The sequence of the various Inaba and Ogawa strains may explain the ability for serotype conversion to occur in some strains and not in others. The change from Ogawa to Inaba is more common than the converse which is extremely rare (Shrivastava and White, 1947; Bhaskaran and Gorrill, 1957; Sheehy *et al.*, 1966). It is easy to see how a simple change can lead to the inactivation of *rfbT* and a consequent shift in serotype from Ogawa to Inaba. The reverse change from Inaba to Ogawa is thus less frequent since a precise reverting mutation is required in order to restore the gene such that a functional RfbT protein is produced. From the literature it appears that not all Inaba strains can be converted to Ogawa (Sack and Miller, 1969; Sakazaki and Tamura, 1971; Salazar-Lindo *et al.*, 1991). This can be explained by the types and number of mutations within *rfbT*. Some Inaba strains have more than one change in the sequence and thus reversion of these strains to Ogawa would be very difficult since multiple precise changes would have to occur. It is also unlikely that deletion mutations could be reverted. Thus, the discrepancies in the literature (Bhaskaran and Gorrill, 1957; Sack and Miller, 1969; Sakazaki and Tamura, 1971;) as to the possibility of Inaba to Ogawa changes are probably simply due to different types of *rfbT* mutations in the strains being tested. It would be of particular interest to obtain the current *V. cholerae* O1 strains from the South American epidemic and look at their *rfbT* genes, since the epidemic strain has recently undergone serotype conversion from the Inaba to the Ogawa form (Salazar-Lindo *et al.*, 1991).

7.2.2 Involvement of the *rfb* locus in virulence of *V. cholerae*

Studies on *rfb* transposon insertion mutants with a total defect in O-antigen biosynthesis, have shown that the flagella are poorly functional and there are no toxin co-regulated pili (TCP) on the surface of the cell. The transposon insertion mutants of strain

569B (Ward and Manning, 1989) are highly attenuated in the infant mouse cholera model, presumably due to the absence of this colonization factor (Taylor *et al.*, 1987; Sharma *et al.*, 1989) and the reduced motility of these strains. It has been shown that defects in TcpA show marked attenuation when compared to wild type *V. cholerae* (Taylor *et al*,. 1987; R. Faast, personal communication). It appears that complete LPS is required for the correct assembly of TCP since large amounts of the major structural subunit TcpA accumulate in the periplasm. The O-antigen mutants show much reduced swarming on soft agar overlay indicating that incomplete LPS results in a defect in motility. This is possibly due to incorrect flagella assembly or a down regulation of flagellar genes triggered by the change in LPS (Komeda *et al.*, 1977). Alternatively it may be related to the fact that the *V. cholerae* flagella is normally sheathed by the O-antigen. The loss of pili and flagella has been reported in deep-rough mutants of *E. coli* K-12 but not in mutants only affecting the O-antigen. In this case the loss of the glucose-I in the LPS was associated with the loss of flagella, the loss of the pili is probably due to incorrect pilus assembly (Parker *et al.*, 1992).

In contrast to the *rfb* transposon insertion mutants, the *rfbT* mutant is unaffected in O-antigen biosynthesis, except for serotype specificity. The mutant is unaltered in all other respects. Strain O17 and its *rfbT* mutant V874, represent the first true isogenic Ogawa and Inaba strains to be tested for the role of serotype specificity in *V. cholerae* O1 pathogenesis. In naturally occurring Inaba and Ogawa strains it is not possible to rule out that other changes have also occurred. The virulence studies indicate that the B antigen does not affect the virulence of the organism *per se*, in that there is no change in the number or in the rate of death of the infant mice. However, it is tempting to speculate that serotype conversion may play a role in avoiding the immune response of the host. Evidence for this comes from the observation that a relapse in the disease is often associated with a change in serotype (Gangarosa *et al.*, 1967) and that a change in serotype often follows years of comparatively low incidence of cholera (Venkatraman, 1947). In the recent epidemic of cholera in Peru (South America) the very distinctive epidemic strain has changed serotype from Inaba to

Ogawa (Salazar-Lindo *et al.*,1991; K. Wachsmith personal communication). This resulted in increased incidence of cholera of the new serotype. The role of the immune response in selecting serotype convertants is supported by blocking antibody production with cyclophosphamide. In this case the serotype of the excreted organisms does not alter with time (Sack and Miller, 1969). These observations strongly suggest that the B antigen may be important in vaccine development. Serotype specificity does not affect virulence *per se* and converting a strain from one serotype to another is a straightforward but precise event. Consequently it should be possible to readily gain permission to use serotype converted forms of strains which have already been tested in humans (many of which are Inaba strains) (Herrington *et al.*, 1988; Levine *et al.*, 1988; Holmgren *et al.*, 1989; Clemens *et al.*, 1990). This would bypass much of the initial trial work required to test organisms for human therapeutic use.

7.2.3 Transcription and translation of *rfbT*

Sequence analysis of the 5' region preceding *rfbT* has revealed a promoter-like sequence homologous to the *E. coli* consensus sequence for σ^{70} promoters (Rosenberg and Court, 1979; Hawley and McClure, 1983). There is a defined -10 and -35 region with optimal spacing of 17nt. Primer extension carried out on RNA from *E. coli* and *V. cholerae* precisely defined the start of the message to be 99nt from the translational start and demonstrated that the promoter is recognized in both organisms. The +1 predicted from the primer extension was 15 nt from the promoter predicted from the sequence. This spacing is less than optimal and may explain the lack of message in the Northern blot and the problems encountered in the detection of RfbT in *V. cholerae* cells. The presence of a promoter has been confirmed by cloning into the promoter detection vector pPM3024. This reduced the likelihood that the message detected in the primer extension was the result of processing of a

larger mRNA transcript. To further confirm the presence of a promoter in this region RNA polymerase footprinting could be done. To confirm the message start it is possible to do phosphate exchange reaction to determine whether the message detected was either full length or processed.

The 99nt long un-translated 5' end of the mRNA has the potential to form a strong secondary structure (ΔG -27.9Kcal/mol). Both the ribosome binding site and the initiation start codon are involved in forming this secondary structure. This would lead to a reduced rate of translation of the mRNA and may indicate a form of translational control (Hall *et al.*, 1982; McCarthy *et al.*, 1985). This secondary structure may also account for the difficulties encountered while trying to over express RfbT in *E. coli*, and also in the detection in *V. cholerae*.

7.2.4 The RfbT protein

The 3kb *Cla*I fragment contains three complete open reading frames for RfbR, RfbS and RfbT proteins. The RfbR protein does not appear to be involved in either serotype determination or O-antigen production, since an 8bp deletion within the gene has been detected in both Ogawa and Inaba strains. *rfbS* appears to be translationally coupled to *rfbR* and its product plays no role in serotype specificity and probably plays no role in O-antigen production. However, no chromosomal mutations have been isolated to confirm this. RfbT has been shown to be solely responsible for the expression of the B antigen. Initial attempts at over-production produced very little or no detectable protein. The T7 promoter/RNA polymerase system (Tabor and Richardson, 1985) yielded significant amounts of protein although RfbT was almost insoluble and could not easily be purified from the membrane. This is presumably due to the formation of intracellular inclusion bodies which co-fractionate with the membrane (Schoner *et al.*, 1985; Tommassen, 1986). This protein was used for

immunization to generate a specific antiserum and for N-terminal analysis to confirm the correct translational initiation site. The RfbT protein was localized to the inner membrane in *E. coli* using a strain which over-expressed the protein, but this localization could be misleading due to the insoluble nature of the overproduced RfbT (Tomassen, 1986). By using the antiserum raised to partially purified RfbT the protein could be localized in *V. cholerae*, where it was detected exclusively in the inner membrane fraction of both strains O17 (Ogawa) and 569B (Inaba). The membrane localization of both the 32kDa O17-form and 27kDa 569B-form indicates that the membrane anchoring domain(s) is within the first 27kDa. The region between residues 86 and 98 is hydrophobic and could represent such a membrane spanning domain. A further region, from residue 121 to 130 also has this potential. These domains are predicted to form a beta-sheet region and nine amino acids are enough to transverse the membrane (Engelman and Zaccai, 1980; Engelman *et al.*, 1980; Paul and Rosenbush, 1984). The distribution of the arginine and lysine residues in RfbT suggests that the protein has a large periplasmic domain, with only residues 100 to 120 in the cytoplasm (Fig. 7.1). This is based upon observations of other cytoplasmic membrane proteins in which the arginine and lysine residues are found predominantly in the periplasmic space (von Hejine, 1986). This correlates with the suggestion that the O-antigen is modified with the B antigen post-synthetically, since the B antigen is not required for O-antigen production. If the modification occured during O-antigen synthesis then removing the B antigen by inactivating *rfbT* should lead to the accumulation of incomplete O-antigen repeat units. Thus, the B antigen should first appear in the periplasm upon O-antigen modification. The topology of RfbT with respect to the cytoplasmic membrane is of great interest and could be probed by using *phoA* and *lacZ* gene fusions to *rfbT* (Manoil and Beckwith, 1985, 1986; Taylor *et al.*, 1987, 1989; Manoil *et al.*, 1990).

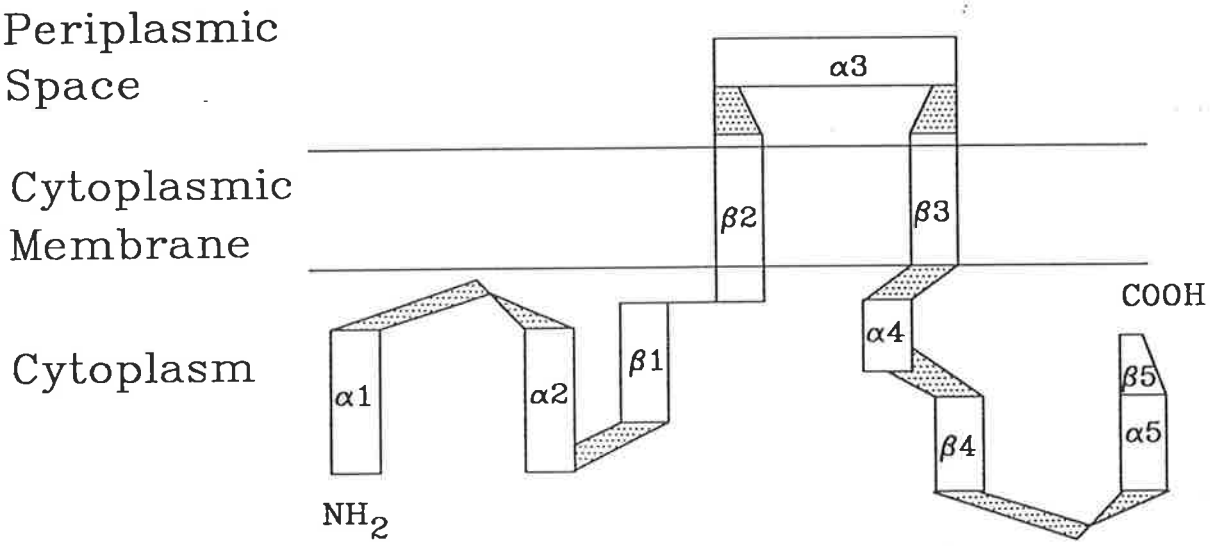
The other two proteins within the 3kb *Cla*I fragment, RfbR and RfbS, appear to be only weakly expressed in the T7 promoter/RNA polymerase system. Without the use of plasmids lacking RfbR and RfbS the possible protein products seen in the T7 expression

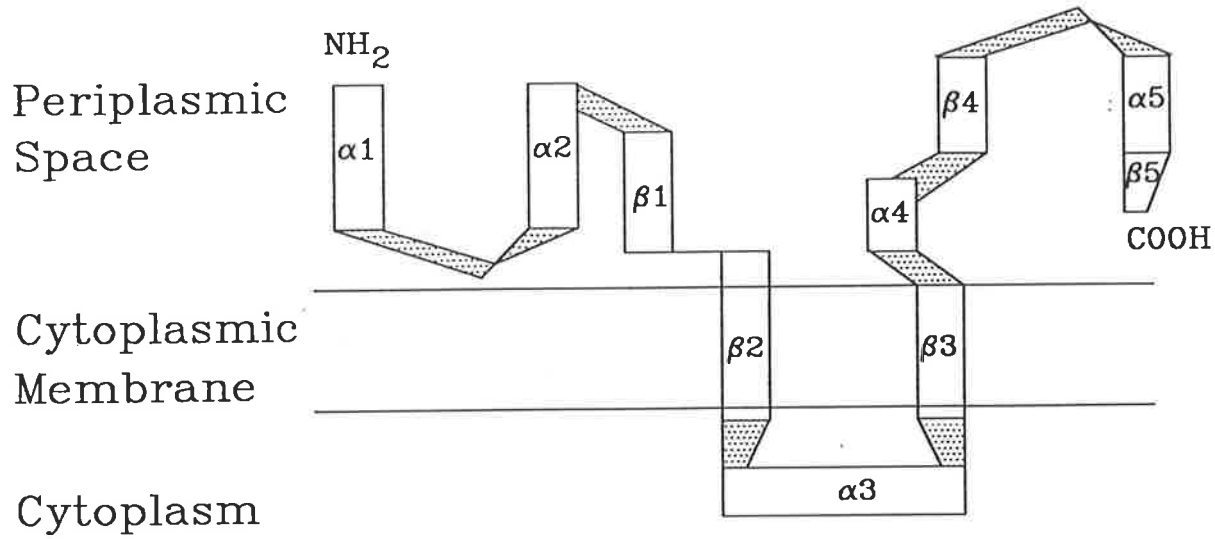
Figure 7.1: Proposed model of the membrane topology of RfbT.

The two possible membrane topology of RfbT was determined using the secondary structure and hydropathy plot. The majority of RfbT must either be in the cytoplasm (top) or periplasmic (bottom). Using the rule from von Hejine (1986) the majority of the protein should be in the periplasmic space.

Domain	Residue no.
α1	1-23
α2	36-59
β1	65-77
β2	81-95
α3	96-119
β3	120-139
α4	148-155
β4	207-225
α5	255-273
β5	275-286

This membrane topology is speculative and the actual topology could be evaluated genetically using *lacZ* and *phoA* gene fusions (Manoil *et al*,. 1990).


Periplasmic
Space
Cytoplasmic
Membrane
Cytoplasm
α3
β2
β3
COOH
α4
β5
α5
β4
α1
α2
β1
NH2


NH2
Periplasmic
Space
α1
α2
β1
β4
α5
β5
α4
COOH
Cytoplasmic
Membrane
β2
β3
α3
Cytoplasm

system are only speculative. The reason for the poor expression is probably due to the fact that *rfbR* and *rfbS* are translationally coupled with the upstream *rfb* genes which are not present on the constructs used. Therefore there are no translational signals available for *rfbR* and *rfbS*. Examination of the sequence does not reveal a good RBS prior to either *rfbR* or *rfbS*.

7.2.5 Characterization of the A, B and C antigens

The three antigens expressed on the *V. cholerae* O1 LPS have not been chemically or structurally defined. The use of serotype-converted strains may help in the identification of these antigens. The serotype converted strains and the *rfbT* mutant V874 only vary in their expression of the B and C antigens. Thus, there are no other detectable alterations in the LPS which may be present in naturally occurring Inaba and Ogawa strains, so any differences seen when analysing the LPS from the serotype-converted strains must be due to the expression of the B and/or C antigen(s).

The Inaba strains 569B and BM69 were serotype-converted to express all three antigens A,B and C. Some of these strains were used to extract LPS (J. Redmond Macquarie Uni. Sydney). The LPS was chemically degraded (Fig. 7.2) and analysed by NMR and HIA assays. The cleavage of the fatty acids from the LPS by hydrazine treatment did not destroy the three antigens when tested by HIA assays. All other treatments destroyed the antigens indicating that these antigens are extremely labile. No difference could be detected in the serotype-converted strains compared to the parent when analysed by NMR using either intact or degraded LPS (J. Redmond, personal communication). The reasons for this may be that the B antigen is a single epitope per LPS molecule and is thus present below the level of detection, or that this antigen is a very subtle change, such as an optical isomer (Guhathakurta *et al.*, 1986) or a modification in the type of linkage between the sugars of the

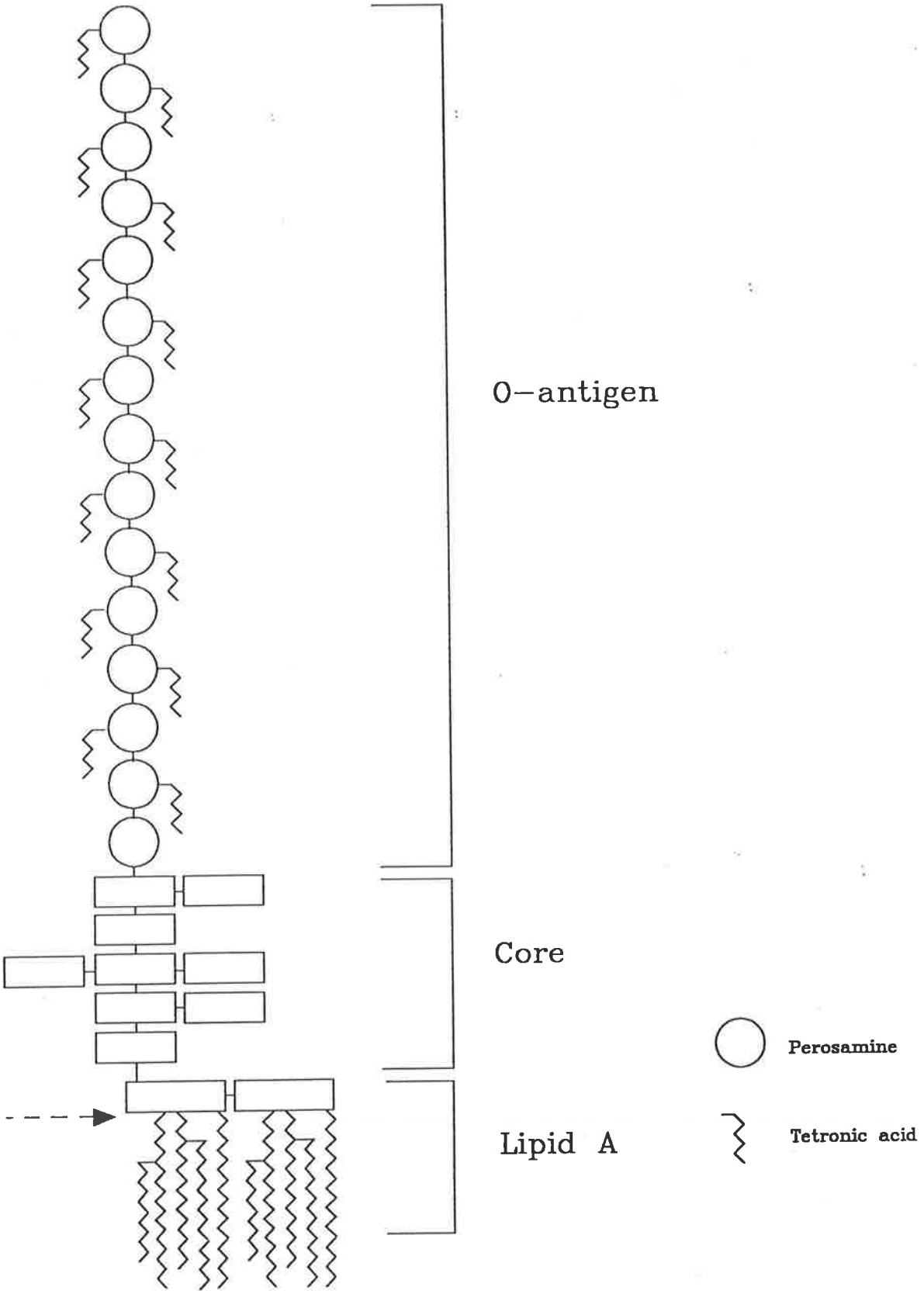
Figure 7.2: Schematic representation of *V. cholerae* O1 LPS.

The LPS of *V. cholerae* is made up of three distinct regions: The O-antigen, the core-oligosaccharide and the lipid A. The LPS was extracted from acetone dried cells and subjected to various chemical treatments.

The hydrazine treatment cleaves the LPS molecule at the fatty acids in the lipid A (dashed arrow) and at the tetronate substituting the perosamine residues. This treatment is designed to cleave of all fatty acid residues.

Periodate treatment is specifically designed to remove the tetronate without affecting the remaining fatty acids.

These treatments were carried out by J. Redmond (Macquarie University Sydney).


O–antigen
Core
Lipid A
Perosamine
Tetronic acid

O-antigen. Analysis of wild type Inaba and Ogawa strains by silver staining reveals no difference in the banding pattern of the O-antigen (Gosh and Campbell, 1985). This is further supported by silver stain analysis following SDS-PAGE of LPS from serotype-converted strains, indicating that the B antigen does not significantly alter the molecular weight of the O-antigen. Identification of these antigens may be possible with further work using other degradation protocols in conjunction with HIA assays, NMR and the use of the *rfbT* mutant.

Bacteriophages which use LPS as their receptor usually have tail fiber associated enzymes which degrade the polysaccharide chain (Lindberg, 1977). Thus the use of *V. cholerae* bacteriophages such as CP-T1 and VcII could also be expected to sequentially degrade the LPS yielding soluble O-antigen fragments. These soluble fragments can be fractionated and can be tested for the presence of the A, B and C antigens by HIA. In conjunction with silver staining and western blotting of the insoluble fraction the position of the antigens on the O-antigen may be defined i.e. whether the B and C antigens are on the distal perosamine or randomly distributed along the O-antigen. The soluble material generated in this way could be further analysed by NMR to possibly define the antigens themselves.

7.2.6 Expression of the B antigen

Since Inaba strains have high levels of C antigen and no B antigen, and Ogawa strains have lower levels of C and high levels of B, the expression of these antigens may be related. It is possible that the B antigen is a modified form of the C antigen, or that the B antigen partially masks the C antigen on Ogawa cells. To examine this possibility the level of expression of the three antigens A, B and C was examined using HIA assays and immunogold electronmicroscopy. HIA assays using purified LPS from wild type and

serotype-converted strains indicated that although, Inaba strains have higher levels of C antigen, this does not change in the serotype converted strains which express all three antigens. Thus, the B antigen does not appear to be a modified form of C nor does the B antigen mask the C antigen. This suggests that the B antigen is independent of the expression of the other two antigens, A and C. The serotype-converted strains appear to express lower levels of the B antigen compared to wild type Ogawa strains. This is due to,plasmid instability. The level of A antigen remains constant in all strains regardless of whether or not they contain the cloned *rfbT* genes. The A antigen is thus the true common or group specific antigen (Holme and Gustafsson, 1985).

The HIA assays were carried out using two fold serial dilution of the antigen (purified LPS), which limits the detection sensitivity to a two fold difference in the expression of the antigens. For this reason the results of the HIA assays were checked by immunogold electronmicroscopy. The use of monoclonal antibodies in immunogold electron-microscopy allows more precise quantitation of the antigens. These results indicate that the expression of the B antigen is independent of the C antigen, since the level of the C antigen does not alter significantly upon the expression of the B antigen in the serotype-converted strains. The serotype-converted strains express lower levels of the B antigen, which may be due to plasmid instability and thus loss of the Ogawa *rfbT* gene. The plasmid stability is approximately 60% after overnight incubation. It has been observed in other species, such as *Shigella flexneri* and *Salmonella typhimurium*, that Ap^R plasmids can be unstable (R. Morona personal communication).

The most interesting finding was that the *rfbT* mutant, V874, expressed much higher levels of the C antigen when compared to its wild type parent O17. This result contradicts the HIA and immunogold studies on the other strains and indicates that the expression of the C and B antigen are somehow linked, either due to conversion of C or by masking of the C antigen. This contradiction is in part due to the lack of plasmid stability. To confirm this

result it is nesessary to construct a chromosomal serotype converted strain from Inaba to Ogawa and subsequently check the expression of the B and C antigens.

7.3 Concluding Remarks and Future work

The mechanism of *V. cholerae* O1 serotype variation has been the subject of controversy for many years. Initial reports found serotype conversion occurred only from the Ogawa to the Inaba form,and suggestions of the reverse switch were met with scepticism. The switch from Inaba to Ogawa is now accepted, as a low frequency event although its relevance in the pathogenesis of the organism is still open to question.

In this study, it has been shown conclusively that the switch from the Inaba to Ogawa form and the consequent expression of the B antigen is mediated by a single 32kDa protein (RfbT). The gene encoding this protein is found at the end of the *rfb* gene cluster of *V. cholerae* which encodes the genes for the enzymes required for O-antigen biosynthesis. The *rfbT* gene appears to be distinct from the other *rfb* genes in a number of ways. Its low G+C content of 32% suggests it may have been acquired from a non-vibrio source, since we have seen only one other protein with such a low G+C content (TcpN)(Ogierman and Manning, 1992). It is possible that the low G+C content which causes the codon usage of RfbT to be distinct is a form of translational control. This correlates with the fact that *rfbT* has its own promoter which is independent of the rest of the *rfb* operon and the 2^{o} structure of the mRNA prior to *rfbT*. The less than optimal spacing of the -10 region possibly plays a major transcriptional role. Furthermore the possibility of co-transcription from the other *rfb* genes cannot be ruled out. Thus, there may well be both translational and transcriptional regulation.

Cloning and sequencing of the *rfbT* genes from a variety of Inaba and Ogawa strains has revealed a number of interesting points:

1. the *rfbT* gene is highly conserved in Ogawa strains and

2. the *rfbT* gene from four Inaba strains have random mutations leading to truncated RfbT proteins.

3. This has permitted a possible mechanism for serotype conversion to be proposed.

From the sequence data it can be seen that a simple base change can convert a strain of the Ogawa serotype to Inaba. However, for the reverse switch a precise or a number of changes have to occur, depending on the nature of the mutation(s) in the Inaba strain. This may explain why some Inaba strains appear to be able to serotype convert to Ogawa whereas others, such as deletion mutations, cannot. Recent work examining strains from the South American epidemic has shown that the serotype switch can occur in nature (K. Wachsmith, personal communication).

The use of specific antiserum to RfbT has allowed the protein to be localized to the inner membrane. It is tempting to speculate that the modification of the O-antigen by the addition of the B antigen occurs at either the inner membrane prior to export of the LPS or in the periplasm. The use of *lacZ* and *phoA* fusions to *rfbT* will help in determining the membrane topology of this protein and more accurately implicate in which cellular compartment the modification occurs.

Since the expression of the B antigen is not necessary for the export of the LPS to the cell surface, i.e. it seems to be a postsynthetic modification, it provides an excellent marker for following O-antigen synthesis and transport. These are areas which are extremely poorly understood even in organisms such as *E. coli* and *S. typhimurium*, where there are extensive data (Muelhradt *et al.*, 1974; Bayer *et al.*, 1975; Osborn, 1979; Rothfield *et al.*, 1987; Raetz, 1990; Osborn *et al.*,1991). The synthesis and incorporation of the B antigen could be followed using a strain in which *rfbT* was under the control of an inducable regulatable promoter. Upon induction of *rfbT*, localization of the B antigen could be performed on cell sections using immunogold electronmicroscropy with the anti-B monoclonal antibody. Transport of the B antigen to the cell surface could be traced with time after *rfbT* induction.

Mutations affecting the ability to synthesize O-antigen have a marked effect on the virulence of *V. cholerae* O1, but a mutation leading to the loss of the B antigen has no effect on virulence in the infant mouse cholera model. It is possible that altered expression of the B antigen may be used as a way of evading the immune response of the host. Manning *et al.* 1986 showed that antibodies to O-antigen were protective and that there was cross protection between serotypes. Homologous protection was better than heterologous and this implies that the B and C antigens must be protective.

To date, the use of isogenic strains ($RfbT^+$ and $RfbT^-$) for the preparation of LPS for chemical degradation has not identified any of the three antigens. It appears as though the three antigens A,B and C are very labile, and are readily destroyed during chemical modification. Expression of the B antigen does not involve a major change that can be detected by either silver staining or NMR analyses. It would be of interest to use the O-antigen from the *rfbT* mutant in the NMR since it shows a complete lack of the B antigen but very high levels of the C antigen. The mutant (V874) thus shows a greater degree of change in the expression of the C antigen compared to the plasmid-mediated serotype converted strains. Furthermore the level of the B antigen on the O17 parent is higher than on the plasmid-converted strains. The difference in the level of expression may be due to plasmid loss in the serotype-converted strains. Thus, not all of the organisms are actively producing the B antigen.

The use of HIA assays and immunogold electronmicroscopy indicated that the C and B antigens are independent in their expression. However problems associated with these experiments were:

1. the sensitivity of the HIA assay, and
2. the plasmid stability in plasmid converted strains.

To overcome these problems ideally a strain would have to be serotype-converted by chromosomally integrating the *rfbT* gene from an Ogawa strain into an Inaba strain (as mentioned previously). The data from the V874 *rfbT* mutant contradicts the HIA and

immunogold studies but probably represents a more accurate picture of serotype conversion. For this reason the expression of the B and C antigen is likely to be linked.

The system of serotype conversion in *V. cholerae* O1 is distinct from any of the previously discussed organisms such as *Sh. flexneri*, *S. typhimurium*, *N. gonorrhoeae* and *H. influenzae* (Chapter 1). In *V. cholerae* the serotype change is not associated with a specific mechanism but rather by random mutations within the *rfbT* gene which are selected by the immune response of the host.

This thesis has resolved the paradoxical nature of the interconversion between the serotypes of *V. cholerae*. This conversion has been shown to be mediated by the acquisition or reversion of mutations in the *rfbT* gene, whose product probably functions as the final modifier of the LPS molecule prior to its export to the cell surface. These studies provide a concrete basis for future work to aid in the elucidation of LPS biogenesis.

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