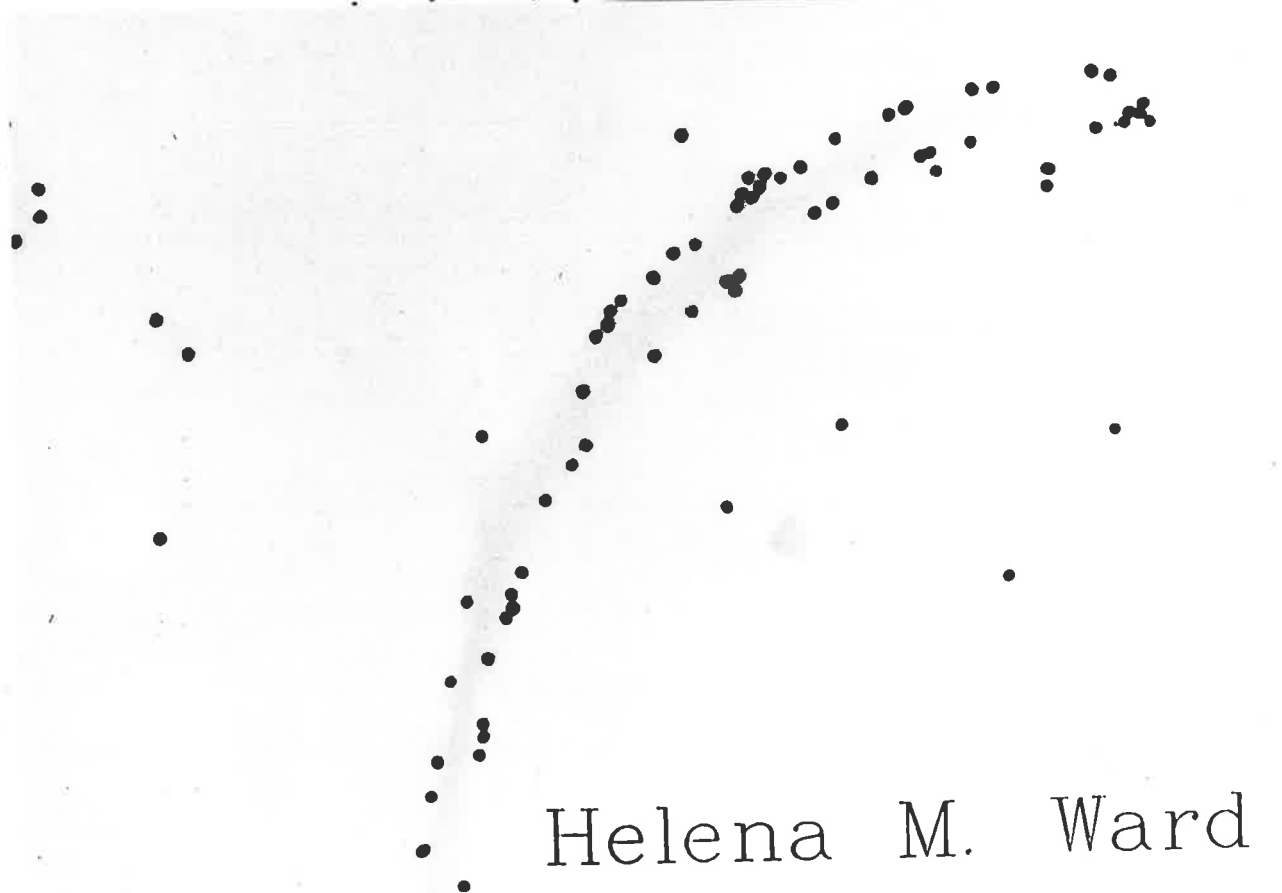# Serotype variation

in *Vibrio cholerae*

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Serotype Variation in <u>Vibrio</u> <u>cholerae</u>

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STATEMENT

I state that 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 any other person, except where due reference is made in the text.

Helena Ward
September, 1988

ACKNOWLEDGEMENTS

LIST OF ABBREVIATIONS

ACL	:	Antigen carrier lipid/undecaprenol phosphate
Ap	:	ampicillin
Ara	:	arabinose
BA	:	Bacterial agglutination
BHI	:	Brain heart infusion
bp	:	base pairs
Cml	:	chloramphenicol
Etn	:	ethanolamine
Gal	:	galactose
Glc	:	glucose
GlcNAc	:	N-acetyl-glucosamine
HA	:	Haemagglutination assay
Hep	:	L-glycero-D-mannoheptose
HIA	:	Haemagglutination inhibition assay
kb	:	kilobases
KDO	:	2-keto-Deoxyoctonic Acid
km	:	kanamycin
lac	:	lactose
LPS	:	Lipopolysaccharide
McAb	:	Monoclonal antibody
Man	:	mannose
MDal	:	Megadalton
M	:	Molecular weight
NB	:	Nutrient Broth
nm	:	nanometer
N.M.R.	:	Nuclear magnetic resonance
O-Ag	:	O-antigen
O-PS	:	O-specific side chain polysaccharide
PxB	:	polymixin B
Rha	:	rhamnose
Spc	:	spectinomycin
SRBC	:	Sheep red blood cells
Str	:	streptomycin
Tc	:	tetracycline
Tn	:	Transposon
ts	:	temperature sensitive

ABSTRACT

Serotype variation in <u>Vibrio</u> <u>cholerae</u>

The genes encoding the biosynthesis of the O-antigen of the lipopolysaccharide (LPS) of <u>V</u>. <u>cholerae</u> O1 have previously been cloned and expressed in <u>E</u>. <u>coli</u>K-12. This study has sought to characterize these clones and analyse the expression of the O-antigen specific determinants in these clones and in <u>V</u>. <u>cholerae</u> and to define the chromosomal region associated with serotype specificity.

Using both polyclonal and monoclonal antibodies to the various O-antigenic determinants it has been possible to show by haemagglutination inhibition assays and by immuno-electron microscopy with protein A-gold, that the cloned genes in <u>E</u>. <u>coli</u>K-12 are expressing LPS of the appropriate serotype. These results demonstrate that Ogawa cells express small amounts of the Inaba antigen (C), but that the Ogawa antigen (B) is specific. These antigens are not detected on strains lacking the O-antigen.

The cloned genes have been studied by restriction analysis, Southern hybridizations and by electron microscopy of homo- and heteroduplexes. These data enable a map of the contiguous chromosomal DNA to be derived and by deletion analysis and subcloning of the DNA, the minimal region necessary for expression of the <u>V</u>. <u>cholerae</u> O-antigen in <u>E</u>. <u>coli</u>K-12 could be defined as >16kb of a 19.5kb <u>Sst</u>I fragment, and by analogy with other organisms represents the <u>rfb</u> region.

A number of independent Tn5 and Tn2680 insertion mutations affecting LPS expression have been isolated in Inaba and Ogawa V. cholerae strains. These mutants have been characterized by bacterial agglutinations, resistance to vibriophages, sensitivity to dyes, detergents and antibiotics and by SDS-PAGE of whole membrane and LPS preparations. The minimal SstI piece has been used as a probe to map, by Southern hybridization, the sites of insertion of the various Tn mutations. Most insertions map within this region, but several map in adjacent DNA, suggesting that the actual rfb region is close to 25kb in V. cholerae and that E. coliK-12 can complement the loss of this extra region. Other mutations map in genes outside of rfb which are presumably associated with other aspects of LPS biosynthesis.

The Tn insertion mutants have provided suitable markers to enable the construction of donor strains. These have enabled the rfb locus to be mapped on the V. cholerae chromosome by conjugation to between ilv and arg and this corresponds to a locus associated with serotype variation, previously described as oag.

CHAPTER 1

INTRODUCTION.

1.1   Review of LPS structure and biosynthesis

   1.1.1 LPS Structure - Introduction

LPS is one of the major constituents of the outer membrane of Gram negative bacteria and analysis of LPS from a variety of bacterial species has revealed a number of common structural features. Three major structural regions have been defined, as shown in Fig. 1.1. These are the lipid A which is inserted in the outer membrane, the core sugars attached to lipid A, and the O-antigen or O-polysaccharide linked to the core. The O-specific poly-saccharide is that part of the LPS molecule which extends outward from the cell surface and carries much of the serological O-specificity of Gram negative cells. The core oligosaccharide has been studied in LPS from Salmonella, E. coliK-12 and other Gram negative bacteria (Jann and Westphal, 1975; Lüderitz et al., 1971). In many of these core oligosaccharides, the sugars galactose, glucose, heptose and N-acetyl glucosamine have been found. The core oligosaccharide is linked to lipid A by an acid labile linkage with KDO.

   O-polysaccharides from Gram negative bacteria

Before discussing the biosynthesis of LPS, the struc-ture of the O-specific polysaccharide from a number of bacterial species will be described.

Salmonella: One of the earliest studies on Salmonella LPS was performed by Kauffman (1961). Analysis of Salmonella groups A, B, D and E revealed the presence of a repeat unit of mannose- rhamnose- galactose. This backbone may be substituted at various sites with sugars such as abequose, tyvelose and paratose (Lüderitz et al., 1971; Jann and Westphal, 1975). For instance, in S. typhimurium (O group B) the basic repeat unit in the O-antigen is a tetramer consisting of abequose, mannose, rhamnose and galactose (Mäkelä and Stocker, 1984). The Kauffman-White classification allows the various Salmonella to be assigned to particular O groups.

Subsequent studies (Lüderitz et al., 1966) revealed that these classifications can be correlated with the carbohydrate composition of the O-antigen. Thus, the A and D group O-antigens can be differentiated from B group O-antigen (containing abequose), by the presence of paratose and tyvelose, respectively. Additional side groups may be present on the O-antigen and these often contribute to new antigenic specificities (Lüderitz et al., 1971). Glucosyl residues and O-acetyl groups are examples of side groups which may be associated with Salmonella LPS (Robbins and Wright, 1971).

A recent study of Salmonella groups A, B and D has been reported, in which the rfb regions from S. typhi and S. paratyphi A were cloned and compared with those of S. typhimurium (Verma et al., 1988). Southern hybridization and heteroduplex analysis of rfb DNA revealed that the

three groups shared considerable homology. Thus, the presence of the identical trisaccharide backbone in these three groups reflects a fundamental degree of relatedness amongst these Salmonella species.

A number of Salmonella O-antigens have quite different backbone structures, such as groups C, G, N, U and L. The various O-antigens in these groups do not appear to be related (Lüderitz et al., 1971; Jann and Westphal, 1975). O-antigens from C1 group Salmonella lack rhamnose completely, with a repeat unit consisting of four mannose residues and one N-acetylglucosamine, whereas the O-antigens from S. greenside and S. adelaide closely resemble those of E. coli O55 and O111, respectively.

The core oligosaccharide was found to be identical in all Salmonella and consists of KDO, heptose and a pentosaccharide (Fig. 1.1) (Lüderitz et al., 1971; Robbins and Wright, 1971; Jann and Jann 1984; Mäkelä and Stocker, 1984).

Citrobacter: Many serological cross-reactions have been described between Citrobacter and Salmonella (Edwards and Ewing, 1966). Chemical analyses of the LPS have shown that some cross-reacting Citrobacter strains have the same chemical composition as those of the Salmonella strains (Jann et el., 1978; Yuan and Harecker, 1968).

Klebsiella: The LPS of Klebsiella have been classified into 12 O groups. Seven of the O-specific polysaccharides are homopolysaccharides, that is, the repeat unit consists of one sugar only (Nimmich and Korten,

1970). For instance, groups 03 and 05 have mannose as the sole sugar constituent of the repeat unit. A number of Klebsiella antigens are very similar to certain E. coliK-12 antigens.

Escherichia coliK12: The first classification of O groups in E. coli was outlined by Kauffman (1954, 1961) and resulted in the description of groups 01 to 0110. Subsequent analysis allowed the various types of E. coli O-antigen to be divided into three groups (Ørskov and Ørskov, 1977; Jann and Jann, 1984).

The first group includes the neutral homopolysaccharides, which consist of only two groups 08 and 09. Both groups contain O-antigens consisting entirely of mannan polymers and are very similar to the 03 and 05 groups of Klebsiella, respectively (Ørskov and Ørskov, 1977). The second group consists of neutral heteropolysaccharides which have repeat units composed of up to six different sugar constituents, which can include unusual amino sugars.

One strain from this group, 0111, produces O-antigen in two forms, one attached to the lipid A-core and the other "free" O-antigen (Goldman et al., 1982). Serotypes 0100 and 0113 have been reported to have similar arrangements of O-antigen (Jann et al., 1970).

The most recent group of E. coli serotypes to be identified was that containing the acidic polysaccharides (Ørskov and Ørskov, 1977; Achtman et al., 1983). The O-antigens of these strains contain acid residues includ-

ing hexuronic acids, neuraminic acid, and hexolactic acids. Hexolactic acids are structurally similar to muramic acid, one of the major components of peptido-glycan. Examples of E. coli serotypes with acidic polysaccharides are O58 and O124, which have identical O-antigens to Sh. dysenteriae 5 and 3, respectively (Dmitriev et al., 1975) and are associated with dysentry.

There is also a level of heterogeneity in the core structures of E. coli LPS. Five different core types have been identified (Mäkelä and Stocker, 1984). The structures of these E. coli cores differ from that found in Salmon-ella, mainly in the hexose region. For example, E. coli types R1, R3 and R4 lack the terminal N-acetylglucosamine which is present in the Salmonella core (Ørskov and Ørskov, 1977; Jansson et al., 1981; Mäkelä and Stocker, 1984).

Shigella LPS

Sh. flexneri: represent a serologically heterogeneous group. The Sh. flexneri serotypes are defined on the basis of type antigens (designated by Roman numerals) which are shared between members of the same serotype and group antigens (Arabic numerals) which are shared by members of different serotypes (Carlin and Lindberg, 1987).

The LPS of Sh. flexneri can be divided into two regions, the common basal structure and the O-side chains. The basal polysaccharide chain is common to all serotypes, except serotype 6, and consists of KDO, heptose, glucose,

galactose and N-acetylglucosamine. This is identical to the core sugar structure from E. coli R3 (Simmons and Romanowska, 1987).

The O-side chains in Sh. flexneri contain six to eight repeating pentosaccharide units. These include a primary unbranched chain (the variant Y antigen) and secondary side chains of α-D-glucosyl and O-acetyl residues.

The variant Y-structure consists of tetrasaccharide repeat units including L-Rha and β-D-GlcNAc with the following formula.

$$\rightarrow 3)-\beta-D-GlcNAc^-(1\rightarrow 2)-\alpha-L-Rha\ I^-(1\rightarrow 2)-\alpha-L-Rha\ II^-$$
$$(1\rightarrow 3)-\alpha-L-Rha\ III-(1\rightarrow$$
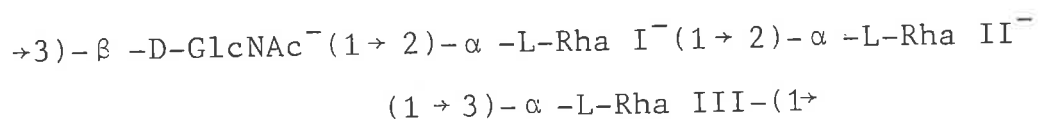
(Jann and Jann, 1984)

The secondary side chain determinants consist of glucosyl or O-acetyl groups substituted at various positions on the primary variant Y chain (Simmons and Romanowska, 1987). Sh. flexneri serotype 6 has been shown to have a unique LPS structure compared with the other groups. There are a number of different biotypes within this serotype (Brenner, 1976). Serotype 6 is thought to cross-react with other Sh. flexneri serotypes, due to a common 2-O-substituted α-L-rhamnosyl residue (Simmons and Romanowska, 1987).

Recent studies on Sh. flexneri LPS have involved the use of monoclonal antibodies (McAbs) directed against various components of the Sh. flexneri O-antigen (Carlin

and Lindberg, 1987). Such analysis has allowed studies of the group antigen 1 to be made. This epitope was previously uncharacterized and was shown by agglutination assays to be common to Sh. flexneri and Sh. dysenteriae type 1. This antigenic determinant named 4X has not yet been defined on a structural basis.

Sh. dysenteriae I has been associated with a severe form of dysentry. A 6 MDal plasmid has been associated with LPS biosynthesis in Sh. dysenteriae I W30864. It should be noted that this group includes at least five species (Watanabe and Timmis, 1984). The O-antigen of Sh. dysenteriae I W30864 has been shown to consist of tetrasaccharide repeat units made up of L-Rha, D-Gal and GlcNAc in a 2:1:1 ratio. The core is linked to the galactose residue (Dmitriev et al., 1976; Sturm et al., 1986b). Sh. dysenteriae species of other serotypes do not appear to carry the 6 MDal plasmid (Watanabe et al., 1984).

In Sh. sonnei all the virulent strains form a single serotype, unlike other Shigella species (Sansonetti et al., 1981). The LPS of Sh. sonnei forms a characteristic cell surface antigen known as Form I antigen. The Form I antigen contains O-side chains consisting of disaccharide repeat units. These repeat units include two unusual amino sugars: $2-NH_2$-deoxy-L-altruonic acid and 2-acetamido-4-NH_2-$2,4,6$-trideoxy-D-galactose (Kopecko et al., 1981; Seid et al., 1984). Genetic studies have shown the Form I antigen is encoded by a 120 MDal plasmid. Loss of this plasmid is associated with conversion of the smooth,

virulent, Form I Sh. sonnei to the rough avirulent Form II type (Formal et al., 1981; Kopecko et al., 1981; Sanson-etti et al., 1981).

Sh. boydii consists of 15 serotypes. The structure of one of these serotypes, 6, has been determined and is composed of repeat units which include Gal, Glu, Rha and Man in a ratio of 2:1:1:1 (Dmitriev et al., 1975).

1.1.2 Antigen carrier lipid

The antigen carrier lipid (ACL) is a C55-polyiso-prenoid alcohol, undecaprenolphosphate. This molecule is also associated with capsules, teichoic acids and peptido-glycan biosynthesis (Mäkelä and Stocker, 1984; Rick, 1987; Raetz, 1987). The ACL is involved in a cycle at the inner surface of the cytoplasmic membrane in which monosacchar-ides are transferred from ACL-P and oligo- and polysacchar-ides are transferred from ACL-P-P- derivatives.

1.1.3 Biosynthesis of lipid A-core

The core of the LPS can be divided into inner and outer regions as shown in Fig. 1.1. The inner core consists of lipid A, L-glycero-D-mannoheptose and KDO. KDO links lipid A to the polysaccharide part of LPS and is sometimes substituted with phosphate and ethanolamine (Jann and Jann, 1984; Raetz, 1987).

Lipid A represents the hydrophobic region of the LPS molecule and its structure appears to be well conserved amongst Gram negative bacteria (Raetz, 1987). Lipid A

Figure 1.1: Structure of <u>S</u>. <u>typhimurium</u> lipopolysaccharide showing
             the inner and outer core. The chemotypes of mutants
             blocked at various stages of core biosynthesis are
             indicated by broken lines. Reproduced from Rick (1987).

Abbreviations: Abe: abequose; Man: D-mannose; Rha: L-rhamnose; Gal:
   D-galactose; GlcNAc: N-acetyl-D-glucosamine; Glc: D-glucose;
   Hep: L-glycero-D-mannoheptose; KDO: 3-deoxy-D-mannooctulosonic
   acid; Etn: ethanolamine; P: phosphate.
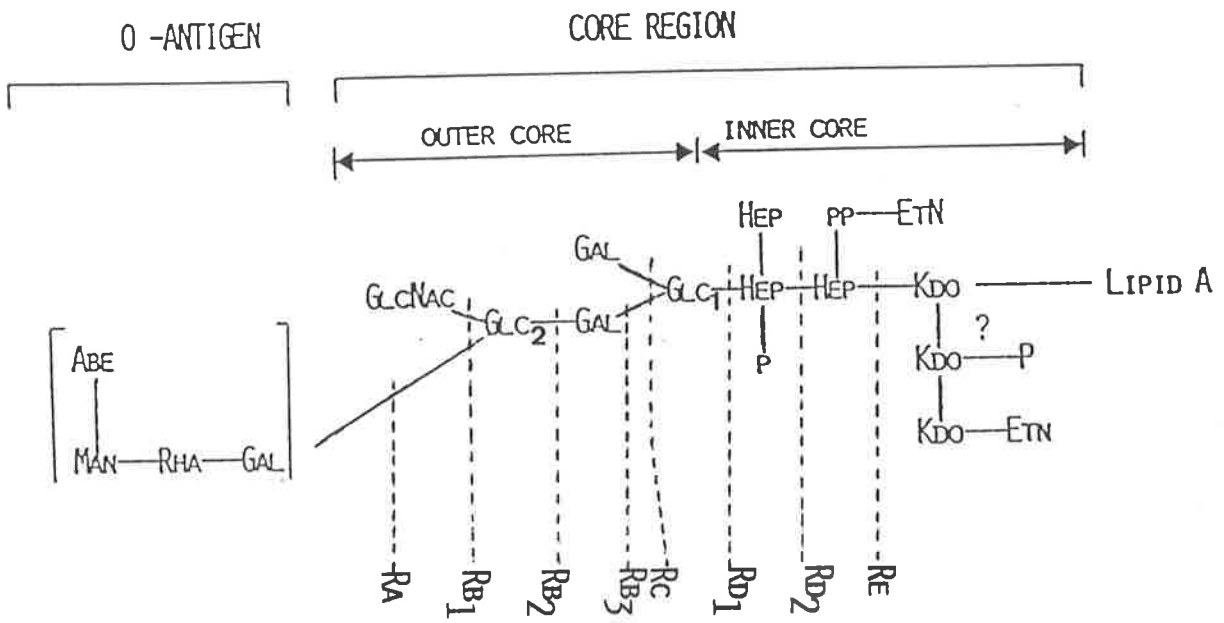
consists of a glucosamine disaccharide linked at $\beta 1 \rightarrow 6$ and phosphorylated at positions 1' and 4'. This disaccharide molecule is acetylated with four β-OH myristic acid residues and two short chain fatty acids (Lüderitz et al., 1973; Rick and Young, 1982; Mäkelä and Stocker, 1984).

The biosynthesis of lipid A takes place in a series of reactions which start from UDP-N-acetylglucosamine (UDP-N-GlcNAc). This pathway occurs in the cytoplasm. UDP-GlcNAc is acylated, then hydrolysed to produce 2,3 diacyl-Glc-1-P. This is catalysed in the disaccharide lipid A molecule by the disaccharide synthetase (Crowell et al., 1986).

KDO occurs as a linear trisaccharide in which the terminal KDO residue is substituted with heptose (Brade et al., 1983; Brade and Rietschel, 1984). The biosynthesis of KDO takes place in three sequential reactions starting from D-ribulose-5-P. The free KDO produced is then converted to CMP-KDO which acts as the donor of KDO residues for the inner core (Lehmann and Osborn, 1977).

Transfer of KDO to lipid A in vivo occurs before the addition of saturated fatty acid residues (Walenga and Osborn, 1980).

L-glycero-D-mannoheptose is synthesized from sedo-heptulose-7-phosphate (Coleman, 1983). It is thought that ADP-L-glycero-D-mannoheptose acts as the donor of residues to the inner core.

Biosynthesis of the outer core involves a series of membrane bound glycosyltransferases. These enzymes catalyse the sequential transfer of sugars from nucleotide sugar donors to the non-reducing end of the growing PS chain. The outer core in S. typhimurium and E. coliK-12 consists of a branched pentosaccharide (Fig. 1.1) (Osborn and Rothfield, 1971; Lüderitz et al., 1971; Jansson et al., 1981).

Incorporation of the proximal glucose residue of the outer core occurs before completion of the heptose region of the inner core. This pattern may reflect some aspect of regulation of LPS biosynthesis (Rick, 1987).

1.1.4 Synthesis of O-specific polysaccharide and polymerization of repeat units

There are three major steps in the synthesis of repeat units in the O-polysaccharide. First, the oligosaccharide repeat unit is synthesized on the antigen carrier lipid undecaprenol phosphate (ACL). Next the finished oligosaccharides, still bound to ACL, are polymerized to form a chain of repeat units. In the final stage, the polymerized repeat units are translocated from the ACL to the lipid A-core.

Step 1: Biosynthesis of repeat unit

In this stage, monosaccharides are transferred sequentially from nucleotide carriers to the phosphate mono-ester of ACL

e.g., UDP-galactose → gal-1-P-ACL → gal-P-P---ACL
$$\downarrow$$
UMP.
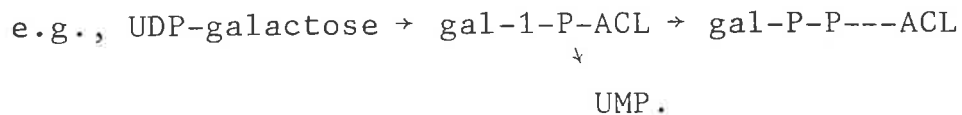
In the synthesis of the repeat unit, mannose-rhamnose- galactose, galactose-1-phosphate is transferred from the nucleotide precursor to produce galactose-diphosphate-ACL. The subsequent transfer of rhamnose and mannose, respectively, from their appropriate nucleotide precursors leads to the formation of the complete repeat unit attached to the carrier (Nikaido, 1970; Jann and Jann, 1984).

Step 2: Polymerization of repeat units

The growing polymer chain is attached to a molecule of ACL through a pyrophosphate linkage. Polymerization occurs by elongation at the reducing end of the poly-saccharide chain (Kanegasaki and Wright, 1970; Osborn and Weiner, 1968). This means that the growing polysaccharide chain is transferred to a new monomer unit (also attached to ACL), which allows enzymes and substrates to remain closely bound to the membrane.

The enzymes which are involved in the synthesis of repeat units have been shown to be membrane bound, and the ACL is also associated with the cytoplasmic membrane (Kanegasaki and Wright, 1970).

1.1.5 Translocation of O-polysaccharide to lipid A-core

The transfer of the O-polysaccharide to the completed lipid A-core, is catalysed by a ligase. This enzyme must

have a dual specificity as it is able to recognize both structures in the reaction. This ligase or translocase as it is also known, catalyses the transfer of the repeat unit chain from the ACL to lipid A-core with subsequent release of a phosphorylated ACL molecule. The ACL is then able to be recycled after dephosphorylation to the mono-phosphate form. This step can be specifically inhibited by bacitracin (Nikaido, 1965; Osborn and Weiner, 1968).

1.1.6 Post-polymerization modifications

The non-essential modifications of the O-polysacchar-ide make a significant contribution to the structural and antigenic variation within the O groups of Gram negative bacteria. Such modifications include substitution with O-acetyl groups or glucosyl groups. For example, in $\underline{S}$. $\underline{typhimurium}$ glucosylation of C-4 in galactose creates α-glucosyl-1,4-galactose. This creates the antigenic factor, 12_2 (Jann and Westphal, 1975; Lüderitz $\underline{et}$ $\underline{al}$., 1971). These late additions to the LPS are not essential for the completion of LPS synthesis (Mäkelä and Stocker, 1984).

Similar modifications may be caused by bacteriophage genes, such changes are known as antigenic conversion. Many phage use the O-antigen chains as receptors on the cell surface, and modifications which change phage sensitivity often affect particular monosaccharides which are hydrolysed by phage tail enzymes (Lindberg, 1977).

1.1.7 Site of lipopolysaccharide biosynthesis and translocation to the outer membrane

The biosynthesis of LPS occurs at the cytoplasmic face of the inner membrane. It has been shown in pulse chase experiments by Osborn et al. (1972) that both core and O-antigen transferases are associated with the inner, cytoplasmic membrane. Consistent with this is the observation that LPS is synthesized on the cytoplasmic face of the inner membrane and subsequently transported to the periplasmic space (Mulford and Osborn, 1983). The immunoelectron microscopy studies of Mulford and Osborn provided evidence of the location of newly synthesized LPS at the periplasmic face of the inner membrane before translocation to the outer membrane. The mechanism of LPS translocation was proposed to occur by a series of steps as follows: i) separate syntheses of core LPS and O-antigen chains at the inner, cytoplasmic membrane; ii) separate transfer of core LPS and O-antigen chains to the periplasmic face of the inner membrane; iii) attachment of O-antigen to core LPS and iv) transfer of LPS molecules to the outer membrane. The final step in this pathway may take place at the Bayer junctions, which are regions where the cytoplasmic and outer membranes are in very close apposition (Bayer, 1975).

1.2   Genetics of LPS biosynthesis in Salmonella

1.2.1 Genetics of the inner core

Studies of the genetics of the LPS core biosynthesis have been facilitated by the use of mutants blocked at

various stages. Such strains are called rfa mutants and
are capable of synthesizing complete O-polysaccharide
which is unable to attach to lipid A-core (Rick and
Osborn, 1977; Lehmann et al., 1977). Such rfa mutants have
been further classified according to the core structure
and a number of rfa chemotypes are shown in Fig. 1.1.

Biosynthesis of the inner core has been difficult to
study because complete mutations in lipid A and KDO
expression appear to be lethal. However, conditional
lethal mutants in lipid A synthesis have recently been
isolated in E. coliK-12 (Crowell et al., 1986). Thus, it
has been possible to identify two genes, 1pxA which
encodes an UDP-GlcNAc acyltransferase and 1pxB which
determines the lipid A disaccharide synthetase (Raetz,
1987).

Temperature sensitive mutants have also been isolated
which have defects in KDO biosynthesis (Rick and Osborn,
1977). Two genes kdsA and kdsB have been identified which
encode a KDO-8-P-synthetase and KDO-CMP synthetase, respec-
tively (Rick and Young, 1982; Sanderson and Roth, 1983).

A number of rfa genes have been described which are
associated with biosynthesis of the heptose region of the
inner core. The rfaD gene encodes the epimerase which
converts ADP-D-glycero-mannoheptose to ADP-L-glycero-D-
mannoheptose (Coleman, 1983). Addition of phosphate to the
heptose region follows addition of the first glucose
residue of the outer core and is catalysed by the rfaP
gene (Mühlardt, 1970; Hämmerling et al., 1973). The rfaA,

rfaC, rfaE and rfaF genes are also associated with bio-synthesis of the heptose region of the inner core, but their precise roles have not yet been determined (Rick, 1987).

1.2.2 Genetics of the outer core

Knowledge of the outer hexose region of the core is fairly complete and the five transferases required to assemble the outer core have been identified. Most of the rfa genes map at 79 min on the S. typhimurium chromosome, between cys and pyrE. Other genes involved in core synthesis which have other roles in the cell, such as galE, galU and pgi map outside this region (Osborn, 1968; Wallin et al., 1983).

The rfaH gene has been shown to decrease the level of galactosyl transferase activity and appears to have a regulatory role in E. coliK-12 and S. typhimurium (Creeger et al., 1984). Table 1.1 lists the known rfa genes in S. typhimurium LT2 and their functions, if known.

1.2.3 Genetics of O-polysaccharide biosynthesis

The genetics of the O-polysaccharide have been studied mainly in S. typhimurium and E. coliK-12. Variation of the O-polysaccharide within the Salmonella genus was originally studied using agglutination with anti-sera raised against various different strains (Kauffman, 1961; Lüderitz et al., 1968; Wilkinson et al., 1972; Hancock and Reeves, 1976; Yu and Mizushima, 1982;

Table 1.1

rfa gene	phenotype of mutant	function
rfaL	Complete core, no attachment of O-Ag	-translocation of O-Ag from ACL to complete core (with rfbT)
rfaK	Semi-rough; the number of the O-side chains is reduced	Attachment of N-acetylglucosamine to C-2 of glucose
rfaJ	Lack of distal glucose of core oligosaccharide	Possibly structural gene for glucosyl transferase
rfaI	Lack main chain galactose unit, but has branch galactose	Galactosyl transferase
rfaB	Lacks branch galactose in core	Galactosyl transferase
galE	Unable to convert UDP-glucose to UDP-galactose	Galactose epimerase
rfaH	Rc phenotype core lacks galactose and distal sugars	Regulatory protein controlling galactosyl transferase
rfaP	Lack phosphates in core heptose region Core stops at glucose I	
rfaG	Deep rough; Lack glucose I, but have normal heptose region	Glucosyl transferase
rfaF rfaE rfaD rfaC	Defects in formation of heptose region of core	Transferases
rfaF rfaD		Heptose II transferase ADP-heptose epimerase

Coleman and Deshpande, 1985). Thus, the O-polysaccharide is also known as the O-antigen.

The O-polysaccharide is a linear polymer of repeating oligosaccharide units. These repeat units usually consist of three to six monosaccharides. Mutants which are blocked in O-polysaccharide synthesis are usually selected by resistance to 'smooth' or O-antigen specific bacteriophage and screening for rough mutants with a complete core.

The entire rfb region has been cloned as a series of overlapping fragments and preliminary analysis of the genes has been performed (Nikaido, 1970; Rick, 1987; Gabriel, 1987). The rfb region maps between his and metG on the Salmonella chromosome (Mäkelä and Stocker, 1969, 1984), and is cotransducible with his. Sanderson and Roth (1983) mapped the rfb region to 42' on the chromosome.

The rfb genes are tightly clustered and this arrangement is typical of genes in an operon. Studies by Levinthal and Nikaido (1969) of deletion mutations extending from his into rfb suggested that the two gene clusters are transcribed in opposite directions. However, analysis of nonsense mutations in rfbD, rfbH and rfbN revealed that expression of genes to the right of the mutated locus, was significantly reduced (Levinthal and Nikaido, 1969; Mäkelä and Stocker, 1984). Such a result would be consistent with transcription of the rfb region occurring from left to right, towards the his operon.

Analysis of various rfb deletion mutants by Nikaido et al. (1967) allowed a partial gene order to be established. Brahmbhatt et al. (1986) described cloning of part of the rfb region from Salmonella using part of the adjacent his operon as a probe (Barnes, 1981). Further cloning and mapping allowed a complete physical map of the rfb region of S. typhimurium LT2 to be constructed (Brahmbhatt et al., 1988).

Strains containing deletions extending from the his operon into the rfb region were further analysed and the endpoints of the various deletions localized. This has made it possible to assign approximate positions for the various rfb genes located near each deletion endpoint. The rfbH gene which encodes abequose synthetase may actually consist of three or more genes forming an operon. Two large gaps, which may encode a number of rfb genes, exist in the 30kb rfb region. Potential regulatory sites have not yet been identified and so the detailed genetic control of the rfb region is still unknown (Brahmbhatt et al., 1988).

Assembly of the O repeat units takes place by sequential transfer of monosaccharide residues from the nucleotide precursors to receptors on the undecaprenol carrier (ACL) (Robbins and Wright, 1971). The transfer of the first residue occurs to ACL- P and a diphosphate linkage is created by transfer of a sugar P from the nucleotide carrier. In all the subsequent transfer reactions the monosaccharide is transferred to the

previous sugar without addition of a phosphate residue. In S. typhimurium the first sugar is galactose and the transfer of galactose-phosphate is reversible. The transfer reactions have been studied in mutants unable to make the various monosaccharide precursors. Genes encoding the galactose - P transferase, rfbN, and the rhamnose transferase have been detected. Other genes involved in the synthesis of the O-polysaccharide are shown in Table 1.2, with the appropriate function (Brahmbhatt et al., 1986; 1988).

Table 1.2

Gene	Function
rfbB	dTPP-glucose oxidoreductase
rfbL	phosphomannomutase
rfbK	modifies the product of rfbL
rfbE	synthesis of abequose
rfbG	synthesis of abequose
rfbH	synthesis of abequose
rfbF	synthesis of abequose
rfbA	synthesis of rhamnose
rfbD	synthesis of rhamnose
rfbM	synthesis of mannose

1.2.4 Polymerization of O-units

After synthesis of the O-units on the ACL, they are polymerized to a long polysaccharide chain which is still attached to the ACL (Robbins et al., 1966). This step occurs in the cytoplasmic membrane and it has been shown that the intermediates and participating enzymes are membrane bound (Robbins and Wright, 1971; Mulford and Osborn, 1982).

Mutants deficient in polymerization have been described in Salmonella groups B and E. Such mutants have a single O-unit on their LPS molecules and exhibit a 'semi-rough' phenotype. The gene responsible for polymerization of the O-units is called rfc and is located outside the rfb operon, between gal and trp, in Salmonella (Naide et al., 1965).

1.2.5 Attachment of O-polysaccharide to lipid A-core

The attachment of O-polysaccharide to the lipid A-core is thought to take place in the cytoplasmic membrane (Robbins and Wright, 1971). Transfer of O-polysaccharide requires the presence of a complete core and completed O-units. However, the finished LPS molecules are not uniform in structure, as heterogeneity of the chain lengths has been observed. Electrophoretic analysis of LPS molecules reveals that a large proportion is without O-side chains (Hitchcock and Brown, 1983). The remaining LPS molecules were shown to contain side chains which contained from 3 to 40 O-units.

Two genes have been characterized which are involved in attachment of O-polysaccharide to the core. These are the rfaL and rfbT genes which contribute to the dual specificity of the O-antigen ligase. Mutations in either of these genes leads to the same rough phenotype characterized by complete core and O-polysaccharide linked to the ACL. It has been suggested that rfaL could encode an enzyme recognizing the core in Salmonella LPS and rfbT encode an enzyme binding to complete O-units. The rfbT

gene product would need to be specific for each O group, whereas rfaL could be common to all Salmonellae (Mäkelä and Stocker, 1984; Rick, 1987).

1.2.6 Genetics of rfe-dependent O-antigen biosynthesis

In S. montevideo, S. minnesota and two E. coli sero-types, 08 and 09, the biosynthesis of O-antigen is dependent upon the rfe gene, rather than rfc. The rfe gene maps close to ilv, near the rfa locus, on the Salmonella chromosome (Mäkelä and Stocker, 1969).

Mutations at the rfe locus in these strains leads to an Ra phenotype, with a complete core, but no O-antigen associated with ACL (Mäkelä and Stocker, 1984). The rfe gene is also associated with biosynthesis of the Entero-bacterial common antigen (Mäkelä and Meyer, 1974). The rfe gene may direct a modification of ACL or possibly be involved in product of a different carrier molecule (Jann and Jann, 1984). The latter possibility is supported by the fact that peptidoglycan synthesis is unaffected in rfe mutants.

In E. coli 08 and 09, O-antigens consist completely of mannose polymers; the rfe gene in these strains may encode mannosyl transferases. Polymerization of the mannose residues may occur at the non-reducing end of the polymer (Jann and Jann, 1984; Mäkelä and Stocker, 1984). This contrasts with rfc mediated polymerization of O-antigen repeat units in which elongation of the polymer occurs at the reducing end, close to the outer membrane

(Kanegasaki and Wright, 1970; Robbins and Wright, 1971). This was demonstrated by pulse chase experiments in S. anatum using labelled UDP-Gal.

1.2.7 Genetics of LPS biosynthesis in other Gram negative bacteria

Studies of the LPS in various Gram negative bacteria have revealed that the genetic basis of LPS biosynthesis has some basic similarities.

E. coli

The wild-type E. coliK-12 strain contains no O-antigen and has presumably lost the ability to synthesise O-side chains in the course of evolution in the laboratory (Mäkelä and Stocker, 1984). Studies of the E. coliK-12 core by Prehm et al. (1976) have shown that there is a considerable degree of heterogeneity in the various K-12 strains analysed. This is thought to be due to the fact that E. coliK-12 wild-type strains are actually leaky rfa mutants. Many of the core structures are incomplete because glycosyltransferases which play a role in the final stages of core biosynthesis cannot operate effective-ly. Analysis of various derivatives of E. coliK-12 revealed that early strains picked up an rfbD1 mutation (Bachmann, 1987) however, the precise nature of this mutation is unknown. These strains also sometimes include galK mutations which, if polar, could lead to effects on galE and thus also interfere with O-antigen expression.

E. coliK-12 can attach rhamnose to the core and contains the genes for the dTPP-rhamnose pathway which map near the his operon (Ørskov and Ørskov, 1962; Nikaido, 1965). Thus, rhamnose may have been part of the O-antigen of the smooth ancestor strain of E. coliK-12. Presumably, E. coliK-12 has a mutation at the rfb gene cluster, which leads to the rough phenotype (Jann and Jann, 1984).

The rfa locus of E. coliK-12 has been mapped near pyrE and cysE, and is similar to that of Salmonella. The E. coli rfa genes can complement some defective rfa genes in Salmonellae (Bachmann and Low, 1980).

Although it has been well characterized in Shigellae species, for instance, that extra-chromosomal elements can encode genes associated with LPS biosynthesis, it was thought that only chromosomal genes were involved in the biosynthesis of E. coli LPS. However, it was observed by Riley et al. (1987), that the O-antigen in an entero-pathogenic E. coli0111:NM strain was determined by plasmid genes. Loss of the 54 MDal plasmid is associated with loss of O-antigen expression.

A number of E. coli serotypes have neutral O-poly-saccharide and exhibit a similar structural complexity to that seen in Salmonellae.

The location of the rfb genes in E. coliK-12 is linked to the his operon, as in Salmonella. The genes encoding the O-antigen polymerase, have not yet been identified.

A number of different core types have been identified in E. coliK-12, which differ from the Salmonella core, particularly in the hexose region (Ørskov and Ørskov, 1977; Jansson et al., 1981; Raetz, 1987). The synthesis and genetic determination of the core appear quite similar to that observed in Salmonella. An rfa locus has been identified, which can complement certain rfa genes of S. typhimurium (Mayer et al., 1976).

Shigella:

Sh. flexneri consists of a serologically heterogeneous group. The O-specific side chains include two regions, the unbranched variant Y chains (consisting of L-rhamnose and N-acetyl-glucosamine) and the type-specific secondary side chains (of α-D-glucosyl and O-acetyl residues). The Y chains have been shown to consist of tetrasaccharide repeating units (Simmons and Romanowska, 1987). The biosynthesis of the lipid A-core is encoded by rfa genes, and the repeat units of the variant Y chain by the rfb genes. The rfe gene directs the polymerization of the repeat units. Attachment of the O-specific side chains to the lipid A-core is catalysed by a ligase encoded by the genes rfaL and rfbT. The rfb genes in Sh. flexneri have been mapped near the his locus and represent one of the chromosomal regions associated with virulence, when transferred to E. coliK-12 (Sansonetti et al., 1983).

It was shown by Kopecko et al. (1980) that Sh. sonnei Form I cells which are the smooth, virulent phase, contain a 120 MDal plasmid. This plasmid was shown to be directly

associated with the presence of Form I antigen O-antigen side chains on the cell surface. Form II cells lacking the 120 MDal plasmid exhibited a rough phenotype and were non-invasive and avirulent.

Sansonetti et al. (1981) extended these studies by tagging the Form I plasmid with transposons and analysing the phenotypes of strains containing these plasmids.

The Form I O-antigen genes were transferred into the S. typhi strain Ty21a by conjugal mobilization, using an F'$_{ts}$ lac::Tn3 plasmid (Formal et al., 1981). Detailed chemical analysis of the resulting strain indicated that the Form I antigen was not actually attached to the S. typhi core, but instead existed as a polymer on the cell surface (Seid et al., 1984). This result may indicate that there are significant differences between the core structures of Salmonella and Sh. sonnei.

The biosynthesis of Sh. dysenteriae O-antigen requires genes from plasmid as well as chromosomal DNA. Watanabe et al. (1984) showed that Sh. dysenteriae I strains contained a 9kb plasmid, pHW400.

The his[+] chromosomal region appeared to modify the E. coliK-12 lipid A-core so that the O-antigen material could then be attached. The genes necessary for O-side synthesis are carried on the Shigella chromosome in the rfb locus, near his. The 9kb plasmid plays a role in core modific-ation and synthesis of O-side chain monomers. A 3kb fragment of this plasmid was cloned and shown to specify

functions needed for O-antigen production and defined as rfp (Watanabe et al., 1984). Hale et al. (1984) studied the expression of Sh. dysenteriae O-antigen in E. coliK-12. They concluded that the transfer of both the 9kb plasmid and a Sh. dysenteriae chromosomal region (co-transducible with his) were necessary for production of O-antigen.

Sturm and Timmis (1986) have described the cloning of the rfb region of Sh. dysenteriae by selecting for the closely linked marker, his. The region necessary for O-antigen biosynthesis has been localized to an 8.9kb fragment. Both the plasmid O-antigen gene, rfp and the chromosomal rfb genes have been cloned into pACYC184 to produce a single hybrid plasmid. The presence of these two sets of genes was sufficient to direct synthesis of Sh. dysenteriae O-antigen when the plasmid was transferred to E. coliK-12.

Analysis of the recombinant plasmid carrying only rfp showed that this gene has a 41 MDal protein product (Sturm et al., 1986a). Transfer of this plasmid into E. coliK-12 resulted in the addition of a galactose residue to the LPS core. Thus, it was concluded that the rfp gene has a role in transfer and linkage of galactose to the lipid A-core. The second and third residues are both rhamnose and their addition is determined by the chromosomal rfb cluster. The minimal coding region of the rfb locus has been estimated between 6.4 and 7.5kb and at least six determinants for O-antigen production have been identified and a minimum of

two operons are involved. A number of LPS intermediates have been detected by studying the expression of Sh. dysenteriae O-antigen in E. coliK-12. As these intermediates were found on the cell surface, it is possible that the O-antigen repeat unit may be synthesized differently in Sh. dysenteriae, from that of Salmonella and E. coli, in which repeat units are polymerized on ACL and then transferred to lipid A-core (Sturm et al., 1986b).

One class of mutants studied had LPS consisting of one repeat unit linked to lipid A-core, thus exhibiting a semi-rough phenotype. Such mutants may be defective in polymerization of repeat units and so could indicate the presence of an rfc-like gene within the rfb gene region. Such an arrangement is quite different from that in Salmonella in which the rfc gene maps in a different part of the chromosome from the rfb locus.

1.3   Vibrio cholerae Lipopolysaccharide (LPS)

1.3.1 Classification and subtypes

Vibrio cholerae (V. cholerae) is a Gram negative organism which is responsible for cholera, a disease still endemic in some parts of the world. V. cholerae belonging to the O:1 serotype are the only bacteria which are associated with the disease and these organisms can be further sub-classified. There are two biotypes of V. cholerae, which can be differentiated on the basis of a number of characteristics (Table 1.3). The 'Classical' biotype refers to the original strains of V. cholerae, described by Pacini in 1854.

Table 1.3

	Classical	E1 Tor
Polymixin B	S	R
Haemolysin	-	+
Mukherjee type IV phage	S	R
Voges-Proskauer	-	+

Vibrios classified as E1 Tor were first isolated in 1905 (Gotschlich, 1906) from pilgrims at the E1 Tor quarantine station. The E1 Tor vibrios could not be separated from the Classical O group I vibrios by agglutination (Burrows et al., 1946) and were shown to be associated with a diarrhoeal disease which was indistinguishable from that caused by Classical vibrios (Doorenbos, 1938). It was noted by Gardner and Venkatraman (1935) that the E1 Tor vibrios differed from the Classical strains in a number of characteristics, mainly haemolytic activity and a positive reaction in the Voges-Proskauer test. Doorenbos (1938) concluded that the Classical vibrios represented the "epidemic" type and the E1 Tor vibrios were a modified version which were an "endemic" type. Thus, it was concluded by Doorenbos that the Classical and E1 Tor vibrios were actually variants of one another, rather than separate species. However, it was not until 1962 that the World Health Organisation defined the disease caused by E1 Tor vibrios as cholera (Gallut, 1974). Further studies by Hugh (1965) of the various characteristics of the two types of O1 vibrios, indicated that the differences noted did not justify the classification of two separate species. Thus, Gallut (1974) recommended that the E1 Tor

vibrio be regarded as a biotype of V. cholerae, after the seventh pandemic of cholera in 1971 was shown to be entirely due to V. cholerae E1 Tor.

The two biotypes share three serotypes which were first described by Kabeshima (1918) who classified V. cholerae 01 into original (Inaba) and variant (Ogawa) types, using agglutination tests with homologous and heterologous anti-sera. Nobechi (1923) identified a third serotype which he described as an intermediate between Ogawa and Inaba and which he named Hikojima. Nobechi also proposed antigenic formulae for the serotypes. Thus, Inaba were classified as AX, Ogawa as B(X) and Hikojima as A(B)X. Gardner and Venkatraman (1935) confirmed that the Inaba, Ogawa and Hikojima serotypes belonged to V. cholerae O group I and proposed the existence of another antigenic component, C, and reclassified Nobechi's scheme as follows. Inaba, AX, Hikojima, ABX and Ogawa, BCX. The suggestion of another antigen, C, was made by Heiberg, in order to account for discrepancies in Nobechi's absorption data. Burrows et al. (1946) confirmed the finding of Nobechi (1923) and Heiberg (1935), but changed the antigenic formulae, so that X was designated as A and A as C (Table 1.4).

Table 1.4

Ogawa	A	B	
Inaba	A		C
Hikojima	A	B	C

Shousha (1931) carried out a study of the chemical nature of the serotype specific antigens and showed that they were associated with an acid-stable, heat-stable polysaccharide fraction isolated from vibrios. Thus, Shousha was able to conclude that the immunological specificity of the V. cholerae serotypes lies in the O-antigen of the LPS.

Sakazaki and Tamura (1971) studied the three serotypes of V. cholerae 01 and showed that the serotype specific antigens were not inactivated by treatment with acid, alcohol or heat. Thus, they concluded that the various serotype antigens are associated with the O-somatic antigen of V. cholerae 01, that is, the O-antigen of the LPS. Using absorbed sera, Sakazaki and Tamura (1971) carried out a series of cross agglutination tests and showed that the Ogawa O-antigen includes all the factors present in Inaba, plus an additional unique factor. Thus, it was proposed that Ogawa cells have a small amount of 'C' antigen on the surface, while Inaba cells contain a higher proportion of 'C' antigen (Figure 1.2). Redmond et al. (1973) were able to confirm this proposal by demonstrating that absorption of an anti-Ogawa serum with Inaba cells still left 5-10% of activity against Ogawa cells. Thus, it was concluded that Ogawa cells had a unique factor (B antigen) which was not present on Inaba cells.

This scheme was slightly modified by Sakazaki (1971). Using absorbed sera, Sakazaki concluded that the Ogawa O-antigen possesses all the factors present in Inaba, plus

Figure 1.2: Diagrammatic representation of the relative amounts of A, B and C antigens on the different _V. cholerae_ serotypes. Reproduced from Sakazaki and Tamura (1971).

A B C　　A B C　　A　　C

Ogawa　　Hikojima　　Inaba
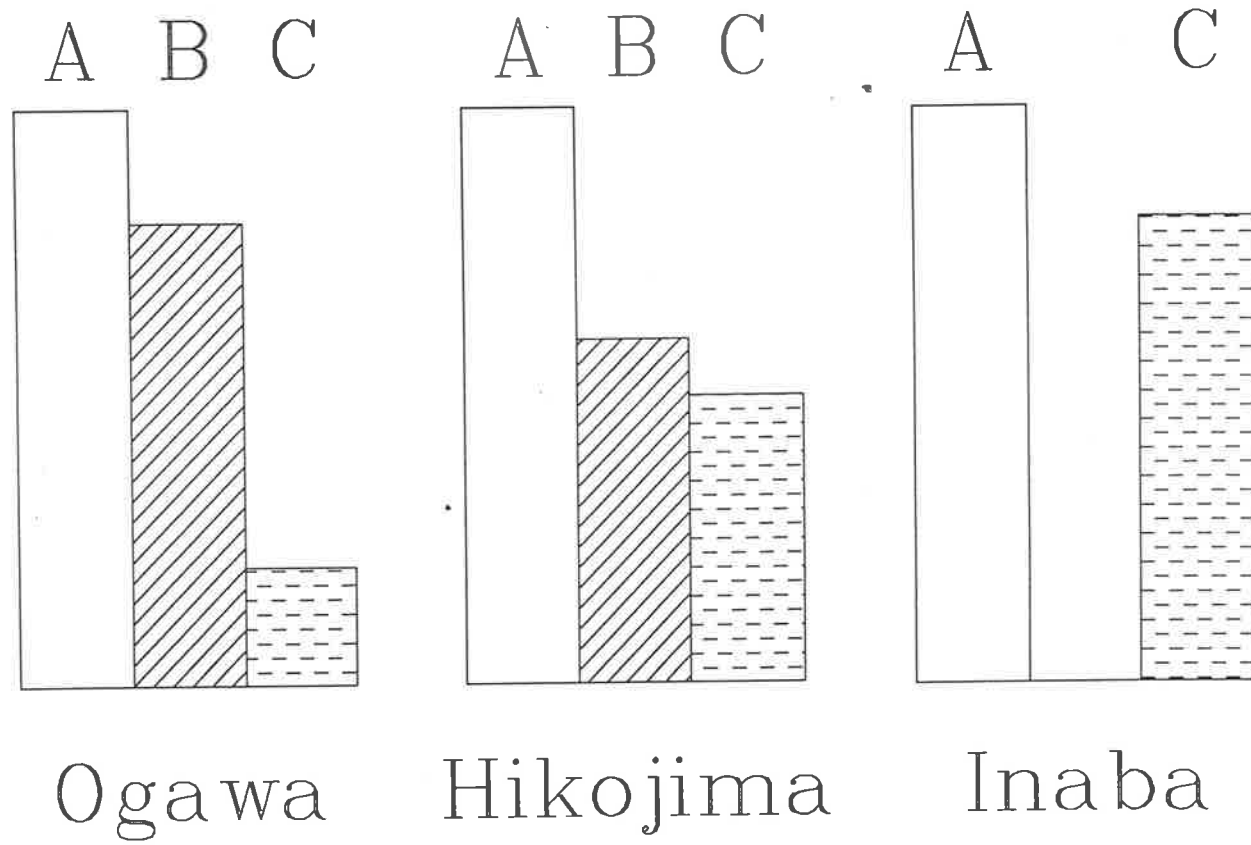
a unique factor. Thus, it was proposed that Ogawa cells possessed a small amount of 'C' antigen on their surface (Fig. 1.2). Redmond et al. (1973), have confirmed this proposal, by demonstrating that absorption of an Ogawa anti-serum with Inaba cells still left 5-10% of activity against Ogawa cells.

1.3.2 V. cholerae LPS-chemical composition

The LPS of V. cholerae has been studied extensively, particularly at the chemical level. Such studies have revealed that there are a number of significant differences between the LPS of V. cholerae and that of other common Gram negative bacteria.

The LPS molecules of V. cholerae have a similar overall structure to those of Salmonella and E. coliK-12. Thus they consist of a lipid A region, a core carbohydrate region and the O-antigen. These regions will be discussed in some detail.

LIPID A: The lipid A of V. cholerae LPS consists of a β 1-6 linked diglucosamine unit (Fig. 1.3). There are phosphate residues associated with the non-reducing ends of the glucosamine residues and with the glycosidic hydroxyl (-OH) group attached to the C-1 atom (Raziuddin 1977; Raziuddin and Kawasaki, 1976; Broady, Rietschel and Lüderitz, 1981).

A number of fatty acids are bound to the hydroxyl and amino groups of the glucosamine residues (Armstrong and Redmond, 1973). Three of these fatty acids are involved in

Figure 1.3: Chemical structure of lipid A from V. cholerae LPS.
Reproduced from Broady et al. (1981).
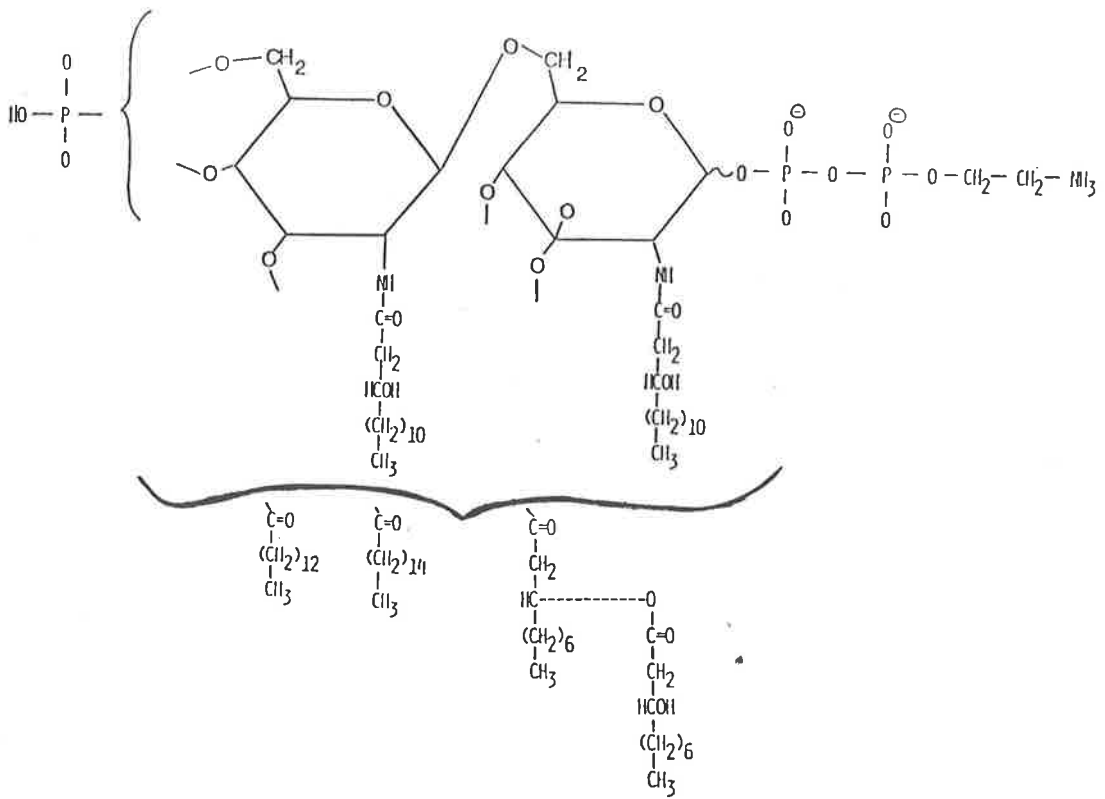
ester linkages and one in an amide linkage. Table 1.5 lists these fatty acids and gives their chemical formulae. 3-hydroxy myristic acid is the longest 3-OH fatty acid in V. cholerae LPS (Hisatsune et al., 1979). This fatty acid is present in the LPS of all Gram negative bacteria except Brucella. The major non-hydroxy fatty acids found in V. cholerae LPS are myristic and palmitic acid. Considerable amounts of odd-numbered fatty acids have been found in V. cholerae lipid A, mainly C15:0 and C17:0.

Table 1.5

Fatty Acid	Formula	Linkage
Palmitic	C16:1	ester
Oleic	C18:1	"
3-OH Lauric	3-OH-C12:0	"
3-OH Myristic	C14:1	amide

It has also been suggested (Hisatsune et al., 1979), that lipid A may be a branched structure of several polyglucosamine chains of various lengths, linked by phosphodiester bonds.

Core sugars: Studies of degraded LPS (Raziuddin, 1977) showed that it could be separated into two fractions, I and II, on the basis of molecular weight. The sugar composition of these fractions is shown in Table 1.6. Fraction I corresponds to the O-polysaccharide or O-antigen, and fraction II consists of sugars associated with the lipid A-core.

Table 1.6

Fraction I	Fraction II
Glucose	Glucose
Mannose	Glucosamine
Rhamnose	D-Perosamine
Fructose	Heptose
Glucosamine	Fructose
Perosamine	Phosphate
	Ethanolamine phosphate

It was stated for some years that the V. cholerae core did not contain KDO (Hisatsune et al., 1976; Nakano et al., 1977; Raziuddin, 1977; Raziuddin, 1980), but studies by Brade (1985) have revealed that KDO can be detected after harsh hydrolytic treatment of the cells. Studies by Kaca et al., (1986) have shown that fructose can be quantitatively released from LPS by mild acid hydrolysis, and that periodate oxidation destroys fructose. This indicates that fructose does not form the link between the core and lipid A as proposed by Jann et al., (1973). It is likely that KDO- phosphate links the polysaccharide and lipid A components of V. cholerae LPS, as observed in a number of other studies, however, it is unusual in that it contains only a single KDO residue.

O-polysaccharide: Studies of LPS usually involve extraction of the LPS from bacterial cell walls using hot phenol and then using acetic acid hydrolysis to separate the preparation into lipid A-core and O-polysaccharide fractions (Westphal and Jann, 1965). The different solubilities of these fractions in chloroform can also be utilized in the extraction procedure (Galanos et al.,

Table 1.7[a]

Strain	Preparation	Lipopolysaccharide (% weight)									
		P	Glc	Hep	Fru	Man	Rha	GlcN	Quin	Pero	Ethanolamine phosphate
Inaba (569B)	O-PS	0	3.2	0	1.8	4.2	2.8	6.2	3.8	3.0	0
	C-PS	5.2	16.4	19.8	6.5	0.8	1.2	0	0	1.2	3.8
Ogawa (NIH41)	O-PS	0	2.8	0	2.7	6.2	3.8	7.2	2.6	3.2	0
	C-PS	5.6	11.3	13.2	5.2	Tr	Tr	0	0	1.2	5.2

a) Taken from Raziuddin (1980).

Abbreviations: O-PS: O-specific side chain polysaccharide; C-PS: core polysaccharide; P: phosphorus; Glc: glucose; Hep: heptose; Fru: fructose; Man: mannose; Rha: rhamnose; Glc: glucosamine; Quin: quinovosamine; Pero: perosamine; Tr, trace amounts.

1969). Hydrochloric acid can be used to hydrolyse the polysaccharide fraction and chromatography on G-50 allows purification of two further fractions (Raziuddin, 1977). These have been shown to correspond to the core region and the O-antigen region.

Table 1.7 gives a summary of the sugar content of the O-polysaccharide of both Inaba and Ogawa serotypes (Raziuddin, 1980). The most important features to be noted are as follows. Although V. cholerae LPS does include a number of 'common' sugars such as mannose, glucose and heptose, it contains no galactose. The LPS of both serotypes contains a high molecular weight polysaccharide which is extremely acid resistant (Jackson and Redmond, 1971; Redmond et al., 1973). This polymer has a molecular weight of $\simeq 10,000$ and n.m.r. spectroscopy has indicated that it consists solely of about sixty perosamine residues arranged in a regular $\alpha(1\rightarrow2)$ linked linear chain. Minor sugars were shown to be present at the reducing end of the perosamine polymer and these are thought to represent the core region of the LPS. The linear chain of perosamine residues may form a 'backbone' in the LPS molecule and be substituted at various positions with the other sugars known to be associated with the O-polysaccharide.

In addition to perosamine, there are other unusual amino sugars which have been identified in V. cholerae LPS. Quinovosamine (Fig. 1.4) was first isolated and identified by Jann et al. (1973). Raziuddin (1980) confirmed the presence of quinovosamine in Inaba and Ogawa

Figure 1.4: Structural formulae for the amino sugars in V. cholerae LPS.

A) Perosamine

B) Quinovosamine

C) 4-NH$_2$-4-deoxy-L-Arabinose

D) Repeat unit of V. cholerae O-antigen, consisting of (1→2) linked 4-amino-4,6-dideoxy-α-D-mannopyranosol residues. The amino groups are acylated with 3-deoxy-L-glycero-tetronic acid. Reproduced from Kenne et al. (1982)

A

PEROSAMINE

4-NH$_2$-4,6-DIDEOXY-MANNOSE
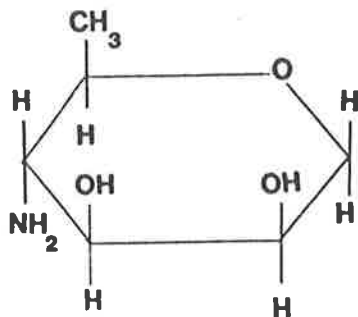
B

QUINOVOSAMINE

2-NH-2,6-DIDEOXY-D- GLUCOSE

C
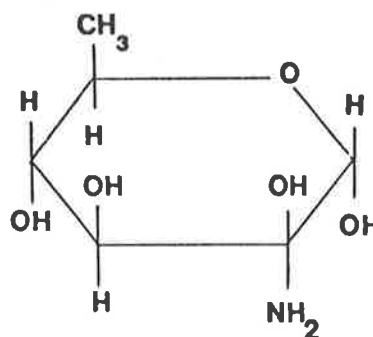
4-NH$_2$-4-DEOXY-L-ARABINOSE
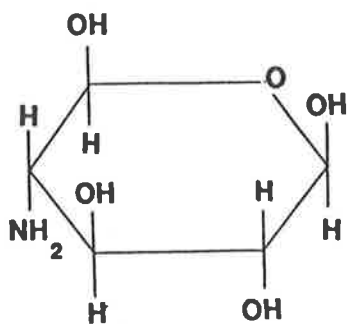
D
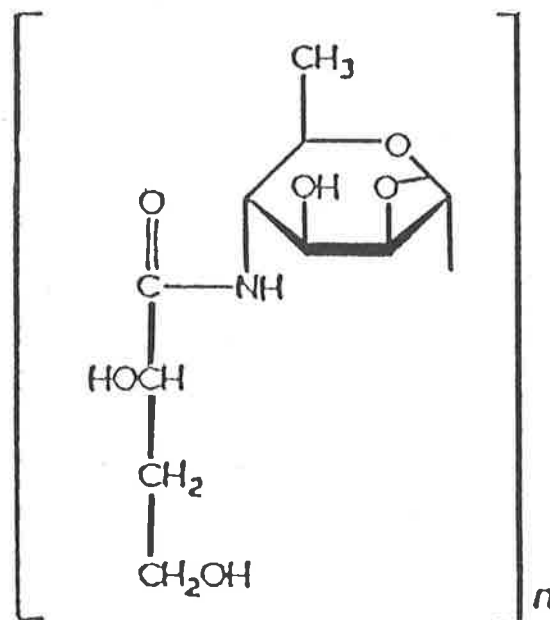
strains of V. cholerae. Subsequent fractionation of the LPS into core-polysaccharide and O-antigen preparations revealed that quinovosamine was present only in O-specific side chain polysaccharide and completely absent from core sugars (Table 1.7), (Raziuddin, 1980).

However, studies by Hisatsune et al. (1980) indicated that quinovosamine was located in the LPS core in 569B (Inaba), or closely associated with the outer core. Hisatsune also reported the chemical composition of a hypotoxigenic mutant, tox101-TI-N4, which lacked perosamine but did have quinovosamine, whereas analysis of a rough V. cholerae strain revealed that this strain lacked both amino sugars. Thus, Hisatsune et al. (1980) suggested that the sequence of sugars in 569B LPS may be: D-perosamine polymer → quinovosamine → core PS → lipid A. Quinovosamine was detected exclusively in the O-antigen by Kabir (1982), thus confirming the findings of Jann et al. (1973).

Subsequent studies involved chemical analysis of an Ogawa strain, P1418 and an Inaba strain, UV601, which had been derived from P1418 (Hisatsune et al., 1985). In this study perosamine and quinovosamine were quantitated in both Ogawa and Inaba LPS and no significant difference was found between serotypes.

The structure of the V. cholerae O-antigen was proposed to consist of 18 repeating units of perosamine, connected to the core region by a quinovosamine di-saccharide unit (Fig. 1.4) (Hisatsune et al., 1985).

The other amino sugar, $4-NH_2-4$-deoxy-L-arabinose (Fig. 1.4), has been detected only in the LPS of Ogawa cells (Redmond, 1978). NMR spectroscopy using purified $4-NH_2-4$-deoxy-L-arabinose as a standard, allowed positive identification of this sugar in the O-antigen from Ogawa lipopolysaccharides. The amount of $4-NH_2-4$-deoxy-L-arabinose present was estimated from thin layer chromatography to be 5-10% of the LPS. Although the presence of this amino sugar in Ogawa cells was confirmed by Kabir (1982), other studies have failed to detect it (Hisatsune et al., 1982). This discrepancy has not yet been resolved.

1.3.3 Structural studies of V. cholerae LPS

Studies by Raziuddin (1977) revealed that the LPS of V. cholerae could be separated into two fractions, I and II, by chromatography (Table 1.6). Chemical analysis of these fractions indicated that the phosphorus and sugar contents differed between fractions I and II. Thus, it was possible to conclude that the high M_r fraction I corresponded to O-antigen, while the low M_r fraction II consisted of core polysaccharides. Kenne et al. (1979) used NMR spectroscopy of the V. cholerae O-antigen to show that the backbone of this structure was a homopolysaccharide. It was suggested by Kenne et al. that this was composed of perosamine residues linked through O-2 to form a linear polymer. Redmond (1979) extended these studies and demonstrated the regular $\alpha (1 \rightarrow 2)$ linkage of perosamine residues. Gel chromatography of the polymer indicated a M_r of $\simeq 10,000$ which would correspond to an unbranched chain

of ≃60 perosamine residues. Redmond (1979) suggested that the minor sugars would be present at the reducing end of this perosamine polymer and thus represent the core region. Methylation analysis of V. cholerae LPS by Hisatsune (1976) revealed that glucose was the terminal sugar on the O-antigen of both Ogawa and Inaba cells.

Further analysis by Kenne et al. (1982), showed that upon mild acid treatment, two fractions of M_r 9000 and 900 could be extracted, representing the O-antigen plus core and core from incomplete LPS molecules, respectively. NMR spectroscopy showed that the O-antigen consisted of $\alpha(1 \to 2)$ linked 4-NH$_2$-4,6-dideoxy-α-D-mannopyranosol residues (perosamine) (Fig. 1.4). Thus, these studies agreed with the results from Redmond (1979). Methylation analysis combined with NMR spectroscopy by Hisatsune et al. (1985) also detected the perosamine homopolymer in the O-antigen of V. cholerae LPS and confirmed the $\alpha(1 \to 2)$ linkage between sugars. Hisatsune et al. (1985) and Kenne et al. (1982) showed that the amino groups of these perosamine residues are acylated with 3-deoxy-L-glycero tetronic acid.

The work of Sen et al. (1979) also indicated the V. cholerae O-antigen has a branched structure of manno-pyranosol residues with glucose and heptose residues as terminal sugars at the non-reducing end of the perosamine polymer. Kabir (1982) extended this study by analysing the interaction of LPS from V. cholerae Ogawa 395 with a number of lectins. Thus, Kabir showed that the terminal glucose residues were α-linked and he also determined the

presence of N-acetyl-D-glucosamine as a terminal sugar linked to the polysaccharide backbone by an $\alpha(1 \rightarrow 3)$ bond.

A series of studies have been made which attempt to correlate the O-antigen structure with particular antigenic specificities. Partial chemical structures have been proposed for both the Ogawa and Inaba O-antigen (Sen et al., 1980; Majumdar et al., 1982, 1983). Hydrolysis of the polysaccharide from Inaba yielded five oligosaccharides:

In-1.   D-glucuronic acid, D-glycero-L-mannoheptose
In-2.   D-glucose   "
In-3.   D-glucose, D-glucosamine   "
In-4.   D-mannose   "
In-5.   D-mannose, D-glucuronic acid.

The oligosaccharides from Ogawa G-2102 contain the following monosaccharides:

Og-1.   D-glucuronic acid, L-glycero-D-glucoheptose
Og-2.   D-glucose   "
Og-3.   D-glucose, D-glucosamine   "

D-glycero-L-mannoheptose is thus associated only with Inaba cells and L-glycero-D-glucoheptose with Ogawa cells. Experiments on the immunochemical properties of these oligosaccharides, have allowed an immunodominant role to be assigned to particular sugars (Guhathakurta et al., 1986). Studies using monosaccharides in inhibition of precipitation reactions, indicated that glucuronic acid, followed by glucosamine were the most effective inhibitors. This result was consistent, regardless of serotype. The two heptoses were shown to inhibit the homologous serotype from which they were derived. When the oligo-

saccharides were tested, In-1, 3 and 5, and Og-1 and 3 significantly inhibited the precipitation reaction. In all cases the oligosaccharides were better inhibitors than their constituent monosaccharides. The Ogawa oligosaccharides were all derived from O-specific polysaccharides, whereas the Inaba oligosaccharides were prepared from a total polysaccharide fraction. Thus, it is possible that some of the Inaba sugars may be from the core region of the LPS. Possibly the heptoses unique to each serotype may be involved in the serotype specific antigens, B and C, but no evidence is yet available which would confirm this hypothesis.

Gustafsson et al. (1982) have described the isolation of a McAb directed against the core of V. cholerae LPS. Subsequently, McAbs were isolated which reacted with the A, B or C antigens of V. cholerae O-antigen, in both slide agglutinations and ELISA inhibition assays (Gustafsson and Holme, 1983).

These four different McAbs were then used to characterize the V. cholerae core and O-side chain (Gustafsson and Holme, 1985). LPS was extracted from cells of each of the three serotypes and subjected to mild acid hydrolysis. Gel chromatography allowed the identification of two separate polysaccharide fractions. One fragment of M_r 900 reacted only with the anti-core McAb and so it is likely to represent the core polysaccharide. The fraction with a M_r of 9,000 reacted with McAbs directed against the O-specific antigens A, B and C. Anti-core McAbs also

reacted with this fraction. Thus it was concluded that the 9,000 M_r fraction includes the core and O-antigen polysaccharides. Immuno-blotting experiments, using LPS from various strains, confirmed these results and showed that the major bands seen in LPS gels consist of LPS molecules carrying O-side chains. Rocket immunoelectrophoresis was performed using the McAbs and the results indicated that the 'A' antigen is present as multiple determinants, which would correlate with the evidence for the 'A' antigen consisting of a perosamine polymer.

No rockets were detected when anti-B and anti-C antibodies were used with the 9,000 M_r fragment. This may indicate that the B and C antigens are present as single determinants on the appropriate polysaccharide chain (Gustafsson and Holme, 1985).

1.3.4 V. cholerae rough strains

One of the first detailed studies of rough mutants in V. cholerae was performed by Shimada and Sakazaki (1973). Based on serology of V. cholerae strains, it was shown that some strains did not agglutinate with the appropriate homologous anti-serum. Thus, rough mutants were identified which, although indistinguishable from smooth vibrios in colony morphology, were shown to differ in the chemical composition of the LPS. Subsequent studies of rough mutants and their corresponding parent strains, showed that the rough strains lacked both quinovosamine and perosamine in their LPS (Hisatsune et al., 1979; Hisatsune and Kondo, 1980). It was concluded that these two amino-sugars

in smooth form <u>V</u>. <u>cholerae</u> are involved in the O-1
specificity of the polysaccharide side chain. Passive
haemolysis - inhibition studies of smooth and rough
strains showed that LPS from the different rough strains
exhibited the same extent of inhibition in homologous and
heterologous systems. That is, a strong cross-reactivity
was observed between anti-sera raised to various rough
strains and so it can be concluded that there is a common
'rough antigen' (Hisatsune <u>et</u> <u>al</u>., 1978).

1.4   Antigenic conversion and phase variation

1.4.1 Introduction

Many pathogens have been observed to alter or
completely lose particular surface characteristics. These
phenotypic changes can be divided into two main categories.

1) <u>Phase changes</u> - A particular characteristic is either
   expressed or is not.

2) <u>Antigenic variation</u> - one of a number of variable
   phenotypes is expressed.

The genetic variations which underlie these changes
have been studied in both prokaryote and eukaryote systems
and in most cases, involve genomic rearrangements, rather
than the gain or loss of extrachromosomal elements. The
role which environmental signals have on variation in
these systems is unknown.

In this section, a number of examples of both phase
and antigenic conversion will be discussed. Such systems

may provide possible models on which to base the phenomenon of serotype variation in V. cholerae.

1:4.2 Antigenic conversion and phase variation in prokaryotic systems

The classic and best analysed example of phase variation in bacteria is that of phase variation in Salmonella. There are two structural genes which code for flagellin, the major protein of the flagella. These genes, called H1 and H2, map in different regions of the genome and lead to the production of two antigenically distinct types of flagella. The frequency of 'switching' between the two phases has been estimated at 10^{-3} and 10^{-5}/cell/generation (Silverman and Simon, 1980).

Heteroduplex studies of the DNA involved in flagella biosynthesis have revealed that a 970 bp region of non-homology existed and so it was suggested that a DNA rearrangement of this segment was occurring which led to switching of the DNA phases. In Fig. 1.5 the arrangement of the flagella genes and control regions can be seen. The invertible region includes a structural gene, hin. This gene encodes an invertase which catalyses the switching of the 970 bp region (Zieg et al., 1978).

In vitro studies on phase variation have shown that the site specific inversion requires host proteins in addition to the bacterial invertase. Recombination occurs within two palindromic 26 bp sequences which are at the boundaries of the invertible element. A recombinational enhancer sequence (a 60bp region in the N-terminal coding

Figure 1.5: The structure of the H2 chromosomal region of *S. typhimurium*. Expression of the two flagellar serotypes is regulated by the orientation of the hin gene which includes the promoter for the H2 gene. Inversion of hin occurs via recombination at hixL and hixR. The enhancer site within hin is shown. Reproduced from Johnson and Simon (1987).
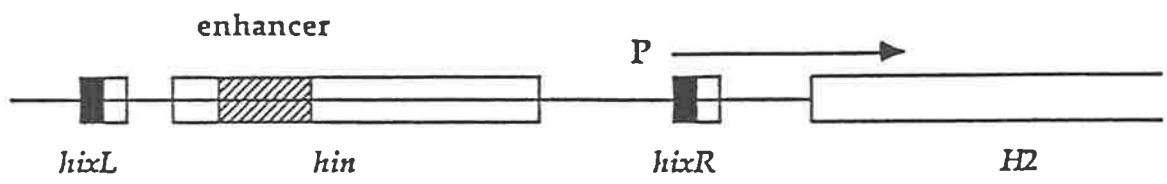
region of the hin gene) causes a high rate of re-combination of the invertible sequence (Mertens et al., 1984; Plasterk et al., 1984).

The two host proteins involved in the recombination process include HU, the major histone-like protein of E. coliK-12 and Factor II, which is a basic protein and binds to functional domains in the enhancer sequence (Koch and Kahmann, 1986). The HU protein may have a role in promoting unwinding of DNA in the presence of topoisomerase I, and so stabilize protein-DNA complexes formed at the recombination sites (Johnson et al., 1986; Meyer and Haas, 1988).

Other examples of antigenic variation have been described, which are characterized by invertase functions homologous to hin. In Mu phage, host specificity is controlled by the variant expression of tail fibre protein. The inversion of the G segment containing variable regions of these proteins, is catalysed by the gin product (Plasterk et al., 1984; Mertens et al., 1984). This product shares considerable homology with Hin in both its recombination site and amino acid sequence. Another similarity with phase variation in Salmonella is, that the gin gene of Mu requires a host factor for full invertase activity of the Gin protein (Kahmann et al., 1985). This host protein, FIS (factor for inversion stimulation), binds to a Sis sequence within the gin gene.

The Pin invertase in E. coli and Cin invertase in phage P1 are both homologous to the Hin and Gin proteins.

The various invertases can complement each other and all require the presence of host factors for full recombination activity (Johnson et al., 1986; Koch and Kahmann, 1986).

Cells of Neisseria gonorrhoeae express pili which have been proposed to play a role in anchoring the gonococci to mucosal cell surfaces during infection, however, there is no direct evidence that pilin is the adhesion molecule (Meyer and Haas, 1988). The expression of pili is subject to phase variation, in which expression is switched on or off and antigenic variation, in which structurally and antigenically different proteins are produced (Meyer et al., 1982, 1984).

The genome of N. gonorrhoeae MS11 has numerous copies of the pilin structural gene, however, most of these are incomplete (Meyer et al., 1982; Haas and Meyer, 1986). Several of these loci have been mapped to a fragment of ≃ 50kb and include the expression loci pilE1 and pilE2 and a number of silent loci, pilS1 through pilS7, upstream of the expression loci (Haas and Meyer, 1986).

The pilE1 and pilE2 loci contain complete pilus structural genes, whereas the pilS1 locus includes pilin genes which are truncated. PilS1 consists of six individual variant pilus gene copies which are tandemly arranged. All six gene copies are missing fractions of the 5' sequence which encodes the N-terminal region of the pilin protein. This region from amino acids 1 to 44 is identical in all pilins so far analysed and so may not contribute at all to

antigenic variation but is thought to be essential for pilus polymerization (Meyer et al., 1984; Haas and Meyer, 1986).

It has been shown by Hagblom et al. (1985) that pilin genes contain a conserved 5' region, a semi-variable control region and a hypervariable 3' region.

Sequence variations have also been observed in both silent and expressed pilin genes. These variations are restricted to six small regions known as "mini-cassettes" in N. gonorrhoeae MS11. Other strains such as N. gonorrhoeae P9 appear to have a similar arrangement of mini-cassettes (Haas and Meyer, 1986; Meyer, 1987; Meyer and Haas, 1987). These fragments are flanked by highly conserved regions. Short repetitive sequences link together the different pilin gene copies in the pilS1 locus. These repetitive regions may facilitate recombination between different pilus loci.

Antigenic variation in gonococcal pili occurs by gene conversion in which one or more mini-cassettes are transferred from a silent pilin gene copy to an expression copy, with the silent copy remaining unaltered. This was shown by analysis of variant gonococci descended from a single parent strain, using oligonucleotide probes complementary to mini-cassettes in the pilS1 gene. Signals were detected in the expression genes as well as the silent genes, indicating that genes from the pilS1 locus had transferred genes to pilE1. The termination points of this conversion are located in the conserved regions

flanking the mini-cassettes (Hagblom et al., 1985; Swanson et al., 1985; Haas and Meyer, 1986; Segal et al., 1986). This process is recA dependent and has been proposed to be at least in part due to transformation (So et al., 1988).

N. gonorrhoeae pili also undergo phase variation in which coarse genetic rearrangements are involved. Segal et al. (1985) concluded that this phase variation is due to deletion of pilin sequences from expression sites. However, recent studies by Haas et al. (1987) showed another class of pilus variants, termed P^S, could be isolated in the N. gonorrhoeae strain MS11. These P^S strains secreted truncated pilin into the extracellular environment (Meyer, 1987; Haas et al., 1987). Northern hybridization analysis of the P^S variants and their P^+ parent strains revealed that the pilE1 locus was transcribed and translated, irrespective of the pilation phase of the cells, and it was concluded that phase variation from P^+ to P^S was due to a reassortment of mini-cassettes in the expression locus (Haas et al., 1987; Seifert et al., 1988). Other pilus variants isolated produced a larger pilin molecule (termed L-pilin) and were shown to have an extra 500bp in their pilE1 expression copy (Meyer, 1987). The variation from P^+ to P^- variants producing L-pilin does not seem to be associated with precise gene conversion mechanisms. The study of P^- variants by Bergstrom et al. (1986) revealed the isolation of another class of strains in which the variation was suggested to result from expression gene deletions or the introduction of a translation stop signal in the expression gene (Bergstrom et al., 1986).

The opacity proteins of N. gonorrhoeae are subject to both antigenic and phase variation. The opa genes which encode them have been found linked to the pilin loci (Stern et al., 1986). All the genes are constitutively transcribed and the control of antigenic variation is at the translational level.

The expression of the opacity genes is regulated by the coding repeat (CR) which consists of a variable number of CTCTT pentamers within the signal sequence coding region. The addition or removal of one of these CRs during DNA replication changes the reading frame of the remainder of the gene and so affects the level of production of the particular opacity protein. However, even transcripts from out of frame genes are translated at a low level frequency suggesting either slippage by RNA polymerase or the translation apparatus (Stern et al., 1986; Meyer and Haas, 1988).

E. coli - phase variation in Type I fimbriae

The Type I fimbriae of E. coli are involved in colonization of bacteria to epithelial surfaces. Expression of these fimbriae is phase variable, that is, cells alternate between a fimbriate and non-fimbriate state. It has been shown that phase variation results from the inversion of a 300bp DNA segment, located immediately upstream of the fimA gene. This invertible segment contains the promoter for the fimA gene (Klemm, 1986).

Phase variation of Type I fimbriae is recA independent and not related to the Hin recombinase of Salmonella. Two regulatory genes, fimB and fimE, have been located upstream of fimA. The fimB gene product switches the invertible sequence into the 'on' position, so that the promoter points towards fimA. The fimE gene directs the switch into the off position and so the promoter is then in the opposite orientation, with respect to the fimA gene. As both the fimB and fimE proteins are very basic, it has been suggested that they are likely to bind to DNA (Abraham et al., 1985; Ratiner, 1985).

In Bordetella pertussis a reversible phase change occurs between the virulent and avirulent states. Tn5 mutagenesis has shown that the virulence factor genes are not linked and are not co-ordinately expressed. The variation in B. pertussis is influenced by growth conditions, such as temperature, magnesium and nicotinic acid concentration. It has been suggested that a positive inducer is required for the expression of the virulence genes, and that environmental signals are involved in its expression. The precise nature of the regulation in B. pertussis is unknown (Weiss and Falkow, 1984; Robinson et al., 1986).

Borrelia recurrentis causes a disease characterized by relapsing fever. Twenty-five different serotypes have been detected. Within any particular serotype, 10^{-3} to 10^{-4} new variants appear each cell generation. The appearance of these new serotypes is spontaneous and reversible (Stoenner et al., 1982).

A number of variable major proteins (VMPs) have been identified on the surface of B. hermsii. These proteins contain the serotype specific epitopes, and are subject to antigenic variation. The DNA rearrangements which lead to this variation have some similarities with the system described for pilin in Neisseria (Meyer and Haas, 1987).

Both silent and expressed copies of the VMPs have been detected, which have similar sequences, but differ in the flanking DNA upstream of the genes. Although all serotypes may contain copies of silent VMP genes, a particular serotype will contain an extra copy of one VMP gene. This corresponds to that copy which is being expressed and is responsible for the serotype. The VMP genes are carried on linear plasmids and the silent and expressed copies, with associated expression sequences are on separate plasmids. The silent genes are present on lower copy number plasmids and the expression plasmid containing the extra copy of one VMP gene is on a plasmid of higher copy number. This second VMP is the one that is transcribed. The switch between serotypes results from site specific recombination between a site upstream of the VMP gene and one at the border of the expression sequence (Plasterk et al., 1986).

1.4.3 Serotype conversion in V. cholerae

The first report of serotype conversion was by Kabeshima (1918), who defined the V. cholerae serotypes. Kabeshima reported 'mutations' in the serotypes in strains stored under laboratory conditions. Shrivastava and White

(1947), reported the isolation of Inaba strains from Ogawa cells grown in the presence of α-Ogawa serum. As similar experiments with Inaba cells grown with α-Inaba serum yielded only rough forms, it was initially postulated that the antigenic change could occur in only one direction, that is, Ogawa to Inaba. It was hypothesized that this was simply the result of the loss of the 'B' antigen.

An example of serotype conversion in vivo was reported by Gangarosa et al. (1967). A patient infected with V. cholerae (E1 Tor Ogawa) was shown to carry the Inaba and Hikojima serotypes, all on different days. The appearance of the different serotypes preceded a clinical relapse, suggesting that, in vivo, serotype variation may occur in response to the production of host antibodies directed against the original serotype. It should be noted that the strain originally isolated as Hikojima reacted as an Ogawa serotype in subsequent analyses. It is therefore not possible to determine if the strain was wrongly sero-typed originally or if it 'switched' to Ogawa.

Studies using germ-free mice (Sack and Miller, 1969), have also shown that serotype variation in V. cholerae can occur in vivo. The significant points to emerge from this study were first, that reciprocal conversions could occur between Ogawa and Inaba serotypes and second, that rough to smooth reversions can occur during infection. Thus, it appears that as well as loss or gain of a single antigenic determinant, the capacity to synthesize the entire O-antigen can somehow be changed so that only the core of the LPS molecule is produced.

In the germ-free mice, the phenomenon of Hikojima instability was again observed and Hikojima isolates were serotyped as Ogawa after subculture. Other serotypically converted vibrios were shown to be stable upon serial transfer.

Another aspect of the studies with germ-free mice concerned the correlation of serotype variation in vivo with the host immune response. It was shown that cyclophosphamide (which suppresses antibody formation) significantly retarded or, in some cases, prevented serotype variation. Vaccination, prior to infection with a particular serotype, accelerated conversion to an alternative serotype. Such evidence suggests that serotype conversions observed in vivo are related to the presence of serotype specific antibodies (Sack and Miller, 1969).

Sakazaki and Tamura (1971) performed a detailed study of the variation between Ogawa and Inaba serotypes. A total of thirteen different V. cholerae strains were used and O-antigen variation was studied with the aid of monospecific Ogawa and Inaba anti-sera. No variation from Inaba to Ogawa was observed. It was suggested that variation of serotypes in vitro occurs in one direction only, that is, from Ogawa to Inaba.

Cross-agglutination and absorption studies have indicated that Ogawa cells may contain some 'C' antigen on their surface and that Inaba strains represent cells which have lost the ability to synthesize the 'B' antigen (Redmond et al., 1973). It was also suggested that the

Hikojima serotype represents an intermediate stage between Ogawa and Inaba strains and the relative amounts of antigens 'B' and 'C' may vary in different Hikojima strains (Sakazaki and Tamura, 1971).

1.5   Mapping oag in V. cholerae

Conjugal transfer of chromosomal DNA in V. cholerae is mediated by the naturally occurring conjugative plasmid, P (Bhaskaran, 1960; Parker and Romig, 1972; Johnson and Romig, 1978), and would appear to be similar to that observed in E. coliK-12, due to the F-factor. The major differences are that recombination frequencies are much lower in V. cholerae (about 10^{-6}/donor cell) and unselected donor markers appear at a lower rate than in E. coliK-12 F^+xF^- matings (Parker et al., 1971). It has been reported (Parker et al., 1979) that the P-factor does not stably integrate into the chromosome to produce "Hfr" strains.

Early mapping experiments in V. cholerae were performed by Bhaskaran (1960) who used auxotrophic strains derived from the Classical strain, 162. By selecting for one auxotrophic marker from each parent and then scoring unselected markers in the recombinants, Bhaskaran was able to order a number of markers:

... str ... pur-1 ... ilv-1 ... oag ... arg-1 ... leu-1 ... his-1

Thus, the oag locus encoding serotype specificity was linked to ilv-1 and arg-1. The linkage map of strain 162 has been extended by Parker et al. (1971, 1972). Bhaskar-

an's work was confirmed and a total of 27 mutant alleles in three distinct linkage groups were mapped. Further analysis by Parker et al. (1978) utilized both linkage analysis and crossover class analysis and gene orders obtained from the two methods were similar. It was not possible from these data to show linkages between the alleles of the three linkage groups, so genetically the map of V. cholerae was concluded to be linear.

Mapping of markers in El Tor strains was studied by Johnson and Romig (1979) using improved donor strains, which were constructed by introducing Tn1 (encoding Ap^R) into both the chromosome and the P-factor. This effectively created "portable regions of homology" which could promote integration of the P-factor into the chromosome. Such strains could be shown to transfer chromosomal genes at high frequencies from origins which were specified by the site of Tn1 insertion. Johnson and Romig called these donor strains "Tfr" for transposon-facilitated-recombination. Thus, the Tfr strains were characterized by moderately high, polarized chromosomal transfer and efficient transfer of the hybrid P-factor, making them more similar to F' "intermediate donors" than to Hfr donors (Scaife, 1967). It was concluded by Johnson and Romig that their Tfr donors consisted of a mixed population with few Hfr donors and mainly P^+ donors. Using different P::Tn1 plasmids in which Tn1 was inserted in opposite orientations, the direction of chromosomal transfer could be reversed. A series of matings was performed in the El Tor strain MAK757 and its derivatives, resulting

in a circular genetic map of the E1 Tor chromosome, in which the gene orders obtained agreed closely with that previously described for the Classical strain 162.

Subsequent work by Sublett and Romig (1981), used the Tfr system in the Classical strain 162, confirming the data of Parker (1971, 1978) and demonstrating that the pro-2 and his-1 markers were linked. These two markers were terminal markers on the chromosome which previously had been unlinked and consequently the genetic map of the Classical strain 162 was shown to be circular (Fig. 1.6).

The Tfr system was also used by Green et al. (1983). Tn5 (km^R) or Tn10 (Tc^R) were introduced into both the chromosome and the P-factor. Whereas Johnson and Romig used super-infection with an incompatible plasmid to eliminate their Tn1 vector, Green et al. (1983) and Newland et al. (1984) used temperature sensitive F' factors as suicide delivery vehicles. Another feature of their work was application of the Tfr system to recent clinical isolates of V. cholerae. Green et al. (1983) reported the mapping of the E1 Tor biotype markers hly, pmx and cha, using E1 Tor Tfr donor strains and auxotrophic Classical recipient strains. The linkage analysis revealed that these three determinants were closely linked to leu and also that a segment of the E1 Tor genetic map (pro-201 ... pyrA ... 201 ... leu-1 ... ura-201) was inverted with respect to the map of the Classical strain, 162.

Figure 1.6:   Genetic map of the V. cholerae chromosome from (A)
              Classical strain 162 and (B) E1 Tor strain GN6300
              based on published linkage data (Sublett and Romig,
              1981; Newland et al., 1984). The double line
              corresponds to an inverted order of markers between
              the Classical and E1 Tor biotypes.

A

str
htx
rif
lys-6,11
ilv-1
aro-11
arg-1
spc
cys-5,11,C
uro-6
ser-10
leu-1
pro-2
his-1
aro-10
tox-1
OAG
asp-1
trp-1
thy-20
met-10::Tn1
met-2

B

str
htx
rif
ilv-5
val-201
pur-201
lys-1,201
arg-7
spc
cys-201
ile-201
xds
pro-201,202,203
pyrA-201
hLy
leu-1
pmx,cha
ser
ura-201
aro-202,201
tox
his-2
ctx
nal
trp-2
OAG
thy
met-4,201
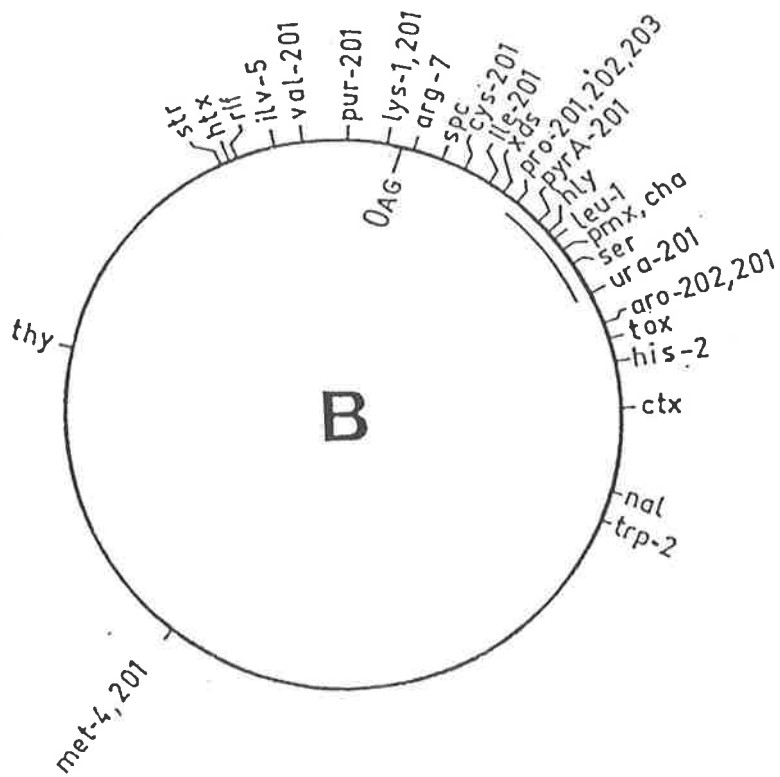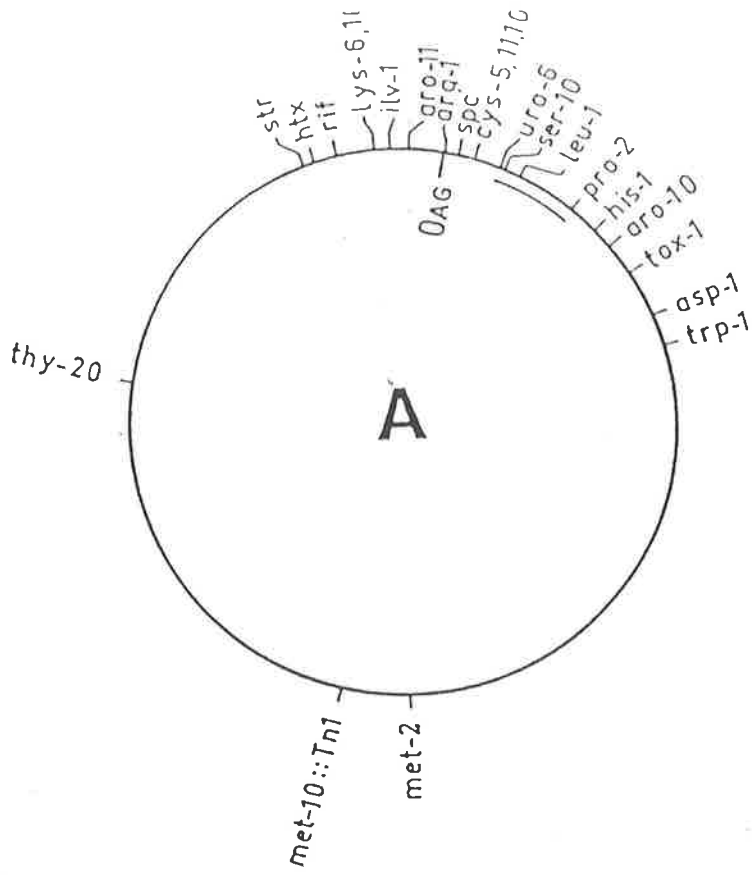
1.6   Objectives of this study

The phenomenon of serotype variation in <u>V</u>. <u>cholerae</u> 01 has been reported by a number of workers, but the mechanism responsible for this process has not yet been determined. The aims of this study are to analyse the expression of <u>V</u>. <u>cholerae</u> serotype antigens on the cell surface and also to characterize the <u>rfb</u> region encoding these specificities. Thus, the objectives are to analyse serotype variation in <u>V</u>. <u>cholerae</u> 01 at both the cellular and molecular level.

CHAPTER 2

MATERIALS AND METHODS.

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) was the general growth medium for $\underline{E.\ coli}$ strains. $\underline{V.\ cholerae}$ strains were grown in Brain Heart Infusion (BHI) (Difco) prepared as directed by the manufacturers. NA is nutrient agar, which is blood base agar (Difco) prepared without the addition of blood.

Antibiotics were added to broth and solid media at the following final concentrations: ampicillin, 25 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml; tetracycline, 10 μg/ml for $\underline{E.\ coli}$, and 4 μg/ml for $\underline{V.\ cholerae}$ strains.

Incubations were at 37°C unless otherwise specified. Normally liquid cultures were grown in 20 ml McCartney bottles, or 125 ml side-arm flasks. Optical densities (OD) were measured at 650 nm using a Unicam Instruments spectrophotometer.

2.2   Chemicals and reagents

Chemicals were Analar grade. Phenol, sodium dodecyl sulphate (SDS), and sucrose were from BDH Chemicals. Tris and Trizma base were from Sigma.

Antibiotics were purchased from Sigma (ampicillin, kanamycin sulphate), and Calbiochem (tetracycline, chloramphenicol).

The following electrophoresis grade reagents were obtained from the sources indicated: acrylamide and ammonium persulfate (Bio-Rad), ultra pure N,N'-methylene-bisacrylamide and urea were from BRL.

2.3   Antibiotic resistance testing

Oxoid "Multodiscs" S1 and S2 were used to test alterations in the pattern of resistance or sensitivity to a range of antibiotics. A nutrient agar plate was overlaid with 5 ml of soft nutrient agar containing 2×10^7 bacteria. On to this was then layered the "Multodisc" and the plates were incubated overnight at 37°C. The zone of inhibition of growth was measured. Similar procedures were followed when using individual Oxoid antibiotic discs. These discs contained Vancomycin (30 μg), Bacitracin (10 i.v.), Novobiocin (30 μg) or Oxytetracycline (30 μg).

2.4   Enzymes

The following enzymes were obtained from Sigma: deoxyribonuclease I (DNase I), ribonuclease A (RNase A) and lysozyme. Pronase was from Boehringer Mannheim.

Restriction endonucleases BamHI, ClaI, HpaI, PstI, SalI and XbaI, were purchased from Boehringer Mannheim. EcoRI, HindIII were from Biolabs. KpnI was from Amersham, while SstI was obtained from BRL.

Table 2.1: Vibrio cholerae strains

Strain	Biotype	Serotype/Phenotype	Source
1621	E1 Tor	Ogawa	J.E. Ogg
T51	E1 Tor	Inaba	K. Bhaskaran
O29	Classical	Ogawa	J.E. Ogg
O17	E1 Tor	Ogawa	K. Bhaskaran
569B	Classical	Inaba	I. Huq
Kasauli R	Classical	Rough	T. Desmarchelier
569B-165	Classical	NAG	K. Bhaskaran
CA411	Classical	Ogawa	K. Bhaskaran
AA140	Classical	Ogawa	D. Sharma
Z175	Classical	Inaba	D. Sharma
V637	E1 Tor	Ogawa F'_{ts} $\underline{lac}$, $\underline{trp}$,::Tn$\underline{5}$	This study
V638	E1 Tor	$\underline{rfa}$ O::Tn$\underline{5}$- 1	This study
V640		$\underline{rfb}$ O::Tn$\underline{5}$- 1	"
V641		$\underline{rfb}$ O::Tn$\underline{5}$- 2	"
V642		$\underline{rfb}$ O::Tn$\underline{5}$- 3	"
V643		$\underline{rfb}$ O::Tn$\underline{5}$- 4	"
V644		$\underline{rfb}$ O::Tn$\underline{5}$- 5	"
V660	Classical	Inaba F'_{ts} $\underline{lac}$, $\underline{trp}$,::Tn$\underline{5}$	This study
V661		$\underline{rfb}$-I::Tn$\underline{5}$- 6	"
V663		$\underline{rfb}$-I::Tn$\underline{5}$- 7	"
V665		$\underline{rfb}$-I::Tn$\underline{5}$- 8	"
V667		$\underline{rfb}$-I::Tn$\underline{5}$- 9	"
V669		$\underline{rfb}$-I::Tn$\underline{5}$-10	"
V671		$\underline{rfa}$-I::Tn$\underline{5}$- 2	"
V677	Classical	V667 - $\underline{ThyA}$	"
V687	Classical	V677 + P- factor	"
V201	E1 Tor	O17 (R_{ts}-1::Tn$\underline{2680}$)	"
V223	E1 Tor	$\underline{rfa}$-O::Tn$\underline{2680}$-1	"
V224	E1 Tor	$\underline{rfa}$-O::Tn$\underline{2680}$-2	"
V225	E1 Tor	$\underline{rfa}$-O::Tn$\underline{2680}$-3	"

Table 2.2: Escherichia coliK-12 strains

Strain	Phenotype/Genotype	Source
LE392	F⁻, hsd R514 (r⁻, m⁻), supE44, supF28, lacY1, or (lac IZY)6, gal k2, gal T22, metB1, trpR55,	B. Bachmann
DH1	F⁻ gyrA96, recA1, relA1, endA1, thi-1, hsdR17, supE44,	B. Bachmann
C600	F⁻ thr-1, leu-6, tonA1, lacY1, supE44, thi-1	W. Arber
E198	pRU669(R$_{ts}$1::Tn1725) km^R Cm^R	N. Willetts
V333	pPM1001 in DH1	P. Manning
V399	pPM1002 in DH1	"
V455	pPM1003 in DH1	"
V472	pPM1004 in DH1	"
V512	pPM1006 in DH1	This study
EX101	pEVX7 in DH1	R. Morona
EX53	pEVX10 in LE392	"
EX84	pEVX12 in LE392	"
V552	V472 + pPM1003	This study
E328	F'$_{ts}$ lac, trp::Tn5 km^R	S. Formal
E167	P1910 (R$_{ts}$1::Tn2680) km^R	N. Willetts

Table 2.3: Bacteriophages

Bacteriophages	Source
CP – T1	J. Ogg
Classical typing phages	
Vc I	B.C. Deb
Vc II	B.C. Deb
Vc III	B.C. Deb
Vc IV	B.C. Deb
E1 Tor typing phages	
e1	B.C. Deb
e2	B.C. Deb
e3	B.C. Deb
e4	B.C. Deb
e5	B.C. Deb

DNA modifying enzymes were purchased from Biolabs (T4 DNA ligase), Amersham (T4 DNA polymerase) and Boehringer Mannheim (DNA polymerase I).

2.5 Bacterial strains and bacteriophages

Vibrio cholerae strains are listed in Table 2.1. Strains of the E1 Tor biotype were distinguished from the classical biotype by resistance to the antibiotic Polymixin B (15μg/ml) and sensitivity to biotype specific typing phages. Table 2.2 describes the E. coli strains used in this study. For long-term storage cultures were stored lyophilized in ampoules or as glycerol cultures frozen at -70°C. Working stocks were glycerol cultures stored at -20°C. Glycerol cultures were prepared by re-suspending bacterial growth from plates in a mixture of 0.4 ml 80% (v/v) glycerol plus 0.6 ml 1% peptone (Difco, Bacto-peptone).

The bacteriophages used are listed in Table 2.3. Bacteriophage CP-T1 was generously provided by J.E. Ogg. The Classical and E1 Tor biotype typing plage are designated VcI-IV and e1-5, respectively, and were provided by B.C. Deb.

2.6 Bacteriophage methods

2.6.1 Plaque assay

Indicator bacteria (0.1 ml), from a freshly grown culture, were pre-incubated with bacteriophage (0.1 ml of neat or 100-fold serial dilutions) at 37°C for 5 min. 4 ml

soft agar (44°C) was then added and poured as an overlay on to a NA plate. The plates were incubated inverted at 37°C.

2.6.2 Phage-inactivation of LPS

The phage neutralizing capacity of LPS preparations was determined by incubating 10^4 plaque forming units (pfu) with various concentrations of LPS in a total volume of 1 ml in nutrient broth at 37°C for 60 min. Each sample was assayed and the phage-inactivating capacity expressed as the concentration of LPS that resulted in a 50% decrease in the titre of the phage during a 60 min incubation at 37°C.

2.7 Selection of phage resistant mutants

Strains containing the temperature sensitive plasmids were grown to exponential phase, with aeration at 30°C. Phage resistant mutants were selected by plating out the cells in the presence of various dilutions of phage. Typically, 0.1 ml of cells were added to 0.1 ml phage (5×10^8 pfu) and mixed after 10' at 37°C, 4 ml of soft agar was added and the contents overlaid on NA-Km plates. The plates were incubated overnight at 42°C. After purification the transposon insertion mutants were grown at 37°C.

2.8 Conjugations

Matings were performed as filter matings. Donor and recipient strains were grown to exponential phase at 37°C. 0.5 ml of donor cells was mixed with 0.5 ml recipient

cells, washed once in saline and resuspended in 200µl. The cells were spread onto a Millipore 0.45µM cellulose acetate filter and placed onto an NA plate which was incubated overnight at 37°C. The cells were then re-suspended in 1 ml NB and 200µl aliquots were spread onto selective plates.

2.9   Electron microscopy

DNA was spread in 0.5 M ammonium acetate at neutral pH following the method of Kleinschmidt and Zahn (1959). Electron micrographs were taken in a Philips EM301 transmission electron microscope.

2.9.1 Preparation of heteroduplex DNA

DNA hybridization and spreading was performed according to Inman (1974), with modifications as in Morris et al. (1975) and Morelli et al. (1978). Electron micrographs were taken in a Philips EM301 transmission electron microscope.

2.9.2 Preparation of Protein A-gold

Colloidal gold (10nm or 15nm diameter) was treated with dextran, activated with glutaraldehyde and then coupled to Protein (Pharmacia), according to the method of Hicks and Molday (1983).

2.9.3 Immuno-gold electron microscopy

Immuno-gold labelling was performed using an adapt-ation 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 60kv. All incubation and wash solutions consisted of PBS, pH 7.2. Cells were initially resuspended in PBS with 1 mM $MgCl_2$ and all subsequent wash solutions included 1% bovine serum albumin.

2.10 Transformation procedure

This is basically a modification of the procedure of Brown et al. (1979). E. coliK-12 strains were made competent for transformation with plasmid DNA as follows: an overnight shaking culture (in NB) was diluted 1:20 into BHI and incubated with shaking until the culture reached an OD_{650} of 0.6. The cells were chilled on ice for 20 min, pelleted at 4°C in a bench centrifuge, resuspended in half volume of cold 100 mM $MgCl_2$, centrifuged again, and resuspended in a tenth volume of cold 100 mM $CaCl_2$. This was allowed to stand for 60 min on ice, after which time the cells were used as competent. Competent cells (0.2 ml) were mixed with DNA (volume made to 0.1 ml with 10 mM Tris-HCl, 1 mM 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, then 3 ml BHI was added, and this was then incubated with shaking at 37°C for 1-2 hr. The culture was

plated on to selection plates directly or concentrated by centrifugation, and plated. Cells with sterile buffer were run as a control.

2.11   DNA extraction procedures

2.11.1 Plasmid DNA isolation

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

Method 1: rapid plasmid preparation by the boiling method of Holmes and Quigley (1981) was performed as follows: Cells from 1 ml of a 10 ml shaking overnight culture were pelleted in an Eppendorf 5414 centrifuge for 30 sec, resuspended in 50µl STET buffer (5% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris HCl pH 8.0). Lysozyme (5µl of 5 mg/ml) was added and the suspension left at room temperature for several min. Samples were then placed in boiling water for 35 sec, and immediately spun for 10 min in an Eppendorf centrifuge. The chromosomal pellet was removed and plasmid DNA in the supernatant precipitated with 0.6 volumes of propan-2-ol, at -20°C for 10 min. DNA was pelleted by centrifugation in an Eppendorf centrifuge for 10 min, washed once with 1 ml 70% ethanol, dried in vacuo and dissolved in 20 µl TE buffer. This method was also scaled up for use with 10 ml cultures.

Method 2: Triton X-100 cleared lysates were prepared by a modification of the procedure of Clewell and Helinski (1969, 1970) from 10 ml overnight cultures following sedimentation of cells in a bench centrifuge. Cells were

resuspended in 0.4 ml 25% sucrose, 50 mM Tris-HCl pH 8.0. Lysozyme (50 µl, 10 mg/ml freshly prepared in H_2O) and 0.25 M EDTA pH 8.0 (50 µl) were added to cells in micro-fuge tubes, and left to stand on ice for 15 min. 0.5 ml TET buffer (50 mM Tris-HCl, 66 mM 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 (15000 rpm, 20 min, 4°C, SS34, Sorvall). The supernatant was extracted twice with TE saturated phenol, and twice with diethyl ether. Plasmid DNA was precipitated by the addition of an equal weight of propan-2-ol, and standing at -70°C for 30 min. The precipitate was collected (10 min, Eppendorf 5414 centrifuge), washed once with 1 ml 70% ethanol, dried and resuspended in 50 µl TE buffer.

Method 3: large scale plasmid purification was performed by the three step alkali lysis method (Garger et al., 1983). Cells from a 1l culture were harvested (6000 rpm, 15 min, 4°C, GS-3 Sorvall) and resuspended in 24 ml solution 1 (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA). Freshly prepared lysozyme (4 ml of 20 mg/ml in solution 1) was mixed with the cell suspension and incubated at room temperature for 10 min. Addition of 55 ml of solution 2 (0.2 N NaOH, 1% SDS) followed by 5 min incubation on ice resulted in total lysis of the cells. After the addition of 28 ml of solution 3 (5 M potassium acetate pH 4.8) and incubation on ice for 15 min, protein, chromosomal DNA and high molecular weight RNA were removed by centrifugation (8000 rpm, 20 min, 4°C in a GSA rotor,

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 top aqueous phase was precipitated with 0.6 volume of propan-2-ol at room temperature for 10 min, and collected by centrifugation, (10000 rpm at 4°C for 35 min in a Sorvall GSA rotor). After washing in 70% ethanol, the pellet was dried _in vacuo_ and resuspended in 4.6 ml TE. Plasmid DNA was purified from contaminating protein 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 g needle attached to a 1 ml syringe. The ethidium bromide was extracted using CsCl saturated isopropanol. CsCl was then removed by dialysis overnight against 2 litres of TE at 4°C. DNA was stored at 4°C.

2.11.2 Preparation of _V. cholerae_ genomic DNA

Cells from a 20 ml shaking overnight culture were pelleted in a bench centrifuge for 10 min and washed once with TES buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 50 mM NaCl). The pellet was then resuspended in 2 ml 25% sucrose, 50 mM Tris-HCl pH 8.0, and 1 ml lysozyme (10 mg/ml in 0.25 M EDTA pH 8.0) was added and the mixture incubated on ice for 20 min. TE buffer (0.75 ml) and 0.25 ml lysis solution (5% sarkosyl, 50 mM Tris-HCl, 0.25 M EDTA pH 8.0) were added together with 10 mg solid pronase. The mixture was gently mixed, transferred to a 50 ml Ehrlenmeyer flask and incubated at 56°C for 60 min. This was followed by two extractions with each of TE

saturated phenol and diethyl ether. The genomic DNA was then precipitated from the solution by the addition of two volumes of cold 95% ethanol. The precipitate was washed twice with 70% ethanol, dried in vacuo for 60 min and allowed to resuspend in 1 ml TE buffer. This was achieved by heating to 56°C for several minutes. This generally yielded high molecular weight DNA at concentrations ranging from 0.5 - 1 mg/ml.

2.11.3 DNA quantitation

The DNA concentration 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).

2.12 DNA manipulation procedures

2.12.1 Digestion of DNA by restriction endonucleases

Cleavage reactions of the restriction enzymes HindIII, BamHI, EcoRI, PstI, SstI and XbaI were performed using SPK buffer (10X stock is 200 mM Tris-HCl, pH 8.0, 50 mM $MgCl_2$, 5 mM dithioerythritol, 1 mM EDTA, 500 mM KCl and 50% glycerol). The remaining restriction digests were carried out as described in Davis et al. (1980). 0.1- 0.5 µg of DNA or purified restriction fragments were incubated with 1 unit of each restriction enzyme in a final volume of 20 µl, at 37°C, for 1-2 hours. The re- actions were terminated by heating at 65°C for 10 minutes. Prior to loading on to a gel a one-tenth volume of tracking dye (15% Ficoll, 1 mg/ml bromophenol blue) was added.

2.12.2 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% agarose gels (Seakem HGT), 13 cm long, 13 cm wide, and 0.7 cm thick. Gels were run at 100 V for 4-5 hr in TBE buffer (67 mM Tris base, 22 mM boric acid, and 2 mM EDTA, final pH 8.8). After electrophoresis the gels were stained in distilled water containing 2 µg/ml ethidium bromide. DNA bands were visualized by trans-illumination with UV light and photographed on 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% was used for separation of restriction fragments. DNA bands were excised and the agarose melted at 65°C. Five volumes of 20 mM Tris-HCl, 1 mM EDTA pH 8.0 buffer was added and the agarose extracted with phenol:water and then phenol:chloroform (both 1 g/ml). Residual phenol was removed with chloroform and the DNA precipitated with two volumes of ethanol and one-tenth volume of 3 M sodium acetate pH 5.0.

2.12.3 Size determination of restriction fragments

The sizes of restriction fragments were determined by comparing their relative mobilities on the gels with those of DNA molecules of known size. The standards used were λ DNA cleaved with HindIII (Philippsen et al., 1978), and Bacillus subtilis phage SPP1 DNA cut with EcoRI (Ratcliff et al., 1979). The sizes of large fragments were calcul-

ated from the sum of the sizes of their sub-fragments obtained after digestion with a second enzyme.

Lambda DNA was graciously provided by Dr R. Morona.

2.12.4 Nick translation method

Nick translation reactions with DNA polymerase I were modified from the procedure described by Maniatis et al. (1982) and carried out as follows:

25 μCi α -[^{32}P]dCTP (1700 Ci/mmole in ethanol) was dried in vacuo in a microfuge tube, resuspended with 80 μl water, 10 μl 10x nick translation buffer (500 mM Tris-HCl pH 7.2, 100 mM MgCl$_2$, 1 mM DTT, 500 μg/ml BSA) and 1 μl each of 2 mM dATP, dGTP, dTTP. DNA (1 μg) to be nick translated was added. To this mixture 1 μl DNase (0.1 μg/ml) was added and the whole was incubated at 37°C for 10 min. DNA polymerase I (5 units) was then added into the reaction mix, and allowed to incubate at 16°C for 2 hr. DNA was separated from unincorporated label by centrifugation through a small column of Sepharose CL-6B (Pharmacia).

2.12.5 Southern transfer and hybridization

Bidirectional transfer of DNA from agarose gels to nitrocellulose paper (Schleicher and Schüll) was performed using the procedure of Southern (1975) as described by Maniatis et al. (1982).

Prior to hybridization with radiolabelled probe, filters were incubated for 4 hr at 44°C in a prehybridiz-

ation solution containing 50% formamide, 50 mM sodium phosphate buffer, pH 6.4, 5x SSC (0.34 M NaCl, 75 mM sodium citrate, pH 7.0) 5x Denhardt's reagent and 83 μ g/ml single stranded Herring sperm DNA (Sigma) (Maniatis et al., 1982). Prehybridization fluid was discarded and replaced with fresh hybridization buffer (as for prehybridization solution, with the exclusion of Herring sperm DNA). Denatured probe (approximately 10^6 cpm) 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 2x SSC, containing 0.1% SDS. This was followed by two further washes in 0.2x SSC. After drying in air (15 min, room temperature) the filters were covered in plastic wrap and placed on film for autoradiography at -70°C with intensifying screens.

2.13   SDS polyacrylamide gel
                 electrophoresis (SDS-PAGE)

2.13.1 Linear gels

SDS-polyacrylamide-gel-electrophoresis was performed on either 11-20% gradients (for proteins) or straight 20% or 25% 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°C for 3 min prior to loading. Gels were generally electrophoresed at 100 V for 5 hr (11-20% gradient gels) or 10 mA constant current for 16 hr (25% PAGE gels). Protein staining was achieved by incubation with gentle agitation for 60 min with Coomassie

Brilliant Blue G250 in 50% (v/v) methanol, 10% (v/v) acetic acid. Destaining was with several changes of 7.5% (v/v) acetic acid, 10% (v/v) methanol, 10% (v/v) ethanol and gentle agitation for 24 hr.

2.13.2 Exponential gels

Exponential gradients (7.5% to 25%, wt/vol) containing 4M Urea, were generated using an XPO77 gradient maker (Hoefer Scientific Instruments, San Francisco, Calif.). After polymerization, a stacking gel containing 5% acrylamide and 0.13% bisacrylamide was cast, according to the method of Kusecek et al. (1984).

2.14   LPS silver staining

Silver staining of LPS in polyacrylamide gels was performed using a modification of the method described by Tsai and Frasch (1982). The following procedure was used: i) fixation overnight in 40% ethanol, 10% acetic acid; ii) oxidation for 5 min with 0.7% periodic acid in 40% ethanol, 10% acetic acid; iii) 4 washes at 30 min each; iv) staining for 10 min, in a solution containing 28 ml 0.1 N NaOH, 2 ml concentrated NH_4OH and 5 ml 20% $AgNO_3$ in a total volume of 150 ml; v) developing in a solution of 50 mg citric acid and 0.5 ml formaldehyde in 1l. 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 less than 18 megaohms/cm was used to rinse all glassware and in preparation of solutions.

2.15   Autoradiography

SDS-PAGE gels were dried on Whatman 3MM chromatography paper at 60°C for 2 hr on a Bio-Rad gel drier. For [33P]-phosphate, autoradiography was performed at room temperature for 1-7 days without intensifying screens using Kodak XR-100 film. For autoradiography with [32P], the gels were exposed to film for 6-72 hr at -70°C, using intensifying screens.

2.16   Small scale cell envelope isolation

Whole membrane material was isolated from 10 ml mid-exponential phase cultures by the method in Manning et al. (1982). The cultures were centrifuged for 10 min in a bench centrifuge and the cells washed with 10 ml 30 mM Tris-HCl pH 8.1. The pellet was resuspended in 0.2 ml 20% sucrose, 30 mM Tris-HCl pH 8.1, and the suspension transferred to SM24 tubes (Sorvall) on ice. After the addition of 20 μl lysozyme (1 mg/ml in 0.1 M EDTA pH 7.3) incubation was continued on ice for 30 min. The cells were then placed in an ethanol-dry ice bath for 30 min. After thawing, 3 ml of 3 mM EDTA pH 7.3 was added and the cells sonicated in a Branson sonicator (four, 15 sec pulses on 50% cycle). Membrane material was pelleted by centrifugation (20000 rpm, 60 min, 4°C, Sorvall) and resuspended in 100 μl 1x sample buffer (Lugtenberg et al., 1975). Samples were stored at -20°C. 10-15 μl amounts were loaded on to SDS-polyacrylamide gels.

2.17   Isolation of lipopolysaccharide

LPS was extracted from cells with hot 90% phenol/ water (w/v) using the procedure of Westphal and Jann (1965). Briefly, a 100 ml bacterial suspension (24 mg/ml dry weight) was heated to 68°C. An equal volume of 90% phenol/water (prewarmed to 68°C) was mixed into the cell suspension and continuously stirred at 68°C for 30 min. The mixture was allowed to cool and the phases were separated by centrifugation (5000 rpm, 25 min, 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 5l of water, overnight at 4°C. Insoluble material was pelleted by centrifugation (5000 rpm for 5 min at 4°C in an SS34 rotor, Sorvall). The LPS was precipitated with 5-6 volumes of cold ethanol and a small quantity of sodium acetate powder. The precipitate was collected by centrifugation (5000 rpm for 30 min at 4°C in a GSA rotor) and resuspended in 25 ml of water and 0.65 ml 1 M $MgCl_2$. A small quantity (approximately 1 mg) of DNase I 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, (5000 rpm for 10 min at 4°C in an SS34 rotor). The LPS was then pelleted by high speed centrifugation in a Beckman L8-80 ultracentrifuge (37000 rpm for 2 hr at 15°C in a 60 Ti rotor). The pellet was then

suspended in 0.5 ml water, of which 0.1 ml was used for dry weight estimation of LPS content. The LPS concentration was subsequently adjusted to 10 mg/ml. LPS preparations were stored at 4°C.

2.18 Bactericidal assay

2.18.1 Complement preparation

Fresh guinea-pig blood was allowed to clot for 30 min at 37°C, followed by an additional 60 min at 4°C. The clear serum was removed with a sterile Pasteur pipette and centrifuged at 2500 rpm for 10 min in a bench centrifuge at room temperature.

Cross-reacting antibodies against V. cholerae were removed from the guinea-pig serum by absorption with 5×10^9 organisms/ml for 60 min at 4°C. The cells were separated from the serum by centrifugation (15 min, 10000 rpm, SS34, Sorvall). The serum was then passed through a Millex filter (45 μm), and stored in aliquots at -20°C.

2.18.2 Complement-mediated bactericidal assay

Bactericidal assays using guinea-pig serum and a monoclonal antibodies provided by Dr S. Neoh (Flinders Medical Centre), were performed in the following manner. A mid-exponential phase culture was diluted 10^4-fold in peptone-saline (1% peptone, 0.5% saline, pH 7.5). 0.2 ml of diluted cell suspension was mixed with 0.1 ml undiluted guinea-pig serum and 0.7 ml saline. To 0.1 ml of the cell/guinea-pig serum mixture was added 0.1 ml of various

dilutions of antibody. After incubation at 37°C for 90 min, 0.1 ml of this mixture was spread on to a nutrient agar plate and incubated overnight at 37°C. The plates were then counted and % survival determined.

2.19   Preparation of whole cell lysates (WCL)

Whole cell lysates (WCL) were prepared using a modification of the method of Hitchcock and Brown (1983). Cells were grown overnight in nutrient broth 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% SDS, 4% β-mercaptoethanol, 10% glycerol, 1 M Tris, (pH 6.8) and 0.1% bromophenol blue. Lysates were heated at 100°C for 10 min. 25 µg of Proteinase K (Boehringer Mannheim, GmBH, West Germany) solubilized in 10 µl of lysing buffer was added to each sample and incubated at 60°C for 60 min. Samples were stored at -20°C.

2.20   Haemagglutination inhibition assay (HIA)

A haemagglutination assay was done 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 anti-serum. Trays were incubated at 37°C for 60 min and the end point determined. Four haemagglutinating units of antibody were used in the HIA.

The HIA was performed as follows. The antigen being tested (usually 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   Preparation of anti-sera

Anti-sera were produced using live organisms and the following schedule was followed. In the initial immunization, 1 ml containing 10^7 cells was mixed with 1 ml of 4% sodium alginate. 0.5 ml of this suspension was injected subcutaneously at 4 sites on the rabbit. Before removing the needle from each site, 0.3 ml of 0.4% $CaCl_2$ was injected. This procedure involved immunization with approximately 2.5×10^6 cells per injection.

The rabbit was subsequently boosted by intravenous injections of organisms ranging from 3×10^7 to 10^9/ml. The first boosting injection was given one week after immunization and three more injections were given over two weeks. The rabbit was testbled from the ear and the serum tested by HA. After resting the rabbit for a month, the animal was again boosted.

All sera obtained were tested by HA, pooled, heat inactivated and stored at $-20°$ with 0.02% azide added.

2.22   Preparation of formalin fixed cells

Overnight cultures were subcultured and grown to 2×10^9 cells/ml. The cells were washed once in saline and resuspended to 10^{10} cells/ml. A sample was kept for a

viable count and 1% formalin was added to the cells. The cells were incubated at $37^{\circ}C$ for 60 min with occasional shaking. The cells were then washed 3 to 4 times with saline and a sample plated out to check the proportion of viable cells remaining. The $O.D._{650}$ of all samples was measured and cells were adjusted to the same concentration $(2 \times 10^{10} /ml)$.

2.23   Preparation of monoclonal antibodies

Hybridoma cells were obtained from Dr Sim Neoh (Flinders Medical Centre) in ampoules stored in liquid N_2. The cells were resuspended in RPMI medium containing 10% foetal calf serum (FCS). The cells were then thawed in a $37^{\circ}C$ waterbath and washed twice in medium with 2% FCS. The cells were counted using a haemocytometer and 10^6 cells were injected intra-peritoneally into Balb/c mice. The mice had been primed with Pristane. Ascites cells from the mice were passaged at intervals of one to two weeks and the ascites fluid removed. The ascitic fluid was tested by HA, pooled and stored at $-20^{\circ}C$.

McAbs H4 and α-A were kindly provided by Prof. Tord Holme and Dr J. Sugiyama, respectively.

CHAPTER 3

CHARACTERIZATION OF V.cholerae
SEROTYPE-SPECIFIC ANTIGENS.

3.   CHARACTERIZATION OF V. cholerae
            SEROTYPE-SPECIFIC ANTIGENS

3.1   Introduction

In order to study the LPS of V. cholerae 01 and, in particular, the phenomenon of serotype variation, it was necessary to characterize the presence of the various antigens using McAbs. Such antibodies have the advantage over polyclonal anti-sera in that each McAb is directed against only one antigenic determinant and so is absolutely specific. Thus, the use of McAbs directed against the A, B and C antigens enables the distribution of each of these factors to be analysed separately on the cell surface of V. cholerae 01. In this chapter several McAbs directed against V. cholerae 01 LPS have been characterized and then used to analyse serotype-specific antigens on both V. cholerae and E. coliK-12 carrying the cloned V. cholerae LPS biosynthesis genes.

3.2   Characterization of McAbs

The McAbs 13-B and 20-B were produced from hybridomas (as described in Materials and Methods) and compared with a series of McAbs provided by Professor Tord Holme. Bacterial agglutination and haemagglutination assays were performed to determine the specificities of the various antibodies and their relative titres (Table 3.1). It can be concluded that 13-B is directed against an antigen present on Inaba cells. This antibody also reacted with Ogawa cells, but only at a much lower titre, indicating

Table 3.1: Bacterial agglutination and haemagglutination titres with monoclonal and polyclonal α-sera

Anti-serum		BA			HA	
		569B	017	E. coliK-12	569B[b]	017[b]
	13-B	512[a]	32	<2	1024[c]	<2
	20-B	4	32	<2	128	128
α-A	H4	64	64	<2	32	32
α-B	H8	<2	64	<2	<2	1024
α-C	C6	128	32	<2	128	<2
	α-569B	512	256	<2	1024	512
	α-017	1024	2048	<2	512	1024

a) Reciprocal of highest dilution of α-serum which agglutinated cells.

b) LPS used to sensitize SRBC.

c) Reciprocal of highest dilution of α-serum which agglutinated appropriately sensitized SRBC.

that this particular antigen was present in lesser amounts on Ogawa than on Inaba cells. Thus, it is likely that 13-B reacts with the 'C' antigen. As 20-B reacts equally well with Ogawa and Inaba it would appear that it is directed against the A antigen, which is common to all serotypes. The H8 antibody reacts only with Ogawa cells, and its specificity is therefore designated α-B.

The McAb 13-B was also tested in a bactericidal assay to confirm its ability to bind to and agglutinate live V. cholerae O1 cells (Fig. 3.1). Thus, 13-B was shown to react with recent clinical isolates of V. cholerae O1 in complement mediated lysis, as well as with 569B and O17. 20-B reacted equally well in the bactericidal assay with Ogawa and Inaba cells, confirming its specificity for the common A antigen.

The McAbs were used in an HIA to classify the antigens expressed by E. coliK-12 containing the LPS plasmids (Table 3.2). V333 inhibited the Inaba system only, whereas V399 and V455 inhibited the Ogawa system. Thus, it can be concluded that the cloned LPS genes are able to express in E. coliK-12, the serotype antigens of their respective parents, and so it follows from this observation that the genes necessary to synthesize the A, B and C antigens have been cloned.

3.3 Distribution of V. cholerae serotype antigens

Previous studies on the V. cholerae serotypes have provided information on the serotype antigens in partic-

Figure 3.1: Bactericidal activity of McAbs directed against various V. _cholerae_ strains.

□:13-B   vs   569B, Z175
▲:13-B   vs   O17, AA140
■:13-B   vs   V226

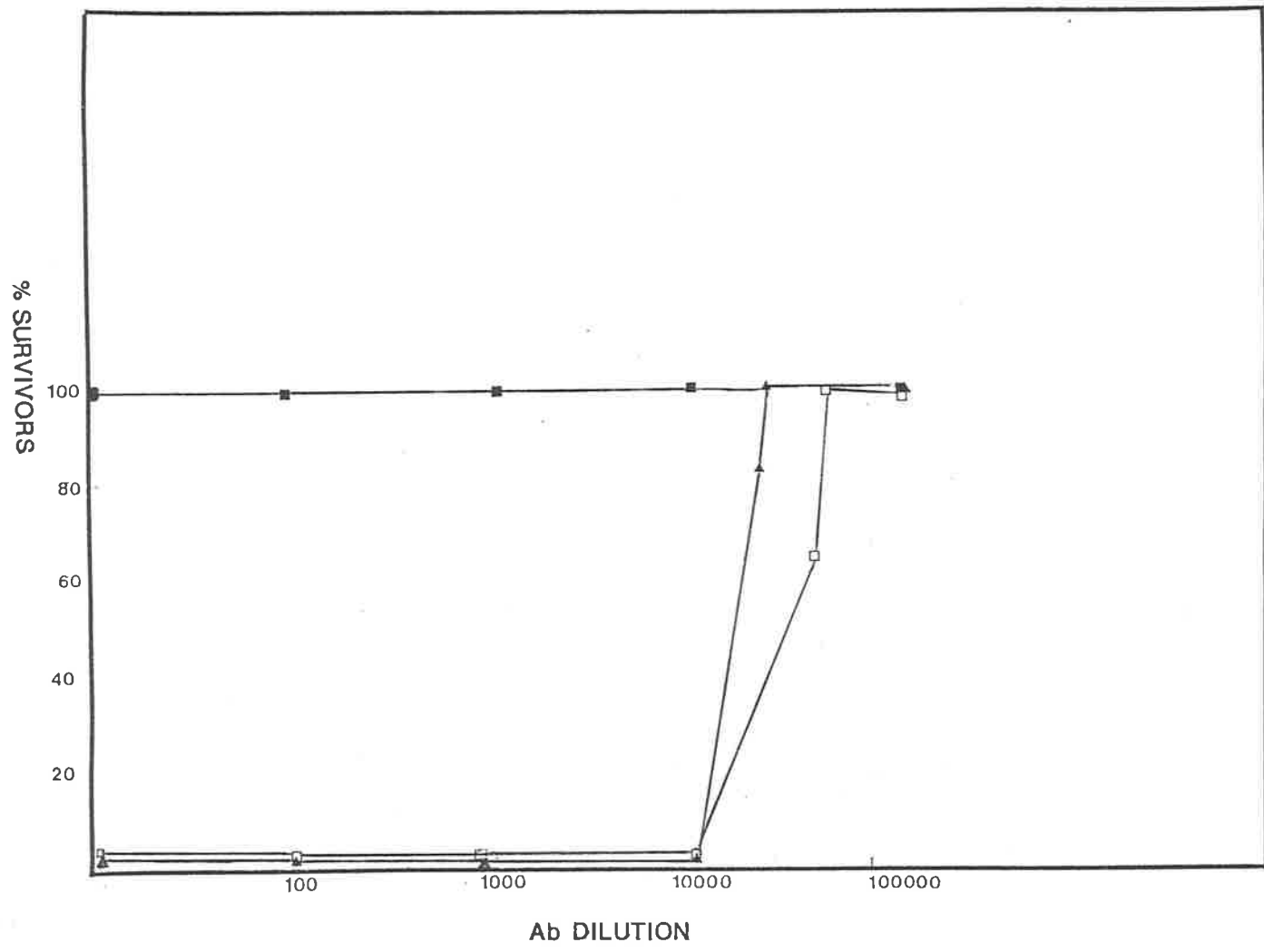□:20-B   vs   569B, AA140, O17, Z175
■:20-B   vs   V226

Table 3.2

Monoclonal antibody	Strain	569B[a]	017[b]
α-C 13B	569B	4[b]	
	017	1000	
	E. coliK-12	1000	
	V333	4	
	V399	500	
	V455	1000	
α-B H8	569B		1000
	017		16
	E. coliK-12		1000
	V333		1000
	V399		16
	V455		63

a) LPS used to sensitize SRBC.

b) Concentration of cell envelope material in μg/μl required to inhibit haemagglutination of sensitized SRBC.

ular cell populations (Sakazaki and Tamura, 1971; Sack and Miller, 1969; Gangarosa et al., 1967; Shrivastava and White, 1947). Such analyses are somewhat limited, as it is not possible to determine if all cells have identical levels of serotype antigen expression. Thus, this section examines the distribution of serotype-specific antigens on individual cells in V. cholerae 01 and also E. coliK-12 harbouring the various LPS clones.

Immuno-gold labelling of cells with McAbs followed by electron microscopy, was used to analyse the distribution of the various antigens. An α-A McAb was used to examine the presence of A antigen on V. cholerae strains 569B and CA411 (Fig. 3.2A and B). Although probe bound to both types of cells, it can be seen that CA411 (Ogawa) bound for more protein A-gold than 569B (Inaba). It is not known whether there is a true serotype difference in A expression or whether the result obtained is only characteristic of these two strains. Immuno-gold labelling of the three O-antigen clones (Fig. 3.2C, D and E) revealed that the McAb did bind to these strains.

The expression of B antigen was studied using the McAb H8 (Fig. 3.3). A high degree of binding of protein A-gold was observed to CA411, and virtually none to 569B (Fig. 3.3A and B). Thus, the B antigen was detected exclusively on Ogawa cells. Analysis of the V. cholerae O-antigen clones reflected the results found with CA411 and 569B. V333 bound very little probe (Fig. 3.3C), while V399 and V455 included cells which bound large amounts of probe

Figure 3.2: Electron micrographs showing binding of α-A McAb (used at
a 1 in 25 dilution) to V. cholerae strains and E. coli
K-12 harbouring the O-antigen clones. The gold particles
were 15 nm in diameter.

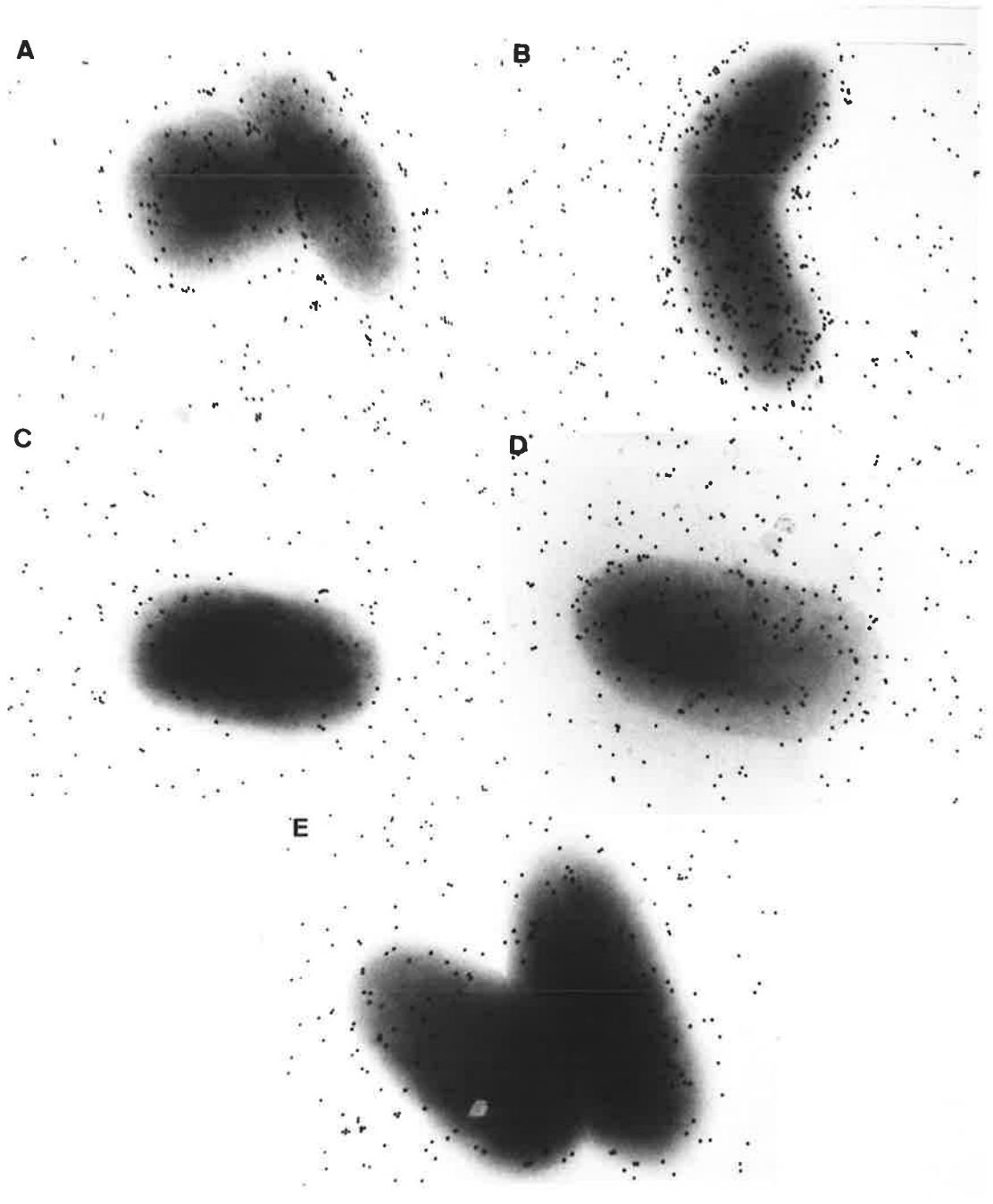|	
A	569B
B	CA411
C	V333
D	V399
E	V455

Figure 3.3:   Electron micrographs showing binding of α–B McAb (used at a 1 in 50 dilution) to V. cholerae strains and E. coliK-12 harbouring the O-antigen clones. The gold particles were 10 nm in diameter.

        A   569B
        B   CA411
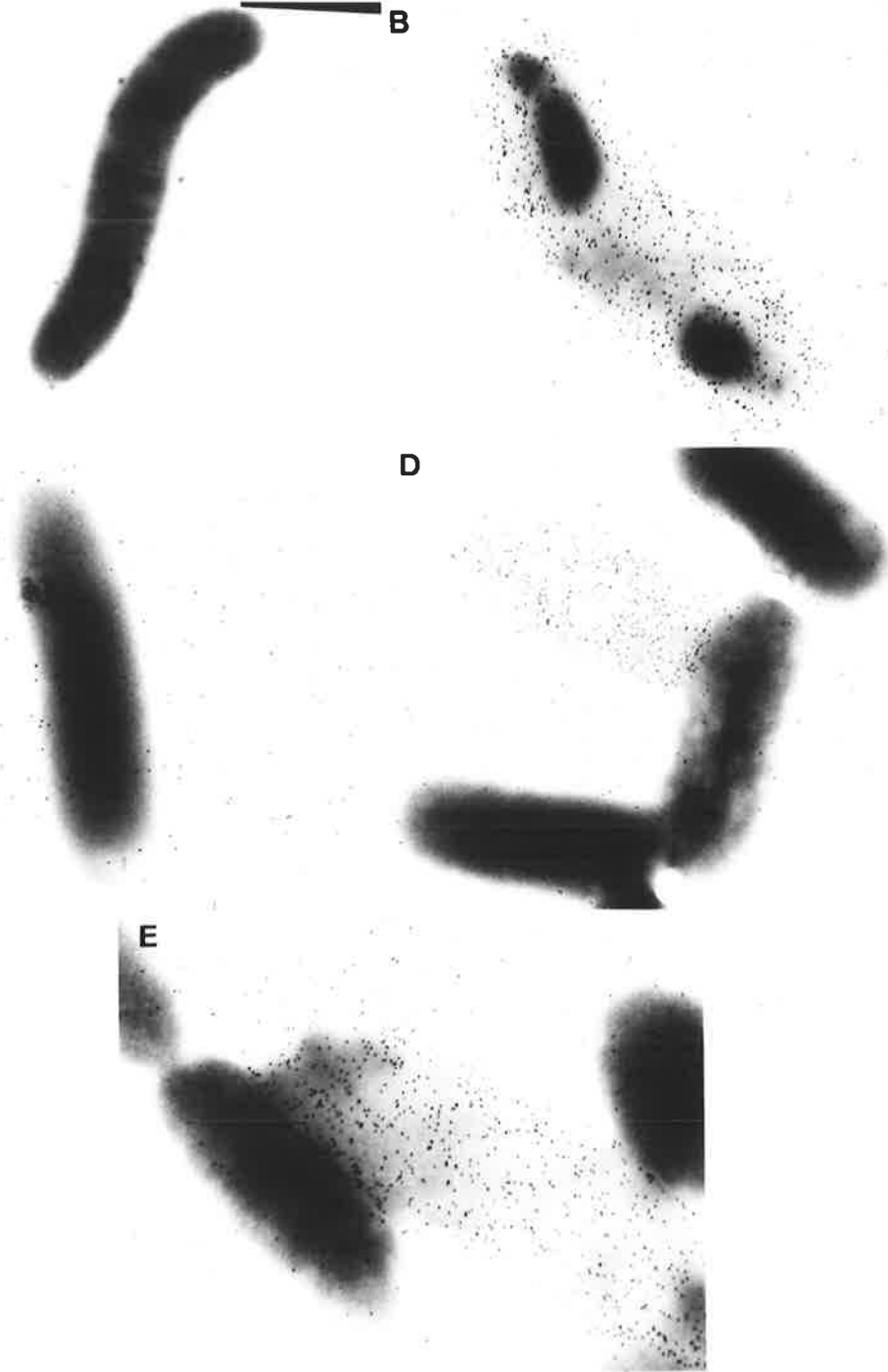   C   V333
        D   V399
   E   V455

A   B

C   D

E

(Fig. 3.3D and E). However, analysis of V399 and V455 also revealed cells which bound small amounts of probe, thus indicating that these clones actually represented a heterogeneous population of cells. It can be seen that those cells which bound very low amounts of protein A-gold have a different morphology from the other cells, as they appear more transparent. It is possible that these cells are undergoing cell death or lysis. Alternatively, they may represent cells in the population which have lost the plasmid and so can no longer express V. cholerae O-antigen on their surface. It has been observed that the various O-antigen clones are somewhat unstable and the use of ampicillin resistance to select for the plasmids may lead to the emergence of ampicillin sensitive colonies as β-lactamases degrade ampicillin in the media.

The McAb 13-B was used to study expression of C antigen (Fig. 3.4). Analysis of 569B and CA411 revealed that C antigen was detected on the majority of cells in both serotypes (Fig. 3.4A, B and C). The amount of protein A-gold bound was quantitated by measuring particles in a fixed area for a number of cells from both 569B and CA411. These results were analysed statistically (Table 3.3) and reveal that there are significantly more particles bound to Inaba cells compared with Ogawa. This would indicate that the scheme of antigen distribution proposed by Sakazaki and Tamura (1971), (see Chapter 1, Fig. 1.2), can be interpreted as a population of Ogawa cells in which all cells express a small amount of C antigen.

Figure 3.4: Electron micrographs showing binding of α-C McAb (used at a 1 in 1000 dilution) to V. cholerae strains and E. coli K-12 harbouring the O-antigen clones. The gold particles were 10 nm in diameter, in A-F, and 15 nm in G and H. V226 and E381 were photographed at a higher magnification than the other strains.

A   569B
B   569B - flagellum
C   CA411
D   V333
E   V399
F   V455
G   V226 - Kasauli R
H   E381
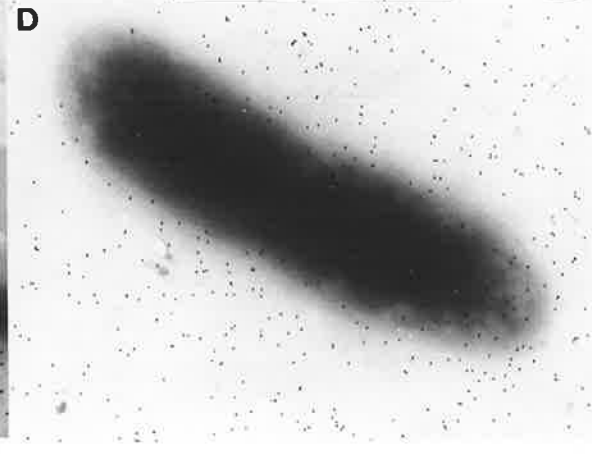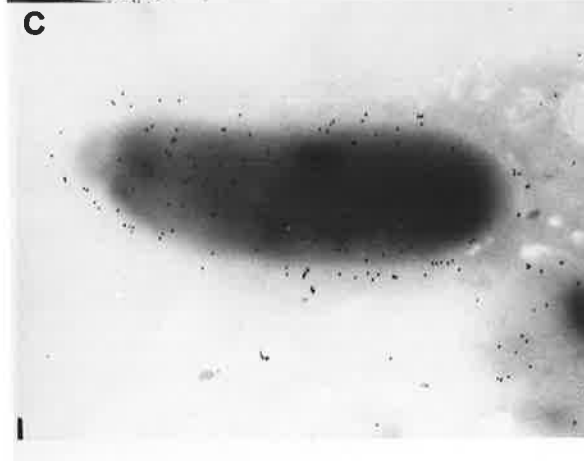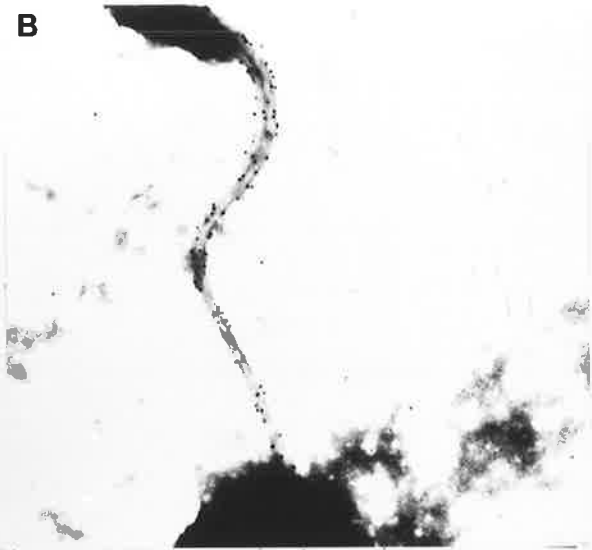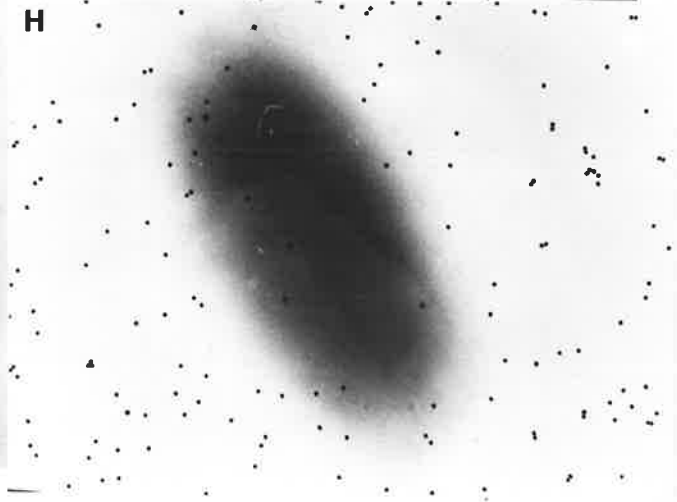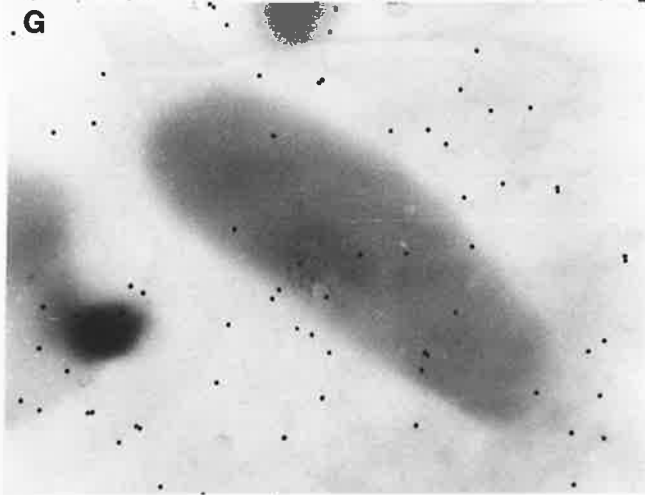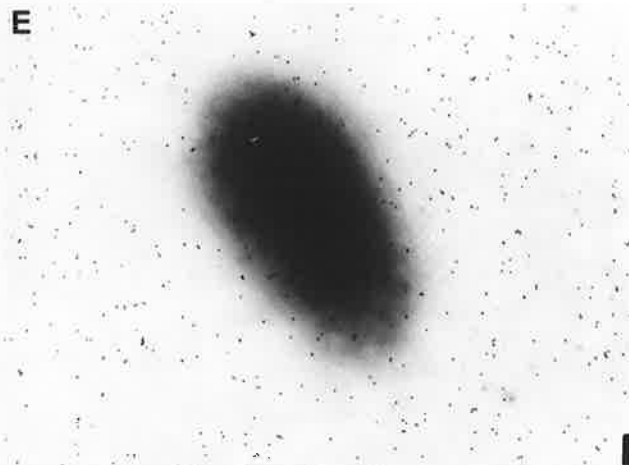
Analysis of 569B with the McAb 13-B also revealed protein A-gold particles bound to the flagellum (Fig. 3.4B). This result confirms earlier observations that the outer sheath of the <u>V. cholerae</u> flagellum is composed of outer membrane material, including LPS (Attridge and Rowley, 1983; Fuerst and Perry, 1988).

Finally, the McAb 13-B was used to analyse the various O-antigen clones (Fig. 3.4D, E and F). Although some binding of protein A-gold was observed, the background was high, as seen in results obtained with the α-A and α-B McAbs. Control strains V226 (Kasauli R) and E381 were also used with this McAb, to ensure that binding of 13-B was specific for <u>V. cholerae</u> O-antigen (Fig. 3.4G and H).

Table 3.3

Strain	Sample	Number[a] counted	S.D.[b]	Average
569B	1	14.8	1.92	
	2	11.6	4.16	14.05
	3	16.0	7.65	
	4	13.8	3.49	
CA411	1	6.4	1.67	
	2	5.2	3.49	6.10
	3	10.2	2.28	
	4	2.6	0.89	

a) Number counted refers to molecules of Protein A-gold bound. Measurements were made by counting molecules bound in a $2cm^2$ area of an enlarged photo of the electron micrographs. Figures represent the average over five squares counted in one cell.

b) S.D. — Standard deviation.

3.4   Conclusion

The presence of the serotype-specific antigens on V. cholerae O1 was originally defined using various absorbed anti-sera to classify cells into particular serotypes (Gardner and Venkatraman, 1935). Thus, the presence of some C antigen on the surface of Ogawa cells was defined only at the level of the entire bacterial population, rather than single cells.

In this chapter the distribution of these antigens has been analysed using McAbs. Immuno-gold staining has revealed that most Ogawa cells appear to contain a small amount of C antigen on their surface, in comparison with Inaba cells which have greater amounts of C antigen. Thus, it can be concluded that the switching of Ogawa to Inaba must involve changes in expression such that the B antigen is no longer expressed and the C antigen is then pre-dominantly produced. The possibility that the appearance of Inaba cells from Ogawa results from a pre-existing subpopulation of AC (i.e., Inaba) cells, however, cannot be ruled out by the results described.

The distribution of the B antigen has also been analysed and this antigen has been shown by immuno-gold labelling to be exclusively associated with Ogawa cells.

It has also been demonstrated that the cloned V. cholerae LPS genes direct the synthesis in E. coliK-12 of O-antigen expressing the appropriate serotype. Thus, the genes responsible for the differences between the Ogawa and Inaba serotypes are included in the cloned DNA.

It cannot, however, be determined at this stage if the genes involved in switching of the serotypes have been cloned. It would be necessary to develop an accurate and sensitive assay to detect sero-conversion in V. cholerae, which could then be applied to both V. cholerae and the cloned rfb genes.

CHAPTER 4

CHARACTERIZATION OF THE O-ANTIGEN

CLONES, pPM1001, pPM1002, AND pPM1003.

4.	CHARACTERIZATION OF THE O-Antigen CLONES
pPM1001, pPM1002 and pPM1003

4.1	Introduction

The molecular cloning of the genes determining O-antigen biosynthesis from strains 569B (Inaba) and 017 (Ogawa), and their expression in E. coliK-12, has been described previously (Manning et al., 1986). Genomic fragments of approximately 40kb generated by partial digestion with the restriction endonuclease, Sau3a, were cloned into the BamHI site of the cosmid vector, pHC79. In this way, the plasmids, pPM1001, from 569B, and pPM1002 and pPM1003, from 017, were derived. Silver staining and Western blot analysis of cell envelopes run on SDS-poly-acrylamide gels, revealed that each of these plasmids produced V. cholerae O-antigen in E. coliK-12. Restriction endonuclease cleavage of the plasmids indicated that the three plasmids shared a common region of about 15kb, with at least a further 5kb of DNA common to the Ogawa plasmids.

This chapter describes the detailed characterization of these three plasmids and their derivatives. The DNA has been analysed by restriction analysis, Southern hybridiz-ation and electron microscopy. This has facilitated the generation of a physical map of the DNA in the vicinity of the locus determining O-antigen biosynthesis.

4.2   Electron microscopic analysis of the clones

4.2.1 Homoduplex analysis

DNA from plasmids pPM1001, pPM1002 and pPM1003 was treated with ultra-violet light to introduce single strand nicks, denatured and then allowed to renature, so that intrastrand annealing could occur. Such homoduplexes of pPM1001 resulted in three different patterns which are shown in Fig. 4.1.   Table 4.1 summarizes the measurements of the various regions in the homoduplexes. Patterns 1 and 3 were most frequent in homoduplexes of pPM1001, whereas pattern 2 was relatively rare. All patterns show the double stranded region A, which corresponds to the two copies of the vector pHC79. Pattern 1 indicates that small regions of non-homology occur at the ends of the large inverted repeat sequences, as shown by loopouts of single-stranded DNA, B' and B''. These loopouts are not detected in pattern 2, suggesting that rearrangement can occur within pPM1001, that is, the isolated cosmid structure is unstable. It can also be seen in patterns 1 and 2 that the inverted repeat arrangement, detected by restriction analysis, can be confirmed. No such arrangement is seen in homoduplexes of any of the other plasmids. It is possible that pattern 2 represents molecules which have undergone some further rearrangement. The occurrence of pattern 3 with pPM1001 may possibly be due to molecules which have only undergone partial intrastrand annealing.

Figure 4.1: Homoduplex analysis of pPM1001. DNA was denatured and
then allowed to reanneal. Three patterns (1, 2 and 3)
were observed. The upper half of the figure shows the
electron micrographs on the left and diagrammatic
representations of the homoduplexes on the right. The
thick regions represent double stranded DNA and the
thin lines, single stranded areas.

The lower half of the figure shows interpretations of
the homoduplexes. The double stranded region correspon-
ding to the two copies of the vector (A) and the
inverted repeat region (C) are indicated in pattern 1.
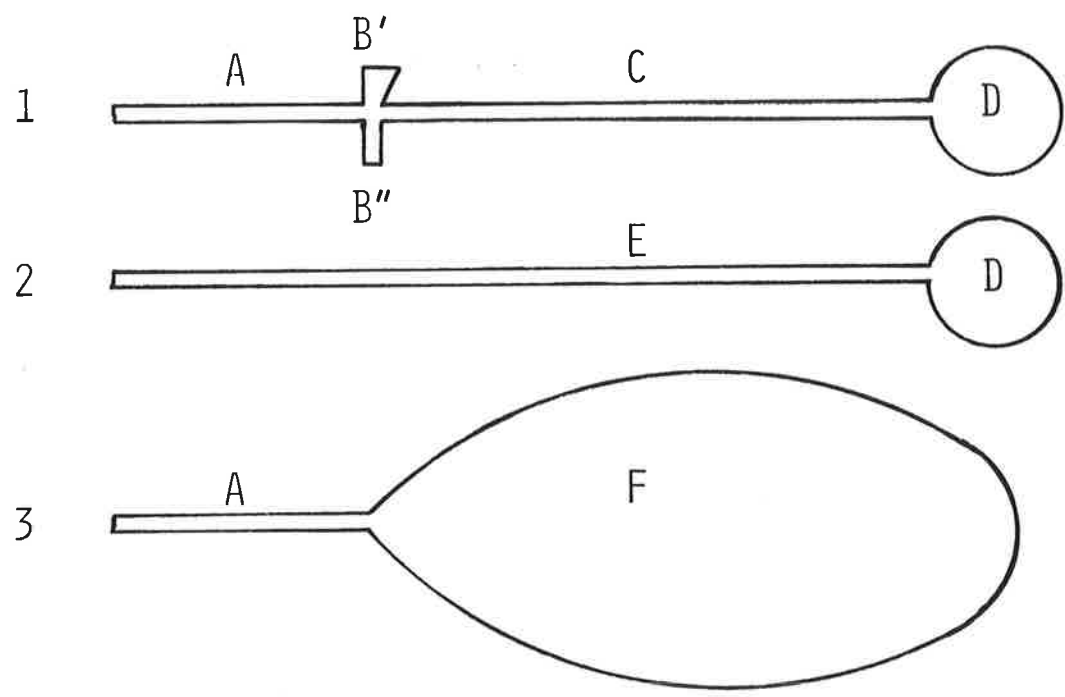The sizes of the various regions are given in Table 4.1

1

2

3

A   B′   C   D

B″

E   D

A   F
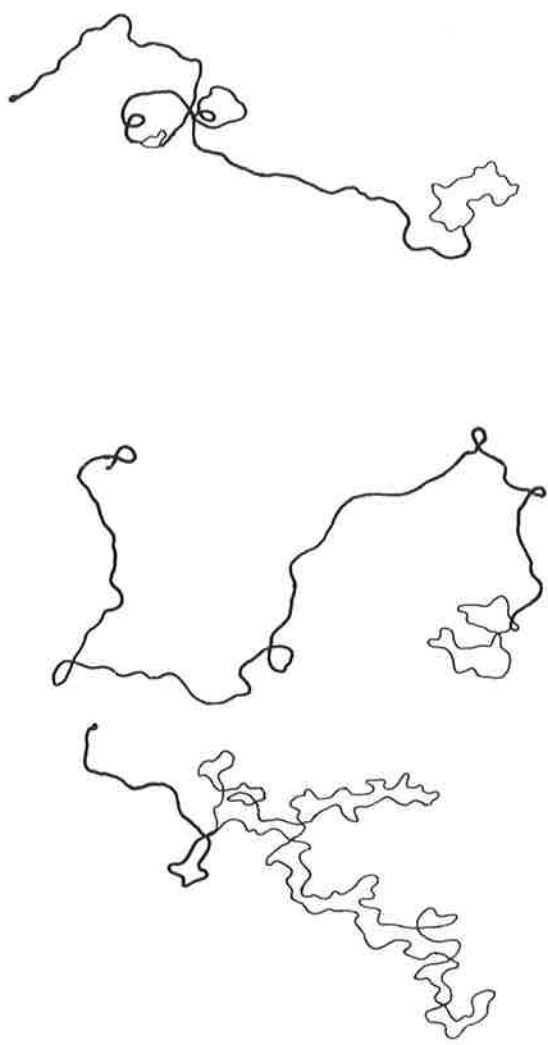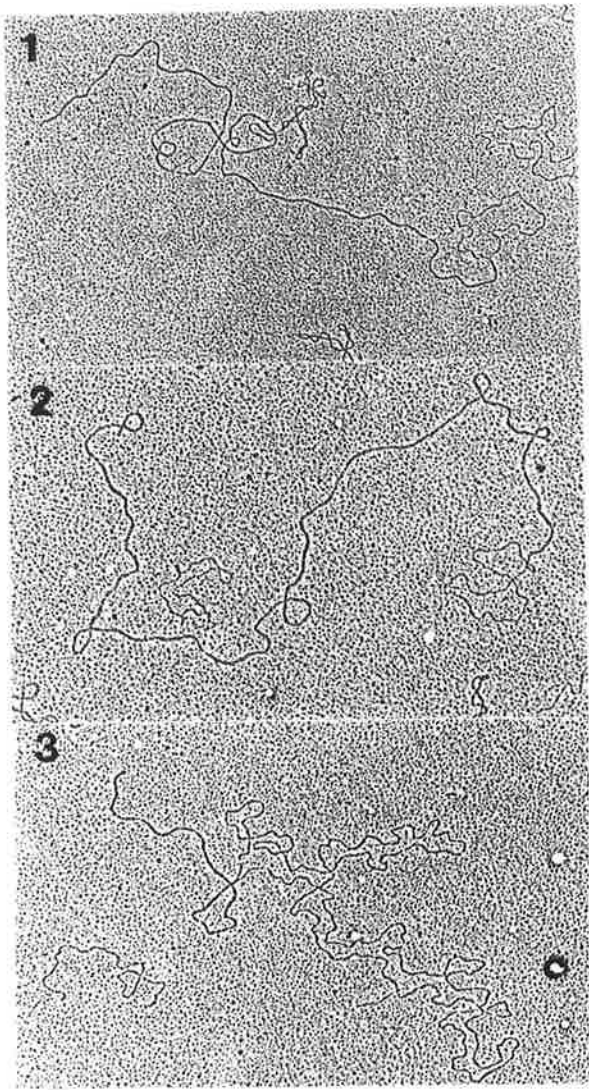
Table 4.1

Plasmid	Region[a]	Size (kb)
pPM1001	A_1	6.2
	B'	0.86
	B''	0.36
	C	13.4
	D	5.3
	E	19.6
	F_1	34
pPM1002	A_2	6.2
	F_2	32
pPM1003	A_3	6.4
	F_3	31
pPM1005	A_5	6.5
	F_5	32

a) The subscripts for the regions A and F refer to the corresponding
    plasmid: 1,   pPM1001
              2,   pPM1002
              3,   pPM1003
              5,   pPM1005

It is difficult to explain the presence of pattern 2. It can be concluded the B' and B" regions are identical in these molecules, but it is not known how such an arrangement has occurred. Perhaps replication of pPM1001 is affected by the extensive inverted repeat arrangement and so the wrong region may be copied at B' or B".

Homoduplexes of the plasmids, pPM1002, pPM1003 and pPM1005 produced molecules corresponding to Fig. 4.1 pattern 3. This pattern clearly shows the two copies of the vector, pHC79 (A), but indicates that there is no other intrastrand homology.

It should be noted that pHC79 is a cosmid cloning vector derived from pBR322 (Hohn and Collins, 1980). Thus, pHC79, like pBR322, has a single unidirectional origin of replication (Bolivar et al., 1977). All the O-antigen clones have two copies of pHC79 'head to head' and therefore two oriV sequences acting against each other. Possibly, this contributes to instability of these plasmids, as the two origins may affect the copy number of the clones.

4.2.2 Heteroduplex analysis

In order to assess the degree of relatedness between the various clones and to define the end points of homology, heteroduplex analyses have been performed using pPM1001 and pPM1003, and pPM1002 and pPM1003. In addition, the plasmids pEVX12 and pEVX10 (which include the central SalI fragments of pPM1001 and pPM1002, respectively, sub-

cloned into pACYC184), were also compared. In order to avoid ambiguities regarding the identity of particular plasmids, additional heteroduplexes were performed in which one of the plasmids had been digested with a suitable restriction endonuclease. Some of these hetero-duplexes are shown in Fig. 4.3 with diagrammatic represen-tations. A summary of the sizes is given in Table 4.2.

Heteroduplexes of pEVX12 and pEVX10 have allowed a precise measurement of the region of homology between pPM1001 and pPM1002. It can be seen (Fig. 4.3B) that homology breaks down between the inverted repeats of pPM1001 and each of the other plasmids. It can also be concluded that the plasmids contain additional cloned DNA which continues beyond this junction.

Analysis of pPM1002 and pPM1003 (Fig. 4.3C) hetero-duplexes indicates that there is a large stretch of homologous DNA of 24.3kb. The additional cloned DNA at either end is non-homologous as shown by the single stranded regions, 1, 3, 4 and 5. These regions can be correlated with the restriction maps of the plasmids, pPM1002 and pPM1003 (see Fig. 4.10). It is not possible from these analyses to say whether or not this DNA is contiguous in the V. cholerae chromosome. However, this information can be obtained from the Southern hybrid-izations above.

Figure 4.2: Heteroduplex analyses of the various LPS clones.

        (A)   pEVX10 and pEVX12

        (B)   pPM1001 and pPM1003

        (C)   pPM1002 and pPM1003

Electron micrographs of the heteroduplexes are shown with a drawing
of the molecules reproduced below each photo.
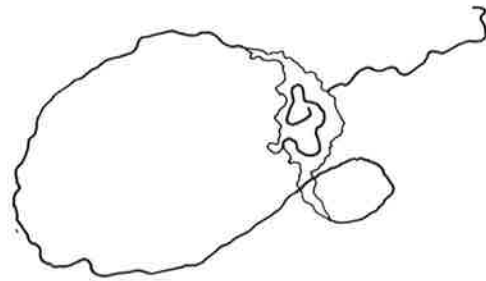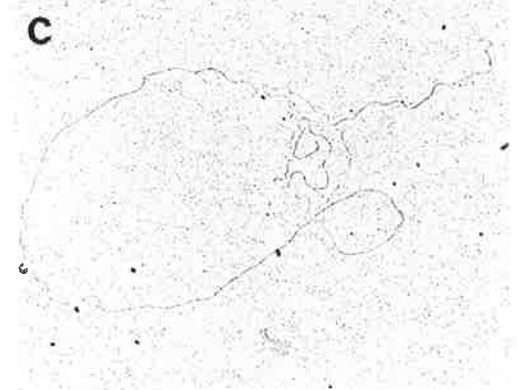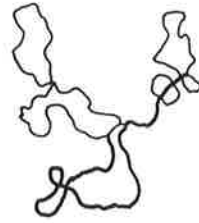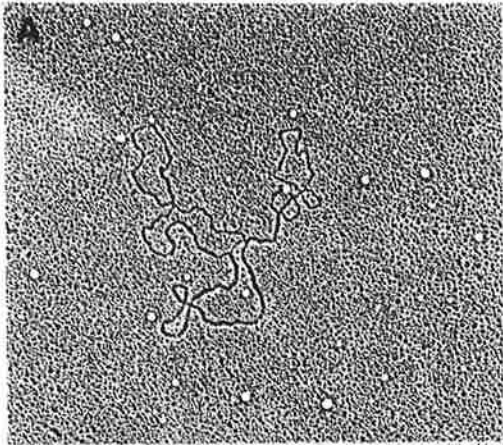
Figure 4.3:   Heteroduplex analyses. Interpretation of the hetero-
              duplexes shown in Fig. 4.2. The sizes of the various
              regions are given in Table 4.2.

a
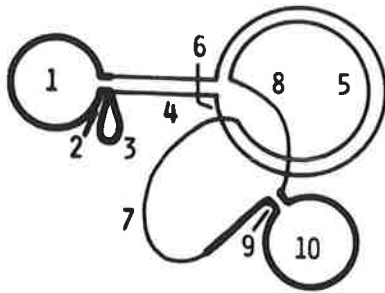
b
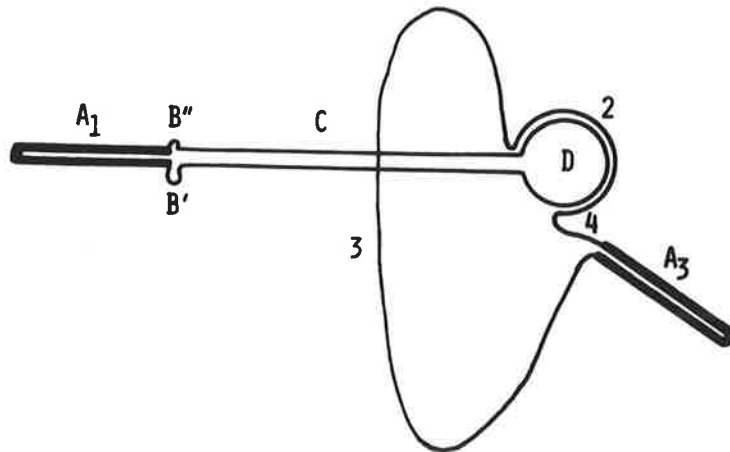
c
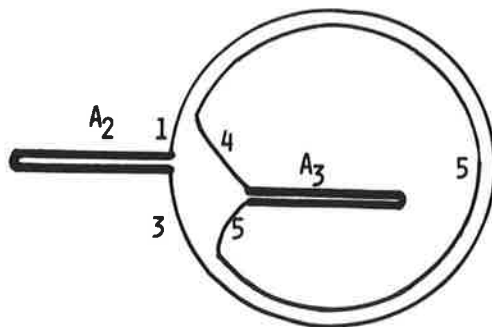
Table 4.2

Plasmids[a]	Region	Size (kb)
(A) pEVX12 and pEVX10	1	2.8
	2	0.12
	3	1.19
	4	1.02
	5	5.4
	6	0.26
	7	2.6
	8	1.3
	9	0.05
	10	2.6
(B) pPM1001 and pPM1003	1	1.8
	2	3.2
	3	25.9
	4	2.5
(C) pPM1002 and pPM1003	1	2.6
	2	23.4
	3	4.8
	4	3.8
	5	2.5

a) A, B and C correspond to the appropriate panels in Figure 4.9.

4.3   Southern hybridization analysis
                    of the clones

4.3.1 Analysis of pPM1001

Preliminary analysis of pPM1001 has implied the
presence of two large inverted repeat regions within the
cloned DNA (Manning et al., 1986). These repeat regions
extend outward from the SalI sites flanking the central
SstI site towards the HpaI sites near the ends of the V.
cholerae DNA (Fig. 4.4). This arrangement was not detected
in the Ogawa clones. However, all three plasmids contain
two copies of the vector, pHC79, which are arranged in a
'head to head' fashion.

Southern hybridization analysis of whole genomic V.
cholerae DNA was performed to assess whether or not the
inverted repeat regions were an artefact. If the arrange-
ment of DNA in pPM1001 was present in the chromosome, then
this could have implications in the control of expression
of the genes for O-antigen biosynthesis and possibly
serotype conversion.

The plasmid, pEVX12 was used to probe the cloned DNA
and whole genomic DNA from V. cholerae. This plasmid was
chosen as it contains the 7kb SalI fragment from pPM1001
which extends into both the inverted repeat regions. Thus
it should immediately confirm whether or not the repeats
are artefactual. Plasmid and genomic DNA were digested
with both BamHI and SstI, because these enzymes will
produce asymmetric fragments of about 8kb and 11.5kb from

Figure 4.4:   Restriction map of pPM1001. The horizontal arrows
correspond to the inverted repeat regions. The
location of pEV12, which includes the cloned SalI
fragment from pPM1001, is shown. The thick lines
represent vector (pHC79) DNA. The fragments produced
by digestion of pPM1001 with SstI and BamH1 and are
able to be detected using the SalI fragment of pEVX12
as a probe are indicated.
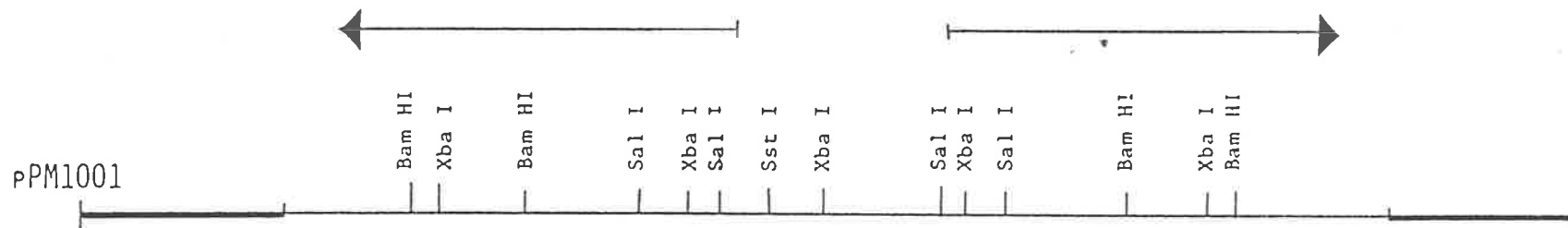
pPM1001

Bam HI Xba I Bam HI Sal I Xba I Sal I Sst I Xba I Sal I Xba I Sal I Bam H! Xba I Bam HI

pEVX12

8kb   11.5kb

1 kB

the central region of pPM1001. The detection of these two fragments in the chromosome would imply that the inverted repeat arrangement in pPM1001 is real. The results of this experiment (Fig. 4.5) show that only the 11.5kb piece can be detected in the chromosomal DNA, indicating that the 8kb BamHI-SstI fragment and, thus, the inverted repeat is actually an artefact in pPM1001. In agreement with the data obtained with the chromosomal DNA, this 8kb fragment was not detected in either pPM1002 or pPM1003. The Southern hybridization also revealed an additional 1.4kb fragment in each of the chromosomal DNA preparations tested. This corresponds to the small SstI fragment at the left end of pPM1002, and implies that it is present as contiguous DNA in the chromosome.

Further Southern hybridization analysis of V. cholerae genomic DNA using whole pPM1002 or pPM1003 as probes has been performed. Probing of SstI digests demonstrates that the 1.8kb and 6.0kb fragments at the right end of pPM1003 are also contiguous chromosomal DNA (Fig. 4.6).

In order to analyse the chromosomal DNA at the left end and define the extent of contiguous cloned DNA, genomic and plasmid DNA were digested with SalI, transferred to nitrocellulose and the filter probed with pPM1002. The results of this Southern hybridization (Fig. 4.7) show that pPM1002 binds to a 7kb SalI piece in chromosomal DNA preparations from the strains tested. Thus, the contiguous chromosomal DNA can be extended to

Figure 4.5:   Southern hybridization analysis of whole genomic DNA.
               DNA was digested with BamH1 and SstI and electrophores-
               ed on 0.8% gels. After transfer to nitrocellulose, the
               filter was probed with nick translated pEVX12 DNA. The
               open arrowheads indicate bands detected in the genomic
               DNA. Sizes are shown in kb.
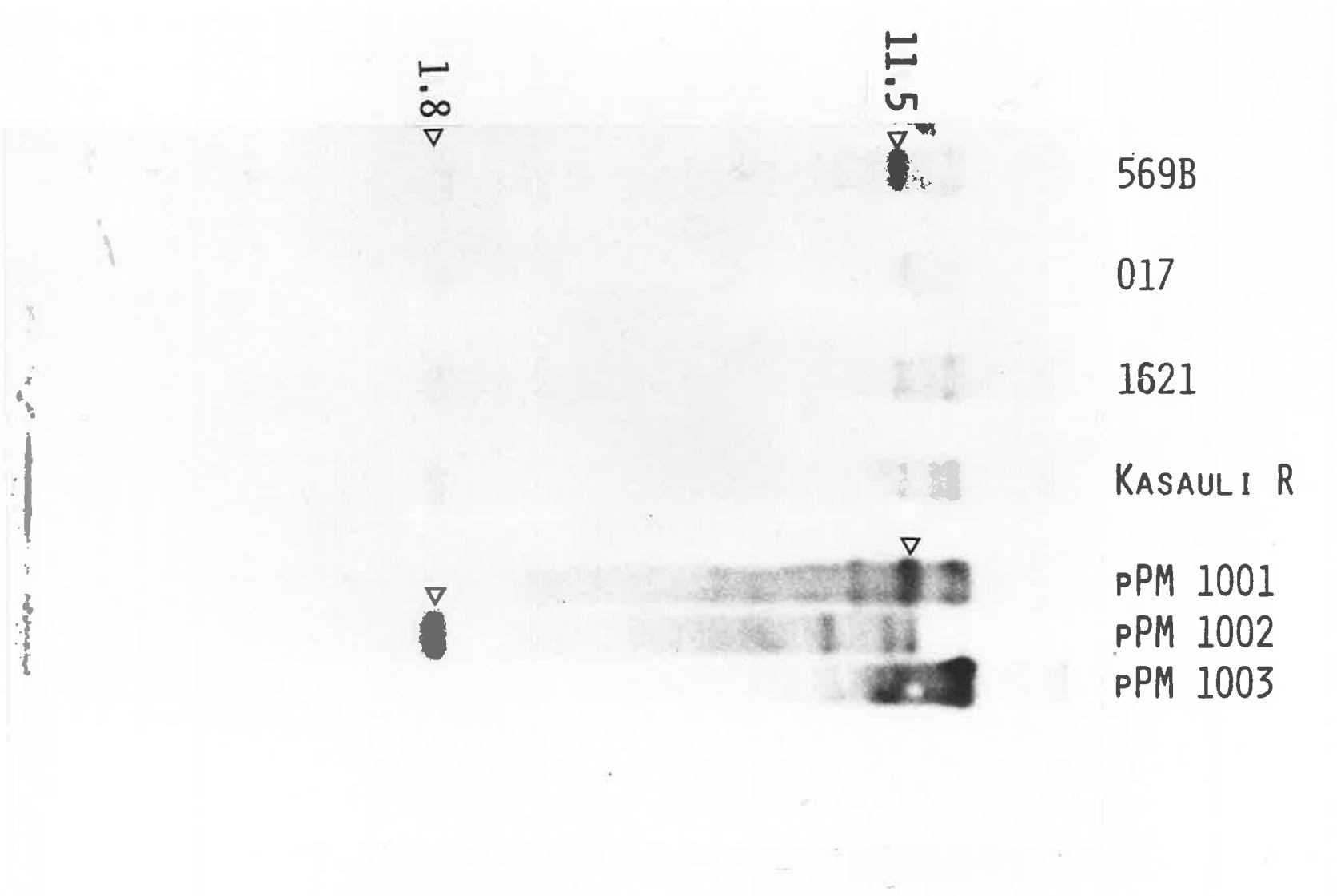
1.8 ▽

11.5 ▽

▽

▽

569B

017

1621

Kasauli R

pPM 1001

pPM 1002

pPM 1003

Figure 4.6: Southern hybridization analysis of whole genomic DNA. DNA was digested with SstI, electrophoresed on 0.8% gels and transferred to nitrocellulose. The filter was probed with nick translated pPM1003. Bands detected in chromosomal DNA are indicated by open arrowheads and the sizes are shown in kb.
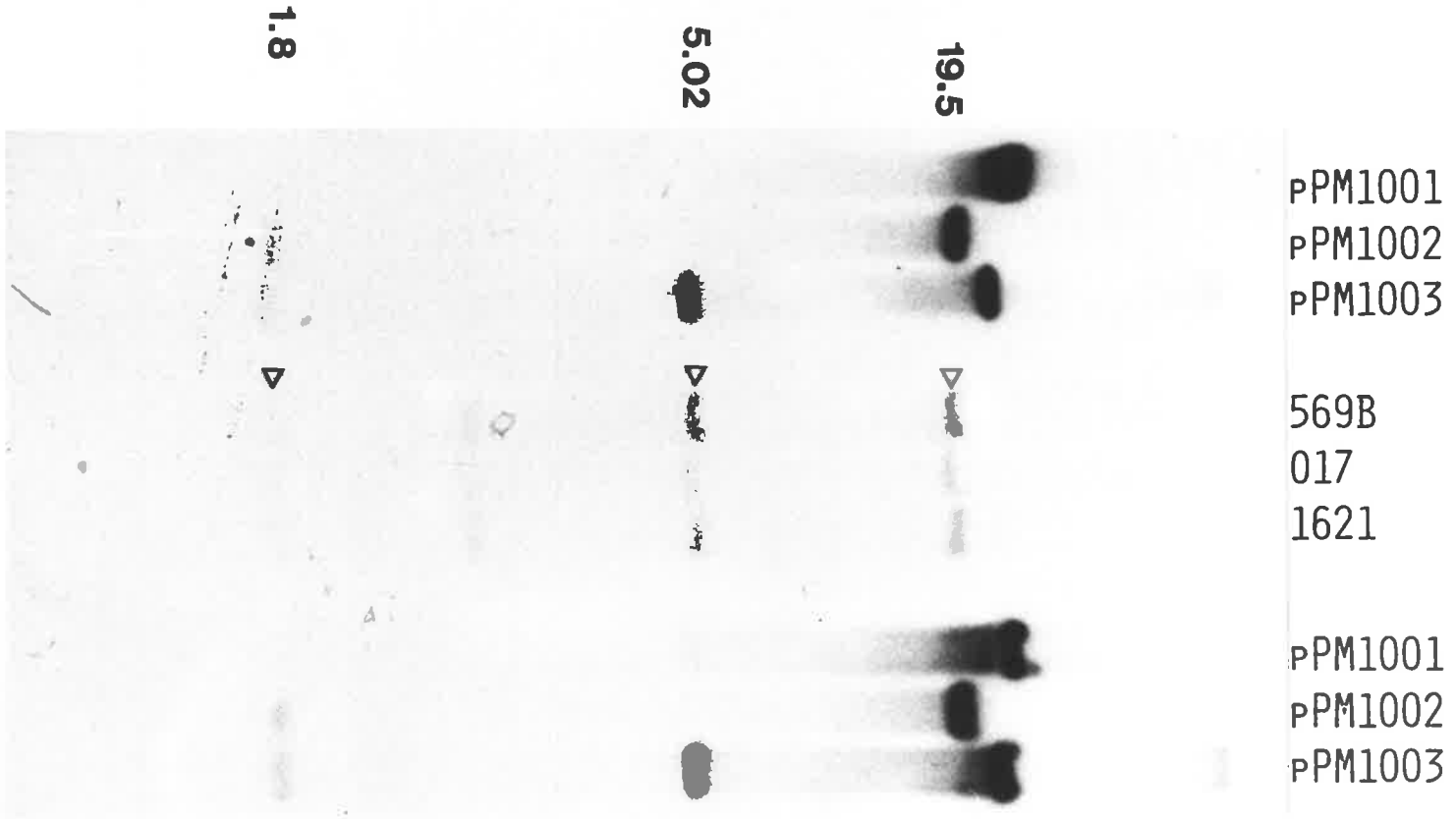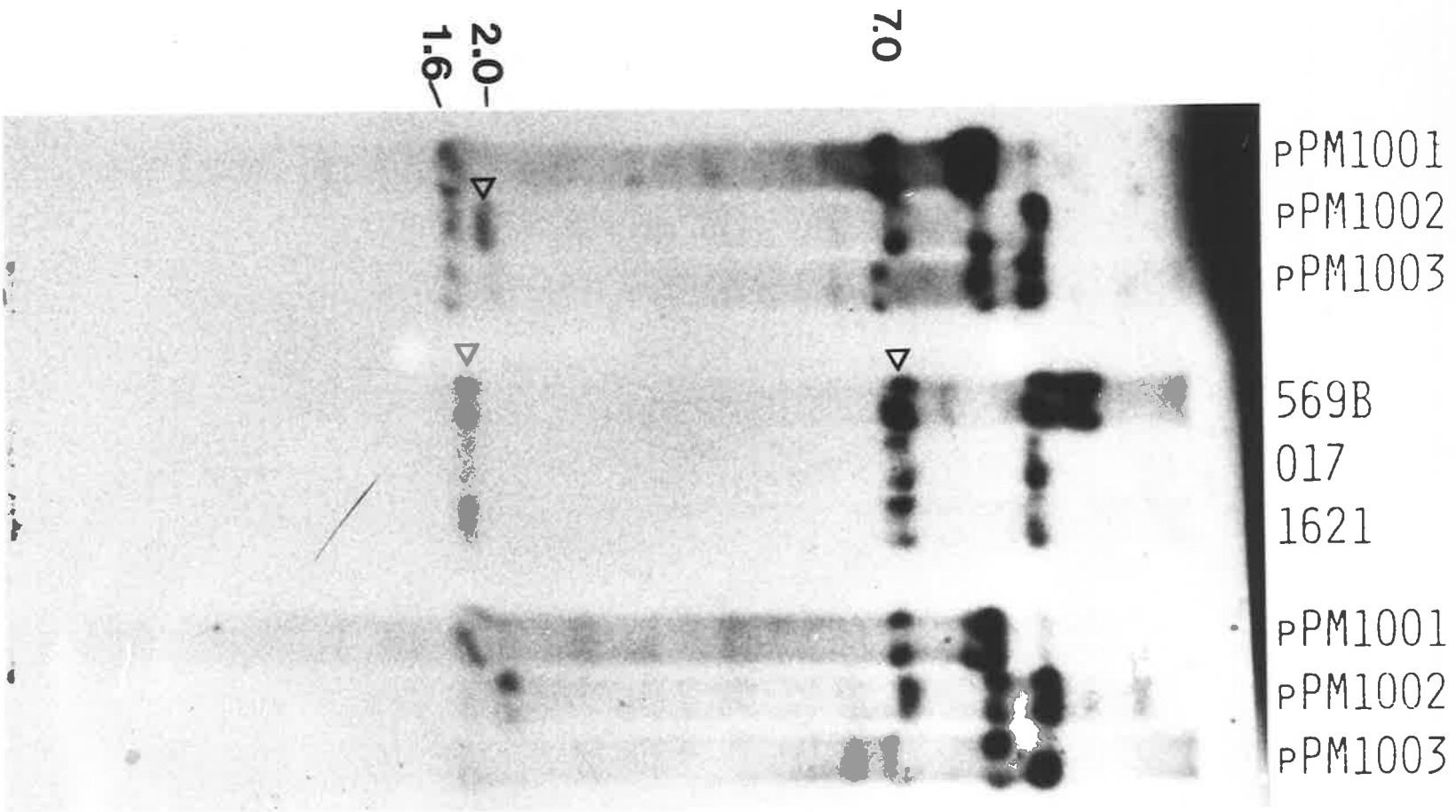
Figure 4.7:   Southern hybridization analysis of whole genomic DNA,
              digested with SalI. After electrophoresis on a 0.8%
              agarose gel, and transfer to nitrocellulose, the DNA
              was probed with nick translated pPM1002. The open
              arrowheads indicate bands detected in the genomic DNA
              and sizes are shown in kb.

include the region up to the SalI site at the left end of the cloned DNA in pPM1002.

Southern hybridization analysis of BamHI digests of genomic DNA was performed using pEVX7 (the large SstI fragment of pPM1002 cloned into pUC18), as the probe. (See Fig. 4.7 for a diagram of the various plasmids used as probes in Southern hybridizations.) It can be seen (Fig. 4.8) that pPM1002 hybridizes to both the 10kb and 3.8kb BamHI fragments, but that the 7.0kb BamHI fragment seen in digests of pPM1002 (but not in pPM1003) was not detected in any of the chromosomal digests. This information has facilitated the localization of the BamHI sites within pPM1002 and one of these lies at the junction of the two copies of pHC79 in pPM1002.

Southern hybridization analysis of V. cholerae chromosomal DNA digested with HindIII and probed with pEVX7, demonstrates that the HindIII patterns of the V. cholerae strains were identical, regardless of serotype (Fig. 4.9). These data, coupled with the Southern hybridizations discussed previously, lead to the conclusion that there are no readily detectable differences in the DNA in the region encoding O-antigen biosynthesis of the Ogawa and Inaba serotypes. However, based on these Southern hybridization data, in conjunction with previous restriction mapping (Manning et al., 1986) and the EM data obtained, it is possible to construct a map of contiguous chromosomal DNA in the vicinity of the genes encoding biosynthesis of the O-antigen of V. cholerae (Fig. 4.10).

Figure 4.8: Southern hybridization analysis of whole genomic DNA, digested
with either SalI (left side of gel) or BamHI (right side of
gel). After electrophoresis on a 0.8% agarose gel and
transfer to nitrocellulose, the DNA was probed with nick
translated pPM1002. The open arrowheads indicate bands
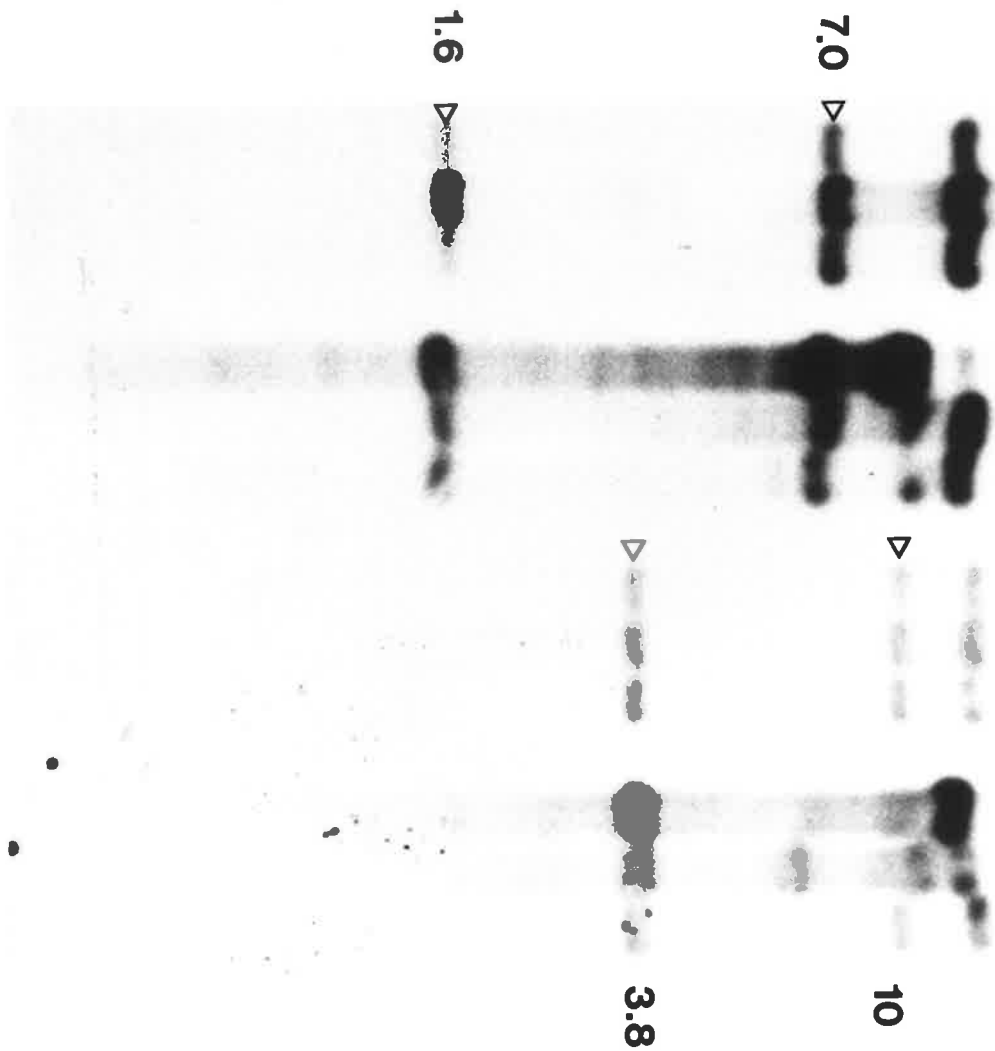detected in genomic DNA and the sizes are shown in kb.

Figure 4.9: Southern hybrization analysis of V. cholerae chromo-
somal DNA. prepared from various strains. DNA was
digested with HindIII, eletrophoresed on a 0.8%
agarose gel and transferred to nitrocellulose. The
filter was hybridized with nick translated pEVX7. The
sizes (in kb) of the HindIII fragments detected in the
genomic DNA are shown.

4.9 2.9 2.4 1.9 1.6 1.3 1.2 1.06
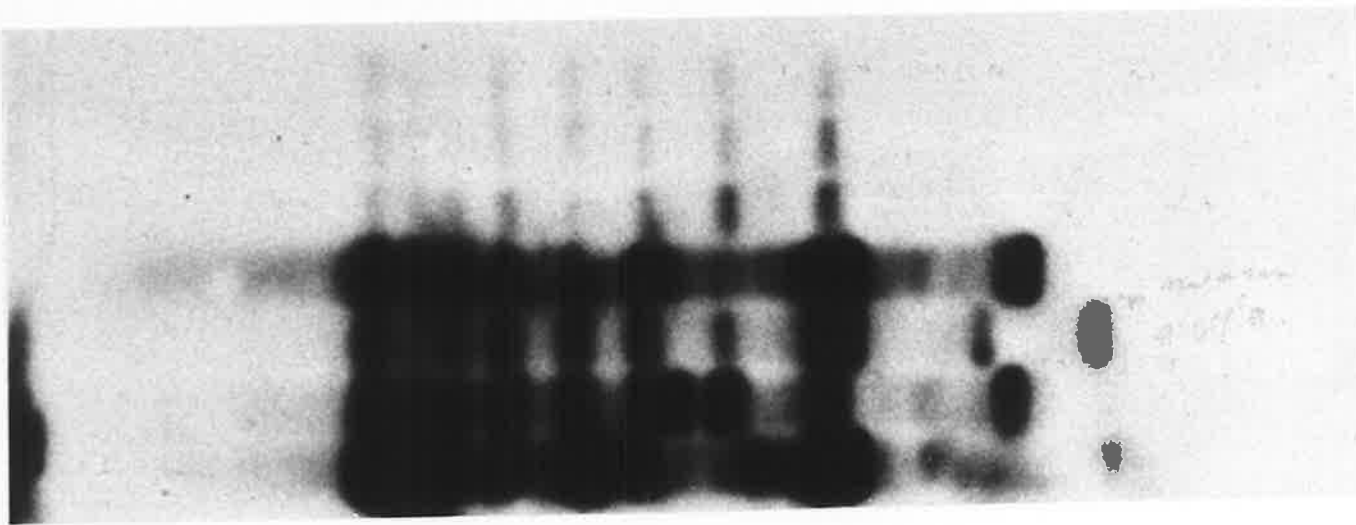
017
569B
1621
pPM1001
pPM1002
pPM1003
¡pEVX7

Figure 4.10: Restriction map of rfb region of the V. cholerae chromosome. The rfb clones pPM1001, pPM1002 and pPM1003 are shown and the regions of cloned DNA included in pEVX7 and pEVX12 are indicated. The area indicated by dashed lines represents the maximum region likely to be necessary for expression of V. cholerae 01 O-antigen in E. coliK-12.

pPM1001

pPM1002

pPM1003

pEVX7

pEVX12

Sal I
Sst I
Sst I
Eco RI
Cla I
Xba I
Eco RI
Cla I
Sal I
Xba I
Eco RI
Sal I
Cla I
Eco RI
Cla I
Bam HI
Xba I
Bam HI
Eco RI
Kpn I
Hpa I
Eco RI
Sst I
Hpa I
Sst I
Hpa I
Bam HI
Sst I
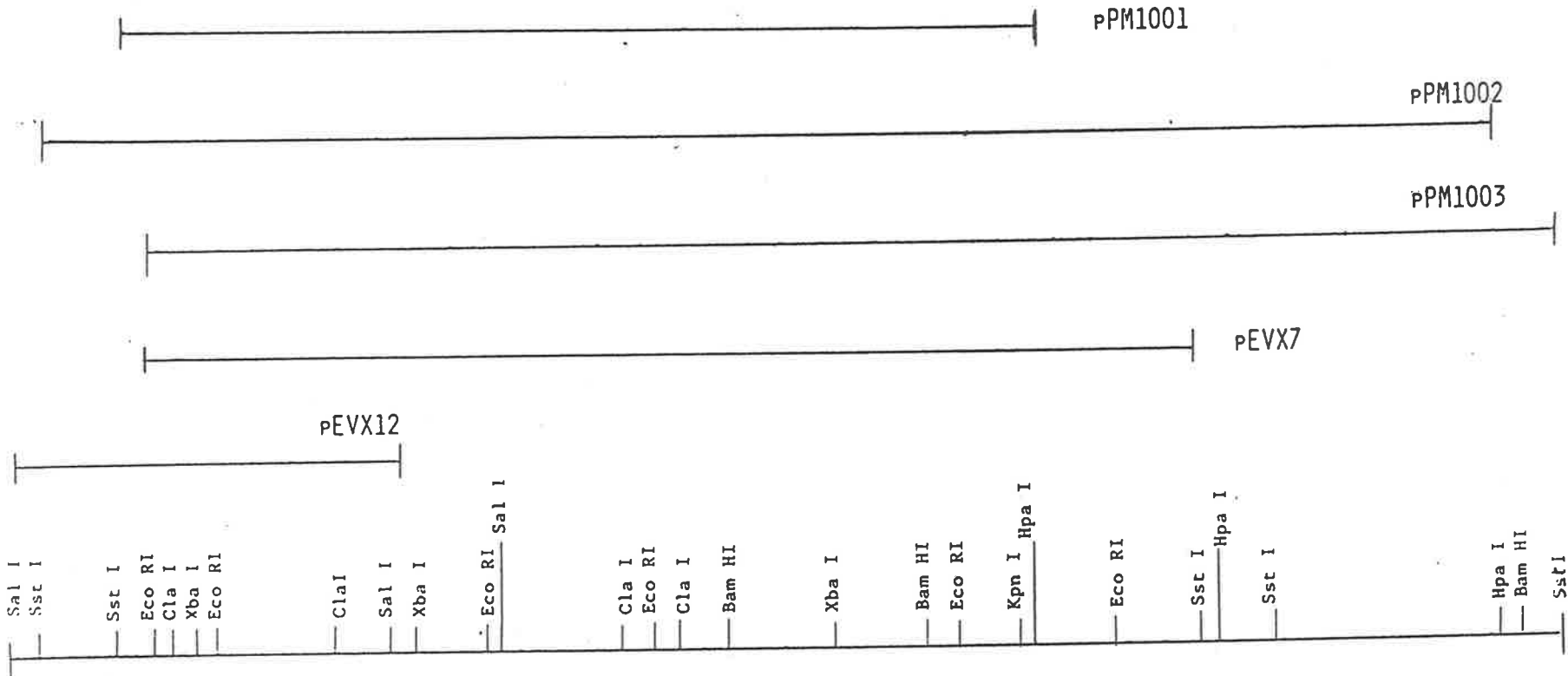
RFB GENES

1 KB

4.4 Definition of minimal coding region for expression of V. cholerae O-antigen in E. coliK-12

The expression of O-antigen on the various clones was studied in a number of ways. The aim of this section was firstly to analyse the type of LPS being made by the clones and secondly to define further the region of DNA necessary to encode O-antigen in E. coliK-12.

4.4.1 Analysis of O-antigen expression in V512

Although the Southern hybridization analyses provided information on the arrangement of contiguous DNA in the V. cholerae chromosome, it was not possible from these data to conclude how much of the cloned DNA was necessary for O-antigen expression in E. coliK-12.

Analysis of the Inaba plasmid, pPM1001, was made difficult because of the inverted repeat structure. Studies of deletion derivatives and subclones of this plasmid have been done, but are difficult to interpret. To help identify and eliminate unimportant regions of the DNA, an SstI deletion derivative of pPM1003 was constructed, by digesting the DNA to completion with SstI and then religating. The plasmid was then transformed back into E. coliK-12. The aim of this experiment was to remove the 5.0kb and 1.6kb SstI fragments from pPM1003 so that the effect of this deletion on O-antigen expression could be assessed. The resultant plasmid, pPM1006 was shown by restriction endonuclease analysis to lack both of the small SstI fragments (data not shown).

The expression of O-antigen in V512 (E. coliK-12 [pPM1006]) has been studied in several ways. Small scale membrane preparations extracted with Sarkosyl to remove proteins (SDS-PAGE followed by silver staining) showed that V512 is still capable of producing amounts of O-antigen (Fig. 4.11) comparable with those produced due to pPM1003. This result suggests that pPM1006 was able to express V. cholerae O-antigen even though it lacked 6.6kb of contiguous DNA at the right end of the cloned DNA, indicating that these SstI fragments are not essential for O-antigen production in E. coliK-12.

This permits the region necessary for O-antigen expression to be defined at the right end, as extending to the first of the SstI sites in this region. It could thus be predicted from these data and from the electron microscopic results that the large SstI fragment would be sufficient for expression of O-antigen in E. coliK-12.

The large SstI fragment of pPM1002 has been cloned in both orientations into the SstI site of pUC18. The two plasmids, pEVX6 and pEVX7, which resulted from this cloning, were both shown to encode production of O-antigen in E. coliK-12 (Yeadon, J. and Morona, R., personal communication). Thus, it would appear from the analysis of pPM1002 and pPM1003 that the large SstI fragment is sufficient for expression of V. cholerae O-antigen in E. coliK-12.

Figure 4.11:   SDS-PAGE analysis of whole cell lysates from V512,
V. cholerae strains 569B and O17 and the O-antigen
clones. 10µl of each lysate was loaded and the gels
were silver stained to detect LPS. The V333 whole
cell lysate was prepared from cells at a lower
concentration than the other strains, thus very
little O-antigen can be seen for this sample.

4.4.2 Cotransformation analysis

In order to analyse the expression of the B and C antigens in the O-antigen clones, cotransformation studies were done. In these experiments the plasmids, pPM1002 or pPM1003, were transformed into cells containing pPM1004. The latter plasmid includes the largest NaeI fragment of pPM1001 cloned into pSC101. It was necessary to use this, rather than pPM1001, because pPM1004 belongs to a different incompatibility group from that of pPM1002 and pPM1003. Thus, pPM1004 is able to stably co-exist with either of these plasmids. The aim of this section was to determine if one of the B or C antigens would be dominant over the other.

DNA of the plasmids pPM1002 and pPM1003 was transformed, separately into V472 (E. coliK-12 [pPM1004]). The resulting strains were checked by restriction endonuclease analysis of plasmid DNA.

Outer membrane preparations were made from these strains and LPS expression assayed in an HIA (Table 4.3). Both of the strains produced expressed both B and C antigens on their surface. Thus, it can be concluded that there is a co-dominance effect when Inaba and Ogawa plasmids are present in the same cell. Neither plasmid would appear to exhibit an inhibitory effect upon the other, as both O-antigens can be expressed simultaneously.

Table 4.3

Antibody	Strain	569B[a]	017[a]
α – C	017	>1000[b]	
	569B	16	
	V472	4	
	V455	>1000	
	V552	32	
	E381	>1000	
α – B	017		16
	569B		>1000
	V472		>1000
	V455		62.5
	V552		62.5
	E381		>1000

a) LPS used to sensitize SRBC.

b) Concentration of cell envelope material in $\mu g/\mu l$ required to inhibit haemagglutination of sensitized SRBC.

4.5   Summary and conclusions

This chapter has described a detailed analysis of the cloned DNA necessary for encoding the biosynthesis of the O-antigen of <u>V</u>. <u>cholerae</u> in <u>E</u>. <u>coli</u>K-12.

A combination of Southern hybridization and electron microscopic analysis has allowed the construction of a contiguous chromosomal map of this region of the <u>V</u>. <u>cholerae</u> genome. This information is summarized in Fig. 4.7.

Deletion of the <u>SstI</u> fragments from pPM1003 and subsequent analysis of the resultant plasmid, pPM1006, has allowed definition of the minimum and maximum coding regions required for <u>V</u>. <u>cholerae</u> O-antigen biosynthesis in <u>E</u>. <u>coli</u>K-12. Thus, the large <u>SstI</u> fragment appears to be sufficient to encode <u>V</u>. <u>cholerae</u> O-antigen expression in this background (Fig. 4.7), but analysis of the extent of DNA included in the various plasmids indicates that the minimum region needed extends from the left-hand <u>EcoRI</u> site to the <u>HpaI</u> site.

Finally, cotransformation of Inaba and Ogawa plasmids into <u>E</u>. <u>coli</u>K-12 revealed that both types of serotype antigens can be expressed in the same <u>E</u>. <u>coli</u>K-12 cell. Thus, in this situation neither serotype exerts a dominant effect upon the other. However, it is important to consider the properties of the two vectors used, pSC101 (low copy number) and pHC79 (high copy number), as the different copy numbers may lead to unequal expression of particular serotype antigens.

CHAPTER 5

TRANSPOSON MUTAGENESIS OF
V.cholerae LPS GENES.

5.   TRANSPOSON MUTAGENESIS OF
                         V. cholerae LPS GENES

5.1   Introduction

The study of the genes associated with LPS bio-
synthesis in various Gram negative bacteria has been
greatly facilitated by the use of mutants which have
defects in particular steps of the pathway. Analysis of
the effect of such mutations and genetic mapping of the
mutations has enabled workers to deduce the arrangement of
genes and the detailed structure of the LPS in Salmonella
typhimurium, for instance (Mäkelä and Stocker, 1969, 1984;
Jann and Jann, 1984; Nikaido et al., 1966; Brahmbhatt et
al. 1986, 1988). The study of V. cholerae LPS has been
difficult for a number of reasons. As described in Chapter
1, the composition of V. cholerae LPS has been determined,
but the exact configuration and order of sugars are, as
yet, unknown. Rough strains have been isolated in V.
cholerae, but defined mutations in the genes encoding LPS
biosynthesis have not been described. The aim of the
experiments described in this chapter was to use trans-
poson mutagenesis to isolate a series of isogenic mutants
which have the transposon inserted in the O-antigen bio-
synthesis genes and are defective in LPS production. Such
strains would be useful in genetic analysis and provide a
basis for analysing the structure of the LPS in V.
cholerae.

5.2   Isolation of transposon insertion mutants

5.2.1 Introduction

The isolation of defined bacterial mutants, using transposon insertions into particular genes or control regions, has been described in a number of different systems.

Such mutants can be particularly useful since the site of transposon insertion can usually be mapped precisely and correlated with the phenotype of the mutant. It is important in mutagenesis experiments to have a method of selection or screening in order to differentiate mutants from the parent strain. In the isolation of LPS mutants, phage resistance is often used as the selection procedure, since numerous phage have been shown to use parts of the LPS as all or part of their receptor (Wilkinson et al., 1972; Ames et al., 1974; Tamaki et al., 1971; Yu and Mizushima, 1982; Boman and Monner, 1975).

By virus neutralization studies the choleraphage CP-T1 has been demonstrated to use the O-antigen as its receptor on the cell surface of V. cholerae 01 (Guidolin and Manning, 1985). The LPS of V. cholerae strains, regardless of biotype or serotype, was able to neutralize the infective capacity of CP-T1. Mutants of V. cholerae, isolated on the basis of resistance to CP-T1, were shown by virus neutralization and silver staining of LPS, to completely lack the O-antigen. These results confirmed the role of O-antigen as a receptor for this phage. Such

studies have provided the basis for the following section of work, in which isolation of transposon mutants in the region of the chromosome responsible for O-antigen bio-synthesis is achieved by selecting for phage resistance.

5.2.2 Neutralization of bacteriophage VcII

Although CP-T1 uses O-antigen as its receptor in the outer membrane, it does not plaque very efficiently on the Classical Inaba strain, 569B. Since this was the strain of choice for isolating mutants, an alternative phage was sought. The Classical phage VcII was chosen as it has been shown by e.o.p. studies to require O-antigen as a compon-ent of its receptor (Guidolin and Manning, 1985). One of the Classical typing phage, VcII, which plaques well on 569B, was tested in virus neutralization studies, using purified LPS from a number of V. cholerae strains. Various concentrations of LPS were mixed with samples of VcII, incubated at 37° C and then assayed on 569B. The results of such an experiment are shown in Fig. 5.1. It can be seen that the LPS from only the smooth V. cholerae strains tested was able to neutralize the activity of VcII. It should be noted that the phage neutralizing capacity of LPS does not depend upon the biotype of the strain. Thus, as observed in previous studies (Imbesi and Manning, 1982), biotype specificity of the choleraphage exists and is probably due to restriction of the phage DNA, since this experiment illustrates that VcII is able to use LPS from either biotype as a receptor, although it only plaques on Classical strains.

Figure 5.1:   Inactivation of bacteriophage VcII with purified LPS
              from V. cholerae strains, 569B, 017, (●——●);
              1621, (△———△); 029, (▲———▲) and Kasauli R;
              569B-165, (■———■).
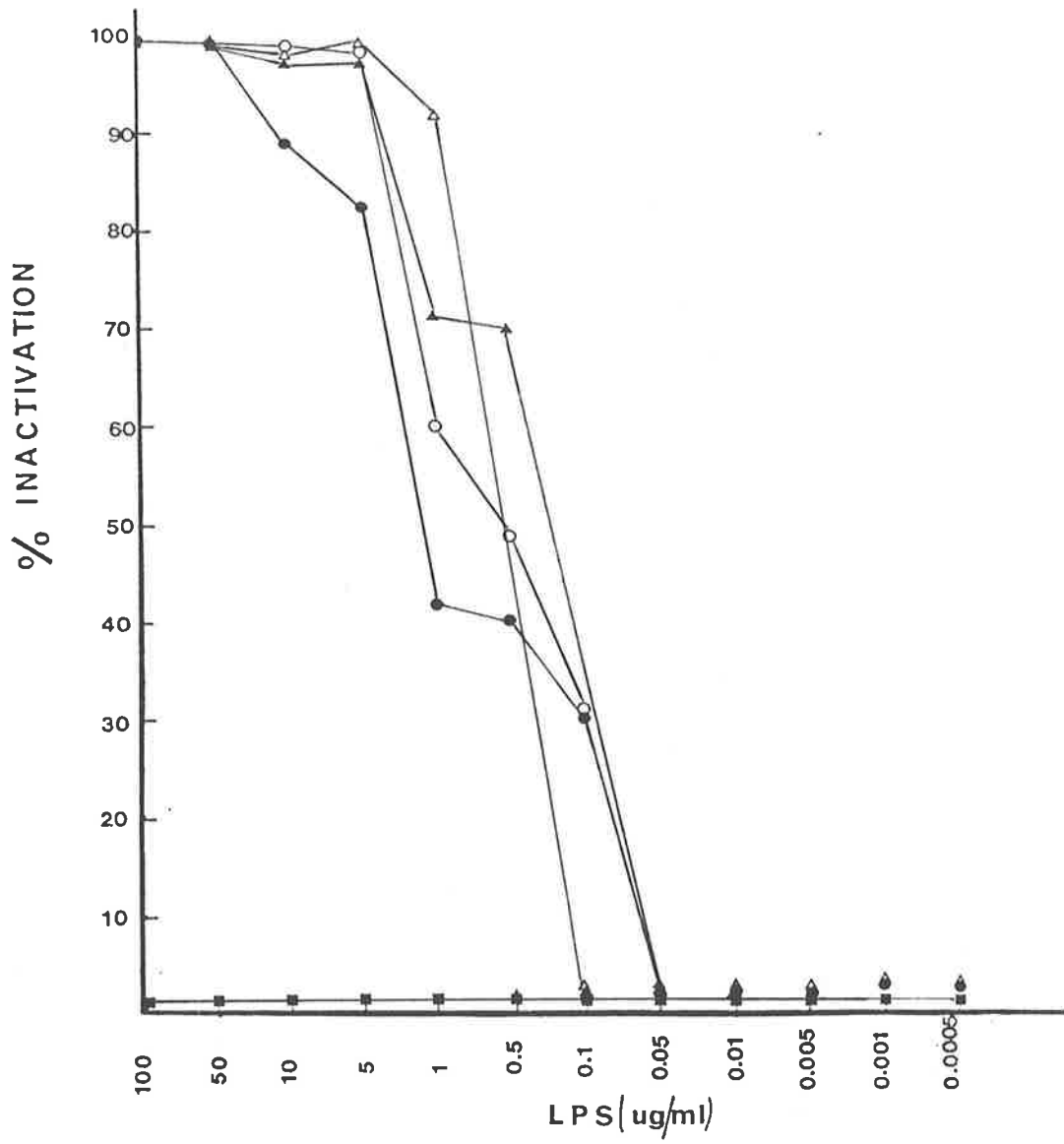
LPS from the rough cholera strain, Kasauli R, was unable to neutralize the activity of VcII. In order to confirm that proteins in the outer membrane have no role in the attachment of VcII to the cells, LPS from the hybrid strain 569B-165 was used. This strain is thought to express the LPS of the non-cholera vibrio strain 165, and the surface proteins of 569B (Bhaskaran, 1971). Fig. 5.1 indicates that no phage neutralizing activity was observed with the LPS from 569B-165. Therefore, it can be concluded that the O-antigen is an essential and probably efficient component of the VcII receptor. Thus, selection of resistance to VcII would be predicted to be a suitable means of obtaining LPS mutants from the Classical strain, 569B.

As a control, the phage inactivating ability of these strains was also tested with bacteriophage CP-T1 (Fig. 5.2). Similar results were obtained, indicating that CP-T1 also uses the O-antigen of V. cholerae LPS as part of its receptor on the cell surface (Guidolin and Manning, 1985).

5.2.3 Isolation of transposon mutants affecting LPS biosynthesis in V. cholerae

Transposon mutagenesis with Tn2680 was performed using the plasmid, $R_{ts}1$, which has a temperature sensitive replicon. This plasmid was transferred into V. cholerae 017 by conjugation and chromosomal transposon insertions were isolated by selection for resistance to CP-T1 and km (carried by Tn2680), at $42°C$. In order to ensure that independent transposon insertions were isolated, only one

Figure 5.2:   Inactivation of bacteriophage CP-T1 with purified LPS
               from V. cholerae strains, 5698, 017, (●——●);
               1621, (△——△); 029, (▲——▲) and Kasauli R;
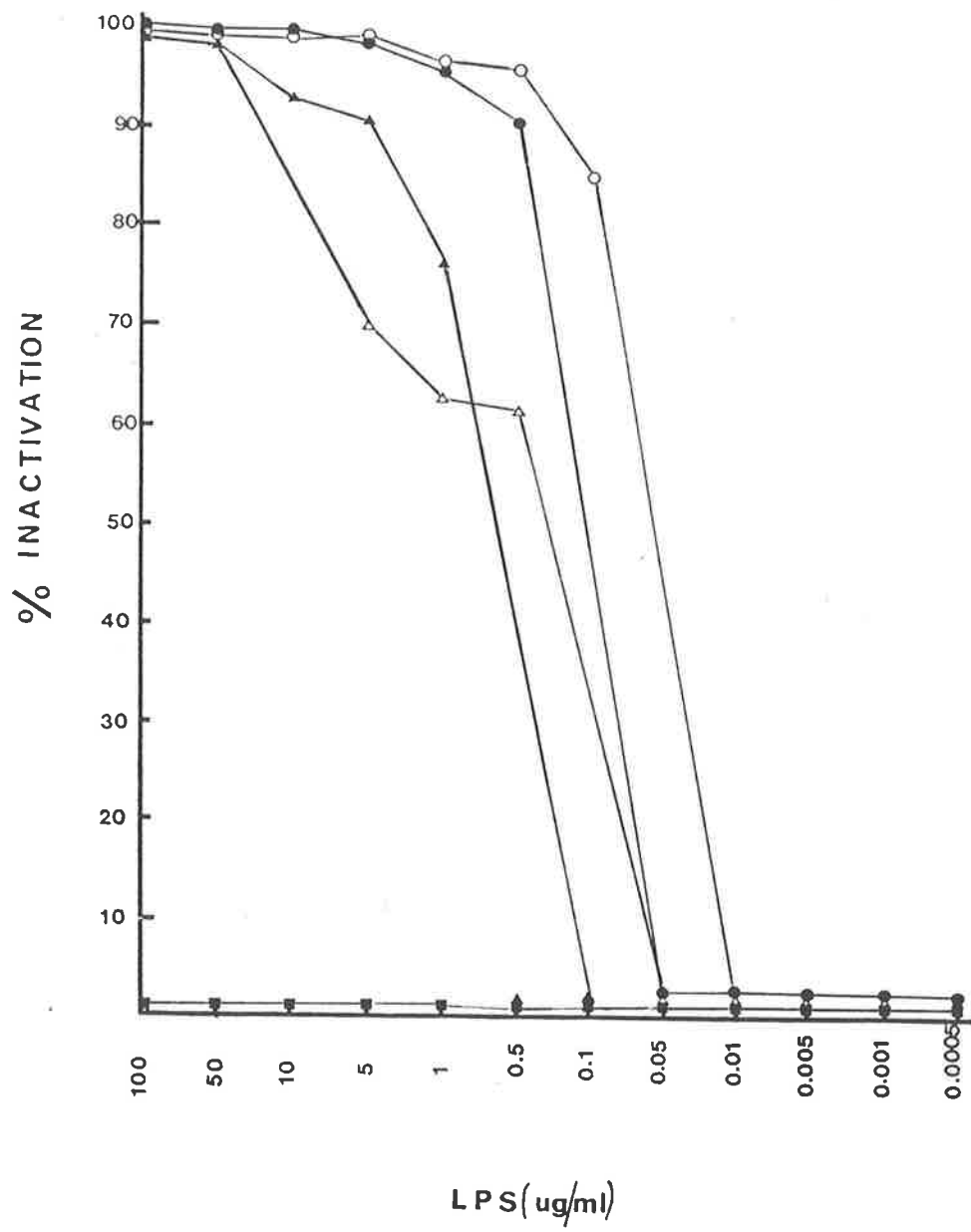   569B-165, (■——■).

colony was taken from cultures from independent trans-
conjugants. After purification, the transposon insertion
mutants were maintained at 37°C. All the mutants were
screened for the degree of phage resistance by checking
the efficiency of plating (e.o.p.) of CP-T1. The results
of this experiment are shown in Table 5.1. Three indepen-
dent Tn2680 mutations were isolated, all of which were
completely resistant to CP-T1. These mutants also exhibit-
ed a rough phenotype as they auto-agglutinated in broth
cultures and had a rough colony morphology on solid media.
Although rough mutants have been described previously in
V. cholerae (Shimada and Sakazaki, 1973; Hisatsune and
Kondo, 1980), it has not been reported that such strains
exhibited a rough appearance on plates.

Another series of transposon insertion mutants has
been isolated using Tn5 which also encodes km^R. In order
to isolate these mutants, the plasmid F'$_{ts}$ lac::Tn5 was
transferred into the V. cholerae strains 569B and 1621, by
conjugation. The same procedure that was used for the
isolation of Tn2680 mutants was followed except that phage
VcII was used with 569B and CP-T1 with 1621. The pheno-
types of these mutants and the e.o.p. of CP-T1 and VcII
are shown in Table 5.2. Six independent transposon
insertion mutants were chosen for study from each of 569B
and 1621.

Table 5.1: Efficiency of plating on CP-T1

Strain	E.O.P. of CP-T1
017	1
V223	$< 10^{-8}$
V224	$< 10^{-8}$
V225	$< 10^{-8}$
V226	$< 10^{-8}$

Table 5.2: Efficiency of plating on VcII or CP-T1

Strain	VcII	CP-T1
569B	1	
V661	$< 10^{-7}$	
V663	$< 10^{-7}$	
V665	$< 10^{-7}$	
V667	$< 10^{-7}$	
V669	$< 10^{-7}$	
V671	$< 10^{-7}$	
1621		1
V638		$< 10^{-8}$
V640		$< 10^{-8}$
V641		$< 10^{-8}$
V642		$< 10^{-8}$
V643		$< 10^{-8}$
V644		$< 10^{-8}$

5.3   Characterization of transposon
insertion mutants

5.3.1 Serological testing of mutants

In order to confirm serologically that the different transposon insertion mutants did not express O-antigen on their surface, the various strains were tested in bacterial agglutinations. Two different anti-sera were used in this assay. A McAb directed specifically against the 'C' antigen was used (as described in Chapter 3) and also a polyclonal α-rough serum which had been raised against Kasauli R. The results of bacterial agglutinations with mutants isolated from 1621 are shown in Table 5.3. No agglutination of the mutants was observed with the α-C McAb however, all mutants agglutinated with the α-rough serum.

Thus, it can be concluded that the mutants no longer express V. cholerae O-antigen on their surface and that "rough" determinants, probably associated with core oligosaccharide, are exposed on these cells. Table 5.4 shows the data for the transposon insertion mutants isolated from 569B.

It was not possible to obtain such information on the Tn2680 mutants derived from 017, as these mutants auto-agglutinated to such a degree that it was extremely difficult to use the cells in suspension however, colony blots using α-C and α-rough antibodies showed that the Tn2680 mutants did not express C antigen. These strains did react with the α-rough serum (data not shown).

Table 5.3: Bacterial agglutination

Strain	Anti-serum	
	α -Ogawa	α -Kasauli R
1621	2^8 a	2^5
V638	< 2	2^{10}
V640[b]	$-$	$-$
V641	< 2	2^{10}
V642	< 2	2^9
V643	< 2	2^{11}
V644	< 2	2^{11}
Kasauli R	< 2	2^{11}

a) Bacterial cells were used at 4×10^9 cells/ml and the titre is the reciprocal of the lowest dilution which gave agglutination.

b) This strain auto-agglutinated.

Table 5.4: Bacterial agglutination

Strain	Anti-serum	
	α -Inaba	α -Kasauli R
569B	2^{10} a	2^6
V661	2^2	2^{11}
V663	< 2	2^{11}
V665	< 2	2^{11}
V667	< 2	2^{11}
V669	< 2	2^{11}
V671	2^7	2^{10}
Kasauli R	< 2	2^{11}

a) Bacterial cells were used at 4×10^9 cells/ml and the titre is the reciprocal of the lowest dilution which gave agglutination.

## 5.3.2 Characterization of the LPS on the transposon mutants

The LPS of the transposon insertion mutants was examined by SDS-PAGE followed by silver staining (Figs 5.3 and 5.4). Most of the mutants exhibit the characteristic pattern of a rough cholera strain and lack material corresponding to LPS substituted with O-antigen, giving a similar pattern to that obtained with the rough strain, Kasauli R. The broad band detected in the lower half of the gels is more diffuse than the corresponding region from the smooth parent strain. This is expected since when there is no O-antigen produced in the rough strains, all the LPS molecules (lipid A plus core) will be unsubstituted. However, only a small proportion of molecules will be unsubstituted in the O-antigen proficient parent strain.

At least one of the mutants V225 (Fig. 5.4) produces a small amount of O-antigen and may represent a "semi-rough" phenotype (see section 5.4). It seems possible that the Tn2680 insertion in this particular mutant has a polar effect on a regulatory region leading to a decrease in transcription of the gene(s) for one or more of the enzymes.

5.3.3 Phage cross-resistance patterns

It has been described earlier that the various transposon insertion mutants were all completely resistant to the choleraphage used in selection. In order to determine if the various mutants could be further differentiated, cells were cross-streaked against the biotype specific

Figure 5.3:   SDS–PAGE on a 20% gel followed by silver staining of whole cell lysates from $\underline{V}$. $\underline{cholerae}$ transposon insertion mutants derived from strain 1621. Whole cell lysates were prepared from cultures containing $2x10^9$ cells/ml. 10μl samples were loaded.

lipid A-core
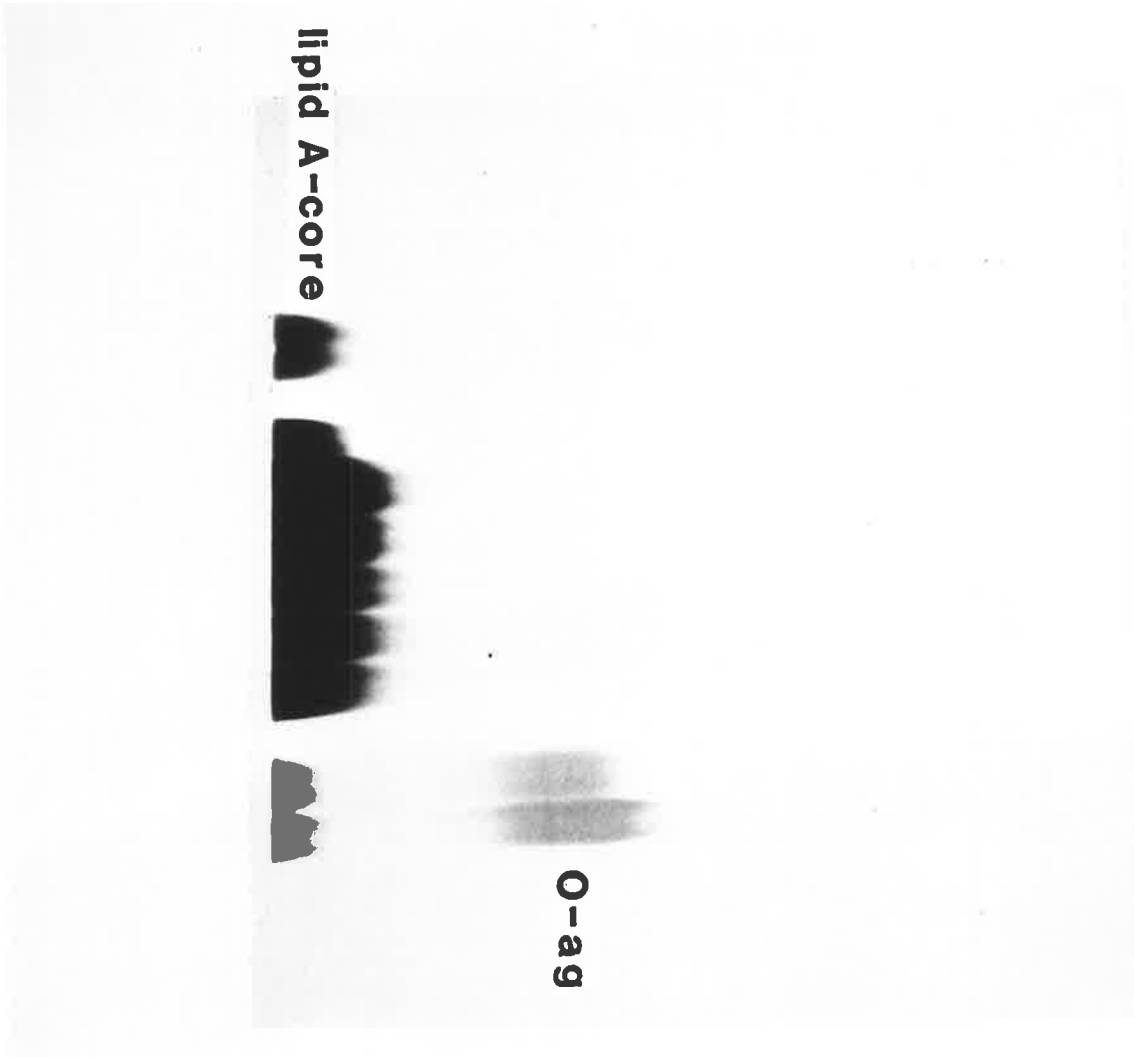
KASAULI R

V638

V640
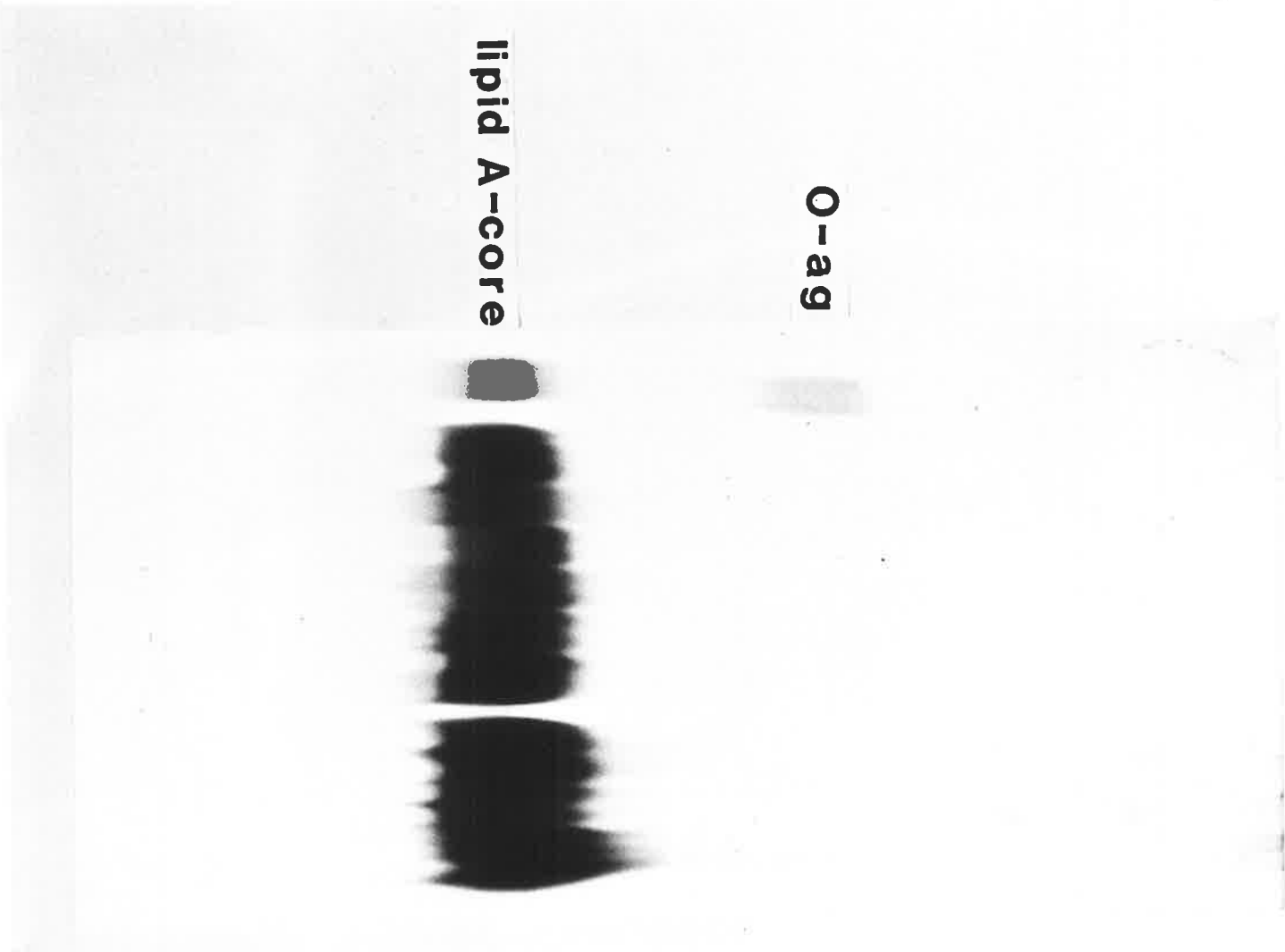
V641

V642

V643

V644

1621

O-ag

Figure 5.4:   SDS-PAGE on a 20% gel followed by silver staining of
whole   cell   lysates   from   V.   cholerae   transposon
mutants   derived   from   569B   and   017.   10μl   of   each
sample was loaded.

typing phage. Tables 5.5 and 5.6 show the results obtained from cross-streaking the mutant strains derived from 017 and 1621 against the various E1 Tor typing phage. A number of the mutants (V640-V643) show partial resistance to the ET1 phage. Although it has been inferred that ET1 does use the O-antigen at least as part of its receptor (Guidolin and Manning, 1985), the exact determinant involved in phage attachment has not been identified. Thus, CP-T1 and ET1 may differ in their requirements for O-antigen. The varying degrees of resistance to ET1 may reflect differences in the types of LPS mutations in these strains.

5.3.4 Whole cell membrane analysis of transposon mutants

It has been observed that LPS mutations can lead to changes in the protein pattern in the membranes of such mutant strains (Nikaido, 1976; Koplow and Goldfine, 1974; Ames et al., 1974). This may be due to either the translocation to the outer membrane or the complete synthesis of the outer membrane proteins being dependent on the presence of complete LPS molecules (Koplow and Goldfine, 1974). The changes observed may either be a gain or a loss in particular protein bands. It was suggested by Ames et al. (1974) that those proteins lost in such mutants may require LPS in order to be anchored to the outer membrane.

In order to analyse the protein composition of the various transposon insertion mutants, whole cell membranes were prepared and analysed by SDS-PAGE. Figures 5.5 and 5.6 show the results obtained with the Tn5 insertion

Table 5.5: E1 Tor Tn5 mutants: efficiency of plaquing of E1 Tor phage

Phage	ETI	ETII	ETIII	ETIV
Strain				
1621	1.0	1.0	1.0	1.0
V638	1×10^{-2}	7.8×10^{-4}	5×10^{-3}	1.4×10^{-3}
V640	5×10^{-2}	5.9×10^{-3}	3.6×10^{-3}	4.2×10^{-2}
V641	4.2×10^{-2}	1×10^{-2}	4.5×10^{-4}	3.4×10^{-2}
V642	3.4×10^{-2}	1.8×10^{-3}	1.2×10^{-3}	8.1×10^{-4}
V643	5.6×10^{-2}	1×10^{-2}	1.8×10^{-3}	2.2×10^{-2}
V644	1.1×10^{-2}	7.8×10^{-4}	5.7×10^{-3}	0.32

Table 5.6: Classical Tn5 mutants: efficiency of plaquing of Classical phage

Phage	VcI	VcII	VcIII	VcIV
Strain				
569B	1.0	1.0	1.0	1.0
V661	5.5	0	0	1×10^{-8}
V663	1.15	0	0	4×10^{-9}
V665	2.35	0.78	0	1.7×10^{-7}
V667	1.8	0	0	8×10^{-9}
V669	13	0	5.2×10^{-3}	7.2×10^{-8}
V671	0.95	0	0	4×10^{-9}

Figure 5.5:   SDS-PAGE patterns of cell envelope proteins of $\underline{V}$. $\underline{cholerae}$ strain 1621 and its Tn$\underline{5}$ insertion derivatives. Proteins were electrophoresed on 11% gels and stained with Coomassie Blue G-250. The closed arrowhead indicates the protein band which is present in larger amounts in the transposon insertion mutants, compared with the parent strains.
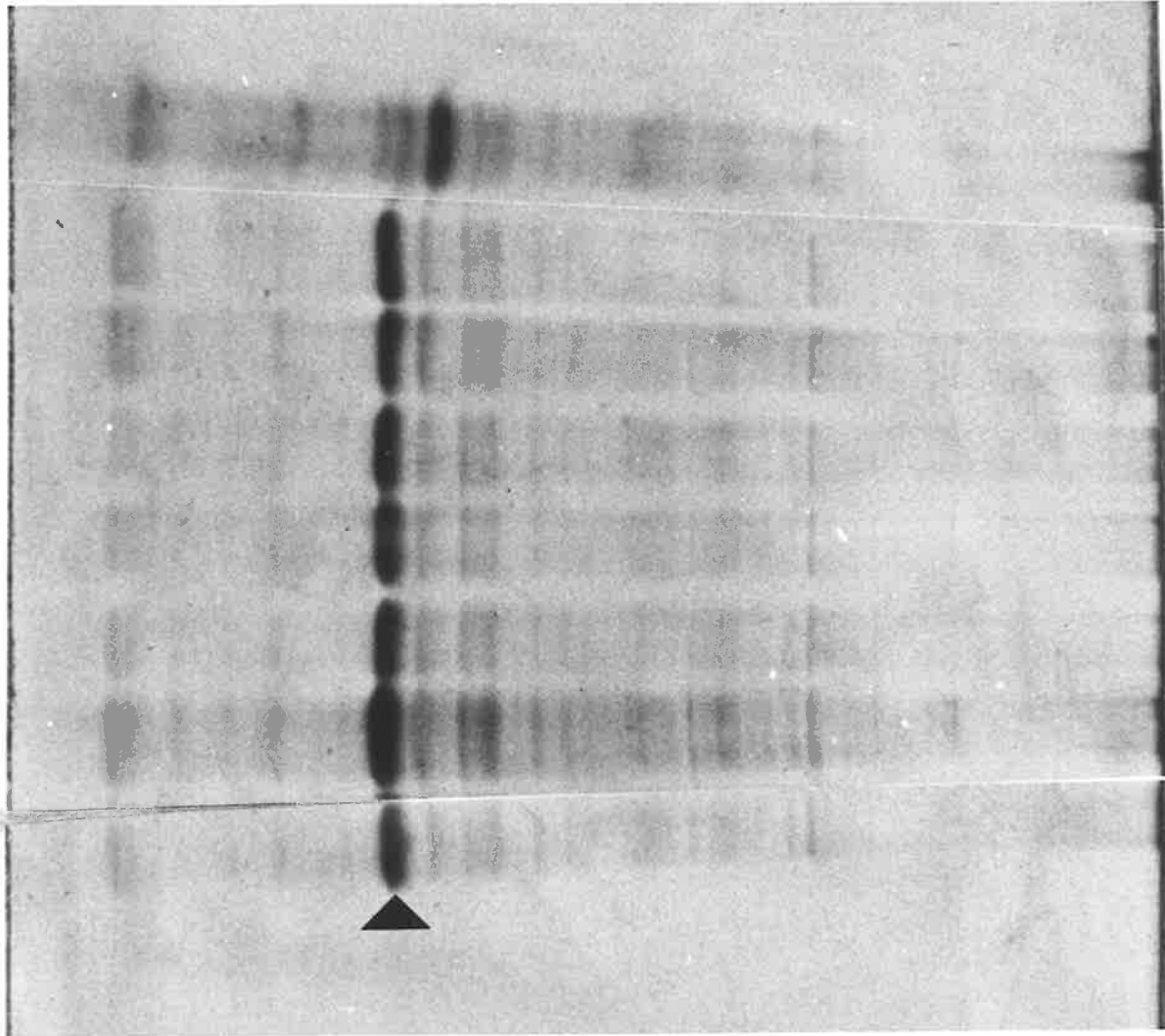
1621

V638

V640

V641

V642

V643

V644

Kasauli r

Figure 5.6: SDS–PAGE patterns of cell envelope proteins of V. cholerae strain 569B and its Tn5 insertion derivatives. Proteins were electrophoresed on 11% gels and stained with Coomassie Blue G–250. The closed arrowhead indicates the protein band which is present in larger amounts in the transposon insertion mutants, compared with the parent strains.

569B

KASAULI R

V661

V663

V665

V667

V669

V671

Figure 5.7:   SDS—PAGE patterns of cell envelope proteins of V. cholerae strain O17 and its Tn5 insertion derivat— ives. Proteins were electrophoresed on 11% gels and stained with Coomassie Blue G—250. The closed arrow— head indicates the protein band which is present in larger amounts in the transposon insertion mutants, compared with the parent strains.

017
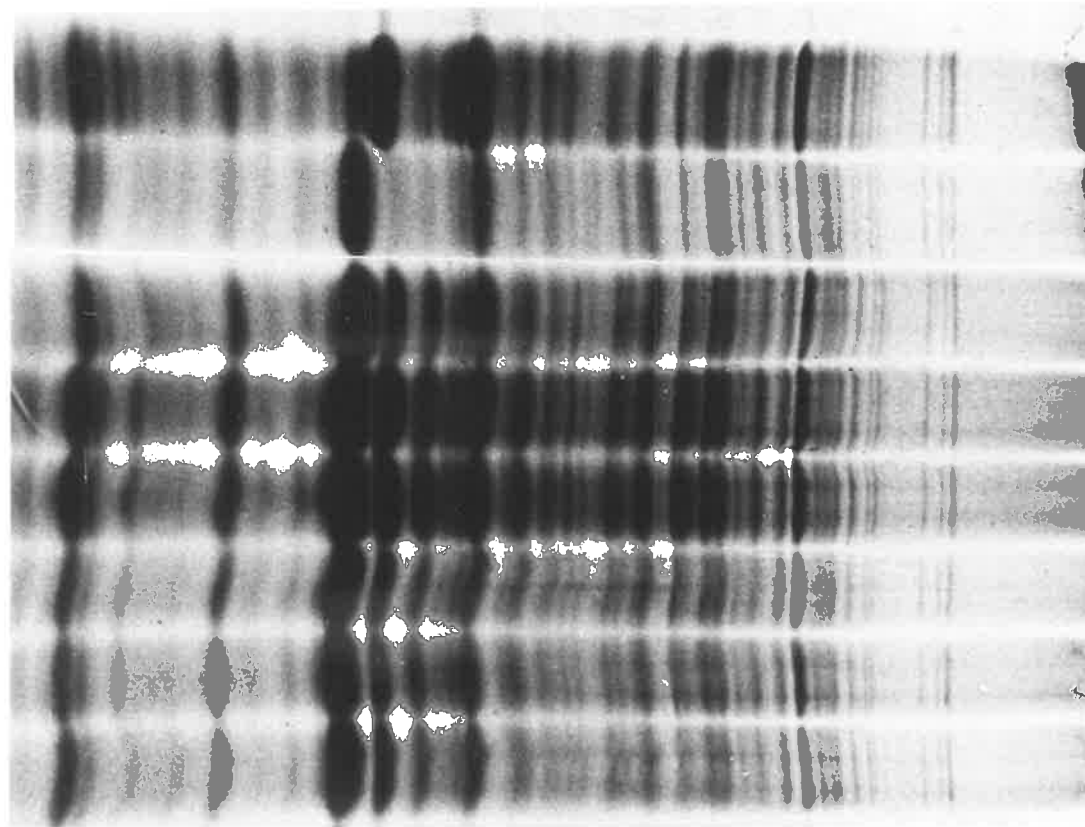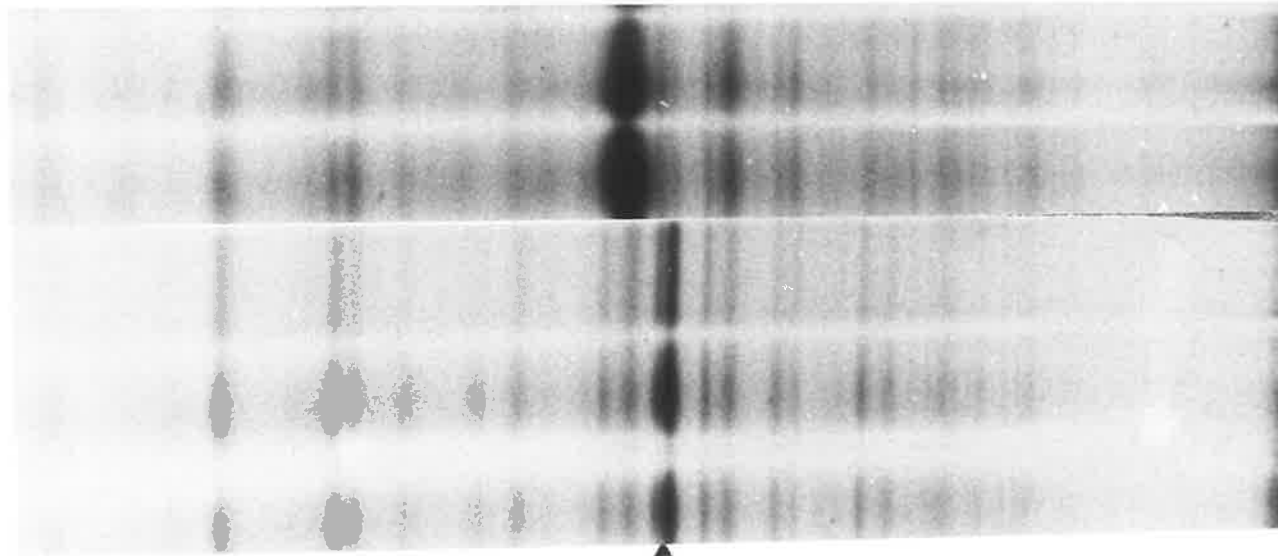569B
V223
V224
V225

mutants. It can be seen that both the Ogawa (Fig. 5.5) and Inaba (Fig. 5.6) derived mutants show an altered protein profile when compared with the appropriate parent strain. All the mutant strains appear to overproduce a protein of 23kDal. The spontaneous type mutant, V226 (Kasauli R), also exhibits this pattern, thus ruling out any association between Tn5 and the protein band observed. The Tn2680 mutants (Fig. 5.7) do not exhibit a similar pattern to that seen with the Tn5 mutants. This result correlates with other evidence which suggests that, in these strains, Tn2680 has not inserted in the rfb region and confirms that these strains exhibit different characteristics from the Tn5 mutants.

5.3.5   Southern hybridization analysis

In order to analyse the genetic basis of the transposon insertions, it was necessary to compare the DNA of the mutant strains with that of the parent strains. Southern hybridization analysis was performed, using pEVX7 DNA as the probe.

In Figs 5.8-5.11 a series of autoradiographs obtained from analysis of the Tn5 mutants derived from 1621 is shown. These results show that the majority of the mutants isolated from strain 1621 do have changes in their DNA pattern corresponding to insertions of Tn5 in particular chromosomal fragments. Strains V638 and V644 exhibit identical patterns to those of the parent strain, 1621. Although it is not possible to map the Tn5 insertions from the HindIII digestion (as the HindIII sites have not been

Figure 5.8:   Southern hybridization analysis of whole genomic DNA
              from 1621 and its Tn5 insertion derivatives, digested
              with HindIII. After electrophoresis on a 0.8% gel,
              the DNA was transferred to nitrocellulose paper and
              hybridized with nick translated pEVX7 DNA. The bands
              were visualized by autoradiography. The closed arrow-
              heads indicate bands which have altered mobility due
              to insertion of Tn5. The sizes of the appropriate
              restriction fragments from the V. cholerae rfb region
              are shown.

V638
V640
V641
V642
V643
V644

1621

1.06 1.2 1.3 1.6 1.9 2.4 2.9 4.9
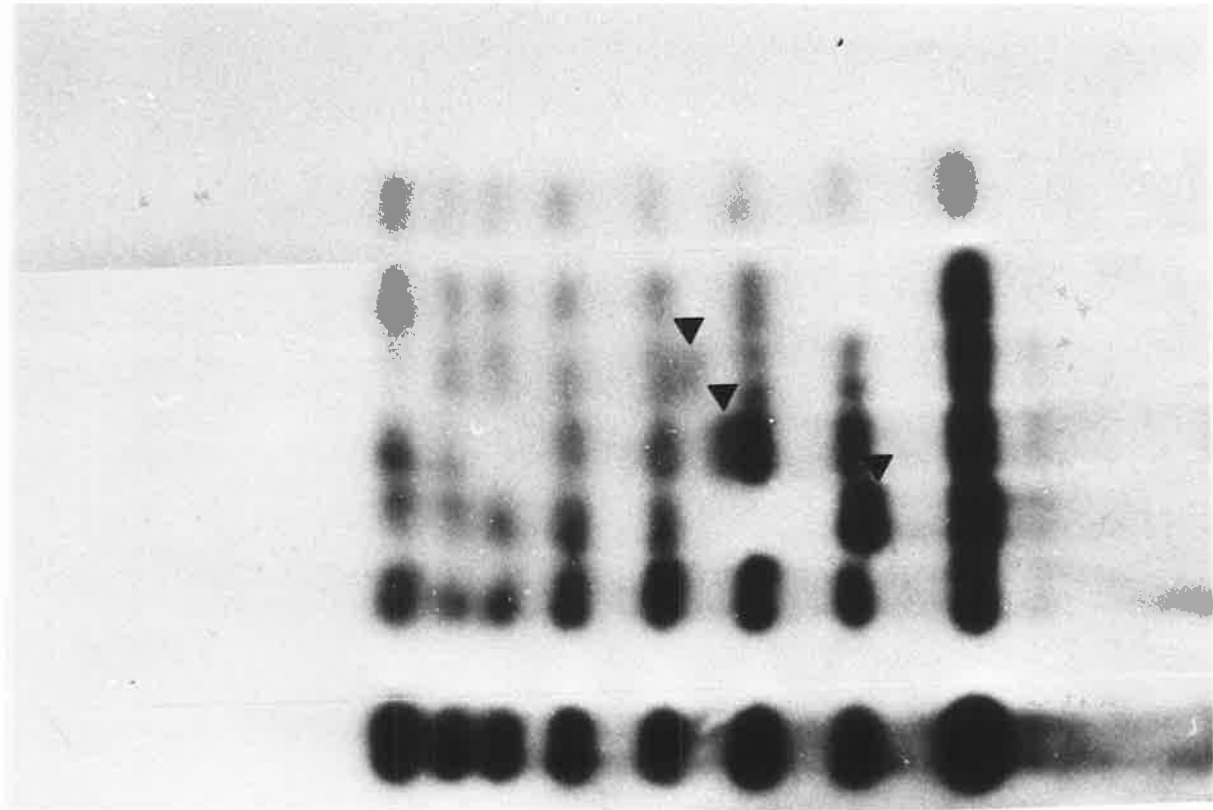
Figure 5.9:   Southern hybridization analysis of whole genomic DNA
               from 1621 and its Tn5 insertion derivatives, digested
               with ClaI. After electrophoresis on a 0.8% gel, the
               DNA was transferred to nitrocellulose paper and
               hybridized with nick translated pEVX7 DNA. The bands
               were visualized by autoradiography. The closed arrow-
               heads indicate bands which have altered mobility due
               to insertion of Tn5. The sizes of the appropriate
               restriction fragments from the V. cholerae rfb region
               are shown.
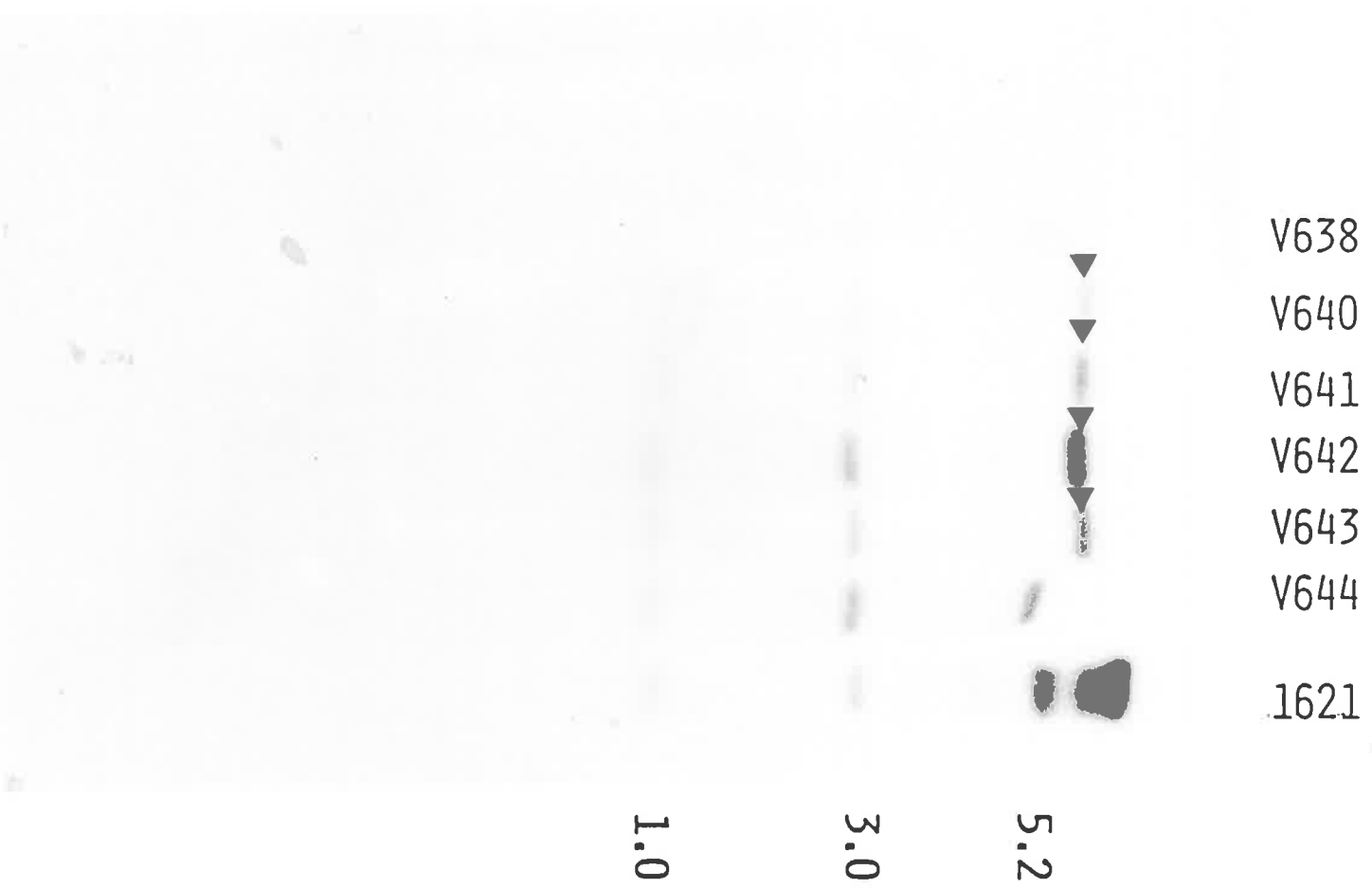
Figure 5.10: Southern hybridization analysis of whole genomic DNA from 1621 and its Tn5 insertion derivatives, digested with EcoRI. After electrophoresis on a 0.8% gel, the DNA was transferred to nitrocellulose paper and hybridized with nick translated pEVX7 DNA. The bands were visualized by autoradiography. The closed arrowheads indicate bands which have altered mobility due to insertion of Tn5. The sizes of the appropriate restriction fragments from the V. cholerae rfb region are shown.

The smaller arrows indicate bands due to partial restriction digests of the chromosomal DNA by EcoRI.

V638
V640
V641
V642
V643
V644

ᴘEVX7

1621

1.2
3.0
3.1
4.9
5.8

Figure 5.11:

(A) Southern hybridization analysis of whole genomic DNA from
     1621 and its Tn5 insertion derivatives, digested with BamHI.
     After electrophoresis on a 0.8% gel, the DNA was transferred
     to nitrocellulose paper and hybridized with nick translated
     pEVX7 DNA. The bands were visualized by autoradiography. The
     closed arrowheads indicate bands which have altered mobility
     due to insertion of Tn5. The sizes of the appropriate
     restriction fragments from the V. cholerae rfb region are
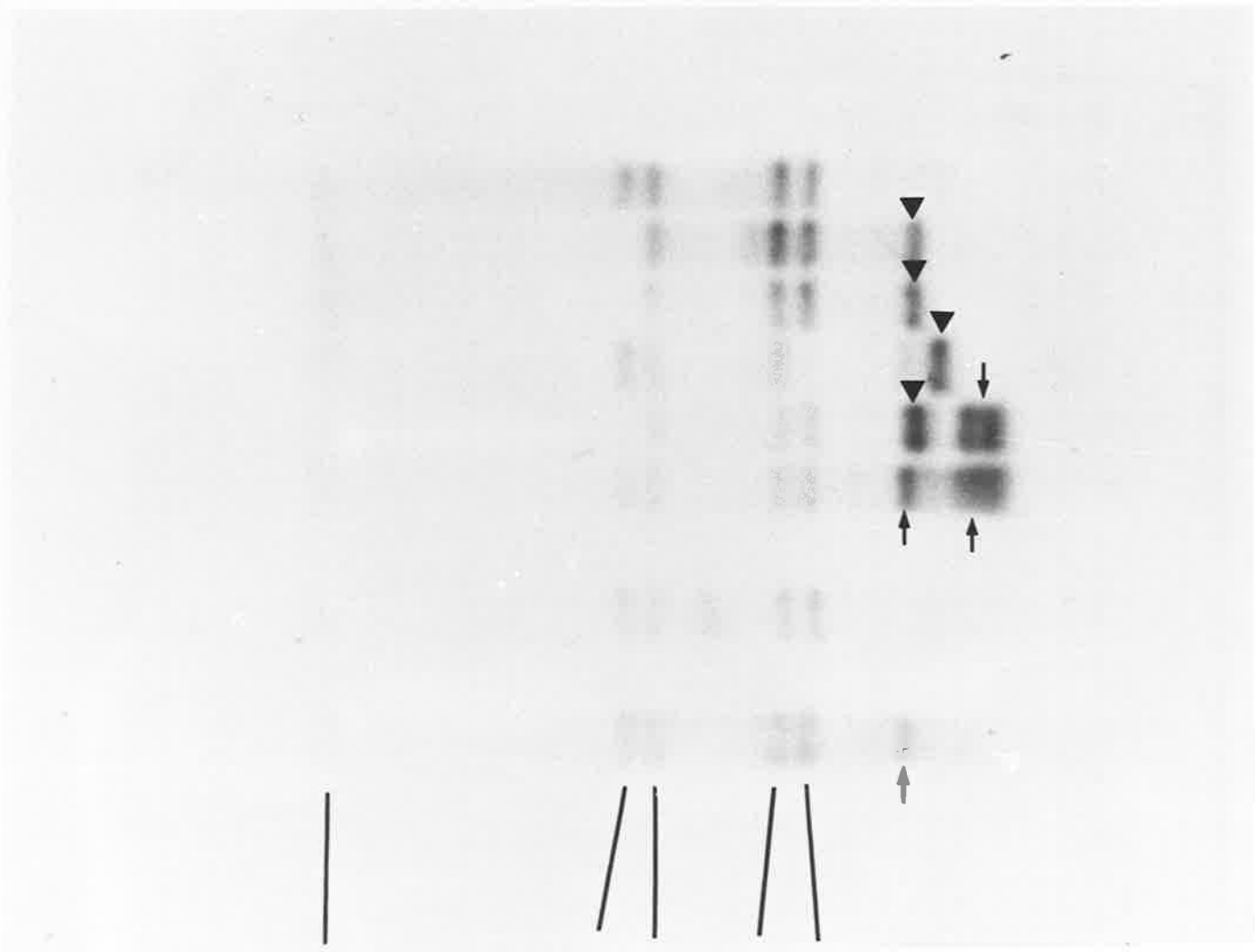   shown.

(B) Further analysis of V644 DNA was digested with BamHI or SstI,
     electrophoresed on a 0.8% gel and transferred to nitro-
     cellulose. The filter was hybridized with nick translated
     pPM1003.

B

V644 RM101 017   V644 RM101 017

13.8
9.6

◁ 19.5

◁ 5.02

3.8

Bam H1   Sst 1
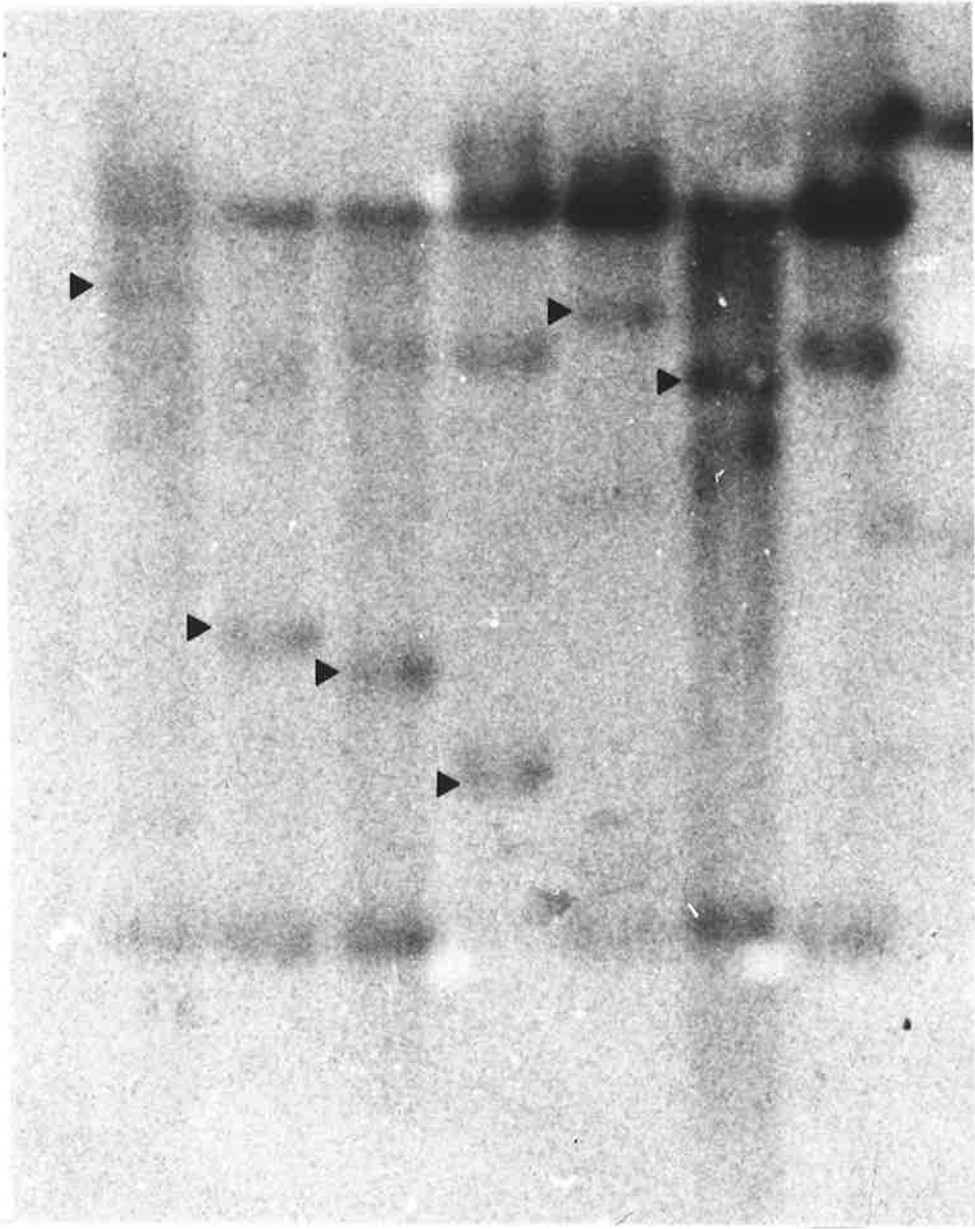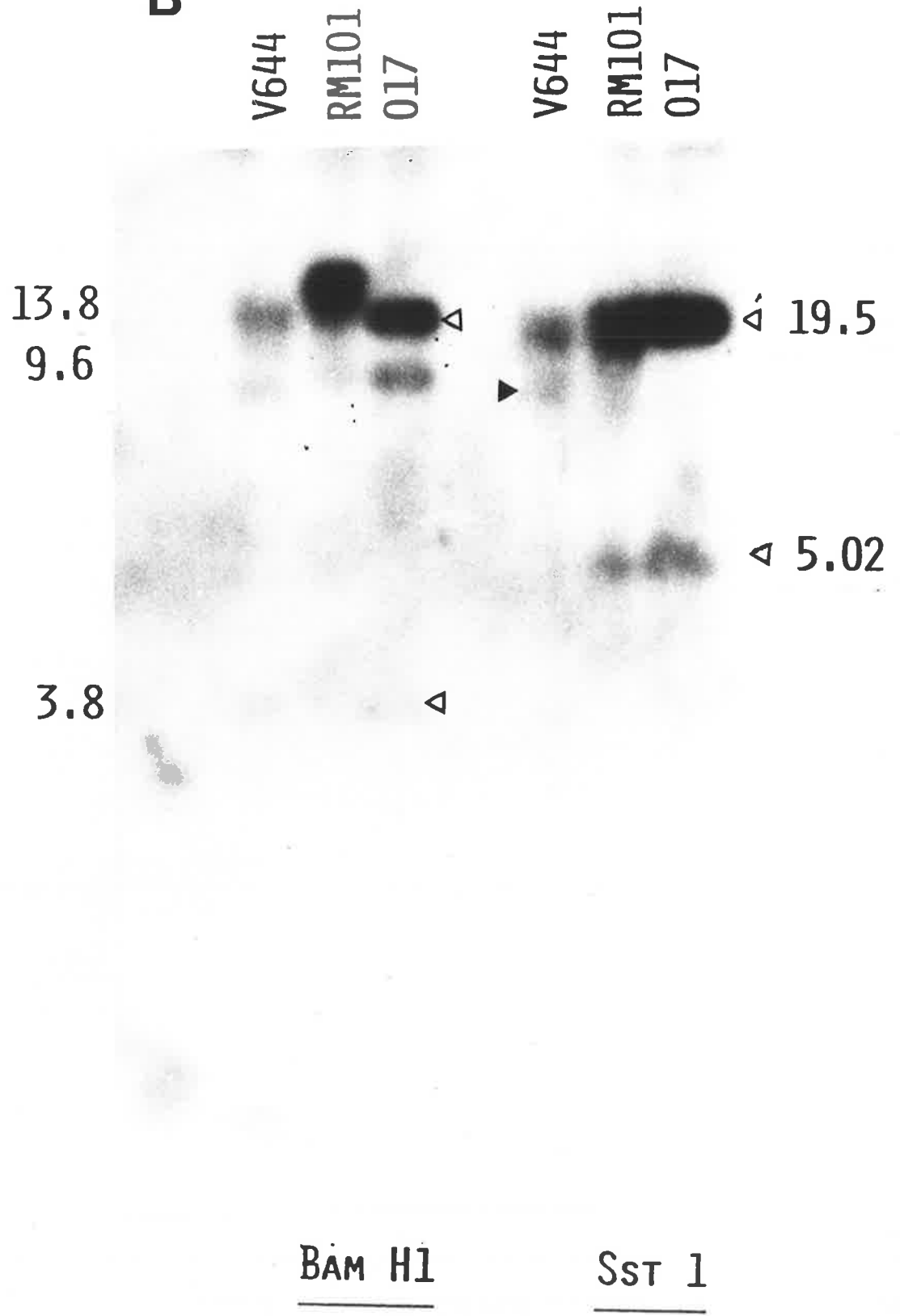
completely mapped in pEVX7), this experiment does allow the identification of independent mutations, that is, the insertion of Tn5 into different chromosomal fragments.

Southern hybridization of the DNA digested with several other restriction enzymes (EcoRI, ClaI and BamHI) (Figs 5.9-5.11) allowed the sites of Tn5 insertion in the different mutants to be precisely mapped. The strain V644 differed in its BamHI pattern (Fig. 5.11A) from the parent, 1621, yet no changes were detected with the other restriction enzymes used. Further analysis was performed on V644 (Fig. 5.11B). Hybridization of SstI digested DNA with pPM1003 showed that Tn5 had inserted into the 5.02kb Sst fragment. The Tn5 mutants, V661 to V671, derived from 569B, were analysed in a similar series of Southern hybridizations (Figs 5.12-5.15).

The sites of insertions of Tn5 in the genomes of 1621 and 569B are summarized in Fig. 5.16. It can be seen that the majority of insertions map in the region corresponding to the right end of the cloned rfb DNA, namely in the 11.7kb ClaI fragment.

5.4 Characterization of transposon insertions which map outside rfb

5.4.1 Southern hybridization analysis

A number of transposon insertion mutants V638, V671 and the Tn2680 insertions, V223, V224, V225, have been isolated which map outside the rfb region as defined by the SstI fragment in pEVX7 and pEVX6. In order to confirm

Figure 5.12: Southern hybridization analysis of whole genomic DNA from 569B and its Tn5 insertion derivatives, digested with HindIII. After electrophoresis on a 0.8% gel, the DNA was transferred to nitrocellulose paper and hybridized with nick translated pEVX7 DNA. The bands were visualized by autoradiography. The closed arrowheads indicate bands which have altered mobility due to insertion of Tn5. The sizes of the appropriate restriction fragments from the V. cholerae rfb region are shown.
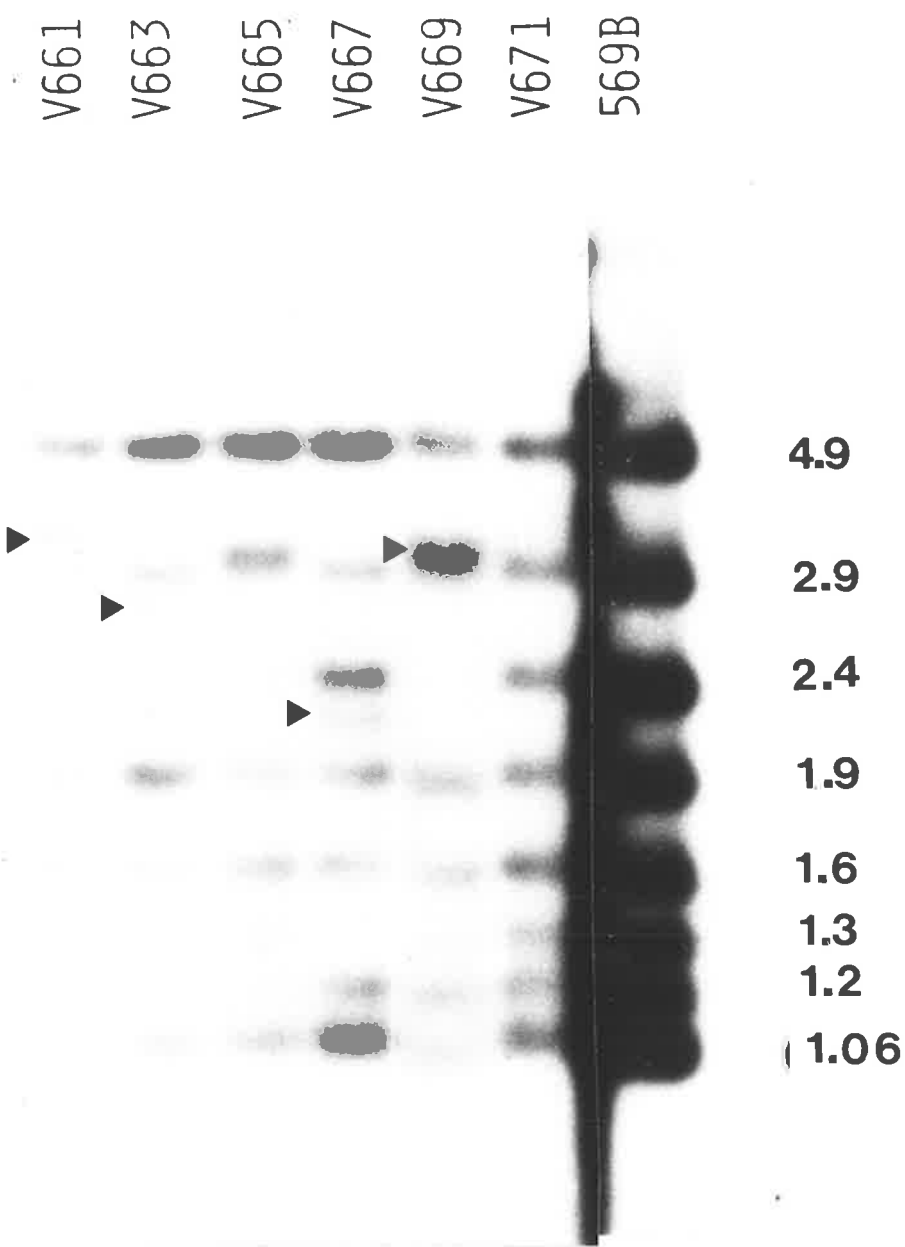
Figure 5.13: Southern hybridization analysis of whole genomic DNA
from 569B and its Tn5 insertion derivatives, digested
with ClaI. After electrophoresis on a 0.8% gel, the
DNA was transferred to nitrocellulose paper and
hybridized with nick translated pEVX7 DNA. The bands
were visualized by autoradiography. The closed arrow-
heads indicate bands which have altered mobility due
to insertion of Tn5. The sizes of the appropriate
restriction fragments from the V. cholerae rfb region
are shown.
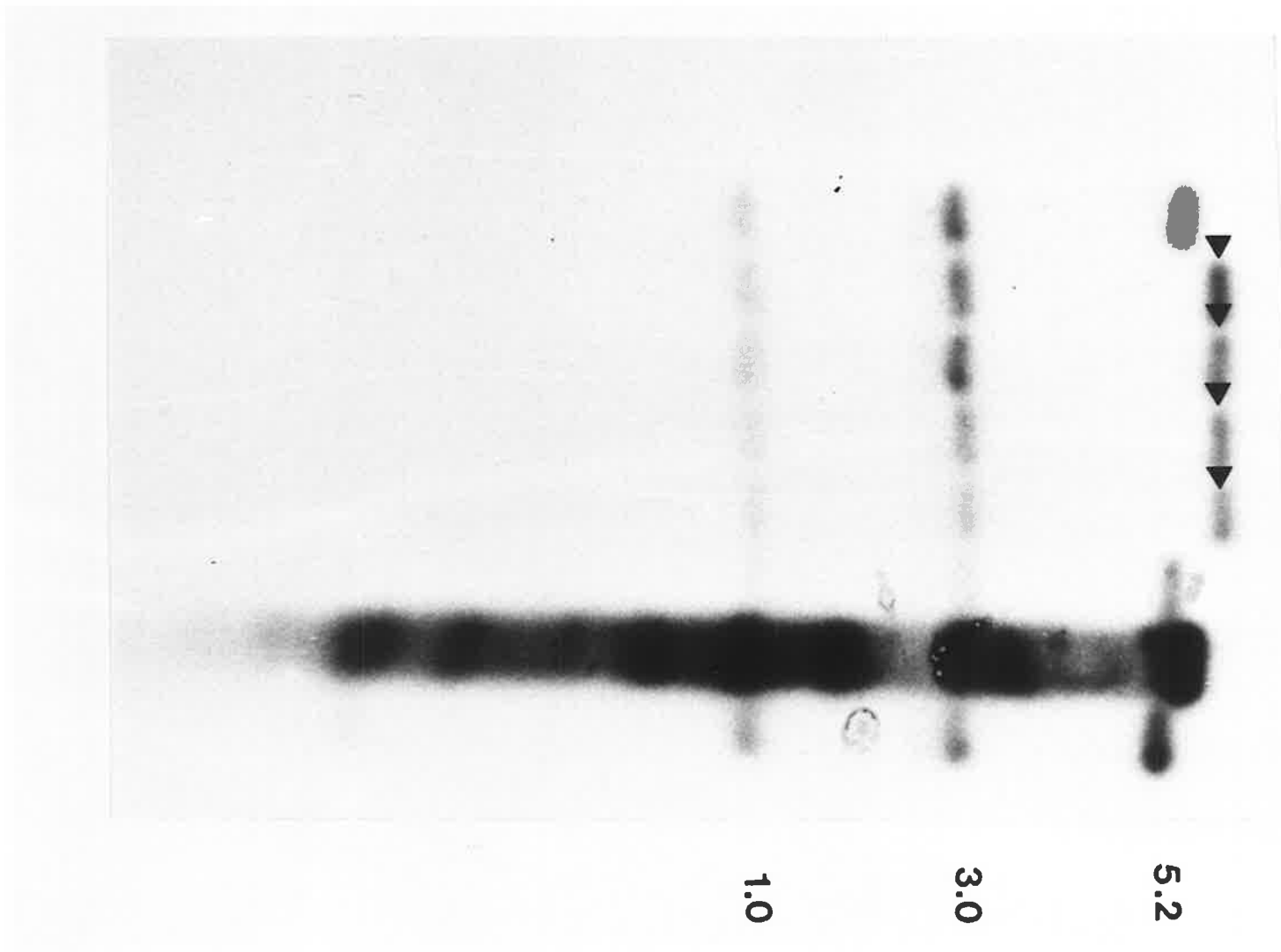
V661
V663
V665
V667
V669
V671
pEVX7
569B

1.0   3.0   5.2

Figure 5.14: Southern hybridization analysis of whole genomic DNA
from 569B and its Tn5 insertion derivatives, digested
with EcoRI. After electrophoresis on a 0.8% gel, the
DNA was transferred to nitrocellulose paper and
hybridized with nick translated pEVX7 DNA. The bands
were visualized by autoradiography. The closed arrow-
heads indicate bands which have altered mobility due
to insertion of Tn5. The sizes of the appropriate
restriction fragments from the V. cholerae rfb region
are shown.
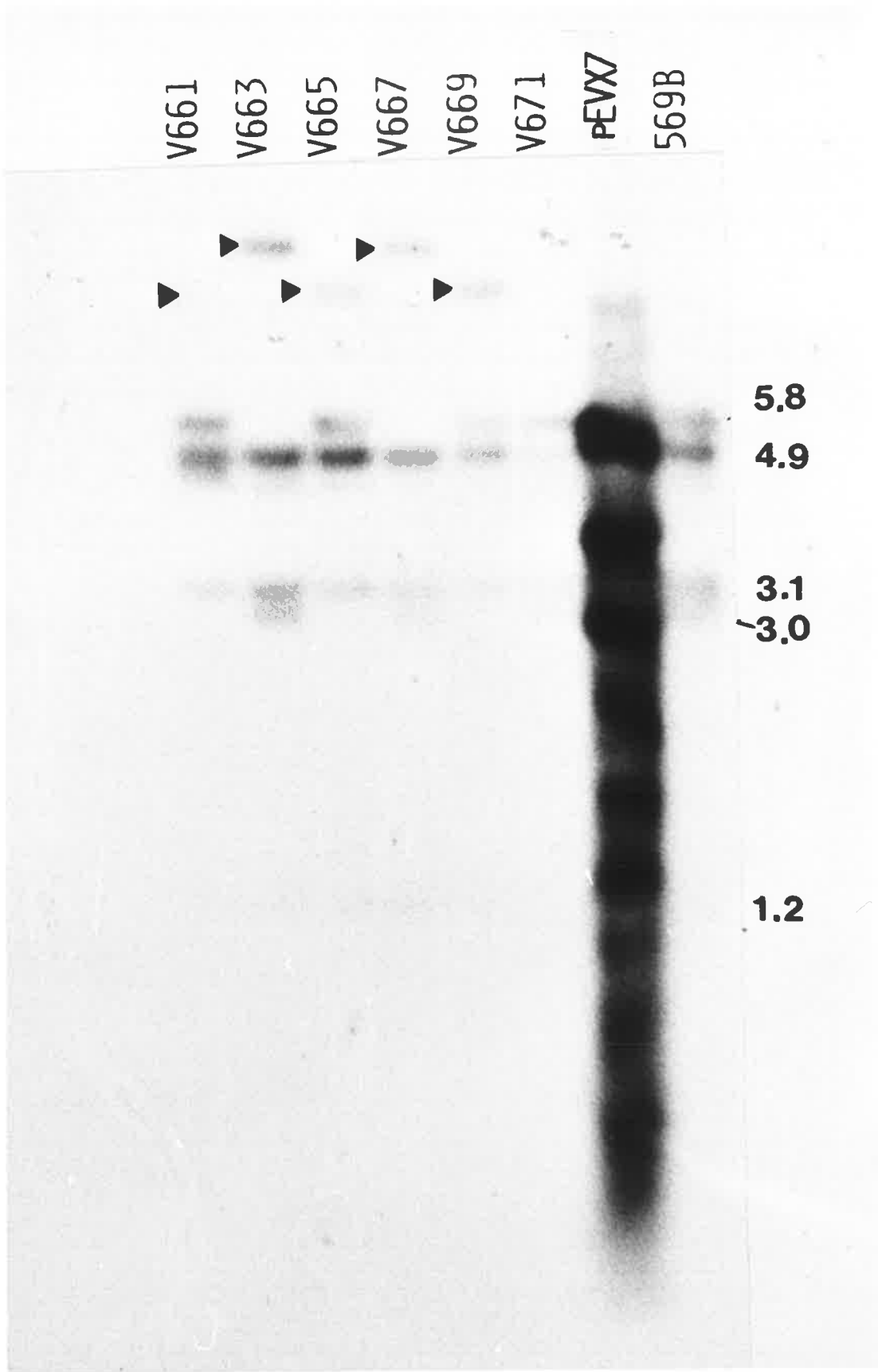
Figure 5.15: Southern hybridization analysis of whole genomic DNA from 569B and its Tn5 insertion derivatives, digested with BamHI. After electrophoresis on a 0.8% gel, the DNA was transferred to nitrocellulose paper and hybridized with nick translated pEVX7 DNA. The bands were visualized by autoradiography. The closed arrowheads indicate bands which have altered mobility due to insertion of Tn5. The sizes of the appropriate restriction fragments from the V. cholerae rfb region are shown.
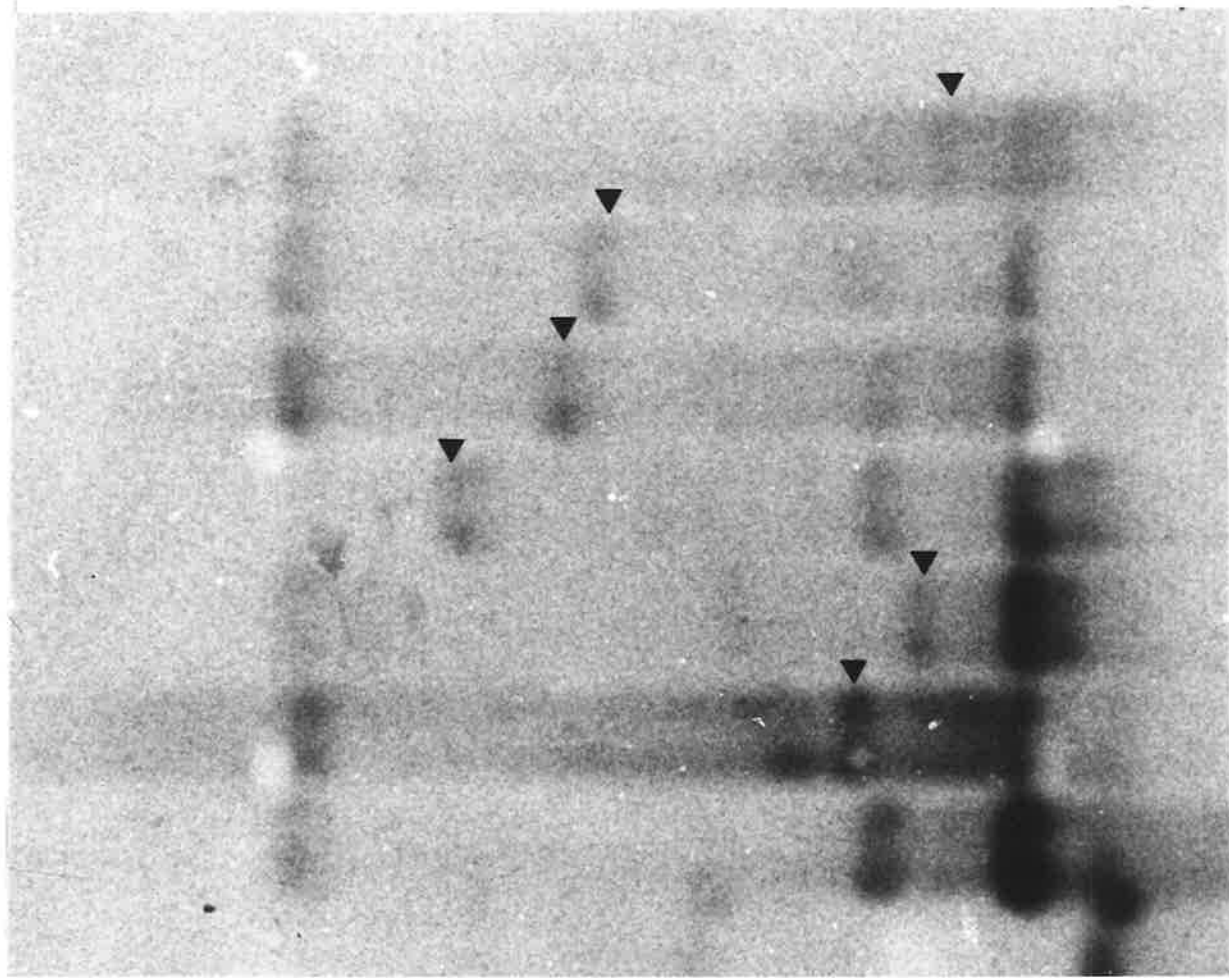
Figure 5.16: Restriction map of the rfb region of the V. cholerae
chromosome, showing locations of Tn5 insertions
mapped by Southern hybridization using pEVX7 as a
probe. Closed arrowheads indicate the site of Tn5
insertions from (A) 1621 and (B) 569B. The numbers
correspond to the various Tn5 insertion strains as
follows:

Number	Strain
1	V640
2	V641
3	V642
4	V643
5	V644
6	V661
7	V663
8	V665
9	V667
10	V669

SalI Sstl Sstl EcoRI ClaI XbaI EcoRI ClaI SalI XbaI EcoRI SalI ClaI EcoRI ClaI BamHI XbaI BamHI EcoRI KpnI HpaI EcoRI Sstl HpaI Sstl HpaI BamHI Sstl

pPM1003

pEVX7

3   4 21   5

A

9 76 8

B

10
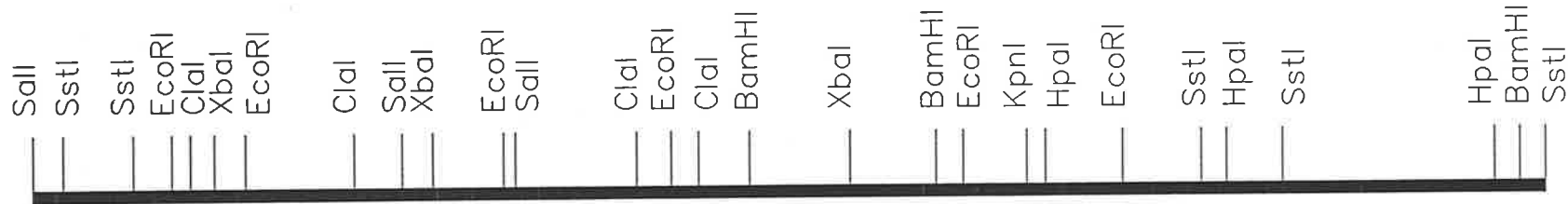
1 kb
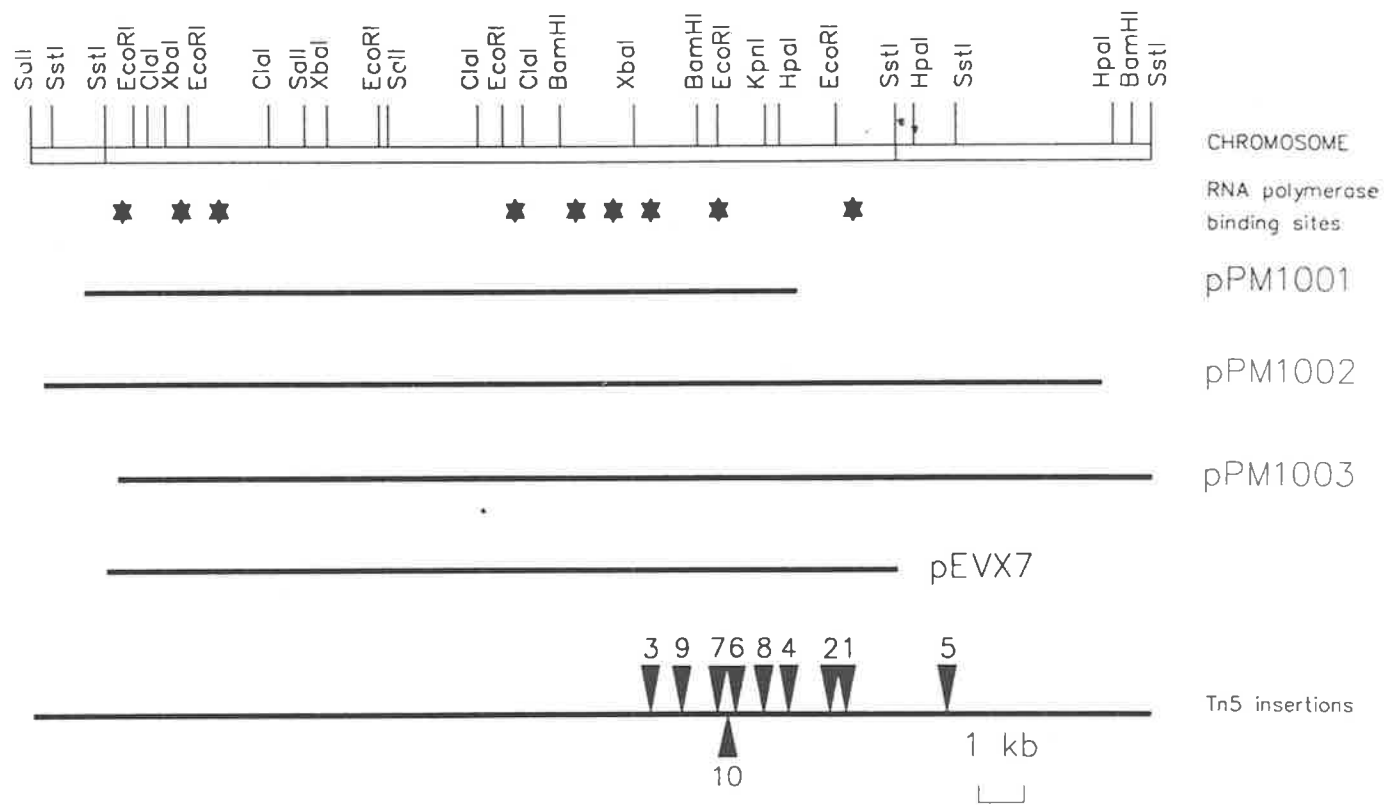
that the transposon insertions did not lie in the flanking DNA outside this minimal SstI piece, Southern hybridization analysis was performed using pPM1003 as the probe (Fig. 5.17). This plasmid includes an additional 7kb of contiguous chromosomal DNA at the right end which is not present in pEVX7. The pattern of fragments observed using this probe was identical to that of the parents, except in V638, indicating that the site of transposon insertion does not lie in the cloned rfb region of V. cholerae 01. V638 showed an altered EcoRI restriction pattern, compared with the parent V. cholerae strain, when probed with pPM1003. Thus, it can be concluded that the Tn5 insertion in V638 maps outside the rfb region defined by pEVX7, but within the extra DNA included in pPM1003.

5.4.2 Analysis of LPS and whole membrane preparations by SDS-PAGE

The LPS of the non-rfb mutants was analysed by SDS-PAGE of whole cell lysates treated with proteinase K (Fig. 5.18), using a series of defined E. coli rfa mutants (Boman and Monner, 1979) as controls. When a 15% polyacrylamide gel was used, O-antigen was not detected in any of the mutants and their lipid A-core region could not be differentiated in terms of mobility from that of the parent V. cholerae strain. However, this system could detect changes in the LPS of deep rough mutants of E. coli (i.e., heptoseless) from that of the appropriate strain.

Further analysis of the LPS was performed using a 7.5-25% hyperbolic polyacrylamide gradient gel. Such gels

Figure 5.17:   Southern hybridization analysis of whole genomic DNA from
                the transposon insertion mutants which map outside rfb. DNA
                was digested with (A) HindIII, (B) XbaI, (C) HindIII and (D)
                EcoRI.   After transfer to nitrocellulose the DNA was
                hybridized with either nick translated pEVX7 (A) or pPM1003,
                (B), (C) and (D). The sizes of the chromosomal HindIII and
                XbaI restriction fragments are shown in kb.   In panel (D),
                the dot indicates the position of the restriction fragment
                which is missing due to Tn5 insertion. The closed arrowhead
                indicates the restriction fragment resulting from this Tn5
                insertion.

D
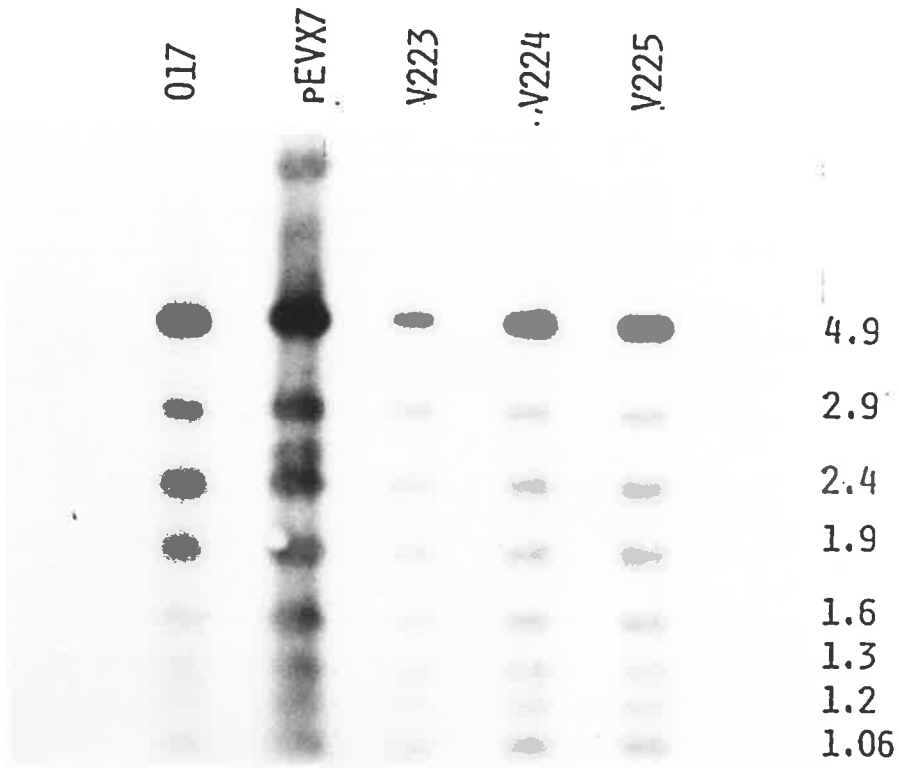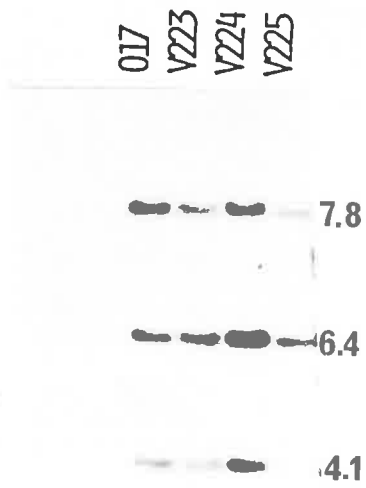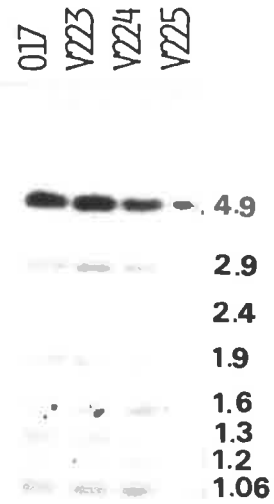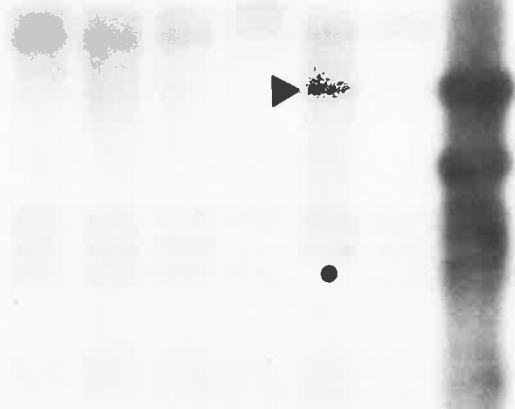
Figure 5.18: SDS-PAGE of whole cell lysates from Tn2680 insertion
mutants and defined E. coli rfa mutants. LPS was
visualized by silver staining after electrophoresis
on 15% gels. 10 μl samples were loaded. The O-antigen
and lipid A-core regions are indicated.

lipid A-core

O-ag

V223
V224
V225

KASAULI R

O17
YA21
YA216
D21
D21E7

D21F1

KA56
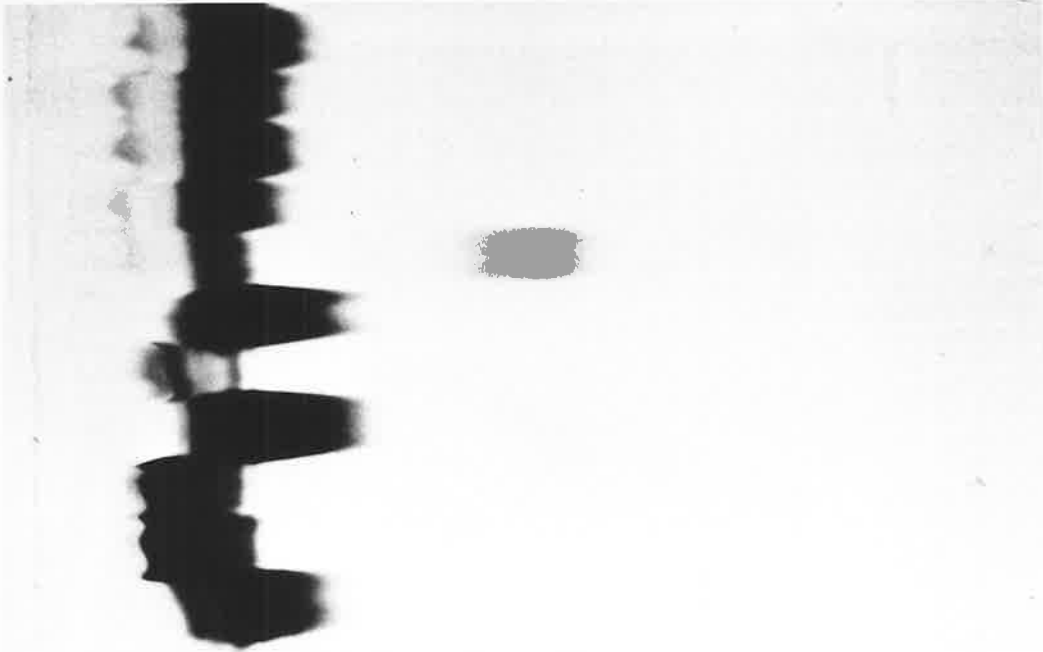
have proven useful in characterization of pathogenic E. coli strains and the identification of doublets in the LPS banding pattern (Kusecek et al., 1984). The lipid A-core of V. cholerae did not run as a diffuse region in these gels (as observed in linear gels or exponential gradients), but appeared as two major bands. The composition of these regions is unknown. However, despite the altered resolution of the lipid A-core region, no differences in mobility of these regions were observed, when the V. cholerae mutants were compared with the parent strain (Fig. 5.19). This gel system, however, was able to differentiate the various E. coli rfa mutants.

Thus, SDS-PAGE of the LPS from these transposon insertions has not revealed any differences in banding patterns or mobility of the lipid A-core material compared with either the rfb::Tn5 mutants or the smooth parent.

The protein patterns of these strains were also studied using SDS-PAGE of whole membrane preparations (Fig. 5.7, see section 5.3.4). It can be seen that the Tn2680 mutants exhibit a distinctive protein pattern which differs from that observed in the Tn5 mutants. The three Tn2680 strains overproduce a protein of 43kDal. This protein is thought to be one of the porins present in the V. cholerae outer membrane (Manning et al., 1982), so it is possible that the LPS mutation in these strains has affected the synthesis of one of these porins and has allowed the preferential incorporation of this species of porin. The Tn5 mutants V671 and V638 have an identical

Figure 5.19: SDS–PAGE on a 7.5–25% exponential gel, followed by silver staining of whole cell lysates from Tn2680 mutants and defined E. coli rfa mutants. 10µl of each sample was loaded.
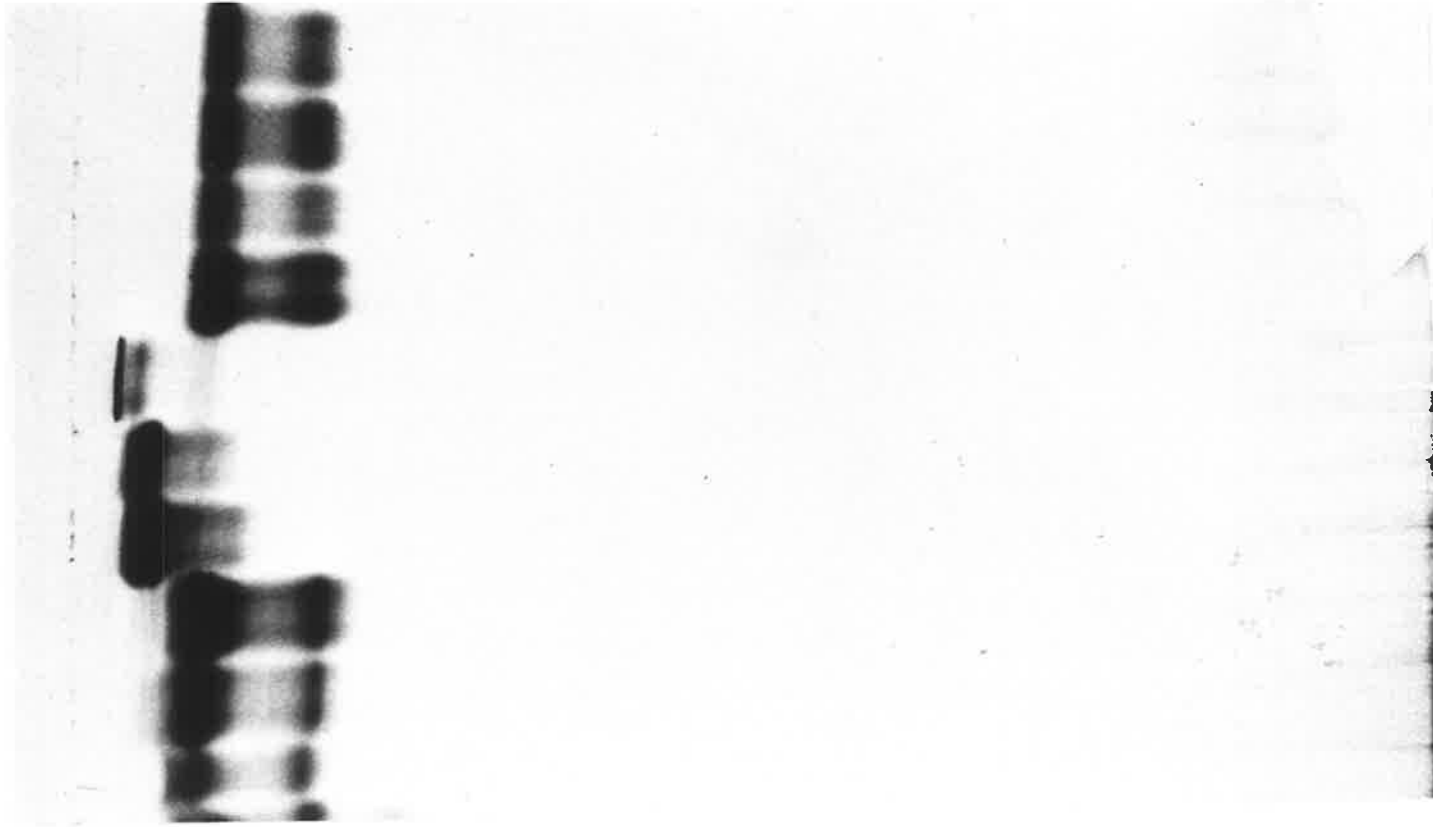
V223
V224
V225
KASAULI R
YA216
D21
D21ε7
YA21
KA56
V638

protein when compared with the Tn5 mutants which have been mapped to the cloned rfb region.

5.4.3 Analysis of sensitivity to dyes, detergents and antibiotics

It has been observed that some LPS mutations in E. coli and S. typhimurium, particularly those in the rfa genes affecting core biosynthesis, lead to altered sensitivity to various chemicals. This effect is thought to be due to an altered ratio of the components of the outer membrane (Roantree et al., 1969; Tamaki et al., 1971; Wu, 1972; Ames et al., 1974). It has been noted that there are significant increases in the phospholipid content of deep rough mutants (Nikaido, 1977) which could lead to the formation of phospholipid bilayer domains in the outer membrane and this, in turn, could contribute to increased permeability to hydrophobic agents. An alternative interpretation comes from Nikaido and Vaara (1985) who have proposed that LPS-LPS interactions play a major role in preventing the penetration of hydrophobic chemicals into smooth strains. Thus, in rough mutants these strong lateral forces in the outer membrane are reduced and so the permeability of the cells is increased.

In this section the effect of transposon insertions in the V. cholerae LPS genes, on outer membrane permeability, has been examined. The strains were tested using Multodiscs (Difco) or individual discs impregnated with a range of dyes, detergents and antibiotics, and the zone of growth inhibition was measured (Tables 5.7, 5.8 and 5.9).

Table 5.7: Sensitivity to dyes, detergents and antibiotics

Drug	Conc. (μg/disc)	Zone of Inhibition – (mm)[a]							Partition[b] Coefficient
		569B	V638	V640	V641	V642	V643	V644	
Rifampicin	0.05	14	12	15	13	15	10	15	8.8
Polymixin B	0.25U	8	8	8	8	8	7	8	–[c]
Novobiocin	10	22	19	22	16	22	29	22	> 20
Deoxycholate	10	< 6	< 6	< 6	< 6	< 6	< 6	< 6	1.09
Methylene Blue	0.005	< 6	< 6	< 6	< 6	< 6	< 6	< 6	0.02
Crystal Violet	0.005	9	8	8	< 6	< 6	< 6	8	14.4
SDS	5	12	< 6	< 6	< 6	< 6	< 6	< 6	0.02
Ampicillin	2	< 6	< 6	< 6	< 6	< 6	< 6	< 6	0.01
Bacitracin	10	< 6	< 6	< 6	< 6	< 6	< 6	< 6	0.12
Vancomycin	30	< 6	< 6	< 6	< 6	< 6	< 6	< 6	0.01
Tetracyline	5	23	20	25	24	20	20	20	0.09

a) The diameter of the disc (6mm) has not been subtracted.

b) Partition coefficients taken from Coleman and Lewe, 1979.

c) Value not available.

Table 5.8: Sensitivity to dyes, detergents and antibiotics

Drug	Conc. (μg/disc)	Zone of Inhibition – (mm)[a]							Partition[b] Coefficient
		1621	V661	V663	V665	V667	V669	V671	
Rifampicin	0.05	16	18	15	15	13	13	13	8.8
Polymixin B	0.25U	10	8	10	10	10	10	10	–[c]
Novobiocin	10	19	23	19	19	20	19	19	>20
Deoxycholate	10	6	6	6	6	< 6	< 6	< 6	1.09
Methylene Blue	0.005	< 6	< 6	< 6	< 6	< 6	< 6	< 6	0.02
Crystal Violet	0.005	8	8	8	8	8	8	8	14.4
SDS	5	10	12	12	12	12	10	10	0.02
Ampicillin	2	< 6	< 6	< 6	< 6	< 6	< 6	< 6	0.01
Bacitracin	10	< 6	< 6	< 6	< 6	< 6	< 6	< 6	0.12
Vancomycin	30	< 6	< 6	< 6	< 6	< 6	< 6	< 6	0.01
Tetracycline	5	23	21	23	23	19	23	22	0.09

a) The diameter of the disc (6mm) has not been subtracted.

b) Partition coefficients taken from Coleman and Lewe, 1979.

c) Value not available.

Table 5.9: Sensitivity to dyes, detergents and antibiotics

Drug	Conc. (μg/disc)	Zone of Inhibition – (mm)[a]					Partition[b] Coefficient
		017	V223	V224	V225	V226	
Rifampicin	0.05	14	16	14	13	11	8.8
Polymixin B	0.25U	6	6	8	11	21	–[c]
Novobiocin	10	15	24	25	29	15	> 20
Deoxycholate	10	< 6	< 6	< 6	< 6	< 6	1.09
Methylene Blue	0.005	7	< 6	< 6	< 6	< 6	0.02
Crystal Violet	0.005	9	9	< 6	9	9	14.4
SDS	5	< 6	< 6	< 6	< 6	< 6	0.02
Ampicillin	2	13	13	13	15	15	0.01
Bacitracin	10	< 6	< 6	< 6	< 6	< 6	0.12
Vancomycin	30	< 6	< 6	< 6	< 6	< 6	0.01
Tetracycline	5	18	22	23	22	18	0.09

a) The diameter of the disc (6mm) has not been subtracted.

b) Partition coefficients taken from Coleman and Lewe, 1979.

c) Value not available.

V223, V224 and V225 (which all contain Tn2680 insertions) show increased sensitivity to both tetracycline and novobiocin (Table 5.9). Novobiocin is a very hydrophobic antibiotic and such changes are known to be associated with core mutations in E. coliK-12 (Tamaki et al., 1971; Coleman and Deshpande, 1985) and in S. typhimurium (Roantree et al., 1977; Wilkinson et al., 1972; Sukupolvi et al., 1985). In E. coliK-12, mutations leading to novobiocin supersensitivity have been mapped to the rfa region on the chromosome (Tamaki et al., 1971; Hancock and Reeves, 1976). Thus, the altered sensitivity of the Tn2680 mutants may reflect a mutation affecting the biosynthesis of the LPS core in V. cholerae. The actual nature of these mutations is unknown, but possibly these strains have lower levels of phosphate in the core, as Tamaki et al. (1971) correlated sensitivity to novobiocin in E. coliK-12 with the absence of phosphate diester bridges in the core.

The Tn5 mutants analysed did not show any significant alteration in sensitivity to the chemicals tested. Thus, the strains V671 and V638 do not show similar characteristics to the Tn2680 strains.

5.5   Conclusion

This chapter has described the isolation and characterization of a series of Tn5 and Tn2680 mutants, which were all selected on the basis of phage resistance to either CP-T1 or VcII. SDS-PAGE analysis has revealed that all these strains have altered LPS patterns compared with the parent strain and that the majority lack O-antigen.

This was confirmed by serological analysis. The protein patterns of the mutants differ from those of the parent, indicating changes in the composition of the outer membrane.

Southern hybridization analysis has enabled most of the Tn5 insertions to be mapped and these lie within (or just outside) the rfb region of V. cholerae, as defined by the cloned 19.5kb SstI fragment. Since V644 maps to the right of the minimal SstI fragment, it can be concluded that the functions encoded in this region can be provided by E. coliK-12. Although E. coliK-12 is rough, it does still contain some rfb genes and E. coliK-12 deletion strains have been constructed which lack the rfb region (Sunshine and Kelly, 1971). Possibly these genes are involved in sugar biosynthesis and so can complement V. cholerae rfb genes.

Preliminary analysis of the transposon insertions which lie outside rfb, has been performed. The Tn2680 mutants have a number of interesting properties which distinguish them from the other strains. These include autoagglutination, the formation of rough colonies, increased sensitivity to tetracycline and novobiocin and a distinctive outer membrane protein pattern. All of these characteristics have been associated with core (i.e., rfa) mutations in E. coliK-12 and S. typhimurium (Ames et al., 1974; Koplow and Goldfine, 1974; Tamaki et al., 1971; Coleman and Deshpande, 1985; Roantree et al., 1977; Wilkinson et al., 1972; Sukupolvi, et al., 1985). Thus, it is

proposed that these strains are putative _rfa_ mutants, with alterations in the lipid A-core of _V. cholerae_ LPS. However, it should be noted that SDS-PAGE failed to reveal any differences in the mobility of the core from these mutants. Perhaps this reflects a fundamental difference in the structure or composition of the _V. cholerae_ core, compared with those of _E. coli_ and _Salmonella_.

The Tn_5_ mutants which map outside _rfb_, showed none of the properties in common with the Tn_2680_ mutants, and were similar to the _rfb_::Tn_5_ strains in protein patterns and cultural characteristics. Conjugational mapping (see Chapter 6) of one of these strains revealed that it did not map at _rfb_, thus it can be concluded that a third locus involved in LPS expression has been identified. Further chemical and genetic analysis will be necessary to determine the genes and functions affected in these strains.

CHAPTER 6

MAPPING OF LPS-ASSOCIATED
GENES IN V.cholerae.

6.   MAPPING OF LPS-ASSOCIATED
       GENES IN V. cholerae

6.1   Introduction

Several markers have already been mapped on the V. cholerae chromosome using P$^+$ cells or Tfr strains as donors (Bhaskaran, 1960; Parker et al., 1979; Johnson and Romig, 1979; Sublett and Romig, 1981; Green et al., 1983; Newland et al., 1984). These data have made it possible to derive a circular genetic map of the V. cholerae chromosome which includes three major linkage groups. The oag locus encoding serotype specificity has been shown to be linked to ilv-1 and map between ilv-1 and arg-1 (Bhaskaran, 1960; Parker et al., 1979).

This chapter describes detailed mapping of the rfb region of V. cholerae, using strains which have transposon insertions in the regions associated with LPS biosynthesis. The aim of this section was to determine whether the rfb region, which has been characterized in the previous chapters, corresponds to the oag locus described by Bhaskaran (1960).

6.2   Mapping rfb-Tfr system

Preliminary mapping of the rfb region was attempted using the Tfr system of Green et al. (1983), using V519 as the donor (Table 6.1). Conjugants were selected as arg$^+$ recombinants and screened for transfer of unselected markers. No transfer of rfb to the recipient strain was

observed (Table 6.2) and analysis of V519 indicated that this strain was resistant to CP-T1. Southern hybridization using pEVX7 as the probe on the donor strain, V519, showed that the HindIII restriction pattern was identical to control smooth V. cholerae strains, thus there is no major insertion or deletion in the rfb region of V519. It is therefore not possible at this stage to determine the reason for the lack of transfer in the rfb region by the strain V519. Due to the failure of the Tfr system of conjugation in mapping rfb, it was proposed that the P-factor of V. cholerae be used to mobilize chromosomal DNA, as described in the following section.

Table 6.1

Donor	Biotype	Genotype	Source
V519	E1 Tor	Spc-201, pro::Tn5-201	R.K. Holmes
V689	Classical	rfb-I::Tn5-8, P::Tn3 Str	This study
V700	E1 Tor	rfb-O::Tn5-4, P::Tn3 Str	This study
Recipient			
V688	E1 Tor	rfb-O::Tn10	This study
V692	Classical	ilv-1, arg-1, his-1 Spc Rif Ogawa	This study

Table 6.2

| Selected Marker | Linkage to | Linkage frequency | | R.D.[a] |
		Number	Fraction	
arg-1	ilv-1	47	0.094	2.36
	his-1	0	–	–
Total: 500	tc^R	0	–	–

a) R.D. = ln $(\frac{1}{L.F.})$,

where R.D. represents relative distance and L.F., linkage frequency. Linkage frequency is defined as the ratio of unselected donor markers to selected donor markers (from Parker et al., 1979).

6.3 Mapping rfb-P$^+$ system

In order to map the rfb locus relative to the ilv-1 and arg-1 loci, a series of matings was performed using V689 (rfb-I::Tn5-7), which was converted to a donor by the introduction of the P-factor (carrying Tn3). This made it possible to screen for the transfer of rfb by plating recombinants onto media containing km. The recipient strain (V692) was auxotrophic (ilv-1, arg-1, his-1) and was Spc^R and Rif^R (Table 6.1).

Recombinants were plated on minimal medium with arginine and histidine, to select ilv$^+$ cells, and then screened for various unselected markers (Table 6.3).

Recombinants which were km^R were assumed to have incorporated the donor rfb locus. This could be confirmed by screening the recombinants for resistance to the

choleraphage VcII. As all km^R recombinants were also VcII resistant, it can be concluded that transfer of Tn5 to the recipient can be correlated with transfer of the donor rfb region.

From Table 6.3 it can be seen that the rfb locus is linked to ilv-1 and maps at a R.D. of 0.79 from this gene. The three-factor cross analysis enables a gene order to be established for ilv-1, arg-1 and rfb (Table 6.4). The number of recombinants expressing only ilv-1 and arg-1 is low compared with those classes which have either ilv-1 or all three markers. Thus, it can be concluded that rfb maps between ilv-1 and arg-1.

The rfb locus was also mapped relative to the arg-1 gene. Recombinants were initially selected on minimal glucose plates containing isoleucine-valine and histidine. Donors were counterselected with rifampicin. A total of 1,106 recombinants were scored (Table 6.5). rfb is linked to arg at a R.D. of 0.52, which is very similar to the result obtained when ilv$^+$ recombinants were analysed. Thus, it can be concluded that rfb maps at the same distance from arg and ilv.

Three-factor cross analysis of the data from Table 6.5 was performed to check the order of the genes ilv, arg and rfb (Table 6.6). The recombinant class, arg rfb, was more frequent than the two classes which included ilv. Thus it can be concluded that, in agreement with the data obtained with the ilv$^+$ recombinants, rfb maps between arg and ilv.

Table 6.3

| Selected Marker | Linkage to | Linkage frequency | | R.D.[a] |
		Number	Fraction	
ilv-1	arg-1	34	0.028	3.57
	his-1	21	0.017	4.07
Total: 1,200	Str	199	0.165	1.8
	km^R(rfb::Tn5)	542	0.45	0.79

a) R.D. $= \ln \left(\dfrac{1}{\text{L.F.}}\right)$,

where R.D. represents relative distance and L.F., linkage frequency. Linkage frequency is defined as the ratio of unselected donor markers to selected donor markers (from Parker et al., 1979).

Table 6.4

Selection	Counter selection	Recombinant class	Number
		ilv-1	441
ilv-1	Spc^R	ilv-1, arg-1	3
Total: 1,200		ilv-1, rfb	505
		ilv-1, arg-1, rfb	31
		ilv-1, his-1	10
		ilv-1, arg-1, his-1	11
		ilv-1, str-1	171
		ilv-1, str-1, arg-1	28

Table 6.5

| Selected Marker | Linkage to | Linkage frequency | | R.D.[a] |
		Number	Fraction	
arg-1	ilv-1	139	0.125	2.08
	his-1	49	0.044	3.12
Total: 1,106	Spc^R	726	0.656	0.42
	Str^R	113	0.102	2.28
	km^R	652	0.589	0.52

a) $R.D. = \ln\left(\frac{1}{L.F.}\right)$,

where R.D. represents relative distance and L.F., linkage frequency. Linkage frequency is defined as the ratio of unselected donor markers to selected donor markers (from Parker et al., 1979).

Table 6.6

Selection	Counter selection	Recombinant class	Number
arg-1	rif^R	arg-1	269
		arg-1, ilv-1	9
Total: 1,106		arg-1, rfb	529
		arg-1, ilv-1, rfb	137
		arg-1, his-1	18
		arg-1, his-1, ilv-1	31
		arg-1, Str	4
		arg-1, Str, ilv-1	109

The final series of experiments involved the use of the strain V671 as a donor. This Tn5 mutant has been shown to map outside the rfb region by Southern hybridization analysis (Chapter 5). Thus, conjugational mapping was used to determine the chromosomal location of the Tn5 insertion in this strain. Table 6.7 shows the R.D. data obtained when recombinants were selected for arg or ilv.

Table 6.7

| Selected Marker | Linkage to | Linkage frequency | | R.D.[a] |
		Number	Fraction	
arg-1	ilv-1	3	0.04	3.2
	his-1	1	0.013	4.3
Total: 75	km^R	14	0.18	1.71
ilv-1	arg-1	30	0.16	1.83
	his-1	1	0.005	5.2
Total: 179	km^R	148	0.82	0.19

a) $R.D. = \ln \left(\dfrac{1}{L.F.} \right)$,

where R.D. represents relative distance and L.F., linkage frequency. Linkage frequency is defined as the ratio of unselected donor markers to selected donor markers (from Parker et al., 1979).

Thus, it can be seen that Tn5 is linked to ilv-1 at a R.D. of 0.19, but at a R.D. of 1.71 to arg-1, and so maps closer to ilv-1. The three-factor cross analysis (Tables 6.8 and 6.9) indicates that the recombinant class ilv-1, km^R was more frequent than the other classes, whereas

Table 6.8

Selection	Counter selection	Recombinant class	Number
arg-1	rif^R	arg-1	58
		arg-1, ilv-1	2
Total: 75		arg-1, his-1	1
		arg-1, ilv-1, his-1	0
		arg-1, km^R	10
		arg-1, ilv-1, km^R	4
		arg-1, his-1, km^R	0

Table 6.9

Selection	Counter selection	Recombinant class	Number
ilv-1	Spc^R	ilv-1	0
		ilv-1, arg-1	6
Total: 179		ilv-1, his-1	0
		ilv-1, arg-1, his-1	25
		ilv-1, km^R	106
		ilv-1, arg-1, km^R	42
		ilv-1, his-1, km^R	0

arg-1, km^R recombinants occurred at a lower frequency than arg-1 recombinants. It can therefore be concluded that Tn5 maps close to ilv-1 in V671, but is not located between ilv-1 and arg-1.

The data from these experiments are summarized in a linear map of part of the V. cholerae chromosome (Fig 6.1).

6.4   Conclusion

This chapter has described the mapping of the rfb locus of V. cholerae using P$^+$ donor strains to mobilize chromosomal DNA. The rfb locus has been mapped with respect to both arg-1 and ilv-1 using linkage data and also three-factor cross analysis. From Fig. 6.1 it can be seen that rfb maps between ilv-1 and arg-1 and is more closely linked to arg-1 than to ilv-1. The order of genes mapped in these experiments agrees with that obtained by earlier workers (Bhaskaran, 1960; Parker et al., 1971, 1979). The relative distances, however, differ considerably from those described in previous publications.

As the experiments described in this chapter were performed with a P$^+$ derivative of strain 569B, it is possible that such variations are due simply to differences between this strain and those used by Bhaskaran (1960) and Parker et al. (1971, 1979).

In addition to precisely mapping the rfb locus on the V. cholerae chromosome, it has also been possible to conclude from these results that rfb is the oag region defined by Bhaskaran (1960), and originally associated

Figure 6.1: A genetic map of the V. cholerae 01 chromosome showing the linkage of rfb to ilv-1 and arg-1. Values represent relative distances (R.D.) calculated from Tables 6.2, 6.4 and 6.6. The location of the non-rfb mutant is indicated by an asterisk.
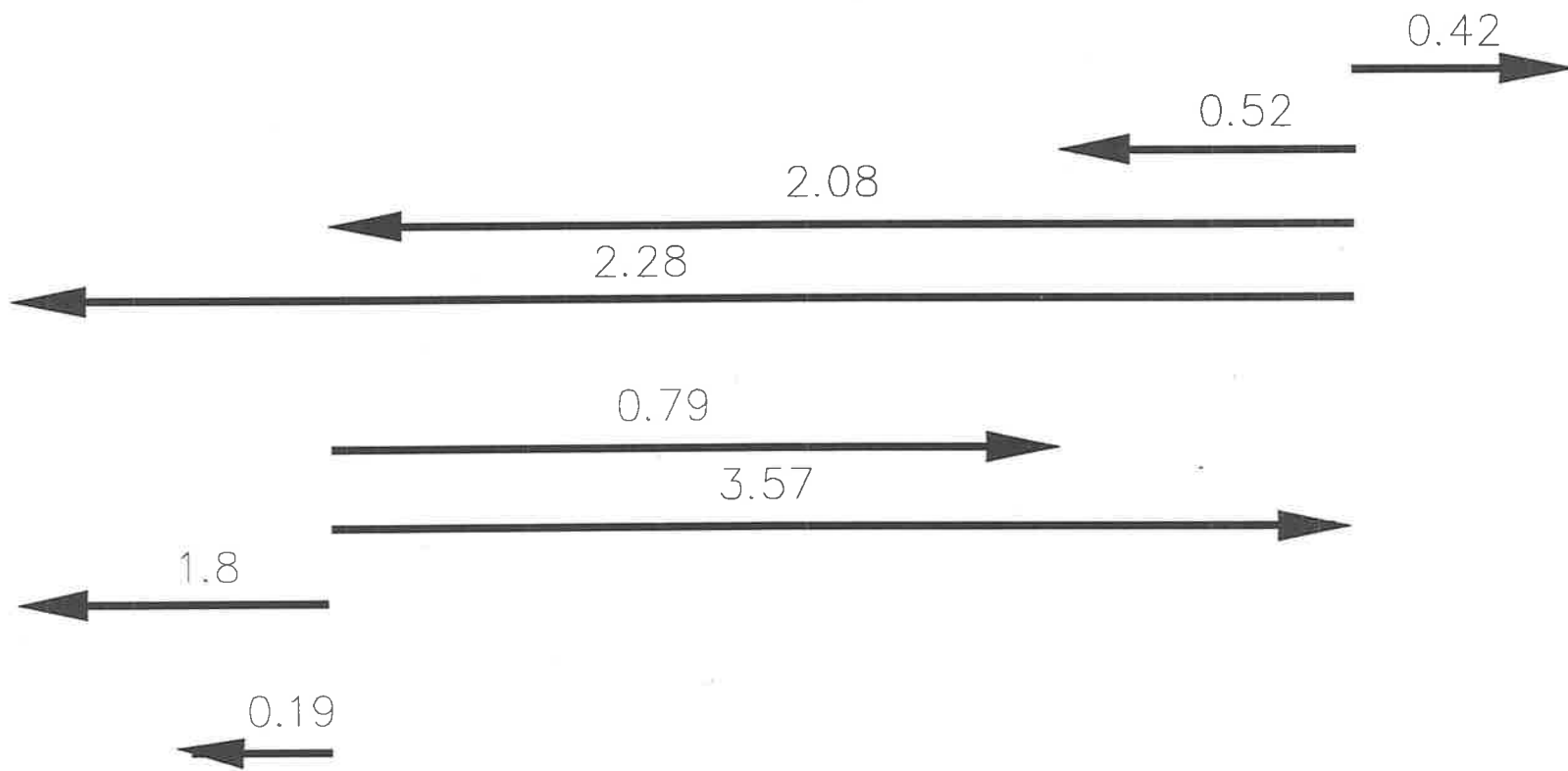
with serotype-specificity of V. cholerae LPS. The rfb
locus described in this chapter includes the chromosomal
region sufficient and necessary to encode V. cholerae
O-antigen production in a heterologous host such as E.
coliK-12 (Manning et al., 1986).

This rfb region has been transferred to recipient V.
cholerae strains by conjugation. As rfb maps in the same
chromosomal region as oag, it can be concluded that they
represent the same locus, which includes the DNA necessary
to encode O-antigen biosynthesis and determine the sero-
type specificity of V. cholerae LPS. Thus, it has been
shown that both rfb-I and rfb-O map in the same region on
the V. cholerae chromosome and thus the loci encoding
serotype specificity are equivalent to the oag locus
defined by Bhaskaran (1960).

Another locus separate from rfb, which is involved in
O-antigen expression in V. cholerae, has been identified.
This locus maps near ilv-1.

CHAPTER 7

DISCUSSION.

7.   DISCUSSION

7.1   Introduction

The LPS of $\underline{V}$. cholerae 01 has been characterized at the chemical level, but no detailed structure for either the O-antigen or the core oligosaccharide has yet been defined (Kenne $\underline{et}$ $\underline{al}$., 1979; Redmond, 1975, 1978, 1979). Three serotypes have been identified which are associated with the $\underline{V}$. cholerae O-antigen and a locus $\underline{oag}$ encoding serotype specificity has been mapped on the chromosome (Bhaskaran, 1958, 1959; Parker $\underline{et}$ $\underline{al}$., 1971, 1979). In this study, the $\underline{rfb}$ region of $\underline{V}$. cholerae 01 has been characterized at the physical and genetic level. The two major serotypes have been analysed in a number of ways, including HIA, immunoelectron microscopy and Southern hybridization. A series of transposon insertion mutants have been isolated which map within the $\underline{rfb}$ region and the LPS and outer membrane proteins of such mutants have been characterized.

These $\underline{rfb}$::Tn$\underline{5}$ strains have been used as donors in P-mediated conjugation to map the $\underline{rfb}$ locus on the $\underline{V}$. cholerae chromosome. A series of mutants have also been isolated which appear to map outside $\underline{rfb}$. These have been analysed and define at least one other locus associated with LPS biosynthesis, distinct from $\underline{rfb}$.

7.2   Chromosomal organization of rfb genes

The use of cloned rfb genes as probes in Southern hybridizations has allowed the chromosomal organization of the rfb region in V. cholerae 01 to be determined. It was shown that the large inverted repeats present in one of these clones, pPM1001, were an artefact, as such an arrangement could not be detected in total genomic DNA from V. cholerae 01. It is difficult to speculate on how such an artefact could arise in the recA background used in construction of the cosmid banks. Similarly, the fact that all the rfb plasmids include two copies of the cloning vector pHC79, has not yet been satisfactorily explained, however, such an arrangement does lead to a reduction in the copy number of the cosmid clone. This suggests that a high gene dosage is detrimental to the cell and, certainly, V. cholerae derivatives harbouring cloned rfb DNA are very unstable. It was noted from immuno-gold studies that the clones exhibited variability in the amount of probe bound. This may reflect the instability of the O-antigen plasmids, as some cells may have lost the plasmid and so are no longer expressing V. cholerae O-antigen.

The Ogawa plasmids, pPM1002 and pPM1003 were used to analyse the extent of contiguous DNA in the V. cholerae 01 chromosome. Thus, the region extending from the SalI site at the left end of pPM1002 through to the SstI site at the right end of pPM1003 represents a contiguous length of DNA in the V. cholerae Inaba and Ogawa chromosomes (Fig. 7.1).

Figure 7.1: Restriction map of the <u>V</u>. <u>cholerae</u> <u>rfb</u> region, showing locations of Tn<u>5</u> insertions, (▲) and proposed RNA Polymerase binding sites (✱).

CHROMOSOME

RNA polymerase binding sites

pPM1001

pPM1002

pPM1003

pEVX7

Tn5 insertions

1 kb
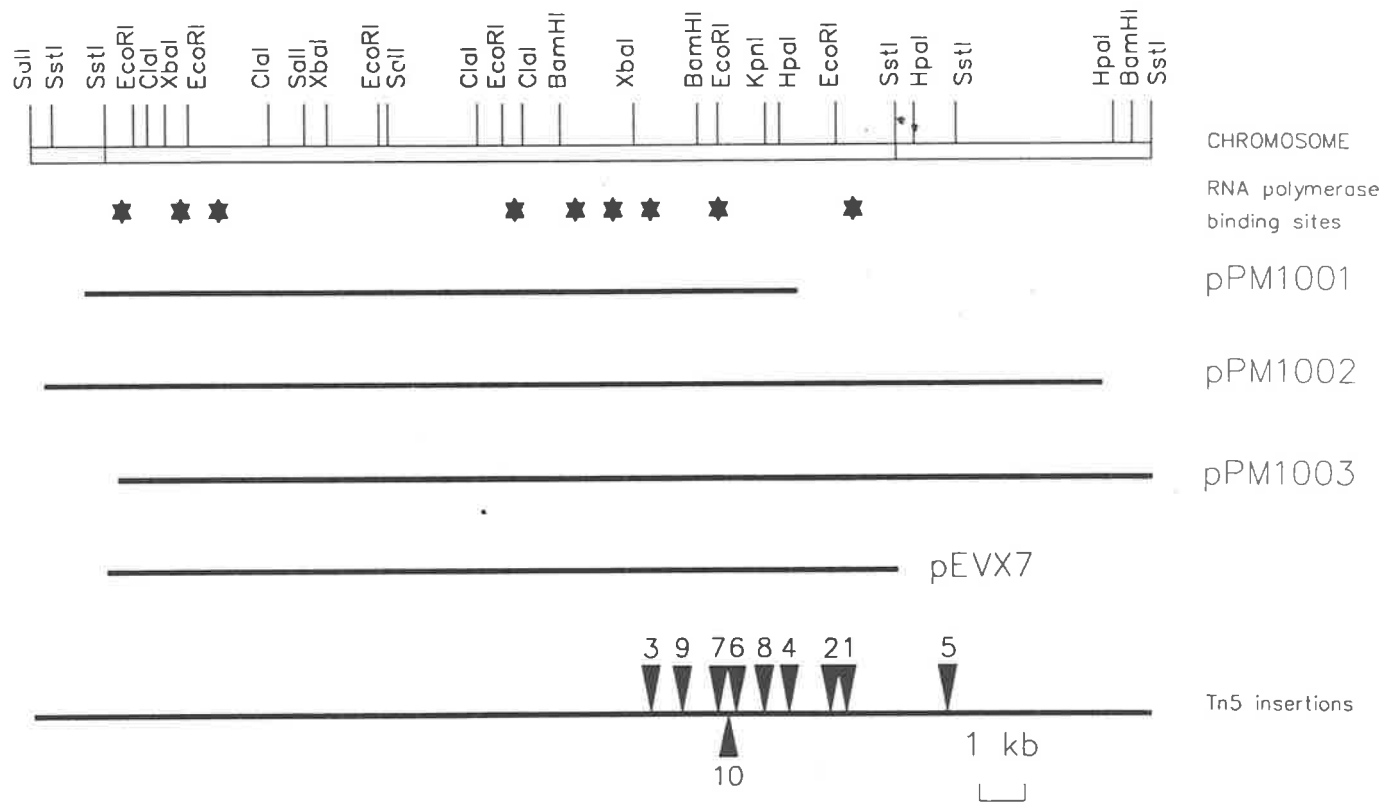
Heteroduplex analysis of the various rfb clones has allowed the extent of DNA common to all three plasmids to be precisely defined and enhanced the results obtained by Southern hybridization analysis.

Characterization of the rfb clones with McAbs revealed that O-antigen of the appropriate serotype was being produced. This indicates that in addition to the genes necessary to synthesize the A antigen, those for the serotype-specific B and C antigens have also been cloned. Studies of O-antigen expression encoded on the 19.5kb SstI fragment of pPM1002 (and pPM1003) indicated that this region is sufficient to encode synthesis of V. cholerae 01 Ogawa O-antigen in E. coliK-12. Since the amount of DNA required for Inaba biosynthesis in pPM1001 is less than this, then the genes for the Inaba O-antigen must also reside in this fragment although they are not necessarily expressed. From the endpoints of the cloned DNA in the various plasmids which express V. cholerae O-antigen in E. coliK-12, and deletions which eliminate expression (Manning et al., 1986), it can be concluded that at least 16kb of the DNA contained within this minimal SstI fragment is required for O-antigen biosynthesis. This is sufficient to encode 15 average sized genes.

From the chemical analyses of V. cholerae LPS, it is possible to propose a potential pathway for O-antigen biosynthesis and so suggest enzymes which are likely to be involved. The major component of V. cholerae O-antigen, perosamine ($4-NH_2-4,6$-dideoxy-mannose) is probably initial-

ly synthesized from glucose. This could occur via a fructose intermediate as postulated by Gabriel (1987) or epimerization of glucose to form mannose. Production of 4-keto-6-deoxy-mannose would require an oxide-reductase enzyme. A series of oxidoreductases involved in biosynthesis of 3,6-dideoxy sugars have been characterized in Yersinia pseudotuberculosis and S. typhimurium. The rfbG product, which converts glucose to 4-keto-6-deoxy-D-glucose has a M_r of 40,000 (Matsuhashi, 1966; Rubenstein and Strominger, 1974). Thus, a gene of $\simeq$ 1.2kb would be required to encode this enzyme if a similar product is present in the V. cholerae rfb region. The final step in the postulated pathway for perosamine synthesis would involve reductive animation at C-4 to produce perosamine (Fig. 7.2). Although it could be proposed that at least three genes are necessary for this series of reactions, it should be noted that some steps, particularly the initial epimerization reaction, may form part of other housekeeping processes in the cell (such as peptidoglycan and capsule synthesis) and so map outside rfb. Such a situation has been noted in S. typhimurium in which only the genes which complete synthesis of the nucleoside precursors of the O-antigen actually map within rfb (Jann and Jann, 1984; Rick, 1987).

The production of the purported Ogawa specific sugar, 4-HN$_2$-4-deoxy-L-arabinose (Redmond, 1978), can also be derived from glucose (Fig. 7.3). Such a pathway would involve conversion of glucose to glucuronic acid, presumably catalysed by a UDP-glucose dehydrogenase. This

Figure 7.2:   Possible sugar biosynthesis pathway for perosamine.
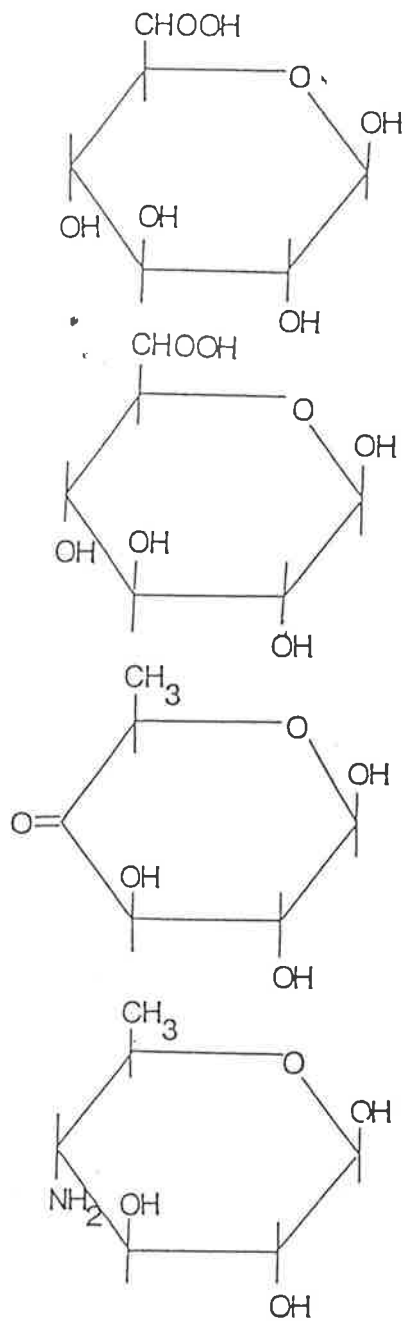
GLUCOSE

↓

MANNOSE
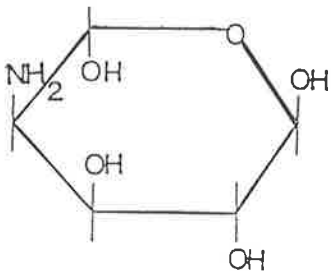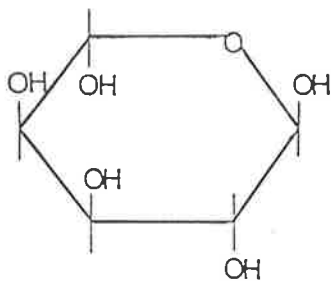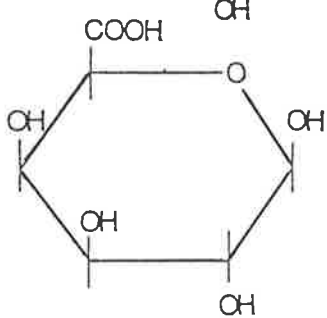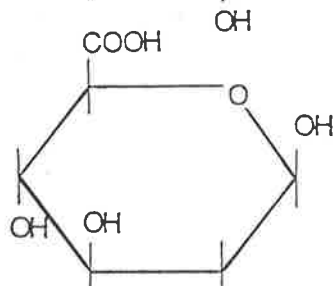
↓

4-KETO 4-DEOXY MANNOSE

↓

PEROSAMINE

Figure 7.3: Possible sugar biosynthesis pathway for 4-NH$_2$-4-deoxy-L-arabinose.
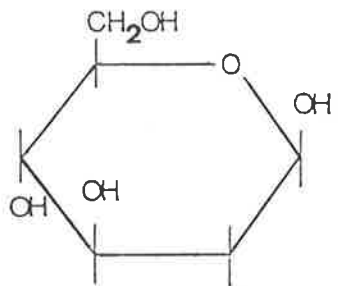
GLUCOSE

GLUCURONIC ACID

GALACTURONIC ACID

4-NH$_2$4-DEOXY
GALACTURONIC ACID

ARABINOSE
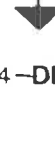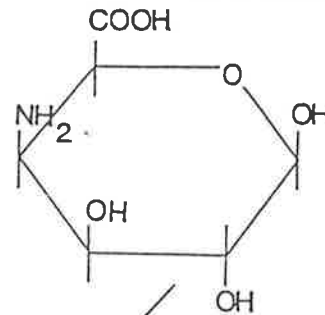
4-NH$_2$-4-DEOXY-L-ARABINOSE

reaction has been characterized in E. coli and a UDP-glucose dehydrogenase of M_r 47,000 has been identified (Gabriel, 1987). Thus, a gene of $\simeq 1.5$kb would be needed to encode this enzyme. The second step in the proposed pathway is epimerization to form galacturonic acid, which may be catalysed by galactose-4-epimerase. This enzyme has been purified from S. typhimurium (Rick, 1987) and has a M_r of 40,000, thus a gene of $\simeq 1.2$kb would be required. However, it should be noted that preliminary testing of V. cholerae strains on tetrazolium indicator plates showed no fermentation of galactose (data not shown), suggesting that V. cholerae may not have galactose epimerase activity. Shibaev (1986) has suggested that $4-NH_2-4$-deoxy-L-arabinose may be formed from D-fructose, thus eliminating the requirement for a galactose epimerase.

The last reaction in the proposed pathway (Fig. 7.3) involves the action of a decarboxylase and then the addition of an amino group at C-4 to produce $4-NH_2-4$-deoxy-L-arabinose. An alternative to these final steps is also shown, in which the amino group is added to galacturonic acid before removal of the COOH group.

The third distinctive amino sugar in V. cholerae O-antigen is quinovosamine ($2-NH_2$,2,6-dideoxy-glucose). Shibaev (1986) has proposed that such monosaccharides may be formed from epimerization of the galactose form, derived from UDP-GlcNAc. This pathway may be similar to that involved in biosynthesis of fucose in S. typhimurium (Gabriel, 1987).

As well as the enzymes necessary to synthesize the various sugars in the V. cholerae O-antigen, the rfb region probably includes genes encoding glycosyltransferases which can assemble these sugars into the O-antigen. As it has been observed that perosamine actually forms a polymer which acts as the backbone of the V. cholerae O-antigen, a perosamine polymerase will be necessary to create the $\alpha 1 \rightarrow 2$ linkages between the perosamine units. In addition the amino groups of this perosamine polymer are acylated with 3-deoxy-L-glycero-tetronic acid, thus an acyl transferase is required.

It is not yet possible to predict the precise amount of DNA required to encode the biosynthetic enzymes in V. cholerae O-antigen production. The synthesis of the various amino sugars may involve a number of operons. However, it is difficult to speculate on the number of genes required as studies in S. typhimurium and Y. pseudotuberculosis have shown that some sugars (e.g., galactose) are mainly encoded by genes outside rfb, whereas all the biosynthetic functions needed for rhamnose and abequose are encoded by rfb genes (Nikaido et al., 1967; Matsuhashi and Strominger, 1967). Chemical analysis of the LPS of V. cholerae strains and the various Tn5 and Tn2680 insertion mutants will be necessary in order to determine the number of discrete genes and operons involved in the V. cholerae rfb region.

Restriction analysis and Southern DNA hybridizations have so far failed to detect any differences at the

genomic level, between serotypes. Thus, it would appear that there are no major DNA rearrangements which distinguish the V. cholerae 01 Inaba and Ogawa serotypes. Studies of co-expression of plasmids containing the Inaba and Ogawa genes have shown that both the B and C antigens can be expressed in the same cell. This codominance effect indicates that, under these conditions, the Ogawa and Inaba plasmids have no inhibitory effect upon O-antigen expression of the heterologous serotype. However, it should be noted that the Inaba genes were present on a low copy number plasmid (pSC101), whereas the Ogawa plasmid was based on a high copy number vector, so the observation may be complicated by the effects of different gene dosages and plasmid stabilities. For instance, a repressor which is only present at a very low level may be unable to inhibit expression of a particular gene if that gene were multicopy.

Thus, although it has been demonstrated that the genes encoding the biosynthesis of the serotype-specific antigens lie within the minimal SstI fragment, and are expressed as the specific serotype, the genes responsible for switching between serotypes may map outside this region. To resolve this, it may be necessary to sequence the Ogawa and Inaba rfb genes in order to determine the precise nature of the difference between these serotypes. It may then be possible to predict whether the genes mediating the serotype switching have been cloned.

7.3   Isolation and characterization
               of Tn5 insertions in rfb

The demonstration that VcII, like CP-T1 (Guidolin and Manning, 1985), uses the V. cholerae 01 O-antigen as its receptor, has enabled resistance to this phage to be used as a direct selection for mutants in LPS biosynthesis. Tn5 mutagenesis, followed by selection for either CP-T1 or VcII resistance has allowed the isolation of a series of independent mutants which no longer express complete LPS on their surface.

The sites of Tn5 insertion have been mapped by Southern hybridization analysis and the majority map in the right end of the 19.5kb SstI fragment (Fig. 7.1). Possibly transposon insertions in the left end of the SstI piece are lethal and so would not be detected. If, for instance, intermediates in LPS biosynthesis remained bound to ACL (undecaprenol phosphate) in the cytoplasmic membrane, this would eventually block recycling of ACL. This, in turn, would prevent peptidoglycan synthesis, which also relies on the ACL for assembly of the dimer units, and so cell lysis would result. Thus, it is proposed that the left end of the cloned DNA includes genes responsible for assembly of O-antigen subunits onto ACL, whereas the right end contains those genes necessary to synthesize the various sugars needed. Mutations in the sugar biosynthesis genes are unlikely to be lethal since ACL cannot be occupied if the sugar is not present. However, the enzyme responsible for loading the first

sugar onto the ACL could also map on the right, since such a mutant could also not lead to the ACL being occupied.

It should be noted that Tn1725 mutagenesis of the cloned V. cholerae 01 rfb region has recently been performed and three insertions have been mapped at the left end of the SstI fragment near the second ClaI site (M. Brown, personal communication). Thus, insertions in this region may not be lethal when the rfb genes are in E. coliK-12, as some rfb functions may be provided by the host. A similar observation was made in Sh. dysenteriae, in which transposon mutagenesis of particular determinants had no effect on LPS expression in E. coliK-12, but lead to LPS defects in rfb-deleted strains of E. coli (Sturm et al., 1986b).

Several potential promoters within the 19.5kb SstI fragment have been defined by RNA-polymerase binding studies and electron microscopy (G. Morelli, personal communication) (Fig. 7.1). These results suggest that at least five promoters exist, inferring that there are several operons within the rfb region in the 19.5kb SstI fragment (of V. cholerae 01). It would be of interest to correlate the sites of transposon insertion with the various functions and so identify particular rfb genes. Initially this could be performed by chemical analysis of the LPS from the various Tn5 and Tn2680 mutants to determine the sugar composition. Comparison of the LPS of these mutants with that of the parent strain could allow the transposon insertions to be correlated with particular

defects in O-antigen synthesis. Further experiments such as minicell analysis to study the effects of transposon mutagenesis on protein expression can only be performed if the mutagenized rfb region is cloned from the V. cholerae 01 chromosome into a plasmid. This could be accomplished by sub-cloning of the region into one of the mobilizable vector plasmids containing the mob site from RP4 (Simon, 1984) in the appropriate host strain containing the RP4 plasmid integrated into the chromosome, which can provide the transfer functions of RP4 in trans. Thus, the plasmid can be mobilized into V. cholerae. The presence of the rfb genes on both the plasmid and the chromosome would presumably provide sufficient homology for recombination to occur, thus resulting in the presence of the rfb::Tn5 region on the plasmid. It would then be possible to study the effects of the Tn5 insertions on the O-antigen bio-synthesis in another background, such as E. coliK-12.

Analysis of whole membranes has revealed that the various transposon insertion mutants have altered protein patterns, as well as defects in O-antigen expression. Such results are suggestive of interactions between the bio-synthesis of LPS and proteins and their incorporation into the outer membrane.

A number of proteins in E. coliK-12 and S. typhi-murium LT2 have been shown to be dependent upon the presence of LPS for stability or full function in the outer membrane. The OmpA protein, for instance, requires LPS for it to be able to efficiently function as a phage

receptor in E. coli (Nikaido and Vaara, 1985). Similarly, the OmpC protein in E. coliK-12 interacts with LPS to act as the receptor for the bacteriophage T4 (Yu and Mizushima, 1982). Studies of heptose-deficient mutants of E. coliK-12 by van Alphen et al. (1976) revealed that up to three major outer membrane proteins were missing, indicating that these proteins rely on LPS for integration into the membrane. Such studies have shown that LPS plays an important role in the structural integrity of the outer membrane in E. coliK-12 and so mutations affecting LPS also lead to changes in protein composition and membrane permeability.

Analysis of S. typhimurium has shown that LPS mutants lacking the glucose I unit and other deep rough strains were deficient in four of the major outer membrane proteins (33kDal, 34kDal, 35kDal and 36kDal) and at least three of these are known to form aqueous pores in the outer membrane (Ames et al., 1974; Nakae, 1976). It was concluded that these proteins were not covalently linked to LPS, but required LPS to be properly anchored in the outer membrane.

Further studies by Roantree et al. (1977) correlated the results obtained by Ames et al. (1974) with alterations in permeability of the outer membrane of S. typhimurium, thus indicating that LPS mutations lead to major changes in the structure and properties of the outer membrane.

A number of Tn5 mutants isolated here showed altered sensitivity to antibiotics, indicating that the permeability of the outer membrane was altered as a result of the LPS mutations. Such effects have been well characterized in E. coli and S. typhimurium and mechanisms leading to increased sensitivity to hydrophobic chemicals have been proposed. Tamaki et al. (1971) have suggested that a decrease in phosphate in rough E. coliK-12 mutants, affected the cross-linking of the LPS molecules by phosphodiester bridges. Such a possibility has not been investigated in V. cholerae. Methods such as ^{33}P-labelling of LPS could be used to compare the phosphate content of the various mutants and that of the parent strains.

The greater sensitivity of S. typhimurium LPS mutants to hydrophobic antibiotics has been studied by Nikaido (1976) and Roantree et al. (1977).

Nikaido proposed that the loss of some proteins in the outer membrane of S. typhimurium produced localized areas of phospholipid bilayers which allowed hydrophobic molecules to enter the cell. However, in a more recent study (Nikaido and Vaara, 1985), the possibility that strong lateral LPS-LPS interactions prevent penetration by hydrophobic molecules in smooth strains, was also considered. It would be of interest to analyse the outer membrane of the V. cholerae LPS mutants to determine if there are any regions of phospholipid bilayer present.

7.4   Mapping of the rfb region

The transfer of the P factor to rfb::Tn5 mutants has provided donor strains which have enabled the rfb locus to be mapped on the V. cholerae chromosome. This locus was mapped to a region between ilv-1 and arg-1, and it can be concluded that rfb and oag represent the same locus. Thus, the DNA encoding both O-antigen biosynthesis and serotype specificity (in Ogawa and Inaba strains) have been mapped to the one region on the chromosome.

It is interesting to note that rfb in V. cholerae maps in a different region of the chromosome compared with other well-characterized Gram negative bacteria. Thus, the rfb gene cluster maps adjacent to the his operon in S. typhimurium LT2 (Sanderson and Roth, 1983), E. coliK-12 (Ørskov and Ørskov, 1962), and Sh. flexneri (Petrovskaya and Licheva, 1982).

In Sh. dysenteriae, it has been shown that a chromosomal region, mapping near his, and a 9kb plasmid are necessary for O-antigen synthesis (Watanabe and Timmis, 1984; Sturm and Timmis, 1987), whereas in Sh. sonnei, the Form I antigen is completely encoded by a 120MDal plasmid, rather than chromosomal genes (Sansonetti et al., 1981).

Possibly this arrangement reflects a different evolutionary origin for rfb in V. cholerae 01, compared with these bacteria. It should be noted that the species mentioned are all Enterobacteriaceae, as most of the analysis of rfb regions and their location on the chromo-

some has been performed in this group of bacteria. One way of analysing this would be to use the cloned V. cholerae 01 rfb DNA as a probe to detect the degree of homology with rfb regions from other bacterial species. No detectable homology exists to E. coliK-12, however, it would be interesting to compare the V. cholerae 01 rfb region with those from other Vibrionaceae. Initial Southern hybridization analysis has shown there is no homology of V. cholerae rfb with that of Aeromonas hydrophila (data not shown). Comparison with other Vibrionaceae has not yet been performed.

7.5   Characterization of transposon insertions
                which map outside rfb

A series of transposon insertion mutants which did not map within rfb was isolated. Three of these strains contained Tn2680 insertions and were shown to have increased sensitivity to tetracycline and novobiocin compared with the parent, 017. These mutants also exhibited an extremely rough colony morphology on solid media, autoagglutinated readily in liquid cultures and exhibited a distinctive cell envelope protein pattern. Thus, they have a number of the characteristic properties of rfa mutants (Boman and Monner, 1975; Roantree et al., 1977; Coleman and Leive, 1979; Coleman and Deshpande, 1985). However, analysis of the lipid A-core from these strains by SDS-PAGE and chemical analysis of the core sugars indicated no difference in core length or composition compared with the parent V. cholerae strain.

It has been proposed that these strains be classified as putative rfa mutants.

It has not been possible to map other loci affecting O-antigen expression, as all attempts to map the site of Tn2680 insertions by conjugation were unsuccessful. It appears that transfer of P::Tn3 into the strains was successful and that another factor must be responsible for the failure to isolate recombinants. Perhaps the difficulties experienced with conjugational mapping in the Tn2680 strains are due to a mutation which affects both LPS expression and P-factor functions. Such mutations have been described in S. typhimurium LT2 and E. coliK-12. The rfaH gene in S. typhimurium LT2 (which maps at 84 min, some distance from the major rfa gene cluster) leads to production of heterogenous core chemotypes and also affects F-factor function, including the formation of mating aggregates with F^- cells (Sanderson and Stocker, 1981). Similarly, in E. coliK-12 the sfrB mutation results in reduced synthesis of full length LPS and lowered expression of the transfer functions of F (Beutin et al., 1981). It has been proposed that the rfaH and sfrB genes act as transcription anti-terminators for a number of operons encoding cell envelope components (Beutin et al., 1981; Sanderson and Stocker, 1981).

Two Tn5 insertions were isolated which mapped outside the 19.5kb SstI fragment. One of these, V638, was shown to map in an EcoRI fragment outside this region as detected in Southern hybridization analysis using pPM1003 as a probe. These data, combined with that obtained for V644,

indicate that although the 19.5kb fragment is sufficient to encode O-antigen expression in E. coliK-12, some functions are presumably provided by E. coli genes. Thus, the region involved for O-antigen biosynthesis in V. cholerae 01 has been extended at the right end to include at least another 7kb of DNA.

The other Tn5 insertion mutant, V671, was not mapped within rfb at all, yet still exhibited similar properties, such as cell envelope patterns, to the Tn5 mutants. Thus, it can be concluded that a third locus affecting O-antigen expression has been identified.

7.6   Immuno-gold electron microscopic studies of V. cholerae serotype antigens

McAbs directed against serotype-specific antigens have been used to analyse the distribution of the A, B and C antigens on V. cholerae strains and E. coliK-12 harbouring the O-antigen clones. Thus, it was shown that both Ogawa and Inaba cells express A antigen, but the clones showed very poor expression of A. Analysis of the B antigen revealed that B is exclusively expressed on Ogawa cells. Electron micrographs of the clones revealed that some cells of the Ogawa clones, V399 and V455, expressed high amounts of B while other cells had virtually no probe bound, thus indicating that some cells may have lost the plasmid. Finally, expression of C antigen was studied and it was shown that significantly more C is expressed on Inaba cells compared with Ogawa. These results also revealed that C antigen is expressed by virtually all the

cells of the Ogawa strain CA411. Thus, the presence of C in Ogawa LPS is not due to a subpopulation of Inaba cells. This result has implications for the mechanism of sero-conversion in V. cholerae, as it effectively rules out theories of antigenic switching which are based on a pre-existing subpopulation of Inaba cells, within an Ogawa strain. An alternative mechanism may involve the loss of a particular determinant, B, in the switch from Ogawa to Inaba. This may lead to unmasking of the C antigen on the cell surface, if B actually consists of a modification of the C antigen (such as an O-acetyl group). Such a theory could explain the low level of C antigen present on Ogawa cells, as not all the C may be modified to produce B. It will be necessary to undertake detailed chemical analyses of V. cholerae strains and the various Tn5 mutants before this theory can be tested.

It is not known why expression of the serotype antigens, particularly A and C, was very poor on most of the O-antigen clones examined. Possibly O-antigen is synthesized by the cells, but not efficiently translocated to the outer membrane in E. coliK-12. It should be noted that assays of serotype antigen expression were done using isolated LPS or whole membrane preparations, thus O-antigenic material in the periplasm or cytoplasm would have been assayed, as well as complete LPS molecules. Analysis of sonicated cells with immuno-gold electron microscopy may reveal whether or not significant levels of V. cholerae O-antigen are associated with other cellular locations in the E. coliK-12 clones.

One final point to be noted from the immuno-gold labelling of V. cholerae is the labelling of the flagellar sheath which was observed with the McAb directed against C. This confirmed the presence of LPS antigenic determinants on the flagellar sheath, which was reported recently using similar methods to those described in Chapter 3 (Fuerst and Perry, 1988). The inability to detect flagellar antigens with the other McAbs has not yet been satisfactorily explained.

7.7   Concluding remarks

This study has described the characterization of the rfb region of V. cholerae 01. A series of transposon insertions in these regions have been isolated which affect O-antigen expression. The use of these strains as donors in conjugations has allowed the rfb region to be mapped on the V. cholerae chromosome. From the location of this region and the properties of the cloned genes it can be concluded that rfb is equivalent to oag and so the regions encoding O-antigen expression and serotype specificity have been mapped to the same location. This is also the region where serotype variation or conversion must occur.

A number of transposon insertion mutants have been identified which map outside rfb. Some of these, containing Tn2680 mutations, have characteristics associated with rfa mutants in other bacteria. Thus, it has been possible to identify at least one other locus apart from rfb, which is involved in LPS expression.

The different V. cholerae serotypes have been characterized in a number of ways. The chromosomal DNA of the serotypes has been compared by Southern hybridization and heteroduplex studies with no differences being detected. However, as the cloned rfb DNA encodes the expression of the A, B and C antigens it would be predicted that some difference will be present in the DNA. Possibly a small region, which has not been detected by the methods used, is involved. Analysis of smaller subcloned fragments using restriction enzymes with 4bp cleavage specificity may detect such small changes in the DNA.

The use of McAbs has allowed characterization of the serotype antigens of V. cholerae 01, in particular their distribution on individual cells. It was shown by immunogold electron microscopy that all Ogawa cells contain a small amount of C antigen on their surface, whereas Inaba cells express significantly more C antigen. This result does not correlate with theories of antigenic conversion which propose a subpopulation of Inaba cells within the Ogawa cell population. Further analysis of V. cholerae strains and Tn5 and Tn2680 mutants will enable the basis for seroconversion to be determined.

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APPENDIX.

APPENDIX

Publications

Material contained in this thesis has been published and accepted for publication in the following papers:

Ward, H.M., Morelli, G., Kamke, M., Morona, R., Yeadon, J., Hackett, J.A. and Manning, P.A. (1987) A physical map of the chromosomal region determining O-antigen biosynthesis in Vibrio cholerae 01. Gene 55, pp.197-204.

Ward, H.M. and Manning, P.A. (1988) Mapping of chromosomal loci associated with lipopolysaccharide synthesis and serotype specificity in Vibrio cholerae 01 by transposon mutagenesis using Tn 5 and Tn 2680. Submitted for publication.

Ward, H.M., Thomas, C. and Manning, P.A. (1988) Analysis of serotype specific antigens on Vibrio cholerae using immuno-gold electron microscopy. Manuscript in preparation.