The Role of the Tol import System in the Surface Expression of O7-Specific Lipopolysaccharide

by

Joe Albert Gaspar
Department of Microbiology and Immunology

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Abstract

The TolA protein is a major component of the Tol import system involved in the internalisation of bacteriocins and bacteriophages across the outer membrane. We present evidence in this work that the TolA protein is also involved in governing the surface expression of O-specific polysaccharide. *tolA* and *tolQ* mutants of *Escherichia coli* K-12 showed a reduced O7 LPS expression directed by the plasmid pJHCV32. We ruled out a direct effect of TolA in the transcription of O7 LPS genes and demonstrated an accumulation of O7 LPS precursors in the inner membrane of a *tolA* mutant. *tolQ* and *tolA* mutants constructed in the wild-type *E.coli* O7 strain VW187 displayed a reduced growth rate and dramatic morphological changes suggesting defects in the formation of cell wall and septation. These morphological changes disappeared upon complementation with cloned *tol* genes or disruption of the O7 subunit biosynthesis genes.

Keywords: Tol proteins, lipopolysaccharide, outer membrane, export, *Escherichia coli*.

Dedication

To my loving parents, Jose and Isabel Gaspar, for their support and encouragement over the years.

To the late Dr. John A. Thomas who laid out the initial ground work for this project. I never met John personally, but through the continuation of his work and through the stories relayed of him by others, I got to know him indirectly and consider him a friend.

Acknowledgement

The accumulation of work presented in this thesis is a direct reflection of the contribution of others throughout my studies within the Department of Microbiology and Immunology. I would like to thank my supervisor, Dr. Miguel Valvano, for the encouragement, advice and enthusiasm he has shown throughout the course of my project. I have appreciated the technical advice and patience from Cristina Marolda which proved to be invaluable. I would also like to acknowledge other members of our laboratory: Lydia Dafoe, Joanna Brooke, Lalit Singh, Amal Amer and all the undergraduate students that have passed through our laboratory for their friendship and encouragement over the years.

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List of Abbreviations

Ap^R	ampicillin resistance
ATP	adenosine 5'-triphosphate
bp	base pair
Ci	Curie
Cm^R	chloramphenicol resistance
Da	daltons
DEPC	diethyl pyrocarbonate
DIG-dUTP	digoxigenin-d11-deoxyuridine 5"-triphosphate
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Gal*p*	galactose-1-phophate
GlcNAc	N-acetylglucosamine
KDO	keto-deoxy-octusolanic acid
Km^R	kanamycin resistance
LPS	lipopolysaccharide
PCR	polymerase chain reaction
Rif^R	rifampicin resistance
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sm^R	streptomycin resistance
Spc^R	spectinomycin resistance
Tc^R	tetracycline resistance
Tris	tris(hydroxymethyl)aminomethane
U	enzymatic units
Und-P	undecaprenol phosphate (bactoprenyl lipid)

Chapter One

Introduction

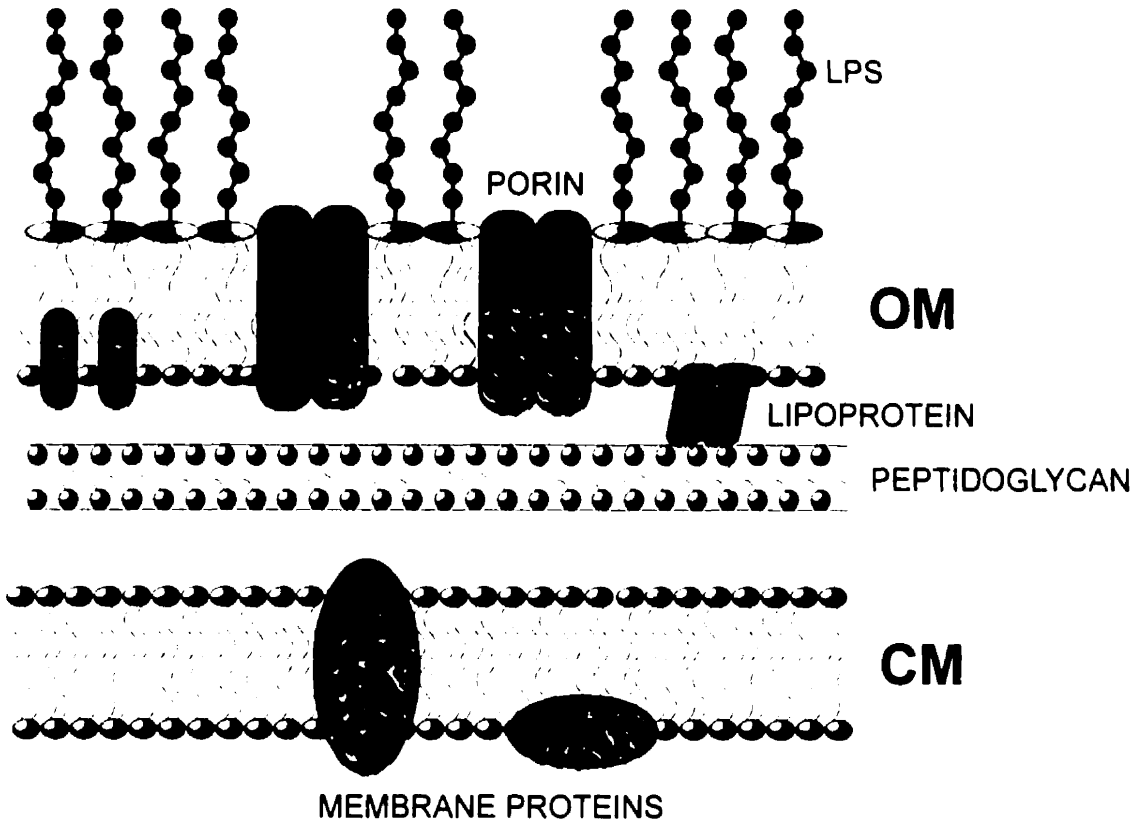
1.1 The Cell Envelope of Gram-Negative Bacteria

Gram-negative bacteria possess a unique envelope consisting of a cytoplasmic membrane surrounded by a peptidoglycan layer and an outer membrane (Lugtenberg and Van Alphen, 1983; Nikaido and Vaara, 1985). The structure of a gram-negative bacterial cell envelope is illustrated in Figure 1.

The cytoplasmic membrane consists of equivalent amounts of both phospholipids and proteins. Although phospholipid composition varies between different strains and is dependent on growth conditions, the cytoplasmic membrane is composed primarily of the three major phospholipids species: phosphatidylethanolamine (70-80%), phosphatidylglycerol (15-25%) and cardiolipin (5-10%) (Kadner, 1996). The cytoplasmic membrane possesses enzyme complexes necessary for nutrient transport, oxidative phosphorylation and electron transfer. It is also the site of

Figure 1. Gram negative bacterial cell envelope of *Escherichia coli.*

The outer leaflet of the outer membrane is composed primarily of lipopolysaccharide. The solid circles represent polymerised O-antigen subunits that are anchored to the outer membrane via the lipidA-core moiety. Abbreviations used are: LPS, lipopolysaccharide; OM outer membrane; CM, cytoplasmic membrane.


LPS
PORIN
OM
LIPOPROTEIN
PEPTIDOGLYCAN
CM
MEMBRANE PROTEINS

biosynthesis of many envelope components such as phospholipids, lipopolysaccharides and both membrane-bound and periplasmic proteins.

A peptidoglycan (murein) monolayer surrounds the cytoplasmic membrane. It is composed of alternating β(1→4) linked N-acetylglucosamine and N-acetylmuramic acid residues that are in turn cross-linked by an amide bond to a small peptide. This structure forms a rigid network which enables the cell to maintain its shape and withstand cytosolic osmotic pressure. The peptidoglycan layer is associated to the outer membrane via peptidoglycan associated lipoproteins which are anchored to the inner leaflet of the outer membrane and both covalently and non-covalently attached to the peptidoglycan.

The periplasmic space is located between the cytoplasmic and outer membranes and is filled with oligosaccharides and proteins. These oligosaccharides are composed of approximately 9 glucose residues and are involved in osmoregulation. The proteins can be divided into the following five classes based on their function: (i) catabolic enzymes that convert nutrients for transport across the cytoplasmic membrane, (ii) high affinity binding proteins essential for nutrients transport, (iii) proteins required for the degradation or modification of deleterious compounds such as heavy metals and antibiotics, (iv) proteins assisting in the process of protein secretion and/or protein folding of secreted proteins, and (v) a variety of proteases.

The outer membrane acts as a functional and physical barrier preventing the leakage of periplasmic proteins and protecting the cell from potentially harmful agents. The outer membrane is composed primarily of integral membrane proteins, phospholipids on the periplasmic leaflet, lipopolysaccharide (LPS) on the extracellular leaflet and enterobacterial common antigen (ECA) on the cell surface. ECA is an acidic polysaccharide that is anchored to the outer membrane by a single covalent bond to either phospholipids (ECA_{PG}, some *E.coli* group-II K-antigens) or lipidA-core (ECA_{LPS}, some *E.coli* group-I K-antigens) (Whitfield and Valvano, 1993).

1.2 Contribution of LPS to Properties of the Outer Membrane

Lipopolysaccharide (LPS) is an amphipathic glycosylated lipid which forms the outer leaflet of the outer membrane in gram negative bacteria. LPS is comprised of 3 components: lipidA, core and O-antigen. The lipidA-core regions are highly conserved among *Enterobacteriaceae* while the O-antigen is subject to a high degree of variability (Lugtenberg and Van Alphen, 1983).

LipidA is a polar lipid consisting of six to seven saturated fatty acid residues substituted on a glucosaminyl-β-(1→6) glucosamine backbone (Nikaido, 1996). Interactions between adjacent fatty acyl chains of lipidA anchor the LPS molecule to the outer membrane. The tightly packed saturated hydrocarbon chains and the large ratio of hydrocarbon chains to a

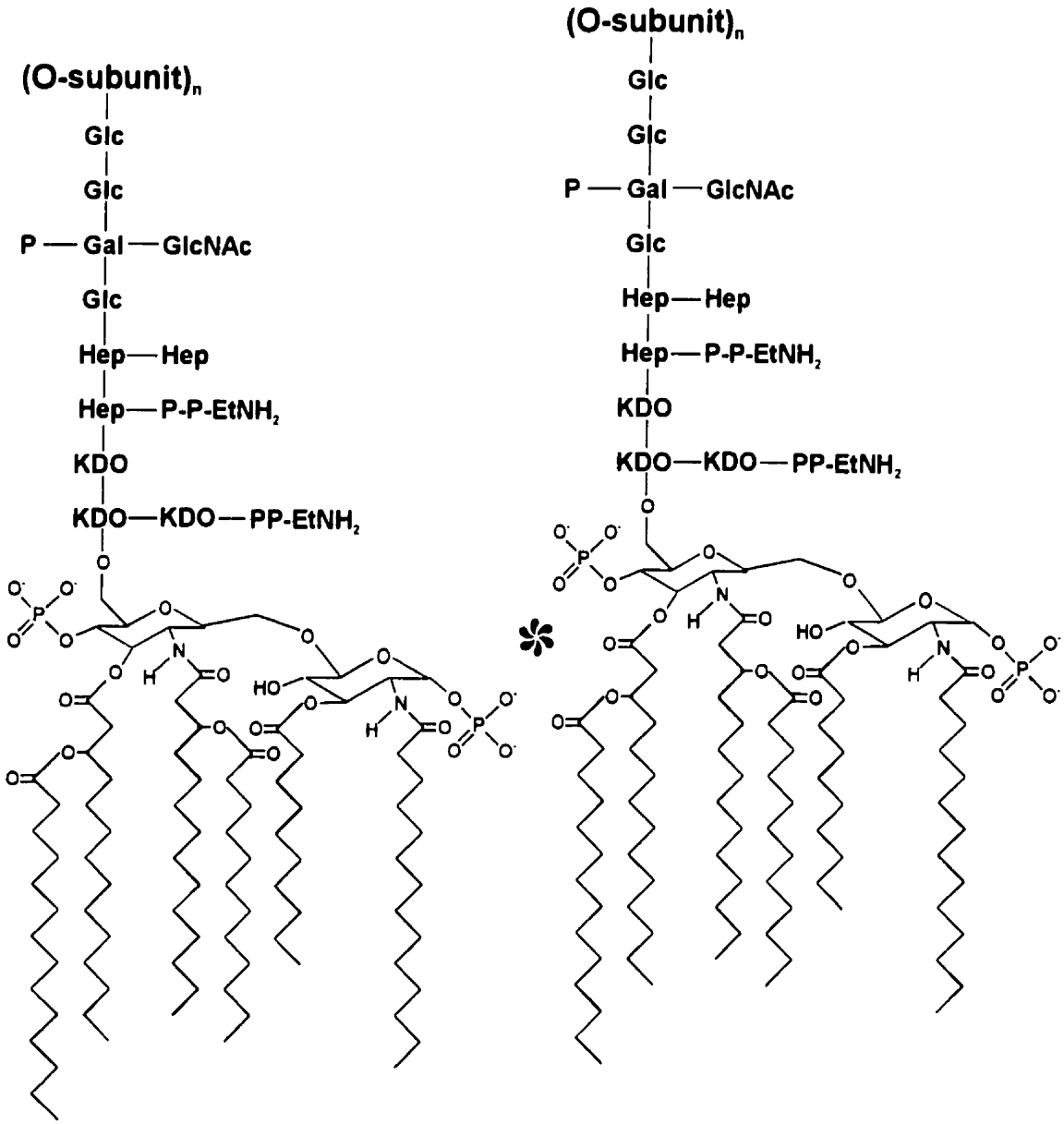
single head group produces a decrease in outer membrane fluidity in comparison to a phospholipid bilayer (Nikaido, 1994).

The core component of LPS can be subdivided into two domains, the inner and the outer core. The outer core is structurally variable as a consequence of different sugar components and glycosidic linkages. The structure of the inner core is highly conserved with only slight variability arising from phosphate substitution of heptose residues (Schnaitman and Klena, 1993). The phosphate groups of the inner core and lipidA confer a net negative charge to the surface of the bacterial cell. The high electrostatic repulsion forces between adjacent LPS molecules are stabilised by ionic interactions between LPS and Ca^{2+} and Mg^{2+}. These divalent cations have been reported to bind to the phosphoryl groups of LPS and the carboxyl group of KDO in the inner core (refer to Figure 2) (Hancock et al., 1994).

The hydrophobic and ionic interactions between adjacent LPS molecules stabilise the outer membrane to form a barrier which prevents the entry of high molecular weight compounds (i.e. nucleases, proteases, peptidases, etc.), hydrophobic antibiotics (i.e. erythromycin, novobiocin, rifampicin), hydrophobic dyes (i.e. methylene blue, brilliant green), and detergents (i.e. deoxycholate, sodium dodecyl sulphate, bile salts) (Nikaido and Vaara, 1985). Hydrophobic probes partition poorly into the hydrophobic portion of LPS. The rate of diffusion of these probes across the outer

Figure 2. Typical structure of lipopolysaccharide observed in *E.coli.*

LipidA is represented symbolically, core is represented in abbreviated nomenclature, and O-antigen is represented by the n^{th}-polymerisation of O-subunits. The abbreviations for the sugars found in the core are Glc, glucose; GlcNAc, N-acetylglucosamine; Gal, galactose; Hep, heptose; KDO, 2-keto-3-deoxyoctulosonic acid; P, phosphate, PP-$EtNH_2$, pyrophosphorylethanolamine. The proposed divalent cation binding site is indicated by the asterisk (*). (Hancock et al., 1994)


(O-subunit)n
Glc
Glc
P — Gal — GlcNAc
Glc
Hep — Hep
Hep — P-P-EtNH2
KDO
KDO — KDO — PP-EtNH2
(O-subunit)n
Glc
Glc
P — Gal — GlcNAc
Glc
Hep — Hep
Hep — P-P-EtNH2
KDO
KDO — KDO — PP-EtNH2

membrane bilayer is approximately 50 to 100 times slower than the rate of diffusion across a phospholipid bilayer (Plesiat and Nikaido, 1992).

1.3 Mechanisms of Transport of Molecules Across the Outer Membrane

Nutrients required for cellular metabolism must traverse the outer membrane by one of the following mechanisms: (i) passive diffusion, (ii) specific diffusion or (iii) receptor mediated import.

Passive diffusion of low molecular weight, hydrophilic molecules occurs through outer membrane porins such as OmpF, OmpC and PhoE. These trimeric proteins form non-specific, water filled channels that span the outer membrane. Alternating hydrophobic and hydrophilic residues of porin proteins produce a β-barrel structure containing a hydrophilic channel and a hydrophobic exterior surface. The hydrophilic interior prevents the diffusion of hydrophobic solutes through the channel.

Some hydrophilic solutes penetrate non-specific porins at negligible rates. Therefore to accelerate their diffusion, specific channels are required. LamB, a porin-like protein, forms a specific channel to facilitate diffusion of maltose and malodextrins across the outer membrane.

Large molecules are not capable of entering the porin channels (exclusion limit of 600Da) and thus require a receptor mediated uptake

system to be internalised. This is a two step process that involves (i) binding of the ligand to an outer membrane high affinity receptor followed by (ii) energy dependent translocation across the outer membrane. The translocation step involves accessory protein components that are part of either the TonB or the Tol import system.

Bacteriophages and colicins (proteins secreted by bacteria that are toxic to other related bacteria) utilise cell surface receptors and these transport systems to their advantage to gain access to the bacterial interior. Bacteria that become insensitive to the deleterious effects of the colicins and/or bacteriophages contain mutation(s) in genes which encode for the phage/colicin receptors or the translocation machinery. Mutants with a defect in the ability of the ligand to bind to the surface receptors are termed resistant. Mutants with a defect in the translocation of the ligand across the outer membrane without compromising binding to the receptor are termed tolerant.

The target and mode of action of colicins varies from DNA cleavage (colicin E2), RNA cleavage (colicin E3), bacterial lysis (colicin M) or the ability to form ion permeable channels (colicins A, B, E1, K, N, Ia, Ib) (Webster, 1991). Colicins can be subdivided into two groups, A and B, depending on the translocation system they require. The activity of the TonB transport system governs entry of the group B colicins (B, D, G, H, Ia, Ib, N, Q, V).

Group A colicins (A, E1, E2, E3, K, L, N, S4) require a distinctive transport system in gram-negative bacteria termed the Tol import system (Braun and Herrmann, 1993).

1.4 The TonB Import System

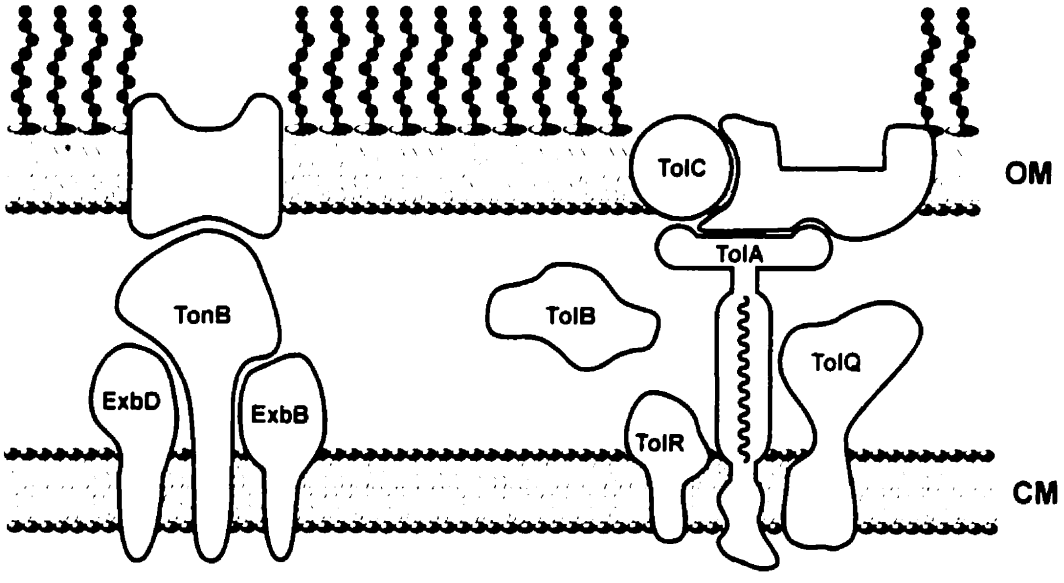
The TonB import system is required for the translocation of iron siderophores (aerobactin), vitamins (vitamin B_{12}), group B colicins, certain bacteriophages and antibiotics (albomycin) (Braun, 1989). All these molecules bind to multi-specific outer membrane receptors (such as IutA, BtuB, FhuA, Tsx) and are internalised in an energy dependent manner.

The TonB import system consists of the three proteins: TonB, ExbB and ExbD (Figure 3a). TonB is anchored to the cytoplasmic membrane and extends into the periplasm where it is able to interact with outer membrane proteins. ExbB is a cytoplasmic protein which proteolytically stabilises TonB and ExbD (Braun and Herrmann, 1993). ExbD is a periplasmic protein that is also anchored to the cytoplasmic membrane. The transport of vitamin B_{12} across the outer membrane requires TonB system proteins and an electrostatic potential across the cytoplasmic membrane. Since TonB spans the periplasmic space, this suggests that TonB may act as an energy transducer between the cytoplasmic and outer membranes (Postle, 1993). TonB dependent receptors and group B colicins possess a conserved

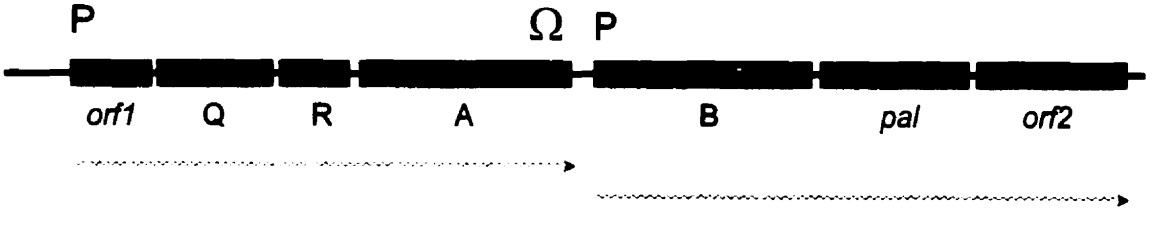
Figure 3. Structural and genetic organisation of the Tol import system

Panel a: Schematic representation of the structural organization of both the TonB and Tol transport systems. Abbreviations are OM, outer membrane; CM, cytoplasmic membrane. Panel b: The *E.coli tol* gene cluster. Abbreviations are: P, promoter; Ω, transcription terminator; *orf1*, open reading frame 1; Q, *tolQ*; R, *tolR*; A, *tolA*; B, *tolB*; *pal*, peptidoglycan associated lipoprotein, *orf2*, open reading frame 2; arrows represent mRNA transcription (Webster, 1991)

a


TolC
OM
TolA
TonB
TolB
TolQ
ExbD
ExbB
TolR
CM

b


P
Ω
P
orf1
Q
R
A
B
pal
orf2

sequence motif, designated the TonB box. Mutations in the TonB box or synthetic TonB box pentapeptides induce *tonB* mutant phenotypic properties suggesting that the receptor proteins directly interact with the TonB protein (Mende and Braun, 1990; Tuckman and Osburne, 1992).

1.5 Tol Import System

The Tol import system is comprised of the proteins TolQ, TolR , TolA, TolB and TolC. (Figure 3). The Tol proteins are encoded by 7 contiguous genes *orf1-tolQRAB-pal-orf2* located at 17 minutes of the *E.coli* genetic map and a separate gene designated *tolC* at 68 minutes. The gene cluster is organised into two transcriptional units *orf1→tolA* and *tolB→orf2*.

Extensive studies have resulted in the identification and localisation of the Tol proteins (Sun and Webster, 1987). TolR and TolA are periplasmic proteins anchored to the cytoplasmic membrane by a single N-terminus membrane spanning domain (Levengood et al., 1991; Levengood and Webster, 1989). TolA can be subdivided into three domains (i) the membrane spanning domain (amino terminal 34 residues), (ii) alpha helix domain (230 residues) and (iii) the active globular domain (carboxy terminal 120 residues). This structure theoretically allows TolA to span the periplasm and interact with the outer membrane proteins, but to date no outer membrane protein or component has been shown to interact directly with TolA (Levengood-

Freyermuth et al., 1993). TolQ is also a membrane protein but it is anchored to the cytoplasmic membrane by three membrane spanning domains (Kampfenkel and Braun, 1992; Vianney et al., 1996). TolB is initially inserted into the cytoplasmic membrane and then post transcriptionally cleaved to yield the functionally active periplasmic protein (Isnard et al., 1994). TolC is an outer membrane protein and PAL is a lipoprotein that interacts with the peptidoglycan layer and the outer membrane (Guihard et al., 1994). There is no known function for *orf1* and *orf2* located within the *tol* gene cluster.

Initially it was postulated that Tol proteins form a complex due to their structural similarities to the TonB system. TolQ and TolR share 25% identity and 75% similarity to their counterparts in the TonB import system, ExbB and ExbD respectively (Eick-Helmerich and Braun, 1989). Cross-complementation experiments revealed that TolQ and TolR are partially similar in function to ExbB and ExbD. Overexpression of TolQ or TolR in *exbB* or *exbD* mutants restores the sensitivity of these mutants to group B colicins but the reverse does not hold true (Braun, 1989). Sucrose gradient centrifugation of membrane preparations results in the separation of cytoplasmic and outer membrane on the basis of density. The membrane bound Tol proteins co-fractionate at an intermediate density to both membranes that corresponds to the theoretical zones of adhesion between

the cytoplasmic and outer membrane (Bourdineaud et al., 1989; Guihard et al., 1994). Null gene mutation in the third membrane spanning domain of TolQ suggest that TolQ and TolR interact with each other (Lazzaroni et al., 1995). Direct evidence of Tol protein interaction has been demonstrated by cross-linking and immunoprecipitating TolA, TolR and TolQ (Derouiche et al., 1995) and TolB and PAL (Bouveret et al., 1995).

The only known function of the Tol import system is that it is required for sensitivity of group A colicins and infection of certain filamentous bacteriophages (Sun and Webster, 1986; Sun and Webster, 1987). Various pleiotropic phenotypes are associated with *tol* mutants. These include hypersensitivity to detergents, bile salts, hydrophobic antibiotics, periplasmic leakiness and expression alterations of membrane proteins (summarised in Table 1). The TolQ, TolR and TolA proteins are believed to be the primary proteins of the transport system due to their requirement for the import of the greatest spectrum of groupA colicins and filamentous bacteriophages (Webster, 1991). Protein deletion analysis has shown that the C-terminal region of TolA is required for the *in vitro* N-terminal binding of colicin A and that the binding of colicin A to the surface receptor OmpF is required for translocation (Benedetti et al., 1991; Fourel et al., 1990). Although a putative TolA box has not been discovered, this data suggests that the TolA protein interacts with a surface receptor in the translocation of colicin A across the

Table 1. Characteristics of *tol* mutations in genes of the system

Gene Mutation	Phage/Colicin Tolerance	Pleiotropic Phenotype
tolA	+/+	Hypersensitive to SDS, DOC, EDTA and certain antibiotics. Periplasmic proteins leak into extracellular medium
tolB	-/+	Similar to *tolA*
tolC	-/+	Hypersensitive to SDS, DOC and certain antibiotics. Lower OmpF expression.
tolQ	+/+	Hypersensitive to SDS, DOC, EDTA and certain antibiotics
tolR	+/+	Similar to *tolQ*
pal	-	Similar to *tolA*
orf1	-	None
orf2	-	None

outer membrane. Also, *tol* mutants show an increase in cell surface expressed colanic acid. An inverse relationship has been shown to exist between the expression of *tol* genes and the regulation of capsule synthesis by the regulatory protein RscC (Clavel et al., 1996).

The hypersensitivity of *tol* mutants towards hydrophobic agents and the increase of colanic acid production in *tol* mutants to compensate for outer membrane perturbations suggest that *tol* genes may play an important role in maintaining the structural integrity of the *E.coli* cell envelope (Lazzaroni et al., 1995). However, the physiological role of the Tol system is not understood in biochemical terms.

Preliminary evidence in our laboratory indicated that strains containing mutations in the *tol* genes, specifically *tolA* and *tolQ*, exhibited a decrease surface expression of O-specific LPS (Thomas and Valvano, 1993). The aim of this project was to elucidate the role of the Tol import system in relationship to the synthesis/processing of the O-antigen component of lipopolysaccharide.

1.6 Biosynthesis and Processing of O-specific Polysaccharide

The structure of O-specific polysaccharide (or O-antigen) consists of a polymerised repeating oligosaccharide subunit usually composed of 3-6 sugars. Differences in the sugar composition, sequence, and linkages gives

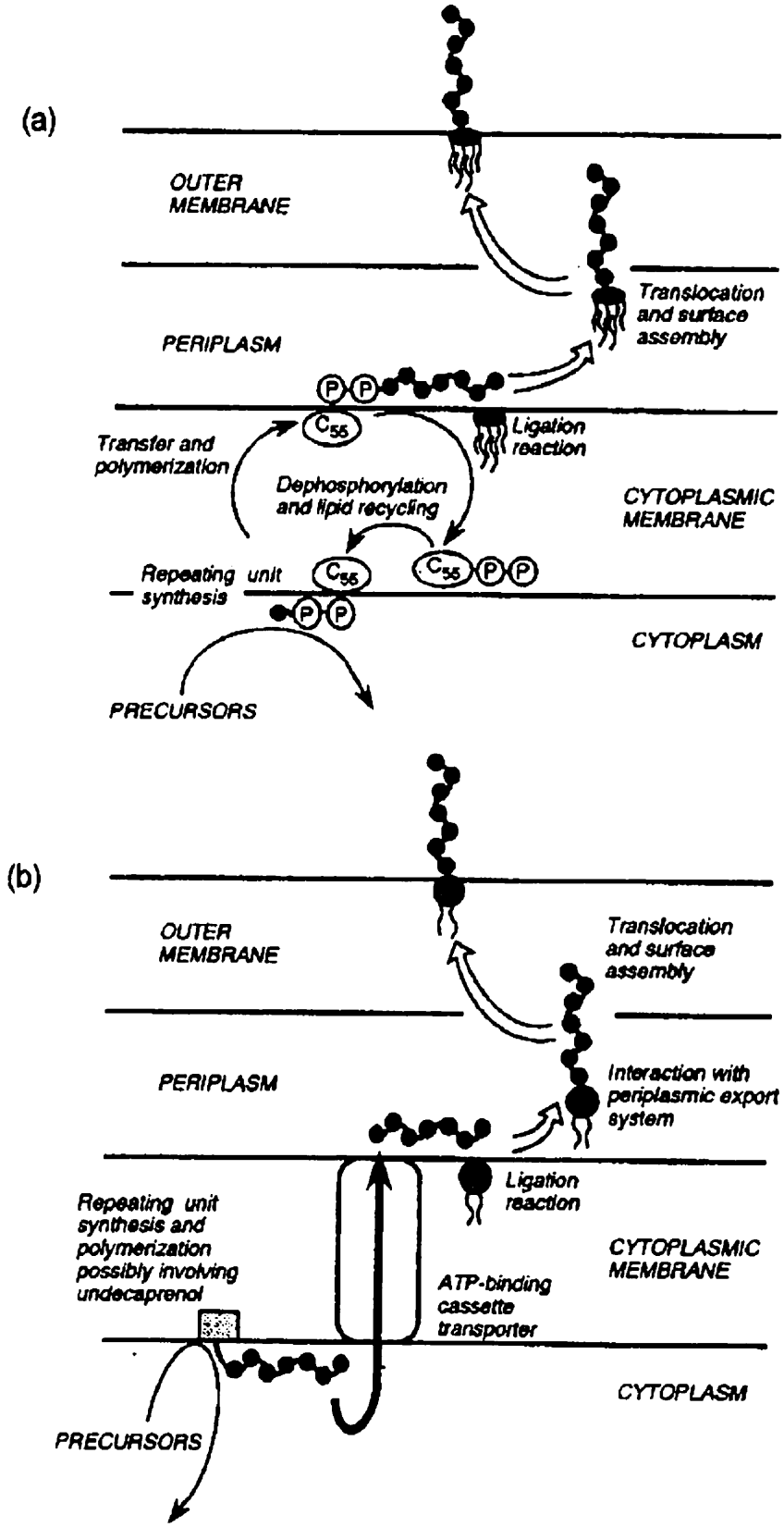
rise to the structural variability which exists between different antigenic LPS molecules (Whitfield and Valvano, 1993).

O-polysaccharide biosynthesis and export can be divided into four different stages: (i) the biosynthesis of the nucleotide sugar precursors, (ii) the assembly of the individual O-antigen subunit (iii) the polymerisation of the O-polysaccharide, and (iv) the ligation of the polysaccharide to the lipidA-core moiety followed by the transport of the complete LPS molecule to the surface of the outer membrane. Sugar nucleotide precursors which are synthesised in the cytoplasm serve as the substrate for the biosynthesis of the O-antigen subunits. These precursors are not transferred directly to a growing lipopolysaccharide molecule but are rather assembled on a intermediate lipid carrier C_{55}-polyisoprenoid derivative, undecaprenol phosphate (und-P) (Whitfield and Keenleyside, 1995). This lipid acts as a platform for the complete assembly of the O-polysaccharide and is also involved in the synthesis of ECA and peptidoglycan.

O-antigen can be synthesised via two different pathways (Figure 4). Heteropolysaccharides are polymerised on the periplasmic surface of the cytoplasmic membrane at the reducing end and homopolysaccharides are polymerised on the cytoplasmic surface of the cytoplasmic membrane at the non-reducing end. Although, both pathways differ in their location and direction of polymerisation, they share common precursors and intermediates.

Figure 4. Model representations of cell surface polysaccharide biosynthesis in Gram-negative bacteria

Surface polysaccharides are synthesised by two separate pathways dependent on the composition of the polymer: (a) heteropolysaccharides or (b) homopolysaccharides. The solid circles represent the O-antigen subunit. Undecaprenol and phosphate groups are represented by C_{55} and P respectively. Diagram taken from (Whitfield and Valvano, 1993)


(a)
OUTER MEMBRANE
Translocation and surface assembly
PERIPLASM
P P
C55
Ligation reaction
Transfer and polymerization
Dephosphorylation and lipid recycling
CYTOPLASMIC MEMBRANE
Repeating unit synthesis
C55
C55 P P
P P
CYTOPLASM
PRECURSORS
(b)
OUTER MEMBRANE
Translocation and surface assembly
PERIPLASM
Interaction with periplasmic export system
Ligation reaction
Repeating unit synthesis and polymerization possibly involving undecaprenol
CYTOPLASMIC MEMBRANE
ATP-binding cassette transporter
CYTOPLASM
PRECURSORS

O-antigen biosynthesis is initiated by the transfer of a nucleotide sugar to Und-PP located on the cytoplasmic surface of the cytoplasmic membrane. Two such transferases have been identified in gram-negative bacteria and they are Rfe and RfbP. Rfe has been demonstrated to be essential for the transfer of N-acetylglucosamine (GlcNAc) residues to Und-PP from the nucleotide precursor UDP-N-acetylglucosamine in *E.coli* serotypes O7, O18, O75 and O111 (Alexander and Valvano, 1994). In addition to initiation of the former heteropolysaccharide, Rfe is also essential for the initiation of enterobacterial common antigen and the initiation of homopolysaccharides of *E.coli* O8 and O9 (Jann et al., 1985) and *Klebsiella pneumoniae* (Clarke and Whitfield, 1992). The role of Rfe is not fully understood in relationship to homopolymeric O-antigen synthesis because GlcNAc is not one of the sugars present in the polysaccharide. It has been proposed that Rfe has a regulatory function in the biosynthesis of O8 and O9 by forming a glycosylated lipid acceptor (Whitfield, 1995). The other glycosyltransferase RfbP, has been shown to mediate the transfer of galactose residues from UDP-Gal*p* to Und-PP in *Salmonella enterica* (Osborn and Tze-Yuen, 1968). Both Rfe and RfbP are predicted to be integral membrane proteins because they must interact with the Und-PP acceptor to initiate O-antigen synthesis and they have similar hydropathy profiles (Schnaitman and Klena, 1993).

Immediately after the initiation of heteropolysaccharides synthesis, transferases encoded from the *rfb* gene cluster progressively transfer the remaining nucleotide sugars to form a complete O-antigen subunit. Since polymerisation has been shown to occur on the periplasmic face of the cytoplasmic membrane, the lipid linked O-unit must be translocated across the cytoplasmic membrane (McGrath and Osborn, 1991). The translocation ("flipping") of the O-unit to the periplasmic surface of the cytoplasmic membrane occurs by an unknown mechanism but a gene product from the *rfb* cluster, RfbX, has been proposed to perform this function (Liu et al., 1996). The O-antigen is then polymerised, by the enzyme Rfc, in a blockwise fashion at the reducing end as the growing O-antigen polymer is transferred to Und-PP containing a single O-subunit. This process which involves the sequential polymerisation of the O-antigen in a blockwise fashion insures that the fidelity of the complex heteropolysaccharide is maintained (Whitfield, 1995). The naked Und-PP is then recycled back to the cytoplasmic surface for further use in O-antigen assembly.

The degree of polymerisation of the O-antigen is mediated by the Rol protein. Once the O-antigen reaches a predetermined length it is ligated by RfaL to the independently synthesised lipidA-core moiety to form a complete LPS molecule. The enzymes involved in O-antigen synthesis RfbP (or Rfe), Rfc, RfbX, Rol and RfaL are all integral membrane proteins and are

postulated to operate in a complex as a result of their sequential action and high affinity (Whitfield, 1995). Once the LPS molecule is complete, it is transported from the periplasmic face of the cytoplasmic membrane to the extracellular surface by an unknown mechanism.

In the case of homopolysaccharides, the polymerisation does not occur on the periplasmic surface but rather on the cytosolic surface. The O-antigen is polymerised at the non-reducing end and the monomers are directly transferred to a growing O-antigen chain which is attached to Und-PP. Once the O-antigen reaches a predetermined length, the nascent polymer is translocated across the cytoplasmic membrane by a dedicated ATP-binding cassette transporter. The O-antigen is then ligated to a lipidA-core molecule by RfaL and the remaining steps are identical to heteropolysaccharide synthesis.

1.7 Research Objectives

Initial observations made in our laboratory demonstrated that *tolQ*, *tolR* and *tolA* are essential for the translocation of a bacteriocin made by *Enterobacter cloacae* (Cloacin DF13) across the outer membrane (Thomas and Valvano, 1992). Since the Tol import system is highly conserved among gram-negative bacteria, we reasoned that the Tol system must play an important role in normal cell metabolism. It is highly improbable that such a system would

have evolved for the sole purpose of transporting deleterious compounds (i.e. colicins and bacteriophages) across the outer membrane.

We observed that *tolA* and *tolQ* mutants of *E.coli* K-12 (transformed with a plasmid containing the O7-specific LPS genes) and wild-type strain VW187 (endogenously expresses O7-specific LPS genes) showed a decrease in LPS production compared to the isogenic parental strains. We postulate that the Tol system may function in the processing or synthesis of O-antigen.

The purpose of this work was to elucidate the physiological role of the Tol import system in relationship to the biosynthesis and assembly of O-antigen. The model system is the O7-antigen that contains a repeating unit oligosaccharide of five different sugars : N-acetylglucosamine, galactose, mannose, viosamine, and rhamnose (L'vov et al., 1984). Previous work in our laboratory has resulted in the biochemical and genetic characterisation of the genes involved in the synthesis of the O7-units (Marolda et al., 1990). *E.coli* K-12 cannot express O-antigen but it can express O7 if transformed with the recombinant plasmid pJHCV32 containing the O7 biosynthesis gene cluster cloned from the *E.coli* O7:K1 strain VW187 (Valvano and Crosa, 1989).

Studies presented in this thesis involved (i) the use of the pJHCV32 plasmid to investigate the expression of O7-LPS in *E.coli* K-12 wild type and

tol mutants, (ii) the construction and characterisation of *tol* mutations in the O7 wild-type *E.coli* strain VW187, and (iii) the biochemical characterisation of the processing of the O7-unit in a *tolA* mutant and its isogenic parent *E.coli* K-12 strain.

Chapter Two

Material and Methods

2.1 Reagents

The chemicals used in this work are reagent grade unless noted otherwise. Reagents were purchased from BDH Chemicals (Toronto, Ontario), BioRad (Richmond, California), Boehringer-Mannheim Canada Limited (Dorval, Quebec), Fisher Scientific (Fairhaven, New Jersey), J.T. Baker (Phillipsburg, New Jersey), or Sigma Chemical Company (St. Louis, Missouri). Restriction enzymes and DNA modifying enzymes were purchased from Boehringer-Mannheim, New England Biolabs (Beverley, Massachusetts) or Pharmacia Limited (Baie d'Urfe, Quebec) and used as recommended by the supplier. Media reagents were purchased from Difco (Detroit, Michigan) or BBL Microbiology Products (Cockeysville, Maryland) and antibiotics were purchased from Sigma. Radioisotopes were purchased from Amersham Canada (Oakville, Ontario) or ICN (Montreal, Canada). Sterile distilled water was used to prepare all solutions unless otherwise noted.

2.2 Bacterial Strains and Plasmids

Culture strains were maintained and manipulated using standard bacteriological techniques. Cultures were stored long-term at -70°C in Luria-Bertani (LB) broth medium containing 20%(w/v) glycerol. Bacterial cultures were typically grown in 5mL LB broth or on agar plates overnight at 37°C. In some experiments micro-organisms were also grown in M9 minimal media supplemented with 0.5%(w/v) D-glucose, D-mannose or glycerol. Selective pressure with the appropriate antibiotics was utilised to screen and maintain both transformants and mutants through cell division. The final concentration of antibiotics in media used in this study are as follows: ampicillin (100µg/mL), chloramphenicol (30µg/mL), kanamycin (50µg/mL), nalidixic acid (20µg/mL), rifampicin (50µg/mL), spectinomycin (80µg/mL) and tetracycline (20µg/mL). All bacterial strains and plasmids used in this study are described in Table 2 and Table 3 respectively.

Typically the isolation of whole cells and cellular components (i.e. DNA, cellular membranes, etc.) was accomplished by centrifugal precipitation. Samples were microcentrifuged (Eppendorf® 5415C Microcentrifuge, Brinkman Instruments, Westbury, New York), centrifuged (Beckman J2-21 Centrifuge, Palo Alto, California) or ultracentrifuged (Beckman L8-55 Ultracentrifuge, Palo, Alto, California) depending on the size of the sample or

Table 2. *Escherichia coli* strains used in this study

Strains	Relevant properties	Source or Reference
A592	C600 *tolA1*	(Nagel de Zwaig and Luria, 1967)
BW19851	RP4-2::Mu-1*kan*::Tn*7*/*creB510* *hsdR17 endA1 zbf-5* *uidA*(Δ*MluI*)::*pir*(wt) *recA1 thi*	(Metcalf et al., 1994)
C600	*thr leu tonA lacY supE thi*	Lab stocks
CLM11	SØ874 *manA*::pMAV020, Ap^R	This work
JAG1	MV501, *tolQ*::Ω, Sm/Spc^R	This work
JAG2	MV501, *tolA*::Ω, Sm/Spc^R	This work
JAG3	CLM11, *tolQ*::Ω, Sm/Spc^R, Ap^R	This work
JAG4	CLM11, *tolA*::Ω, Sm/Spc^R, Ap^R	This work
MV501	VW187, *rfe*	(Alexander and Valvano, 1994)
P90C	*ara* Δ*(lac-pro) thi*	(Bradley and Whelan, 1989)
P90C*tolQ*	*ara* Δ*(lac-pro) thi tolQ13*	(Bradley and Whelan, 1989)
SØ874	*lacZ trp* Δ*(sbcB-rfb) upp rel rpsL*	(Neuhard and Thomassen, 1976)
SY327-λ *pir*	λ *pir araD* Δ*(lac-pro) argE recA56* Rif^R *nalA*	(Miller and Mekalanos, 1988)
VW187	E.coli O7:K1, prototrophic	(Valvano and Crosa, 1984)

Table 3. Plasmids used in this study

Plasmids	Relevant properties	Source or Reference
pCM131	1.2-kbp *EcoRI-HindIII* fragment from pJHCV32 carrying the O7promoter transcriptionally fused to the promoterless *lacZ* gene in pFZY1, Ap^R	(Marolda, 1996)
pGP704	Cloning vector, *oriR6K* Ap^R *mobRP4*	(Miller and Mekalanos, 1988),
pJG1	pTPS202 with the Km^R gene cassette inserted in the *ScaI* site, Km^R, Ap^S	This work
pJG11	*tolA*::Ω and *nptI-sacBR* into pGP704, Ap^R, Km^R, Sm/Spc^R	This work
pJG2	pTPS202 with a deletion of the single *NotI* site within *tolA*	This work
pJHCV32	*rfb*O7 cosmid, Tc^R, O7+	(Valvano and Crosa, 1989)
pJT10	*tolQ*::Ω and *nptI-sacBR* into pGP704, Ap^R, Km^R, Sm/Spc^R	This work
pColN-284	Colicin N plasmid from *E.coli* strain 284	(Reeve, 1979)
pMAV020	0.85-kbp of the *manA* gene cloned in the *EcoV* site of pGP704	This work
pMAV11	*rfe* gene cloned in pACYC184	(Alexander and Valvano, 1994)
pTPS202	8.7-kbp *BglII* fragment carrying *orf1-tolQRAB* genes cloned in pJIH12, Ap^R	(Sun and Webster, 1986)
pUM24Cm	*ntpI-sacBR* cassette in pBR329, Cm^R, Km^R	(Ried and Collmer, 1987)

relative centrifugal force required to precipitate the cells or cellular components.

Absorbance and optical density measurements were taken using an ultra violet/visible wavelength spectrophotometer (4050 LKB Biochrom UltraspecII, LKB Ltd., Cambridge, England).

2.3 Assessment of *tol* mutants

Since *tol* mutants have a compromised outer membrane, these mutants are hypersensitive to detergents. The *tol* phenotype was typically assessed by plating a culture on LB plates containing 0.1%(w/v) sodium deoxycholate (Levengood-Freyermuth et al., 1993). Under these conditions, *tol* mutants did not grow.

In some cases, the *tol* phenotype was assessed by examining colicin N resistance since colicin N requires both the OmpF receptor and an intact Tol import system for translocation across the outer membrane. A crude preparation of colicin N was prepared using the following protocol (Cavard and Lazdunski, 1979; Massotte and Pattus, 1989).

An overnight culture of W3100 pColN-284 was diluted to a final volume of 1L and grown to mid-exponential growth phase (OD_{600nm}=0.5). MitomycinC (0.3µg/mL) was added to the growing culture to induce the production of colicin N. After 2 hours, the cells were harvested by centrifugation (5,000×*g*,

10 minutes, 4°C), washed once with cold water, and colicin N was extracted by stirring the cells in 200mL saline buffer (0.7M NaCl, 10mM phosphate, pH6.8). The colicin extract was filter sterilised, aliquoted in 10mL tubes and stored at -70°C. Sensitivity to colicin N was assayed by preparing serial dilutions of freshly thawed colicin, depositing 100µL samples of each dilution onto a freshly prepared overlay plate containing actively growing cells, incubating plates at 37°C and examining the bacterial lawn for zones of inhibited growth.

2.4 Recombinant DNA Techniques

2.4.1 Small Scale Plasmid Preparation

Small scale plasmid preparations were prepared from an overnight bacterial culture grown in 5mL LB broth at 37°C. An 1.5mL aliquot was placed into a centrifuge tube and the cells were harvested by centrifugation (16,000×*g*, 1 minute, RT). This step was repeated an additional 2 times to harvest the complete culture in a single tube.

The cell pellet was resuspended in 200µL Solution I (50mM glucose, 25mM Tris-HCl, 10mM EDTA, 4mg/mL lysozyme, 100µg/mL RNAase, pH8.0) and incubated at room temperature for 5 minutes. Next, 400µL freshly prepared solution II (0.2N NaOH, 1% SDS) was added to the cell suspension, mixed gently and placed on ice for 5 minutes. The solution was neutralised

by the addition of 300µL ammonium acetate (7.4M, pH4.8), mixed by inversion, and followed by a second incubation on ice for 5 minutes.

Cellular debris and chromosomal DNA were removed by centrifugation (16,000×*g*,10 minutes, RT). The supernatant was decanted into a second centrifuge tube and 500µL isopropanol was added to precipitate plasmid DNA. After 15 minutes at room temperature, the preparation was centrifuged (16,000×*g*, 10 minutes, RT) and the supernatant was discarded. The plasmid DNA pellet was washed 3 times with 70% ethanol, vacuum dried for 10 minutes and resuspended in 25µL distilled water or TE (10mM Tris-HCl, 1mM EDTA).

2.4.2 Large Scale Plasmid Preparation

The protocol from Goto et al. (1984) was slightly modified for the preparation of large quantities of plasmid DNA by cesium chloride density gradient centrifugation.

Overnight bacterial cultures were diluted (1:100) to a final volume of 1L and grown to late exponential growth (OD_{600nm}=1.0) with vigorous shaking at 37°C. Cells were harvested by centrifugation (5,000×*g*, 10 minutes, 4°C) and the supernatant was discarded.

The bacteria were combined and resuspended in 2mL TE suspension buffer (10mM Tris-HCl, 1mM EDTA, 4mg/mL lysozyme, 250µg/mL RNAase,

pH8.0) and chilled on ice for 5 minutes. Next, 2.5mL EDTA (0.25M, pH8.0) was mixed with the cell suspension by gentle swirling and incubated for an additional 5 minutes on ice. A dual phase mixture was formed by gently layering 16mL lysis mix (1%(w/v) Brij58, 0.04%(w/v) sodium deoxycholate, 62.5mM EDTA, 50mM Tris-HCl, pH7.4) on top of the bacterial cell suspension. The bacteria were lysed by vigorous shaking (3 or 4 times) and the lysate was centrifuged (39,000×*g*, 30 minutes, 2°C) to precipitate cellular debris.

The non-viscous fluid was carefully decanted into a graduated cylinder and the exact volume was measured so that additional reagents could be added in the proper proportions. A volume of 10%(w/v) sarkosyl was added to the lysate to bring the final concentration of detergent to 1% and was incubated for 10 minutes at 30°C. Next the lysate was diluted with sodium chloride (5M) to bring the salt concentration to 0.5M. Solid polyethylene glycol was dissolved to a concentration of 10%(w/v) and chilled overnight at 4°C.

The plasmid DNA was precipitated by centrifugation (5,000×*g*, 5 minutes, 4°C) and the pellet was resuspended in 3mL TE buffer containing sarkosyl (0.76mL, 10%) and EDTA (0.24mL, 0.5M). Next, 4.5g cesium chloride was completely dissolved in the plasmid suspension before the

addition of 0.2mL ethidium bromide (10mg/mL in TE). The solution was protected from direct light to avoid the introduction of secondary mutations. The density was adjusted to 1.393 using a refractometer, the samples were distributed into 5mL Quick Seal (Beckman, Palo Alto, California) tubes and centrifuged (150,000×*g*, VTi65 rotor, 24 hours, 20°C).

The plasmid band was detected using long wave ultraviolet light and collected from the gradient by bottom puncture. The ethidium bromide was extracted by washing the preparation with isopropanol saturated with CsCl until the colour of the ethidium bromide disappeared. The plasmid preparation was dialysed against TE buffer for 3 hours at 4°C to remove the cesium chloride.

In some experiments, plasmid DNA was extracted using a commercial kit purchased from Qiagen Inc. (Chatsworth, California).

2.4.3 Recombinant DNA Techniques

Unless otherwise indicated, all cloning procedures outlined throughout the course of this work are described in Maniatis et al. (1982).

2.4.3.1 Restriction Enzyme Digestion

Restriction endonuclease digests of DNA were completed under the conditions recommended by the manufacturer. Typically, the digests were

performed in a final volume of 10 to 20µL containing approximately 0.5 to 2.0µg DNA, the appropriate restriction buffer, and 10U of the restriction enzyme.

Complete digestion of the sample was confirmed by electrophoresis. Aliquots of digested DNA samples were combined with 10X DNA stop dye (1%(w/v) SDS, 0.1%(w/v) bromophenol blue, 10mM EDTA, 50% glycerol, pH8.0) and loaded into 0.5 to 1.5%(w/v) agarose gels containing ethidium bromide (1µg/µL). The agarose was dissolved in TBE running buffer (89mM Tris-HCl, 89mM borate, 2mM EDTA, pH8.0). Gels were electrophoresed in a Wide Mini-Sub™ Cell apparatus (BioRad laboratories Ltd., Mississauga, Ontario) and run at a constant amperage (70-80mA). Gel photographs were taken using a Polaroid MP4 Land Camera with Polaroid-Negative 4x5 instant Sheet 55 film.

2.4.3.2 Enzyme Inactivation in DNA Samples

DNA samples used for cloning after restriction endonuclease digestion were treated with 95%(v/v) phenol (saturated with 0.1M Tris-HCl, 2%(v/v) β-mercaptoethanol, pH8.0) and chloroform (4%(v/v) isoamylalcohol). The volume of the DNA samples were adjusted to 50µL with distilled water, phenol and chloroform were added in a 1:1:2 ratio relative to the DNA digest, and the

samples were mixed by tapping. The samples were centrifuged (16,000×*g*, 5 minutes, RT) to separate the organic and aqueous phases.

The aqueous phase was carefully removed and delivered into a clean centrifuge tube. The plasmid preparation was washed twice with ether (saturated with TE buffer, 4°C) to remove remaining phenol-chloroform from the aqueous phase. Sodium acetate (3M, pH4.8, to a final concentration 0.3M) and two volumes of double distilled ethanol (-20°C) were mixed with the plasmid preparation and the DNA was precipitated by incubation for at least one hour at -20°C. The solution was centrifuged (16,000×*g*, 10 minutes, RT) to collect the DNA and the plasmid pellet was washed with 70%(v/v) ethanol (4°C) three times and intermittently centrifuged (16,000×*g*, 1 minute, RT) between washes. The pellet was dried over a drying agent under vacuum, the plasmid DNA was resuspended in distilled water and stored at 4°C until required for further manipulation.

2.4.3.3 Synthesis of Blunt Ends

Generation of blunt ends was accomplished by complementary filling of 5'-overhanging ends or removal of 3'-overhanging ends utilising the Klenow fragment of DNA polymerase I. The DNA solution was diluted to 50µL and one unit of Klenow fragment, deoxynucleotidetriphophate solution (20µM ATP, 20µM CTP, 20µM GTP, 20µM TTP) and NT buffer (0.06M Tris-HCl,

0.01M $MgCl_2$) were added. The solution was mixed by gentle tapping, centrifuged (16,000×*g*, 15 seconds, RT), and incubated for 30 minutes at 37°C. The sample was treated with phenol-chloroform as described previously.

2.4.3.4 Vector Dephosphorylation

To prevent the reannealing of vector fragments in ligation reactions, the vector ends were dephosphorylated. The removal of 5'-phosphate groups was accomplished by adding 1.5U of calf alkaline phosphatase and 5µL alkaline phosphorylase buffer (0.05M Tris-HCl, 0.1mM EDTA, pH8.5) to a 50µL DNA sample. The solution was incubated for 30 minutes at 37°C and treated by the phenol-chloroform as described previously.

2.4.3.5 Ligation of DNA Fragments

Ligation of DNA fragments was performed in a similar manner for both sticky-end overhangs and blunt-end ligation. The ligation mixture (total volume 15µL) contained the two DNA fragments, 1U of T4 DNA ligase and ligation buffer. Compatible end ligation buffer (0.05M Tris-HCl, 10mM $MgCl_2$, 10mM dithiothreitol, 50µg/mL bovine serum albumin, pH7.6) or blunt end ligation buffer (0.0825M Tris-HCl, 6.25mM $MgCl_2$, 6.25mM dithiothreitol, 125µg/mL bovine serum albumin, pH7.5) were added to the respective ligation mixtures and incubated at 15°C overnight.

2.4.4 Bacterial Transformations

Bacterial transformations were typically performed by calcium chloride method described by Hanahan (1983) or by electroporation following the protocol from Dower et al. (1988).

2.4.4.1 Calcium Chloride Transformation

The transformation procedure by calcium chloride method was performed in the following manner. A dilution (1:50) of an overnight bacterial culture was prepared in 25mL fresh LB broth (containing 20mM $MgCl_2$). The culture was incubated for 3 hours at 37°C with shaking. The cells were harvested by centrifugation (5,000×*g*, 10 minutes, 4°C) and the bacterial pellet was resuspended in a cold calcium chloride solution (100mM $CaCl_2$, 10mM MOPS, 0.5% glucose, pH6.5) insuring the cell suspension remained on ice at all times. The cells were harvested again by centrifugation (5,000×*g*, 10 minutes, 4°C) and resuspended in 0.2mL calcium chloride solution. The plasmid DNA sample (approximately 1µg) was added to 250µL of the cell suspension and incubated for 45 minutes on ice. The solution was then incubated for two minutes at 42°C, 1mL LB broth was added to the transformation mixture and the culture was incubated for 2 hours at 37°C. The transformation was plated on LB agar plates containing the appropriate antibiotics and incubated overnight at 37°C.

2.4.4.2 Electroporation of Plasmid DNA

Bacteria were prepared for transformation by electroporation in the following manner. A dilution (1:50) of an overnight bacterial culture in 25mL fresh LB broth was prepared and incubated for 3 hours at 37°C with vigorous shaking until late log phase. The culture was cooled on ice for 15 minutes and harvested by centrifugation (5,000×*g*, 10 minutes, 4°C). The supernatant was discarded and the cells were resuspended in 10mL sterile, cold distilled water (4°C) and precipitated again by centrifugation (5,000×*g*, 10 minutes, 4°C). The supernatant was discarded, the cells were resuspended in 0.5mL sterile cold 10%(w/v) glycerol and transferred to a centrifuge tube. The cell suspension was centrifuged (16,000×*g*, 30 seconds, RT), the supernatant was discarded and the pellet was resuspended in 150µL cold sterile 10%(w/v) glycerol. The bacterial suspension was aliquoted into 40µL samples and stored at -20°C until required. A thawed cell aliquot was mixed with approximately 1µg plasmid DNA and placed into a 10mm cold cuvette (4°C). The sample was pulsed with a Gene Pulser apparatus (BioRad Laboratories Ltd. , Mississauga, Ontario) set at a voltage 1.25V, resistance 200Ω, and capacitance 2.5mF. The electroporated cell suspension was mixed with 1mL SOC (2% Bacto-tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM D-glucose) and the culture was incubated for 1-2

hours at 37°C. The transformation was plated on LB agar plates containing the appropriate antibiotics and incubated overnight at 37°C.

2.4.5 Conjugation Experiments

Plasmids were also introduced by conjugation into bacteria. This strategy was employed to introduce a suicide vector designed to integrate by homologous recombination into a target gene. This concept will be described further in the Results and Discussion section. The protocol involved growing overnight cultures of the recipient and mating strain on separate selective plates. The bacteria were collected from the two plates using a sterilised loop, thoroughly mixed on the surface of a LB agar plate, and incubated overnight at 37°C. The conjugation paste was harvested from the plate, resuspended in LB broth and a series of serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) were prepared. An 100μL aliquot of each dilution was plated on selective plates, the plates were incubated overnight and colonies were then isolated and screened.

2.5 Lipopolysaccharide Extraction and Detection

2.5.1 Extraction

LPS was extracted by a modified hot phenol-water method as described in Marolda et al. (1990).

Bacteria were collected from the surface of an overnight LB agar plate using a cotton swab and resuspended in 2mL PBS buffer (8.7mM Na_2HPO_4, 1.3mM NaH_2PO_4, 145mM NaCl, pH7.6). The optical density (OD_{600nm}) was measured and the turbidity of the suspension was adjusted to OD_{600nm}=2.0. A volume of the cell suspension (1.5mL) was transferred to a centrifuge tube, the cells were pelleted by centrifugation (16,000×*g*, 1minute, RT) and the supernatant was discarded. The cell pellet was resuspended in 150µL lysis buffer (2%(w/v) SDS, 4%(v/v) β-mercaptoethanol, 0.5M Tris-HCl, pH6.8) and boiled for 10 minutes. The cell lysate was allowed to cool for 2 minutes at room temperature, 10uL proteinase K (20mg/mL) was mixed by vortex and incubated for 1 hour at 60°C. Next, 150µL pre-warmed (55°C) 95%(v/v) phenol was added to the lysate and the mixture was incubated for 15 minutes at 70°C with intermittent vortexing every 5 minutes. The mixture was placed on ice for 10 minutes, centrifuged (16,000×*g*, 10 minutes, RT) and the aqueous phase was transferred to a clean centrifuge tube. The aqueous phase was washed with 500µL ether, the samples were centrifuged (16,000×*g*, 1minute, RT), the ether layer was aspirated and 100µL stop mix (2%(w/v) SDS, 4%(v/v) β-mercaptoethanol, 10%(w/v) glycerol, 1M Tris-HCl, 0.01%(w/v) bromophenol blue, pH6.8) was added to the LPS samples. The samples were frozen at -20°C and boiled just prior to gel loading.

2.5.2 Quantification of Proteins

To ensure comparable loading of samples in each well of SDS-PAGE, the protein content of the each sample was typically determined by colorimetric method as described by Lowry et al. (1951). The samples were then normalised on the basis of protein concentration and equivalent volumes were loaded in each well.

Reagent for the quantification of proteins were freshly prepared on the same day from stock solutions. Reagent C was prepared by mixing 25mL Reagent A (2.5ml-1M NaOH, 2.5mL-20%(w/v) Na_2CO_3, 25mL distilled H_2O) with 0.5mL Reagent B (250μL-1%(w/v) $CuSO_4 \cdot 5H_2O$, 250μL-2%(w/v) sodium citrate). A standard curve was obtained by aliquoting a protein stock solution (1mg/mL (w/v) bovine serum albumin) in increments of 4μL from 0μL to 36μL in 1mL disposable cuvettes and the volume was adjusted to 200μL. An aliquot of each protein sample was also diluted to 200μL so that the measurement of the protein concentration would fall within the range of the standard curve.

When all the samples were prepared, 1mL Reagent C was added to each of the samples, mixed well, and allowed to stand for 10 minutes at room temperature. Next, 100μL Reagent D (1N Folin-Ciocalteu's phenol reagent) was added to the protein dilutions and mixed immediately. After 30 minutes at room temperature, the A_{660nm} was measured. Linear regression was used to

plot a line of best fit through the standard curve, and unknown protein concentrations were extrapolated from the curve.

2.5.3 KDO Assay

The amount of LPS can be quantified by measuring the amount of 2-keto-3-deoxyoctanoic acid (KDO) in the inner core. To release the KDO from the LPS, a 50μL aliquot of LPS was mixed with 50μL sulphuric acid (0.04N) and boiled for 20 minutes. The volume of the samples were adjusted to 200μL with sulphuric acid (0.02N), 250μL periodate solution (0.025N periodate, 0.125N NH_4SO_4) was mixed with the LPS sample and incubated for 20 minutes at room temperature. Next, 500μL sodium arsenate (0.2N in 0.5N HCl) was added, incubated for 2 minutes at room temperature before the addition of 2mL thiobarbituric acid (0.3%(w/v), pH2.0). The solution was mixed, heated for 20 minutes and the A_{548nm} was recorded. One nanomole per mL KDO corresponds to an A_{548nm} of 0.019 in a cuvette with a path length of 1cm . All readings were normalised against the protein content of the LPS sample.

2.5.4 Electrophoresis of LPS

LPS extracts were analysed by Tris-Glycine (12%(w/v) acrylamide, 2.6%(w/v) bis-acrylamide) or Tricine (16%(w/v) acrylamide, 2.6%(w/v) bis-acrylamide) sodium dodecyl sulphate polyacrylamide gel electrophoresis as described by

Schagger and von Jagow (1987). Pre-cast Novex™ polyacrylamide gels (Helix Technologies, Scarborough, Ontario) were used in this study. Samples were electrophoresed in a Xcell II™ Mini-Cell Apparatus (Helix Technologies, Scarborough, Ontario) utilising Tris-Glycine running buffer (24mM Tris base, 192mM glycine, 1%(w/v) SDS, pH8.3) or Tricine running buffer (100mM Tris base, 1M tricine, 1%(w/v) SDS, pH8.3) at a constant 125V for approximately 90 to 120 minutes. LPS was visualised typically by silver staining or immunoblotting.

The silver stain procedure for developing SDS-PAGE involved placing the electrophoresed gel in 200mL fix solution (60%(v/v) methanol, 10%(v/v) acetic acid) for 15 minutes followed by soaking the gel overnight in fresh fix solution. The gel was then progressively soaked in 7.5%(v/v) acetic acid for 30 minutes, 0.2%(w/v) periodic acid for 30 minutes, washed with distilled water for 2 hours, and stained for 15 minutes. The stain solution was prepared by mixing the following reagents in sequential order: 148mL distilled water, 42mL 0.36%(w/v) sodium hydroxide, 2.8mL concentrated ammonium hydroxide, and the dropwise addition of 8mL 19.4%(w/v) silver nitrate to prevent the precipitation of silver hydroxide. The gel was washed for an additional 15 minutes and soaked in developing solution (10%(v/v) methanol, 0.02%(w/v) formaldehyde, 0.05%(w/v) citric acid) until the LPS bands were

visible. The gel was rinsed with copious amounts of water to prevent further darkening of the stained LPS.

For immunoblotting, the LPS was transferred from the electrophoresed gel to a nitro-cellulose support matrix using the Novex™ Western Transfer Apparatus (Helix Technologies, Scarborough, Ontario). The transfer chamber was filled with buffer (12mM Tris, 96mM glycine, 20%(v/v) methanol, pH8.3) and the gel was exposed for 1-2 hours to a constant voltage of 30V. The blot was removed from the transfer apparatus and incubated with 50mL blocking solution (3%(w/v) bovine albumin antiserum, 0.9%(w/v) NaCl, 10mM Tris-HCl, pH7.4) for 30 minutes at room temperature on a rocking platform (Bellco Biotechnology, Vinland, New Jersey). The blot was washed three times with distilled water and rocked overnight at room temperature in a dilution (1:50) of O7-specific polyclonal rabbit antiserum. The next day the antiserum was recovered and the blot was washed three times with distilled water. A dilution of horse-radish peroxidase conjugated to protein A (0.1mg/ml HRP-protein A, 150mM NaCl, 50mM Tris-HCl, 5mM EDTA, 0.05% Triton X-100. pH7.4) was added to the blot and incubated for 2 hours at room temperature with gentle rocking. The blot was washed again with distilled water, placed in 60mL buffer (10mM Tris-HCl, 150mM NaCl, pH7.2) and developed by the simultaneous addition of BioRad HRP Colour Development Reagent (60mg 4-chloro-1-naphthol dissolved in 20mL methanol) and 60μL hydrogen peroxide.

The stained immunoblot was washed with copious amounts of distilled water to stop further colour development.

2.6 Measurement of mRNA Transcriptional Levels

2.6.1 Reverse Transcriptase Chain Reaction Amplification

The isolation of RNA from culture samples was performed as described in the Qiagen RNeasy Total RNA Purification System. An overnight culture was diluted (1:50) to 250mL and grown to late logarithmic phase (OD_{600nm}=1.0). The cells were harvested by centrifugation (5,000×*g*, 10 minutes, 4°C), resuspended in 100μL of TE buffer (containing 4μg/mL lysozyme) and lysed under high denaturing conditions. The cell lysate was applied to the RNeasy spin column and the column was rinsed with a series of wash buffers supplied by the manufacturer. RNA adsorbed to the silica gel-based membrane within the column and was eluted with DEPC-treated water (0.1%(v/v) diethyl pyrocarbonate). The composition of the supplied lysate and wash buffers is unknown due to proprietary infringements.

The quantity of RNA in each extraction was determined by measuring the absorbance at A_{260nm}. Purity was assessed by comparing the relative ratios between A_{260nm} and A_{280nm}. Samples that had a A_{260nm}:A_{280nm} higher than 1.6 were considered to be fairly pure and were used for the synthesis of the complementary DNA strand (cDNA).

The samples were diluted in 10μL RNAase-free distilled water (treated with 0.1%(v/v) diethylpyrocarbonate) to a final concentration of 0.1μg RNA per 1μL of water. Next, 2μL hexanucleotide mix (0.5M Tris-HCl, 0.1$MgCl_2$, 1mM dithiotherythretol, 2mg/ml BSA, random primers, pH7.2) was added to the RNA solution, incubated for 10 minutes at 70°C, and then quickly chilled on ice. The contents were collected by centrifugation and the following reagents were added: 4μL 5xRT buffer(0.25M Tris-HCl, 40mM $MgCl_2$, 150mM KCl, pH8.5), 2μL dithiothreitol (100mM), 1μL PCR nucleotide mix (10mM ATP, 10mM CTP, 10mM GTP and 10mM TTP) and 20U RNAase inhibitor. The contents were mixed gently and incubated for 2 minutes at 37°C to equilibrate the temperature of the solution before the addition of 20U reverse transcriptase. The solution was mixed gently and incubated for 1 hour at 42°C and stored on ice until ready to use.

Polymerase chain reaction (PCR) amplification of the cDNA was accomplished using specific primers (described in Chapter 4). The PCR reaction was performed by aliquoting 10μL cDNA sample into a PCR tube, 1μL of each primer, 10μL 10xPCR buffer (100mM Tris-HCl, 15mM $MgCl_2$, 500mM KCl, pH8.3), 2μL PCR Nucleotide mix (10mM ATP, 10mM CTP, 10mM GTP and 10mM TTP) and 3U Taq DNA polymerase. The solution was diluted to a final volume of 100μL and two drops of mineral oil was added to the

surface of the solution to prevent the reaction mixture from evaporating during the PCR amplification process. The fragment was then amplified using the following temperature cycling conditions: 1 minute at 94°C, 1 minute at 55°C, and 2 minutes at 72°C for thirty cycles. The final stage was set for 5 minutes at 72°C and the samples were then analysed by agarose gel electrophoresis.

2.6.2 β-Galactosidase Assay

The β-galactosidase activity was measured from cultured bacteria. The substrate o-nitrophenyl-β-D-galactoside (ONPG) is converted to galactose and *o*-nitophenol, an intense yellow compound which can be measured spectrophotometrically at A_{420nm}.

A 5mL bacterial dilution (1:25) was prepared from an overnight culture and grown for a period of 2 to 3 hours at 37°C with vigorous shaking. To obtain an indication of the amount of growth which occurred over this incubation period, the OD_{660} was measured from a culture aliquot diluted in PBS buffer (8.7mM Na_2HPO_4, 1.3mM NaH_2PO_4, 145mM NaCl, pH7.6). To measure the amount of β-galactosidase activity, a cell suspension was prepared by aliquoting 100μL of bacteria into 900μL minimal media containing chloramphenicol (50μg/mL) to halt further microbial growth. The bacteria were lysed by vigorous shaking with 100μL lysis solution (1.25%(w/v) SDS,

12.5%(v/v) toluene, 2.5mM $MnCl_2$, 62.5% β-mercaptoethanol). At time zero, 200μL chromogen substrate solution (4mg/mL ONPG, 0.1M phosphate buffer, pH7.0) was added and after exactly ten minutes had elapsed, the enzymatic activity of β-galactosidase was terminated by the addition of 500μL stop solution (20%(w/v) Na_2CO_3). To determine the amount of β-galactosidase activity , the reaction mixture was diluted with 500μL distilled water and the absorbance (A_{420} and A_{550}) was measured spectrophotometrically. The total units of β-galactosidase activity was determined by using the following formula (Miller and Mekalanos, 1988):

$$\beta - galactosidase\ activity = 1000\left[\frac{A_{420} - (1.75 \times A_{550})}{(Time \times Volume) \times OD_{660}}\right]$$

2.7 Light and Electron Microscopy

Observations of bacterial cells were accomplished using standard microscopic techniques. Light microscope observation were made by using phase contrast optics. Preparations for electron microscopy were resuspended in PBS buffer (8.7mM Na_2HPO_4, 1.3mM NaH_2PO_4, 145mM NaCl, pH7.6), placed on grids washed several times, stained negatively with 1%(w/v) ammonium molybdate, and examined utilising a transmission electron microscope (Phillips 300 Electron Microscope).

2.8 Radioactive Labelling of O7-subunits

2.8.1 Radiolabelling Incorporation

The following protocol for the labelling of O-antigen was adapted from Marino *et al.* (1985).

Overnight cultures were grown in M9 minimal medium supplemented with glycerol as the sole carbon source. Cultures were diluted the next day to an OD_{600nm}=0.25 and were grown to mid log phase (OD_{600nm}=0.5) with vigorous shaking at 37°C. At this point, ^{3}H-mannose (10μCi) was added to the growing cell culture, incubated for an additional 2 minutes at 37°C, and then transferred to an ice-cold water bath to stop growth. The cells were harvested by centrifugation (5,000×*g*, 10 minutes, 4°C), resuspended in 10mL ice-cold suspension buffer (10mM Hepes, 25%(w/v) sucrose, 4μg/mL RNAase, 4μL DNAase, pH7.4) and passed through a French Press 3 times at 10,000psi. The cell lysate was centrifuged (5,000×*g*, 10 minutes, 4°C) to precipitate any unbroken cells and then layered on a 60%(w/w) sucrose cushion (10mM Hepes, pH7.4). The cushion was centrifuged at (100,000×*g*, SW41 rotor, 3 hours, 6°C) to concentrate the cellular membranes at the interface of the sucrose cushion and the interface was collected by bottom puncture. The concentrated membrane was adjusted to a sucrose concentration of 25%(w/v) with 10mM Hepes, diluted to 2mL with 25%(w/v)

sucrose (10mM Hepes, pH7.4) and layered onto a 30%-60%(w/v) sucrose step gradient. The sucrose gradient was prepared by successively layering 1.5mL aliquots of decreasing sucrose concentrations (55%, 50%, 45%, 40%, 35%, 30%(w/v) sucrose in 10mM Hepes, pH7.4) onto a 1.0mL 60%(w/v) sucrose cushion. A small piece of cork on the surface of the building gradient prevented the mixture of adjacent sucrose layers (Vareli and Frangou-Lazaridis, 1996)

The gradient was centrifuged (220,000×*g*, SW41 rotor, 24 hours, 4°C) and fractionated into approximately 20 fractions by bottom puncture.

2.8.2 Fraction Analysis

The incorporation of ^{3}H-mannose in each of the fractions was assessed by measuring the amount of radiolabel present in a small aliquot of each collected fraction in a scintillation counter (Beckman LS7500 Scintillation Counter, Palo Alto, California). Protein was quantified spectrophotometrically (A_{280nm}) in each of the individual fractions. Protein peaks were indicative of the elution of separated cytoplasmic and outer membranes from the gradient. The relative incorporation of radiolabel counts in the outer membrane as compared to the inner membrane was used to determine the amount of 07 LPS biosynthesis intermediate which accumulated in the cytoplasmic membrane.

Lactate dehydrogenase, a cytoplasmic membrane enzyme, was used as a marker to locate the fraction which contains the cytoplasmic membrane from the sucrose gradient. Since the activity of lactate dehydrogenase declines quite rapidly if stored at 4°C or at -20°C, this assay was performed on fresh samples. The activity of lactate dehydrogenase was measured by spectrophotometrically monitoring the conversion of NAD^+ to NADH in an incubation mixture containing 50μL β-NAD (172mM) and 1.2mL substrate buffer (112mM Tris, 56mM lithium lactate, 170mM KCl, pH9.1). The reaction was initiated by the addition of 50μL sucrose gradient fraction and enzymatic rate was monitored by recording A_{339nm} at the time intervals of 0.5, 1, 2, 5, and 10 minutes. The catalytic activity was determined using the following formula (the time and absorbance differential was calculated between one of the time intervals and 0.5 minutes) (Bergmeyer et al., 1983).

$$catalytic\ activity = \frac{\Delta A_{339nm} \times V_{Assay}}{\varepsilon \times \ell \times \Delta t}$$

$$= \frac{\Delta A_{339nm}}{4.84 \times \Delta t}\ \mu mol / \min(U)$$

Chapter Three

The TolA protein is Required for O7-LPS Surface Expression

3.1 Introduction

The primary focus of this project was to elucidate the function of the *tol* genes in the expression of O-antigen associated lipopolysaccharide.

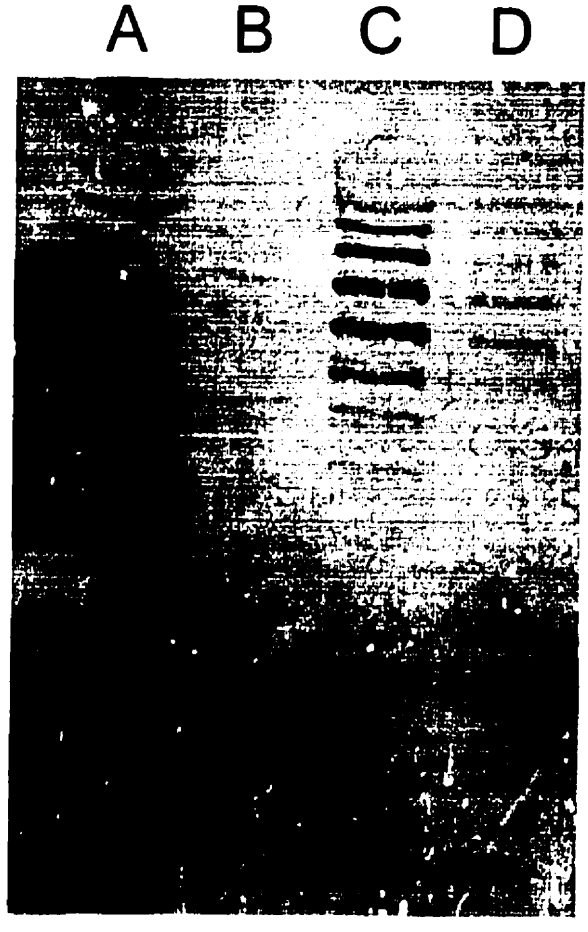
Initial experiments conducted to determine whether the Tol system plays a role in the expression of O-specific LPS were obtained by comparative analysis of lipopolysaccharide migratory profiles. LPS samples were prepared from the *E.coli* K-12 *tol* mutants and their respective parental strains and analysed by SDS-polyacrylamide gel electrophoresis. *E.coli* K-12 strains do not endogenously express O-antigen due to mutation(s) in the *rfb* gene cluster (Liu and Reeves, 1994). However, *E.coli* K-12 can support the synthesis of O-antigen by the introduction of a plasmid containing O-specific *rfb* genes. In the following experiments, the cosmid pJHCV32 containing the cloned heteropolysaccharide O7-specific *rfb* genes from the wild-type strain VW187 was utilised for the expression of O7-antigen in *E.coli* K-12.

3.2 Effects of *tol* mutations on O7-specific LPS Expression in *E.coli* K-12

To study the effect of the Tol import system in the expression of O-antigen, LPS was extracted from *E.coli* K-12 strains C600(pJHCV32), P90C(pJHCV32), A592(pJHCV32) and P90C*tolQ*(pJHCV32) and analysed by Tris-Glycine SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblot. LPS samples isolated from these strains (Figure 5) produce a ladder banding pattern that is characteristic of the variation in O-antigen heterogenic polymerisation. Samples were typically standardised on the basis of KDO concentration or total protein content. Lane intensity comparisons between the *tol* mutants, A592(pJHCV32) in lane B and P90C*tolQ*(pJHCV32) in lane D, illustrates a definitive reduction in O7-specific LPS expression relative to their respective isogenic parental strains C600(pJHCV32) in Lane A and P90C(pJHCV32) in Lane C. By visual observation, the *tolA* mutation results in approximately 10-fold decrease in O7-LPS expression while the *tolQ* mutation exhibits a 2-fold reduction. No effects were found in the O7-LPS expression of *tolR*, *tolB*, and *tolC* mutants (data not shown).

Figure 5. O7-specific LPS migratory profiles from *tol* mutants and their respective parental strains

Western blot of LPS extracts from *E. coli* strains containing pJHCV32. LPS samples were standardised on the basis of total cellular protein, analysed by 14%(w/v) acrylamide SDS-PAGE, transferred to nitro-cellulose and reacted with O7-specific rabbit polyclonal antiserum. Lanes: A, C600(pJHCV32); B, A592(pJHCV32); C, P90C(pJHCV32); D, P90C*tolQ*(pJHCV32).


A
B
C
D

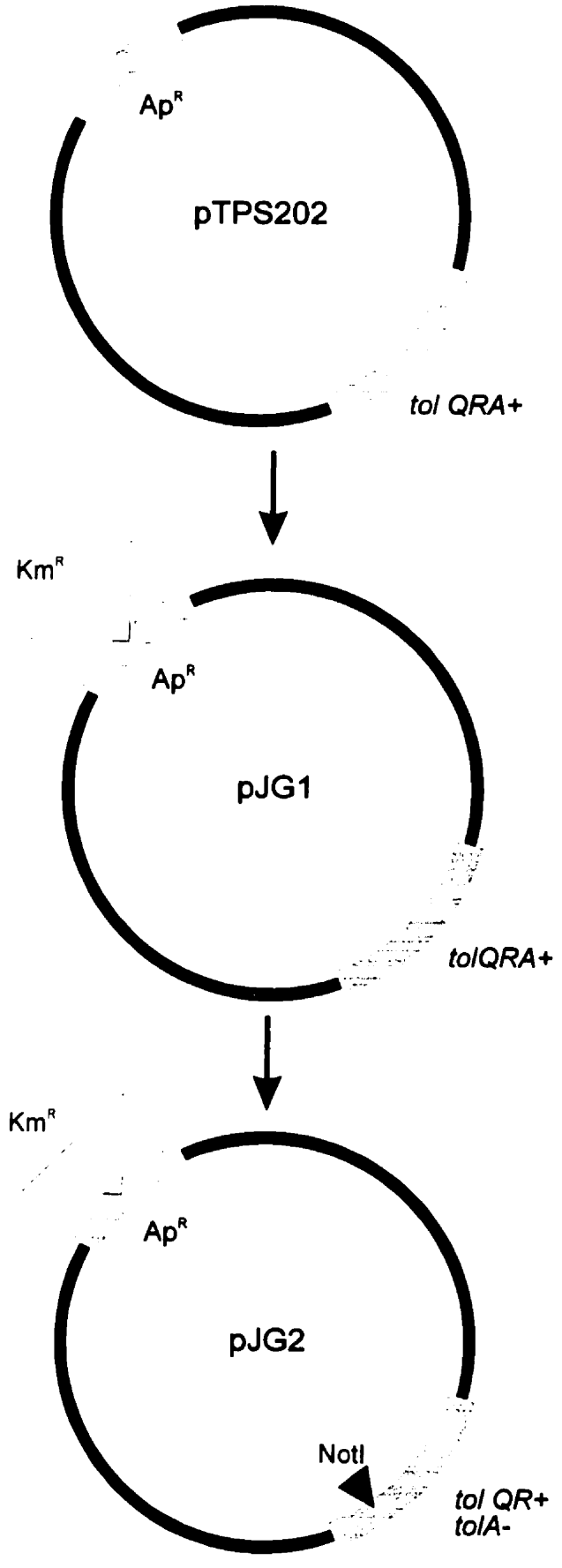
3.3 Complementation of *tol* mutants

The plasmid pTPS202 (Figure 6), a derivative of pBR322 containing an 8.7kbp *BglII* fragment with the *tol* gene cluster (*orf1tolQRAB*), has been shown in our laboratory to complement *tolQ* and *tolA* mutations in P90C*tolQ* and A592 respectively (Thomas and Valvano, 1993). This has been assessed by the elimination of the *tol*-associated pleiotropic phenotypes such as restored sensitivity to colicins (Cloacin DF13, colicin N) and restored resistance to hydrophobic detergents (deoxycholate). In addition, pTPS202 can restore the O7 phenotypic expression in *E.coli* K-12 (pJHCV32) *tol* mutants.

The strain P90C*tolQ* contains a mutation in codon 36 of *tolQ* which has been shown to reduce the expression of TolA by approximately 50% (Vianney et al., 1996). To determine if the decrease in O7-LPS expression in P90CtolQ is due to a polar effect on the expression of the downstream gene *tolA*, a plasmid was constructed which over-expresses *tolQ* and *tolR* and has a non-functional *tolA* gene. A derivative of pJG1 containing a frame-shift mutation in codon 292 of *tolA*, was constructed and designated as pJG2 (Figure 6). The frame-shift mutation was introduced into *tolA* by the digestion of pJG1 with *NotI* restriction enzyme which internally cleaves the *tolA* gene. The overhanging ends were filled with Klenow polymerase, religated with T4 DNA

Figure 6. Construction of pJG1 and pJG2 plasmids

Plasmid pTPS202 can complement mutation in genes encoding for TolQ, -R, and -A. pJG1 was derived from pTPS202 by the insertion of a kanamycin resistance gene cassette into the ScaI site of the ampicillin resistance gene. pJG2 was derived from pJG1 by the insertion of a frame-shift mutation in the NotI site of the *tolA* gene.


Ap^R
pTPS202
tol QRA+
Km^R
Ap^R
pJG1
tolQRA+
Km^R
Ap^R
pJG2
NotI
tol QR+
tolA-

ligase and the ligation mixture was transformed into *E.coli* DH5α. A plasmid preparation was isolated from DH5α(pJG2) and the presence of the frame-shift mutation was confirmed by the absence of the *NotI* restriction site. pJG2 was transformed into strains A592 and P90C*tolQ* to assess the functional properties of this new construct. As expected, pJG2 was unable to complement the *tolA* phenotype in A592 as assessed by sensitivity to deoxycholate and reduced expression of O7-antigen. In the case of P90C*tolQ*, pJG2 partially restored sensitivity to deoxycholate but failed to restore O7 phenotypic expression. Data collected in these experiments suggests that TolA is directly involved in the expression of O7-antigen and TolQ is indirectly involved as a consequence of a polar effect on the expression of *tolA*.

3.4 Effects of *tol* mutations on LipidA-core

LPS is composed of two covalently linked, independently synthesised components: lipidA-core and O-antigen (Figure 4). The expression of lipidA-core is necessary for the co-expression of O-antigen but the reverse is not true. Since the expression of O-antigen is dependent on its association with lipidA-core, the observed decrease of O7-LPS in *tolA* and *tolQ* mutants may be associated with either a decrease in lipidA-core or an alteration in the lipidA-core structure which prevents the formation of the covalent bond.

3.4.1 Qualitative and Quantitative Assessment of LipidA-core

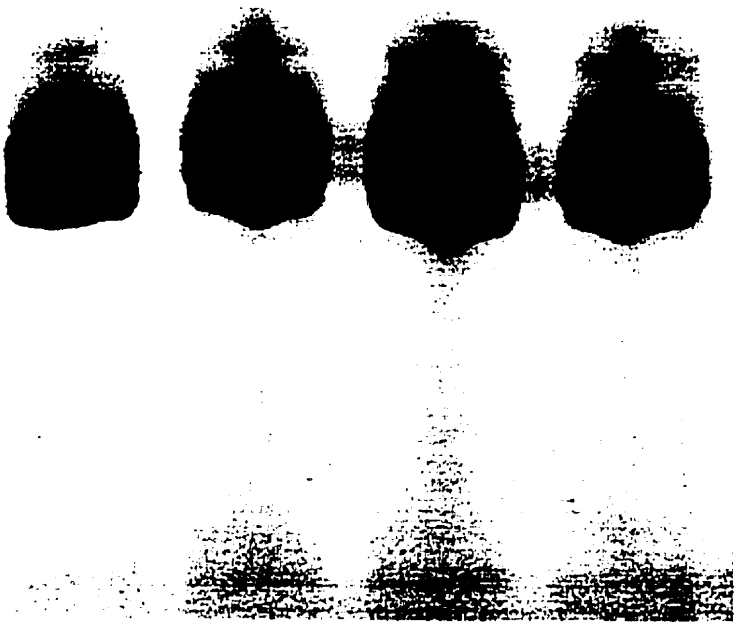
To determine if the decrease in LPS expression in *tol* mutants was due to a structural defect of lipidA-core, the structure was analysed by Tricine SDS-PAGE. LPS was extracted from *tol* mutants A592(*tolA*) and P90C*tolQ* and their respective parental strains C600 and P90C. The lipid-A core samples were electrophoresed and developed by silver staining (Figure 7). Comparisons between different lipidA-core migratory profiles revealed that the lipidA-core isolated from the *tol* mutants and their isogenic parental strains were identical indicating that the lipidA-core isolated from each strain are structurally similar. LipidA-core samples isolated from *E.coli* K-12 strains are chemically heterogeneous exhibiting three bands in Tricine SDS-PAGE gels. This heterogeneity in migration reflects a chemical modification of the core. The three bands are believed to correspond to (i) a substitution of partial O-units from bactoprenyl lipid to lipidA-core, (ii) a lipidA-core precursor for O-antigen attachment and (iii) a form of rough LPS, termed lipooligosaccharide (LOS) (Schnaitman and Klena, 1993).

The relative intensity of the lipidA-core bands suggested that no apparent differences in the amount of lipidA-core existed in *tolQ* and *tolA* mutants. To obtain a quantitative measurement rather than a qualitative assessment of the total amount of lipidA-core in each sample, a keto-deoxy-octusolanic acid (KDO) assay was performed. KDO is a sugar which is

Figure 7. LipidA-core migratory profiles from *tol* mutants and their respective parental strains

Silver-stained SDS-Tricine polyacrylamide gel of LPS extracts from *E. coli* K-12 strains not containing plasmids encoding for the *rfb* genes (required for O-antigen synthesis). Lanes: A, P90C; B, P90C*tolQ*; C, C600; D, A592.

A B C D



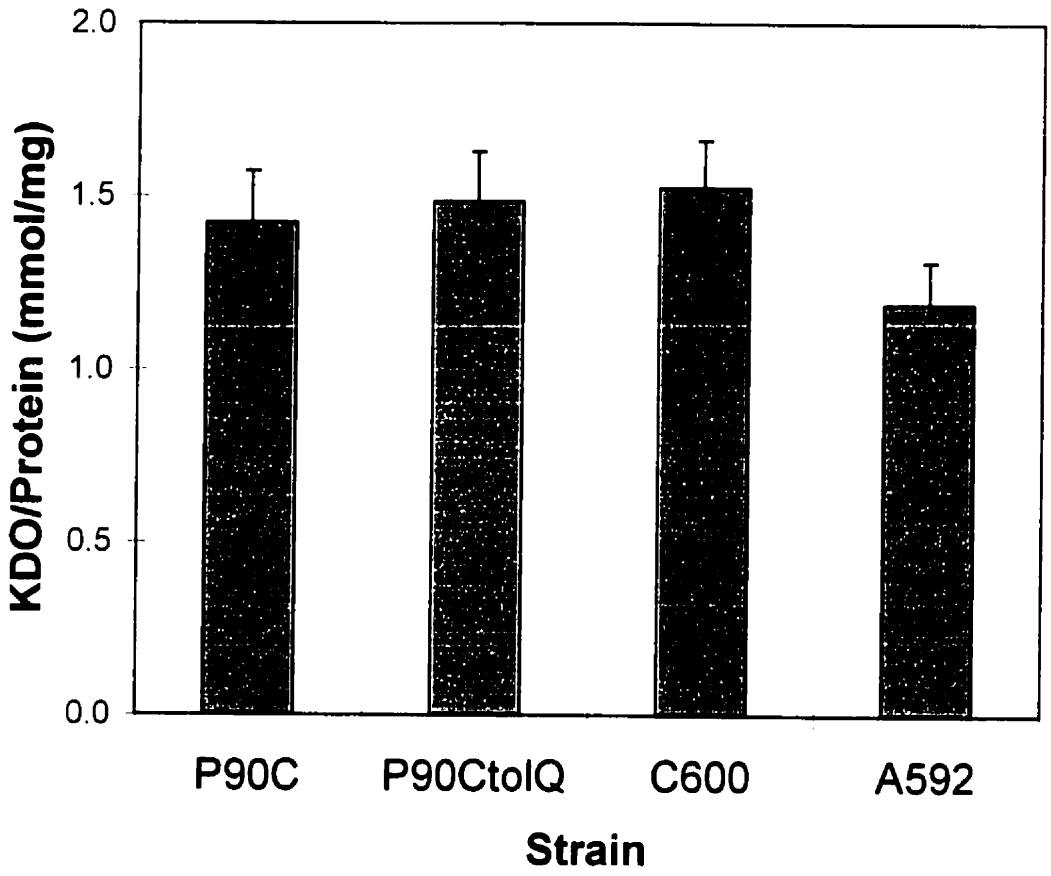
unique to the core moiety and present in stoichiometric quantities. Therefore, a measurement of KDO in a prepared LPS sample could be directly correlated to the amount of LPS present in the sample. The quantity of KDO in strains C600, P90C, A592 and P90C*tolQ* was measured and normalised against the total protein content of the LPS sample (Figure 8) (Armitage and Berry, 1994). The data collected from this experiment suggests that there is a statistically significant reduction (P=0.032) of about 25% less lipidA-core in the *tolA* mutant and no statistically significant reduction (P>0.05) of lipidA-core in the *tolQ* mutant as calculated by comparison of two-sample means (z-test). Therefore, the *tolA* mutation is associated with a reduced amount of lipidA-core with no apparent structural differences in the lipidA-core of the parental strain. However, the reduction in the amount of lipidA-core cannot account for the practically complete disappearance of O7-polysaccharide in the *tolA* mutant.

3.5 TolA and O7-antigen Gene Transcription

The dramatic effect of *tolA* on the expression of O7-antigen could be due to a down regulation of the O7 biosynthetic genes at the transcriptional level. Initial attempts to detect transcriptional levels of the O7-specific mRNA in *E.coli* K-12 *tol* mutants and their parental strains transformed with pJHCV32 were performed by the classical method of Northern blot hybridisation. An

Figure 8. Quantification of lipidA-core in *tol* mutants and their respective parental strains

The error bars are representative of the standard deviation between measurements.


2.0
1.5
1.0
0.5
0.0
KDO/Protein (mmol/mg)
P90C
P90CtolQ
C600
A592
Strain

anti-sense mRNA probe was synthesised in vitro which annealed to an internal region of the *rfbM* gene transcript. This DIG-dUTP labelled probe was used for hybridisation with total RNA prepared from the various *E.coli* K-12 strains. The template plasmid used to synthesise the labelled probe was used as a positive control in the hybridisation experiment. In these preliminary experiments, O7-specific mRNA was undetectable even under low stringency conditions (data not shown). Since O-antigen genes are expressed at relatively low levels and since the probe was designed to anneal to the 3'-end of the mRNA, the inability to detect any message may have been due to rapid degradation of the mRNA.

Therefore to assess the transcription of the O7-specific mRNA more sensitive procedures were employed such as reverse transcriptase polymerase chain reaction (RT-PCR) and a reporter gene (β-galactosidase) enzymatic assay.

3.5.1 Detection of O7 transcript by RT-PCR

To obtain a semi-quantitative assessment of the expression levels of O7 antigen gene transcription, reverse transcriptase PCR was utilised. Total RNA was isolated from *tol* mutants A592(pJHCV32) and P90C*tolQ*(pJHCV32) and their respective parental strains C600(pJHCV32) and P90C(pJHCV32). The samples were standardised on the basis of total RNA. To maximise the

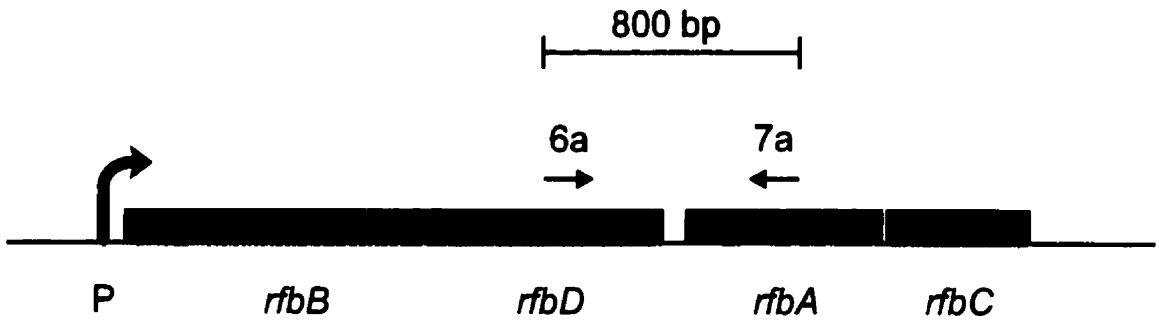
probability of detecting message that is relatively unstable, a small region at the 5'-end of the O7 mRNA was amplified to reduce the possibility of not detecting the mRNA as a result of degradation from the 3'-end to the 5'-end. The synthetic oligonucleotide primer 7a (5'-GACACCATAACGTTCAG-3') was designed to anneal internally to the third gene (*rfbD*) of the single O7 transcriptional unit and reverse transcriptase was used to synthesise the complementary DNA strand which spans the 5'end of the messenger RNA from the O7 promoter. The cDNA strand was amplified by Taq DNA polymerase with primer 7a and primer 6a (5'-CGCGTAGCAGTAGACAA-3') which anneals internally to the second gene (*rfbA*) in the O7-specific cDNA (Figure 9, Panel a).

The amplified PCR fragments were electrophoresed (Figure 9, Panel b) in an agarose gel. As expected, a DNA fragment that migrated a distance that corresponding to an 800bp DNA fragment was detected in each lane. There was no discernible difference in the intensity of the PCR product between A592(pJHCV32) and C600(pJHCV32) and between P90C*tolQ*(pJHCV32) and P90C(pJHCV32). A second unaccountable PCR product, corresponds to a fragment of 1.2kbp, was also detected in each of the reactions but was not further characterised.

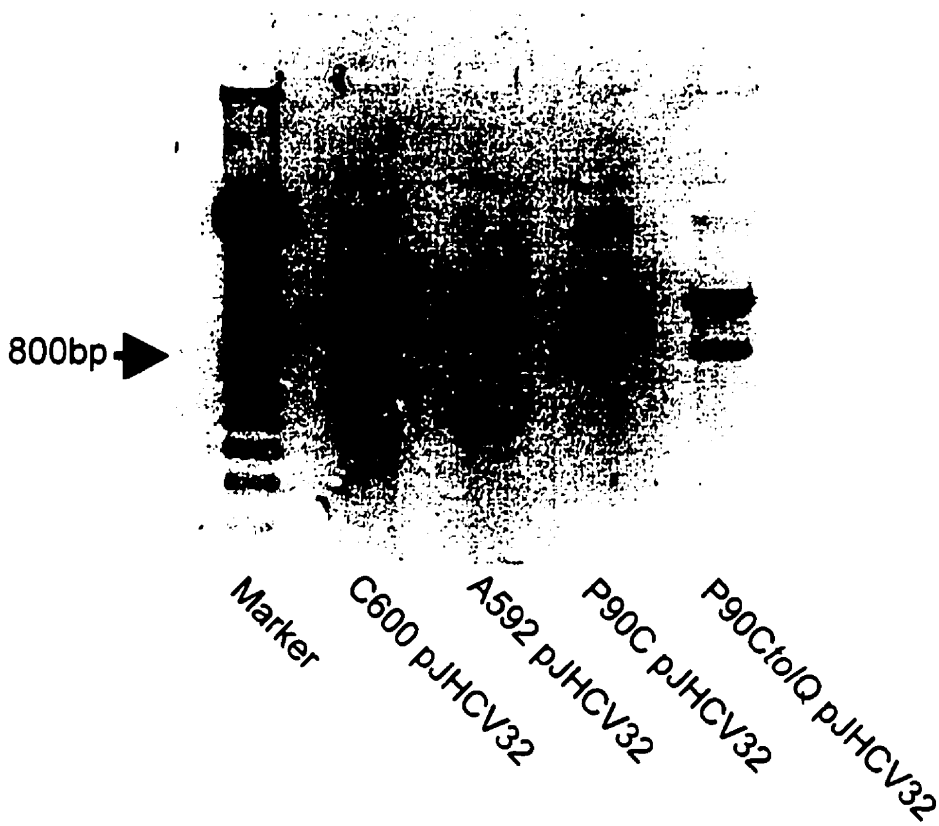
Figure 9. Reverse transcriptase polymerase chain reaction (RT-PCR) in the detection O7-*rfb* gene transcription

Panel a: Organisation of the 5' region of *rfb* gene cluster in *E.coli* VW187. Primers 6a and 7a were used to amplify a 800bp region at the 5'-end of the O7-lipopolysaccharide *rfb* gene cluster. Panel b: Agarose gel stained with ethidium bromide. The marker consists of 100bp incremental fragments.

A


800 bp
6a
7a
P
rfbB
rfbD
rfbA
rfbC

B


800bp
Marker
C600 pJHCV32
A592 pJHCV32
P90C pJHCV32
P90CtoIQ pJHCV32

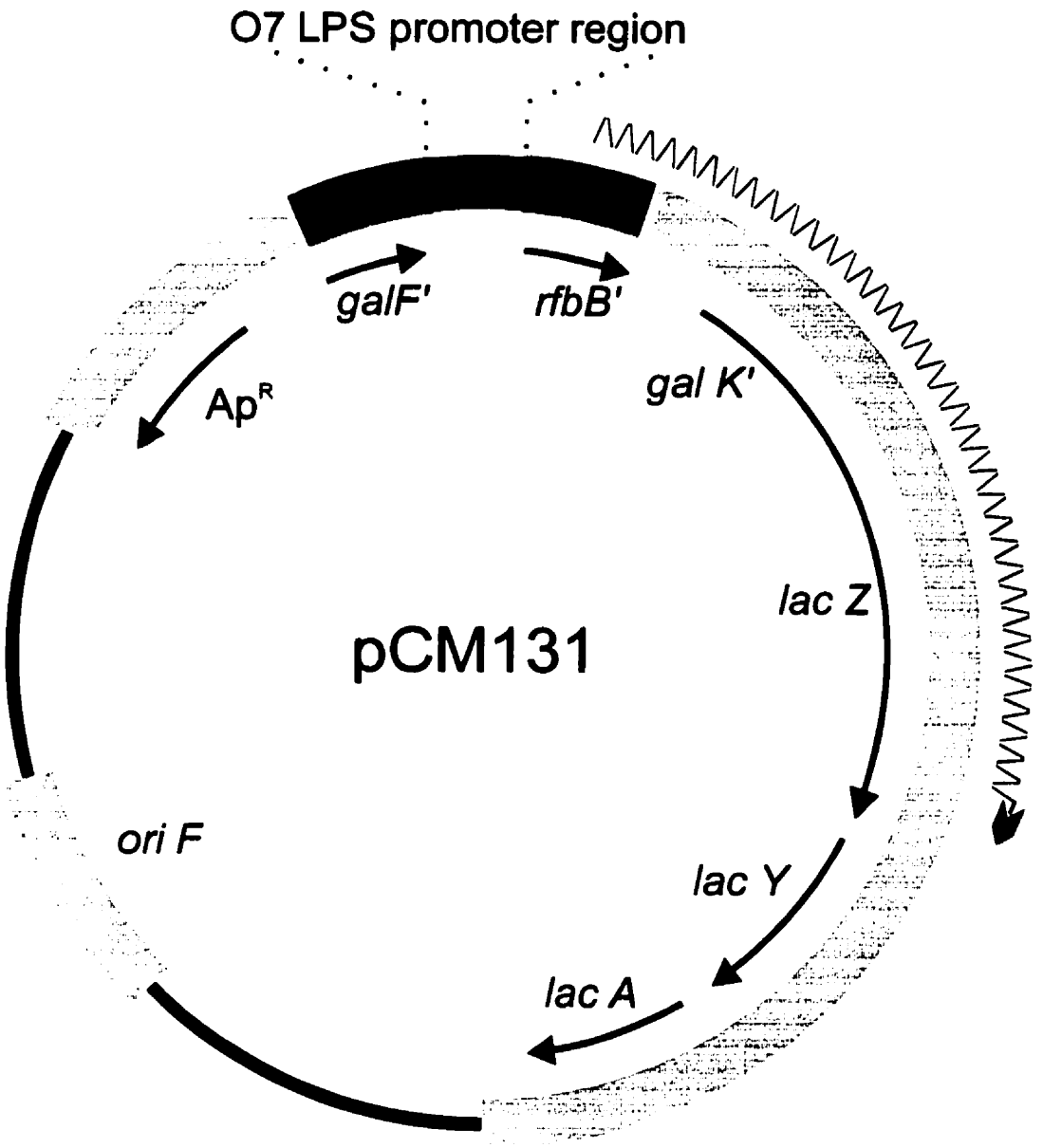
3.5.2 Quantification by β-Galactosidase

To measure the transcriptional levels of the O7 genes in *tol* mutants and their parental strains, a β-galactosidase reporter plasmid was utilised. The plasmid pCM131 (Figure 10), constructed in our laboratory by Cristina Marolda, contains a transcriptional fusion between the O7 promoter and the promoterless *lac* operon. pCM131 is a low copy plasmid with 1-2 copies per chromosome and as a consequence of the low copy number, pCM131 emulates chromosomal O7 transcriptional levels. Therefore a measurement of β-galactosidase activity present in the transformed K-12 strains could be directly correlated to the transcriptional activity of the O7 promoter.

To avoid differences that may occur due to plasmid instability or over expression of *tolQ* and *tolR* in strains that have a *tolA* or *tolA*$^+$ genotype, we decided to use the isogenic pair A592(pJG1, pCM131) and A592(pJG2, pCM131) to conduct these experiments. The plasmid designated as pJG1 was constructed from pTPS202 by the replacement of the ampicillin gene with a kanamycin resistance marker (Figure 6). This was accomplished by the digestion of pTPS202 with restriction enzyme *ScaI* which cleaves the plasmid within the ampicillin gene. Next, the 1.4kbp *HincII* kanamycin cassette from pUC4K was inserted into the ampicillin gene by religation. This new construct was transformed into *E.coli* strain DH5α and the cells were plated on LB agar

Figure 10. Structure of the reporter plasmid pCM131

This low copy plasmid was constructed so that the O7-lipopolysaccharide promoter is transcriptionally fused with *lacZ* gene. Therefore the activity of β-galactosidase activity directly correlates to the transcriptional level from the O7 promoter. Arrows indicate the direction of gene transcription. The wavy arrow represents the transcriptional unit from the O7 promoter.


O7 LPS promoter region
galF'
rfbB'
gal K'
Ap^R
lac Z
pCM131
ori F
lac Y
lac A

plates supplemented with kanamycin. The loss of the ampicillin marker was assessed by replica plating. To confirm that pJG1 could complement the *tolA* and *tolQ* mutations, plasmid prepared from DH5α(pJG1) was transformed into strain A592 and P90C*tolQ*. The transformants were screened for sensitivity to Cloacin DF13 and resistance to deoxycholate. The construction of pJG2 was described earlier in this chapter.

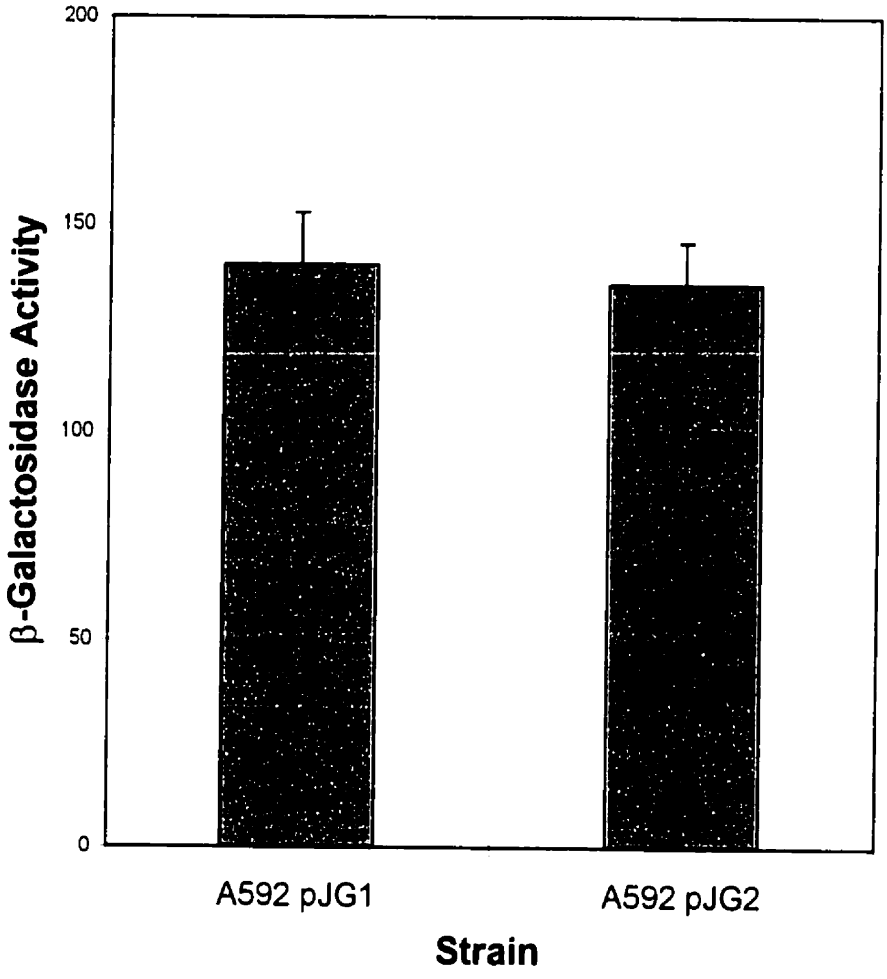
In order to assay the β-galactosidase activity resulting from initiation of transcription from the O7 promoter, A592(pJG1, pCM131) and A592(pJG2, pCM131), were grown to mid-log phase in the presence of 1%(w/v) glucose to inhibit the endogenous production of β-galactosidase. This experiment was conducted in triplicate from three independently inoculated cultures, and the β-galactosidase activity was measured as described in the material and methods. Comparisons between the two isogenic strains revealed the O7 promoter strength was equivalent in the *tolA* mutants and the complemented strain (Figure 11).

3.6 Conclusions

Initial observations indicate that the Tol import system plays a role in the surface expression of O7-specific LPS. Although *tolQ* and *tolA* mutants exhibit a decrease in surface expressed O-antigen, the mutant strains can

Figure 11. β-galactosidase activity from *tolA* mutant and its isogenic parental strain

The error bars are representative of the standard deviation between measurements.


200
150
100
50
0
β-Galactosidase Activity
A592 pJG1
A592 pJG2
Strain

form a complete O7-antigen as judged by its reactivity with the O7 antiserum and by similar migratory profiles of O7-specific LPS by SDS-PAGE.

The genes *tolA* and *tolQ* comprise an operon structure where *tolA* is the last gene in the transcriptional unit and transcription is initiated upstream from *tolQ* (Vianney et al., 1996). Upon complementation of the *tolQ* gene in P90C*tolQ* with pJG2, neither the pleiotropic phenotype is eliminated nor the expressional levels of O7 synthesis restored to that of the parental strain, suggesting that polar mutations in *tolQ* compromise the expression of the *tolA* gene located downstream. Therefore, lower expression of *tolA*, whether as a result of *tolA* mutations or a polar effect of *tolQ* mutations, are most likely the primary cause of the observed decrease in O7-LPS surface expression.

The first hypothesis proposed to explain the O7-LPS decrease in *tol* mutants was that this effect may be caused by either an altered structure or a decrease in expression of lipidA-core. LipidA-core samples isolated from *tol* mutants and their parental strains migrate the same distance and are homogenic in composition suggesting that the structure of lipidA-core is not affected in *tol* mutants. The KDO assay results indicates that a decrease in the amount of lipidA-core occurs in the *tolA* mutant, however this decrease cannot explain the marked decrease of surface expressed O-LPS. Therefore, we conclude that the effect of TolA is predominantly affecting the expression of the O7-antigen.

The results obtained from the RT-PCR and β-galactosidase reporter gene assay indicate that the transcription of O7 genes is not down-regulated in *tolA* mutants. Since the *tolA* mutation does not compromise the synthesis of O-antigen at the transcriptional level, we postulate that its effect is exerted at a post-transcriptional level, probably in steps involved with the assembly and processing of O-antigen.

Chapter Four

The TolA protein is Involved in the Processing of O7-subunits

4.1 Introduction

Previous experiments have shown that the expression of O7-specific LPS mediated by pJHCV32 in *E.coli* K-12 is substantially lower in comparison to the wild-type *E.coli* strain VW187 (Marolda et al., 1990; Valvano and Crosa, 1989). This observation may be attributed to the relative instability of the cosmid clone pJHCV32 in *E.coli* K-12 strains. In addition, the decrease in O-specific LPS in *tolQ* and *tolA E.coli* K-12 mutants may be due to trivial causes such as a decrease in plasmid stability. In order to gain more insight into the affect of *tol* mutations in a strain which expresses normal levels of O-antigen it was necessary to construct *tolA* and *tolQ* mutants in the wild-type strain VW187.

4.2 Construction of *tol* mutants in *E.coli* VW187

A gene replacement technique was used to create specific *tol* mutations in the *E.coli* wild type strain VW187 . This process involves the introduction of a

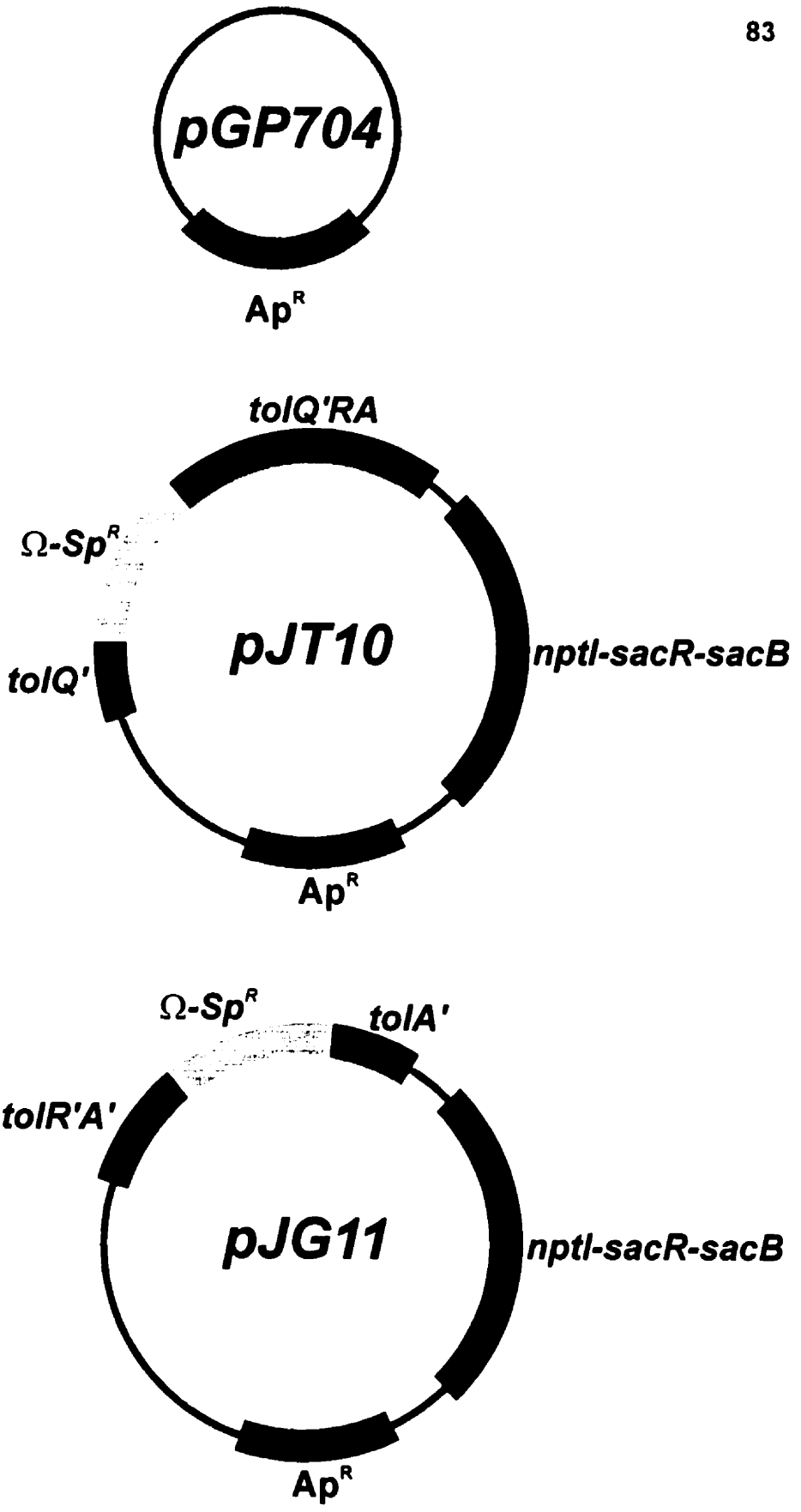
suicide vector, with specific properties, into the target strain followed by the selection of vector integration into the chromosome. The vector must posses a non-functional target gene (i.e. gene segments flanking an antibiotic marker) to promote homologous recombination into the chromosome. The vector must not be able to replicate within the target strain to ensure that the maintenance of the selection marker through cell division is due to vector integration and not vector replication. Since the integration of the vector results in the replication of the target gene, one functional and the other non-functional, the vector must also posses another selection marker to screen for a secondary cross-over event that excises the vector from the site of integration. The resulting mutant contains a single selective marker inserted into the chromosomal target gene.

4.2.1 Construction of Suicide Plasmids

Two suicide vectors, pJG11 and pJT10, were constructed to inactivate the *tolA* and *tolQ* genes respectively (Figure 12). These vectors are derivatives of pGP704 which can only be maintained in the host bacterial cell if the *pir* replication factor is supplied in trans (Miller and Mekalanos, 1988). These plasmids also contain the cloned *B.subtilis sacB* gene which serves as a positive selection marker for the second cross-over event. Levansucrase, the gene product of *sacB*, is lethal to several genera of gram-negative bacteria

Figure 12. Structure of mutagenic plasmids pJT10 and pJG11

These suicide plasmids were constructed in three stages (i) the insertion of the Ω interposon (Sm/Sp^R) within the coding regions of either *tolQ* or *tolA* genes; (ii) the cloning of the mutated gene fragment into the suicide plasmid pGP704; and (iii) the insertion of the *sacBR/nptI* (Km^R)cassette.


pGP704
Ap^R
tolQ'RA
Ω-Sp^R
pJT10
tolQ'
nptI-sacR-sacB
Ap^R
Ω-Sp^R
tolA'
tolR'A'
pJG11
nptI-sacR-sacB
Ap^R

when grown in the presence of 5%(w/v) sucrose (Steinmetz et al., 1985).

The plasmid pJG11 was constructed by inserting an 1.7kbp *NruI* fragment from pTPS202 containing the complete *tolA* and partial *tolR* genes into the *EcoRV* of pGP704. To inactivate the *tolA* gene, a spectinomycin interposon was isolated from pHP45Ω by digestion with *SmaI* and the 2.1kbp fragment was inserted into the unique *NotI* restriction site internal to the *tolA* gene. The Ω-spectinomycin interposon possesses strong translational and transcriptional terminators at either end of the fragment to prevent the transcription of downstream genes thus producing a polar mutation. Lastly, the 2.0kbp *BamHI* cassette from pUM24Cm containing the *sacBR-nptI* genes was inserted into the *BglII* site of pGP704. The *nptI* gene encodes for kanamycin resistance and serves as a positive marker for the selection of the final construct. Each sub-clone of pJG11 was transformed into the background strain *E.coli* SY327-λ *pir* which has a chromosomal copy of the *pir* replication factor.

The *tolQ* suicide plasmid, pJT10, was constructed in a similar manner by John Thomas, a previous student in our laboratory.

4.2.2 Introduction of *tolA* and *tolQ* mutations into *E.coli* VW187

pJG11 and pJT10 plasmid DNA were isolated and used to transform independently the conjugative strain *E.coli* BW19851. The introduction of the

suicide plasmid into the VW187-Nal^R was accomplished by conjugation with BW19851(pJG11) or BW19851(pJT10) as described in Chapter 2 (Material and Methods). Once the plasmid is transferred into the target strain, the plasmid may integrate into the chromosome due to homology between the functional chromosomal gene and the non-functional vector gene (Figure 13). The second cross-over event results in the replacement of the target gene with the spectinomycin Ω cassette flanked by the 5' and 3' regions of the target gene.

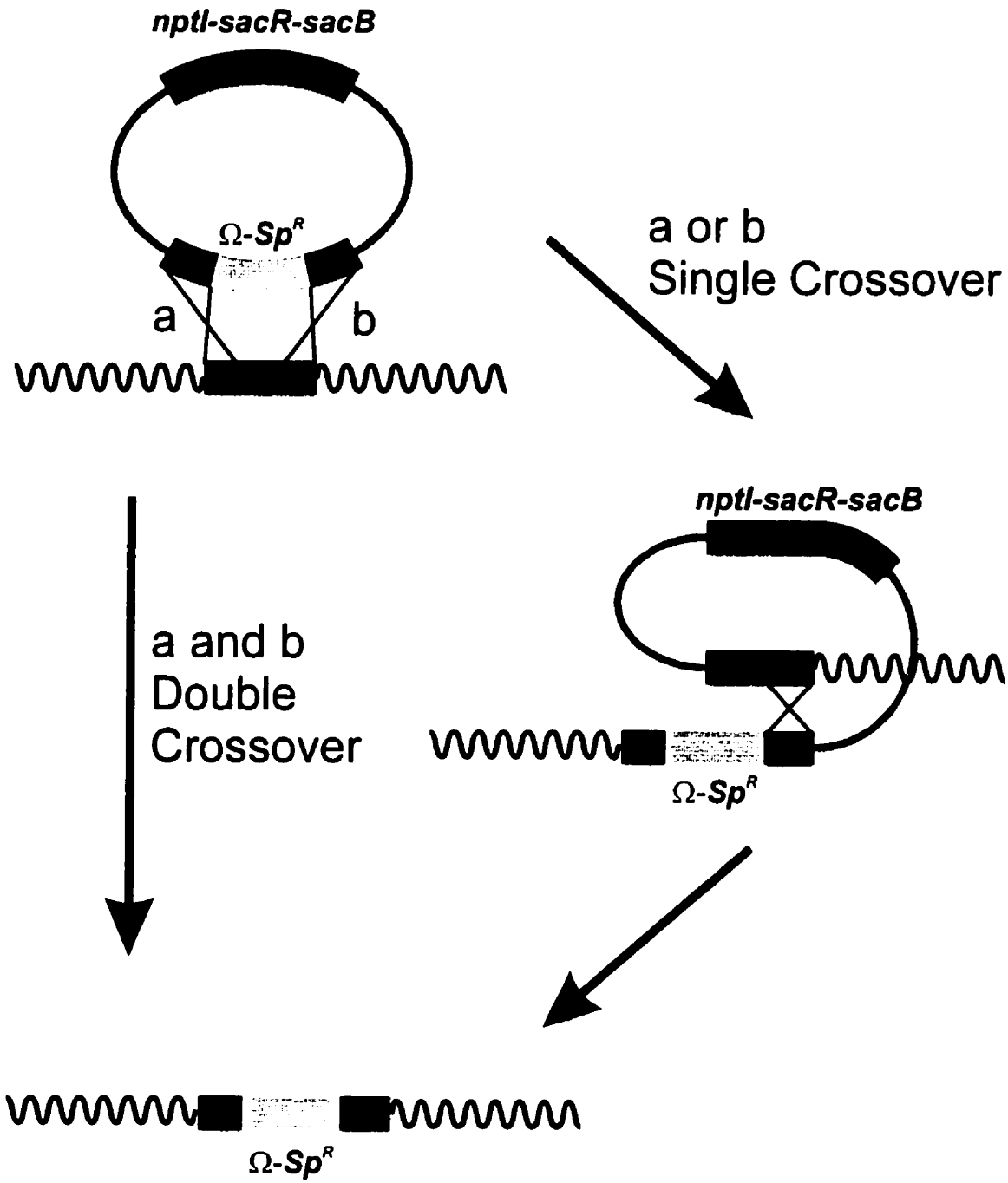
The conjugation mixture was plated on LB plates supplemented with nalidixic acid (target strain marker), spectinomycin (integration marker) and sucrose (vector excision marker). Colonies were screened by replica plating for sensitivity to both ampicillin and kanamycin. To confirm the presence of putative *tol* mutations, the colonies were also screened for sensitivity to colicin N and sensitivity to deoxycholate.

4.2.3 Characterisation of VW187 *tol* mutants

LPS extracts from VW187, VW187*tolQ*, and VW187*tolA* were analysed by Tris-Glycine SDS-PAGE. The *tol* mutants exhibited an overall reduction in surface expressed O7-LPS of approximately 50% and 95% for the *tolQ* and *tolA* mutants respectively (Data not shown).

Figure 13. Strategy employed to construct *tolA* and *tolQ* mutants in *E.coli* target strains

The mutagenesis process that is based on the integration of the mutated gene in the chromosome by homologous recombination, followed by the selection of double crossover events by plating bacteria on plates containing 5% sucrose and 80 μg/ml Sp. As a result of the crossover and the selection strategy, colonies are isolated that contain the mutated gene replacing the wild-type gene.


nptI-sacR-sacB
Ω-Sp^R
a
b
a or b
Single Crossover
a and b
Double
Crossover
nptI-sacR-sacB
Ω-Sp^R
Ω-Sp^R

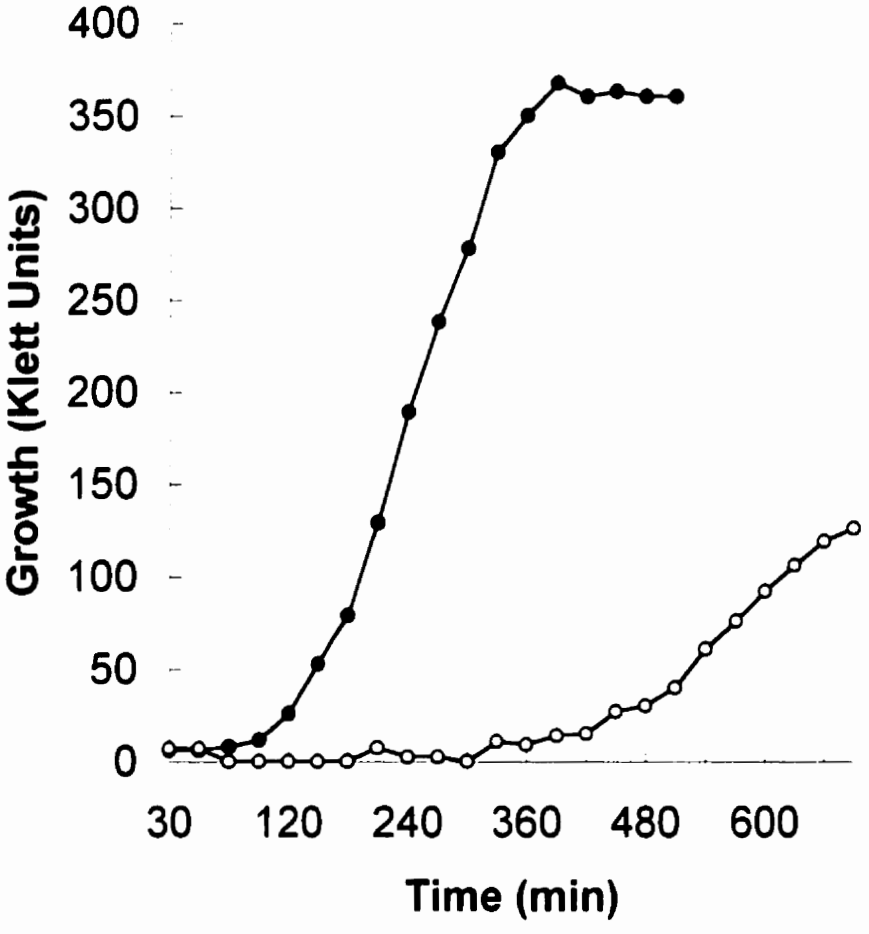
Interestingly, after a few passages the *tolA* mutant lost the ability to produce O7-antigen. Several attempts were made to obtain a stable *tolA* mutant in VW187 but each attempt resulted in the similar outcome. The presence of the *tolA* mutation was confirmed by the assessment of deoxycholate sensitivity and colicin tolerance. Therefore, secondary mutations may have developed to relieve the potentially lethal effect of O7 synthesis in a *tolA* mutant.

4.2.4 Growth Rate of VW187*tolQ*

During the preparation of bacterial cultures for LPS extraction, the observed rate of growth of VW187*tolQ* was substantially lower than the parental strain VW187. The growth rate of these two strains was assessed by measuring the optical density of the actively growing cultures over consecutive time intervals. Both cultures were grown in LB broth containing no antibiotics to exclude the possibility of antibiotic dependant effects on the growth rate of VW187*tolQ*. The optical density of both cultures was standardised at time zero and Figure 14 illustrates the growth rate of these strains. VW187 grows very quickly with a doubling time of approximately one hour. VW187*tolQ* on the other hand grows very slowly and experiences a fairly lengthy lag phase before cell growth is observed. This is in sharp contrast with the similar growth rate of A592 and P90C*tolQ* with respect to their respective parent

Figure 14. Growth curve of VW187 and VW187*tolQ*

Closed circles represent VW187, open circles represent VW187*tolQ*.


400
350
300
250
200
150
100
50
0
Growth (Klett Units)
30
120
240
360
480
600
Time (min)

strains in *E.coli* K12 (Thomas and Valvano, 1993). Therefore, O-antigen synthesis in the VW187*tolQ* mutant imposes a growth constraint.

4.3 Characterisation of *tol* mutants in *E.coli* VW187

The previous experiments indicated that the introduction of *tolA* mutations in the wild-type strain VW187 resulted in secondary mutations abolishing O-antigen synthesis and a *tolQ* mutant producing O7 LPS exhibited a reduction in growth rate. Therefore synthesis of O7 LPS exerts a deleterious effect in *tolA* and *tolQ* mutants. In order to prevent these effects, *tol* mutants can be constructed in a derivative of VW187 in which the synthesis of O-antigen can be controlled by complementation. The *E.coli* strain MV501(VW187*rfe*) contains a mutation in the *rfe* gene which encodes for the first transferase involved in the biosynthetic initialisation of heteropolymeric O-antigen subunits. Upon complementation of the *rfe* mutation with pMAV11, O7-antigen synthesis is restored (Alexander and Valvano, 1994).

Therefore the gene replacement strategy described above was used to construct *tolQ* and *tolA* mutants in MV501. These new mutants were designated JAG1 and JAG2, respectively, and displayed a growth rate comparable to that of the parent strain MV501.

4.3.1 Morphological Alterations of *tolA* and *tolQ* mutants VW187

To observe the effects of *tol* mutations in strains which endogenously express O-antigen, a series of complementation assays were performed. JAG1 and JAG2 were transformed with pMAV11 alone to complement the *rfe* mutation and thus restore O7-antigen synthesis, or transformed with pJG1 and pMAV11 to complement both the *tol* and *rfe* mutations.

JAG1 and JAG2 transformed with pMAV11 exhibited a decrease in the growth rate and altered cell morphology. Colonies grown on agar plates had a 'fried egg' appearance showing a central round dome surrounded by an irregularly shaped flat halo. These cells examined by light microscopy (phase contrast) were non-motile and elongated in comparison to their parental strains. JAG2(pMAV11) were approximately 10 times longer than VW187 and JAG1(pMAV11) also exhibited an elongated phenotype of approximately 3 fold. When the *tol* mutations were complemented in these strains, JAG1(pJG1, pMAV11) and JAG2(pJG1, pMAV11), the strains were phenotypically identical to VW187. The observed morphological changes are summarised in Table 4.

These transformants were also examined by electron microscopy. The elongated morphology of JAG2(pMAV11) is illustrated in Figure 15 (Panel c and Panel d). The cells appear to be filamentous in nature in comparison to JAG2 in Panel a and VW187*rfe* in Panel b. In addition, the cellular envelope

Table 4. Summary of properties of *tol* mutants in VW187

Strain	Relevant Genotype	Sensitivity to DOC	LPS Banding Pattern	Cell Morphology
VW187	wild-type	R	normal O7-LPS bands	short rods
VW187*rfe*	*rfe*	R	LipidA-core band	short rods
JAG1	*rfe tolQ*	S	LipidA-core band	short rods
JAG1(pMAV11)	*rfe*$^+$ *tolQ*	S	reduced amount of O7-LPS	long rods
JAG1(pMAV11, pJG1)	*rfe*$^+$ *tolQ*$^+$	R	normal O7-LPS bands	short rods
JAG2	*rfe tolA*	S	LipidA-core band	short rods
JAG2(pMAV11)	*rfe*$^+$ *tolA*	S	reduced amount of O7-LPS	very long rods
JAG2(pMAV11, pJG1)	*rfe*$^+$ *tolA*$^+$	R	normal O7-LPS bands	short rods

Figure 15. Cell morphology of *tolA* mutant in VW187*rfe*

Cells were negatively stained with 1%(w/v) ammonium molybdate, and examined by transmission electron microscopy at a low magnification. Panel a, JAG2; Panel b, VW187*rfe*; Panel c, and d, JAG2(pMAV11). The long cells in Panel c and d show indications of damage to the cell envelope as suggested by the lack of uniform staining of the bacterial surface.


a
b
3µm
c
d

of JAG2(pMAV11) appears to be compromised in comparison to the parental strain JAG2. The strains VW187, VW187*rfe*, JAG2(pJG1) and JAG2(pJG1, pMAV11) all have similar morphological appearances when examined under light or electron microscopy. The morphological changes observed together with the reduction in growth rate suggests defects in the synthesis of the cell wall material that is essential to form the septum prior to cell division.

4.4 Radiolabelling of O-specific precursors

The previous experiments suggested that expression of O7 LPS in *tolQ* and *tolA* mutants interferes with cell division. This could be due to an accumulation of O-antigen precursors in the inner membrane attached to the bactoprenyl phosphate which in turn could cause a depletion of free bactoprenyl phosphate for peptidoglycan synthesis. An accumulation of O-antigen precursors in the cytoplasmic membrane can be detected by specifically labelling the O7-antigen subunit and then separating the cytoplasmic and outer membrane on the basis of buoyancy density (Osborn et al., 1972). Tritium labelled mannose was utilised to label the O7-subunit since mannose is one of the five primary sugars present in the O7-subunit.

In order to increase the incorporation of the radiolabelled mannose directly into the O7-subunit, a strain possessing specific mutations that would

increase the incorporation of the added label in O-antigen synthesis was required. The model strain for this experiment cannot produce colanic acid since mannose can be enzymatically converted to fucose a component of capsule polysaccharide. Colanic acid synthesis would interfere with studies on the accumulation of radiolabelled mannose on the cytoplasmic membrane since CPS is also assembled in the cytoplasmic membrane. A secondary requirement for maximum incorporation of mannose into the O-subunit would be that the strain was a *manA* mutant. This gene encodes for an enzyme which converts mannose-6-P to fructose-6-P, a intermediate sugar of the glycolysis pathway, resulting in the dilution of the label to cellular metabolism (Figure 16).

4.4.1 Accumulation of O-antigen Precursors in Cytoplasmic Membrane

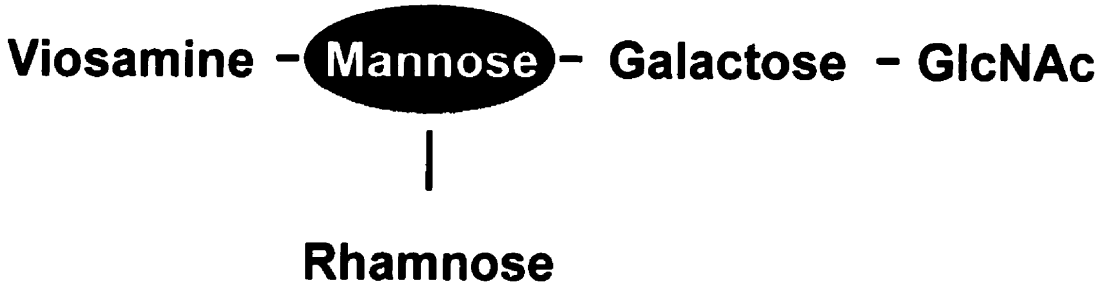
To ensure exclusive incorporation of the radiolabel into the O7-subunit, the strain CLM11 which possesses a deletion in the colanic acid genes and a mutation in the *manA* gene was utilised for these studies. The expression of O7-antigen expression has been demonstrated to be mannose dependent in strain CLM11(pJHCV32).

The plasmids pJT10 and pJG11 were used to construct the isogenic strains JAG3 and JAG4, containing mutations in *tolQ* and *tolA* respectively. These strains were transformed with pJHCV32 and radiolabelling experiments

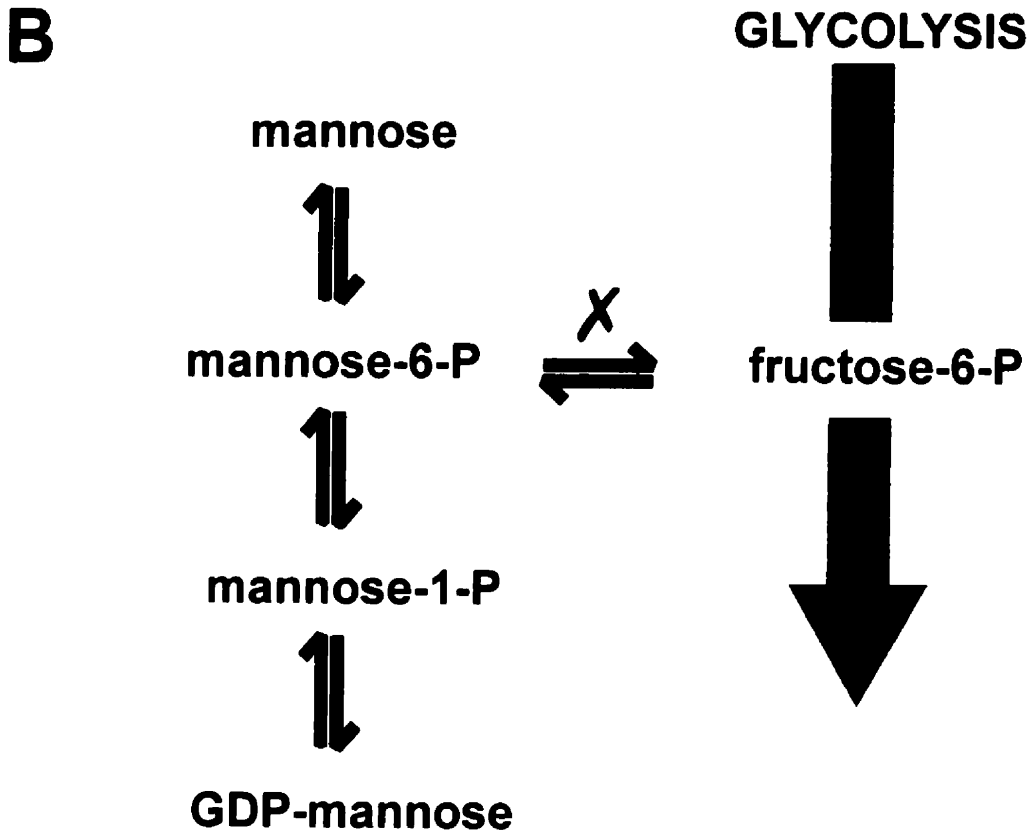
Figure 16. O7-subunit structure and metabolic pathway of mannose

Panel a: The chemical structure of the O7-subunit. The mannose residue is unique to the O7 subunit and does not occur in the core LPS or the colanic acid capsule. Panel b The metabolic pathway of mannose utilisation. The X indicates the reaction catalysed by the phosphomannose isomerase (*pmi/manA*). A mutation in the *manA* gene will result in a block in the utilisation of mannose and an increase incorporation of labelled mannose in the O7 LPS. GDP-mannose is a direct precursor for O7_antigen synthesis.

A


Viosamine
Mannose
Galactose
GlcNAc
Rhamnose

B


GLYCOLYSIS
mannose
mannose-6-P
fructose-6-P
mannose-1-P
GDP-mannose

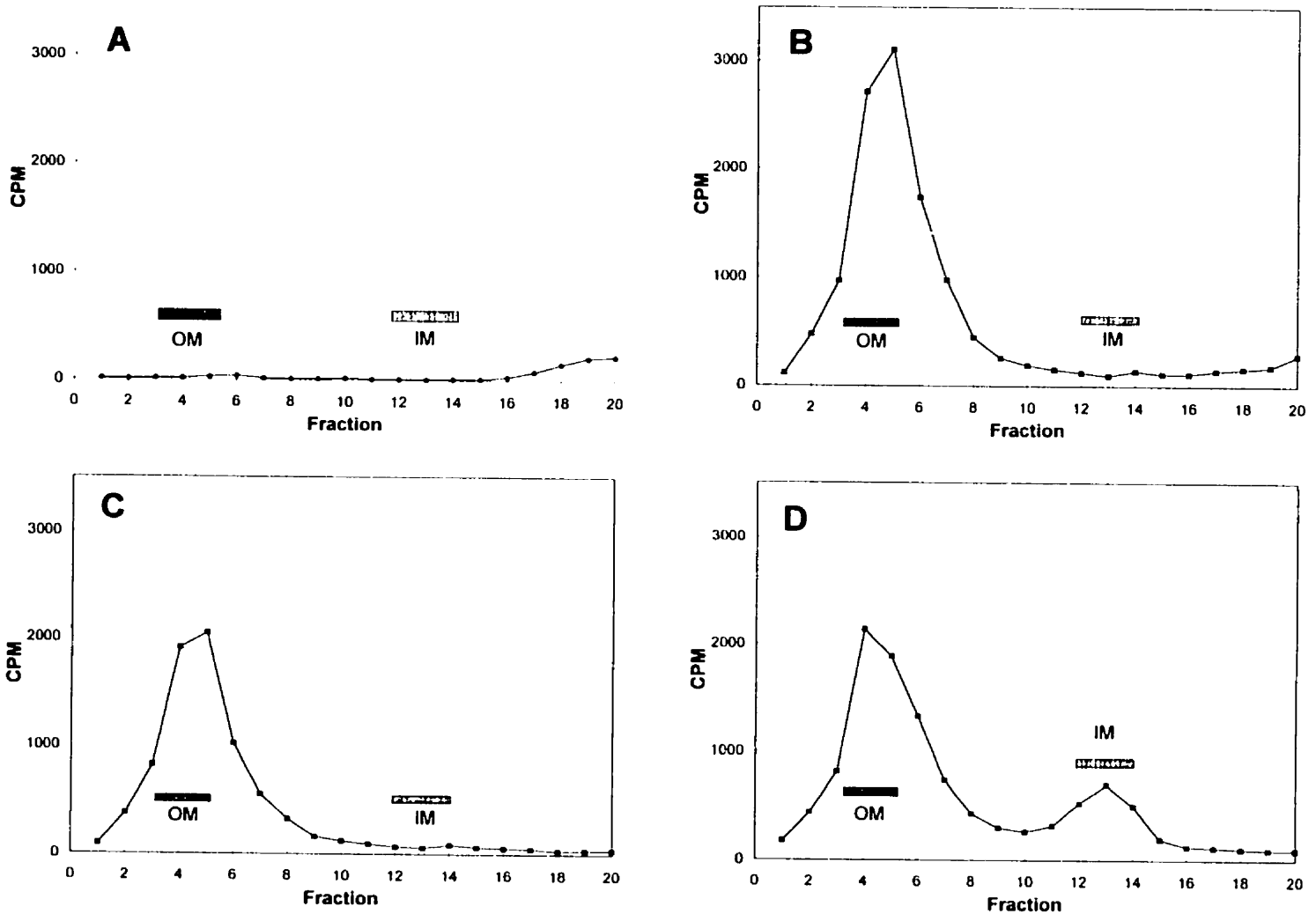
were carried out as described in the material and methods. Maximum incorporation of tritium mannose occurred within two minutes of adding the label as assessed by trichloroacetic acid precipitation.

Initial experiments revealed that there was an accumulation of radiolabelled mannose in the eluted sucrose fraction which corresponds to the cytoplasmic membrane from JAG3(pJHCV32) and JAG4(pJHCV32) but the incorporation levels of the labelled mannose were quite low (approximately 1%). We reasoned that this might be due to the poor expression of *rfb* genes from the cosmid pJHCV32. To induce our model systems to increase O-antigen production, the strains were transformed with pMAV11 which overexpresses *rfe* and theoretically should increase the initiation of O-subunit synthesis.

The results from these later experiments are summarised in Figure 17. The negative control (Panel a) illustrated that there is very little mannose incorporation in CLM11 alone. The positive control (Panel b) illustrates that there is a high incorporation of label in the outer membrane fractions (3 to 6) of CLM11(pJHCV32). Panels c and d illustrate the labelling profile from the *tolA* mutants JAG4(pJHCV32) and JAG4(pJHCV32, pMAV11). There is s decrease in total label incorporated into the outer membrane in both *tolA* mutants in comparison to the positive control. Also, JAG4(pJHCV32, pMAV11) exhibited an accumulation of O-antigen precursors in the

Figure 17. Incorporation of tritium labelled mannose into the outer an inner membrane fractions

Horizontal bars indicate the fractions containing outer (OM) and inner (IM) membrane peaks after fractionation in sucrose gradients. Panel a, control experiment using CLM11 (*manA*); Panel b, CLM11 containing pJHCV32 ($O7^+$ cosmid); Panel c, JAG4 (CLM11; *tolA*) containing pJHCV32; Panel d, JAG4 containing pJHCV32 and pMAV11(*rfe*$^+$).


A
B
C
D
CPM
Fraction
OM
IM
0
1000
2000
3000
0 2 4 6 8 10 12 14 16 18 20

cytoplasmic membrane fractions (12 to 14). The presence of cytoplasmic membrane in fractions 12 to 14 was confirmed by lactate dehydrogenase enzymatic activity. Outer membrane porins were detected in fractions 3 to 6 as assessed by the abundance of 35-40kDa proteins in SDS-PAGE gels stained with Coomassie blue.

4.5 Conclusions

The gene replacement technique proved to be an immensely powerful tool in the construction of *tol* mutants in the wild type strain VW187. A decrease in LPS expression in VW187 after the introduction of the *tol* mutation indicated that the decrease in LPS expression in K-12 mutants was not due to the instability of the cosmid pJHCV32 in the bacterial cell but rather was a direct result of *tol* mutations.

The lethality of *tolA* mutations in VW187 and reduced growth rate of VW187*tolQ* suggested that the proper expression of O-antigen was dependent on the Tol system. Mutations in *tol* genes of *Pseudomonas aeruginosa* have also been shown to be lethal (Dennis et al., 1996) indicating that this phenomenon is not specific to *E.coli* VW187.

Attempted *tolA* mutations in VW187 were unsuccessful unless accompanied by secondary mutations that eliminated O7 synthesis. Also, the expression of O7 genes was associated with altered cell morphology in *tol*

mutants in VW187. These observations led us to postulate that TolA has a function in the processing or assembly of O7-polysaccharide. The morphological changes were phenotypically similar to those observed in mutation affecting normal cell wall synthesis and septation (Spratt, 1975). A partial block in the translocation of single O-antigen subunits across the cytoplasmic membrane, the polymerisation of O-antigen at the periplasmic surface of the cytoplasmic membrane or the ligation of the O-antigen to lipidA-core would result in the sequestering of bactoprenyl lipid for O-antigen synthesis. Since O-antigen and peptidoglycan biosynthesis both require bactoprenyl lipid, sequestering of bactoprenyl could conceivably compromise the recycling of bactoprenyl phosphate. This would in turn limit the ability of the bacterial cell to structurally synthesise a proper peptidoglycan layer, and could explain the cell morphology changes.

The accumulation of labelled mannose in JAG4(pJHCV32, pMAV11) in the cytoplasmic membrane fraction indicated that a block in the processing or assembly of O7-intermediates occurs in the cytoplasmic membrane of *tolA* mutants. This effect was only observed if the bacterial system produces large amounts of O-antigen. In parallel to this observation, the K-12 strain A592(pJHCV32) did not exhibit any morphological changes but when this strain was induced to produce O-antigen by the overexpression of *rfe*, the

cells also became elongated although not to the same extent as in the *tolA* mutant of VW187.

The lower expression of O7-LPS, morphological changes of *tol* mutants and the accumulation of labelled O-antigen precursors in the cytoplasmic membrane indicate that the Tol import system, and more specifically TolA, is involved in the processing or assembly of O-antigen LPS.

Chapter Five

Concluding Remarks

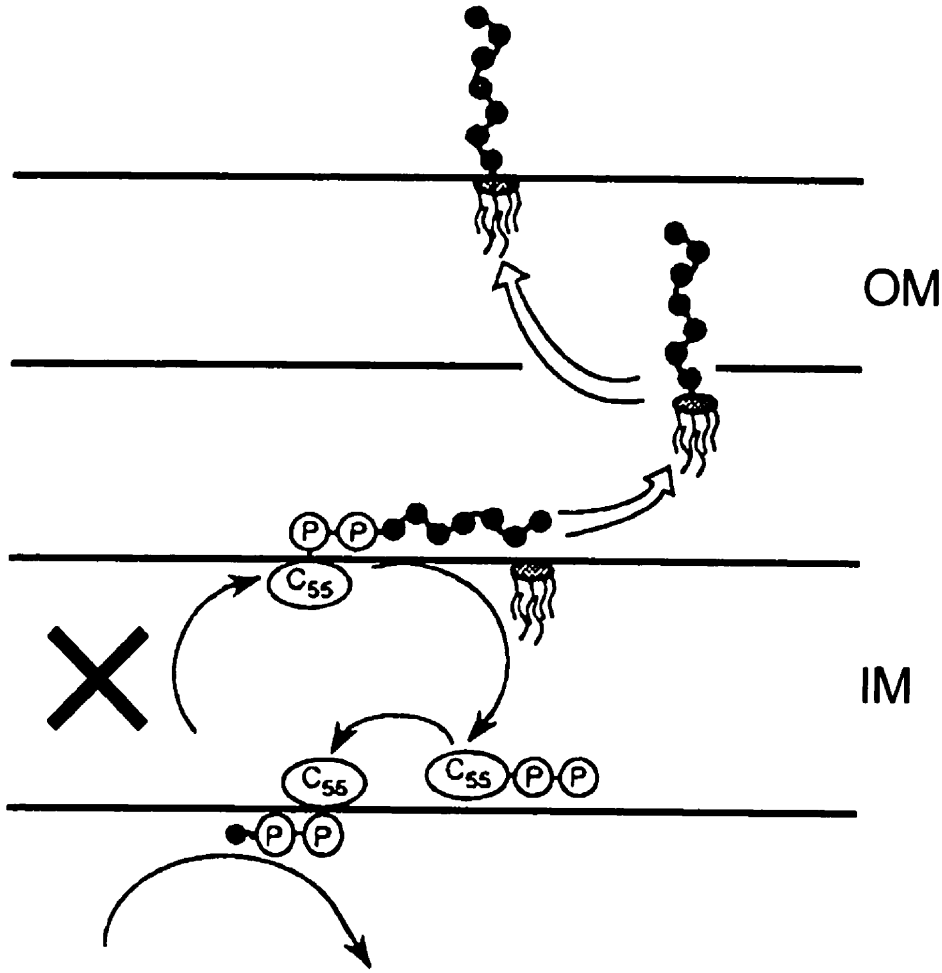
The experiments presented in this work demonstrate that the TolA protein plays a role in governing the surface expression of O7-specific LPS. The reduction of O7-LPS does not appear to be the result of a defect in the formation of lipidA-core nor the consequence of a reduced transcription of the O7 biosynthetic genes. Also, the mutant strains can form a complete O7-antigen, as judged by its reactivity with the O7-antiserum and by the similar migration of O7-specific LPS bands in SDS-PAGE. Therefore, we postulate that TolA has a function in the processing of O7-polysaccharide. This is supported by the fact that cells with a *tolA* defect (due to a *tolA* mutation or a *tolQ* mutation with a polar effect on TolA expression) display dramatic morphological changes with the concomitant expression of the O7 biosynthesis genes. These morphological changes were phenotypically similar to those observed in mutations affecting the normal septation and synthesis of cell wall components. Furthermore, an abnormal accumulation of radiolabelled O7-LPS biosynthesis precursors in the inner membrane was detected, indicating a block in the transfer of O7-LPS intermediates to the

outer membrane which occurs very rapidly in normal cells (Marino et al., 1991; McGrath and Osborn, 1991). A possible explanation for these findings is an involvement of TolA in the process of translocation of O7-subunits from the cytoplasmic to the periplasmic site of the inner membrane, but it is also possible that TolA may be involved in other aspects of O7 processing such as O-polymerisation and ligation of the O7-polysaccharides to the lipidA-core. If the TolA defect causes a block in the translocation of O7-subunits, it would be conceivable to expect an accumulation of bactoprenyl-bound O7-monomers which could not be delivered to lipidA-core units to complete the LPS molecules (Figure 18). This would compromise the recycling of bactoprenyl phosphate for other uses in the cell such as the synthesis of cell wall, and could explain the cell morphology changes due to reduced formation of peptidoglycan. Experiments to directly measure the total peptidoglycan content of *tol* mutants expressing O7-LPS may support this hypothesis.

Further experiments are required to examine in detail the appropriate site of TolA function in O7-LPS processing. Isolation of bactoprenyl lipid by butanol extraction followed by thin layer chromatography analysis might be used to identify the O-antigen associated bactoprenyl species that accumulates in the inner membrane. Immunolabelling of spheroplasts or membrane vesicles (right side out and inside out) can be used to determine

Figure 18. Model proposing the effect of TolA in the synthesis of O-antigen synthesis

We propose that TolA protein is directly or indirectly involved in the process of translocation of the O7 subunit precursor molecules (attached to undecaprenol-P) across the inner membrane. The TolA block (indicated with an X) will result in a reduction in the amount of free undecaprenol-P available which in turns causes a defect in the synthesis of cell wall peptidoglycan (PG). PG precursors also require undecaprenol-P for its synthesis and assembly.


OM
IM
P
P
C55
C55
C55
P
P
P
P

whether the accumulation of O-antigen precursors occurs on the cytoplasmic or periplasmic face of the inner membrane. Co-immunoprecipitation of Tol proteins may identify proteins involved in the synthesis of O7-LPS that directly interact with the Tol import system. These experiments are important to further elucidate the role of the Tol transport system in the processing of O7-LPS.

Several lines of evidence indicate that TolQ, -R, and -A proteins interact physically forming a complex (Derouiche et al., 1995; Guihard et al., 1994). However, we have shown in this work that *tolQ* and *tolR* genes do not appear to be involved in the O7-LPS surface expression defect, except for the fact that polar mutations in these genes compromise the expression of the *tolA* gene located downstream from *tolQR* (Vianney et al., 1996). It is possible that the function(s) of defective *tolQ* and *tolR* may be compensated by their homologous genes *exbB* and *exbD*, respectively (Braun, 1989; Eick-Helmerich and Braun, 1989; Eick-Helmerich et al., 1987). The construct and examination of double mutants in strain VW187 with defects in both *exbB/tolQ* and *exbD/tolR* will be required to examine this possibility.

The Tol import system has been described as being associated with the translocation of DNA from filamentous bacteriophages and bacteriocins across the outer membrane (Webster, 1991). Mutations in various *tol* genes are not only associated with a defect in the import of these molecules but also

with various pleiotropic effects, especially a reduction in the content of outer membrane proteins, increased susceptibility to detergents and hydrophobic compounds in general, and in the case of *tolA* mutations, a leakage of periplasmic proteins (Webster, 1991). These phenotypes suggest that the Tol import system plays an important role in maintaining the integrity of the outer membrane but its exact function(s) remain to be elucidated. Our discovery that TolA is involved with surface expression of O polysaccharide supports a role for the Tol system in export of outer membrane components. We envisage the Tol proteins as a multi-protein complex sensing the integrity of the outer membrane and providing a co-ordination with the inner membrane for general functions related to the export and assembly of outer membrane macromolecules.

References

Alexander, D. C., and Valvano, M. A. (1994). Role of the *rfe* gene in the biosynthesis of the *Escherichia coli* O7-specific lipopolysaccharide and other O-specific polysaccharides containing N-acetylglucosamine. J Bacteriol *176*, 7079-84.

Armitage, P., and Berry, G. (1994). Statistical methods in medical research, 3rd Edition (Oxford, Boston: Blackwell Scientific Publications).

Benedetti, H., Lazdunski, C., and Lloubes, R. (1991). Protein import into *Escherichia coli*: colicins A and E1 interact with a component of their translocation system. Embo J *10*, 1989-95.

Bergmeyer, H. U., Bergmeyer, J., and Grassl, M. (1983). Enzymes 1 : oxidoreductases, transferases. In Methods of enzymatic analysis, H. U. Bergmeyer, ed. (Weiheim, Basel, Deerfield Beach: Verlag Chemie).

Bourdineaud, J. P., Howard, S. P., and Lazdunski, C. (1989). Localization and assembly into the *Escherichia coli* envelope of a protein required for entry of colicin A. J Bacteriol *171*, 2458-65.

Bouveret, E., Derouiche, R., Rigal, A., Lloubes, R., Lazdunski, C., and Benedetti, H. (1995). Peptidoglycan-associated lipoprotein-TolB interaction. A possible key to explaining the formation of contact sites between the inner and outer membranes of *Escherichia coli*. J Biol Chem *270*, 11071-7.

Bradley, D. E., and Whelan, J. (1989). *Escherichia coli tolQ* mutants are resistant to filamentous bacteriophages that adsorb to the tips, not the shafts, of conjugative pili. J Gen Microbiol *135*, 1857-1863.

Braun, V. (1989). The structurally related *exbB* and *tolQ* genes are interchangeable in conferring *tonB*-dependent colicin, bacteriophage, and albomycin sensitivity. J Bacteriol *171*, 6387-90.

Braun, V., and Herrmann, C. (1993). Evolutionary relationship of uptake systems for biopolymers in *Escherichia coli*: cross-complementation between the TonB-ExbB-ExbD and the TolA-TolQ-TolR proteins. Mol Microbiol *8*, 261-8.

Cavard, D., and Lazdunski, C. J. (1979). Purification and molecular properties of a new colicin. Eur J Biochem *96*, 519-24.

Clarke, B. R., and Whitfield, C. (1992). Molecular cloning of the rfb region of *Klebsiella pneumoniae* serotype O1:K20: the *rfb* gene cluster is responsible for synthesis of the D-galactan I O polysaccharide. J Bacteriol *174*, 4614-21.

Clavel, T., Lazzaroni, J. C., Vianney, A., and Portalier, R. (1996). Expression of the *tolQRA* genes of *Escherichia coli* K-12 is controlled by RcsC sensor protein involved in capsule synthesis. Mol Micro *19*, 19-25.

Dennis, J. J., Lafontaine, E. R., and Sokol, P. A. (1996). Identification and characterization of the *tolQRA* genes of *Pseudomonas aeruginosa*. J Bacteriol *178*, 7059-68.

Derouiche, R., Benedetti, H., Lazzaroni, J. C., Lazdunski, C., and Lloubes, R. (1995). Protein complex within *Escherichia coli* inner membrane. TolA N-terminal domain interacts with TolQ and TolR proteins. J Biol Chem *270*, 11078-84.

Dower, W. J., Miller, J. F., and Ragsdale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res *16*, 6127-45.

Eick-Helmerich, K., and Braun, V. (1989). Import of biopolymers into *Escherichia coli*: nucleotide sequences of the *exbB* and *exbD* genes are homologous to those of the *tolQ* and *tolR* genes, respectively. J Bacteriol *171*, 5117-26.

Eick-Helmerich, K., Hantke, K., and Braun, V. (1987). Cloning and expression of the *exbB* gene of *Escherichia coli* K-12. Mol Gen Genet *206*, 246-51.

Fourel, D., Hikita, C., Bolla, J. M., Mizushima, S., and Pages, J. M. (1990). Characterization of *ompF* domains involved in *Escherichia coli* K-12 sensitivity to colicins A and N. J Bacteriol *172*, 3675-80.

Goto, N., Shoji, A., Horiuchi, S., and Nakaya, R. (1984). Conduction of nonconjugative plasmids by F' lac is not necessarily associated with transposition of the gamma delta sequence. J Bacteriol *159*, 590-6.

Guihard, G., Boulanger, P., Benedetti, H., Lloubes, R., Besnard, M., and Letellier, L. (1994). Colicin A and the Tol proteins involved in its translocation are preferentially located in the contact sites between the inner and outer membranes of *Escherichia coli* cells. J Biol Chem *269*, 5874-80.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol *166*, 557-80.

Hancock, R. E. W., Karunaratne, D. N., and Bernegger-Egli, C. (1994). Molecular organization and structural role of outer membrane macromolecules. In Bacterial cell wall, J. M. Ghuysen and R. Hackenbeck, eds. (Amsterdam, New York: Elsevier Science), pp. 263-79.

Isnard, M., Rigal, A., Lazzaroni, J. C., Lazdunski, C., and Lloubes, R. (1994). Maturation and localization of the TolB protein required for colicin import. J Bacteriol *176*, 6392-6.

Jann, K., Pillat, M., Weisgerber, C., Shibaev, V. N., and Torgov, V. I. (1985). Biosynthesis of the O9 antigen of *Escherichia coli*. Synthetic glycosyldiphosphomoraprenols as probes for requirement of mannose acceptors. Eur J Biochem *151*, 393-7.

Kadner, R. J. (1996). Cytoplasmic Membrane. In Escherichia coli and Salmonella. Cellular and molecular biology, F. C. Neidhardt, R. Curstiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter and H. E. Umbarger, eds. (Washington, DC: ASM Press), pp. 58-87.

Kampfenkel, K., and Braun, V. (1992). Membrane topology of the *Escherichia coli* ExbD protein. J Bacteriol *174*, 5485-7.

Lazzaroni, J. C., Vianney, A., Popot, J. L., Benedetti, H., Samatey, F., Lazdunski, C., Portalier, R., and Geli, V. (1995). Transmembrane alpha-helix interactions are required for the functional assembly of the *Escherichia coli* Tol complex. J Mol Biol *246*, 1-7.

Levengood, S. K., Beyer, W. F., Jr., and Webster, R. E. (1991). TolA: a membrane protein involved in colicin uptake contains an extended helical region. Proc Natl Acad Sci U S A *88*, 5939-43.

Levengood, S. K., and Webster, R. E. (1989). Nucleotide sequences of the *tolA* and *tolB* genes and localization of their products, components of a multistep translocation system in *Escherichia coli*. J Bacteriol *171*, 6600-9.

Levengood-Freyermuth, S. K., Click, E. M., and Webster, R. E. (1993). Role of the carboxyl-terminal domain of TolA in protein import and integrity of the outer membrane. J Bacteriol *175*, 222-8.

Liu, D., Cole, R. A., and Reeves, P. R. (1996). An O-antigen processing function for Wzx (RfbX): A promising candidate for O-unit flippase. J Bacteriol *178*, 2102-2107.

Liu, D., and Reeves, P. R. (1994). *Escherichia coli* K12 regains its O antigen. Microbiology *140*, 49-57.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randal, R. J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 265-267.

Lugtenberg, B., and Van Alphen, L. (1983). Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim Biophys Acta *737*, 51-115.

L'vov, V. L., Shashkiv, A. S., Dimitriev, B. A., Kochtkov, N. K., Jann, B., and Jann, K. (1984). Structural studies of the O-specific side chain of the lipopolysaccharide from *Escherichia coli* O:7. Cabrohydr Res *126*, 249-259.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular cloning : a laboratory manual (Cold Spring, New York: Cold Spring Harbor Laboratory Press).

Marino, P. A., McGrath, B. C., and Osborn, M. J. (1991). Energy dependence of O-antigen synthesis in *Salmonella typhimurium*. J Bacteriol *173*, 3128-33.

Marino, P. A., Phan, K. A., and Osborn, M. J. (1985). Energy dependence of lipopolysaccharide translocation in *Salmonella typhimurium*. J Biol Chem *260*, 14965-70.

Marolda, C. L. (1996). Unpublished Data.

Marolda, C. L., Welsh, J., Dafoe, L., and Valvano, M. A. (1990). Genetic analysis of the O7-polysaccharide biosynthesis region from the *Escherichia coli* O7:K1 strain VW187. J Bacteriol *172*, 3590-9.

Massotte, D., and Pattus, F. (1989). Colicin N and its thermolytic fragment induce phospholipid vesicle fusion. FEBS Lett *257*, 447-50.

McGrath, B. C., and Osborn, M. J. (1991). Evidence for energy-dependent transposition of core lipopolysaccharide across the inner membrane of *Salmonella typhimurium*. J Bacteriol *173*, 3134-7.

McGrath, B. C., and Osborn, M. J. (1991). Localization of the terminal steps of O-antigen synthesis in *Salmonella typhimurium*. J Bacteriol *173*, 649-54.

Mende, J., and Braun, V. (1990). Import-defective colicin B derivatives mutated in the TonB box. Mol Microbiol *4*, 1523-33.

Metcalf, W. W., Jiang, W., and Wanner, B. L. (1994). Use of the rep technique for allele replacement to construct new *Escherichia coli* hosts for maintenance of R6K gamma origin plasmids at different copy numbers. Gene *138*, 1-7.

Miller, V. L., and Mekalanos, J. J. (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J Bacteriol *170*, 2575-83.

Nagel de Zwaig, R., and Luria, S. E. (1967). Genetics and physiology of colicin-tolerant mutants of *Escherichia coli*. J Bacteriol *94*, 1112-1123.

Neuhard, J., and Thomassen, E. (1976). Altered deoxyribonucleic pools in P2 eductants of *Escherichia coli* K-12 due to deletion of the *dcd* gene. J Bacteriol *126*, 999-1001.

Nikaido, H. (1996). Outer Membrane. In Escherichia coli and Salmonella. Cellular and molecular biology, F. C. Neidhardt, R. Curstiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter and H. E. Umbarger, eds. (Washington, DC: ASM Press), pp. 29-47.

Nikaido, H. (1994). Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science *264*, 382-8.

Nikaido, H., and Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. Microbiol Rev *49*, 1-32.

Osborn, M. J., Gander, J. E., Parisi, E., and Carson, J. (1972). Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. J Biol Chem *247*, 3962-72.

Osborn, M. J., and Tze-Yuen, R. Y. (1968). Biosynthesis of bacterial lipopolysaccharide. VII. Enzymatic formation of the first intermediate in biosynthesis of the O-antigen of *Salmonella typhimurium*. J Biol Chem *243*, 5145-52.

Plesiat, P., and Nikaido, H. (1992). Outer membranes of gram-negative bacteria are permeable to steroid probes. Mol Microbiol *6*, 1323-33.

Postle, K. (1993). TonB protein and energy transduction between membranes. J Bioenerg Biomembr *25*, 591-601.

Reeve, J. (1979). Use of minicells for bacteriophage-directed polypeptide synthesis. Methods Enzymol *68*, 493-503.

Ried, J. L., and Collmer, A. (1987). An *nptI-sacB-sacR* cartridge for constructing directed, unmarked mutations in gram-negative bacteria by marker exchange-eviction mutagenesis. Gene *57*, 239-46.

Schagger, H., and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem *166*, 368-79.

Schnaitman, C. A., and Klena, J. D. (1993). Genetics of lipopolysaccharide biosynthesis in *enteric* bacteria. Microbiol Rev *57*, 655-82.

Spratt, B. G. (1975). Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K12. Proc Natl Acad Sci U S A *72*, 2999-3003.

Steinmetz, M., Le Coq, D., Aymerich, S., Gonzy-Treboul, G., and Gay, P. (1985). The DNA sequence of the gene for the secreted *Bacillus subtilis* enzyme levansucrase and its genetic control sites. Mol Gen Genet *200*, 220-8.

Sun, T. P., and Webster, R. E. (1986). *fii*, a bacterial locus required for filamentous phage infection and its relation to colicin-tolerant *tolA* and *tolB*. J Bacteriol *165*, 107-15.

Sun, T. P., and Webster, R. E. (1987). Nucleotide sequence of a gene cluster involved in the entry of E colicins and single stranded DNA of infecting filamentous bacteriophages into *Escherichia coli*. J Bacteriol *169*, 2667-2674.

Thomas, J. A., and Valvano, M. A. (1993). Role of *tol* genes in cloacin DF13 susceptibility of *Escherichia coli* K-12 strains expressing the cloacin DF13-aerobactin receptor IutA. J Bacteriol *175*, 548-52.

Thomas, J. A., and Valvano, M. A. (1992). *tolQ* is required for cloacin DF13 susceptibility in *Escherichia coli* expressing the aerobactin/cloacin DF13 receptor IutA. FEMS Microbiol Lett *70*, 107-11.

Tuckman, M., and Osburne, M. S. (1992). *In vivo* inhibition of TonB-dependent processes by a TonB box consensus pentapeptide. J Bacteriol *174*, 320-3.

Valvano, M. A., and Crosa, J. H. (1984). Aerobactin iron transport genes commonly encoded by certain ColV plasmids occur in the chromosome of a human invasive strain of *Escherichia coli* K1. Infect Immun *46*, 159-67.

Valvano, M. A., and Crosa, J. H. (1989). Molecular cloning and expression in *Escherichia coli* K-12 of chromosomal genes determining the O7 lipopolysaccharide antigen of a human invasive strain of *E. coli* O7:K1. Infect Immun *57*, 937-43.

Vareli, K., and Frangou-Lazaridis, M. (1996). Pouring gradients using a cork. Biotechniques *21*, 237-8.

Vianney, A., Muller, M. M., Clavel, T., Lazzaroni, J. C., Portalier, R., and Webster, R. E. (1996). Characterization of the tol-pal region of *Escherichia coli* K-12: Translational control of *tolR* expression by TolQ and identification of a new open reading frame downstream of *pal* encoding a periplasmic protein. J Bacteriol *178*, 4031-4038.

Webster, R. E. (1991). The *tol* gene products and the import of macromolecules into *Escherichia coli*. Mol Microbiol *5*, 1005-11.

Whitfield, C. (1995). Biosynthesis of lipopolysaccharide O antigens. Trends Microbiol *3*, 178-85.

Whitfield, C., and Keenleyside, W. J. (1995). Regulation of expression of group IA capsular polysaccharides in *Escherichia coli* and related extracellular polysaccharides in other bacteria. J Ind Microbiol *15*, 361-71.

Whitfield, C., and Valvano, M. A. (1993). Biosynthesis and expression of cell-surface polysaccharides in gram-negative bacteria. Adv Microb Physiol *35*, 135-246.