LIPOPOLYSACCHARIDE SYNTHESIS AND CHARACTERIZATION OF A CORE OLIGOSACCHARIDE KINASE ESSENTIAL TO OUTER MEMBRANE STABILITY

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ABSTRACT

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University of Guelph, 2001

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In *Escherichia coli* and *Salmonella enterica*, genes for assembly of the lipopolysaccharide (LPS) core region are clustered on the chromosome in three adjacent operons. The central of these operons encodes glycosyltransferases and other enzymes responsible for assembling one of seven distinct core structures. In *E. coli* with core type R1, there are nine "*waa*" genes in the central core biosynthesis operon. In order to assign function to each of these genes, non-polar mutations were constructed in each, and the resulting mutant LPS core structures were determined by chemical methods.

In *E. coli* and *S. enterica*, phosphoryl substituents in the LPS core region are essential for the formation of a stable outer membrane. Of particular note, therefore, mutations in four genes characterized by the above approach were shown to affect the degree of core phosphorylation. Two of these genes (*waaY* and *waaP*) were presumed to be involved directly in phosphotransfer reactions based on their weak homology to eukaryotic kinases. The remaining two genes (*waaQ* and *waaG*) were shown to encode glycosyltransferases acting near phosphorylation sites, and are thus likely required to fulfill the substrate specificity requirements of the phosphorylating enzymes. Mutant derivatives with altered core phosphorylation were analyzed for outer membrane stability

by novobiocin and SDS sensitivity testing, and were shown to be more susceptible to these agents than the parent strain.

In general, decreased core phosphate substitution resulted in greater membrane instability. In particular, mutation of *waaP*, resulting in a core completely devoid of phosphate, caused a dramatic increase in antibiotic susceptibility. To assess how WaaP and LPS core phosphorylation influence the biology of an intracellular pathogen, a *waaP* mutant was constructed using an invasive strain of *S. enterica*, and this mutant was tested for virulence using mouse models of infection. The results were unequivocal; mutation of *waaP* completely abrogated virulence. Given these data, the WaaP protein provides a novel potential target for the development of antibiotics. To further characterize WaaP, the protein was purified, and its enzymatic activity was reconstituted *in vitro*. Purified WaaP was shown to catalyze the incorporation of ^{33}P from [γ-^{33}P]ATP into phosphate-deficient acceptor LPS.

Acknowledgements

A very special thank you...

to Mom and Dad, for your love and support,

and to Wendy, for more than words can ever say.

Thanks also to my advisor, Dr. Chris Whitfield, for being a mentor, role model, and friend. And to my friends in the lab, both past and present, I'll miss you—you made the work fun, and were there to commiserate with me when it wasn't!

Best of luck to all!

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List of Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
Ap	ampicillin
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BLAST	basic local alignment search tool
Cam	chloramphenicol
CFU	colony-forming units
CMP	cytidine monophosphate
dH_2O	deionized water
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FAB-MS	fast atom bombardment mass spectrometry
Gal	galactose
Glc	glucose
GLC-MS	gas-liquid chromatography-mass spectrometry
GlcN	glucosamine
GlcNAc	*N*-acetylglucosamine
Gm	gentamicin
Hep	L-*glycero*-D-*manno*-heptose
Hex	hexose
Kdo	3-deoxy-D-*manno*-oct-2-ulosonic acid
Km	kanamycin

LOS	lipooligosaccharide
LPS	lipopolysaccharide
MIC	minimum inhibitory concentration
MS	mass spectrometry
NBT	nitro blue tetrazolium
Ni-NTA	nickel-nitrilotriacetic acid
NMR	nuclear magnetic resonance
P	phosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEtN or EtNP	2-aminoethyl phosphate
PPEtN or EtNPP	2-aminoethyl diphosphate
PSI-BLAST	position-specific iterated-BLAST
Rha	rhamnose
SDS	sodium dodecyl sulfate
UDP	uridine diphosphate

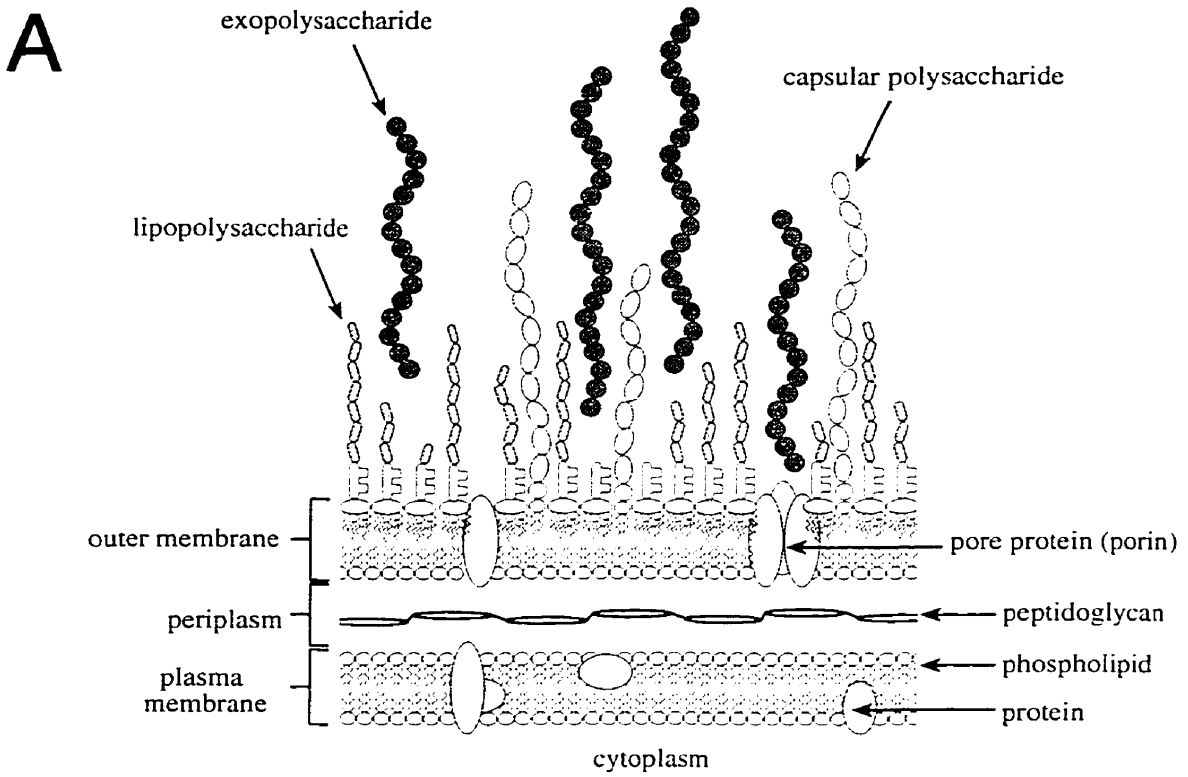
Chapter 1. Introduction

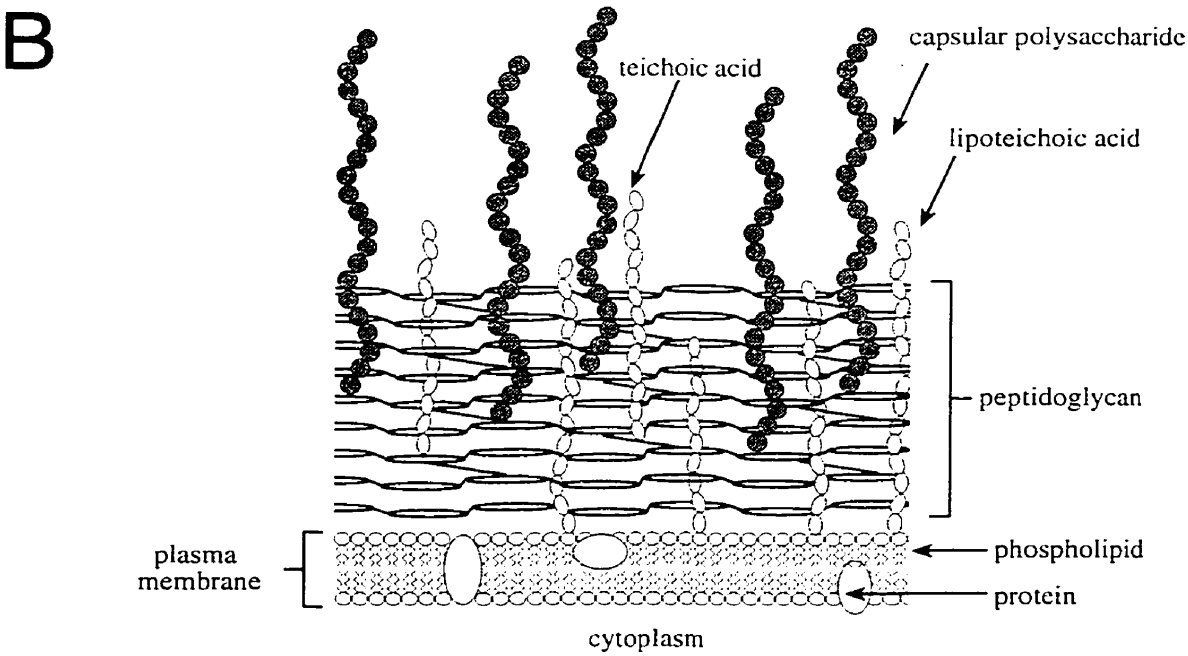
1.1. Overview:

Bacteria are classified into two broad groups based on their response to a staining procedure developed by Christian Gram in 1884. The differential staining response of Gram-negative and Gram-positive bacteria reflects fundamental differences in the composition and organization of their respective cell envelopes (see Figure 1.1). One major difference is that Gram-positive bacteria have a multi-layered, crosslinked polymer of peptidoglycan surrounding their plasma membranes, whereas the peptidoglycan surrounding Gram-negative bacteria is essentially a monolayer. Another critical distinction is that Gram-negative bacteria further encapsulate themselves with a second membrane exterior to their peptidoglycan, whereas no comparable structure is observed in most of their Gram-positive counterparts (Salton, 1994).

Given these marked differences in cell envelope structure, it is perhaps not surprising that the Gram stain reaction also tends to correlate with susceptibility to a variety of antibacterial agents. In general, Gram-negative bacteria are intrinsically more resistant to the effects of antibiotics and antibacterial compounds. In terms of peptidoglycan cell wall thickness, this generalization seems counterintuitive—why should Gram-positive bacteria, with their thicker cell walls, be more susceptible to these antibacterial agents? In fact, while the peptidoglycan layer surrounding the cell is mechanically strong, it offers little resistance to the diffusion of small molecules such as

Figure 1.1. Schematic cross-section of the Gram-negative (panel A) and Gram-positive (panel B) bacterial cell envelopes. Notable differences include the thickness of the peptidoglycan cell wall, and the presence of a second (outer) membrane in the Gram-negative cell envelope whereas Gram-positive bacteria have just one. Although both Gram-negative and -positive bacteria express different polysaccharides and polymers on their cell surfaces, these polymers are often thought to fulfill similar roles.


A
exopolysaccharide
capsular polysaccharide
lipopolysaccharide
outer membrane
pore protein (porin)
periplasm
peptidoglycan
plasma membrane
phospholipid
protein
cytoplasm


B
capsular polysaccharide
teichoic acid
lipoteichoic acid
peptidoglycan
plasma membrane
phospholipid
protein
cytoplasm

antibiotics because its meshwork is quite porous (Demchick and Koch, 1996; Pink *et al.*, 2000; Scherrer *et al.*, 1977; Scherrer and Gerhardt, 1971). As it turns out, the higher intrinsic resistance of Gram-negative bacteria can only be rationalized in terms of the permeability restrictions imposed by their outer membranes (reviewed in Nikaido, 1994).

The Gram-negative outer membrane is an asymmetric, lipid bilayer interspersed with proteins. Like the plasma membrane, its inner leaflet is composed of glycerophospholipids: primarily phosphatidylethanolamine, with smaller amounts of phosphatidylglycerol and cardiolipin. By contrast, the lipid of its outer leaflet is made up almost exclusively of lipopolysaccharide (LPS) molecules. LPSs are amphiphilic glycolipids, whose structural features help give rise to the selective permeability and barrier function of the outer membrane.

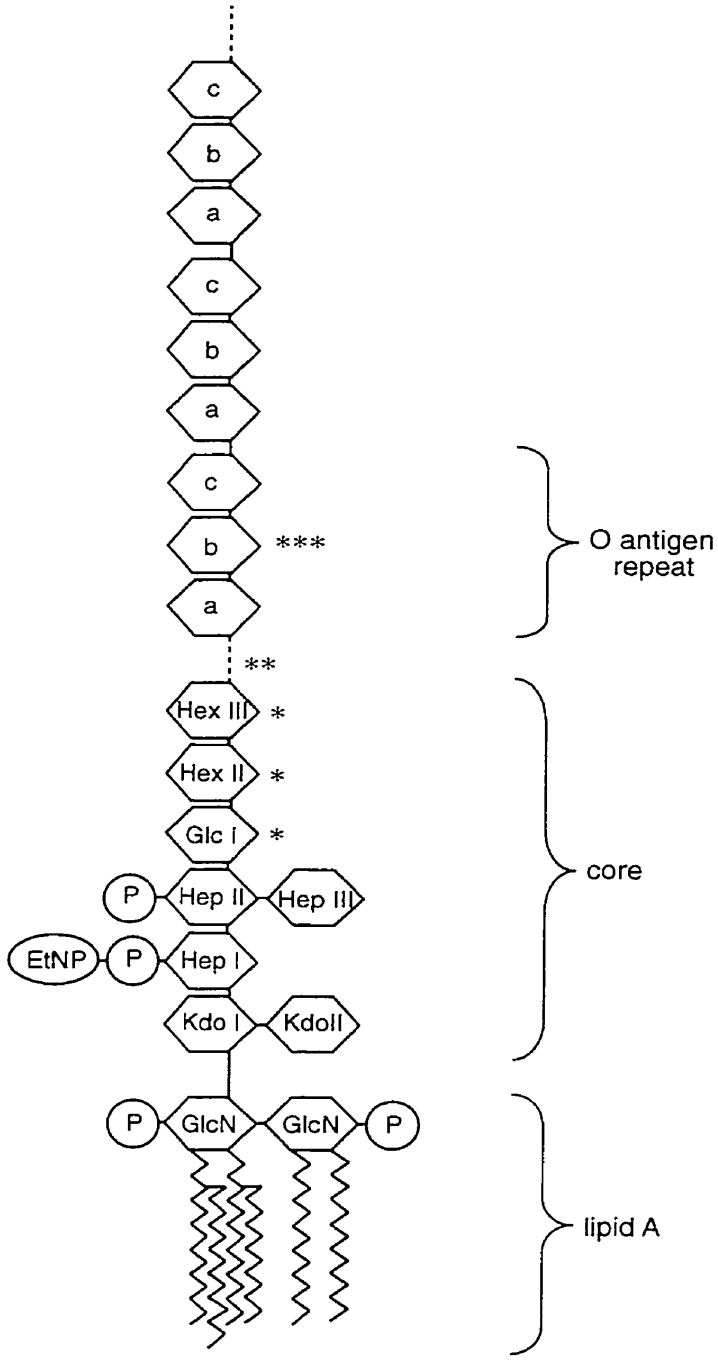
This thesis will first review what is known regarding the structure, function, and biosynthesis of the LPS molecule, highlighting where appropriate how this knowledge has been (or might be) exploited by the pharmaceutical industry for drug discovery. After a general overview of LPS as a whole, the focus will then shift to a specific subsection of the LPS molecule called the core oligosaccharide. The core oligosaccharide plays a key role in the formation of a stable outer membrane, but there were many important open questions at the outset of this work regarding its biosynthesis. The research reported in this thesis helps to clarify the steps involved in the biosynthesis of this critical core region of the LPS molecule. Of particular

importance, this thesis describes the identification and characterization of a kinase enzyme involved in core biosynthesis, called WaaP, which is essential for outer membrane stability. The WaaP kinase is further shown to be required for virulence *in vivo*, and is thus suggested to represent a potential new target in the LPS biosynthetic pathway for the development of antibacterial agents.

1.2. LPS structure and function:

The LPS of *Escherichia coli* and *Salmonella enterica* has been studied extensively, and serves as the model system for this work and many other studies on LPS biosynthesis. For the sake of discussion, the LPS molecule in these bacteria is conceptually divided into three distinct regions: 1) a hydrophobic membrane anchor designated lipid A; 2) a short chain of sugar residues with multiple phosphoryl substituents, referred to as the core oligosaccharide; and 3) a structurally diverse, serospecific polymer composed of oligosaccharide repeats, termed the O antigen (see Figure 1.2). The LPSs of other genera such as *Haemophilus* and *Neisseria* differ somewhat from that described above. These bacteria lack a typical long-chain, repeating-unit O antigen; instead, they possess short, serospecific oligosaccharides extending from the core. As a result, the LPS of *Haemophilus* and *Neisseria* is often referred to as lipooligosaccharide (LOS) to better reflect this structure. In general, however, the basic tripartite LPS framework holds true for all characterized Gram-negative bacteria.

Figure 1.2. Generalized structure of the LPS of *E. coli* and *S. enterica*. Core sugar residues are designated by sugar abbreviation and number to facilitate identification. Abbreviations are as follows: P, phosphate; GlcN, glucosamine; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; PEtN or EtNP, 2-aminoethyl phosphate; Hep, L-*glycero*-D-*manno* heptose; Glc, glucose; Hex, hexose. (*): Depending upon the core type, there can be sugar substituents branching out from the main carbohydrate backbone at these locations. (**): The linkage point for O antigen to the core can vary. (***): The number of O antigen repeats generally numbers between 1 and 40. The repeat unit shown is 3 sugars in length, but this too can vary (usually between 2 and 6).


c
b
a
c
b
a
c
b
a
***
**
Hex III
Hex II
Glc I
*
*
*
P
Hep II
Hep III
EtNP
P
Hep I
Kdo I
KdoII
P
GlcN
GlcN
P
O antigen repeat
core
lipid A

1.2.1. Lipid A:

The predominant form of lipid A found in *E. coli* and *S. enterica* is shown in Figure 1.3 (Takayama and Qureshi, 1992), although many modifications to this structure have been characterized (see section 1.2.4. Regulated LPS modifications). Of note, the free form of lipid A does not occur naturally in living cells, but it can be released from the core of completed LPS molecules by mild acid hydrolysis since the ketosidic linkage between the first sugar of the core oligosaccharide and lipid A is more labile than typical glycosidic bonds. The lipid A backbone is a disaccharide of glucosamine residues linked β,1′→6, and phosphorylated at the 1- and 4′-positions. This backbone structure is multi-acylated with *R*-3-hydroxymyristate at the 2-, 3-, 2′-, and 3′-positions. In *E. coli* and *S. enterica*, the addition of laurate and myristate to the non-reducing end of lipid A (generating the acyloxyacyl moieties shown in Figure 1.3) does not occur until after addition of the first two sugars of the core oligosaccharide (Brozek and Raetz, 1990), which is assembled on the hydroxyl group at the 6′-position. Interestingly, the minimal LPS structure required for the viability of *E. coli* and *S. enterica* in the laboratory consists of a completely acylated lipid A and the first two 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) sugars of the core (Kdo_2-lipid A, see Figure 1.3). Indeed, only temperature-sensitive mutants defective in lipid A biosynthesis (Galloway and Raetz, 1990) or Kdo precursor formation (Rick and Osborn, 1977; Rick and Young, 1982) have been isolated, and these mutations are bactericidal at the non-permissive temperature.

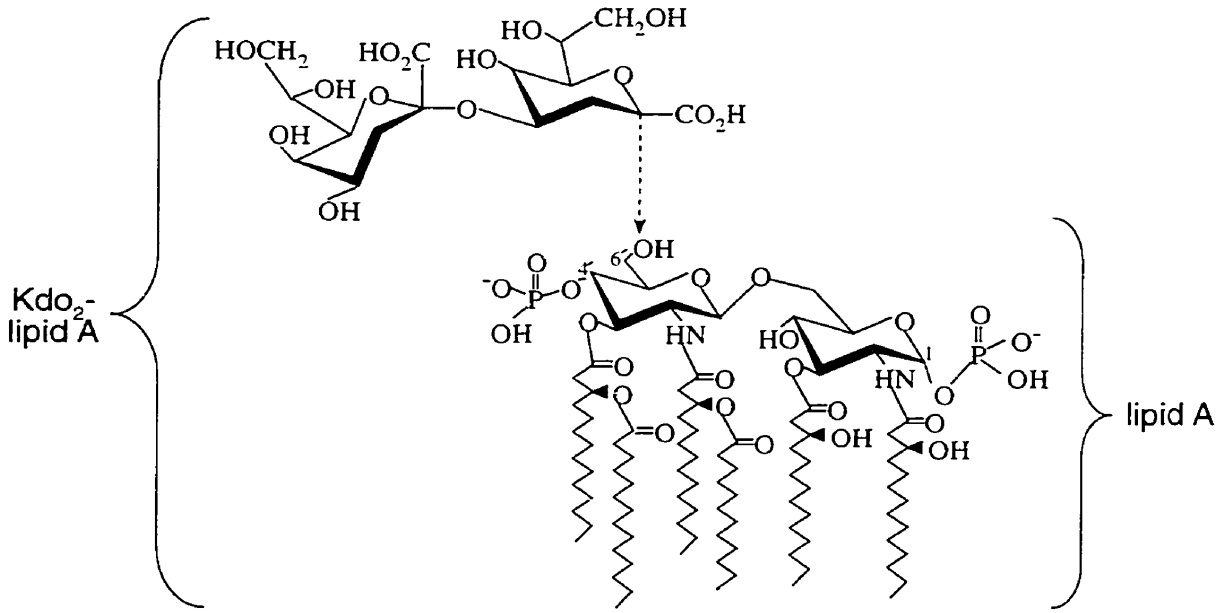


Figure 1.3. Structure of the predominant form of lipid A found in *E. coli* and *S. enterica*, which is released from intact LPS by hydrolysis in mild acid. Kdo_2-lipid A (lipid A together with the first two sugars of the core oligosaccharide) is the minimal LPS structure required for the viability of *E. coli* under laboratory conditions.

What then, if anything, does the unique structure of this minimal Kdo_2-lipid A species contribute to the outer membrane permeability barrier? Firstly, it has been shown that even a 30 percent reduction in lipid A content renders *E. coli* hypersensitive to antibiotics to which it is normally resistant, such as erythromycin and rifampicin (Galloway and Raetz, 1990). Presumably this effect is due to a compromised outer membrane permeability barrier (Vuorio and Vaara, 1992), so lipid A must play a critical role that cannot simply be substituted for by an excess of glycerophospholipids. One of the major structural contributions of lipid A appears to be the low fluidity of its resulting monolayer (Seydel *et al.*, 1993). In contrast to glycerophospholipids, the acyl chains attached to the lipid A diglucosamine backbone are all saturated, allowing for much tighter packing in the outer leaflet. In addition, there are at least six acyl chains covalently linked to a single lipid A head group, whereas there are only two fatty acids per glycerophospholipid—and in general, the larger the number of hydrocarbon chains per head group, the less fluid the monolayer. Indeed, the interior of the outer membrane bilayer has been shown experimentally to have very low fluidity, and hydrophobic probe molecules have been shown to diffuse across the outer membrane at a rate that is fifty to one hundred times slower than that observed across typical glycerophospholipid bilayers (Plesiat and Nikaido, 1992; Vaara *et al.*, 1990).

Aside from its function as a membrane anchor for the LPS molecule, lipid A is also known to be responsible for the variety of biological effects observed in mammals during Gram-negative sepsis. In fact, "LPS" has

become synonymous with "endotoxin", as a reflection of these biological properties of the lipid A portion of the molecule. Examples of lipid A-related bioactivities include induction of endotoxic shock, generation of fever, and non-specific activation of host lymphocytes and macrophages (reviewed in Bone, 1993; Raetz, 1993). How does lipid A elicit these effects? Cells of the mammalian innate immune system respond to lipid A via a protein kinase cascade that leads to nuclear factor kappa B (NF-κB) activity and cytokine production (Muller *et al.*, 1993). The initial binding/sensing of lipid A is mediated by the serum protein called LPS binding protein (LBP), and by soluble or membrane-bound forms of CD14 (Wright *et al.*, 1990). Recently, mammalian TLR4 (Toll-like receptor 4) has also been identified as a key component in the LPS/lipid A signaling pathway (reviewed in Beutler, 2000). Given these data, small molecules designed to inhibit lipid A biosynthesis could prove to be effective antibiotics on a number of counts: 1) lipid A is essential for viability, 2) decreased levels of lipid A compromise the outer membrane barrier function, and 3) decreased levels of lipid A lessen the threat of a severe host inflammatory response during sepsis. Indeed, such inhibitors to lipid A biosynthesis are actively being pursued (see section 1.3.1. Biosynthesis of lipid A).

Also of potential interest to the pharmaceutical industry is the fact that minor modifications to lipid A structure can convert it from an active mediator of inflammation, into a potent endotoxin antagonist. For example, some lipid A precursors (Golenbock *et al.*, 1991) and certain naturally-

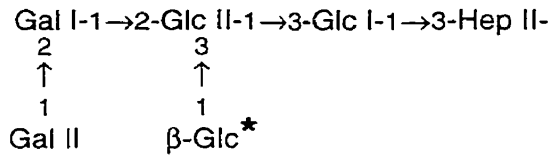
occurring pentaacylated lipid A variants from *Rhodobacter* species (Golenbock *et al.*, 1991; Takayama *et al.*, 1989) are able to inhibit the cell-stimulating activity of endotoxin. Further, using the *Rhodobacter capsulatus* lipid A as a model, organic chemists have created a synthetic lipid A antagonist, called E5531 (Christ *et al.*, 1995), which has recently shown very promising results in blocking endotoxin challenge in human clinical trials (Bunnell *et al.*, 2000). These observations raise interesting questions as to precisely what structural features of lipid A are responsible for eliciting the immune response, especially since such closely related structures can have dramatically different effects. Initial attempts to correlate lipid A structure with biological activity using a variety of synthetic lipid A and related compounds yielded a set of guidelines, but no hard and fast rules (reviewed in Takada and Kotani, 1992). However, recent data suggest that the overall shape and intrinsic conformation of the lipid A may be more important in determining lipid A bioactivity than any specific primary structure (Brandenburg *et al.*, 2000; Schromm *et al.*, 2000; Seydel *et al.*, 2000).

1.2.2. Core oligosaccharide:

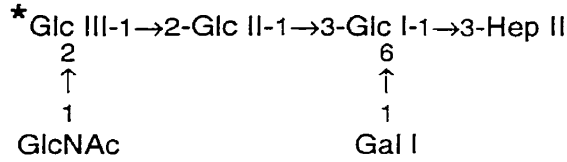
There are five known core structures for *E. coli* (designated R1, R2, R3, R4, and K-12) and two for *S. enterica* (referred to by their respective prototype serovars, Typhimurium and Arizonae IIIa). In general, the structure of the lipid A-distal region of the core (outer core) is more variable than the lipid A-proximal region (inner core). These structural differences in the outer core serve as the basis for distinguishing between core types (see Figure 1.4). For

Figure 1.4. Outer core structures of *E. coli* and *S. enterica* (reviewed in Holst, 1999). All glycoses are in the α-anomeric configuration unless otherwise indicated. Asterisks denote the core residues to which O antigen is ligated. The O antigen attachment site has been proven chemically for *S. enterica* serovars Typhimurium (Nikaido, 1969) and Arizonae IIIa (Olsthoorn *et al.*, 1998), while in *E. coli* it has only been defined for core types K-12 (Feldman *et al.*, 1999), R1 (Heinrichs *et al.*, 1998(b)), and R2 (Gamian *et al.*, 1992). In *E. coli* R3 and R4, the O antigen attachment site is still uncharacterized.

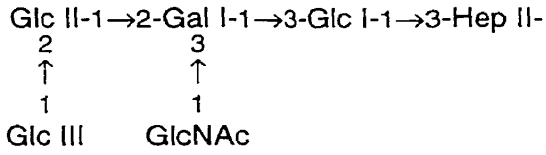
E. coli R1



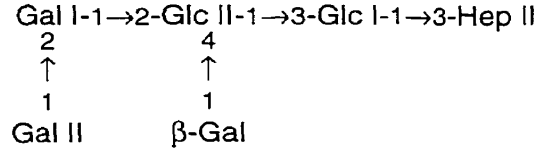
E. coli R2



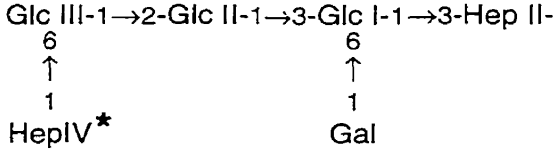
E. coli R3



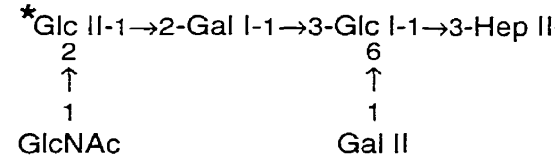
E. coli R4



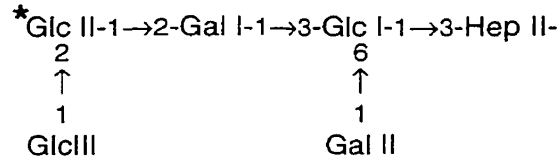
E. coli K-12



S. enterica sv. Typhimurium



S. enterica sv. Arizonae IIIa



E. coli, the R1 core type is predominant among clinical isolates, but notably R3 is more prevalent among enterohaemorrhagic *E. coli* isolates (Amor *et al.*, 2000; Appelmelk *et al.*, 1994; Currie and Poxton, 1999; Gibb *et al.*, 1992). For the entire genus *Salmonella*, it was long believed that there was only one core type, the Typhimurium core. However, a second core structure was recently identified in *S. enterica* serovar Arizonae IIIa (Olsthoorn *et al.*, 1998), but to date its occurrence is limited to that one strain. In *E. coli* and *S. enterica*, the outer core oligosaccharide provides the attachment site for preformed O antigen, but other than that it does not appear to play a direct role in virulence.

The inner region of the core oligosaccharide is highly conserved, and generally comprises L-*glycero*-D-*manno*-heptose (Hep) residues and the unique sugar 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) (see Figure 1.5). The presence of phosphoryl substituents in this region of the molecule is considered to be a key feature for the formation of a stable outer membrane in *E. coli* and *S. enterica* (Helander *et al.*, 1989; Parker *et al.*, 1992(a)). These phosphoryl substituents are postulated to be critical to outer membrane integrity because their negative charge allows neighbouring LPS molecules to be crosslinked by divalent cations, such as Ca^{2+} or Mg^{2+} (Nikaido and Vaara, 1985; Vaara, 1992). Bridging of LPS molecules in this way imparts stability to the Gram-negative outer membrane by decreasing its fluidity, and also helps to make it an effective permeability barrier. This proposed strengthening of the outer membrane by the crosslinking of LPS molecules is corroborated by

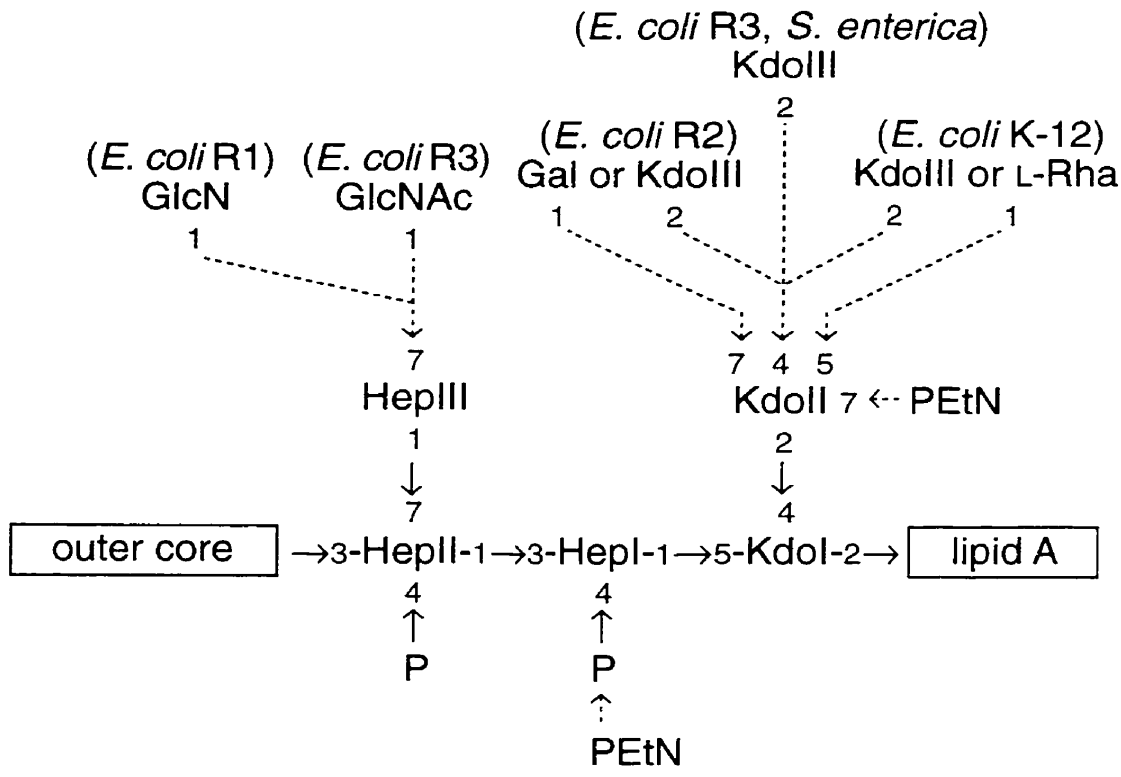


Figure 1.5. Structure of the inner core oligosaccharide from *E. coli* and *S. enterica*. The dashed arrows indicate modifications that are either non-stoichiometric, or that are confined to specific core types.

the observation that chelation of divalent cations with ethylenediamine-tetraacetic acid increases outer membrane permeability, and causes LPS molecules to be released from the cell (Vaara, 1992). In terms of pharmaceutical development, because the inner core structure is essential and highly conserved, it has received considerable attention both as a target for novel drug development (see section 1.3.2. Biosynthesis of the core oligosaccharide), and as a potential common protective antigen for the preparation of vaccines (Bennett-Guerrero *et al.*, 2000; Di Padova *et al.*, 1993; Stanislavsky *et al.*, 1997).

1.2.3. O antigen:

O antigens are the immunologically dominant surface epitopes of *E. coli* and *S. enterica*. These antigenic polysaccharides are made up of repeating oligosaccharide units, and are attached via specific sugars in the outer core to the lipid A membrane anchor. Variability within the repeat unit (in the type, linkage, and substitution of its component sugars) makes possible an enormous number of O antigen structures, and results in the vast serological diversity observed for *E. coli* and *S. enterica*. For example, more than 170 different O antigens have been identified in *E. coli* (Hull, 1997), and approximately 60 different O antigens are characterized for *S. enterica* (Reeves, 1993).

The length of the O antigen polymers in *E. coli* and *S. enterica* is often regulated by the product of the *wzz* gene (formerly *rol* or *cld*) (reviewed in Whitfield *et al.*, 1997), with the number of repeats per O antigen usually

numbering between one and forty. The chain length of the O antigen is important because it influences the ability of *E. coli* to resist complement-mediated serum killing (reviewed in Joiner, 1988; Whitfield *et al.*, 1994). Also, the extension of the O antigen polymer from the outer membrane surface may be important for 'masking' the immunostimulatory effects of the underlying lipid A (Rietschel *et al.*, 1992). Given the role of O antigen in evasion of the complement system, O antigen biosynthesis also presents some interesting avenues for drug discovery. Although the range of potential O antigen structures is enormous, there are conserved steps in its biosynthetic pathway. Particularly attractive targets include the conserved initiating steps or the final ligation step in which completed O antigen is attached to the core (see section 1.3.3. Biosynthesis of O antigen).

1.2.4. Regulated LPS modifications:

It is also noteworthy, however, that the structure of an LPS molecule can be modified in many ways. For example, in *E. coli* and *S. enterica* the acylation pattern of lipid A is known to be growth temperature-dependent (Wollenweber *et al.*, 1983). The structure of the predominant form of lipid A shown in Figure 1.3 is that which is observed during growth at 30°C. However, when *E. coli* is grown at 12°C, this structure changes, with the laurate acyl substitution being replaced to large extent by palmitoleate (Carty *et al.*, 1999). This replacement of laurate with palmitoleate presumably helps to maintain optimal fluidity in the outer membrane at lower temperatures.

S. enterica and *Pseudomonas aeruginosa* have also been shown to specifically modify the lipid A portion of their LPSs in response to other environmental cues. In these bacteria, many such lipid A modifications are controlled by the actions of the PhoP-PhoQ two-component regulatory system, which senses and responds to growth under Ca^{2+}- and Mg^{2+}-limited conditions. This system was first characterized for *S. enterica*; under conditions of low Ca^{2+} and Mg^{2+}, the sensor kinase PhoQ phosphorylates the transcriptional response regulator PhoP, which results in the activation or repression of as many as 40 different genes (García Véscovi *et al.*, 1996). Of note, these conditions are thought to simulate the environment within host tissues and macrophage phagosomes (Guo *et al.*, 1997), indicating that lipid A modifications may be occurring at key stages during the infection process. Indeed, in the case of *P. aeruginosa*, isolates from cystic fibrosis patients with chronic airway infections have been shown to express a modified form of lipid A that contributes to pathogenesis (Ernst *et al.*, 1999). Interestingly, *E. coli* K-12 also has the enzyme systems required to synthesize many of these modified lipid A derivatives, but they appear to be latent and no longer under the regulatory control of PhoP-PhoQ (Zhou *et al.*, 1999). In *E. coli* K-12, these lipid A modifications are only observed when the cells are grown in medium containing the phosphatase inhibitor VO_3^-, but the mechanism of this artificial induction is not clear (Zhou *et al.*, 1999). Whether these enzyme systems are still fully functional and regulated in wild-type *E. coli* isolates remains to seen.

Examples of lipid A modifications in *S. enterica* that are directly regulated by PhoP-PhoQ include the formation of a heptaacylated version of lipid A by the addition of palmitate (Guo *et al.*, 1998), and the replacement of the lipid A acyl group myristate with *S*-2-hydroxymyristate (Guo *et al.*, 1997). The PhoP-PhoQ-activated gene responsible for the regulated addition of palmitate has recently been identified as *pagP*, and interestingly, PagP activity has been localized to the outer membrane (Bishop *et al.*, 2000). This observation is remarkable because lipid A biosynthesis occurs at the inner face of the plasma membrane, thus suggesting that lipid A is subject to post-biosynthetic modification. Further corroborating such post-biosynthetic modifications to lipid A, a PhoP-PhoQ-activated lipid A deacylase activity (encoded by *pagL*) has also been identified and localized to the outer membrane (Trent *et al.*, 2001). Finally, the PhoP-PhoQ-regulated gene responsible for the generation of *S*-2-hydroxymyristate has been recently identified as *lpxO*, however the activity of the corresponding LpxO protein has not been localized in the cell (Gibbons *et al.*, 2000). Given the precedent of PagP and PagL, it is tempting to speculate that LpxO might also function as a post-biosynthetic lipid A modifying enzyme located in the outer membrane.

The PhoP-PhoQ system also effects lipid A modifications indirectly via activation of a second two-component regulatory system, PmrA-PmrB. In addition to signals sensed by PhoP-PhoQ, PmrA-PmrB is also known to respond independently to growth at mildly acidic pH (Soncini and Groisman, 1996) and to high concentrations of iron (Kox *et al.*, 2000). Examples of lipid A

modifications in *S. enterica* that are directly regulated by PmrA-PmrB include the addition of aminoarabinose or 2-aminoethyl phosphate (PEtN) to lipid A phosphates (Gunn *et al.*, 1998). These LPS modifications are known to be important for resistance to cationic antimicrobial compounds, such as polymyxin (Roland *et al.*, 1993; Shafer *et al.*, 1984). Resistance to these cationic compounds is thought to be effected by decreasing the net negative charge of the LPS molecule, thus reducing electrostatic interactions between the compound and the outer membrane. One mechanism by which the PmrA-PmrB system works to decrease LPS negative charge is by the addition of aminoarabinose or PEtN to lipid A (Gunn *et al.*, 1998), as discussed above. However, there is circumstantial evidence suggesting that modification of core phosphate residues may also be important to polymyxin resistance. Mutants of *E. coli* which are resistant to polymyxin are not only observed to substitute their lipid A phosphates with aminoarabinose (Nummila *et al.*, 1995), but they also appear to show increased PEtN substitution of core phosphates as well (Helander *et al.*, 1994). This PEtN substitution in the LPS core could also play a role in polymyxin resistance by decreasing the negative charge of the bacterial cell surface. Whether the PmrA-PmrB regulatory system is directly involved in the modification of core phosphates with PEtN remains to be seen.

In fact, the only confirmed report to date of a regulated covalent modification of the *E. coli* LPS core region is the PEtN substitution of the inner core KdoII residue (see Figure 1.5) (Kanipes *et al.*, 2001). The addition of

this non-stoichiometric modification was shown to be Ca^{2+}-dependent, but does not appear to involve the PhoP-PhoQ or PmrA-PmrB system since other divalent cations such as Mg^{2+} cannot substitute for this Ca^{2+} requirement. (PhoP-PhoQ does not appear to discriminate between these two metal ions, although two distinct cation binding sites have been identified in the PhoQ sensor (García Véscovi *et al.*, 1997).) The uncharacterized regulatory mechanism responsible for this phenomenon works via induction of a phosphatidylethanolamine-dependent PEtN transferase, but the structural gene encoding this activity has yet to be identified.

Finally, the O antigen portion of the LPS molecule and serospecific domain of LOSs are also subject to change. For example, the LOSs of *Neisseria* (van Putten, 1993; van Putten and Robertson, 1995), *Helicobacter* (Appelmelk *et al.*, 1998), and *Haemophilus* (Weiser and Pan, 1998) have all been shown to undergo phase variation. Phase variation appears to function as an adaptive mechanism in these bacteria, allowing them to alter or modify their cell surface polysaccharide structures in response to changing environments during the disease process. In the specific case of *N. gonorrhoeae*, the serospecific oligosaccharide is substituted differentially with sialic acid at varying stages of infection. The unsubstituted form allows for better entry into epithelial cells, at which point the bacterium converts to a highly sialylated oligosaccharide form which is more resistant to serum (van Putten, 1993). Overall, the ability of *E. coli*, *S. enterica*, and other pathogens to

modulate the structure of their LPSs suggests that LPS plays both a structural and functional role in the biology of these organisms.

1.3. LPS Biosynthesis:

LPS biosynthesis occurs by two distinct, yet convergent pathways: one for the lipid A-core unit, and another for the polysaccharide O antigen. After independent synthesis, the two parts are ligated together to complete the LPS molecule. It is not known how completed LPSs are translocated to their final destination in the outer membrane. The various aspects of LPS biosynthesis are the subject of recent reviews (Raetz, 1996; Whitfield, 1995), and are summarized briefly as follows.

1.3.1. Biosynthesis of lipid A:

The enzymology of lipid A biosynthesis in *E. coli* has in large part been resolved by work done in the Raetz laboratory (reviewed in Raetz, 1996; Raetz, 1990; with more recent developments cited below). The first steps in lipid A biosynthesis (see Figure 1.6) are catalyzed by the highly selective cytoplasmic acyltransferases LpxA and LpxD, and by the deacetylase LpxC. First, LpxA adds *R*-3-hydroxymyristate to the 3-OH of UDP-*N*-acetylglucosamine (UDP-GlcNAc). The product of this reaction is then *N*-deacetylated by LpxC, and immediately acylated by LpxD at the free amino group with another *R*-3-hydroxymyristate. Some of the resultant UDP-2,3-diacylglucosamine is cleaved at the pyrophosphate bond to give 2,3-diacyl-glucosamine-1-phosphate (lipid X). Lipid X is then combined with one of its uncleaved precursors by LpxB to give a β,1′→6-linked disaccharide.

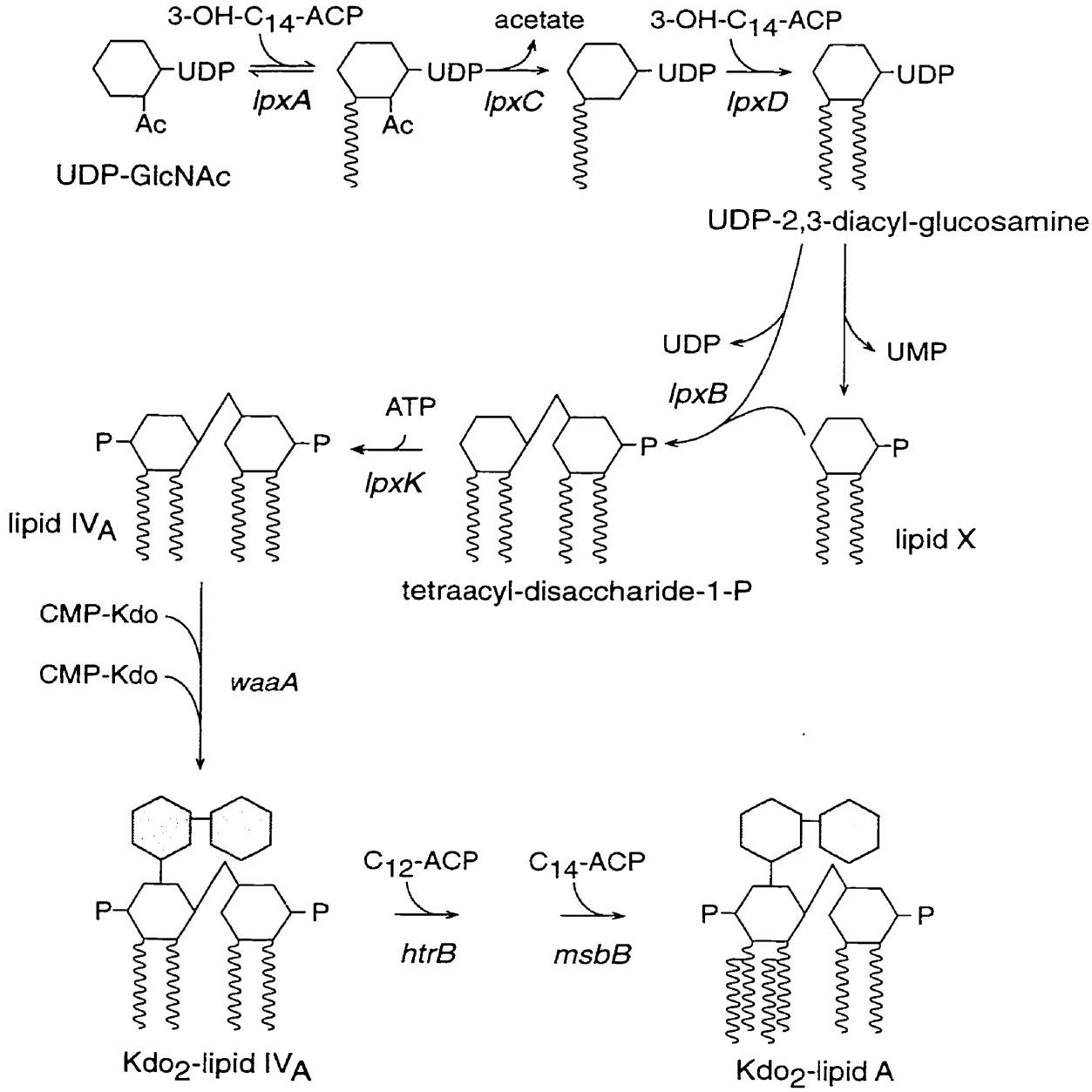


Figure 1.6. Lipid A biosynthetic pathway. See text for details.

Phosphorylation at the disaccharide 4′-position by LpxK (Garrett *et al.*, 1997) yields lipid IV_A, which serves as an acceptor for the first two Kdo residues of the inner core in *E. coli* and *S. enterica*. After the two Kdo additions, the final acylation steps performed by HtrB and MsbB (Clementz *et al.*, 1997) complete the molecule.

Two reactions in the lipid A biosynthetic pathway have thus far received particular attention in terms of antibacterial research: 1) the initial acylation of UDP-GlcNAc by LpxA, and 2) the deacetylation step catalyzed by LpxC. In an effort to facilitate the rational design of inhibitors of the LpxA acyltransferase, the protein has been crystallized, and its three-dimensional structure has been solved (Raetz and Roderick, 1995). In addition, site-directed mutagenesis studies have been carried out on LpxA (Wyckoff and Raetz, 1999) to further accelerate inhibitor design, but to date no candidate inhibitors have been reported. However, targeting the deactylase encoded by *lpxC* has proven to be somewhat more fruitful. A number of compounds have been reported which inhibit LpxC from *E. coli* (Onishi *et al.*, 1996; Wang *et al.*, 2000), or from a broad range of Gram-negative bacteria (Jackman *et al.*, 2000). The most potent of these compounds (L-161,240) is bactericidal to *E. coli* with a minimum inhibitory concentration of 1 μg/ml, and was shown to cure mice challenged with a lethal dose of *E. coli* (Onishi *et al.*, 1996). However, the frequency of resistance to compound L-161,240 is approximately 10^{-8} to 10^{-9} in *E. coli*, and two different classes of resistant mutants have thus far been identified (Rafanan *et al.*, 2000).

1.3.2. Biosynthesis of the core oligosaccharide:

The glycosyltransferases responsible for core extension are peripheral membrane proteins associated with the inner leaflet of the plasma membrane (Rick, 1987). In *E. coli* and *S. enterica*, transfer of the first two Kdo residues of the inner core is catalyzed by a single, bifunctional enzyme called WaaA (formerly KdtA) (Belunis and Raetz, 1992). As discussed previously (section 1.3.1 Biosynthesis of lipid A), for *E. coli* and *S. enterica* the addition of these first two Kdo residues is required before the final lipid A acylation steps can occur. *P. aeruginosa* differs somewhat in this respect, in that fully acylated lipid A is synthesized prior to the addition of Kdo (Goldman *et al.*, 1988; Mohan and Raetz, 1994). While no compounds have been developed that can interfere directly with the WaaA enzyme, more success has been attained in pursuing the biosynthesis of its sugar-nucleotide precursor as a drug target.

The activated sugar-nucleotide precursor for Kdo addition is CMP-Kdo, and the enzymology for the biosynthesis of this unique sugar-nucleotide is well established (Goldman *et al.*, 1986) (see Figure 1.7B). Given the restricted occurrence of this sugar-nucleotide and its requirement for the completion of lipid A, the biosynthesis of this precursor has attracted considerable attention as a target for drug design. Indeed, a number of inhibitors targeted to Kdo biosynthesis have been reported (Birck *et al.*, 2000; Du *et al.*, 1999; Goldman *et al.*, 1987; Hammond *et al.*, 1987). The earliest of these compounds (Goldman *et al.*, 1987; Hammond *et al.*, 1987) were Kdo analogs that served as competitive inhibitors of the CMP-Kdo synthase encoded by *kdsB* (see

Figure 1.7. Biosynthesis of sugar-nucleotide precursors required for the assembly of the LPS inner core oligosaccharide of *E. coli* and *S. enterica*. (A): Biosynthesis of ADP-L-*glycero*-D-*manno*-heptose (adapted from Valvano *et al.*, 2000). A specific enzyme has not been identified for the dephosphorylation of the D-*glycero*-D-*manno*-heptose 7-phosphate intermediate, but this reaction can be catalyzed by a non-specific phosphatase (e.g. alkaline phosphatase) *in vitro* (Brooke and Valvano, 1996). Although Domains I and II of the RfaE protein are expressed as a single polypeptide, they are also able to fulfill their distinct functions as separate proteins. (B): Biosynthesis of CMP-Kdo (adapted from Raetz, 1996). A specific enzyme has not been identified for the dephosphorylation of the Kdo 8-phosphate intermediate, but this reaction can also be catalyzed by a non-specific phosphatase (e.g. alkaline phosphatase) *in vitro* (Clementz and Raetz, 1991).

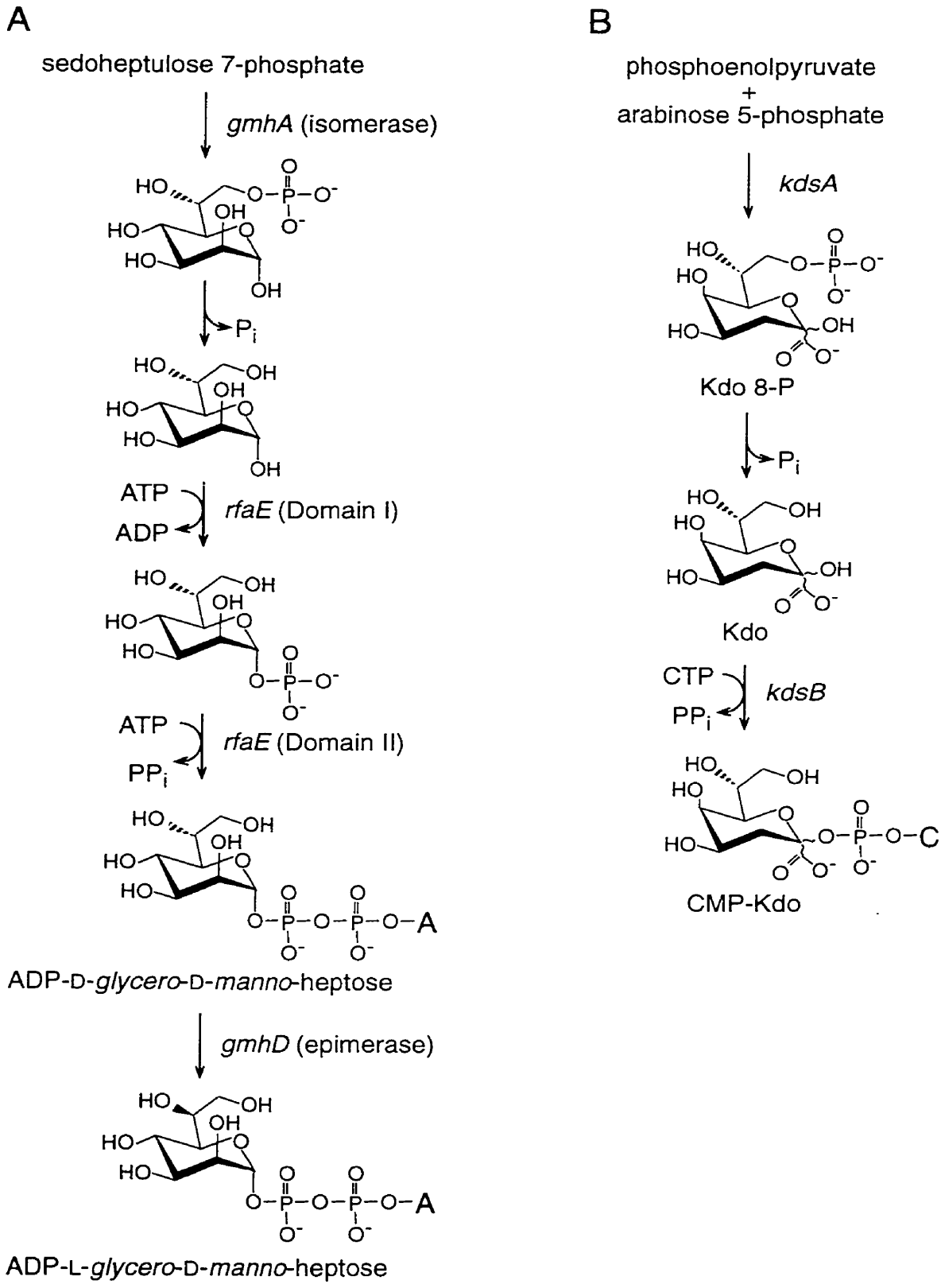

A
sedoheptulose 7-phosphate
gmhA (isomerase)
Pi
ATP
ADP
rfaE (Domain I)
ATP
PPi
rfaE (Domain II)
ADP-D-glycero-D-manno-heptose
gmhD (epimerase)
ADP-L-glycero-D-manno-heptose
B
phosphoenolpyruvate
+
arabinose 5-phosphate
kdsA
Kdo 8-P
Pi
Kdo
CTP
PPi
kdsB
CMP-Kdo

Figure 1.7B). Unfortunately, these Kdo analogs permeated poorly across the bacterial cell envelope, and in order to achieve penetration to the target enzyme they needed to be derivatized with short peptides to promote their uptake via the oligopeptide permease system (Goldman *et al.*, 1987; Hammond *et al.*, 1987). However, this mechanism of uptake allows too easy a route to resistance, and these compounds have not found clinical application. More recently, compounds have been developed that target the Kdo 8-phosphate synthase encoded by *kdsA* (Birck *et al.*, 2000; Du *et al.*, 1999). In general, these KdsA inhibitors have been designed as bisubstrate or transition-state analogues, but their application is currently limited by their hydrophilicity, susceptibility to hydrolysis, and complicated syntheses.

After completion of Kdo_2-lipid A, extension of the inner core oligosaccharide then proceeds by sequential addition of sugar residues from activated sugar-nucleotide precursors by specific glycosyltransferases. For example, the conserved main-chain Hep region of the LPS inner core (HepII-[1→3]-HepI-[1→ , refer back to Figure 1.5) found in all *E. coli* and *S. enterica* core types is assembled by the sequential action of the WaaC (Kadrmas and Raetz, 1998; Sirisena *et al.*, 1992) and WaaF (Gronow *et al.*, 2000) heptosyltransferases using ADP-Hep as a substrate. Homologs of WaaC and WaaF exist in a variety of clinically relevant Gram-negative species, and given the specificity of this reaction and its conservation among many core types and genera, the WaaC and WaaF enzymes might provide potential targets for inhibitor design. The biosynthesis of the unique sugar-nucleotide

precursor for these heptosyltransferases (see Figure 1.7A) also represents a viable target, although some of the details of its enzymology still need to be resolved (Valvano *et al.*, 2000, and references therein).

Synthesis of the more variable outer core proceeds in similar fashion to that for the inner core, except that the enzymes involved are less conserved. Thus the biosynthesis of the outer core offers less opportunity for broad-based therapeutic intervention. Upon completion of the lipid A-core structure, it is subsequently translocated to the outer leaflet of the plasma membrane by a process which requires both ATP and the proton motive force (Marino *et al.*, 1985; McGrath and Osborn, 1991(a)). Export across the plasma membrane is proposed to occur via the recently identified MsbA protein, an ATP-binding cassette transporter (Polissi and Georgopoulos, 1996; Zhou *et al.*, 1998). It is not known to what degree the translocation process is conserved among different genera.

1.3.3. Biosynthesis of O antigen:

O antigen synthesis occurs separately on a lipid carrier molecule called undecaprenol phosphate (a C_{55}-polyisoprenoid derivative), which is embedded in the plasma membrane (Wright *et al.*, 1967). An initiation reaction first transfers a sugar-phosphate residue from an activated precursor onto the lipid carrier. This initiation step primes the carrier lipid for subsequent O antigen biosynthesis, perhaps sequestering the undecaprenol phosphate from its other essential roles in the cell (e.g. its requirement in peptidoglycan biosynthesis, see Higashi *et al.*, 1967). In fact, the priming sugar

is sometimes not even a part of the O antigen repeat unit structure. Despite the vast number of characterized O antigen structures, only two types of initiating enzyme have been discovered: WecA (formerly Rfe) and the WbaP family of enzymes (reviewed in Whitfield, 1995). WecA initiates polymer synthesis with the addition of GlcNAc-1-phosphate (Meier-Dieter *et al.*, 1992), while the WbaP family of enzymes prime the carrier lipid with Glc-1-phosphate or Gal-1-phosphate (Jiang *et al.*, 1991). WecA and WbaP are predicted to be integral membrane proteins with similar hydropathy profiles (Klena and Schnaitman, 1993), perhaps reflecting a shared requirement for interaction with undecaprenol phosphate. The conservation of this initiating reaction may thus present an opportunity to target inhibitors toward O antigen biosynthesis, but this line of research has yet to be explored.

Subsequent chain elongation generally occurs by one of two mechanisms. In the case of heteropolysaccharide and branched O antigens, individual undecaprenol-linked O antigen repeat units are assembled at the cytoplasmic face of the membrane, then transferred to the periplasmic side by a reaction involving the Wzx (formerly RfbX) protein (Feldman *et al.*, 1999), and finally added to the reducing terminus of the growing polysaccharide chain by the O antigen polymerase, Wzy (formerly Rfc) (Daniels *et al.*, 1998; Naide *et al.*, 1965). By contrast, simple, linear O antigens are polymerized completely within the cytoplasm by sequential addition of sugar residues to the non-reducing terminus of the lipid-linked intermediate, and the entire

O antigen is subsequently transported to the periplasmic side of the membrane via the action of an ATP-binding cassette-transporter.

In both pathways, completed O antigen is covalently linked to preformed lipid A-core by the action of the ligase enzyme, which is presumed to be WaaL. Direct biochemical evidence for WaaL function is lacking, but WaaL mutants have been shown to accumulate undecaprenol pyrophosphate-linked O antigen units at the periplasmic face of the plasma membrane (McGrath and Osborn, 1991(b); Mulford and Osborn, 1983). WaaL proteins are poorly conserved at the primary amino acid sequence level, but are predicted to have very similar secondary structure and membrane topologies, being characterized by the possession of eight or more transmembrane domains and a relatively large (approximately 80 amino acid) periplasmic loop, which represents the potential catalytic domain. Further, it has been shown by cross-complementation studies that WaaL enzymes can accommodate heterologous O antigens of vastly differing structures. Again, the O antigen ligation step presents an interesting potential therapeutic target, although much still needs to be learned about the mechanism of action of this complex protein.

1.4. Focus on the core oligosaccharide, historical perspective and genetics:

Much of what is known about the structure, function, and biosynthesis of LPS comes from the discovery of mutants defective in particular steps of its biosynthetic pathway. In the early 1920s, a mutant phenotype designated 'rough' was described for isolates of *S. enterica*. The 'rough' designation

referred to the mutants' atypical colony morphology in comparison to the shiny or 'smooth' wild-type colonies on solid media. Other characteristics of the isolated rough mutants included a tendency to autoagglutinate, avirulence, and loss of their specific somatic antigen (O antigen). The most likely explanation for the phenotypic appearance of these mutants is that the loss of their highly hydrated O antigen polysaccharide from the cell surface resulted in a drier and hence rougher-looking colony morphology.

The first genetic analyses mapped the site of these rough mutations to two distinct loci, originally called *rouA* and *rouB* (Subbaiah and Stocker, 1964) but later renamed *rfa* and *rfb* respectively. The *rfa* locus maps near min 81 on the *S. enterica* (Sanderson and Roth, 1988) and *E. coli* K-12 (Bachmann, 1990) linkage maps, while the *rfb* locus maps near min 45. The *rfa* locus was inferred by chemical and serological analyses (Beckmann *et al.*, 1964), and by enzymatic studies (Nikaido *et al.*, 1964), to encode the genes responsible for synthesis of the core oligosaccharide. The *rfb* locus, on the other hand, was shown to encode genes involved in the synthesis of the LPS O antigen repeat unit (Nikaido *et al.*, 1966). Mutations in both of these loci thus result in loss of O antigen, but for different reasons: rough mutations in the *rfa* locus result in truncated core oligosaccharides that cannot serve as acceptors for O antigen, while rough mutations in the *rfb* locus are unable to properly synthesize or assemble the O antigen repeat unit. Recently, the system of nomenclature for genes involved in LPS biosynthesis has again been revised (Reeves *et al.*, 1996), both for clarity and to accommodate the increasingly large number of

characterized genes. Under the newly proposed scheme, genes for the glycosyltransferases encoded by the former *rfa* and *rfb* loci have been renamed using the form *wa*** and *wb***, respectively (where the asterisks represent variable letters).

Clearly, any mutation within the core biosynthetic pathway that affects the addition of a main chain sugar will also affect the addition of all subsequent sugars, including the O antigen, and result in the rough phenotype. However, a subset of *E. coli* and *S. enterica* core mutants with highly truncated core oligosaccharides (usually missing one or both core Hep residues) exhibit an even more dramatic phenotype called "deep-rough" (reviewed in Heinrichs *et al.*, 1998(c); Schnaitman and Klena, 1993). Characteristics of the deep-rough phenotype include 1) hypersensitivity to detergents and hydrophobic antibiotics (Roantree *et al.*, 1977; Sanderson *et al.*, 1974; Tamaki *et al.*, 1971), 2) the appearance of phospholipid in the outer leaflet of the outer membrane bilayer (Kamio and Nikaido, 1976; Smit *et al.*, 1975), 3) leakage of periplasmic proteins into the culture medium (Chatterjee *et al.*, 1976; Lindsay *et al.*, 1972), and 4) a marked decrease in the protein content of the outer membrane (Ames *et al.*, 1974; Koplow and Goldfine, 1974; Lugtenberg *et al.*, 1976). It has also been shown that the LPS from deep-rough mutants cannot support the proper folding of some outer membrane proteins (de Cock *et al.*, 1999; Sen and Nikaido, 1991). Many of the characteristics associated with this pleiotropic phenotype are thought to arise from the lack of inner core phosphate groups, which are lost in these highly truncated core

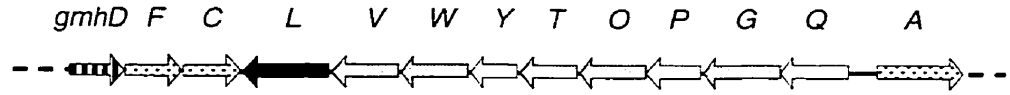
mutants (refer back to Figure 1.5). The loss of these critical phosphate groups would prevent the crosslinking of LPS molecules by divalent cations, thus destabilizing the outer membrane (reviewed in Nikaido and Vaara, 1985).

Unfortunately, the initial isolation of rough and deep-rough core mutants of *S. enterica* relied heavily on the use chemical mutagenesis and selection by bacteriophages with specificities for different LPS structures. As such, much of what was known about the genetics of core biosynthesis at the outset of this thesis relied on poorly-characterized mutant strains that potentially carried multiple or polar mutations. However, with the advent of improved PCR technologies and automated sequencing, it became possible to revisit much of this earlier work with improved genetic data in hand.

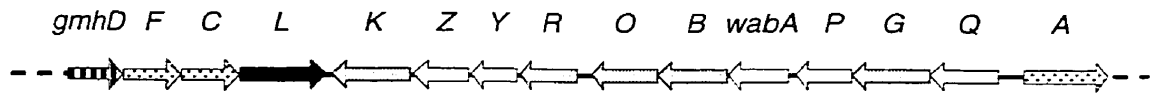
With this goal in mind, the genetic loci responsible for core oligosaccharide biosynthesis in *E. coli* R1-R4 were recently sequenced in their entirety (Heinrichs *et al.*, 1998(a); Heinrichs *et al.*, 1998(c)). The corresponding sequence for *S. enterica* was compiled from several published fragments (Klena *et al.*, 1993; MacLachlan *et al.*, 1991) and new sequence data (Heinrichs *et al.*, 1998(a)), and for *E. coli* K-12 these data were simply retrieved from its completed genome sequence (Blattner *et al.*, 1997). Figure 1.8 shows the comparative chromosomal organization of these loci. There are three operons in each locus, which are arranged clockwise on the circular chromosomal map as (1) an operon beginning with *gmhD* (formerly *rfaD*), followed by (2) a long, central operon beginning with *waaQ* (formerly *rfaQ*), and lastly (3) a two-gene operon beginning with *waaA* (formerly *kdtA*)

Figure 1.8. Physical maps of the genetic loci involved in core oligosaccharide biosynthesis (Heinrichs *et al.*, 1998(a)). These loci are found at approximately 81 min on the *E. coli* K-12 and *S. enterica* linkage maps.

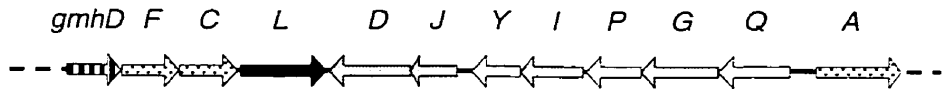
E. coli R1:



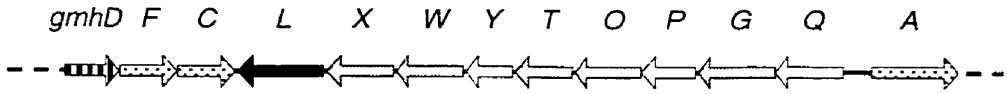
E. coli R2:



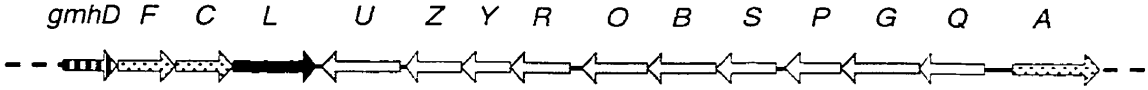
E. coli R3:



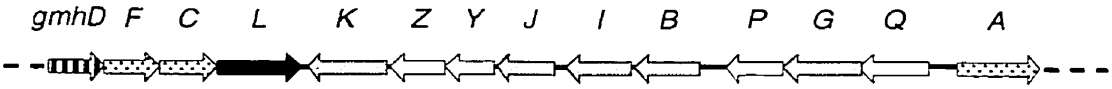
E. coli R4:

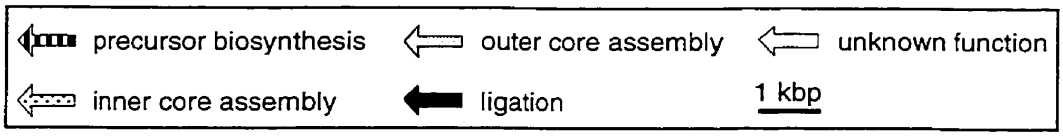


E. coli K-12:



S. enterica sv. Typhimurium:





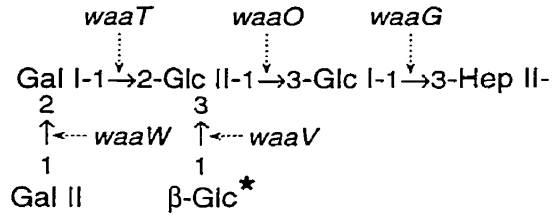
(Roncero and Casadaban, 1992). (The second gene in the *waaA* operon is not involved in core biosynthesis (Geerlof *et al.*, 1999), and so it has been omitted from Figure 1.8.) At the completion of sequencing, limited biochemical data was available for the assignment of function to these genes. To summarize what was known at the time, the first two Kdo residues of the inner core were known to be added by WaaA (Belunis and Raetz, 1992), followed by the addition of the first Hep residue by WaaC (Kadrmas and Raetz, 1998), the second Hep residue by WaaF (Gronow *et al.*, 2000), and the first glucose (Glc) residue of the outer core by WaaG (Müller *et al.*, 1972). However, by combining the known outer core chemical structures with this new sequence data for each of the different core types, tentative gene assignments were made at the time of sequencing as shown in Figure 1.9. These assignments were based on the limited known biochemical data, and some cross-complementation studies, but relied very heavily on sequence homology.

1.5. Unresolved issues in LPS inner core biosynthesis:

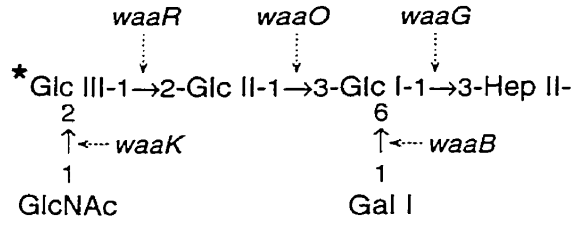
Despite tentative assignment of function to many of the genes involved in core biosynthesis at their time of sequencing, there were still a number of genes within the core biosynthesis loci to which no biological function could be ascribed. Of these unassigned genes, *waaY*, *waaQ*, and *waaP* were well-conserved and present in all of the sequenced loci (refer back to Figure 1.8). Given their high degree of conservation, candidate functions for these gene products were expected to be conserved among all the different core types. (The assumption was made that these genes were involved in core

Figure 1.9. Assignment of function to genes involved in the biosynthesis of the outer core oligosaccharides of *E. coli* and *S. enterica* (reviewed in Heinrichs *et al.*, 1998(a)).

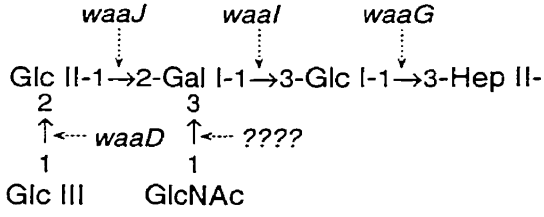
E. coli R1



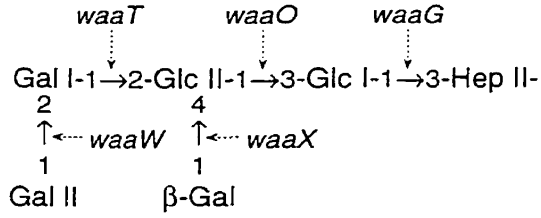
E. coli R2



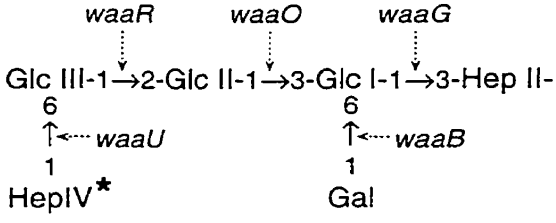
E. coli R3



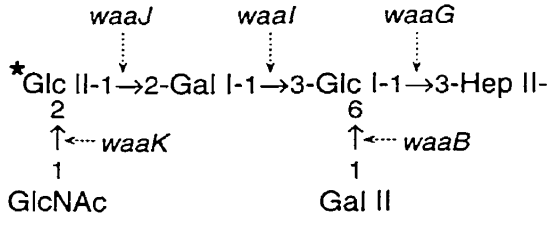
E. coli R4



E. coli K-12



S. enterica sv. Typhimurium



biosynthesis, and that their placement within these operons was not simply happenstance). Thus, it was postulated at the time that these gene products were somehow involved in the biosynthesis of the conserved inner core region.

Indeed, although the role of the conserved *waaY* gene was completely enigmatic at the time, there was some evidence to support a role for *waaQ* and *waaP* in inner core biosynthesis. Firstly, *waaQ* was observed to share some sequence homology with *waaC* and *waaF* (encoding the HepI and HepII transferases, respectively), so it was suggested that WaaQ might be involved in the transfer of the conserved inner core branch HepIII residue (Parker *et al.*, 1992(a)) (see Figure 1.10). In addition, previous studies with *S. enterica* serovars Minnesota (Dröge *et al.*, 1968; Mühlradt *et al.*, 1968) and Typhimurium (Helander *et al.*, 1989), and with *E. coli* K-12 (Parker *et al.*, 1992(a)) had implicated *waaP* in the phosphorylation of both HepI and HepII (see Figure 1.10). But confounding the issue, in *E. coli* K-12, *waaP* was also implicated in the addition of HepIII, for which the *waaQ* gene product seemed a more likely candidate (see Figure 1.10). Interpretation of these data, however, was complicated by their reliance on strains with poorly-defined or polar mutations. Also confusing the assignment of function to *waaP* was that despite its potential involvement in inner core phosphorylation, the predicted WaaP protein showed no primary sequence similarity to known kinases or other phosphotransferases. However, mutation of *waaP* in these bacteria was reported to cause characteristics of the deep-rough phenotype,

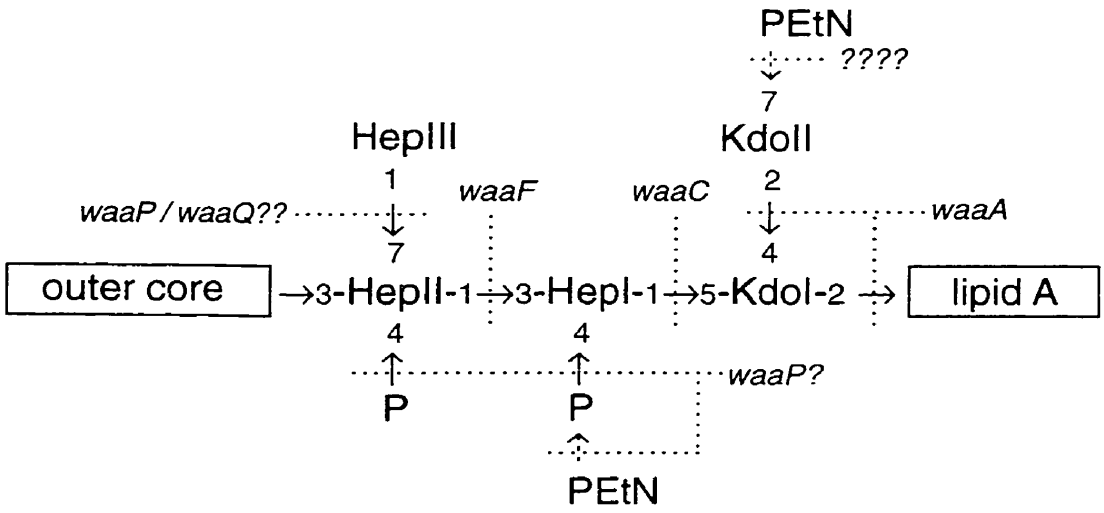


Figure 1.10. Summary of the known and predicted activities of core biosynthetic genes involved in assembly of the conserved inner core of *E. coli* and *S. enterica*. Non-stoichiometric substitutions are indicated by dashed arrows.

making the characterization of its gene product a significant goal towards a better understanding the Gram-negative outer membrane barrier function.

1.6. Thesis research objectives:

The first goal of this study was to identify unequivocally the genes involved in the phosphoryl modification of the inner core Hep residues. While earlier studies had implicated at least one gene (*waaP*) in this process, the reliance of those studies on strains with poorly-defined or polar mutations necessitated a more precise approach. To this end, the first objective was to make defined, non-polar, chromosomal mutations in candidate genes, and to determine the resulting core structures by chemical methods. In this way, single mutations could be correlated to defined changes in core oligosaccharide structure.

Building on this initial work, the second goal of this research was to better characterize the proteins encoded by these genes. Given the importance of core phosphorylation to membrane stability, it was anticipated that better definition of these reactions might present novel opportunities for therapeutic intervention. Therefore, the second objective was to purify key proteins and reconstitute their enzymatic activities using purified components *in vitro*. These *in vitro* assays could then be used for high through-put screening of small compound libraries, to find inhibitors for the corresponding reactions. The ultimate goal would be to develop these inhibitory compounds as potential leads for the development of new classes of antibacterial agents.

Chapter 2. Materials and Methods

2.1. Bacterial strains and plasmids:

The bacterial strains in this study are summarized in Table 2.1, and plasmids are summarized in Table 2.2.

2.2. Media and growth conditions:

Cultures were routinely grown in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, MI) or on LB-agar plates, at 30°C or 37°C. Growth media were supplemented with antibiotics (Sigma Chemical Co., St. Louis, MO) at the following concentrations when necessary: ampicillin (Ap; 100 µg/mL), gentamicin (Gm; 15 µg/mL), kanamycin (Km; 50 µg/mL) or chloramphenicol (Cam; 30 µg/mL).

2.3. DNA methods:

2.3.1. General cloning techniques:

Restriction endonucleases were purchased from either Canadian Life Technologies, Inc. (Burlington, ON), New England Biolabs (Mississauga, ON), or Roche Molecular Biochemicals (Laval, QC), and were used according to the manufacturer's instructions. T4 DNA ligase and Klenow were purchased from New England Biolabs and Canadian Life Technologies, respectively. Agarose gel electrophoresis was performed in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8), using 0.7% (w/v) agarose gels. Agarose gels were stained with ethidium bromide (20 µg/mL in H_2O) to allow DNA visualization under UV light. When necessary, DNA fragments were isolated from agarose gels using the GENECLEAN kit from Bio/Can Scientific (Mississauga, ON).

Table 2.1. Summary of bacterial strains used in this study.

Strain	Description	Reference or Source:
F470	*E. coli* R1 core prototype strain; used for cloning of core biosynthesis genes and construction of defined core mutations; rough mutant and acapsular derivative of an O8:K27 parent	(Schmidt *et al.*, 1969)
SGSC225	*S. enterica* serovar Typhimurium wild-type strain; used for cloning of core biosynthesis genes and construction of defined core mutations	Salmonella Genetic Stock Centre
ATCC 14028	*S. enterica* serovar Typhimurium wild-type strain; used to construct specific mutations in core biosynthesis genes and for subsequent virulence testing	American Type Culture Collection
W3100	K-12 IN(*rrnD-rrnE*)*1 rph-1*; K-12 strain; used as a source of *groESL*, encoding molecular chaperones	*E. coli* Genetic Stock Center
DH5α	K-12 ϕ80d *lacZ*ΔM15 *endA1 recA1 hsdR17* ($r_K^- m_K^-$) *supE44 thi-1 gyrA96*	(Sambrook *et al.*, 1989)

	relA1 Δ(*lacZYA-argF*) U169 F^-; host for general cloning strategies	
BL21(DE3)	F^- *ompT gal* [*dcm*] [*lon*] $hsdS_B$ ($r_B^-m_B^-$) λDE3; used for high level recombinant protein expression from the T7 promoter	Novagen
CWG296 [a]	*waaP::aacC1* derivative of F470; Gm^R	this study, (Yethon *et al.*, 1998)
CWG297 [a]	*waaQ::aacC1* derivative of F470; Gm^R	this study, (Yethon *et al.*, 1998)
CWG303 [a]	*waaG::aacC1* derivative of F470; Gm^R	this study, (Heinrichs *et al.*, 1998(b))
CWG308 [b]	*waaO::aacC1* derivative of F470; Gm^R	this study, (Heinrichs *et al.*, 1998(b))
CWG309 [b]	*waaT::aacC1* derivative of F470; Gm^R	this study, (Heinrichs *et al.*, 1998(b))
CWG310 [b]	*waaW::aacC1* derivative of F470; Gm^R	this study, (Heinrichs *et al.*, 1998(b))

CWG311 [b]	*waaV::aacC1* derivative of F470; Gm^R	this study, (Heinrichs *et al.*, 1998(b))
CWG312 [b]	*waaY::aacC1* derivative of F470; Gm^R	this study, (Yethon *et al.*, 1998)
CWG304 [a]	*waaP::aacC1* derivative of ATCC 14028; Gm^R	this study, (Yethon *et al.*, 2000(a))
JSG435	derivative of ATCC 14028 carrying the *pmrA505* allele, which confers a PmrA-constitutive phenotype	(Gunn and Miller, 1996)
JSG778 [c]	derived by P22 transduction of the *waaP::aacC1* allele into JSG435	this study, (Yethon *et al.*, 2000(a))

[a] strain created as part of this thesis work

[b] strain created by Dr. D. E. Heinrichs

[c] strain created by Dr. J. S. Gunn

Table 2.2. Summary of plasmids used in this study.

Plasmid	Description	Reference
1. Plasmids used and created for the generation of chromosomal mutations:		
pBluescriptII SK(+)	General cloning vector; Ap^R; Stratagene	Stratagene
pUCGM	Derivative of pUC18/19 carrying the *aacC1* gene (gentamicin resistance cassette); Ap^R Gm^R	(Schweizer, 1993)
pMAK705	suicide vector for chromosomal allelic exchange; temperature sensitive; Cam^R	(Hamilton *et al.*, 1989)
pJY1	R1C11-R1K5 PCR product (*waaP* and flanking DNA from F470) cloned into *Hin*cII site of pBluescriptII SK(+) (opposite orientation of *lacZ*); Ap^R	this study
pJY2	855 bp *Sma*I fragment of pUCGM containing *aacC1* cloned into the *Nru*I site of pJY1 (same orientation as *waaP*); Ap^R Gm^R	this study
pJY3	2563 bp *Xho*I-*Hin*dIII fragment of pJY2 containing disrupted *waaP* from	this study

	F470 cloned into the *Sal*I-*Hin*dIII sites of pMAK705; Cam^R Gm^R	
pJY10	ST11-ST26 PCR product (*waaP* and flanking DNA from SGSC225) cloned into *Hin*cII site of pBluescriptII SK(+) (opposite orientation of *lacZ*); Ap^R	this study
pJY11	855 bp *Sma*I fragment of pUCGM containing *aacC1* cloned into the 4814 bp *Nru*I fragment of pJY10 (same orientation as *waaP*); Ap^R Gm^R	this study
pJY12	2730 bp *Xho*I-*Hin*dIII fragment of pJY11 containing disrupted *waaP* from SGSC225 cloned into the *Sal*I-*Hin*dIII sites of pMAK705; Cam^R Gm^R	this study
pJY7	R1C14-KDTA2 PCR product (*waaQ* and flanking DNA from F470) cloned into *Hin*cII site of pBluescriptII SK(+) (opposite orientation of *lacZ*); Ap^R	this study
pJY8	855 bp *Sma*I fragment of pUCGM containing *aacC1* cloned into the *Nru*I site of pJY7 (same orientation as *waaQ*); Ap^R Gm^R	this study

pJY9	2760 bp *Xho*I-*Hin*dIII fragment of pJY8 containing disrupted *waaQ* from F470 cloned into the *Sal*I-*Hin*dIII sites of pMAK705; Cam^R Gm^R	this study
pJY13	R1C12-KDTA3 PCR product (*waaG* and flanking DNA from F470) cloned into *Hin*cII site of pBluescriptII SK(+) (opposite orientation of *lacZ*); Ap^R	this study
pJY14	855 bp *Sma*I fragment of pUCGM containing *aacC1* cloned into the filled-in (Klenow) *Sfu*I site of pJY13 (same orientation as *waaG*); Ap^R Gm^R	this study
pJY15	3300 bp *Xho*I-*Hin*dIII fragment of pJY14 containing disrupted *waaG* from F470 cloned into the *Sal*I-*Hin*dIII sites of pMAK705; Cam^R Gm^R	this study
pDEH115	R1C6-R1K10 PCR product (*waaY* and flanking DNA from F470) cloned into *Eco*RV site of pBluescriptII SK(+) (opposite orientation of *lacZ*); Ap^R	Dr. D. E. Heinrichs, unpublished
pDEH118	855 bp *Sma*I fragment of pUCGM containing *aacC1* cloned into the	Dr. D. E. Heinrichs,

	*Sna*BI site of pDEH115 (same orientation as *waaY*); Ap^R Gm^R	unpublished
pDEH119	*Pst*I-*Sal*I fragment of pDEH118 containing disrupted *waaY* from F470 cloned into the *Pst*I-*Sal*I sites of pMAK705; Cam^R Gm^R	Dr. D. E. Heinrichs, unpublished

2. Plasmids used and created for protein expression studies:

pET-28a(+)	IPTG-inducible expression vector; Km^R	Novagen
pET-30a(+)	IPTG-inducible expression vector; Km^R	Novagen
pBAD18	Arabinose-inducible expression vector; Ap^R	(Guzman *et al.*, 1995)
pBAD24	Arabinose-inducible expression vector; Ap^R	(Guzman *et al.*, 1995)
pBAD33	Arabinose-inducible expression vector; Cam^R	(Guzman *et al.*, 1995)
pJY4	JY1-JY2 PCR product (*waaP* from F470), digested *Nde*I-*Bam*HI, cloned into the *Nde*I and *Bam*HI sites of pET-30a(+); for high-level expression of WaaP from the T7 promoter; Kan^R	this study
pWQ909	*Xba*I-*Hin*dIII fragment from pJY4	this study,

	containing *waaP* and an optimally positioned Shine-Dalgarno sequence, cloned into the *Xba*I-*Hin*dIII sites of pBAD18; for expression of WaaP from the P_{BAD} promoter; Ap^R	(Yethon *et al.*, 1998)
pWQ910	JY1-JY2 PCR product (*waaP* from F470), digested *Nde*I-*Bam*HI, cloned into the *Nde*I and *Bam*HI sites of pET-28a(+); for high-level expression of His_6-tagged WaaP from the T7 promoter; Kan^R	this study, (Yethon and Whitfield, 2001)
pWQ912	*Xba*I-*Hin*dIII fragment from pWQ910 containing *waaP* and an optimally positioned Shine-Dalgarno sequence, cloned into the *Xba*I-*Hin*dIII sites of pBAD18; for expression of His_6-tagged WaaP from the P_{BAD} promoter; Ap^R	this study, (Yethon and Whitfield, 2001)
PJY22	JY3-JY4 PCR product (*groESL* from W3100), digested *Nde*I-*Hin*dIII, cloned into the *Nde*I and *Hin*dIII sites of pET-30a(+); for expression of GroES and GroEL from the T7	this study

	promoter; Kan^R	
pWQ911	*Xba*I-*Hin*dIII fragment from pJY22 containing *groESL* and an optimally positioned Shine-Dalgarno sequence, cloned into the *Xba*I-*Hin*dIII sites of pBAD33; for expression of GroES and GroEL from the P_{BAD} promoter; Cam^R	this study, (Yethon and Whitfield, 2001)
pWQ913	for expression of His_6-tagged WaaP(D162A); derived by PCR-based site-directed mutagenesis of pWQ912 using the JY5-JY6 primer pair; Ap^R	this study, (Yethon and Whitfield, 2001)
pJY29	*Xba*I-*Hin*dIII fragment from pWQ913 encoding His_6-tagged WaaP(D162A) behind an optimally positioned Shine-Dalgarno sequence, cloned into the *Xba*I-*Hin*dIII sites of pET-30a(+); for expression of His_6-tagged WaaP(D162A) from the T7 promoter; Kan^R	this study
pJY30	for expression of His_6-tagged WaaP(H167N); derived by PCR-based site-directed mutagenesis of pWQ912 using the JY7-JY8 primer pair; Ap^R	this study

pJY31	*Xba*I-*Hin*dIII fragment from pJY30 encoding His_6-tagged WaaP(H167N) behind an optimally positioned Shine-Dalgarno sequence, cloned into the *Xba*I-*Hin*dIII sites of pET-30a(+); for expression of His_6-tagged WaaP(H167N) from the T7 promoter; Kan^R	this study
3. Other plasmids:		
pWQ3	pRK404 derivative containing the O antigen biosynthetic cluster from *Klebsiella pneumoniae* O1; Tet^R	(Clarke and Whitfield, 1992)
pWQ903	pBAD24 derivative for expression of WaaG from the P_{BAD} promoter; Ap^R	(Heinrichs *et al.*, 1998(b))
pWQ904	pBAD24 derivative for expression of WaaO from the P_{BAD} promoter; Ap^R	(Heinrichs *et al.*, 1998(b))
pWQ905	pBAD24 derivative for expression of WaaT from the P_{BAD} promoter; Ap^R	(Heinrichs *et al.*, 1998(b))
pWQ906	pBAD24 derivative for expression of WaaW from the P_{BAD} promoter; Ap^R	(Heinrichs *et al.*, 1998(b))
pWQ907	pBAD24 derivative for expression of WaaV from the P_{BAD} promoter; Ap^R	(Heinrichs *et al.*, 1998(b))

Plasmid DNA was prepared using QIAprep Miniprep Spin Columns (Qiagen Inc., Santa Clarita, CA). Plasmids were introduced into *E. coli* strains by electroporation (2.5 kV, 15 μFD, 400 Ω) using a Gene Pulser from Bio-Rad Laboratories (Richmond, CA). Electrocompetent cells for transformation were prepared by the method of Binotto (Binotto *et al.*, 1991).

2.3.2. PCR and DNA sequencing:

PCR amplifications were carried out in a Perkin-Elmer (Mississauga, ON) GeneAmp PCR System 2400 thermocyler. *Pwo*I DNA polymerase (Roche Molecular Biochemicals) was used according to the manufacturer's recommendations, with amplification conditions optimized for each primer pair. Template genomic DNA was purified using the InstaGene kit (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions. Primers used for PCR amplification are summarized in Table 2.3. Primers were synthesized using a Perkin-Elmer 394 DNA synthesizer at the Guelph Molecular Supercentre (University of Guelph, Guelph, ON). DNA sequencing was also performed at the Guelph Molecular Supercentre, using an ABI 377 DNA sequencing apparatus (Perkin-Elmer).

2.3.3. Generation of non-polar chromosomal insertion mutations:

Individual genes of the R1 core oligosaccharide biosynthesis region were independently mutated by insertion of a non-polar gentamicin-resistance cassette (the *aacC1* gene from Tn*1696*). Briefly, each gene (with some flanking DNA) was individually PCR-amplified, cloned into pBluescript® II SK(+) (Stratagene), and sequenced to ensure error-free

Table 2.3. Primers used for PCR amplification.

Primer	Sequence (5'-3') [a]	Description
Primers used to amplify target genes for allelic exchange:		
R1C11	GCAATTACAAAACGGAAATA	*E. coli* R1 *waaP*
R1K5	GTTGCAGGTCGGTTCAGACT	*E. coli* R1 *waaP*
ST11	TTGGCATAAAGACATGAGAT	*S. enterica waaP*
ST26	TGGCATCGCTACCCGAATCT	*S. enterica waaP*
R1C14	CGCTCCGCTTCAGTCTGATA	*E. coli* R1 *waaQ*
KDTA2	TTCCCGTTTTTGACTTAAAT	*E. coli* R1 *waaQ*
R1C12	GCAGGTCAATTACCGAAATT	*E. coli* R1 *waaG*
KDTA3	TTATTAAATGCCCGTGTGAA	*E. coli* R1 *waaG*
R1C6	CGTGTGCTGCGGGTCACTT	*E. coli* R1 *waaY*
R1K10	TGCAAAACGCCTCAACATTC	*E. coli* R1 *waaY*
Primers used to verify chromosomal mutations:		
R1C10	CCGTTAATTGGTTTGTTTTG	*E. coli* R1 *waaP*
R1K4	GTTATTCCGGCGGCAGATGT	*E. coli* R1 *waaP*
ST7	CGGCGCGCTTTGCCAACCAT	*S. enterica waaP*
ST25	GGAATGCCCGGATAACTTTG	*S. enterica waaP*
R1C13	GCTTTCTCTGGCAGACTGTA	*E. coli* R1 *waaQ*
KDTA	TTCGGTTGCAGGTAAGGTTC	*E. coli* R1 *waaQ*
R1C11	GCAATTACAAAACGGAAATA	*E. coli* R1 *waaG*

KDTA3	TTATTAAATGCCCGTGTGAA	*E. coli* R1 *waaG*
R1C6	CGTGTGCTGCGGGTCACTT	*E. coli* R1 *waaY*
R1K9	AACGGGATAATGAATTATAT	*E. coli* R1 *waaY*
Primers used to make protein expression plasmids:		
JY1	TGTGGATccAAATAGTGGGCACTCA	*waaP* end; *Bam*HI
JY2	GGGTGGTCcatATGGTTGAACTTAA	*waaP* start; *Nde*I
JY3	GGAGAGTTAcatATGAATATTCG	*groES* start; *Nde*I
JY4	AGGTGCAGGAAgcttACATCATG	*groEL* end; *Hin*dIII
Primers used for site-directed mutagenesis:		
JY5	CAGATATAACAGgCACGGTGGTTAATGCCC	WaaP D162A
JY6	GGGCATTAACCACCGTGcCTGTTATATCTG	WaaP D162A
JY7	CGTGACTGTTATATCTGTaATTTCCTGCTGC	WaaP H167N
JY8	GCAGCAGGAAATtACAGATATAACAGTCACG	WaaP H167N

[a] Underlined sequences indicate restriction sites engineered for cloning. Small case letters indicate mismatches between the primer and target sequences.

amplification. The *aacC1* gene from plasmid pUCGM (Schweizer, 1993) was then inserted into an appropriate restriction site (near the middle) of the coding region of each gene. The DNA fragment containing the insertionally-inactivated gene was subsequently cloned into the suicide delivery vector pMAK705 (Hamilton *et al.*, 1989), and the resulting plasmid was transformed into the parent strain (F470 for *E. coli*, ATCC 14028 for *S. enterica*) by electroporation. (See Table 2.2 Summary of plasmids used in this study, for complete details.)

The pMAK705 plasmid encodes resistance to Cam, and contains a temperature-sensitive origin of replication that allows the vector to replicate at 30°C but not at 44°C. Therefore, chromosomal gene replacement could be effected by growing the transformed parent strain at 30°C, then shifting it to the non-permissive temperature (44°C) and selecting for retention of gentamicin-resistance concomitant with the loss of the plasmid marker (Cam-resistance). Such a phenotype could only occur by homologous recombination onto the chromosome with subsequent excising of the plasmid and wild-type gene: a so-called double crossover event. Mutations were verified by PCR amplification of the mutated gene from the chromosome using the primers specified in Table 2.3.

2.3.4. Computer analyses:

Homology searches of nucleotide and amino acid sequences in the National Center for Biotechnology Information databases were performed using the BLAST (basic local alignment search tool) or PSI (position-specific

iterated)-BLAST server analysis programs (Altschul *et al.*, 1990; Altschul *et al.*, 1997). Pairwise nucleotide sequence alignments and percent identity scores were obtained using the N_ALIGN program of the PC/GENE® software package (IntelliGenetics, Inc., Mountain View, CA) with an open gap cost of 25 and a unit gap cost of 5. Multiple protein alignments and percentage identity and similarity scores were obtained using CLUSTAL_W (http://www.clustalw.genome.ad.jp). Routine DNA and protein sequence manipulations were performed using the MacVector™ and AssemblyLIGN™ software packages (International Biotechnologies, Inc., New Haven, CT).

2.4. Methods for the analysis of LPS:

2.4.1. Small-scale LPS preparations:

Small-scale LPS preparations were made by the method of Hitchcock and Brown (Hitchcock and Brown, 1983). Briefly, cell suspensions (standardized by OD_{600}) were harvested by centrifugation, washed once with saline, then centrifuged again. The washed cell pellet was resuspended in 100 µL of lysis buffer (2.0% (w/v) SDS, 10% (v/v) glycerol, 1 M Tris-HCl (pH 6.8), 0.1% (w/v) bromophenol blue) and heated for 30 min at 100°C. Proteinase K was added to 0.5 mg/mL (final), and samples were incubated at 55°C for 2 h.

2.4.2. SDS-PAGE conditions for LPS analysis:

LPS samples prepared by the method of Hitchcock and Brown (see section 2.4.1 Small-scale LPS preparations) were run on commercially prepared gradient (10-20%) tricine SDS-PAGE gels from Novex (Helixx Technologies Inc., Scarborough, ON) according to the manufacturer's

directions. LPS was subsequently visualized by the silver staining method of Tsai and Frasch (1982), or by the Western immunoblot method of Towbin and coworkers (Towbin *et al.*, 1979).

For silver staining, gels were incubated overnight in fixative (10 mL glacial acetic acid, 84 mL 95% (v/v) ethanol, 116 mL dH_2O), then oxidized in fixative containing 0.7% (w/v) periodic acid for 30 min. Excess periodic acid and the remaining fixative were removed by extensive washing in dH_2O (5 times over the course of 1 h), and the gels were then incubated for 30 min in silver stain (28 mL 0.1 M NaOH, 2 mL NH_4OH, 1 g $AgNO_3$ dissolved in 5 mL dH_2O and added slowly, 115 mL dH_2O), and washed again as above. Finally, gels were immersed in developer (1.8% (v/v) fomaldehyde, 0.005% (w/v) citric acid) until bands appeared, at which point the developing reaction was stopped by repeated washing in dH_2O.

For Western immunoblotting, samples from SDS-PAGE gels were transferred by electrophoresis onto nitrocellulose (Roche) in carbonate buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, 20% (v/v) methanol) at 45 V for 45 min. The resulting blots were washed briefly in phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g K_2HPO_4, to 1 L with dH_2O, pH 7.4) and blocked in 5% (w/v) skim milk in PBS for 1 h. Primary antibody (rabbit polyclonal) was diluted 1:2000, and applied to the blot mixed with skim milk-PBS. The blot was incubated overnight and then washed 3 times in wash buffer (PBS containing 0.05% (v/v) Tween 20), rinsed briefly in PBS, and incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary

antibody (Cedarlane Laboratories Ltd., Hornby, ON) for 1 h. Blots were washed again as above, and developed using the chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate (Roche; 0.016% (w/v)) and nitro blue tetrazolium (Sigma; 0.033% (w/v)) in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5). The developing reaction was stopped by repeated washing in dH_2O.

2.4.3. Large-scale LPS preparations:

Large-scale LPS purifications for chemical analyses were done by hot phenol/water extraction of cells (Westphal and Jann, 1965). Bacteria were grown in a 60 L fermentor, in brain-heart infusion broth (Difco Laboratories) at 37°C, and harvested after 16.5 h (performed by D. Griffith, Institute for Biological Sciences, National Research Council, Ottawa, ON). The cell pellet was resuspended in H_2O by mechanical stirring and heated to 70°C, then pre-warmed phenol was added to a final concentration of 50%. (Phenol and H_2O are miscible at 70°C but immiscible at room temperature.) Once the extraction had proceeded for 15 min at 70°C, it was cooled on ice, and centrifuged for 3 h at 5000 × g to separate the aqueous and organic phases. The upper aqueous phase was removed, dialyzed for 3 days against H_2O, then flash frozen in round-bottomed flasks and lyophilized overnight. The resultant white powder was resuspended in a minimal volume of H_2O, treated with DNase and RNase for 1 h at 37°C, and finally treated with proteinase K for 2 h at 37°C. Cellular debris was removed by centrifugation at 7000 × g for 30 min,

and LPS was recovered from the supernatant by ultracentrifugation at 100 000 × g for 16 h.

2.4.4. Preparation of water-soluble core oligosaccharides:

Purified LPS from large-scale preparations (approximately 50 mg) was treated with 4 mL of 2% (v/v) acetic acid at 100°C for 2 hours to cleave the acid-labile glycosidic linkage between the first Kdo residue of the core oligosaccharide and lipid A. The water-insoluble lipid A was removed by centrifugation (5000 × g, 30 min), and the supernatant was passed through a column of BioGel P-2 (1 m × 1 cm) with water as eluent. The core-containing fractions were collected and lyophilized.

2.4.5. Sugar composition analysis of core oligosaccharides:

Sugar composition analysis was performed by the alditol acetate method (Sawardeker *et al.*, 1967). Briefly, samples (approximately 1 mg) of core oligosaccharide were hydrolyzed in 4 M trifluoroacetic acid (TFA) at 100°C for 4 h. The TFA was removed by evaporating to dryness under a stream of N_2, and the resulting monosaccharides were then reduced in water with sodium borodeuteride overnight. Following neutralization with glacial acetic acid, the reaction mixture was evaporated to dryness, and borate esters were removed by distillation with methanol/acetic acid (95:5) (3 times, 2 mL). The product was subsequently acetylated with acetic anhydride using residual sodium acetate as the catalyst (100°C for 2 h), dried, resuspended in CH_2Cl_2, and run over a column of anhydrous Na_2SO_4 to remove any traces of water. Characterization of the alditol acetate derivatives was performed by gas-liquid

chromatography (GLC)-mass spectrometry (MS) using a Hewlett-Packard chromatograph equipped with a 30 m DB-17 capillary column (210°C (30 min) to 240°C at 2°C/min). Mass spectrometry in the electron impact mode was recorded using a Varian Saturn II mass spectrometer.

2.4.6. Methylation linkage analysis of core oligosaccharides:

Methylation linkage analyses were performed to determine the core oligosaccharide structure of each mutant strain (Ciucanu and Kerek, 1984). Interpretation of these data was based upon the previously published structure of the R1 core (Holst and Brade, 1992). Briefly, samples (~ 1 mg) of core oligosaccharide were methylated with iodomethane in dimethyl sulfoxide, and the methylated products were recovered from the reaction mixture by CH_2Cl_2 extraction (2.5 mL) against H_2O washing (3 times, 5 mL each wash). At this point, a small amount of sample was removed for fast atom bombardment (FAB)-MS where applicable (see section 2.4.7. Fast atom bombardment-mass spectrometry). The remainder of the sample was hydrolyzed, reduced, and acetylated at the subsequently exposed hydroxyl functions, as described for the sugar composition analysis (see section 2.4.5. Sugar composition analysis). The permethylated alditol acetate derivatives were fully characterized by GLC-MS in the electron impact mode using a column of DB-17 operated isothermally at 190°C for 60 min.

2.4.7. Fast atom bombardment-mass spectrometry:

A fraction of the methylated sample from the initial steps of the linkage analysis was taken for positive ion FAB-MS, which was performed

using a Jeol JMS-AX505H mass spectrometer with glycerol/thioglycerol as the matrix, and a tip voltage of 3 kV (performed by K. Chan, Institute for Biological Sciences, National Research Council, Ottawa, ON).

2.4.8. ^{31}P-NMR spectroscopy:

^{31}P-NMR spectra were recorded with a Brüker DRX 400 MHz instrument at 161.98 MHz with *ortho*-phosphoric acid as the external reference (0.0 ppm) and with p1=30 in the proton-decoupling mode (performed by Dr. M. Monteiro, Institute for Biological Sciences, National Research Council, Ottawa, ON). Prior to performing the NMR experiments, the samples were lyophilized three times in $^{2}H_2O$ (99.9%). The p^{2}H was adjusted with triethylamine when necessary.

2.5. Analysis of mutant phenotypes:

2.5.1. SDS and novobiocin sensitivity testing:

Two-fold serial dilutions of SDS (200 → 0.1 mg/mL) and novobiocin (200 → 1.6 µg/mL) were made in 5 mL volumes of LB. Each series of tubes was inoculated (5 µL) from an overnight culture of the strain to be tested, and then incubated with shaking at 37°C. Growth was scored as positive if after 8 hours the culture was visibly turbid (i.e. OD_{600}>0.2). Trials were performed in triplicate, and repeated on two separate occasions.

2.5.2. Analysis of outer membrane protein profiles by SDS-PAGE:

Equal numbers of cells (standardized by OD_{600}) from 100 mL overnight cultures were harvested by centrifugation, resuspended in 20 mM Tris (pH 8), then completely lysed by passage through a French pressure cell (cell pressure

of 200 MPa, flow rate of 1 mL/min). After removal of cell debris (5000 × *g*, 10 min), the total membrane fractions were collected by centrifugation (100000 × *g*, 2 h), then resuspended in 2% Sarkosyl. The detergent Sarkosyl selectively solubilizes the inner membrane, while leaving the outer membrane intact (Filip *et al.*, 1973). The Sarkosyl-insoluble outer membrane fraction was collected by centrifugation (14000 rpm, 30 min), washed a second time in 2% (v/v) Sarkosyl, and centrifuged again (14000 rpm, 30 min). The resulting pellet was resuspended in 1 mL of 20 mM Tris (pH 8) and analyzed by SDS-PAGE (see section 2.6.2. SDS-PAGE conditions for protein analysis).

2.5.3. Mouse virulence studies:

Inbred mouse strains C57BL/6J and A/J were bred and maintained in the Montreal General Hospital Research Institute under conditions specified by the Canadian Council on Animal Care. Mice between 8 and 12 weeks of age were challenged with either ATCC 14028 or CWG304 by inoculation in the caudal vein with 0.2 mL of physiological saline containing 10^2, 10^3, or 10^4 colony forming units (CFU) of *S. enterica*. The inoculum of *S. enterica* was prepared by growing the bacteria for 2 hours at 37°C in tryptic soy broth followed by enumeration of the CFU by incubating serial 10-fold dilutions on tryptic soy agar at 37°C for 16 hours (Sebastiani *et al.*, 1998; Vidal *et al.*, 1995). The degree of CWG304 virulence was established *in vivo* by survival analysis, and by measuring the number of CFU in the spleen and liver of surviving mice 21 days post inoculation. The number of viable Salmonellae in the spleens and livers of the infected animals was determined by plating

serial 10-fold dilutions of organ homogenates in physiological saline on tryptic soy agar (Govoni *et al.*, 1999). (These studies were performed by L. Laroche and Dr. D. Malo at the Centre for the Study of Host Resistance, McGill University, Montreal, QC, Canada.)

Oral and intraperitoneal infection of 16-18 g BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were accomplished as follows. For oral infections, mice deprived of food or water for at least 4 hours were pre-fed 20 µL of 10% sodium bicarbonate 30 minutes prior to oral inoculation with 20 µL of stationary phase bacteria (~ 3-6 × 10^6 CFU) diluted in PBS. Intraperitoneal infection was performed with 100 µL of stationary phase bacteria (~ 60-100 CFU) diluted in PBS. Mouse survival was monitored for three weeks. (These studies were performed by Dr. J. S. Gunn, Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA.)

2.5.4. Sensitivity to serum killing:

Parent and mutant strains were incubated with fresh mouse serum (obtained from Dr. W. Woodward, University of Guelph, Guelph, Ontario, Canada) for 2 hours at 37°C, and then serial dilutions were plated on LB agar plates, incubated overnight at 37°C, and the CFU were counted.

2.6. Protein methods:

2.6.1. Materials:

Materials for protein purification and activity assays were purchased from the following suppliers: nickel-nitrilotriacetic acid agarose (Ni-NTA

agarose) (Qiagen); PD-10 desalting columns (Amersham Pharmacia, Uppsala, Sweden); [γ-^{33}P]ATP (3000 Ci/mmol) (NEN Life Science Products) and other assay reagents (Tris, $MgCl_2$, Triton X-100, dithiothreitol) (Sigma); EcoLite scintillation fluid (ICN). LPS acceptor for the WaaP assay was purified from defined *waaP* mutant strain CWG296 (Yethon *et al.*, 1998) by hot phenol/water extraction of cells (Westphal and Jann, 1965) (see section 2.4.3. Large-scale LPS preparations).

2.6.2. SDS-PAGE conditions for protein analysis:

Samples for SDS-PAGE were resuspended in SDS-PAGE sample buffer (2.0% (w/v) SDS, 4% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 1.0 M Tris-HCl (pH 6.8), 0.1% (w/v) bromophenol blue) and solubilized at 100°C for 15 min. Discontinuous Tris-glycine SDS-polyacrylamide gels were cast as described by Sambrook *et al.* (Sambrook *et al.*, 1989), using a 5% stacking gel and a 12% resolving gel, and were run in a Bio-Rad MiniPROTEAN 3 apparatus at 150 V to completion. Gels were stained with Coomassie Brilliant Blue R250 to visualize protein bands, as described by Sambrook *et al.* (Sambrook *et al.*, 1989).

2.6.3. Protein expression and preparation of cell-free extracts:

LB broth containing Cam (30 μg/mL) and Km (50 μg/mL) was inoculated (1:20) from an overnight culture of BL21(DE3)pWQ910/pWQ911, and grown to an $OD_{600} \equiv 0.8$ at 37°C with shaking at 200 rpm. L-Arabinose (0.02%) was then added to induce GroES and GroEL expression, and cells were grown for an additional 45 min, at which point IPTG (0.5 mM) was added to

induce expression of WaaP. After 4.5 h, cells were harvested by centrifugation (15 min, 5000 × *g*, 4°C), then frozen and stored overnight at -20°C. The cell pellet was thawed on ice the next day, and resuspended in a minimal volume (4 mL/g wet weight) of lysis buffer (50 mM sodium phosphate, pH 8, 350 mM NaCl, 10 mM imidazole, 2% Triton X-100, 10 mM β-mercaptoethanol). Lysozyme (1 mg/mL) was added to initiate lysis, and both DNase I (5 µg/mL) and RNase A (10 µg/mL) were added to reduce viscosity. After 45 min, the sample was sonicated on ice (6 × 10 sec bursts, with 20 sec cooling periods) to ensure complete lysis, then centrifuged (30 min, 25000 × *g*, 4°C) to remove cellular debris.

2.6.4. Immobilized metal affinity chromatography:

One mL of Ni-NTA agarose (50% slurry, Qiagen) was added to every 6 mL of cleared lysate, and batch binding was allowed to proceed for 1 h (4°C, with shaking at 200 rpm). The lysate/Ni-NTA agarose mixture was loaded into a disposable plastic column (5 mL) and the unbound material from the cleared lysate was allowed to flow through. The column was washed with 3 × 5 mL of wash buffer (50 mM sodium phosphate, pH 8, 350 mM NaCl, 30 mM imidazole, 0.5% Triton X-100), then the His_6-tagged WaaP was eluted in 4 × 0.5 mL of elution buffer (50 mM sodium phosphate, pH 8, 250 mM NaCl, 200 mM imidazole, 0.5% Triton X-100). The second 0.5 mL elution typically contained the highest concentration of His_6-tagged WaaP.

2.6.5. High performance anion exchange chromatography:

The enzyme eluted from the Ni-NTA agarose column was further purified by using subtractive anion exchange chromatography to bind the remaining contaminating proteins. Protein eluted from the Ni-NTA agarose column was first exchanged into a lower ionic strength loading buffer (25 mM sodium phosphate, pH 8, 150 mM NaCl, 0.5% Triton X-100) using disposable PD-10 columns (containing Sephadex G-25) as recommended by the manufacturer (Amersham Pharmacia). The sample was then applied (0.8 mL/min) to a Beckman Q HyperD 20 column (equilibrated in 40 mM sodium phosphate, pH 8, 50 mM NaCl), and the WaaP-containing flow-through was collected in 0.5 mL fractions.

2.6.6. Assay conditions for WaaP activity:

Unless otherwise indicated, reaction mixtures contained 50 mM Tris, pH 8.5, 17.5 mM $MgCl_2$, 0.5% Triton X-100, 0.5 mM dithiothreitol, 1 μCi [γ-^{33}P]ATP (3000 Ci/mmol) diluted to 1 μM with cold ATP, and 1 mM acceptor LPS. The enzyme source was added last to initiate the reaction, and comprised 10% of the reaction volume. Reactions were incubated at 35°C for 30 min, then stopped by the addition of an equal volume of stop solution (1 M acetic acid, 0.1 M NaH_2PO_4, 25 mM $MgCl_2$). LPS was collected in a pellet by centrifugation (15 min, 14000 rpm), and washed three times in stop solution to remove unincorporated radiolabel. (For each wash, the LPS pellet was completely resuspended by vortexing for about 5 min.) After the third wash, the LPS pellet was resuspended in water, and the incorporated radiolabel was

quantified in EcoLite scintillation fluid (ICN) using a Packard TRI-CARB Liquid Scintillation Analyzer (Model 2000CA).

2.6.7. Analysis of WaaP reaction products by gel filtration chromatography:

Radiolabelled LPS was pooled from 10 reaction mixtures, and resuspended in 250 µL of 2% (v/v) acetic acid. The LPS/acetic acid suspension was then incubated at 100°C for 90 min in order to cleave the acid-labile ketosidic linkage between the core oligosaccharide and lipid A. Insoluble lipid A was removed by centrifugation (15 min, 14 000 rpm). The water-soluble core oligosaccharides were then applied to a column of Sephadex G-25 (1 × 40 cm) with water as eluent. The column void volume and included volume were determined using Blue Dextran and KCl, respectively. Eluted fractions (0.85 mL) were collected and analyzed for radioactivity by liquid scintillation counting.

2.6.8. Site-directed mutagenesis:

Plasmid pWQ912 encoding wild-type His_6-tagged WaaP was PCR amplified in its entirety using the complementary mutagenic primers indicated in Table 2.3 and the following temperature program: 1) one cycle at 95°C for 2 min; 2) 16 cycles at 95°C for 30 sec, 55°C for 1 min, and 68°C for 11 min. The amplified plasmid was purified, then digested with *Dpn*I (specific for the methylated parental DNA template, thus selecting for plasmid containing the mutation). The *Dpn*I-digested sample was transformed into electrocompetent DH5α cells, and plasmid was purified from the resulting

colonies, then sequenced to ensure only the desired mutation had been introduced into the *waaP* coding region.

Chapter 3. Results

3.1. Assignment of function to genes involved in outer core biosynthesis:

To confirm the predicted activities of *waa* genes involved in the assembly of the R1 outer core (refer back to Figure 1.8), non-polar chromosomal insertion mutations were made in each of the genes encoding putative outer core glycosyltransferases, and the resulting LPS core structure from each mutant strain was determined by chemical methods. The data from these experiments have been published in "The assembly system for the outer core portion of R1- and R4-type lipopolysaccharides of *Escherichia coli*" (Journal of Biological Chemistry, 1998, vol. 273, 29497-29505). Mutation of the *waaO*, *waaT*, *waaW*, and *waaV* genes was performed by co-author Dr. D. E. Heinrichs. Mutation of the *waaG* gene, and chemical analysis of all of the LPS core oligosaccharides were performed as part of this thesis. Co-authors Dr. M. A. Monteiro and Dr. M. B. Perry provided advice and assistance with the LPS structural analyses.

3.1.1. Transfer of GlcI in core type R1:

In all of the *E. coli* and *S. enterica* core types, the first outer core sugar residue is GlcI (refer back to Figure 1.9), which is linked $\alpha,1\rightarrow3$ to HepII of the inner core. The product of the *waaG* gene is involved in the addition of this conserved GlcI residue in *S. enterica* serovar Typhimurium (Müller *et al.*, 1972), and the LPS core structure of an *E. coli* K-12 *waaG* mutant is in agreement with this activity (Parker *et al.*, 1992(a)). Homologs of *waaG* are

present in all of the sequenced core biosynthesis loci, and the predicted WaaG proteins from each locus share total similarities of greater than 90%.

Consistent with the predicted assignment of WaaG as the GlcI transferase, LPS from strain CWG303 (*waaG::aacC1* derivative of F470) migrates faster than F470 LPS in SDS-PAGE (see Figure 3.1A). Complementation of CWG303 with the R1 *waaG* gene *in trans* results in an LPS with mobility equivalent to that of F470 (see Figure 3.1B). Methylation linkage analysis (see Table 3.1) of the CWG303 core oligosaccharide shows a complete absence of hexose sugars, further confirming the assignment of WaaG as the glucosyltransferase responsible for the addition of GlcI in core type R1.

3.1.2. Transfer of GlcII in core type R1:

In *E. coli* core types K-12, R1, R2, and R4, the second main chain sugar residue is GlcII, which is linked α,1$\rightarrow$3 to GlcI (refer back to Figure 1.9). The product of the *waaO* gene is predicted to be involved in the addition of GlcII (Heinrichs *et al.*, 1998(b); Pradel *et al.*, 1992). The predicted WaaO proteins from each core type share total similarities greater than 60%.

Insertional inactivation of the *waaO* gene of F470 (strain CWG308) results in a truncated LPS molecule that migrates slower in SDS-PAGE than CWG303 LPS (see Figure 3.1A), but faster than the LPS from the parent F470. Complementation of CWG308 with the R1 *waaO* gene *in trans* returns the LPS mobility in SDS-PAGE to that of F470 (see Figure 3.1B). Methylation linkage analysis of the core oligosaccharide from CWG308 indicates that the

Figure 3.1. SDS-PAGE analysis showing the effect of insertion mutations within genes of the *waa* cluster of *E. coli* F470 on LPS migration. Panel A: silver-stained SDS-PAGE of separated LPS samples of F470 and mutant derivatives. These strains produce only rough LPS, therefore only the relevant portion of the gel is shown. Both strain designations and the mutated gene name are shown. Panel B: silver-stained SDS-PAGE of separated LPS samples showing restoration of the wild-type LPS mobility when mutant stains are complemented with plasmids carrying functional copies of the corresponding mutated gene. Complementation confirms that the constructed mutations are indeed non-polar, and that no other unanticipated mutations have been introduced during the mutagenesis protocol.

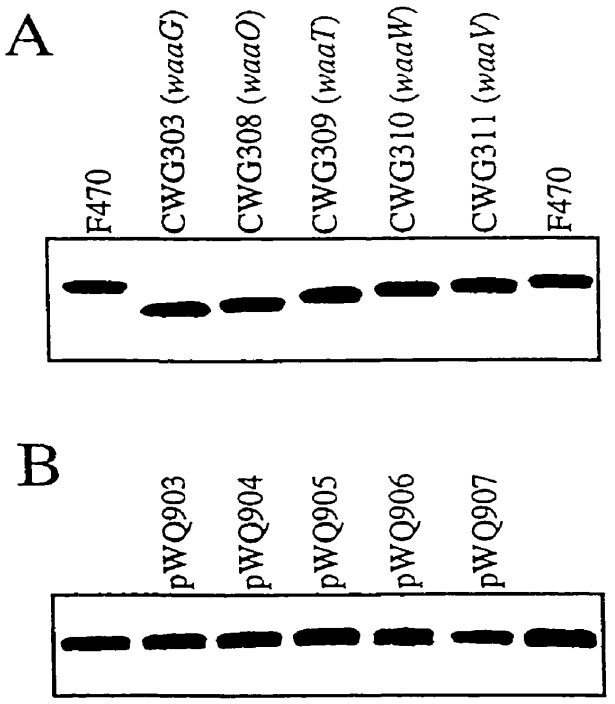

A
F470
CWG303 (waaG)
CWG308 (waaO)
CWG309 (waaT)
CWG310 (waaW)
CWG311 (waaV)
F470
B
pWQ903
pWQ904
pWQ905
pWQ906
pWQ907

Table 3.1. Linkage analysis of the outer core region of the core oligosaccharides from *E. coli* F470 and *waa* mutant derivatives.

Strain	Outer core linkages detected	Approximate molar ratio	Outer core structure
F470	Glc-(1→	1.0	Gal I-(1→2)-Glc II-(1→3)-Glc I-
	Gal-(1→	1.0	$^{2}\uparrow$ $^{3}\uparrow$
	→3)-Glc-(1→	1.0	^{1}Gal II ^{1}β-Glc
	→2)-Gal-(1→	1.0	
	→2,3)-Glc-(1→	1.0	
CWG303 (*waaG*)	none		-
CWG308 (*waaO*)	Glc-(1→	1.0	Glc I-
CWG309 (*waaT*)	Glc-(1→	1.0	Glc II-(1→3)-Glc I-
	→3)-Glc-(1→	1.0	
CWG310 (*waaW*)	Gal-(1→	1.0	
	Glc-(1→	0.1	Gal I-(1→2)-Glc II-(1→3)-Glc I-
	→2)-Glc-(1→	1.0	$^{3}\uparrow$
	→3)-Glc-(1→	1.0	^{1}β-Glc [a]
	→2,3)-Glc-(1→	0.1	
CWG311 (*waaV*)	Gal-(1→	1.0	Gal I-(1→2)-Glc II-(1→3)-Glc I-
	→2)-Glc-(1→	1.0	$^{2}\uparrow$
	→2)-Gal-(1→	1.0	^{1}Gal II
	→3)-Glc-(1→	1.0	

[a] The β-linked Glc side branch in CWG310 is only present in approximately 10 percent of the core oligosaccharide molecules

only hexose sugar present is a terminal Glc, which can only arise from an outer core structure consisting of only GlcI (see Table 3.1). These data confirm WaaO as the glucosyltransferase responsible for the addition of GlcII in core type R1.

3.1.3. Transfer of GalI in core type R1:

In *E. coli* core types R1 and R4, the third main chain sugar residue is GalI, which is linked α,1→2 to GlcII (refer back to Figure 1.9). Since this structure is found only in the R1 and R4 core types, the corresponding gene encoding this activity should only be found in these core clusters. *waaT* and *waaW* are the only genes which are unique to both the R1 and R4 core clusters (refer back to Figure 1.8), and either one could potentially fulfill this role. However, based on the position of *waaT* within the operon (immediately downstream of *waaO* encoding the GlcII transferase, see section 3.1.2 Transfer of GlcII in core type R1), *waaT* was tentatively assigned as the transferase for GalI (Heinrichs *et al.*, 1998(b)).

Insertional inactivation of the *waaT* gene of F470 (strain CWG309) results in a truncated LPS molecule that migrates slower in SDS-PAGE than CWG308 LPS (see Figure 3.1A), but faster than the LPS from the parent F470. Complementation of CWG309 with the R1 *waaT* gene *in trans* returns the LPS mobility in SDS-PAGE to that of F470 (see Figure 3.1B). Methylation linkage analysis of the CWG309 core oligosaccharide indicates that CWG309 contains both terminal and 3-linked Glc, but no Gal, which can only arise from an outer core structure consisting of only GlcI and GlcII (see Table 3.1).

These data confirm that WaaT is the galactosyltransferase responsible for the addition of GalI in core type R1.

3.1.4. Transfer of GalII in core type R1:

In *E. coli* core types R1 and R4, GalII is attached to the core via an α,1→2 linkage to GalI (refer back to Figure 1.9). Again, since this structure is found only in the R1 and R4 core types, the corresponding gene encoding this activity should only be found in these core clusters. As discussed above, *waaT* and *waaW* are the only genes which are unique to both the R1 and R4 core clusters (refer back to Figure 1.8), and either one could potentially fulfill this role. However, based on its position within the operon (downstream of *waaT*), *waaW* was tentatively assigned as the transferase for GalII (Heinrichs *et al.*, 1998(b)).

Insertional inactivation of the *waaW* gene of F470 (strain CWG310) results in a truncated LPS molecule that migrates slower in SDS-PAGE than CWG309 LPS (see Figure 3.1A), but faster than the LPS from the parent F470. Complementation of CWG310 with the R1 *waaW* gene *in trans* returns the LPS mobility in SDS-PAGE to that of F470 (see Figure 3.1B). Methylation linkage analysis of the CWG310 core oligosaccharide indicates that CWG310 contains terminal Gal, and both 2- and 3-linked Glc, which can only arise from an outer core structure consisting of only GlcI, GlcII, and GalI (see Table 3.1). The missing GalII residue is as predicted, but interestingly the amount of side-branch substitution on GlcII was also greatly reduced (but not eliminated) in this mutant. This effect is clearly due to a single mutation in *waaW* as

wild-type core oligosaccharide is restored in CWG310 complemented by *waaW* alone (see Figure 3.1B). Efficient addition of the GlcII side-branch (a β-linked Glc residue) in core type R1 is therefore concluded to be dependent on the prior addition of GalII by WaaW.

3.1.5. Transfer of the β-linked Glc side-branch in core type R1:

The *E. coli* R1 and R4 core types are differentiated solely by the type of side-branch substituent on their main chain GlcII residues. In *E. coli* R1, GlcII is substituted with Glc, linked β,1-3, while in *E. coli* R4, GlcII is substituted with Gal, linked β,1-4 (refer back to Figure 1.8). The predicted product of the *waaV* gene of the R1 core biosynthesis locus shows limited similarity to a number of known and putative β-glycosyltransferases (e.g. the *cps14I* gene of *Streptococcus pneumoniae* encoding a β1,3-*N*-acetylglucosaminyltransferase involved in serotype 14 capsule biosynthesis; 30% identity, 50% similarity). Given the presence of a single β-linked Glc in the core oligosaccharide of R1-type LPS, it was considered likely that the product of the *waaV* gene encodes the required β,1→3-glucosyltransferase activity.

Insertional inactivation of the *waaV* gene of F470 (strain CWG311) results in a truncated LPS molecule that migrates slower in SDS-PAGE than CWG310 LPS (see Figure 3.1A), but faster than the LPS from the parent F470. This phenotype can be complemented by introduction of the R1 *waaV* gene *in trans* (see Figure 3.1B). Methylation linkage analysis of the CWG311 core oligosaccharide indicates that CWG311 contains all of the expected core sugar derivatives except one that corresponds to terminal Glc; this situation can

only arise if the β-linked Glc side branch is absent (see Table 3.1). Further, the ^{1}H NMR spectrum of F470 core oligosaccharide contains a signal at 4.7 ppm (characteristic of a β-linked hexopyranose residue), while no such signal is evident in the spectrum from CWG311 core oligosaccharide (data not shown). Taken together, these data confirm that *waaV* encodes the glucosyltransferase responsible for the addition of the β1,3-linked Glc side-branch. Based on all of the data above, this substitution would appear to be the final sugar added in the assembly of the outer core oligosaccharide of R1-type LPS. With the assignment of function to WaaV, all of the transferases for the assembly of the outer portion of the core oligosaccharide of R1-type LPS have been identified.

3.2. Assignment of function to genes involved in inner core biosynthesis:

To confirm the prediction that certain *waa* genes were involved in the assembly of the conserved *E. coli* and *S. enterica* inner core region (refer back to Figure 1.8), non-polar chromosomal insertion mutations were made in each of the target genes, and the resulting LPS core structure from each of these mutant strains was determined by chemical methods. The data from these experiments have been published in "Involvement of *waaY*, *waaQ*, and *waaP* in the modification of *Escherichia coli* lipopolysaccharide, and their role in the formation of a stable outer membrane" (Journal of Biological Chemistry, 1998, vol. 273, 26310-26316). Mutation of the *waaY* gene was performed by co-author Dr. D. E. Heinrichs. The remainder of the work reported in this article was performed as part of this thesis.

3.2.1. Assignment of *waaY* gene function:

Pairwise nucleotide alignments of the *waaY* coding region from *E. coli* K-12, R1, R2, R3, and R4, and *S. enterica* serovar Typhimurium showed identities ranging from 59 to 99%. Multiple alignment of the predicted WaaY proteins showed high total similarity (85.3%) but much lower identity (43.1%). In BLASTP searches of the available databases, WaaY showed no significant homologies to other characterized proteins. However, using the PSI-BLAST database analysis program, queries with the various WaaY protein sequences suggested weak (~15% identity, ~25% similiarity) but biologically relevant similarities to a large number of protein kinases (data not shown). These circumstantial data suggested a potential role for WaaY in phosphorylation reactions.

To test this possibility, strain CWG312 was derived by insertion of the *aacC1* cassette into the middle of the *waaY* open reading frame. The LPS from the resulting mutant migrated similarly to that of the F470 parent in SDS-PAGE (data not shown), precluding any significant effect of the mutation on core extension. The core oligosaccharide of the LPS from CWG312 was then subjected to methylation linkage analysis. Since phosphorylated sugars give rise to permethylated alditol acetate derivatives that are too polar to elute from the GLC column, such sugars are not detected by this analysis. This proved useful for the analysis of core phosphorylation because it allowed the loss of phosphoryl substituents from HepI and HepII to be monitored as the

appearance of either a 3-substituted or 3,7-disubstituted heptose derivative peak in the GLC chromatogram, respectively.

Methylation linkage analysis of the CWG312 core oligosaccharide resulted in a GLC chromatogram that was identical to that from the F470 parent (compare Figure 3.2, panels A and B) except for the appearance of a peak (see Figure 3.2B, peak 9) corresponding to a 3,7-disubstituted heptose derivative. Based on the structure of the F470 core, this derivative must result from non-phosphorylated HepII (see Table 3.2). ^{31}P-NMR spectroscopy further corroborated the loss of phosphate from HepII of the CWG312 core. The ^{31}P-NMR spectrum of F470 (see Figure 3.2E) at pH 8.5 shows a signal at 5 ppm indicative of a phosphomonoester (phosphate on either HepI or HepII), and two peaks near -10 ppm that are characteristic of a diphosphodiester (2-aminoethyl diphosphate (PPEtN) on HepI) (Helander *et al.*, 1994). In the F470 spectrum, the ratio of phosphate to PPEtN is greater than that observed in the CWG312 spectrum (compare Figure 3.2, panels E and F). The observed shift in the ratio of phosphate to PPEtN is explained by the loss of phosphate from HepII, leaving only phosphate and PPEtN on HepI. Therefore, given the disappearance of phosphate from HepII in the CWG312 core, and the biologically significant sequence similarity of WaaY to some kinases, WaaY was concluded to be involved in the phosphorylation of HepII. The structure of the deduced CWG312 core is summarized in Table 3.2.

Figure 3.2. Methylation linkage analyses and ^{31}P-NMR spectroscopy of the core oligosaccharides from F470 and *waa* mutant derivatives. Panels A-D: GLC chromatograms from the methylation linkage analyses of A, F470; B, CWG312; C, CWG297; and D, CWG296. The numbered peaks correspond to the permethylated alditol acetate derivatives from 1) terminal Glc (5.8 min); 2) terminal Gal (6.2 min); 3) 2-substituted Glc (7.3 min); 4) 3-substituted Glc (7.5 min); 5) 2-substituted Gal (7.8 min); 6) terminal Hep (8.6 min); 7) 2,3-disubstituted Glc (9.2 min); 8) 3-substituted Hep (11.0 min); and 9) 3,7-disubstituted Hep (14.0 min). The approximate molar ratios of these derivatives are shown in Table 3.2. Panels E-H: ^{31}P-NMR spectra of the core OSs from E, F470; F, CWG312; G, CWG297; and H, CWG296. The signal at 5 ppm is indicative of a phosphomonoester (phosphate on either HepI or HepII), and the two peaks near -10 ppm are characteristic of a diphosphodiester (PPEtN on HepI).

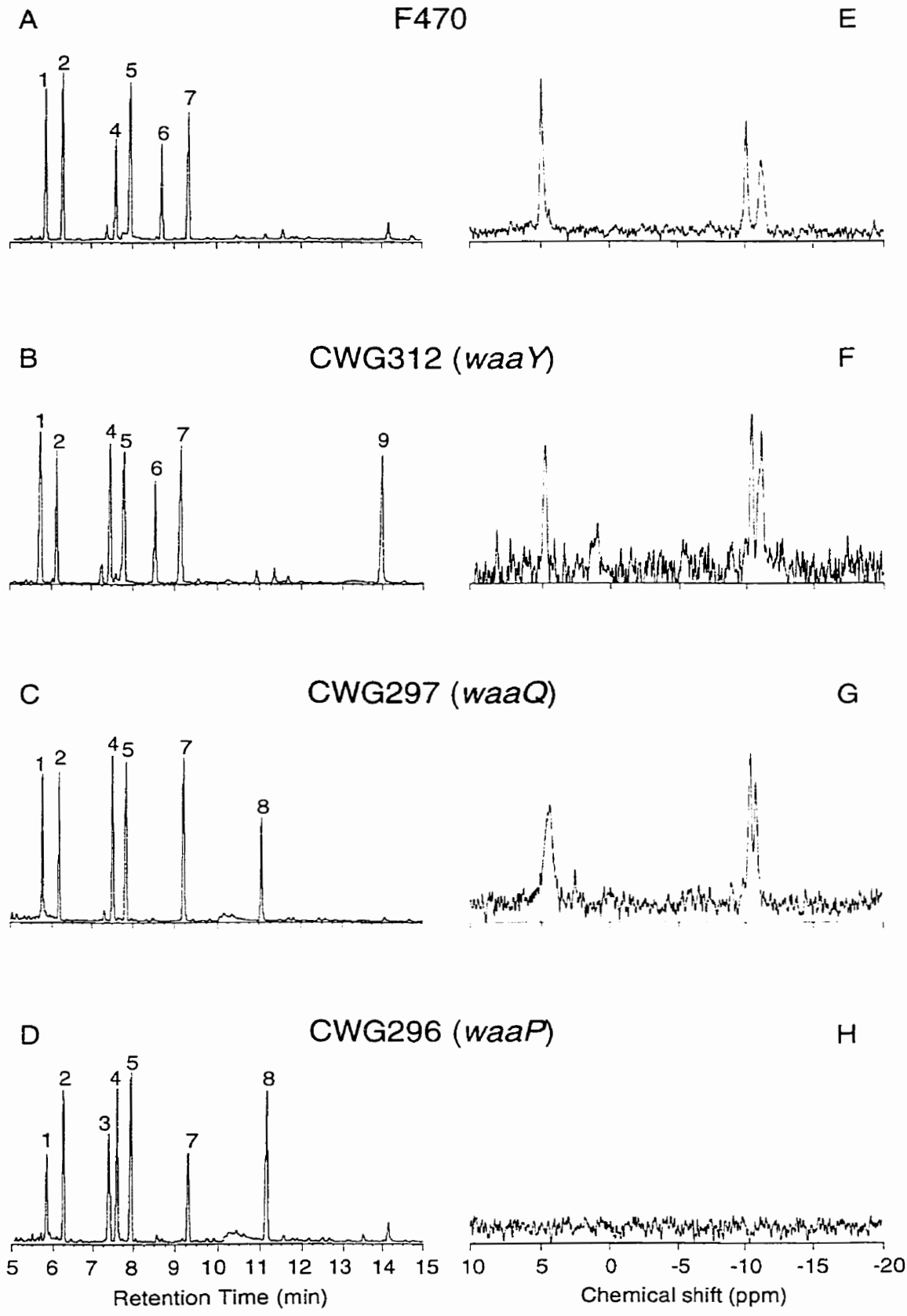

A
F470
E
1
2
4
5
6
7
B
CWG312 (waaY)
F
1
2
4
5
6
7
9
C
CWG297 (waaQ)
G
1
2
4
5
7
8
D
CWG296 (waaP)
H
1
2
3
4
5
7
8
5 6 7 8 9 10 11 12 13 14 15
Retention Time (min)
10 5 0 -5 -10 -15 -20
Chemical shift (ppm)

Table 3.2. Linkage analysis of the core oligosaccharides from *E. coli* F470 and its *waaY*, *waaQ*, and *waaP* mutant derivatives.

Strain	GLC Peak [a]	Linkage	Approximate molar ratio	Core structure
F470	1	Glc-(1→	1.0	Hep III 1 ↓ 7 Gal I-(1→2)-Glc II-(1→3)-Glc I-(1→3)-Hep II-(1→3)-Hep I- 2 3 4 4 ↑ ↑ ↑ ↑ 1 1 P P/PPEtN Gal II β-Glc
	2	Gal-(1→	1.0	
	4	→3)-Glc-(1→	0.7	
	5	→2)-Gal-(1→	1.3	
	6	Hep-(1→	0.6	
	7	→2,3)-Glc-(1→	1.0	
CWG312 (*waaY*)	1	Glc-(1→	0.9	Hep III 1 ↓ 7 Gal I-(1→2)-Glc II-(1→3)-Glc I-(1→3)-Hep II-(1→3)-Hep I- 2 3 4 ↑ ↑ ↑ 1 1 P/PPEtN Gal II β-Glc
	2	Gal-(1→	0.8	
	4	→3)-Glc-(1→	1.1	
	5	→2)-Gal-(1→	1.0	
	6	Hep-(1→	0.7	
	7	→2,3)-Glc-(1→	1.1	
	9	→3,7)-Hep-(1→	1.1	

CWG297	1	Glc-(1→	0.9	
(*waaQ*)	2	Gal-(1→	1.0	Gal I-(1→2)-Glc II-(1→3)-Glc I-(1→3)-Hep II-(1→3)-Hep I-
	4	→3)-Glc-(1→	1.1	2 ↑ 3 ↑ 4 ↑
	5	→2)-Gal-(1→	1.0	1 1 P/PPEtN
	7	→2,3)-Glc-(1→	1.1	Gal II β-Glc
	8	→3)-Hep-(1→	0.7	
CWG296	1	Glc-(1→	0.5	
(*waaP*)	2	Gal-(1→	0.9	
	3	→2)-Glc-(1→	0.7	Gal I-(1→2)-Glc II-(1→3)-Glc I-(1→3)-Hep II-(1→3)-Hep I-
	4	→3)-Glc-(1→	1.0	2 ↑ 3 ↑
	5	→2)-Gal-(1→	1.1	1 1
	7	→2,3)-Glc-(1→	0.6	Gal II β-Glc [b]
	8	→3)-Hep-(1→	1.1	

[a] Refer to Figure 3.2 for GLC peak numbering. [b] The β-linked Glc side branch in CWG296 is only present in approximately 50 percent of the core oligosaccharide molecules

3.2.2. Assignment of *waaQ* gene function:

Pairwise nucleotide alignments of the *waaQ* coding region from *E. coli* K-12, R1, R2, R3, and R4, and *S. enterica* serovar Typhimurium showed identities ranging from 68 to 99%. Multiple sequence alignment of the predicted WaaQ proteins reflected the homology observed at the nucleotide level with a total similarity of 88% (64% identity). The predicted WaaQ proteins showed limited homology to both WaaC (41% total similarity, 11% identity) and WaaF (50% total similarity, 16% identity), the HepI (Kadrmas and Raetz, 1998) and HepII (Brabetz *et al.*, 1997; Sirisena *et al.*, 1994) transferases, respectively. These data suggested a potential role for WaaQ in the transfer of the branch HepIII residue.

To test this possibility, strain CWG297 was derived by insertion of the *aacC1* cassette into the middle of the *waaQ* open reading frame. The LPS from the resulting mutant migrated similarly to that of the F470 parent in SDS-PAGE (data not shown), precluding any significant effect of the mutation on core extension. The core oligosaccharide of the LPS from CWG297 was then subjected to methylation linkage analysis.

Methylation linkage analysis of the CWG297 core resulted in the GLC chromatogram shown in Figure 3.2C. Of particular note, the terminal heptose derivative (resulting from HepIII) was completely absent in the CWG297 spectrum (note the absence of peak 6 in Figure 3.2C). The disappearance of the HepIII side branch was also confirmed by FAB-MS (performed by K. Chan, Institute for Biological Sciences, National Research Council, data not shown).

Given the similarity of WaaQ to WaaC and WaaF (the HepI and HepII transferases respectively) and the complete absence of HepIII in the CWG297 core OS, it was concluded that *waaQ* encodes the transferase for the branch HepIII residue. However, a peak corresponding to a 3-substituted heptose derivative unexpectedly appeared in the CWG297 GLC spectrum from the methylation linkage analysis (see Figure 3.2C, peak 8), indicating loss of the phosphoryl substituent from either HepI or HepII (both are 3-substituted after the loss of HepIII). To resolve which heptose residue was lacking its phosphoryl substituent, the ^{31}P-NMR spectrum of the CWG297 core OS was compared to the spectra from F470 and CWG312 (*waaY*). Given the similar phosphate to PPEtN ratios for CWG297 and CWG312 (compare Figure 3.2, panels F and G) it was concluded that the same phosphoryl substituent (phosphate on HepII) was absent in both strains. If the *waaQ* mutation had affected phosphoryl substitution of HepI, the ratio of phosphate relative to PPEtN would have increased rather than decreased. The observed loss of phosphate from HepII in CWG297 does not contradict the assignment of WaaQ as the HepIII transferase since WaaY had already been assigned as the enzyme responsible for the phosphorylation of HepII (see section 3.2.1 Assignment of *waaY* gene function). Rather, the transfer of HepIII by WaaQ appears to be a prerequisite to the phosphorylation of HepII catalyzed by WaaY. The structure of the deduced CWG297 core is summarized in Table 3.2.

3.2.3. Assignment of *waaP* gene function:

Pairwise nucleotide alignments of the *waaP* coding region from *E. coli* K-12, R1, R2, R3, and R4, and *S. enterica* serovar Typhimurium showed identities ranging from 72 to 98%. Multiple sequence alignment of the predicted WaaP proteins reflected the homology observed at the nucleotide level with a total similarity of 93% (76% identity). In BLASTP searches of the available databases, WaaP showed no significant similarity to other characterized proteins, as previously reported (Parker *et al.*, 1992(b); Schnaitman and Klena, 1993). However, the recently developed PSI-BLAST database analysis program again identified weak (~10% identity, ~25% similarity) but biologically relevant similarities shared between the various WaaP proteins and a large number of protein kinases (discussed further in section 3.5.6 Site-directed mutagenesis of WaaP). These circumstantial data suggested a potential role for WaaP in phosphorylation reactions.

To test this possibility, strain CWG296 was derived by insertion of the *aacC1* cassette into the middle of the *waaP* open reading frame. The LPS from the resulting mutant migrated similarly to that of the F470 parent in SDS-PAGE (data not shown), precluding any significant effect of the mutation on core extension. The core oligosaccharide of the LPS from CWG296 was then subjected to methylation linkage analysis and ^{31}P-NMR.

^{31}P-NMR spectroscopy showed a complete lack of phosphorus in the CWG296 core (see Figure 3.2H), indicating that mutation of *waaP* resulted in the loss of all phosphoryl substituents from both HepI and HepII. The GLC

chromatograms from the CWG296 and F470 methylation linkage analyses were then compared to determine any other effects of the *waaP* mutation on the core oligosaccharide structure (see Figure 3.2, panels D and E). Of note, the terminal heptose derivative from HepIII was almost completely absent in the CWG296 core OS (note the absence of peak 6 in Figure 3.2D). This lack of terminal heptose was also observed by FAB-MS (performed by K. Chan, Institute for Biological Sciences, National Research Council, data not shown), and implicated the *waaP* mutation in the loss of HepIII in addition to the above-mentioned deficiency in core phosphorylation. The absence of phosphoryl substituents and of HepIII was further corroborated by the appearance of a large peak in the CWG296 GLC chromatogram (see Figure 3.2D, peak 8) corresponding to a 3-substituted heptose derivative from HepI and HepII. The calculated molar ratio of this derivative (see Table 3.2) was somewhat lower than expected, but the observed signal was clearly twice that from CWG297 (compare peak 8 in Figure 3.2C and 3.2D, and see Table 3.2), as expected based on their predicted structures. (The prolonged retention time of the 3-substituted heptose derivative on the GLC column may account for this slight discrepancy in molar ratios.) Finally, the amounts of terminal glucose (from the β-Glc side branch) and of 2,3-disubstituted glucose (from GlcII) were noticeably decreased in CWG296 (compare peaks 1 and 7 in Figure 3.2A and 3.2D, and see Table 3.2). The decrease in 2,3-disubstituted glucose was offset by the appearance of an approximately equal amount of 2-substituted glucose

(note the appearance of peak 3 in Figure 3.2D), reflecting GlcII lacking the β-Glc substitution.

In summary, mutation of *waaP* resulted in a core oligosaccharide which was devoid of all phosphoryl substituents and the branch HepIII residue, and which appeared to contain a lower percentage of β-Glc substituted GlcII. Since WaaY and WaaQ had already been assigned in this study as the enzymes responsible for the phosphorylation of HepII and the transfer of HepIII respectively (see section 3.2.1 Assignment of *waaY* gene function and section 3.2.2 Assignment of *waaQ* gene function), and since WaaV had been previously assigned as the β-Glc transferase (see section 3.1.5 Transfer of the β-linked Glc side-branch in core type R1), it was concluded that the enzyme encoded by *waaP* was responsible for the phosphoryl substitution of HepI. This conclusion is supported by the sequence similarity of WaaP to known kinases, first reported in this study. The loss of other substituents and sugar residues from the CWG296 core OS does not contradict this assignment, but suggests that the activity of WaaP is a prerequisite to the efficient functioning of other enzymes.

3.3. Phenotypic analysis of core mutants:

The series of defined inner and outer core mutants was subsequently analyzed to determine whether mutations to specific *waa* genes had adverse effects on membrane stability. Initially, minimum inhibitory concentration (MIC) testing for sensitivity to the hydrophobic antibiotic novobiocin and the detergent SDS were performed as a crude measure of membrane stability. The

outer membranes of mutants displaying a dramatic increase in susceptibility to these agents were further analyzed by SDS-PAGE to look for gross compositional changes in outer membrane protein content. The data from these experiments have been published in "Mutation of the lipopolysaccharide core glycosyltransferase encoded by *waaG* destabilizes the outer membrane of *Escherichia coli* by interfering with core phosphorylation" (Journal of Bacteriology, 2000, vol. 182, 5620-5623). Analysis of phosphorylation in the *waaG* core oligosaccharide was performed by co-author Dr. E. Vinogradov. The remainder of the work reported in this article was performed as part of this thesis.

3.3.1. Novobiocin and SDS sensitivity testing:

The MIC data for novobiocin and SDS sensitivity testing are summarized in Table 3.3. Strains with core mutations distal to *waaO* are not listed because they are identical to the wild-type. As shown in Table 3.3, mutation of *waaY* (CWG312, lacking phosphate on HepII) caused only a slight increase in susceptibility to both compounds, as did mutation of the HepIII transferase encoded by *waaQ* (CWG297, also lacking phosphate on HepII because the presence of HepIII is a prerequisite for the functioning of WaaY). Mutation of *waaP* (CWG296, lacking all core phosphate) caused a drastic increase in susceptibility to both compounds. Given the effects of the *waaY*, *waaQ*, and *waaP* mutations on core phosphorylation, and the previously noted correlation of LPS core phosphorylation to membrane stability

Table 3.3. MIC of SDS and novobiocin for F470 and derivatives [a]

Strain	MIC	
	SDS (mg/ml)	novobiocin (μg/ml)
F470 (parent)	>200	200
CWG312 (*waaY*)	200	100
CWG297 (*waaQ*)	200	100
CWG296 (*waaP*)	0.1	6.3
CWG303 (*waaG*)	0.2	100
CWG308 (*waaO*)	>200	200

[a] Strains with core mutations distal to *waaO* are not listed because they are identical to the wild-type.

(Nikaido and Vaara, 1985), these results were rationalized in terms of the decreased core phosphate content.

On the other hand, mutation of *waaG* in strain CWG303 (truncated after HepII) caused a slight increase in susceptibility to novobiocin, but a major (1000-fold) increase in susceptibility to SDS. It should be noted, however, that the change in SDS sensitivity of CWG303 might not be as large as the impression given by the decrease in the MIC: the critical micelle concentration of SDS is 7.2 mM (approximately 2 mg/mL), so the concentration of monomeric SDS does not increase linearly beyond this point. Nevertheless, the increased susceptibility to SDS is significantly more than that observed for strains CWG312 and CWG297.

Given that WaaG had already been identified as the glucosyltransferase for GlcI (see section 3.1.1 Transfer of GlcI in core type R1), it was hard to rationalize the decreased outer membrane stability of the *waaG* mutant. To investigate the structural basis for the CWG303 phenotype, its LPS was further analyzed to determine the extent of its core phosphate substitution. The detailed analysis of CWG303 core phosphorylation was performed by Dr. E. Vinogradov, and the results are summarized as follows. NMR and electrospray MS showed that the CWG303 core lacked all phosphate on HepII, and that the level of phosphoryl substitution on HepI was only 40 percent that of the wild-type (Yethon *et al.*, 2000(b)). This corresponds to an 80 percent total reduction in heptose phosphorylation, given that the modification of HepI and HepII is known to be nearly stoichiometric in the wild-type (from

the structural studies above). The non-stoichiometric substitution of HepI phosphate with PEtN was unaffected. Therefore, given the unanticipated effect of the *waaG* mutation on core phosphorylation, the CWG303 phenotype can also be rationalized in terms of decreased core phosphorylation. In summary, derivatives of F470 with mutations affecting core phosphorylation were all shown to display increased susceptibility to novobiocin and/or SDS, and by inference to have reduced outer membrane stability. Indeed, it was observed that the greater the decrease in LPS core phosphorylation, the greater the corresponding destabilization of the outer membrane.

3.3.2. Analysis of outer membrane protein profiles by SDS-PAGE:

Supersensitivity to hydrophobic agents is a characteristic often associated with the pleiotropic phenotype called "deep-rough", which is exhibited by mutants with severely truncated LPS core regions (*i.e.* lacking inner core heptose residues). Another characteristic of the deep-rough phenotype is a marked decrease in the protein content of the outer membrane (reviewed in Heinrichs *et al.*, 1998(c); Schnaitman and Klena, 1993). Indeed, in the case of deep-rough mutants, a proposed explanation for the increased susceptibility to hydrophobic compounds is that the void left by missing outer membrane proteins is filled by phospholipids, resulting in patches of phospholipid bilayer in the outer membrane (Nikaido and Vaara, 1985). Previous studies done with only partially characterized mutants suggest that the deep-rough phenotype is due mainly to the loss of core phosphoryl substituents (Helander *et al.*, 1989; Parker *et al.*, 1992(a)), and that

it can be caused by mutation of the *waaP* gene alone. Therefore, to investigate whether the defined *waaG* and *waaP* mutants (those with the most dramatic antibiotic susceptibilities) exhibited the outer membrane protein defect, outer membrane fractions were isolated from the parent strain, F470, and from the defined mutant strains CWG303 (*waaG*) and CWG296 (*waaP*), and their protein profiles were examined by SDS-PAGE (see Figure 3.3).

Based on earlier studies (Helander *et al.*, 1989; Parker *et al.*, 1992(a)), it was expected that CWG296 (*waaP*) would show the outer membrane protein defect, but it was unknown what effect mutation of *waaG* would have in strain CWG303. Surprisingly, the outer membrane protein profiles from F470, CWG296, and CWG303 appeared identical (see Figure 3.3). Of particular note, the ratio of porins (OmpF and OmpC) to OmpA is constant, which is not the case in earlier work done with partially characterized mutants (Helander *et al.*, 1989; Parker *et al.*, 1992(a)).

3.4. *waaP* mutants of *S. enterica* are avirulent:

Given the severe antibiotic and detergent sensitivity of CWG296 (*waaP*) described above (see section 3.3.1 Novobiocin and SDS sensitivity testing), it was hypothesized that this mutation would also adversely affect virulence. However, the F470 parent of CWG296 is already an avirulent mutant with rough LPS, and is thus not suitable for virulence studies. Therefore, to assess the contribution of WaaP and LPS core phosphorylation to the biology of an intracellular pathogen, a *waaP* mutation was made in a virulent strain of

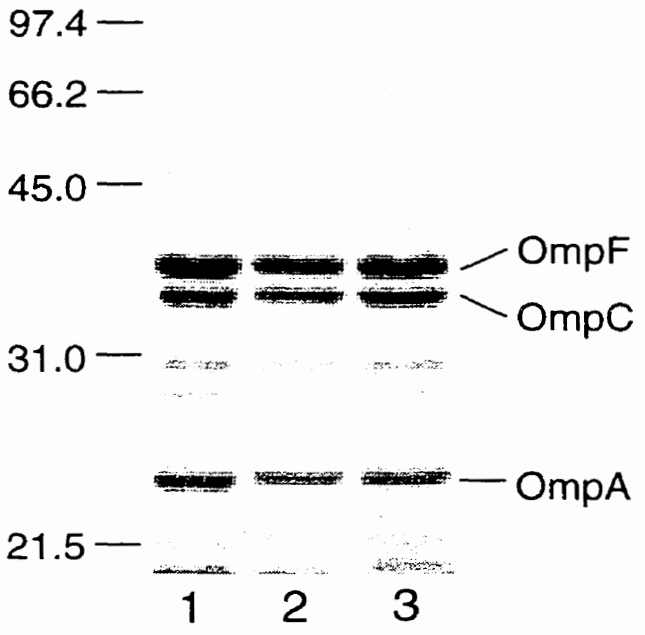


Figure 3.3. SDS-PAGE gel (12.5% polyacrylamide) of Sarkosyl-insoluble outer membrane fractions. Lane 1, F470 (parent); lane 2, CWG303 (*waaG*); lane 3, CWG296 (*waaP*). Molecular mass markers (in kilodaltons) are indicated to the left of the gel, and major proteins are labeled on the right.

S. enterica (ATCC 14028), and the virulence of the mutant was subsequently tested in a variety of accepted mouse infection models. The data from these experiments have been published in "*Salmonella enterica* serovar Typhimurium *waaP* mutants show increased susceptibility to polymyxin and loss of virulence in vivo" (Infection and Immunity, 2000, vol. 68, 4485-4491). Animal experiments were performed by co-authors L. Laroche, Dr. D. Malo, and Dr. J. S. Gunn. Dr. J. S. Gunn also performed the polymyxin susceptibility testing. Matrix-assisted laser desorption ionization-time-of flight (MALDI-TOF) MS of isolated lipid A was performed by co-author Dr. R. K. Ernst. The remainder of the work in this article was performed as part of this thesis.

3.4.1. Structure of the LPS core oligosaccharide of a *waaP* mutant of *S. enterica*:

Earlier studies performed with genetically uncharacterized *S. enterica* "*waaP* mutants" indicated that the core oligosaccharide of such mutant strains was truncated after the first glucose residue of the outer core (Helander *et al.*, 1989). To determine whether such was the case for our defined mutant strain (CWG304) or whether the core defect would more closely resemble that observed in *E. coli* R1, we examined the CWG304 and parent (ATCC 14028) LPSs by SDS-PAGE (see Figure 3.4). A typical ladder-like pattern of smooth LPS bands is visible for the parent strain, and also (although to a lesser extent) for CWG304 (see Figure 3.4A and 3.4B, lanes 1 and 2). Both strains show the same high molecular weight modal cluster of smooth LPS bands (see Figure 3.4B, lanes 1 and 2). However, the CWG304 profile using gradient Tricine SDS-PAGE gels also shows an intense band that migrates slightly faster than

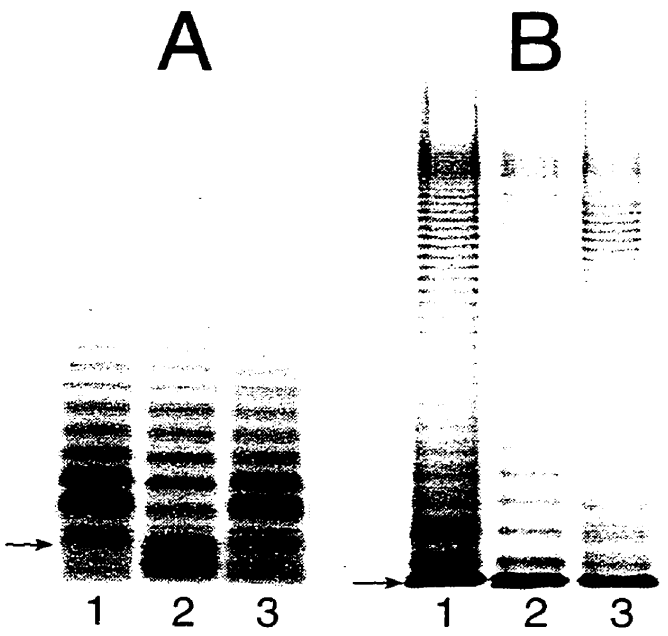


Figure 3.4. Silver-stained SDS-PAGE gels showing the LPS profile of strains ATCC 14028 (lane 1), CWG304 (lane 2), and CWG304 complemented with the *waaP* open reading frame from *E. coli* F470 (lane 3). Panel A shows the samples run on a 10-20% Tricine SDS-PAGE gel, while panel B shows the same samples run on a standard 12% SDS-PAGE gel. The gel system in panel A gives better resolution of low molecular weight LPS (i.e. LPS lacking O antigen), while the standard gel system shows that the modality of O antigen expression is unaffected in the mutant strain. The migration of Ra-LPS (lipid A and complete core) in each gel system is indicated by an arrow. Note that Ra-LPS comigrates with LPS molecules with one O antigen repeat in the gel system in panel B, so the amount of free (uncapped) lipid A-core is misleading. The extent of capping is clear in panel A.

any band in the parent profile (see Figure 3.4A, lane 2). These observations indicate that CWG304 produces a full-length (complete) core capped with O antigen, but that a portion of its LPS molecules is prematurely truncated in the core. The wild-type LPS banding pattern could be restored to CWG304 (see Figure 3.4A and 3.4B, lane 3) by complementation with plasmid pWQ909, carrying *waaP* from *E. coli* F470, further confirming the identical functioning of WaaP in both organisms. This was the expected result given that the predicted WaaP proteins of *S. enterica* and *E. coli* F470 are 81.5% identical (90.6% similar).

Phosphorylation of the inner core heptose region in CWG304 was then examined by ^{31}P-NMR and methylation linkage analysis. The ^{31}P-NMR spectra of the core oligosaccharides from the wild-type parent and the *waaP*-null strain are shown in Figure 3.5. Again, the signal at approximately 5 ppm in the parent spectrum corresponds to the phosphate residues on HepI and HepII, while the doublet at approximately -10 ppm corresponds to PPEtN on HepI (Helander *et al.*, 1994; Helander *et al.*, 1997; Olsthoorn *et al.*, 1998; Yethon *et al.*, 1998). The complete lack of phosphate in the CWG304 core is shown by the disappearance of all phosphorus signals (see Figure 3.5).

Methylation linkage analysis of the LPS core oligosaccharides from ATCC 14028 and CWG304 further confirmed the predicted structure of the mutant core (see Table 3.4). Derivatives from HepI or HepII were not evident for ATCC 14028 LPS because the phosphoryl substituents attached to these residues make their derivatives too polar to elute from the gas-liquid

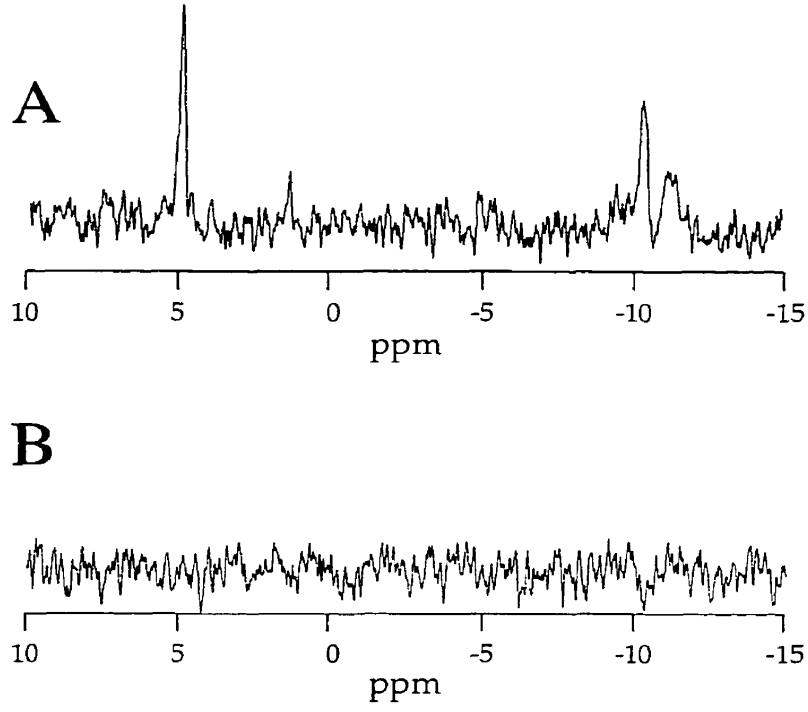


Figure 3.5. ^{31}P-NMR spectra of the core oligosaccharides from ATCC 14028 (A), and CWG304 (B). The signal at 5 ppm is indicative of a phosphomonoester (P on either HepI or HepII), and the two peaks near -10 ppm are characteristic of a diphosphodiester (PPEtN on HepI).

Table 3.4. Methylation linkage analysis of the core oligosaccharide heptose region from the LPSs of ATCC 14028 and CWG304

Strain	Heptose region residues detected [a]	Structure of the heptose region
ATCC 14028	Hep-(1→	Hep III 1↓ [b] 7 →3)-Hep II-(1→3)-Hep I-(1→ 4 4 ↑ ↑ P P / PPEtN
CWG304 (*waaP*)	→3)-Hep-(1→	→3)-Hep II-(1→3)-Hep I-(1→ [c]

[a] Phosphorylated residues are not detected by this analysis (see text).

[b] Published structure (Holst and Brade, 1992; Olsthoorn *et al.*, 1998).

[c] Deduced structure, taking into account both methylation linkage analysis and ^{31}P-NMR data (see Figure 3.5).

chromatography column, as established previously in *E. coli* (Yethon *et al.*, 1998). Analysis of the CWG304 core showed the disappearance of the HepIII derivative and the appearance of a derivative corresponding to 3-substituted heptose (see Table 3.4). The 3-substituted heptose derivative reflects both non-phosphorylated HepI and non-phosphorylated HepII lacking the branch HepIII residue. Together with our ^{31}P-NMR results, these data confirm that the structural defect caused by the *waaP* mutation in *S. enterica* is identical to that observed in *E. coli* R1.

3.4.2. Sensitivity of an *S. enterica waaP* mutant to antimicrobial compounds:

To confirm the predicted sensitivity of CWG304 to SDS and novobiocin, sensitivity testing to these compounds was performed as described previously (see Table 3.5). As shown in Table 3.5, CWG304 exhibited more than a 125-fold increase in susceptibility to SDS, and more than a 30-fold increase in susceptibility to novobiocin as compared to the parent strain. Complementation with plasmid pWQ909, carrying *waaP* from *E. coli* F470, restored wild-type levels of resistance to these compounds. The MIC results for polymyxin susceptibility testing are also summarized in Table 3.5.

Polymyxins are cyclic, amphipathic, lipopeptide antibiotics that act preferentially on Gram-negative bacteria (Fidai *et al.*, 1997; Vaara, 1992). The peptide portion of polymyxins is cationic, and interacts strongly with the negatively-charged LPS on the surface of the Gram-negative outer membrane. Not surprisingly, then, resistance to polymyxins is generally correlated with modifications of LPS that render it less electronegative. Examples of such LPS

Table 3.5. MIC testing of *S. enterica* ATCC 14028 and derivatives for susceptibility to novobiocin, SDS, and polymyxin.

	MIC		
Strain	Novobiocin (μg/mL)	SDS (mg/mL)	Polymyxin (μg/mL)
ATCC 14028 (parent)	200	200	1.0
CWG304 (*waaP::aacC1*)	6.3	1.6	0.01
CWG304 + pWQ909	200	100	0.4
JSG435 (*pmrA505*)	—	—	4.0
JSG778 (*pmrA505, waaP::aacC1*)	—	—	0.5

modifications include the PmrA-PmrB regulated substitution of lipid A phosphates with aminoarabinose or PEtN (see section 1.2.4 Regulated LPS modifications). Therefore, two possible outcomes were envisioned to sensitivity testing with polymyxin. In one predicted scenario, the loss of phosphoryl substituents would increase resistance to polymyxin by decreasing the LPS negative charge. In the second scenario, the previously noted sensitivity of *waaP* mutants to hydrophobic agents might decrease polymyxin resistance due to polymyxin-outer membrane interactions of a hydrophobic nature.

In the absence of the *waaP* defect, the PmrA-constitutive strain JSG435 shows increased resistance to polymyxin (compare ATCC 14028 and JSG435), as reported previously (Gunn *et al.*, 1998). (Strain JSG435 carries the *pmrA505* allele, which expresses a mutated version of the PmrA regulator that is constitutively active, resulting in a lipid A that is highly substituted with aminoarabinose (Gunn *et al.*, 1998).) The *waaP* mutation, however, causes a clear increase in polymyxin sensitivity. In the wild-type background, the polymyxin MIC is decreased by 100-fold (compare ATCC 14028 and CWG304), while a somewhat smaller decrease (8-fold) is observed in the PmrA-constitutive background (compare JSG435 and JSG778).

To address whether the changes in polymyxin susceptibility were related to, or independent of lipid A aminoarabinose substitution, the lipid A of each LPS was analyzed by MALDI-TOF mass spectrometry (performed by Dr. R. K. Ernst, Departments of Medicine and Microbiology, University of

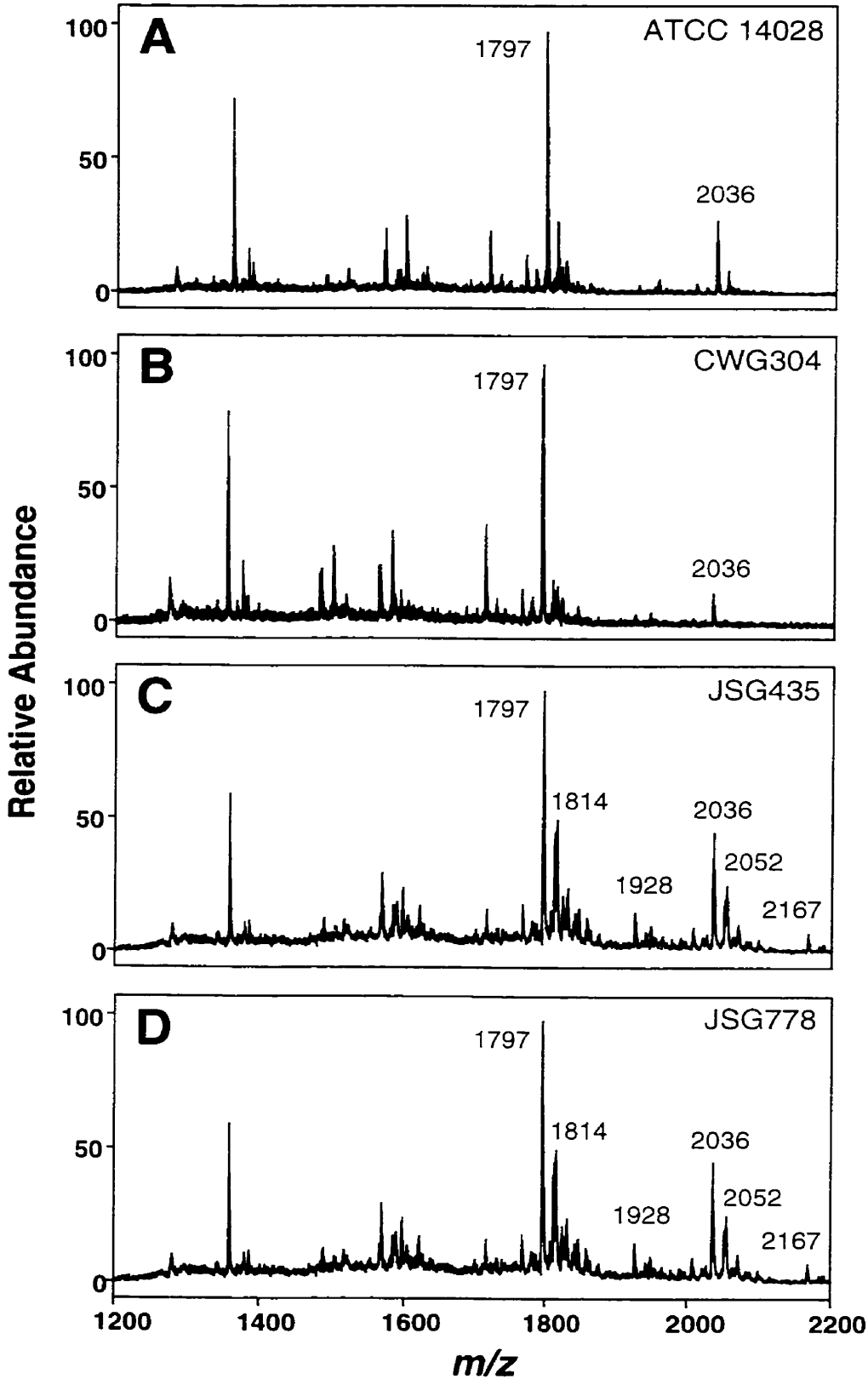
Washington, Seattle). The mass spectra of the lipid A isolated from ATCC 14028 and CWG304 show no significant differences in the amount or nature of lipid A modifications (see Figure 3.6). It was therefore concluded that the contribution of core phosphate residues to polymyxin resistance is distinct from the effects of lipid A aminoarabinose modification.

3.4.3. Effect of *waaP* mutation on growth and virulence:

Given the outer membrane defect caused by the *waaP* mutation, we performed growth curves for ATCC 14028 and CWG304 to determine if mutation of *waaP* affected basic growth characteristics. The growth curves of the wild-type and *waaP* mutant (in Luria-Bertani broth at 37°C) were identical (data not shown), precluding a significant effect on normal cell growth.

To then assess whether the virulence of CWG304 was altered *in vivo*, three different mouse strains (C57BL/6J, A/J, and BALB/c) were used in infection assays. Both C57BL/6J and BALB/c are extremely susceptible to *S. enterica* infection due to a mutation in the *Nramp1* gene (Vidal *et al.*, 1995), while the wild-type strain A/J is naturally more resistant to infection. In the first experiment, both A/J and C57BL/6J mice were infected by intravenous injection in the caudal vein. The resistant A/J mice were unaffected by challenge with either 10^2 or 10^3 CFU of ATCC 14028. At 10^4 CFU, 3 of 5 mice died after 15 days. The remaining mice survived over the 21 day course of the experiment. As for the highly susceptible C57BL/6J mice, when challenged with the ATCC 14028 parent, all died within 5 days at 10^3 or 10^4 CFU, and within 6 days at 10^2 CFU (as expected). By contrast, all of the mice challenged

Figure 3.6. Characterization of structural modifications of *S. enterica* lipid A by negative-ion MALDI-TOF mass spectrometry. All values given are average masses rounded to the nearest whole number for singly charged, deprotonated molecules $[M-H]^-$. (A) ATCC 14028 (parent) lipid A, with the major signal representing the hexa-acylated form (at *m/z* 1797) and the hepta-acylated form containing palmitate (at *m/z* 2036) indicated. (B) CWG304 (*waaP::aacC1*) lipid A, showing ions at *m/z* 1797 and 2036 as described above. (C) JSG435 (*pmrA505*) lipid A, showing the hexa- and hepta-acylated forms (as above), as well as modification by the addition of aminoarabinose (*m/z* 1928 and 2167, respectively) or by the addition of a hydroxyl group (*m/z* 1814 and 2052, respectively). (D) JSG778 (*pmrA505*, *waaP::aacC1*) lipid A, showing ions at *m/z* 1797, 1814, 1928, 2036, 2052 and 2167 as described above. (These experiments were performed by Dr. R. K. Ernst, Departments of Medicine and Microbiology, University of Washington, Seattle.)


A
ATCC 14028
1797
2036
B
CWG304
1797
2036
C
JSG435
1797
1814
2036
2052
1928
2167
D
JSG778
1797
1814
2036
2052
1928
2167
100
50
0
Relative Abundance
1200
1400
1600
1800
2000
2200
m/z

with the *waaP* mutant strain CWG296 survived (both mouse strains, all doses, for the duration of the experiment).

The surviving A/J mice were euthanized on day 21, and spleen and liver homogenates were plated to determine the extent of persisting *S. enterica* infection (see Table 3.6). In mice challenged with ATCC 14028, the bacteria could still be isolated from the liver and spleen homogenates in significant numbers. However, CWG304 bacteria were virtually cleared by day 21, and could only be detected in very small numbers in the spleen at the highest dose administered (see Table 3.6).

Given the somewhat decreased amount of O antigen expression in strain CWG304, as observed by SDS-PAGE (refer back to Figure 3.4), it was possible that the mutant's loss of virulence could be attributed to an increase in susceptibility to complement-mediated serum killing. O antigen is known to be an important factor in the ability of many bacteria to evade complement (Joiner, 1988). To determine whether bacteria injected into the bloodstream were simply cleared by complement-mediated cell lysis, the parent and *waaP* mutant strains were incubated with fresh mouse serum for 2 hours at 37°C, and then serial dilutions were plated on LB agar plates. No difference in CFU was observed between the two strains, indicating that complement-mediated killing is not a factor in inhibiting CWG304 infection.

In a second experiment, BALB/c mice were challenged with ATCC 14028 and CWG304, both orally and by intraperitoneal injection to determine whether the loss of virulence of CWG304 was influenced by route of

Table 3.6. Average number of CFU in the spleens and livers of A/J mice challenged intravenously with the indicated doses of ATCC 14028 or CWG304. Surviving mice were sacrificed 21 days post infection.

	Average CFU/spleen		Average CFU/liver	
Dose (CFU)	ATCC 14028	CWG304	ATCC 14028	CWG304
10^4	3468 ± 3352 [a]	9 ± 5	142 ± 46	0
10^3	56 ± 13	0	32 ± 17	0
10^2	58 ± 9	0	31 ± 3	0

[a] Only 2 of 5 mice survived until day 21 at this dose. All other results are averages from 5 mice. Results are reported as averages ± standard deviations.

infection. These infection models are the most widely accepted for *S. enterica* virulence studies, and LD_{50} values have been determined for both methods (6.5×10^4 and <10, for oral and intraperitoneal infection respectively) (Behlau and Miller, 1993). The results from the oral and intraperitoneal infection experiments show that the virulence defect of CWG304 does not depend on how the inoculum is administered: all of the mice infected with CWG304 (N=5-10) survived at a dose 10 times the LD_{50} (intraperitoneally) or 100 times the LD_{50} (orally), while all of the mice challenged with the wild-type ATCC 14028 died at these doses.

3.5. Reconstitution of WaaP activity *in vitro* using purified components:

Given the importance of WaaP-catalyzed core phosphorylation to membrane stability and virulence, it was anticipated that better definition of this reaction might present novel opportunities for therapeutic intervention. Therefore, the second major objective of this thesis was to purify WaaP and reconstitute its enzymatic activity using purified components *in vitro*. The data from these experiments have been published in "Purification and characterization of WaaP from *Escherichia coli*, a lipopolysaccharide kinase essential for outer membrane stability" (Journal of Biological Chemistry, 2001, vol. 276, 5498-5504). All of the work reported in this article was performed as part of this thesis.

3.5.1. Expression of WaaP:

The plasmid construct for overexpression of recombinant WaaP (pWQ910) was designed to incorporate an N-terminal His_6-tag to facilitate

subsequent purification. The addition of this N-terminal His_6-tag was shown to have no effect on the ability of WaaP to complement the CWG296 *waaP* mutant phenotype, thus validating the use of the His_6-tagged derivative for purification. Expression of His_6-tagged WaaP from plasmid pWQ910 resulted in a significant amount of protein, as seen by the appearance of an intense band located at ~33 kDa in Coomassie Blue-stained SDS-PAGE gels (see Figure 3.7). The predicted molecular weight of WaaP is 31049 Da, and of His_6-tagged WaaP is 33212 Da, a difference which could be observed by SDS-PAGE (see Figure 3.7). The identity of the overexpressed protein was further confirmed by Western immunoblotting with monoclonal antibodies specific for the His_6-tag (see Figure 3.7). Reactivity with the His_6-tag-specific monoclonal antibody also demonstrated that the N-terminus of the protein was not processed post-translationally, consistent with the prediction from the *waaP* sequence. Unfortunately, when the WaaP-expressing cells were lysed and fractionated by differential centrifugation (into insoluble matter, membranes, and cytosol), the recombinant protein was found almost exclusively in insoluble inclusion bodies (see Figure 3.8).

Many standard variables were tested to increase the yield of soluble WaaP based on published successes with other recombinant proteins (Blackwell and Horgan, 1991; Sawyer *et al.*, 1994; Schein and Noteborn, 1988). For example, various growth media were tested (LB, Terrific Broth, Tryptic Soy Broth), different growth temperatures were tested (15°C, 25°C, 30°C, 37°C, and 45°C), the duration of induction was varied (30 min, 1 h, 2 h, 3 h, 5 h),

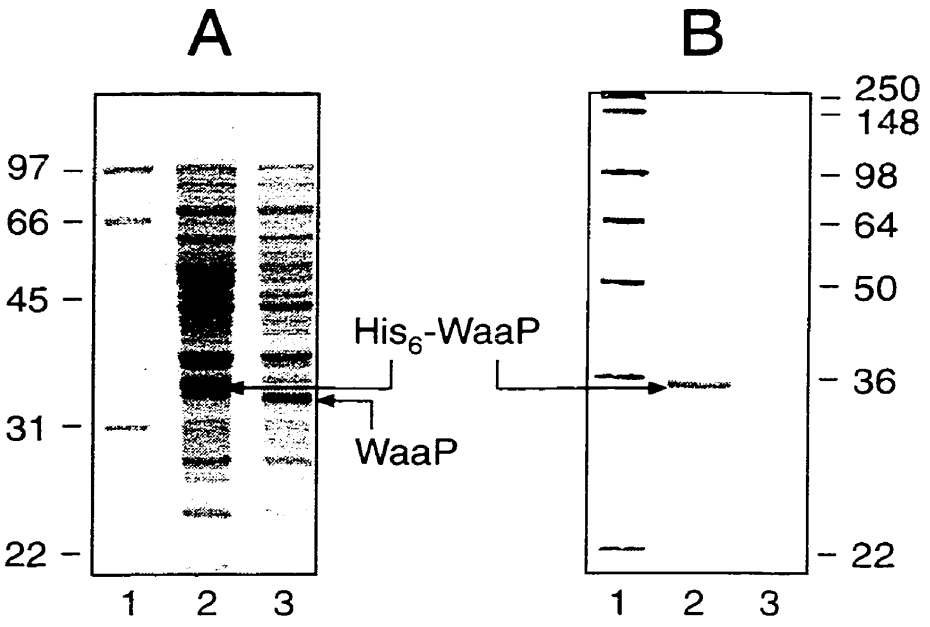


Figure 3.7 Panel A: Coomassie Blue-stained SDS-PAGE gel of whole-cell lysates of BL21(DE3) expressing the His_6-tagged (lane 2) and native (lane 3) versions of WaaP. Panel B: Corresponding Western immunoblot of the gel in panel A, probed with a monoclonal antibody specific for the His_6-tag. (Prestained molecular weight markers were used in panel B to enable visualization on the blot.)

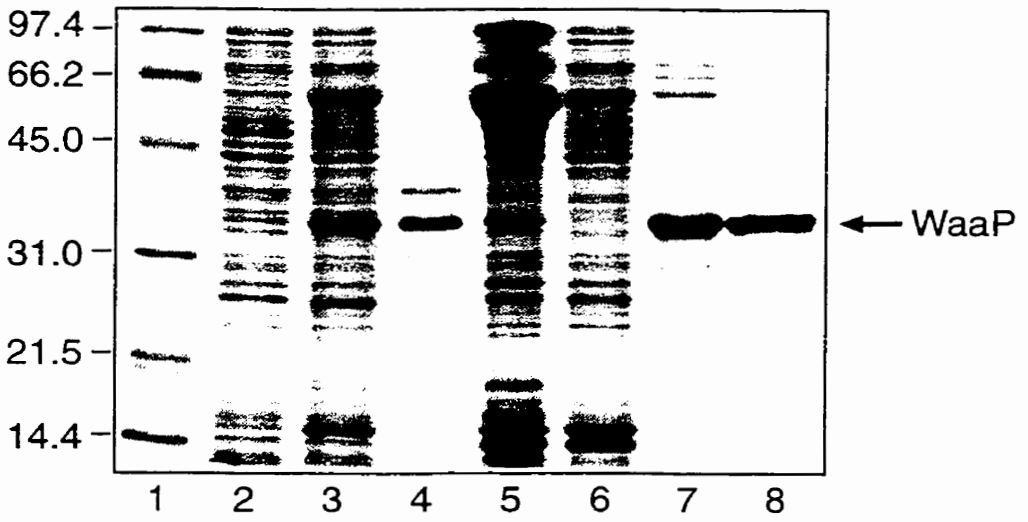


Figure 3.8. Coomassie Blue-stained SDS-PAGE gel of fractions generated during the expression and purification of His_6-tagged WaaP from strain BL21(DE3)pWQ910/pWQ911. Plasmid pWQ910 is a derivative of pET-28a(+) for the IPTG-inducible expression of WaaP, while plasmid pWQ911 is a derivative of pBAD33 for the L-arabinose-inducible co-expression of the GroES and GroEL molecular chaperones (see section 2.6.3 Protein expression and preparation of cell-free extracts).
Lane 1, molecular weight standards; lane 2, pre-induced whole-cell lysate; lane 3, arabinose- and IPTG-induced whole-cell lysate; lane 4, insoluble material from cell lysate; lane 5, cleared lysate; lane 6, flow-through from Ni-NTA agarose affinity chromatography column; lane 7, protein eluted from Ni-NTA agarose affinity chromatography column; lane 8, flow-through from anion-exchange chromatography column.

cultures were induced at different times (OD_{600nm} = 0.6, 0.8, 1.0, 1.2), and a range of different inducer concentrations was used (0.005-0.1 mM IPTG or 0.002-0.02% arabinose). In addition, removal of the His_6-tag, expression from different promoters, and compatible solute accumulation were attempted in an effort to increase soluble protein yield, but none of these treatments provided substantial improvement.

In a final attempt to increase the yield of soluble WaaP, a plasmid construct was designed that would allow for co-expression of the *E. coli* GroES and GroEL chaperones (based on the work of Amrein *et al.*, 1995). GroES and GroEL are members of the heat shock protein family that bind and stabilize proteins at intermediate stages of folding, assembly, and translocation across membranes (Hartl, 1996). Since the WaaP expression plasmid pWQ910 had a kanamycin resistance marker and a pBR origin of replication, therefore the chaperone expression plasmid was based on pBAD33, which has a chloramphenicol resistance marker and a compatible origin of replication from pACYC (Guzman *et al.*, 1995). Not only can these two plasmids be maintained simultaneously in a bacterial cell, but expression from each is controlled independently (expression from pBAD plasmids requires L-arabinose induction, while expression from pET plasmids requires IPTG induction). The ability to express chaperones independently of WaaP allowed for accumulation of chaperones prior to the induction of WaaP. Using this co-expression system, it was possible to achieve sufficient soluble protein expression to allow for the purification of the protein under native

conditions, although the majority of protein still remained insoluble (see Figure 3.8). Subcellular fractionation also indicated a tendency for the recombinant His_6-tagged WaaP to associate with membranes, so Triton X-100 was added to the lysis buffer to help extract any membrane-associated WaaP and achieve the highest possible yield of soluble protein. The association of recombinant WaaP with membranes was not unexpected; indeed the enzymes involved in LPS core assembly are predicted to function as peripheral membrane proteins at the cytoplasmic face of the inner membrane (Schnaitman and Klena, 1993) where they have access to both their cytoplasmic substrates (sugar nucleotides or ATP) and their lipid acceptor molecules.

3.5.2. Purification of WaaP:

The initial purification of recombinant His_6-tagged WaaP was achieved via immobilized metal-affinity chromatography (see Figure 3.8). Given that the predicted pI of the WaaP protein is very high (approximately 9.8), the next logical step was to use cation-exchange chromatography. However, the protein tended to precipitate rapidly during the buffer exchange required prior to loading on the cation-exchange column. To circumvent this problem, the enzyme was further purified by using subtractive anion exchange chromatography to bind the remaining contaminating proteins, allowing WaaP to be collected in the flow-through (see Figure 3.8). In this way, the protein could be kept in a higher ionic strength buffer to minimize the precipitation problem. The purity of the protein collected in the flow-through

from the anion-exchange column was over 95% as judged by SDS-PAGE (see Figure 3.8). Using purified His_6-tagged WaaP, an assay was subsequently developed to monitor its activity (refer back to section 2.6.6 Assay conditions for WaaP activity). The protein was then purified a second time to collect enzyme purification data, as shown in Table 3.7. After the final step in the purification protocol, the specific activity of the His_6-tagged WaaP was 25.8-fold higher than the crude extract, with a total activity yield of 0.53 % (see Table 3.7).

Of note, the presence of 10 mM β-mercaptoethanol in the lysis buffer was found to greatly increase the activity of the purified protein (more than a 10-fold increase in ^{33}P incorporation). Further, the purified enzyme could be stored in 50% glycerol at -20°C, without significant loss of activity for over 30 days.

3.5.3. Optimization of WaaP assay conditions:

The kinase activity of purified WaaP was measured using a range of Mg^{2+} concentrations from 0 to 25 mM, as shown in Figure 3.9A. The enzyme displays an absolute requirement for Mg^{2+}, which cannot be substituted with Ca^{2+}. Maximal kinase activity was observed at 17.5 mM $MgCl_2$. The activity of WaaP was then tested over a pH range from 4.5 to 9.0, as shown in Figure 3.9B. The kinase was shown to be inactive at pH 4.5, with maximal activity between pH 8.0 and 9.0.

Table 3.7. Purification of His_6-tagged WaaP from BL21(DE3)pWQ910/pWQ911. WaaP activity was measured using the assay described in section 2.6.6 (Assay conditions for WaaP activity).

Sample	[Protein] (mg/ml)	Total protein (mg)	Specific Activity (nmol/min/mg)	Total activity (nmol/min)	Purification (-fold)	Yield (%)
Cleared lysate	18.8	148	0.334	49.5	1	100
Ni-NTA	0.491	1.28	3.893	4.97	11.7	10
Anion-exchange	0.00395	0.0304	8.606	0.261	25.8	0.53

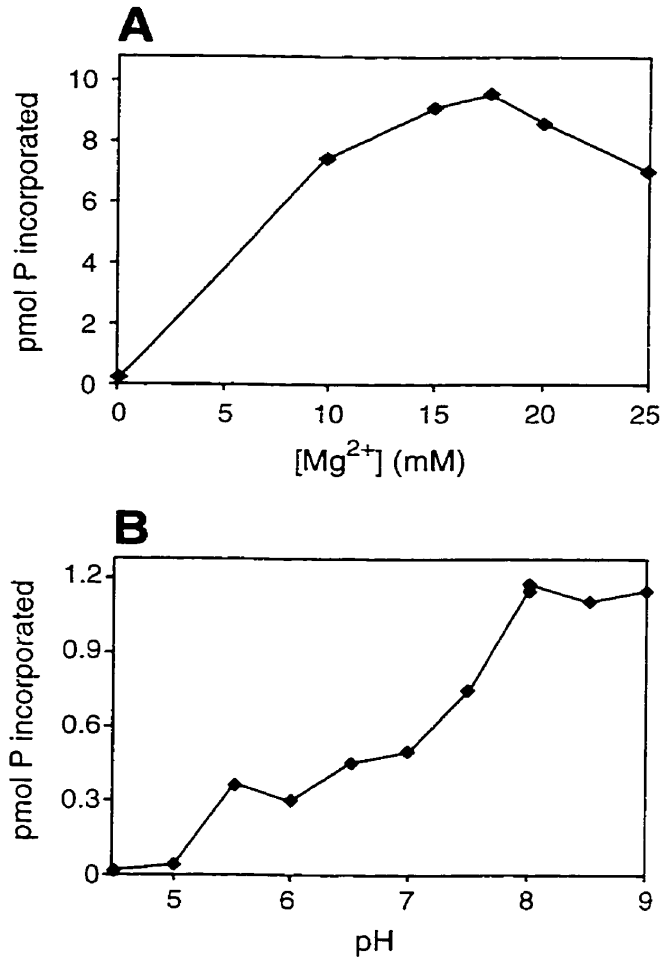


Figure 3.9. Effect of Mg^{2+} concentration and pH on kinase activity. Panel A: WaaP activity was measured under standard assay conditions over a range of $MgCl_2$ concentrations, as indicated. Mg^{2+} was absolutely required for activity, and optimal activity was observed at 17.5 mM $MgCl_2$. However, due to the binding of Mg^{2+} by LPS, the free concentration of Mg^{2+} is not known. Panel B: The pH dependence of the reaction was demonstrated under standard assay conditions using 75 mM sodium acetate (pH 4.5, 5.0, 5.5), 75 mM sodium phosphate (pH 6.0, 6.5, 7.0, 7.5, 8.0), or 75 mM diethanolamine-HCl (pH 8.0, 8.5, 9.0). The enzyme was inactive at pH 4.5, and optimally active between pH 8.0 and 9.0.

3.5.4. Kinetic properties of purified WaaP:

The activity of WaaP was shown to be linearly dependent on both time and protein concentration (see Figure 3.10). When the concentration of ATP in the reactions was held constant at 2.5 mM and the concentration of LPS was varied (see Figure 3.11A), the K_M for the LPS acceptor was calculated to be 76 μM. Likewise, when the concentration of LPS acceptor was held constant at 1 mM and the concentration of ATP was varied (see Figure 3.11B), the K_M for ATP was determined to be 0.13 mM. In both cases, the apparent V_{max} for the reaction was ~ 3.7 nmol/min/mg. Using these data, the turnover number (k_{cat}) for WaaP under these reaction conditions was calculated to be 0.13/min.

3.5.5. Characterization of reaction products by gel filtration chromatography:

When the acceptor LPS purified from our defined *waaP* mutant strain, CWG296, is run on an SDS-PAGE gel and stained with silver, two distinct molecular weight species are observed (see inset of Figure 3.12). The structure of the larger of these two species is known to consist of complete lipid A-core lacking the usual modifications (P, PEtN, and HepIII) on the heptose region of the core (see section 3.2.3 Assignment of *waaP* gene function). The smaller molecular weight species is a truncated form of the larger, terminated after GlcI as determined by co-migration with LPS from a defined *waaO* mutant on SDS-PAGE (data not shown). To determine whether WaaP was capable of phosphorylating both forms of LPS from the *waaP* mutant strain, the LPS reaction products were first hydrolyzed in mild acid to remove the insoluble lipid A portion of the molecule. After hydrolysis and centrifugation,

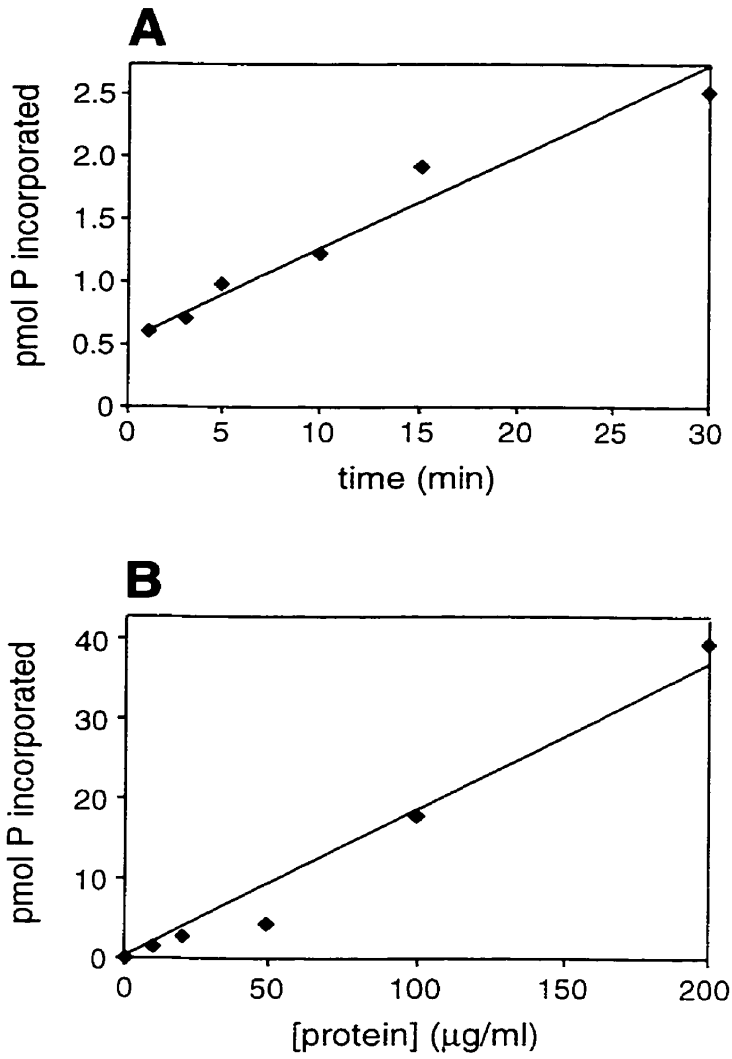


Figure 3.10. Dependence of WaaP activity on time and protein concentration. The activity of WaaP was shown to be linearly dependent on both time (from 0 to 30 min, panel A) and protein concentration (from 0-200 µg/mL, panel B).

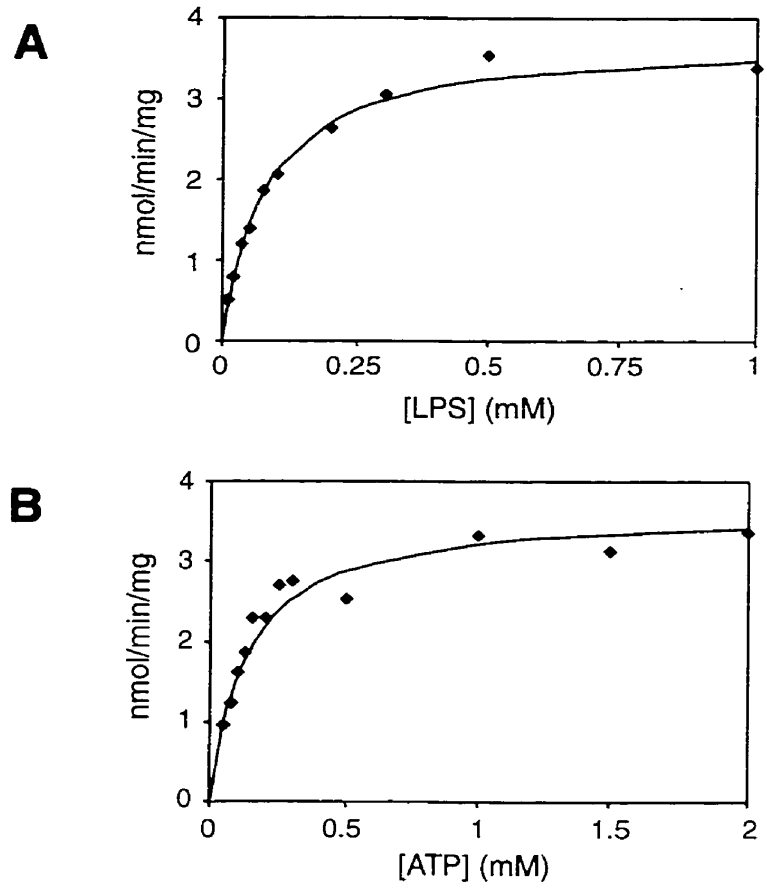


Figure 3.11. Kinetic properties of purified WaaP. Standard assay conditions were used in these experiments, except that the substrate concentrations were varied as indicated. In panel A, the concentration of ATP was held constant at 2.5 mM and the concentration of LPS was varied. The apparent K_m for the LPS acceptor was calculated to be 76 μM. In panel B, the concentration of LPS acceptor was held constant at 1 mM and the concentration of ATP was varied. The apparent K_m for ATP was determined to be 0.13 mM. In both cases, the apparent V_{max} for the reaction was ~ 3.7 nmol/min/mg. Values were determined using EnzFitter v. 1.05 software (Biosoft), and lines were drawn using a non-linear least squares fitting to the following equation:

$v = (V_{max}[S])/(Km + [S])$.

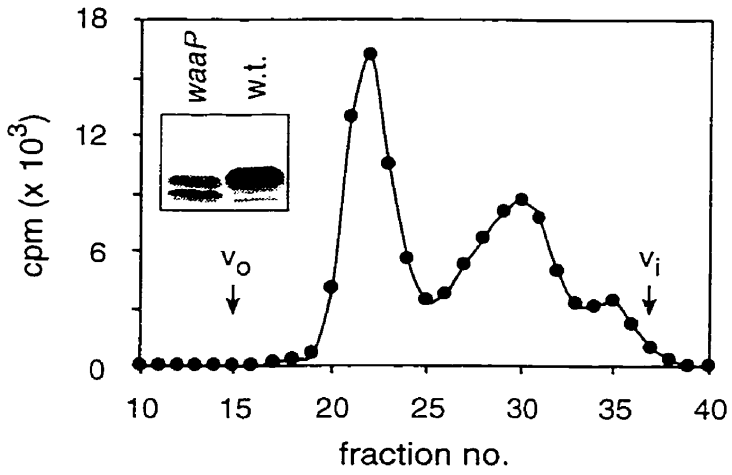


Figure 3.12. Separation of radiolabelled core oligosaccharides by chromatography on Sephadex G-25. Oligosaccharides were generated by mild acid hydrolysis of the LPS reaction products from assays performed under standard conditions. The two peaks of radioactivity correspond to the core oligosaccharides from the two major LPS species that are evident in SDS-PAGE profiles of *waaP* mutant LPS (see inset).

approximately 95% of the radioactivity was recovered in the supernatant as soluble core oligosaccharides. The soluble core oligosaccharides were then separated on a column of Sephadex G-25 (see Figure 3.12). Two distinct peaks of radioactivity were observed within the fractionation range of the column (M_r of 100-5000 for dextrans), indicating that WaaP is indeed capable of phosphorylating both the larger and smaller molecular weight LPSs isolated from the *waaP* mutant strain (see Figure 3.12). The small third peak which occurs at the column included volume likely corresponds to a small amount of ^{33}P being hydrolyzed from the reaction products during the acid hydrolysis.

3.5.6. Site-directed mutagenesis of WaaP:

Alignment of the predicted WaaP proteins from *E. coli* and *S. enterica* shows greater than 80% identity (90% similarity), while the *P. aeruginosa* WaaP homolog is approximately 55% identical (70% similar) to those of *E. coli* and *S. enterica* (see Figure 3.13). Of particular note, residues 159 to 171 (NHRDCYICHFLLH) and 184 to 192 (SVIDLHRAQ) (*E. coli* R1 residue numbering) are absolutely conserved among all of the WaaP homologs. On the other hand, alignment of the *E. coli* WaaP protein with eukaryotic protein kinases typically shows only about 10-15% identity (25-30% similarity) (see Figure 3.14). It is interesting, however, that the majority of conserved amino acids in Figure 3.14 coincide with residues that are known in eukaryotic protein kinases to be important for catalysis, based on both sequence similarity (Hanks and Quinn, 1991) and crystal structure data (Knighton *et al.*, 1991; Madhusudan *et al.*, 1994).

Figure 3.13. Multiple sequence alignment of the predicted WaaP proteins from *E. coli* R1, R2, R3, R4, K-12, *S. enterica* serovar Typhimurium (Se), and *P. aeruginosa* (Pa). The overall identity is approximately 55%, with a total similarity of approximately 70%. Considering just the WaaP proteins from *E. coli* and *S. enterica*, the similarity is much higher (80% identity, 90% similarity).

```
R1        1 -MVELKEPFATLWRGKDPFEEVKTLQGEVFRELETRRTLRFEMAGKSYFL
R2        1 -MVELEEPLATLWRGKDAFAEVKKLNGEVFRELETRRTLRFELAGKSYFL
R3        1 -MVELKEPFATLWRGKDPFEEVKTLQGEVFRELETRRTLRFEMAGKSYFL
R4        1 -MVELKEPFATLWRGKDPFEEVKTLQGEVFRELETRRTLRFEMAGKSYFL
K12       1 -MVELKEPLATLWRGKDAFAEVKKLNGEVFRELETRRTLRFELSGKSYFL
Se        1 -MVELKAPLTTLWRGKDAFEEVKTLQGEVFRELETRRTLRFELDGKSYFL
Pa        1 MRLVLEEPFKRLWNGRDPFEAVEALQGKVYRELEGRRTLRTEVDGRGYFV
          1  ...*..*...**.*.*.*..*..*.*.*.****.*****.*..*..**.

R1       50 KWHRGTTLKEIIKNLLSLRMPVLGADREWNAIHRLRDVGVDTMYGVAFGE
R2       50 KWHKGTTLKEIIKNLLSLRMPVLGADREWHAIHRLHDVGVDTMHGIGFGE
R3       50 KWHRGTTLKEIIKNLLSLRMPVLGADREWNAIHRLRDVGVDTMYGVAFGE
R4       50 KWHRGTTLKEIIKNLLSLRMPVLGADREWNAIHRLRDVGVDTMYGVAFGE
K12      50 KWHKGTTLKEIIKNLLSLRMPVLGADREWHAIHRLSDVGVDTMKGIGFGE
Se       50 KWHKGTSLKEIVKNLISLRMPVLGADREWHAIHRLHELGVDTMHGVGFGE
Pa       51 KIHRGIGWGEIAKNLLTAKLPVLGARQEWQAIRRLHEAGVATMTAVAYGE
         51 *.*.*....**.***.....*****..**.**.**...**.** ....**

R1      100 KGMNPLTRTSFIITEDLTPTISLEDYCADWATNPPDVRVKRMLIKRVATM
R2      100 KGLNPLTRTSFIITEDLTPTISLEDYCADWAVNPPDVHIKRMLIARVATM
R3      100 KGMNPLTRTSFIITEDLTPTISLEDYSADWATNPPDVRVKRMLIKRVATM
R4      100 KGINPLTRTSFIITEDLTPTISLEDYCADWATNPPDVRVKRMLIKRVATM
K12     100 KGLNPLTRASFIITEDLTPTISLEDYCADWAVNPPDIRVKRMLIARVATM
Se      100 KGVNPLTRTSFIITEDLTPTISLEDYCADWAVNPPDAQVKWMIIKRVATM
Pa      101 RGSDPARQHSFIVTEELAPTVDLEVFSQDWRERPPPPRLKRALVEAVARM
        101 .*..*....***.**.*.**..**....**. .**....*......**.*

R1      150 VRDMHAAGINHRDCYICHFLLHLPFSGKEEELKISVIDLHRAQLRTRVPR
R2      150 VRKMHAAGINHRDCYICHFLLHLPFTGREDELKISVIDLHRAQIRAKVPR
R3      150 VRDMHAAGINHRDCYICHFLLHLPFSGKEEELKISVIDLHRAQLRTRVPR
R4      150 VRDMHAAGINHRDCYICHFLLHLPFSGKEEELKISVIDLHRAQLRTRVPR
K12     150 VRKMHTAGINHRDCYICHFLLHLPFTGREDELKISVIDLHRAQIRAKVPR
Se      150 VRKMHAGGINHRDCYICHFLLHLPFTGREEDLKISVIDLHRAQIRQHVPL
Pa      151 VGDMHRAGVNHRDCYICHFLLHTDKPVSADDFRLSVIDLHRAQTRDATPK
        151 *..**..*.**********.....*********.* ..*.

R1      200 RWRDKDLIGLYFSSMNIGLTQRDIWRFMKVYFAAPLKDILKQEQGLLSQA
R2      200 RWRDKDLIGLYFSSMNIGLTQRDIWRFMKVYFGMPLRDIYRLEIDLLKKA
R3      200 RWRDKDLIGLYFSSMNIGLTQRDIWRFMKVYFAAPLKDILKQEQGLLSQA
R4      200 RWRDKDLIGLYFSSMNIGLTQRDIWRFMKVYFAAPLKDILKQEQGLLSQA
K12     200 RWRDKDLIGLYFSSMNIGLTQRDIWRFMKVYFGMPLRKILSLEQNLLNMA
Se      200 RWRDKDLIGLYFSSMNIGLTQRDIFRFMREYFSLPLREILQKESGLIHQA
Pa      201 RWRNKDLAALYFSALDIGLTRRDKLRFLRTYFRRPLREILRDEAGLLAWM
        201 ***.***..****...****.**..**...**. **..*.. *..*. ..

R1      250 EAKATKIRERTIRKSL--
R2      250 RIKAGKIEARTIRKNL--
R3      250 EAKATKIRERTIRKSL--
R4      250 EEKATKIRERTIRKSL--
K12     250 SVKAERIKERTQRKGL--
Se      250 DVKAARIKERTIRKNL--
Pa      251 ERQAEKLYERKQRYGDLL
        251 . .* ....*..*. .
```

Figure 3.14. Alignment of WaaP from *E. coli* to cAMP-dependent protein kinase (cAPK) from *Mus musculus* (mouse). Identical residues are indicated by single letter code, and similar residues are indicated by a "+". The WaaP and cAPK sequences show only 12% identity (23% similarity) over the length of the alignment, however the following list denotes potentially important similarities. 1) cAPK residues 50-55 contain the nucleotide-binding motif GXGXXG, of which Gly 52 (Gly 44 in WaaP) is an invariant residue among eukaryotic protein kinases. 2) cAPK residue Lys 72 (Lys 58 in WaaP) is an invariant residue, that forms a salt bridge with the invariant residue Glu 91 (Glu 70 in WaaP). The cAPK Lys 72 residue is also involved in hydrogen bonding to the α and β phosphates of ATP. 3) cAPK residues Glu 121 and Glu 127 (Glu 114 and Glu 123 in WaaP) are involved in hydrogen bonding to adenosine. 4) cAPK residue Asp 166 (Asp 162 in WaaP) is an invariant residue that potentially functions as the enzyme's catalytic base, and is shown in this study to be essential for WaaP activity. 5) cAPK residue Asp 184 (Asp 187 in WaaP) is an invariant residue required to orient the γ-phosphate of ATP for efficient transfer to the substrate. The potential roles of conserved residues in cAPK were derived from crystal structure data (Knighton *et al.*, 1991; Madhusudan *et al.*, 1994).

```
WaaP    42 MAGKSYFLKWHRGT------TLKEIIKNLLSLRMPVLGADREWNAIHRLRDVGVDTMYGV
             G    +   +          +K + K+ +     +     E    + L  V    +  +
cAPK    50 GTGSFGRVMLVKHKESGNHYAMKILDKQKVVKLKQIEHTLNEK---RILQAVNFPFLVKL

WaaP    96 AFGEKGMNPLTRTSFIITEDLTPTISLEDYCADWATNPPDVRVKRMLIKRVATMVRDMHA
            F  K          +++ E +      E +               R     +      +H
cAPK   107 EFSFKDN----SNLYMVMEYVAGG---EMFSHLRRIGRFSEPHARFYAAQIVLTFEYLHS

WaaP   156 AGINHRDCYICHFLLHLPFSGKEEELKISVIDLHRAQLRTRVPRRWRDKDLIGLYFSSMN
             +  RD      L+             I V D        RV  R          Y
cAPK   160 LDLIYRDLKPENLLIDQ-------QGYIQVTDFG---FAKRVKGRTWTLCGTPEYLAPEI
```

To test the biological significance of these sequence similarities, a nucleic acid change was engineered into the *waaP* open reading frame in plasmid pWQ912 that resulted in the replacement of Asp 162 with Ala (plasmid pWQ913). Asp 162 was chosen specifically because it is absolutely conserved among all the known WaaP homologs (within the sequence NHRDCYICHFLLH, see above), and because sequence similarity predicts that it functions as the WaaP catalytic base. In this role, the WaaP Asp 162 residue would accept a proton from the 4-OH of HepI, thus leaving an oxyanion capable of nucleophilic attack at the γ-phosphate of ATP. Recent data have questioned the proposed general-base role of this residue in eukaryotic protein kinases (Cole *et al.*, 1995), but there is no doubt that it is essential for catalysis. The replacement of Asp 162 with Ala would thus be expected to completely abrogate WaaP activity. When plasmid pWQ913 encoding WaaP (D162A) was introduced into our defined *waaP* mutant strain, CWG296, it was unable to complement the mutant's antibiotic and detergent supersensitivity (data not shown). Further, purified WaaP (D162A) showed negligible kinase activity in our *in vitro* assay system (data not shown), clearly indicating that Asp 162 is an essential residue.

One notable difference between the WaaP primary amino acid sequence and that of eukaryotic protein kinases is at residue 167 (see Figure 3.14): in WaaP this residue is an absolutely conserved His, while in eukaryotic kinases this residue is an absolutely conserved Asn that is involved in chelation of the Mg^{2+} ion that spans the α and γ phosphates of ATP. To test

whether the catalytic efficiency of WaaP might be improved by modifying the protein to more closely resemble its eukaryotic counterparts, a second nucleic acid change was engineered into the *waaP* open reading frame in plasmid pWQ912 that resulted in the replacement of His 167 with Asn (plasmid pJY30). When plasmid pJY30 encoding WaaP (H167N) was introduced into our defined *waaP* mutant strain, CWG296, it was able to complement the mutant's antibiotic and detergent supersensitivity somewhat, but not to wild-type levels (data not shown). Further, purified WaaP (H167N) showed reduced kinase activity in our *in vitro* assay system (approximately 5% of wild-type activity). Clearly, this absolutely conserved His residue within the invariant NHRDCYICHFLLH WaaP sequence is important for catalysis, but reflects important differences between WaaP structure and function and that of eukaryotic kinases.

Chapter 4. Discussion

4.1. Assignment of function to *waa* genes by insertion mutagenesis:

As discussed in Chapter 1, differences in the central *waaQ* operon of the core oligosaccharide biosynthesis loci of *E. coli* and *S. enterica* result in the production of at least seven distinct core structures (five for *E. coli* and two for *S. enterica*). Although the genetics of LPS core biosynthesis in *E. coli* K-12 and *S. enterica* sv. Typhimurium had been studied previously, definitive assignment of glycosyltransferase activity to specific gene products was often lacking. The insertion mutagenesis study of the R1 core biosynthesis region reported in Chapter 3 is the first example of a single system where all of the glycosyltransferases required for the assembly of the outer core have been unambiguously assigned (see section 3.1 Assignment of function to genes involved in outer core biosynthesis). In addition, several highly conserved genes, which previously had speculative or unknown activities, were assigned to functions in the biosynthesis of the inner core (see section 3.2 Assignment of function to genes involved in inner core biosynthesis). As a result of the work in Chapter 3, a function has now been ascribed to every gene in the central *waaQ* operon of the core biosynthesis region of the *E. coli* R1 prototype strain, F470 (see summary in Figure 4.1). Given that the overall organization of the R1 *waa* cluster is similar to those of *E. coli* K-12, R2, R3, R4, and *S. enterica* sv. Typhimurium, this framework provides a solid basis for the assignment of activity to transferases from other core types based on homology.

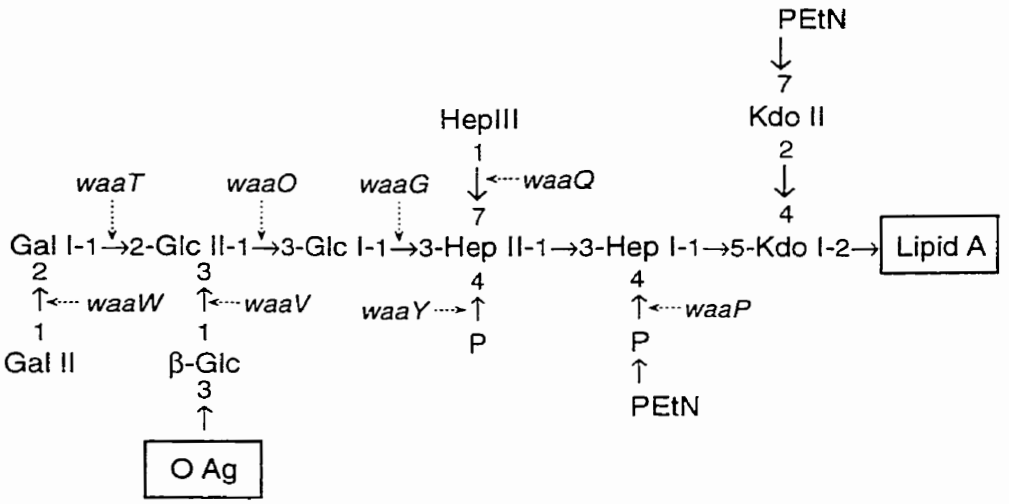


Figure 4.1. Summary of gene assignments from Chapter 3.

Specifically addressing the first research objective of this thesis, which was to identify unequivocally the genes involved in the phosphoryl modification of the inner core Hep residues, the data presented in Chapter 3 identify WaaY as the enzyme which phosphorylates HepII, and WaaP as the enzyme responsible for the addition of phosphate to HepI. Moreover, the deduced structures of the core oligosaccharides from the *waaY*, *waaQ*, and *waaP* mutants suggest a strict sequence of events in the decoration of the heptose region of the core: 1) WaaP adds phosphate to HepI; 2) WaaQ adds HepIII to HepII; and 3) WaaY adds phosphate to HepII.

The conclusion that the activity of WaaP is a prerequisite to the functioning of both WaaQ and WaaY is based on the fact that the core of CWG296 (*waaP*) does not have HepIII or a phosphate substituent on HepII even though functional copies of the *waaQ* and *waaY* genes are present on the chromosome. Likewise, the activity of WaaQ is concluded to be required prior to the functioning of WaaY because the core oligosaccharide of CWG297 (*waaQ*) does not have the phosphate substituent on HepII even though the *waaY* gene in this mutant is fully functional. These results can be explained by a fastidious substrate requirement in each sequential reaction, such that WaaQ can only effect the transfer of HepIII to a lipid A-core acceptor with phosphate on HepI, and WaaY can only phosphorylate HepII if the lipid A-core acceptor has both HepIII and a phosphoryl substituent on HepI. Alternatively, these data could reflect a requirement for specific protein-protein interactions to provide a functional multi-enzyme complex.

Two separate experiments could potentially resolve whether substrate specificity or protein-protein interactions are the cause of the strict sequence of core modifications catalyzed by WaaP, WaaQ, and WaaY. In one experiment, allelic exchange mutagenesis could be used to replace the *waaP* gene encoding the wild-type protein with one encoding WaaP (D162A). This point mutation eliminates WaaP function (see section 3.5.6 Site-directed mutagenesis of WaaP), but would allow for expression of otherwise normal, full-length WaaP to participate in any protein-protein interactions. Determination of the LPS core structure from this mutant would show whether WaaQ and WaaY could function in the absence of phosphate on HepI but in the presence of WaaP protein. The second experiment requires purification of WaaY and reconstitution of its enzymatic activity *in vitro*. If it can be demonstrated that pure WaaY can catalyze the incorporation of phosphate into acceptor LPS purified from a *waaY* mutant derivative, these same assay conditions could then be used to test whether WaaY is capable of incorporating phosphate into acceptor LPS purified from defined *waaQ* and *waaP* mutants. If pure WaaY can use LPS purified from a *waaY* mutant, but cannot use LPSs purified from the *waaQ* and *waaP* mutants, then the conclusion would be that substrate specificity is the main factor responsible for the strict sequence of core modifications.

Multiple sequence alignments of the predicted WaaP, WaaQ, and WaaY proteins from *E. coli* K12, R1, R2, R3, and R4, and *S. enterica* serovar Typhimurium also showed an interesting trend. As reported in Chapter 3, the

percent identity shared between the WaaP sequences is very high at ~75%, but then drops to ~64% and ~43% for the WaaQ and WaaY sequence alignments respectively. Given that the sites of their respective activities are now known, the observed decrease in sequence identity from WaaP to WaaQ to WaaY can be correlated with an increased interaction of the WaaQ and WaaY proteins with the more variable outer core. This is especially plausible for WaaY because of its involvement in the transfer of phosphate to HepII, which is immediately adjacent to the outer core. Alternatively, this decrease in sequence identity from WaaP to WaaQ to WaaY could reflect an increased interaction of the WaaQ and WaaY proteins with the different enzymes that assemble the different outer core structures.

In light of the data presented here, re-evaluation of previous LPS structural studies performed using mutants of *E. coli* K-12 (Parker *et al.*, 1992(a)) further confirms the assignment of WaaY and WaaP function. Parker and co-workers created a polar mutation in *E. coli* K-12 that eliminated function of all genes downstream of *waaQ* in the *waaQ* operon, then determined the core oligosaccharide structures (by tandem mass spectrometry) of the resulting mutant and of the mutant complemented with both *waaG* and *waaP* (Parker *et al.*, 1992(a)). As expected, the strain without a functional copy of the *waaG* and *waaP* genes was truncated after HepII and had no phosphoryl substituents or branch HepIII residue in the heptose region. Complementation of the mutant with a fragment carrying *waaG* and *waaP*, however, resulted in a core with GlcI and with both HepIII and

phosphate or PPEtN on HepI, but no phosphate on HepII. Again, these results are as expected given the absence of a functional *waaY* gene on the chromosome (due to the polarity of the mutation). Correct and complete interpretation of these previous data was not possible prior to the unequivocal gene assignments reported here.

The data presented in Chapter 3 also provide an explanation for the multiple functions attributed to *waaP* in the literature. For example, the suggested involvement of WaaP in the transfer of HepIII in *E. coli* K-12 (Parker *et al.*, 1992(a)) has now been resolved: WaaQ is the HepIII transferase, but requires the prior functioning of WaaP. It had previously been suggested, based solely on limited predicted protein sequence similarities, that the product of *waaQ* might encode the HepIII transferase (Schnaitman and Klena, 1993). This study provides the first genetic and structural data to support this claim. Another reported effect of *waaP* mutations in *S. enterica* serovars Minnesota (Mühlradt *et al.*, 1968) and Typhimurium (Helander *et al.*, 1989) is a truncation of the core distal to GlcI; however, the precisely defined, non-polar *waaP* mutation constructed in this study clearly shows that core extension is complete (although there is a slight reduction in the efficiency of branch β-Glc substitution). These earlier "*waaP*" mutants of *S. enterica* were obtained using chemical mutagenesis and phage selection, and are all reported to be leaky, with small amounts of phosphate still detectable in the core (Helander *et al.*, 1989). Therefore, the core truncation observed in these

previous "*waaP*" mutants of *S. enterica* is most likely explained as a polar effect that eliminates the function of genes downstream in the operon.

4.2. Interpretation of mutant phenotypes:

The presence of phosphoryl substituents in the inner core region of the LPS molecule is considered to be a key feature for the formation of a stable outer membrane in *E. coli* and *S. enterica* (reviewed in Heinrichs *et al.*, 1998(c); Nikaido and Vaara, 1985; Schnaitman and Klena, 1993; Sukupolvi and Vaara, 1989), as discussed in Chapter 1. These phosphoryl substituents are postulated to be critical to outer membrane integrity because their negative charge allows neighbouring LPS molecules to be crosslinked by divalent cations, such as Ca^{2+} or Mg^{2+} (Nikaido and Vaara, 1985; Vaara, 1992). Bridging of LPS molecules in this way imparts stability to the Gram-negative outer membrane by decreasing its fluidity, and helps to make it an effective permeability barrier. The contribution of core phosphoryl substituents to outer membrane stability is clearly shown by the novobiocin and SDS sensitivity testing of *waa* mutants defective in core phosphorylation, as reported in Chapter 3.

The lack of phosphate on HepII in CWG312 (*waaY*) and in CWG297(*waaQ*) results in a minor increase in the sensitivity of these strains to these hydrophobic agents, while the phenotype resulting from mutation of *waaG* (strain CWG303, with a complete lack of phosphate on HepII and only 40 percent phosphate substitution at HepI) is more dramatic than that observed for CWG312 and CWG297. However, the total absence of

phosphoryl substituents in the core oligosaccharide of CWG296 (*waaP*) results in the greatest increase in susceptibility to novobiocin (32-fold), and SDS (>1000-fold). The relationship between core phosphorylation and membrane stability thus shows a clear trend: the greater the decrease in core phosphorylation, the greater the decrease in outer membrane barrier function.

The observed phenotype of CWG303 (*waaG*) was initially somewhat perplexing until it was shown that the *waaG* mutation indirectly affected core phosphate substitution. In hindsight, the effect of the *waaG* mutation on core phosphorylation can be rationalized given the proximity of GlcI to the core heptose phosphorylation sites; the WaaG-catalyzed reaction might be a requirement for WaaP and WaaY substrate specificity. This suggestion fits well with the much earlier observation that the *in vitro* phosphorylation of LPS (using LPS from an undefined phosphate-deficient mutant and crude lysates from wild-type bacteria) proceeds much faster when GlcI is present in the LPS core acceptor (Mühlradt, 1971). It is still unknown why an 80% reduction in core phosphorylation should drastically affect the MIC of SDS, but not that of novobiocin.

Finally, the observation that CWG303 (*waaG*) and CWG296 (*waaP*) both show wild-type outer membrane protein profiles was somewhat surprising given results from previous studies (Helander *et al.*, 1989; Parker *et al.*, 1992(a)). These earlier studies concluded that all of the characteristics of the deep-rough phenotype (see section 1.4 Focus on the core oligosaccharide,

historical perspective and genetics), including a drastic reduction in outer membrane protein content, could be attributed to the loss of core phosphoryl substituents caused by mutation of *waaP* alone. Indeed, a proposed explanation for the increased susceptibility of deep-rough mutants to hydrophobic agents suggests that the void left by missing outer membrane proteins is filled by phospholipids, resulting in patches of phospholipid bilayer in the outer membrane through which these hydrophobic compounds can readily penetrate (Nikaido and Vaara, 1985). The discrepancy between the results presented in Chapter 3 and those of previous studies (Helander *et al.*, 1989; Parker *et al.*, 1992(a)) suggests that the mutants used in those earlier studies possibly carried additional uncharacterized mutations, or that the results of those studies were influenced by differences in genetic backgrounds. Given the unaltered outer membrane protein content, the susceptibility of CWG303 to SDS and the susceptibility of CWG296 to SDS and novobiocin cannot be explained simply by the loss of outer membrane proteins and their replacement by phospholipid bilayer patches in the outer membrane. However, these data do not preclude the appearance of phospholipid bilayer patches in the outer membrane as a direct consequence of LPS changes. To resolve this issue, the presence of phospholipid in the outer membrane could be tested directly by using a macromolecular labelling reagent such as dansyl chloride to label intact cells (any exposed ethanolamine head groups on phosphatidylethanolamine would be labelled) (Schindler and Teuber, 1978).

4.3. Virulence defect of a *waaP* mutant of *S. enterica*:

Based on the severe antibiotic and detergent sensitivity of *E. coli* CWG296 (*waaP*), it was hypothesized that this mutation would adversely affect virulence. Unfortunately, the original F470 parent of CWG296 is already an avirulent rough mutant (chosen as such to facilitate the structural chemistry required to properly define *waaP* function). Therefore, the *waaP* mutation was recreated in an invasive strain of *S. enterica* to test its effect on virulence, as reported in Chapter 3 (see section 3.4 *waaP* mutants of *S. enterica* are avirulent).

Strain CWG304 is the first *S. enterica waaP* mutant to be characterized both genetically and structurally. As discussed above, earlier "*waaP* mutants" of *S. enterica* serovar Typhimurium were obtained using chemical mutagenesis and phage selection, and are all reported to be leaky, with small amounts of phosphate still detectable in the core (Helander *et al.*, 1989). CWG304, however, is known to be non-leaky because of the nature of the mutation, and the complete lack of phosphate in its core region as observed by ^{31}P-NMR. As such, our *waaP* mutant of *S. enterica* refutes the earlier conclusion that such mutations in *S. enterica* must be leaky to maintain viability (Helander *et al.*, 1989). Further, the *waaP::aacC1* mutation could be transduced by P22 into a clean *S. enterica* serovar Typhimurium background, giving a strain with the same phenotype (performed by Dr. J. S. Gunn, data not shown). This argues against the possibility of unlinked, secondary, compensating mutations. Strain CWG304 therefore represents a valuable tool

for dissection of the various factors involved in antibiotic sensitivities (i.e. changes in core phosphorylation versus core truncation and the presence/absence of O antigen). Interestingly, our *waaP* mutant strain shows a slightly decreased efficiency of core completion and capping with O antigen, but this difference is not enough to cause any increase in susceptibility to complement-mediated serum killing. There is no obvious change in maximal O-chain length or modal distribution of O antigen. Therefore, the *waaP* mutant is essentially a smooth strain that expresses the antibiotic susceptibility characteristic of the deep-rough phenotype, clearly demonstrating that it is lack of core phosphate and not core truncation that causes this trait.

The effect of various LPS core defects on antibiotic susceptibilities in *S. enterica* has been examined previously (Roantree *et al.*, 1977), but the genes mutated were not precisely defined. Therefore, it is difficult to compare our results directly with these earlier studies. However, *S. enterica* mutants with highly truncated cores were previously shown to be more susceptible to numerous antibiotics, including polymyxin (Roantree *et al.*, 1977). In light of the findings reported here, it is concluded that LPS core phosphate residues are an essential element to the outer membrane barrier function of *S. enterica*, and in particular to polymyxin resistance.

The interaction between cationic antimicrobial peptides and the Gram-negative outer membrane is complex (reviewed in Hancock and Chapple, 1999; Nikaido and Vaara, 1985; Vaara, 1992). Indeed, there appear to be distinct

modifications required for resistance to different cationic antimicrobial compounds (Gunn and Miller, 1996; Guo *et al.*, 1998). The increased polymyxin susceptibility of *S. enterica waaP* mutants brings into question the current dogma that polymyxin resistance is mediated by LPS charge modulation alone. It appears instead, at least in the case of a *waaP* mutation, that detrimental hydrophobic interactions between polymyxin and the outer membrane can outweigh the benefits associated with a decrease in LPS net negative charge. This does not mean that electrostatic interactions are not important. It has been proposed, for example, that polymyxin functions by a detergent-like mechanism, requiring numerous polymyxin molecules to aggregate into clusters at the outer membrane surface (Wiese *et al.*, 1998). Clearly, electrostatic interactions would favour the accumulation of polymyxin molecules at the outer membrane surface, and thus facilitate the formation of such clusters. However, our studies and the work of others (Srimal *et al.*, 1996) suggest that hydrophobic interactions may play a critical role in the polymyxin-LPS interaction.

Our data must be interpreted with caution, however, because there is evidence that other defects (not related to LPS structure) in the outer membrane of deep-rough mutants might influence the membrane barrier function in unexpected ways. For example, there are regions of phospholipid bilayer in the outer membranes of deep-rough mutants (Kamio and Nikaido, 1976), although the extent of these regions is not known. One hypothesis proposed by Nikaido and Vaara (Nikaido and Vaara, 1985) suggests that these

regions of phospholipid bilayer are responsible for the increased susceptibility of deep-rough mutants to hydrophobic agents. Therefore, the increased polymyxin sensitivity of our *S. enterica waaP* mutant might be the result of polymyxin simply passing through phospholipid enriched domains in the outer membrane, and not interacting with LPS at all. However, the surface area occupied by these phospholipid domains is estimated to be small (Nikaido and Vaara, 1985), and given the fact that a PmrA-constitutive strain can still upregulate polymyxin resistance despite carrying *waaP* mutation (compare CWG304 and JSG778 in Table 3.5), this is likely not a complete explanation.

Finally, regardless of the exact mechanism by which mutation of *waaP* affects the outer membrane, it is clear that such a mutation leads to avirulence *in vivo*. Loss of virulence was demonstrated by both the survival of C57BL/6J and BALB/c mice, and the decrease in CFU in the spleens and livers of infected A/J mice (see Table 3.6). In addition, the virulence defect was shown for intravenous, intraperitoneal, and oral administration of the inoculum. Given the greatly compromised barrier function of the mutant outer membrane, and its avirulence in our mouse infection model, we believe that the *waaP* gene product represents a valid potential target for the development of novel therapeutics. Interestingly, a WaaP homolog was recently identified in *P. aeruginosa*, an important opportunistic pathogen in the lungs of individuals infected with cystic fibrosis. The initial identification of WaaP in *P. aeruginosa* was made based on homology (>60% similarity) to

WaaP from *E. coli* and *S. enterica*. Of particular note, all attempts at generating chromosomal *waaP* mutants of *P. aeruginosa* proved unsuccessful unless a wild-type copy of the gene was provided *in trans*, suggesting that WaaP is essential for the viability of *P. aeruginosa* (Walsh *et al.*, 2000). Consistent with this observation, *P. aeruginosa* mutants lacking inner core Hep or phosphate have never been isolated (Walsh *et al.*, 2000). Therefore, an inhibitor targeted against WaaP would likely be active against a range of bacteria extending beyond members of the Enterobacteriaceae.

Another potential therapeutic application arising from this research is the possibility of using *waaP* mutants of *S. enterica* (such as CWG304) as live vaccine delivery strains. Strains of *Salmonella* are particularly attractive live-vaccine candidates for two reasons: 1) they can be delivered orally, and 2) they can be used as efficient carriers of heterologous antigens (reviewed in Everest *et al.*, 1995). Such live vaccines have already been attempted for use in protecting both humans (Hohmann *et al.*, 1996; Hone *et al.*, 1992) and agriculturally important livestock such as chickens (Cooper *et al.*, 1990), calves (Jones *et al.*, 1991; Smith *et al.*, 1984), swine (Roof and Doitchinoff, 1995), and sheep (Brennan *et al.*, 1994). Given that *waaP* mutants of *S. enterica* are avirulent (see above), mutation of *waaP* alone, or in conjunction with a second attenuating mutation, could provide a useful strain for live vaccine delivery.

4.4. *In vitro* phosphorylation of LPS with purified WaaP:

The second major research objective of this thesis was to better characterize key proteins involved in core phosphorylation. Phosphorylation of the LPS core region is essential for outer membrane stability in *E. coli* and *S. enterica*, and has been studied over the course of many years (reviewed in Heinrichs *et al.*, 1998(c); Mäkelä and Stocker, 1984; Schnaitman and Klena, 1993). Indeed, the activity of an LPS core phosphorylating enzyme was reported as early as 1969, using acceptor LPS from an undefined phosphate-deficient mutant of *S. enterica* and crude lysates from wild-type bacteria as the enzyme source (Mühlradt, 1969). Unfortunately, the kinase implied by these early studies was never actually identified, nor was it confirmed that ATP was the direct donor of phosphate. More recently, a single gene called *rfaP* (now *waaP*) was implicated in all the phosphoryl modifications of the *E. coli* LPS core (Parker *et al.*, 1992(a)). However, the conclusions from these studies were necessarily limited by their reliance on strains with polar mutations and also in hindsight by the strict sequential action of the two core kinases, WaaP and WaaY. The direct involvement of the *waaP* gene product in the phosphorylation of the *E. coli* LPS core at HepI was only established with confidence based on work presented in this thesis.

The data presented in Chapter 3 provide the first biochemical characterization of WaaP activity, and prove unequivocally that WaaP is indeed an LPS core heptose kinase. Purified protein was used to determine the optimal assay conditions and catalytic properties of WaaP. Optimal

activity was observed between pH 8.0 and 9.0, and like most kinases, the enzyme displayed an absolute requirement for Mg^{2+}. The apparent K_M values for the WaaP-catalyzed reaction were calculated to be 0.13 mM for ATP, and 76 μM for LPS (determined at saturating concentrations of the second substrate). The turnover number calculated for WaaP under these reaction conditions (k_{cat} = 0.13/min) is clearly too low to reflect any real biological situation given that, for *E. coli*, the number of LPS molecules per cell is estimated to be about 2×10^6 (Raetz, 1996) and its doubling time is less than 30 minutes under optimal growth conditions. The low value for k_{cat} may indicate that the protein is not fully active upon purification, or alternatively may signify a need to further optimize the assay system. In this study, the site of WaaP phosphorylation was only localized to the soluble core oligosaccharide portion of the LPS that was released by mild acid hydrolysis. However, given the known structure of the *E. coli* core (Holst and Brade, 1992; Vinogradov *et al.*, 1999) and the core structures resulting from defined *waaY* and *waaP* mutations (loss of phosphate on HepII and loss of phosphate on both HepII and HepI, respectively) (Yethon *et al.*, 1998), it is possible to conclude that WaaP is in fact a heptose-specific LPS core kinase, and is responsible for phosphorylation at O-4 of HepI. Further, the WaaP enzyme is known to catalyze the addition of phosphate to only HepI (and not HepII), since LPS purified from a defined *waaY* mutant strain (with phosphate on HepI but no phosphate on HepII) could not serve as an acceptor for the WaaP enzyme (data not shown).

These data thus help to clarify the sequence of events in core biosynthesis that result in the observed heterogeneity of phosphoryl modifications on HepI. It was previously speculated that all LPS molecules might initially be modified by the addition of PPEtN to HepI, with a subset of these molecules subsequently undergoing cleavage to liberate PEtN and leave just phosphate (Schnaitman and Klena, 1993). Now, however, with the assignment of WaaP as the HepI kinase, the more likely scenario is clearly that WaaP catalyzes the transfer of phosphate to HepI with a subset of molecules being further substituted at this phosphate residue with PEtN by a currently unidentified enzyme. The determination of the gene responsible for this PEtN modification is of particular interest, given the potential role of PEtN core modification in resistance to polymyxin and modulation of surface charge as discussed in Chapter 1 (Helander *et al.*, 1994; Nummila *et al.*, 1995).

Given the importance of WaaP to membrane stability (Yethon *et al.*, 1998) and its requirement for virulence *in vivo* (Yethon *et al.*, 2000(a)), inhibitors to the WaaP-catalyzed reaction could potentially function as novel antimicrobial compounds. As a starting point for the design of inhibitors, it would be useful to know the minimal structure that is capable of being phosphorylated by WaaP. The finding that WaaP is capable of phosphorylating both the high and low molecular weight LPS species isolated from a *waaP* mutant strain helps to better define this minimal acceptor; apparently the outer core glycoses distal to GlcI are not strictly required for activity (although they may affect efficiency, refer back to Figure 3.10). This

finding is supported by the observation that LPS isolated from a defined *waaG* mutant strain (truncated after HepII) carries the phosphate modification on HepI, albeit at only 40 percent of wild-type levels (see section 3.3.1 Novobiocin and SDS sensitivity testing). As for the structure of the minimal acceptor on the lipid A-proximal side of HepI, preliminary experiments using a synthetic acceptor consisting of a disaccharide of α1,3-linked Hep residues (mimicking the structure found in the inner core) conjugated to fluorescein showed no activity in the WaaP kinase assay (data not shown). These data suggest that at least KdoI (and possibly a more complex structure involving the glucosamine backbone of lipid A) is also required for recognition and phosphorylation by WaaP.

Finally, it is noteworthy that WaaP was never assigned putative kinase function based on sequence homology alone, even though the sequence of the *E. coli waaP* gene has been known for some time (Parker *et al.*, 1992(b)), and homologs have since been identified in *S. enterica* (Klena *et al.*, 1993) and *P. aeruginosa* (Walsh *et al.*, 2000). The complicating factor has always been that the primary sequences of these predicted proteins bear such limited semblance to characterized kinases that similarities cannot be detected with typical BLAST searches (Altschul *et al.*, 1990). However, with the advent of the PSI-BLAST search tool (Altschul *et al.*, 1997) it was observed that WaaP did show homology to kinases when considering only key residues throughout the length of the entire protein (see section 3.2.3 Assignment of *waaP* gene function). We tested the biological significance of one of these key

similarities by specifically mutating the WaaP Asp 162 residue, predicted by homology to function as the enzyme's catalytic base. As expected for mutation of a residue essential to catalysis, the WaaP (D162A) protein showed no activity *in vivo* or *in vitro*. It is suggested therefore, given this conservation of catalytic residues, that WaaP may have a similar mechanism of action to other eukaryotic protein kinases, although given results with a second WaaP derivative (H167N) there are clearly some important differences. Interestingly, the crystal structure of the APH(3')-IIIa aminoglycoside kinase from *Enterococcus*, which catalyzes the phosphorylation of a broad spectrum of aminoglycoside antibiotics, was recently solved (Hon *et al.*, 1997). Like WaaP, the APH(3')-IIIa protein displays virtually no sequence similarity to eukaryotic kinases except at key residues, such as the conserved Asp mutated in this study. Yet despite this lack of sequence homology, the APH(3')-IIIa three-dimensional structure is strikingly similar to that of eukaryotic protein kinases (Hon *et al.*, 1997). Future determination of the crystal structure of WaaP could give important insights into the functioning of this novel enzyme and provide important leads for inhibitor design.

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