THE FUNCTIONAL ROLE OF LIPOPOLYSACCHARIDE IN THE CELL
ENVELOPE AND SURFACE PROTEINS OF PSEUDOMONAS AERUGINOSA

A Thesis
Presented to
The Faculty of Graduate Studies
of
The University of Guelph

by
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In partial fulfillment of requirements
for the degree of
Doctor of Philosophy
July, 2004

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ABSTRACT

THE FUNCTIONAL ROLE OF LIPOPOLYSACCHARIDE IN THE CELL ENVELOPE AND SURFACE PROTEINS OF PSEUDOMONAS AERUGINOSA

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University of Guelph, 2004

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Pseudomonas aeruginosa cell-surface structures that have been identified to be important during the initial stages of colonization of cystic fibrosis airways include pili, flagella, and lipopolysaccharide (LPS). The major goal of this research is to use a multidisciplinary approach to investigate the relationship between the biosynthesis of LPS and glycosylation of pili and flagella in P. aeruginosa, and to further define the role of LPS in bacterial interactions with epithelial cells. The interplay between biosynthesis of LPS and proteoglycans was investigated by examining the glycosylation of pilin and flagellin in LPS mutants. Mutations were constructed in wbpM and wbpL in strain 1244. The wbpM mutant was deficient in B-band LPS whereas the wbpL mutant did not produce A- or B-band LPS. Pilin isolated from both mutants was nonglycosylated and complementation of the mutants with their respective genes restored both the wild-type LPS phenotype and glycosylation of pilin. Analysis of flagellins isolated from wild-type PAK, a wbpO and rmlC mutant indicated that the wbpO mutant produced flagellin with one covalently attached deoxyhexose, whereas flagellin from the wild type is glycosylated with sugar residues with a mass of ca. 2540 Da. Since wbpO is required for synthesis of UDP-N-acetylglucosaminuronic acid (UDP-GlcNAcA), the data obtained
suggested that UDP-GlcNAcA or a UDP-GlcNAcA-derivative is directly attached to the 
deoxyhexose residue. Biosynthesis of the LPS core-oligosaccharide was investigated and 
characterization of *wapH*, which encodes a glucosyltransferase, is described. Analysis of 
the PAO1 *wapH* mutant revealed that *wapH* is critical for assembly of the core 
oligosaccharide that is capped with long-chain polysaccharides, as well as the core 
oligosaccharide glycoform that is unsubstituted. LPS mutants, including the *wapH* 
mutant, were assessed for adhesion and internalization within bronchial epithelial cells. 
Evidence was obtained to show that A-band PS and outer-core oligosaccharide play a 
role in the ability of this microbe to attach to epithelial cells. The terminal glucose and 
rhamnose of the unsubstituted outer-core oligosaccharide region are not required for 
maximal internalization of *P. aeruginosa*. These findings will have a profound impact on 
understanding the function of carbohydrate-based interactions between pathogen and host 
during *P. aeruginosa* infections.
ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Joseph Lam for giving me the opportunity to conduct research in his laboratory. I am very grateful for his guidance and the opportunities to attend various conferences over the years. I would like to thank my advisory committee members Dr. Chris Whitfield and Dr. Dev Mangroo for all of their assistance during my studies. I would also like to thank the members of my examination committee, Dr. Herbert Schweizer, Dr. Andrew Preston, Dr. Chris Whitfield, and Dr. Dev Mangroo for their comments and constructive criticisms. All of these individuals have taught me the love of learning, the value of networking, and the profound impact of being able to answer one simple but important question, "So What?"

I would like to thank Dr. Lori Burrows, Dr. Carole Creuzenet and Dr. Karen Poon for all their guidance and mentorship. Thanks to Dianne Moyles, our transmission electron microscope specialist and to Dr. Dyanne Brewer at the Biological Mass Spectrometry Facility. Thank you Rohan Van Twest for teaching me the ways of the microbial world. I am very grateful to all members of the Lam lab for their support and assistance, as well as my fellow colleagues within the Microbiology Department.

I would also like to thank my parents, Joseph and Mary Matewish, for their love and encouragement. Thank you to my husband James Vanderleeuw for his love and patience. Finally, I am extremely grateful to the Canadian Cystic Fibrosis Foundation for funding this research and for my studentship.
STATEMENT OF CONTRIBUTION

Matrix-Assisted Laser Desorption Ionization Time-of-Flight and electrospray mass spectrometry of the intact flagellin proteins and flagellin trypsin digests were performed by Dr. Dyanne Brewer at the Biological Mass Spectrometry Facility (Department of Molecular Biology and Genetics, University of Guelph, Guelph, ON). Electron microscopy of P. aeruginosa and flagellin were performed by Dianne Moyles (Microbiology Department, University of Guelph). Technical assistance in construction of the PAO1 wapH mutant and trypsin digestion of the flagellin proteins were provided by Robert Urbanic and Hamed Ghanei according to experiments designed by the author.
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LIST OF ABBREVIATIONS

*aacC1* - gene encoding *N*-aminoglycoside acetyltransferase, gentamicin resistance cassette

Ala - alanine

ACP - acyl carrier protein

AMP - adenosine 5'-monophosphate

ATP - adenosine 5'-triphosphate

bp - basepair

CAMPs - cationic antimicrobial peptides

CF - cystic fibrosis

CFTR - cystic fibrosis transmembrane regulator protein

CFU - colony forming units

CMP - cytidine 5'-monophosphate

CONH₂ - carbamoyl

CTP - cytidine 5'-triphosphate

Da - dalton

DNA - deoxyribonucleic acid

dTDP - deoxythymidine diphosphate

ELISA - enzyme-linked immunosorbent assay

FucNAc - *N*-acetylfucosamine (Fuc2NAc; 2-acetamido-2,6-dideoxy-D-galactose)

g - gram

GalN - D-galactosamine

GalNAc - *N*-acytylegalactosamine (Gal2NAc; 2-acetamido-2-deoxy-D-galactose)
GalNAcA - N-acetylgalactosaminuronic acid (Gal2NAcA; 2-acetamido-2-deoxy-D-galacturonic acid)

GDP - guanosine 5'-diphosphate

Glc - D-glucose

GlcN - D-glucosamine

GlcNAc - N-acetylglucosamine (Glc2NAc; 2-acetamido-2-deoxy-D-glucose)

GlcNAcA - N-acetylglucosaminuronic acid (Glc2NAcA; 2-acetamido-2-deoxy-D-glucuronic acid)

h - hour

Hep - heptose

HUVECS - human umbilical vein endothelial cells

IATS - International Antigenic Typing Scheme

IL-8 - interleukin 8

IPTG - isopropyl-β-D-thiogalactoside

kb - kilobase

kDa - kilodalton

Kdo - 3-deoxy-D-manno-octulosonic acid

L - litre

LOS - lipooligosaccharide

LPS - lipopolysaccharide

MALDI - matrix assisted laser desorption ionization

Man - mannose

ml - millilitre
mM - millimole/litre
MEM - Minimal essential media
min - minutes
mAb - monoclonal antibodies
MS - mass spectrometry
NAD - nicotinamide adenine dinucleotide
OH - hydroxyl
O-PS - O-antigen polysaccharide
ORF - open reading frame
OS - oligosaccharide
P - phosphate
PBS - phosphate buffered saline
PCR - polymerase chain reaction
PGM - phosphoglucomutase
pI - isoelectric point
PMM - phosphomannomutase
PS - polysaccharide
PseudoCAP - Pseudomonas aeruginosa Community Annotation Project
psi - pounds per square inch
Rha - rhamnose
SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis
rpm - revolutions per minute
TBS - tris-buffered saline
TOF - time-of-flight
Und - undecaprenol
μg - microgram
μM - micromole/litre
UDP - uridine 5′-diphosphate
UTP - uridine 5′-triphosphate
QuiNAc - N-acetylquinovosamine (Qui2Nac; 2-acetamido-2,6-dideoxy-D-glucose)
Xyl - xylose
Chapter 1. Introduction

1.1 Pseudomonas aeruginosa – microbiology and medical significance

*Pseudomonas aeruginosa* is a gram-negative, nonsporulating, rod-shaped bacterium with a size of 0.3-0.5 μm in width and 1-2 μm in length. The secretion of two water-soluble pigments, namely the fluorescent-green pigment pyoverdin and the blue-green pyocyanin (which is the origin of the name *aeruginosa*-full of copper rust or verdigris, hence green) are diagnostic for identifying this bacterium from cultures. While the optimal growth temperature of this species is 37°C, it can thrive in a broad range of temperatures between 10°C and 40°C. Although *P. aeruginosa* is classified as an obligate aerobe with a respiratory type of metabolism, growth can occur anaerobically using nitrate as an alternative electron acceptor. A remarkable feature of *P. aeruginosa* is its nutritional versatility. This organism is capable of utilizing over 76 different organic compounds as its sole source of carbon and energy (Palleroni, 1984).

This bacterium is ubiquitous in the environment and can be isolated from most natural habitats including water, soil, plants, and sewage. It is often found in the hospital setting including respiratory equipment, cleaning solutions, medicines, disinfectants, sinks, and floors, and accounts for about 10% of human nosocomial infections (Pollack, 1995; Kluytmans, 1997; Wiblin, 1997). Differentiation of clinical isolates is important for epidemiological studies and is facilitated by numerous typing schemes, including biotyping, antibiogram analysis, O-antigen serotyping, pyocin typing, and bacteriophage typing (Rhame, 1980). These phenotyping methods are being replaced with more accurate and sensitive genotyping/DNA fingerprinting methods that include pulsed-field gel electrophoresis (Fanci et al., 2003), ribotyping (Denamur et al., 1991), and PCR-
based techniques such as amplified fragment length polymorphism analysis (AFLP) (De Vos et al., 1993) and random amplified polymorphic DNA analysis (RAPD) (Mahenthiralingam et al., 1996).

_Pseudomonas aeruginosa_ is an opportunistic pathogen and generally requires a defect or alteration in the normal host defenses to establish an infection. Injury, trauma, surgery, burns, or indwelling devices such as intravenous lines or urinary catheters make the host susceptible to _P. aeruginosa_ colonization. In humans, this pathogen can cause life-threatening infections in individuals who are immunocompromised, and those suffering from burn wounds, cancer, cystic fibrosis, and AIDS. The range of diseases includes pneumonia, bacteremia, and infections of the urinary tract and surgical sites (Pollack, 1995; Lyczak et al., 2000). _P. aeruginosa_ is an important nosocomial pathogen. The National Nosocomial Infection Surveillance (NNIS) system of the Centers for Disease Control and Prevention (CDC) collected surveillance data between 1986 and 1998 and reported that _P. aeruginosa_ was the second most common cause of nosocomial pneumonia (14% of isolates), the third most common cause of urinary tract infections (7%), the fourth most common cause of surgical site infections (8%), the seventh most frequently isolated pathogen from the bloodstream (2%), and the fifth most common isolate (9%) overall from all sites (NNIS, 1998).

One disease in which _P. aeruginosa_ is the leading cause of death is cystic fibrosis (CF). CF is an autosomal recessive disorder that results from mutations in the gene that encodes the cystic fibrosis transmembrane conductance regulator protein (CFTR). CFTR is expressed in the apical membrane of selected epithelial cells and functions as a cyclic AMP-regulated chloride ion channel (Collins, 1992) and has been proposed to transport
water, urea, formate, adenosine triphosphate, glutathione, and organic anions, as well as influencing a large number of other cellular functions (reviewed in Kunzelmann, 1999; Widdicombe, 2000; Greger et al., 2001). Expression of abnormal CFTR or no CFTR in the plasma membrane of epithelial cells of the respiratory tract of CF patients results in various physiological changes in their lung environment including impaired host defenses. The major cause of death in CF patients is the progressive deterioration of respiratory function caused by chronic infections with *P. aeruginosa* and concomitant inflammation. Although intense research has revealed key aspects of *P. aeruginosa*-CF host interactions (reviewed in Lyczak et al., 2002), why the CF lung is hypersusceptible to *P. aeruginosa* infection is still not well understood.

The ability of *P. aeruginosa* to cause a wide variety of diseases can be attributed to three main factors: (i) its physiological adaptability and ubiquitous nature, (ii) its intrinsic resistance to antimicrobial drugs; a result of the overall decrease in outer-membrane permeability (Hancock and Brinkman, 2002) combined with multidrug efflux pumps (Poole and Srikumar, 2001), and (iii) its large number of virulence factors (Table 1.1). The pathogenesis of *P. aeruginosa* infections can be divided into three distinct stages: bacterial colonization, local invasion, and dissemination followed by systemic disease (Van Delden and Iglewski, 1998). Colonization of damaged epithelium is the first step to establishing an infection. The first line of host defense against bacterial invasion is the skin and mucous membranes and when damaged by injury or, for example, compromised by the insertion of a urinary catheter or intravenous line, bacteria attach to the damaged epithelial cells by cell-associated factors such as pili, flagella and
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<td>Tang et al. (1996)</td>
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<td></td>
<td></td>
<td>Feldman et al. (1998)</td>
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<td></td>
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<td>Scharfman et al. (2001)</td>
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<tr>
<td>Lipopolysaccharide</td>
<td>Colonization of tissues, Adherence and invasion of epithelial cells</td>
<td>Cryz et al. (1984)</td>
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<td></td>
<td></td>
<td>Tang et al. (1996)</td>
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<td></td>
<td></td>
<td>Pier et al. (1996a)</td>
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<td></td>
<td></td>
<td>Zaidi et al. (1999)</td>
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<tr>
<td>O antigen</td>
<td>Serum resistance, resistance to phagocytosis</td>
<td>Dasgupta et al. (1994)</td>
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<td></td>
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<td>Engels et al. (1985)</td>
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<td></td>
<td></td>
<td>Hancock et al. (1983)</td>
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<tr>
<td>Lipid A</td>
<td>Endotoxic properties</td>
<td>reviewed in Alexander and Retschel (2001)</td>
</tr>
<tr>
<td>Alginate/biofilm</td>
<td>Antiphagocytic, adherence to epithelium, antibiotic resistance</td>
<td>reviewed in Govan and Deretic (1996)</td>
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<tr>
<td></td>
<td></td>
<td>Costerton et al. (1999)</td>
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<tr>
<td><strong>Extracellular products</strong></td>
<td></td>
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<tr>
<td>Exotoxin A</td>
<td>Cytotoxicity, tissue invasion, cellular damage</td>
<td>Komatsu et al. (1998)</td>
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<tr>
<td></td>
<td></td>
<td>Plotkowski et al. (2002)</td>
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<td></td>
<td></td>
<td>Pillar and Hobden (2002)</td>
</tr>
<tr>
<td>Proteases:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i) LasB and LasA elastase</td>
<td>Damage of lung tissue, blood vessels</td>
<td>reviewed in Galloway (1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Komori et al. (2001)</td>
</tr>
<tr>
<td>ii) Alkaline protease</td>
<td>Tissue damage, increased vascular permeability, disruption of respiratory cilia</td>
<td>Holder and Haidaris (1979)</td>
</tr>
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<td></td>
<td></td>
<td>Hingley et al. (1986)</td>
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<td>Molla et al. (1989)</td>
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</tbody>
</table>
Table 1.1 Summary of *P. aeruginosa* virulence factors, continued

<table>
<thead>
<tr>
<th>Virulence factor</th>
<th>Proposed or demonstrated biologic effects</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>Type III secretion system:</td>
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<td></td>
<td></td>
<td>Sundin et al. (2001)</td>
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<td></td>
<td></td>
<td>Sato et al. (2003)</td>
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<tr>
<td>Extracellular products</td>
<td></td>
<td></td>
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<tr>
<td>Rhamnolipid</td>
<td>Ciliostatic, impairs mucociliary clearance</td>
<td>Stutts et al. (1986)</td>
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<td></td>
<td>Read et al. (1992)</td>
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<tr>
<td>Phospholipase C</td>
<td>Destruction of pulmonary surfactant, dissemination within host tissues, cell injury</td>
<td>Coutinho et al. (1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Meyers et al. (1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wiener-Kronish et al. (1993)</td>
</tr>
<tr>
<td>Siderophores (Pyochelin, Pyocyanin, Pyoverdin)</td>
<td>Enhances survival in low-iron environments</td>
<td>reviewed in Vasil and Ochsner (1999)</td>
</tr>
</tbody>
</table>
lipopolysaccharide (Woods et al., 1980; Pier, 1985; Doig et al., 1988; Fletcher et al., 1993; Gupta et al., 1994; Zaidi et al., 1996; Feldman et al., 1998).

After initial attachment and replication, local tissue invasion proceeds with the development of bacterial microcolonies or biofilms (Lam et al., 1980; Costerton et al., 1999; Hoiby et al., 2001). The infection process evolves into either an acute infection characterized by the production of high levels of extracellular virulence factors or to a chronic infection characterized by persistent infection accompanied by the production of low amounts of extracellular products (Van Delden and Iglewski, 1998; Smith and Iglewski, 2003). Van Delden and Iglewski (1998) propose that in an acute infection, the bacterial biofilm uses cell-to-cell signaling systems (quorum-sensing systems; las and rhl) to coordinate the mass production of high levels of extracellular virulence factors that overwhelm host defenses. These extracellular products include exotoxin A, LasA and LasB elastase, alkaline protease, rhamnolipids, and exoenzymes secreted by the type III secretion system. These products are primarily responsible for tissue-damaging effects and also impair host defenses. Many think the same extracellular products that are responsible for local tissue damage mediate bloodstream invasion and dissemination of P. aeruginosa from the local site of infection.

The versatile physiology and adaptability that make P. aeruginosa successful as a pathogen are in part reflected by its rather large genome. At 6.3 million base pairs (Pseudomonas Genome Project Strain PAO1; Stover et al., 2000; http://www.pseudomonas.com) it is the fourth largest among the 147 complete microbial genomes that have been sequenced to date (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/micr.html). The PAO1 genome contains
5,570 open reading frames (ORFs; Stover et al., 2000) and annotation efforts by the Pseudomonas Genome Project [http://www.pseudomonas.com; Stover et al., 2000] and the Pseudomonas aeruginosa Community Annotation Project [PseudoCAP; http://www.cmdr.ubc.ca/bobh/PAAP.html] have revealed that the PAO1 genome contains the highest proportion of regulatory genes observed for a bacterial genome. There are a very large number of genes involved in catabolism, transport and efflux of organic compounds as well as four potential chemotaxis systems. Shotgun sequencing of the genomes of three other P. aeruginosa strains, two from late-stage infections of cystic fibrosis patients and one from an aquatic environment, has also been accomplished (Spencer et al., 2003). Comparison of these sequence data with that from PAO1 reveals that most of the PAO1 genome represents a core backbone sequence while the genomes of the three isolates possess additional genetic material that accounts for at least 10% of their genomes. Strain-specific gene islands appear to be the primary mode of genetic variation among the different strains of P. aeruginosa (Spencer et al., 2003). These gene islands include PAGI-1 (for “P. aeruginosa genomic island 1”), which is proposed to play a role in evading the host immune response (Liang et al., 2001), a flagellar glycosylation gene island (Arora et al., 2001), and the recently described 11 groups of gene clusters at the O-antigen biosynthetic locus (Raymond et al., 2002).

1.2 Overview of Pseudomonas aeruginosa lipopolysaccharide

Lipopolysaccharide (LPS) of *P. aeruginosa* is a major constituent of the outer leaflet of the outer membrane of the Gram-negative bacterial cell wall. LPS is composed of three distinct regions: (i) lipid A, (ii) core oligosaccharide (OS), which can further be divided into inner and outer core, and (iii) the long chain O-antigen polysaccharides (PS). The hydrophobic lipid A anchors the LPS molecule in the outer membrane and lipid A is covalently attached to the core OS. *P. aeruginosa* produces two chemically distinct forms of long-chain PSs that are attached to the lipid A-core, an O-antigen PS and a common antigen PS and these molecules are called B-band and A-band LPS, respectively (Rocchetta et al., 1999). In the sections that follow I will first provide an overview of our current understanding of LPS biosynthesis in *P. aeruginosa*, and second, present the current hypotheses of how LPS is presumed to contribute to the pathogenicity of *P. aeruginosa* infections.

1.2.1 Lipid A biosynthesis

1.2.1.1 Structure of lipid A

The structure of lipid A in LPS of many different *P. aeruginosa* strains, including PAO1 and PAK, which are standard wild-type laboratory strains, has been determined by many groups (Bhat et al., 1990; Kulshin et al., 1991; Karunaratne et al., 1992; Ernst et al., 1999). The lipid A molecule consists of a β-1,6-linked D-glucosamine disaccharide [β-D-GlcN-(1→6)-D-GlcN]. Positions 4’ and 1 of the β-D-GlcN-(1→6)-D-GlcN disaccharide are phosphorylated and the attachment site of the core oligosaccharide is at position 6’. The diglucosamine backbone is substituted to varying degrees with fatty acid chains and
as a result, hexaacyl, pentaacyl and tetraacyl species are produced. The major lipid-A species that is synthesized, however, is the pentaacyl lipid A form that contains two amide-bound 3-O-acylated (R)-3-hydroxydodecanoic acid groups [12:0(3-OH)] at positions 2 and 2’ of the GlcN disaccharide and one ester-bound (R)-3-hydroxydecanoic acid group [10:0(3-OH)] at position 3’ (Fig. 1.1 A). The 3-hydroxy group of the two amide-linked 12:0(3-OH) residues are acylated by either dodecanoic (12:0) or (S)-2-hydroxydodecanoic acid [12:0(2-OH)]. This can lead to structural heterogeneity within the lipid-A molecule, and consequently, _P. aeruginosa_ has been found to synthesize the pentaacyl lipid-A form with three acylation patterns (Kulshin et al., 1991). One form of lipid A is synthesized with the two amide-linked 12:0(3-OH) residues acylated by two 12:0 groups. The other lipid A forms contain 12:0(2-OH) at GlcN I and 12:0 at GlcN II or with the reverse distribution, whereas lipid A carrying two 12:0(2-OH) residues at both GlcN I and GlcN II do not appear to be synthesized.

_Pseudomonas aeruginosa_ synthesizes different forms of lipid A in response to conditions encountered during different types of human infections. Ernst et al. (1999) identified several penta-acylated forms of lipid A from _P. aeruginosa_ isolated from CF individuals. One is the penta-acylated form that is similar in structure to those reported earlier by Bhat et al. (1990), Kulshin et al. (1991), and Karunaratne et al. (1992). Another lipid A form which has the same penta-acylated structure is modified by the addition of palmitate, whereas the other lipid A forms are characterized by the addition of palmitate as well as substitutions of aminoarabinose (Fig. 1.1 B). These lipid-A forms are also observed when _P. aeruginosa_ is grown under magnesium-limiting growth conditions.
Figure 1.1 Various structures of *P. aeruginosa* lipid A. (A) The predominant penta-acylated form of lipid A found in *P. aeruginosa* strains, including PAK and PAO1 when grown in high-magnesium conditions. (B) Modified lipid A found in LPS from *P. aeruginosa* CF-clinical isolates containing aminoarabinose (4-amino-4-deoxy-L-arabinose) at the 1 and 4′ phosphates and a palmitate at the 3-hydroxydecanoic acid, bound via an ester linkage to the 3′ carbon. (C) The non-CF dominant penta-acylated lipid A form found in blood and bronchiectasis isolates when grown in Luria-Bertani media. C16 refers to the 16-carbon chain length of the palmitate (hexadecanoic acid) substitution. The structures were illustrated based on data from Bhat et al. (1990), Kulshin et al. (1991), Karunaratne et al. (1992), and Ernst et al. (1999).
Ernst et al. (1999) reported that different structures of lipid A are synthesized in non-CF clinical isolates. The dominant penta-acylated lipid A form isolated from minimally passaged non-CF strains (isolated from sepsis and bronchiectasis) was markedly different in the fatty acid distribution than the dominant penta-acylated lipid A species isolated from PAK, PAO1 and the CF isolates (Fig. 1.1 C). Firstly, the dominant penta-acylated form of lipid A in the non-CF isolates contains 3-OH-C10:0 at the 3 position, whereas the lipid A from the CF isolates and PAK did not have this fatty acyl chain. Secondly, the non-CF lipid A lacked the 2-OH-C12:0 at the 2’ position; and thirdly, the addition of palmitate and aminoarabinose to this penta-acylated lipid A form was not observed in any of the non-CF clinical isolates (Ernst et al., 1999). Therefore, these studies demonstrate that different isolates of *P. aeruginosa* have the remarkable ability to synthesize a variety of lipid A structures in response to changes in its environment *in vivo*.

1.2.1.2. Genes of lipid-A biosynthesis

The molecular genetics and biochemistry of lipid A biosynthesis are best characterized in *E. coli* and have been thoroughly reviewed by Raetz and Whitfield (2002). In *P. aeruginosa*, many genes that are highly homologous to those involved in *Escherichia coli* lipid A biosynthesis have been identified. The gene products of *lpxA* and *lpxC* are required for the first two and most conserved steps and have been characterized at the biochemical level (Williamson et al., 1991; Hyland et al., 1997; Dotson et al., 1998; Wyckoff et al., 1998). The other genes thought to be involved in the remaining steps of lipid A biosynthesis in *P. aeruginosa* have been annotated and assigned putative functions based on homology of their encoded proteins with enzymes of *E. coli* lipid A.
biosynthesis (Pseudomonas aeruginosa Community Annotation Project-PseudoCAP; [http://www.pseudomonas.com/AnnotationListByFunction.asp?Function=Cell%20wall%20LPS%20capsule]; The Pseudomonas Genome Project; Stover et al., 2000). There are, however, two significant differences between synthesis of P. aeruginosa lipid A when compared to lipid A of E. coli. First, there are variations in the structure of P. aeruginosa lipid A in comparison to that of E. coli, which include differences in acyl chain length and distribution. These structural differences may account for the presence of unique enzymes in P. aeruginosa that have not yet been identified because they do not share significant homology with other characterized proteins. Second, the final steps in biosynthesis of lipid A differ between enteric and non-enteric bacteria. In enteric bacteria, once lipid IV$_A$ is made, the addition of two 3-deoxy-D-manno-octulosonic acid residues to lipid IV$_A$ precedes the addition of the fifth and sixth acyl chains (Brozek and Raetz, 1990). In P. aeruginosa, the lipid IV$_A$ is first acylated with two dodecanoic acid chains, which are O-linked to the acyl chains at the 2 and 2' positions before the addition of the 3-deoxy-D-manno-octulosonic acid residues (Goldman et al., 1988; Mohan and Raetz, 1994). Based on their homology to E. coli LpxL (Clementz et al., 1996), putative proteins encoded by PA0011 and PA3242 of the P. aeruginosa PAO1 genome have been annotated to have the function of lauroyltransferases that add the two acyl groups. To date, however, there is no experimental data to support these assignments.

A PhoP-PhoQ two-component regulatory system in P. aeruginosa described by Macfarlane et al. (1999) has been implicated in the regulation of genes required for addition of aminoarabinose and palmitate to lipid A in magnesium-starvation conditions and is supported by structural data obtained by Ernst et al. (1999). At present, the genes
presumably turned on by the PhoP-PhoQ system that mediate the transfer of palmitate to lipid A in *P. aeruginosa* have not been identified. Studies comparing the biophysical properties of the PhoQ sensors from *P. aeruginosa* and *E. coli* show that the PhoQ proteins from these two organisms differ in their structural response to divalent cations. PhoQ from *P. aeruginosa* may recognize additional signals or respond differently than *E. coli* and presumably *S. enterica* PhoQ in certain environments (Lesley and Waldburger, 2001). Furthermore, the distinct mechanisms of signal detection between *P. aeruginosa* and *E. coli* suggest that lipid A modifications in these two organisms may be regulated differently.

1.2.2 Core Oligosaccharide

1.2.2.1 Structure of the core oligosaccharides

*Pseudomonas aeruginosa* produces two distinct core OS glycoforms. One of these core glycoforms is the acceptor molecule for the covalent attachment of the long chain A- or B-band PS while the other core form remains uncapped (Fig. 1.2). The two core glycoforms differ in the outer-core region whereas the inner core remains conserved. The inner core is composed of two residues of 3-deoxy-D-manno-octulosonic acid (Kdo) and two residues of L-glycero-D-manno-heptose (L,D-Hep). The inner core also contains a unique O-carbamoyl substitution at C-7 of the L,D-Hep residue located proximal to the outer core (Beckmann et al., 1995). The substitution of a sugar by a carbamoyl residue is unique in bacterial LPS and the only other report of a carbamoylated sugar is the terminal N-acylated and N-methylated glucosamine residue of the Nod factor produced by *Azorhizobium caulinodans* (Mergaert et al., 1993). 7-O-carbamoyl-L,D-Hep has also been identified in the LPS of other *P. aeruginosa* strains including rough mutants.
Figure 1.2 Structures of the core oligosaccharides from *P. aeruginosa* strain PAO1. Two distinct core oligosaccharides have been elucidated. One of them is devoid of O antigen and is referred to as “uncapped” core oligosaccharide (A). Note the L-rhamnose in a α-1,6 linkage to an α-D-glucose in the main chain. The other core oligosaccharide contains O antigen linked to L-rhamnose in an α-1,3 linkage to β-D-glucose in the branch chain (B). Glc, glucose; Rha, rhamnose; GalN, galactosamine; Ala, alanine; CONH$_2$, carbamoyl group; P, phosphate; Hep, L-glycero-D-manno-heptose; Kdo, 3-deoxy-D-manno-octulosonic acid. The structures were drawn based on data from Sadovskaya et al. (1998; 2000).
A. Core oligosaccharide

\[
\begin{align*}
\text{\textalpha-d-Glc} & \\
1 & \\
\downarrow & \\
6 & \\
\beta-d-Glc & \\
1 & \\
\downarrow & \\
3 & \\
\beta-d-Glc-(1\rightarrow2)-\alpha-l-Rha-(1\rightarrow6)-\alpha-d-Glc-(1\rightarrow4)-\alpha-d-GalN-(1\rightarrow3)-l-\alpha-d-Hep-(1\rightarrow3)-l-\alpha-d-Hep-(1\rightarrow5)-Kdo-(2\rightarrow6)-lipid A & \\
\uparrow & \\
2 & \\
L-Ala & \\
\vspace{.5cm}
\end{align*}
\]

\text{outer core} \hspace{.5cm} \text{inner core}

B. O-antigen-capped core oligosaccharide

\[
\begin{align*}
\text{\textalpha-d-Glc} & \\
1 & \\
\downarrow & \\
6 & \\
\beta-d-Man2N-ac3AmA-(1\rightarrow4)-\beta-d-Man2N-ac3N-acA-(1\rightarrow3)-\beta-d-FucN-ac-(1\rightarrow3)-\alpha-l-Rha-(1\rightarrow3)-\beta-d-Glc & \\
\uparrow & \\
1 & \\
\downarrow & \\
3 & \\
\alpha-d-Glc-(1\rightarrow4)-\alpha-d-Gal-(1\rightarrow2) & \\
\uparrow & \\
L-Ala & \\
\vspace{.5cm}
\end{align*}
\]

\text{outer core}
(PAC605, PAC557, PAC608, and R5) and wild-type strains (Fisher immunotypes 1, 2, and 7) and other *Pseudomonas* species including *P. fluorescens*, *P. syringae*, and *P. wieringae* (Beckmann et al., 1995). Consequently, the presence of the carbamoyl substituent could serve as a diagnostic marker for *Pseudomonas* spp. of the RNA group I described by Palleroni (1993). One distinguishing feature of the *P. aeruginosa* inner core is the remarkably high degree of phosphorylation associated with the heptose region. Determination of the phosphorylation patterns in this region has proven to be difficult and early studies have reported as many as 10 phosphate groups associated with an LPS molecule (Wilkinson, 1981; Wilkinson, 1983; Nikaido and Hancock, 1986). The exact phosphorylation sites were not identified until an accurate core oligosaccharide structure of a mutant derived from strain PAO1 was described by Masoud et al. (1994). The location of two phosphate groups was found to occur at C-2 and C-4 of L,D-HepI (HepI being the first Hep residue linked to Kdo-lipid A). These phosphates are present in stoichiometric amounts and similar results have been reported in numerous studies (Masoud et al., 1995; Sadovskaya et al., 1998; Sánchez Carballo et al., 1999; Sadovskaya et al., 2000) on other *P. aeruginosa* strains. A third phosphorylation substitution has been found at C-6 of either L,D-HepI (Sadovskaya et al., 1998) or L,D-HepII (Sánchez Carballo et al., 1999). In a recent study by Knirel et al. (2001), four major phosphorylation sites were identified on the LPS of a CF clinical isolate and these phosphates were found at C-2 and C-4 of HepI and C-4 and C-6 of HepII.

The LPS outer-core region contains four D-glucose (D-Glc), one L-rhamnose (L-Rha) and one *N*-(L-alanyl)-D-galactosamine. The outer-core occurs as two glycoforms differing in the position of L-Rha and one D-Glc residue. Although both core types
contain an L-Rha residue linked to D-Glc, the uncapped core glycoform contains L-Rha in an α1,6 linkage to an α-D-Glc in the main chain, whereas the second core glycoform capped with O antigen contains L-Rha in an α-1,3 linkage to β-D-Glc in the branch chain. This α-1,3 linked L-Rha is the attachment point for the O antigen in strain PAO1 (serotype O5) (Sadovskaya et al., 2000). In a study by Bystrova et al. (2002), they observed two core glycoforms in P. aeruginosa strain 170041, classified as Immunotype 1 or serotype O6. The chemical structure of the core OS and linkage of the L-Rha of the core capped in the LPS of this strain is the same as that reported previously by Sadovskaya et al. (2000) for PAO1 (serotype O5). One difference between the uncapped core OS of strain PAO1 and 170041 is that the core of 170041 lacks the terminal D-Glc residue. The terminal D-Glc found in the outer core of PAO1 (serotype O5) was also shown to be absent in the outer core of strain PAK (which also produces serotype O6 O antigen) (Masoud et al., 1995) and is consistent with the structural data presented for strain 170041, immunotype 1 (serotype O6). This structural distinction between the outer-core OS of serotypes O5 and O6 was substantiated by immunochemical data whereby an outer core-specific monoclonal antibody, mAb 101, reacted to a core LPS band of O5 but not O6 in Western immunoblots (de Kievit and Lam, 1994).

In a recent study, Knirel et al. (2001) investigated the structure of the core LPS of a rough, CF-clinical isolate. It is important to note that LPS from chronic CF clinical isolates of P. aeruginosa is usually devoid of O antigen PS (Hancock et al., 1983; Penketh et al., 1983; Fomsgaard et al., 1988; Lam et al., 1988). These researchers noted that CF clinical isolate 2192 synthesizes two different core glycoforms even in the absence of the O-antigen PS (Knirel et al., 2001) and the presence of two distinct core
forms is a characteristic similar to that observed in strain PAO1. However, it is of interest to note that the core glycoform required for attachment of the O antigen was produced despite the absence of capping by O-PS in the LPS preparation from the clinical isolate. Comparison of the two LPS core glycoforms between isolate 2192 and strain PAO1 revealed that the core glycoform available for O attachment has the same structure; however, the uncapped core from the CF isolate lacks the terminal β-D-Glc residue attached at C-2 of the L-Rha of the main chain that is found in PAO1. A new finding in the core OS of clinical isolate 2192 is the O-acetylation that occurs at four sites, although the location of only one O-acetylation site was found at the L-Rha residue (Knirel et al., 2001). To date, O-acetylation of core LPS has been found in other bacteria including *Pseudomonas fluorescens* (Knirel et al., 1996) with the only other report of this modification in the *P. aeruginosa* species in strain 170041 (Bystrova et al., 2002). It is apparent that O-acetylation of core OS is nonstoichiometric, and at present, the exact positions of the O-acetyl groups and the role of O-acetylation in the LPS core are unknown.

The elucidation of the complete structures of the two distinct core glycoforms has a profound impact on our understanding of core biosynthesis in *P. aeruginosa*. Previously, many groups reported partial structures. Alternatively, by using LPS mutants elucidation of complete structures of truncated core OS for *P. aeruginosa* serogroups O2, O3, O5 and O6 were attained (Drewry et al., 1975; Rowe and Meadow, 1983; Masoud et al., 1994; Masoud et al., 1995; Sadovskaya et al., 1998; Sánchez Carballo et al., 1999; de Kievit and Lam, 1997). The study by Sadovskaya et al. (1998) was the first to have accomplished the elucidation of the chemical structure of the outer-core region in a fully
assembled O-chain containing LPS. The knowledge of the existence of two core OS types facilitated a better understanding of the different structural data obtained from LPS mutants, and more importantly, it allowed our group to pursue functional characterization of the genes involved in biosynthesis, and assembly of the LPS core using genetic and biochemical approaches.

1.2.2.2 Genetic organization of core oligosaccharide biosynthesis genes

Earlier studies by our laboratory showed that an operon consisting of \( waaFwaaCwapGwaaPwapPwapQ \) is associated with the production of \( P.\ aeruginosa \) core OS (de Kievit and Lam, 1997; Matewish et al., 1999; Walsh et al., 2000). By using a similarity search program called BLAST (Basic Local Alignment Search Tool; National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/), we compared the nucleotide sequence of the operon to the whole genome sequence of \( P.\ aeruginosa \) PAO1 and found that these genes are part of a larger gene cluster. The PAO1 core OS gene cluster (Fig. 1.3) appears to contain four transcriptional units that span the region corresponding to PA5012 to PA4996 of the PAO1 genome (PseudoCAP; http://www.cmdr.ubc.ca/bobh/PAAP.html; The Pseudomonas Genome Project; Stover et al., 2000). At the 5’ end of the core cluster, \( waaF \) (PA5012) is the first gene of the major operon that contains 12 contiguous ORFs, which include \( waaFwaaCwapGwaaPwapPwapQ \). These first six genes are involved in inner-core biosynthesis.
Figure 1.3 The genetic organization of the core-oligosaccharide biosynthetic gene cluster of *P. aeruginosa* strain PAO1. The predicted genes are designated by their open reading frame number (for example, PA4996-PA5012), which has been assigned based on analysis of the PAO1 genome by Stover et al. (2000). Those proteins whose function has been characterized experimentally or have significant homology to known proteins in the database have also been assigned a gene name, for example, *waaF, waaC, wapG, waaP, wapP, wapQ, wapR, waaL, msbA* and *hldE*. The function of the gene products of this core cluster are described in detail in section 1.2.2.3 and 1.2.2.4.
How the last six ORFs of the *waaF* operon contribute to core OS biosynthesis is not well understood. The ORF after *wapQ*, PA5006, encodes a protein with some kinase-like features and it is hypothesized to be involved in phosphorylation of the inner-core region. Investigations in our laboratory and annotation information (PseudoCAP) have revealed that PA5005 and PA5004 may encode a putative carbamoyltransferase and glycosyltransferase, respectively. The next ORF, PA5003, is annotated (PseudoCAP) to encode a protein that is 48% similar to the protein encoded by *mig-14* of *S. enterica* serovar Typhimurium, which may be a putative transcriptional activator (Valdivia and Falkow, 1997). The *mig-14* gene is a host-induced virulence gene under the control of the PhoP/PhoQ two-component system in *S. enterica* serovar Typhimurium. This gene is required for fatal infection in a model of enteric fever (Valdivia et al., 2000) and was shown to be necessary for resistance of *S. enterica* serovar Typhimurium to antimicrobial peptides (Brodsky et al., 2002). Resistance to antimicrobial peptides in *Salmonella* is frequently associated with modifications in the LPS molecule (Gunn et al., 1998; Trent et al., 2001), however, changes in expression of outer-membrane proteins have also been shown to confer resistance (Guina et al., 2000). Attempts in our laboratory to create a null mutation in PA5003 in *P. aeruginosa* PAO1 have been unsuccessful, implicating that the gene product may be essential for viability in *P. aeruginosa* (M. J. Matewish, F. Bojani and J. S. Lam, unpublished data). This result is in contrast to those obtained for *S. typhimurium*, where a *mig-14* mutant has been generated (Brodsky et al., 2002). The mechanism by which *mig-14* contributes to pathogenicity in *S. enterica* is not yet known. In *P. aeruginosa*, the start codon of PA5003 is four nucleotides downstream of the stop codon of ORF PA5004, which we show in chapter 5 of this thesis to encode a putative
outer-core OS glycosyltransferase. These two genes appear to belong to the same transcriptional unit and in chapter 5 data supports the role of ORF PA5004 in core OS assembly. We speculate that in *P. aeruginosa*, PA5003 is involved in LPS-core OS assembly. The last two ORFs of the waaF operon, PA5002, PA5001, have no significant homology to any known proteins. Therefore, it remains to be determined as to what role these ORFs play in LPS-core biosynthesis.

Located at 158-basepairs downstream of the last ORF of the waaF operon is a single gene, *wapR* (PA5000), which encodes a rhamnosyltransferase involved in outer-core assembly (Poon et al., 2003). This gene is followed by two divergently transcribed operons, each of which contains two genes. The two-gene operon closest to *wapR* contains ORFs PA4998 and PA4999. Although PA4998 has no proposed function, PA4999 most likely encodes WaaL, the O-antigen ligase. Using a prediction of transmembrane helices in proteins program (TMHMM version 2.0; Center for Biological Sequence Analysis, The Technical University of Denmark; http://www.cbs.dtu.dk/services/TMHMM-2.0/; Sonnhammer et al., 1998; Krogh et al., 2001), the protein encoded by PA4999 has 11 membrane spanning domains and its hydropathy profile is similar to those obtained from known WaaL proteins from *E. coli* and *S. enterica* (Heinrichs et al., 1998a). The second two-gene operon contains two ORFs (PA4997 and PA4996), which encode proteins similar to *E. coli msbA* and *hldE*. *E. coli* MsbA has been shown to be an essential ABC transporter for lipid A (Zhou et al., 1998; Doerrler et al., 2001; Doerrler and Raetz, 2002) while *hldE* encodes a bifunctional D-β-D-heptose-7-phosphate kinase/ D-β-D-heptose-1-phosphate adenyllyltransferase required for synthesis of ADP-L,D-Hep, the activated nucleotide donor for heptose residues for the
inner core (Kneidinger et al., 2002; Valvano et al., 2002). The genes and their proposed functions in core OS biosynthesis are summarized in Table 1.2 and will be described in further detail in the following sections.

Regulation of transcription elongation of PS gene clusters in many different organisms is controlled by the RfaH protein and the ops element (operon polarity suppressor) (reviewed in Bailey et al., 1997). RfaH is a processive elongation factor and the ops element is the nucleic acid recognition site for RfaH (Bailey et al., 2000). It is hypothesized that this system modifies the RNA polymerase complex to increase its processivity and allows transcription to proceed over long distances. The ops element is a single 8-basepair motif, 5'--ggcgcttag-3', which has been found upstream of polysaccharide gene clusters from E. coli, Salmonella spp., Shigella flexneri, Yersinia enterocolitica, Vibrio cholerae, and Klebsiella pneumoniae (Bailey et al., 1997), as well as in the RP4 fertility operon of P. aeruginosa (Nieto et al., 1996). We suspect that the P. aeruginosa waaF operon of 12 genes may need antitermination because of its large number of genes, however, an ops consensus sequence could not be located upstream of the waaF operon.

1.2.2.3 Enzymes for inner-core oligosaccharide synthesis

In P. aeruginosa strain PAO1, an operon containing three genes, pyrG, kdsA and eno is involved in biosynthesis of the Kdo residue, which is first sugar of the inner core (Walsh et al., 1999). One of the steps in Kdo synthesis involves the condensation of phosphoenolpyruvate and arabinose-5-P to form Kdo-8-P and this reaction is catalyzed by a Kdo-8-P synthase called KdsA (Rick and Osborn, 1977). Kdo-8-P is activated to cytidine monophosphate Kdo (CMP-Kdo) with cytidine triphosphate (CTP) as the

24
<table>
<thead>
<tr>
<th>Gene function</th>
<th>Gene name</th>
<th>PAO1 genome ORF</th>
<th>Demonstrated or proposed function of gene product</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner-core biosynthesis</td>
<td><em>kdsA</em></td>
<td>PA3636</td>
<td>3-deoxy-D-manno-octulosonic acid 8-P synthase</td>
<td>Walsh et al. (1999)</td>
</tr>
<tr>
<td></td>
<td><em>pyrG</em></td>
<td>PA3637</td>
<td>CTP synthase</td>
<td>Walsh et al. (1999)</td>
</tr>
<tr>
<td></td>
<td><em>eno</em></td>
<td>PA3536</td>
<td>Phosphopyruvate hydratase, 2-phosphoglycerate dehydratase</td>
<td>Walsh et al. (1999)</td>
</tr>
<tr>
<td></td>
<td><em>waaF</em></td>
<td>PA5012</td>
<td>Heptosyltransferase II</td>
<td>de Kievit and Lam (1994)</td>
</tr>
<tr>
<td></td>
<td><em>waaC</em></td>
<td>PA5011</td>
<td>Heptosyltransferase I</td>
<td>de Kievit and Lam (1994)</td>
</tr>
<tr>
<td></td>
<td><em>wapG</em></td>
<td>PA5010</td>
<td>N-acetylgalactosyltransferase</td>
<td>Matewish et al. (1999)</td>
</tr>
<tr>
<td></td>
<td><em>waaP</em></td>
<td>PA5009</td>
<td>Phosphorylation of HepII</td>
<td>Walsh et al. (2000)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Zhao et al. (2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PA5008</td>
<td>Phosphorylation of HepII</td>
<td>Walsh and Lam (2002)</td>
</tr>
<tr>
<td></td>
<td><em>wapP</em></td>
<td>PA5007</td>
<td>Phosphorylation of HepII</td>
<td>Walsh et al. (2000)</td>
</tr>
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<td></td>
<td><em>wapQ</em></td>
<td>PA5005</td>
<td>Carbamoyl transferase</td>
<td>A.G. Walsh and J.S. Lam, unpublished data</td>
</tr>
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<td></td>
<td><em>wapO</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer-core biosynthesis</td>
<td><em>wapH</em></td>
<td>PA5004</td>
<td>Glucosyltransferase</td>
<td>Chapter 5</td>
</tr>
<tr>
<td></td>
<td><em>wapR</em></td>
<td>PA5000</td>
<td>Rhamnosyltransferase</td>
<td>Poon et al. (2002)</td>
</tr>
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<td></td>
<td><em>migA</em></td>
<td>PA0705</td>
<td>Rhamnosyltransferase</td>
<td>Yang et al. (2000)</td>
</tr>
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<td></td>
<td><em>algC</em></td>
<td>PA5322</td>
<td>Phosphomannomutase and phosphoglucomutase; Biosynthesis of UDP-D-glucose</td>
<td>Poon et al. (2001)</td>
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<td></td>
<td><em>rmlBDAC</em></td>
<td>PA5161-PA5164</td>
<td>Biosynthesis of dTDP-L-rhamnose</td>
<td>Zielinski et al. (1991)</td>
</tr>
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<td></td>
<td><em>waaL</em></td>
<td>PA4999</td>
<td>Ligase</td>
<td>Zielinski et al. (1992)</td>
</tr>
<tr>
<td></td>
<td><em>rfaL</em></td>
<td></td>
<td></td>
<td>Ye et al. (1994)</td>
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<td></td>
<td></td>
<td></td>
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<td>Olvera et al. (1999)</td>
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<td></td>
<td></td>
<td>Rahim et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P. Abeyratne and J. S. Lam, unpublished data</td>
</tr>
</tbody>
</table>
nucleotide donor and CMP-Kdo serves as the activated sugar donor for transfer of Kdo residues to lipid A by the putative Kdo transferase, encoded by PA4988 (PseudoCAP). PyrG is a CTP-synthetase, which catalyzes the transfer of ammonia to UTP to form CTP. Eno is involved in formation of phosphoenolpyruvate. P. aeruginosa PyrG, KdsA and Eno are 69%, 69% and 77% identical to PyrG, KdsA and Eno of E. coli, respectively. P. aeruginosa kdsA was shown to rescue a 42°C temperature-sensitive S. typhimurium kdsA mutant for growth at 42°C. The Kdo-8-P synthase activity of E. coli carrying P. aeruginosa kdsA on a plasmid was almost 6-fold higher than that of a control E. coli strain carrying a vector with no gene (Walsh et al., 1999). P. aeruginosa pyrG can complement the growth of an E. coli pyrG mutant that is auxotrophic for cytidine. The pyrG operon is located outside the waaF gene cluster and a sigma-70 like promoter upstream of pyrG initiates transcription of the genes in this operon (Walsh et al., 1999).

The first two genes of the waaF operon, waaF and waaC, encode heptosyltransferases I and II that are required for assembly of the heptose region of the inner core (de Kievit and Lam, 1997). WapG has homology to E. coli WaaG, which is a UDP-glucose (heptosyl) LPS α1,3-glucosyltransferase, that transfers D-Glc from UDP-D-Glc to HepII of the inner core (Yethon et al., 2000). The first sugar of the outer core in E. coli is D-Glc, in contrast, the first outer-core residue in P. aeruginosa is N-(L-alanyl)-D-galactosamine. The homology between WaaG and WapG may be based partially on a common α-1,3 linkage of the first hexose residue to the acceptor molecule, HepII-inner-core. We hypothesize that WapG transfers D-GalNAc from UDP-D-GalNAc to L,D-HepII of the inner core. Subsequently, D-GalNAc is deacetylated, followed by the addition of L-alanine, as by yet, unidentified enzymes. Although many different strategies were used, a
wapG mutant could not be generated. *P. aeruginosa* wapG is unable to complement the LPS defect in an *E. coli* waaG mutant, demonstrating that despite the sequence similarity between WapG and WaaG at the protein level, these enzymes likely have different glycosyltransferase activities (Matewish et al., 1999).

The heptose region of the inner core in *P. aeruginosa* has been shown to be substituted with up to four phosphate moieties and so far, three genes, *waaP*, *wapP* and *wapQ* have been implicated in phosphorylation (Walsh et al., 2000). Amino acid sequence analysis shows WaaP, WapP and WapQ to contain consensus sequences of kinase-2 motifs. By performing nuclear magnetic resonance and methylation linkage analysis our group has demonstrated that WaaP is responsible for the addition of one phosphate to O4 of HepI (Walsh et al., 2000). The WaaP protein has been purified and the detailed kinetics of its heptose kinase activity were measured using an enzyme-linked immunosorbent assay (ELISA) developed in our laboratory (Zhao and Lam, 2002; Zhao et al., 2002). BLAST searches revealed that *P. aeruginosa* WaaP shares homology with eukaryotic-type protein kinases that belong to the serine/threonine kinase and the tyrosine kinase family. WaaP has significant identity in the conserved, functional residues within Hank’s designation of “motifs” commonly found in protein kinases (Hanks and Quinn, 1991), which include, for example, the amino acid residues that are thought to interact with the phosphate groups of ATP, the phosphate donor. The hypothesis that WaaP could also be a protein kinase in addition to a sugar kinase was substantiated by its ability to phosphorylate an exogenous tyrosine-containing substrate (Zhao and Lam, 2002). WaaP is capable of a third enzyme activity, auto-phosphorylation. Purified WaaP was found to exhibit auto-phosphorylation activities when assayed in a self-phosphorylation
chemiluminescence-based ELISA using an anti-phosphotyrosine antibody. MALDI-TOF mass spectrometry and proteolytic peptide mapping studies of the full-length purified WaaP indicate that eight tyrosine residues are phosphorylated. The hypothesis that WaaP utilizes a catalytic mechanism similar to that of eukaryotic type protein kinases was validated by site-directed mutagenesis of key catalytic residues and subsequent complementation assays. Therefore, from the results of detailed biochemical characterization studies, *P. aeruginosa* WaaP is capable of three distinct activities, sugar kinase activity, protein kinase activity and self-phosphorylating tyrosine kinase activity (Zhao and Lam, 2002). *P. aeruginosa* WaaP also has homology to *E. coli* WaaP. Although both proteins have heptose kinase activity, these enzymes appear to be structurally different (Yethon and Whitfield, 2001; Zhao and Lam, 2002; Zhao et al., 2002). Also, the *E. coli* WaaP protein does not contain the typical pattern of conserved domains that are characteristic of tyrosine kinases and no data has been reported to demonstrate protein tyrosine kinase activity for *E. coli* WaaP. Altogether, the additional protein kinase and auto-phosphorylating activities that *P. aeruginosa* WaaP has, in addition to the lethality of the waaP mutation in *P. aeruginosa* suggest that the mechanism by which the inner core heptose is phosphorylated in *P. aeruginosa* may be different than that of *E. coli* or that *P. aeruginosa* WaaP may have an additional role in core assembly.

The functions of *P. aeruginosa* WaaC, WaaF, WaaP and WapP in inner-core assembly was demonstrated by cross complementation of *S. typhimurium* waaC, waaF and waaP mutants, respectively, rather than by generating and characterizing defined *P. aeruginosa* mutants in those respective genes. Repeated attempts to create null mutations
in *P. aeruginosa* waaC, waaF, wapG, waaP and wapP were unsuccessful (de Kievit and Lam, 1997; Matewish et al., 1999; Walsh et al., 2000). A chromosomal *P. aeruginosa* waaP mutant was made only when the mutant was concomitantly carrying a copy of *waaP in trans* (Walsh et al., 2000). In addition, despite numerous attempts by various research groups using different mutagenesis approaches, *P. aeruginosa* LPS mutants lacking inner core L,D-Hep or phosphate have never been isolated. These observations suggest that L,D-Hep-linked phosphate is essential for viability. The inability to generate a *P. aeruginosa* wapG mutant is consistent with this hypothesis and is further substantiated by comparing to the phenotype of an *E. coli* waaG mutant. The LPS from an *E. coli* waaG mutant is not only truncated after the inner-core heptose residues, but there is also an 80% total reduction in heptose phosphorylation (Yethon et al., 2000). In *E. coli*, the addition of the first glucose residue of the outer core is required for complete phosphorylation of the inner core and is likely the reason why a *P. aeruginosa* wapG mutant cannot be constructed.

The observation of lethal consequences in *P. aeruginosa* inner-core mutants is in contrast to *E. coli* and *Salmonella*, where LPS mutants that lack the heptose region of the inner core are viable, though they exhibit a “deep-rough” phenotype. Some of the characteristics of this phenotype include changes in surface hydrophilicity and hypersensitivity to hydrophobic antibiotics, dyes and detergents (reviewed in Schnaitman and Klena, 1993; Nikaido, 2003). The phosphate moiety likely participates in ionic interactions with divalent cations thereby causing cross-linking between the LPS molecules and serves to stabilize the outer membrane. Cross-linking LPS molecules may be especially important in *P. aeruginosa* since this bacterium is highly susceptible to lysis.
by agents that chelate divalent cations, such as ethylenediamine tetra-acetic acid (Gray and Wilkinson, 1965; Eagon et al., 1965). Thus, attempts to perform detailed characterization of the inner core genes of P. aeruginosa has been hampered by the inability to create LPS null mutants. These mutants would be extremely important for two reasons. First, the phenotype of the mutant equivocally shows direct involvement of the gene product in LPS biosynthesis. Second, LPS isolated from the mutant provides the specific substrate molecules for in vitro assays and consequently, facilitates biochemical characterization.

1.2.2.4 Genes for outer-core oligosaccharide biosynthesis

The major sugar residues of the outer-core OS are D-Glc and L-Rha and the nucleotide-activated sugar donors for addition of these sugars by glycosyltransferases to the inner core are UDP-D-Glc and dTDP-L-Rha. AlgC is a key enzyme in polysaccharide synthesis in P. aeruginosa because it is required for synthesis of both UDP-D-Glc and dTDP-L-Rha and these nucleotide donors provide sugar residues not only for assembly of the core OS (Coyne et al., 1994; Giraud and Naismith, 2000), but also for the biosynthesis of O-antigen PS (Goldberg et al., 1993) and rhamnolipids (Olvera et al., 1999). The algC gene encodes a bifunctional enzyme that has both phosphoglucomutase (PGM) and phosphomannomutase (PMM) activity. The PGM activity catalyzes the conversion of glucose 6-phosphate to glucose 1-phosphate (Glc-1-P), which is a common intermediate between the biosynthetic pathways for UDP-D-Glc and dTDP-L-Rha. The conversion of Glc-1-P to UDP-D-Glc is catalyzed by GalU, a UDP-D-glucose pyrophosphorylase, while the conversion of Glc-1-P to dTDP-L-Rha requires numerous reaction steps catalyzed by the rmlBDAC operon. Synthesis of dTDP-L-Rha in bacteria
has been characterized at the biochemical level (Graninger et al., 1999; Giraud and Naismith, 2000) and the three-dimensional structure of RmlA from *P. aeruginosa* as well as the enzymatic mechanism of this protein has been solved (Blankenfeldt et al., 2000a; 2000b). Mutations in *rmlC* and *galU* in *P. aeruginosa* have been shown to abrogate the production of complete core OS (Rahim et al., 2000; Dean and Goldberg, 2002). The second enzyme activity of AlgC, the PMM activity, catalyzes the conversion of mannose 6-phosphate to mannose 1-phosphate, leading to the formation of GDP-D-mannose and GDP-D-mannuronic acid. These are required for A-band PS (Ye et al., 1994) and alginate synthesis (Zielinski et al., 1991; Ye et al., 1994). It is of interest to note that *algC, galU*, and the *rml* operon are all located outside the *waaF* core gene cluster.

Our genetic data suggest that the assembly of the two distinct outer-core glycoforms requires six glycosyltransferases, in addition to the glycosyltransferase encoded by *wapG*, which transfers the first hexose sugar to lipid A-inner core. Two genes, *wapR* and *migA*, which encode two of the six glycosyltransferases are currently under investigation in our laboratory. *WapR* encodes the α1,3-rhamnosyltransferase that adds the rhamnose residue to the core that is required for attachment of long chain PS whereas *migA* encodes the α1,6-rhamnosyltransferase required for assembly of the uncapped core glycoform. These functional assignments are based on the evidence of the LPS phenotypes and complementation results observed in mutant constructs of *wapR, migA* in a PAO1 background, and *migA* in a PAK background (Yang et al., 2000; Poon et al., 2003). Interestingly, *wapR* is located within the core LPS gene cluster while *migA* maps outside this locus. The gene, *migA*, was identified as a mucus inducible gene whose promoter was specifically inducible during growth in respiratory mucus obtained from
CF patients (Wang et al., 1996). A subsequent study revealed that \textit{migA} is highly expressed in the CF lung environment and is regulated by the RhII/RhIR quorum sensing regulatory system (Yang et al., 2000). Although two consensus \textit{las}-box-like sequences were identified upstream of \textit{migA}, it is not known which one or if both of the \textit{las}-box-like sequences are required for \textit{migA} expression. High-level expression of \textit{migA} results in the loss of the core-plus-one O-antigen form of LPS (also referred to as semi-rough LPS), whereas a \textit{migA} mutation results in an increased amount of semi-rough LPS (Yang et al., 2000). Expression of the MigA protein is also increased during biofilm formation (deLancey Pulcini and Camper, 2002) and other groups have shown that there are significant changes in LPS expression in \textit{P. aeruginosa} during a biofilm mode of growth (Giweracman et al., 1992; Beveridge et al., 1997). Up-regulation of the \textit{migA} gene in the CF lung environment appears to increase the production of the uncapped form of core OS and this may promote a cell-surface change that favours survival of the bacterium in this environment.

Why \textit{migA} is located outside the core-gene cluster and why it appears to be separately regulated is not completely understood. In \textit{E. coli} (Heinrichs et al., 1998c), \textit{S. enterica} (Kaniuk et al., 2002) and \textit{K. pneumoniae} (Regué et al., 2001), the \textit{waa} locus contains the majority of genes, and more importantly for our comparison, contains all the glycosyltransferases involved in assembly of the core OS. In \textit{Vibrio cholerae}, the core OS \textit{wax} gene cluster has been characterized at the genetic level and assignment of the genes required for synthesis of the known core structures allowed them to account for most of the predicted glycosyltransferases required in this cluster (Nesper et al., 2002). Similar to our observations with the \textit{P. aeruginosa} core gene cluster, in \textit{V. cholerae} the
gene assignments are such that the wav gene cluster encodes five proteins of unknown function, and yet three additional transferases are still required for complete assembly. Therefore, possibly in both \textit{P. aeruginosa} and \textit{V. cholerae}, the genes for the outer core glycosyltransferases do not share similarity to other known glycosyltransferases or they map outside this locus.

There are organisms where the core OS genes are not clustered or have a unique regulatory element. Biosynthesis of the core OS of \textit{Yersinia enterocolitica} serotype O:3 involves at least two unlinked genetic loci, the \textit{rfa} gene cluster and the \textit{trs} operon, for synthesis of the inner core and outer core, respectively (Skurnik et al., 1995; Skurnik and Zhang, 1996). Some of the proteins encoded by the \textit{trs} operon are similar to those encoded by the O-antigen gene clusters of other Enterobacteriaceae. Based on these novel observations, the \textit{trs} operon is hypothesized to be an O-antigen gene cluster “relic” that has maintained a role in core OS although it has lost its ability to produce O antigen. A unique regulatory element for core modification has been observed in \textit{Yersinia pestis}, where modification of the outer region of the lipooligosaccharide (LOS) by one sugar residue is under the control of the \textit{phoP/phoQ} regulatory system. Hitchen et al. (2002) constructed a \textit{phoP} mutant and compared the chemical structures of the LOS and found that the wild-type strain produced two distinct LOS molecular species differing in a terminal galactose or heptose residue. The \textit{phoP} mutant was restricted to expression of a single molecular LOS form, one containing the terminal heptose. Interestingly, the addition of the galactose residue is associated with increased resistance to polymyxin B. Altogether, there appears to be a variety of mechanisms by which core OS and LOS is synthesized and it is apparent from these examples that one cannot assume that all genes
involved in core OS are located in one locus, nor are they controlled by one regulatory element and promoter. Clearly, the development of sensitive techniques to perform structural analysis of small quantities of LPS that can be isolated under a variety of conditions has allowed us to see that the process of the biosynthesis and assembly of the core OS region is similar to that of the lipid A molecule, in that under different environmental conditions, it is modified to enhance survival of the bacterium. Since the core OS region of *P. aeruginosa* is crucial for the outer-membrane integrity, studies investigating core OS modifications due to environmental influences warrants further investigation.

The information from structural elucidation of the two core OS glycoforms and whole genome sequence annotation are presently our most valuable tools for further investigation into the mechanisms of core oligosaccharide biosynthesis in *P. aeruginosa*. A number of questions that need to be addressed in future research include (1) What are the mechanisms by which the inner core is phosphorylated? (2) How do changes in the core oligosaccharide by MigA enhance survival of *P. aeruginosa in vivo*? (3) Where are the genes that encode the rest of the outer-core glucosyltransferases? It would be interesting to determine if the ORFs of unknown function at the 3’ end of the *waaF* operon encode the three glucosyltransferases required for synthesis of the outer-core OS, because it would lead to the identification of a novel family of glycosyltransferases.
1.2.3 B-band polysaccharide (O antigen)

1.2.3.1 Structure of O polysaccharides

*Pseudomonas aeruginosa* produces O-antigen polysaccharide (O-PS) that is referred to as B-band LPS (Rivera et al., 1988). The O-PS consists of linear polymers of di- to penta-saccharide repeating units. A prominent feature of the *P. aeruginosa* O antigen is that it contains uronic acids, amino sugars and some rare sugars. For example, residues typically found in *P. aeruginosa* O antigen include: N-acetylated 6-deoxyhexosamines (D-quinovosamine, D- and L-fucosamine), 2,4-diamino-2,4-dideoxy-D-quinovose (D-bacillosamine), and acidic monoamino and diaminor residues, D- and L-galactosaminuronic acid, 2,3-diamino-2,3-dideoxyuronic acids and 5,7-diamino-3,5,7,9-tetrahydroxynonulosonic acids (pseudaminic acid) (Knirel and Kochetkov, 1994). The chemical structures of many *P. aeruginosa* O-PS have been studied extensively (reviewed in Knirel, 1990; Knirel and Kochetkov, 1994) and the O-PS of serotypes O5, O6 and O11 are shown in Figure 1.4. Structural information of the O-PS and data from immunochemical investigations has lead to the identification of 31 distinct O-antigen chemotypes (Knirel et al., 1988; Stanislavsky et al., 1988). The B-band O antigen is highly immunogenic and differences in the chemical structure of the O-PS are responsible for the serogroup specificity of the respective strains (Knirel et al., 1988).

Many different serological typing schemes that distinguish *P. aeruginosa* O antigens have been described and these include schemes by Habs (1957), Sandvik (1960), Verder and Evans (1961), Meitert (1964), Wokatsch (1964), Lanyi (1966), Fisher (Fisher et al., 1969) Akatova and Smirnova (1982), and Homma (1976). The classification scheme currently used by most laboratories is the International Antigenic Typing Scheme (IATS),

35
A. Serotype O5
\[
\rightarrow 4)\beta-D-Man(2N\text{A}c3N)A-(1\rightarrow 4)\beta-D-Man(2N\text{A}c3N\text{A}c)A-(1\rightarrow 3)\alpha-D-FucN\text{A}c-(1\rightarrow 3)\text{CH}_3C=\text{NH}
\]

B. Serotype O6
\[
\rightarrow 4)\alpha-D-GalN\text{A}cA-(1\rightarrow 4)\alpha-D-GalN\text{F}oA-(1\rightarrow 3)\alpha-D-\text{QuiN}\text{A}c-(1\rightarrow 2)\alpha-L-\text{Rha}-(1\rightarrow 3)\text{OAc}\quad \text{NH}_2 \quad \text{NH}_2
\]

C. Serotype O11
\[
\rightarrow 3)\alpha-L-FucN\text{A}c-(1\rightarrow 3)\beta-D-FucN\text{A}c-(1\rightarrow 2)\beta-D-\text{Glc}-(1\rightarrow)
\]

**Figure 1.4** O-antigen structure for serotype O5, O6 and O11. Man(2N\text{A}c3N\text{*})A, 2-acetamido-3-acetamidino-2,3-dideoxy-mannuronic acid (\text{*} represents CH\text{\text{3}}C=\text{NH}); Man(2N\text{A}c3N\text{A}c)A, 2,3-acetamido-D-mannuronic acid; FucN\text{A}c, N-acetyl\text{fucosamine}; GalN\text{A}cA, N-acetyl\text{galactosaminuronic acid}; GalN\text{F}oA, 2-deoxy-2-formamido-galacturonic acid; \text{QuiN}\text{A}c, N-acetyl\text{quinovosamine}; \text{Rha}, \text{rhamnose}; and Glc, \text{glucose}. The O-acetyl and amino group substitutions are abbreviated as -OAc and -NH\text{\text{2}}, respectively. Structures are based on Knirel et al. (1988) and Knirel and Kochetkov (1994).
which describes 20 reference serotype strains of *P. aeruginosa* (Liu et al., 1983; Liu and Wang, 1990). A comprehensive review of how the different classification schemes compare in type numbers and antigenic constituents of the O antigens has been provided by Stanislavsky and Lam (1997). Serotyping of *P. aeruginosa* strains is normally performed using polyclonal antiserum that has been adsorbed to improve specificity against a specific serotype. To provide improved specificity and sensitivity monoclonal antibodies specific for the first 17 of the 20 IATS serotypes have been produced (Lam et al., 1987a; 1987b), while polyclonal antiserum against IATS serotypes 18-20 are also available (Liu and Wang, 1990).

1.2.3.2 Overview of O-antigen biosynthesis

Biosynthesis of B band O-antigen LPS in *P. aeruginosa* is based on the Wzy-dependent assembly model of O-antigen biosynthesis, which has been extensively reviewed (Whitfield, 1995; Raetz and Whitfield, 2002). It begins in the cytoplasm with the synthesis of nucleoside diphosphate (NDP)-activated sugar precursors. Different biosynthetic pathways generate the complex sugar residues that are found in *P. aeruginosa* PS. Once synthesized, the nucleotide-activated sugars act as donor molecules for the sequential addition of sugar residues by glycosyltransferases onto undecaprenol phosphate, to generate individual O units. These single O units are then translocated from the cytoplasmic face of the inner membrane to the periplasmic face by an O-unit translocase or flippase protein called Wzx (Burrows and Lam, 1999). The O units are polymerized into the O-antigen PS by the oligosaccharide transferase activity of the O-antigen polymerase, Wzy (de Kievit et al., 1997). The chain length regulator protein called Wzz regulates the length of the PS generated by the Wzy protein (Burrows et al.,
The majority of the genes encoding these proteins as well as the enzymes involved in the biosynthesis of the nucleotide activated precursor sugars are located in one O-antigen biosynthetic gene cluster (Rocchetta et al., 1999). After assembly of the PS, the O-antigen ligase, WaaL, transfers the O antigen to the core-lipid A to make the finished LPS molecule that is then transported to the outer leaflet of the outer membrane.

The genetics of B-band biosynthesis in *P. aeruginosa* has been extensively reviewed (Rocchetta et al., 1999; Lam et al., 2004). Therefore, the following sections will provide only a brief overview of the genetic basis for the generation of the 20 different serotypes along with a few key findings from the most recent studies. In many of the early studies, assignment of the putative function of the gene products of the O-antigen gene cluster mainly relied on: (i) similarity to other known proteins (most of which have not been characterized at the biochemical level), (ii) LPS phenotypes of null mutants, and (iii) data from cross-complementation experiments with genes from other organisms. In contrast, within the last five years the focus has been to apply biochemical approaches to further elucidate the function of these proteins.

### 1.2.3.3 Biosynthesis of O-antigen polysaccharide

In general, O-antigen biosynthesis in *P. aeruginosa* is best understood in strain PAO1 (serotype O5). Strains producing O6 and O11 O antigens, however, are the most frequently encountered serotypes isolated from the hospital setting and the environment (Farmer et al., 1982; Pitt, 1988; Bert and Lambert-Zechevsky, 1996; Vachee et al., 1997). The gene names within the O-antigen gene clusters for *P. aeruginosa* serotypes O5 (Burrows et al., 1996), O6 (Bélanger et al., 1999), and O11 (Dean et al., 1999) have been
assigned in accordance with bacterial polysaccharide synthesis gene nomenclature (Reeves et al., 1996). The genes that encoded the O-antigen chain length regulator, flippase and O-antigen polymerase, were named wzz, wzx and wzy, respectively. All of the other genes involved in biosynthesis of the O unit in O5 were designated wbpA through wbpM (Fig. 1.5 A). In the O-antigen gene cluster for serotype O6, wzz, wzx, wbpL and wbpM showed very strong homology to those characterized in serotype O5, therefore, these genes have been given the same names and presumed to have the same function. The other O-antigen genes in the cluster are designated wbpO through wbpV (Fig. 1.5 B). The serotype O11 gene cluster contains wzz, wzx, wxy, wbpL and wbpM whereas the other genes are designated whjA through whjF by Dean et al. (1999) (Fig. 1.5 C). The proposed functions for the majority of the gene products have been described previously (Burrows et al., 1996; Bélanger et al., 1999; Dean et al., 1999).

Nucleotide sequencing of the O-antigen biosynthetic loci from all 20 IATS reference strains was achieved by Raymond et al. (2002). The boundaries of the 20 B-band gene islands lie within a 20-bp sequence at the 5' end and within the wbpM gene at the 3' end. The 20 B-band gene islands have mol% G+C contents ranging from 46-55. This is in marked contrast to the overall mol% G+C content of the P. aeruginosa PAO1 genome, which is 67 (Stover et al., 2000). The wbpM gene, however, has a mol% G+C content of approximately 62. This unusual discrepancy in base composition of the B-band genes is consistent with the theory that these gene clusters have been acquired by horizontal transfer from other bacterial species (Rocchetta et al., 1999), whereas the wbpM gene may be an ancestral P. aeruginosa gene.
**Figure 1.5** The genetic organization of the B-band biosynthetic gene clusters for serotypes O5, O6 and O11. The genes are represented by arrows. Genes involved in biosynthesis of the O unit in O5 are designated *wbpA* through *wbpM* (A), for serotype O6, *wbpO* through *wbpV* (B) and for serotype O11, *wbjA* through *wbjF* (C). The genes that encode the O-antigen chain length regulator, flippase and O-antigen polymerase are designated *wzz*, *wzx* and *wzy*, respectively. The black arrows designate genes involved in biosynthesis of the activated nucleotide precursor sugars, grey arrows represent the genes involved in polymerization and assembly, hatched arrows represent genes that encode glycosyltransferases and white arrows represent open reading frames whose function have not been shown to correlate with LPS biosynthesis. All three loci are shown on the same scale; bar equals 1 kb. The assignment of gene functions are based on data from Bélanger et al. (1999), Burrows et al. (1999), Dean et al. (1999), and Raymond et al. (2002).
A. O2, O5, O16, O18, O20

himD/ihiB orfA wzz₁  wb₅A  wb₅B  wb₅C  wb₅D  wb₅E  wzy  wzx  hisH  hisF  wb₅G  wb₅H  wb₅I  wb₅J  wb₅K  wb₅L  SI/209  wb₅M

B. O6

himD/ihiB orfA wzz₁  wb₅O  wb₅P  wb₅Q  wb₅R  wzx  wb₅S  wb₅T  wb₅U  wb₅V  wb₅L  wb₅M

C. O11

himD/ihiB orfA wzz₁  wzx  wbj₅A  wzy  wbj₅B  wbj₅C  wbj₅D  wbj₅E  wbj₅F  wb₅L  wb₅M

1 kb
Analysis of the 20 O-antigen gene clusters by Raymond et al. (2002) revealed 11 distinct gene clusters that are highly divergent from one another at the DNA sequence level. Of particular interest is B-band gene islands of serotypes O2, O5, O16, O18, and O20, which are 98% identical, and belong to a single group. This observation substantiated previous results of Southern hybridization (Burrows et al., 1996) and serotyping with serotype-specific and cross-reactive monoclonal antibodies showing cross-reactivity among these serotypes (Lam et al., 1992). The chemical variability of the O-antigen structures within these groups is most likely due to additional genes that map outside the B-band locus. Modifications of the O-PS have been shown to occur by the action of lysogenic bacteriophages. For example, bacteriophage D3, which lysogenizes *P. aeruginosa* strain PAO1, causes two major changes in the O5 O antigen. Firstly, the C-4 position of the FucNAc residue is O-acetylated (characteristic of serotype O20), and the linkage between the O units is changed from α1-4 to β1-4 (change from serotype O5 to O16) (Kuzio and Kropinski, 1983). The bacteriophage D3 genome contains three genes required for “serotype conversion”, an inhibitor of the α-polymerase (*iap*); an O-acetylase (*oac*); and a β-polymerase (*wzy*β) (Newton et al., 2001). The phage-encoded Iap inhibitor is capable of inhibiting B-band LPS containing α-linked O units in different *P. aeruginosa* strains. For this reason there is considerable interest in understanding the mechanism by which Iap inhibits Wzy. The interaction of Iap with Wzy provides a unique approach to investigating the functional domain(s) of the O-antigen polymerase. Since Iap is predicted to span the cytoplasmic membrane and interact with Wzy, which is predicted to contain 11 membrane-spanning domains, the challenge in future investigations will be the design of experimental parameters by which to deal with the
hydrophobic nature of these proteins during protein expression, purification, and how to maintain their native molecular characteristics when designing \textit{in vitro} systems by which to examine the interactions of these proteins.

\textbf{1.2.3.4 Conserved proteins of the B-band gene clusters}

Raymond et al. (2002) observed that there is a certain degree of conservation in the organization of the genes among the 20 B-band gene islands. This is an intriguing observation considering the differences in chemical structures of the sugar residues that exists between the O serotypes. Located at the 5' end of each gene cluster is a \textit{wzz} gene, which encodes the O-antigen chain length regulator protein. Two distinct \textit{Wzz} proteins, \textit{Wzz1} and \textit{Wzz2}, have been identified in \textit{P. aeruginosa} strain PAO1 (serotype O5) (Burrows et al., 1997; Daniels et al., 2002). \textit{Wzz1} is located at the 5' end of the B-band gene cluster, while \textit{wzz2} maps outside this locus. \textit{Wzz1} regulates the production of two preferred O-antigen chain lengths in serotype O5 that equate to 12-16 and 22-30 O-antigen repeat units, whereas \textit{Wzz2} imparts the chain length of approximately 40-50 repeats. Daniels et al. (2002) investigated the relative distribution of O5-specific \textit{Wzz1} and \textit{Wzz2} within the 20 serotypes and found that serotype strains O1, O2, O5, O16, O18 and O20 express the \textit{Wzz1} protein, while \textit{Wzz2} is produced by all 20 IATS reference strains. \textit{Wzz1} and \textit{Wzz2} from strain PAO1 were overexpressed and purified to near homogeneity (Daniels et al., 2002). These preparations were used to develop polyclonal antibodies against each \textit{Wzz} protein. \textit{In vivo} protein cross-linking followed by Western immunoblotting indicated that \textit{Wzz1} forms dimers, whereas \textit{Wzz2} forms octamers. The observation that the two different \textit{Wzz} proteins were able to oligomerize in the absence of each other leads Daniels and his colleagues (2002) to hypothesize that \textit{Wzz1} and \textit{Wzz2}
may form part of two distinct O-antigen biosynthetic complexes. Clearly, since no
interactions between Wzy and Wzz were reported, more evidence is needed to resolve the
hypothesized interaction between these two proteins.

The exact mechanism by which Wzz proteins function in controlling modal chain
length is not completely understood. Two different models have been proposed to
describe the Wzz-dependent chain length regulation and both models predict the Wzz
proteins to work together with the O-antigen polymerase (Wzy) to control O-PS chain
length (Bastin et al., 1993; Morona et al., 1994). Furthermore, it has been postulated that
modal chain length is determined at the ligation step (Whitfield et al., 1997). This would
require the interaction of Wzz and Wzy with the O-antigen ligase (WaaL) so that PS of
the preferred chain length are ligated to the lipid A core. In contrast to this postulate,
Daniels et al. (2002) showed that modal chain length-distributed O-PS antigen in P.
aeruginosa strain PAO1 (serotype O5) occurs on the growing
undecaprenylpyrophosphate-bound O-antigen polymer, before ligation to the core-lipid
A. In addition, by performing immunoprecipitation, this group has provided the evidence
to show that both Wzz1 and Wzz2 proteins are associated with the growing O-antigen
polymer, which is covalently attached to the undecaprenyl phosphate (Daniels et al.,
2002). Although the molecular mechanisms of this process still need to be elucidated, the
use of other approaches including overexpression of the proteins, protein purification,
and the use of biophysical methods to measure protein-protein interactions should prove
useful in clarifying the molecular interactions. One major difficulty in this endeavour is
the expression of Wzy in amounts that can be detected (Daniels et al., 1998; C. Daniels
and J. S. Lam, unpublished data), purified and used in an in vitro system.
There is little direct experimental evidence to support or reject the hypothesis that the proteins involved in the Wzy-assembly pathway function as a multi-protein complex. Furthermore, there is little experimental data that describes the molecular mechanisms underlying the interactions between these proteins and the interactions with their respective substrate molecules, including core-lipid A, O units and PS. Key players in this system to consider for future investigations include Wzy, Wzz1, Wzz2, Wzx, as well as the Iap inhibitor, and purified substrate molecules. Specific aims in these studies include investigating protein-protein interactions, with emphasis on identifying functional domains. It is important to note that despite the numerous reports on these proteins, the detailed molecular mechanism of their proposed specific activities is not known. Challenges in these investigations include establishing systems by which to study multi-protein interactions with proteins that are predicted to contain numerous membrane-spanning domains, such as Wzy. These proteins are difficult to overexpress, and since some of these proteins are predicted to be very hydrophobic, they will most likely require different sets of conditions for assaying their function than those described for investigating the enzyme kinetics of the more soluble biosynthetic enzymes that have been purified and assayed that will be described in the next section.

1.2.3.5 Biosynthetic pathways of hexose residues of the O antigen

Biosynthetic pathways have been proposed for the synthesis of the nucleotide precursors for the sugar residues of serotypes O5 (Burrows et al., 1996), O6 (Bélanger et al., 1999) and O11 (Dean et al., 1999). To test these hypotheses, proteins from selected genes were overexpressed, purified, and tested for their function using in vitro enzyme assays. For example, a p-dimethylaminobenzaldehyde colorimetric assay for the
detection of N-acetylated nucleotide-activated sugars was developed for analyzing
the activity of WbpP of serotype O6 (Creuzenet et al., 2000). In addition, a number of
techniques including capillary electrophoresis, mass spectrophotometry and nuclear
magnetic resonance were used for separation and identification of closely related
compounds including epimers and isomers of sugar-nucleotides. Using the
aforementioned techniques, the enzymes involved in the pathways for the biosynthesis of
N-acetyl dideoxy/deoxy hexoses including UDP-N-acetyl-D-galactosamine (UDP-D-GalNAc) (Creuzenet et al., 2000), UDP-N-acetyl-D-galactosaminuronic acid (UDP-D-GalNAcA) (Zhao et al., 2000), UDP N-acetyl-D-quinovosamine (UDP-D-QuinNAc)
(Creuzenet and Lam, 2001), UDP-N-acetyl-L-fucosamine (UDP-L-FucNAc) (Kneidinger
et al., 2003) and UDP-N-acetyl-L-quinovosamine (UDP-L-QuinNAc) (Kneidinger et al.,
2003) have been characterized at the biochemical level.

Three significant milestones have been made towards achieving a better
understanding of O-antigen LPS biosynthesis in P. aeruginosa. First, is the sequence data
of the 20 O-antigen gene islands (Raymond et al., 2002). Second, is the detailed chemical
structure of the corresponding 20 O-PS antigens (Knirel, 1990; Knirel and Kochetkov,
1994), and third, is the establishment of biochemical approaches that can be applied to
investigating enzyme kinetics. Altogether, these three components provide the foundation
to develop a model system that will be extremely powerful to study the biosynthesis of
NDP-activated sugar precursors that are required for synthesis of various carbohydrates
in a variety of microbes, such as those made by prokaryotic glycosylation systems, where
mechanisms of synthesis are less understood. The significance of this work reaches
beyond the scope of P. aeruginosa LPS biosynthesis because many of the P. aeruginosa
enzymes involved in sugar nucleotide biosynthesis are representative members of families of similar enzymes found in other medically important bacteria.

1.2.4 A-band polysaccharide (common antigen)

1.2.4.1 Structure of A-band polysaccharide

A-band PS is a D-rhamnose homopolymer arranged as repeating trisaccharide units of \([\rightarrow 2-\alpha-D-Rha-(1\rightarrow3)-\alpha-D-Rha-(1\rightarrow3)-\alpha-D-Rha-(1\rightarrow)]_n\) (Arsenault et al., 1991) with a chain length of approximately 23 repeat units (Yokota et al., 1987). A-band PS is also referred to as the “common antigen” as it is commonly isolated from *P. aeruginosa* specimens obtained from the lungs of CF patients. A study by Lam et al. (1989) demonstrated that in a group of 250 clinical isolates from patients with CF, 68% of the isolates produced A-band LPS. LPS from serial *P. aeruginosa* isolates from a single patient were analyzed and during the course of the infection, serotypeable isolates (those producing B-band LPS) became nontypeable (lost O-antigen LPS) and the major antigen on the cell surface was A-band PS. The majority of chronic *P. aeruginosa* isolates from CF patients produce A-band LPS regardless of whether these strains are completely rough or produce small amounts of B-band PS. In addition, 14 of the 20 IATS serotype strains produce A-band LPS (Lam et al., 1989; Currie et al., 1995). Interestingly, the same D-rhamnmosylated homopolymer has been isolated from other opportunistic pathogens including *Burkholderia cepacia* (Cerantola and Montrozier, 1997) and *Stenotrophomonas maltophilia* (Winn and Wilkinson, 1998) and various other bacteria (Khirel et al., 1986; 1987; Khirel and Kochetkov, 1994).
1.2.4.2 Organization of A-band polysaccharide genes

Assembly of the A-band rhamnan homopolymer in *P. aeruginosa* proceeds via the ABC-transporter-dependent pathway and has been reviewed by Rocchetta et al. (1999). According to this model (Whitfield, 1995), which has recently been reviewed by Raetz and Whitfield (2002), synthesis of the complete homopolymer proceeds processively within the cytoplasm by specific glycosyltransferases and is assembled onto Und-P. The initiating glycosyltransferase of A-band PS in *P. aeruginosa* is encoded by *wbpl*, which is located in the B-band O-antigen gene cluster (Rocchetta et al., 1998a). Subsequent transfer of the homopolymer to the periplasmic side of the inner membrane occurs via the ABC-transporter system. The ABC transporter system of A-band PS in *P. aeruginosa* consists of two proteins: Wzm, a predicted integral membrane protein with six membrane-spanning domains, and Wzt, a hydrophilic protein containing an ATP-binding motif (Rocchetta and Lam, 1997). The chain length of O-antigen homopolymers in *K. pneumoniae* O5 (Lindberg et al., 1972) and *E. coli* O8 (Iredell et al., 1998) appear to be regulated by a mechanism that involves the presence of a novel 3-0-methyl sugar at the terminal position. In *P. aeruginosa*, a 3-0-methyl rhamnose is present at the terminal end (Arsenault et al., 1991) and thus, A-PS length may be controlled in a similar manner. Ligation of the polymer to the core-lipid A requires the O-antigen ligase that is encoded within the core gene cluster.

The eight genes encoding the proteins involved in synthesis of the D-rhamnan homopolymer are clustered in one locus. The operon is organized such that the genes involved in synthesis of D-rhamnose, *rmd, gmd, wbpW*, are located at the 5' end, followed by the genes that encode the ABC-transporter system (*wzm* and *wzt*) and next, the genes
encoding the glycosyltransferases, \textit{wbpX}, \textit{wbpY} and \textit{wbpZ}. The proposed functions of the gene products are derived from analysis of the phenotypes of defined mutants and cross-complementation studies with homologous proteins from other organisms. Since these studies have been thoroughly reviewed by Rocchetta et al. (1999), only the most recent developments in this area of biosynthesis will be described here.

1.2.4.3 Biosynthesis of D-rhamnose

The nucleotide activated sugar donor, GDP-D-rhamnose (GDP-D-Rha), is required for A-band PS and is synthesized from GDP-D-mannose (GDP-D-Man) (Lightfoot and Lam, 1993; Rocchetta et al., 1998b). The enzymes required for conversion of GDP-D-Man to GDP-D Rha are Gmd and Rmd, GDP-mannose-4,6-dehydratase and GDP-4-keto-6-deoxy-D-mannose reductase, respectively. Gmd converts GDP-D-Man into GDP-4-keto-6-deoxy-D-mannose, which is reduced to GDP-D-Rha by Rmd. Metabolic studies of Gmd in \textit{P. aeruginosa} have provided evidence to support its proposed function as a GDP-mannose-4,6-dehydratase (Tatnell et al., 1994) and the 1.55 Å crystal structure of Gmd in a ternary complex with its cofactor NAD(H) and product GDP-mannuronic acid has been reported (Snook et al., 2003). Detailed characterization of \textit{P. aeruginosa} Rmd in synthesis of GDP-D-Rha has been demonstrated by coexpression of the \textit{H. pylori gmd} and \textit{P. aeruginosa rmd} in a yeast expression system (Maki et al., 2002), where the structure of synthesized GDP-D-Rha was verified by HPLC, MALDI-TOF-MS and $^1$H NMR. Detailed biochemical characterization of Gmd and Rmd from \textit{Aneurinibacillus thermoaerophilus} (Kneidinger et al., 2001), \textit{H. pylori} (Wu et al., 2001), and \textit{E. coli} (Mattila et al., 2000) further substantiate their proposed enzyme activities.
The rationale for detailed enzymology studies of the biosynthesis of nucleotide-activated rhamnose, whether the precursor is dTDP-L-Rha (found in *P. aeruginosa* LPS core) or GDP-D-Rha, is that both L- and D-Rha are found in the cell surface polysaccharides of many pathogenic bacteria. D-Rha is found not only in homopolymers of pathogens mentioned in section 1.2.4.1, but it is also present in the LPS of plant pathogens like *Xanthomonas campestris* (Hickman and Ashwell, 1966), *Pseudomonas syringae* (Smith et al., 1985) and human pathogens like *Campylobacter fetus* (Senchenkova et al., 1996) and *H. pylori* (Kocharova et al., 2000). To date, neither rhamnose nor the genes responsible for its synthesis have been identified in humans. The enzymes of these pathways may be potential antimicrobial therapeutic targets, as drugs inhibiting these enzymes are unlikely to interfere with metabolic pathways in humans.

1.3 The role of lipopolysaccharide in host-pathogen interactions

1.3.1 Lipid A

Lipid A is the endotoxic component of the LPS molecule that mediates inflammation and tissue damage associated with Gram-negative septicaemia. Immunoactivation in mammalian systems by lipid A, including LPS/lipid-A recognition and signaling systems in mammalian phagocytes has been thoroughly reviewed by Alexander and Rietschel (2001). Early studies have reported the pentaacyl form of lipid A that is produced by *P. aeruginosa* when grown under conventional bacterial culture media is less toxic for mammalian cells than the hexaacyl lipid A that is produced by *E. coli* (Kropinski et al., 1985). Several lines of evidence support the hypothesis that is due
to differences in structure of the lipid A between the two organisms (Rietschel et al.,

The discovery of CF-specific hexa-acylated lipid A that contains palmitate and
aminoarabinose substitutions (which are not found in the penta-acylated form of lipid A
that is produced by laboratory-derived strains and environmental isolates) has prompted
investigations into its biological activity, since one of the characteristic features of the P.
aeruginosa-CF lung interaction is chronic bacterially induced inflammation. Ernst et al.
(1999) reported that CF-specific lipid A has a greater ability to produce proinflammatory
cytokines by human umbilical vein endothelial cells than the lipid A isolated from
clinical bronchiectasis isolates and laboratory grown P. aeruginosa strains. Toll-like
receptor 4 (TLR4) has been implicated in transducing the signal that leads to the
production of proinflammatory mediators in response to LPS (Beutler, 2000; Poltorak et
and showed that TLR4 transmits strong proinflammatory signals in response to CF-
specific hexa-acylated lipid A but not to the penta-acylated form. More importantly, this
group demonstrated that human TLR4 discriminates between the CF hexa-acylated lipid
A and the penta-acylated lipid A through an 82-amino-acid region in the TLR4 protein.
These findings may have significant implications for P. aeruginosa infection of the CF
lung. There is data to suggest that CF individuals are initially infected with
environmentally acquired P. aeruginosa strains (Speert and Campbell, 1987; Burns et al.,
2001). These strains produce the penta-acylated lipid A form, and the inability of the
immune system to respond vigorously to this lipid A form, in addition to the other defects
in innate immunity in CF individuals (Bals et al., 1999; Guggino, 1999) may contribute to the ability of *P. aeruginosa* to colonize and establish infection.

In *P. aeruginosa*, the PhoP-PhoQ system has been shown to control resistance to aminoglycosides, polymyxin B, and cationic antimicrobial peptides (CAMP) (Ernst et al. 1999; Macfarlane et al., 1999; 2000). Data presented in the study by Ernst et al. (1999) support the hypothesis that PhoP-PhoQ mediates resistance to some of these compounds through the addition of palmitate and aminoarabinose to lipid A. A mutation in *phoQ* has been shown to decrease the virulence of *P. aeruginosa* in a burned mouse model by 100-fold when compared to the wild-type strain (Brinkman et al., 2001). Altogether, lipid A modifications that are found in CF-specific lipid A forms may contribute to the survival of *P. aeruginosa* during infection of CF airways by promoting resistance to CAMPs and other active components of the innate immune system, in addition to generating increased inflammatory responses that lead to the destruction of host tissues.

### 1.3.2 Core oligosaccharide

One area of CF microbiology concerns the consequences of mutations in the *CFTR* gene and initial infection of the CF airways with *P. aeruginosa*. The basis for hypersusceptibility of the CF lung to this organism is poorly understood, and so are the defects in innate defenses that arise as a consequence of the mutations within *CFTR*. Pier et al (1996a) hypothesized that internalization of *P. aeruginosa* within epithelial cells lining the respiratory tract and subsequent shedding of these cells may be a mechanism for clearance of bacteria from the respiratory tract. This hypothesis was based on observations made by other groups who reported this to be an antimicrobial defense mechanism for protecting against bladder infections (Aronson et al., 1979; Aronson et al.,
1988; Dalal et al., 1994; Mulvey et al., 1998). Therefore, investigations were initiated by Pier and his colleagues (1996a) to test the hypothesis that this is a clearance mechanism for *P. aeruginosa* in the airways, and that this host defense is compromised in the CF lung.

The first question addressed was whether internalization of *P. aeruginosa* was dependent on the expression of the CFTR protein. Using invasion assays, Pier et al. (1996a; 1996b) demonstrated that epithelial cells expressing the most common CFTR mutation (ΔF508 allele of the CFTR) were defective in the internalization of *P. aeruginosa* when compared to airway cells expressing the wild-type CFTR allele. The decrease in bacterial internalization by the ΔF508 CFTR cell line was found to be specific for *P. aeruginosa*, since other pathogens including human clinical isolates of *E. coli*, *Burkholderia cepacia*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* type XIV were internalized equally well by both cell lines. To identify the ligand that appeared to be specific to *P. aeruginosa* internalization by cells expressing wild-type CFTR, *P. aeruginosa* strains with mutations in genes required for production of pili, flagella, LPS, and alginate were tested for invasion, however, the strains that were deficient in the production of complete outer-core OS were the only strains that showed a significant decrease in invasion (Pier et al., 1996a; 1996b). Pier et al. (1997) used both *in vitro* and *in vivo* models to verify that CFTR is the receptor for *P. aeruginosa* core OS. In one experiment, murine cells expressing human wild-type CFTR ingested 30-100 times as many *P. aeruginosa* as cells lacking CFTR or expressing mutant ΔF508 CFTR protein. Inhibition-of-ingestion assays using monoclonal antibodies raised against a synthetic peptide that corresponds to the first predicted extracellular loop domain of CFTR,
resulted in concentration-dependent inhibition of bacterial internalization. An in vitro binding assay showed direct binding between a synthetic peptide of the extracellular loop domain of CFTR and the complete-core OS of P. aeruginosa, but not to incomplete-core OS or E. coli core OS. These studies lead to a most intriguing role for the core OS, which is as a bacterial ligand for CFTR-mediated internalization within airway epithelial cells.

The physiological significance of CFTR-mediated internalization of P. aeruginosa has been assessed using animal models. Pier et al. (1996a) evaluated the internalization of P. aeruginosa within epithelial cells and clearance of bacteria from the lungs using a neonatal mouse model of lung infection. Using this model, this group was able to show that inhibition of internalization of this microbe by the addition of purified complete-core OS along with the bacterial inoculum resulted in significantly more bacteria in the lungs when compared to the mice inoculated with bacteria alone. Inhibition of ingestion of bacteria within epithelial cells in the lung tissue was also observed when a synthetic peptide corresponding to the CFTR extracellular loop was added with the bacterial inoculum. Therefore, it was hypothesized that bacterial internalization (mediated by core OS-CFTR interactions) may be an important host defence mechanism (Pier et al., 1996a; 1997). The proposal is that in normal healthy individuals, bacteria in the airways are internalized by epithelial cells and subsequent desquamation of these cells leads to removal of invaded bacteria. Defective internalization of this microbe within the bronchioles of lungs of CF individuals would significantly increase the numbers of bacteria, which could attach and colonize the airways. This hypothesis is supported by Schroeder et al. (2001) who evaluated bacterial internalization and clearance from the lungs of wild type and transgenic CF mice.
Transgenic CF mice show significantly less ingestion of LPS-smooth *P. aeruginosa* by lung cells and significantly greater numbers of bacteria within the lungs at 4.5 hours after infection, when compared to that of the wild-type mice.

More recently, the *P. aeruginosa* core OS-CFTR interaction has been proposed to be involved in coordinating an innate immune response to *P. aeruginosa* infection. CFTR expressed in both cultured human airway epithelial cells and lung epithelial cells of transgenic mice can recognize and extract LPS from the outer membrane of this bacterium (Schroeder et al., 2002). This interaction was shown to activate nuclear transcription factor NF-κB translocation. NF-κB regulates a large variety of genes involved in apoptosis, cell growth, responses to inflammatory and stress signals, and the immune response (Epinat and Gilmore, 1999; Mercurio and Manning, 1999). Following with the original hypothesis of epithelial cell desquamation for removal of internalized bacteria, Cannon et al. (2003) showed *P. aeruginosa*-induced apoptosis to be significantly less in cultured human bronchial epithelial cells that have mutations in the CFTR alleles. Furthermore, lungs from *P. aeruginosa* infected CF mice showed no apoptosis 3 h after infection, whereas apoptotic cells are readily detected in wild-type mice.

Decreased *P. aeruginosa* internalization and delayed apoptosis of cells expressing mutant CFTR may contribute to the prolonged persistence of bacteria within CF airways, allowing for proliferation of this organism and colonization of lung tissues. Given the complexity of both the CF lung environment and the microbiology of CF lung infections, how much does a defect in this clearance mechanism, simple ingestion and desquamation
of epithelial cells containing *P. aeruginosa*, really contribute to the overall pathology of the initial infection of CF airways?

*P. aeruginosa* is a leading cause of bacterial keratitis associated with contact lens wear (Wang et al., 1998; Cheng et al., 1999) and *P. aeruginosa* LPS has been shown to be an essential virulence factor for corneal infections (Preston et al., 1995). The role of LPS core OS-mediated invasion of corneal epithelial cells has been investigated and it was determined that the minimal LPS structure necessary for maximal adherence and invasion of *P. aeruginosa* within corneal epithelial cells *in vitro* and whole mouse eyes *in situ* is the outer-core OS portion of the LPS molecule containing an exposed terminal glucose residue (Zaidi et al., 1996). Zaidi and colleagues (1999) provided evidence to support that core OS-CFTR interaction also mediates invasion within corneal epithelial cells. *P. aeruginosa* can survive and replicate within the cell following internalization (Fleischig et al., 1995) and strains possessing complete-core OS show maximal survival and reproduction intracellularly (Evans et al., 2002). Altogether, these studies suggest that the core OS of *P. aeruginosa* plays a role in corneal infections by mediating invasion and increases intracellular survival. It is proposed that these mechanisms allow the bacteria to hide intracellularly, evading host defences such as complement, antibodies, phagocytosis and antimicrobial drugs that do not readily penetrate mammalian cells. It is rather fascinating that the outcome of *P. aeruginosa* internalization mediated by core OS-CFTR binding is very different in the two different types of infection. In the CF lung, it appears to serve as a mediator of innate immunity, whereas in the cornea, it may contribute significantly to the successful colonization and infection of host tissues.
Although these studies certainly implicate the core OS as a ligand for CFTR-mediated internalization of *P. aeruginosa* by airway epithelial cells, many aspects of this interaction remain poorly understood. For example, the role of LPS in adherence is not clear. Although one study showed that the outer-core OS is required for maximal invasion, the role of LPS in bacterial binding could not be determined (Pier et al., 1996a). Attachment of bacteria to epithelial cells is thought to be a prerequisite to invasion, and many groups have reported that *P. aeruginosa* adheres to a wide variety of eukaryotic cells. However, one area of some controversy is whether the expression of CFTR on epithelial cells affects bacterial adhesion. Many studies using various cell culture systems including primary and cultured cells have shown that *P. aeruginosa* bind significantly greater to cells expressing defective or no CFTR than cells expressing wild-type CFTR (de Bentzmann et al., 1996; Bryan et al., 1998; Davies et al., 1999) whereas others report no increase in adherence to CF cells (Plotkowski et al., 1992; Cervin et al., 1994). Clearly, the role of LPS in adherence and whether adherence influences internalization needs to be clarified.

The core OS appears to play a role in biofilm formation and the biofilm mode of growth is a significant feature in the pathogenesis of *P. aeruginosa* infections (reviewed in Govan and Deretic, 1996; Donlan and Costerton, 2002). Many studies have shown a correlation between biofilm formation and upregulation of *migA*, which encodes the rhamnosyltransferase required for assembly of the uncapped core OS glycoform (or rough LPS). *migA* is highly expressed in the sputum from CF patients (Yang et al., 2000) where *P. aeruginosa* grows as biofilms (Lam et al., 1980; Singh et al., 2000). In addition, *migA* is regulated by the *rhlI/rhlR* quorum-sensing system (Yang et al., 2000), which is
also required for biofilm differentiation (Davies et al., 1998). Increased expression of
*migA* in *P. aeruginosa* leads to the loss of the “semi-rough” LPS (core OS covalently
linked to one O-antigen unit) and appears to correlate with a biofilm mode of growth
(Yang et al., 2000) that is characteristic of *P. aeruginosa* infection of the CF airways.
Direct experimental evidence, however, to show the relationship between changes in core
OS during biofilm formation is still clearly lacking, and some of the challenges in this
area of study includes the ability to detect and monitor small changes in LPS when
bacteria are grown in a biofilm system.

Changes in LPS that have been reported to occur *in vivo*, other than the CF-
specific lipid A substitutions, are CF-specific changes in the expression of O antigen. *P.
aeruginosa* produces smooth LPS during initial colonization of the airways and during
chronic infection *P. aeruginosa* produces predominantly rough LPS (loss of O antigen)
(Lam et al., 1989). Chronic *P. aeruginosa* isolates from CF patients either lack B-band O
antigen entirely or express smaller amounts (Hancock et al., 1983; Penketh et al., 1983;
Fomsgaard et al., 1988; Pitt, 1988; Lam et al., 1988). It is also intriguing that the loss of
smooth LPS appears to coincide with the production of alginate and biofilm mode of
growth. The mechanism or relationship, if any, between down-regulation of O-antigen
synthesis and the switching on of alginate production is not known. Some researchers
have proposed that the loss of O antigen occurs through the acquisition of mutations
within the O-antigen gene cluster (Evans et al., 1994; Spencer et al., 2003). Our
hypothesis, however, is that upregulation of *migA* results in an increase in the production
of the rough core OS (core glycoform that is unable to be capped by O antigen) and a
gradual decrease in the second form of core OS, which is the glycoform that can be
capped with O antigen. Upregulation of *migA* may be an adaptive response to changing the outer membrane to confer a survival advantage *in vivo*, during biofilm, and this hypothesis is currently under investigation in our laboratory.

### 1.3.3 B-band and A-band polysaccharide

The O-antigen PS is antiphagocytic and confers resistance to complement-mediated killing (Hancock et al., 1983; Dasgupta et al., 1994). O antigen is required for virulence as shown in a murine burn wound sepsis model where the LD$_{50}$ for the O-antigen deficient mutant was at least 1 000-fold higher than that of the wild-type strain (Cryz et al., 1984). In a separate study, Tang et al. (1996) used a neonatal mouse model and demonstrated that an LPS mutant was unable to initiate a respiratory tract infection whereas the wild-type strain caused acute pneumonia, bacteremia and death.

Many studies have demonstrated that *P. aeruginosa* produces different forms of LPS during the biofilm mode of growth. Giwercman et al. (1992) isolated LPS from CF isolates of *P. aeruginosa* cultured in biofilm and in broth (planktonically grown) and observed that the core-LPS fraction was more prominent in the biofilm LPS. Makin and Beveridge (1996a) propose that A- and B-band expression strongly influence cell-surface charge and hydrophobicity. This contributes to the overall strength of bacterial attachment to various surfaces, which is an important initial step in biofilm development. In the Makin and Beveridge (1996a) study, cells that produced B-band LPS had the lowest surface hydrophobicity, lowest surface charge, and adhered to glass (hydrophilic) surfaces most effectively. Strains that produced only A-band LPS or rough LPS had the highest surface hydrophobicity, possessed a more electronegative surface and adhered to polystyrene (hydrophobic) surfaces. Their data also suggested that the main surface-
charge determining groups reside in the core region of the LPS molecule. Beveridge et al. (1997) showed that *P. aeruginosa* strain PAO1 (which produces both A- and B-band LPS when grown planktonically) grew on DEAE Sepharose beads as biofilms and after five days they stopped producing O antigen. Different LPS mutants were assessed for their ability to bind to the positively charged DEAE Sepharose beads and rough strains (lacking A- and B-band LPS) attached to the beads stronger than the parent PAO1 strain. Flemming et al. (1998) observed similar results in that strains deficient in B-band LPS had a greater capacity to form biofilms on stainless steel and glass surfaces. Preliminary data from our laboratory has shown that O antigen is necessary for the initial stages of microcolony formation when cells are grown in glass laminar flow cells (Rocchetta et al., 1999). Altogether, these studies indicate that LPS is important in the initial binding of *P. aeruginosa* to abiotic surfaces and may influence initial biofilm formation.

In the studies by Makin and Beveridge (1996a), Flemming et al. (1998), and Rocchetta et al. (1999): (i) defined LPS mutants were not always used, and (ii) conclusions were based on results obtained from comparing A-band, B-band and core-LPS mutants derived from different parental strains. Also, strain differences and LPS effects on flagella-mediated motility and pili-mediated twitching motility, both of which significantly contribute to initial biofilm formation were not always investigated. Regardless, their data suggest that B-band LPS may dominate initial interactions with abiotic surfaces and the major charge-determining sites of the LPS reside in the core-lipid-A regions.

A-band LPS appears to be constitutively expressed and is commonly found in clinical and environmental isolates. McGroarty and Rivera (1990) showed that the size
and amount of common antigen from *P. aeruginosa* strain PAO1 does not change dramatically under different growth conditions. In a separate study, A-band LPS production from bacteria grown in various media, temperatures, and stress conditions was either unaffected or increased slightly (Makin and Beveridge, 1996b). Bacterial strains producing only A-band LPS have the highest surface hydrophobicity when compared to LPS mutants that produce B-band or no long chain PS (Makin and Beveridge, 1996b). Whether A-band PS induced surface hydrophobicity affects interactions of *P. aeruginosa* with host cells *in vivo* is not yet known. During chronic infection of the airways of CF patients, A-band LPS becomes the dominant LPS form expressed (Lam et al., 1989), however, this is also when the bacteria are growing as biofilms and producing the extracellular polysaccharide alginate. Anti-A-band antibodies have been found within CF patients, however, alginate can block the efficacy of such antibodies (Hatano et al., 1995). In conclusion, *P. aeruginosa* produces A-band PS during most growth conditions *in vitro* and *in vivo*, but how exactly A-band PS confers a survival advantage, is not known.

1.4 Non-lipopolysaccharide cell-surface carbohydrates: prokaryotic protein glycosylation

Many recent developments in the area of prokaryotic glycobiology have revealed that the genes, enzymes, and molecular mechanisms involved in biosynthesis of LPS are similar to those involved in synthesis of the glycan moieties that are covalently attached to proteins such as pilin and flagellin. To date, the archaeal and eubacterial S-layer glycoproteins are the best-studied glycoproteins (Schaffer et al., 2001). There are many
studies describing the structure, function, and biosynthesis of S-layer glycoproteins (Moens and Vanderleyden, 1997; Sleytr and Beveridge, 1999; Schaffer and Messner, 2001), in addition to the development of many specialized techniques dedicated for structural elucidation of proteoglycans (Schaffer et al., 2001). Many pathogenic bacteria have also been reported to possess glycoproteins and among the Gram-positive organisms include proteins of *Streptococcus sanguis* (Erickson and Herzberg, 1993) and *Mycobacterium tuberculosis* (Dobos et al., 1996). Glycoproteins identified in Gram-negative bacteria include the OspA and OspB minor proteins of *Borrelia burgdorferi*, MOMP minor proteins of *Chlamydia trachomatis*, and TibA and AIDA of *E. coli* (reviewed in Benz and Schmidt, 2002).

One of the best-described bacterial glycoproteins is the glycosylated pilin of *Neisseria meningitidis*. The approach used to study pilin glycosylation is similar to that of LPS and includes (i) determination of the chemical structure of the glycan, (ii) identification of the genes required for synthesis of the glycan, and (iii) understanding the structure-function relationship of the glycan and its biological role. The pilin from *N. meningitidis* strain C311 is glycosylated at serine 63 with a rather unusual trisaccharide structure, β-Gal-(1→4)-α-Gal-(1→3) [2,4-diacetamido-2,4,6-trideoxyhexose] (Stimson et al., 1995). The genes involved in synthesis of the pilin glycan in this strain are located in three different genetic loci. Two of these loci, *pglA* (Jennings et al., 1998) and *pgIBCD* (Power et al., 2000), are specific to synthesis of the pilin glycan, whereas *galE*, the third locus, which encodes a UDP-galactose-4-epimerase, is required for both pilin glycosylation and synthesis of the lipooligosaccharide (Stimson et al., 1995). *pglABCD* encodes proteins that are similar to those involved in LPS biosynthesis. For example,
PglA is a galactosyltransferase, PglB is a putative bifunctional enzyme with acetyltransferase and glycosyltransferase domains, PglC is a putative aminotransferase, and PglD is a putative dehydratase. These proteins are thought to be involved in synthesis of the unusual 2,4-diacetamido-2,4,6-trideoxyhexose. The actual mechanism by which the glycan is attached to the pilin during assembly of the pilus is not understood. The biological role of pilin glycosylation in this organism is not clear, however, Gubish et al. (1982) found that galactosidase treatment of isolated pili significantly reduced their attachment to Chinese hamster ovary cells and Marceau and Nassif (1999) reported that the presence of a glycan at serine 63 on N. meningitidis pilin was shown to be required for the production of a truncated monomer of S pilin (soluble pilin).

To date, the flagellin and pilin subunits that compose the flagella and pili, respectively, of P. aeruginosa have been shown to be glycosylated (Castric, 1995; Brimer and Montie, 1998). It is important to note, however, that glycosylation of pilin and flagellin is not found in all strains of P. aeruginosa. For example, in some strains neither of these structures are glycosylated, whereas other strains may only have glycosylation of one of these structures. Castric (1995) surveyed different P. aeruginosa strains and found that the majority of clinical isolates tested positive for glycosylated pilin. So far, in P. aeruginosa, the only proteoglycan with a detailed characterized structure is the pilin glycan of strain 1244. Pilin from P. aeruginosa 1244 is glycosylated at serine 148 (Comer et al., 2002) with a trisaccharide molecule: \(\alpha-5N\betaOHC_47NFmPse-(2\rightarrow4)\beta-Xyl-(1\rightarrow3)\beta-FucNAc\) (Castric et al., 2001). An essential component of the glycosylation apparatus required for pilin glycosylation in strain 1244 has also been identified and is encoded by pilO (Castric, 1995; DiGiandomenico et al., 2002). Secondary structure
programs predict that PilO contains nine hydrophobic regions and large portions of these hydrophobic regions are of β-structure and of adequate length to span the membrane lipid core. Castric (1995) proposes that PilO resides in the cytoplasmic membrane where it could function catalytically on the periplasmic side to transfer carrier lipid-bound glycan units to pilin monomers. The function of PilO, the genes required for synthesis of the glycan, and the biological role of pilin glycosylation in *P. aeruginosa* remains to be determined.

Although some strains of *P. aeruginosa* produce glycosylated flagellin (Brimer and Montie, 1998), the detailed chemical structure of these glycans is not yet known. Arora et al. (2001) identified a “flagellin glycosylation island” and showed that inactivation of either one of the two flanking genes present on this 14-gene cluster island abolished glycosylation. The gene products of the “glycosylation island” were characterized based on their similarity to other proteins in the databases. Since the chemical structure of the flagellin glycan is not known, no biosynthetic pathways, mechanisms or putative functions of the gene products in biosynthesis of a glycan structure were reported in this study.

Prokaryotic flagellin glycosylation has been well studied in *Campylobacter jejuni*. Structural analysis of the glycosylated flagellin of *C. jejuni* strain 81-176 shows that the flagellin is modified on 19 serine/threonine residues with an *O*-linked 5,7-diacetamido-3,5,7,9-tetradeoxy-L-glycero-L-manno-nonulosonic acid (pseudaminic acid) (Thibault et al., 2001). This glycosylation represents 10% of the total mass of the protein and imparts an approximate 6 000 Da shift from the molecular mass of the protein that is predicted from the DNA sequence. Interestingly, the flagellin protein in *P. aeruginosa* strain PAK,
encoded by *fliC*, also has a predicted mass based on its sequence of 41 000 Da, but the mass of the flagellin isolated from the bacterial-cell surface has a mass determined by SDS-PAGE to be 45 000 Da (Totten and Lory, 1990; Brimer and Montie, 1998). The pseudaminic acid (Pse5Ac7Ac) substituent of *C. jejuni* flagellin is closely related to the 5-N-3 hydroxybutyryl-7-N-formylpseudaminic acid reported to be part of the pilin glycan of *P. aeruginosa* strain 1244 (Castric et al., 2001). In *C. jejuni*, a cluster of genes called the *pgl* locus is involved in protein glycosylation (Szymanski et al., 1999). Although the gene products of the *pgl* cluster in *C. jejuni* show significant levels of sequence homology to enzymes involved in LPS and capsule synthesis, the *pgl* genes do not appear to be involved in LPS synthesis in *C. jejuni*. Interestingly, some of the gene products from the *pgl* cluster have been shown to modify the LPS core OS when transformed into an *E. coli* background (Szymanski et al., 1999), demonstrating the dual functionality of these gene products in protein glycan and LPS biosynthesis.

This brief introduction to glycosylation of pilin and flagellin in prokaryotes provides only an overview of some well-characterized systems and this topic has recently been reviewed (Benz and Schmidt, 2002; Power and Jennings, 2003). The most significant findings in prokaryotic glycosylation that are relevant to my research is: first, molecular characterization of the genes that encode the biosynthetic enzymes and glycosyltransferases in the synthesis of glycans, whether it be for flagellin or pilin glycosylation for example, have striking similarity to those involved in lipopolysaccharide biosynthesis, and second, little is known about protein glycosylation in *P. aeruginosa*. 

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1.5 Hypothesis and research objectives

**Rationale** - glycosylation of *P. aeruginosa* pilin and flagellin proteins is poorly understood. The structure of the pilin glycan in strain 1244 is similar to that of serotype O7 O antigen. The genes involved in pilin glycosylation had not been described, nor were the genes involved in biosynthesis of the O antigen of serotype O7. The structure of the flagellin glycan in *P. aeruginosa* strain PAK was unknown, and although a gene cluster had been implicated to be involved in synthesis of the flagellin glycan, there are examples in different prokaryotes where protein glycosylation requires more than one genetic locus. Furthermore, structural studies indicate that many sugar residues found in prokaryotic proteoglycans are highly complex, and the reported structures of the 20 serotypes of *P. aeruginosa* O-antigen PS clearly demonstrates the ability of this bacteria to synthesis complex sugar residues through the O-antigen biosynthetic machinery.

Although many studies have demonstrated core OS-CFTR mediated internalization of *P. aeruginosa* within epithelial cells *in vitro* and *in vivo*, the *P. aeruginosa* LPS mutants used for those investigations produced either complete core OS or no outer-core OS. For the invasion studies that showed core OS-mediated internalization of *P. aeruginosa* into corneal epithelial cells (Zaidi et al., 1996), the detailed chemical structure of the core OS from the mutants used in these studies has not been reported and some of the mutants are not genetically defined. Thus, identification of the specific outer-core OS residues and the core glycoform required for bacterial internalization has not been investigated. In addition, the role of LPS in attachment of the bacterium to epithelial cells of bronchial origin is not known. Further investigations are warranted to define the role of A-band and B-band LPS in attachment and to more clearly
define the region of the outer-core OS that is required for maximal internalization of *P. aeruginosa* within host cells.

**Hypothesis** - the first hypothesis is that genes involved in synthesis of the O-antigen LPS are required for glycosylation of pilin and flagellin proteins. The second hypothesis is that different forms of LPS contribute to the ability of *P. aeruginosa* to attach to and become internalized within human bronchial epithelial cells.

**Research objectives** - (i) to identify and characterize genes involved in LPS biosynthesis and to generate genetically defined LPS mutants, (ii) to examine the state of glycosylation in pilin and flagellin proteins produced by well-characterized *P. aeruginosa* LPS mutants, and (iii) to assess a panel of mutants deficient in the different forms of LPS for their ability to attach to and become ingested by human bronchial epithelial cells.

**Approaches** - a multidisciplinary experimental approach was used to achieve these objectives. Genetic tools developed by Schweizer and Hoang (1995) were used to generate chromosomal mutations in genes required for LPS biosynthesis and an immunoochemical approach using a panel of LPS-specific mAbs previously generated in this laboratory was used to characterize the phenotypes of the LPS mutants. To investigate the effect of LPS mutations on flagellin glycosylation, a combination of techniques were applied, including MALDI-TOF mass spectrometry, glycosylation and deglycosylation assays. Fluorescence microscopy and a tissue culture system with human epithelial cells were used for determining association and ingestion of the different LPS mutants by these cells.

The results from my investigations led to the novel finding that the LPS gene clusters in *P. aeruginosa* strain 1244 and PAK are required for glycosylation of pilin and
flagellin, respectively. Furthermore, combining the knowledge derived from this research and previously published results (de Kievit and Lam, 1997; Stover et al., 2000; Walsh et al., 2000), I was able to identify and characterize a gene involved in core OS biosynthesis and clarified the role of the different LPS forms in the interactions of *P. aeruginosa* with human bronchial epithelial cells.

Chapter 2. Material and Methods

2.1 Microbiology

2.1.1 Bacterial strains, plasmids, growth conditions. The bacterial strains and plasmids used are listed in Table 2.1. *E. coli* and *P. aeruginosa* strains were routinely grown at 37°C in Miller’s LB broth (Invitrogen, Burlington, ON) unless otherwise stated. For all gene replacement experiments, *Pseudomonas* isolation agar (Difco Laboratories; Becton, Dickinson and Co., Franklin Lakes, NJ) was used for selection of *P. aeruginosa* transconjugants after mating experiments. When appropriate, antibiotics (Sigma-Aldrich Canada Ltd., Oakville, ON) were used to supplement media at the indicated concentrations: ampicillin at 100 μg·ml⁻¹, kanamycin at 50 μg·ml⁻¹, chloramphenicol at 30 μg·ml⁻¹, gentamicin at 15 μg·ml⁻¹, and tetracycline at 15 μg·ml⁻¹, for *E. coli*, carbenicillin at 500 μg·ml⁻¹, tetracycline 80 μg·ml⁻¹, and gentamicin at 300 μg·ml⁻¹ for *P. aeruginosa*. IPTG (20 mM) and X-Gal (40 μg·ml⁻¹) (Invitrogen) were added to solid media to determine loss of *lacZ* α-complementation in cloning experiments utilizing the appropriate vector-host combinations.

2.1.2 Bacterial motility assay. *P. aeruginosa* strains were grown overnight at 37°C on Miller’s LB agar containing 1.5% Difco granulated agar (Invitrogen).
Table 2.1. Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Strains/plasmids</th>
<th>Genotypic or phenotypic characteristics</th>
<th>Reference/Source</th>
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</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>E. coli</em></td>
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<tr>
<td>SM10</td>
<td>thi-1 thr leu tonA lacY supE recA RP4-2-Tc::Mu, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Simon et al. (1993)</td>
</tr>
<tr>
<td>DH5α</td>
<td>Δ80dlacZAM15 Δ(lacZYA-argF) U169 recA1 endA1 hisD17(ri&lt;sup&gt;-&lt;/sup&gt;, mc&lt;sup&gt;-&lt;/sup&gt;) phoA supE44 λ&lt;sup&gt;-&lt;/sup&gt; gyrA96 thi-1 relA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>HB101</td>
<td>supE44 hsdS20(μ&lt;sup&gt;−&lt;/sup&gt; m&lt;sup&gt;−&lt;/sup&gt;) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 F&lt;sup&gt;−&lt;/sup&gt; St&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Boyer and Roulland-Dussoix (1969)</td>
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<tr>
<td><strong>P. aeruginosa</strong></td>
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<tr>
<td>1244</td>
<td>Wild-type strain, produces A-band LPS (A&lt;sup&gt;+&lt;/sup&gt;) and B-band LPS (B&lt;sup&gt;−&lt;/sup&gt;), serotype O7</td>
<td>Ramphal et al. (1984)</td>
</tr>
<tr>
<td>1244 wbpL</td>
<td>A-band deficient (A&lt;sup&gt;−&lt;/sup&gt;), B-band LPS deficient (B&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>1244 wbpM</td>
<td>A&lt;sup&gt;−&lt;/sup&gt; B&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>PAK</td>
<td>Wild-type strain, A&lt;sup&gt;+&lt;/sup&gt; B&lt;sup&gt;+&lt;/sup&gt;, serotype O6</td>
<td>ATCC33354; Liu et al. (1983)</td>
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<td>Bélanger et al. (1999)</td>
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<tr>
<td>PAK wbpP</td>
<td>A&lt;sup&gt;−&lt;/sup&gt; B&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Bélanger et al. (1999)</td>
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<td>A&lt;sup&gt;−&lt;/sup&gt; B&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Bélanger et al. (1999)</td>
</tr>
<tr>
<td>PAK rml&lt;sup&gt;C&lt;/sup&gt;</td>
<td>Truncated core, A&lt;sup&gt;−&lt;/sup&gt; B&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Rahim et al. (2000)</td>
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<td>PAO1</td>
<td>Wild-type strain, A band&lt;sup&gt;+&lt;/sup&gt;, B band&lt;sup&gt;−&lt;/sup&gt;, serotype O5</td>
<td>Hancock and Carey (1979)</td>
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<td>PAO1 wapH</td>
<td>Truncated core; A&lt;sup&gt;−&lt;/sup&gt; B&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Chapter 5</td>
</tr>
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<td>PAO1 rmd</td>
<td>A&lt;sup&gt;−&lt;/sup&gt; B&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Rahim et al. (2000)</td>
</tr>
<tr>
<td>PAO1 wbpL</td>
<td>A&lt;sup&gt;−&lt;/sup&gt; B&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Rocchetta et al. (1998a)</td>
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<td>PAO1 wbpM</td>
<td>A&lt;sup&gt;−&lt;/sup&gt; B&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Burrows et al. (1996)</td>
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<td>PAO1 wapR</td>
<td>A&lt;sup&gt;−&lt;/sup&gt; B&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Poon et al. (2002)</td>
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<td>PAO1 migA</td>
<td>Truncated core; A&lt;sup&gt;−&lt;/sup&gt; B&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Poon et al. (2001)</td>
</tr>
<tr>
<td>PAO1 rml&lt;sup&gt;C&lt;/sup&gt;</td>
<td>Truncated core; A&lt;sup&gt;−&lt;/sup&gt; B&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Rahim et al. (2000)</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pBluescript II SK</td>
<td>2.9 kb cloning vector, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>PDI Biosciences</td>
</tr>
<tr>
<td>pEX18Ap</td>
<td>5.8 kb gene-replacement vector with multiple cloning sites from pUC18, sacB&lt;sup&gt;R&lt;/sup&gt;, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Hoang et al. (1998)</td>
</tr>
<tr>
<td>pEX100T</td>
<td>5.8 kb gene-replacement vector, orit&lt;sup&gt;T&lt;/sup&gt;, sacB&lt;sup&gt;R&lt;/sup&gt;, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Schweizer and Hoang (1995)</td>
</tr>
<tr>
<td>pUCGM</td>
<td>Contains the 879 bp gentamicin cassette (Gm&lt;sup&gt;R&lt;/sup&gt;), Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Schweizer (1993)</td>
</tr>
<tr>
<td>pUCP26</td>
<td>4.9 kb pUC18-derived broad-host-range cloning vector, Tec&lt;sup&gt;+&lt;/sup&gt;</td>
<td>West et al. (1994)</td>
</tr>
<tr>
<td>pAK1900</td>
<td>4.75 kb cloning vector, pGEM-3Zf(+) derivative with pRO1600 orIT&lt;sup&gt;R&lt;/sup&gt;, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>A. Kropinski</td>
</tr>
<tr>
<td>pET-30a(+)</td>
<td>5.4 kb vector used for protein expression, contains C-terminal His·Tag sequence</td>
<td>Novagen</td>
</tr>
<tr>
<td>pCR&lt;sup&gt;R&lt;/sup&gt;II-TOPO</td>
<td>topoisomerase I-activated pCR-Blunt II-TOPO vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pFVAB1-SK</td>
<td>3 kb Xhol fragment in pBluescript II SK; contains wbpL&lt;sub&gt;1244&lt;/sub&gt; and wbpM::Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Chapter 3</td>
</tr>
</tbody>
</table>
Table 2.1. Bacterial strains and plasmids continued.

<table>
<thead>
<tr>
<th>Strains/plasmids</th>
<th>Genotypic or phenotypic characteristics</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFVAB2-18Ap</td>
<td>1.5 kb <em>KpnI-KpnI</em> insert in pEX18Ap containing <em>wbpL</em>&lt;sub&gt;1244&lt;/sub&gt;, with a Gm&lt;sup&gt;R&lt;/sup&gt; cassette cloned into the <em>NruI</em> site of <em>wbpL</em>&lt;sub&gt;1244&lt;/sub&gt;</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>pFVAB3-26</td>
<td>1.5 kb <em>KpnI-KpnI</em> fragment in pUCP26 for complementation; contains <em>wbpL</em>&lt;sub&gt;1244&lt;/sub&gt;</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>pFV163-26</td>
<td>3.6 kb <em>XbaI-SalI</em> insert in pUCP26 for complementation; contains <em>wbpM</em>&lt;sub&gt;PA01&lt;/sub&gt;</td>
<td>Burrows et al. (1996)</td>
</tr>
<tr>
<td>pFV616-26a</td>
<td>1.5 kb <em>Spel-HindIII</em> insert in pUCP26; contains <em>wbpO</em> under control of the <em>lacZ</em> promoter for complementation</td>
<td>Bélanger et al. (1999)</td>
</tr>
<tr>
<td>pWAPH-TO</td>
<td>3.6 kb PCR product containing <em>wapH</em> cloned into pCR&lt;sup&gt;®&lt;/sup&gt;-II-TOPO</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>pWAPH-Ap</td>
<td>1.3 kb <em>KpnI-EcoRI</em> fragment containing <em>wapH</em> cloned into the <em>KpnI-EcoRI</em> sites of pEX18Ap</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>pWAPH-Gm</td>
<td>840 bp <em>SmaI-SmaI</em> fragment containing the gentamicin cassette cloned within the <em>EcoRV</em> site of pWAPH-Ap; used for generating <em>wapH</em> mutation</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>pWAPH-His19</td>
<td>1.2 kb PCR product containing <em>wapH</em> and a histidine-tag coding sequence cloned into the <em>SmaI</em> site of pAK1900; used for complementation</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>pWAPH-30a</td>
<td>1.14 kb <em>NdeI-HindIII</em> fragment cloned into the <em>NdeI-HindIII</em> sites within pET-30a; used for protein expression</td>
<td>Chapter 5</td>
</tr>
</tbody>
</table>
Motility plates made with 0.3% Difco-Bacto agar (Invitrogen) were made as described by Rashid and Kornberg (2000). Bacteria from the Miller’s LB agar were transferred to the motility plates with a sterile toothpick. The toothpick was inoculated at the tip and the bacteria stabbed into the swim agar. The motility plates were wrapped in saran wrap to prevent dehydration and incubated at 30°C for 18 h. Motile bacterial cells move away from the site of the inoculum within the agar and motility was assessed by measuring the diameter of the bacterial circle.

2.1.3 Electron microscopy. A 200 mesh formvar carbon coated grid was floated on top of a 10 μl sample of bacteria that was grown overnight shaking at 150 rpm, 37°C in Miller’s LB broth. The grid was left on the sample for ca. 30 s. The grid was removed and floated on top of 20 μl of water, then touched to a Whatman filter No.1 to remove excess fluid, and repeated twice. The grid was then floated on 10 μl of a 2% uranyl acetate solution. Excess uranyl acetate was removed by touching the grid to a Whatman filter. Samples were examined using a Philips 400 TEM microscope operating at 100 kV and images captured with a 35 mm camera (Microbiology Department, University of Guelph).

2.2 DNA molecular biology

2.2.1 DNA preparation, transformations, and nucleotide sequencing.

Restriction and modification enzymes were used according to the manufacturer’s instructions and were obtained from New England BioLabs Inc. (Mississauga, ON), Amersham Biosciences Inc. (Piscataway, NJ, QC), Boehringer Mannheim (Laval, QC), and Invitrogen. DNA plasmid preparations were made using either the standard alkaline lysis method (Sambrook et al., 1989) or the High pure plasmid isolation kit (Boehringer
Mannheim). Plasmid DNA was transformed into *E. coli* by the CaCl₂ method (Huff et al., 1990). Bacterial electrotransformation was performed with electrocompetent *E. coli* (Binotto et al., 1991) and with electrocompetent *P. aeruginosa* based on the methods of Berry and Kropinski (1986) and Farinha and Kropinski (1990) with a Gene Pulser (Bio-Rad Laboratories Inc., Mississauga, ON) as recommended by the manufacturer. Chromosomal DNA was isolated from *P. aeruginosa* using DNAzol reagent (Invitrogen) according to the manufacturer’s instructions. All nucleotide sequencing reactions and determination was performed at the Guelph Molecular Supercentre (Laboratory Services, University of Guelph) using the d-Rhodamine Terminator Cycle Sequencing Ready Reaction Kit (PerkinElmer, Woodbridge, ON) unless otherwise stated.

**2.2.2 Computational analysis of genes and gene products.** Nucleotide sequence analysis was performed using the GENE RUNNER program for Windows (Hastings Software; Hastings, NY). Nucleotide and amino acid homology searches were determined using the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information; [http://www.ncbi.nlm.nih.gov/BLAST/]; Altschul et al., 1990). Homologous proteins, protein families and conserved domains were identified using: (i) Position Specific Iterated and Pattern Specific Iterated BLAST (PSI- and PHI-BLAST; National Center for Biotechnology Information; [http://www.ncbi.nlm.nih.gov/BLAST/]; Altschul et al., 1997), (ii) The Pfam Protein Families Database (Wellcome Trust Sanger Institute; [http://www.sanger.ac.uk/Software/Pfam/help/index.shtml]; Bateman et al., 2002), (iii) NCBI Domain Conserved Database (A Conserved Domain Database and Search Service, v1.62; [http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml]), and (iv) the
Carbohydrate-Active Enzymes server ([http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html]; Coutinho and Henrissat, 1999). Nucleotide and protein alignments were performed using ClustalW (European Bioinformatics Institute; [www.ebi.ac.uk/clustalw/]; Thompson et al., 1994), and prediction of transmembrane helices in proteins was determined using the TMHMM program (TMHMM Server v. 2.0; Center for Biological Sequence Analysis; The Technical University of Denmark, [http://www.cbs.dtu.dk/services/TMHMM-2.0/]; Sonnhammer et al., 1998; Krogh et al., 2001).

2.2.3 Preparation of gene probes and Southern blot hybridizations. To confirm correct insertion of the gentamicin cassette within the various P. aeruginosa mutants generated, Southern hybridizations were performed based on the methods described by Schweizer and Hoang (1995). Chromosomal DNA was isolated using DNAzol reagent (Invitrogen) according to the manufacturer’s instructions from P. aeruginosa strains including the 20 IATS serotypes, PAO1, PAK, 1244 and their respective mutants. DNA was digested with restriction endonucleases as described and then subjected to electrophoresis in 0.7% agarose gels. The DNA fragments were transferred onto a Zeta-probe membrane (Bio-Rad Laboratories Inc.) by capillary action according to instructions given in the DIG System User’s Guide for Filter Hybridization manual (Boehringer Mannheim). All DNA probes were labelled using a nonradioactive digoxigenin-dUTP labelling system (Boehringer Mannheim) as described by the manufacturer’s instructions. Gene probes wbpM and aacC1 (aacC1 encodes 3-N-aminoglycoside acetyltransferase, also designated as the gentamicin-resistance cassette) consisted of a 2.12 kb SalI-ScaI and a 0.85 kb KpnI-KpnI DNA fragment, which
contained each gene, respectively. Southern blots were performed under high stringency conditions as described by the manufacturer (Boehringer Mannheim).

**2.2.4 Generation of *P. aeruginosa* strain 1244 *wbpM* mutant.** *P. aeruginosa* 1244 *wbpM* mutants were generated by the gene replacement strategy as described by Schweizer and Hoang (1995) using a previously generated *wbpM*<sub>PAO1</sub> knockout construct (Burrows et al., 1996). Briefly, this construct contains *wbpM* from *P. aeruginosa* strain PAO1 interrupted at the *NruI* site with a gentamicin-resistance cassette cloned into the gene replacement vector pEX100T. Homologous recombination of *wbpM*<sub>PAO1</sub> allele containing the gentamicin resistance cassette with *wbpM*<sub>1244</sub> on the chromosome was confirmed by Southern blot analysis.

**2.2.5 Cloning of *P. aeruginosa* 1244 *wbpL* and construction of *wbpL*<sub>1244</sub> knockout mutant.** To verify the presence of the gentamicin resistance cassette in the 1244 *wbpM* knockouts, two separate DNA probes containing the *wbpM*<sub>PAO1</sub> gene and the *aacC1* gene, were used in Southern blots. These two DNA probes were found to hybridize to a 3.1-kb *XhoI* fragment of 1244 *wbpM* DNA in Southern hybridizations. This fragment should contain *wbpL*, which is located 5' to *wbpM* in strain PAO1. Thus, the following strategy was developed to clone *wbpL*<sub>1244</sub>. Genomic DNA from the 1244 *wbpM* mutant was digested with *XhoI* and separated by electrophoresis on a 0.7% agarose gel. DNA bands containing *XhoI* fragments between 2.5-3.5 kb were isolated from the gel using GeneClean (Mo Bio Laboratories Inc., Solana Beach, CA), ligated to the cloning vector pBluescript II SK and transformed into *E. coli* DH5α by CaCl<sub>2</sub> transformation. Transformants containing a plasmid with the 3.1-kb *wbpM* insert were selected by growth on Miller's LB agar containing 15 µg·ml<sup>-1</sup> gentamicin. The presence of *wbpL* was
verified by performing sequence analysis of the 3.1-kb insert of pFVAB1-SK. To
generate the *wbpL* knockout construct, a 1.5-kb *KpnI* fragment containing *wbpL* was
then cloned into the replacement vector, pEX18Ap and a 879 bp gentamicin resistance
cassette from pUCGM was then inserted into the unique *NruI* site within *wbpL*, and
the new construct was designated as pFVAB2-18Ap. The successful construction of 1244
*wbpL* mutants was confirmed by Southern blot analysis.

**2.2.6 PCR amplification of the PAK *fliC* gene.** The *fliC* gene from *P.
aeruginosa* wild-type strain PAK and its *wbpO* and *rmlC* mutants was isolated by PCR
amplification using the forward primer 5'-
AAGCGCCGATAAAGATCGAATGCGAAC-3' and the reverse primer 5'-
AAGCTTGAGCGACGAGGCTCA-3' using *Pwo* enzyme (Roche Diagnostics Canada,
Laval, QC). The primers were designed based on the published sequence of *fliC* from
Totten and Lory (1990). Genomic DNA from the PAK strains was isolated using DNAzol
reagent (Invitrogen) according to the manufacturer’s instructions. The PCR reaction
consisted of 100 ng of genomic DNA, 50 pmole of each primer, 0.25 nM each dNTP, 2.0
mM MgCl₂, and 5 μl *Pwo* DNA polymerase PCR buffer (10X without MgCl₂; Roche
Diagnostics Canada) in a total of 50 μl. A 5 min denaturation at 95°C was performed
before the addition of 1.5 units of *Pwo* DNA polymerase. This was followed by 25 cycles
of 30 s at 95°C, 30 s at 52°C and 1 min at 72°C. A final elongation step of 7 min at 72°C
was included. The 1.3 kb PCR product containing *fliC* was purified using the PCR
purification kit (Boehringer Mannheim) as described by the manufacturer. Nucleotide
sequencing of the purified PCR product was performed using the forward and reverse
primers described above in addition to two other internal forward primers, 5'
ACGCAACGACGATCTCCCTGG-3' and 5'-TTGCAAACACCACATCAACAACCTG-3'.

2.2.7 Generation of *P. aeruginosa* PAO1 *wapH* mutant. For construction of the PAO1 *wapH* mutant, a DNA fragment containing *wapH* was PCR amplified from genomic DNA isolated from *P. aeruginosa* strain PAO1 using the upstream primer 5'-TGGAATTCTGCGCATCGCCACCGC-3' and the downstream primer 5'-GCAGATCTAGTGCCAGGGCTGGCGAT-3'. The PCR reaction consisted of 100 ng of genomic DNA, 50 pmole of each primer, 0.25 nM of each dNTP, 1.5 mM MgCl$_2$, and 5 µl *Pwo* DNA polymerase PCR buffer (10X without MgCl$_2$) in a total reaction volume of 50 µl. A 4 min denaturation at 94°C was performed before the addition of 1.5 units of *Pwo* DNA polymerase. This was followed by 10 cycles of 1 min at 94°C, 30 s at 60°C and 1 min at 68°C, followed by 20 cycles of 1 min at 94°C, 30 s at 60°C and 1.5 min plus 20 s (per cycle). A final elongation step of 7 min at 68°C was included. The 3.6 kb PCR product generated was cloned into the pCR®II-TOPO vector (Invitrogen). This construct, designated pWAPH-TO, was digested with *Kpn*I and *Eco*RI, which generated a 1.3 kb fragment containing *wapH*. This fragment was cloned into the *Kpn*I-*Eco*RI sites of pEX18Ap and the resultant plasmid was designated pWAPH-Ap. The 840 bp gentamicin cassette was isolated from pUCGM using the *Sma*I sites and this cassette was cloned into the *EcoRV* site within the *wapH* coding sequence within pWAPH-Ap. This construct, called pWAPH-Gm, was used to generate a *wapH* mutant in strain PAO1 by homologous recombination according to the method of Schweizer and Hoang (1995). To confirm that allelic replacement had occurred, forward oligonucleotide primer 5'-AATGCGCAGATCAAGTTCCGCGAGCG-3' and reverse primer 5'-
AAGATAGATGGCAGCGAACTCGGCCG-3' were used to amplify \textit{wapH} from potential mutants. The conditions used for PCR amplification were the same as described for PCR amplification of \textit{wapH} for generating the knockout construct. PCR-amplification reactions were performed with a Gene Amp 2400 PCR System Thermocycler (PerkinElmer).

\textbf{2.2.8 \textit{In vivo} complementation of \textit{P. aeruginosa} \textit{wapH} mutants.} For complementation experiments, \textit{wapH} with the C-terminal His-Tag coding sequence was used and PCR amplified from the pWAPH-30a plasmid using the following upstream and downstream primers, 5'-TAATACGACTCACTATAGGGATTTG-3' and 5'-ACCAAGGGGTATGCTAGTTATTGC-3', respectively. The PCR reaction consisted of 50 ng of plasmid DNA, 50 pmole of each primer, 0.25 nM of each dNTP, 1.5 mM MgCl$_2$, and 5 µl \textit{Pwo} DNA polymerase PCR buffer (10X without MgCl$_2$) in a reaction volume of 50 µl. A 5 min denaturation at 95°C was performed before the addition of 1.5 units of \textit{Pwo} DNA polymerase. This was followed by 25 cycles of 1 min at 95°C, 30 s at 54°C and 57 s at 72°C. A final elongation step of 7 min at 72°C was included. The 1.2 kb PCR product generated by \textit{Pwo} DNA polymerase was purified using the High Pure PCR Template Purification Kit (Roche Diagnostics Corp.) according to the manufacturer’s instructions and then ligated into \textit{P. aeruginosa-E. coli} shuttle vector, pAK1900, which was previously digested with \textit{SmaI}. This construct was designated pWAPH-His19 and the \textit{wapH} insert of this plasmid was checked by nucleotide sequencing using T7 forward and reverse universal primers, 5'-TAATACGACTCACTATAGGC-3', and 5'-GATCAATAACGAAAGTCGCCA-3', respectively.
2.2.9 Cloning of \textit{wapH} in the PET system. The \textit{wapH} gene was PCR amplified from genomic DNA isolated from \textit{P. aeruginosa} strain PAO1 using the following upstream and downstream primers, 5'-

\text{TATATATCTCATATGACTCGGTCGGCTGAACCTCG-3'} \text{ and 5'-}

\text{TATATAAGCTTTCCGCATGCAGACTCCGAAGCGAAGC-3'}, respectively. The PCR reaction consisted of 100 ng of genomic DNA, 50 pmole of each primer, 0.25 nM of each dNTP, 1.5 mM MgCl₂, and 5 \mu l \textit{Pwo} DNA polymerase PCR buffer (10X without MgCl₂) in a reaction volume of 50 \mu l. A 5 min denaturation at 95°C was performed before the addition of 1.5 units of \textit{Pwo} DNA polymerase. This was followed by 25 cycles of 1 min at 95°C, 30 s at 57°C and 47 s at 72°C. A final elongation step of 7 min at 72°C was included. The 1.14 kb PCR product was cut with \textit{NdeI} and \textit{HindIII} and ligated into pET-30a, which was previously digested with \textit{NdeI} and \textit{HindIII}. This construct resulted in the incorporation of a coding sequence for a C-terminal histidine tag at the end of the \textit{wapH} coding sequence. The ligation mixture was transformed into \textit{E. coli} JM109.

Transformants were screened for the presence of pET-30a containing \textit{wapH} by PCR amplification of the insert of pET-30a from whole cells as described by Sambrook et al. (1989) using the T7 promoter and T7 terminator primers described in section 2.2.8. The \textit{wapH} gene in pET-30a construct was confirmed by nucleotide sequencing and was designated pWAPH-30a. This construct was transformed into \textit{E. coli} expression strain BL21 (DE3) (Novagen).
2.3 Analysis of LPS

2.3.1 SDS-PAGE and Western immunoblotting. LPS was prepared from whole cell lysates of *P. aeruginosa* by the method of Hitchcock and Brown (1983). LPS samples were subjected to electrophoresis on 12.5% glycine SDS-PAGE gels prepared according to Hancock and Carey (1979) based on the standard discontinuous buffer system of Laemmli (1970), or by commercially prepared Novex® 10-20% tricine SDS-PAGE gels (Invitrogen) with tricine running buffer prepared according to the manufacturer’s instructions. This tricine system is based on that developed by Schaegger and von Jagow (1987) and tricine replaces glycine in the running buffer. LPS was visualized by silver staining using the method of Dubray and Bezard (1982) for glycine SDS-PAGE and Tsai and Frasch (1982) for tricine gels. For Western immunoblots, LPS separated by SDS-PAGE was transferred to BioTrace® NT nitrocellulose (Life Sciences, Ann Arbor, MI) and the membranes blocked with 3% skim milk. For detection of *P. aeruginosa* LPS, blots were immersed in hybridoma-culture supernatants containing monoclonal (mAb) MF15-4 (specific for B-band LPS of serotype O5) (Lam et al., 1987a), or mAb N1F10 (specific for A-band LPS) (Lam et al., 1989), or mAb 101 (specific for LPS-outer core) (de Kievit and Lam, 1994). After incubation with the corresponding first antibodies, blots were washed and then incubated with goat anti-mouse F(ab’)_2-alkaline phosphatase conjugate (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) at the dilution suggested by the manufacturer. Following a series of washes, as described by de Kievit and Lam (1994), the blots were developed using a substrate consisting of 30 mg of Nitro Blue Tetrazolium (NBT) and 15 mg 5-bromo-4-
chloro-3-indolylphosphate (BCIP; Sigma-Aldrich Canada Ltd.) in 100 ml of 0.1 M sodium bicarbonate buffer (pH 9.8).

2.3.2 LPS extraction using hot aqueous phenol. LPS was extracted from whole cells with aqueous phenol by the method developed by Westphal and Lederitz (1954) with the following modifications. *P. aeruginosa* strains were grown in 1.5 L of Miller’s LB broth in 6 L erlenmeyer flasks (a total of 10 flasks were used per strain) for 18 h at 37°C, shaking at 175 rpm. Bacterial cells were collected by centrifugation at 8000 x g for 15 min and the cell pellet was lyophilized. Approximately 30 g of dry cell pellet was ground into a fine powder and resuspended in 300 ml of water that was preheated to 70°C. To this cell suspension, 350 ml of 95% phenol (Fisher Scientific Ltd., Nepean, ON) preheated to 70°C was added and this suspension was vigorously stirred for 3 h in a round bottom flask situated within a 70°C hot water bath. The mixture was allowed to cool for ca. 4 h at room temperature and packed on ice overnight to promote phase separation. The suspension was centrifuged at 12 000 x g for 20 min to separate the aqueous and phenol layer and the aqueous layer containing the LPS was removed and clarified with 5 more rounds of centrifugation. The clarified aqueous phase was dialyzed against running tapwater for 72 h and lyophilized.

2.4 Analysis of protein

2.4.1 Pilin isolation. Pilin was isolated from *P. aeruginosa* strains based on the method described by Frost and Paranchych (1977) with the following modifications. *P. aeruginosa* strains were inoculated into 100 ml of Miller’s LB and incubated for 6 h at 37°C with shaking at 150 rpm. Growth of bacteria on solid medium involved spreading 10 ml of this culture onto 1 L of Miller’s LB agar in aluminum trays (27 cm by 40 cm by
0.8 cm). The trays were covered with aluminum foil lids and incubated overnight at 37°C. Bacteria from 12-16 trays were harvested by scraping the surface of the agar and resuspending the cells (ca. 70-100 g wet weight) in 1 L of standard saline citrate buffer (0.15 M sodium chloride and 0.15 M sodium citrate adjusted to pH 7.0 with NaOH). The bacterial suspension was stirred for 2 h at 4°C. Pili were removed with shear force by blending 200-ml portions for 3 min on the high-speed setting of a Waring blender. The bacteria were then removed from this suspension by centrifugation at 12 000 x g for 20 min. The supernatant containing the pili was carefully removed and centrifuged again, usually 4-5 times, until no pellet was observed in the bottom of the centrifuge tube. The concentration of NaCl in the supernatant solution was then slowly adjusted to 0.5 M. Polyethylene glycol (PEG 600; Fisher Scientific Ltd.) was then added to a final concentration of 1% w/v and the solution stirred for 20 h at 4°C. Pili precipitated under these conditions and was collected by centrifugation at 12 000 x g for 30 min. For the wild-type strain, the pili-containing pellet was resuspended in water and dialyzed over a 48-h period at 4°C with changes in water every 6 h. The pili-containing pellets isolated from the LPS mutants and complemented mutants were resuspended in a 10% (w/v) ammonium sulfate (pH 4.0). This solution was stirred gently at 4°C for 1 h and then allowed to stand at 4°C overnight with no stirring. The pili precipitated under these conditions and was collected by centrifugation at 12 000 x g for 30 min. The pellet was resuspended in water and dialyzed as described above. This process was found to remove some of the contaminating protein in the pilin preparations. After dialysis, the pilin samples were lyophilized overnight to concentrate the sample, thawed in a water-ice mixture, and distributed into 30 μl volumes and then stored at -80°C.
2.4.2 Flagellin isolation. Flagella was purified from the different *P. aeruginosa* strains based on the procedure described by Montie et al. (1982) with the following modifications. *P. aeruginosa* strains were inoculated onto Miller’s LB agar and incubated overnight at 37°C. A single colony was then used to inoculate 3 ml of Davis minimal broth (Difco Laboratories) supplemented with 0.01 mM glucose, 0.8 mM MgSO₄, 0.015 mM thiamine, and 0.04 mg·ml⁻¹ casamino acids. The culture was incubated overnight at 37°C with shaking at 175 rpm. These cultures were used to inoculate 100 ml of supplemented Davis minimal broth, which was incubated at 37°C, 150 rpm, for 6 h. Ten milliliters of this culture was used to inoculate 1 L of prewarmed supplemented minimal broth, and a total of 10 L was grown for 12-14 h at 37°C, shaken at 150 rpm. Bacteria were collected by centrifugation at 8 000 x g at 4°C for 15 min and gently resuspended in 400 ml of 50 mM sodium phosphate buffer (Sambrook et al., 1989) containing 10 mM MgCl₂ at room temperature with gentle stirring for 30 min. The bacterial suspension was then stirred gently at 4°C overnight. Flagella were sheared off the bacteria by blending the bacterial suspension in a Waring blender at high speed for 1.5 min. Bacteria and contaminating proteins were removed by five cycles of centrifugation at 14 000 x g, at 4°C for 30 min. The flagella in the supernatant were recovered by 20% ammonium sulphate precipitation as described by Brimer and Montie (1998) with the following changes. After each addition of 5% ammonium sulfate, the suspension was stirred gently at room temperature for 5-6 h. The flagellin were collected by centrifugation at 12 000 x g for 30 min. The flagella-containing pellet was resuspended in 10 ml of 50 mM sodium phosphate buffer and dialyzed over a 48 h period with four changes in water at 4°C. The flagella preparation was then lyophilized. Flagella stock suspensions were made in water
or 50 mM sodium phosphate buffer depending on the experiment and frozen at -20°C. The flagellin preparation was assessed for purity by SDS-PAGE analysis. The preparation of flagella from the *P. aeruginosa* LPS mutants was more difficult than from the wild-type strain since the LPS mutants were more susceptible to cell lysis during the initial steps of the isolation procedure, resulting in large amounts of contaminating protein. If the sample contained many contaminating proteins, the sample was brought up to a volume of 200 ml in sodium phosphate buffer and the flagella was again recovered by repeated ammonium sulphate precipitation. Flagella prepared in this manner were about 90% pure and were typically contaminated with pilin. These samples were used for all of the analyses including the glycosylation assay, deglycosylation assay, isoelectric focusing, and mass spectroscopy.

2.4.3 Analysis of proteins by SDS-PAGE. Purified proteins, including flagellin, pilin, WapH, and the low range molecular weight markers (Bio-Rad Laboratories) were resuspended in protein sample buffer consisting of 12.5% 0.5 M Tris-HCl, 0.4% SDS buffer (pH 6.8), 10% glycerol, 2% SDS and 0.02% bromophenol blue. All samples were heated at 100°C for 10 min just before electrophoresis, on 10% or 12.5% glycine gels that were prepared based on the Laemmli system (Laemmli, 1970) or commercially prepared NuPAGE® Novex 4-12% Bis-Tris gels (Invitrogen; NuPAGE® system is based upon a Bis-Tris-HCl buffered (pH 6.4) polyacrylamide gel, with a separating gel that operates at pH 7.0; [http://www.invitrogen.com/content.cfm?pageid=3468]) that were used according to the manufacturer’s instructions. Proteins were stained with Coomassie brilliant blue R-250 solution (Sambrook et al., 1989).
**2.4.4 Isoelectric focusing.** Isoelectric focusing (IEF) was performed as follows using an XCell Surelock™ Mini-Cell (Invitrogen). Samples containing flagella were mixed with an equal volume of IEF sample loading buffer (Invitrogen) and loaded onto Novex® IEF gels with a range of pH 3-7 (Invitrogen) and electrophoresis was performed according to the manufacturers instructions (Novex pre-cast gel electrophoresis guide, Invitrogen, http://www.invitrogen.com/content/sfs/manuals/electrophoresisguide_man.pdf). After electrophoresis, the IEF gel was fixed in 12% trichloroacetic acid for 30 min and then washed with distilled water over a period of 45 min with the water changed every 5 min. The IEF gel was stained with SimplyBlue™ Safestain (Invitrogen) and washed according to the manufacturer’s instructions. For the Western immunoblotting experiment, proteins were transferred from the IEF gel after electrophoresis to Biotrace™ nitrocellulose using a Bio-Rad transfer cell for 1 h at 150 mA using tris-glycine transfer buffer as described by the manufacturer (Invitrogen).

**2.4.5 Adsorption of nonspecific antibodies from anti-FliC antiserum.** Rabbit antiserum to *P. aeruginosa* strain PAK flagellin was a gift from Dr. Reuben Ramphal (Department of Medicine/Infectious Diseases, University of Florida, Gainesville, FL). To increase the specificity, the antiserum was adsorbed against proteins isolated from *P. aeruginosa* strain PAK-N1, a mutant that does not produce flagella. Whole cell protein lysates were prepared from strain PAK-N1 as follows: 5 ml of a bacterial culture grown overnight in Miller’s LB broth, 37°C, was centrifuged 13 000 x g for 2 min and the pellet was resuspended in protein sample buffer and boiled in a water bath for 10 min. This protein preparation was resolved on a 10% glycine SDS-PAGE gel and transferred to
Biotrace™ nitrocellulose. To confirm proteins had bound to the nitrocellulose, the membrane was stained with Ponceau S as described by Harlow and Lane (1988). The nitrocellulose was cut into 0.5 x 8 cm pieces. The original antiserum (15 µl) was diluted in 7.5 ml of phosphate-buffered saline (PBS; 0.125 M NaCl, 0.0015 M KH₂PO₄, 0.008 M Na₂HPO₄·12H₂O, 0.007 M KCl, pH 7.0) and sodium azide was added to a final concentration of 0.02%. Five nitrocellulose strips were incubated in the 7.5 ml antiserum for 4 h at room temperature on a CLAY ADAMS Brand Nutator (Becton Dickinson and Co.). The strips were then removed, 5 new strips added, and the antiserum was again incubated as described. This process was repeated 5 times with fresh strips each time and the antiserum was stored at -20°C. The specificity of the antiserum for FliC was tested by Western immunoblotting using flagellin prepared from the wild-type PAK and PAK-N1 (serves as a negative control).

2.4.6 Western immunoblotting of PAK flagellin using anti-FliC antibodies.

Flagellin protein was resolved on 10% glycine gels or IEF gels as described earlier, and the proteins were transferred to Biotrace™ using the Bio-Rad Western transfer apparatus at 40 V for 4 h. The membrane was removed from the blotter and rinsed twice for 1 min using PBS. The membrane was blocked in PBS containing 3% bovine serum albumin (BSA) for 30 min and then incubated overnight at room temperature in the adsorbed anti-FliC antiserum on a rotating platform. The membrane was thoroughly washed in PBS for 10 min each, six times. Secondary goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad Laboratories) was used at a 1:3,000 dilution in PBS and incubated for 2 h at room temperature. The membrane was washed in PBS, for 10 min each, five
times. The immunoblot was developed by the addition of developing buffer and a solution of NBT and BCIP as described in section 2.3.1.

2.4.7 Flagellin glycosylation detection. Flagellins were resolved on a 10% glycine gel and transblotted onto nitrocellulose as described in the earlier section on Western immunoblotting of flagellin protein. The glycosylation detection protocol of O’Shannessy et al. (1987) was used with the following modifications. All incubations took place at room temperature. After transfer of the proteins to the nitrocellulose, the membrane was washed with 10 ml PBS (9 mM sodium phosphate, 27 mM sodium chloride, pH 7.2) for 10 min. The membrane was incubated in 15 mM sodium periodate (Sigma-Aldrich Canada Ltd.) in 50 mM sodium acetate buffer (50 mM sodium acetate, 50 mM acetic acid, pH 5.5) for 30 min in the dark. The membrane was washed for 10 min with PBS and repeated three times and then incubated in 5 mM biotin hydrazide (Sigma-Aldrich Canada Ltd.) in 50 mM sodium acetate buffer for 1 h. The nitrocellulose was washed three times, 10 min per wash in tris-buffered saline (TBS; 50 mM Tris, 27 mM sodium chloride, pH 7.2) and then blocked for 30 min in TBS containing 3% BSA. The membrane was washed three times, 10 min per wash in TBS and incubated for 2 h in a 1:1,000 dilution of alkaline phosphatase streptavidin (Vector Laboratories Inc. Burlingame, CA) in TBS. This was followed by three washes for 10 min each in TBS. The membrane was developed in the same developer, NBT and BCIP solutions as described for the development of Western immunoblots for LPS in section 2.3.1. The nonglycosylated WbpD protein used as a negative control was a gift from Cory Wenzel (University of Guelph; Wenzel, 2004) whereas transferrin was purchased from Sigma-Aldrich.
2.4.8 Deglycosylation assay. Sugar residues were removed from glycosylated flagellin using the GlycoFree™ Deglycosylation Kit (Glyco Inc; Novato, CA) as per the manufacturer’s instructions. This procedure is based on the chemical deglycosylation of proteoglycans using anhydrous trifluoromethanesulfonic acid to remove both N- and O-linked glycans as described by Edge et al. (1981).

2.4.9 Mass spectrometry of flagellins. Lyophilized flagellin protein was first resuspended in water, and then a 1:1 mixture of protein solution to matrix solution was made (matrix solution - sinapinic acid in 50% acetonitrile (ACN), 0.1% TFA and 1 µl was spotted onto the MALDI plate. Some of the flagellin samples were desalted directly on the MALDI plate by pipetting 2 µl of water onto the spot and removing after 1 min using a gel-loading tip. Analysis of intact flagellin proteins was performed using a Reflex III MALDI-TOF instrument (Bruker, Germany) equipped with a 337 nm nitrogen laser (Biological Mass Spectrometry Facility, Department of Molecular Biology and Genetics, University of Guelph). The instrument was externally calibrated using various protein standards for different flagellin samples and these included cytochrome C, bovine serum albumin, trypsinogen, and alcohol dehydrogenase (all standards were obtained from Sigma-Aldrich Canada Ltd.). Mass spectrometry of intact flagellins was performed in the linear detection mode, in the positive mode, with laser energy of 35-40%.

The protocol for in-gel digestion was provided by Dr. Dyanne Brewer of the Biological Mass Spectrometry Facility (Department of Molecular Biology and Genetics, University of Guelph [http://www.uoguelph.ca/mbgwww/bmsf/Ingeldigestday1.htm, http://www.uoguelph.ca/mbgwww/bmsf/Ingeldigestday2.htm]) and was performed as described here briefly. Flagellins were subjected to electrophoresis on 10% glycine SDS-
PAGE gels and the gel was stained with Coomassie brilliant blue and destained. The flagellin protein band and a non-protein containing region were excised from the gel and sliced into smaller pieces. The gel bands were rinsed thoroughly in water. A 50 mM NH₄HCO₃/50% ACN solution was added to cover the gel slices in a siliconized eppendorf tube and the mixture was then vortexed for 10 min. This suspension was centrifuged for 30 s at 13 000 x g; the supernatant was removed and discarded, and this step was repeated 5 to 6 times. To each tube, 100 µl of 100% ACN was added and incubated for 5 min. This suspension was centrifuged and the supernatant discarded. The gel particles were covered with 100 µl of 10 mM DTT (in 100 mM NH₄HCO₃) and incubated at 50°C for 30 min. Samples were centrifuged for 30 s at 13 000 x g and all liquid was removed. Next, 100 µl of ACN was added to shrink the gel particles and the excess fluid was removed. The particles were alkylated by adding 100 µl of 55 mM iodoacetamide (in 100 mM NH₄HCO₃) and incubated at room temperature for 30 min in the dark. Samples were centrifuged and all liquid was removed. The gel particles were washed with 100 µl of 100 mM NH₄HCO₃ for 15 min with occasional vortexing. The samples were then centrifuged and all liquid was removed. To shrink the particles, 100 µl of ACN was added and samples were incubated for 5 min, and the liquid was removed. The samples were dried in a Speedvac for 20 min. For the trypsin digestion, 20 µl of sequencing grade trypsin (Sigma-Aldrich Canada Ltd.) was added and the samples were left to rehydrate for 1 h at room temperature. This was followed with the addition of 50 µl of 100 mM NH₄HCO₃ and the gel particles were incubated for an additional 18 h at 37°C.
For peptide extraction, the samples were vortexed briefly and centrifuged. Then 50 µl of water was added, vortexed for 2 min and centrifuged again. The gel particles were sonicated for 10 min in a bath sonicator. The samples were vortexed briefly and centrifuged for 30 s. The supernatant was then transferred to a new tube containing 5 µl of 5% formic acid in 50% ACN. Another extraction was performed by adding 75 µl of 5% formic acid in 50% ACN to the original tube containing the gel pieces, these samples were vortexed for 2 min, centrifuged, and sonicated for 5 min. The supernatant was collected and combined with the supernatant from the first extraction. The volume of the samples was reduced to 10-15 µl using the Speedvac.

A C18 ZipTip (Millipore Corp., Nepean, ON) was equilibrated in 0.1% TFA in water. The peptide solution was then aspirated into the ZipTip about 15 times to allow for maximal binding of peptides to the column matrix. To elute the peptides, 4 µl of elution solution (50:50 ACN/water with 0.1% TFA saturated with α-cyano-4-hydroxycinnamic acid) was added to new siliconized tubes. This solution was aspirated and dispensed through the ZipTip about 10-15 times to elute the peptides and 1 µl of this desalted sample was added directly onto the MALDI plate in duplicate. Tryptic peptide digests were also analyzed using an Agilent LC-MSD instrument and the HPLC with autosampling device was coupled to a single quadrupole detector with a binary pump and the variable wavelength detector (VWD) set at 220 nm, for analysis in the positive ion mode. Peptides were separated using an Agilent ZORBAX Extend C-18 (300 Å) column (length, 150 mm; inside diameter, 4.6 mm; particle size, 3.5 µm) with online degasser. A linear gradient of 5-65% ACN (1% acetic acid) over 30 min at a rate of 1 ml·min⁻¹ was used. External calibration was performed prior to sample analysis in positive ion mode.
using calibration solution from Agilent (Scientific Instrument Services, Inc., Ringoes, NJ).

2.4.10 **Expression and purification of WapH.** For protein expression, *E. coli* BL21 (DE3) containing pWAPH-30a was grown overnight at 37°C with shaking at 175 rpm in 5 ml of Miller’s LB broth in the presence of ampicillin. The next morning, the 5 ml culture was used to inoculate 250 ml of Miller’s LB broth with ampicillin. The culture was grown at 37°C and when the OD<sub>600</sub> reached 0.6, the culture was removed to room temperature for 20 min and then isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM. Expression was allowed to proceed for 6 h at 30°C, shaking at 175 rpm. The cells were harvested by centrifugation at 5 000 x g for 20 min at 4°C, at which time the supernatant was discarded and the pellet was frozen at -20°C. The frozen pellet was resuspended in cold 20 mM sodium phosphate buffer, 0.5 M NaCl (pH 7.4) and then passaged through a French pressure cell four times (1 000 psi). After sequential centrifugation at 10 000 x g (20 min, 4°C) and 100 000 x g (1 h, 4°C), the supernatant was cleared by filtration through a 0.4-μm membrane and this protein sample was then used for protein purification by affinity chromatography. A 5-ml HiTrap column (Amersham Pharmacia Biotech Inc.) was loaded with nickel ions (NiCl₂, 5 mg·ml⁻¹), washed with water and equilibrated in 20 mM sodium phosphate buffer, 0.5 M NaCl, 10 mM imidazole (pH 7.4). The column was loaded with the protein sample that had been adjusted to contain 10 mM imidazole. The column was washed with 50 ml 20 mM sodium phosphate buffer, 0.5 M NaCl, 50 mM imidazole. The proteins were eluted in stepwise increments of buffer containing 100, 150, 200, 250, 300, 350, 400, 450, and 500 mM imidazole. The presence of the histidine-tagged protein was confirmed by Western
immunoblotting using the monoclonal antibody His-probe (H-3; Santa Cruz Biotechnology, Inc; Santa Cruz, CA) at a 1:1,000 dilution and the method of detection used was according to that described in the Western and Colony Blot protocols manual (Qiagen Inc., Mississauga, ON).

2.5 Cell culture

2.5.1 Culture of epithelial cell lines. The IB3 cell line is adenovirus 12-SV40 hybrid-transformed human bronchial epithelial cells from a CF patient (ΔF508/W1282X). The C38 cell line is IB3-derived, stably expressing a transfected functional CFTR (Zeitlin et al., 1991). The IB3 and C38 cell lines were kindly provided by Dr. Greg Downey (University of Toronto, Toronto, ON; originally from Dr. P. Zeitlin, John Hopkins University, Baltimore, MD). The IB3 and C38 cell lines were maintained in LHC-8 media (Biofluids, Rockville, MD) supplemented with 5% (v/v) fetal calf serum, 100 μg·ml⁻¹ streptomycin, and 100 units·ml⁻¹ penicillin. Cell lines were passaged following harvest with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA·4Na) and washing with unsupplemented media. The transformed human bronchial epithelial cell line 16HBE14o- (Cozens et al., 1992) was kindly provided by Dr. Neil Sweezy (University of Toronto; originally from Dr. D. C. Gruenert, University of California, San Diego, CA). This cell line was maintained in Earle’s minimal essential media (MEM) supplemented with 5% fetal calf serum, 2 mM L-glutamine, 100 μg·ml⁻¹ streptomycin, and 100 units·ml⁻¹ penicillin. Epithelial cells were grown as culture stocks in 75 cm² Corning tissue culture flasks (Fisher Scientific Ltd.) at 37°C with 5% carbon dioxide and usually fed every other day. The cells were passaged about every five days when the cells reached about 80% confluency. For passaging of the 16HBE14o- cells, growth media was removed and 2.5
ml of trypsin-EDTA (0.25% trypsin, 1mM EDTA-4Na) was added and the flask was put into a 5% CO₂, 37°C incubator until cells came off the bottom of the flask, which took ca. 5 min. Then 5 ml of fresh media was added to the flask to dilute the trypsin solution. The cells were centrifuged at 3 000 x g for 3 min and the supernatant removed. Five milliliters of fresh media was added and the cell pellet was gently resuspended, counted using a hemocytometer and then seeded into new flasks or tissue culture plates. All tissue culture media and reagents (mentioned in this section) used for maintenance and propagation of cells were purchased from Invitrogen unless otherwise indicated.

2.5.2 Cytotoxicity assays. Cytotoxicity of the bacterial inoculum for each the different epithelial cell lines was assessed in a semiquantitative manner using the Trypan Blue exclusion assay described by Fleiszig et al. (1996) with some modifications. For the IB3 and C38 cell lines, confluent monolayers containing ca. 1x 10⁵ cells (the number of cells within one well of a 96-well plate) were washed three times with antibiotic-free MEM and infected with varying amounts of P. aeruginosa strain PAO1 and E. coli HB101 resuspended in MEM supplemented with 5% (v/v) fetal calf serum. The inoculum consisted of these bacterial strains at the following colony forming units (CFU): 1 x 10⁶, 2.5 x 10⁶, 5 x 10⁶, 1 x 10⁷, 2.5 x 10⁷, 5 x 10⁷, 1 x 10⁸, which corresponded to a multiplicity of infection (MOI; number of bacteria per epithelial cell) of 10, 25, 50, 100, 250, 500, 1000, respectively. The plates were centrifuged for 5 min at 600 x g at room temperature in a clinical centrifuge (GPKR centrifuge; Beckman Coulter Inc., Fullerton, CA) and the infection was monitored for a total of 4 h. At 30 min intervals, Trypan Blue was added to three wells that corresponded to each MOI along with three control wells with no bacteria. The percentage of cells that took up the stain was estimated by direct
microscopic examination using a Zeiss inverted microscope with a 35 mm camera attached (Microscopy Services, Laboratory Services, University of Guelph). A cytotoxicity score was based on the percentage of stained cells (1, <1%; 2, 1 to 5%; 3, 5 to 10%; 4, >15%). Each MOI was assessed in triplicate and the average score was used in data analysis. Since infection inoculums for the 16HBE14o- cells have been described for use in a similar in vitro adherence and invasion assays (Plotkowski et al., 1999), preliminary experiments confirmed the bacterial inoculum of 5 x 10^7 CFU per well (of a 24-well plate) had no cytotoxic effect on the 16HBE14o- bronchial cells.

2.5.3 Preparation of bacteria for ingestion and association assays. Bacteria were inoculated from frozen glycerol stocks onto Miller’s LB agar and grown at 37°C overnight. The next day, bacteria were inoculated into Miller’s LB broth, incubated at 37°C, shaken at 200 rpm for 10-12 h. Bacteria from this culture was washed in sterile PBS (0.125 M NaCl, 0.0015 M KH₂PO₄, 0.008 M Na₂HPO₄·12H₂O, 0.007 M KCl, pH 7.0) and diluted to an OD₆₅₀ of 0.1. The actual CFU of bacteria at OD₆₅₀ of 0.1 was determined to be ca. 1.0 x 10^8 CFU·ml⁻¹ as determined by viable plate counts. After washing bacterial cells in PBS, the bacteria were resuspended in MEM containing fetal calf serum and dilutions of this stock were made according to the number of epithelial cells that were counted per well to achieve an initial MOI in the range of 10-20 depending on the experiment.

2.5.4 Association and invasion assays. Tissue culture plates of 24-well (Linbro, Flow Laboratories, Inc., McLean, VA) or 96-well MICROTEST™ tissue culture plates (Becton Dickinson and Co., Franklin Lakes, NJ) were coated with 300 µl or 75 µl, respectively, with a collagen solution. The collagen solution was prepared by adding

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collagen (Worthington Biochem Corp. Lakewood, NJ) to water (250 mg·ml\(^{-1}\)), and this suspension was allowed to stir for 30 min at 37°C. It was then sterilized by autoclaving. After adding collagen to the tissue culture plates, the collagen-coated plates were allowed to sit for 14-30 days. For adherence and invasion assays performed in tissue culture plates, the collagen solution was first removed and the wells washed with sterile media. Cells were seeded at ca. 1 x 10\(^5\) cells per well in supplemented LCH-8 or MEM. The media was changed every 48 h until cells grew to confluence. Monolayers were washed in antibiotic-free MEM three times and the volume of bacterial cell suspension added per well for a 96-well or 24-well plate was 200 \(\mu\)l or 500 \(\mu\)l, respectively. The infected cells were incubated in 5% CO\(_2\) at 37°C for the designated times, 2 h for the association assays and 4 h for the invasion assays unless otherwise designated. For the association assays, supernatants from the wells were removed after the association incubation and the monolayers were washed five times in unsupplemented MEM. After the last wash was removed, sterile PBS containing 0.5% Triton X-100 was added to each well and incubated for 5 min at room temperature. Aliquots of the cell lysates were serially diluted in sterile PBS and plated on Miller’s LB agar plates to quantitate viable bacteria. Association is used interchangeably with the term “attachment” in this study. Association here is defined as the total number of bacteria that have attached and invaded the epithelial cells. Invasion assays were performed as follows. After the association incubation, nonadherent bacterial cells were washed off the cultured epithelial cells by five washes with unsupplemented MEM. After the last wash was removed, MEM supplemented with fetal calf serum and 400 \(\mu\)g·ml\(^{-1}\) amikacin was added to each well and then the monolayers were incubated for another 3 h in 5% CO\(_2\), at 37°C. The antibiotic-
containing media was removed and the wells were washed three times in antibiotic-free MEM. The last wash was aspirated and sterile PBS, 0.5% Triton X-100 solution was added and invasive bacteria were quantitated by viable plate counts as previously described for the association assay. All media was prewarmed to 37°C before use on the monolayers except for the lysis solution. Integrity of the monolayers was monitored throughout the assays by direct examination using a Wilovert inverted microscope (Will-Wetzlar, Germany).

2.5.5 Inhibition of association assay. LPS core OS was isolated from *P. aeruginosa* strain PAO1 *wbpL* using the aqueous-phenol method as described in section 2.24. The intact core OS was dialyzed in water before lyophilization. A stock concentration of LPS was made in water and small aliquots were added to the MEM supplemented with fetal calf serum and the bacterial inoculum to generate final concentrations of 1 μg and 10 μg of purified core OS per well. The rest of the experiment was performed as described in section 2.30 for the association assay. A Trypan Blue exclusion assay was performed in parallel with the inhibition assay. At the end of the 2 h association incubation, Trypan Blue dye was added to triplicate wells containing the same bacterial inoculum and LPS concentrations as for the inhibition assay. The integrity of the epithelial cell monolayers was assessed by direct examination using a Wilovert inverted microscope (Will-Wetzlar, Germany) to monitor the effect of the concentration of LPS used on the cultured cells. The inhibition experiment was performed twice, on two separate days.

2.5.6 Statistical analysis. The bacterial association and internalization assays were repeated more than three times and these experiments were carried out on separate
days. Association and invasion data are reported as the results of a single experiment unless otherwise designated. Three to nine replicates were obtained per point and statistical assessment of the differences between the means was performed using ANOVA or Student’s t test. P values < 0.05 were considered significant. Data are presented as mean ± standard error. These results are reflective of data obtained in replicate experiments performed on separate days. For these analyses, SigmaPlot 2002 for Windows Version 8.0 and Microsoft®Excel 2000 software programs were used.

2.5.7 Fluorescence microscopy examination of P. aeruginosa associating with epithelial cells. Collagen-coated glass coverslips were prepared as follows. A collagen solution of 250 mg·mL⁻¹ in water was stirred and warmed at 37°C for 30 min. This solution was autoclaved and round glass coverslips (Fisher Scientific Ltd.) was added to this solution, which was autoclaved again, and allowed to sit for two weeks prior to use. The collagen-coated glass coverslips were placed within the wells of a 24-well plate and ca. 1.5 x 10⁵ epithelial cells were seeded per well and fed every other day until confluency. Preliminary experiments were performed to determine the optimal conditions that allowed for maximal bacterial staining with minimum nonspecific background fluorescence and optimal visualization of the membrane of the epithelial cells using SP-DilC₁₈(3) (Molecular probes, Invitrogen). Association assays were performed as described earlier. After the final wash step to remove nonadherent bacteria, the coverslips were incubated in 100 µl of a 5 µM solution of SP-DilC₁₈(3) diluted in unsupplemented MEM (prewarmed to 37°C), for 4 min at 37°C in 5% CO₂. The coverslips were incubated for 10 min more at 4°C. This solution was then removed and cells were fixed in 3.7% (w/v) paraformaldehyde in PBS at 37°C for 30 min and then overnight at 4°C. The cells
were washed 4 times (15 min per wash) in PBS and then incubated in B-band specific mAb MF15-4 containing 1% BSA overnight at room temperature. The monolayers were washed 4 times in PBS (15 min each wash) and then incubated in fluorescein-conjugated F(ab')2 fragment goat anti-mouse IgG (Jackson Immunoresearch Laboratories Inc.) at a 1:50 dilution in PBS-1% BSA for 2 h at room temperature. Samples were washed 4 times, 15 min each with PBS and mounted on microscope slides with Vectashield (Vector Laboratories, Burlingame, CA) as a mounting reagent. Fluorescent images were taken using a Leica TCS SP2 confocal laser-scanning microscope with Leica confocal software (Version 2.5.1227a; Confocal Microscope Facility, Department of Botany, University of Guelph). The images were collected as Z-series and figures shown are a result of a 3-D reconstruction using scion software (Scion Corp., Frederick, ML; [http://www.scioncorp.com/]) and Adobe Photoshop.

2.5.8 Detection of the CFTR protein on the IB3 and C38 cell lines. IB3 and C38 epithelial cells were grown on glass coverslips as described previously. Once the cells reached confluency, the coverslips were washed twice with prewarmed PBS (pH 7.4) and the sample was fixed with freshly prepared 3.7% paraformaldehyde in PBS solution for 15 min at room temperature followed by three washes in PBS, 15 min each. To allow for penetration of the primary antibody, the epithelial cell membranes of different samples were first permeabilized. Samples were treated with 0.1% Triton X-100 in PBS for 5 min at room temperature, or a solution of 80% acetone (v/v) in water for 5 min at -20ºC, or 0.1% saponin in PBS, or PBS alone for 30 min at room temperature. After the permeabilization step, cells were washed in PBS three times, 15 min each, and incubated for 30 min at room temperature in PBS-1% BSA. For the samples treated with
saponin, washes in PBS, the blocking step and incubations in antibodies were all carried out in the continuous presence of 0.1% saponin. The primary antibodies used were: (i) CFTR mAb AB-1 (TAM18) (Medicorp., Montréal, QC; Coltrera et al., 1999); (ii) mAb MA1-935, specific to the first extracellular loop of human CFTR (Affinity Bioreagents Inc., Golden, CO); (iii) antiserum against recombinant human CFTR, a gift from Dr. C. Bear (Department of Physiology, Faculty of Medicine, University of Toronto, Toronto, ON; immunogen was purified recombinant CFTR; Bear et al., 1992). Samples were incubated in primary antibodies (diluted 1:100, 1:500, 1:1 000 in PBS-1% BSA) overnight at room temperature, followed by four washes (15 min each) in PBS-1% BSA. Monolayers were incubated for 1-2 h at room temperature in secondary antibody (diluted 1:50, 1:100, 1:250, 1:500 in PBS-1% BSA). The secondary antibodies used were fluorescein (FITC)-conjugated affiniPure F(ab')2 fragment goat anti-mouse IgM (Jackson Immunoresearch Laboratories, Inc.) and rhodamine red™-X-conjugated affiniPure goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc.). Cells were then washed four times in PBS (15 min each wash), and then covered with Vectashield and mounted on microscope slides. Samples were examined using a Zeiss Photomicroscope III microscope equipped with a transmitted light DIC system, epi-fluorescence condenser III RS and an illuminator 100 (Zeiss) and image capturing and analysis were performed with Northern Eclipse™ image analysis software (version 5.0; Empix Imaging Inc.; Image Analysis Centre, Botany Department, University of Guelph) and a Leica TCS SP2 confocal laser scanning microscope equipped with Leica confocal software (Version 2.5.1227a; Confocal Microscope Facility, Botany Department, University of Guelph).
Chapter 3. Involvement of WbpM\textsubscript{07} and WbpL\textsubscript{07} in lipopolysaccharide biosynthesis and glycosylation of pilin in \textit{Pseudomonas aeruginosa} strain 1244 (serotype O7)\textsuperscript{a}

\textsuperscript{a} The abstract and introduction of this chapter have been revised slightly from the original manuscript by Antonio DiGiandomenico,\textsuperscript{1+} Mauricia J. Matewish,\textsuperscript{2+} Amy Bisaillon,\textsuperscript{2} John R. Stehle,\textsuperscript{1} Joseph S. Lam\textsuperscript{2} and Peter Castric\textsuperscript{1}, 2002, Glycosylation of \textit{Pseudomonas aeruginosa} 1244 pilin: glycan substrate specificity. Mol. Microbiol. 46:519-530, to report only my own contribution to the paper.

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A brief summary of the results obtained by Antonio DiGiandomenico, John R. Stehle, and Dr. Peter Castric are included in Appendix I. The discussion is from the original manuscript, since the results obtained from both laboratories together provide a fundamental understanding of the relationship between O-antigen biosynthesis and pilin glycosylation in \textit{P. aeruginosa} strain 1244.
3.1 Abstract

The pilin glycan and O-antigen unit of *Pseudomonas aeruginosa* strain 1244 have the same structure (α-5NβOHC₄7NFMpse-(2→4)-β-Xyl-(1→3)-β-FucNAc) and we wanted to determine if this trisaccharide had a common metabolic origin. Two genes that are found within the O-antigen gene cluster from *P. aeruginosa* strain PA01, *wbPM* and *wbPL*, are required for synthesis of UDP-D-FucNAc and initiation of O-antigen synthesis, respectively. *wbPM* and *wbPL* homologues were identified in strain 1244 and a gene replacement strategy was used to generate 1244 *wbPM* and *wbPL* mutants. These mutants did not produce O-antigen LPS but complementation with plasmids containing functional *wbPM* or *wbPL* genes fully restored their ability to produce O-antigen. Pilin isolated from the wild type and LPS mutants were resolved by SDS-PAGE, stained with Coomassie Brilliant blue stain. The apparent molecular mass of the pilin isolated from the *wbPM* and *wbPL* mutants was 16 kDa whereas the mass of the pilin isolated from the parent strain was 18 kDa. The mass discrepancy between the pilin isolated from the parent and LPS mutants suggested a loss of the glycan moiety of the pilin. Transformation of *wbPM* and *wbPL* mutants with complementation plasmids containing *wbPM* and *wbPL*, respectively, allowed the mutants to make glycosylated pilin similar to that produced by the wild-type strain. These results show that the pilin glycan of *P. aeruginosa* is produced from the O-antigen biosynthetic pathway.
3.2 Introduction

*Pseudomonas aeruginosa* pili are the dominant adhesins involved in the initial phase of attachment to host tissues. Pili mediate adherence to many epithelial cell types *in vitro* including human buccal cells (Woods et al., 1980; Doig et al., 1988), lung pneumocytes (Chi et al., 1991; Farinha et al., 1994), exfoliating tracheal cells (Zoutman et al., 1991), human airway cells (Feldman et al., 1998), and cystic fibrosis airway cells (Saiman and Prince, 1993). Pilus-deficient strains are significantly reduced in their ability to cause epithelial cell damage, to persist at the site of infection, and to disseminate throughout the host (Woods et al., 1980; Sato et al., 1988; Farinha et al., 1994; Tang et al., 1995). The epithelial-cell receptors recognized by *P. aeruginosa* pili are the common cell-surface glycosphingolipids asialo-GM1 and asialo-GM2, and to a weaker extent lactosyl ceramide and ceramide trihexoside (Saiman and Prince, 1993; Gupta et al., 1994; de Bentzmann et al., 1996; Comolli et al., 1999). The pili of *P. aeruginosa* are the prototypes of the Type 4 family of pili (Hahn, 1997) and are composed of pilin monomers, which are encoded by pilA. A defining characteristic of these pilin proteins is the N-methylation of the amino-terminal residue phenylalanine (Paranchych and Frost, 1988; Strom et al., 1993).

Glycosylation of prokaryotic proteins is now known to occur in a variety of organisms and the best-studied example is the archeal and eubacterial S-layer glycoproteins (Schaffer and Messner, 2001). A number of examples of glycosylated surface proteins from pathogens have been reported. Among Gram-positive organisms, proteins of *Streptococcus sanguis* (Erickson and Herzberg, 1993) and *Mycobacterium tuberculosis* (Dobos et al., 1996) are glycosylated. Examples of glycosylation of cell-
surface proteins among Gram-negative pathogens include the AIDA-A adhesion of *E. coli* (Benz and Schmidt, 2001), the 40 kDa MOMP minor proteins of *Chlamydia trachomatis* (Kuo et al., 1996), the flagellins of *Campylobacter* species (Doig et al., 1996; Thibault et al., 2001) and *P. aeruginosa* (Brimer and Montie, 1998), and the pili of *Neisseria meningitidis* (Stimson et al., 1995) and *N. gonorrhoeae* (Parge et al., 1995).

The structure of the pilin glycan of *P. aeruginosa* 1244 is α-5NβOHC₄⁷NFmPse-(2→4)-β-Xyl-(1→3)-β-FucNAc (Castric et al., 2001). Each pilin subunit has one glycan moiety covalently attached to the serine residue at 148 at the β carbon through the C-1 of FucNAc (Castric et al., 2001; Comer et al., 2002). The identical trisaccharide forms the repeating unit of the IATS serotype O7 O antigen (Liu et al., 1983; Knirel, 1990) and is the serogroup to which strain 1244 belongs. These findings suggested that the pilin glycan and the O antigen of this organism have a common synthetic origin.

A protein that has been implicated in the attachment of this trisaccharide to the pilin protein is PilO (Castric, 1995). In strain 1244, the *pilO* gene is directly downstream of the pilin structural gene, *pilA* and genetic evidence was provided to relate the presence of pilin glycosylation to expression of the *pilO* gene. Hydropathy plots based on the method of Kyte and Doolittle (1982) revealed that PilO contains nine membrane-spanning domains. It is hypothesized that PilO resides in the cytoplasmic membrane to transfer carrier lipid-bound oligosaccharide subunits to the emerging pilin proteins (Castric, 1995), however, to date, there is no experimental evidence to support this hypothesis.

The genes required for biosynthesis of the O-antigens of *P. aeruginosa* serotypes O5, O6, and O11 have been characterized (Burrows et al., 1996; Bélanger et al., 1999;
Dean et al. 1999), and two genes, \textit{wbpM} and \textit{wbpL}, are essential for the initial steps of O-antigen biosynthesis. \textit{wbpM} encodes a bifunctional UDP-GlcNAc C-6 dehydratase/C-4 reductase that is essential for formation of the nucleotide precursor UDP-QuiNAc (Creuzenet and Lam, 2001). UDP-QuiNAc is thought to be converted into UDP-FucNAc by an epimerization step and UDP-FucNAc is the precursor for the first sugar residue of the serotype O7 O-antigen unit. \textit{WbpL}, a bifunctional glycosyltransferase, is required for the initiation of both A-band PS and B-band PS synthesis (Rocchetta et al., 1998a).

\textit{WbpL} initiates the assembly of individual O-antigen units by the transfer of FucNAc from UDP-FucNAc to undecaprenol phosphate. Consequently, mutations in \textit{wbpM} in \textit{P. aeruginosa} strains PAO1 (serotype O5) and PAK (serotype O6) result in the complete loss of O-antigen production, whereas null mutations in \textit{wbpL} completely abolish production of both A-band and B-band LPS. Therefore, \textit{wbpL} and \textit{wbpM} are ideal targets for constructing null mutants to investigate the role of O-antigen biosynthesis in the glycosylation of strain 1244 pilin.

I provide evidence that the pilin glycan is a product of the O-antigen biosynthetic pathway. This was performed using defined \textit{wbpM} and \textit{wbpL} mutants derived in the \textit{P. aeruginosa} strain 1244 background. The \textit{wbpM} mutant was deficient in B-band LPS while the \textit{wbpL} mutant did not produce A- or B-band LPS. Pilin isolated from both mutants was nonglycosylated and complementation of the mutants with their respective genes restored both the wild-type LPS phenotype and glycosylation of pilin. These results indicate that in \textit{P. aeruginosa} 1244, synthesis of the pilin glycan is directed by the O-antigen gene cluster. This is the first report to describe that the O-antigen gene cluster
directs synthesis of a glycan required for both O-antigen biosynthesis and pilin glycosylation.

3.3 Results

3.3.1 Generation of \textit{P. aeruginosa} 1244 \textit{wbpM} and \textit{wbpL} mutants

As \textit{wbpL} and \textit{wbpM} are involved in the initial steps of O-antigen biosynthesis, mutants of these genes in \textit{P. aeruginosa} 1244 were generated to investigate whether the O7 O antigen and the pilin glycan were products of the same metabolic pathway. The nucleotide sequence for the \textit{wbp} cluster in strain 1244 was unknown. However, based on the findings from a previous study (Burrows et al., 1996), it was known that a highly conserved \textit{wbpM} gene is localized at the 3’ end of the \textit{wbp} cluster, and that \textit{wbpL} is localized upstream of \textit{wbpM} in a non-conserved serotype-specific region. The \textit{wbpL} gene in strain 1244 (\textit{wbpL}_{1244}) could not be identified in Southern blot analysis using the PAO1 \textit{wbpL} as a gene probe, even using low-stringency conditions (Fig. 3.1 A).

Therefore, taking advantage of the highly conserved nature of \textit{wbpM} at the nucleotide level, a 1244 \textit{wbpM} null mutant was first generated following an established method (Schweizer and Hoang, 1995) using an allelic replacement construct containing the \textit{wbpM} gene from \textit{P. aeruginosa} strain PAO1 (serotype O5) interrupted with a gentamicin resistance cassette (Burrows et al., 1996).

Southern blot analysis was performed on \textit{XhoI}-digested chromosomal DNA from \textit{P. aeruginosa} strain PAO1, 1244 wild type, and the 1244 \textit{wbpM} mutant using \textit{wbpM}_{PAO1} as a gene probe (Fig. 3.1 B). The \textit{wbpM}_{PAO1} gene probe is a 2.12 kb fragment that contains almost the entire \textit{wbpM} gene. The \textit{wbpM} gene contains one \textit{XhoI} site, therefore
Figure 3.1  Southern hybridizations were performed on XhoI-digested genomic DNA from strain PAO1, wild type 1244, and the 1244 wbpM mutant using three separate DNA probes. A wbpL probe-reactive fragment was observed in genomic DNA isolated from strain PAO1, but not from strain 1244 (A). Southern hybridization of this membrane was performed under low stringency conditions. Panel (B) shows wbpM probe reactivity with 4.3 kb and 1.6 kb XhoI fragments in PAO1, with 2.2 kb and 1.6 kb XhoI fragments in wild type 1244, and with 3.0 kb and 1.6 kb in the 1244 wbpM mutant. Panel (C) shows a aacC1 probe-reactive fragment at 3.0 kb in the XhoI digested chromosomal DNA from the 1244 wbpM mutant. Lambda DNA digested with HindIII was used for molecular markers and was probed with corresponding labeled DNA.
Southern blot hybridization performed on XhoI-digested chromosomal DNA showed that the \textit{wbpM}_{PAO1} probe hybridized to two DNA fragments from each strain. A \textit{wbpM} probe-reactive fragment of 1.6 kb was identified in all three strains. Based on the available nucleotide sequence of the PAO1 \textit{wbp} cluster (Burrows et al., 1996; Stover et al., 2000), it was deduced that the 1.6 kb fragment corresponds to the 3' end of the \textit{wbpM} gene (Fig. 3.2). \textit{wbpM} probe-reactive DNA fragments of sizes 4.3 kb, 2.2 kb and 3.1 kb were also observed from PAO1, 1244 wild type and the \textit{wbpM} mutant, respectively (Fig. 3.1 B).

Analysis of the nucleotide sequence of the PAO1 \textit{wbp} gene cluster shows there to be 723 nucleotides upstream of the \textit{XhoI} site within \textit{wbpM} that corresponds to the 5' end of the gene. Therefore, \textit{wbpM} probe-reactive DNA fragments 4.3 kb, 2.2 kb and 3.1 kb correspond to the 5' end of \textit{wbpM} with upstream DNA from each of the different strains (Fig. 3.2). \textit{XhoI}-digested chromosomal DNA from the 1244 \textit{wbpM} mutant was again subjected to electrophoresis. \textit{XhoI} fragments within a 2.5 kb to 3.5 kb region were isolated and ligated together with \textit{XhoI}-digested DNA from cloning vector, pBluescript. The ligation mixture was transformed into \textit{E. coli} DH5\textalpha{} and transformants containing the \textit{XhoI} fragment containing the 5' end of \textit{wbpM} and upstream DNA were selected for by growth of the transformants on gentamicin, since these DNA fragments also contained the gene for gentamicin resistance (located at the \textit{NruI} site located 261 nucleotides from the \textit{wbpM} gene start codon). Nucleotide sequencing analysis of the 3 kb \textit{XhoI} fragment from the 1244 \textit{wbpM} mutant revealed an ORF at the 5' end composed of 1041 nucleotides, which encodes a 346-amino-acid protein with 58% identity and 74% similarity to WbpL from \textit{P. aeruginosa} strain PAO1. In addition, the protein encoded by this ORF contained five conserved motifs characteristic of the WbpL-type family of
Figure 3.2 Strategy for identifying \textit{wbpM} and \textit{wbpL} in strain 1244 based on comparison of location of these two genes in PAO1, 1244 wild type and the 1244 \textit{wbpM} mutant. The marked \textit{XhoI} fragments represent the results from the Southern hybridization analysis.
glycosyltransferases (Rocchetta et al., 1998a), which belong to a larger family of UDP-GlcNAc/MurNAc: polyisoprenol-P GlcNAc/MurNAc-1-P transferases (Lehrman, 1994). This ORF was called \( wbpL_{1244} \) and a 1244 \( wbpL \) null mutant was successfully constructed using the gene replacement method described earlier (Schweizer and Hoang, 1995).

### 3.3.2 \( wbpL \) and \( wbpM \) mutants are deficient in LPS production

Analysis of the LPS from 1244 \( wbpM \) and \( wbpL \) mutants showed that they are both deficient in B-band production, whereas the \( wbpL \) mutant is also deficient in A-band production (Fig. 3.3). Complementation experiments transforming \( wbpM \) (pFV163-26; \( wbpM_{PAO1} \)) and \( wbpL \) (pFVAB3-26; \( wbpL_{1244} \)) into their respective mutants restored the phenotype to that of the parent. The GenBank accession numbers for these genes are AY095101 for \( wbpL_{1244} \) and AF508154 for \( wbpM_{1244} \).

### 3.3.3 Pilin glycosylation of \( P. aeruginosa \) 1244 LPS mutants

If the 1244 pilin glycan originates in the O-antigen pathway, it would be expected that mutants defective in biosynthesis of this polysaccharide would produce altered pilin glycosylation. To test this, pilin isolated from 1244 \( wbpM \) and \( wbpL \) mutants were analyzed by SDS-PAGE to determine whether they produced pilin of a size consistent with glycosylation. Pilin from these mutants had an apparent molecular mass of 16 kDa, whereas pilin from the wild-type strain was 18 kDa (Fig. 3.4). The molecular mass of the pilin protein from the LPS mutants is consistent with the size of nonglycosylated pilin. The molecular mass of mature pilin predicted from the nucleotide sequence of the \( pilA \) gene is 15 653 Da (Castric et al., 1989). Complementation of these mutants with their
Figure 3.3 Analysis of LPS from the parent 1244, \textit{wbpM} and \textit{wbpL} mutants.

Silver-stained SDS-PAGE gel (A) and Western immunoblots using the O7 B-band specific mAb MF29-2 (B) and A-band specific mAb N1F10 (C). The 1244 \textit{wbpM} and \textit{wbpL} mutants are both deficient in B-band LPS, whereas the \textit{wbpL} mutant is also deficient in A-band LPS. LPS of the \textit{wbpM} and \textit{wbpL} mutants is restored to that of the parent phenotype when complemented with pFV163-26 (\textit{wbpM}_{PAO1}) and pFVAB3-26 (\textit{wbpL}_{1244}), respectively.
Figure 3.4 Coomassie stained SDS-PAGE gel of pilin isolated from *P. aeruginosa* 1244 and the 1244 *wbpL* and *wbpM* mutants. The apparent molecular mass of the pilin isolated from the LPS mutants (16 kDa) was less than that of the parent (18 kDa) and was similar to the mass predicted for the mature nonglycosylated protein (15 653 Da). Complementation of these mutants with pFVAB3-26 (*wbpL*<sub>1244</sub>) and pFV163-26 (*wbpM*<sub>PAO1</sub>), respectively, restored the pilin to an apparent mass similar to the pilin from the wild-type strain.
respective genes resulted in production of pilin with the same molecular mass as glycosylated 1244 pilin produced by the wild type strain. To verify these results, our collaborators, Antonio DiGiandomenico and Dr. Peter Castric (Department of Biological Sciences, Duquesne University, Pittsburgh, PA) analyzed pilin from cell extracts using Western immunoblotting with mAb 6.45, which is specific for 1244 pilin protein (Castric and Deal, 1994). They observed that the pilin from each of the LPS mutants was of an apparent molecular mass consistent with nonglycosylated pilin, whereas that from the complemented mutants had an apparent molecular mass similar to glycosylated pilin (Appendix I). In addition, our collaborators analyzed pilin by isoelectric focusing, and the isoelectric point of the pilin from the wbpM and wbpL mutants was similar to that of nonglycosylated pilin. In contrast, the complemented mutants produced pilin with acidic isoelectric points similar to that of glycosylated pilin (Appendix I). These data show that 1244 mutants defective in either the N-acetyl fucose pathway (wbpM) or in the transferase responsible for attachment of this sugar to the undecaprenol carrier lipid (wbpL) were unable to produce glycosylated pilin. Altogether, these results support the proposal (Castric et al., 2001) that the O-antigen biosynthetic pathway is the synthetic source of the pilin glycan.

3.4 Discussion

The structural similarity between the *P. aeruginosa* 1244 pilin glycan and the serotype O7 O-antigen repeating unit suggested that this saccharide was derived from the O-antigen biosynthetic pathway (Castric et al., 2001). This was substantiated in the present paper by two lines of evidence. In the first, defined 1244 mutants defective in
specific steps of the O-antigen biosynthetic pathway were unable to glycosylate pilin, whereas complemented mutants regained that ability. In the second, the expression of cloned O-antigen biogenesis gene clusters in strain 1244 resulted in heterologous pilin glycosylation.

An important question arising from these results concerns how pilus and O-antigen biosynthesis intersect, in terms of both metabolism and cellular location, to produce pilin glycosylation. These pathways are multistep reactions that begin in the cytoplasm and culminate at the cell exterior (Mattick et al., 1996; Darzins and Russell, 1997; Rocchetta et al., 1999). Evidence presented here indicates that a mutant \( \text{wbpL} \), was unable to support pilin glycosylation. It is hypothesized that \( \text{WbpL} \) initiates the assembly of individual O-antigen units by the transfer of FucNAc from UDP-FucNAc to undecaprenol phosphate (Und-P) (Rocchetta et al., 1998a). There are significant regions of protein similarity between \( \text{WbpL} \) and \( \text{E. coli WecA} \) (Rocchetta et al., 1999a). Genetic and biochemical data support the conclusion that \( \text{E. coli WecA} \) catalyzes the transfer of glucose-1-P from UDP-glucose to Und-P (Meier-Dieter et al., 1990; Meier-Dieter et al., 1992; Rick et al., 1994). To date, there is no biochemical evidence to support that \( \text{WbpL} \) recognizes Und-P as a substrate for its proposed glycosyltransferase reaction. However, cross-complementation data from Rocchetta et al. (1998a) reveal that \( \text{wbpL} \) can complement a \( \text{wecA} \) mutation and initiate O-antigen biosynthesis in an O-antigen deficient, \( \text{wecA} \) mutant strain of \( \text{E. coli K-12} \) when carrying plasmids that contained the O-antigen gene cluster for \( \text{Klebsiella pneumoniae O1 O-antigen} \) concomitantly with the \( \text{P. aeruginosa wbpL} \) gene. The loss of both O antigen and A-band LPS in \( \text{wbpL} \) mutants and (iii) data from cross-complementation experiments (Burrows et al., 1996; Rocchetta
et al., 1998a; Bélanger et al., 1999) are consistent with the putative assignment of WbpL as as initiating glycosyltransferase. This suggests that the pilin glycosylation reaction requires the first sugar residue to be attached to Und-P. The nonglycosylated phenotype of pilin isolated from the wbpL mutant, along with the relation of the glycan structure to the O-antigen (Castric et al., 2001), suggest that the glycosylation precursor may be an undecaprenol-bound repeating unit. If this is the case, pilin glycosylation could occur at the cell membrane in either the cytoplasm or, as a result of the Wzx-mediated transfer of the repeating unit (Rocchetta et al., 1999) in the periplasm. In this situation, the pilin cytoplasmic membrane pool (Watts et al., 1982) would provide the protein substrate. If pilin glycosylation occurs in the periplasm, it would be topologically similar to the dolichol pathway of protein glycosylation in eukaryotes (Burda and Aebi, 1999), with the periplasmic space functioning in a manner analogous to the endoplasmic reticulum. This general strategy has been proposed previously for the attachment of the glycosaminoglycan and the sulphated oligosaccharides to the surface layer protein of Halobacterium halobium (Lechner and Wieland, 1989).

Wherever these pathways converge, it is clear that PilO is the only factor required for pilin glycosylation that is not a component of either O-antigen or pilus biogenesis. It will be necessary to determine the mechanism of action, as well as specific details of glycan and pilin substrate specificity, to clarify the role of PilO in pilin glycosylation. Although much work remains to be done in PilO characterization, patterns of glycan substrate specificity are apparent from the work presented here, where it is shown that glycosylation could use eight different O antigens, including E. coli O157:H7, as glycosylation substrate. The fact that the O antigens used by PilO in this study (Fig. 3.5)
Serotype  Structure

*Pseudomonas aeruginosa*

O2: \( \rightarrow 4\)-\(-\beta\)-D-Man(2Nac3N)A-(1→4)-\(-\alpha\)-L-Gul(2Nac3Nac)A-(1→3)-\(-\beta\)-D-FucNac-(1→3)\[CH_2\text{C}=\text{NH}\]

O5: \( \rightarrow 4\)-\(-\beta\)-D-Man(2Nac3N)A-(1→4)-\(-\beta\)-D-Man(2Nac3Nac)A-(1→3)-\(-\alpha\)-D-FucNac-(1→3)\[CH_2\text{C}=\text{NH}\]

O6: \( \rightarrow 3\)-\(-\alpha\)-L-Rha-(1→4)-\(-\alpha\)-D-GalNacA-(1→4)-\(-\alpha\)-D-GalNFmA-(1→3)-\(-\alpha\)-D-QuiNac-(1→3)\[OAc\text{ NH}_2\]

O7: \( \rightarrow 4\)-\(-\alpha\)-Pse(5N7NFm)-(2→4)-\(-\beta\)-D-Xyl-(1→3)-\(-\beta\)-D-FucNac-(1→6)\[OC\text{CH}_2\text{CH(OH)}\text{CH}_3\text{ OAc}\]

O11: \( \rightarrow 2\)-\(-\beta\)-D-Glc-(1→3)-\(-\alpha\)-L-FucNac-(1→3)-\(-\beta\)-D-FucNac-(1→3)\[OAc\]

O13: \( \rightarrow 2\)-\(-\alpha\)-L-Rha-(1→3)-\(-\alpha\)-L-Rha-(1→4)-\(-\alpha\)-D-GalNacA-(1→3)-\(-\beta\)-D-QuiNac-(1→3)\[OAc\]

O16: \( \rightarrow 4\)-\(-\beta\)-D-Man(2Nac3N)A-(1→4)-\(-\beta\)-D-Man(2Nac3Nac)A-(1→3)-\(-\beta\)-D-FucNac-(1→3)\[CH_2\text{C}=\text{NH}\]

*Escherichia coli*

O157: \( \rightarrow 3\)-\(-\alpha\)-L-Fuc-(1→4)-\(-\beta\)-D-Glc-(1→3)-\(-\alpha\)-D-GalNac-(1→2)-\(-\alpha\)-D-PerNac-(1→)

**Figure 3.5** Chemical structures of the O-antigen repeating units from seven serotypes of *P. aeruginosa* (Knirel, 1990) and *E. coli* O157:H7 (Perry et al., 1986).
vary so greatly in structure, size, and overall charge raises the intriguing question as to how PilO would recognize these molecules. Although common sugars and linkages are seen among certain related repeating units (for example, serotypes O2, O5 and O16), no major structures are shared by all groups. The results presented suggest that *P. aeruginosa* does not use A-band polysaccharide or peptidoglycan biosynthesis for pilin glycosylation, as no apparent pilin glycosylation occurs in the absence of O-antigen biosynthesis. Further, a single glycan isoform is produced by pilin glycosylation (Castric et al., 2001), indicating that polymerized O-antigen repeating units do not serve as a glycosylation substrate. These findings suggest that recognition of the glycosylation substrate may be based on a combination of molecule size and the presence of some common structural elements. For example, if the glycan is transferred to pilin via undecaprenol phosphate, PilO may recognize portions of this molecule in addition to (or instead of) the O-antigen repeating unit.

As prokaryotes produce relatively few polysaccharides, and so have limited routes for biosynthesis of these molecules, it would not be surprising to find that these organisms must use pathways dedicated to saccharide production for protein glycosylation. This appears to be the case in examples such as pilin glycosylation by *N. meningitidis* (Jennings et al., 1998; Power et al., 2000; Kahler et al., 2001) and flagellin glycosylation by *P. aeruginosa* (Arora et al., 2001). In *H. halobium*, a common pathway produces sulphated oligosaccharides used for glycosylation of the S-layer flagellin of this organism (Wieland et al., 1985; Lechner and Wieland, 1989). An analogous situation may occur in the multiple glycosylation of *Campylobacter* proteins (Logan et al., 1989; Guerry et al., 1996). It is therefore unusual to find that pilin glycosylation in *P.*
*Pseudomonas aeruginosa* 1244 uses a major pathway (O-antigen biosynthesis) that normally operates to fulfill a separate function. An obvious disadvantage to this arrangement is that the pilin-bound O-antigen repeating unit presents an additional antigenic target in which at least some of the antibodies directed against the lipopolysaccharide O antigen, a major target of the immune response, would also be expected to recognize the pilus. This would be important as a monoclonal antibody reacting with the 1244 pilin glycan has been shown to block twitching motility by this organism (Castric et al., 2001). On the other hand, utilization of the O-antigen pathway for pilin glycosylation would represent a saving of energy expended for biosynthesis. A further advantage could be found in the low glycan substrate specificity of PilO. This property would guarantee that a *pilAO* operon entering the genome of a type IV pilin-producing species by reciprocal recombination would be able to code for pilin that had immediate access to the glycosylation substrate. Horizontal transfer of the ability to form glycosylated pili could then be carried out using small segments of information without the necessity for the mobilization of an entire pathogenicity island. This low specificity would also guarantee that variations in O-antigen biosynthesis (which could also be introduced by horizontal transfer), such as the addition or removal of functional groups, would not interfere with pilin glycosylation.

Previous work (Comer et al., 2002) has shown that *P. aeruginosa* 1244 pili are able to stimulate glycan-specific antibodies. Further, the inhibition of twitching motility, a process important to *P. aeruginosa* virulence, by an anti-glycan monoclonal antibody (Castric et al., 2001) indicates that this structure is a potential vaccine target. Of further significance is the finding that antibodies raised against the 1244 pilus glycan recognize LPS from this organism (Comer et al., 2002). This suggests that pilus immunization
could be used to raise a protective LPS-specific B-cell response without the
disadvantages of LPS toxicity and the requirement for LPS purification. The ability of
PilO to attach *E. coli* O157 O-antigen to 1244 pilin suggests that repeating units from a
variety of Gram-negative pathogens could be used for pilin glycosylation. This raises the
possibility of a pilus/O-antigen bioconjugate vaccine produced by expression of a
pathogen O-antigen cluster in a *P. aeruginosa* strain capable of glycosylating pilin. Such
an effort will first require the determination of the range of glycan substrate specificity
for PilO and the compatibility of pathogen O-antigen cluster expression with *P.
aeruginosa*. 
Chapter 4. A lipopolysaccharide biosynthetic enzyme, WbpO, is required for flagellin glycosylation in *Pseudomonas aeruginosa* strain PAK

4.1 Abstract

In the previous chapter, it was shown that the O-antigen biosynthetic locus of *P. aeruginosa* strain 1244 is required for pilin glycosylation. Therefore, it was of interest to investigate whether the O-antigen *wbp* gene cluster was involved in flagellin glycosylation in *P. aeruginosa* strain PAK. Flagellin purified from three PAK lipopolysaccharide mutants, *wbpL*, *wbpP*, and *wbpO*, were examined. Wild-type PAK and the *wbpL* and *wbpP* mutants produced flagellin with an apparent molecular mass of 45 kDa as determined by SDS-PAGE analysis, whereas that from the *wbpO* mutant was 42 kDa. Based on the nucleotide sequence of the PAK *fliC* gene, which encodes the flagellin structural protein, nonglycosylated flagellin is predicted to have a mass of 40 036 Da. A biotin-hydrazone glycosylation assay showed a strong positive glycosylation reaction for flagellin isolated from strain PAK, whereas a very weak reaction was observed for the flagellin from the *wbpO* mutant. By performing isoelectric focusing on gels, flagellin from the *wbpO* mutant had a pI of 4.8, whereas the pI of the wild-type flagellin was 4.6. Analysis of flagellins isolated from wild-type PAK, the *wbpO* mutant and an *rmlC* mutant by MALDI-TOF mass spectrometry indicated that the *wbpO* mutant produced flagellin with one covalently attached sugar residue, which may be L-rhamnose or another residue which requires the dTDP-4-dehydrorhamnose 3,5-epimerase activity of the RmlC protein. In contrast, flagellin from wild-type PAK is glycosylated with sugar residues with a total mass of ca. 2 540 Da. Previous reports from our laboratory had
shown that \textit{wbpO} is essential for O-antigen synthesis in strain PAK. WbpO is a UDP-\textit{N}-acetylglucosamine C6 dehydrogenase that is required for synthesis of UDP-\textit{N}-acetylglucosaminuronic acid (UDP-GlcNAcA). The data obtained in this study suggested that UDP-GlcNAcA is either required for the glycan, or serves as an intermediate substrate in the synthesis of a complex sugar residue that is attached to the sugar residue located proximal to the flagellin protein.

4.2 Introduction

\textit{Pseudomonas aeruginosa} produces single polar flagella, which has been implicated as an important virulence factor in pulmonary disease (Tang et al., 1996; Feldman et al., 1998). The primary function of the flagella is bacterial motility. However, flagella is also an adhesin for eukaryotic cell receptors. Flagellar proteins have been shown to specifically bind to secreted mucins (Arora et al., 1998; Scharfman et al., 2001) as well as membrane-tethered Muc1 mucin, which is abundantly expressed by respiratory epithelial cells (Lillehoj et al., 2002). These interactions are believed to be physiologically relevant to colonization of the respiratory tract and lungs by \textit{P. aeruginosa} in patients with cystic fibrosis and nosocomial pneumonia.

The flagellar filament of \textit{P. aeruginosa} is composed of flagellin subunits that are encoded by \textit{fliC}. Each strain produces a flagellum composed of either a-type or b-type flagellin subunits (Lanyi, 1970), which are differentiated based on the following criteria: (i) the primary amino acid sequence (Spangenberg et al., 1996), (ii) their reactivity with specific polyclonal antibodies (Ansorg, 1978; Montie and Anderson, 1988), and (iii) their molecular mass (Allison et al., 1985). The \textit{fliC} gene has been sequenced and compared
among the different a-type and b-type producing strains (Spangenberg et al., 1996).
Analysis of fliC from eight different b-type producing strains revealed that fliC is highly
conserved with only two nucleotide substitutions. These fliC genes encode flagellins with
a molecular mass of ca. 53 kDa (Brimer and Montie, 1998) with the deduced and
apparent molecular masses in good agreement with each other. Examination of the a-type
fliC from 12 different strains showed 57 nucleotide substitutions of which 39 occurred
within a variable central region. Thus, fliC genes among different a-type producing
strains encode a more heterogeneous group of proteins when compared to that of the b-
type flagellins. The deduced molecular mass of these a-type flagellins based on their
amino acid sequence is around 40-41 kDa, however, SDS-PAGE analysis shows their
apparent molecular mass to be between 45-52 kDa. The size discrepancy between the
predicted and apparent molecular mass of the a-type flagellins is due to post-translational
modifications (Brimer and Montie, 1998). For example, the deduced molecular mass of
P. aeruginosa strain PAK a-type flagellin is 41 kDa (Totten and Lory, 1990), but the
apparent molecular mass of flagellin isolated from whole bacterial cells as determined by
SDS-PAGE is 45 kDa. Results from glycosylation and deglycosylation assays showed the
5 kDa difference to be due to the presence of a glycan moiety (Brimer and Montie, 1998).

Prokaryotes can synthesize a wide variety of sugar residues and many of these
sugars were originally identified in the lipopolysaccharides of Gram-negative bacteria.
Some of the sugar residues found in protein glycans are similar to or the same as those
found in LPS, LOS and capsules. Therefore, it is not surprising that in some pathogenic
bacteria, genes required for LPS biosynthesis are also required for protein glycosylation.
To date, the extent of the inter-relationship between LPS and protein glycan synthesis
within different prokaryotes varies. For instance, in *C. jejuni*, a general protein
glycosylation locus is required for glycosylation of multiple proteins, including flagellin,
but this locus is not involved in synthesis of the LPS (Szymanski et al., 1999). Pilin
glycosylation in *N. meningitidis* requires the participation of three different unlinked loci.
One of these, *galE*, is required for both pilin glycan and LOS synthesis (Stimson et al.,
1995). In contrast, the O-antigen gene cluster in *P. aeruginosa* 1244 appears to synthesize
the complete glycan for both its LPS molecule and pilin glycan (DiGiandomenico et al.,
2002; Chapter 3). The extent to which genes, mechanisms, and sugar biosynthetic
pathways are shared between LPS biosynthesis and the production of protein glycans is
not clear. However, one place for these two different biosynthetic pathways to come
together would be in the sharing of nucleotide activated sugar molecules, which would
serve as sugar donors for each pathway.

The genetics of flagellin glycosylation in *P. aeruginosa* strain PAK has been
investigated by Arora et al. (2001), and they identified a “flagellin glycosylation island”
containing 14 genes. These genes encode proteins that are similar to those involved in the
synthesis of polysaccharides and include a nucleotide-sugar aminotransferase, an
acetyltransferase, and a glycosyltransferase. To verify the role of these genes in flagellin
glycosylation, this group demonstrated that inactivation of the genes at each end of the
glycosylation cluster abolished flagellin glycosylation. The genes within this genetic
cluster, however, were not assigned putative functions with respect to synthesis of the
flagellin glycan because the detailed chemical structure of the PAK flagellin glycan had
not been elucidated at the time of the report.
Recently, Schirm et al. (2004) reported *P. aeruginosa* PAK flagellin to have two *O*-linked glycan moieties containing up to 11 monosaccharides. Their data supported the identification of rhamnose as the proximal residue *O*-linked to the flagellin protein. The oligosaccharide chains found on the flagellin were shown to exhibit considerable heterogeneity in both sugar composition and length within the central region and the residues found in this region included pentoses, hexoses, deoxyhexoses, hexuronic acids, and deoxyhexoses with amino and formyl substitutions. Major components identified were mannose, glucose, and 4-amino-4,6-dideoxyglucose (viosamine). Since *P. aeruginosa* is capable of synthesizing complex sugar residues for its O antigen, the identification of complex sugar residues in the flagellin glycan is not unexpected. One prominent feature of *P. aeruginosa* O antigens is that they contain uronic acids, amino sugars and some rare sugars (Knirel and Kochetkov, 1994). It would be of interest to determine the extent of the sharing of biosynthetic pathways for donor nucleotide activated sugars between O-antigen biosynthesis and protein glycosylation in this organism. This information may provide clues for understanding the evolution, development, acquisition and relationship, if any, between “glycosylation gene clusters” and “O-antigen gene clusters”, both of which have recently been described as “pathogenicity islands” (Arora et al., 2001; Raymond et al., 2002; Spencer et al., 2003).

The genes responsible for biosynthesis of the O-antigen of *P. aeruginosa* strain PAK (serotype O6) have been characterized and designated as the *whp* gene locus (Bélanger et al., 1999). The structure of the O antigen of strain PAK (serotype O6) is a tetrasaccharide repeat of \(\rightarrow-\alpha-D-3\text{ O-acetyl-6 amino-GalNAcA-(1-4)-}\alpha-D-6\text{-amino-2-deoxy-2-formamido-D-galacturonic acid-(1\rightarrow3)-}\alpha-D-2\text{-acetamido-2,6-dideoxy-D-}\)
glucose-(1→2)-α-L-Rha-(1→] (Knirel, 1990). Null mutations in three of the genes within
the O6 O-antigen wbp cluster, wbpL, wbpP, and wbpO, have been generated and analysis
of the LPS phenotypes from these mutants show that these genes are essential for O-
antigen production (Bélanger et al., 1999). WbpL is the initiating transferase of O-antigen
biosynthesis and transfers the first sugar of the O-repeat unit, QuiNAc, from UDP-
QuiNAc to the undecaprenol phosphate lipid carrier. WbpP and WbpO are required for
synthesis of the complex nucleotide activated sugar donor molecules, N-
acetylgalactosaminuronic acid containing O-acetyl and amino substituents, and 2-deoxy-
2-formamido-galacturonic acid containing an amino group. The role of WbpP and WbpO
in synthesis of these sugars has been demonstrated by detailed biochemical studies.
WbpP is an UDP-N-acetylglucosamine (UDP-GlcNAc) C4 epimerase that converts UDP-
GlcNAc to UDP-N-acetylgalactosamine (UDP-GalNAc) (Creuzenet et al., 2000) and
WbpO is an UDP-GalNAc dehydrogenase, which converts UDP-GalNAc to UDP-
GalNAcA (Zhao et al., 2000). A second genetic locus, rmlBDAC, is also required for O-
antigen biosynthesis (Rahim et al., 2000) as it encodes biosynthetic enzymes necessary
for synthesis of dTDP-L-rhamnose (Giraud and Naismith, 2000). Therefore, the genes
encoding well-characterized proteins with respect to LPS biosynthesis, wbpL, wbpP,
wbpO, and rmlC are logical candidates to be used to investigate the relationship between
LPS biosynthesis and glycosylation of P. aeruginosa strain PAK flagellin.

In this chapter the hypothesis that LPS biosynthetic enzymes are required for
flagellin glycosylation in P. aeruginosa strain PAK was tested. Here I provide evidence
that the flagellin glycan contains a sugar residue that is synthesized by the O-antigen wbp
gene cluster and that this sugar is linked to the proximal residue in the glycan moiety.
Furthermore, these results indicate that in addition to the previously identified glycosylation gene island, biosynthesis of the flagellin glycan in strain PAK requires genes located in two other genetic loci, the \textit{rml} and \textit{wbp} gene clusters.

4.3 Results

4.3.1 Molecular mass of flagellin isolated from PAK and LPS mutants

The \textit{fliC} gene, which encodes the flagellin protein, was PCR amplified from genomic DNA from strain PAK and the nucleotide sequence was analyzed to determine the amino acid sequence of the translated protein. The predicted amino acid sequence of FliC from strain PAK used in our laboratory had 11 amino acid substitutions at amino acid positions 303-313 and six substitutions at position 321-326 when compared with the amino acid sequence of FliC, Genbank accession no. A37853, from Totten and Lory (1990; sequence revised 1999) (Fig. 4.1). When compared to the partial amino acid sequence of PAK FliC (Genbank accession no. AAC09389) submitted by Arora et al. (1999), FliC from our PAK strain had 12 amino acid substitutions. Interestingly, these 12 amino acid substitutions in the sequence submitted by Arora et al. (1999) is in a different region than the FliC submitted by Totten and Lory (1999). The predicted molecular mass of PAK FliC based on primary amino acid sequence using Protparam tool (ExPASy Molecular Biology Server of the Swiss Institute for Bioinformatics [http://ca.expasy.org/tools/protparam.html]) is 40 036 Da.

In testing the hypothesis that genes involved in O-antigen biosynthesis are also required for synthesis of sugar residues of the flagellin glycan, one would anticipate that mutants defective in biosynthesis of LPS would produce altered flagellin glycosylation.
**Figure 4.1** Amino acid comparison of the a-type flagellin sequence of the PAK FliC protein used in this study [strain source-Woods et al. (1997)] with two other PAK FliC proteins that are designated by their accession numbers A37853 and AAC09389. Identical amino acids are marked by asterisks; similar amino acids are marked by dots. The FliC protein with accession number AAC09389 is only a partial sequence. Regions with amino acid substitutions between PAK FliC used in this study and that of FliC (accession number A37853) are highlighted in grey and include the regions corresponding to amino acids 303-313 and 321-326. Alignment was performed by the ClustalW program (European Bioinformatics Institute [http://www.ebi.ac.uk/clustalw/#]; Pearson and Lipman (1988); Pearson (1990)).
Results from SDS-PAGE and Western immunoblotting showed that flagellin from the \textit{wbpL} and \textit{wbpP} mutant had an apparent molecular mass of 45 kDa, which was identical to the mass of the wild-type flagellin. In contrast, flagellin from the \textit{wbpO} mutant was 42 kDa (Fig. 4.2 A). To further substantiate the role of \textit{wbpO} in post-translational modification, flagellin was isolated from the PAK \textit{wbpO} mutant complemented with \textit{wbpO} on a plasmid (pFV616-26a). When provided \textit{in trans}, this plasmid has been shown to restore O-antigen synthesis in the \textit{wbpO} mutant (Bélanger et al., 1999). The transconjunctive strain PAK \textit{wbpO/} pFV616-26a produced flagellin with the same molecular mass as the glycosylated flagellin from the parent. Western immunoblotting of the PAK flagellins with rabbit polyclonal antiserum raised against purified PAK flagellin showed a strong reaction with the 45 kDa flagellin proteins isolated from the parent, \textit{wbpL}, and \textit{wbpP} mutant strains as well as the smaller sized flagellin isolated from the \textit{wbpO} mutant, but not to PAO1 flagellin, which served as a negative control (Fig. 4.2 B). These data indicated that the PAK \textit{wbpO} mutant produced flagellin with an altered glycosylation phenotype, which suggests that \textit{wbpO} is required for synthesis of the flagellin glycan.

The requirement for the production of dTDP-L-rhamnose in flagellin glycan biosynthesis was investigated by examining the electrophoretic mobility of flagellin isolated from a PAK \textit{rmlC} mutant with that from the wild type. Flagellin from the \textit{rmlC} mutant had an apparent molecular mass of 42 kDa (Fig. 4.3). To further verify that the faster mobility of flagellin from the \textit{rmlC} and \textit{wbpO} mutants was due to the loss of a glycan moiety, flagellin from wild-type PAK and the \textit{wbpO} mutant was chemically deglycosylated using trifluoromethanesulfonic acid as described by Edge et al. (1981).
Figure 4.2 Analysis of flagellins isolated from *P. aeruginosa*. (A) SDS-PAGE followed by staining with Coomassie brilliant blue, and (B) Western immunoblotting using the anti-FliC polyclonal antiserum. The wild-type flagellin migrates as a diffuse band indicative of glycosylation. Flagellin from the *whpO* mutant showed a molecular mass smaller than the flagellin isolated from the parent strain. PAK *whpO* carrying plasmid pFV616-26a, which contains a copy of wild-type *whpO*, produced flagellin proteins with a mass similar to that of the parent strain. Flagellin isolated from strain PAO1 was included as a negative control for the immunoblotting experiment.
Figure 4.3 Comparison of flagellins isolated from wild-type PAK and the rmlC mutant with chemically deglycosylated flagellin from PAK and the wbpO mutant by Coomassie blue staining after separation by SDS-PAGE. The apparent molecular mass of flagellin from the rmlC mutant was similar to that observed for the deglycosylated flagellins, suggesting loss of glycosylation in this LPS mutant strain.
Deglycosylated flagellin from these two strains had an apparent molecular mass similar to that of the flagellin isolated from the PAK \textit{rmlC} mutant (Fig. 4.3). These data suggest that \textit{rmlC} and \textit{wbpO} synthesize sugar nucleotides necessary for complete synthesis of the glycan.

### 4.3.2 Flagellin from the \textit{wbpO} mutant has altered isoelectric point

To determine if there are charge differences between the glycosylated flagellin from the wild-type PAK and the flagellin from the \textit{wbpO} mutant, flagellin purified from PAK and the LPS mutants was analyzed by IEF gels with a pH gradient of 3-7. Based on the amino acid sequence of the PAK FliC protein, the isoelectric point (pI) of this protein is predicted to be 4.94 using the ProtParam program (ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics; [http://ca.expasy.org/]). The predicted pI does not include any charge effects that may be contributed by the glycan moiety. In IEF gel electrophoresis, flagellin from the wild type, \textit{wbpL} and \textit{wbpP} mutants resolved as multiple isoforms ranging from a pI of 4.6-4.8, with a predominant isoform with a pI of 4.6 (Fig. 4.4 A). Flagellin from the \textit{wbpO} mutant also appeared to have multiple isoforms, however, the predominant isoform was less acidic, with a pI of 4.8, when compared to that of the parent strain. Flagellin proteins isolated from the complemented mutant, \textit{wbpO/ pFV616-26a}, exhibited an IEF banding profile similar to that of the wild-type flagellin. To verify that the different flagellin isoforms observed on the IEF gels were flagellin, proteins from the IEF gels were transblotted onto nitrocellulose and a Western immunoblot was performed using antiserum to PAK FliC. In all lanes, the predominant protein band had a strong positive reaction (Fig. 4.4 B) whereas the minor
Figure 4.4 Isoelectric focusing of PAK flagellins. (A) Simply-blue stained IEF gel, and (B) Western immunoblot using anti-FliC antiserum. Flagellins were separated in an ampholyte mixture of pH 3-7. Flagellin from the wbpO mutant corresponds to the predominant protein band that reacted with the anti-FliC antiserum, and this flagellin has a significant difference in charge as compared to the flagellin from the other strains. The pI of the wbpO mutant flagellin is similar to the pI predicted for the nonglycosylated FliC protein. Flagellin proteins isolated from the complemented mutant, wbpO/ pFV616-26a, exhibited an IEF banding profile similar to that of the wild-type flagellin.
protein bands that isofocused around the predominant band showed weak positive reactions. Flagellin isolated from the \textit{wbpO} mutant was considerably less acidic than the flagellin of the parent strain. Also, the pI of the \textit{wbpO} mutant flagellin was closer to the pI that was predicted for the nonglycosylated FliC protein.

4.3.3 Flagellin glycosylation phenotypes of PAK and LPS mutants

The observed differences between the apparent molecular mass and charge of the flagellin from the wild type and \textit{wbpO} mutant suggest a change in, or lack of, glycosylation in the flagellin produced by the LPS mutant. Flagellin produced by the \textit{wbpO} mutant has an apparent molecular mass of ca. 42 kDa, as determined by SDS-PAGE, and this is greater than the 40-kDa mass predicted from the amino acid sequence of PAK FliC (without the glycan), indicating that flagellin from the \textit{wbpO} mutant may still contain covalently linked sugar residues. A biotin-hydrazide glycosylation assay of the flagellin proteins was performed and the assay showed strong positive reactions with the flagellin isolated from all strains except for the \textit{wbpO} mutant (Fig 4.5). Close examination of the nitrocellulose reveals a possible weak positive reaction to the \textit{wbpO} mutant flagellin, which could not be reproduced in the figure at a level sufficient for visibility. Negative and positive protein controls were included in the assay. Nonglycosylated protein WbpD and glycoprotein transferrin gave a negative and positive reaction, respectively. Results from the glycosylation assay suggest that flagellin from the \textit{wbpO} mutant may still contain one or a small number of covalently linked sugar residues that result in an ‘incomplete’ glycan moiety. However, the extent of glycosylation is clearly not the same as that seen in the flagellin from the wild-type strain.
Figure 4.5 Biotin-hydrazide glycosylation assay of flagellins isolated from *P. aeruginosa* PAK and its LPS mutants. Plasmid pFV616-26a contains wild-type *wbpO*. Negative and positive controls included in the glycosylation assay were purified WbpD and the glycoprotein transferrin, respectively. Flagellin isolated from the *wbpO* mutant showed a very weak positive reaction and flagellin from the *wbpO* mutant carrying *wbpO in trans* (pFV616-26a) showed a positive reaction similar to that of the parent strain.
4.3.4 Mass spectrometry analysis of *P. aeruginosa* flagellins

MALDI-TOF MS analysis of the intact flagellin proteins was performed by Dr. Dyanne Brewer at the Biological Mass Spectrometry Facility (Department of Molecular Biology and Genetics, University of Guelph). Flagellin purified from strain PAK, the *wbpO* mutant and the *rmlC* mutant showed a peak ([M+H]⁺ ion) at a mass-to-charge ratio (m/z) of 42 576, 40 180 and 40 041 (±10), respectively (Fig. 4.6). The molecular mass of the nonglycosylated FliC protein was predicted to be 40 036 Da based on primary amino acid sequence. Therefore, these data indicated that the wild-type flagellin contains covalently bound material corresponding to a total mass of ca. 2 540 Da, whereas the flagellin from the *wbpO* mutant possibly contained one sugar with a corresponding mass of ca. 144 Da. This mass is consistent with the addition of a deoxyhexose residue. In contrast, MALDI-TOF MS analysis of flagellin isolated from the *rmlC* mutant showed a peak at a m/z value of 40 041 (±10), The corresponding mass of this flagellin is similar with that predicted for the nonglycosylated form of the FliC protein. *rmlC* is required for synthesis of dTDP-L-rhamnose, which has a predicted molecular mass of 146 Da.

Chemically deglycosylated flagellin from wild-type PAK and the *wbpO* mutant was also analyzed by MALDI-TOF MS. Data from these experiments showed peaks at a value of m/z of 40 029 (±10) and 40 027 (±10), respectively (Fig. 4.7). This data further indicates that the additional masses observed in the flagellin from PAK and the *wbpO* mutant are glycan moieties, as there is considerable evidence that suggests that post-translational modifications other than glycosylation are stable to trifluoromethanesulfonic acid treatment (reviewed in Edge et al., 2003). These data support the hypothesis that *rmlC* and *wbpO* are required for synthesis of nucleotide-activated sugars that act as donor
Figure 4.6 MALDI-TOF mass spectrum of intact flagellin from *P. aeruginosa* wild-type PAK (A), the *wbpO* mutant (B) and the *rmlC* mutant (C). The flagellin from the different strains show peaks of \( m/z \) of 42576, 40180 and 40041, respectively. The Reflex III MALDI-TOF instrument was externally calibrated with trypsinogen for the flagellin isolated from the wild type and *wbpO* mutant, whereas alcohol dehydrogenase was used for the PAK *rmlC* flagellin. The \( m/z \) values of ca. 15000 and 13910 that also appear in the mass spectra correspond to contaminating protein. Abbreviation a.i., abundance intensity.
Figure 4.7 MALDI-TOF mass spectrum of flagellin after chemical deglycosylation with trifluoromethanesulfonic acid. Deglycosylated flagellin from PAK (A) and the wbpO mutant (B) show peaks at m/z of 40 029 (±10) and 40 027 (±10), respectively, and is consistent with the size that is predicted for the nonglycosylated FliC protein. The m/z values of 36 849.100 in both mass spectra correspond to an internal calibrant, alcohol dehydrogenase.
molecules for sugar residues of the flagellin glycan. Alternatively, the products of the RmlC and WbpO-mediated enzyme reactions are intermediates for the synthesis of more complex sugar residues. The mass spectrometry data presented here suggests that the deoxyhexose sugar that is derived from pathway involving RmlC is attached directly to the flagellin, whereas the sugar that is derived from the pathway that utilizes WbpO, is the second residue of the glycan.

To assign more precisely the location of the glycosylation sites on the PAK FliC protein, flagellin from both the wild type and wbpO mutant strains as well as these flagellins chemically deglycosylated were digested with trypsin. Technical assistance in trypsin digestion of the flagellin proteins was provided by Hamed Ghanei (University of Guelph) according to experiments designed by the author and protocols that were provided by Dr. Dyanne Brewer (Biological Mass Spectrometry Facility, Department of Molecular Biology and Genetics, University of Guelph) as outlined in the materials and methods. Trypsin digests were analyzed by MALDI-TOF MS by Dr. Dyanne Brewer. Comparison of the experimentally obtained tryptic peptide m/z values with theoretical peptide m/z values [M+H⁺], which were determined by the PeptideMass program (ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics [http://ca.expasy.org/tools/peptide-mass.html]; Wilkins et al., 1997; 1998) was performed using the Profound search engine (http://prowl.rockefeller.edu/profound_bin/WebProFound.exe) as well as direct visual comparison and analysis. Three sets of tryptic digests of flagellin isolated from wild-type PAK, the PAK wbpO mutant and a control lane from the gel were analyzed on three separate occasions. Our analysis of one set of samples from one experiment revealed
coverage of the flagellin protein from PAK and the \textit{wbpO} mutant to be 77\% and 68\%, respectively. Analysis from the other two sets of digests revealed similar coverage and by evaluating the information from all of these experiments, five peptides could not be identified. The deglycosylated flagellins were also subjected to trypsin digestion to possibly determine if there were any peptides that might be identified in these samples that would assist in the identification of potential glycopeptide candidates. Sequence coverage obtained for the deglycosylated flagellin from the wild type and the \textit{wbpO} mutant was 68\% and 64\%, respectively. Since the Reflex III MALDI-TOF instrument used for this study has a detection range of a \textit{m/z} value of 800 - 4 000 and the mass of three tryptic peptides not identified by MALDI-TOF analysis were predicted to be < 800 Da, further analysis was performed by Dr. Dyanne Brewer using electrospray MS, which has a detection range of a \textit{m/z} value of 50 - 1 500. Three tryptic peptides corresponding to amino acids 211-221 (1 089.5 Da), 289-295 (747.4 Da) and 296-302 (746.4 Da) could not be identified by MALDI-TOF or electrospray MS and represent potential candidates for peptides that may contain glycosylation site(s) of the PAK FliC protein (Fig. 4.8). One of the reasons as to why these three peptides could not be identified by these two methods is that they do not ionize very well. All three peptides contain threonine or both serine and threonine residues, which represent potential O-linked glycosylation sites.

Analysis of the tryptic peptides from the deglycosylated flagellin of the PAK \textit{wbpO} mutant by MALDI-TOF MS revealed a peak in the mass spectrum with an \textit{m/z} value of 1 563.440. This value was comparable to the predicted value of 1 562.7479 for the tryptic peptide corresponding to amino acids 207-221, with a missed trypsin cleavage site. The tryptic peptide corresponding to amino acids 207-221 contains a potential
MALTVNNTIA  SLNTQRNLNN  SSASLNSTSLQ  RLSTGSRINS  AKDDAAGLQI  50
ANRLTSEQVNG  LNVAIKNAND  GISLAQTAEG  TALQCSNILQ  RMRDLSLQSA  100
NGSNSSDSDRT  NALGEVKQLQ  KELDRISNTT  TFGGRKDLLDG  SFGVASFQVG  150
SAANEIIISVG  IDEMSAESLN  GTYFKADGGG  AVTAATASGT  VDIAIGITGG  200
SAVNVKVDMK  GNTEAEQAAAA  KIAAAVNDAN  VGIGAFSDGD  TISYVSKAGK  250
DGSGAITSAV  SGGVIADTGS  TGVGTAAGVA  PSA  TAFKTN  DTVAKIDIST  300
AGQAQSAVVLV  IDEAIKQIDA  QRADLGAVQN  RFDNTINNLK  NIGENVSAAR  350
GRIEDTDFAA  ETANLTKNQV  LQQAGTAILA  QANQLPQSVL  SLLR  400

M_r: 40 036 Da

Figure 4.8 Primary amino acid sequence of the PAK FliC protein. Peptides that were identified by MALD-TOF or electrospray MS are shown in boldface type, whereas peptides that were missing from the analysis (shown in boxes) contain potential serine or threonine residues (highlighted in red) for O-linked glycosylation sites.
trypsin cleavage site after residue 210, which if cleaved, results in two fragments corresponding to amino acids 207-210 and 211-221. Incomplete digestion of flagellin protein with trypsin may result in missed cleavages, and the m/z value of 1 563.440 is consistent with the 207-221 fragment with a missed cleavage. The mass spectrum data obtained from analysis of the tryptic peptides from the flagellin from the PAK wbpO mutant and the control digest did not show this peak at m/z of 1 563.440. The control digest contains tryptic peptides from protein isolated from a polyacrylamide gel slice taken from an empty lane in the same gel that was used to isolate the flagellin. It serves as a control for protein contamination which may occur at various stages during the procedure and this gel slice is processed in the same manner as the polyacrylamide gel slices containing flagellin protein. The presence of a peptide with a corresponding m/z value of 1 563.440 in the trypsin-digested deglycosylated flagellin sample and the absence of it in the trypsin-digested flagellin from the PAK wbpO mutant provides further evidence that tryptic peptide 211-221 may be a potential region for glycosylation in the wbpO mutant flagellin. This peptide has a threonine residue at position 214 and this represents a potential glycosylation site. If the tryptic peptide 211-221 contained one deoxyhexose residue, as shown by the data in the previous sections, the predicted monoisotopic mass of this glycopeptide would correspond to a m/z value of 1 235.575 (mass of the peptide [1 089.5171] plus deoxyhexose [146.0579]). Close examination of the peaks and corresponding m/z values from the mass spectrum obtained from analysis of the wbpO mutant flagellin revealed a peak with a m/z value of 1 236.805 (±1.237) which was not observed in the mass spectra obtained from analysis of the control digest or that from the wild-type strain (Fig. 4.9). This m/z value is consistent with the
Figure 4.9  MALDI-TOF mass spectrum of peaks that correspond to the tryptic peptides detected within the range of a mass-to-charge ratio (m/z) of 1 200 to 1 300. A peptide from the tryptic digest of flagellin isolated from the PAK *wbpO* mutant at *m/z* 1236.80 (A) was not observed in the tryptic digest of flagellin from wild-type PAK (B) or the control digest (C). Abbreviation a.i., abundance intensity. The Reflex III MALDI-TOF instrument was externally calibrated with ACTH (18-39) human (CLIP) which has a corresponding molecular mass of 2465.7 Da.
corresponding mass of a glycopeptide at amino acids 211-221. In addition, a \( m/z \) value of 1236.805 was not detected in the analysis of the tryptic peptides from the deglycosylated flagellin. Altogether, these results indicate that rhamnose is covalently attached to FliC within the peptide that corresponds to amino acids 211-221 of the flagellin isolated from the PAK \( wbpO \) mutant. These data, however, do not exclude the possibility that the other two tryptic peptides, corresponding to amino acids 289-295 and 296-302, within the flagellin produced by the wild-type strain contains sugar residues.

4.3.5 Swimming motility of PAK and the LPS mutants

To see if the difference in glycosylation status of the \( wbpO \) mutant affects the function of the flagellum, flagella-mediated swimming motility was examined by measuring the ability of motile bacterial cells to migrate away from the point of inoculation within motility media. The \( wbpO \) mutant migrated slightly less than that of the parent strain (Fig. 4.10). The motile phenotype of the other LPS mutants was examined and the \( wbpP \) mutant also showed a slight decrease in swimming motility, whereas the \( wbpL \) mutant showed the greatest decrease in motility. O antigen and A-band LPS production in the PAK \( wbpL \) mutant (which showed the most significant change in motility) can be restored by complementation with \( wbpL \) in trans (pFV605-26) (Bélanger et al., 1999). The complemented mutant, \( wbpL/ \) pFV605-26, showed partial restoration of the swimming phenotype. Strain PAK-N1 was included as a negative control for flagella-mediated motility as this strain does not produce flagella. These results suggest that a mutation in \( wbpO \) does affect flagella-mediated swimming motility. However, since the other LPS mutants also showed various degrees of defective motility, the change in
Figure 4.10 Flagella-mediated swimming motility of *P. aeruginosa* strain PAK and its isogenic LPS mutants, *wbpL*, *wbpP*, and *wbpO*. Strain PAK-N1 does not produce flagella and is included as a negative control for swimming motility. Bacteria were inoculated into the center of the soft agar plates and images were taken 18 h after incubation at 30°C. Although the diameter of the migrating bacteria seems apparent, the lines drawn on the agar plates indicates the actual edge of the swimming bacteria within the agar. All of the LPS mutants showed decreased motility when compared to that of the parent strain with the *wbpL* mutant showing the greatest decrease. The PAK *wbpL* mutant transformed with a plasmid containing the *wbpL* gene (pFV605-26) is included and shows partial restoration of the motile phenotype.
swimming phenotype of the wbpO mutant is not due to changes in flagellin glycosylation alone. Other factors, for instance, the loss of long-chain O antigen and A-band polysaccharide on the cell surface could also affect swimming motility in P. aeruginosa.

4.3.6 Electron microscopy examination of P. aeruginosa flagella

Since there were differences in bacterial motility and glycosylation of the flagellin proteins, whole cells of P. aeruginosa PAK and the LPS mutants were examined by transmission electron microscopy using negative staining to examine the flagella. Electron microscopy was performed by Dianne Moyles (Microbiology Department, University of Guelph). The wbpL, wbpP, and wbpO mutants were observed to produce single, polar flagella (Fig. 4.11). Examination of the width of the flagellar filaments between the wild type and the wbpO mutant showed no significant differences in structure and the flagella appeared lined in both strains. These electron microscopy studies show that all of the strains tested, including the wbpO mutant, which has altered flagellin glycosylation, appeared to have normal flagellar assembly.
Figure 4.11 Electron microscope analysis of *P. aeruginosa* flagella. A representative bacterial cell (PAK *wbpO* mutant) showed a single flagella located at the pole of the cell (indicated by the arrow, the other filamentous structure is pilus) (A). Bar, 200 nm. Closer examination of the flagella from wild-type PAK (B) and the PAK *wbpO* mutant (C) showed no significant differences in the structure of the flagellar filament. Bar, 50 nm.
4.4 Discussion

The interrelationship between LPS biosynthesis and protein glycosylation has been shown to vary significantly among different prokaryotes (Stimson et al., 1995; Szymanski et al., 1999; DiGiandomenico et al., 2002). This investigation into the role of LPS genes in glycosylation of flagella in *P. aeruginosa* strain PAK leads to the following conclusions: (i) flagellin glycosylation in strain PAK requires an LPS biosynthetic enzyme encoded by *wbpO*, which is located in the O-antigen gene cluster and maps outside the "flagellin glycosylation island" previously identified by Arora et al. (2001), (ii) the nucleotide activated sugar donor produced by the WbpO-enzyme reaction is channeled into assembly of LPS as well as flagellin glycan synthesis, (iii) a third locus, *rmlC* is also required for the flagellin glycan for synthesis of a deoxyhexose residue, and, (iv) the entire O-antigen unit is not required for the flagellin glycan.

Many lines of evidence presented here show that WbpO, an enzyme required for O-antigen biosynthesis, is also required for flagellin glycosylation in *P. aeruginosa* strain PAK. These include the significant differences in size, charge, and glycosylation status of the flagellin isolated from the *wbpO* mutant when compared to that of the wild-type strain. These specific criteria have been used by various groups to show glycosylation differences among pilin and flagellin proteins within different Gram-negative bacteria (Doig et al., 1996; Brimer and Montie, 1998; Arora et al., 2001). The finding that *wbpO* is involved in flagellin glycosylation is quite interesting because another locus, called the "flagellin glycosylation gene island", has previously been identified in *P. aeruginosa* strain PAK (Arora et al., 2001). Using techniques similar to those used in this study, this glycosylation gene island was shown to be required for flagellin glycosylation. Therefore,
in strain PAK, two unlinked genetic loci are involved in flagella glycosylation and one of them, \textit{wbpO}, is also required for LPS synthesis. This is similar to pilin glycosylation in \textit{N. meningitidis} where a biosynthetic enzyme encoded by \textit{galE}, which produces UDP-galactose, is required for both LOS and pilin glycosylation (Stimson et al., 1995). \textit{galE} is unlinked to four other loci that are also required for glycosylation (reviewed in Power and Jennings, 2003). In \textit{P. aeruginosa}, the interplay between LPS biosynthesis and protein glycosylation in strain PAK is quite different than that found in \textit{P. aeruginosa} strain 1244. In 1244, the O-antigen cluster directs synthesis of the O antigen and the O-antigen unit is covalently linked to the 1244 pilin protein (DiGiandomenico et al., 2002).

Although the number of genes, and therefore the extent of involvement of the LPS genes in glycosylation of pilin and flagellin differ between the two \textit{P. aeruginosa} strains, the interrelationship that is reported here for LPS biosynthesis and flagellin glycosylation in strain PAK suggests that similar to pilin glycosylation, synthesis of the flagellin glycan is dependent on expression of the O antigen.

The fact that WbpO is required for both the flagellin glycan and the O antigen in PAK indicates that the product of the WbpO-catalyzed reaction is incorporated into both carbohydrate structures. The O antigen of strain PAK (serotype O6) is a repeating tetrasaccharide unit composed of \(\alpha\)-L-rhamnose, \(N\)-acetyl-\(\alpha\)-D-2,6-dideoxy-glucosamine (\(N\)-acetyl-\(\alpha\)-D-quinovosamine), and two \(\alpha\)-D-galactosaminuronic acid residues, one is formylated, whereas the second is acetylated (Knirel, 1990). The function of WbpO in synthesis of the two \(N\)-acetyl-D-galactosaminuronic acid (GalNAcA) residues of the O antigen has been investigated by our laboratory. The GalNAcA derivatives of the O antigen have been proposed to be derived from a nucleotide-activated form, namely,
UDP-GalNAcA. A two-step biosynthetic pathway has been proposed for the synthesis of UDP-GalNAcA (Bélanger et al., 1999). Based on this model, UDP-GlcNAc undergoes an epimerization reaction by WbpP to produce UDP-GalNAc. WbpO converts this product in an oxidation reaction at C6 to form UDP-GalNAcA. To test this model, WbpP and WbpO have been overexpressed and purified. Detailed enzymology studies confirmed the function of WbpP as an UDP-GlcNAc C4 epimerase (Creuzenet et al., 2000) and WbpO as a C6 dehydrogenase (Zhao et al., 2000). Kinetic investigation of WbpO showed a preference of WbpO for UDP-GalNAc over UDP-GlcNAc as the substrate and WbpO is specific for N-acetylated substrates. If WbpO is involved in the synthesis of a nucleotide activated sugar donor, such as UDP-GalNAcA, this molecule could be an intermediate substrate in the synthesis of a more complex residue that is eventually incorporated into the growing flagellin glycan.

Based on the current pathway proposed for synthesis of the GalNAcA derivatives of the O antigen, with WbpO acting on the reaction product of WbpP, it was predicted that a *wbpP* mutant would have a similar glycosylation phenotype to that of the *wbpO* mutant, since both genes are thought to be involved in synthesis of the UDP-GalNAc derivatives. The results from this study indicated that the *wbpP* mutation had no apparent effect on flagellin glycosylation and this observation is inconsistent with this pathway. Recently, exciting new data from our laboratory, however, supports the proposal of a different pathway for the synthesis of GalNAcA derivatives, which is consistent with the results from this study. The enzyme kinetics obtained for WbpO by Zhao et al. (2000) was based on the activity of refolded WbpO protein, as the soluble protein was inactive. It was also shown that the refolded protein had a different secondary structure than that
predicted for the native WbpO. Recently, a different approach was taken to overexpress and purify WbpO and the soluble protein was shown to be active (W. Miller, C. Q. W. Wenzel and J. S. Lam, unpublished data). Kinetic investigation of this purified WbpO shows that it has a preference for UDP-GlcNAc over UDP-GalNAc as a substrate, and converts UDP-GlcNAc to UDP-GlcNAcA, which is in contrast to what was shown by Zhao et al. (2000), who demonstrated that refolded WbpO had a preference for converting UDP-GalNAc to UDP-GalNAcA. These new data suggest that WbpO first converts UDP-GlcNAc to UDP-GlcNAcA, and this nucleotide sugar donor can be used directly or as an intermediate for synthesis of a different sugar residue by the “glycosylation genes” and then incorporated into the growing glycan of the flagellin. In light of this new data, more studies are needed to clarify the exact sequence in the biosynthetic pathway of the nucleotide activated sugar donors that are required for the GalNAc or GlcNAc derivatives that are found in the O antigen and flagellin glycan.

Data from this study indicates that the proximal sugar residue that is covalently attached to the flagellin protein is a deoxyhexose residue and that this sugar requires the activity of RmlC. MALDI-TOF MS analysis of the flagellin isolated from the wbpO mutant indicated that this flagellin contained an additional mass that corresponded to deoxyhexose residue. Results obtained from examination of the flagellin isolated from a PAK rmlC mutant using SDS-PAGE and MALDI-TOF MS analysis support this conclusion, since the molecular mass of the flagellin was found to be similar to that of the deglycosylated parent flagellin as well as that predicted for the nonglycosylated FliC protein. Analysis of flagellin isolated from the PAK rmlC mutant carrying rmlC on a plasmid revealed partial restoration of the glycosylation phenotype (M. J. Matewish and
J. S. Lam, unpublished data). In addition, analysis of the flagellin from the \emph{wbpO} mutant, as well as the flagellin from the \emph{wbpO} mutant after chemical deglycosylation indicated the mass difference to correspond to that of a deoxyhexose. These results are consistent with those reported recently by Shirm et al. (2004), who investigated the structure of the flagellin glycan produced by \emph{P. aeruginosa} strain PAK. Based on data obtained by analysis of tryptic digests of PAK flagellin by nanoelectrospray mass spectroscopy, it was determined that a deoxyhexose was the first sugar residue attached to the flagellin. Analysis of the monosaccharides of the glycan by alditol acetate derivatization followed by gas chromatography-mass spectrometry (GC-MS) revealed the major components of the glycan to be rhamnose, mannose, glucose, 4-amino-4,6-dideoxyglucose (viosamine). They also reported the presence of trace amounts of ribose and arabinose. Analysis of flagellin from another \emph{P. aeruginosa} strain, JJ692, revealed a simpler glycosylation scenario than that observed in strain PAK. Using a combination of GC-MS and nanoelectrospray mass spectroscopy to analyze the flagellin, it was concluded that there were two rhamnose residues, both of which were \emph{O}-linked at two different sites within the flagellin. The authors in this study used these data as further evidence to conclude that in strain PAK, the deoxyhexose identified to be \emph{O}-linked to the flagellin was most likely a rhamnose residue. In the present investigation, flagellin from a genetically defined PAK \emph{rmlC} mutant was examined. Rahim et al. (2000) characterized the \emph{rmlBDAC} operon in \emph{P. aeruginosa} at the genetic level and knockout mutations in the \emph{rmlC} gene followed by structural analysis of the LPS isolated from the \emph{rmlC} mutants provided evidence that this gene is involved in synthesis of dTDP-L-rhamnose. The \emph{rmlABCD} operon of \emph{S. enterica} serovar Typhimurium has been characterized genetically and the gene products studied
using biochemical approaches. This system serves as a model for investigations into the synthesis of dTDP-L-rhamnose. Many studies strongly support the assignment of RmlC as a dTDP-4-dehydrorhamnose 3,5-epimerase and have shown this enzyme catalyzes the conversion of dTDP-6-deoxy-D-xylo-4-hexulose to dTDP-6-deoxy-L-lyxo-4-hexulose (Kornfeld and Glaser, 1961; Glaser and Kornfeld, 1961; Jiang et al., 1991; Schnaitman and Klena, 1993; Reeves, 1994; Graninger et al., 1999). This product is then converted to dTDP-L-rhamnose by the enzyme activity of RmlD (Graninger et al., 1999). By analyzing the flagellin from a PAK rmlC mutant, this study equivocally demonstrated that the product of the RmlC reaction is required for synthesis of the first proximal sugar of the PAK flagellin.

The nature of the glycosylation of the flagellin in strain PAK was found by Schirm et al. (2004) to have oligosaccharides of various lengths and complex microheterogeneity. Using data obtained from GC-MS analysis of the flagellin glycan and mass measurements of glycan fragment ions, they designed a structural model to account for the different combinations of masses of sugars that could best describe the data they observed. They concluded that the glycans contain hexoses, deoxyhexoses, amino sugars and formyl deoxyhexose residues. Interestingly, amino and formyl substituted sugar residues are present in the O-antigen unit of PAK, specifically the two galactosaminuronic acid (α-D-GalNAcA) derivatives: N-acetylgalactosaminuronic acid containing O-acetyl and amino substituents, and 2-deoxy-2-formamido-D-galacturonic acid containing an amino group (Knirel and Kochetkov, 1994). Schirm et al. (2004) concluded that other than rhamnose, no other O-chain specific monosaccharides were found to be present in the glycan. However, identification of the actual sugar residues
was accomplished by GC-MS and the presence of uronic acids was not determined in this study. In addition, the model and assignment of sugars was designed based on fitting in combinations of sugars that would account for the masses of the many different oligosaccharide chains observed. One interesting observation is the presence of ribose in their flagellin glycan preparations. The authors did not discuss whether this could be a result of RNA contamination. The method of flagellin isolation in the study by Schirm et al. (2004) was based on the shearing of flagella from whole cells followed by ultracentrifugation as originally described by Totten and Lory (1990). However, in the Totten and Lory (1990) study, in addition to ultracentrifugation they also used anion-exchange chromatography to further purify the flagellin preparation and this procedure was not mentioned or described in the methods section of Schirm et al. (2004). One concern is the presence of LPS in the flagellin samples. Experiments in our laboratory have shown that Western immunoblotting of flagellin [isolated using the same method of Totten and Lory (1999) without a chromatography separation method] resulted in a positive reaction to monoclonal antibodies specific to O-antigen LPS and is indicative of contamination of the flagellin sample with LPS (M. J. Matewish and J. S. Lam, unpublished data). The removal of LPS from the flagellin samples was not discussed. Clearly, further detailed structural analysis of the flagellar glycan is warranted to reveal if the same GalNAcA derivatives of the O antigen are found in the flagellin glycan. Conversely, if these GalNAcA derivatives are not found in the glycoprotein, the structural information will lead to the development of an exciting new biosynthetic pathway whereby WbpO is involved in synthesis of a common intermediate that is channelled into both LPS and flagellin glycosylation.
To determine the extent of the interrelationship between LPS and flagellin glycan synthesis, the glycosylation status of flagellin from the PAK wbpL mutant was examined. WbpL is a key glycosyltransferase that initiates assembly of the entire O-antigen unit (Rocchetta et al., 1998a; Bélanger et al., 1999). Data presented here indicated that a mutation in wbpL had no effect on flagellin glycosylation. This is consistent with the recent results obtained by Schirm et al. (2004), who have determined the size of the PAK flagellin glycan to be up to eleven monosaccharides in size, whereas the O-antigen unit contains four sugar residues. Also, the presence of a separate glycosylation gene island containing fourteen genes that is also required for flagellar glycosylation suggests that it contains the majority of the genes required for assembly and synthesis of the glycan moiety. This result is quite different than that reported for the role of wbpL in pilin glycosylation of P. aeruginosa strain 1244. A mutation in wbpL results in mutants that produce nonglycosylated pilin (DiGiandomenico et al., 2002). Further studies by DiGiandomenico et al. (2002) show that the O-antigen unit synthesized by the O-antigen wbp gene cluster is covalently attached to the pilin protein. In P. aeruginosa strain PAK, the O-antigen tetrasaccharide unit is not attached to the flagellin protein.

Using a combination of MALDI-TOF and electrospray MS, three peptides were identified as potential candidates to contain the amino acid residue for the covalent attachment of the flagellin glycan in P. aeruginosa strain PAK. Although we were unable to determine equivocally the exact linkage site(s), the data collected so far suggests that the flagellin of this strain may not have the same glycan attachment sites as that observed for the flagellin from the PAK strain used in the study by Schirm et al. (2004). This group, however, used a powerful combination of mass spectrometry techniques including
capillary liquid chromatography-nanoelectrospray quadrupole time-of-flight MS followed by MS-MS. Since very little is known about the mechanism of flagellin glycosylation and the genes required for this process, further experiments are required to identify the exact location of the glycosylation sites within the flagellin of the PAK strain used in our laboratory.

There is much evidence to indicate that bacteria that produce polar flagella, such as *Campylobacter* spp (Logan et al., 2002), *H. pylori* (Schirm et al., 2003), *A. caviae* (Gryllos et al., 2001), and *P. aeruginosa* (Brimer and Montie, 1998) produce glycosylated flagella. In contrast, the biological role of glycosylation and the effect glycosylation has on assembly and function of the flagella are not clearly defined at present. In *P. aeruginosa* PAK, the *whpO* mutant was less motile than the parent strain, but this phenotype was observed for the other LPS mutants, which were not defective in flagellin glycosylation. This indicates that the production of different forms of LPS may affect motility. Examination of the flagella by electron microscopy revealed that all strains produced intact polar flagella that appeared to be assembled properly. These observations are consistent with those made by Arora et al. (2001) who reported that mutations in the genes within the flagellin glycosylation island in strain PAK result in mutants which have no motility defects. However, mutations in genes involved in flagella glycosylation in *H. pylori* result in a nonmotile phenotype, no structural flagella filament and only minor amounts of flagellin protein (Schirm et al., 2003). Nonmotile phenotypes have also been reported for glycosylation mutants of *Campylobacter* spp (Linton et al., 2000; Thibault et al., 2001). So far, the biological role of flagella glycosylation in *P. aeruginosa* is not known.
In conclusion, this investigation provides evidence that \textit{wbpO}, a gene within the \textit{wbp} O-antigen biosynthetic gene cluster, is involved in the synthesis of a sugar residue that is incorporated into the flagellin glycan in strain PAK. Elucidation of the detailed chemical structure of the flagellin glycan and further investigation into the biosynthetic pathways for the O-antigen GalNac or GlcNAc derivatives should reveal the relationship between the reaction product of WbpO, the O antigen and the flagellin glycan.
Chapter 5. WapH is a putative glucosyltransferase required for synthesis of the core oligosaccharide region of the lipopolysaccharide of *Pseudomonas aeruginosa*

5.1 Abstract

The lipopolysaccharide of *P. aeruginosa* is composed of lipid A, core oligosaccharide and O polysaccharide or O antigen. A second form of LPS, composed of lipid A and core oligosaccharide that is not substituted with long chain polysaccharide, is also produced. The two core oligosaccharide forms have a slightly different structure in the outer-core region. A genetic locus consisting of waaFwaaCwapGwaaPwapPwapQ, which is required for production of the inner-core region, belongs to a larger operon containing six additional open reading frames (ORFs). One of these ORFs, PA5004, designated here as *wapH*, encodes a protein that resembles glycosyltransferases. In this study, I test the hypothesis that WapH is a glucosyltransferase required for synthesis of the core oligosaccharide. Non-polar chromosomal *wapH* mutants were generated in *P. aeruginosa* strain PAO1 using an allelic replacement strategy. SDS-PAGE and Western immunoblotting with LPS-specific monoclonal antibodies showed that the *wapH* mutant produced LPS that was truncated in the outer-core region. This mutant produced small quantities of high-molecular weight B-band polysaccharide that was attached to core-lipid A, however, no core-lipid A linked A-band PS could be detected. WapH was expressed in the pET system as a C-terminal histidine-tagged protein. SDS-PAGE analysis of WapH along its purification showed the protein to have apparent molecular mass of 44 kDa, which is consistent with its predicted mass. *wapH* with a C-terminal histidine tag provided in trans in a PAO1 *wapH* mutant was shown to restore synthesis of
LPS to the parent phenotype. This is the first outer-core OS glucosyltransferase gene to be characterized for *P. aeruginosa*. This study shows that wapH is critical for complete assembly of the outer-core region of both types of core oligosaccharides, that which is capped with long chain A- and B-band LPS and the unsubstituted form.

5.2 Introduction

*Pseudomonas aeruginosa* is the primary cause of chronic lung infections in individuals with cystic fibrosis (Lyczak et al., 2002) and bacterial keratitis associated with contact lens wear (Coster and Badenoch, 1987; Schein et al., 1989). Studies which have examined the initial stages of infection of this microbe with bronchial and corneal epithelial cells have shown the core OS region of the LPS molecule to be an important bacterial ligand for *P. aeruginosa*-epithelial cell interactions (Pier et al., 1996a; 1996b; Zaidi et al., 1996). The identification of the core OS-CFTR protein interaction has provided the impetus to further investigate the consequences of this interaction on pathogenesis. Internalization of *P. aeruginosa* mediated by core OS-CFTR binding within the epithelial cells of the respiratory tract appears to serve as a mediator of innate immunity (Pier et al., 1996a; Schroeder et al., 2001; 2002; Cannon et al., 2003), whereas in the cornea, it may contribute significantly to the successful colonization and infection of host tissues (Fleischig et al., 1994; Zaidi et al., 1999).

To investigate the role of LPS in pathogenicity of this organism, researchers have relied on comparing wild-type strains to those strains defective in the production of LPS for testing in a number of *in vitro* assays and *in vivo* animal models. Therefore, LPS
mutants are valuable tools for investigation into the interactions of *P. aeruginosa* with host cell receptors such as CFTR.

*Pseudomonas aeruginosa* produces two distinct forms of core OS. One core OS form is designated the "uncapped" or unsubstituted form, and constitutes the rough LPS on the bacterial cell surface which is not capped with A or B-band PS. The second core OS form is substituted with long chain PS. The inner-core region of the core OS molecule is highly conserved among different *P. aeruginosa* strains (de Kievit and Lam, 1994; Sadovskaya et al., 1998; Knirel et al., 2001; Bystrova et al., 2003; Kooistra et al., 2003). In contrast, the outer-core region of the uncapped and capped core OS differs slightly in sugar composition and linkages (Fig. 5.1). In strain PAO1, the uncapped outer-core OS is composed of four D-glucose, one L-rhamnose and one N-(L-alanyl)-D-galactosamine, whereas the outer-core region of the core OS that is capped or substituted with O antigen contains one less glucose residue. In addition, the two outer-core regions differ in the position and linkage of L-rhamnose. In the substituted core OS form, L-rhamnose is the attachment point for the O antigen (Sadovskaya et al., 1998; 2000). Detailed chemical structures of core OS LPS from other *P. aeruginosa* strains, including a cystic fibrosis clinical isolate, 2192, (Knirel et al., 2001), which produces rough LPS, strain 170041, classified as Immunotype 1 or serotype O6 (Bystrova et al., 2002) and strain 170023, serotype O12 (Bystrova et al., 2003) have been reported. These strains produce two distinct core OS glycoforms similar in structure to those reported for strain PAO1 (serotype O5) (Sadovskaya et al., 1998; 2000). Therefore, these core OS structures seem to be produced by *P. aeruginosa* regardless of whether the strains are producing O-antigen and are not dependent on the serotype of O antigen produced. A conserved
**Figure 5.1** Structures of the two distinct core oligosaccharides from *P. aeruginosa* strain PAO1 and depiction of the structures for the mutant strains. The uncapped or unsubstituted core oligosaccharide is devoid of O antigen (A). Note the L-rhamnose in a α1,6-linkage to an α-D-glucose in the main chain. The substituted core oligosaccharide contains O antigen linked to L-rhamnose in an α1,3-linkage to β-D-glucose in the branch chain (B). Glc, glucose; Rha, rhamnose; GalN, galactosamine; Ala, alanine; CONH$_2$, carbamoyl group; P, phosphate; Hep, L-glycero-D-manno-heptose; Kdo, 3-deoxy-D-manno-octulosonic acid. The structures were drawn based on data from Jarrell and Kropinski (1981), Rahim et al. (2000), Sadovskaya et al. (1998; 2000), and K. K. H. Poon and J. S. Lam (unpublished data). The depiction of the proposed structure of the core OS from the PAO1 *wapH* mutant is based on data obtained in this study.
A. Core oligosaccharide

\[ \beta-D-Glc-(1\rightarrow2)-\alpha-L-Rha-(1\rightarrow6)-\alpha-D-Glc-(1\rightarrow4)-\alpha-D-GalN-(1\rightarrow3)-L-\alpha-D-Hep-(1\rightarrow3)-L-\alpha-D-Hep-(1\rightarrow5)-Kdo-(2\rightarrow6)-lipid\ A \]

PAO1 \( wapH \)
PAO1 \( migA \)
PAO1 \( rmlC \)

\[ \alpha-D-Glc \]
\[ \beta-D-Glc \]
\[ L-Ala \]

outer core

inner core

B. O-antigen-capped core oligosaccharide

\[ \beta-D-Man2Nac3AmA-(1\rightarrow4)-\beta-D-Man2Nac3NacA-(1\rightarrow3)-\beta-D-FucNac-(1\rightarrow3)-\alpha-L-Rha-(1\rightarrow3)-\beta-D-Glc \]

PAO1 \( wapR \)
PAO1 \( wapH \)
PAO1 \( rmlC \)

\[ \alpha-D-Glc \]
\[ \alpha-D-Glc-(1\rightarrow4)\alpha-D-GalN-(1\rightarrow2) \]

\[ L-Ala \]

outer core
structural feature in the outer-core regions of the two different core types in strain PAO1 is a central tetrasaccharide structure, which is composed of three glucose residues attached to a central N-((L-alanyl)-D-galactosamine residue. Although slight modifications in the core OS have been observed in the different strains, such as the addition of acetyl groups (Bystrova et al., 2003), this tetrasaccharide structure is common to the outer-core of both core OS forms of PAO1 (serotype O5) and was also shown to be present in the outer core of strains 170041, 170023, PAK (Masoud et al., 1995), and PAC1R (Rowe and Meadow, 1983).

Although we have extensive knowledge of the detailed chemical structure of the core OS, relatively little is known regarding the assembly of the outer-core OS region. Based on genetic and structural data obtained thus far, seven glycosyltransferases are needed for assembly of the outer-core (Lam et al., 2004). Two genes, wapR and migA, encode the rhamnosyltransferases required for the two core glycoforms (Yang et al., 2000; Poon et al., 2003) and are currently under investigation in our laboratory. A glycosyltransferase encoded by wapG is thought to be required for addition of the first sugar of the outer-core OS, presumably N-acetyl-galactosamine, which is further modified to generate N-((L-alanyl)-D-galactosamine (Matewish et al., 1999). Based on the structure of the PAO1 core OS by Sadovskaya et al. (1998; 2000), complete assembly of the two forms of outer-core OS requires an additional four glucosyltransferases, none of which have been identified. This investigation describes the cloning and characterization of ORF PA5004, which has been designated wapH, of P. aeruginosa strain PAO1. In this chapter, I provide evidence to show that wapH encodes a putative glucosyltransferase that is required for the assembly of the uncapped and capped core-OS glycoforms.
5.3 Results

5.3.1 Identification of \textit{wapH}, a gene encoding a putative glucosyltransferase

Based on information from the PAO1 genome sequence (\textit{Pseudomonas aeruginosa} Community Annotation Project [http://www.pseudomonas.com]; Stover et al., 2000) and work by de Kievit and Lam (1997) and Walsh et al. (2000), a gene cluster encoding enzymes for biosynthesis of the LPS core OS has been described (Fig. 5.2; Lam et al., 2004). The core OS gene cluster is flanked at the 5’ and 3’ ends by \textit{waaF} and \textit{hldE}, respectively. Sequence analysis of this cluster identified an ORF consisting of 1134 nucleotides, PA5004, which was designated \textit{wapH}. The ATG initiation codon of \textit{wapH} overlaps 15 bp with the stop codon of the preceding ORF, which is proposed to encode the LPS-core carbamoyltransferase. A recognizable ribosomal binding site (AGGAGG) was located 13 nucleotides upstream of the \textit{wapH} ATG start codon.

Analysis of the amino acid sequence reveals that WapH shares similarity to WaaG of \textit{E. coli} (50% total similarity, 16% identity; Heinrichs et al., 1998b; accession number AAC69657) and WapG of \textit{P. aeruginosa} (39% total similarity, 20% identity; Matewish et al., 1999; accession number AAD33103). These proteins function as the first hexosyltransferases in the synthesis of their respective LPS-outer core OS. \textit{E. coli} WaaG is a UDP-glucose:(heptosyl) LPS \(\alpha1,3\)-glucosyltransferase, and \textit{P. aeruginosa} WapG, a putative UDP-galactosamine: (heptosyl) LPS \(\alpha1,3\)-galactosyltransferase. These proteins belong to a larger grouping of Family 1 inverting glycosyltransferases based on BLAST analysis (CAZy: Carbohydrate-Active enZYmes [http://afmb.cnrs-mrs.fr/CAZY/]; Coutinho et al., 2003). Both \textit{E. coli} WaaG and \textit{P. aeruginosa} WapH are predicted to be a retaining glucosyltransferases. \textit{In vitro} glycosyltransferase assays and structural data
Figure 5.2 The genetic organization of the core-oligosaccharide biosynthetic gene cluster of *P. aeruginosa* strain PAO1. The predicted genes are designated by their open reading frame number (for example, PA4996-PA5012), which has been assigned based on analysis of the PAO1 genome by Stover et al. (2000). Those proteins whose function has been characterized experimentally or have significant homology to known proteins in the database have also been assigned a gene name; waaF, waaC (de Kievit and Lam, 1997); wapG (Matewish et al., 1999), waaP wapP wapQ (Walsh et al., 2000), wapR (Poon et al., 2001; 2002), waaL (P. Abeyrathne and J. S. Lam, unpublished data), msbA (Doerrler and Raetz, 2002) and hldE (Kneidinger et al., 2002).
support the function of WaaG in its predicted α1,3-glucosyltransferase activity (Muller et al., 1972; Kadam et al., 1985; Heinrichs et al., 1998b). Thus, members of Family 1 inverting glycosyltransferases includes retaining enzymes as well. UDP-glucosyltransferase GtfB also belongs to this family and the crystal structure for this protein has been reported (Mulichak et al., 2001). GtfB and WaaG both utilize UDP-D-glucose as a donor molecule, however their substrate molecules differ. The C-terminal domain of GtfB contains a glycine fingerprint sequence (G-X-G-XX-G), which is characteristic of the classical Rossmann fold and is an important element in dinucleotide binding. This motif is not observed in the C-terminal domain of WapH. There is amino acid similarity spread throughout amino acids 181 to 346 of WapH to the domain that is characteristic of protein members within Family 1 (Fig. 5.3). This domain lies within the C terminus of the Family 1 protein members, and those members that are most similar to WapH in this Family 1 glycosyltransferase domain, other than E. coli WaaG, have not been characterized at the genetic or biochemical level. Secondary structure predictions show similar patterns of alpha-helices and random coil structures between WaaG and WapH at the C-terminal half of the proteins (Fig. 5.4). The amino acid sequence of WapH shows the presence of a hydrophobic region between amino acids 287 and 300 (based on analysis using the DAS transmembrane prediction server; [http://www.sbc.su.se/~miklos/DAS/]) and suggests that this putative glycosyltransferase is an inner membrane-bound protein. The glycosyltransferases that assemble the core OS onto lipid A are hypothesized to be located on the cytoplasmic face of the cytoplasmic membrane (Raetz and Whitfield, 2002) and the presence of this hydrophobic domain is consistent with its presumed location in the bacterial cell.
Figure 5.3  Amino acid alignment between the C-terminal regions of WapH and WaaG of *E. coli*, a UDP-glucose:(heptosyl) LPS α1,3-glucosyltransferase, that belongs to glycosyltransferase Family 1. (Yellow box) C-terminal domain characteristic of Family 1 glycosyltransferases; (*) identical amino acid; (:) conserved substitution; (.) semi-conserved substitution. Sequence alignment was performed using the program ClustalW and the Family 1 domain region was identified visually based on sequence data obtained from the Pfam (Protein families database of alignments and HM) and CAZy (Carbohydrate-Active enZYmes) web servers. The crystal structure of one member of Family 1, GtfB of *Amycolatopsis orientalis* (accession number U84349) contains a glycine fingerprint sequence (G-X-G-XX-G), which is characteristic of the classical Rossmann fold and is an important element in dinucleotide binding. This motif is not observed in the C-terminal domain of either WapH or WaaG.
Figure 5.4 In silico secondary prediction for WapH is similar to that of the C-terminal portion of *E. coli* WaaG. Predictions were performed using the PSIPRED protein structure prediction program obtained from PSI-BLAST (Position Specific Iterated - BLAST; Altschul et al., 1997). Green cylinders, alpha-helix (H); yellow arrows, beta-strand (E); black lines, random-coil (C). Note that both predictions contain a similar pattern of alpha-helixes and beta strands over the last two thirds of the proteins.
5.3.2 Construction of PAO1 wapH mutants

A wapH nonpolar mutant was generated in strain PAO1 using a well-established gene replacement strategy created by Schweizer and Hoang (1995). A copy of wapH with its coding sequence interrupted with a gentamicin resistance cassette was exchanged with the intact wild-type copy of wapH on the chromosome by homologous recombination. Technical assistance in construction of the PAO1 wapH mutant was provided by Robert Urbanic according to experiments designed by the author. To confirm that potential mutants contained the gentamicin cassette within wapH, wapH was PCR amplified from parent and mutant genomic DNA. A PCR product of 2.0 kb was obtained from the parent DNA whereas a 2.85 kb product was obtained from the potential wapH mutants (Fig. 5.5). The 2.85 kb product corresponded to the wapH insert (2.0 kb) plus the gentamicin cassette (0.85 kb), demonstrating that the wapH isogenic mutants had undergone gene replacement. To confirm that a single mutation had occurred and could account for the mutant phenotype observed, wapH was cloned into a Pseudomonas-E.coli shuttle vector, pAK1900, to generate plasmid pWAPH-His19, and this plasmid was transformed into the wapH mutants.

5.3.3 PAO1 wapH mutants produce truncated core oligosaccharide

LPS from the wapH mutants was analyzed by SDS-PAGE and Western immunoblotting with LPS-specific monoclonal antibodies. The wapH mutant produced a core OS of lower molecular mass than that produced by the parent as observed by silver-stained SDS-PAGE (Fig. 5.6). Our laboratory has generated a mAb, designated 101, which recognizes the terminal D-glucose of the main backbone of the uncapped
Figure 5.5 The PAO1 \textit{wapH} mutant contains a copy of \textit{wapH} that is disrupted by insertion of a gentamicin cassette and no wild-type copy of \textit{wapH}. Lanes 2 and 3 contain PCR products amplified from genomic DNA isolated from wild-type PAO1 and a PAO1 \textit{wapH} mutant using primers as described in materials and methods. Note that the ca. 2-kb product amplified from genomic DNA from PAO1 is consistent with the expected size of the PCR product containing a wild-type copy of \textit{wapH} and that the ca. 2.9-kb product amplified from genomic DNA from the PAO1 \textit{wapH} mutant is consistent with the size of the DNA fragment with \textit{wapH} containing the gentamicin cassette (0.85 kb) insertion.
Figure 5.6 Analysis of proteinase K whole cell lysates from \textit{P. aeruginosa} strain PAO1 and its isogenic \textit{wapH} mutant by silver-stained SDS-PAGE and Western immunoblotting using LPS-specific antibodies. The PAO1 \textit{wapH} mutant produced truncated core OS. The Western immunoblot showed a positive reaction for the presence of O antigen in the LPS produced by the \textit{wapH} mutant. However, O antigen production was less than that produced by the parent strain and low molecular weight O antigen, including semi-rough LPS was not produced at all by the mutant. The histidine-tagged version of \textit{wapH} carried in vector pAK1900 (pWAPH-His19) provided \textit{in trans} in the PAO1 \textit{wapH} mutant restored synthesis of LPS similar to that observed from the parent strain. The \textit{wapH} mutant carrying vector pAK1900 with no gene insert serves as a negative control. The silver stain and Western immunoblots were all performed on LPS that was first resolved on Novex\textsuperscript{®} 10-20\% gradient tricine gels. The location of the core OS plus one O-antigen unit and complete core OS banding patterns are based on data from de Kiefit and Lam (1994), de Kiefit et al. (1995; 1997), Sadovskaya et al. (1998), Rocchetta et al. (1998a), and Sadovskaya et al. (2000). Two intense bands are observed in the silver-stained gel and correspond to proteinase K resistant proteins.
Silver-stained SDS-PAGE

Western immunoblot
mAb 101
(outer-core specific)

Western immunoblot
mAb MF15-4
(B-band specific)

Western immunoblot
mAb N1F10
(A-band specific)
outer-core OS of PAO1 LPS and is an immunodominant epitope within the core region (de Kievit and Lam, 1994). In Western immunoblots, mAb 101 did not react with the truncated core LPS band produced by the *wapH* mutant (Fig. 5.6). An identical Western immunoblot reacted with inner-core specific mAb 7-4 showed a positive reaction with the core OS from both the parent strain and the *wapH* mutant (data not shown). These data suggest that a mutation in *wapH* does not affect inner-core biosynthesis, but affects the assembly of the outer-core region of the core-OS form that remains uncapped. In the *wapH* mutant, this core OS form is truncated in the main backbone chain, with the loss of at least the terminal glucose residue.

The *wapH* mutant did not produce “semi-rough” LPS, a term which describes the population of LPS molecules which is made up of core OS plus one O-antigen unit. This form of LPS in *P. aeruginosa* has been characterized genetically (de Kievit et al., 1995; 1997), immunochemically (de Kievit and Lam, 1994) and structurally (Sadovskaya et al., 1998; 2000). This semi-rough LPS is produced by the parent strain but was not seen in the LPS isolated from the *wapH* mutant as visualized in the silver-stained SDS-PAGE gel (Fig. 5.6). This suggests that the *wapH* mutant does not produce B-band LPS. Western immunoblotting with B-band specific mAbs revealed that the *wapH* mutant produced significantly less B-band PS than the parent strain, with the loss of lower-molecular weight O-antigen LPS. Upon close examination of the immunoblot, the *wapH* mutant also produced trace amounts of A-band PS, which is difficult to visualize in the figure.

Introduction of *wapH* on plasmid pWAPH-His19 restored the ability of the *wapH* mutant to synthesize complete core OS and to produce A- and B-band LPS similar to that of the parent (Fig. 5.6). The PAO1 *wapH* mutant carrying pAK1900 with no gene insert serves
as a negative control to demonstrate that the restoration of the LPS phenotype is due to the \textit{wapH} gene.

Previous reports have shown that a PAO1 \textit{rmlC} mutant which produces truncated core OS also produces A-band and B-band PS that is presumed to be linked to undecaprenol phosphate (Rahim et al., 2000), whereas Sánchez Carballo et al. (1999) examined a core LPS mutant designated H4, and observed it to be linked to lipid-A core. To determine if the A-band and B-band PS observed in LPS from the \textit{wapH} mutant in the Western immunoblots are attached to either core-lipid A or the carrier-lipid molecule, undecaprenol, LPS was isolated from the parent, mutant and complemented strain using the hot-aqueous phenol method. The pyrophosphate linkage between undecaprenol pyrophosphate and the nascent PS is labile and disrupted by hot-aqueous phenol extraction (Kent and Osborn, 1968). Silver-stained SDS-PAGE clearly shows that the \textit{wapH} mutant does not produce semi-rough LPS (Fig. 5.7 A), and LPS isolated from the \textit{wapH} mutant using this method showed a positive reaction on Western immunoblots with B-band-specific antibodies (Fig. 5.7 B). These data indicated that the B-band PS produced by the mutant is covalently attached to lipid A-core OS. However, the B-band PS produced is in much smaller amounts than that produced by the wild-type strain and only high molecular weight O antigen is observed. In contrast, there does not appear to be any lipid A-core-linked A-band PS (Fig. 5.7 C). Altogether, these results suggest that a mutation in \textit{wapH} also affects assembly of the second form of core OS, which serves as an acceptor molecule for the attachment of long chain A- and B-band PS.
Figure 5.7 Analysis of LPS isolated from *P. aeruginosa* by the hot-aqueous phenol method. Silver-stained SDS-PAGE gel (A) analysis showed that PAO1 *wapH* produced truncated core OS and no semi-rough LPS. Corresponding Western immunoblots probed with B-band specific mAb MF15-4 (B) and A-band specific mAb N1F10 (C) indicated that the PAO1 *wapH* mutant produced significantly less O antigen than the parent strain and no A-band LPS. Restoration of A-band and B-band LPS similar to that of the wild-type strain was observed when the PAO1 *wapH* mutant carried *wapH in trans* (pWAPH-His19).
5.3.4 Comparison of LPS from PAO1 \textit{wapH} with other LPS-core mutants

LPS isolated from the LPS mutants PAO1 \textit{rmlC}, PAO1 \textit{migA}, PAO1 \textit{wapR} and PAO1 \textit{algC} (AK1012), was compared to that of the \textit{wapH} mutant. Gradient (10-20\%) tricine SDS-PAGE gel analysis was used since it provides excellent resolution of low molecular-mass molecules. As indicated by the relative position of the core OS bands, the molecular mass of the \textit{wapH} core OS was slightly greater than the mass of the core OS from the \textit{algC} mutant and less than that of the \textit{rmlC} mutant (Fig. 5.8). The \textit{algC} mutant produces truncated core OS that contains no glucose or rhamnose, whereas the unsubstituted core OS from the \textit{rmlC} mutant is missing two residues, a glucose and rhamnose (Fig. 5.1). This data substantiates our previous observation, that the unsubstituted core OS produced by the \textit{wapH} mutant is missing at least three sugar residues, the terminal glucose and rhamnose, in addition to another glucose residue attached to \textit{N-}(\text{L}-alanyl)-galactosamine.

5.3.5 Cloning and expression of \textit{wapH} in \textit{E. coli}

\textit{WapH} is predicted to have one transmembrane domain and to have a molecular mass of 42 197 Da as predicted from the primary amino acid sequence using the ProtParam program (ExPASy; Molecular Biology Server of the Swiss Institute of Bioinformatics [http://ca.expasy.org/]). PCR amplification of the \textit{wapH} gene of strain PAO1 and its insertion downstream of the T7 promoter in the expression vector pET-30a, yielded the plasmid pWAPH-30a. Nucleotide sequencing of this construct confirmed an ORF of 1.179 kb encoding a predicted protein of 392 amino acids. This sequence was
**Figure 5.8** Comparison of LPS isolated from PAO1 *wapH* with that from other core LPS mutants that have known chemical structures by performing SDS-PAGE using 10-20% gradient tricine gels followed by silver staining. The parent strain PAO1 serves as a control for the identification of complete-core OS and the semi-rough LPS (or core plus one O-antigen unit form of LPS) (Sadovskaya et al., 1998; 2000). Both wild-type PAO1 and the PAO1 *wapR* mutant produce complete-core OS (Poon et al., 2001). The truncated core OS from the *rmlC* mutant, *migA* mutant and strain AK1012 have been previously characterized (Jarrell and Kropinski, 1981; Sadovskaya et al., 1998; Rahim et al., 2000, Yang et al., 2000, Poon et al., 2001). The relative position of the core OS bands from the parent and different LPS mutants in comparison to that of the *wapH* mutant suggest that this mutant is missing at least three sugar residues.
identical to that submitted by Stover et al. (2000) for ORF PA5004 plus the extra nucleotide coding sequence for the addition of the C-terminal hexa-histidine tag.

Induction, lysis and centrifugation of *E. coli* BL21 λDE3 cells carrying the pWAPH-30a plasmid yielded insoluble and soluble fractions. Analysis of these fractions by SDS-PAGE revealed that a high level of recombinant protein was expressed by BL21 λDE3 cells carrying pWAPH-30a, but a large amount of WapH protein was present in the membrane-associated fraction (pellet after ultracentrifugation) when the induction was carried out at 30°C for 6 h (Fig. 5.9 A). Using an affinity chromatography column loaded with nickel ions, a one step purification was sufficient to produce WapH in high yield from the soluble fraction. Protein eluted from the column was nearly homogenous as seen by SDS-PAGE analysis when eluted in buffer containing 300 mM imidazole. Western immunoblotting using an anti-His antibody showed a positive reaction with the purified protein (Fig. 5.9 B). The recombinant protein has an apparent molecular mass of 43.7 kDa as estimated by SDS-PAGE, and this is similar to the theoretical value (42 197 Da) calculated from its predicted amino acid sequence. These results show that under the described experimental parameters, WapH can be overexpressed and purified to near homogeneity with a high yield in the soluble fraction. The establishment of a set of conditions by which to express and purify WapH is the first step towards examining the activity of the enzyme *in vitro*. 
Figure 5.9 Expression and purification of WapH with a C-terminal histidine tag in *E. coli* BL21 λDE3. Protein samples were loaded onto 10% SDS-PAGE and visualized by Coomassie Blue R-250 staining (A) and Western immunoblotting with anti-His antibody (B). M_r, low range molecular weight standard; vector control, expression strain *E. coli* BL21 λDE3 containing plasmid pET30a (with no wapH insert); Pre-induction, *E. coli* BL21 λDE3 containing wapH inserted into vector pET30a before induction with IPTG; Post-induction, after induction with 1 mM IPTG; Pellet, pellet following high-speed centrifugation (100 000 x g, 1 h); Cell lysate, soluble fraction following high-speed centrifugation. The soluble fraction was loaded onto an affinity chromatography column and lanes marked 50, 100, 200, and 300 represent protein eluted in those concentrations of imidazole (mM). The recombinant WapH-His protein had an apparent molecular mass of 43 700 Da and is consistent with the theoretical value (42 197 Da) of the WapH protein calculated from its predicted amino acid sequence plus the histidine residues.
5.4 Discussion

Assembly of the core-OS region of the LPS molecule of *P. aeruginosa* is poorly understood. Therefore, the main objective of this study was to generate and characterize a genetically defined outer-core OS *P. aeruginosa* LPS mutant. Based on the similarity of WapH to glycosyltransferases involved in core OS assembly of other bacteria, it was hypothesized that *wapH* encoded a glycosyltransferase. Many different lines of evidence presented in this study support the assignment of WapH as the putative outer-core α1,4-glucosyltransferase that is required for assembly of both the uncapped and capped core-OS glycoforms.

To assess the role of *wapH* in core OS assembly, null mutations were constructed in the *wapH* gene in strain PAO1 (serotype O5). The results presented here indicate that the *wapH* mutant was unable to synthesize the complete outer-core region of the uncapped form of core OS. Evidence that showed the loss of the terminal glucose residue of the uncapped core OS was obtained by Western immublotting with the outer-core specific mAb 101. In Western immunoblots, mAb 101 did not react with core LPS that was isolated from the PAO1 *wapH* mutant. This antibody has been shown to be specific for the terminal glucose residue of the uncapped core OS (de Kievit and Lam, 1994). Evidence to support the specificity of mAb 101 to the terminal glucose of the outer-core region include results from studies which assessed its reactivity to rough LPS antigens in both enzyme-linked immunosorbent assays (ELISAs) and Western immunoblots. Data from competition ELISAs using monosaccharides as competing antigens also substantiate the specificity of the outer-core specific monoclonal antibody. It is intriguing that the *wapH* mutation also affected synthesis of the core OS glycoform that contains the
ligation site for B-band and A-band PS. This was shown by the complete loss of expression of both A-band LPS and low-molecular weight forms of the B-band LPS including semi-rough LPS (core plus one O-antigen unit form of LPS). Examination of LPS isolated using the hot-water phenol method indicated that the wapH mutant still produced high-molecular weight B-band LPS, but clearly, in a lower quantity than that of the parent strain, which was indicative of a small population of substituted core-OS forms that could accept long chain PS. This result was unexpected, since the PAO1 wapH mutant produced an uncapped core OS that was more truncated than that of the PAO1 rmlC mutant (Rahim et al., 2000).

Two other LPS core mutants, namely H4, derived from PAK (Dasgupta et al., 1994; Sánchez Carballo et al., 1999), and PAC611, derived from PAC1R (Rowe and Meadow, 1983) have been reported to have a B-band LPS phenotype similar to that seen for the wapH mutant. Detailed structural analysis of the core OS from both of these mutants has been performed, and although evidence was provided to show that the O antigen is linked to a core lipid-A moiety rather than undecaprenol, the site of attachment of this O antigen in relation to the truncated core OS was not investigated in these earlier studies. One explanation offered for the presence of the higher molecular weight PS found in the H4 mutant was that it is genetically unstable (Sánchez Carballo et al., 1999). The genetic locus responsible for the LPS phenotype in both the H4 and PAC611 mutant is not known. In contrast, the knockout PAO1 wapH mutant generated in this study has a defined mutation and was shown to be non-polar since the mutation could be successfully complemented with wapH in trans. The PAO1 wapH mutant produces truncated core OS and high-molecular weight B-band LPS similar to that observed for the LPS isolated
from strains H4 and PAC611. The B-band LPS phenotypes of these mutants may reflect how a truncation in the outer core region of the uncapped core OS affects assembly of the capped core OS glycoform, particularly with respect to synthesis of the ligation site.

Examination of the LPS phenotype of the PAO1 waph mutant leads to the hypothesis that waph encodes a putative glucosyltransferase that adds a glucose in an α1,4-linkage to N-(L-alanyl)-galactosamine. The absence of the α1,4-linked glucose in the outer-core OS may result in a core OS form that is a less than suitable acceptor molecule for recognition by the glycosyltransferase that attaches rhamnose in a α1,3-linkage, the O-antigen attachment site. Data from two previous studies, which examined the LPS phenotypes of a PAO1 rmlC and PAO1 migA mutant also support this hypothesis. A mutation in rmlC abrogates the production of dTDP-L-rhamnose, and as a result, no rhamnose is added onto the two core OS forms, and no A- or B-band LPS is expressed on the cell surface (Rahim et al., 2000). Structural information obtained by using sugar composition and methylation linkage analysis of the core OS LPS from a PAO1 rmlC mutant unequivocally showed the presence of a terminal glucose linked to the β1,3-linked glucose in the absence of the α1,3-linked rhamnose. This observation suggests that addition of the α1,3-rhamnose is not required for addition of the terminal glucose attached in a α1,6-linkage to the β-D-glucose. Furthermore, structural data obtained in the study by Rahim et al. (2000) showed that in the absence of the α1,3-linked rhamnose, the α1,4-linked glucose is still present. migA encodes a second rhamnosyltransferase which adds a α1,6-linked rhamnose into the uncapped core OS form. A mutation in migA results in the formation of an uncapped core OS form missing a terminal glucose and α1,6-linked rhamnose, but the ligation site within the capped form
of the core OS is unaffected (Yang et al., 2000). Altogether, phenotypic analysis of the LPS produced from the wapH, rmlC, and migA mutant in the PAO1 background support our assignment of the putative α-1,4 glucosyltransferase activity of WapH.

Characterization of the LPS phenotype of the wapH mutant, together with structural and immunochemical information available for a number of other P. aeruginosa core LPS mutants (Jarrell and Kropinski, 1981; de Kievit and Lam, 1994; Sadovskaya et al., 1998; Rahim et al., 2000; Sadovskaya et al., 2000, Yang et al., 2000; Poon et al., 2001) suggest that the outer-core region is assembled as follows: the addition of the β1,3-linked glucose to \( N-(\text{L-alanyl})-\text{D-galactosamine} \) is first, followed by the addition of a terminal glucose in an α1,6-linkage. Subsequently, another glucose residue is added in a α1,4-linkage to the substituted \( N-(\text{L-alanyl})-\text{galactosamine} \). This conserved structure would then be available for the addition of rhamnose in either an α1,3- or α1,6-linkage to different glucose residues, and addition of these rhamnose residues will then determine the fate of the core OS, whether it can be capped with O antigen or remain as an uncapped form. This hypothesis fits very well with a theory that is currently being investigated in our laboratory, that is, the regulation of expression of migA and wapR, which encode the two core OS rhamnosyltransferases, control the proportion of the two different core OS forms that are synthesized (Yang et al., 2000; Poon et al., 2003, K. K. H. Poon and J. S. Lam, unpublished data).

This proposed model of core-OS assembly for P. aeruginosa is based on the hypothesis that addition of the α1,4-linked glucose is required for efficient attachment of the α1,3-linked rhamnose, which is the O-antigen attachment site. An assembly mechanism similar to this has been proposed for synthesis of the R1 core OS type in E.
coli. Heinrichs et al. (1998b) examined the assembly of the outer-core OS in the E. coli R1 core type and reported also an unusual phenotype for a glycosyltransferase mutant, called strain CWG310, which has a mutation in the glycosyltransferase gene designated waaW. The outer-core OS region of the E. coli R1 core OS is composed of a trisaccharide hexose backbone, referred to as HexI-HexII-HexIII (with HexI being the sugar attached to the heptose of the inner core). The HexII and HexIII residues each have a sugar substitution, with the HexII sugar substitution being the site of attachment for the O antigen. In the E. coli R1 core type, it was observed that a mutation in the waaW gene, which encodes the glycosyltransferase that adds on the HexIII sugar substitution, results in the production of truncated core OS with no O antigen. The outer-core region lacked substitutions at both HexII and HexIII, which would explain the absence of the O antigen, since it requires the HexII substitution. It was proposed that addition of the HexII substitution requires prior modification of HexIII with its substituent, and that their results reflected the complex requirements in the acceptor molecule for individual glycosyltransferases (Heinrichs et al., 1998b; Whitfield et al., 1999). Analysis of LPS from the E. coli waaW mutant lead to the conclusion that addition of a sugar substituent, not directly linked to the sugar of O-antigen attachment, is necessary for addition of the sugar residue that acts as the ligation site. Data obtained in the current study suggest that in P. aeruginosa PAO1, the addition of the α1,3-linked rhamnose, requires prior modification of N-(L-alanyl) galactosamine with the α1,4-linked glucose. The production of high-molecular weight B-band LPS could reflect that the core OS is still being recognized by the rhamnosyltransferase, but less efficiently, resulting in smaller quantities of O antigen rather than complete abrogation.
The requirement of side-chain sugars for complete assembly of the outer-core OS in *E. coli* K-12 and R2 core types has also been reported. The K-12 and R2 core types have a galactose substitution on HexI (Pradel et al., 1992). Although it was predicted that a mutation in *waab*, the gene encoding the galactosyltransferase for the side branch substitution, would result only in the loss of this side branch residue, the LPS phenotype observed corresponded to truncated core OS containing only the HexI residue. These data lead the researchers to conclude that the HexI substituent may be part of the acceptor molecule that acts as a substrate for the HexII glycosyltransferase. Alternatively, it has been postulated that the glycosyltransferases function together in a complex and therefore, the absence of the protein itself, not the core OS molecule, affected the function of other glycosyltransferases (Schnaitman and Klena, 1993).

A defined mutation in *wapH* has been made and studies are currently underway in our laboratory to examine the chemical composition and linkage of the sugars present in the PAO1 *wapH* core OS. It would be important to examine the biochemical properties of this putative glycosyltransferases to verify its tentative assignment. To achieve this objective, purified WapH protein would be the best source of the enzyme for *in vitro* transferase assays rather than whole cell lysates. Therefore, in this study, overexpression and purification experiments were initiated. The results indicated that WapH could be expressed in an *E. coli* expression strain and the expressed protein was of a molecular mass consistent with that predicted from the amino acid sequence. The WapH protein was monitored throughout the purification procedure by SDS-PAGE and it was observed that although a large proportion of the overexpressed WapH protein associated with the membrane fraction of the lysed bacterial cells, there was sufficient soluble protein to
facilitate purification and a substantial amount of soluble WapH protein was obtained. Complementation of the \textit{wapH} mutation with a construct that expressed WapH with a C-terminal histidine tag indicated that the histidine tag does not affect the activity of WapH \textit{in vivo}. Although further experiments will be required to determine whether WapH expressed and purified under the conditions described in this chapter will be active \textit{in vitro}, this work has provided two essential components for investigating the putative glycosyltransferase activity at the biochemical level. One component is purified core OS from the \textit{wapH} mutant, which can be used as an acceptor molecule for an \textit{in vitro} transferase assay. The second component is the establishment of a set of initial conditions by which to overexpress and purify WapH, which provides the groundwork by which to test for glycosyltransferase activity.

This study is the first to report the identification and characterization of a putative glucosyltransferase that is required for outer-core OS assembly of both core OS forms in \textit{P. aeruginosa}. Further studies are necessary to elucidate the function of WapH and to evaluate the proposed model of assembly. The use of genetically defined \textit{P. aeruginosa} core LPS mutants will be valuable for investigating specific host-pathogen interactions, such as initial colonization stages where LPS has been shown to be an important bacterial factor.
Chapter 6: Interaction of *Pseudomonas aeruginosa* with human bronchial epithelial cells

6.1 Abstract

Core oligosaccharide of *P. aeruginosa* has been implicated as a ligand that associates with human airway epithelia. However, the precise region of the core that participates in pathogen-host interactions and the degree to which other LPS regions might play a role in interaction with airway tissues have not been defined. Thus, the objective of this study was to evaluate the ability of the PAO1 *wapH* mutant, a genetically defined outer-core OS mutant derived from a PAO1 background, to associate and become internalized within epithelial cells. LPS mutants previously generated in our laboratory were also used for comparison. The mutants that did not produce A-band or complete core OS showed a significantly lower level of association with human bronchial epithelial cells when compared to that of the parent strain. Distinct differences in adherence patterns between the parent strain and the A-band mutant, PAO1 *rmf*, could be discerned using fluorescence microscopy. The addition of purified complete-core OS could inhibit the association of PAO1 to epithelial cells. In the ingestion assays, the level of PAO1 *wapH* mutant bacteria being ingested was not significantly different than that of the parent strain. The use of defined core LPS mutants allowed us to determine that the terminal glucose and rhamnose sugars of the uncapped core OS are not required for maximal ingestion of *P. aeruginosa*. 
6.2 Introduction

*Pseudomonas aeruginosa* is the most important pathogen in CF disease (reviewed by Lyczak et al., 2002), especially since the leading cause of death in CF patients is pulmonary failure due to chronic lung infections with *P. aeruginosa* and the ensuing chronic inflammation. It is not completely understood why CF patients are hypersusceptible to infection by *P. aeruginosa*. Results from studies that have examined the initial interactions of *P. aeruginosa* with airway epithelial cells support the hypothesis that CF individuals are unable to mount an effective inflammatory response during initial infection. It is proposed that in normal healthy individuals, a mechanism for clearing bacteria from the respiratory tract involves ingestion of the organisms within the epithelial cells lining the airways. Internalization of *P. aeruginosa* is mediated by the cystic fibrosis transmembrane regulator protein (CFTR) and the core OS region of the LPS molecule (Pier et al., 1996a; 1996b; 1997). LPS core OS-CFTR binding initiates NF-κB nuclear translocation (Schroeder et al., 2002) and is presumably followed by desquamation and eventual apoptosis of the infected cells (Cannon et al., 2003). These events may be crucial for innate immune resistance to *P. aeruginosa* and are compromised in CF patients due to defects in the CFTR protein.

LPS has been implicated as a ligand for attachment of *P. aeruginosa* to epithelial cells. Expression of outer-core OS is necessary for maximal association and entry of *P. aeruginosa* into primary cultures of rabbit corneal epithelial cells and whole mouse eyes *in situ* (Zaidi et al., 1996). Binding inhibition assays and scanning electron microscopy of organ cultures of whole mouse eyes showed that *P. aeruginosa* was inhibited from corneal binding by exogenous LPS. Using solid-phase binding and thin-layer
chromatography assays, LPS was shown to bind to glycolipid gangliotetraosylceramide (asialo GM1) from mouse and bovine corneal epithelial cells (Gupta et al., 1994). However, the role of LPS in attachment of *P. aeruginosa* to epithelial cells derived from the respiratory tract is not clear. Interestingly, the expression of asialo GM1 on the cell surface of CF-derived epithelial cells is increased when compared to cells expressing wild-type CFTR (de Bentzmann et al., 1996). Therefore, whether there is a significant contribution to attachment of *P. aeruginosa* to CF airway epithelial cells by way of LPS-asialo GM1 interactions is not known.

To determine which part of the LPS molecule is important for initial interactions with host cells, many investigators have used LPS mutants in adherence and invasion assays. Pier et al. (1996a; 1997) tested two sets of LPS mutants and wild-type controls for invasion of cultured airway epithelial cells. One set consisted of the wild-type strain PA01 and two LPS mutants derived from PA01, AK44 and PA01 *algC*. The second set consisted of parent strain PAC1R and mutants PAC605 and PAC1R *algC*. AK44 and PAC605 are “rough” strains. They produce complete-core OS and no A- or B-band LPS. The *algC* mutants produce “incomplete”-core OS, which consists of the inner-core OS region plus *N*-(L-alanyl)-D-galactosamine. Internalization values for AK44 and PAC605 were comparable to those of their respective parent strains. In contrast, the two *algC* mutants showed a significant decrease. Although these studies provide evidence that the outer-core portion of the core OS is required for maximal cell entry, the LPS mutants used to arrive at these conclusions, strains AK44 and PAC605, are genetically undefined. Furthermore, it is still not known what sugar residues of the outer-core OS are critical for ingestion.
The importance of having a panel of genetically defined LPS core mutants with corresponding structures being elucidated cannot be overstated. Only two defined outer-core LPS null mutants in *P. aeruginosa* have been reported. These are PAO1 *rmlC* (Rahim et al., 2000) and PAO1 *algC* (Coyne et al., 1994). These mutants are able to restore their LPS to parental phenotypes when transformed with a plasmid carrying a functional copy of the relevant gene in trans. *algC* encodes phosphoglucomutase, and this enzyme catalyzes the interconversion of glucose 1-phosphate and glucose 6-phosphate. Glucose 1-phosphate is an intermediate sugar that is channelled into the biosynthetic pathway of UDP-D-glucose and dTDP-L-rhamnose (reviewed in Giraud and Naismith, 2000) and mutations in *algC* result in strains that produce incomplete outer-core OS that contains no glucose or rhamnose. The use of *algC* mutants and the ability to isolate purified LPS from these mutants for experiments have been critical in showing the importance of the outer-core OS in host cell interactions. Using invasion assays, Pier et al. (1996a; 1996b; 1997) and Zaidi et al. (1996) tested LPS core mutants that have been isolated by selection for resistance to LPS-specific bacteriophages (strain AK44; Kropinski et al., 1977) or for resistance to aeruginocin (strain PAC557, Koval and Meadow, 1977). Although the structure of these mutants has been determined (Kropinski et al., 1979; Rowe and Meadow, 1983) it is important to note that conclusions are being made on the data obtained from assessing invasion of LPS mutants that are genetically undefined.

The purpose of this study was to assess the ability of the outer-core LPS mutant, *P. aeruginosa* strain PAO1 *wapH*, to associate and become internalized by bronchial epithelial cells. Other isogenic outer-core LPS mutants were also used in the study for
assessing the region of the core OS that are significant for bacteria-host interactions. Data from this study indicate that the LPS-core OS and A-band PS influence the attachment of this microbe to bronchial epithelial cells, and the glucose residues present within the truncated outer-core OS of the newly generated core mutant PAO1 wapH are necessary for maximal ingestion of *P. aeruginosa* within bronchial epithelial cells.

6.3 Results

6.3.1 Effect of bacterial inoculum on epithelial cells

Before the interactions of the LPS core mutants with the epithelial cells can be investigated, it is prudent that the bacterial inoculum sizes and their effect on the cells be clearly defined. Although Imundo et al. (1995) had used an MOI = 100 (MOI: multiplicity of infection, number of bacteria per epithelial cell) for these epithelial cell strains, they used a different cell culture system so it was necessary to examine the effect of our bacterial strains on IB3 and C38 in the collagen-coated cell culture plates. Cytotoxicity assays were performed with *P. aeruginosa* strain PAO1 and a negative control *E. coli* HB101 strain to determine the maximum number of bacteria that could be applied to the monolayers before compromising epithelial cell integrity. Bacterial inocula in the range of $1 \times 10^6$ to $1 \times 10^8$ colony forming units (CFU) were applied to IB3 epithelial monolayers (ca. $1 \times 10^5$ cells). Qualitatively, as determined by direct microscopic examination, cytotoxicity was identified as blebbing and rounding up of epithelial cells from the confluent monolayer, increasing regions of dead and dying cells and the presence of visible “holes” in the monolayers. Quantitatively, cytotoxic effects were measured by the Trypan Blue dye exclusion assay. This assay provides a direct
measure of cell viability. It relies on the alteration in membrane integrity as determined by the uptake of the large-sized dye by dead cells. The percent of nonviable epithelial cells adjusted for normal epithelial cell interactions when incubated in MEM supplemented with fetal calf serum was less than 5%. Trypan Blue exclusion assay scores obtained for the IB3 cell line when infected with PAO1 showed that epithelial cells inoculated with $2.5 \times 10^7$ CFU, infected for 4 h showed no significant signs of cell destruction. In contrast, within the first 15 min of infection, monolayers infected with $1 \times 10^8$ CFU showed obvious cell destruction and at $5 \times 10^7$ CFU, integrity of the monolayer was compromised after 3 h (Fig. 6.1). These data indicated that an inoculum of *P. aeruginosa* less than $2.5 \times 10^7$ (MOI = 250) could be used to infect the epithelial cells for a 4-h infection without cytotoxic effects. Based on the above observations, an infection inoculum corresponding to an MOI of 10-20 was routinely used for subsequent experiments.

### 6.3.2 Effect of centrifugation on bacterial association and internalization within epithelial cells

Centrifugation of bacteria onto the epithelial monolayers may initiate infection sooner and serves to synchronize contact between the bacteria and monolayer, which may promote bacterial attachment and penetration. The effect of centrifugation on the association and ingestion of *P. aeruginosa* to the IB3 epithelial cells was evaluated. A bacterial inoculum of ca. $2 \times 10^6$ CFU of wild-type strain PAO1 was used to infect IB3 epithelial cells (ca. $1 \times 10^5$). Association and ingestion assays were performed with duplicate plates in which one cell culture plate was centrifuged at 600 x g before the
Figure 6.1 Trypan Blue exclusion assay results obtained for *P. aeruginosa* PAO1 infecting IB3 cells. Assay scores showed that within the first 15 min, monolayers infected with $1 \times 10^8$ CFU showed obvious cell destruction and at $5 \times 10^7$ CFU, integrity of the monolayer was compromised after 3 h. Epithelial cells inoculated with $2.5 \times 10^7$ CFU and infected for 4 h showed no significant signs of cell destruction. Bars represent the mean of three determinations and error bars represent the standard error. Results that have no error bars indicate that no apparent variability in cytotoxicity was observed in multiple observations. Toxicity was assigned a score between 1 and 4 based on an estimation of the percentage of cells that had taken up the trypan blue dye (1, no cytotoxicity; 4, staining observed for > 15% of cells).
association incubation. After 2 h of incubation, levels of adherent PAO1 from the centrifuged monolayers (1.7 x 10^6 ± 3.4 x 10^4 CFU) was similar to adherent PAO1 on the noncentrifuged epithelial cells (1.8 x 10^6 ± 1.9 x 10^4 CFU) (P = 0.09, Student’s t-test). For the ingestion assays the bacteria were allowed to associate and invade for 4 h. Internalization of PAO1 within the centrifuged monolayers (4.5 x 10^4 ± 8.4 x 10^3 CFU) was similar to that of PAO1 within the noncentrifuged cells (2.7 x 10^4 ± 4.5 x 10^3 CFU) (P = 0.1, Student’s t-test).

6.3.3 Association of *P. aeruginosa* with epithelial cell lines IB3 and C38

Differences have been observed in association of *P. aeruginosa* with CF-derived airway epithelial cells when compared to epithelial cells expressing normal CFTR protein have been observed (Imundo et al., 1995; Davies et al., 1997). Therefore, the first aim was to determine the level of association of wild-type strain PAO1 to IB3, a bronchial epithelial cell line derived from a CF patient with the genotype (ΔF508/W1282X), and C38, the IB3 cell line transfected with CFTR. Association assays were carried out with epithelial cell monolayers grown in collagen-coated cell culture plates and association of PAO1 to the IB3 cell line was similar to that of the C38 cell line (P > 0.05, Student’s t-test; data not shown).

6.3.4 Association of *P. aeruginosa* A-band and B-band LPS mutants with epithelial cells

To investigate whether long chain A-band and B-band LPS affected association of *P. aeruginosa*, association assays were performed using IB3 bronchial epithelial cells. P.
aeruginosa strain PAO1 and isogenic LPS mutants differing in the production of A- and B-band LPS were evaluated for their ability to adhere to epithelial cells. Results from association assays indicated that wild-type strain PAO1 and the B-band LPS mutant, PAO1 wbpM, showed similar levels of association (P > 0.05, Student’s t-test; data not shown). However, the PAO1 rmd mutant, which is deficient in the production of A-band PS, and the PAO1 wbpL mutant, which is A-band and B-band deficient, both showed less association with the bronchial epithelial cells when compared to values for the parent strain (Fig. 6.2). The adherence patterns of wild-type PAO1 and the rmd mutant with epithelial cells grown on glass coverslips were further investigated using fluorescence microscopy. The binding patterns of P. aeruginosa to the epithelial monolayers were surveyed over a 9-h period. It was observed that during infection, wild-type PAO1 appeared to form microcolonies whereas the rmd mutant adhered to the IB3 cell line as isolated bacteria (Fig. 6.3). Closer examination of the bacteria interacting with the monolayers during a 6-h infection with a lower MOI showed similar results (Fig. 6.4). To determine whether the decrease in attachment of the A-band LPS deficient mutant was specific to P. aeruginosa interaction with the IB3 cell line, I assessed binding of the same strains to human bronchial epithelial cells, 16HBE14o-. In these experiments, the rmd mutant showed decreased binding when compared to the parent strain (Fig. 6.5). These data indicate that maximal association of P. aeruginosa with epithelial cells correlated with the expression of A-band LPS on the bacterial surface and this form of LPS may play a role in the binding of P. aeruginosa to epithelial cells as well as contributing to bacteria-bacteria interactions on the epithelial cell surface.
**Figure 6.2** Association of *P. aeruginosa* strain PAO1 and isogenic LPS mutants PAO1 *wbpL* and PAO1 *rmd* with cultured IB3 epithelial cells.

Epithelial cells (1 x 10^5) grown for 48 h were inoculated with ca. 2 x 10^6 CFU of each strain with an association incubation of 2 h. Bars represent the means of three determinations and error bars represent the standard error. A-band LPS deficient bacterial strains PAO1 *wbpL* and PAO1 *rmd* adhered less to the IB3 cells when compared with the wild-type strain, which produces both A- and B-band LPS (*P* < 0.05, analysis of variance, Student’s *t*-test).

* Results are significantly different from the wild-type strain.
Figure 6.3 Fluorescence microscopy images of *P. aeruginosa* strain PAO1 and its isogenic *rmd* mutant (A-band deficient) infecting bronchial epithelial cell line IB3. The infected monolayer was examined over a 9-h period and images shown were taken at 1 h, 2 h, 4 h, and 9 h postinfection. Wild-type bacteria were labeled with B-band specific mAb MF15-4 and the *rmd* mutant was labeled with core OS-specific mAb 101 and secondary goat anti-mouse FITC-conjugated antibodies. Over the time of infection the parent strain appeared to form microcolonies on the epithelial cell surface whereas the *rmd* mutant appeared to be mono-dispersed. Epithelial cells were grown on collagen-coated glass coverslips. Each of the fluorescence images are one field of view with an original magnification of ×400 and are representative of three different samples taken at each designated time point.
Figure 6.4  The adherence of *P. aeruginosa* PAO1 and the *rmd* mutant to IB3 epithelial cells, visualized with laser-scanning confocal microscopy. At 2, 4, and 6 h postinfection, the cell monolayers were reacted with the (red) dye SP-DiIC$_{18}(3)$, which is specific for vacuolar and plasma membranes, and the adherent bacteria were visualized with the mAb MF15-4 followed by anti-mouse FITC conjugate. At 2 h postinfection, wild-type bacteria could be seen as microcolonies on the cell surface whereas the *rmd* bacteria attached over the surface as individual cells. Epithelial cells were grown to confluence on collagen-coated glass coverslips. Each image is a composite 3-D reconstruction of a Z-series taken at a magnification $\times 1000$. 
Figure 6.5 Association of *P. aeruginosa* PAO1 and the A-band LPS mutant PAO1 *rmd* to human bronchial 16HBE14o- epithelial cells.

Epithelial cells (7.5 x 10^5) grown in collagen-coated plates were infected with 1.5 x 10^7 CFU of each strain with an association incubation of 2 h. There was a significant difference between association of PAO1 and the *rmd* mutant (*P* = 0.0001, Student’s *t*-test) to the bronchial epithelial cells. Bars represent means and error bars represent the standard error.
6.3.5 Association of *P. aeruginosa* core OS mutants with epithelial cells

LPS-core OS has been implicated to play a role in the attachment of *P. aeruginosa* to corneal epithelial cells. To address whether the core OS is a ligand for attachment to epithelial cells of bronchial origin, association assays were used to evaluate the binding of core-LPS mutants. Both the PAO1 *rmlC* and *wapH* mutants showed a significant decrease in adherence to IB3 epithelial cells when compared to the wild-type strain and the PAO1 *wbpL* mutant (Fig. 6.6). The *wbpL* and *rmlC* mutants are completely deficient in cell surface expression of A-band and B-band PS. The *wbpL* mutant produces complete-core OS (Rocchetta et al., 1998a), whereas the *rmlC* mutant produces a truncated core OS that is missing two outer-core residues, glucose and rhamnose (Rahim et al., 2000). The *wapH* mutant produces an outer-core OS which is more truncated than that of the *rmlC* mutant. These data suggest that the outer-core region of the LPS molecule influences attachment of *P. aeruginosa* to bronchial epithelial cells.

To investigate further the role of core OS in association, we tested the ability of intact LPS core OS isolated from a PAO1 *wbpL* mutant to inhibit association of wild-type PAO1 to IB3 epithelial cells. The addition of ≥1 μg of intact core OS along with the bacterial suspension at the beginning of the association assay significantly inhibited epithelial cell association of *P. aeruginosa* (*P* = 0.004, analysis of variance) (Fig. 6.7). A Trypan Blue exclusion assay was performed in parallel with the inhibition experiment and addition of the core LPS at the designated concentrations had no significant cytotoxic effects on the integrity of the epithelial cell monolayer.
Figure 6.6 Association of *P. aeruginosa* wild-type strain PAO1 and isogenic LPS core OS mutants to IB3 cells. IB3 epithelial cells (1 x 10⁵) grown for 48 h were inoculated with ca. 1 x 10⁶ CFU of each strain with an association incubation of 2 h. The *wbpL* mutant showed less adherence than the parent strain (*P* = 0.02, Student’s *t*-test), however, the *rmlC* and *wapH* mutants were less adherent than the *wbpL* mutant (*P* = 0.0001, analysis of variance). These data indicate that the outer-core OS influences association of *P. aeruginosa* to the IB3 epithelial cells. Bars represent means and error bars the standard error.
Figure 6.7 Inhibition of association of *P. aeruginosa* strain PAO1 to IB3 epithelial cells in the presence of complete-core LPS isolated from strain PAO1 *wbpL*. LPS was purified using the hot-water aqueous phenol method. IB3 cells were infected with a bacterial inoculum of ca. 1.5 x 10⁶ CFU and 1 µg or 10 µg of purified core OS. Association occurred over a 2.5 h period. Points represent means, and error bars represent the standard errors. Doses of ≥ 1 µg of intact core OS per well significantly inhibited association (*P* = 0.004, analysis of variance). Inhibition of association for PAO1 in the presence of 1 µg purified core OS was similar to that observed with the addition of 10 µg (*P* > 0.05, Student’s *t*-test).
6.3.6 Internalization of *P. aeruginosa* core LPS mutants within IB3 epithelial cells

For the invasion assays, nonadherent bacteria were washed off and adherent organisms were killed by treatment of the epithelial monolayers with an antibiotic, which is usually gentamicin. However, since the LPS mutants carry a gentamicin cassette in their chromosome, amikacin was used instead of gentamicin to kill the adherent bacteria, and an amikacin-kill incubation time was established. To measure internalization of *P. aeruginosa* within the IB3 epithelial cells, a 3-h amikacin-kill incubation time at a final concentration 400 μg·ml⁻¹ was used to kill adherent organisms. LPS mutants PAO1 *wbpL*, PAO1 *rmlC* and AK1012 were assessed for ingestion under these conditions and the *rmlC* mutant, which produces truncated core OS (missing the terminal glucose and rhamnose of the uncapped core) was as invasive as both the parent strain and *wbpL* mutant, which has complete-core OS (Fig. 6.8). In the previous chapter, a *wapH* mutant with defined core OS deficiency was constructed. Examination of the LPS phenotype showed this mutant to produce the most truncated outer-core OS when compared to the other isogenic core LPS mutants derived from a PAO1 background. This outer-core OS mutant showed levels of ingestion similar to that of the parent strain. Internalization of strain AK1012 was significantly less than the other core OS mutants tested. Strain AK1012 is a mutant derived from PAO1 and has a mutation within the *algC* gene (Jarrell and Kropinski, 1976; Coyne et al., 1994). This mutant produces an incomplete core OS that is missing all of the outer-core glucose and rhamnose residues. A noninvasive strain, *E. coli* HB101 was included as a negative control (Boyer and Roulland-Dussoix, 1969). *E. coli* strain HB101 showed very low levels of “ingestion” as measured within our experimental system. This strain serves as an internal control to help establish the
Figure 6.8 Internalization of *P. aeruginosa* isogenic core LPS mutants within IB3 epithelial cells. IB3 epithelial cells (1 x 10^5) grown for 48 h were inoculated with ca. 1 x 10^6 CFU with an invasion incubation of 4 h. The PAO1 *wapH* mutant was internalized equally well as the parent strain within the IB3 cells, and the core LPS mutants PAO1 *wbpL* and PAO1 *rmlC* showed similar levels of ingestion (*P = 0.3*, analysis of variance). A significant decrease in ingestion was observed for strain AK1012, which produces incomplete core OS, when compared to the other *P. aeruginosa* strains (*P < 0.05*, analysis of variance). Based on our analysis of the structure of the *wapH* mutant core OS, the glucose residues in the outer core located closest to *N*-(L-alanyl)-galactosamine are necessary for parental levels of ingestion.
background sensitivity of the assay on a particular day. Postinfection controls for each experiment was performed and this data indicated that on average in a 4 h infection, growth of the individual strains in the tissue culture medium during the course of the experiment, as defined by the growth index (ratio of the counts at 4 h postinfection to the inoculum) was in the range of 2.1 to 3.5. Altogether, these results indicated that (i) the terminal glucose and rhamnose of the uncapped core OS are not required for maximal invasion, and (ii) the \textit{wapH} mutant, which produced the most truncated outer-core OS of the panel of LPS mutants tested, contained the outer-core sugars necessary for levels of ingestion within bronchial epithelial cells that were comparable to the parent strain.

6.3.7 Ingestion of wild-type PAO1 and the \textit{wapH} mutant within IB3 and C38 cells

\textit{P. aeruginosa} core OS has been shown to bind to the CFTR protein on normal airway epithelial cells (Pier et al., 1996; 1997). Thus, in addition to assessing the invasion phenotype of the \textit{wapH} mutant with that of the parent strain, it was necessary to evaluate the ingestion of these strains between the IB3 and C38 cells. These are an isogenic pair of bronchial epithelial cells, with the IB3 cell line derived from a CF patient and the C38 cell line carrying a wild-type copy of the gene encoding the CFTR protein. The epithelial cells and bacterial inoculum were standardized and no significant differences in internalization was observed between the \textit{wapH} mutant and the parent strain under the conditions of the cell culture invasion assay used in this study (Fig. 6.9). The distribution of the CFTR protein on the epithelial cell surfaces was examined using fluorescence microscopy. Epithelial cells were grown on collagen-coated glass coverslips and to detect CFTR, CFTR-specific antibodies CFTR Ab-1 (TAM18), CFTR Ab-3, and polyclonal
Figure 6.9 Internalization of *P. aeruginosa* within IB3 (transformed human bronchial epithelial cells from a CF patient with genotype ΔF508/W1282X) and C38 cell line (IB3-derived, stably expressing a transfected functional CFTR). Epithelial cells (1 x 10⁵) were infected with 1.5 x 10⁶ CFU and invasion incubation was 4 h. Wild-type PAO1 and the *wapH* mutant showed similar levels of internalization in the IB3 cell line when compared to the C38 cells (*P > 0.05*, analysis of variance).
anti-CFTR serum were used in conjunction with secondary goat anti-mouse and goat anti-rabbit FITC-conjugated and rhodamine-conjugated antibodies. The concentrations of primary and secondary antibodies used for this study were varied and various fixation and permeabilization methods were attempted. Permeabilization agents were used to allow better access of the CFTR-specific antibodies to the CFTR protein. The CFTR protein contains many transmembrane spanning domains and is situated within the plasma membrane, and although there are some extracellular loops, not all of it exposed on the cell surface. Thus, a set of samples were processed with no permeabilization agent, whereas other samples were treated separately with several different permeabilization agents, including a more selective detergent-like molecule, saponin, which is supposed to leave much of the membrane intact (Willingham and Pastan, 1985). Fluorescence was observed in both cell lines (which was not a result of nonspecific binding of the secondary antibody, as controls with secondary antibody alone showed no fluorescence). We were unable to detect observable differences in binding of the CFTR-specific antibodies to the C38 cell line when compared to the IB3 cell line when grown on collagen-coated glass coverslips using fluorescence microscopy techniques (data not shown). This suggests that the CFTR protein in the C38 cells may not be efficiently targeted to the cell membrane under the cell culture conditions used.
6.4 Discussion

A panel of genetically and structurally defined isogenic LPS mutants was assessed for their ability to associate with and become internalized within bronchial epithelial cells. The conclusive observations made from this investigation are as follows: (i) A-band PS and outer-core OS contribute to the ability of *P. aeruginosa* to attach to epithelial cells of bronchial origin, and (ii) the outer-core OS structure of the PAO1 *wapH* mutant is required for internalization of *P. aeruginosa* within cultured bronchial cells to levels comparable to the wild-type strain.

To better understand why CF lungs are so susceptible to *P. aeruginosa* infection, many researchers have tested the hypothesis that this microbe has greater adherence to CF-derived epithelial cells than to epithelial cells from normal individuals, based on the differences in expression of receptors. Imundo et al. (1995) showed that proteins located in the apical membranes of CF cells were sialylated at a lower proportion as compared to non-CF cells. They found a higher concentration of asialoganglioside (aGM1) glycolipid on the apical surface of CF cells, when compared to normal cells. Inhibition assays using anti-aGM1 antibodies as well as purified aGM1 effectively displaced *P. aeruginosa* from binding to epithelial cells, demonstrating that *P. aeruginosa* binds to aGM1. Using the same cell lines that were used in this study, bronchial epithelial cells IB3 (derived from a CF patient) and C38 (IB3 rescued with transfection by CFTR gene), Imundo et al. (1995) demonstrated that *P. aeruginosa* attachment was significantly greater in the IB3 cell line. In this study, we found no significant difference in the association of PAO1 to the IB3 when compared to that of the C38 cells. Dr. Imundo’s group cultured their cells on transwell filters, measured specific binding using a radioactive assay, and an MOI close
to 100 for the bacterial inoculum. In this investigation, the cells were grown on collagen-coated cell culture plates, adherence was quantitated by viable plate counting, and low MOI's of 10 to 20 were used. The different results obtained in these two studies may be due to the different cell culture systems used, as well as the methods of quantitation.

I did not observe differences in the invasion of *P. aeruginosa* between the different cell lines. Plotkowski et al. (1999) has reported that proper localization of the CFTR protein to the apical cell surface occurs when cells are grown in cell culture systems that allow for the formation of polarized cells and tight cellular junctions. They showed that when cells are grown on thin collagen films in plates, similar to the system that we used, CFTR is not expressed on the cell surface. Interestingly, Pier et al. (1996a), who reported significant differences between invasion of *P. aeruginosa* to CF-derived cells and those transfected with the gene for CFTR, used a cell culture system similar to that used in this study. Clearly, different methods of cell culture are enough to influence the expression of cell surface receptors interactions with *P. aeruginosa*.

Comparison of the binding of the various LPS mutants revealed that the presence of A-band PS enhanced the ability of *P. aeruginosa* to attach. A previous report by Gupta et al. (1994) implicated LPS in binding of *P. aeruginosa* to epithelial cells. LPS binding to aGM1 was demonstrated by this group using solid-phase binding and thin layer chromatography assays. In addition, exogenous O10 LPS inhibited binding of *P. aeruginosa* to scarified corneal epithelium in organ culture binding assays. However, the LPS used in their study was commercially purchased serotype O10 O-antigen LPS, and the presence or absence of A-band LPS in this preparation was not indicated. In contrast to these findings, I did not find that the presence of B-band influenced attachment of *P. aeruginosa*.
*aeruginosa* to bronchial cells. These results are consistent with those reported by Zaidi et al. (1996), who tested a B-band LPS mutant, AK1401, and observed that it was as adherent as the parent strain to corneal epithelial cells. The observation that A-band PS enhances bacterial association was interesting, since during the course of CF chronic lung infection with *P. aeruginosa*, A-band PS becomes the dominant long chain PS expressed, and O antigen is no longer produced (Lam et al., 1989).

Zaidi et al. (1996) reported that core OS plays a role in attachment of *P. aeruginosa* to corneal epithelial cells. However, there have been no reports describing how core OS influences adherence of *P. aeruginosa* to bronchial epithelial cells. In this study, inhibition of association assays were performed using purified complete core OS isolated from a PAO1 *wbpL* mutant, and the results showed that core OS also influences the ability of *P. aeruginosa* to bind to epithelial cells of bronchial origin. The role of core OS in adherence was further substantiated by the significant decrease in association of the *rmlC* mutant to bronchial cells when compared to the *wbpL* mutant. Both the *rmlC* and *wbpL* mutants are A-band and B-band deficient, with the only difference being that the *rmlC* mutant has a truncated core OS.

Using various microscopy techniques, we observed that wild-type bacteria associated with each other into microcolonies, whereas the A-band LPS mutant remained mono-dispersed over the epithelial cell surface. A-band PS may contribute to bacteria-bacteria interactions or bacteria-epithelial cell interactions to promote attachment to the cell surface. Imundo et al. (1995) reported that individual bacteria were observed to adhere to the epithelial cell surface at low concentrations, and at higher concentrations *P. aeruginosa* were seen adhering to each other. This report did not mention the MOI’s that
were used for those experiments and so the number of bacteria defined as low and high bacterial inoculum that resulted in these observations is unclear. The method of microscopy and micrographs of this phenomenon were not presented. These authors, however, commented that this self-adherence is a “phenomenon known to occur in vivo”. In their study they tried to use bacterial inocula that would result in only a few bacteria per cell to avoid “self-agglutination”. We observed self-adherence in the wild-type strain, but not the A-band mutant. In addition, self-adherence was seen in relatively low numbers of infecting bacteria in our study and thus, may very well be a natural mechanism for colonization and establishment of infection.

Studies by Pier et al. (1996a; 1996b), which describe the interaction of *P. aeruginosa* with epithelial cells derived from the respiratory tract, reported that the core OS was a ligand for ingestion. LPS mutants were used that produced either complete-core OS or incomplete-core OS. The incomplete-core OS mutants were deficient in all of the outer-core sugar residues except for N-(L-alanyl)-galactosamine. LPS mutants used in these studies, PAO1-AK44 and PAC557, were critical for implicating the LPS-core OS in ingestion. However, the mutations in these two mutants have not been mapped. Based on partial characterization of the sugar constituents in these strains, both produce complete core OS with no A- or B-band PS (Kropinski et al., 1979; Rowe and Meadow, 1983). In our study, a knockout mutant, PAO1 *wbpL*, was used. This mutant produces a complete core OS and is devoid of A-band and B-band LPS (Rocchetta et al., 1998a). The results obtained in this study are in agreement with the results obtained by Pier et al. (1996a) and Zaidi et al. (1996), in that within our cell culture system, the complete-core OS is required for maximal internalization within bronchial epithelial cells.
The present study shows that the terminal glucose and rhamnose residue located in the main chain of the uncapped core OS form are not required for maximal ingestion of *P. aeruginosa* within bronchial epithelial cells. Invasion assays showed that the core OS mutant, PAO1 *rmlC*, was as invasive as the wild-type strain to epithelial cells. It is truncated in the outer-core region and the uncapped core OS form lacks the terminal glucose and the α1,6-linked rhamnose, whereas the capped core OS form is missing the α1,3-linked rhamnose (Rahim et al., 2000). These results are consistent with those obtained by Zaidi et al. (1996), who used a similar approach and tested a panel of PAC1R-derived LPS mutants for internalization within corneal epithelial cells. They concluded from their data that a terminal glucose residue linked to *N*-(L-alanyl)-galactosamine had to be present on the LPS core for efficient epithelial cell ingestion. This conclusion was based on the observation that core LPS mutant PAC611 was as invasive as the parent strain for corneal epithelium. PAC611 produces a truncated outer-core OS composed of two sugars, *N*-(L-alanyl)-galactosamine and one glucose residue (Rowe and Meadow, 1983).

One concern when comparing our data with that reported by Pier et al. (1996a; 1996b) and Zaidi et al (1996), two groups who have contributed significantly to understanding the role of LPS in bacteria-host interactions, is that the core OS structures reported for the PAC1R series of mutants, namely PAC611 and PAC557 (Rowe and Meadow, 1983) are very different than that reported for PAO1 (Sadovskaya et al., 1998; 2000). According to their interpretation, wild-type PAC1R produces only one core OS form, which contains one rhamnose directly attached to the *N*-(L-alanyl)-galactosamine via an α1,6-linkage, and the O antigen is attached through a glucose that is α1,3-linked to
N-(L-alanyl)-galactosamine. This is quite different than that reported for PAO1 where there are two distinct core OS glycoforms and the O antigen is linked through a rhamnose in a side branch chain. Recently, LPS core OS structures for three different *P. aeruginosa* strains have been reported (Knirel et al., 2001; Bystrova et al. 2002; Bystrova et al., 2003) and these studies indicate the presence of two distinct populations of core OS glycoforms with structures that are more consistent to that reported for strain PAO1.

In conclusion, this chapter describes how the different forms of LPS influence the attachment and ingestion of *P. aeruginosa* within human bronchial epithelial cells when cultured in a collagen-coated tissue culture plate system. By including the PAO1 *wapH* mutant in this investigation, we were able to further define the sugar residues in the outer-core OS necessary for maximal internalization within bronchial epithelial cells. In addition, these investigations have provided a foundation by which to examine, in our laboratory, the interactions of *P. aeruginosa* with cultured epithelial cells.
Chapter 7. Conclusions

Cell-associated structures including LPS, flagella, and pili of *P. aeruginosa* have been implicated as important virulence factors for initial interactions with host tissues. Recent studies have shown that pili and flagella in a number of pathogenic bacteria contain covalently bound sugar residues. The presence of these carbohydrates on protein structures that are in direct exposure to host cells suggests that these sugars play a role in interactions with the host. The initial steps of glycan biosynthesis are presumed to be similar to that of LPS-like carbohydrate structures, and so the question arises as to whether there is any relationship between synthesis of LPS and protein glycans in *P. aeruginosa*. Very little information had been reported for protein glycosylation in this organism and this prompted us to apply our extensive knowledge of the molecular genetics of LPS biosynthesis to better understand the interplay between synthesis of protein glycans and LPS.

*Pseudomonas aeruginosa* wild-type strain 1244 produces glycosylated pilin (Castric, 1995; Castric et al., 2001) and mutations in ‘key’ LPS genes, *wbpL* and *wbpM*, within this strain were generated. Pilin isolated from both LPS mutants was found to be nonglycosylated and complementation of the mutants with their respective genes restored both the wild-type LPS phenotype and glycosylation of pilin. In strain 1244, the O-antigen gene cluster directs biosynthesis of the O-antigen unit, which is covalently attached to the pilin protein. A glycan with this metabolic origin has not been reported for any other prokaryotic glycoprotein. Although Castric (1995) has shown pilin glycosylation to be common among *P. aeruginosa* clinical isolates, it is not yet known whether the O-antigen gene cluster is the origin of the pilin glycan in different *P.*
*Pseudomonas aeruginosa* strains. One key component of the 1244 pilin glycosylation apparatus is the PilO protein (Castric 1995; DiGiandomenico et al., 2002). PilO is rather unique since it has the ability to transfer O-antigen units with different structures to the pilin protein. Altogether, these results contribute to the understanding of pilus structure and function, and have important implications in pilus and LPS targeted vaccine design.

Examination of the glycosylation status of flagellin isolated from strain PAK and a number of isogenic LPS mutants revealed a different relationship. Our results showed that *wbpO* and *rmlC*, which are located in the O-antigen *wbp* gene cluster, and the *rmlBDAC* locus, respectively, are essential for synthesis of the PAK flagellin glycan. *wbpO* and *rmlC* are required for synthesis of UDP-d-N-acetylglucosaminuronic acid and dTDP-L-rhamnose, respectively. Rhamnose was identified to be the first sugar in the glycan and is covalently attached to the protein, whereas the *N*-acetylglucosaminuronic acid derivative is the second residue. In contrast to what was observed for pilin glycosylation, the involvement of LPS genes in synthesis of the PAK flagellin glycan appears to be limited to the sharing of individual nucleotide activated sugar molecules. These findings are very interesting since they indicate that two other loci are involved in biosynthesis of the flagellin glycan in addition to the flagellin glycosylation gene island described by Arora et al. (2001). The biological significance of the flagellar glycans in *P. aeruginosa* remains unknown. Our findings help to relate the genetics of biosynthesis to the structure of the glycan, which will allow a more comprehensive approach to defining the structure-function aspect of flagellar glycosylation in ligand-receptor interactions. Flagellin is one of the most potent stimuli of the host innate immune response via the Toll-like receptor, TLR-5 (Hayashi et al., 2001; Jacchieri et al., 2003). Therefore it is of
interest to determine if the glycan moiety may prevent this type of ligand-receptor interaction in flagellins that are glycosylated. Further studies are required to determine how to best evaluate the importance of glycosylation of pilus and flagellar structures in the pathogenesis of CF airway infections.

The importance of carbohydrate-based interactions is clearly reflected in studies that have demonstrated that the LPS core OS-CFTR interaction leads to host cell signaling that generates an innate immune response which clears the bacteria from the airways (Pier et al., 1996a; 1996b; 1997; Schroeder et al., 2001; 2002; Cannon et al., 2003). In the last five years we have acquired extensive knowledge of the chemical structure of the core-OS region, however, biosynthesis of the outer-core region was poorly understood. The lack of knowledge in this area and the importance of this structure in modulating the host immune response provided the impetus for us to investigate the assembly of the outer-core OS and to further define the precise region of the outer-core OS that participates in pathogen-host interactions and the degree to which other LPS regions might play a role in these interactions.

This investigation leads to the characterization of a putative outer-core glucosyltransferase. A chromosomal mutation within the \textit{wapH} allele was generated and analysis of the LPS phenotype of the mutant indicated that this gene was required for assembly of the outer-core region of both the uncapped core OS glycoform and the core-OS form substituted with long chain PS. Characterization of the phenotype of this outer-core mutant has provided valuable insight into how the two different outer-core OS glycoforms may be assembled in \textit{P. aeruginosa}. A model of outer-core OS assembly has been proposed: a glucose residue is added to \textit{N}-\textit{L}-alanyl-galactosamine in a $\alpha$1,3-
linkage, a second glucose is added to this first glucose in a β1,6-linkage, followed by the addition of the third glucose to the substituted N-(L-alanyl)-galactosamine in a α1,4-linkage. Whether this outer-core structure becomes an uncapped or capped core OS glycoform is determined at this stage of assembly, for this structure is the acceptor molecule for the addition of rhamnose in either a α1,3- or α1,6-linkage to their respective glucose residues. We hypothesize that differential expression of migA and wapR, which encode the two core-OS rhamnosyltransferases, controls the production of the two distinct core-OS types, and consequently influences the amount of long chain LPS that is expressed on the cell surface. Modification of the LPS cell-surface coat by this mechanism has been hypothesized to promote a survival advantage in the CF lung environment (Yang et al., 2000) and this hypothesis is currently being tested in our laboratory. Further experiments including methylation linkage analysis of the core OS sugars from the wapH mutant and characterization of the glucosyltransferase activity will be required to verify the identity of WapH as the α1,4-glucosyltransferase.

The use of genetically and structurally defined P. aeruginosa LPS mutants allowed us to more clearly define the role of the different LPS forms during the initial stages of infection of bronchial epithelial cells. A-band PS and outer-core OS were shown to contribute to the ability of P. aeruginosa to attach to bronchial epithelial cells and the conserved region of the outer-core OS (α-D-Glc-(1→6)-β-D-Glc-(1→3)-α-D-GalN) appears to be the minimum outer-core region required for maximal internalization of P. aeruginosa within cultured bronchial cells. These studies have furthered our understanding of the initial interactions by defining the specific region of the outer-core OS that acts as a ligand for internalization within epithelial cells. Altogether, these
findings indicate that interactions involving bacterial surface carbohydrates and receptors on epithelial cells contribute to the initial encounter of a bacterial cell with host issues. The detailed molecular mechanisms of the binding of core OS to the CFTR protein that lead to bacterial clearance remain unclear. Our advanced knowledge of the genetics, structure, and assembly of the core-OS molecule provides the foundation by which to pursue investigations into the molecular mechanisms underlying the core OS-CFTR interaction.

References

Abeyrathne, P. and Lam, J.S. 2004. (University of Guelph.) Unpublished data.


Daniels, C. and Lam, J.S. 2004. (University of Guelph.) Unpublished data.


Epinat, J.C. and Gilmore, T.D. 1999. Diverse agents act at multiple levels to inhibit the

Erickson, P.R. and Herzberg, M.C. 1993. Evidence for the covalent linkage of
carbohydrate polymers to a glycoprotein from Streptococcus sanguis. J. Biol.
Chem. 268: 23780-23783.

Specific lipopolysaccharide found in cystic fibrosis airway Pseudomonas

strains with lipopolysaccharide defects exhibit reduced intracellular viability after

Evans, D.J., Pier, G.B., Coyne, M.J., Jr. and Goldberg, J.B. 1994. The rfb locus from
Pseudomonas aeruginosa strain PA103 promotes the expression of O antigen by
both LPS-rough and LPS-smooth isolates from cystic fibrosis patients. Mol.

Incidence and molecular epidemiology of Pseudomonas aeruginosa bacteremias
Microbiol. 26: 353-361.

Farinha, M.A., Conway, B.D., Glasier, L.M., Ellert, N.W., Irvin, R.T., Sherburne, R. and
Paranchych, W. 1994. Alteration of the pilin adhesin of Pseudomonas aeruginosa
PAO results in normal pilus biogenesis but a loss of adherence to human


Heinrichs, D.E., Yethon, J.A., Amor, P.A. and Whitfield, C. 1998b. The assembly system for the outer core portion of R1- and R4-type lipopolysaccharides of *Escherichia...


Kropinski, A.M., Chan, L., Jarrell, K. and Milazzo, F.H. 1977. The nature of

Kropinski, A.M., Chan, L.C. and Milazzo, F.H. 1979. The extraction and analysis of
lipopolysaccharides from _Pseudomonas aeruginosa_ strain PAO, and three rough

Kropinski, A.M.B., Jewel, B., Kuzio, J., Milazzo, F. and Berry, D. 1985. Structure and
function of _Pseudomonas aeruginosa_ lipopolysaccharide. Antibiot. Chemother. 36:
58-73.

1991. Structural characterization of the lipid A component of _Pseudomonas
aeruginosa_ wild-type and rough mutant lipopolysaccharides. Eur. J. Biochem.
198: 697-704.

Kunzelmann, K. 1999. The cystic fibrosis transmembrane conductance regulator and its

Kuo, C., Takahashi, N., Swanson, A.F., Ozeki, Y. and Hakomori, S. 1996. An N-linked
high-mannose type oligosaccharide, expressed at the major outer membrane
protein of _Chlamydia trachomatis_, mediates attachment and infectivity of the


Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of


Miller, W., Wenzel, C.Q.W. and Lam, J.S. 2004. (University of Guelph.) Unpublished data.


Plotkowski, M.C., Povoa, H.C., Zahm, J.M., Lizard, G., Pereira, G.M., Tournier, J.M.
and Puchelle, E. 2002. Early mitochondrial dysfunction, superoxide anion
production, and DNA degradation are associated with non-apoptotic death of
human airway epithelial cells induced by Pseudomonas aeruginosa exotoxin A.

diseases. Edited by G.L. Mandell, J.E. Benett and R. Dolin. Churchill

between lipopolysaccharide and toll-like receptor 4 revealed by genetic

Poole, K. and Srikumar, R. 2001. Multidrug efflux in Pseudomonas aeruginosa:
components, mechanisms and clinical significance. Curr. Top. Med. Chem. 1: 59-
71.

WapR from Pseudomonas aeruginosa. CBDN Ann. Meet. 2002.(Closed poster
session).

rhamnosyltransferase involved in core oligosaccharide biosynthesis from
242.

rhamnosyltransferases involved in core oligosaccharide biosynthesis in

262


Schweizer, H.P. and Hoang, T.T. 1995. An improved system for gene replacement and

The O-specific polysaccharide chain of Campylobacter fetus serotype B
lipopolysaccharide is a D-rhamnan terminated with 3-O-methyl-D-rhamnose (D-

E.P. 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected

enterocolitica serotype O:3 involved in lipopolysaccharide outer core

Skurnik, M. and Zhang, L. 1996. Molecular genetics and biochemistry of Yersinia


Smith, A.R., Zamze, S.E., Munro, S.M., Carter, K.J. and Hignett, R.C. 1985. Structure of
the sidechain of lipopolysaccharide from Pseudomonas syringae pv.

Smith, R.S. and Iglewski, B.H. 2003. P. aeruginosa quorum-sensing systems and

dehydrogenase: a key enzyme of alginate biosynthesis in P. aeruginosa.
Biochemistry 42: 4658-4668.


Appendix I

Involvement of WbpM<sub>O7</sub> and WbpL<sub>O7</sub> in lipopolysaccharide biosynthesis and glycosylation of pilin in Pseudomonas aeruginosa strain 1244 (serotype O7)<sup>a</sup>

<sup>a</sup> A brief summary of the data obtained by Antonio DiGiandomenico, John R. Stehle, and Dr. Peter Castric is provided below and supports my observations in Chapter 3 that mutations in wbpL and wbpM result in nonglycosylation of pilin protein. The methods and results provided in this appendix have been taken from the original manuscript by Antonio DiGiandomenico, Mauricia J. Matewish, Amy Bisaillon, Joseph S. Lam and Peter Castric, 2002, Glycosylation of Pseudomonas aeruginosa 1244 pilin: glycan substrate specificity. Mol. Microbiol. 46:519-530.

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Materials and Methods

Pilin preparation. Glycosylated and nonglycosylated pilin was prepared from P. aeruginosa 1244 as previously described (Castric et al., 2001).

SDS-PAGE and isoelectric focusing. LPS was separated on 12.5%T Tris-glycine SDS-PAGE gels while pilin samples utilized 15%T resolving gels. LPS bands were visualized by the silver-staining method of Dubray and Bezard (1982). Pili samples to be subject to isoelectric focusing were treated with 2% β-octylglucoside for 15 min at
room temperature. This material was separated in a pH gradient of from either 3.5 or 2.5 to 9.0 using a Pharmacia PhastSystem apparatus in which the gels had been equilibrated with 1.0 % β-octylglucoside.

**Immunoblot analysis.** Pilin separated by SDS-PAGE was transferred to nitrocellulose paper by electroblotting, while diffusion blotting was used with pilin separated by isoelectric focusing. These blots were blocked and developed as described previously (Castric et al., 1989). LPS or pilin were detected using mAb 11.14 (specific for the 1244 pilin glycan and IATS serotype O7 O-antigen) (Castric et al., 2001), mAb O11 (specific for IATS serotype O11) (ERFA, Westmount, QC, Canada), a polyclonal serum specific for *E. coli* O157 O-antigen (Difco, Detroit), or mAb 6.45 (specific for 1244 pilin protein) (Castric and Deal, 1994). Detection of antibody reactions has been described previously (Castric et al., 1989).

**Results**

**Pilin glycosylation state of 1244 *wbpM* and *wbpL* mutants**

If the 1244 pilin glycan originates in the O-antigen pathway, it would be expected that mutants defective in biosynthesis of this polysaccharide would produce altered pilin glycosylation. To test this, the *wbpM* and *wbpL* 1244 mutants were analyzed by immunoblot to determine whether they produced pilin of size and charge consistent with glycosylation. The pilin from each of these mutants was of an apparent molecular weight consistent with nonglycosylated pilin (Fig. 1 A). Complementation of these mutants resulted in production of pilin with the same molecular weight as authentic glycosylated
Figure 1 Immunoblot analysis of pilin produced by *P. aeruginosa* 1244 *wbpM* and *wbpL* mutants. Western blot of cell extracts using anti-1244 pilin monoclonal 6.45 as probe (A). Immunoblot of pilin separated by electrofocusing using anti-1244 pilin monoclonal 6.45 (B) or anti-1244 pilin glycan monoclonal 11.14 (C) as probe. NG pilin is nonglycosylated 1244 pilin and G is glycosylated pilin from the same strain. The 1244 *wbpM* and *wbpL* mutants both produce only nonglycosylated pilin.

Complementation of these mutants with pFV163-26 (*wbpM*<sub>PAOI</sub>) and pFVAB3-26 (*wbpL*<sub>1244</sub>), respectively restored the ability to form glycosylated pilin.
1244 pilin. To confirm these results pilin produced by these mutants was separated by
onelectric focusing, blotted and probed with monoclonal antibodies, specific in one case
for 1244 pilin protein and in the other for pilin glycan. It has previously been
demonstrated that nonglycosylated 1244 pilin focuses at approximately pH 6.25, while
glycosylated pilin has a pI of 4.75 (Castric, 1995). The pIs of the pilin from the \( wbpM \)
and \( wbpL \) mutants were identical and matched that of nonglycosylated pilin, whereas the
complemented mutants produced pilin with acidic isoelectric points indistinguishable
from that of glycosylated pilin (Fig. 1 A). In addition, the pilins from the complemented
strains reacted with the glycan-specific monoclonal antibody 11.14, whereas mutant
pilins were non-reactive (Fig. 1 C). These data showed that 1244 mutants defective in
either the \( N \)-acetyl fucose pathway (\( wbpM \)) or in the transferase responsible for
attachment of this sugar to the undecaprenol carrier lipid (\( wbpL \)) were unable to produce
glycosylated pilin. Altogether, these results support the proposal (Castric et al., 2001) that
the O-antigen biosynthetic pathway is the metabolic source of the pilin glycan.

**Heterologous pilin glycosylation by P. aeruginosa 1244**

If the O-antigen biosynthetic pathway is the source of the glycan, and the glycan
is identical with the O-antigen repeating unit, a question concerning pilin glycan substrate
specificity arises. The high degree of variability in \( P. aeruginosa \) O-antigen structure
(Knirel, 1990) suggests that either there is more than one glycosylating system available
to this organism, depending on O-antigen, or there is one type of glycosylation machinery
that is nonspecific with regards to glycan structure.
To examine the glycan substrate specificity question, heterologous cloned O-antigen biosynthetic clusters from *P. aeruginosa* and *E. coli* were expressed in strain 1244 and the pilin produced by these strains were tested for glycosylation. To do this, the cloned O-antigen biosynthetic clusters from a number of different serotypes was mobilized into strain 1244 and the pilin was analyzed by Western immunoblotting analysis using O-specific and pilin-specific antibodies, isoelectric focusing, and MALDI-TOF MS. Altogether, the results presented in the rest of this study provide concrete evidence that strain 1244 can use glycans that are significantly different in size, structure and charge from the homologous substrate.

Previous work has shown that PilO is an absolute requirement for pilin glycosylation in *P. aeruginosa* 1244 (Castric, 19995). As cloned heterologous O-antigen biosynthesis clusters can serve as a glycan source in strain 1244, it might be possible for PilO from this strain to be able to glycosylate 1244 pilin in heterologous strains if no other pilin-specific glycosylation elements were required. To investigate this, a cloned 1244 structural gene (*pilA*) was expressed in eight *P. aeruginosa* strains heterologous for O-antigen, either in the presence or absence of *pilO*. The data indicated that the 1244 pilin could be glycosylated with a glycan containing the same structure as the host strain O antigen, but only when the strain also carried *pilO* on a plasmid. These results not only confirm the lack of glycan specificity in pilin glycosylation, but demonstrate the vital role PilO plays in this process. In addition, these experiments suggest that PilO is the only requirement for pilin glycosylation that is not an integral part of another cell function.
References


Discovering a novel gene cluster from *Pseudomonas aeruginosa* that can modify the core region of *Escherichia coli* lipopolysaccharide

**Preamble**

One of my research objectives was to identify glycosyltransferases involved in LPS-core OS assembly in *P. aeruginosa*. Studies were performed to identify genes that would encode putative core OS glycosyltransferases and this lead to the identification of a novel genetic locus containing genes that were predicted to encode proteins similar to those involved in LPS biosynthesis. The hypothesis that this new gene cluster was involved in biosynthesis of the LPS of *P. aeruginosa* was tested and the results indicated that this gene cluster was not involved in the synthesis of LPS that is expressed when *P. aeruginosa* is grown under standard laboratory conditions. The results did not provide conclusive evidence to identify the function of the genes and so this investigation was removed from the main body of the thesis. However, the results from this investigation strongly indicate that this gene cluster may be involved in protein glycosylation and warrants the report of this work here.
Abstract

*Pseudomonas aeruginosa* invades various eukaryotic cells, including human bronchial and corneal epithelium. To identify genes required for invasion, a PAO1 bacterial artificial chromosome (BAC) library was transformed into *Escherichia coli* and transformants were screened for their ability to invade HEp-2 cells. A BAC subclone, called pINV8, conferred an invasive phenotype in *E. coli*. SDS-PAGE analysis of the LPS from *E. coli* carrying pINV8 showed a modified core oligosaccharide. This 8.5 kb genetic locus was designated *galEwlpABCDEF*, and the genes are predicted to encode proteins that are similar to enzymes involved in LPS biosynthesis and protein glycosylation. Based on complementation experiments, *galE* and *wlpA* was shown to encode proteins with UDP-galactose-4-epimerase and galactosyltransferase activity, respectively. Null mutants of *galE* and *wlpA* were generated and analysis of the LPS prepared from these mutants by SDS-PAGE and Western immunoblotting showed no significant changes in the LPS banding profiles as compared to wild-type PAO1. To determine if this gene cluster is involved in glycosylation of pilin or flagellin, pilin and flagellin isolated from the mutants were examined and these proteins showed no difference in molecular weight when compared to that of the parent strains. An invasion assay was used to evaluate the invasive phenotype of the *P. aeruginosa* mutants and no significant difference in invasion was observed between the wild type and mutant strains. *In silico* analysis of the ORFs flanking the wlp locus shows that it is located within a cluster of genes that encode proteins that are predicted to be involved in a protein secretion system. We speculate that the *wlp* cluster is involved in synthesis of an as of yet, unidentified glycoprotein.
Introduction

The initial interactions between *P. aeruginosa* and host tissues thought to be critical for colonization is attachment and invasion. Many cell-surface structures that contribute to the adherence of *P. aeruginosa* to eukaryotic cells include alginate (Baker and Svanborg-Eden, 1989), components of the flagellar apparatus (Simpson et al., 1995), pili (Woods et al., 1980) and LPS (Zaidi et al., 1996). In recent years, transmission electron microscopy and various experimental models have revealed that *P. aeruginosa* is internalized by many different cell types, including human respiratory epithelial cells (Pier et al., 1996a), corneal epithelial cells (Fleischig et al., 1995), Mandin-Darby canine kidney cells (Fleischig et al., 1997), and Caco-2 cells (Pereira et al., 1997). After internalization, *P. aeruginosa* can survive and replicate intracellularly (Chi et al., 1991; Fleischig et al., 1995; Plotkowski et al., 1999; Evans et al., 2002). Internalization by host cells has been observed in many animal models (Pier et al., 1996b; Schroeder et al., 2001), thus, invasion represents a potential pathogenic mechanism for this organism.

The LPS-core OS has been implicated as a ligand for internalization within epithelial cells (Pier et al., 1996a; 1996b; Zaidi et al., 1996). Assembly of the core OS is not well understood, and some of the genes required for assembly of the outer-core region have not yet been identified. Therefore, bacterial invasion assays were used to identify bacterial factors required for invasion of *P. aeruginosa* within epithelial cells. Using a similar invasion assay, Miller and Falkow (1988) identified the *inv* and *ail* genes of *Yersinia enterocolitica*. These genes promote adherence and invasion to host cells. In the study by Miller and Falkow (1988), a DNA library from *Y. enterocolitica* was transformed into a non-invasive *E. coli* strain and *E. coli* transformants were screened for
an invasive phenotype within human pharyngeal epithelial cells (HEp-2) using a gentamicin exclusion invasion assay. A bacterial artificial chromosome (BAC) library has been generated for *P. aeruginosa* strain PAO1 and has clones representing more than 9.5-fold physical coverage of the PAO1 genome (Dewar et al., 1998). This PAO1 BAC library would be ideal to use in a similar assay to identify genes required for *P. aeruginosa* invasion.

The purpose of this study was to identify and characterize genes from *P. aeruginosa* involved in internalization within host cells. *P. aeruginosa* BAC library was transformed into *E. coli* and transformants were screened for an invasive phenotype within HEp-2 cells. A gene cluster, designated *wlp*, conferred an invasive phenotype upon *E. coli* and the genes within this locus encode proteins similar to those involved in LPS biosynthesis. *E. coli* transformed with the *wlp* locus produced a modified core OS. This gene cluster, however, does not appear to play a role in synthesis of LPS in *P. aeruginosa*, but instead, may be involved in synthesis of a glycoprotein.

**Materials and Methods**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. *Salmonella enterica* sv Typhimurium, referred to hereafter as *S. typhimurium*, was routinely grown at 37°C in Miller’s LB broth (Invitrogen) unless otherwise stated. When appropriate, ampicillin (Sigma-Aldrich Canada Ltd) was used to supplement media at 100 μg·ml⁻¹ for *S. typhimurium*. All other growth conditions are described in section 2.1.1.
Table 1. Bacterial strains and plasmids.

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<td><em>mrcA Δ(mrr-hsdRMS-mrcBC) φ80dlacZAΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara, leu) 7697 galiU galK λ- rpsL supG</em></td>
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<td>DH10B</td>
<td><em>supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1</em></td>
<td>Gibco BRL</td>
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<tr>
<td>DH5α</td>
<td>K-12; thr-1 leuB6 Δ(gpt-proA)66 hisG4 argE3 thi-1 rfbD1 lacY ara-14 galK2 xyl-5 mil-1 mgl-51 rpsL31 kdgK51 supE44</td>
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<td>AB1133</td>
<td>AB1133 derivative, <em>wecA::Tn10-48</em></td>
<td>Meier-Dieter et al. (1990)</td>
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<td>21548</td>
<td>K-12; <em>lacZ trp ΔsbeB-rfb upp rel rpsL</em></td>
<td>Meier-Dieter et al. (1990)</td>
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<td>S$^+$874</td>
<td><em>thi-1 thr leu tonA lacY supE</em> recA RP4-2-Tc::Mu, Km$^R$*</td>
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<td>SM10</td>
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<td>Simon et al. (1993)</td>
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<td><strong>P. aeruginosa</strong></td>
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<td>PAO1</td>
<td>Wild-type strain, B band$^+$, A band$^*$, serotype O5</td>
<td>Hancock and Carey (1979)</td>
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<td>PAO1 <em>galE</em></td>
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<tr>
<td>PAO1 <em>wlpA</em></td>
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<td>This study</td>
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<td><strong>S. typhimurium</strong></td>
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<td>SL3770</td>
<td>Produces smooth LPS</td>
<td>Roantree et al. (1977)</td>
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<td>SL1306</td>
<td><em>galE503</em>, produces rough LPS</td>
<td>MacLachlan and Sanderson (1985)</td>
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<td><strong>Y. enterocolitica</strong></td>
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<td>8081c</td>
<td>Invasion strain containing <em>inv</em> and <em>ail</em> locus</td>
<td>Portnoy et al. (1981)</td>
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<td><strong>Plasmids</strong></td>
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<td>Single copy F-derived vector</td>
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<td>Phagemid with CoE origin of replication, <em>Ap$^R$</em></td>
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<td>2.9 kb cloning vector, <em>Ap$^R$</em></td>
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<td>pUCP26</td>
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<td>PEX100T</td>
<td>5.8 kb gene-replacement vector, <em>oriT</em>, <em>sacB</em>, <em>Ap$^R$</em></td>
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Table 1. Bacterial strains and plasmids continued.

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<td>pINV1</td>
<td>2.3 kb SalI fragment cloned into pBluescript II SK, contains galE</td>
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<td>pINV1-TGm</td>
<td>2.3 kb SalI fragment cloned into pEX100T, contains Gm&lt;sup&gt;R&lt;/sup&gt; cassette in the KpnI site within galE</td>
<td>This study</td>
</tr>
<tr>
<td>pINV2</td>
<td>1.5 kb EcoRI fragment cloned into pUCP26, contains wlpA</td>
<td>This study</td>
</tr>
<tr>
<td>pINV8</td>
<td>8.5 kb HindIII fragment from pHK27-4 cloned into pTZ18R, contains wlpABCDEF</td>
<td>This study</td>
</tr>
<tr>
<td>PINV3-TGm</td>
<td>3.1 kb HindIII-BamHI fragment from pINV8 cloned into pEX100T, contains a Gm&lt;sup&gt;R&lt;/sup&gt; cassette inserted in the SalI site of wlpA</td>
<td>This study</td>
</tr>
<tr>
<td>pMAV11</td>
<td>1.4 kb ClaI-Aval fragment in pACYC184; contains wecA&lt;sub&gt;Ec&lt;/sub&gt;</td>
<td>Alexander and Valvano (1994)</td>
</tr>
<tr>
<td>pWQ20</td>
<td>1.5 kb PstI fragment in pTrc99A; contains wbbO (rfb&lt;sup&gt;F&lt;/sup&gt;) from Klebsiella pneumoniae O1:K20</td>
<td>Clarke et al. (1995)</td>
</tr>
<tr>
<td>pJK2363</td>
<td>2.5 kb ClaI-BglII fragment in pGEM4; contains rfpB from Shigella dysenteriae type 1, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Klena et al. (1992)</td>
</tr>
<tr>
<td>pSS37</td>
<td>pACYC184 carrying rfb and rfpAB from S. dysenteriae</td>
<td>Sturm and Timmis (1996)</td>
</tr>
<tr>
<td>pSS37::TnlacZ-T42</td>
<td>pSS37-derivative with rfpB inactivated by a TnlacZ insertion; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Klena and Schnaitman (1993)</td>
</tr>
</tbody>
</table>
Computational analysis of genes and gene products. Analysis of the genes and predicted proteins are as described in section 2.2.2.

DNA preparation and transformations. These were performed as described in section 2.2.

PCR amplification of *P. aeruginosa* strain PAO1 *galE*. DNA sequencing of the 8.5 kb HindIII insert from pINV8 was performed using purified plasmid DNA by the double stranded-DNA method and the cycle sequencing analyzed with an ABI 373A apparatus (Applied Biosystems, Montréal, QC). Since *galE* was truncated during subcloning of pINV8, PCR primers were designed to isolate the entire ORF and were synthesized by Genologics (University of Guelph, Guelph, ON). The upstream primer, 5'-GAATATCAACCAGACCTACAA-3', was designed from the *P. aeruginosa* PAO1 genome sequence ([www.pseudomonas.com](http://www.pseudomonas.com); Stover et al., 2000) and this sequence flanked the 5' end of the complete ORF. The downstream primer, 5'-ATAATAGGAGGCGACGCAGA-3', was designed from the insert of pINV8. Approximately 100 µg of *P. aeruginosa* strain PAO1 genomic DNA was used as a template, with 25 pmole of each primer. Thermocycling reactions were performed in an automated Ercomp Powerblock I system (San Diego, CA). The DNA template was denatured for 5 min at 95°C, followed by the addition of 2.5 units of *Pwo* enzyme (Boehringer Mannheim). The DNA was amplified through 29 cycles of 95°C (1 min), 64°C (30 s), 68°C (1.5 min). After completion of these cycles, there was a final elongation step 68°C (7 min). The 2.3 kb PCR product was sequenced by the Guelph Molecular Supercentre (Laboratory Services, University of Guelph, Guelph, ON) using an ABI automated sequencer (model 377, Applied Biosystems).
**Generation of *P. aeruginosa* strain PAO1 and PAK *galE* and *wlpA* mutants.**

To generate insertional mutations in *galE* and *wlpA*, the 2.3 kb *SalI* insert of pINV1 and a 3.1 kb *HindIII-BamHI* fragment from pINV8 were blunt-ended using Klenow enzyme (Invitrogen) and independently ligated into the *SmaI* site of the suicide vector pEX100T (Schweizer and Hoang, 1995). The gentamicin resistance cassette (Gm\(^R\)) was isolated from the plasmid, pUCGM (Schweizer, 1993) using either the *KpnI* or the *SalI* sites, and then cloned into the unique *KpnI* site within the *galE* coding sequence or the unique *SalI* site within *wlpA*, to generate pINV1-TGm, and pINV8-TGm, respectively. These constructs were then used to generate *P. aeruginosa* *galE* and *wlpA* mutants in strains PAO1 and PAK, by the gene replacement strategy of Schweizer and Hoang (1995).

**Preparation of gene probes and Southern blot hybridizations.** To verify the correct insertion of the gentamicin cassette within the various *P. aeruginosa* mutants generated, Southern hybridizations were performed based on the methods described by Schweizer and Hoang (1995). Chromosomal DNA was isolated using DNAzol reagent (Invitrogen) according to the manufacturer's instructions, from *P. aeruginosa* strains including the 20 IATS serotypes, PAO1, PAK, and their respective mutants. Southern blotting was carried out as described in section 2.2.3.

**Analysis of LPS, proteins, and isolation of pilin and flagellin.** LPS was prepared by the method of Hitchcock and Brown (1983) and analyzed as described in section 2.3.1. For *S. typhimurium* LPS, the first antibody was a 1:2 000 dilution of rabbit antiserum raised against LPS from the smooth strain SL3770 (de Kievit and Lam, 1997) and secondary antibody goat anti-rabbit IgG, F(ab')\(_2\)-alkaline phosphatase conjugate (Jackson ImmunoResearch Laboratories Inc.) was used at the dilutions suggested by the
manufacturer. Pilin and flagellin were isolated from *P. aeruginosa* strains as described in sections 2.4.1 and 2.4.2, respectively, and analyzed by SDS-PAGE as outlined in section 2.4.3.

**Results**

Dr. Levesque and his colleagues (Dr. François Sanschagrin, Marie-Josée Morency, Guy Cardinal, and Dr. Roger C. Levesque from the Microbiologie Moléculaire et Génie des Protéines, Sciences de la Vie et de la Santé, Faculté de Médecine, Pavillon Charles-Eugène Marchand, Université Laval, Ste-Foy, QC) constructed the BAC library and performed screening of the *E. coli* transformants for invasion within HEp-2 cells. This group identified a BAC clone pHK27-4, and a recombinant plasmid pINV8, containing an 8.5 kb *HindIII* fragment from pHK27-4, both of which conferred an invasive phenotype upon *E. coli*

**Characterization of the wlp cluster and adjacent regions**

Sequence analysis of the 8.5 kb *HindIII* insert of pINV8 revealed six ORFs, which correspond to PA1384 to PA1390 of the *P. aeruginosa* PAO1 genome (Pseudomonas genome project; http://www.pseudomonas.com/; Stover et al., 2000). An incomplete ORF at the 5' end consisted of 194 nucleotides that could putatively encode a peptide of 60 amino acids, 37 of which are identical to the C terminus of GalE (UDP-galactose-4-epimerase) of *Bacillus subtilis*. To obtain the complete ORF, the 8.5 kb *HindIII* insert was used as an *in silico* probe against the PAO1 genome database. Analysis of the genome sequence that overlapped with the 5' end of the pINV8 insert allowed the identification of a complete ORF. This ORF is predicted to encode a protein
with 76% similarity (59% identity) to *B. subtilis* GalE and was designated *galE*. The molecular structure of the NADH/UDP-glucose complex of the UDP-galactose-4-epimerase of *E. coli* predicts that 15 amino acids interact with NADH and UDP-glucose (Thoden et al., 1996). Fourteen of these are conserved in *P. aeruginosa* GalE.

The six ORFs downstream of the *galE* homologue have been called *wlpABCDEF* in accordance with the bacterial polysaccharide gene nomenclature (Reeves et al., 1996). These genes were designated *wlp*, for LOS synthesis because the *wlp* cluster transformed into *E. coli* was found to modify the core OS (see below). All six predicted proteins show significant similarity to enzymes involved in polysaccharide biosynthesis and is summarized in Table 2. The genetic organization of the *galE-wlp* gene cluster suggests there are four separate operons. The genes from *galE* through to *wlpF* are transcribed in the same direction and there are discernable Shine Dalgarno sequences for *galE*, *wlpA*, *wlpC*, *wlpD*, and *wlpF*. *galEwlpAwlpB* appear to belong to a three-gene operon, followed by a 147-bp intergenic region before a two-gene operon composed of *wlpCwlpD*. A 96-bp intergenic region separates *wlpCwlpD* from *wlpE*, which is followed by another intergenic region of 186 bp before the start codon of *wlpF*. The stop codon of *wlpF* overlaps with a start codon of what appears to be a partial ORF. Comparison of the 3’ end of the 8.5 kb sequence to the PAO1 genome revealed *wlpF* to be the start of a three-gene operon. The average mol% G+C of *galEwlpAwlpB* is 53, which is ca. 10% lower than the average G+C of *P. aeruginosa* (65-67%) (Stover et al., 2000). The average mol% G+C of *wlpCDEF* and the two downstream ORFs is approximately 61, which is more consistent with that of the *P. aeruginosa* genome.
Table 2  Similarity-based annotation of ORFs forming the wlp cluster

<table>
<thead>
<tr>
<th>Gene</th>
<th>PA ORF</th>
<th>GC Content (%)</th>
<th>Lengtha</th>
<th>Related proteins</th>
<th>Accession No.</th>
<th>Length</th>
<th>Functionb</th>
<th>Identity/ Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Name</td>
<td>Organism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GalE</td>
<td><em>Bacillus subtilis</em></td>
<td>CAA67713</td>
<td>339</td>
<td>UDP-galactose-4-epimerasec</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GalE</td>
<td><em>Escherichia coli</em></td>
<td>AAD50491</td>
<td>339</td>
<td>UDP-galactose-4-epimerasec</td>
</tr>
<tr>
<td>galE</td>
<td>PA1384</td>
<td>56</td>
<td>337</td>
<td>RfpB</td>
<td><em>Shigella dysenteriae</em></td>
<td>AAC60480</td>
<td>377</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PglA</td>
<td><em>Neisseria meningitidis</em></td>
<td>AAC35426</td>
<td>376</td>
<td>galactosyltransferasec</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>WlaG</td>
<td><em>Campylobacter jejuni</em></td>
<td>CAA72356</td>
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<td>galactosyltransferasec</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RfaK</td>
<td><em>Neisseria meningitidis</em></td>
<td>AAC44648</td>
<td>354</td>
<td>N-acetylglucosamine transferasec</td>
</tr>
<tr>
<td>wlpA</td>
<td>PA1385</td>
<td>51</td>
<td>374</td>
<td>Wzt</td>
<td><em>Burkholderia pseudomallei</em></td>
<td>AAD05459</td>
<td>469</td>
<td>ATP-binding protein of ABC transporterd</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KpsT</td>
<td><em>E. coli</em></td>
<td>AAC16668</td>
<td>421</td>
<td>ATP-binding protenec</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>None with significant similaritye</td>
<td></td>
<td>BAA07748</td>
<td>431</td>
<td>ATP-binding proteinc</td>
</tr>
<tr>
<td>wlpC</td>
<td>PA1387</td>
<td>59</td>
<td>518</td>
<td>Glycosyl transferase 2 familyf</td>
<td></td>
<td></td>
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<td>wlpD</td>
<td>PA1388</td>
<td>59</td>
<td>242</td>
<td>PA1391</td>
<td><em>P. aeruginosa</em></td>
<td>none</td>
<td>591</td>
<td>Putative glyosyltransferased</td>
</tr>
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<td>wlpE</td>
<td>PA1389</td>
<td>63</td>
<td>494</td>
<td>MigA</td>
<td><em>P. aeruginosa</em></td>
<td>AAC4786</td>
<td>299</td>
<td>Rhamnosyltransferased</td>
</tr>
<tr>
<td>wlpF</td>
<td>PA1390</td>
<td>62</td>
<td>344</td>
<td>LgtA</td>
<td><em>N. meningitidis</em></td>
<td>AAM33852</td>
<td>348</td>
<td>N-acetylglucosamine transferasec</td>
</tr>
</tbody>
</table>

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a Number of amino acid residues

b Functions have been assigned to these proteins as a result of experimental evidence (c) or on the basis of sequence similarity and data pertaining to the gene cluster to which it belongs (d)

c No related proteins of significant similarity were found using the non-redundant Genbank CDS translations based upon the Expect value set with a significance threshold of 0.1.

d Although protein belongs to this family based on similar motifs, there is no experimental evidence to suggest exact function

e No accession no. has been assigned to ORF PA1391 of the PA01 genome yet
**WlpA.** This protein is most similar to *Shigella dysenteriae* RfpB, a galactosyltransferase involved in O-antigen biosynthesis. RfpB transfers galactose from UDP-galactose to undecaprenyl pyrophosphate-linked N-acetylglucosamine (GlcNAc-PP-Und) (Gohmann et al., 1994). WlpA is also homologous to galactosyltransferases from many different organisms and the majority of its closest homologues has been shown to use or are predicted to recognize a nucleotide donor containing galactose or a galactose derivative, whereas the acceptor molecule for the different homologues varies significantly. For example, WlpA is similar to PglA of *N. meningitidis*, a galactosyltransferase involved in the biosynthesis of a pilin-linked trisaccharide. PglA is required for the covalent attachment of galactose to 2,4,diacetamido-2,4,6-trideoxyhexose. WlpA belongs to glycosyl transferase group 1, a protein family described in CAZy-Carbohydrate Active enZYmes database (http://afmb.cnrs-mrs.fr/CAZy/acc.html; Coutinho and Henrissat, 1999) and the proteins in this family share significant sequence homology in the C terminus.

**WlpB.** wlpB encodes a putative protein that contains a domain of approximately 215 amino acids at its N terminus that includes the ATP-binding motif (Walker A and B domains) as well as other conserved regions that define ATP-binding proteins that belong to the family of proteins required for ABC (ATP binding cassette) transporters. This highly conserved 215-amino acid domain differentiates this family of ATP-binding proteins from other ATP-binding proteins that only have Walker motifs, which are not ABC transporters (reviewed in Higgins, 1992). WlpB is similar to the ATP-binding proteins of the ABC-transporters that are used for O-antigen synthesis in many bacteria. WlpB has 59% similarity (40% identity) to Wzt of *P. aeruginosa*, which is the ATP-
binding protein involved in ABC-transporter dependent O antigen assembly of A-band PS (Rocchetta and Lam, 1997). The ABC-transporter for O-antigen assembly is usually composed of two-components, a hydrophilic protein (the ATP-binding protein; Wzt homologues) and an integral membrane protein (Wzm homologues). To determine if any of the other genes encoded the second component of a putative ABC transporter, the Wzm homologue, hydropathy profiles were generated for the proteins encoded by wlpABCD\textit{E}FF and were compared to those generated for Wzm from \textit{P. aeruginosa} and \textit{E. coli} O9. Wzm-like proteins have a characteristic hydropathy profile and the Wzm from \textit{E. coli} O9 and \textit{P. aeruginosa} have seven and six membrane-spanning domains, respectively (Rocchetta and Lam, 1997). The secondary structure predictions of the Wlp proteins had no similarity to those generated for the two known Wzm proteins. There are ABC-transporter fusion proteins reported to contain the ATP-binding domain at one terminus and the integral membrane component domain at the opposite terminus (Higgins et al., 1992). The predicted secondary structure of WlpB, however, shows no transmembrane spanning domains. WlpB does not appear to be a fusion protein of the ATP binding domain with the integral membrane protein, and a Wzm homologue does not appear to be encoded by any of the ORFs within this cluster.

\textbf{WlpC, WlpD, WlpE.} Primary amino acid sequence alignments for WlpC, WlpD, and WlpE with proteins in the databases show that (i) WlpC does not have significant similarity to any known proteins, and (ii) WlpD and WlpE have small regions of homology to other putative glycosyltransferases, but the function of these homologues have not been shown experimentally. Conserved protein motifs were identified in WlpD and WlpE by using the NCBI Conserved Domain Search, which includes reverse position
specific BLAST (Altschul et al., 1997). WlpD belongs to glycosyl transferase group 2 (CAZy) and the similarity of WlpD to other glycosyltransferases in this family is restricted to a region in the N terminus between amino acids 35 to 120. WlpE belongs to glycosyl transferase group 1 and the similarity to transferases in this family lies in the C terminus region between amino acids 300 to 450. Interestingly, WbpE is 33% identical and 53% similar to the protein encode by PA1391, which is the ORF directly downstream of wbpF.

**WlpF.** The protein encoded by *wlpF* is 51% identical and 65% similar to *P. aeruginosa* MigA, an α1,6-rhamnosyltransferase required for assembly of the uncapped outer-core region of the core OS molecule (Yang et al., 2000; Poon et al., 2003). Both WlpF and MigA belong to the glycosyl transferase group 2 (CAZy). WlpF is also 60% similar to *N. meningitidis* LgtA. LgtA has been characterized biochemically to catalyze the transfer of N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc) from their respective UDP-nucleotide sugar donors to substrate molecules that contain galactose residues to synthesize both GlcNAc β (1→3) Gal and GalNAc β (1→3) Gal linkages (Blixt et al., 1999).

**In silico analysis of the regions that flank the wlp cluster.** Using the *wlp* cluster as an *in silico* probe against the PAO1 genome sequence using the NCBI BLAST server revealed some interesting features of the gene products encoded by the ORFs that flank the 5' and 3' end of the cluster. Upstream of *galE* is PA1383, which encodes a protein with three membrane-spanning domains according to the TMHMM prediction program. This protein is characterized by PseudoCAP, TIGRFAMs and Pfam to have secondary protein structure similar to that of *S. enterica* FlgA, a protein involved in
assembly of the flageller P-ring. PA1383 and galE are transcribed in the same direction but are separated by an intergenic region of 152 bp (Pseudomonas Genome Project; Stover et al., 2000).

Directly upstream of PA1383 is PA1382, which encodes a protein of 759 amino acids (PseudoCAP) with six probable membrane-spanning domains. This protein belongs to a large superfamily of proteins called Secretins, which form multimeric outer membrane proteins and mediates transport of macromolecules across the outer membrane of Gram-negative bacteria (Genin and Boucher, 1994). The Secretin family contains proteins of type III secretion pathways, such as InvG of S. typhimurium (Crago and Koronakis, 1998), PilQ, which is required for biogenesis of type IV pili in P. aeruginosa (Bitter et al., 1998) and PulD, the secretin of the pullulanase-specific secretion system of Klebsiella oxytoca (Nouwen et al., 2000). Secretin proteins have two domains, an N-terminal periplasmic domain (Guilvout et al., 1999) and a C-terminal domain that contains several amphipathic β-strands similar to transmembrane segments and this domain is predicted to be embedded in the outer membrane (Bitter et al., 1998). The protein encoded by PA1382 contains a highly conserved motif consisting of (V/I)PXL(S/G)XIPXXGXL in the C terminus, which is characteristic of this family (Genin and Boucher, 1994). The C terminus is the region of homology to its family members and it is this domain that is responsible for channel formation.

At the 3' end of the wlp cluster, wlpF (PA1390) is the first gene of a three-gene operon in which the second ORF, PA1391, encodes a putative glycosyltransferase, and PA1392 encodes a hypothetical protein with a predicted signal peptide as determined by PseudoCAP.
Distribution of the *wlp* locus among different *P. aeruginosa* strains

It was of interest to determine if the *wlp* locus was conserved in other *P. aeruginosa* strains. The 8.5 kb *Hind*III fragment was used as a DNA probe for Southern hybridization studies against *Hind*III-digested chromosomal DNA isolated from the 20 IATS serotype strains of *P. aeruginosa*. An 8.5-kb probe reactive DNA fragment was identified in the chromosomal DNA isolated from IATS serotype strains O1, O3, O5, O6, O7, O8, O16, O17, O18, and O20 (data not shown).

*P. aeruginosa galE encodes a UDP-galactose-4-epimerase*

The significant similarity shared between the *P. aeruginosa* putative GalE protein and the GalE (EC 5.1.3.2) of other bacteria suggested that the *P. aeruginosa* homologue may have UDP-galactose-4-epimerase activity, catalyzing the reversible conversion of UDP-glucose to UDP-galactose (Adhya, 1987). The 8.5 kb insert of plasmid pINV8 did not contain the entire *galE* gene and so PCR primers were designed with the forward primer based on the genome sequence and the reverse primer from the insert of pINV8. A 2.3 kb fragment containing the entire *galE* was PCR-amplified from PAO1 chromosomal DNA. The size of the PCR product was consistent with that predicted from the genome sequence. *P. aeruginosa galE* was cloned into pBluescript II SK to generate pINV1 (Fig. 1), which was transformed into *S. typhimurium* *galE* mutant strain SL1306. *S. typhimurium* SL1306 produces significantly less long-chain PS when the cells are grown in Luria-Bertani media, because production of UDP-galactose by GalE is required for synthesis of both the outer-core OS and O antigen. LPS from SL1306, SL1306 (pINV1; *galEp*) and the wild-type strain SL3770 was analyzed using silver-stained SDS-PAGE and Western immunoblotting with rabbit polyclonal antiserum against SL3770 (Fig. 2).
Figure 1 Restriction maps of the BAC clone pHK27-4, and subclones created in this study, pINV8, pINV1 and pINV2. The double slash (/) seen in pHK27-4 represents a DNA insert of 93 kb cloned into the vector pBeloBAC11. An 8.5 kb HindIII fragment from pHK27-4 was cloned into pTZ18R to generate pINV8 which contains six ORFs, wlpABCDEF. The direction of transcription of the genes are indicated by the arrows and the first two shaded genes, galE and wlpA encode UDP-galactose-4-epimerase and a galactosyltransferase, respectively. pINV1 contains the complete coding sequence of galE and pINV2 contains wlpA cloned into pBluescript II SK and pUCP26, respectively. Insertion of the gentamicin cassette (Gm⁸) into the galE and wlpA coding sequences for generation of chromosomal mutants is shown, although the inserts were first cloned into the suicide vector pEX100T.

Abbreviations for restriction sites: B, BamHI; E, EcoRI; H, HindIII; K, KpnI; S, SalI.
Figure 2  Complementation of a S. typhimurium galE mutant, SL1306, with P. aeruginosa galE. Silver-stained gel after LPS resolved by SDS-PAGE showed restoration of long-chain LPS in SL1306 when carrying P. aeruginosa galE (pINV1) that is comparable to LPS produced by the wild-type strain SL3770 and reacts with anti-SL3770 serum. Strain SL1306 transformed with pPH6.2 is a positive control plasmid which carries galE from Pasteurella haemolytica.
SL1306 produced significantly less O-antigen PS. Small amounts of O antigen are still produced because of the incorporation of trace amounts of galactose present in the media. The transformed strain SL1306 (pINV1; galEPAOI) produced smooth LPS that was comparable to that of the parent strain, SL3770. In addition, the restored LPS from the complemented mutant reacted with anti-SL3770 antibodies. This complementation data suggested that P. aeruginosa GalE has UDP-galactose-4-epimerase activity.

The wlp cluster encodes functional glycosyltransferases

Three proteins predicted to be encoded by pINV8, WlpA, WlpD, and WlpE, have homology to glycosyltransferases involved in LPS synthesis. To determine if the gene products of pINV8 were involved in synthesis of an LPS structure, LPS of E. coli DH5α (pBluescript; vector control) was compared to that of E. coli (pINV8) using tricine SDS-PAGE. The lipid A-core OS of E. coli (pINV8) had a core OS band of higher molecular mass than the core OS of the vector control strain (Fig. 3). This indicated the addition of sugar residues by the wlp gene cluster to the E. coli core OS. To assess the function of the gene product of wlpA in core modification, a 1.5 kb EcoR I fragment carrying wlpA was subcloned from pINV8 into vector pUCP26 to generate pINV2 (Fig. 1). Comparison of the LPS banding profiles from E. coli (pINV2), E. coli (pINV8) and E. coli (pBluescript) using silver-stained tricine gels showed that E. coli (pINV8) produced two distinct modified-core oligosaccharides, both of which migrated with a larger molecular mass than the predominant core OS band produced by E. coli carrying the vector alone (Fig. 3). E. coli (pINV2) also produced a modified core OS with a larger molecular mass than that from the E. coli parent. One of the pINV8-modified core OS bands migrated with the same relative mobility as the WlpA-modified core band. The second pINV8-modified
Figure 3  LPS-core oligosaccharides isolated from E. coli DH5α transformants visualized by silver-stained tricine gels. Two distinct core modifications are observed in E. coli (pINV8). One is a core OS plus three sugar modification (arrow indicates this “Wlp-modified core”) and the second is a core OS plus two sugar modification, which is shown as an intense band of lower molecular mass that has the same relative mobility as the “WlpA-modified core” produced by E. coli (pINV2).
core species migrated with a slightly greater molecular mass. To determine the relative sizes of the WlpA-modified core and the higher-molecular mass “Wlp”-modified core, LPS of *E. coli* (pINV8) and *E. coli* (pINV2) were analyzed by SDS-PAGE along with LPS from *E. coli* K-12 (pWQ20). Plasmid pWQ20 contains *Klebsiella pneumoniae* O1 *wbbO*, which directs the formation of a WbbO-modified core OS in *E. coli*, consisting of lipid A-core plus three-sugar residues [β-D-Gal-(1→3)-α-D-Gal-(1→3)-β-D-GlcNAc] (Clarke et al., 1995). The WbbO-modified core had the same relative mobility as the minor “Wlp”-modified core (data not shown). These results suggest that the minor “Wlp”-modified core produced by *E. coli* (pINV8) consisted of a core OS plus three sugar residues, which arose from the combined activities of presumably WlpA and other Wlp proteins.

_**wlpA encodes a putative galactosyltransferase that may use GlcNAc-PP-Und as an acceptor molecule**_

WlpA is similar to *S. dysenteriae* RfpB, a galactosyltransferase involved in the synthesis of type 1 O antigen (Gohmann et al., 1994). *E. coli* strain DH5α carrying *S. dysenteriae rfpB* (*rfpBsa*) on a plasmid (pJK2363) produces an RfpB-modified core OS consisting of *E. coli* core OS plus two sugars [α-D-Gal-(1→3)-β-D-GlcNAc] (Fält et al., 1993). RfpB modifies the *E. coli* DH5α core OS by synthesizing an incomplete O unit that is covalently linked to the native core OS via the Wzy-dependent pathway for O-antigen biosynthesis. The mechanism is as follows: RfpB transfers a galactose residue to GlcNAc-PP-Und to form α-D-Gal-(1→3)-β-D-GlcNAc-PP-Und. This disaccharide carrier lipid is translocated by the native O-antigen flippase, Wzx, from the cytoplasmic face of the inner membrane to the periplasmic face, where the disaccharide is ligated to lipid-A.
core (Klena and Schnaitman, 1993; Clarke et al., 1995). To determine if WlpA has similar glycosyltransferase activity and mechanism of sugar addition as RfpB in an E. coli background, LPS profiles from E. coli (pINV2) was compared to that of E. coli (pJK2363) using silver-stained tricine SDS-PAGE (Fig. 4). The WlpA-modified core band showed the same relative mobility as the RfpB-modified core band.

Modification of E. coli core by RfpB has been shown to require WecA, a UDP-GlcNAc:undecaprenyl phosphate GlcNAc-1-phosphate transferase (Meier-Dieter et al., 1992). WecA catalyzes the transfer of the first sugar residue, GlcNAc, to the lipid carrier, undecaprenol pyrophosphate to form GlcNAc-PP-Und. This is the acceptor molecule for the transfer of galactose by RfpB to form Gal-GlcNAc-PP-Und. To examine whether WlpA recognizes GlcNAc-PP-Und as an acceptor molecule, the LPS of E. coli 21548, a wecA mutant, was compared to that of E. coli 21548 (pINV2) and E. coli 21548 (pJK2363) using tricine SDS-PAGE. A silver stain of the gel showed no obvious changes in the molecular mass of the core OS in either of the transformants (data not shown), suggesting that in E. coli DH5α, WlpA-catalyzed modifications are WecA dependent. WlpA likely adds a sugar residue to GlcNAc-PP-Und, rather than directly to the cognate-core OS. Co-mobilization of wecA, (on plasmid pMAV11) and pINV2 into E. coli 21548 restored the WlpA-modified band (data not shown).

To determine if E. coli Wzx was required to translocate the disaccharide generated by WlpA and WecA across the inner membrane to the acceptor core OS, pINV2 was transformed into E. coli K-12 rfb delete strain Sφ874, which is deficient in wzx (Neuhard and Thomassen, 1976). SDS-PAGE analysis of LPS from Sφ874 and Sφ874 (pINV2) revealed the absence of the WlpA-modified core band (data not shown).
Figure 4  LPS-core oligosaccharides analyzed by silver-stained tricine gels.

LPS from *E. coli* DH5α (pINV2; *wlpA*) has the same relative mobility as the RfpB-modified core band of *E. coli* K-12 (pJK2363; *rfpB*).
WlpA was unable to modify the core OS in *E. coli wecA* and *wzx* mutants and this suggests that *P. aeruginosa* WlpA is a putative glycosyltransferase that most likely recognizes GlcNAc-PP-Und as the acceptor molecule for its transferase reaction.

The functional similarity of WlpA with RfpB suggests that it could replace RfpB in synthesis of *S. dysenteriae* type 1 O antigen. Plasmid pSS37, which carries *rfpB* and the *rfb* gene cluster from *S. dysenteriae*, can direct the formation of type 1 O antigen in *E. coli* strain AB1133, with the O repeat consisting of Rha-Rha-Gal-GlcNAc (Klena and Schnaitman, 1993). In plasmid pSS37::TnlacZ-T42, *rfpB* is inactivated. When pSS37::TnlacZ-T42 is mobilized into AB1133, no O antigen is produced. The LPS from AB1133 carrying both pSS37::TnlacZ-T42 and pINV2 was compared with that of AB1133 and AB1133 (pSS37) by silver-stained SDS-PAGE (Fig. 5). *P. aeruginosa wlpA* was able to complement the *rfpB* mutation in AB1133 carrying pSS37::TnlacZ-T42, as shown by the production of long chain LPS. Collectively, these results indicate that WlpA transfers galactose to GlcNAc-PP-Und.

**Generation of *P. aeruginosa* PAO1 galE and wlpA mutants**

The genes involved in synthesis of the outer-core OS of *P. aeruginosa* had not been identified at the time of the study, thus, I wanted to determine if *galE* and *wlpA* were involved in production of this structure. Null mutants of *galE* and *wlpA* were constructed following the gene replacement system of Schweizer and Hoang (1995). Since *galE* appeared to be the first gene in the cluster, a nonpolar mutation was constructed by inserting the gentamicin cassette in opposite orientation with respect to the *galE* gene, which would also interrupt transcription of the genes downstream of *galE*, including *wlpA* and *wlpB*, provided that they were transcribed together. Southern blot
Figure 5 Silver-stained gel of LPS produced by *E. coli* strain AB1133 and AB1133 carrying pSS37, which contains *S. dysenteriae* *rfpB* and *rfb* O-antigen cluster. Note that *P. aeruginosa* pINV2 carrying *wlpA* can complement a mutation in *rfpB*. 
analysis was performed on EcoRI-digested genomic DNA from wild-type PAO1 and three wlpA mutants using the gentamicin resistance gene, aacC1 and wlpA as separate gene probes. The aacC1-DNA probe hybridized to a 4 kb-EcoRI DNA fragment of the wlpA mutants. The wlpA-DNA probe reacted to the same 4 kb fragment of the mutants and hybridized to a smaller 3.1 kb fragment of the parent strain (data not shown). PCR analysis was used to confirm the correct genotype of the PAO1 galE mutants. The primers used to PCR-amplify galE from the chromosome was used to PCR-amplify galE from three potential PAO1 galE mutants. One PCR product of 3.1 kb was obtained, which is consistent with the size of the galE-PCR product plus the 850-bp aacC1 gene (data not shown).

**LPS phenotype of PAO1 galE and wlpA mutants**

LPS from PAO1 galE and PAO1 wlpA mutants were compared to that of wild-type PAO1 using SDS-PAGE analysis and Western immunoblotting using B-band specific monoclonal antibody (mAb) MF15-4, A-band specific mAb N1F10 and outer-core specific mAb 101 (Fig. 6). The core-OS region of the LPS was examined by silver staining of the tricine gels after SDS-PAGE. A band, B band, and core OS from the parent and mutant strains appeared similar. Although it was shown that *P. aeruginosa* galE and wlpA are involved in biosynthesis and modification of core LPS in *S. typhimurium* and *E. coli*, respectively, loss of these genes does not appear to affect the synthesis of *P. aeruginosa* LPS when grown in standard laboratory growth conditions. These conditions are defined in this study to be growth in Miller’s LB broth at 37°C, shaking in an incubator at 200 rpm overnight or on Miller’s LB agar at 37°C overnight.
Figure 6 Analysis of LPS isolated from *P. aeruginosa* strain PAO1 and mutants. LPS was separated on 12.5% glycine SDS-PAGE and visualized by silver staining (A) and Western immunoblotting using B-band specific mAb MF15-4 (B), A-band specific mAb N1F10 (C), and outer-core specific mAb 101 (D). The core region of the LPS was visualized by silver-staining a 10-20% gradient tricine polyacrylamide gel (E). No significant differences in LPS banding profiles were observed.
Pilin and flagellin glycosylation status of PAO1 galE and wlpA mutants

The alternative hypothesis that the wlp gene cluster is required for glycosylation of either pilin or flagellin was tested. Crude pilus extracts isolated from wild-type PAO1, galE and wlpA mutants were subjected to SDS-PAGE using tricine gels. The predicted molecular mass of PAO1 pilin, based on primary amino acid sequence of the protein encoded by pilA, is 15 511 Da (Sastry et al., 1985; Stover et al., 2000). Posttranslational modifications of pilin and flagellin proteins in P. aeruginosa have been shown to result in an increase in their mass as determined by SDS-PAGE analysis (Castric, 1995; Brimer and Montie, 1998). The mass of PAO1 pilin as determined by SDS-PAGE was in agreement with that predicted (Fig. 7 A). To further substantiate that galE and wlpA were not involved in glycosylation of this protein, pilin isolated from the P. aeruginosa mutants appeared to migrate with the same mass as pilin from the parent strain.

The predicted molecular mass of wild-type PAO1 b-type flagellin, encoded by fltC, is 49 242 Da (accession no. P72151; submitted by Wahl and Baker, 1996; Stover et al., 2000). Flagellin isolated from the parent and mutant strains migrated to approximately this size, with no apparent differences in mass (Fig. 7 B). Mutations in the galE and wlpA genes of P. aeruginosa strain PAO1 do not appear to affect the mass of these two different proteins, suggesting that these genes do not play a role in glycosylation of pilin and flagellin in strain PAO1.

LPS and flagellin phenotypes of strain PAK galE and wlpA mutants

The flagellin of P. aeruginosa strain PAK had recently been shown to be glycosylated (Brimer and Montie, 1998). To determine if galE and the wlp cluster are present in the genome of P. aeruginosa strain PAK, Southern blot analysis using high
Figure 7 Analysis of pilin (A) and flagellin (B) isolated from *P. aeruginosa* strain PAO1 and its derivatives using glycine polyacrylamide gels stained with Coomassie brilliant blue. There were no observable differences in the molecular mass of the pilins and flagellins isolated from the PAO1 *galE* and PAO1 *wlpA* mutants when compared to the parent strain.
stringency conditions was performed. PAO1 \( wlpA \) and \( galE \) gene probes hybridized to similar size DNA fragments from PAK chromosomal DNA (data not shown). Since these genes were highly conserved in strain PAK, the chromosomal knock-out plasmid constructs used for generating the PAO1 \( galE \) and \( wlpA \) mutants, \( pINV1\)-TGm and \( pINV3\)-TGm, respectively, were used to generate null mutations in strain PAK using the same allelic exchange strategy described earlier (Schweizer and Hoang, 1985). Southern blot analysis was used to verify the correct genotypes of the PAK \( galE \) and PAK \( wlpA \) mutants (data not shown).

The effect of these mutations on the LPS phenotype and molecular mass of the flagellin proteins were examined. LPS from the PAK \( galE \) and PAK \( wlpA \) mutants were compared to that of the parent strain using glycine and tricine SDS-PAGE and Western immunoblotting with O6-serotype specific mAb 0253gD6, A-band specific mAb N1F10, and inner-core specific mAb 7-4. LPS from PAK \( galE \) and PAK \( wlpA \) mutants appeared similar to that of the wild-type strain (Fig. 8). Flagellin was isolated from these three strains and the molecular mass of these proteins was determined by SDS-PAGE. The deduced molecular mass of the flagellin protein encoded by \( fliC \) from strain PAK is 40 036 Da. However, flagellin isolated from strain PAK has been shown to have a mass, as determined by SDS-PAGE, to be 45 kDa (Totten and Lory, 1990) with the increase in size as a result of glycosylation (Brimer and Montie, 1998). Flagellin isolated from the parent strain, PAK \( galE \) and PAK \( wlpA \) mutants all had an apparent molecular mass of ca. 45 k Da (Fig. 9 A and B), which is ca. 5 kDa greater than that of the predicted size for the FliC protein, confirming the posttranslation modification in the flagellin observed by Brimer and Montie (1998). Although the flagellin isolated from the mutants appear to
Figure 8 Analysis of LPS isolated from *P. aeruginosa* strain PAK and mutants. LPS was separated on 12.5% glycine polyacrylamide gels and visualized by silver staining (A) and Western immunoblotting using B-band specific mAb 025g3d6 (B), A-band specific mAb N1F10 (C), inner-core specific mAb 7-4 (D). The core region of the LPS was visualized by silver-stained 10-20% gradient tricine polyacrylamide gel (E).
Figure 9 Flagellin proteins isolated from *P. aeruginosa* and analyzed by SDS-PAGE followed by Coomassie brilliant blue staining (A), Western immunoblot with FliC antiserum (B) and isoelectric focusing gel (C) in an ampholyte mixture of pH 3-7.
migrate slightly faster, flagellin was isolated many times from the different strains and these slight migration differences could be accounted for by the different methods of preparation, amount of protein loaded, and extent of dialysis of the purified protein, particularly when purified using polyethylene glycol precipitation. Flagellins were subjected to isoelectric focusing (IEF) to determine if there was any significant differences in the isoelectric point of the flagellin isolated from the wild-type PAK in comparison to that isolated from the PAK galE mutant. This may arise from the loss of charged sugar residues if galE was involved in synthesis of sugar residues required for glycosylation. No apparent differences in the IEF profiles were observed (Fig. 9 C). Similar to our results obtained with PAO1, the wlp cluster, although present in the genome from strain PAK, does not appear to be involved in LPS biosynthesis when grown under laboratory conditions or in flagellin glycosylation.

**Internalization of P. aeruginosa PAO1 and its mutants within epithelial cells**

The *P. aeruginosa* wlp cluster was identified based on its ability to cause *E. coli* to become internalized within HEp-2 cells. Thus, it was necessary to determine if these genes played a significant role during invasion of *P. aeruginosa* within eukaryotic cells. *P. aeruginosa* PAO1 and its isogenic galE and wlpA mutants were tested in an invasion assay for their ability to invade human bronchial epithelial cells (cell line 16HBE14o-). Invasion assays revealed that the PAO1 galE and wlpA mutants invaded the bronchial epithelial cells equally as well as the parent strain (Fig. 10), indicating that the *wlp* genes do not contribute significantly to internalization of *P. aeruginosa* by human airway epithelial cells.
**Figure 10** *Pseudomonas aeruginosa* ingestion by human bronchial epithelial cells (16HBE14o-). This data shows the results obtained from one experiment and similar results were obtained when the experiment was repeated two more times on different days. *E. coli* HB101 is included as a negative invasive control. Bars represent means and error bars the standard errors. No significant differences in the invasion phenotype were observed in the mutant strains when compared to the parent strain.
Discussion

A novel gene locus, the \textit{wlp} cluster, from \textit{P. aeruginosa} strain PAO1 was identified in this study based on its ability to confer an invasive phenotype in \textit{E. coli}. The \textit{wlp} genes were capable of causing the production of a modified form of LPS in \textit{E. coli}. The main conclusions from this study are: (i) the \textit{wlp} cluster encodes proteins that are similar to those involved in LPS biosynthesis and protein glycosylation, (ii) two genes in the \textit{wlp} cluster encode functional glycosyltransferases, (iii) the \textit{galE} gene located at the 5' end of the locus encodes an enzyme that has UDP-glucose-4-epimerase activity, (iv) this genetic locus is not required for LPS biosynthesis in \textit{P. aeruginosa} strains PAO1 or PAK when grown in standard laboratory conditions, and (v) this locus is present in many, but not all serotype strains of \textit{P. aeruginosa}. \textit{E. coli} transformed with the \textit{wlp} genes produced modified LPS structures. Other studies have reported that expression of heterologous LPS-like genes in \textit{E. coli} also modify the core OS to produce a low-molecular-mass LOS-like species. For example, \textit{E. coli} produces chimeric LPS when transformed with genes from \textit{Haemophilus influenzae} that are involved in LOS biosynthesis (Phillips et al., 2000). Structural analysis of the chimeric LPS showed that \textit{E. coli} carrying \textit{lsgCDEFG} on a plasmid produced a core OS capped with a tetrasaccharide. The tetrasaccharide contained GlcNAc as the first sugar residue and similar to the findings with the \textit{wlp} cluster, the addition of sugars to the \textit{E. coli} core by the \textit{lsg} genes also require \textit{E. coli} WecA. In contrast, the \textit{lsg} genes expressed in its native host, \textit{H. influenzae}, direct the sequential addition of three sugar residues directly onto the core OS to produce LOS. Fry et al. (1998) identified a locus from \textit{C. jejuni} strain 81116 (O:6 serotype) that encodes proteins involved in LPS biosynthesis. When expressed in \textit{E. coli}, these genes were
shown to synthesize O side-chains that were reactive with O:6 typing serum and this gene cluster was identified to be involved in LOS biosynthesis. Szymanski et al. (1999) identified a similar gene cluster in *C. jejuni* strain 81-176, which belongs to the O:23 and O:36 serogroup, and the LPS core of *E. coli* DH5α carrying some of these genes on a plasmid was modified such that the core OS became immunoreactive with O:23 and O:36 antisera. Further studies (Szymanski et al., 1999; Linton et al., 2001) demonstrated that this locus was involved in a “general” glycosylation pathway and flagellin glycosylation. Therefore, expression of chimeric LPS forms in *E. coli* mediated by plasmid-encoded genes does not necessarily indicate that the transformed genes are only involved in synthesis of LPS. This strategy, however, may provide data to support the identification of the acceptor and donor molecules for glycosyltransferases in a heterologous, well-characterized background such as *E. coli*.

*Pseudomonas aeruginosa* wlp genes were shown to be involved in the production of a low-molecular mass LOS structure when expressed in *E. coli*. It was further shown that production of Wlp-mediated chimeric-OS structures in *E. coli* required WecA. WecA is a glycosyltransferase that initiates O-antigen synthesis in many enteric bacteria (Alexander and Valvano, 1994; Clarke et al., 1995; Zhang et al., 1997). In *E. coli* O8, WecA initiates assembly of the O unit by transferring GlcNAc from UDP-GlcNAc to Und-P (Rick et al., 1994). The product of this reaction, GlcNAc-PP-Und, acts as an acceptor molecule for another glycosyltransferase to add on the second sugar residue of the O unit. In Wzy-dependent O-antigen assembly, the O unit is flipped by Wzx from the cytoplasmic face of the inner membrane to the periplasmic face and there the O unit is ligated to the core OS (reviewed in Raetz and Whitfield, 2002). We presented evidence
that production of the Wlp-mediated chimeric LPS structures also required the flippase, Wzx. The requirement of WecA and Wzx for Wlp-mediated core modification in *E. coli* suggests that in this particular background, the Wlp transferases assemble an O unit and the native core OS acts as a scaffold for the addition of this structure.

The role of the wlp cluster in the synthesis of an O unit is proposed only in the *E. coli* background because core modifications by the wlp cluster requires enzymes, such as Wzx, which are coded for by *E. coli*’s cryptic Wzy-dependent O-PS locus. This is clearly demonstrated by the lack of core modification in *E. coli* strain Sφ874 carrying pINV8. In this strain, the entire O-PS locus is deleted. Results from this study showed that two of the wlp genes encode glycosyltransferases. WlpA most likely recognizes UDP-galactose as a substrate donor and an acceptor molecule that contains a GlcNAc residue. Although there are three putative glycosyltransferases encoded by pINV8, only two of these could be shown to be functional in the *E. coli* background. It may be that the activated nucleotide donor or the proper acceptor molecule for the third glycosyltransferase is not synthesized in this *E. coli* strain.

To date, O-antigen polysaccharides use one of three pathways for assembly: (i) Wzy-dependent, (ii) ABC-transporter dependent, and (iii) synthase-dependent (reviewed in Raetz and Whitfield, 2002). *wlpB* encodes a putative ATP-binding protein that is characteristic of those used in ABC-transporter systems. The majority of ABC-transporter dependent polysaccharides are synthesized by genetic loci that contain genes encoding both the Wzt and Wzm homologues. *wlpB* encodes a putative Wzt component, however, our results showed that the *wlp* genes do not encode the second membrane component, a Wzm protein. Of the many O-antigen gene clusters that utilize an ABC-
transporter-dependent pathway, including *Y. enterocolitica* O:3 (Zhang et al., 1993), *E. coli* O9 (Kido et al., 1995), and *Brucella melitensis* 16M (Godfroid et al., 2000), all of them contain a *wzm* and *wzt* gene pair. In contrast, the Wzy-dependent O-PS pathway is directed by a gene cluster that usually contains *wzx*, *wzy*, and *wzz*, which encode the flippase, polymerase, and chain length regulator, respectively (Raymond et al., 2002; reviewed in Raetz and Whitfield, 2002). Since the *wlp* cluster does not appear to encode *Wzx*, *Wzy* or *Wzz* homologues, it is unlikely that the *wlp* locus synthesizes a PS via the Wzy-dependent pathway. Also, the *wlp* cluster does not appear to encode a putative synthase homologue that is required for the third assembly pathway.

Two notable exceptions where assembly and transport genes for O-antigen synthesis are located within genetic loci that are involved in synthesis of shorter oligosaccharides are the *wbc* (*trs*) operon of *Y. enterocolitica* strain 6471/76-e, which encodes an outer-core OS (Skurnik et al., 1995) and the *lsa* gene cluster of *H. influenzae*, that is involved in LOS synthesis (Phillips et al., 2000). Although these genes direct the synthesis of core OS and LOS, located at the 5' end of these clusters is a gene encoding a putative flippase (*Wzx* protein). Alternatively, the presence of an incomplete ABC-transporter in the *P. aeruginosa* *wlp* genes may suggest that this locus is a nonfunctional, cryptic O-PS gene cluster. Non-functional O-antigen gene clusters have been identified in *P. aeruginosa* (Dean and Goldberg, 2000, Raymond et al., 2002), however, these genetic loci are punctuated with insertion elements and none have been identified in the *wlp* locus.

Several lines of evidence presented here indicate that the *P. aeruginosa* *wlp* genes are involved in synthesis of a protein glycan rather than an O antigen or core OS. Is the
wlp cluster part of a larger locus involved in protein glycosylation? One of the conserved features that Power and Jennings (2003) identified in many prokaryotic glycosylation clusters is that in the majority of the systems where the location of the target protein is known, the genes responsible for glycosylation are adjacent to the protein they glycosylate. Examination of the ORFs flanking the wlp cluster led to some interesting observations. Two large ORFs upstream of galE encode proteins with many putative membrane-spanning domains and both are likely to be involved in type II or type III secretion systems. Both of these secretion pathways are responsible for the extracellular secretion of toxins, hydrolytic enzymes, and various other macromolecules. One of these proteins belongs to the PulD or Secretin protein family and this protein forms the pore in the type II secretion apparatus located in the outer membrane. The presence of a PulD homologue suggests that this protein may be a channel-forming protein in a type II secretion system, which interestingly, in this type of secretion pathway also uses an ATP-binding component (Lory, 1998). Of further interest is that wlpF is the first gene of a three-gene operon where the last gene encodes a hypothetical protein with a signal peptide sequence, which could potentially be secreted by this system.

In many pathogenic bacteria, galE, which is required for synthesis of UDP-galactose, is associated with both LPS biosynthetic gene clusters and glycosylation gene islands. In N. meningitidis, galE is required for biosynthesis of the pilin glycan and LOS (Stimson et al., 1995). There is a galE gene at the 5’ end of the general protein glycosylation locus of C. jejuni (Szymanski et al., 1999). Y. enterocolitica strain 6471/76-c has a unique wbc (trs) operon directly upstream of its galE gene (Skurnik et al., 1995). The wbc cluster directs formation of a unique “outer-core” that is synthesized on a lipid
carrier in a mechanism similar to that of an O-antigen unit, and this “outer core” is then transferred to the “inner core”. This study shows that \textit{galE} is at the 5' end of a locus predicted to have eight genes in its cluster encoding LPS-biosynthetic type enzymes. In \textit{P. aeruginosa} strain PAO1 and PAK, the only galactose residue found in the LPS that might be derived from UDP-galactose is \textit{N-}(\textit{L}-alanyl)-D-galactosamine, which is present in the core OS of both strains. Our analysis of the LPS from \textit{P. aeruginosa galE} mutants indicated that this \textit{galE} is not involved in synthesis of this core-OS residue.

My results do not preclude the possibility that the \textit{wlp} genes are required for protein glycosylation in \textit{P. aeruginosa}. WlpA has marked similarity to \textit{N. meningitidis} PglA, which is required for synthesis of the pilin-linked trisaccharide Gal (β1→4) Gal (α1→3) 2,4-diacetimido-2,4,6-trideoxyhexose (Jennings et al., 1998). PglA transfers galactose from UDP-galactose to the trideoxyhexose-lipid carrier. The association of the \textit{wlp} cluster with the \textit{galE} gene, and thus synthesis of UDP-galactose, which is not needed for LPS biosynthesis, may suggest that \textit{galE} is needed for synthesis of another glycan. Interestingly, the \textit{wlp} genes were shown to be conserved in ten out of twenty \textit{P. aeruginosa} strains tested. Results obtained in this study indicated that GalE and WlpA of \textit{P. aeruginosa} are not involved in pilin or flagellin glycosylation in \textit{P. aeruginosa} strains PAO1 or PAK. However, strain PAO1 produces at least 15 glycoproteins located in its outer membrane (Nouwens et al., 2000) whose glycosylation status was not examined in our study. In conclusion, the location of the \textit{wlp} genes flanked by proteins involved in a potential protein secretion system as well as a protein containing a signal peptide sequence strongly support that the \textit{wlp} locus may be involved in glycosylation of an as of yet, unidentified protein.
ACKNOWLEDGMENTS

We would like to thank Dr. C. Whitfield and Dr. D. Heinrichs for valuable
discussions on LPS glycosyltransferases and Dr. C. Whitfield and Dr. J. Klina for the
provision of useful strains and constructs.

References

cellular and molecular biology. Edited by F.C. Neidhardt, J.L. Ingraham, K.B.
Low, B. Magasanik, M. Schaechter and H.E. Umbarger. American Society for

Alexander, D.C. and Valvano, M.A. 1994. Role of the rfe gene in the biosynthesis of the
*Escherichia coli* O7-specific lipopolysaccharide and other O-specific

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and

Baker, N.R. and Svanborg-Eden, C. 1989. Role of alginate in the adherence of


Formation of oligomeric rings by XcpQ and PilQ, which are involved in protein
transport across the outer membrane of *Pseudomonas aeruginosa*. Mol.

of the *Neisseria meningitidis* lgtA gene in *Escherichia coli* and characterization of


Plotkowski, M.C., de Bentzmann, S., Pereira, S.H., Zahm, J.M., Bajolet-Laudinat, O.,
 Rogers, P. and Puchelle, E. 1999. Pseudomonas aeruginosa internalization by
 human epithelial respiratory cells depends on cell differentiation, polarity, and

 rhamnosyltransferase involved in core oligosaccharide biosynthesis from
 Microbiology.

Power, P.M. and Jennings, M.P. 2003. The genetics of glycosylation in Gram-negative

 Biochem. 71: 635-700.

 Y., Kaul, R., Clendenning, J.B. and Olson, M.V. 2002. Genetic variation at the O-
 antigen biosynthetic locus in Pseudomonas aeruginosa. J. Bacteriol. 184: 3614-
 3622.

Reeves, P.R., Hobbs, M., Valvano, M.A., Skurnik, M., Whitfield, C., Coplin, D., Kido,

Rick, P.D., Hubbard, G.L. and Barr, K. 1994. Role of the rfe gene in the synthesis of the


glycoprotein substituted with digalactosyl 2,4-diacetamido-2,4,6-trideoxyhexose. Mol. Microbiol. 17: 1201-1214.


Yang, H., Matewish, M., Loubens, I., Storey, D.G., Lam, J.S. and Jin, S. 2000. migA, a quorum-responsive gene of Pseudomonas aeruginosa, is highly expressed in the
cystic fibrosis lung environment and modifies low-molecular-mass lipopolysaccharide. Microbiology **146**: 2509-2519.

