Evaluation of PilO Substrate Specificity Using Normally Non-Glycosylated Proteins in Pseudomonas Aeruginosa

Matthew A. Henkel

Follow this and additional works at: https://dsc.duq.edu/etd

Recommended Citation

This Immediate Access is brought to you for free and open access by Duquesne Scholarship Collection. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of Duquesne Scholarship Collection. For more information, please contact phillipsg@duq.edu.
EVALUATION OF PILO SUBSTRATE SPECIFICITY USING
NORMALLY NON-GLYCOSYLATED PROTEINS
IN PSEUDOMONAS AERUGINOSA

A Thesis
Submitted to the Bayer School
of Natural and Environmental Sciences

Duquesne University

In partial fulfillment of the requirements for
the degree of Master of Science

By
Matthew A. Henkel

May 2009
Copyright by

Matthew A. Henkel

2009
EVALUATION OF PILO SUBSTRATE SPECIFICITY USING NORMALLY NON-GLYCOSYLATED PROTEINS IN PSEUDOMONAS AERUGINOSA

By

Matthew A. Henkel

Approved March 27, 2009

___________________________             ___________________________
Dr. Jana Patton-Vogt          Dr. Philip E. Auron
(Thesis Committee)                (Chair, Department of Biological
and Environmental Sciences)

Dr. Joseph R. McCormick
(Thesis Committee)

Dr. David W. Seybert
(Dean, Bayer School of Natural
and Environmental Sciences)
ABSTRACT

EVALUATION OF PILO SUBSTRATE SPECIFICITY USING NORMALLY NON-GLYCOSYLATED PROTEINS IN PSEUDOMONAS AERUGINOSA

By

Matthew A. Henkel

May 2009

Thesis Supervised by Dr. Peter A. Castric

P. aeruginosa 1244 (PA1244) possesses an O-linked glycosylation system by which the glycosyltransferase, PilO, transfers a single preassembled O-antigen repeating unit to the C-terminal serine residue of pilin as a posttranslational modification. Previous work has shown that the group II pilin of PA103 can be engineered for glycosylation by extending a C-terminal serine away from the surface with a short stretch of alanine residues (Horzempa et al., 2006a). As an extension of that study, several normally non-glycosylated proteins [PA683 pilin, PA1244 PilA-E. coli (EC) alkaline phosphatase (PhoA) fusion, and EC PhoA] were engineered with C-terminal amino acid extensions and assessed for glycosylation by PilO-mediated glycan modification. Migration patterns on SDS-PAGE and an electrofocusing gel revealed that the TfpY accessory protein does not directly confer a posttranslational modification to group III PA683 pilin. The addition of two C-terminal peptide extensions, an –AAS tripeptide and a 15 amino acid
PA1244 derived-peptide tail, to PA683 pilin allowed glycosylation in an LPS polymerization mutant, PA103 \(\text{wzy}_{P_aO11}::\text{aacC1}\), when complemented with PilO. A PA1244 PilA-EC PhoA fusion and EC PhoA engineered with the C-terminal –AAS tripeptide extension were unable to be glycosylated in strain 1244 or PA103 \(\text{wzy}_{P_aO11}::\text{aacC1}\) complemented with PilO. These results suggest the necessity of pilin-specific structures, or the necessity of a membrane anchor for enhancement of PilO substrate recognition. Additionally, larger peptide tail extensions should be considered for testing in future work. Overall, these findings provide further information on glycosylation substrate specificity and suggest the ability to glycosylate normally non-glycosylated proteins in an effort to produce potential conjugate vaccine components.
ACKNOWLEDGEMENTS

I would first like to thank my thesis advisor, Dr. Peter Castric, for his guidance, support, and patience. My time under his advisement has proved to be a valuable academic experience, culminating around the knowledge I have gained as a scientific researcher and a lesson in persistence that I will never forget. I would also like to thank the entire faculty and staff of Duquesne University’s Department of Biological Sciences who have, in one way or another, helped make my experience here an enjoyable one. Of all of the faculty and staff I am indebted too, I would like to extend a special thank you to my committee members, Dr. Jana Patton-Vogt and Dr. Joseph McCormick. I would like to specifically thank Dr. McCormick for his thoughtful willingness to troubleshoot any and all procedural problems about which I sought his guidance. Also, I would like to extend my deepest gratitude to Dr. Patton-Vogt for generously providing me with additional financial support and constant (and very much appreciated) guidance. I would like to thank all of the members of the Castric laboratory that I had the pleasure of working alongside, namely, Joseph Horzempa, Mohammed Qutyan, and Michael Quinn. Thank you for all of your help, guidance, and laughs along the way. Additionally, I would like to extend my gratitude to Dr. Alan Seadler for his constant interest in my research, his help with all of my biotech-related questions, and for providing funding for my research. I would like to thank Dr. Lisa Ludvico for her academic support and guidance. I would also like to thank Dr. Nancy Trun for thoughtfully checking up on my progress from time to time.
Lastly, but certainly not least, I would like to extend my whole-hearted thanks to my family and friends. Their love, patience, guidance, support, and willingness to put up with me in general, has played the most important part in getting me to graduate school, through graduate school, and on to whatever may be next.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Abstract</th>
<th>iv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>vi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xiii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xiv</td>
</tr>
</tbody>
</table>

## I. INTRODUCTION

A. *Pseudomonas aeruginosa* ........................................ 1

B. *Pseudomonas aeruginosa* and Pathology ........................... 3

1. Cystic Fibrosis ........................................ 6

2. Severe Burns ........................................ 8

3. Contact Lens Wear ........................................ 9

C. Virulence Factors ........................................ 10

1. Antibiotic Resistance ........................................ 10

2. Cell Behavior ........................................ 12

   Quorum Sensing ........................................ 12

   Biofilm Formation ........................................ 12

3. Secreted Proteins ........................................ 13

   Exotoxin A ........................................ 13

   Elastase ........................................ 14

   Protease IV ........................................ 14

   Alkaline Protease ........................................ 15

   Phospholipase C ........................................ 15
Type III Secretion

4. Non-Protenacious Secreted Factors
   Alginate
   Rhamnolipids
   Pyocyanin
   Hydrogen Cyanide

5. Surface Structures
   Flagella
   LPS and Pili

D. LPS
   1. LPS Structure
   2. O-antigen Biosynthesis
   3. O-antigen Polymerization
   4. LPS Virulence

E. Type IV Pili
   1. Pilin
   2. Pilin Polymerization
   3. Pili Virulence

F. Glycosylation of *Pseudomonas aeruginosa* 1244 Pilin
   1. Characterization of Pilin Glycan
   2. Glycosylation Pathway
   3. Characterization f PilO
   4. Specificity of the Glycosylation Reaction
II. MATERIALS AND METHODS

A. Media and Bacterial Strains

B. Homology Modeling

C. Plasmid Construction

1. Construction of p683pila

2. Construction of p683pila-tpfY

3. Construction of p683aas

4. Construction of p683pep

5. Construction of pPilAPhoA

6. Construction of pPilAP

7. Construction of pPilAPaas

8. Construction of pECAP

9. Construction of pECAPaas

D. Transformation of Strains

1. Chemical

2. Electroporation

E. Alkaline Phosphatase Periplasmic Activity Assay

F. Extraction of Soluble and Insoluble Cell Fractions

G. Extraction of Periplasmic Fraction

H. Isolation of Pili
I. Western Blot

1. Sample Preparation
2. SDS-PAGE Gel Preparation
3. Blotting Procedure

J. Isoelectric Focusing

K. Generation of Polyclonal Anti-PA683 Pilin Antibodies

1. Antigen Preparation
2. Immunization
3. Serum Collection

III. RESULTS

A. Homology modeling and identification of surface properties of PA1244 PilA, PA683 PilA, and EC PhoA

B. Can a structurally different, Group III, pilin be engineered to accept the O-antigen repeating unit of lipopolysaccharide?

Is PA683 pilin already being modified?

Can PA683 pilin be engineered for glycosylation?

C. Can a protein fused to pilin be engineered to accept the O-antigen repeating unit of lipopolysaccharide?

D. Can a non-pilin protein be engineered to accept the O-antigen repeating unit of lipopolysaccharide?

IV. DISCUSSION

A. The role of TfpY as a group III pilin accessory protein

B. Glycosylation of Group III pilin engineered with
C-terminal peptide extensions………………………………………107

C. Inability of the minimal –AAS peptide extension to permit glycosylation
    of a PA1244 PilA- E. coli alkaline phosphatase fusion protein………108

D. Inability of the minimal –AAS peptide extension to permit glycosylation
    of E. coli alkaline phosphatase……………………………………109

E. Significance to vaccine design……………………………………110

F. Were my thesis goals achieved? …………………………………..111

V. REFERENCES 113
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Bacterial Strains and Plasmids</td>
<td>58</td>
</tr>
<tr>
<td>Table 2</td>
<td>Primer List</td>
<td>60</td>
</tr>
<tr>
<td>Table 3</td>
<td>Antibodies and Visualization</td>
<td>75</td>
</tr>
<tr>
<td>Table 4</td>
<td>Periplasmic phosphatase activity of alkaline phosphatase constructs in PA1244 and PA103 wzy_{PdO11}::aacC1 strain backgrounds</td>
<td>98</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure 1:</th>
<th>Several skin ulcerations infected with <em>Pseudomonas aeruginosa</em></th>
<th>Page 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2:</td>
<td>Microscopy image of <em>Pseudomonas aeruginosa</em> pilus and flagellum structures</td>
<td>21</td>
</tr>
<tr>
<td>Figure 3:</td>
<td>Illustration of the basic structure of LPS</td>
<td>23</td>
</tr>
<tr>
<td>Figure 4:</td>
<td>Predicted molecular structure of <em>P. aeruginosa</em> 1244 LPS</td>
<td>24</td>
</tr>
<tr>
<td>Figure 5:</td>
<td>General steps of O-antigen subunit biosynthesis in <em>P. aeruginosa</em> 1244</td>
<td>26</td>
</tr>
<tr>
<td>Figure 6:</td>
<td>General steps of LPS O-antigen polymerization in <em>P. aeruginosa</em> 1244</td>
<td>29</td>
</tr>
<tr>
<td>Figure 7:</td>
<td>Homology model of the tertiary structure of PA1244 PilA protein</td>
<td>32</td>
</tr>
<tr>
<td>Figure 8:</td>
<td>Operon differences between Type IV Pilin Groups I, II, and III</td>
<td>34</td>
</tr>
<tr>
<td>Figure 9:</td>
<td>Illustration of the general protein machinery responsible for the production of a type IV pilus</td>
<td>36</td>
</tr>
<tr>
<td>Figure 10:</td>
<td>PA1244 pilin aminoglycan produced by total pilin digestion</td>
<td>40</td>
</tr>
<tr>
<td>Figure 11:</td>
<td>Proposed pathway for pilin glycosylation</td>
<td>41</td>
</tr>
<tr>
<td>Figure 12:</td>
<td>Topology of PilO</td>
<td>44</td>
</tr>
<tr>
<td>Figure 13:</td>
<td>Predicted surface properties of PA1244 pilin monomer, PA683 pilin monomer, and <em>E. coli</em> alkaline phosphatase homodimer</td>
<td>83</td>
</tr>
<tr>
<td>Figure 14:</td>
<td>Disulfide loop regions of PA1244 pilin and PA683 pilin</td>
<td>84</td>
</tr>
<tr>
<td>Figure 15:</td>
<td>C-terminal peptide extensions used to study glycosylation substrate specificity of <em>P. aeruginosa</em></td>
<td>84</td>
</tr>
<tr>
<td>Figure 16:</td>
<td>Molecular weight and electrofocusing-based polyacrylamide gel analysis of PA683 pilin for modification by TfpY</td>
<td>87</td>
</tr>
</tbody>
</table>
Figure 17: Western blot analysis of mutant PA683 pilins expressed in PA103 wzyPaO11::aacC1

Figure 18: Western blot analysis of PA1244 PilA-EC PhoA fusion expressed in E. coli

Figure 19: Western blot analysis of mutant PA1244 PilA-EC PhoA fusion expressed in PA1244

Figure 20: Chemiluminescent blot analysis of mutant PA1244 PilA-EC PhoA fusion expressed in PA1244

Figure 21: Western blot analysis of mutant PA1244 PilA-EC PhoA fusion expressed in PA103 wzyPaO11::aacC1

Figure 22: Western blot analysis of mutant E. coli alkaline phosphatase in E. coli

Figure 23: Western blot analysis of mutant E. coli alkaline phosphatase in PA1244

Figure 24: Isoelectric focusing analysis of mutant E. coli alkaline phosphatase in PA1244

Figure 25: Western blot analysis of mutant E. coli alkaline phosphatase in PA103 wzyPaO11::aacC1
I. INTRODUCTION

A. Pseudomonas aeruginosa

*Pseudomonas aeruginosa* is a gram-negative, rod-shaped eubacterium commonly characterized by its ubiquitous inhabitance and opportunistic pathogenicity. *P. aeruginosa* is a member of the class Gamma Proteobacteria and the family Pseudomonadaceae. Motility is conferred by the presence of flagella and pili, which promotes diverse habitation and success as a pathogen. Colonized growth emits a discernible “grape-like” odor.

The earliest suspected reports of *P. aeruginosa* infection occurred around 1860, but this bacterium was not identified as *P. aeruginosa* until about 35 years later (Colwell, 1965; Anzai et al., 2000; Lyczak et al., 2000; El-Banna, 2007). *P. aeruginosa* was first isolated in 1982 by Gessard and named *Bacillus pyocyaneus* because of the characteristic blue-colored purulence at infection sites (Figure 1) (Lyczak et al., 2000). *Pyocyaneus* can be etymologically broken down to mean “blue pus.” The root word is “cyan,” which means blue, and the prefix, “pyo-,” which means pus. The secreted compound responsible for this blue pus was identified and aptly named pyocyanin by Fordos, as cited by Gamgee and Gaby (Gamgee, 1880; Gaby, 1945). Pyocyanin is a blue colored, redox-active phenazine pigment that serves as a *P. aeruginosa* virulence factor by activating reactive oxygen species (ROS) (O'Malley et al., 2003; Lau et al., 2004). Various sources have cited the cytotoxicity of this compound (O'Malley et al., 2003; Lau et al., 2004; Allen et al., 2005; Reszka et al., 2006). As another unique physiological characteristic, *P. aeruginosa* has the ability to respire under anaerobic conditions using
nitrate as the terminal electron acceptor (Schreiber et al., 2007). Recently, nitrate respiration has been shown to play a role in the potentiation of pathogenicity in the cystic fibrosis lung, which harbors readily accessible and sufficient levels of nitrate for respiration (Palmer et al., 2007; Van Alst et al., 2007).

Figure 1. Several skin ulcerations infected with *Pseudomonas aeruginosa*. The blue-green color is a physical characteristic of *P. aeruginosa* infections caused by the production of pyocyanin (White & Cox, 2006).

The *Pseudomonas aeruginosa* genome is 6.3 million base pairs and contains 5,570 predicted open reading frames (Stover et al., 2000; LaBaer et al., 2004). As one of the largest bacterial genomes, the potential proteome is anticipated to near the complexity of simple eukaryotes such as *Saccharomyces cerevisiae* (Stover et al., 2000; LaBaer et al., 2004). It is speculated that the proteomic depth of this large bacterial genome is what allows *P. aeruginosa* to survive in a variety of environments (Stover et al., 2000). This facultative anaerobe has been shown to inhabit various soil environments, fresh water, marine water, other organisms (i.e. plants, nematodes, moths, mice), people, and human-made products (Glessner et al., 1999; Matar et al., 2005; Wade et al., 2005).

As an infectious microbe, the *P. aeruginosa* genome possesses a plethora of genes devoted to virulence. These virulent effectors will be discussed in more detail below. Specifically, *P. aeruginosa* has demonstrated infectivity in plants, insects, and animals
(Mahajan-Miklos et al., 1999; Jander et al., 2000; Rahme et al., 2000; Gallagher & Manoil, 2001; Choi et al., 2002). Although *P. aeruginosa* can be found in the gut of healthy individuals (6-12 %), it should be noted that this bacterium is not native flora and fauna of the human digestive tract (Shooter, 1971; Griffith et al., 1989; Ohara & Itoh, 2003; Vedantam & Hecht, 2003; Sears, 2005; Rekha et al., 2006). Most significantly, *P. aeruginosa* is an opportunistic pathogen, only infecting those susceptible through compromised immune defenses. Due to natural resistance of *P. aeruginosa* to common antimicrobials and disinfectants, this pathogenic microbe readily gains access to susceptible individuals at medical treatment facilities (Lyczak et al., 2000; Romao et al., 2005). In particular, *P. aeruginosa* has been isolated from hospital staff members, patients, wash areas, water, and virtually all medical equipment (Beatty et al., 2005; Matar et al., 2005).

**B. *Pseudomonas aeruginosa* and Pathology**

It is the combination of tolerance for a diverse array of habitats and the presence of multiple virulence factors that make *P. aeruginosa* an opportunistic pathogen. *P. aeruginosa* is considered an opportunistic pathogen because it is able to cause disease in individuals that are unable to launch a protective immune response against normally non-pathogenic foreign microbes, but is generally a non-threat to healthy individuals. Individuals most readily afflicted by *P. aeruginosa* infections are those who suffer from cystic fibrosis, chronic obstructive pulmonary disorder (COPD), cancers, immune deficiency disorders, and severe burns. These patient populations tend to have
insufficient immune responses against \textit{P. aeruginosa}, propagating the severity of infection.

Infections by \textit{Pseudomonas} generally lead to familiar pathological symptoms and conditions characteristic of other bacterial infections. Some general symptoms of \textit{P. aeruginosa} infection include fever, headache, blue colored purulence, chills, nausea, diarrhea in new borns, and slight pain (Bobo \textit{et al.}, 1973; Ratnam \textit{et al.}, 1986; Baruchin \textit{et al.}, 1996; Lyczak \textit{et al.}, 2000). Infectivity may arise in vulnerable individuals from intravenous drug use, hospital stays after invasive surgery, or from machines that assist in breathing.

As mentioned earlier, \textit{P. aeruginosa} has been shown to inhabit a wide range of habitats, from the clothing and skin of our fellow \textit{homo sapiens} to almost all medical equipment. Due to its high prevalence in hospitals, \textit{P. aeruginosa} is a leading cause of gram-negative nosocomial infections (Weinstein, 1998). Nosocomial infections are those acquired from hospitals. \textit{P. aeruginosa} is the second leading cause of nosocomial pneumonia infections, accounting for 20\% of these infections in hospitals nationwide (Beatty \textit{et al.}, 2005; Matar \textit{et al.}, 2005). \textit{P. aeruginosa} does not only cause nosocomial pneumonia infections, it is capable of infecting all types of tissue, and accounts for about 10-29\% of all nosocomial tissue infections in the United States (Vincent, 2003; Beatty \textit{et al.}, 2005; Matar \textit{et al.}, 2005). The first leading cause of nosocomial infections can be attributed to infection by the gram-positive \textit{Staphylococcus aureus} (Vincent, 2003).

Tissues commonly susceptible to infection by \textit{Pseudomonas aeruginosa} are the heart, blood, bones, joints, central nervous system, eyes, ears, urinary tract, lungs, gastrointestinal tract, and skin (Foca, 2002). \textit{P. aeruginosa} infection affecting the heart
can lead to endocarditis, which usually affects the valves of the heart and can evolve into more serious heart conditions (Venkatesan et al., 2005). *P. aeruginosa* infection of the blood can lead to a condition of bacteria in the blood called bacteremia, which can further progress to septic shock. Bacteremia is especially dangerous to those who acquire a *P. aeruginosa* infection because the blood may spread the infection to other tissues (Mencacci et al., 2006; Lodise et al., 2007). *P. aeruginosa* infections have been found in bones and joints, as well as in prosthetic replacements (Calza et al., 2002; Sauer et al., 2005). Infection in the central nervous system may lead to the development of meningitis as a result of an inflammatory response (Schina et al., 2006; Huang et al., 2007). *P. aeruginosa* infection of the ear can cause swimmer’s ear (Wang et al., 2005).

Immunocompromised patients that rely on respirators or portable oxygen supplies, such as those with COPD, are extremely susceptible to lung infection leading to pneumonia. A Brazilian study of a university hospital showed that *P. aeruginosa* ventilator-acquired pneumonia occurred in 22% of patients on respiratory devices (Guimaraes & Rocco, 2006). As with respirators, hospital patients can also acquire a *Pseudomonas* urinary tract infection from catheters (Jones et al., 2006; Shigemura et al., 2006). *P. aeruginosa* infections can potentiate in the digestive tract by route of percutaneous endoscopic gastrostomy tubes, nasogastric tubes, or nosocomial infections after surgery, such as an appendectomy or implantation of an occluded bile duct stent (Kasatpibal et al., 2005; Segal et al., 2006; Demirbag et al., 2007). *P. aeruginosa* can also cause a skin rash in immunocompetent individuals often referred to as “hot tub folliculitis,” from the most common site of exposure (Yu et al., 2007).
*Pseudomonas* infection can be detected by laboratory cultures taken from the site of infection. These infections are extremely difficult to treat due to *P. aeruginosa’s* general resistance to antibiotics. As a result, extreme standards of sanitation in conjunction with a combination of antibiotics are usually administered to treat *Pseudomonas* infections (Baltch & Smith, 1985).

1. **Cystic Fibrosis**

   Although a very serious threat to all immunocompromised individuals, *P. aeruginosa* pathogenesis has been characterized most thoroughly in cystic fibrosis respiratory infections, severe burn infections, and ocular keratitis. *P. aeruginosa* infections account for greater than 90% of deaths in cystic fibrosis patients (Corech et al., 2005). Cystic fibrosis is a genetic disorder that displays aberrant mucosal secretion in the respiratory tract, pancreas, small intestine, and male reproductive tract (Wine, 1995; Sheppard & Ostedgaard, 1996; Rosenstein & Zeitlin, 1998). Cystic fibrosis results from a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene located on chromosome 7 (Riordan et al., 1989; Stanke et al., 2008). This gene encodes an apical epithelial membrane-embedded chloride channel that is responsible for the transport of chloride, bicarbonate, and glutathione upon cAMP activation (Stanke et al., 2008). Cystic fibrosis patients are immunocompromised because their respiratory tract is unable to provide essential immune responses. Because cystic fibrosis patients must receive more frequent medical attention, they are predisposed to an increased risk of developing a nosocomial pneumonia or respiratory tract infection.
Studies have shown that CFTR mutations affect a cystic fibrosis patient’s ability to clear \textit{P. aeruginosa} infections. For example, an aberrant CFTR mutation, alone, increases inflammatory responses and provides a suitable environment for the establishment of a \textit{P. aeruginosa} infection (Pier \textit{et al.}, 1996; Lyczak \textit{et al.}, 2000; van Heeckeren \textit{et al.}, 2005). The CFTR protein may serve as an epithelial cell immune-receptor for \textit{P. aeruginosa} clearance (Schroeder \textit{et al.}, 2002). Specifically, Pier \textit{et al.} identified the core oligosaccharide of \textit{Pseudomonas aeruginosa} as the ligand for the CFTR protein (Zaidi \textit{et al.}, 1996; Schroeder \textit{et al.}, 2002). When this gene is mutated, as in cystic fibrosis, \textit{P. aeruginosa} is not internalized by the epithelial cells, and may more easily establish colonization (Pier \textit{et al.}, 1996; Pier \textit{et al.}, 1997; Schroeder \textit{et al.}, 2001; Cannon \textit{et al.}, 2003). Once colonization is facilitated, \textit{P. aeruginosa} secretes an exopolysaccharide called alginate, which results in the transition from a non-mucoid to a mucoid growth phenotype (DeVries & Ohman, 1994). This mucoid phenotype, along with the mutant CFTR aberrant exocrine phenotype, interferes with ciliary clearance and the delivery of host defenses (Read \textit{et al.}, 1992; Cowely \textit{et al.}, 1997; Noone \textit{et al.}, 2004). The inability to physically clear the respiratory tract harbors trapped immune cells which eventually lyse and become part of the mucoid matrix (Kirchner \textit{et al.}, 1996). Additional factors, such as flagellum, pili, and O-antigen have been shown influential in the establishment of \textit{P. aeruginosa} infections in cystic fibrosis patients, while multiple excreted proteases have been shown influential in the maintenance of infection in this patient population (Lyczak \textit{et al.}, 2000).
2. Severe Burns

As with cystic fibrosis, severe burn patients often fall victim to the omnipresent existence and multiple virulence factors of *P. aeruginosa*. For severe burn victims, loss of skin tissue means the loss of a principal anatomic barrier of the innate immune system. Although flagella and pili play an important role in facilitation and spread of infection, secreted enzymes such as, exotoxin A, elastases, phospholipase C, type III secretion proteins, and alkaline protease all have been shown to be important in the maintenance of infection by compromising host defenses. In general these proteases have been shown to break down collagen in healing skin epithelium, degrade immunoglobulins, degrade complement proteins, and indirectly affect the ability of the host to recruit immune cells to burn sites (Lyczak *et al.*, 2000). It has been shown that an inherent characteristic of thermal injury is the decrease of polymorphonuclear cell Fc receptors (Jeyapaul *et al.*, 1984). Fc receptors bind immunoglobulins to target a foreign antigen for clearance. Not only is *P. aeruginosa* destroying immunoglobulins at the site of infection, but with the decrease in Fc receptors, immunoglobulin-mediated defenses are greatly diminished. One study showed that administration of host-derived polyclonal antibodies to infection sites restored ability to fight infection in a murine burn model (Felts *et al.*, 1999). It is the inability to deliver host immune defenses to the site of infection that ultimately manifests the scenario of systemic spread and subsequent death in severe burn victims (Ha & Jin, 1999).
3. Contact Lens Wear

*P. aeruginosa* infection of the eye threatens eyesight of even healthy individuals. *P. aeruginosa* infection of the cornea is also referred to as ulcerative keratitis. The cornea is the outer, clear layer of epithelial cells that serves as a barrier between the inner-eye anatomy (pupil, iris, lens, etc.) and the external environment. Because the cornea is the outer-most tissue layer of the eye, it must rely on specific innate immune responses, such as tear production and the blink reflex, in addition to standard immune responses, to combat the readily encountered pathogens from the external environment. The most commonly affected are those that wear contacts. Multiple studies have shown that about 40% of all contact lens-related cases of keratitis are a result of *P. aeruginosa* (Ormerod & Smith, 1986; Yu *et al.*, 2007; Watt & Swrbrick, 2007). Studies have also shown that contact lenses can decrease secreted IgA, mucins, and overall effective tear production, leading to increased susceptibility to bacterial infection (Versura *et al.*, 1987; Cheng *et al.*, 1996; Pearce *et al.*, 1999; Hori *et al.*, 2006; Berry *et al.*, 2008). Upon *P. aeruginosa* inoculation of the eye, virulence factors such as flagella, pili, alkaline protease, exotoxin A, and protease IV have all been shown to establish and maintain infection (Gupta *et al.*, 1994; Gupta *et al.*, 1996; Engel *et al.*, 1998; Fleiszsig *et al.*, 2001; Pillar & Hobden, 2002; Zhang *et al.*, 2003). *P. aeruginosa* LPS has been shown to play a special role in maintenance of infection. As mentioned earlier, the CFTR protein is suspected to act as an immune-receptor of epithelial cells to internalize and diminish establishment of infection. In the cornea epithelial cells, CFTR is also expressed and shown to interact with *P. aeruginosa* LPS core. However, unlike apical epithelial cells of the respiratory tract that can safely be removed from the infection site after *P. aeruginosa*
internalization, corneal epithelial internalization does not yield the same host success.
The corneal epithelium recruits phagocytes and other polymorphonuclear cells to the site
of infection, where these immune cells, in an attempt to clear the infection through
phagocytosis and release of various proteases and reactive oxygen species, cause
devastating damage to the surrounding corneal tissue, compromising vision (Zaidi et al.,
1996; Lyczak et al., 2000). For contact lens users, this corneal cell binding and
internalization can occur as quickly as 24 hours after lens wear (Ladage et al., 2004).

It is the presence of numerous virulence factors that allow *P. aeruginosa* to
exploit and evade weakened immune defenses. The next section will provide a brief
overview of some of the most well characterized virulence factors of *P. aeruginosa*.

### C. Virulence Factors

Although *P. aeruginosa* has access to human hosts through a multitude of
environmental media, *P. aeruginosa* could not survive as an opportunistic pathogen
without the presence of virulence factors to colonize, damage, and evade host defense
responses.

#### 1. Antibiotic Resistance

The most clinically-intimidating virulent characteristic that *P. aeruginosa*
possesses is antibiotic resistance. Specifically, *P. aeruginosa* has been shown to be
resistant to classes of antibiotics such as β-lactams, chloramphenicol, glycopeptides,
quinolones, and tetracyclines (Li et al., 1995; Aeschlimann, 2003; Hocquet et al., 2007).
*P. aeruginosa* has evolved anti-microbial drug resistance through adaptations of the cell membrane and mutations in gene production and function. The outer membrane, along with accompanying LPS layer, produces a barrier of low permeability to many extracellular molecules (Rocchetta *et al.*, 1999; Lambert, 2002). Alginate, a secreted polysaccharide that promotes bacterial colonization, has been shown to bind cationic molecules, conferring antibiotic resistance to cationic aminoglycosides (Lambert, 2002).

Additionally, various membrane-embedded porins play a part in transferring hydrophilic molecules across the lipid bilayers. In particular, loss of the *oprD* gene, which encodes a porin protein used for the acquisition of positively charged amino acids, has been shown to block the uptake of cationic glycopeptide anti-microbials (Lambert, 2002). OprF is the major outer membrane porin of *P. aeruginosa*. Although it possesses a larger channel size than other enteric porins, OprF has been shown to be structurally and functionally similar to the major *E. coli* outer membrane protein, OmpA (Hancock, 1984; Hancock & Brinkman, 2002). Despite the channel size of OprF and elevated prevalence in the outer membrane, it has been shown that less than 1% of these proteins are functional. This decrease in porin activity is believed to be an additional factor that influences the low permeability of *P. aeruginosa*’s outer membrane (Benz & Hancock, 1981; Nicas & Hancock, 1983; Hancock, 1984; Nestorovich *et al.*, 2006). *P. aeruginosa* produces β-lactamase enzymes that degrade β-lactam antibiotics (Rocchetta *et al.*, 1999; Lambert, 2002). *P. aeruginosa* also possesses four membrane-embedded drug-efflux pump complexes, MexA-MexB-OprM, MexC-MexD-OprJ, MexE-MexF-OprN, and MexX-MexY-OprM, which have been shown to be randomly overexpressed in up to 75% of mutant clinical isolate strains (Aeschlimann, 2003). The latest findings suggest
genetic mutations that increase the resistance of *P. aeruginosa* to anti-microbials, such as over-production of drug-efflux pumps, do not appear to compromise overall fitness of the organism (Hocquet *et al.*, 2007).

2. Cell Behavior

*Quorum Sensing*

Quorum sensing allows neighboring cells to communicate through the secretion of species-specific signals and other chemotactic extracellular signals. A bacterial cell, such as *Pseudomonas*, can produce and excrete a homoserine lactone-based autoinducer. As cell density increases, the intracellular levels of this autoinducer build up. At a specific threshold concentration, the autoinducer activates a transcriptional activator protein, which activates thousands of synchronized genes in the cells of a growing bacterial colony. In *P. aeruginosa*, two cell-to-cell signaling pathways work together to produce virulence factors and expand colonization. These two cell-to-cell signaling pathways are (1) the *las* pathway and (2) the *rhl* pathway. These quorum sensing systems lead to the spread of colonization, biofilm formation, and overall synchronization of global gene regulation (Van Delden & Iglewski, 1998; Whiteley *et al.*, 1999; Schuster *et al.*, 2003).

*Biofilm Formation*

When *P. aeruginosa* forms a biofilm, the cells are in an altered growth state where they are imbedded in an exopolysaccharide-based matrix, mainly composed of alginate, DNA, and immune cell debris, with synchronized gene expression, loss of LPS
O-antigen, and no motility (Rocchetta et al., 1999; Parsek & Singh, 2003; Allesem-Holm et al., 2006; Bjarnsholt & Givskov, 2007). A defining characteristic of biofilms, as a virulence factor, is the expression of membrane protein lectin B (LecB) (Tielker et al., 2005). *P. aeruginosa* uses LecB to bind to host cells, such as lung epithelial cells. One extracellular signaling molecule that is directly involved in biofilm formation, N-3-oxododecanoyl homoserine lactone (OdDHL), has been shown to possess the ability to disrupt T-cell proliferation, leading to the inhibition of T-cell differentiation and cytokine secretion (Tateda et al., 2003; Ritchie et al., 2005).

The formation of a biofilm offers *P. aeruginosa* resistance to antibiotics and host defenses (Allesen-Holm et al., 2006; Garcia-Medina et al., 2005; Sarkisova et al., 2005). In general, it has been shown that early contact with the respiratory epithelium allows *P. aeruginosa* to form biofilm-like colonization, which may contribute to prolonged or chronic infections through the physical retardation of host clearing mechanisms as well as the many secreted signals that interact with the respiratory epithelium on a molecular and cellular level (Garcia-Medina et al., 2005; Yeh et al., 2005).

### 3. Secreted Proteins

**Exotoxin A**

Exotoxin A (ETA) is a secreted cytotoxin that enters host cytoplasm via receptor-mediated endocytosis and targets protein synthesis (Alcorn et al., 2004; Yates et al., 2005). Specifically, ETA blocks protein synthesis at the translational level by ADP ribosylating translational elongation factors. However, recent studies have shown that the ability of ETA to halt protein synthesis in a host cell is not the defining function of
ETA targeted cell destruction (Jenkins et al., 2004). Specifically, Jenkins et al. showed that exotoxin A functions in human mast cell death by inducing apoptosis via caspase cascade pathways and decreasing expression of anti-apoptotic proteins (Jenkins et al., 2004).

**Elastase**

*P. aeruginosa* also secretes a number of proteases such as elastase, protease IV, and alkaline protease that cleave proteins necessary for host survival (Alcorn et al., 2004). Two prominent elastase proteins are LasA and LasB. These proteins are regulated by the Las and Rhl quorum-sensing pathways, interact with the type III secretion system by regulating exo-enzyme expression, and play a role in host epithelial cell invasion (Cowell et al., 2003). Alcorn et al. have shown that elastase from *P. aeruginosa* has the ability to degrade pulmonary surfactant D, impairing immune responses, in cystic fibrosis patients (Alcorn et al., 2004). Since cystic fibrosis patients express low levels of this protein as is, elastase serves as a key virulence feature of *P. aeruginosa* in these patients (Beatty et al., 2005).

**Protease IV**

Protease IV cleaves at C-terminal regions of lysine-containing target proteins (Engel et al., 1998). In particular protease IV has been shown capable of cleaving some immunologically significant proteins such as complement components, immunoglobulins, plasmin, plasminogen, fibrinogen, surfactant protein A, surfactant protein B, and surfactant protein D (Engel et al., 1998; Caballero et al., 2001; Malloy et
al., 2005). Not only does protease IV enhance *P. aeruginosa* virulence during lung infection with the degradation of surfactant proteins, it has also been shown to enhance virulence in *P. aeruginosa* keratitis by degradation of corneal and intrastromal ocular tissues (O’Callaghan et al., 1996; Malloy et al., 2005). Additionally, in corneal infection, protease IV has shown to be immunoevasive (Thibodeaux et al., 2005).

**Alkaline Protease**

Alkaline protease is an exo-protease that exerts its most virulent effects in *P. aeruginosa* keratitis, and often in conjunction with protease IV and elastase (Howe & Igluewski, 1984; O’Callaghan et al., 1996; Bandara et al., 2006). In a mouse corneal infection model, alkaline protease secretion was shown to be necessary for initiation and maintenance of infection (Howe & Igluewski, 1984). Alkaline protease, along with elastase, has been shown to play a role in the invasive infiltration into ocular tissues (Bandara et al., 2006). To a greater extent than elastase, an alkaline protease possessing additional N-terminal leucine-lysine residues has been shown to target key proinflammatory cytokines, IL-6 and IL-8, thereby providing *P. aeruginosa* with another immunoevasive virulence mechanism (Matheson et al., 2006).

**Phospholipase C**

Phospholipase C (PLC) is an excreted *P. aeruginosa* virulence enzyme that generally targets the cleavage of phosphate groups from phospholipid molecules. *P. aeruginosa* secretes PLC in several pathogenically favorable forms, hemolytic phospholipase C (PlcH), non-hemolytic phospholipase C (PlcN), and the bioinformatics-
discovered phospholipase C (PlcB) (Dubouix et al., 2004; Barker et al., 2004; Okino & Ito, 2007). PlcH targets phosphatidylcholine (PC), lyso-PC, and sphingomyelin (Okino & Ito, 2007). PlcN targets PC and phosphatidylserine (PS) (Okino & Ito, 2007). PlcB targets PC and phosphatidylethanolamine (PE) (Barker et al., 2004; Okino & Ito, 2007). Hemolytic phospholipase C has the potential to exert its degradative effects on a variety of host tissues (Lisa et al., 1994; Wieland et al., 2002). Specifically, PlcH has been shown to increase vascular and systemic damage in P. aeruginosa infected mice (Wieland et al., 2002). In vivo mouse studies have shown PlcH to be responsible for vascular epithelial necrosis, peritoneal epithelial necrosis, and to target the liver, kidneys, and spleen (Berk et al., 1987; Meyers et al., 1992). In vitro studies suggest that PlcH is cytotoxic to human leukocytes (Meyers et al., 1992). Non-hemolytic phospholipase C has not been shown to have any virulent effects on host tissue (Wieland et al., 2002). PlcB is used by P. aeruginosa as a chemotactic sensor for twitching motility towards PC and PE (Barker et al., 2004).

**Type III Secretion**

The basic structures of the type III secretion system consists of a basal, flagellum-like membrane-embedded complex of proteins and a secretory pilus-like needle made of a single polymerized protein monomer, which is collectively referred to as an injectisome complex (Cornelis, 2006; Quinaud et al., 2007). The type III secretion system allows P. aeruginosa to inject cytotoxic proteins, exotoxin S, exotoxin T, exotoxin U, and exotoxin Y, directly into host cytoplasm. Collectively, these toxins inactivate immune cells, damage host tissue, and overall, impair immune responses (Galan & Collmer, 1999;
Epelman et al., 2004; Ader et al., 2005; Corech et al., 2005; Rietsch et al., 2005; Vance et al., 2005; Cowell et al., 2005; Cuzick et al., 2006; Shafikhani et al., 2008; Soong et al., 2008). ExoS is a multi-functional enzyme that possesses a N-terminal Rho GTPase activating (RhoGAP) domain, and an ADP ribosyl transferase (ADPRT) domain, and region that interacts with Cbl-b ubiquitin ligase of host cells (Soong et al., 2008). ExoS has been shown to inhibit phagocytosis (Galan & Collmer, 1999), have significant cytotoxic effects on epithelial tissue (Soong et al., 2008), and to regulate type III secretion activation upon encountering host tissue (Cisz et al., 2008). ExoT is a bifunctional enzyme that also possesses an N-terminal RhoGAP domain and a C-terminal ADPRT domain (Shafikhani & Engel, 2006). ExoT has been shown to be cytotoxic to epithelial tissue (Galan & Collmer, 1999; Shafikhani et al., 2008) and target epithelial healing by disrupting cytokinesis through its ADPRT domain (Shafikhani & Engel, 2006). ExoU is a phospholipase that has been shown to cause cytotoxicity and induce pro-inflammatory host reactions leading to tissue infiltration and colonization (Galan & Collmer, 1999; Cuzick et al., 2006; Shafikhani & Engel, 2006). ExoY is an adenylate cyclase that has been shown to distort the actin cytoskeleton of host cells through its adenylate cyclase activity, expanding colonization (Cowell et al., 2005; Shafikhani & Engel, 2006). ExoS and ExoT have also been shown to disrupt actin cytoskeletal structure and infiltrate host tissue through the use of their RhoGAP domains (Cowell et al., 2005). A more recent study demonstrates that ExoS, T, and Y can disrupt host epithelium by interactions with cytoskeletal tight junctions, actin binding proteins, and actin (Cowell et al., 2005; Soong et al., 2008). Another recent study has demonstrated that cystic fibrosis lung infections typically possess P. aeruginosa strains that tend to
express ExoS, while blood samples from individuals with *P. aeruginosa* bacteremia tend to express ExoU (Wareham & Curtis, 2007).

4. Non-Proteinaceous Secreted Factors

_Alginate_

In addition to exolipid compounds, *P. aeruginosa* also secretes an extracellular polysaccharide called alginate. The virulence of alginate is functionally characterized by its role in the lung tissue of cystic fibrosis patients, where it is alters the *P. aeruginosa* phenotype from a non-mucoid to a mucoid colonial environment. The release of alginate turns the respiratory tract into a mucoid colonized environment, which leads to promotion of further colonization and biofilm formation. As neutrophils are attracted and accumulate at the site of infection, their own elastase, along with various excreted *Pseudomonas* cytotoxins, lead to a synergistic scenario of lysed host tissue, which in turn, contributes to the composition of the mucoid biofilm matrix (Amitani _et al._, 1991; Allen _et al._, 2005). Interestingly, hydrogen peroxide release by neutrophils has been shown to increase transition of *P. aeruginosa* to a mucoid phenotype (Mathee _et al._, 1999).

Alginate and associated biofilm matrix, in conjunction with the characteristic CFTR gene aberration of cystic fibrosis, responsible for excess mucus build up in the respiratory tract, manifests a fatal scenario of insufficient clearance, respiratory failure, and subsequent asphyxiation (Cystic Fibrosis Foundation). A recent study, utilizing a mouse model, suggests that alginate secretion increases host pro-inflammatory responses, leading to tissue damage and subsequent inability of the host to clear *P. aeruginosa* from the sight of infection (Song _et al._, 2003; Remminghorst & Rehm, 2006). Although
alginate, alone, does not appear to cause tissue damage (Abusriwil & Stockely, 2007), it
has been shown to interfere with polymorphonuclear cell chemotaxis and subsequent
clearing mechanisms (Cabral et al., 1987; Krieg et al., 1988; Jensen et al., 1990;
Pedersen et al., 1990; Leid et al., 2005).

**Rhamnolipids**

During colonization *P. aeruginosa* secretes two hemolytic biosurfactants called
rhamnolipids (monorhamnolipid and dirhamnolipid) that have been shown to disrupt the
function and facilitate cellular infiltration of human respiratory epithelial cells as a
virulent physiological process (McClure & Schiller, 1992; Pamp and Tolker-Nielsen,
2007; Zullianello et al., 2006). Although both rhamnolipids appear to have cytolytic
capabilities, dirhamnolipid has been shown to be the more virulent of the two.
Specifically, dirhamnolipid has been shown to induce more cellular membrane disruption
and lysis of human monocyte-derived macrophages (McClure & Schiller, 1992). It has
been suggested that this disruption and lysis occurs as a result of rhamnolipid
intercalation between phosphatidylcholine molecules of the membrane’s lipid bilayer
(Ortiz et al., 2006).

**Pyocyanin**

As mentioned earlier, pyocyanin serves as a virulence factor by activating harmful
reactive oxygen species (O'Malley et al., 2003). Reactive oxygen species are oxygen
ions or oxygen-containing molecules whose oxygen atom has lost an electron. With the
loss of an electron, this oxygen species becomes highly reactive with neighboring atoms
in an attempt to gain back that lost electron to form a complete electron-shell configuration. Reactive oxygen species cause damage when they interact with the atoms of macromolecules, such as nucleic acids, lipids, and proteins, of host origin, disrupting their structures and functions. Pyocyanin has been shown to induce tissue damage by necrosis in mice with P. aeruginosa lung infections (Lau et al., 2004). Additionally, more recent evidence demonstrates the ability of pyocyanin to impair acute inflammatory responses by activating neutrophil apoptosis (Allen et al., 2005).

**Hydrogen Cyanide**

*P. aeruginosa* uses a cyanide synthase enzyme to convert glycine to CO₂ and hydrogen cyanide (HCN) under low oxygen and high cell densities approaching stationary phase (Castric, 1975; Castric, 1977; Castric et al., 1979; Castric, 1983; Castric, 1994). Induction of the cyanide synthase operon (*hcnABC*) has been shown to be under regulation by an anaerobic regulator of arginine deiminase and nitrate reductase (ANR), and the two quorum sensing pathways, *las* and *rhl* (Pessi & Haas, 2000). HCN is an extremely cytotoxic compound because it readily ionizes, freeing cyanide, which directly inhibits the function of cytochrome c oxidase (Jones et al., 1984; Jensen et al., 1984). Aerobic cellular respiration relies on this enzyme to transfer electrons to oxygen in the last step of the electron transport chain, producing energy in the form of ATP. In 2001, Gallagher and Manoil demonstrated the lethal effects of hydrogen cyanide production by *P. aeruginosa* in *C. elegans* (Gallagher & Manoil, 2001). Specifically, they showed that a hydrogen cyanide synthase (*hcnC*) null strain was unable to cause lethal infection (Gallagher & Manoil, 2001). Most recently, the virulent effects of hydrogen cyanide
production has been assessed in humans. In 2008, Ryall et al. examined *P. aeruginosa* infected lung tissue of cystic fibrosis and non-cystic fibrosis bronchiectasis patients and were able to show cyanide ion accumulation and subsequent decrease in lung function (Ryall et al., 2008).

5. Surface Structures

*Flagella*

*P. aeruginosa* has a number of pathogenic features that allow motility. One physiological attribute of *P. aeruginosa* that allows it to inhabit such diverse environments is the flagellum (Figure 2). The presence of a flagellum allows swimming motility (Mattick, 2002; Arora et al., 2006).

![Microscopy image of *Pseudomonas aeruginosa* pilus and flagellum structures](Image provided by Erica Jewell).
Flagella are propeller-like organelles made of polymerized flagellin monomer units that extend indiscriminately from the cell surface. A molecular motor is responsible for the kinetic propulsion of the flagellum, which results in the swimming motion (Bardy et al., 2003; Doyle et al., 2004). In addition to swimming motility, flagella also provide motility by swarming. Swarming motility is a synchronized form of motility utilized by \textit{P. aeruginosa} on semisolid medium. Swarming appears to require a battery of specific conditions such as the presence of flagella, type IV pili, rhamnolipid production, certain amino acids, and a nitrogen source (Köhler et al., 2000; Caiazza et al., 2005). These locomotive attributes are valuable to the microorganism for purposes of chemotaxis, nutrition, pathogenesis, and survival (Mahenthiralingam & Speert, 1995; Feldman et al., 1998; Soscia et al., 2007).

In addition to swimming and swarming motility, flagella serve \textit{P. aeruginosa} as a bacterial adhesin. Specifically, flagellin has been shown to have binding affinity for Muc1, a mucin found on respiratory epithelial cells (Arora et al., 1998; Lillehoj et al., 2002). Although flagellin’s ability to bind mucins may play a leading role in colony establishment, \textit{P. aeruginosa} flagella have also been shown to be a physiological requirement for internalization by host phagocytic cells (Mahenthiralingam & Speert, 1995). In 2006, Tseng et al. published a study that demonstrated the ability of \textit{P. aeruginosa} flagellin to induce a host innate immune response. Flagellin was shown to induce the expression of NF-\textit{kappa}B leading to an inflammatory response in human tracheal epithelium cells (Tseng et al., 2006). A previous study of respiratory infection has indicated that flagella activate host inflammatory responses through epithelial cell toll-like receptors, TLR2 and TLR5 (Adamo et al., 2004). Most recently, in a mouse
respiratory model, flagella interaction with TRL5 has been shown to be sufficient for induction of innate defenses (Ramphal et al., 2008).

**LPS and Pili**

LPS and pili are two important virulence factors that pertain specifically to the current research. Because of their fundamental application to my thesis project, they have each been given their own sections and discussed in depth below.

**D. LPS**

1. LPS Structure

Lipopolysaccharide is the primary surface structure of gram-negative bacteria. The LPS layer is made up of lipid A, a core oligosaccharide, and in some instances, such as *P. aeruginosa*, an O-antigen repeating unit (Figure 3). Lipid A serves to anchor the

![Figure 3. Illustration of the basic structure of LPS.](image)
LPS complex to the outer cell membrane, while the O-antigen repeating unit is attached to the core oligosaccharide following assembly on an undecaprenol-phosphate molecule (Marolda et al., 2004; Nilsson et al., 2006). The lipid A structure is fairly conserved across gram negative bacteria (Figure 4) (de Kievit & Lam, 1994). In general, lipid A is synthesized in the cytoplasm through a series of anabolic steps, of which, primarily involve the addition of phospholipids to uridine diphosphate (UDP)-N-acetylglucosamine (GlcNAc). Lipid A is then covalently linked to two 2-keto-3-deoxyoctonate (Kdo) molecules and transported to the outer membrane. The Kdo molecules serve to covalently link lipid A to the core oligosaccharide on the cell surface (Raetz & Whitfield, 2002).

Figure 4. Predicted molecular structure of *P. aeruginosa* 1244 LPS. Illustration generated by ChemDraw software (Courtesy of Dr. Joseph Horzempa).
The core oligosaccharide is broken into lipid A-proximal and lipid A-distal divisions, referred to as the inner and outer core, respectively (Figure 4). The portion that forms a covalent linkage to Kdo-lipid A is referred to as the inner core and typically consists of Kdo, conserved L-glycero-D-manno-heptose residues, and phosphate moieties (de Kievit & Lam, 1994; Masoud et al., 1995; Raetz & Whitfield, 2002). The portion of the core that covalently binds the O-antigen repeating unit is referred to as the outer core and typically consists of D-glucose, 2-amino-2-deoxy-D-galactose, L-rhamnose, and L-alanine (Figure 4) (de Kievit & Lam, 1994; Masoud et al., 1995). In particular, strains of Pseudomonas can be identified by the immunological properties of LPS O-antigens. Although other serotyping systems exist, P. aeruginosa strains are most commonly identified by the International Antigen Typing System (IATS). The IATS is a serotyping system that is based on anti-sera agglutination of heat-stable P. aeruginosa O-antigens (Liu et al., 1983). The LPS of P. aeruginosa is made up of two bands of O-antigens, the A-band and the B-band (Rivera et al., 1988; Rivera & McGroarty, 1989). The A-band contains shorter, more conserved chains composed of the neutral D-rhamnan sugar, while the B-band consists of the diverse O-antigen repeating structures identified by the IATS serotyping system (Lightfoot & Lam, 1991; de Kievit & Lam, 1994; Rocchetta et al., 1999; Raetz & Whitfield, 2002). In P. aeruginosa 1244, it appears that the B-band O-polysaccharides are derived from central metabolic pathways such as the pentose phosphate cycle and glycolysis.
2. O-Antigen Biosynthesis

The general steps of O-antigen subunit biosynthesis are known for *P. aeruginosa* 1244 (Figure 5). The serotype O7 gene cluster was structurally elucidated by Raymond *et al.* in a 2004 publication identifying multiple O-antigen loci in *P. aeruginosa*.

(Raymond *et al.*, 2002). The gene product of *wbpM* serves as a bifunctional UDP-GlcNAc C6 dehydratase/C4 reductase. This reductase mediates the formation of UDP-\(\beta\)-D-\(N\)-acetyl-D-quinosamine (QuiNAc) which is the nucleotide precursor of the first O-antigen sugar residue, UDP-\(\beta\)-D-\(N\)-acetyl\(\beta\)-fucosamine (FucNac). UDP-QuiNAc is derived from...
UDP-GlcNAc, which most likely exists as a downstream metabolite of fructose-6-phosphate from the pentose phosphate pathway (Kneidinger et al., 2003; Druzhinina et al., 2005). The gene product of wbpL encodes a bifunctional glycosyltransferase that subsequently transfers the FucNAc sugar residue from the UDP-FucNAc nucleotide precursor to an undecaprenol-phosphate molecule in the inner cell membrane (DiGiandomenico et al., 2002). As mentioned earlier, DiGiandomenico et al. showed that PA1244 wbpM and wbpL mutants lacked the ability to produce the LPS O-antigen repeating unit. The remaining two sugar residues (Xylose and Pseudaminic acid) are added onto the inner membrane embedded undecaprenol-phosphate (Und-P) facing the cytoplasm. UDP-xylose is a derivative of UDP-glucuronic acid (GlcUA), which is a known metabolite of glycolysis (Harper & Bar-Peled, 2002; Seifert, 2004; Hung et al., 2007). Interestingly, UDP-GlcUA, along with mannuronic acid, serve as key components in the production of alginate (Hung et al., 2007). As UDP-FucNAc, UDP-5NβOHC₄₇NPse is also a derivative of UDP-GlcNAc thought to have derived from fructose-6-phosphate of the pentose phosphate cycle (Goon et al., 2003). Each sugar residue is sequentially transferred to undecaprenol-phosphate as a linked nucleotide UDP precursor. Once all three sugar residues are added to undecaprenol-phosphate (now undecaprenol-pyrophosphate+FucNAc+Xyl+5NβOHC₄₇NPse), a Wzx flipase enzyme is required to position the Und-PP/O-antigen complex in the outer leaflet of the inner-cell membrane, facing the periplasm. A study by Marolda et al. published in 2004, demonstrated that Wzx flipase activity, in Salmonella, occurs through recognition of the primary Und-PP sugar residue. Once the Und-PP/O-antigen complex has been flipped to
the outer leaflet of the inner membrane by Wzx, the repeating unit enters one of two pathways discussed below.

3. O-Antigen Polymerization

With regards to the formation and addition of the LPS O-antigen repeating unit to the core oligosaccharide, the gene products required at each step have been well characterized (Figure 6). For the LPS, once the O-antigen is flipped to the periplasm by Wzx flipase, Wzy is responsible for O-antigen polymerization (Marolda et al., 2004). Once polymerized by Wzy, Wzz regulates the final O-antigen chain length. Once the proper repeating unit is synthesized, WaaL functions as a ligase to link the O-antigen repeat unit to the core oligosaccharide (Abeyrathne et al., 2005).

The mechanism by which LPS is translocated from the site of assembly (inner membrane) to its final destination (surface of the outer membrane) is less understood in P. aeruginosa. The most knowledge about this process has been obtained from studies in E. coli (Tefsen et al., 2005; Sperandeo et al., 2007; Ruiz et al., 2008). In E. coli, it has been suggested that an ABC transporter, LptBFG, uses energy to remove LPS from the inner membrane en route to the outer membrane (Sperandeo et al., 2007; Ruiz et al., 2008). However, the question still remains as to how LPS transverses the periplasmic space to contact the outer membrane. Two possible scenarios have been proposed. One
scenario suggests that a periplasmic chaperone, LptA in *E. coli*, transports LPS to the outer membrane (Sperandeo *et al.*, 2007; Ruiz *et al.*, 2008). The second scenario suggests that LPS reaches the outer membrane through direct inner membrane-outer membrane contact sites, possibly mediated by a protein intermediate (Tefsen *et al.*, 2005). Once in contact with the outer membrane, proteins LptD and LptE are believed to finalize the orientation the LPS at the surface of the outer membrane (Bos *et al.*, 2004; Wu *et al.*, 2006; Ruiz *et al.*, 2008).
4. LPS Virulence

As introduced earlier, one of the major virulence factors of *P. aeruginosa* is the outer membrane bound LPS layer. This virulence is attributed to LPS inhibition of host cell communication and the ability to adhere to various surfaces, such as lung tissue (Yeh *et al.*, 2005; Pier, 2007). Recent research on LPS virulence demonstrates that LPS of *P. aeruginosa* serotype 10 decreases the expression of nasal epithelial cell signaling protein, connexin 43, which suggests LPS may play a role in diminishing host cell signaling and the promotion of *P. aeruginosa* colonization (Yeh *et al.*, 2005). LPS core has also been shown to be a potential ligand for the epithelial-expressed CFTR protein, which as mentioned above, has a paradoxical immune effect dependent on the target organ (Zaidi *et al.*, 1996; Lyczak *et al.*, 2000). Adhesive properties of LPS contribute, along with flagella and pili, to *P. aeruginosa* colony establishment. In addition to LPS binding to the CFTR protein, it has also been shown to have affinity for gangliotetraosylceramide, a glycolipid expressed by corneal cells (Gupta *et al.*, 1994). LPS, along with another virulence factor, exotoxin A, has been shown to increase the production of interferon regulatory factor 1 (IRF-1), which mediates an inflammatory response, in pulmonary infections (Weiland *et al.*, 2002). This has significance to cystic fibrosis patients, which often have been shown to have decreased IRF-1 production (Weiland *et al.*, 2002). A recent study utilizing a mouse respiratory model demonstrated that an LPS-induced innate immune response was primarily mediated through TLR4 (Ramphal *et al.*, 2008).

Recent evidence suggests that optimization of the virulent potential of *P. aeruginosa* is regulated, in part, by the lengthening of the O-antigen repeating unit chain (Kintz *et al.*, 2008). Complete loss of the O-antigen has been shown to increase
susceptibility to clearance by host serum (Rocchetta et al., 1999; Priebe et al., 2004).
Interestingly, as mentioned earlier, once biofilm colonization has been established, *P. aeruginosa* is found to have reduced or lost LPS O-antigen expression, promoting evasion of innate immune responses (Rocchetta et al., 1999).

E. Type IV Pili

1. Pilin

In general, type IV pili (Tfp) have been identified in a variety of gram positive and gram negative bacteria. Type IV pili are bundled, rod-like extensions located at one or both ends of the cell that are composed of helically arranged pilin subunits (Touhami et al., 2006). Generally, these strong, yet flexible, filaments can reach 3-4 µm in length and have a diameter of 6 nm (Mattick et al., 1996; Maier et al., 2002; Craig et al., 2003). Type IV pili can be subdivided into two groups, TfpA and TfpB. These two subgroups are mainly based on signal peptide length and mature peptide length (Craig & Li, 2008; Pelicic, 2008). TfpA pilins have leader peptide sequences typically 10 amino acids or less, and a mature peptide sequence of about 150-160 amino acids (Pelicic, 2008). TfpB pilins typically have peptide leader sequences between 15-30 amino acids and mature protein sequences at an extreme maximum of 180-200 amino acids or extreme minimum of 40-50 amino acids (Pelicic, 2008). Both subgroups of Tfp have the same general structural features consisting of a homologous hydrophobic N-terminal α-helical tail and
a globular head containing an anti-parallel β-sheet arrangement and a conserved C-terminal disulfide loop region (Figure 7) (Craig & Li, 2008; Pelicic, 2008).

Figure 7. Homology model of the tertiary structure of PA1244 PilA protein.

The α-helical tail and the disulfide loop region play important roles in pilus functionality. The N-proximal 25-30 residues of Tfp are highly conserved across various bacterial species (Castric & Deal, 1994; Craig et al., 2003; Pelicic, 2008). Structural analysis of pilin subunits from Neisseria gonorrhoeae, N. meningitidis, P. aeruginosa, and Vibrio cholerae have demonstrated that N-terminal α-helix interactions mediate pilus assembly and resulting hydrophobicity of the filament core (Parge et al., 1995; Craig et al., 2003; Helaine et al., 2007; Li et al., 2008). Although there is sequence dissimilarity within the disulfide loop regions of pilin groups, this region has been shown to function
P. aeruginosa possess TfpA which can be further subdivided into five groups, Groups I-V (Castric & Deal, 1994; Kus et al., 2004). These five groups are based on varying primary sequence characteristics, antigenicity, and the presence or absence of defined or undefined accessory proteins (Castric & Deal, 1994; Kus et al., 2004). The current research works with Groups I, II, and III. PA1244 possesses Group I Tfp, while the most common lab strains, such as PAK, PA103, and PAO1, possess Group II Tfp. Group I pilins tend to be more conserved, have a higher GC content, have a larger gene sequence, and possess the pilO accessory protein gene in between the pilA gene and the tRNA\textsuperscript{Thr} sequence in the operon (Castric & Deal, 1994; Kus et al., 2004). Group II pilins have shorter gene sequences than Groups I and III, and do not contain an additional open reading frame between the pilA and tRNA\textsuperscript{Thr} sequences (Castric, 1995; Kus et al., 2004). Group III pilins have longer gene sequences than either, Group I or Group II, pilins (Kus et al., 2004). Group III pilins also possess the open reading frame of an accessory protein of unknown function, TfpY, between the pilA and tRNA\textsuperscript{Thr} sequences (Kus et al., 2004). Figure 8, below, provides a basic illustration of the pilin biosynthetic operon of Groups I, II, and III.
2. Pilin Polymerization

The current model for pilin polymerization is based on studies conducted on enteropathogenic *E. coli* (EPEC), *N. gonorrhoeae*, and *Myxococcus xanthus* pilus formation (Craig *et al.*, 2006; Craig & Li, 2007; Jakovljevic *et al.*, 2008; Pelicic, 2008). This model requires the presence of a cytoplasmic membrane associated protein (BfpD in EPEC, PilF in *N. gonorrhoeae*, PilB in *P. aeruginosa* and *M. xanthus*), an inner membrane protein (BfpE in EPEC, possibly PilG in *N. gonorrhoeae*, and possibly PilC in *P. aeruginosa*), and mature pilin (*Figure 9*) (Burrows, 2005; Craig *et al.*, 2006; Craig & Li, 2007; Jakovljevic *et al.*, 2008; Pelicic, 2008). The Sec machinery is responsible for
prepilin translocation to the inner cell membrane. Once in the cytoplasmic membrane, the PilD endopeptidase is responsible for the cleavage of prepilin’s basic leader sequence (~6-7 amino acids) and N-methylation of the resulting membrane bound pilin subunit, producing mature pilin (Strom & Lory, 1992). Mature pilin can then be pooled in the membrane and recruited for polymerization. Recruitment to the site of polymerization may partially result from free pilin interactions with the hydrophobic tails of recently polymerized pilins (Craig & Li, 2007). The pilus has been shown to be composed of 3 strands of pilin subunits arranged in a helix. At the site of polymerization, pilin subunits are added one at a time to each strand.

The motor ATPase, PilB, is thought to interact with an inner membrane protein, potentially PilC, or a complex of unidentified proteins to form a scaffold for subsequent polymerization. Once a pilin molecule docks this protein scaffold via attraction to pilin already added to the pilus, hydrolysis of ATP to ADP by PilB causes the inner membrane protein to change conformation. This conformational change acts as a molecular piston to punch the pilus upward into the periplasm about 10 Å, leaving room for a new pilin molecule to be recruited (Craig et al., 2006; Craig & Li, 2007; Jakovljevic et al., 2008; Pelicic, 2008). PilB exchanges ADP for ATP causing the inner membrane protein to shift back to its resting conformation, starting the polymerization process all over again (Burrows, 2005; Craig et al., 2006; Craig & Li, 2007; Jakovljevic et al., 2008; Pelicic, 2008). As pilin monomers are added, the pilus bundle is extruded into the extracellular space through the pore forming, PilQ, protein in the outer cell membrane (Figure 9) (Martinez et al., 1998). Although, the role of PilC as the inner membrane protein
providing piston function has not been demonstrated, PilC has been shown to interact with PilB through a cytoplasmic domain at the membrane periphery and is required for PilB polar localization in *P. aeruginosa* (Burrows, 2005).

Craig *et al.* has suggested the reversal of this model for pilin depolymerization, by switching from PilB polymerizing ATPase to PilT depolymerizing ATPase through interaction with the inner membrane protein (Craig *et al.*, 2006; Craig & Li, 2007).
Although PilC has also been suggested to interact with PilT at the cytosolic membrane periphery, no evidence exists to demonstrate the ability of PilC to function as a macromolecular piston (Burrows 2005). PilU has been shown to support retraction by an unknown mechanism, but has also been shown to be a cytosolic membrane associated ATPase, ruling out its role as an inner membrane protein or periplasmic effector (Graupner et al., 2001; Chiang et al., 2005; Chaing et al., 2008). In *P. aeruginosa*, 3 ATPase hexamers (PilB, PilT, PilU) have been shown to play a role in pilin polymerization/depolymerization (Burrows, 2005; Chiang et al., 2008). Studies in *P. aeruginosa*, *P. stutzeri*, *N. meningitidis*, and *M. xanthus* have demonstrated these hexamers to be cytosolic membrane associated proteins (Pujol et al., 1999; Chiang et al., 2005; Jakovljevic et al., 2008). Chaing et al. pointed out that *P. aeruginosa* PilB, PilT, and PilU do not have known transmembrane domains or periplasmic signal sequences (Chiang et al., 2005), supporting this current “motor-piston” model’s need for one or more proteins to transverse the membrane into the periplasmic space and transfer the chemical energy from ATP hydrolysis into mechanical energy for pilus assembly/disassembly.

Taking into account the uncertain identity of ATPase associated effector proteins, an alternate model has been proposed by studies of PilT and PilB in *M. xanthus*. In this model, pilin molecules assemble into a pilus spontaneously, whereby PilB and PilT serve as direct molecular motors or regulators of pilin polymerization/depolymerization (Jakovljevic et al., 2008). In either model, it is clear that cytosolic ATP is required for PilB and PilT to mediate pilus assembly/disassembly (Jakovljevic et al., 2008).
3. Pili Virulence

As opposed to the flagellum of *P. aeruginosa*, which mediates swimming motility, the presence of type IV pili allows twitching motility (Mattick, 2002; Arora et al., 2006). Figure 2, above, illustrates pilus and flagellum structures of *P. aeruginosa*. The motion generated by twitching motility results from depolymerization-polymerization of pilin subunits (Touhami et al., 2006). Type IV pili also serve additional functions for *P. aeruginosa*, such as adherence to moist, solid surfaces and colony formation (Mattick, 2002; Kim et al., 2006). As an adhesin, pili has been shown to bind to abiotic surfaces such as stainless steel, plastics, glass, and various minerals, as well as biotic surfaces such as human epithelial cells, red blood cells, other bacteria, fungi, and yeast (Woods et al., 1980; Gupta et al., 1994; Mattick, 2002; Giltner et al., 2006; Touhami et al., 2006). It has been estimated that pilus-mediated adhesion accounts for about 90% of the attachment capabilities of *P. aeruginosa* (Hahn, 1997).

Recent findings suggest that the glycosylation of pili and flagellum is an optimal virulence enhancing attribute of *P. aeruginosa* pathogenicity (Kus et al., 2004; Smedley et al., 2005; Arora et al., 2006). In particular, Smedley et al. found that the glycosylated type IV pili are more commonly found than non-glycosylated pili in clinical isolates (about 67% in sputum samples), while Kus et al. has shown that glycosylated pili were most common among clinical isolates obtained from cystic fibrosis patients (about 67%) (Kus et al., 2004; Smedley et al., 2005). Specifically, Smedley et al. were able to demonstrate increased virulence of glycosylated pili by showing that a strain producing glycosylated pilin, PA1244, significantly outcompeted a non-glycosylated pilin.
producing strain, 1244G7, for survival in a mouse respiratory model (Smedley et al., 2005).

The ability of virulence factors to evade an acquired (memory) immune response is what dictates the severity of pathogenicity. However, recent evidence demonstrates that the hypervariable regions of \textit{N. gonorrhoeae} type IV pili serve as antigenic epitopes for recognition by host memory eliciting T lymphocytes (Hansen et al., 2007; Plant & Jonsson, 2006). This information, along with recent findings on pilin glycosylation in \textit{P. aeruginosa} 1244 (Horzempa et al., 2008), holds the potential for microbiological vaccine production.

\section*{F. Glycosylation of \textit{Pseudomonas aeruginosa} 1244 Pilin}

\subsection*{1. Characterization of Pilin Glycan}

\textit{P. aeruginosa} 1244 belongs to the IATS serotype O7. As mentioned earlier, IATS is a method of \textit{P. aeruginosa} strain identification based on specified anti-sera agglutination of heat-stable, surface O-antigens (Liu et al., 1983). The O7 LPS O-antigen is characterized by the presence of three sugar residues, 5NβOH\textsubscript{4}7NFmPse, Xyl, and FucNAc. In 2001, Castric \textit{et al.} published results that characterized the PA1244 pilin glycan (Figure 10).
Castric et al. showed that the pilin aminoglycan \((\alpha-5\beta\text{OHC}_4\text{NFmPse-(2} \rightarrow 4\text{-}\beta\text{-Xyl-(1} \rightarrow 3\text{-}\beta\text{-FucNAc-(1} \rightarrow 3\text{-}\beta\text{-Ser})}\) was covalently attached to a serine residue and possessed the same sugar residues as the LPS O-antigen \((5\beta\text{OHC}_4\text{NFmPse, Xyl, FucNAc})\) (Castric et al., 2001). In 2002, Comer et al. demonstrated that the C-terminal 148 serine residue of a pilin monomer serves as the sole attachment site for the O-antigen repeating unit (Comer et al., 2002). In a separate study published by the Castric lab in 2002, DiGiandomenico et al. showed that PA1244 deletion mutants of \(wbpM\), responsible for synthesis of the first sugar (FucNAc) of the O-antigen, and \(wbpL\), responsible for the transfer of the first sugar to an undecaprenol phosphate carrier, were not able to produce the LPS O-antigen repeating unit, nor glycosylated pilin. These findings demonstrate that the pilin glycan and the LPS O-antigen are produced by the same biosynthetic pathway (DiGiandomenico et al., 2002). The glycan/O-antigen repeating unit is preassembled on an undecaprenol phosphate carrier lipid within the cytoplasm, transported to the inner cell membrane, and then transferred to pilin or LPS core within the periplasm. This pathway is demonstrated in Figure 11.
2. Glycosylation Pathway

For pilin glycosylation, once Wzx has flipped the O-antigen to the periplasmic space, prepilin must also be present in the inner cell membrane (Figure 11). Once the prepilin subunit is targeted to the inner cell membrane via the Sec machinery it undergoes posttranslational modification by cleavage of its basic leader sequence and N-methylation by PilD (Strom & Lory, 1992). PilO is responsible for the addition of the O-antigen glycan to pilin in the periplasm (Smedley et al., 2005; Qutyan et al., 2007). PilO transfers the O-antigen repeating unit from Und-P to pilin through the recognition of the O-antigen’s primary sugar moiety, β-D-FucNAc, similar to Wzx flipase recognition of the O-antigen-Und-P complex (Marolda et al., 2004; Horzempa et al., 2006b).

In pilus formation, a PilBC complex located in the inner cell membrane is responsible for polymerization of the pilin subunits, while PilT depolymerizes pilin
subunits. PilQ forms a pore in the outer membrane whereby polymerized pilin subunits are channeled out of the cell forming a pilus (Figure 9) (Martinez et al., 1998).

As mentioned earlier, the pilin subunits that form the pilus are arranged helically by interactions between the alpha-helix tails and the globular head groups of the monomers. This arrangement shields functionally conserved regions of the PilA sequence, while exposing post-translational modifications (such as glycosylation) and hypervariable regions (Craig et al., 2006). Surface exposure of hypervariable regions and modifications, such as glycosylation, have been shown to play an important role in P. aeruginosa pathogenesis and immunogenicity (Smedley et al., 2005; Hansen et al., 2007).

3. Characterization of PilO

As mentioned earlier, P. aeruginosa 1244 possesses group I pilin (Castric & Deal, 1994). In 1995, Castric identified the open reading frame of an accessory protein between the pilA and tRNA\textsuperscript{thr} gene sequences in the group I operon of P. aeruginosa 1244, which was named pilO and shown to be required for PA1244 pilin glycosylation (Castric, 1995). Specifically, Castric showed that recombinant pilA, overexpressed in a pilAO mutant, PA1244N3, was not able to produce pilin that could be recognized by a sugar specific reagent (Castric, 1995). Later studies have further supported the necessity of pilO in the glycosylation of pilin. In 2002, DiGiandomenico et al. showed that a strain of P. aeruginosa that does not contain the pilO ORF, PA103, could produce heterologously expressed PA1244 pilin glycosylated with native O-antigen repeating unit (serotype O11) upon introduction of recombinant pilO (DiGiandomenico et al., 2002). In 2005, Smedley et al. constructed a true pilO delete strain, PA1244G7, which produced
endogenous pilin that could not be recognized with a sugar-specific antibody (Smedley et al., 2005). Collectively, these findings suggest that PilO is the only protein necessary for the covalent linkage of the O-antigen repeating unit to pilin.

PilO has a predicted molecular weight of 51,000 kDa that has recently been shown to localize to the inner cell membrane (Qutyan et al., 2007). As a member of the Wzy_C family, PilO shares similarity in size, number of predicted transmembrane regions, and position of the Wzy_C domain with two other proteins of the Wzy_C family, Wzy and WaaL, responsible for the transfer of O-antigen repeating units from an undecaprenol-phosphate carrier molecule to a substrate in the periplasmic space (Bateman et al., 2004; Qutyan et al., 2007). Through a series of single amino acid deletions/alterations, Qutyan et al. was able to demonstrate that the catalytic regions of PilO lie within the Wzy_C domain and C-proximal 430-461 residues. Qutyan et al. then went on to show PilO topology at the inner cell membrane, through a series of PilO fusions to cytoplasmic LacZ and periplasmic PhoA, which identified the periplasmic location of the catalytic regions (Figure 12) (Qutyan et al., 2007).
Figure 12. Topology of PilO. Qutyan et al. determined the catalytic regions to be located in the periplasm, within the Wzy_C domain (consensus residues 281-301) and the C-proximal hydrophilic region (residues 430-461). The white circles and black diamonds are amino acid residues. Figure from Qutyan et al., 2007.

4. Specificity of the Glycosylation Reaction

Once pilin is embedded in the cytoplasmic membrane, covalent glycan linkage to the C-terminal 148 serine residue may occur through the catalytic domain of PilO within the periplasmic space. As mentioned, the pilin glycan and the O-antigen repeating unit consist of the same three saccharides from the same biosynthetic pathway (DiGiandomenico et al., 2002). *P. aeruginosa* 1244 strains containing deletions in either *wbpM* or *whpL* resulted in the absence of both, glycosylated pilin and O-antigen polymerization (DiGiandomenico et al., 2002). These findings also suggest that the O-
antigen repeating unit and pilin glycan are preassembled on, and subsequently transferred from, an undecaprenol phosphate carrier molecule.

DiGiandomenico et al. demonstrated the low glycan substrate specificity of the glycosylation reaction by separately expressing the O-antigen biosynthesis gene clusters of PA103 and E. coli O157 in PA1244 and recovering pilin glycosylated with the heterologous glycans (DiGiandomenico et al., 2002). Horzempa et al. further addressed the issue of low glycan specificity in a 2006 publication in which a strain of PA103 that was deficient in the addition of the second sugar (Xyl) of the trisaccharide O-antigen repeating unit, in the presence of PA1244 pilAO, produced pilin glycosylated with only the first sugar (FucNAc) of the glycan (Horzempa et al., 2006b). These results in combination with comparative structural similarities of several reducing end O-antigen saccharides of various P. aeruginosa strains indicated that recognition of the glycan substrate lies solely in the reducing end moiety (Horzempa et al., 2006b).

In 2001, Castric et al. identified a serine residue as the site of glycan linkage to pilin (Castric et al., 2001). In 2002, Comer et al. showed that only the 148 serine residue of pilin accepted a glycan (Comer et al., 2002). Further examination by Horzempa et al. in 2006 suggested that a terminal serine residue, in combination with a compatible surface charge, is the sole pilin specific recognition structure for PilO-mediated O-linkage of a glycan (Horzempa et al., 2006a). To arrive at this conclusion, Horzempa et al. tested the glycosylation status of a series of mutant PA1244 and PA103 pilins. Site-directed mutagenesis of DSL cysteine residues to alanines permitted glycosylation of PA1244 pilin. Deletions of several segments of the DSL permitted glycosylation of PA1244 pilin. Insertion of 5 residues with an overall positive charge into the negatively
charged DSL of PA103 pilin with a C-terminal serine permitted glycosylation in a pilAO null strain, PA1244.47, upon complementation with pilO. Extension of a C-terminal serine away from the electronegative DSL of PA103 pilin with a tail of alanine residues permitted glycosylation in PA1244.47 upon complementation with pilO (Horzempa et al., 2006a).

G. Market Potential of PA Vaccination

*P. aeruginosa* is a lethal pathogen to immunocompromised individuals and patients suffering from chronic respiratory disease or injuries. Commonly affected are those suffering from Cystic Fibrosis, Chronic Obstructive Pulmonary Disease (COPD), cancers, immunodeficiency disorders, AIDS, and severe burns. Conjugate vaccine technology holds the potential to provide preventative treatment against *Pseudomonas* infections. A conjugate vaccine covalently links a memory eliciting antigen to a weaker or non-memory inducing antigen. Typically, as in the case of the *P. aeruginosa* vaccine, a non-memory inducing surface glycan (sugar of LPS) is linked to a memory inducing protein (pilin or toxoid), thereby mediating a memory response to the LPS surface glycan-coat of a gram-negative bacterium. Dr. Peter Castric of Duquesne University has designed a *P. aeruginosa* glycoconjugate vaccine, which enables conjugate vaccination antigens to be manufactured *in vivo*. In other words, the pathogen itself becomes a biological production factory of the vaccine. Dr. Castric’s technology takes advantage of *P. aeruginosa*’s physiological mechanism of providing glycosylated pilin to produce covalently linked virulence factors that can function as vaccine components.
Dr. Castric’s *P. aeruginosa* biconjugate vaccine provides immunization against *P. aeruginosa* by stimulating a T-dependent response, in turn, leading to a memory response against the LPS O-antigen surface layer of this gram-negative bacterium. This extremely antigenic LPS O-antigen layer is readily recognized by the immune system, yet is recognized by a T-independent response. This means no memory. When your body establishes memory to a pathogen, it stores up immune information that will allow your body to provide you with long term protection against that pathogen. The *P. aeruginosa* vaccine technology is permissive of immune memory by covalently linking the LPS O-antigen to the pilin protein. This is the basic idea behind all current glycoconjugate vaccines. The pilin protein appears ideal for glycoconjugation because it has previously been shown to elicit a memory response (Ohama *et al.*, 2006), which suggests the ability to confer this memory on the covalently linked glycan subunit (Lesinski & Westerink, 2001). In 2008, Horzempa *et al.* showed that pure glycosylated PA1244 pilin provided protection against a PA1244 LPS-producing mutant that lacked pilus formation in mouse pneumonia and burn models (Horzempa *et al.*, 2008). Although this study only qualifies as preliminary animal work, glycoconjugate vaccines have been shown to provide long-term protection in humans. In 2005, Robbins *et al.* demonstrated their *H. influenzae* type b capsular polysaccharide-tetanus toxin conjugate vaccine provided protection after 9 years following initial administration (Claesson *et al.*, 2005). Other systems have used LPS O-antigens linked to various bacterial proteins such as toxoids (i.e. inactive forms of bacterial toxins from diphtheria or tetanus) (Crowcroft, 1999). Dr. Castric is currently using his own technology to explore the application of vaccination design against other pathogenic gram-negative bacteria, such as *E. coli* O157, the cause of travelers’ diarrhea.
and Jack-in-the-Box disease. In the case of *E. coli* O157, the technology uses *P. aeruginosa* to covalently attach the glycan of O157 to *P. aeruginosa* pilin.

*P. aeruginosa* is a leading cause of lung infections in Cystic Fibrosis patients and nosocomial pneumonia infections. With respect to these pathological threats, the commercial opportunity of the *P. aeruginosa* biconjugate vaccine has modest to globally-intense potential. Cystic Fibrosis is a genetic disorder manifested from a mutation in the CFTR gene. The CFTR gene encodes the CFTR protein, that when defective, effects cellular transport of charged particles resulting in improper mucus production. This mucus is not easily cleared from the body, which allows it to build up and cause serious health problems. Cystic Fibrosis is estimated to effect over 30,000 people in the United States (1:31 Americans) (Wertz *et al.*, 1991; Cystic Fibrosis Foundation, 2009). Moreover, greater than 90% of deaths in Cystic Fibrosis patients result from *P. aeruginosa* infections (Corech *et al.*, 2005).

Additionally, *P. aeruginosa* is one of the four most common gram-negative pathogens responsible for nosocomial infections according to the CDC (Weinstein, 1998). *P. aeruginosa*’s ubiquitous inhabitance and increased resistance to metallo-β-lactamase antibiotics has made *P. aeruginosa* a grave threat to those with compromised immune systems (Zavascki *et al.*, 2006). Statistically, it has been reported that *P. aeruginosa* is responsible for 10% of nosocomial tissue infections and 20% of nosocomial pneumonia infections (Beatty *et al.*, 2005; Matar *et al.*, 2005). Although the acquisition of reliable statistics on the incidence of mortality from *P. aeruginosa* infection was unattainable due to reliability issues, the reported lethality in immunocompromised individuals is extraordinarily high (Obritsch *et al.*, 2005;
\textit{Pseudomonas aeruginosa} Infections, 2006). One study suggested a mortality rate of 67% in patients with multi-drug resistant infections (Obritsch \textit{et al.}, 2005). The \textit{P. aeruginosa} biconjugate vaccination has the potential to be a clinical precautionary necessity for individuals with Cystic Fibrosis, respiratory diseases, immune system disorders, cancer patients undergoing chemotherapy or bone marrow transplants, and burn victims.

Although competing, non-conjugate \textit{P. aeruginosa} vaccinations exist, utilizing outer membrane proteins and flagella, they are still in clinical trials (Baumann \textit{et al.}, 2007; Doring \textit{et al.}, 2007). As for a technology-specific competitor, there are currently no other biconjugate vaccines on the market for \textit{P. aeruginosa}. The Swiss Serum and Vaccine Institute, along with Berna Biotech Ltd. of Switzerland are currently working on multivalent conjugate \textit{P. aeruginosa} vaccines for children with Cystic Fibrosis (Cryz \textit{et al.}, 1997; Zuercher \textit{et al.}, 2006). These technologies use LPS O-antigen linked to toxin A. Djarid \textit{et al.} at Tarbiat Medares University in Iran are also working on a biconjugate \textit{P. aeruginosa} vaccine utilizing an alginate-tetanus conjugate (Kashef \textit{et al.}, 2006). Szu \textit{et al.} at Carolinas Medical Center in North Carolina will soon be entering a phase III clinical trial for a biconjugate vaccine of O157 O-antigen linked to \textit{P. aeruginosa} exotoxin A (Ahmed \textit{et al.}, 2006). These vaccinations use chemical cross-linking techniques to link the two molecules. The 4 major problems associated with chemically cross-linking these vaccine components are; (1) it is expensive, (2) time-consuming, (3) requires growing up large quantities of harmful pathogens, and (4) cross-linking stability is unpredictable (Jones, 2005; Horzempa \textit{et al.}, 2008).

Despite this, multi-conjugate vaccines have reached the market. Sanofi Pasteur, GSK, and Merck market conjugate vaccinations for tetanus, \textit{Haemophilus influenzae} type
b (Hib), diphtheria, meningococcal, and pneumococcal (CDC, Vaccination Price List, 2006). This demonstrates that there is a market opportunity and well established corporations are already involved. However, the vaccines currently on the market are produced by chemical cross-linking. Dr. Castric’s technology eliminates the problems associated with chemical cross-linking. Despite this, the integrity of adequate testability during clinical trials of the current vaccine technology presents itself as a potential challenge. Although preliminary data of the current \textit{P. aeruginosa} vaccine holds promise, competing conjugate technologies progressing through clinical trials have encountered several complications (Döring & Pier, 2008; Horzempa \textit{et al.}, 2008; Johansen & Gøtzsche, 2008). First, increased sanitation and optimized antibiotic therapies may mask the true effectiveness of the vaccine (Döring & Pier, 2008). Second, there is difficulty in acquiring a patient population large enough to produce statistically relevant data during clinical trials (Döring & Pier, 2008). Third, basic research still needs to be done to further uncover the specifics of host immune responses to \textit{P. aeruginosa} (Johansen & Gøtzsche, 2008). However, with the continuous evolution of antibiotic resistant \textit{P. aeruginosa} strains, vaccination is still a very appealing treatment option for the numerous patient populations at risk of infection by this opportunistic microbe. Because multiple groups are exploring \textit{P. aeruginosa} conjugate vaccine development at the preliminary stage, undoubtedly, the breadth of basic immunological knowledge regarding \textit{P. aeruginosa} infection will continue to expand. Additionally, Dr. Castric’s vaccine technology harbors its significance in the potential to easily manufacture vaccine components to any number of pathogenic entities.
At this time Dr. Castric possesses three patents on the *P. aeruginosa* biconjugate vaccine. The first is “Conjugate Vaccine Against Gram-Negative Bacterial Infections,” Patent #: US 6,872,398 B2, the second, “Compositions and Methods for Eliciting an Immune Response to Gram-Negative Bacterial Infections,” Patent #: US 7,132,101 B2 and the third, “Compositions and Methods for Eliciting an Immune Response to Gram-Negative Bacterial Infections,” Patent #: US 7,135,175 B2. These patents cover the methods to produce and use the *P. aeruginosa* vaccine technology. With these patents, the methods and technology can be licensed.

The *P. aeruginosa* conjugate vaccine offers benefits to the market place. Successful immunization against *P. aeruginosa* can have positive impacts on the health of patients and the finances of the health care provider. For patients, the number of hospital visits should decline due to immunity against *P. aeruginosa* infection. For example, if all 30,000 Cystic Fibrosis patients are immunized, this saves a potential 30,000 initial visits to the doctor’s office or hospital as a result of a *P. aeruginosa* infection. The cost and amount of antibiotic treatment should decline because of the need to use multiple antibiotics to treat multi-antibiotic resistant *P. aeruginosa* (Zavascki et al., 2006). Also, this decline in antibiotics use will decrease the progress of *P. aeruginosa* becoming resistant to more antibiotics. The length of hospital stays should decline due to a decrease of *P. aeruginosa* related nosocomial infections, resulting from a decrease in antibiotics resistance. The average cost of hospitalizations due to multi-drug resistant infection was found to be about $54,000 per patient (Obritsch et al., 2005). Multidrug resistant *P. aeruginosa*, statistically, has been shown to prolong hospital stays up to six days, which accumulates an average $7,300 in extra care costs. If these data are
applied to the 30,000 cystic fibrosis patients that inhabit the US, health care providers could save $219 million with this patient population alone (Obritsch et al., 2005).

In 1995, it was estimated that nosocomial infections resulted in 88,000 deaths and cost over $4.5 billion (Weinstein, 1998). As stated earlier, *P. aeruginosa* infection accounts for roughly 30% of nosocomial infections in the United States. Additionally, a study done in India estimated that about 25% of nosocomial infections were caused by *P. aeruginosa* (Agarwal et al., 2006). If a quarter of nosocomial infections are cause by *P. aeruginosa*, the *P. aeruginosa* vaccine will save about $1 billion in expenses. As of 2006, the cost of community-acquired pneumonia, which can result from *P. aeruginosa* infection, was estimated to be about $9.7 billion annually (Harrison’s practice, 2006). If a quarter of community-acquired infections are caused by *P. aeruginosa*, the *P. aeruginosa* vaccine could save about $2 billion dollars in expenses in this area. For health care providers, the elimination of hospital-acquired and community-acquired *P. aeruginosa* infections saves roughly $3 billion in health care costs annually.

The conjugate vaccinations on the market range from $8.00-$120.00 per dose (CDC, Vaccination Price List, 2006). For the projected revenues below I used an average value of $64 per dose. If all Cystic Fibrosis patients were vaccinated, the projected gross revenue on this vaccination is $1,920,000. The use of the vaccines as a preventive measure for those anticipating chemotherapy/immunosuppressant drugs, dependence on mechanical ventilation, and for burn victims, these earnings can be expected to increase. As of December 2006, there were 94,554 patients in the U.S. awaiting organ transplantation (OPTN, 2006). If all of these patients are vaccinated the revenue would increase by $6,051,456. The number of individuals suffering from COPD is estimated to
be about 12 million in the United States and 600 million worldwide (COPD Foundation, 2009). These patients are at a high risk of developing respirator-acquired *P. aeruginosa* infections. In 1990, greater than 50% of COPD patients acquired a respiratory or pulmonary *P. aeruginosa* infection (Yu *et al.*, 1990). This number is expected to have increased due to drug-resistant *Pseudomonas* strains. Immunization of these patients as a preventative measure would boost the projected revenue to $768,000,000 for the U.S. market and $38.4 billion when considering the global market. The number of burn victims each year that require hospitalization is estimated to be about 20,000 (CDC, Mass Casualties Fact sheet; Burns, 2006). Immunization of these patients is projected to be an additional $1,280,000 in revenue.

If the breadth of this vaccination could be expanded to cover other gram-negative pathogens, the return would be even greater. For example, the CDC estimates that there is over 73,000 *E. coli O157* cases reported annually in the United States (CDC, *Escherichia coli O157:H7*, 2008). If vaccine production can be extended to include this pathogen, a vaccination against *E. coli O157* should bring in an additional projected $4,672,000 per year.

Additionally, the CDC also estimates that 90,000 people a year acquire nosocomial infections (Infection Control Related Sentinel Events, 2003). Making the assumptions that 30,000 of these infections are acquired by Cystic Fibrosis patients (90,000 – 30, 000CF = 60,000) and that the vaccine can be extended to other gram-negative bacterial pathogens, this adds an additional projected revenue of $3,840,000 per year. The sum of these projections could provide a total projected revenue of $785,763,456. These estimated returns are based on U. S. incidence alone, not
worldwide. With global incidence of infection caused by gram-negative pathogens the total revenue is over $38.5 billion.

Although it has been shown that key aspects of the technology are applicable to other gram-negative bacteria, the ability of expanding vaccine production to other pathological conditions exists as a challenge of future application. It has yet to be shown to be applicable to gram-positive bacteria, cancers, viruses, or any other non-bacterial protein. Also, Dr. Castric’s vaccine technology may be used to produce biconjugate animal vaccines. For example, *E. coli* O157 and *Salmonella* are constant concerns for cattle and poultry farmers, as well as the consumer. Ability to produce animal-vaccinations also expands this technologies potential market.

Overall, the biconjugate *P. aeruginosa* vaccination appears to be a modest marketing opportunity in the U.S. However, the current global vaccine market is estimated at about $9 billion. At this time, the earnings in the vaccine market are just as successful as the market for conventional drugs (Marx, 2005). In the U.S., it is estimated that 30,000 patients suffer from Cystic Fibrosis, and globally, 600 million patients are estimated to suffer from COPD. If only 10% of this combined patient population receives the *P. aeruginosa* vaccination, Dr. Castric’s technology could still potentially capture over $3.84 billion of the world vaccine market. For a company licensing this technology, this projection is sufficient to support the cost of clinical trials. Globally, the marketing opportunity of this vaccine technology has the potential to be remarkably large.
H. Significance of Current Research----Thesis Goals

The main objective of my thesis research was to study substrate specificity in protein glycosylation reactions. Specifically, the minimal glycosylation requirements will be assessed using a structurally different pilin, a pilin fusion protein, and a bacterial alkaline phosphatase protein in *P. aeruginosa*. The current work is an extension of Horzempa *et al.*’s study on glycosylation substrate specificity of pilin in *P. aeruginosa* strain 1244 (Horzempa *et al.*, 2006a). Overall, Horzempa *et al.* showed that the minimal requirement for glycosylation is a C-terminal serine residue and an electropositive surface charge (Horzempa *et al.*, 2006a). In this study, Horzempa *et al.* were able to glycosylate the normally non-glycosylated PA103 pilin in *P. aeruginosa* by extending a C-terminal serine residue away from the electronegative surface with a “tail” of alanines (Horzempa *et al.*, 2006a). The current research utilized this strategy to assess the compatibility of several distinct mutant proteins in the *P. aeruginosa* glycosylation reaction.

In 2002, DiGiandomenico *et al.* demonstrated the flexibility of the pilin glycosylation reaction by showing the O-antigen repeating unit of *E. coli* O157 could be used to glycosylated PA1244 pilin *in vivo*, and also that PA1244 *pilAO* could glycosylate pilin with native repeating units in other *P. aeruginosa* serotypes (DiGiandomenico *et al.*, 2002). In 2006, Horzempa *et al.* was able to show that this flexibility occurs as a result of the PilO protein’s recognition of the reducing end glycan residue, which possess a similar structure across gram negative O-antigen subunits (Horzempa *et al.*, 2006b).

Collectively, these findings suggest applications to *in vivo* generation of vaccine components. The LPS of gram negative bacteria is the predominant immunogenic
surface structure (Pier, 2007). The saccharide residues that make up the LPS are able to elicit a T-independent immune response, but no immunogenic memory is established (Mond et al., 1995). However, a T-dependent immune response is achieved when LPS saccharides are covalently conjugated to a protein (Avery & Goebel, 1931; Mond et al., 1995; Lesinski & Westerink, 2001; Wourimaa et al., 2001). This memory eliciting response generated towards both components, the saccharide and protein, is the working principle behind glycoconjugate vaccine production. Glycoconjugate vaccinations are currently marketed, however, their production is expensive and time consuming compared to the proposed technology (Jones, 2005; Horzempa et al., 2008). Dr. Castric’s glycoconjugate vaccine technology utilizes P. aeruginosa to covalently link a single O-antigen repeating unit to a pilin molecule via the native glycosylation mechanism, thereby producing multi-valent vaccine components in vivo. Pilin is a prime protein for glycoconjugation because it has previously been shown to elicit a memory response (Ohama et al., 2006), which suggests the ability to confer this memory on the covalently linked saccharide.

In 2008, Horzempa et al. confirmed this notion, by showing that pure glycosylated PA1244 pilin provided protection against a PA1244 LPS-producing mutant that lacked pilus formation in mouse pneumonia and burn models (Horzempa et al., 2008). This study suggests mechanisms by which the vaccine eliminates bacterial infection. A memory response could result in the immune system targeting pili and the O-antigen. Targeting pili would eliminate adhesion and twitching motility, eliminating colonization establishment and expansion. Targeting the O-antigen would target the bacteria for clearance by opsonic phagocytosis.
Fundamentally, the current study may be able to provide further information on pilin glycosylation specificity. Since PA103 pilin was able to be engineered for glycosylation, it is hypothesized that all of the proposed proteins in this study can similarly be engineered for glycosylation. If any number of these mutagenized proteins are able to be glycosylated, these findings would hold practical applications for the research and design of multi-valent vaccine production. Further identification of substrate specificity of the glycosylation reaction in *P. aeruginosa* could potentially allow a variety of bacterial or pathogenic protein epitopes to be covalently conjugated to highly compatible immunogenic LPS O-antigen repeating units, producing memory eliciting antigenic entities to multiple pathogens by a readily inducible microbial physiological mechanism. Successful manipulation of this glycosylation system could expand the breadth of pathogenic targets while maintaining cheap and easily manufactured multi-valent vaccine components. The current research examines the ability to glycosylate proteins other than PA1244 pilin, in *P. aeruginosa*.

**Specific Aims:**

1. Can a structurally different, Group III, pilin be engineered to accept the O-antigen repeating unit of lipopolysaccharide?

2. Can a protein fused to pilin be engineered to accept the O-antigen repeating unit of lipopolysaccharide?

3. Can a non-pilin protein be engineered to accept the O-antigen repeating unit of lipopolysaccharide?
II. MATERIALS AND METHODS

A. Media and Bacterial Strains

All bacterial strains and plasmids used in the study can be found in Table 1. Strains were aerobically grown in Luria Bertani (LB) (0.5% yeast extract, 1% bacto-tryptone, 1% sodium chloride) broth cultures, on LB agar plates, or on CAYE (0.75% casamino acids, 0.15% yeast extract, 2% agar [Silipigni-Fusco, 1987]) agar pans (68 cm X 28 cm X 3 cm). All strains aerobically grown in LB broth cultures were done so at 37°C and 250 rpm. All strains grown on LB agar plates or CAYE agar pans were done so at 37°C. Antibiotic concentrations for *E. coli* were kanamycin at 35-50 µl/ml, ampicillin at 50-100 µg/ml, and tetracycline at 15 µg/ml. Antibiotic concentrations for *P. aeruginosa* were carbenicillin at 250 µg/ml and tetracycline at 50-100 µg/ml. IPTG (Isopropyl-β-D-Thiogalactopyranoside) was added to the media of all strains to induce expression of recombinant genes under control of the *tac* promoter in pMMB66EH, the *lacI* promoter in pRMCD28, and the *trc* promoter in pHK734 at a concentration of 0.5 mM for *E. coli* and 5mM for *P. aeruginosa*.

Table 1. Bacterial Strains and Plasmids

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> Top10*</td>
<td>F- mcrA ∆(mrr-hsdRMS-mcrBC) ∆(araleu) 7697 galU galK rpsL (StrR) endA1 nupG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>E. coli</em> XL-Gold*</td>
<td>endA1 supE44 thi-1 recA1 gryA96 relA1 lacHte</td>
<td>Stratagene</td>
</tr>
<tr>
<td><em>E. coli</em> HB101</td>
<td>supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</td>
<td>Bolivar &amp; Backman, 1979</td>
</tr>
<tr>
<td>PA1244</td>
<td>Wild type, Serotype O7</td>
<td>Ramphal et al., 1984</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------------------------------------------------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>PA1244-N3</td>
<td>Interruption of sigma factor rpoN with Tc cassette, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Ramphal et al., 1991</td>
</tr>
<tr>
<td>PA103</td>
<td>Wild type, Serotype O11</td>
<td>Liu, 1973</td>
</tr>
<tr>
<td>PA103 wzy&lt;sub&gt;PaO11::aacC1&lt;/sub&gt;</td>
<td>PA103 wzy&lt;sub&gt;PaO11::aacC1&lt;/sub&gt;, Interruption of wzy gene with a Gm cassette (aacC1) Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Dean et al., 1999</td>
</tr>
<tr>
<td>PA683</td>
<td>Wild type, Non-Typable Serotype</td>
<td>Seyboldt Thesis, 1996</td>
</tr>
<tr>
<td>pUCP26</td>
<td>4,977bp broad host-range cloning vector, pUC18-derived, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>West et al., 1994</td>
</tr>
<tr>
<td>pUCP26&lt;pilO&gt;</td>
<td>pUCP26 with &lt;pilO&gt; under continuous expression from a tac promoter</td>
<td>Horzempa et al., 2006a</td>
</tr>
<tr>
<td>pMMB66EH</td>
<td>8, 807bp broad host-range expression vector, IPTG inducible tac promoter, Amp&lt;sup&gt;R/Cb&lt;/sup&gt;R</td>
<td>Fürste et al., 1986</td>
</tr>
<tr>
<td>p683&lt;pilA&gt;</td>
<td>pMMB66EH with PA683 &lt;pilA&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>p683&lt;pilA&gt;-tfpY</td>
<td>pMMB66EH with PA683 &lt;pilA&gt;-tfpY</td>
<td>This study</td>
</tr>
<tr>
<td>p683aas</td>
<td>pMMB66EH with mutated PA683 &lt;pilA&gt; that had an ala-ala-ser-stop extension added to the C-terminal arginine residue</td>
<td>This study</td>
</tr>
<tr>
<td>p683pep</td>
<td>pMMB66EH with mutated PA683 &lt;pilA&gt; that had the C-terminal 15 amino acids of PA1244 &lt;pilA&gt;, with the C-proximal cysteine changed to an alanine and addition of a stop codon (TAWKPNYAPANAPKS-stop) to the C-terminal arginine residue</td>
<td>This study</td>
</tr>
<tr>
<td>pRMCD28</td>
<td>PhoA fusion vector, PhoA starts at the 27&lt;sup&gt;th&lt;/sup&gt; amino acid encoding codon, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Daniels et al., 1998</td>
</tr>
<tr>
<td>pPilAPhoA</td>
<td>pRMCD28 with PA1244 pilA-E. coli phoA fusion, KAYRYRR linker, phoA is missing the N-terminal amino acids 1-27</td>
<td>This study</td>
</tr>
<tr>
<td>pPilAP</td>
<td>pMMB66EH with PA1244 pilA-E. coli phoA fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pPilAPaas</td>
<td>pMMB66EH with mutated PA1244 pilA-E. coli phoA fusion that had an ala-ala-ser-stop extension added to the C-terminal lysine residue</td>
<td>This study</td>
</tr>
<tr>
<td>pHK734</td>
<td>E. coli phoA gene under the trc promoter of pTrc99a, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Gift from Jon Beckwith</td>
</tr>
<tr>
<td>pECAP</td>
<td>pMMB66EH with E. coli phoA from pHK734</td>
<td>This study</td>
</tr>
<tr>
<td>pECAPaas</td>
<td>pMMB66EH with mutated E. coli phoA that had an ala-ala-ser-stop extension added to the C-terminal lysine residue</td>
<td>This study</td>
</tr>
</tbody>
</table>
B. Homology Modeling

Homology models were generated using the PHYRE software online (http://www.sbg.bio.ic.ac.uk/phyre/index.cgi). Peptide sequences of PA1244 pilin, PA683 pilin, and bacterial alkaline phosphate were submitted to the PHYRE program. The tertiary homology structural output was analyzed using DS Viewer Pro 6.0 for surface exposed C-terminal residues and surface charge.

C. Plasmid Construction

All primers used in this study can be found in Table 2. All constructs were confirmed with one or a combination of restriction enzyme digestion, PCR screen, or sequencing.

Table 2. Primer List

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter into pilA</td>
<td>GCC CTT TCA CTA GCT TGC TC</td>
<td>Amplify PA683 pilA from genome</td>
</tr>
<tr>
<td>683PilA-F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer G</td>
<td>CAG ATG ATT GCC AGC AAT</td>
<td>Amplify PA683 pilA from genome</td>
</tr>
<tr>
<td>683PilA-R1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xmal-PilA-F</td>
<td>CAT TAG CCC GGG GCC CTT TCA CTA GCT TGC TC</td>
<td>PCR amplify PA683 pilA(-AAS, -Tail) adding Xmal site</td>
</tr>
<tr>
<td>HindIII-PilA-R1</td>
<td>CAT TAG AAG CTT CAG ATG ATT GCC AGC AAT</td>
<td>PCR amplify PA683 pilA adding HindIII site</td>
</tr>
<tr>
<td>HindIII-TfpY-R1</td>
<td>CAT TAG AAG CTT TAC GAA TGA GCT GCT CTA CC</td>
<td>PCR amplify PA683 pilA-tfpY adding HindIII site</td>
</tr>
<tr>
<td>HindIII-683PilA-AAS-R</td>
<td>CAT TAG AAG CTT TTA GCT AGC AGC GCG GCA TTC GCT CGG AGC</td>
<td>PCR amplify PA683 pilA adding C-term - AAS codons and HindIII site</td>
</tr>
<tr>
<td>HindIII-683PilA-Tail-R</td>
<td>CAT TAG AAG CTT TTA GGA TTT CGG GGC ATT AGC CGG AGC GTA GTT GGG CTT CCA AGC TGT GCG GCA TTC GCT CGG AGC</td>
<td>PCR amplify PA683 pilA adding C-term altered PA1244 pilA peptide tail and HindIII site</td>
</tr>
<tr>
<td>683PilA-Cterm</td>
<td>TCG GAA TCC AAT GCG GTT TC</td>
<td>For sequencing, 100bp upstream of 683 pilA C-term</td>
</tr>
<tr>
<td>683PilA-Cterm</td>
<td>TCG GAA TCC AAT GCG GTT TC</td>
<td>For sequencing, 100bp upstream of 683 pilA C-term</td>
</tr>
</tbody>
</table>
### 1. Construction of p683pilA

To make the p683pilA construct, PA683 pilA was amplified from PA683 genomic DNA using PCR and primers, 683PilA-F and 683PilA-R1. Thermocycler conditions for this PCR reaction consisted of a 105°C lid temperature, initial incubation at 94°C for 7 minutes, followed by 30 cycles of 94°C denature for 1 minute, 50°C annealing for 1 minute, and 72°C extension for 2 minutes. The final extension was 72°C for 7 minutes and stored at either 4°C or 21°C. This amplified PA683 pilA sequence was then run on a 0.8% agarose gel and gel extracted using the Qiagen QIAquick® Gel Extraction Kit 250.
(catalog# 28706, Valencia, CA). This gel extracted PA683 \( \textit{pilA} \) fragment was then amplified using primers that contained \textit{XmaI} and \textit{HindIII} sites, \textit{XmaI-PilA-F} and \textit{HindIII-PilA-R1}. Thermocycler conditions for this PCR reaction were the same as the first primer set. This amplified version of the PA683 \( \textit{pilA} \) sequence was then cloned into the pCR\textsuperscript{®}2.1-TOPO\textsuperscript{®} vector using Invitrogen’s TOPO TA Cloning\textsuperscript{®} Kit. The PA683 \( \textit{pilA} \) sequence was cut out of the pCR\textsuperscript{®}2.1-TOPO\textsuperscript{®} vector using \textit{XmaI/HindIII} and ligated into pMMB66EH that was cut with the same restriction enzymes to form the p683\( \textit{pilA} \) construct. PilA expression is under the control of the IPTG-inducible plasmid promoter.

2. Construction of p683\( \textit{pilA-tfpY} \)

To make the p683\( \textit{pilA-tfpY} \) construct, PA683 \( \textit{pilA-tfpY} \) was amplified from PA683 genomic DNA using PCR and primers designed to contain \textit{XmaI} and \textit{HindIII} sites, \textit{XmaI-PilA-F} and \textit{HindIII-TfpY-R1}. Thermocycler conditions for this PCR reaction consisted of a 105°C lid temperature, initial incubation at 94°C for 7 minutes, followed by 30 cycles of 94°C denature for 1 minute, 50°C annealing for 1 minute, and 72°C extension for 2 minutes. The final extension was 72°C for 7 minutes and stored at either 4°C or 21°C. This amplified version of the PA683 \( \textit{pilA-tfpY} \) sequence was then cloned into the pCR\textsuperscript{®}2.1-TOPO\textsuperscript{®} vector using Invitrogen’s TOPO TA Cloning\textsuperscript{®} Kit. The PA683 \( \textit{pilA-tfpY} \) sequence was cut out of the pCR\textsuperscript{®}2.1-TOPO\textsuperscript{®} vector using \textit{XmaI/HindIII} and ligated into pMMB66EH that was cut with the same restriction enzymes to form the p683\( \textit{pilA-tfpY} \) construct. PilA-TfpY expression is under the control of the IPTG-inducible plasmid promoter.
3. Construction of p683aas

To make the p683aas construct, primers designed to flank the ends of the 683 pilA sequence, contain codons for a C-terminal –AAS extension, and contain Xmal and HindIII restriction sites were used to PCR out mutant 683 pilA-AAS from the p683pilA vector. The primers are named Xmal-PilA-F and HindIII-683PilA-AAS-R. Thermocycler conditions for this PCR reaction consisted of a 105°C lid temperature, initial incubation at 94°C for 7 minutes, followed by 30 cycles of 94°C denature for 1 minute, 53.5°C annealing for 1 minute, and 72°C extension for 2 minutes. The final extension was 72°C for 7 minutes and stored at either 4°C or 21°C. This amplified product was then cloned into the pCR®2.1-TOPO® vector using Invitrogen’s TOPO TA Cloning® Kit. The PA683 pilA-AAS sequence was cut out of the pCR®2.1-TOPO® vector using Xmal/HindIII and ligated into pMMB66EH that was cut with the same restriction enzymes to form the p683aas construct. Mutant PilA-AAS expression is under the control of the IPTG-inducible plasmid promoter.

4. Construction of p683pep

To make the p683pep construct, primers designed to flank the end of the 683 pilA sequence, contain codons for a C-terminal – peptide tail mutation (-TAWKPNYAPANAPKS) consisting of the C-terminal 15 amino acids of PA1244 PilA (with the C-proximal cysteine residue changed to alanine), and contain Xmal and HindIII restriction sites were used to PCR amplify mutant 683 pilA with the peptide tail from the p683pilA vector. The primers are named Xmal-PilA-F and HindIII-683PilA-Tail-R. Thermocycler conditions for this PCR reaction consisted of a 105°C lid temperature,
initial incubation at 94°C for 7 minutes, followed by 30 cycles of 94°C denature for 1 minute, 53.5°C annealing for 1 minute, and 72°C extension for 2 minutes. The final extension was 72°C for 7 minutes and stored at either 4°C or 21°C. This amplified product was then cloned into the pCR®2.1-TOPO® vector using Invitrogen’s TOPO TA Cloning® Kit. The PA683 pilA with the peptide tail sequence was cut out of the pCR®2.1-TOPO® vector using EcoRI/HindIII and ligated into pMMB66EH that was cut with the same restriction enzymes to form the p683pep construct. Mutant PilA (with peptide tail) expression is under the control of the IPTG-inducible plasmid promoter.

5. Construction of pPilAPhoA:

To make the pPilAPhoA fusion construct, PA1244 pilA was amplified from the PA1244 genome using PCR and primers, EcoRI-PilA-Promoter to add a 5’-EcoRI site and PilA-HindIII to add a 3’-HindIII site. Thermocycler conditions for this PCR reaction consisted of a 105°C lid temperature, initial incubation at 94°C for 7 minutes, followed by 30 cycles of 94°C denature for 1 minute, 45-65°C annealing gradient for 1 minute, and 72°C extension for 1 minute. The final extension was 72°C for 7 minutes and stores at either 4°C or 21°C. PA1244 pilA was then cloned into the pCR®2.1-TOPO® vector using Invitrogen’s TOPO TA Cloning® Kit. PA1244 pilA was cut out of the pCR®2.1-TOPO® vector using XbaI/HindIII and then ligated, in frame with phoA, into pRMCD28 vector cut with the same restriction enzymes to form the pPilAPhoA fusion construct. Fusion expression is under the control of the IPTG-inducible plasmid promoter.
6. Construction of pPilAP

To make the pPilAP construct, the fusion sequence was amplified from pPilAPhoA using primers designed to flank the ends of the fusion sequence and contain EcoRI and BamHI restriction sites. The primers are named EcoRI-1244PilA-F and BamHI-ECAP-R. Thermocycler conditions for this PCR reaction consisted of a 105°C lid temperature, initial incubation at 94°C for 7 minutes, followed by 30 cycles of 94°C denature for 1 minute, 53.5°C annealing for 1 minute, and 72°C extension for 2 minutes and 30 seconds. The final extension was 72°C for 7 minutes and stored at either 4°C or 21°C. This amplified fusion sequence was then cloned into the pCR®2.1-TOPO® vector using Invitrogen’s TOPO TA Cloning® Kit. The fusion sequence was cut out of the pCR®2.1-TOPO® vector using EcoRI/BamHI and ligated into pMMB66EH that was cut with the same restriction enzymes to form the pPilAP construct. Because the phoA sequence contains several EcoRI sites, partial digestion with this enzyme was performed for 30 seconds at 37°C. Fusion expression is under the control of the IPTG-inducible plasmid promoter.

7. Construction of pPilAPaas

To make the pPilAPaas construct, primers designed to flank the end of the fusion sequence and contain the C-terminal –AAS mutation and XmaI and SalI restriction sites were used to PCR out the fusion from the pPilAPhoA vector. The primers are named XmaI-1244PilA-F and SalI-ECAP-AAS-R. Thermocycler conditions for this PCR reaction consisted of a 105°C lid temperature, initial incubation at 94°C for 7 minutes, followed by 30 cycles of 94°C denature for 1 minute, 53.5°C annealing for 1 minute, and
72°C extension for 3 minutes and 30 seconds. The final extension was 72°C for 7 minutes and stored at either 4°C or 21°C. This amplified version of a mutated fusion sequence was then cloned into the pCR®2.1-TOPO® vector using Invitrogen’s TOPO TA Cloning® Kit. The mutated fusion sequence was cut out of the pCR®2.1-TOPO® vector using XmaI/SalI and ligated into pMMB66EH that was cut with the same restriction enzymes to form the pPilAPaas construct. Mutant fusion-AAS expression is under the control of the IPTG-inducible plasmid promoter.

8. Construction of pECAP

To make the pMMB66EH+E. coli (EC) alkaline phosphatase (AP) construct, pECAP, the EC phoA sequence was cut out of pHK734 using EcoRI/BamHI. pHK734 was a gift from Hiroshi Kadokura and Jon Beckwith (Harvard Medical School Department of Microbiology and Molecular Genetics). Because the phoA sequence contains several EcoRI sites, partial digestion with this enzyme was performed for 30 seconds at 37°C. This cut EC phoA sequence was then ligated into pMMB66EH cut with the same restriction enzymes. PhoA expression is under the control of the IPTG-inducible plasmid promoter.

9. Construction of pECAPAas

Site-directed mutagenesis was used to add an alanine-alanine-serine (-AAS) extension to the C-terminal lysine residue of EC PhoA of pECAP to form the pECAPAas construct. The mutagenic primers are named Ecoli-AAS-F and Ecoli-AAS-R. This site directed mutagenesis was performed using Stratagene’s QuikChange® II XL Site-
Directed Mutagenesis kit, following the instruction manual protocol. However, the PCR reaction was divided into two half-reactions. One half-reaction contained the forward primer and the second half-reaction contained the complimentary primer. These reactions were run for three cycles of the kit thermocycler amplification conditions. After three cycles, the reactions were stopped, the two half-reactions were combined into one reaction tube, and then the mutagenic PCR conditions were started again from the beginning and allowed to run to completion. Since the construct was so large, this technique allowed half-templates to be produced, ensuring proper annealing and replication of the mutagenized product. Mutant PhoA-AAS expression is under the control of the IPTG-inducible plasmid promoter.

D. Transformation of Strains

1. Chemical:

Two chemical transformation protocols were used in this study. Transformation of pCR2.1-TOPO vector (with or without insert) was carried out according to Invitrogen TOPO TA Cloning® Kit’s OneShot® Chemical Transformation protocol. This transformation protocol allowed transformation of plasmid into kit provided Top10® E. coli cells.

All other plasmid constructs were chemically transformed, using the general laboratory protocol, into E. coli HB101 cells. HB101 cells were made competent by aerobically growing them to early log phase and then treating with half the original volume of an ice-cold, filter sterilized solution of 50 mM CaCl₂, 10 mM Tris-HCl pH8.0, incubating on ice for 15 minutes, then spinning to pellet the cells. The cells are then
treated with 1/15 the original volume of an ice-cold, filter sterilized solution of 50 mM CaCl₂, 10 mM Tris-HCl pH 8.0, 10% glycerol and stored in this suspension at -80°C.

Forty-five microliters of competent HB101 cells were aliquoted into a sterile, thin-walled polypropylene culture tube. To these cells, 2.5 µl of transforming DNA was added and incubate on ice for 30 minutes. This suspension was heat shocked at 42°C for 30 seconds, then quickly put on ice for 2 minutes. Two hundred fifty microliters NZYE+ (1% NZ Amine/casein hydrolysate, 0.5% Yeast Extract, 0.5% NaCl, pH 7.5, 12.5 mM MgSO₄, 12.5 mM MgCl₂, 0.4% glucose) broth was added to the cells and incubated for 1 hour at 37°C, 250 rpm. The desired amount of transformation reaction was plated onto selective media and incubated under optimal aerobic conditions.

2. Electroporation:

Strains of *P. aeruginosa* were aerobically grown at 37°C, 250 rpm in LB broth to early log phase. Subsequently, 4 ml of cell culture was distributed into four 1.5 ml microfuge tubes. These cells were pelleted and washed with 300 mM sucrose (room temperature). Cells were resuspended in 100 µl of 300 mM sucrose and transferred into one 1.5 ml microfuge tube. To this cell suspension, approximately 5-7µl of high copy (10-30 µl low copy) transforming DNA was added. This suspension was mixed briefly by gently pipetting up and down and then transferred to an electroporation cuvette. Electroporation, as specified by Choi *et al.*, was carried out with the BIO-RAD Gene Pulser™ set to 25 µF, 200 Ω, 2.5 kV (Choi *et al.*, 2006). One pulse generally took between 3-4 seconds. After this pulse, 1 ml of LB broth was added to the cuvette and gently mixed by briefly pipetting up and down. The transformation reaction was then
transferred to a sterile, thin-walled polypropylene culture tube and incubated for 1 hour at 37°C, 250 rpm (Choi et al., 2006). After incubation, varying volumes (usually 20 µl and 50 µl) of this transformation reaction were plated onto selective media and incubated overnight at 37°C.

E. Alkaline Phosphatase Periplasmic Activity Assay

This assay protocol was adapted from Daniels et al. 1998. All strains were cultured overnight under aerobic conditions with respective antibiotic for selection. Subcultures were diluted to 1:20 with fresh LB, induced at 5 mM IPTG, and incubated at 37°C, 250 rpm for 1.5 hours. The cultures were normalized to an optical density of 0.5-0.6 at A600 using a Spectronic® 20 Genesys™. The cells were pelleted and washed with cold 10 mM Tris-HCl pH8.0, 10 mM MgSO4. The cells were resuspended in 1 ml cold 1M Tris-HCl pH8.0, 1 mM iodoacetamide. Two hundred microliters of cell suspension was added to 800 µl 1M Tris-HCl ph8.0, 0.1 mM ZnCl2, 1 mM iodoacetamide. To this suspension, 50 µl 0.1% SDS and 50 µl chloroform was added to permeabilize the cells (creating spheroplasts). Two hundred seventy-five microliters of cell suspension was added to the well of a 96-well plate in triplicate. The reaction was started with the addition of 25 µl 0.4% p-nitrophenyl phosphate, covered with foil, and incubated at 37°C for 30 minutes. The reaction was stopped with the addition of 20 µl 0.5 M ethylenediaminetetraacetic acid (EDTA) pH8.0. The optical density was taken at an absorbance of 415 nm for color change and 595 nm for cell debris using a BIO-RAD Model 3550 Microplate Reader (courtesy of Dr. Kyle Selcer). Microsoft Excel was used
to calculate periplasmic alkaline phosphatase activity using absorbencies taken at 415 nm, 595 nm, and 600 nm.

\[
\text{Units Activity} = \left[ \frac{\text{OD}_{415} - (1.75 \times \text{OD}_{595})}{\text{Time (min)} \times \text{OD}_{600} \times \text{vol. cells (ml)}} \right] 1000
\]

F. Extraction of Soluble and Insoluble Cell Fractions

*Pseudomonas aeruginosa* strains were grown aerobically overnight at 37°C on LB plates. Plate grown *P. aeruginosa* cells are known to increase piliation (Silipigni-Fusco, 1987). The entire lawn of cells was resuspended in 5 ml of lysis buffer (10 mM K₂HPO₄, 30 mM NaCl, 0.25% Tween-20, 10 mM β-mercaptoethanol, 10 mM EDTA, 10 mM EGTA) and stored at -80°C for 30 minutes. To the thawed cell suspension, 1 ml of lysozyme (100 µg/ml lysis buffer) was added, briefly vortexed, and incubated on ice for 30 minutes. Sonication was then performed on the cell suspension 3 times at 15 second intervals on ice (with 1 minute breaks on ice between sonication intervals). The suspension was then centrifuged at 9,000 rpm with a DuPont Sorvall® SS-34 rotor (Thermo Fischer Scientific, Waltham, MA) for 15 minutes at 4°C. The supernatant, containing the soluble (S) cytosolic/periplasmic and insoluble (IS) membrane cell fractions, was immediately transferred to a new centrifuge tube and kept on ice. The supernatant was then centrifuged at 32,000 rpm with a Beckman SW60Ti rotor (Beckman Coulter, Inc., Fullerton, CA) for 1 hour at 4°C. The supernatant is the soluble fraction (SF) and the pellet is the insoluble fraction (ISF). The ISF pellet was resuspended in 200 µl lysis buffer. Both fractions were stored at -21°C until further preparation or analysis.
Protein concentration was determined using the Pierce BCA™ Protein Assay Kit.

Further preparation of these samples for SDS-PAGE and Western analysis is provided below under “Western Blot: Sample Preparation.”

G. Extraction of Periplasmic Fraction

The current protocol was obtained from Jayashree Rao’s thesis (Rao, 1995), which is an adaptation of the Poole and Hancock periplasmic extraction protocol (Poole & Hancock, 1984). *Pseudomonas aeruginosa* strains were grown aerobically overnight at 37°C on LB plates. Plate grown *P. aeruginosa* cells are known to increase piliation (Silipigni-Fusco, 1987). The entire lawn of cells was resuspended in 20 ml 0.01 M Tris/HCl pH 7.5. This cell suspension was split into two centrifuge tubes, 10 ml per tube. The samples were then centrifuged at 7,500 rpm with a Sorvall® SS-34 rotor for 10 minutes at 4°C. The pelleted cells were resuspended in 10 ml of 0.01 M Tris/HCl pH 7.5. To this suspension, 200 µl of 0.1M EDTA and 1 ml lysozyme (25 mg/ml in 0.01 M Tris/HCl pH 7.5) was added and incubated on ice for 25 minutes. The sample was then centrifuged at 7,500 rpm with a Sorvall® SS-34 rotor for 10 minutes at 4°C. The supernatant (periplasmic fraction) was transferred to a 14 ml Falcon tube and stored at -21°C until further preparation or analysis. Further preparation of these samples for SDS-PAGE and Western analysis is provided below under “Western Blot: Sample Preparation.”
H. Isolation of Pili

Four IPTG-induced, LB agar plate grown cultures (entire lawn) were resuspended in 10 ml fresh LB broth (in centrifuge tubes) after 14-18 hours at 37°C. The cells were vortexed for 2-3 minutes to shear the pili from the cells. As an additional shearing step, the cell suspension was passed through an 18-gauge needle 4 times. The suspension was then centrifuged at 10,000 rpm for 30 minutes at 4°C with a Sorvall® SS-34 rotor. The supernatant (pili) was transferred to a new centrifuge tube and 7.8 ml 5X polyethylene glycol (PEG)/NaCl (15% PEG, 2.5 M NaCl) was added to each sample (for 32.05 g pili suspension). The samples were mixed thoroughly by vortexing, incubated on ice for 2 hours, and then centrifuged again at 10,000 rpm for 30 minutes at 4°C with a Sorvall® SS-34 rotor. The pellet (pili) was resuspended in 1 ml sterile deionized water and transferred to a 1.5 ml microfuge tube. 200 µl 5X PEG/NaCl was added, mixed thoroughly by vortexing, and incubated on ice for 30-45 minutes. The sample was then microfuged at 14, 500 rpm (or at max) in a microcentrifuge for 10 minutes. The pellet (pili) was resuspended in 1 ml sterile deionized water and subjected to dialysis to remove buffers and salts. The sample was transferred to Fisherbrand® regenerated cellulose dialysis tubing (catalog# 21-152-7, Fisher Scientific, Suwanee, GA) and stirred at 4°C overnight in 6 liters of deionized water with 1.5 g sodium azide. The dialyzed sample was then lyophilized in a LABCONCO Freeze Dry System/Freezone® 4.5 (Kansas City, MO) overnight. The lyophilized sample was resuspended in 500-650 µl sterile deionized water and stored at -20°C as a native stock. For SDS-PAGE analysis, the native stock was diluted in a 1:1 ratio with 2X loading buffer and prepared as stated below in the section entitled “Western Blot: Sample Preparation.”
I. Western Blot

1. Sample Preparation:

For whole cell extracts, all broth and plate grown cells were normalized to an optical density reading of 0.5-0.6 at an absorbance of 650 nm. One and one half milliliters of cells was centrifuged at 14,500 rpm (or at max) for one minute in a microcentrifuge to pellet the cells. The cell pellet was then resuspended in 250 µl of 1X Loading Buffer (62.5 mM Tris/HCl pH6.8, 2% SDS, 10% glycerol, 2.5% β-mercaptoethanol, 0.0025% Bromophenol Blue) and incubated on a heat block at 95°C for 10 minutes. Samples were stored at 4°C.

Cell fractions and pili preparations were subjected to dialysis and lyophilization to remove buffer salts/contaminants and further concentrate the sample. All cell fractions were normalized for protein concentration by using the Pierce BCA™ Protein Assay Kit (prod# 23225) and standard curve analysis. Cell fraction/pilin samples and 2X Loading Buffer (125 mM Tris/HCl pH6.8, 4% SDS, 20% glycerol, 5% β-mercaptoethanol, 0.005% Bromophenol Blue) were added in a 1:1 ratio. The samples were then incubated on a heat block at 95°C for 10 minutes. Samples were stored at 4°C.

2. SDS-PAGE Gel Preparation:

Polyacrylamide gels were prepared to concentrations between 10% T and 16% T for protein separation and 5% T for the top stacking layer in 1.0 mm Invitrogen cassettes. Bio-Rad Prestained SDS-PAGE Broad Range Standard (catalog# 161-0318) was used as the general molecular weight ladder for all gels and samples were loaded at 1-30 µl per lane. The gel was run using a Novex X-Cell Surelock™ Mini-Cell (Invitrogen) in 1X
Tris/glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS). The top, stacking phase, of the gel was run at 125 V and the bottom, separation phase, of the gel was run at 175 V with the Bio-Rad Model 200/2.0 power supply until the tracking dye reached the bottom of the cassette. All gels were stained with Coomassie Brilliant Blue R-250 solution (0.1% Coomassie Brilliant Blue R-250, 40% methanol, 10% acetic acid) (Bio-Rad) for 30 minutes to overnight, and then destained with 40% methanol, 10% acetic acid solution three times at 30 minutes per wash. Alternatively, gels were subjected to western blot analysis as described below.

3. Blotting Procedure:

After the samples were run on an SDS-PAGE gel, a Bio-Rad Mini Trans-Blot® Electrophoretic Transfer Cell containing Trans-Blot Buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH8.3) on ice was used to transfer the proteins from the gel to nitrocellulose paper (NitroBind, Cast, Pure Nitrocellulose, 0.45 Micron, 20 cm X 3 m, GE Water and Process Technologies, Fisher Scientific, Suwanee, GA). The running conditions were set at 100 volts with a current maximum of 0.25 A for 23 minutes. The membrane was then blocked for 25 minutes, while shaking, in Killer Filler buffer (10 g casein boiled in 200 ml 0.1 M NaOH added to 1.8 L Phosphate Buffer Saline [PBS; 5.3 L deionized water, 28 g NaCl, 0.7 g KCl, 4.0 g Na₂HPO₄, 0.35 g CaCl₂·2H₂O, 0.53 g MgCl₂·6H₂O] containing 10g bovine serum albumin, adjust pH to 7.4, add 0.2 g Phenol Red and 3.6 g sodium azide). The primary antibody (dilution 10⁻² or 10⁻³, see Table 3 below) was incubated at room temperature for 5-12 hrs while shaking. The blot was then washed three times with PBS for 10 min/wash. The secondary antibody (10⁻³) was then
incubated at room temperature for 1 hour while shaking. The blot was washed again three times with PBS for 10 min/wash. If Fast-Red was used for visualization, an additional 10 minute wash step with 50 mM Tris/HCl pH8.0 was included. Pilin proteins were probed with an alkaline phosphatase-labeled 2\textsuperscript{nd} antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and visualized using the Fast Red reaction mixture, 20 mg Napthol AS-MX Phosphate (Sigma-Aldrich Co., St. Louis, MO) and 40 mg Fast-Red (Sigma-Aldrich Co.) dissolved in 20 ml 50 mM Tris/HCl pH8.0. Alkaline phosphatase-containing constructs were probed using a horseradish peroxidase-labeled 2\textsuperscript{nd} antibody (Kirkegaard and Perry Laboratories) and visualized with the SigmaFast\textsuperscript{TM} 3,3-diaminobenzidine tablet reaction mixture (catalog# D4418-5SET, Sigma-Aldrich Co.) or the SuperSignal\textsuperscript{®} West Dura Extended Duration Substrate reaction (catalog# 34075, Pierce).

Table 3. Antibodies and Visualization

<table>
<thead>
<tr>
<th>1\textsuperscript{st} Antibody</th>
<th>Dilution</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Bacterial Alkaline Phosphatase, Monoclonal</td>
<td>1:1,000</td>
<td>Mouse derived</td>
<td>Sigma-Aldrich Co.</td>
</tr>
<tr>
<td>Anti-\textit{E. coli} Alkaline Phosphatase Polyclonal</td>
<td>1:1,000</td>
<td>Rabbit derived</td>
<td>US Biomax, Inc., Ijamsville, MD</td>
</tr>
<tr>
<td>Anti-\textit{P. Aeruginosa} 1244 O7-Antigen Monoclonal, 11.14</td>
<td>1:1,000</td>
<td>Mouse derived</td>
<td>Sadoff et al., 1985</td>
</tr>
<tr>
<td>Anti-\textit{P. aeruginosa} 103 O11-Antigen Monoclonal</td>
<td>1:100</td>
<td>Mouse derived</td>
<td>ERFA Biotech, Montreal (QC), Canada</td>
</tr>
<tr>
<td>Anti-\textit{P. aeruginosa} 1244 Pilin Monoclonal, 5.44</td>
<td>1:1,000</td>
<td>Mouse derived</td>
<td>Castric &amp; Deal, 1994</td>
</tr>
<tr>
<td>Anti-\textit{P. Aeruginosa} 683 Pilin Polyclonal</td>
<td>1:1,000</td>
<td>Mouse derived</td>
<td>This study</td>
</tr>
</tbody>
</table>

2\textsuperscript{nd} Antibody

<table>
<thead>
<tr>
<th></th>
<th>Dilution</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatase-labeled Anti-Mouse</td>
<td>1:1,000</td>
<td>Goat derived, Affinity purified, Human serum adsorbed, visualized with the Fast Red reaction</td>
<td>Kirkegaard and Perry Laboratories, Gaithersburg, MD</td>
</tr>
</tbody>
</table>
### J. Isoelectric Focusing

Isoelectric focusing allowed assessment of the glycosylation status of mutated proteins by their isoelectric point-specific migration. Addition of a glycan subunit may alter the isoelectric point of the protein and cause it to migrate differently on a gel in comparison to its native, non-glycosylated state. Native cell fractions were prepared as above. Prior to loading the protein samples, the gel (Amersham Biosciences Phast Gel™ IEF 3-9, <0.2% acrylamide, Piscataway, NJ) was soaked in 1.5% n-octyl-β-D-glucopyranoside (BOG), 3.4% ampholytes (AMP)3-9, 0.025% Azide for 15 minutes. All protein samples were loaded in native conformation. The standard marker was BIO-RAD IEF pI 4.45-9.6. Two microliters of each sample was touched to a comb that was used to load the gel. The gel was run on a PhastSystem Separation and Control Unit under the separation command.

Before the gel was finished running, a square of nitrocellulose paper was soaked in 50% methanol for 15 minutes. This nitrocellulose paper was then used to capture the protein from the gel in a diffusion transfer process. In this diffusion transfer of proteins from gel to the blotting paper, the gel was placed in a Petri dish and the nitrocellulose paper was placed on top of the gel (avoiding air bubbles). The Petri dish was then sealed with plastic wrap and let sit at room temperature overnight. The next day, the blotting
paper was peeled off of the gel and subjected to the routine Western blot development as above.

**K. Generation of Polyclonal Anti-683 Pilin Antibodies**

The current protocol, used to generate polyclonal antibodies against *P. aeruginosa* strain 683 pilin, was approved by Duquesne University’s Institutional Animal Care and Use Committee (IACUC). All animal storage and experimentation took place at the USDA approved, Duquesne University Animal Care Facility.

1. **Antigen Preparation**

Day 1: *P. aeruginosa* strain 683 was grown up aerobically in LB broth overnight at 37°C, 250 rpm. Metal culture pans (68 cm X 28 cm X 3 cm) and CAYE agar medium were sterilized in the autoclave. One liter CAYE makes two pans at 500 ml per pan.

Day 2: Sterilized CAYE culture pans were inoculated dropwise with 2.5-3.0 ml of PA683 overnight culture from Day 1, spread evenly over the surface of the pan with a sterile hockey stick, and incubated at 37°C for 14 hours.

Day 3: After 14 hours at 37°C, the cells were harvested with a metal spatula and a sterile cotton swab into 100 ml CAYE broth. The cells were completely resuspended by stirring vigorously for 30 minutes. Pili were sheared from the cells by two excursions through a 50 ml syringe with an 18 gauge blunt end-needle. Cells were divided into four 50 ml centrifuge tubes (25 ml per tube) and centrifuged at 10,000 rpm for 30 minutes with a Sorvall® SS-34 rotor. The supernatant was added to a 200 ml beaker and the pellet was discarded. To the supernatant, 3% PEG (8,000) and 0.5 M NaCl was added and
stirred into solution at room temperature. Once in solution, the suspension was stored on ice for 2 hours. The suspension was then centrifuged again as earlier. The supernatant was discarded and any remaining supernatant fluid was removed with a Kimwipe® (Kimberly-Clark, Dallas, Texas). The pellets were resuspended in 100ml CAYE broth (total volume) and stirred for 1 hour at room temperature. The suspension was centrifuged again as earlier. The pellet was discarded. The supernatant was treated again with PEG to 3% and NaCl to 0.5 M and stirred at room temperature. Once in solution, the suspension was stored on ice for 1 hour and then centrifuged again as earlier. The supernatant was discarded and any remaining supernatant fluid was removed with a Kimwipe®. The pellets were resuspended in 5 ml CAYE broth (total volume) and stored for 1 hour at room temperature. This 5 ml suspension was divided into four 1.5 ml microfuge tubes (1.25 ml per tube) and microcentrifuged at 14,500 rpm (or at max) for 5 minutes. The pellets were discarded. The supernatant was transferred to a new 1.5 ml microfuge tube and 250 µl of 15%PEG/2.5 M NaCl was added per 1,000 µl of supernatant. The suspensions were mixed by vortexing and then incubated on ice for 30 minutes. Each sample was then microcentrifuged as 14, 500 rpm (or at max) for 10 minutes. The supernatant was drained completely and the pellets were stored at 4°C until they were resuspended in a total volume of 1,000 µl sterile deionized water. At this point a polyacrylamide 16% T gel could be run and stained with Coomassie Brilliant Blue R-250 solution to assess the purity of your pili preparation.

The pili suspension was dialyzed for 2 days in 6 liters of deionized water containing 1.5 g sodium azide stirring at 4°C. The protein concentration of the dialyzed pili preparation was then determined by the Pierce BCA™ Protein Assay Kit. The
remaining pili preparation was lyophilized overnight and then resuspended with physiological saline solution (0.9% NaCl) to a concentration of 1 µg/µl. The desired immunization concentration for each mouse was 10 µg antigen/100 µl. To make the final working immunization stock for 8 mice, 80 µg/400 µl was prepared and taken up into a 1cc glass syringe. This glass syringe was then connected to another glass syringe containing 400 µl of Freund’s Incomplete Adjuvant (DIFCO Laboratories, Detroit, MI) by a stainless steel emulsifying needle. The contents were mixed through the emulsion needle until a viscous equilibrated suspension was formed. This prepared immunization stock suspension was then taken to the Duquesne University Animal Care Facility to immunize the mice.

2. Immunization

A total of 6 female Balb/C mice (Hilltop Lab Animals, Scottdale, PA), approximately 6-8 weeks old and weighing 25 g, were used for immunization. The mice received a primary injection on day 1, followed by booster injections of the same concentration on days 7 and 14. The mice were bled (described below) on day 21, 7 days after the final boost. For all injections, the mice were anesthetized intraperitoneally using a 28-gauge needle with 250 µl ketamine HCL/xylazine HCL solution (6.67 mg ketamine HCL/mL; Ketaject® Pheonix Pharmaceutical Inc., St. Joseph, MO and 1.33 mg xylazine HCL/mL; Sigma® Sigma-Aldrich Co.). A 22-gauge needle was used to immunize each mouse subcutaneously at the nape of the neck with 100 µl (10 mg antigen) of the emulsified antigen/adjuvant suspension.
3. Serum Collection

The mice were anesthetized intraperitoneally using a 28-gauge needle with 250 µl ketamine HCL/xylazine HCL solution. A 26-gauge tuberculin needle was used to harvest the blood by intra-cardiac puncture. The blood was stored in 1.5 ml microfuge tubes at 4°C for 1 hour and then microcentrifuged at 3,000 rpm for 1 minute. The sera was drawn off and stored as 100 µl aliquots in 0.5 ml microfuge tubes. The blood was then centrifuged a second time in a microcentrifuge at 5,000 rpm for 1 minute. The sera, again, was drawn off and stored as 100 µl aliquots in 0.5 ml microfuge tubes. The blood was centrifuged a third time at 5,000 rpm for 1 minute and stored as previously. All 3 rounds of sera were stored at -20°C until needed to prepare a working antibody dilution. Serum dilutions of $10^{-3}$ and $10^{-4}$ gave sufficient reactivity for use in immunoblot analysis. The dilution used in the present study can be found in Table 3.
III. RESULTS

A. Homology Modeling and identification of surface properties of PA1244 PilA, PA683 PilA, and EC PhoA. Previous findings have identified the necessity of a C-terminal serine/threonine, a compatible surface charge surrounding this ultimate residue, and periplasmic localization for the glycosylation of pilin in PA1244 (Horzempa et al., 2006a; Qutyan et al., 2008). Homology modeling was performed on PA1244 PilA, PA683 PilA, and the *E. coli* (EC) K12 alkaline phosphatase homodimer to compare and contrast the tertiary structure of a known glycosylated protein, PA1244 PilA, to normally non-glycosylated proteins, PA683 PilA and EC PhoA (Figure 13). Each of the engineered proteins in this study are known to localize to the periplasm, of which, the PA1244 PilA-EC PhoA fusion protein and the PA683 PilA protein, upon transfer by the pilin signal sequence, become embedded in the outer leaflet of the inner membrane (oriented in the periplasmic space), providing colocalization with the catalytic PilO protein to the site of glycosylation. As highlighted by Comer et al. (2002) and Horzempa et al. (2006a), compatible characteristics of substrate glycosylation primarily encompass surface charge and location of the carboxy terminal serine residue (Figure 13). In 2006, Horzempa et al. demonstrated that an electronegative surface charge was inhibitory to the glycosylation reaction, but could be side-stepped if the C-terminal serine residue was surfaced exposed and further extended away from the electronegative surface charge with a short spacer of alanine residues (Horzempa et al., 2006a). The homology modeling revealed that the general surface charge of PA683 pilin and the bacterial alkaline phosphatase protein appear to be predominantly electropositive around the carboxy
terminal region, but more importantly, the C-terminal residues are surface exposed (Figure 13). The general tertiary structure of the PA683 pilin was expected to similarly present a surface exposed carboxy terminal residue as the general model of pilin structure suggests (Craig et al. 2006; Horzempa et al. 2006; Keizer et al. 2001). Each pilin possesses a characteristic, yet distinct, disulfide loop (DSL) region at their C-terminus (Figure 14) that has been previously ruled-out as a structural requirement for pilin glycosylation by necessity of surface charge, alone, in this region (Horzempa et al., 2006a). In attempt to nullify any surface incompatibilities, each construct was engineered by site-directed mutagenesis or by PCR to extend the C-terminal serine away from the surface by a minimum of two alanine residues or the C-terminal 15 amino acids of PA1244 pilin, of which the C-proximal cysteine residue was changed to an alanine (Figure 15). Overall, these homology models reveal group III pilin and E. coli alkaline phosphatase to be attractive candidate proteins to be engineered for glycosylation, which, if successful, may hold practical application to the expansion of potential vaccine components.
Figure 13. Predicted surface properties of PA1244 pilin monomer, PA683 pilin monomer, and *E. coli* alkaline phosphatase homodimer. Tertiary structures of each were generated by homology modeling. Whole structure models have the C-terminal amino acid residue highlighted yellow and labeled with an arrowhead. Zoomed images have the C-terminal amino acid residue highlighted in yellow and display surface charge properties, respectively. For surface charge; blue indicates a positive charge, red indicates a negative charge, and white indicates neutral charge.
PA1244 (residues 127-148). . . CKTIKTPTAWKPNYAPANCPS
PA683 (residues 140-173). . . CASESNAVSGFDRNMPALTGAFTPARFAPSEC

Figure 14. Disulfide loop regions of PA1244 pilin and PA683 pilin. Cysteine residues are in red and underlined. Conserved residues of the DSL region are in red.

<table>
<thead>
<tr>
<th>PA1244 pilA (128-146)</th>
<th>-CKTIKTPTAWKPNYAPANCPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA683 pilA (139-178)</td>
<td>-CASESNAVSGFDRNMPALTGAFTPARFAPSEC</td>
</tr>
<tr>
<td>PA683 pilA-AAB</td>
<td>-CASESNAVSGFDRNMPALTGAFTPARFAPSEC</td>
</tr>
<tr>
<td>PA683 pilA-T1G</td>
<td>-CASESNAVSGFDRNMPALTGAFTPARFAPSEC</td>
</tr>
<tr>
<td>ECAP (631-650)</td>
<td>-VGLIDQIDLYFTMKAALGKL</td>
</tr>
<tr>
<td>ECAP-AAS</td>
<td>-VGLIDQIDLYFTMKAALGKL</td>
</tr>
<tr>
<td>PA683 pilA (139-170)</td>
<td>-LAWKPRYAPANCPSKAYTREDPVLRKRAAQGD/-VGLIDQIDLYFTMKAALGKL</td>
</tr>
<tr>
<td>PA683 pilA-AAS</td>
<td>-LAWKPRYAPANCPSKAYTREDPVLRKRAAQGD/-VGLIDQIDLYFTMKAALGKL</td>
</tr>
</tbody>
</table>

Figure 15. C-terminal peptide extensions used to study glycosylation substrate specificity of P. aeruginosa. The C-terminus with the entire disulfide loop amino acid sequence is given for PA1244 pilin. The disulfide bond forming cysteine residues are underlined for PA1244 pilin and PA683 pilin. The alanine substitution is underlined for PA683 PilA-Pep. C-terminal extensions added to each construct are highlighted in blue. The linker region for the PA1244 pilA-EC phoA fusion is highlighted in red. Residue specifications are in parenthesis.

B. Can a structurally different, Group III, pilin be engineered to accept the O-antigen repeating unit of lipopolysaccharide?

Is PA683 pilin already being modified? Before assessing whether pilin from strain PA683 could be engineered for glycosylation, I first needed to determine whether or not this pilin is already being modified. Preliminary data from the Castric laboratory demonstrated that, although the gene sequence of PA683 pilA (540 nucleotides) is larger than Group I PA1244 pilA (465 nucleotides), PA683 pilin runs at a lower apparent
molecular weight on an SDS-PAGE gel, suggesting an unknown characteristic of tertiary structure (personal communication, with M. C. Quinn). Mature unglycosylated PA1244 pilin has a theoretical molecular weight of 15,640 Daltons (Da), an apparent molecular weight on SDS-PAGE of about 15,600 Da (Castric, 1995), and an actual molecular weight of 16,307 Da as determined by MALDI analysis (Castric et al., 2001). MALDI analysis also revealed the size of the glycan subunit to be about 666 Da (Castric et al., 2001). With this covalently attached glycan, mature glycosylated PA1244 pilin migrates to an apparent molecular weight of 16,900 Da on SDS-PAGE, and was determined by MALDI analysis to be about 16,973 Da (Castric et al., 2001). For these two flavors of PA1244 pilin, molecular weight analysis by SDS-PAGE yields fairly accurate approximations.

SDS-PAGE was performed on purified PA683 pilin to determine the apparent molecular weight value for comparison to the theoretical and known values. The theoretical molecular weight of PA683 pilin is 17,439 Da. This predicted value agrees with the mass of PA683 pilin generated by MALDI-TOF (17,475 Da) (personal communication, with P.A. Castric). Although the predicted/determined molecular weight of PA683 pilin is larger than that of PA1244 pilin, preliminary SDS-PAGE analysis demonstrated that PA683 pilin migrates to a surprisingly smaller size than glycosylated PA1244 pilin (16,900 Da). This SDS-PAGE analysis was repeated in the current study and the same result was reproduced (Figure 16A). Pooled standard curve estimations from several gels suggest PA683 pilin migrates to about 15,000-16,300 Da, which is less than the predicted weight of 17,439 Da and MALDI-TOF generated mass of 17,475 Da (Figure 16A). These findings suggest that strain PA683 pilin is not posttranslationally
modified. The unexpected migration of this protein on an SDS-PAGE gel may be the result of incomplete protein unfolding in the presence of this detergent.

Although MALDI analysis of PA683 pilin has indicated no significant mass deviation from the theoretical value, the role of group III accessory protein, TfpY, in posttranslational modification of PA683 pilin was further explored. In 1995, Castric showed that the PilO accessory protein was necessary for the posttranslational glycosylation of PA1244 pilin (Castric, 1995). The pilO gene is located between the gene sequences of pilA and tRNAthr in the operon of P. aeruginosa strains that produce Group I pilin. P. aeruginosa strains that possess group III pilin, such as PA683, have the ORF of a suspected non-homologous accessory protein, TfpY, of unknown function in place of the pilO gene (Figure 8). To identify a potential role of TfpY in the posttranslational alteration of PA683 pilin size, the gene sequences of pilA and pilA-tfpY were cloned into a pMMB66EH vector and overexpressed in a strain of P. aeruginosa that does not express pilAO, PA1244N3, to produce p683pilA and p683pilA-tfpY. Overexpressed pili were sheared from the cells and subjected to SDS-PAGE analysis (Figure 16B). The SDS-PAGE results showed all PA683 pilin bands migrated to the same size, which suggests that TfpY does not confer a modification that noticeably alters pilin size by this technique.

Next, an electrofocusing gel of purified pili from PA1244N3/p683pilA and PA1244N3/p683pilA-tfpY was used to determine whether TfpY is required for altering the charge of mature pilin (Figure 16C). The predicted isoelectric point (pI) of PA683 pilin is about 4.45. All pilin bands on the gel migrated to this theoretical pI, indicating that TfpY does not attribute an identifiable charge altering modification to pilin (Figure
Collectively, these findings do not identify detectable molecular alterations to PA683 pilin by TfpY and are consistent with the MALDI results that suggest PA683 pilin is not modified.

Can PA683 pilin be engineered for glycosylation? Strain PA683 pilin was classified as group III on the premise of sequence homology with known group III pilins (i.e. PA14) and presence of the adjacent tfpY ORF (unpublished observation; Kus et al., 2004).

Although the general tertiary structure of pilins are conserved, structural differences can be identified between groups within the DSL and α-β loop regions. These differences can be observed between PA1244 and PA683 pilins in Figure 13. As mentioned, previous findings indicate that the normally non-glycosylated PA103 group II pilin can be
engineered for glycosylation in PA1244 by extending a C-terminal serine away from the inhibitory electronegative surface with a short stretch of alanine residues (Horzempa et al., 2006a). To determine whether the normally non-glycosylated PA683 group III pilin could be engineered for glycosylation, an alanine-alanine-serine (A-A-S) peptide extension was added to the C-terminal arginine residue and cloned into pMMB66EH, forming p683aas. This construct was transformed into a mutant PA103-derived strain that does not polymerize LPS surface O-antigen, PA103 \( wzy_{Pa}O11::aacC1 \), along with a pUCP26 vector containing the O-transferase responsible for glycosylation of pilin in PA1244, \( pilO \) (pUCP26 \( pilO \)). Control strains were transformed with the empty pUCP26 vector. PA103 \( wzy_{Pa}O11::aacC1 \) eliminates the presence of the LPS ladder on an anti-O-antigen immunoblot. Plate grown \( P. aeruginosa \) cells are known to increase piliation (Silipigni-Fusco, 1987). When whole cell extracts of plate grown strains were subjected to Western blot analysis, the C-terminal A-A-S peptide extension was demonstrated to be sufficient for the glycosylation of PA683 pilin (Figure 17A). This was determined by mutant pilin migration on SDS-PAGE probed with a polyclonal antibody specific for PA683 pilin (Figure 17A), and by an antibody specific for the PA103 O11 O-antigen glycan (Figure 17B). The low yield of glycosylated mutant pilin compared to the PA1244 glycosylated control pilin suggests the construct may be unstably produced or that glycosylation efficiency is low with this particular mutant. These findings indicate that group III pilin monomers can be engineered for glycosylation with this minimal glycosylation substrate.

There are a series of bands on the anti-PA683 pilin blot that can be explained. In the lane containing p683aas/\( pilO \), the top band is glycosylated mutant pilin, the second band
down is unglycosylated mutant pilin, the third band down is believed to be PA683 pilin with the A-A-S peptide extension cleaved off due to its alignment with control PA683 pilin band, and the lighter bands below this are thought to be more advanced degradation of the mutant PA683 pilin monomer. In addition to the glycosylated mutant pilin band, there are two additional bands on the blot probed with anti-O11 that can be explained. In the lane containing p683aas/pilO, the two bands at the bottom of the gel are an artifact of the PA103 \( wzy_{PaO11}::aacC1 \) system. The lower band is lipid A and the higher band is core plus one O-antigen subunit. As mentioned, this results because the \( wzy \) mutant does not polymerize additional LPS O-antigen subunits. These same bands should have also been observed in the lanes containing p683aas/pUCP26 and pPAC24/pilO, however, the reaction does not appear as strong.

To determine whether a pilin specific sequence would increase glycosylation efficiency in this PA103 \( wzy_{PaO11}::aacC1 \) system, the 15 C-proximal amino acids of PA1244 pilin (with the C-proximal cysteine changed to an alanine) were added to the C-terminal arginine residue of PA683 pilin and cloned into pMMB66EH, forming p683pep. This peptide tail mutation has been previously shown to be a major B-cell epitope of PA1244 pilin (Castric & Deal, 1994). The p683pep construct was transformed into PA103 \( wzy_{PaO11}::aacC1 \) in the presence and absence of PilO expression, and whole cell extracts of plate grown cells were subjected to Western blot analysis. Western blot analysis confirmed the glycosylation of mutant pilin by migration on SDS-PAGE probed with a polyclonal antibody specific for PA683 pilin (Figure 17A), and by detection with an antibody specific for the PA103 O-antigen (Figure 17B). The low yield of glycosylated mutant pilin compared to the PA1244 glycosylated control pilin suggests the
construct may be unstably produced or that glycosylation efficiency is low with this particular mutant. This finding indicated that addition of a pilin-specific glycosylation site permits glycosylation of group III pilin, but does not appear to greatly increase glycosylation efficiency in this system.

There are a series of bands on the blot probed with anti-PA683 pilin that can be explained. In the lane containing p683pep/pilO, the top band is glycosylated mutant pilin, the second band down is unglycosylated mutant pilin, the third band down is believed to be PA683 pilin with the peptide tail mutation partially cleaved off due to its slight offset aligment with control PA683 pilin band, and the lighter bands below this are thought to be more advanced degradation of the mutant PA683 pilin monomer. In addition to the glycosylated mutant pilin and control bands, lipid A and core plus one O-antigen subunit bands can be observed as in Figure 17B.

Although previous findings suggested that glycosylation of pilin does not appear to have an effect of pilus biogenesis (Smedley et al., 2005), functional assays were performed to determine if heterologously expressed group III pilin and the respective mutant epitopes are compatible with the PA1244 pilus biogenesis machinery. When pilin is overexpressed from p683pilA in the pilAO null strain, PA1244N3, surface pili and twitching motility are observed (personal communication, with P.A. Castric). However, when both mutant PA683 pilins are separately overexpressed from p683aas and p683pep in PA1244N3, surface pili and twitching motility are absent (personal communication, with P.A. Castric). These findings suggest that wild type PA683 pilin is tolerated by PA1244 pilus machinery while each mutation prohibits pilus biogenesis.
C. Can a protein fused to pilin be engineered to accept the O-antigen repeating unit of lipopolysaccharide? Although Horzempa et al. (2006a) demonstrated a lack of C-proximal consensus residues, other than serine/threonine, for pilin glycosylation, additional structural characteristics, such as anchorage to the inner cell membrane via hydrophobic tail residues, may be necessary for PilO substrate recognition. To examine this, the PA1244 structural pilin gene, pilA, was fused inframe at the 27th amino acid encoding codon of an E. coli alkaline phosphatase gene via the fusion vector pRMCD28, to form pPilAPhoA. Expression of this construct was first assessed in E. coli (Figure 18). Whole cell extracts of a strain containing pPilAPhoA were subjected to Western
blot analysis and probed with a monoclonal anti-bacterial alkaline phosphatase (BAP) antibody (Figure 18A) and a monoclonal anti-PA1244 pilin antibody, 5.44 (Figure 18B). The fusion was successfully expressed and detected on each blot at the expected molecular weight of about 63.5 kDa, noticeably larger than the *E. coli* PhoA control band (~47 kDa) (Figure 18). The intact fusion is believed to be the single predominant band on both blots. The other bands in the anti-BAP blot fusion lane are believed to be incomplete denaturation (above fusion band) and degradation (below fusion band).

**Figure 18.** Western blot analysis of PA1244 PilA-EC PhoA fusion expressed in *E. coli*. Western blot of IPTG-induced PA1244 PilA-EC PhoA fusion protein (pPilAPhoA), the empty fusion vector (pRMCD28), and an *E. coli* alkaline phosphatase control protein (pHK734) produced by *E. coli* HB101 cell extracts using anti-BAP monoclonal antibody (A) or anti-PA1244 pilin monoclonal antibody 5.44 (B) as a probe. A whole cell extract of HB101 without a vector was used as an additional negative control to show that endogenous alkaline phosphatase did not react with the anti-BAP monoclonal antibody (A). Purified glycosylated PA1244 pilin was used as a positive control for the 5.44 antibody (B).
To determine whether a PA1244 PilA- EC PhoA fusion protein can be engineered for glycosylation, an A-A-S peptide extension was added to the C-terminal lysine residue of PhoA and cloned into pMMB66EH, forming pPilAPaas. Two *P. aeruginosa* systems, PA1244 and PA103 *wzy*<sup>Pa</sup>*o11::aacC1*, were used to assess the glycosylation status of a fusion protein engineered with a minimally pilin-compatible PilO-recognition site (A-A-S). The PA1244 system was used first because it is the model strain for O-linked pilin glycosylation. The PA103 *wzy*<sup>Pa</sup>*o11::aacC1* system was then utilized because it eliminated LPS-band contamination on Western blots probed with anti-O-antigen antibody. As mentioned, previous results from the Castric laboratory suggest the necessity of a C-terminal surface exposed serine residue as the sole acceptor of the glycan subunit (Horzempa et al., 2006a). All non-terminal serine residues are unable to accept a glycan modification, eliminating the potential for serine residues within the fusion peptide sequence to be glycosylated, which would result in a false positive. Functional periplasmic alkaline phosphatase activity was recorded for all alkaline phosphatase containing constructs in both systems, PA1244 and PA103 *wzy*<sup>Pa</sup>*o11::aacC1* (Table 4). This demonstration of periplasmic functionality suggests that alkaline phosphatase is able to achieve a functional conformation and that the prepilin leader sequence is able to successfully target the fusion to the periplasmic face of the inner cell membrane.

Because PA1244 pilin is known to become embedded in the inner cell membrane, insoluble (IS) fractions of cell extracts prepared from plate grown PA1244/pPilAPaas and PA1244/pMMB66EH were subjected to Western analysis. Western blot results show that the mutated fusion was not glycosylated based on migration on SDS-PAGE probed with anti-bacterial alkaline phosphatase (BAP) (Figure 19A), and by the lack of a reaction
with an anti-O7 antibody, 11.14 (Figure 19B). The same result was observed when the blot was re-run and visualized with the more sensitive chemiluminescence technique (Figure 20). These results indicate an inability of the A-A-S peptide extension to tag a PA1244 PilA-EC PhoA fusion protein for glycan acceptance using the native physiological glycosylation mechanism of PA1244.

On the anti-BAP blot of Figure 19A, the faint bands in the control IS fraction PA1244/pMMB66EH lane resulted from sample spill-over from the IS PA1244/pPilAPaas lane. Additionally, in Figure 19B and Figure 20B, the ladder banding pattern can be attributed to anti-O11 antibody reaction with LPS. The detection of LPS ladder may make it difficult to identify a glycosylated band of a given molecular weight. In the present case, the fusion bands migrate to a higher molecular weight than the largest detectable LPS band, eliminating ambiguity. The PA103 \( wzy_{Pa11::aacC1} \) system was used to eliminate the appearance of an LPS ladder on anti-glycan probed blots.
Figure 19. Western blot analysis of mutant PA1244 PilA-EC PhoA fusion expressed in PA1244. Western blot of soluble (S) and insoluble (IS) fractions of IPTG-induced PA1244/pPilAPaas and PA1244/pMMB66EH (empty vector) for mutant PA1244 PilA-EC PhoA-AAS fusion protein using anti-BAP monoclonal antibody as a probe (A) or anti-O7 monoclonal antibody 11.14 as a probe (B). Purified glycosylated PA1244 pilin was used as a positive control for the 11.14 antibody (B).
Figure 20. Chemiluminescent blot analysis of mutant PA1244 PilA-EC PhoA fusion expressed in PA1244. Chemiluminescent blot of IS cell fractions of IPTG-induced PA1244/pPilAPaas and PA1244/pMMB66EH (empty vector) for mutant PA1244 PilA-EC PhoA-AAS fusion protein using anti-BAP monoclonal antibody as a probe (A) and anti-O7 monoclonal antibody 11.14 as a probe (B). Purified glycosylated PA1244 pilin was used as a control for the 11.14 antibody (B).

The original and mutated fusion constructs were then transformed into PA103 wzypa11::aacC1 in the presence of PilO, and whole cell extracts prepared from broth grown cells were subjected to Western blot analysis (Figure 21). Unpublished observations have indicated near identical glycosylation efficiency in broth and plates with this strain. Western blot analysis in this system also demonstrated that the mutated fusion was not glycosylated based on migration on SDS-PAGE probed with anti-BAP (Figure 21A) and anti-PA1244 pilin (5.44) (Figure 21B), and by the lack of detection with an anti-O11 antibody (Figure 21C). These results indicate an inability of the A-A-S
peptide extension to serve as a compatible site for fusion glycan acceptance in the PA103 wzyPaO11::aacC1 glycosylation system.

The anti-BAP and 5.44 blots indicate that the original fusion has a slightly higher molecular weight, which may be due to degradation of the mutant fusion (Figure 21A&B). PhoA degradation appears evident by the presence of two lower weight bands on the anti-BAP blot in the lanes of both, original and unmutated, fusion proteins (Figure 21A). However, the 5.44 blot does not show detection of these degradation products, indicating that the 5.44 immunological epitope of PA1244 pilin is being cleaved from the fusion (Figure 21B). Additionally, the bands observed in the anti-O11 blot (Figure 21C) around 38-40 kDa are unknown, yet established artifacts of this probe, and the absence of lipid A and core plus one O-antigen bands are attributed to running off of the polyacrylamide gel during electrophoresis. Overall, this glycosylation incompatibility in both systems, PA1244 and PA103 wzyPaO11::aacC1, suggests that PilO may require a yet unestablished degree of proximity or accessibility to pilin-specific structures.
Figure 21. Western blot analysis of mutant PA1244 PilA-EC PhoA fusion expressed in PA103 *wzy*<sub>pa</sub>011::*aacC1*. Western blot of IPTG-induced PA103 *wzy*<sub>pa</sub>011::*aacC1*/pPilAP and PA103 *wzy*<sub>pa</sub>011::*aacC1*/pPilAPaas for PA1244 PilA-EC PhoA and mutant PA1244 PilA-EC PhoA-AAS fusion proteins in the presence of PilO (pUCP26*pilO*) using anti-BAP monoclonal antibody as a probe (A), anti-PA1244 pilin monoclonal antibody 5.44 (B), or anti-O11 monoclonal antibody (C) as a probe. Whole cell extracts of PA103 and HB101 cultured with phosphate-limited (PL) media were used as controls for anti-BAP monoclonal antibody (A). Whole cell extract of IPTG-induced PA103 *wzy*<sub>pa</sub>011::*aacC1*/pPAC24*pilO* was used as a control for 5.44 (B) and anti-O11 (C).

<table>
<thead>
<tr>
<th>Strain</th>
<th>PhoA Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA1244/pMMB66EH</td>
<td>0.0</td>
</tr>
<tr>
<td>PA1244/pECAP</td>
<td>2,756.1</td>
</tr>
<tr>
<td>PA1244/pECAPAas</td>
<td>1,772.3</td>
</tr>
<tr>
<td>PA1244/pPilAPaas</td>
<td>2,121.5</td>
</tr>
</tbody>
</table>

Table 4. Periplasmic phosphatase activity of alkaline phosphatase constructs in PA1244 and PA103 *wzy*<sub>pa</sub>011::*aacC1* strain backgrounds.
D. Can a non-pilin protein be engineered to accept the O-antigen repeating unit of lipopolysaccharide? Although the fusion protein was unable to be engineered for glycosylation, the necessity of pilin specific structures was further pursued by attempting to glycosylate E. coli alkaline phosphatase not fused to PA1244 pilin. To determine whether the E. coli alkaline phosphatase protein can be engineered for glycosylation, an A-A-S peptide extension was added to the C-terminal lysine residue of PhoA and cloned into pMMB66EH, forming pECAPaas. Expression of this construct was first assessed in E. coli. Whole cell extracts of HB101/pECAPaas were subjected to Western blot analysis probed with a monoclonal anti-BAP antibody (Figure 22). The mutated PhoA was successfully expressed and detected on the blot at the expected molecular weight of about 47.4 kDa (Figure 22). As observed in the lanes containing unmutated PhoA (pHK734, pECAP) and mutated PhoA (pECAPaas), when these constructs are overexpressed, they were observed as two closely stacked bands. The faint bands below were thought to be further degradation products of PhoA. This banding pattern can be seen when expressed in all strains (HB101, PA1244, and PA103 wzyPaO11::aacC1).
Figure 22. Western blot analysis of mutant *E. coli* alkaline phosphatase in *E. coli*. Western blot of IPTG-induced HB101/pECAP, and HB101/pECAPAas whole cell extracts for EC PhoA and mutant EC PhoA-AAS proteins using anti-BAP monoclonal antibody as a probe. A whole cell extract of IPTG-induced HB101/pHK734 was used as a positive control for wild type PhoA expression and the anti-BAP antibody. A whole cell extract of HB101 without a vector was used as an additional negative control to show that endogenous alkaline phosphatase did not react with the anti-BAP monoclonal antibody.

Again, two *P. aeruginosa* systems, PA1244 and PA103 wzyPaO11::aacC1, were used to assess the glycosylation status of an *E. coli* PhoA protein engineered with a minimally pilin-compatible PilO-recognition site (A-A-S). The PA1244 system was used first because it is the model strain for O-linked pilin glycosylation. The PA103 wzyPaO11::aacC1 system was then utilized because it eliminated LPS-band contamination on Western blots probed with anti-O-antigen antibody. Functional periplasmic alkaline phosphatase activity was recorded for all alkaline phosphatase containing constructs in both, PA1244 and PA103 wzyPaO11::aacC1, systems (Table 4). This demonstration of periplasmic functionality suggests that alkaline phosphatase is able to be targeted to the periplasmic space by its signal sequence in *P. aeruginosa* and achieve a functional conformation. Because PhoA is known to localize to the periplasmic space, periplasmic fractions (PF) of plate grown PA1244/pECAPAas and PA1244/pECAP strains were
subjected to Western blot analysis. Western blot results show that the mutated PhoA was not glycosylated based on migration on SDS-PAGE probed with anti-BAP (Figure 23A), and by lack of a reaction with an anti-O7 antibody (11.14) (Figure 23B). However, the detection of LPS ladder in the anti-O7 blot of Figure 23B made identification of a glycosylated PhoA band difficult. The PA103 wzy\textsubscript{Pa}11::\textit{aacC1} system was used later to eliminate this problem.

![Western blot analysis of mutant \textit{E. coli} alkaline phosphatase in PA1244.](image)

**Figure 23.** Western blot analysis of mutant \textit{E. coli} alkaline phosphatase in PA1244. Western blot of pellet and periplasmic fractions (PF) of IPTG-induced PA1244/pECAP and PA1244/pECAP\textsubscript{aas} for EC PhoA and mutant EC PhoA-AAS proteins using anti-BAP monoclonal antibody as a probe (A) and anti-O7 monoclonal antibody 11.14 as a probe (B). Purified glycosylated PA1244 pilin was used as a control for 11.14 (B). S and IS fractions of IPTG-induced PA1244/pMMB66EH were used as negative controls for both antibodies.

Periplasmic fractions of plate and broth grown PA1244/pECAP\textsubscript{aas} were subjected to protein separation by electrofocusing and subsequent immunoblot analysis (Figure 24). The O7 glycan is known to be acidic. Covalent addition of the O7 glycan to mutated PhoA would cause it to migrate to a more acidic isoelectric point on the pH-
based gel. The mutation, A-A-S, itself, is not expected to alter migration because alanine and serine are uncharged. Immunoblot analysis of electrofocused protein extracts using an anti-BAP antibody (Figure 24A) demonstrated no deviation of migration pattern between mutated and unmutated PhoA, suggesting mutant PhoA is not glycosylated. The multiple bands observed in this blot were thought to be migration of degradation products. The possibility of these bands being glycosylated was ruled out because they were consistent between mutated and unmutated fractions and they do not appear in the 11.14 blot. Immunoblot analysis of electrofocused PhoA proteins using 11.14 (Figure 24B) demonstrated no reaction with the anti-O7 antigen, again, suggesting mutant PhoA was not glycosylated. The smear at the bottom of the 11.14 blot was thought to be an artifact of handling the blot or from minimal LPS contamination. These results indicate an inability of the A-A-S peptide extension to tag the PhoA protein for glycan acceptance using the native physiological glycosylation mechanism of PA1244.
**Figure 24. Isoelectric focusing analysis of mutant E. coli alkaline phosphatase in PA1244.** Immunoblot of electrofocused periplasmic fractions containing ECAP and ECAP-AAS produced by IPTG-induced plate grown PA1244/pECAP, plate grown PA1244/pECAPaas, and broth grown PA1244/pECAPaas using anti-BAP monoclonal antibody as a probe (A) and anti-O7 monoclonal antibody 11.14 as a probe (B). Purified glycosylated PA1244 pilin was used as a control for 11.14 (B).

The unmutated and mutated PhoA constructs were then transformed into PA103 wzyP011::aacC1 in the presence of PilO, and broth grown whole cell extracts were subjected to Western blot analysis (Figure 25). Western blot analysis in this system also demonstrated that mutated PhoA was not glycosylated based on migration on SDS-PAGE probed with anti-BAP (Figure 25A), and by the lack of detection with an anti-O11 antibody (Figure 25B). These results indicate an inability of the A-A-S peptide extension to serve as a compatible site for PhoA glycan acceptance in the PA103 wzyP011::aacC1 glycosylation system. The anti-BAP blot indicates that the mutated PhoA has a slightly higher molecular weight (Figure 25A). The bands observed
in the anti-O11 blot (Figure 25B) around 38-40 kDa are unknown, yet established artifacts of this probe, and the absence of lipid A and core plus one O-antigen bands are attributed to running off of the polyacrylamide gel during electrophoresis. Overall, this glycosylation incompatibility in both, PA1244 and PA103 \( \text{wzy}_{\text{Pa}O11::\text{aac}C1} \), systems suggests the requirement for pilin-specific structures or a longer peptide extension, such as the highly antigenic peptide tail that was engineered on the PA683 pilin above.

Figure 25. Western blot analysis of mutant \( \text{E. coli} \) alkaline phosphatase in PA103 \( \text{wzy}_{\text{Pa}O11::\text{aac}C1} \). Western blot of IPTG-induced PA103 \( \text{wzy}_{\text{Pa}O11::\text{aac}C1}/\text{pECAP} \) and PA103 \( \text{wzy}_{\text{Pa}O11::\text{aac}C1}/\text{pECAPAas} \) cell extracts for EC PhoA and mutant EC PhoA-AAS proteins in the presence of PilO (pUCP26\( \text{pilO} \)) using anti-BAP monoclonal antibody as a probe (A) and anti-O11 monoclonal antibody as a probe (B). Whole cell extracts of PA103 and HB101 cultured with phosphate-limited (PL) media were used as controls for anti-BAP monoclonal antibody (A). Whole cell extract of IPTG-induced PA103 \( \text{wzy}_{\text{Pa}O11::\text{aac}C1}/\text{pPAC24/pilO} \) was used as a control for anti-O11 (B).
IV. DISCUSSION

Glycosylation is a posttranslational modification of protein that has been observed in eukaryotes, archea, and prokaryotes (Abu-Qarn et al., 2008). Two types of glycosylation have been identified, N-linked and O-linked. N-linked glycosylation occurs when a saccharide modification is covalently linked to the amide nitrogen of an asparagine side chain. N-glycosylation generally occurs at a conserved consensus sequence (AsnXxxSer/Thr/Cys, where X and x are any amino acid except proline) and has been reported in eukaryotes, archea, and prokaryotes. O-linked glycosylation occurs when a saccharide is covalently linked to the hydroxy oxygen of a serine or threonine side chain. O-glycosylation has been shown to occur in eukaryotes and prokaryotes with no known consensus sequence identified to date. Interestingly, the study of protein glycosylation in prokaryotes has identified a high incidence of this posttranslational modification in mucosal pathogens such as Campylobacter jejuni, Helicobacter pylori, Neisseria gonorrhoeae, N. meningitidis, and P. aeruginosa (Szymanski & Wren, 2005; Abu-Qarn et al., 2008). Although the specific role of glycosylation in these bacterial pathogens is still unclear, studies have demonstrated that abololition of glycosylation has resulted in diminished ability to survive within a host (Szymanski et al., 2002; Hendrixson & DiRita, 2004; Arora et al., 2005; Smedley et al., 2005; Szymanski & Wren, 2005; Abu-Qarn et al., 2008). Additionally, prokaryotic glycosylation systems have been shown to utilize saccharide residues distinct from those utilized in eukaryotic glycosylation (Abu-Qarn et al., 2008). These two findings appeal to the generation of bioconjugate vaccines targeting the glycan modification.
If a glycan is used as a target of vaccination, it would ideally be conjugated to a highly immunogenic peptide. The mechanism of glycosylation in PA1244 is very appealing to vaccine design because PilO utilizes preassembled LPS O-antigen subunits to glycosylate the highly immunogenic pilin protein, thereby stimulating host immune defenses to pilin, glycan, and LPS surface layer of gram-negative pathogens. Current cross linking methods allow normally non-glycosylated proteins such as toxoids to serve as the protein conjugate to glycan units. The goal of the current research was determine whether or not normally non-glycosylated proteins could be engineered for glycosylation in vivo by exploiting the O-glycosylation machinery of P. aeruginosa.

A. The role of TfpY as a group III pilin accessory protein. MALDI-TOF analysis in conjunction with SDS-PAGE and electrofocusing reveal that PA683 pilin is not modified, and therefore, TfpY confers no direct modification to this surface protein. The unusual migratory pattern of PA683 pilin on SDS-PAGE is interpreted to result from the inability of SDS detergent to completely denature the pilin protein—although other detergents were not tested. The hydrophobicity of SDS may not be able to disrupt hydrophobic residues of the α-helix tail causing the pilin to migrate to a molecular weight lower than expected. Recently, Asikyan et al. (2008) have identified a role for TfpY in pilin polymerization. Specifically, they showed that complementation of a PAO1 pilA mutant with pilA-tfpY, from group III strain PA14, could restore twitching motility and recoverable surface pili, but complementation with pilA alone could not restore these phenotypes. Their results also suggested an antagonistic role to PilT, an accessory protein known to be involved in pilus retraction (Asikyan et al., 2008). However, results
from the Castric laboratory disagree with these findings. Complementation of the pilAO mutant, PA1244N3, with PA683 pilA, alone, was able to restore twitching motility (personal communication, with P.A. Castric) and surface pili. Although band intensity was not quantified for significance, the surface pili in Figure 16B suggests TfpY has no effect on polymerization. In this figure, complementation with PA683 pilA and PA683 pilA-tfpY appeared to yield comparable amounts of surface pili. As future work, the role of TfpY as a pilin accessory protein should continue to be pursued.

**B. Glycosylation of Group III pilin engineered with C-terminal peptide extensions.** Western blot analysis showed that both, -AAS and peptide tail, extensions permitted the larger, more hydrophobic PA683 pilin to be glycosylated in *P. aeruginosa*. These findings, along with similar results with mutant PA103 pilins, suggest pilin-specific recognition by PilO (Horzempa *et al.*, 2006a; personal communication, with M. Qutyan). However, each of the engineered PA683 pilin mutants yielded minimal reaction with primary antibody upon immunoblotting, which indicates that the ability to produce glycosylated PA683 pilin is less efficient then native group I (PA1244) pilin or group II (PA103 mutants) pilin (Horzempa *et al.*, 2006a; personal communication, with P. A. Castric & M. Qutyan). Inefficiency of glycosylation may be directly related to pilin-specific properties such as surface charge, as indicated by Horzempa *et al.* (2006a). Horzempa *et al.* (2006a) identified a significant difference in DSL charges of PA1244 pilin (pH 9.31, basic) and PA103 pilin (pH 4.53, acidic). The DSL of PA683 pilin possesses an intermediate pH of 6.17(slightly acidic), which would suggest the
surrounding tertiary surface environment as the source of charge incompatibility or steric hindrance.

Western analysis of both pilin mutants also revealed what appeared to be significant degradation compared to expression of the unmutated pilin, which suggests these mutations target the pilin for destruction. Future experiments, such as expressing PilO and the mutant pilins in strain 683 and engineering PA683 pilin with a longer C-terminal peptide extension, would be performed in an attempt to optimize the glycosylation of mutant PA683 pilins.

C. Inability of the minimal –AAS peptide extension to permit glycosylation of a PA1244 PilA- *E. coli* alkaline phosphatase fusion protein. Western blot analysis revealed that a PA1244 PilA- *E. coli* (EC) alkaline phosphatase (PhoA) fusion protein with a C-terminal –AAS peptide extension was unable to be glycosylated in *P. aeruginosa*. These findings suggest that PilO is unable to access the C-terminal serine of the mutated fusion. Recognition by PilO may be hindered by the bulky surface structure of EC PhoA, which may or may not be of compatible charge, and/or the lack of accessibility to pilin-specific structures. Dimerization of PhoA monomers can be ruled out based on the established dimerization site demonstrated in Figure 13, which shows the C-termini of both monomers to be surface exposed. With the possibility that the –AAS peptide extension is unstable or is not accessible to PilO, Dr. Mohammed Qutyan engineered the fusion to present a longer peptide extension. When the 15 amino acid peptide epitope of PA1244 used to engineer PA683 pilin was added to the C-terminus of the fusion (pPilApep), glycosylation was achieved. This was accomplished by western
analysis using membrane fractions of broth grown PA103 \( wzy_{Pa011}::aacC1 \) with anti-BAP and anti-O11 antibodies used as probes (personal communication, with M. Qutyan). The longer peptide tail extension was able to extend the glycosylation site far enough away from the bulky surface of EC PhoA to permit interaction with PilO. This finding, however, still does not rule out the necessity of pilin-specific structures or the necessity of membrane localization.

Additionally, western blot analysis revealed that both, original and mutated, fusion proteins were being targeted for degradation in all strains (\( E. coli \), PA1244, and PA103 \( wzy_{Pa011}::aacC1 \)). Degradation only showed up in immunoblots probed with anti-BAP, while no pilin degradation was observed in blots probed with 5.44, which suggests that only the EC PhoA portion of the fusion was being targeted for cleavage. The –AAS peptide extension may be getting targeted for cleavage, which could account for the lack of reaction with the primary antibody probes. MALDI-TOF should be performed as a future experiment to confirm if both, original and mutated, fusions are being expressed as expected.

D. Inability of the minimal –AAS peptide extension to permit glycosylation of \( E. coli \) alkaline phosphatase. Western blot analysis revealed that an \( E. coli \) alkaline phosphatase protein with a C-terminal –AAS peptide extension was unable to be glycosylated in \( P. aeruginosa \). The lack of PilO substrate recognition may have resulted from an inability to access the C-terminal serine residue of the mutated EC PhoA. This may have been due to surface incompatibilities, such as steric hindrance or charge, the need for pilin-specific structures, or the necessity of a membrane anchor. As with the
fusion protein, to circumvent the possibility that the –AAS peptide extension is unstable or is not accessible to PilO, Dr. Mohammed Qutyan engineered EC PhoA to present a longer peptide extension. Periplasmic fractions of broth grown PA103 \( \text{wzy}_{\text{P}0_{11}}::\text{aacC1} \) cells subjected to western analysis revealed that EC PhoA was able to be glycosylated when engineered with a 15 amino acid peptide tail extension (pECAP pep) (personal communication, with M. Qutyan). These findings indicate that the lack of glycosylation of the EC PhoA-AAS construct resulted from surface environment incompatibilities and not on the necessity of pilin-specific structures or a membrane anchor. However, these findings do not rule out the possibility that pilin-specific structures or membrane localization may enhance the glycosylation reaction. In particular, membrane localization may make the substrate more readily accessible to PilO, since PilO is embedded in the same membrane.

As with the fusion constructs, the unmutated and mutated EC PhoA constructs should be subjected to MALDI-TOF analysis to determine whether or not these proteins are being expressed as expected. If the –AAS tripeptide extension is being targeted for cleavage, these results may provide an additional explanation for the lack of reaction with primary antibody probes.

E. Significance to vaccine design. Previous work from the Castric laboratory has demonstrated that PilO has low glycan substrate recognition (DiGiandomenico et al., 2002; Horzempa et al., 2006b). These findings suggest that a variety of O-antigens may be utilized to glycosylate pilin in \( P. \text{aeruginosa} \). The current findings provide further information on glycosylation substrate specificity, which has direct application to the
construction of vaccine components (Horzempa et al., 2006a; Horzempa et al., 2008).
Specifically, other pilins may be used to produce a tripartite pilin vaccine composed of
the pilin monomer, a glycosylation epitope, and an O-antigen subunit. In the current
case, PA683 pilin was engineered with a highly immunogenic PA1244 epitope tail
(Castric & Deal, 1994), and covalently attached O11 O-antigen repeating unit. A vaccine
component of this nature has the potential to induce a protective immune response
against PA683 pilin, PA1244 pilin, and the LPS surface layer of PA103. This same
manipulation may be applied to such pilins as that of N. gonorrhoeae, which has
previously been shown to be expressed in P. aeruginosa (Hoyne et al., 1992). The work
done by Dr. Mohammed Qutyan, which demonstrated that EC PhoA could be
glycosylated with the PA1244 peptide tail extension, further expands the potential for
vaccine design. These data suggest that non-pilin proteins, such as bacterial toxins, may
be expressed in P. aeruginosa and glycosylated with O-antigen subunits. Overall, the
manipulation of the glycosylation machinery of P. aeruginosa portrays the potential to
mix and match various antigens of bacterial pathogens, particularly gram-negatives, for
the production of versatile vaccine components.

F. Were my thesis goals achieved? Yes, my thesis goals were achieved. The original
study intended to assess the ability of a C-terminal –AAS peptide extension to
glycosylate several normally, non-glycosylated proteins in P. aeruginosa. After
completing all –AAS constructs and observing glycosylation of only the PA683 pilin
mutant, it was not in the best interest of time to apply the peptide tail extension (which
also glycosylated PA683 pilin) to the fusion protein or EC PhoA. Because of this high
risk endeavor, I focused on the TfpY study as my exit project, while Dr. Castric’s post-doc, Dr. Mohammed Qutyan, in a collaborative publication attempt, constructed the last two peptide tail constructs, pPilAPpep and pECAPpep.
V. REFERENCES


