LIPID A REGIOSELECTIVITY OF THE ESCHERICHIA COLI
PALMITOYLTRANSFERASE PAGP

By

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A Thesis Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree Master of Science

McMaster University

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MASTER OF SCIENCE (2014)  McMaster University
(Biochemistry)  Hamilton, Ontario

TITLE: Lipid A regioselectivity of the Escherichia coli palmitoyltransferase PagP

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NUMBER OF PAGES: xii, 74
ABSTRACT

The outer membrane palmitoyltransferase PagP possesses regioselectivity for the palmitoylation of the \((R)-3\)-hydroxymyristate chain at position 2 on the proximal glucosamine unit of lipid A. The residues Arg45 and Arg49 in the L1 loop appear to poise their guanidinium groups so as to interact with the proximal and distal phosphate groups at positions 1 and 4’ of lipid A, respectively. Both single and double substitution of these arginine residues with serine has no effect on the folding, stability, phospholipase and palmitoyltransferase activities. Additionally, the arginine to serine substitutions display wild-type regioselectivity and specific activity in the palmitoylation of the biosynthetic precursor lipid IV\(_A\) as indicated by collision induced fragmentation MS/MS. *In vivo*, lipid A analysis in a *msbB/pagP* deletion strain shows no difference in acylation pattern as compared to the wild-type. These results establish both *in vitro* and *in vivo* that the arginine to serine substitutions have no effect on lipid A regioselectivity.
ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor, Dr. Russell Bishop for his incredible amount of compassion, patience and support for me during my masters. He was a fantastic mentor, with a depth of knowledge and insight without which this work would not have been possible. I would like to thank my committee members, Dr. Paul Berti and Dr. Gerry Wright for their invaluable expertise and experience, as well as taking the time out of their busy schedules. I would also like to thank my colleagues in the Bishop lab, both past and present, who have been an absolute pleasure to work with and who’s company and friendship I have deeply enjoyed. I would also like to thank the support staff in the Department of Biochemistry and Biomedical Science at McMaster University, in particular Lisa Kush who was incredibly helpful and sympathetic. Finally, to my friends and family, for all of your love and support, thank you.
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<td>APS</td>
<td>Ammonium persulfate</td>
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<tr>
<td>CAMP</td>
<td>Cationic antimicrobial peptide</td>
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<td>CD</td>
<td>Circular dichroism</td>
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<tr>
<td>DDM</td>
<td>$n$-dodecyl-$\beta$-D-maltopyranoside</td>
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<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
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<tr>
<td>DPPC</td>
<td>Dipalmitoylphosphatidylcholine</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
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<tr>
<td>ESI</td>
<td>Electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-$\beta$-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kdo</td>
<td>3-deoxy-$D$-$\alpha$-manno-$\alpha$-uloseonic acid</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
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<td>LDAO</td>
<td>Lauroyltrimethylamine $N$-oxide</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MD-2</td>
<td>Myeloid differentiation protein 2</td>
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<td>MS</td>
<td>Mass spectrometry</td>
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<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PtdEtn</td>
<td>Phosphotidylethanolamine</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>SOC</td>
<td>Super optimal broth with catabolic repressor</td>
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<td>TCA</td>
<td>Trichloroacetic acid</td>
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<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
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<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
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<tr>
<td>TOF</td>
<td>Time-of-flight</td>
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<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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CHAPTER 1 – INTRODUCTION

1.1 The Outer Membrane

The outer membrane of Gram-negative bacteria is distinctly characterized by an asymmetric bilayer of lipids between the inner and outer leaflets. The inner leaflet is primarily comprised of phospholipids, while the outer leaflet is composed of lipopolysaccharide (LPS) (Raetz and Whitfield, 2002; Raetz et al., 2007). The LPS is composed of the O-antigen polysaccharide, the outer and inner core oligosaccharides, and the biologically active lipid A (endotoxin) hydrophobic anchor, which actually forms the outer membrane (Raetz and Whitfield, 2002; Raetz et al., 2007) (Figure 1). Lipid A consists of a β-1’,6-linked disaccharide of glucosamine, which is phosphorylated at the 1 and 4’ positions and acylated with (R)-3-hydroxymyristate chains at the 2, 3, 2’ and 3’ positions of the proximal and distal glucosamine units, respectively; with secondary acyloxyacylations with laurate and myristate chains on the 2’ and 3’ (R)-3-hydroxymyristate chains of the distal glucosamine unit, respectively (Raetz and Whitfield, 2002; Raetz et al., 2007). The addition of two 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) sugar moieties of the inner core oligosaccharide forms Kdo2-lipid A,
Figure 1: Schematic structure of the *E. coli* cell envelope. The phospholipids represent phosphatidylethanolamine (red) and phosphatidylglycerol (yellow) Abbreviations: LPS, lipopolysaccharide; PPEtn, ethanolamine diphosphosphate; Heptose, L-glycero-D-manno-heptose; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid: MDO, membrane derived oligosaccharide (Figure taken from Raetz *et al.*, 2007).
which represents the absolute essential component of LPS to produce viable cells (Raetz and Whitfield, 2002; Raetz et al., 2007) (Figure 2).

1.2 The Lipid Anchor, Lipid A

The characteristic phosphate groups and acidic sugar units in LPS confer a predominantly negative charge to the bacterial cell surface that is strongly stabilized by bridging magnesium divalent cations (Coughlin et al, 1983). The strong electrostatic and hydrophobic lateral interactions between Kdo₂-lipid A molecules provide an effective permeability barrier against hydrophobic antibiotics and detergents (Nikaido and Vaara, 1985, Nikaido, 2003). However, it also makes the bacteria susceptible to cationic antimicrobial agents produced by the immune system to fend off infection (Epand and Epand, 2011; Raetz et al., 2007). In response to this vulnerability, bacteria have evolved several different enzymatic mechanisms to modify the structure of lipid A to neutralize the negative charges of the phosphate groups (Trent, 2004; Raetz et al., 2007) and to add or remove acyl chains to influence the lipid hydrophobicity (Trent et al, 2001; Kawasaki et al. 2004; Raetz et al., 2007) (Figure 3) to not only resist uptake of these antimicrobial agents but to also attenuate their production by the host immune system (Kawasaki et al. 2004; Raetz et al., 2007), one such modification is palmitoylation of lipid A (Bishop et al., 2000; Raetz et al., 2007).
Figure 2: Structure of Kdo<sub>2</sub>-lipid A. The chemical structure of the lipid anchor of LPS, lipid A; and the first two Kdo sugar units of the inner core oligosaccharide.
Figure 3: Covalent modifications of Kdo₂-lipid A. The covalent modifications of Kdo₂-lipid A in *E. coli* K-12 and *Salmonella* as indicated by the substituents with the dashed bonds and highlighted in red. ArnT and EptA (PmrC) are under the control of PmrA/B. PagP and PagL are regulated by PhoP/Q. LpxO, LpxR, and EptB are not regulated by either PhoP/Q or PmrA/B. Asterisks indicate modification enzymes not found in *E. coli* K-12.
1.3 The Palmitoyltransferase PagP

The *E. coli* outer membrane phospholipid:lipid A palmitoyltransferase, PagP, catalyzes the transfer of a palmitate chain (16 carbon) from the \( sn \)-1 position of a phospholipid, aberrantly migrated into the outer leaflet of the outer membrane due to membrane perturbation, to the free hydroxyl group on the \( N \)-linked (\( R \))-3-hydroxymyristate chain at position 2 of the proximal glucosamine unit of lipid A, producing \( sn \)-1 lyso-phospholipid, which gets translocated back to the inner leaflet of the outer membrane, and palmitoylated-lipid A in the process (Bishop *et al.* 2000; Bishop, 2005) (Figure 4). PagP lies dormant in the outer membrane and is activated in response to outer membrane lipid asymmetry perturbation induced by the immune system during infection (Bishop, 2008); for example by cationic antimicrobial peptides (CAMPs), an amphipathic short chain peptide with a net positive charge that recognize the negatively charged surface of the bacterial cell envelop created by the negatively charged lipid A (Hancock *et al.*, 1995; Epand and Epand, 2011). CAMPs displace bridging magnesium cations responsible for stabilizing lipid A in the membrane, thereby inducing electrostatic repulsion as the CAMPs transverse the outer membrane, allowing phospholipids to translocate into the outer leaflet thereby destabilizing the outer membrane and increasing its permeability (Epand and Epand, 2011). Palmitoylation of lipid A by PagP strengthens the outer membrane by increasing the hydrophobicity of the lipid A molecule and rigidifies the outer leaflet, which then provides resistance to CAMPs in order to evade the host immune system (Guo *et al.*, 1998; Bishop, 2008).
Figure 4: Palmitoyltransferase reaction of PagP. The PagP catalysed transfer of a palmitate chain (16:0) from the sn-1 position of a phospholipid to the free hydroxyl group on the N-linked (R)-3-hydroxymyristate chain at position 2 of the proximal glucosamine unit of lipid A, producing sn-1 lyso-phospholipid and palmitoylated-lipid A in the process.
1.4 Attenuation of the TLR4/MD2 Pathway

In addition to its biophysical effects on the outer membrane, palmitoylated-lipid A attenuates the innate immune response through the toll-like receptor 4 (TLR4) and myeloid differentiation protein 2 (MD-2) signal transduction pathway (Tanamoto and Azumi, 2000; Muroi et al., 2002; Bishop, 2005). The TLR4/MD-2 signal transduction pathway recognizes the lipid A portion of LPS shed from the surface of bacteria during infection, leading to the production of various cytokines and activation of the host immune system (Park et al., 2009; Raetz et al., 2006). During an acute infection overstimulation of the TLR4/MD-2 signal transduction pathway can lead to septic shock and death. Palmitoylated-lipid A binds to the TLR4/MD-2 complex and interferes with dimerization necessary for signal transduction, thereby attenuating the immune response (Miller et al., 2005; Bishop, 2005) (Figure 5). This is also important to vaccine formulation, where a palmitoylated-lipid A derivative, 3-O-desacyl-4’-monophosphoryl lipid A (MPLA) is used as an adjuvant to gently stimulate the innate immune system in order to facilitate production of antibodies by the adaptive immune system for the specific antigen presented in the vaccine (Presing et al., 2002; Hawkins et al., 2004; Casella and Mitchell, 2008).
Figure 5: Structural basis for lipid A binding to the TLR4/MD-2 complex. The crystal structure of the TLR4/MD-2/LPS complex (PDB 3FX1). During infection the TLR4/MD-2 complex recognizes LPS shed from the surface of bacteria. The lipid A portion of LPS binds to MD-2 and facilitates dimerization with TLR4 in order to generate signal transduction. Palmitoylation of the lipid A at R2 interferes with the dimerization surface of the TLR4/MD-2 complex.
1.5 Structure and Function of PagP

The pagP gene was first identified in Salmonella (Gue et al., 1997; Guo et al., 1998) and its E. coli homologue was later purified from the outer membrane and characterized as a phospholipid:lipidA palmitoyltransferase (Bishop et al., 2000). The structure and dynamics of the E. coli PagP have been solved by X-ray crystallography (Ahn et al., 2004; Cuesta-Seijo et al., 2010) and solution nuclear magnetic resonance (NMR) spectroscopy (Hwang et al., 2002; Hwang et al., 2004) to reveal an 8 stranded anti-parallel β-barrel preceded by a short amphipathic α-helix at its N-terminus (Bishop, 2008) (Figure 6). The β-barrel sits in the outer membrane with its barrel axis tilted by ~25° with respect to the membrane normal, as evident by the hydrophobic surface potential, the aromatic belt that demarcates the membrane interface (Bishop, 2008), and experimentally by NMR (Evanics et al., 2006). The active site residues are exposed to the cell’s exterior surface, which explains why PagP remains dormant in the outer membrane as long as lipid asymmetry is maintained (Bishop, 2008). The NMR spectroscopy revealed a dynamic protein that exists between an inactive relaxed (R) state and an active tense (T) state (Hwang et al., 2004).

1.6 The Hydrocarbon Ruler

At the centre of PagP is the palmitate-binding pocket known as the hydrocarbon ruler (Khan et al., 2007), designated by bound detergent molecules in the crystal
Figure 6: Structure of *E. coli* PagP. The annotated crystal structure of *E. coli* PagP in the detergent LDAO (PDB 1THQ) with the highly dynamic L1 loop was modelled in by energy minimized molecular dynamic simulations. The bound detergent molecule, shown as magenta spheres, designates the hydrocarbon ruler and the aromatic belt, shown as black sticks, indicates the membrane interface, tilting the β-barrel ~25° angle to the membrane normal. The catalytically relevant residues, His33, Ser77 and Arg114, are shown as sticks and are exposed to the cell’s exterior surface.
structures (Ahn et al., 2004; Cuesta-Seijo et al., 2010; Bishop, 2008). The hydrocarbon ruler interrogates the phospholipid acyl chain to specify a palmitate chain rather than shorter acyl chains such as myristate (14 carbon) (Khan et al., 2007). The bottom of the hydrocarbon ruler can be modified at Gly88 by site-directed mutagenesis (Khan et al., 2007; Khan et al., 2010a) or chemical modification (Khan et al., 2010b) to shorten the acyl-chain specificity of PagP. By this process it was found that PagP binds the distal six carbon atoms of the acyl chain thereby positioning a palmitate chain in the active site, where as a myristate chain would be too short and position the carbonyl group lower than the active site (Khan et al., 2010b).

1.7 The Crenel and Embrasure

There are two disruptions in the β-barrel hydrogen bonds that allow for lateral access of lipid substrates to the hydrocarbon ruler, termed the crenel and embrasure, produced by Pro127/Pro144 and Pro28Pro50, respectively (Khan et al., 2009). The crenel is between strands F and G and is responsible for phospholipid access. The embrasure is on the opposite side of the enzyme between strands A and B and is responsible for lateral lipid A access (Khan et al., 2009) (Figure 7). Crenel-gating by the L4 loop, specifically by hydrogen bonding between Tyr147 and Leu125, is responsible for sn-1 regioselectivity of the phospholipid donor and blockage of acyl chains larger than palmitate such as stearate chains (18 carbon) (Cuesta-Seijo et al., 2010; Khan and Bishop, unpublished data). Tyr147 and Leu125 positioned at the crenel vestibule specifically
Figure 7: Crenel and embrasure. The annotated crystal structure of *E. coli* PagP in the detergent LDAO (PDB 1THQ) with the highly dynamic L1 loop was modelled in by energy minimized molecular dynamic simulations, shown from opposing sides to highlight the two disruptions in the $\beta$-barrel hydrogen bonds that allow for lateral access of lipid substrates to the hydrocarbon ruler, termed the crenel and embrasure. The crenel is produced by Pro127/Pro144 between strands F and G and is responsible for phospholipid access. The embrasure is produced by Pro28Pro50 on the opposite side of the enzyme between strands A and B and is responsible for lateral lipid A access.
interrogate the sn-1 acyl chain and prevent acyl chains larger than palmitate from maneuvering over the crenel aperture (Khan and Bishop, unpublished data).

1.8 Phospholipid Binding and Crenel Gating

During phospholipid binding at the crenel the L4 loop is displaced as the acyl chain disrupts the hydrogen bonding between Tyr147 and Leu125 and enters the hydrocarbon ruler (Cuesta-Seijo et al., 2010; Khan and Bishop, unpublished data) (Figure 8). The displaced L4 loop pushes the L1 loop into the plane of the membrane due to steric hindrance as evident from the crystal structures of PagP (Cuesta-Seijo et al., 2010). While at the embrasure the large conformational change of the L1 loop shifts Arg45 and Arg49 into the membrane, which we believe is responsible for binding and orienting the proximal and distal phosphate groups of lipid A at the embrasure so that the proximal glucosamine unit is poised for catalysis (Figure 9).

1.9 Lipid A Binding at the Embrasure

The sequence alignment of PagP homologues shows that Arg45 and Arg49 are not conserved residues and are substituted with serine or a hydrophobic amino acid, for example in Yersinia and Bordetella spp. (Figure 10). E. coli PagP palmitoylates the lipid A precursor, lipid IV$_A$ at the 2 position on the proximal glucosamine unit to produce lipid IV$_B$ (Bishop et al., 2000). While Yersinia spp. have an Arg45Ser substitution and
Figure 8: Phospholipid binding and crenel gating. The annotated crystal structures of *E. coli* PagP in the detergent LDAO (left panel) (PDB 1THQ) and SDS/MPD (right panel) (PDB 3GP6) with the highly dynamic L1 loop was modelled in by energy minimized molecular dynamic simulations. The structures focus on the phospholipid binding site at the crenel and demonstrates crenel gating by the L4 loop and the hydrogen bonding between Try147 and Leu125.
Figure 9: Lipid A binding at the embrasure. The annotated crystal structures of *E. coli* PagP in the detergent LDAO (left panel) (PDB 1THQ) and SDS/MPD (right panel) (PDB 3GP6) with the highly dynamic L1 loop was modelled in by energy minimized molecular dynamic simulations. The structures focus on the lipid A binding site at the embrasure and shows the large conformational change of the L1 loop that shifts Arg45 and Arg49 into the plane of the membrane.
Figure 10: Sequence alignment of PagP homologues. Sequence alignment of PagP from five different pathogenic Gram-negative bacteria genera. Sequences are representative from each genus and do not include the signal peptide. The catalytically important residues, His33, Ser77 and Arg114 are shown in red and highlighted in yellow. Absolutely conserved residues are highlighted in blue and structurally similar residues are highlighted in grey. Arg45 and Arg49 are shown in yellow and highlighted in red. The annotated secondary structure is indicated above the alignment.
palmitoylate lipid IVₐ at both position 2 and 3’ on the proximal and distal glucosamine units, respectively (Aussel et al., 2000); whereas Bordetella spp. have both an Arg45Ser and Arg49Leu substitution and palmitoylate lipid IVₐ at just position 3’ on the distal glucosamine unit (Preston et al., 2003; Pilione et al., 2004) (Figure 11). In the cases of Yersinia and Bordetella spp. the palmitoylation at the 3’ position is predicted to stimulate the immune system through activation of the TLR4/MD-2 signal transduction pathway similar to that seen in Pseudomonas aeruginosa (Thaipisuttikul et al., 2014). Our hypothesis is that Arg45 and Arg49 are responsible for E. coli PagP’s regioselectivity for the hydroxymyristate chain at position 2 of the proximal glucosamine unit of lipid A during the palmitoyltransferase reaction and that when these residues are substituted with serine that E. coli PagP will relinquish its regioselectivity for lipid A and behave more like Yersinia PagP.
Figure 11: Structure of lipid IV$_A$ and its palmitoylated products. (A) The tetraacylated, lipid A biosynthetic precursor, lipid IV$_A$; (B) lipid IV$_A$ palmitoylated at the 2 position of the proximal glucosamine unit, referred to as lipid IV$_{B}$; (C) lipid IV$_A$ palmitoylated at the 3’ position of the distal glucosamine unit, referred to as lipid IV$_{B'}$; and (D) lipid IV$_A$ palmitoylated at both the 2 and 3’ positions, referred to as lipid IV$_{B''}$.
CHAPTER 2 – MATERIALS AND METHODS

2.1 Materials

The antibiotics were obtained from Sigma-Aldrich. The spin miniprep, PCR purification, and gel extraction kits were all obtained from Qiagen. The QuickChange site-directed mutagenesis kit was obtained from Stratagene. The high fidelity PCR was performed with a proofreading DNA polymerase from Fisher Thermo Scientific. Restriction endonucleases, alkaline phosphatase, T4 DNA ligase were obtained from Fisher Thermo Scientific. The His-bind resin was obtained from Novagen. The $[^{14}C]$-labelled DPPC and the $[^{32}P]$-phosphate were obtained from PerkinElmer Life Sciences. The detergents were obtained from Anatrace. The lipids were obtained from Avanti Polar Lipids and Peptides International. The TLC plates were obtained from Caledon labs. The solvents and chemicals were obtained from Sigma-Aldrich. All other materials were obtained from commercial sources.
2.2 Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are described in Table 1 and Table 2. Cells were grown at 37°C in solid or liquid Lysogeny Broth (LB) media or Super Optimal broth with Catabolic repressor (SOC) media with aeration. Antibiotics were added when necessary to a final concentration of 100 µg/mL ampicillin, 50 µg/mL kanamycin, and 35 µg/mL chloramphenicol. A single colony was isolated on solid LB media, 1.5% agar plates supplemented with the appropriate antibiotic grown at 37°C overnight and cultured in 10 mL of liquid LB media supplemented with the appropriate antibiotic and grown at 37°C with aeration overnight.

2.3 Site-Directed Mutagenesis

Plasmid DNA was purified using the QIAprep spin miniprep kit (Qiagen). Mutations were created using the oligonucleotide primer sets listed in Table 3. Site-directed mutagenesis was performed using the QuikChange procedure (Stratagene) according to the manufacturer’s instructions. The reaction mixture utilized for mutagenesis was 15 ng of template DNA, 125 ng each of the forward and reverse primers, 1 µL of proofreading PfuTurbo DNA polymerase, 5 µL of supplied 10X reaction buffer, 1 µL of supplied dNTP mix, and distilled water was added to produce a total volume of 50 µL.
### Table 1: *E. coli* strains and sources used in this work.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description and Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>XL1-Blue</td>
<td>F, λ, recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, {F’ proAB, lacIqZ M15,</td>
<td>Stratagene</td>
</tr>
<tr>
<td></td>
<td>Tn10(Tet’)}c</td>
<td></td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F, ompT, hsdSb(rK-mK'), gal, dcm, λ(DE3)</td>
<td>Stratagene</td>
</tr>
<tr>
<td>MC1061</td>
<td>F, λ, araD139, Δ(ara-leu)7697, Δ(lac)X74, galU, galK, hsdR2 (rK-mK+), mcrB1, rpsL.</td>
<td>(Casadaban and Cohen, 1980)</td>
</tr>
<tr>
<td>MC1061-msbB</td>
<td>MC1061 msbB::Tn5 (kan’).</td>
<td>(Kim et al., 2006)</td>
</tr>
<tr>
<td>WJ0124</td>
<td>MC1061 pagp::amp’</td>
<td>(Smith et al., 2008)</td>
</tr>
<tr>
<td>SK1061</td>
<td>MC1061 msbB::Tn5 (kan’), pagp::amp’</td>
<td>(Jia et al., 2004)</td>
</tr>
</tbody>
</table>
Table 2: Bacterial plasmids and sources used in this work.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description and Genotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>pET21a-PagP-HΔS-WT</td>
<td>PCR product carrying pagP cloned into pET21a+ with a C-terminal His6-tag, N-terminal signal sequence removed by PCR</td>
<td>(Hwang et al., 2002)</td>
</tr>
<tr>
<td>pET21a-PagP-HΔS-R45S</td>
<td>Derivative of pET21a-PagP-HΔS-WT</td>
<td>This study</td>
</tr>
<tr>
<td>pET21a-PagP-HΔS-R49S</td>
<td>Derivative of pET21a-PagP-HΔS-WT</td>
<td>This study</td>
</tr>
<tr>
<td>pET21a-PagP-HΔS-R45S/R49S</td>
<td>Derivative of pET21a-PagP-HΔS-WT</td>
<td>This study</td>
</tr>
<tr>
<td>pBad18</td>
<td>Low-copy vector (Amp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>(Guzman et al., 1995)</td>
</tr>
<tr>
<td>pBad18-PagP-WT</td>
<td>E. coli pagP cloned into pBad18</td>
<td>Khan and Bishop, unpublished data</td>
</tr>
<tr>
<td>pBad18-PagP-R45S</td>
<td>Derivative of pBad18-PagP-WT</td>
<td>This study</td>
</tr>
<tr>
<td>pBad18-PagP-R49S</td>
<td>Derivative of pBad18-PagP-WT</td>
<td>This study</td>
</tr>
<tr>
<td>pBad18-PagP-R45S/R49S</td>
<td>Derivative of pBad18-PagP-WT</td>
<td>This study</td>
</tr>
<tr>
<td>pBad18-cm</td>
<td>Low-copy vector (Cm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>(Guzman et al., 1995)</td>
</tr>
<tr>
<td>pBad18-cm-PagP-WT</td>
<td>E. coli pagP-WT sub-cloned into pBad18-cm</td>
<td>This study</td>
</tr>
<tr>
<td>pBad18-cm-PagP-R45S</td>
<td>E. coli pagP-R45S sub-cloned into pBad18-cm</td>
<td>This study</td>
</tr>
<tr>
<td>pBad18-cm-PagP-R49S</td>
<td>E. coli pagP-R49S sub-cloned into pBad18-cm</td>
<td>This study</td>
</tr>
<tr>
<td>pBad18-cm-PagP-R45S/R49S</td>
<td>E. coli pagP-R45S/R49S sub-cloned into pBad18-cm</td>
<td>This study</td>
</tr>
</tbody>
</table>
### Table 3: Oligonucleotide primers used for site-directed mutagenesis.

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>R45S for</td>
<td>5’-TACGACAAAGAAGCCGACCCGTTGGGT-3’</td>
</tr>
<tr>
<td>R45S rev</td>
<td>5’-ACCACGCTCGCTATAGGGGTTGGCGGT-3’</td>
</tr>
<tr>
<td>R49S for</td>
<td>5’-ACGGCCTCCATACCCGTTGGGTTGGCGGT-3’</td>
</tr>
<tr>
<td>R49S rev</td>
<td>5’-ACCGCCACCGCCCTGATGCGGT-3</td>
</tr>
<tr>
<td>R45S/R49S for</td>
<td>5’-AAAGAAGACCCGTTGGGTTGGCGGT-3</td>
</tr>
<tr>
<td>R45S/R49S rev</td>
<td>5’-ACCGCCACCGCCCTGATGCGGT-3</td>
</tr>
</tbody>
</table>

Bold and underlined nucleotides are codon of interest for mutagenesis.

### Table 4: Oligonucleotide primers used for DNA sequencing.

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7 for</td>
<td>5’-TAATACTACGACTACATAGGG-3</td>
</tr>
<tr>
<td>T7 rev</td>
<td>5’-GCTAGTTATGCTAGCGTGC-3</td>
</tr>
<tr>
<td>pBad for</td>
<td>5’-CTGTTTCTCCATACCGGTT-3</td>
</tr>
<tr>
<td>pBad rev</td>
<td>5’-GGCTGAAAATCTTCTCT-3</td>
</tr>
</tbody>
</table>
In the mutagenesis of pET21a-PagP-HΔS-WT and pBad18-PagP-WT, an initial denaturation of 5 minutes at 94°C, was followed by 25 cycles of 94°C for 1 minute, 55°C for 1 minute, and 68°C for 3 minutes. The final extension was at 68°C for 10 minutes, followed by a 4°C hold. Following the mutagenesis, 1 μL of DpnI was added to the reaction in order to digest the methylated parent plasmid, and the reaction mixture was incubated at 37°C for 1 hour. The plasmid DNA was purified using the QiaQuik PCR purification kit (Qiagen). The mutations were verified by sequencing using the oligonucleotide primer sets listed in Table 4.

2.4 DNA Manipulation

The transfer of *E. coli pagP-WT, pagP-R45S, pagP-R49S*, and *pagP-R45S/R49S* from pBad18-PagP to pBad18-cm was accomplished using standard molecular biology procedures. Plasmid DNA was purified using the QIAprep spin miniprep kit (Qiagen), digested with *SacI* and *HindIII*, and resolved by agarose gel electrophoresis. Vector and insert DNA was isolated using the QiaQuick Gel Extraction Kit and ligations reactions were performed using T4 DNA ligase.

2.5 Chemical Transformation

The plasmids were transformed into CaCl₂ chemically competent cells. Plasmid DNA (50 ng) was added to 100 μL of chemically competent cells in pre-chilled tubes
and allowed to incubate on ice for 30 minutes. The cells were then heat-shocked in a 42°C heat block for 45 seconds and incubated on ice for 2 minutes. 900 µL of prewarmed SOC media was then added to the heat-shocked cells and the transformation was incubated at 37°C with aeration for 1 hour. Cells were spread onto LB media, 1.5% agar plates supplemented with the appropriate antibiotic and incubated at 37°C overnight. Single colonies resulting from the transformation were used to inoculate an overnight culture that was then incubated at 37°C with aeration overnight, which was then used to generate 20% glycerol stock for storage at -80°C.

2.6 Expression of PagP

To express and purify PagP in an insoluble denatured state, PagP was expressed without its native signal peptide and with a C-terminal His6-tag in E. coli BL21(DE3) transformed with the expression plasmid pET21a-PagP-HΔS. A single colony was isolated on LB media, 1.5% agar plates supplemented with 100 µg/mL of ampicillin grown at 37°C overnight and cultured in 10 mL of LB media supplemented with 100 µg/mL of ampicillin and grown at 37°C with aeration overnight. The bacterial cells were then sub-cultured in 1 L of LB media supplemented with 100 µg/mL of ampicillin and grown at 37°C with aeration to an optical density at 600 nm of 0.4-0.8 and then induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for an additional 4 hours. The cells were harvested and washed with phosphate buffered saline (PBS) by
centrifugation with a Sorval RC-58 centrifuge in a Sorval SLA-1500 rotor at 8000 rpm for 10 minutes at 4ºC.

2.7 Isolation and Purification of PagP

The cells were then suspended in 20 mL of 50 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) (pH 8.0) and 5.0 mM ethylenediaminetetraacetic acid (EDTA), and lysed by passage through a French Press at 5000-10000 psi. The insoluble material was recovered by centrifugation in a Sorval RC-58 centrifuge in a Sorval SS-34 rotor at 8000 rpm for 10 minutes at 4ºC. The pellets were washed with 20 mL of 50 mM Tris-HCl (pH 8.0) and 2% Triton X-100 followed by 40 mL of 50 mM Tris-HCl at pH 8.0 and then solubilized in 20 mL of 50 mM Tris-HCl (pH 8.0) and 6 M guanidine (Khan et al., 2007). The supernatant was collected after centrifugation and loaded onto a 5 mL His-binding resin (Novagen) that was washed with 3 column volumes of distilled water, charged with 5 column volumes of 50 mM NiSO₄ and equilibrated with 3 column volumes of 100 mM Tris-HCl (pH 8.0), 250 mM NaCl, 6 M guanidine and 5 mM imidazole. The protein was then washed with 10 column volumes of equilibration buffer (100 mM Tris-HCl (pH 8.0), 250 mM NaCl, 6 M guanidine and 5 mM imidazole), followed by 5 column volumes of wash buffer (100 mM Tris-HCl (pH 8.0), 250 mM NaCl, 6 M guanidine and 20 mM imidazole). The protein was eluted in a step-wise gradient using 5 mL each of 35, 50, 75, 100 and 125 mM imidazole (Khan et al., 2007). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) confirmed the
presence of purified PagP in the 100 and 125 mM imidazole fractions, which were pooled and dialyzed against distilled water, and the precipitated protein was collected by centrifugation in a Sorval RC-58 centrifuge in a Sorval SS-34 rotor at 8000 rpm for 20 minutes at 4°C.

2.8 Electrospray Ionization Mass Spectrometry

Prior to detergent refolding, approximately 1 µg of wet pellet precipitated protein sample was dissolved in a 1 mL solution of 1:1 1% formic acid:acetonitrile just prior to injection of 10 µL into an electrospray ionization mass spectrometer (ESI-MS) (Khan et al., 2007). The sample was injected directly into a Waters/Micromass Q-TOF Ultima Global quadrupole time-of-flight mass spectrometer. The spectrum was reconstructed using MassLynx 4.0 MaxEnt 1 moldule.

2.9 Refolding of PagP

The precipitated protein was dissolved in 5 mL of 10 mM Tris-HCl (pH 8.0), 6 M guanidine and diluted dropwise (~1 drop per second) into 10-fold excess (50 mL) of 10 mM Tris-HCl (pH 8.0), 0.5% lauroyldimethylamine N-oxide (LDAO) at room temperature with vigorous stirring and left to stir overnight at 4°C (Khan et al., 2007). The refolded sample was then centrifuged to get rid of any precipitated protein and applied to a 4 mL His-binding resin (Novagen) that was washed with 3 column volumes
of distilled water, charged with 5 column volumes of 50 mM NiSO₄ and equilibrated with 3 column volumes of 10 mM Tris-HCl (pH 8.0), 0.1% LDAO and 5 mM imidazole. The protein was then washed with 10 column volumes of equilibration buffer (10 mM Tris-HCl (pH 8.0), 0.1% LDAO and 5 mM imidazole), followed by 5 column volumes of wash buffer (10 mM Tris-HCl (pH 8.0), 0.1% LDAO and 20 mM imidazole) and then eluted with 2 mL of 10 mM Tris-HCl (pH 8.0), 0.1% LDAO and 250 mM imidazole. The protein was dialyzed against 10 mM Tris-HCl (pH 8.0) and 0.1% LDAO (Khan et al., 2007). Refolded PagP protein concentrations were determined by absorbance at 280 nm in 6 M guanidine using the Edelhoch method (Edelhoch, 1967) and the calculated extinction coefficient (ε₂₈₀) of 82630 M⁻¹cm⁻¹.

2.10 Circular Dichroism Spectroscopy

Samples to be analyzed by circular dichroism (CD) were maintained at a concentration of 0.3 mg/mL in 10 mM Tris-HCl (pH 8.0) and 0.1% LDAO and were analyzed using a cuvette of 1 mm path length. An Aviv 215 Spectrophotometer was linked to a Merlin Series M25 Peltier device for temperature control. For each sample three accumulations were averaged at a data pitch of 1 nm and an averaging time of 5 seconds/nm. The temperature was maintained at 25°C and data sets were obtained from 200-260 nm. Thermal denaturation profiles were obtained by heating the samples from 20°C to 100°C at 218 nm with a temperature slope of 2°C/min and an averaging time of 15 seconds.
2.11 Phospholipase Activity Assay

The phospholipase activity assay was conducted using 20 µM [14C]-labelled DPPC at 4000 cmp/µL to monitor the phospholipase reaction (Figure 12) of 1 mM DPPC with 0.6 ng/µL of PagP in a reaction volume of 25 µL of 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.25% n-dodecyl-β-D-maltopyranoside (DDM) (Khan et al., 2009). The lipids were dried down under a stream of N₂ (g) and dissolved into 22.5 µL of the reaction buffer. The reaction was initiated with the addition of 2.5 µL of 6 ng/µL PagP, conducted at 30°C and terminated by directly spotting 2.5 µL (10 000 cpm/spot) of the reaction mixture onto the origin of a TLC plate at 5, 10, 20, 30 and 60 minutes to determine the initial velocity and visualize the entire reaction profile. The TLC plate was then resolved in chloroform:methanol:water (65:25:4) solvent system equilibrated in a sealed glass tank. The plate was exposed to a PhosphorImager screen overnight to visualize the reaction products using a Molecular Dynamic Typhoon 9200 PhosphorImager, and quantified by ImageQuant software. The phospholipase A₂ control of 4 milliunits/µL was incubated with 10 mM CaCl₂ in place of EDTA.

2.12 Palmitoyltransferase Kinetic Assay

The palmitoyltransferase kinetic assay was conducted using 20 µM [14C]-labelled dipalmitoylphosphatidylcholine (DPPC) (Figure 13) at 4000 cpm/µL or [32P]-labelled Kdo₂-lipid A (Figure 14) prepared as described previously (Khan et al., 2007) to monitor
Figure 12: \[^{14}\text{C}]\text{ monitored phospholipase reaction of PagP.}\) The PagP catalysed hydrolysis of a palmitate chain (16:0) from the \(sn-1\) position of \[^{14}\text{C}]-\text{labelled DPPC}\) producing \[^{14}\text{C}]-\text{labelled free palmitic acid}\) and \[^{14}\text{C}]-\text{labelled } sn-1\text{ lyso-PC}\) in the process.
Figure 13: $[^{14}C]$ monitored palmitoyltransferase reaction of PagP. The PagP catalysed transfer of a palmitate chain (16:0) from the $sn$-1 position of $[^{14}C]$-labelled DPPC to the free hydroxyl group on the $N$-linked $(R)$-3-hydroxymyristate chain at position 2 of the proximal glucosamine unit of lipid A, producing $[^{14}C]$-labelled $sn$-1 lyso-PC and $[^{14}C]$-labelled palmitoylated-lipid A in the process.
Figure 14: $[^{32}\text{P}]$ monitored palmitoyltransferase reaction of PagP. The PagP catalysed transfer of a palmitate chain (16:0) from the $sn$-1 position of DPPC to the free hydroxyl group on the N-linked (R)-3-hydroxymyristate chain at position 2 of the proximal glucosamine unit of $[^{32}\text{P}]$-labelled Kdo$_2$-lipid A, producing $sn$-1 lyso-PC and $[^{32}\text{P}]$-labelled palmitoylated-Kdo$_2$-lipid A in the process.
the palmitoyltransferase reaction between a fixed concentration of 480 µM DPPC (500 µM total) and various concentrations of 50, 100, 150, 200, 250 and 500 µM lipid A, with 0.6 ng/µL of PagP in a reaction volume of 25 µL of 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.25% DDM. The lipids were dried down under a stream of N₂ (g) and dissolved into 22.5 µL of the reaction buffer. The reaction was initiated with the addition of 2.5 µL of 6 ng/µL PagP, conducted at 30°C and terminated by directly spotting 2.5 µL (10 000 cpm/spot or 200 cpm/spot) of the reaction mixture onto a TLC plate at 5, 10 and 15 minutes to determine the initial velocity. The TLC plate was resolved in pyridine:chloroform:88% formic acid:water (50:50:16:5), exposed to a PhosphorImager screen overnight to visualize the reaction products using a Molecular Dynamic Typhoon 9200 PhosphorImager, and quantified by ImageQuant software. The phospholipase A₂ control of 4 milliunits/µL was incubated with 10 mM CaCl₂ in place of EDTA.

2.13 Palmitoyltransferase Activity Assay

The palmitoyltransferase activity assay was conducted using 20 µM [¹⁴C]-labelled DPPC at 4000 cmp/µL to monitor the palmitoyltransferase reaction (Figure 13) between 1 mM DPPC and 100 µM of lipid IVₐ or lipid A, with 0.6 ng/µL of PagP in a reaction volume of 25 µL of 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.25% DDM. The lipids were dried down under a stream of N₂ (g) and dissolved into 22.5 µL of the reaction buffer. The reaction was initiated with the addition of 2.5 µL of 6 ng/µL PagP,
conducted at 30°C and terminated by directly spotting 2.5 µL (10 000 cpm/spot) of the reaction mixture onto the origin of a TLC plate at 5, 10, 20, 30 and 60 minutes to determine the initial velocity and visualize the entire reaction profile. The TLC plate was resolved in either pyridine:chloroform:88% formic acid:water (70:30:16:10) or either pyridine:chloroform:88% formic acid:water (50:50:16:5) solvent system equilibrated in a sealed glass tank for lipid IV_A or lipid A, respectively. The plate was exposed to a PhosphorImager screen overnight to visualize the reaction products using a Molecular Dynamic Typhoon 9200 PhosphorImager, and quantified by ImageQuant software. The phospholipase A2 control of 4 milliunits/µL was incubated with 10 mM CaCl_2 in place of EDTA.

2.14 Mass Spectrometry of Lipid IV_B

Lipid IV_B was prepared similar to the reaction conditions above without the addition of radioactive material to monitor the reaction. The palmitoyltransferase reaction was carried out between 1 mM DPPC and 100 µM of lipid IV_A with 0.6 ng/µL of PagP in a reaction volume of 25 µL of 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.25% DDM. The lipids were dried down under a stream of N_2 (g) and dissolved into 22.5 µL of the reaction buffer. The reaction was initiated with the addition of 2.5 µL of 6 ng/µL PagP, conducted at 30°C overnight. The reaction was terminated by adding 55 µL of chloroform:methanol (1:1) to for a two-phase Bligh/Dyer mixture consisting of chloroform/methanol/water (2:2:1.8), the lower organic phase was extracted and dried.
down under a stream of N\textsubscript{2} (g). The non-radioactive dried lipid films were dissolved in chloroform:methanol (2:1) and analyzed by normal phase liquid chromatography electrospray ionization quadrupole time-of-flight tandem mass spectrometry (LC-ESI-Q-TOF MS/MS) as described previously (Garrett \textit{et al.}, 2011).

2.15 Analysis of Lipid A by TLC

The analysis of lipid A by TLC was conducted using the mild acid hydrolysis procedure (Jia \textit{et al.} 2004). A single colony was isolated on LB media, 1.5% agar plates supplemented with the appropriate antibiotic grown at 37ºC overnight and cultured in 10 mL of LB media supplemented with the appropriate antibiotic and grown at 37ºC with aeration overnight. Then 50 µL of overnight culture was diluted into 5 mL of LB media without antibiotics containing 5 µCi/mL \textsuperscript{32}P-phosphate and was grown at 37ºC with aeration for 4 hours. After 4 hours, 550 µL of 250 mM Na\textsubscript{4}EDTA was added to the +EDTA samples and 550 µL of ddH\textsubscript{2}O was added to the −EDTA samples and allowed to grow for an additional 5 minutes at 37ºC with aeration. The \textsuperscript{32}P-labelled cells were harvested by centrifugation using a clinical centrifuge and washed with 5 mL of PBS. The pellet was resuspended in 0.8 mL of PBS and converted into a single-phase Bligh/Dyer mixture by adding 2 mL of methanol and 1 mL of chloroform. After 10 minutes of incubation at room temperature, the insoluble material was collected by centrifugation. The pellet was washed once with 5 mL of a fresh single-phase Bligh/Dyer mixture, consisting of chloroform/methanol/water (1:2:0.8). This pellet was then
dispersed in 1.8 mL of 12.5 mM sodium acetate (pH 4.5) and 1% SDS, with sonic irradiation in a bath apparatus. The mixture was incubated at 100 °C for 30 min to cleave the ketosidic linkage between Kdo and the distal glucosamine sugar of lipid A. After cooling, the boiled mixture was converted to a two-phase Bligh/Dyer mixture by adding 2 mL of chloroform and 2 mL of methanol. Partitioning was made by centrifugation, and the lower phase material was collected and washed once with 4 ml of the upper phase derived from a fresh neutral two-phase Bligh/Dyer mixture, consisting of chloroform/methanol/water (2:2:1.8). The lower phase lipid A sample was collected and dried under a stream of nitrogen gas. The lipid A sample was dissolved in 100 µL of chloroform/methanol (4:1) and a 200 cpm portion of the sample was applied to the origin of a TLC plate. The TLC plate was resolved in pyridine:chloroform:88% formic acid:water (50:50:16:5) solvent system equilibrated in a sealed glass tank. The plate was exposed to a PhosphorImager screen overnight to visualize the lipid A species using a Molecular Dynamic Typhoon 9200 PhosphorImager, and quantified by ImageQuant software.
CHAPTER 3 – RESULTS

3.1 Preparation of PagP

The wild-type and arginine to serine substitutions of PagP were successfully isolated, purified and refolded in LDAO as described previously (Khan et al., 2007). The wild-type and substitutions of Arg45Ser, Arg49Ser and Arg45Ser/Arg49Ser were validated by ESI-MS as illustrated in Table 5 and the concentration was determined using absorbance at 280 nm using the Edelhoch method (Edelhoch, 1967).

3.2 Circular Dichroism Spectroscopy

The folding and stability of the wild type and the arginine to serine substitutions of PagP was evaluated using CD spectroscopy. The overlapping positive ellipticity at 232 nm corresponding to the intrinsic exciton coupling of PagP and the negative ellipticity at 218 nm corresponding to the $\beta$-barrel formation indicate that all of the arginine to serine substitutes folded properly compared to the wild-type. Interestingly Arg49Ser caused an increase in the negative ellipticity at 218 nm, corresponding to an increase in $\beta$-strand formation by approximately 3-4% as determined by CDPro modeling,
Table 5: Predicted molecular masses from ESI-MS.

<table>
<thead>
<tr>
<th>PagP Protein</th>
<th>Theoretical Mass (Da)</th>
<th>ESI-MS (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PagP-HΔS-WT</td>
<td>20175</td>
<td>20174</td>
</tr>
<tr>
<td>PagP-HΔS-R45S</td>
<td>20106</td>
<td>20105</td>
</tr>
<tr>
<td>PagP-HΔS-R49S</td>
<td>20106</td>
<td>20105</td>
</tr>
<tr>
<td>PagP-HΔS-R45S/R49S</td>
<td>20037</td>
<td>20036</td>
</tr>
</tbody>
</table>
indicating a conformational change as compared to the wild-type PagP (Figure 15). Therefore the arginine to serine substitutions have folded properly compared to the wild-type. The stability of the wild type and the arginine to serine substitutions of PagP was evaluated by thermal denaturation, measuring the negative ellipticity at 218 nm. The thermal melts show the thermal unfolding at approximately ~88°C, same as the wild type PagP (Figure 16). Exciton loss at ~44°C was apparent previously (Khan et al., 2007), but it appears that this transition is masked by noise present in the current CD spectrophotometer used here. Therefore the arginine to serine substitutions have no effect on the stability of PagP relative to the wild-type.

3.3 Phospholipase Activity Assay

PagP possesses an intrinsic phospholipase activity, where it hydrolyses phospholipids into lyso-phospholipid and a free palmitic acid. The phospholipase activity was determined to see if the conformational change induced by the Arg49Ser mutation would increase the inherent activity of PagP relative to the wild type. The phospholipase activity assay shows that the arginine to serine substitutions have no effect on the phospholipase activity of PagP (Figure 17). Therefore the arginine to serine substitutions do not adversely effect the catalytic activity of PagP in vitro and that the conformational change induced by the Arg49Ser mutation does not increase the intrinsic activity of PagP.
Figure 15: CD spectroscopy of PagP. CD spectroscopy of wild-type and arginine to serine substitutes of PagP refolded in 0.1% LDAO, shows the characteristic positive ellipticity of the excitation at 232 nm and negative ellipticity of the $\beta$-barrel at 218 nm of PagP. The Arg49Ser substitution causes an increase in the negative ellipticity at 218 nm corresponding to increase in $\beta$-strand formation.
Figure 16: Thermal unfolding of PagP. Thermal unfolding as followed by the relative molar ellipticity at 218 nm of wild-type and arginine to serine substitutes of PagP refolded in 0.1% LDAO, shows the thermal unfolding at a $T_u = 88^\circ C$. 
**Figure 17: Phospholipase specific activity.** The phospholipase specific activity of the wild-type and arginine to serine substitutions of PagP with 1 mM DPPC and 20 µM [14C]-labelled DPPC to monitor the reaction of 0.6 ng/µL PagP in 100 mM Tris (pH 8.0), 10 mM EDTA, 0.25% DDM at 30°C. The wild-type and arginine to serine substitutes of PagP have specific activities of ~1.5 µmol/min/mg. Error bars represent the mean ± standard deviation of triplicates.
3.4 Palmitoyltransferase Kinetic Assay

Michealis-Menten enzyme kinetics are characterized by a hyperbolic rate vs. [S] curve. However, in some cases enzymes follow sigmoidal enzyme kinetics. This is indicative of cooperative binding as seen in multi-subunit enzymes or allosteric binding that changes the binding affinity of the enzyme for its substrate. Previous work done on the PagP palmitoyltransferase enzyme kinetics using $^{32}$P-labelled Kdo₂-lipid A to monitor the reaction showed hyperbolic Michealis-Menten enzyme kinetics. However when the palmitoyltransferase reaction was monitored using $^{14}$C-labelled DPPC and lipid A the palmitoyltransferase enzyme kinetics produced a sigmoidal curve with respect to lipid A with a $K_M$ of $226\pm28\ \mu M$, a $V_{max}$ of $5.0\pm0.7\ \mu M/min$, and a Hill co-efficient of $3.3\pm1.1$ (Figure 18). When the palmitoyltransferase reaction was repeated using $^{32}$P-labelled Kdo₂-lipid A to monitor the reaction the enzyme kinetics could be modeled by either a hyperbolic Michealis-Menten curve with a $K_M$ of $479\pm148\ \mu M$ and a $V_{max}$ of $4.8\pm0.9\ \mu M/min$ or a sigmoidal curve with a $K_M$ of $196\pm47\ \mu M$, a $V_{max}$ of $2.9\pm0.5\ \mu M/min$, and a Hill co-efficient of $1.6\pm0.4$. Although the sigmoidal fit appears qualitatively superior, the f-test states that the more complex Hill equation does not provide a significantly better fit (Figure 19).
Figure 18: $[^{14}\text{C}]$ palmitoyltransferase enzyme kinetics. The sigmoidal palmitoyltransferase enzyme kinetics of PagP with lipid A, 500 µM $[^{14}\text{C}]$-labelled DPPC to monitor the reaction, and 0.6 ng/µL PagP in 0.25% DDM at 30°C. The kinetics were modelled by the Hill equation $v_0=V_{\text{max}}\times[S]^n/(K_m^n+[S]^n)$, with the kinetic parameters $K_m = 226\pm28$ µM, $V_{\text{max}} = 5.0\pm0.7$ µM/min, and a Hill coefficient of $n = 3.3\pm1.1$. Error bars represent the mean ± standard deviation of triplicates.
Figure 19: $[^{32}\text{P}]$ palmitoyltransferase enzyme kinetics. The palmitoyltransferase enzyme kinetics of PagP with $[^{32}\text{P}]$-labelled Kdo$_2$-lipid A to monitor the reaction, 500 µM DPPC, and 0.6 ng/µL PagP in 0.25% DDM at 30°C. The kinetics can be modelled by either (A) the Michealis-Menten equation $v_0=V_{max}\times[S]/(K_m+[S])$, with the kinetic
parameters $K_M = 479\pm148 \ \mu M$ and $V_{max} = 4.8\pm0.9 \ \mu M/min$ or (B) the Hill equation

$$v_0=V_{max}\times[S]^n/(K_m^n+[S]^n),$$

with the kinetic parameters $K_M = 196\pm47 \ \mu M$, $V_{max} = 2.9\pm0.5 \ \mu M/min$, and a Hill coefficient of $n = 1.6\pm0.4$. However, the f-test states that the more complex Hill equation does not provide a significantly better fit. Error bars represent the mean ± standard deviation of triplicates.
3.5 Palmitoyltransferase Activity Assay

PagP has a low specificity for the acyl chain acceptor, with the ability to palmitoylate both free miscible alcohols as well as a number of immiscible alcohol species including lipid A as well as its biosynthetic precursors lipid IVₐ. Despite PagP having a broad substrate specificity it does still possess a high regioselectivity for its lipid A substrates, palmitoylating the proximal glucosamine unit on the \((R)-3\)-hydroxymyristate chain at position 2 rather than any other free hydroxyl group. This is further evident in the palmitoylation of the lipid A precursor lipid IVₐ which has four free hydroxyl groups posed for secondary acylation on both the proximal and distal glucosamine units of the lipid IVₐ molecule, however PagP still only palmitoylates lipid IVₐ at the proximal glucosamine unit on the \((R)-3\)-hydroxymyristate chain at position 2 producing lipid IVₐ. The palmitoyltransferase activity was determined using both lipid A and the biosynthetic precursor lipid IVₐ. The palmitoyltransferase activity assay shows that the arginine to serine substitutions have no effect on the palmitoyltransferase activity of PagP to either lipid A (Figure 20) or lipid IVₐ (Figure 21). In addition overnight analysis of the palmitoyltransferase reaction of PagP with lipid IVₐ produces a primary spot corresponding to \([^{14}\text{C}]\)-labelled palmitoylated lipid IVₐ or lipid IVₐ as the major product (Figure 22). Therefore, the arginine to serine substitutions do not disrupt the lipid A binding affinity of PagP or its ability to palmitoylate lipid A \textit{in vitro} which one would expect if the arginine residues are involved in lipid A binding and regioselectivity.
Figure 20: Palmitoyltransferase specific activity for lipid A. The palmitoyltransferase specific activity of the wild-type and arginine to serine substitutions of PagP with 100 µM lipid A, 1 mM DPPC and 20 µM [14C]-labelled DPPC to monitor the reaction with 0.6 ng/µL PagP in 0.25% DDM at 30°C. The wild-type and arginine to serine substitutes of PagP have specific activities of ~1.2 µmol/min/mg. Error bars represent the mean ± standard deviation of triplicates.
Figure 21: Palmitoyltransferase specific activity for lipid IVₐ. The palmitoyltransferase specific activity of the wild-type and arginine to serine substitutions of PagP with 100 µM lipid IVₐ, 1 mM DPPC and 20 µM [¹⁴C]-labelled DPPC to monitor the reaction with 0.6 ng/µL PagP in 0.25% DDM at 30°C. The wild-type and arginine to serine substitutes of PagP have specific activities of ~1.2 µmol/min/mg. Error bars represent the mean ± standard deviation of triplicates.
Figure 22: TLC plate of overnight palmitoyltransferase activity assay. The TLC plate of the palmitoyltransferase activity assay after overnight incubation with 100 µM lipid IVₐ, 1 mM DPPC and 20 mM [¹⁴C]-labelled DPPC to monitor the reaction of 0.6 ng/µL PagP in 0.25% DDM at 30°C. The reactions were spotted on the origin of the TLC plate and the products were resolved in a pyridine:chloroform:88% formic acid:water (70:30:16:10) and visualized with a PhosphorImager.
Unfortunately this experiment doesn’t distinguish the lipid IV\textsubscript{A} palmitoylation site the arginine to serine substitutions of PagP are transferring the palmitate chain to.

### 3.6 Mass Spectrometry of Lipid IV\textsubscript{B}

In order to determine the exact position that the arginine to serine substitutions are transferring the palmitate chain to we use collision induced dissociation (CID) tandem mass spectroscopy (MS/MS) (Kussak and Weintraub, 2002). The lack of secondary acyl chains on lipid IV\textsubscript{A} allow for the palmitoylation of either the hydroxymyristate chain at position 2 of the proximal glucosamine unit of lipid IV\textsubscript{A}, to produce lipid IV\textsubscript{B}; or the palmitoylation of the hydroxymyristate chain at position 3’ of the distal glucosamine unit of lipid IV\textsubscript{A}, to produce lipid IV\textsubscript{B’}. The mass spectrometry of the lipid IV\textsubscript{B} acylation product produced a $[\text{M}+\text{H}]^{+}$ with an m/z of 1644, which corresponds to the lipid IV\textsubscript{B} molecule. The lipid IV\textsubscript{B} molecule was then bombarded with N\textsubscript{2} (g) causing characteristic fragmentation (Figure 23) (Kussak and Weintraub, 2002). All of the arginine to serine substitutions produced a B$^{+}$ ion consistent with the palmitate being placed on the proximal glucosamine unit on the (R)-3-hydroxymyristate chain at position 2 producing lipid IV\textsubscript{B} the same as the wild type rather than lipid IV\textsubscript{B’}; since they all gave a B$^{+}$ ion with an m/z of 694 (Figure 24). Therefore, the arginine to serine substitutions do not disrupt the lipid A regioselectivity of PagP \textit{in vitro}. That is not to say that \textit{in vivo} the same is true, as the physical parameters of the lipid bilayer versus the detergent micellular environments are distinctly different and can have profound effects on the substrate
**Figure 23: Fragmentation pattern of lipid IVₐ.** The characteristic fragmentation pattern produced during collision-induced dissociation during MS/MS of the lipid A biosynthetic precursor, lipid IVₐ (Kussak and Weintraub, 2002)
Figure 24: CID MS/MS of lipid IV$_B$. The wild type and arginine to serine substitutions of PagP CID MS/MS produce a lipid IV$_B$ acylation product [M+H$^+$]$^+$ at m/z of 1644 that produces a B$^+$ ion at m/z of 694 consistent with the palmitate being placed on the (R)-3-hydroxymyristate chain at position 2 on the proximal glucosamine unit producing lipid IV$_B$ the same as the wild type rather than lipid IV$_B'$. 
binding and regioselectivity as already seen with the regioselectivity of the phospholipid at the crenel gate (Khan and Bishop, unpublished data).

3.7 Analysis of Lipid A by TLC

To determine if the arginine to serine substitutions can palmitoylate lipid A on either the proximal or distal glucosamine units \textit{in vivo}, a \textit{pagP/msbB} deletion complementation experiment using $^{32}$P-labeling and TLC analysis of the lipid A species was used. The \textit{msbB} gene codes for the enzyme LpxM of the lipid A biosynthesis pathway, which is responsible for the secondary myristylation of Kdo$_2$-lipid IV$_A$ on the (R)-3-hydroxymyristate at position 3’ of the distal glucosamine unit (Figure 25). This frees up the 3’ position for secondary palmitoylation by the endogenously expressed arginine to serine substitutions of PagP in a \textit{pagP} deletion strain. The palmitoylation of wild-type hexa-acylated lipid A produces a hepta-acylated lipid A upon chelation of the bridging magnesium divalent cations by EDTA treatment of the cells. The deletion of the \textit{msbB} gene produces penta-acylated lipid A that when palmitoylated produces hexa-acylated lipid A. The deletion of the chromosomal \textit{pagP} gene abolishes the palmitoylation activity of lipid A during EDTA treatment, upon complementation with an exogenous \textit{pagP} gene under endogenous control introduced via a plasmid the penta-acylated lipid A is palmitoylated producing hexa-acylated lipid A. The arginine to serine substitutions behave the same as the wild type PagP producing only hexa-acylated lipid A, if there were in fact a loss of regioselectivity then the arginine to serine substitutions of
Figure 25: Kdo$_2$-lipid A biosynthesis pathway. The Kdo$_2$-lipid A biosynthesis pathway of *E. coli*, deletion of the *msbB* gene responsible for coding the myristoyltransferase, LpxM, removes the 3’ myristate chain from Kdo$_2$-lipid A allowing for the potential palmitoylation of the 3’ position of the distal glucosamine of Kdo$_2$-lipid A by the arginine to serine substitutes of PagP in a pag$^P$ deletion strain (Figure taken from Raetz *et al.* 2007).
PagP would produce both hexa-acylated lipid A as well as hepta-acylated lipid A in the 
\textit{msbB} deletion strain (Figure 26). In addition the incorporation of the exogenous plasmid 
\textit{pagP} gene, although under the endogenous promoter of PagP and in a low copy number 
plasmid, produces a larger amount of palmitoylated lipid A both in the presence and 
absence of EDTA due to an increase expression of PagP by the gene dosage effect 
(Figure 27).
Figure 26: TLC analysis of *in vivo* lipid A. The lipid A was labelled with $^{32}$P and isolated from cells by mild acid hydrolysis (Jia *et al.*, 2004). Culture of *E. coli* MC1061 (wild-type), WJ0124 ($\Delta$pagP), MC1061-msbB ($\Delta$msbB), SK1061 ($\Delta$pagP,$\Delta$msbB) and SK1061 transformed with pBad18-cm or pBad18-cm-PagP wild-type and arginine to serine substitutions of PagP were grown for 4 hours and treated with or without 25 mM EDTA for an additional 5 minutes. The lipid A isolates were separated by TLC with pyridine:chloroform:88% formic acid:water (50:50:16:5) and visualized with a PhosphorImager. The three species of lipid A that were identified previously by mass spectrometry (Jia *et al.*, 2004) are to the right of the figure and include the lipid A
1,4'-bisphosphate (1-O-P), the 1-pyrophosphate (1-O-P-P), and the 4’-monophosphate (1-OH). The penta-, hexa- and hepta- acylated derivatives of each lipid A species are indicated to the left of the figure.
Figure 27: Quantification of in vivo lipid A palmitoylation. Lipid A was labelled with $^{32}$P and isolated from cells by mild acid hydrolysis (Jia et al., 2004). Culture of E. coli MC1061 (wild-type), WJ0124 ($\Delta$pagP), MC1061-msbB ($\Delta$msbB), SK1061 ($\Delta$pagP,$\Delta$msbB) and SK1061 transformed with pBad18-cm or pBad18-cm-PagP wild-type and arginine to serine substitutions of PagP were grown for 4 hours and treated with or without 25 mM EDTA for an additional 5 minutes. The lipid A isolates were separated by TLC with pyridine:chloroform:88% formic acid:water (50:50:16:5), visualized with a PhosphorImager, and quantified the lipid A palmitoylation as a percentage of the lipid A 1,4'-bisphosphate species.
CHAPTER 4 – DISCUSSION

Our hypothesis that Arg45 and Arg49 are responsible for *E. coli* PagP’s regioselectivity for the hydroxymyristate chain at position 2 of the proximal glucosamine unit of lipid A during the palmitoyltransferase reaction appears to be incorrect. The arginine to serine substitutions had no effect on the folding, stability, phospholipase and palmitoyltransferase activities. As well the arginine to serine substitutions display wild-type regioselectivity and specific activity in the palmitoylation of the biosynthetic precursor lipid IVₐ as indicated by collision induced fragmentation MS/MS. *In vivo*, lipid A analysis in a *msbB/pagP* deletion strain shows no difference in acylation pattern as compared to the wild-type.

4.1 Folding and Stability

The folding and stability of the wild-type and arginine to serine substitutions of PagP was evaluated by CD spectroscopy and showed that the arginine to serine substitutions were properly folded and thermally stable as compared to the wild-type. Interestingly, the Arg49Ser substitution caused an increase in β-strand formation as evident by the negative ellipticity at 218 nm in the CD spectra, indicating a
conformational change as compared to the wild-type. This increase in negative ellipticity at 218 nm was also previously observed during formation of the ternary complex, upon addition of both DPPC and Kdo2-lipid A to PagP in the enzymatically active detergent DDM (Figure 28) (Khan and Bishop, unpublished data). I believe this increase in β-strand formation seen in the Arg49Ser substitution is the same as that seen in the formation of the ternary complex, indicating that the region surrounding Arg49 is responsible for the conformational change upon formation of the ternary complex. However this has to be experimentally validated by determining if the increase in negative ellipticity at 218 nm seen in Arg49Ser can be further increased upon addition of both DPPC and Kdo2-lipid A in the detergent DDM indicating that the conformational change is not the same as that seen in formation of the ternary complex.

4.2 Sigmoidal Kinetics

The palmitoyltransferase kinetics produced a sigmoidal kinetic curve indicative of cooperative binding with respect to lipid A using [14C]-labelled DPPC and produced a hyperbolic Michealis-Menten curve with respect to [32P]-labelled Kdo2-lipid A. The difference in kinetics seen here could be due to either the substrates or the radiolabel used to monitor the reaction. However the sigmoidal cooperative binding can be explained by an allosteric binding of the substrate to the enzyme that increases the binding affinity of lipid A at the substrate binding site. It has been previously shown that PagP binds lipid A at a one to two ratio as determined by stoichiometric analysis of the conformational
Figure 28: CD spectroscopy of PagP ternary complex. The CD spectroscopy of PagP exchanged into 0.25%DDM with 1 mM DPPC and 100 µM Kdo₂-lipid A. In the presence of both DPPC and Kdo₂-lipid A, PagP forms a ternary complex that undergoes a conformational change with an increase in β-strand formation, corresponding to an increase in the negative ellipticity at 218 nm (Khan and Bishop, unpublished data).
change seen in formation of the ternary complex by CD spectroscopy with Kdo$_2$-Lipid A and non-hydrolysable DPPC ether analogue (Figure 29), which shows that the increase in negative ellipticity at 218 nm is only seen when the ternary complex is formed in a 1:2:3 ratio of PagP:Kdo$_2$-lipid A:DPPC-ether analogue (Khan and Bishop, unpublished data). Furthermore it was shown that PagP binds lipid A at a one to two ratio with a binding experiment using [$^{32}$P]-labelled Kdo$_2$-lipid A to monitor binding in the ternary complex (Figure 30), which shows that wild-type palmitoyltransferase binds twice as much [${}^{32}$P]-labelled Kdo$_2$-lipid A in the presence of C16-ether-PC; while the myristoyltransferase Gly88Ser binds twice as much [${}^{32}$P]-labelled Kdo$_2$-lipid A in the presence of C14-ether-PC during formation of the ternary complex, demonstrating a dependence on the specificity of the phospholipid binding site (the hydrocarbon ruler) (Khan and Bishop, unpublished data). These results show that there must be a second allosteric binding site for lipid A during formation of the ternary complex. In addition NMR spectroscopy revealed that PagP is a dynamic protein that exists in between an inactive relaxed (R) state and an active tense (T) state (Hwang et al., 2004), allosteric binding of lipid A could facilitate this transition as seen in other enzymes that exhibit sigmoidal kinetics due to allosteric binding of the substrate.

4.3 Lipid A Regioselectivity

The *in vitro* palmitoyltransferase activity shows that the arginine to serine substitutions have no effect on the palmitoyltransferase activity of PagP. This result
Figure 29: Stoichiometry of PagP ternary complex. The CD spectroscopy of PagP exchanged into 0.25%DDM with various proportional concentrations of non-hydrolysable DPPC ether-analogue (16:0-ether-PC) and Kdo2-lipid A. Upon formation of the ternary complex PagP undergoes a conformational change with an increase in negative ellipticity at 218nm corresponding to an increase in $\beta$-strand formation. This conformational change is seen when PagP is in a 1:2:3 ratio with Kdo2-lipid A and 16:0-ether-PC (Khan and Bishop, unpublished data).
Figure 30: $[^{32}P]$-labelled Kdo$_2$-lipid A binding experiment. The binding experiment with $[^{32}P]$-labelled Kdo$_2$-lipid A in the absence and presence of non-hydrolysable DPPC (16:0-ether-PC) and DMPC (14:0-ether-PC) ether-analogue by filter retention. The wild-type palmitoyltransferase PagP binds twice the amount of $[^{32}P]$-labelled Kdo$_2$-lipid A in the presence of C16-ether-PC while the Gly88Ser myristoyltransferase binds twice the amount of $[^{32}P]$-labelled Kdo$_2$-lipid A in the presence of C14-ether-PC, demonstrating a dependence on the specificity of the phospholipid binding site. Error bars represent the mean ± standard deviation of triplicates (Khan and Bishop, unpublished data).
suggests that the arginine residues are not responsible for binding lipid A as one would expect that substitution of these residues would result in a decrease in binding affinity and therefore palmitoyltransferase activity. The smoking gun is the mass spectra results of the lipid IVB product, which shows that the arginine to serine substitutions produced lipid IVA palmitoylated at the hydroxymyristate chain at position 2 of the proximal glucosamine unit of lipid IVA, to produce lipid IVB rather than palmitoylating the hydroxymyristate chain at position 3' of the distal glucosamine unit of lipid IVA, to produce lipid IVB'. In addition the palmitoyltransferase activity assay for lipid IVA produced only a single spot corresponding to lipid IVB, with no additional spots showing up that would indicate PagP double palmitoylation of lipid IVA, producing lipid IVB'. Finally the in vivo results from the pagP/msbB deletion complementation experiment using 32P-labelling and TLC analysis of the lipid A species shows that the arginine to serine substitutions do not produce hepta-acylated lipid A which would indicate the palmitoylation of both the proximal and distal glucosamine units. The only thing that would complete these results further is to perform MS/MS on the lipid IVB produced from the in vivo experiment, however there is no evidence to suggest that the results would be any different from the in vitro results.

4.4 Conclusions

Though Arg45 and Arg49 appear to be perfectly positioned at the membrane interface and the proposed lipid A binding site at the embrasure it appears that they are
not involved in lipid A binding and regioselectivity. However these results should also be confirmed by substitution of the arginine residues to alanine to ensure that polarity of the residue plays no role in binding and regioselectivity of lipid A. In addition there are a number of other positively charged residues in the L1 loop that could in fact be responsible for binding lipid A, acting like a lasso to bind lipid A from the membrane and pull it over PagP in order to facilitate bringing all the necessary chemical groups and catalytic residues into close proximity of one another, a problem that has plagued our understanding of how PagP functions as an enzyme.
REFERENCES


