A NEW ROLE FOR PAGP IN SIGNAL TRANSDUCTION
A NEW ROLE FOR PAGP IN SIGNAL TRANSDUCTION

By

LAXMAN PANDEY, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfilment of the Requirements

For the Degree

Master of Science

McMaster University

© Copyright by Laxman Pandey, September 2013
MASTER OF SCIENCE (2013) McMaster University

(Biochemistry and Biomedical Sciences) Hamilton, Ontario

TITLE: A new role for PagP in signal transduction

AUTHOR: Laxman Pandey, B.Sc.

SUPERVISOR: Dr. Russell E. Bishop

NUMBER OF PAGES: xii, 86
ABSTRACT

The outer membrane (OM) of Gram-negative bacteria is not a static structure; it can be remodeled in response to environmental conditions that allow bacteria to survive and function in hostile conditions. PagP, an OM enzyme, plays a crucial role in remodeling the OM by transferring a palmitate chain from a phospholipid to the proximal glucosamine unit of lipid A, which anchors lipopolysaccharides (LPS) to the external leaflet of the OM. PagP also affects cytoplasmic events of core biosynthesis in response to OM stress. A putative catalytic triad (Asp 61, His 67 and Tyr 87) at the periplasmic interface is hypothesized to play a role in transducing the signal to influence core biosynthesis. Mutations of a triad residue (Y87F), a catalytical residue (S77A) and wild-type pagP have all been transduced separately to the strains having PagP in inactive (imp+) and active states (imp4213). Correction and validation of all the strains designed for this project has been achieved. A quantitative real-time polymerase chain reaction (qPCR) approach was applied to monitor the expression of candidate genes of PagP-mediated signalling. The decreased transcription of slyB in a strain containing pagP in comparison to the strain with deleted pagP indicates that PagP inhibits slyB transcription. The periplasmic catalytic triad of PagP appears to play a role in the repression of SlyB because the Y87F mutant also showed activated slyB transcription. The effect of PagP over the transcription of extracytoplasmic factor, σE, and glucosyl transferase encoded by waaG was analyzed and ruled out because of their stable transcription in both imp+ and imp4213 strains.
ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my supervisor Dr. Russell Bishop for giving me the opportunity to work in his laboratory. His tireless support, motivation, and immense knowledge guided me to strive for the best.

I would also like to thank my committee members, Dr. Richard Epand and Dr. Michael Surette for giving insightful comments and suggestions for this project.

I am also thankful to all the past and present lab members of Bishop Lab for their support, encouragement and co-operation. I am indebted to Michelle Pinto and Anne-Marie Lacroix from Surette lab for their assistance in working with RNA and qPCR.

Last but not the least, I express my gratitude to my parents and family members for being there for me during good and difficult times.
TABLE OF CONTENTS

Title page.......................................................................................................................i
Descriptive note.............................................................................................................ii
Abstract.........................................................................................................................iii
Acknowledgements.......................................................................................................iv
List of tables.....................................................................................................................viii
List of figures...................................................................................................................ix
List of abbreviations.......................................................................................................x

1: INTRODUCTION........................................................................................................1
  1.1 Gram Negative cell envelope..............................................................1
  1.2 LPS .............................................................................................................4
  1.3 Lipid A biosynthesis and LPS transport.................................................7
  1.4 OM proteins (OMPs).............................................................................11
  1.5 Lipoproteins..............................................................................................12
    1.5.1 SlyB, a putative OM lipoprotein..................................................13
  1.6 The Gram-negative response to envelope stress.................................14
    1.6.1 Extracytoplasmic sigma factor, σE ...............................................14
    1.6.2 Two component regulatory systems...........................................15
  1.7 PagP..........................................................................................................16
    1.7.1 PagP structure..............................................................................16
    1.7.2 Role of PagP as palmitoyl transferase........................................19
    1.7.3 New role for PagP as an apical sensory transducer......................23
1.7.4 Putative Role of the catalytic triad in signalling…………………26

1.8 Objective and approach..............................................................28

2: MATERIALS AND METHODS........................................................................30

2.1 Materials..........................................................................................30

2.2 Methods...........................................................................................34

2.2.1 Primer designing............................................................................34

2.2.2 Plasmid extraction and PCR........................................................36

2.2.3 PCR purification, DpnI digestion, ethanol precipitation of DNA.........36

2.2.4 Electrocompetent cells....................................................................36

2.2.5 Transformation using electroporation method.................................37

2.2.6 Excising the kanamycin cassette.....................................................38

2.2.7 Lipid A isolation and analysis........................................................39

2.2.8 RNA extraction..............................................................................40

2.2.9 On column digestion and purification of RNA.................................41

2.2.10 cDNA synthesis, optimization of annealing temperature and standard curve.........................................................42

2.2.11 qPCR and data analysis................................................................42
3: RESULTS

3.1 Confirmation of pagP as a non-essential gene

3.2 imp4213 (lptD4213) disrupts the permeability barrier and
constitutively activate PagP

3.3 Y87F palmitoylates lipid A to the same degree as the
wild-type PagP

3.4 Correction and validation of strains

3.5 PagP activation does not affect the transcription of
WaaG glucosyl transferase

3.6 PagP controls transcription of slyB

3.7 PagP and stress response regulator (σE)

4: DISCUSSION

5: FUTURE DIRECTIONS

6: REFERENCES
LIST OF TABLES

Table 1. Materials and sources.................................................................30
Table 2. Bacterial strains and plasmids used in this work..................................32
Table 3. Oligonucleotide primers used for gene knockout................................34
Table 4. Oligonucleotide primers used for PCR..............................................35
Table 5. Oligonucleotide primers used for qPCR.............................................35
LIST OF FIGURES

Figure 1. The Gram negative cell envelope..........................................................3
Figure 2. Structure of LPS..................................................................................6
Figure 3. Raetz pathway for biosynthesis of Kdo2-lipid A....................................9
Figure 4. LPS transport.....................................................................................10
Figure 5. PagP structure...................................................................................18
Figure 6. Reaction catalyzed by PagP...............................................................21
Figure 7. OM permeability barrier and PagP......................................................22
Figure 8. Model for PagP-mediated signaling..................................................25
Figure 9. Putative catalytic triad......................................................................27
Figure 10. Confirmation of pagP as a non essential gene.................................45
Figure 11. Lipid A analysis of ΔpagP strains.......................................................46
Figure 12. MacConkey agar plating.................................................................49
Figure 13. Lipid A analysis of NR754 and NR760 strains.................................50
Figure 14. Lipid A analysis of NR754 strains.....................................................53
Figure 15. Lipid A analysis of NR760 strains.....................................................54
Figure 16. PCR verification of NR754 strains.....................................................57
Figure 17. PCR verification of NR760 strains.....................................................58
Figure 18. Transcription of waaG in different mutants of pagP.......................61
Figure 19. Transcription of slyB in different mutants of pagP..........................64
Figure 20. Transcription of rpoE in different mutants of pagP.........................66
Figure 21. Model for pagP control of slyB transcription ..................................72
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACP</td>
<td>Acyl carrier protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CAMPs</td>
<td>Cationic antimicrobial peptides</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>IM</td>
<td>Inner membrane</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kdo</td>
<td>3-deoxy-D-manno-2-octulosonic acid</td>
</tr>
<tr>
<td>L-Ara4N</td>
<td>4-amino-4-deoxy-L-arabinose</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OM</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>OMP</td>
<td>OM protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PPEtn</td>
<td>Phosphoethanolamine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNA-seq</td>
<td>RNA sequencing</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll like receptor 4</td>
</tr>
<tr>
<td>UDP-GlcNAc</td>
<td>Uridine diphosphate-N-acetylglucosamine</td>
</tr>
<tr>
<td>UMP</td>
<td>Uridine monophosphate</td>
</tr>
<tr>
<td>und-PP</td>
<td>Undecaprenyl phosphate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Gram-negative cell envelope

Bacteria are often exposed to unpredictable and hostile environments. To combat such environments, they have evolved with a sophisticated and complex cell envelope [1]. Gram-negative bacteria possess a unique cell envelope that is composed of an inner membrane (IM) and an outer membrane (OM) separated by a gelatinous region called the periplasmic space (fig. 1) [2].

The IM is a conventional phospholipid bilayer that delineates the cytoplasm. Phospholipids present in the IM are phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine and cardiolipin [1]. Proteins with α-helical structure dominate the IM and play a role in selective nutrient uptake, protein translocation, lipid biosynthesis and oxidative phosphorylation [3]. The IM is also involved in generating an electrochemical gradient, used as an energy source for the chemical synthesis of ATP that drives all cellular functions. It limits passive diffusion of most charged compounds but is permeable to small lipophilic molecules [1].

The aqueous cellular compartment between the IM and the OM of the cell envelope is called the periplasm [4]. The periplasmic space contains peptidoglycan and soluble proteins that are involved in nutrient uptake and secretion. The peptidoglycan exoskeleton, present in the periplasm, provides the structural framework for the cell and an anchor for certain OM lipoproteins [5]. It is a mesh like structure formed by
alternating residues of N-acetylglucosamine and N-acetylmuramic acid interconnected by oligopeptides [6].

The OM of Gram-negative bacteria is a unique asymmetric bilayer that provides a selective barrier to prevent the entry of many toxic molecules into the cell. Its inner leaflet is comprised of phospholipids whereas the outer leaflet is composed predominantly of lipopolysaccharides (LPS). The OM is provided with proteins having diverse functions and are of two types: lipoproteins that are anchored to the OM by a modified N-terminal lipid tail and integral proteins that span the membrane as β-barrels [2].
Figure 1. The Gram-negative cell envelope. The Gram-negative cell wall consists of an IM, periplasm and the OM. The IM consists of phospholipids in both leaflets whereas the OM is an asymmetric bilayer with phospholipids on the inner leaflet, and LPS on the outer leaflet. LPS is comprised of lipid A, core oligosaccharides and O-antigen [7].
LPS is an amphipathic molecule that resides in the outer leaflet of the OM. LPS is comprised of three parts: a proximal lipid A anchor, a distal O-antigen polysaccharide and the interconnecting core oligosaccharide (fig. 2) [7].

Lipid A is a saccharolipid that is phosphorylated and acylated by saturated fatty acid chains to make up the outer monolayer of the Gram-negative OM [8]. The lipid A backbone is a $\beta$-1',6 linked disaccharide of glucosamine, but E. coli possesses enzymes for modifying lipid A with L-4-aminoarabinose, phosphoethanolamine and palmitate in response to membrane perturbing agents [9].

Core oligosaccharides are the short chains of sugar residues that are attached to lipid A. Core oligosaccharides are conceptually divided into an inner and outer core. The inner core contains 1-3 Kdo (3-deoxy-D-manno-oct-2-ulosonic acid) residues with 2-3 heptoses (L-glycero-D-manno-heptose) attached [10]. The inner core is structurally conserved which suggests a role in maintaining the stability of the OM. [7].

The outer core consists of hexose residues that are attached to the last heptose residue in the inner core. The structure of the outer core is slightly more diverse. In E. coli, 5 core types exist: R1, R2, R3, R4, and K12 [11]. All the outer core oligosaccharide contains three hexoses and two side chain residues but the order of the hexoses in the backbone and the nature, position, and linkage of the side chain residues can vary among the five core types. R3 and K12 core, related to this study, share similar inner core and first outer core glucose, which is added by the glucosyl transferase encoded by waaG, but differs in the rest of the outer core structure [10].
The O-antigen polysaccharide is a repeating oligosaccharide of 2 to 8 carbohydrates attached to the outer core of LPS [12]. O-antigen provides resistance against the membrane attack complex of the host’s serum complement cascade. The type of sugar residues, their arrangement and their linkage varies among O-antigen and makes them the most variable cell constituent [13]. In fact, 186 different O-antigens have been identified in *E. coli* alone [14]. O-antigen is non essential and is absent in non-pathogenic organisms like *E. coli* K-12 but contributes to the virulence of various pathogenic strains. The presence of O-antigen makes ‘smooth’ LPS and its absence makes ‘rough’ LPS. Accordingly, LPS with one or two O-antigen units is termed ‘semi-rough’ [7].
**Figure 2. Structure of LPS.** LPS is a tripartite molecule consisting of lipid A, core oligosaccharides and O-antigen. The core oligosaccharide is divided into inner and outer core. Laboratory strain *E. coli* K-12 is devoid of O-antigen.
1.3 Lipid A biosynthesis and LPS transport

The lipid A biosynthesis pathway, known as the Raetz pathway, involves 9 enzymatic steps located in the cytoplasm or exposed to the cytoplasm at the membrane interface (fig. 3). UDP-GlcNAc is the key precursor for lipid A biosynthesis through the Raetz pathway. In the first step, LpxA transfers R-3-hydroxymyristoyl group from acyl carrier protein (ACP) to UDP-GlcNAc at position 3 [15]. LpxA has a precise hydrocarbon ruler and is specific to the R-3-hydroxymyristate chain [16]. The acylation of UDP-GlcNAc is reversible and unfavourable [17]. Therefore, subsequent deacetylation by LpxC, a Zn\(^{2+}\) dependent enzyme, is the first committed step in lipid A biogenesis [18].

LpxD catalyzes the addition of the R-3-hydroxymyristate chain to position 2 of glucosamine to form UDP-2,3-diacylglycerol [19]. LpxH, a pyrophosphatase, then cleaves UDP-2,3-diacylglycerol at its pyrophosphate bond to form lipid X and UMP [20]. The characteristic lipid A β-1’,6-glycosidic bond is produced when LpxB condenses a molecule of lipid X with another molecule of UDP-2,3-diacylglycerol [21]. LpxK, a membrane bound kinase, then phosphorylates the 4’ position using ATP to produce lipid IV\(_A\) [22]. The bifunctional enzyme KdtA (WaaA) incorporates two 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) sugars to the 6’ position of the distal glucosamine unit using CMP-Kdo as the Kdo donor. The final two enzymes of lipid A biosynthesis, LpxL (HtrB) and LpxM (MsbB), react only after the addition of the Kdo disaccharide moiety [23]. These two acyl-ACP-dependent enzymes transfer the lauroyl and myristoyl groups to form the acyloxyacyl linkages with the 2’ and 3’ R-3-hydroxymyristoyl groups, respectively [24, 25]. Bacterial strains having only Kdo\(_2\)-lipid A are referred to as deep
rough mutants and are typically not found in nature. Attachment of core sugars and the O-antigen to lipid A generates smooth-LPS, which is necessary for bacterial survival in different ecological niches [26].

Cytoplasmic enzymes necessary for the core oligosaccharide synthesis is encoded by \textit{waa (rfa)} operon [27]. Core oligosaccharides are assembled on Kdo$_2$-lipid A, and then \textit{flipped} to the periplasmic side of the IM via an ABC transporter \text{MsbA} [28]. The O-antigen is assembled separately on undecaprenyl pyrophosphate (Und-PPi) and flips to the periplasmic side of the IM [29]. O-antigen is then ligated to core-lipid A by \text{WaaL} ligase to form the mature LPS. A protein complex, the LPS transport (Lpt) machinery, is responsible for assembling a complete LPS in the outer leaflet of the OM [27]. The Lpt protein complex localized to the IM consists of LptB, LptC, LptF and LptG (YbrK) that form an ABC transporter \textit{is proposed} to be responsible for releasing the LPS molecule from the IM and delivering it to the periplasmic chaperone LptA (YhbN) [30, 31]. Finally, an OM protein LptD (Imp), along with a lipoprotein LptE (RlpB), is responsible for the flipping of LPS from the inner leaflet to the outer leaflet of the OM (fig. 4) [32].
Figure 3. Raetz pathway for Kdo$_2$-lipid A biosynthesis. In *E. coli*, Lipid A is synthesized in the cytoplasm and at the inner leaflet of the IM. Three soluble proteins (LpxA, LpxC and LpxD), two peripheral membrane proteins (LpxH and LpxB), and four integral IM proteins (LpxK, KdtA, HtrB, MsbB) take part in lipid A biosynthesis [9].
Figure 4. LPS transport. Core sugars are attached to the Kdo2-lipid A moiety at the inner leaflet of the IM and flipped to the outer leaflet by MsbA enzyme. O-antigen synthesized on undecaprenyl PP is flipped to the outer leaflet of the IM where it is polymerized and attached to the core-lipid A to form a complete LPS. Complete LPS is then shuttled by the Lpt system to the inner leaflet of the OM where it is flipped to the outer leaflet of the OM by the Imp (LptD)/RlpB complex.
Nearly 20% of all proteins in a cell are present in membranes [33] and these occupy nearly 50% of the membrane mass [34]. Since the OM of Gram-negative bacteria is a highly impermeable structure, the tasks that are crucial for the survival of bacterial cells like solute and protein translocation are provided by the proteins embedded in it [34]. Apart from transporters, OMPs also function as enzymes, receptors, and structural proteins [35].

Integral OMPs of Gram-negative bacteria possess distinctive structural features. Only OMPs with $\beta$-barrel conformation have been discovered so far in the OM [36]. The number of $\beta$ strands observed in known OMP $\beta$-barrels ranges from 8-24 and contains an even number of strands [37]. The minimum amino acid residues needed for the $\beta$-strands to span the membrane is 6, but this might extend up to 25 residues in some $\beta$-strands [34]. The exterior region of the protein that is in contact with the lipid milieu is characteristically hydrophobic, whereas the hydrophilic residues tend to be exposed at the membrane interfaces. Integral OMPs have long extracellular loops and short periplasmic turns [38]. Aromatic girdles present in the $\beta$-strands help in the anchorage of the proteins in the membrane [34]. Both N- and C- termini of proteins are located on the periplasmic side. Transmembrane $\beta$-barrel proteins are often devoid of cysteine residues, which otherwise are processed to form disulfide bonds during secretion and assembly [34].

The OM, being the outermost part of cell, is the first structure to perceive any environmental assaults. Thus, maintaining the integrity of the OM is essential in order for
the bacteria to survive. Few integral OMPs, which can function as enzymes, play a crucial role in maintaining the integrity of the OM. β-barrel enzymes can modify the membrane structures in response to the membrane perturbing signals and maintain the OM barrier. PagP is one of the three integral OMPs, which can function as an enzyme in *E. coli*. PagP responds to the outer membrane assault by palmitoylating the lipid A, which strengthens the permeability barrier of the OM.

### 1.5 Lipoproteins

Lipoproteins are the types of membrane proteins that have lipid-modified cysteines at the amino-terminal [39]. This lipid-modified N-terminus helps in anchoring the hydrophilic proteins to the hydrophobic surface of cell envelope [40]. Some bacterial lipoproteins anchor on the outer surface of the IM, whereas some can be transported by the Lol system to anchor into the inner leaflet of OM. The fate of lipoprotein localization depends on the residues adjacent to the cysteine residue at the N-terminal [41]. *E. coli* alone contains about 100 lipoproteins [39] and bacterial lipoprotein genes constitute 1-3% of the total number of genes [42]. Lipoproteins play a vital role in the biogenesis and the maintenance of the cell surface structures, as well as in transmembrane signal transduction, and nutrient uptake [42].
1.5.1 SlyB, a putative OM lipoprotein

SlyB is a putative OM lipoprotein consisting of 155 amino acids and has a molecular weight of ~15.6 kDa. It is part of the PhoPQ regulon and is conserved in different Gram-negative bacteria [43]. The SlyB sequence shows that it has a Type II single peptide, where amino acids 1-17 represent the signal peptide, and amino acids 18-155 is the SlyB OM lipoprotein [44]. Thus, it is expected to be modified at cysteine (amino acid 18) with an N-palmitoyl group and a thioether-linked diacylglycerol moiety, and is expected to be sent to the OM due to the absence of aspartic acid after cysteine (an IM retention signal) [45].

In *E. coli* and *Salmonella enterica*, transcription of *slyB* is stimulated by low levels of Mg$^{2+}$ via PhoPQ regulatory system [46, 47]. SlyB is the only lipoprotein present in the PhoPQ regulon of both *Salmonella enterica* and *E. coli* [48]. Studies have shown *slyB* mutants in *Burkholderia multivorans* to have altered cell morphology and an increased sensitivity to SDS and EDTA [43]. In *Pseudomonas aeruginosa*, *slyB* expression is dependent on AlgU, a sigma factor that responds to the periplasmic stress [49]. Additionally, SlyB also has been shown to inhibit the activity of PhoPQ regulatory system [50]. Since PagP and SlyB are PhoPQ regulated proteins and both play a role in maintaining the integrity of the OM, they might interact with each other to maintain the permeability barrier of the OM.
1.6 The Gram-negative response to envelope stress

Bacteria encounter harsh conditions in nature and inside their hosts [51]. The bacterial cell envelope, being an outermost structure, is the first one to experience adverse conditions and provides the major line of defense against these threats. The cell envelope is a crucial structure for the survival of the bacteria. Therefore, maintaining and monitoring the cell envelope is essential to maintain its integrity [52]. The signal caused by the envelope perturbing agents is sensed on one side of the membrane, and needs to be transduced to the other parts of the membrane in order to generate a response, adding complexity in the process [53]. To survive in the host environment, and to cause infection, enteric bacteria like *E. coli* have evolved a number of signaling networks, including the alternative extracytoplasmic function sigma factor σ^E_ and the two component systems such as CpxAR and PhoPQ [54].

1.6.1 Extracytoplasmic sigma factor, σ^E_

Bacteria activate the σ^E_ pathway in response to the stresses that interfere with OM protein biogenesis [55]. Anti-sigma factor RseA and its periplasmic counterpart RseB render σ^E_ inactive under normal conditions [56]. Extracytoplasmic stress degrades anti-sigma factor by the sequential action of two IM proteases, DegS and RseP, releasing σ^E_ into the cytoplasm. Activated σ^E_ causes the transcriptional activation of a set of genes involved in OM biogenesis [53].
Production of atypical lipid A by mutation of msbB (LpxM) or htrB (LpxL), which transfers myristoyl and lauroyl groups, respectively, to the distal glucosamine of lipid A, has been shown to increase the expression of the σE regulon [57]. PagP also produces the atypical lipid A by palmitoylating lipid A, thereby converting the regular hexa-acylated lipid A into hepta-acylated lipid A [58]. It has also been shown that this palmitoylated lipid A also activates σE expression, but when cells are grown at 42° [57].

1.6.2 Two component regulatory systems

The two component regulatory system, CpxAR, responds to stress that adversely affects the assembly of surface molecules like pili and certain OM proteins [59]. These environmental cues provide the stimulus for the dissociation of the periplasmic inhibitor CpxP from CpxA and trigger the autophosphorylation of a conserved histidine residue on its cytoplasmic domain. Phosphorylated CpxA then transfers phosphoryl groups to the response regulator CpxR, which ultimately causes the transcription of genes involved in envelope protein maintenance [56].

The PhoPQ two component regulatory system consists of PhoQ as the membrane bound sensor histidine kinase and PhoP as the cognate response regulator. PhoQ binds to divalent cations like Mg^{2+} via its periplasmic domain in its repressed state [38]. Cationic antimicrobial peptides displace the Mg^{2+} from PhoQ [60] and promotes the phosphorylation of PhoP. PhoP then regulates the transcription of genes related to lipid A structure, resistance to antimicrobial peptides and phagosome alteration [61].
The PhoPQ two component system also regulates the OM β-barrel enzyme involved in lipid A modification called PagP. Of the many enzymes involved in the modification of lipid A, PagP is the only known enzyme that is located in the OM of *E. coli*. It catalyzes the transfer of palmitate from phospholipid to lipid A thereby converting hexa-acylated lipid A to hepta-acylated lipid A [58]. This seemingly minor modification neutralizes the challenges of various cationic antimicrobial peptides and attenuates host innate immune signaling through TLR4 [62].

1.7 PagP

1.7.1 PagP structure

Integral OM proteins of Gram-negative bacteria have characteristic β-barrel structures [36]. PagP, being an OM protein, is no exception and has 8 strands to transverse the OM and a short N-terminal amphipathic α-helix on the periplasmic side [63]. Recent research indicates that the α-helix acts as a post assembly clamp, which helps in stabilizing PagP in the membrane once folding is complete [64]. PagP, unlike other OM proteins, has its barrel axis tilted at an angle of ~25º with respect to the membrane normal and the tilted barrel makes apparent the aromatic belt residues [63] (Fig. 5). PagP exists in two dynamically distinct states, termed R (relaxed), which facilitates the substrate entry, and T (tense), which plays a role in catalysis [65].
PagP has the usual flexible extracellular loops, but has an unusual centre. The lower periplasmic-exposed half of the core is hydrophilic as expected, but the upper half is unusually hydrophobic. The upper hydrophobic region provides a lipid binding pocket called the hydrocarbon ruler and a single molecule of bound detergent helps identify its position. Among all the fatty acids present in the membrane lipid pool, PagP selects the saturated 16 carbon palmitate chain to transfer to lipid A [63]. The depth of the hydrocarbon ruler can be modified to select shorter acyl chains by mutating the glycine 88 residue that lies on the base of the hydrocarbon ruler. The Gly88 substitution generated by a combination of site directed mutagenesis and chemical alkylation have produced modified PagP enzymes that transfer acyl chains in single carbon increments from C16 to C10 [66].
Figure 5. PagP structure. The crystal structure of PagP reveals an 8-strand antiparallel β-barrel preceded by an amphipathic N-terminal (N) α-helix. The PagP barrel axis is tilted by ~25° with respect to the membrane normal (red line). The lipid binding pocket revealed by a crystallographic LDAO detergent molecule is shown in grey mesh. Aromatic belt residues are shown in black at the membrane boundaries. Extracellular loops (L) and periplasmic turns (T) interconnect β-strands A-H, leaving the C-terminus (C) in the periplasmic side of the membrane [63].
1.7.2 Role of PagP as palmitoyl transferase

Asymmetric bilayer composition of the OM confines phospholipids on the inner leaflet; however PagP’s active site is located on the outer leaflet. Thus, phospholipids can only access PagP’s lipid binding pocket from the OM outer leaflet. As long as phospholipids remain on the inner leaflet, PagP remains in a dormant state [67]. It seems that PagP does not play a role in the migration of phospholipids from the inner to the outer leaflet, but simply responds to any externally mediated perturbation in the OM lipid asymmetry by palmitoylating lipid A [68].

Cationic antimicrobial peptides, on attacking bacteria, strip off Mg$^{2+}$ ions that bridge adjacent LPS molecules. As a result, negatively charged LPS begin to repel each other, thereby allowing phospholipids to migrate from the inner to the outer leaflet. The breach in the asymmetric distribution of the OM phospholipids allows hydrophobic antibiotics and detergents to enter freely into the periplasm [38]. This stress condition of the OM makes phospholipids available in the outer leaflet, along with LPS, and both substrates can now access the active site of PagP [63].

It has been shown that the phospholipids and lipid A gain access to PagP’s lipid binding pocket through lateral diffusion. PagP possesses in its β-barrel wall several key proline residues, which lack amide protons to donate hydrogen bonds, and thus display weakened hydrogen bonding in the adjacent β-strands. The gateways so formed are called the crenel and embrasure, which provide lateral routes for entry and egress of phospholipid and lipid A, respectively [69].
PagP mitigates the OM stress caused by the attack of antimicrobial peptides by palmitoylating the lipid A. The extra acyl chain resulting from the palmitoyl transferase reaction lowers the fluidity of the OM and helps restore the OM permeability barrier [70]. PagP specifically selects the palmitate chain from the phospholipid sn-1 position for transfer to the hydroxyl group of the R-3 hydroxymyristate chain at position 2 of lipid A (fig. 2 (B)) [58].

Normally, pagP is transcribed by PhoPQ in response to Mg\(^{2+}\)-limitation and/or cationic antimicrobial peptides. When cells are treated with EDTA or cationic antimicrobial peptides, fractions of LPS from the cell surface are stripped and promote the migration of phospholipids into the outer leaflet. This leads to the palmitoylation of lipid A, which occurs independently of de novo protein synthesis [67]. Due to its location and ability to respond to OM stress immediately, PagP represents a form of first-line defense for many Gram-negative bacteria [38].
Figure 6. Reaction catalyzed by PagP: PagP catalyzes the transfer of a palmitate chain from the \( sn-1 \) position of phosphotidylethanolamine (PtdEtn) to lipid A (red) [62].
Figure 7. OM permeability barrier and PagP. PagP is inactive if the permeability barrier of the OM is intact. When Mg$^{2+}$ is stripped off, LPS molecules start to repel each other and phospholipids from the inner leaflet start migrating to the outer leaflet to fill that space. This breach in lipid asymmetry causes the activation of PagP. PagP transfers the palmitate chain from phospholipid to lipid A and thus repairs the permeability barrier.
1.7.3 New role for PagP as an apical sensory transducer

Recently, an OM enzyme, PagP was shown to influence certain cytoplasmic events in *E. coli* O157:H7 [71]. Deficiency of the myristate chain on the distal glucosamine unit of lipid A, due to the deletion of *msbB*, was sufficient to breach the OM lipid asymmetry in *E. coli* O157:H7 so as to activate PagP. The *msbB* mutants also showed increased susceptibility to serum treatment, which suggested a defect in the O-antigen region of LPS. Further analysis of LPS isolated from *msbB* mutants by electrophoresis supports the absence of O-antigen repeats normally present in this serotype of *E. coli*. The nature of the core truncation was revealed by mass spectrometry, gas chromatography of alditol acetates, and NMR spectroscopy to indicate that truncation was occurring at the level of the first outer core glucose residue, which is added in the cytoplasm by WaaG. Surprisingly, when *pagP* was deleted in this *msbB* mutant, the rough LPS with a truncated core oligosaccharide were restored to a smooth LPS bearing O-antigen, which indicates that the core structure had been restored [71]. Variation in the LPS structure due to the absence and presence of *pagP* supports the role of PagP in R3 core truncation.

LPS is known to play a crucial role in the folding of OMPs [72] and there is the possibility that truncation is an artifact of producing an atypical lipid A species that does not support OMP folding. Similarly, the lysophospholipid byproduct of PagP catalysis shuttles back to the cytoplasm for reacylation, and PagP might communicate truncation during this process. However, a catalytically inactive *pagPS77A* allele was equally
capable of producing truncation, indicating that PagP’s palmitoyltransferase activity and, therefore, the production of an atypical lipid A species, is not responsible for truncation. Therefore, PagP is communicating with the cytoplasm through a separate mechanism, likely via a different domain of PagP [71].
Figure 8. Model for PagP-mediated signaling. Myristate deficient lipid A is glycosylated and transported to the OM where it causes the perturbation of lipid asymmetry in *E. coli* O157:H7 and activates PagP. Activated PagP transduces the signal across the cell envelope and reduces the UDP-glucose pool in the cytoplasm, which is manifested as the truncated R3 core. As a result, truncated core is transported to the OM [71].
1.7.4 Putative Role of the catalytic triad in signalling

The periplasmic side of PagP, when inspected closely, reveals a cluster of 3 amino acids i.e. Asp 61, His 67 and Tyr 87, which form a tight charge-relay network protruding from the β-barrel exterior that is buried beneath the α-helix, as observed in the enzyme’s inactive conformational R state. We propose that PagP is activated by phospholipid migration to the outer leaflet and transformed to the conformational T state. It is thus possible that the helix might change its position [73] such that Asp 61, His 67 and Tyr 87 become exposed (Fig. 9(A)). This exposed catalytic triad might proteolytically cleave a lipoprotein and release it into the periplasm to initiate the signal transduction.

The spatial arrangement of these residues strengthens the evidence of working together as a catalytic triad. Asp61, His 67 and Tyr 87 residues of PagP have nearly superimposable arrangement with the catalytic triad of chymotrypsin (Asp 102, His 57, Ser 195) (Fig 9(B)). Chymotrypsin, a serine protease, contains a catalytic serine residue, which serves as a nucleophile and affects the catalysis with its hydroxyl group. The putative catalytic triad of PagP contains a tyrosine instead of serine, but there is not any biochemical reason why the hydroxyl group of tyrosine could not function proteolytically similar to the hydroxyl group of serine. Although, the additional resonance stabilization afforded by tyrosine’s phenyl ring reduces its pKa relative to serine and makes it a weaker nucleophile, serine can also be replaced by cysteine in cysteine proteases, which
has even lower pKa. While Asp 61 and His 67 are highly conserved amongst homologs of \textit{pagP} from 8 genera, Tyr 87 is absolutely conserved [74].

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figures/figure9.jpg}
\caption[A]{A) Position of putative catalytic triad. When PagP is activated, Asp 61, His 67 and Tyr 87 on the periplasmic surface of PagP might be exposed and thereby proteolytically trigger signal transduction.}
\caption[B]{B) Arrangement of PagP putative catalytic triad and Chymotrypsin catalytic triad. Figure shows the nearly superimposable image of putative catalytic triad of PagP (blue) and catalytic triad of chymotrypsin (green).}
\end{figure}
1.8 Objectives and approach

The signalling ability of PagP has been observed in *E. coli* O157:H7, a pathogenic organism [71]. Taking into consideration the type of environment these pathogenic organisms are exposed to, PagP mediated signalling ability might be specific to this strain and might not be the general trend. Therefore, the model organism has been shifted to the laboratory strain, *E. coli* K-12. Since *msbB* is disrupting the OM permeability in *E. coli* O157:H7 and activating PagP, which in turn is affecting the cytoplasmic step of core truncation, it is reasonable to assume that PagP-mediated signalling is activated by aspects of OM stress. Like *msbB*-deficient *E. coli* O157:H7, *E. coli* MC4100 having the *imp4213* allele has a severe permeability defect that incites PagP-mediated lipid A palmitoylation [75]. Imp (LptD) is the OM protein responsible for flipping LPS from the OM inner leaflet to the outer leaflet [76]. *imp* is an essential gene, but an *imp4213* mutation restricts the enzyme’s ability to move LPS into the OM outer leaflet in a non-lethal manner. This severely compromises the OM permeability barrier [75] and constitutively activates PagP.

Regulation of the cytoplasmic events by the OM protein can be exerted at the level of gene expression, through covalent modification or through allosteric regulation. This work will focus on the regulation at the level of transcription by monitoring the transcription of genes in various mutants of PagP. Catalytically inactive (S77A) and signalling deficient (Y87F) mutants have been incorporated into the chromosome of PagP inactivated (NR754) and PagP activated (NR754, *imp4213*) strains. Through the gene
expression approach, efforts will be made to reveal the downstream target of PagP-mediated signalling as well as to uncover the relationship between catalysis and signalling events occurring in the different domains of PagP.
Chapter 2: Materials and Methods

2.1 Materials

Table 1: materials and their sources

<table>
<thead>
<tr>
<th>Materials</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>QIAprep spin minuteprep kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Taq buffers</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Red safe dye</td>
<td>Intron Biotechnology</td>
</tr>
<tr>
<td>Agarose</td>
<td>Bioshop</td>
</tr>
<tr>
<td>Microcentrifuge tube (1.5 and 2 ml)</td>
<td>Diamed</td>
</tr>
<tr>
<td>PCR tubes</td>
<td>Diamed</td>
</tr>
<tr>
<td>PCR purification kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>DpnI</td>
<td>New England Biolab</td>
</tr>
<tr>
<td>Buffer 4</td>
<td>New England Biolab</td>
</tr>
<tr>
<td>Absolute ethanol</td>
<td>Commercial Alcohols</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>Bioshop</td>
</tr>
<tr>
<td>Granulated Agar</td>
<td>Bioshop</td>
</tr>
<tr>
<td>Bacto-tryptone</td>
<td>Becton, Dikinson</td>
</tr>
<tr>
<td>Bacto-yeast extract</td>
<td>Becton, Dikinson</td>
</tr>
<tr>
<td>Bacterial culture plates</td>
<td>Corning Incorporated</td>
</tr>
<tr>
<td>Macconkey agar</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Bioshop</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>IPTG</td>
<td>Bioshop</td>
</tr>
<tr>
<td>Polypropylene tube</td>
<td>BD falcon</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Caledon</td>
</tr>
<tr>
<td>Arabinose</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>
Gel extraction kit  
Gene pulsur cuvette 0.2 cm  
Phosphorus-32 radionucleotide ($^{32}$P)  
Ethylene diamine tetraacetic acid  
Methanol  
Chloroform  
Sodium acetate  
SDS  
$N_2$ gas  
Glass-backed silica gel 60 TLC plates  
Pyridine  
88% formic acid  
DEPC treated water  
Lysozyme  
RNA later  
Trizol  
RNA purification kit  
DNAse digestion kit  
Iscript cDNA synthesis kit  
Ssofast evagreen supermix  
PCR tube strips  
PCR tubes 8-tube strip  
RNase away  
Isopropanol  
TAE Buffer  
$Na_2HPO_4$  
$KH_2PO_4$  
KCl
Table 2: Bacterial strains used in this work

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description and Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655</td>
<td>F, lambda, rph-1</td>
<td>Guyer et al 1981</td>
</tr>
<tr>
<td>BW25113</td>
<td>F-, Δ(araD-araB)567, ΔlacZ4787(:rrnB-3), λ- rph-1, Δ(rhaD-rhaB)568, hsdR514</td>
<td>Datsenko and Wanner 2001</td>
</tr>
<tr>
<td>MC4100</td>
<td>F- (araD139) Δ(argF-lac)169 λ- e14- flhD5301 Δ(fruK-yeiR)725(fruA25) relA1 rpsL150(Str') rbsR22 Δ(fimB-fimE)632(:IS1) deoC1</td>
<td>Casadaban 1976</td>
</tr>
<tr>
<td>MC1061</td>
<td>F-, araD139, (ara-leu)7697, (lac)X74, galU, galK, hsdR2 (rK-mK+), merB1, rpsL</td>
<td>(Casadaban and Cohen 1980)</td>
</tr>
<tr>
<td>WJ0124</td>
<td>MC1061 pagP::amp</td>
<td>Jia et al. 2004</td>
</tr>
<tr>
<td>NR754ΔpagP λInch pagP</td>
<td>MC4100 ara⁺ΔpagP, λInch pagP</td>
<td>This study</td>
</tr>
<tr>
<td>NR754ΔpagP λInch Bad18</td>
<td>MC4100 ara⁺ΔpagP, λInch Bad18</td>
<td>This study</td>
</tr>
<tr>
<td>NR754ΔpagP λInch S77A</td>
<td>MC4100 ara⁺ΔpagP, λInch pagPS77A</td>
<td>This study</td>
</tr>
<tr>
<td>NR754ΔpagP λInch Y87F</td>
<td>MC4100 ara⁺ΔpagP, λInch pagPY87F</td>
<td>This study</td>
</tr>
<tr>
<td>NR760ΔpagP λInch pagP</td>
<td>MC4100 ara⁺imp4213ΔpagP, λInch pagP</td>
<td>This study</td>
</tr>
<tr>
<td>NR760ΔpagP λInch Bad18</td>
<td>MC4100 ara⁺imp4213ΔpagP, λInch Bad18</td>
<td>This study</td>
</tr>
<tr>
<td>NR754ΔpagP λInch S77A</td>
<td>MC4100 ara⁺imp4213ΔpagP, λInch pagPS77A</td>
<td>This study</td>
</tr>
<tr>
<td>NR754ΔpagP λInch Y87F</td>
<td>MC4100 ara⁺imp4213ΔpagP, λInch pagPS77A</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td><strong>pagPY87F</strong></td>
<td><strong>Dr. Brown’s lab</strong></td>
</tr>
<tr>
<td>--------------</td>
<td>--------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>pKD13</td>
<td>Template plasmid used for gene knock out, Kan&lt;sup&gt;r&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>pKD46</td>
<td>Temperature sensitive replication (repA101ts) &amp; conditional replicon (oriR101, requires Pir in trans for plasmid replication); encodes lambda Red genes (exo, bet, gam); native terminator (tL3) after exo gene; arabinose-inducible promoter for expression (P&lt;sub&gt;araB&lt;/sub&gt;); encodes araC for repression of P&lt;sub&gt;araB&lt;/sub&gt; promoter;</td>
<td>Dr. Brown’s lab</td>
</tr>
<tr>
<td>pCP20</td>
<td>It has the yeast Flp recombinase gene, FLP, chloramphenicol and ampicillin resistant genes, and temperature sensitive replication.</td>
<td>Dr. Brown’s lab</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 Primer designing

For gene knockout experiments, kanamycin cassette (1327 base pairs) was amplified using primers that were designed to overlap the 50 base pairs (bp) homologous region present in the upstream and downstream of start and stop codon of pagP. For quantitative PCR (qPCR), special considerations were taken while designing the primers: size of the amplicon 100-200 bp, GC content 50%-60%, length 20-21 bp, melting temperature (Tm) 60°C to 61°C, Tm difference 1°C, maximum 3’ self complimentary 1.

For PCR, the length of the primers was 20-26 bp, melting temperature 55°C-60°C, GC content 40-60% and no secondary structure present in the primers were considered.

Table 3 Oligonucleotide primers used for gene knockout

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KanF</td>
<td>5’-AGCTTTGCTATGCTAGTAGATTTTTGATAAATGTTTTATG GTCACAACTGTCAACACTGAGAATTTAA 3’</td>
</tr>
<tr>
<td>KanR</td>
<td>5’-TACTTATTTTAGCTATTGATTTTAAAGAAGTTACTAACAACCTTC ATTTGTGTCGTAGGGCTGGAGCTCTTC-3’</td>
</tr>
<tr>
<td>KwpF</td>
<td>5’-CGGAAATAAATAGACGAGCTATTCAGATTATTCTTTTATGTTG GGTCTATTAAGGTATGTTAATTGTTAGCCTGTCAACATGAGAATTTA-3’</td>
</tr>
<tr>
<td>KwpR</td>
<td>5’-AGACATCAAATTTACCTTTACATGAAAAATAGACACAAATGTG TGTGCGGTTACCAGTACCCAATTTTGTGTTAGGCTGGAGCTGC TTC-3’</td>
</tr>
</tbody>
</table>
Table 4: Oligonucleotide primers used for PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PagP For</td>
<td>5’-GTTTTATGGTCACAAATGAACGTG-3’</td>
</tr>
<tr>
<td>PagP Rev</td>
<td>5’-CTAAAACCTTCATTTGCTCTCAAAACTG-3’</td>
</tr>
<tr>
<td>dcuC F</td>
<td>5’-TTCCCTGTCTCCAGGCCCAAAAAGTA-3’</td>
</tr>
<tr>
<td>cspE R</td>
<td>5’-TGCCGTTCTTCCGGAGTAATGAAACC-3’</td>
</tr>
</tbody>
</table>

Table 5: Oligonucleotide primers used for qPCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>slyB F</td>
<td>5’-GCGGTGCTGTTCTTGGTGGTT-3’</td>
</tr>
<tr>
<td>slyB R</td>
<td>5’-GCTCGACACCCCTGCGTTTTGT-3’</td>
</tr>
<tr>
<td>rpoE F</td>
<td>5’-CTGGTTGAACGGGTCCAGAAGG-3’</td>
</tr>
<tr>
<td>rpoE R</td>
<td>5’-AACATCACCGACGGACCATAG-3’</td>
</tr>
<tr>
<td>waaG F</td>
<td>5’-GCCGCTGATGTTTGTACGCC-3’</td>
</tr>
<tr>
<td>waaG R</td>
<td>5’-GCTCGAAAGTCGCTCGCTCAA-3’</td>
</tr>
<tr>
<td>dnaQ F</td>
<td>5’-TGGGCTTTTGCAGATGGAAGGAGAG-3’</td>
</tr>
<tr>
<td>dnaQ R</td>
<td>5’-CCAGATCGAGACGGGCTTCATG-3’</td>
</tr>
<tr>
<td>glnD F</td>
<td>5’-CGGACGATCGAGGGCGCAAAAGT-3’</td>
</tr>
<tr>
<td>glnD R</td>
<td>5’-TCGAGGAACAGCGCAACATCG-3’</td>
</tr>
</tbody>
</table>
2.2.2 Plasmid extraction and PCR

Plasmid DNA was isolated using QIAprep Spin Miniprep Kit from Qiagen. The PCR was performed using the following conditions: 1 minute denaturation at 94°C and 33 cycles of amplification [30 seconds denaturation at 94°C, 1 minute annealing varies with the primer, 1 minute extension at 72°C, 5 minutes elongation at 72°C], followed by holding at 4°C. The PCR amplified products were separated on an 1% agarose gel in Tris acetate EDTA buffer, stained with red safe dye and visualized with a UV transilluminator.

2.2.3 PCR purification, DpnI digestion, and ethanol precipitation of DNA

PCR amplified product was purified with the MinElute PCR purification kit from Qiagen. 200 µl of purified PCR product was digested to make it linear with 8 µl of DpnI enzyme (NEB) using Buffer 4 system at 37°C for 1 hour. Digested DNA was precipitated with 1 ml cold absolute ethanol and 0.5 M NaCl and was incubated overnight at -20°C.

2.2.4 Electrocompetent cells

MG1655 was streaked onto a 1.5% Luria-Bertani (LB) agar plates, and single colony was inoculated in 5 ml LB medium and incubated overnight at 37°C. Overnight was then subcultured (1/100) into fresh LB (50 ml) and grown at 37°C to an optical density 600 nm of around 0.4-0.5. Subculture was then transferred to a 50 ml falcon tube and kept on ice. Cells were spun down at 4000 rpm (Sorvall HS4) at 4°C for 20 minutes. Supernatant was removed, and pellets were gently resuspended with 30 ml of cold 10%
glycerol. Washing step with glycerol was repeated three times; each wash led cells to become softer and easier to resuspend. Finally, cells were resuspended in 1 ml of 10% glycerol and were stored at -80°C as 50 μl aliquots in sterile microtubes.

While making electrocompetent cells of MG1655 containing pKD46 plasmid, incubation temperature was brought down to 30˚C. 100 mM filter sterilized arabinose was added during subculture and the rest of the steps were kept same.

### 2.2.5 Transformation using electroporation method

Strain containing pKD46 plasmid was obtained from Dr. Eric Brown’s laboratory. pKD46 plasmid was isolated using a QIAprep Spin Miniprep Kit (Qiagen). 50 μl of competent cells of MG1655 were thawed on ice, and 1 μl of pKD46 plasmid was added to it. Mixture was incubated in ice for 1 minute and moved to 1.5 ml cuvette. Electroporation was done by using *E. coli* gene pulser at 2.5mV. Cells were revived using 700 μl of SOC media at room temperature and were moved back to the tube. Cells were grown in a shaker for 1 hour at 30˚C. 200-300 μl of cells were plated on LB-Ampicillin (50-100 μg/ml) plates and incubated overnight at 30˚C.

While transforming linear kanamycin cassette into MG1655 harboring pKD46 plasmid, ethanol precipitated DNA was centrifuged at 15000 rpm for 15 minutes at 4˚C. Supernatant was discarded, and the pellet was resuspended with 1 ml of 70% ethanol and the centrifugation step was repeated. Pellet was dried completely and resuspended in 10 μl of water. Concentration of the linear cassette was measured with the Nano drop. Around 1 μg of DNA was transformed with the MG1655 comp cells, containing pKD46,
and incubated at 30°C for 3 hours. 200-300 µl of cells were plated on LB-kanamycin (50 µg/ml) plates and incubated overnight at 37°C.

2.2.6 Excising the kanamycin cassette

Cells that have kanamycin, replacing pagP, were identified and made electrocompetent by using the similar protocol mentioned above. The helper plasmid, pCP20 was isolated using a QIAprep Spin Miniprep Kit (Qiagen). Around 400 ng of plasmid was transformed into the competent cells of MG1655 containing kanamycin cassette. The transformed cells were incubated in a shaker at 30°C for 2 hours. 200-300 µl of cells was plated in LB-amp plates containing different concentration of ampicillin (5, 10, 20 and 30 µg/ml).

Plates containing the transformed cells were incubated at 30°C for 36 hours to 48 hours. Some colonies were picked up and streaked onto LB agar plates and incubated overnight at 42°C to activate Flp-recombinase. Colonies grown were streaked on the following plates: LB-ampicillin (30 µg/ml) to check the loss of plasmid; LB-kanamycin (10 µg/ml) to ensure the excision of kanamycin cassette; and on LB agar plates to recover the strains. Colonies grown on LB agar but not in LB-amp and LB-kan were picked up, and the excision of kanamycin cassette was verified by PCR.
2.2.7 Lipid A isolation and analysis

An overnight culture was diluted (1% inoculum) into 5 ml LB containing 5 µCi/ml $^{32}$P. The sub-culture was incubated with shaking at 37 °C for 3-4 hours depending on the growth rate of the strain, after which the cultures were treated with or without 25 mM EDTA for 5 minutes. Radiolabelled cells were harvested by centrifugation and washed once with 5 ml PBS. The supernatant was discarded, and the pellet was resuspended in 0.8 ml PBS and converted into a single-phase Bligh-Dyer mixture by adding 2 ml of methanol and 1 ml of chloroform. The mixture was vortexed gently and incubated at room temperature for 10 minutes; and the resulting precipitate was collected by centrifugation. The supernatant was discarded, and the pellet was washed once with 5 ml of a single-phase Bligh-Dyer mixture (chloroform/methanol/water 1:2:0.8 v/v). The pellet was resuspended in 1.8 ml of NaCH$_3$CO$_2$H (pH 4.5) containing 1% SDS by sonication. In order to cleave the labile ketosidic linkage between the distal lipid A glucosamine residue and the first Kdo sugar of the core oligosaccharide, the solution was incubated at 100 °C for 30 minutes. Once cool, the solution was converted into a two-phase Bligh-Dyer mixture (chloroform/methanol/water 2:2:1.8 v/v) by adding 2 ml of chloroform and 2 ml of methanol. The resulting two-phase solution was partitioned by centrifugation, and the lower phase was transferred to a new test tube, which was washed once with the upper-phase of a fresh two-phase Bligh-Dyer mixture. This mixture was partitioned as before, and the lower phase was transferred to a new test tube, where the lipid A sample was dried using Nitrogen gas and dissolved in 100 µl of a 4:1 chloroform/methanol solution.
The cpm of the $^{32}$P-labelled lipid A samples were determined using a scintillation counter, and a 1000 cpm sample was applied to the origin of a glass-backed Silica Gel 60 TLC plate. The TLC was developed in a glass tank equilibrated with a solvent system consisting of chloroform, pyridine, 88% formic acid and water (50:50:16:5 v/v). The developed plate was air-dried and visualized with a PhosphorImager (Amersham Biosciences).

### 2.2.8 RNA extraction

All the control (NR754) and experimental (NR760) strains were streaked onto LB agar plates and a single colony was inoculated into 5 ml LB media to make overnights. Overnights were subcultured (1:100) and were grown at 37°C to reach mid log phase (optical density (600nm) between 0.6-0.8). 1 ml of subculture was taken and centrifuged at 12000 rpm for 3 minutes, and the supernatant was discarded. Pellet was resuspended in 700 µl of water (RNase free, DEPC treated from Sigma-Aldrich) with 50µl of 100 mg/ml lysozyme. The mixture was incubated at 37°C for 45 minutes and spun down at 13000 rpm at 4°C for 15 minutes. Pellet was resuspended in 1 ml trizol and was vortexed at high speed for 10-20 seconds. The mixture was incubated at room temperature for 5 minutes; 200 µl of chloroform was then added to the mixture and was vortex briefly. After 2-3 minutes of incubation, mixture was centrifuged at 13000 rpm for 20 minutes at 4°C. Aqueous phase was then taken as much as possible without touching the other phase (400 ul).
2.2.9 On column digestion and purification of RNA

One volume of 70% ethanol was added to one volume of aqueous phase and was mixed thoroughly. 700 µl of the mixture was added to the column provided for RNA purification and centrifuged at 9000 rpm for 30 seconds. After discarding the flow through, remaining mixture was added to the column and centrifuged. 350 µl of RW1 buffer was added and centrifuged at 9000 rpm for 30 seconds and flow through was discarded. 10 µl of Dnase I stock solution was added to 70 µl of Buffer RDD and mixed by gently inverting the tube. 80 µl of Dnase I incubation mix was directly added to the Rneasy silica-gel membrane and placed on the bench top (20-30°C) for 15 minutes. 350 µl of RW1 buffer was then added to the mixture and centrifuged, followed by the addition of 500 µl of washing buffer RPE and centrifugation for 30 seconds at 9000 rpm. 500 µl of RPE buffer was again added and centrifuged at 9000 rpm for 2 minutes. Column was replaced and was centrifuged at 13000 rpm for 1 minute. 50 µl of Rnase free water was added and was allowed to stand for 1 minute. Finally, centrifugation was done to elute purified RNA in micro centrifuge tubes and stored at -80°C. RNA concentrations were determined with a NanoDrop, and the integrity of RNA was analyzed by Bioanalyzer.
2.2.10 cDNA synthesis, Optimization of annealing temperature and standard curve

First strand of cDNA was synthesized using iscript cDNA synthesis kit from Bio-Rad. 250 ng RNA from each mutant was converted to cDNA. A tenfold dilution of cDNA was made, and ssofast evagreen supermix along with primers was used to amplify the cDNA. Temperature gradient from 55°C to 65°C was used to figure out the appropriate common annealing temperature for all the primers. Dilution factor of primer sets was identified for determining the standard curve of every primer. Efficiency of all the primers ranges from 95-105%.

2.2.11 qPCR and data analysis

5 µl of ssofast evagreen supermix, 4 ul of cDNA template and 10uM of primers (0.5 µl each) was added to make a total volume of 10 µl. Each samples had three biological replicates and each biological replicates were made into three technical replicates to strengthen the statistical analysis. qPCR was done in CFX96 touch™ Real-time PCR detection system (Bio-Rad). The conditions for the qPCR were as follows: 2 minutes at 98°C followed by a cycle of 2 seconds at 98°C, and 5 seconds at 61°C. The number of cycles used for amplification was 40. Data obtained through qPCR was analyzed by software called cfx manager (Bio-Rad).
Chapter 3: Results

3.1 Confirmation of pagP as a non-essential gene

_E. coli_ possess around 4000 genes and based on the essentiality for the bacteria to survive, these genes have been categorized into essential and non-essential genes [77]. A gene is considered to be “essential”, if its knockout results in lethality and a gene is considered to be “non-essential” if its knockouts are still viable [78]. Non-essential genes in _E. coli_ K-12 have been deleted and compiled in a library called the Keio Collection [79].

_pagP_ is a PhoP activated gene which is located in the 14.13 minutes of the _E. coli_ chromosome. It is 561 base pairs long and encodes an OM protein called PagP [77]. The Keio Collection has categorized _pagP_ as a non-essential gene and _pagP_ deleted strains are available in the Keio library. Strains designed for this project were constructed on _pagP_ deleted strain available in the Keio library.

When the ΔpagP strains designed for this project were tested, _pagP_ was found to be present in the original locus along with the kanamycin cassette that was used to replace _pagP_ (data not shown). It seems _pagP_, which was replaced by antibiotic marker, somehow got reverted and made us hypothesized that _pagP_ is essential to bacteria. To test the essentiality of _pagP_, gene knockout experiments were done to delete it from the chromosomes of MC1061 and BW25113 strains of _E. coli_ K-12.

Gene knockout experiment is an efficient way to determine the essentiality of gene in an organism. This method was applied to delete _pagP_ from the chromosomes of MC1061 and BW25113 and got two bands repeatedly (one corresponding to _pagP_ and
the other corresponding to kanamycin cassette) in an agarose gel, with products of amplification of the primers that hybridize flanking \textit{pagP} (fig 10 (a)). Efforts were made to delete upstream (promoter) and downstream regions of \textit{pagP} and using different antibiotic marker but the agarose gel electrophoresis result was showing similar patterns of bands (data not shown). After repeating the experiment by using new sets of reagents and primers, \textit{pagP} was successfully replaced from the original locus with kanamycin cassette (fig. 10 (b)). This suggested that the \textit{pagP} band that was constantly appearing in the agarose gel electrophoresis results due to an artifact or contamination in some of the reagents.

PagP specific lipid A analysis was also performed to further validate the successful knockout of \textit{pagP} from the chromosome. $^{32}$P labeled lipid A was isolated by mild acid hydrolysis from EDTA treated and untreated cells and resolved by a thin layer chromatography (TLC). Wild-type strain palmitoylated lipid A after the addition of EDTA whereas \textit{pagP} deleted (\textit{\Delta pagP}) strain did not palmitoylate the lipid A even after the addition of EDTA (fig. 11). This implies that PagP is absent in the chromosome of \textit{\Delta pagP} strain.

\textit{pagP} encode an OMP called PagP which is responsible for lipid A modification by adding extra palmitoyl chain to it [58]. PagP normally remains in the dormant condition in the OM, and get activated only after the perturbation of the OM asymmetry [67]. Therefore, PagP is triggered in bacteria only under membrane stress conditions and does not respond during normal cellular growth. Growth and viability of \textit{E. coli \Delta pagP} strain supports the non essentiality of \textit{pagP}. 

44
Figure 10. Confirmation of pagP as a non essential gene. Chromosomes extracted from the wild-type, and ΔpagP strain was amplified by the primers that hybridize flanking pagP and was visualized on a 1% agarose gel. A) Gel shows two bands for BW25113ΔpagP; one corresponding to wild-type and the other higher than wild-type. B) Gel shows a single band higher than pagP corresponding to the presence of kanamycin for BW25113ΔpagP.
Figure 11. Lipid A analysis of ΔpagP strains. Lipid A isolated from both MC1061 and MC1061ΔpagP after mild acid hydrolysis was resolved on TLC and analyzed with phosphorImager. MC1061 shows the increase in intensity of hepta-acylated band with the addition of EDTA and in contrast, MC1061ΔpagP shows equally intense hepta-acylated spot even after the addition EDTA.
3.2 \textit{imp4213 (lptD4213)} disrupts the permeability barrier and constitutively activate PagP

LPS, present in the outer leaflet of the OM, is essential for maintaining the permeability barrier in the OM. Lipid A-core moiety of LPS, synthesized in the cytoplasm, is flipped across the IM by MsbA [80] and gets attached with O-antigen [7]. Protein complexes of Lpt family are responsible for transporting complete LPS from the IM to the outer leaflet of the OM [81]. LptD (Imp), an OM protein, is responsible for flipping the LPS from the inner leaflet to the outer leaflet of the OM [32]. \textit{imp (lptD)} is an essential gene [79], but \textit{imp4213 (lptD4213)} is 8 Base pair deletion, which restricts the enzyme’s ability to move LPS into the outer leaflet of the OM in non lethal manner [82]. Therefore, \textit{imp4213} increases the permeability barrier of the OM by restricting the LPS in the inner leaflet of the OM.

Based on the \textit{imp} allele, two strains have been designed for this project, NR754 (\textit{E. coli MC4100, ara}^+), and NR760 (\textit{E. coli MC4100, ara}^+, \textit{imp4213}). In order to evaluate the state of the OM in these strains, both types of strains were streaked on MacConkey agar plates. MacConkey agar consists of bile salts, and the tolerance of gram-negative enteric bacteria to bile is partly a result of the relatively bile-resistant OM, which hides the bile-sensitive IM. Therefore, MacConkey agar plating helps in selecting against organisms with a compromised permeability barrier. Both strains grew in LB agar medium, but only NR754 strains showed viable colonies in MacConkey agar plate which implies the compromised permeability barrier in NR760 strains (fig.12)
PagP adds up a palmitate chain to lipid A and convert hexa-acylated lipid A to hepta-acylated lipid A which can be differentiated by Thin layer chromatography (TLC). Lipid A was labeled with $^{32}$P and isolated from cells by mild acid hydrolysis. 5 ml culture of all NR754 and NR760 strains were grown for 3 hrs and adjusted with or without 25mM EDTA for an additional 5 minutes. Lipid A was separated by the TLC and visualized with a phosphorImager. For NR754 strains, which has wild-type $imp$, hepta-acylated lipid A appeared in the TLC after the addition of EDTA, but for NR760 strains, which has $imp4213$ allele, palmitoylation was occurring even without EDTA treatment (fig. 13). This implies that PagP is constitutively active in the strains which have $imp4213$ allele.

$imp4213$ reduces the function of Imp to assemble LPS in the OM and only few LPS are available in the OM. As a result, phospholipids, which are normally present in the inner leaflet, migrate to the outer leaflet. Presence of phospholipids patches in the OM create a localized symmetry, thus disrupts the permeability barrier, and allow the access of hydrophobic compounds [82]. Since both the substrates of PagP, phospholipid and lipid A, are now present in the outer leaflet, they can easily gain access to the active site of PagP and make it active [63].
Figure 12. **MacConkey agar plating.** NR754 (imp⁺) and NR760 (imp4213) strains grow in LB agar medium and MacConkey agar plate for 16 hours at 37°C. LB agar plate shows the growth of both strains, whereas MacConkey agar plate shows the growth of NR754 strain only.
Figure 13. Lipid A analysis of NR754 and NR760 strains: Lipid A was isolated from NR754 and NR760 strains after mild acid hydrolysis and analyzed by TLC. The degree of palmitoylation of all the strains was analyzed. NR754 has the minimum amount of palmitoylation that increases to a significant amount after the addition of 25 mM EDTA. NR760, which has permeability defect, shows the hepta-acylated lipid A even without the addition of EDTA and addition of EDTA does not affect the palmitoylation level.
3.3 Y87F mutant palmitoylates lipid A to the same degree as the wild-type PagP

Three amino acid residues (Asp 61, His 67 and Tyr 87), present at the interface of the OM and the periplasm, are arranged in such a way that they can act as a catalytic triad to cleave the peptide bond. Their superimposable image with the catalytic triad of chymotrypsin provides an extra boost for this hypothesis. These amino acid residues were mutated and analyzed the differences in the phenotype of LPS from these mutants. Result showed that D61 and Y87, but not H67, are required for the truncation of R3 core oligosaccharide. The mutant of these residues, Y87F, showed the confirmation, stability and specific activity similar to wild-type PagP (Smith unpublished data).

The palmitoylation of lipid A affects the OM permeability barrier, and, therefore, global gene regulation. In order to compare the gene expression between the two mutants to identify the downstream targets, a signaling deficient mutant with the similar palmitoylating ability is necessary. One of the signalling deficient mutants of the catalytic triad residue, Y87F, fulfilled this characteristic. In order to verify that Y87F palmitoylates lipid A to the same degree as the wild-type enzyme in the control (NR754) and experimental strains (NR760), $^{32}$P lipid A was isolated from EDTA-treated and -untreated cells and resolved by TLC. In NR754 strains, palmitoylation increased up to 49% and 43% after the addition of EDTA for wild-type PagP and Y87F mutant (fig. 14). In NR760 strains, wild-type palmitoylates lipid A to 45% in EDTA untreated cells, which increased up to 55% in EDTA treated cells. Similarly, Y87F has the palmitoylation of 42% and 52% for EDTA treated and untreated cells respectively (fig. 15). It is, therefore,
an ideal construct to use as the signaling-negative control in evaluating PagP-mediated changes in gene expressions.

S77 residue present in the extracellular loops plays a prominent role in the palmitoylation event of PagP. Mutant of this residue, S77A, was analyzed for its role in palmitoylation. $^{32}$P labeled lipid A was extracted from NR754 and NR760 strains, with and without EDTA, and analyzed by TLC. For both type of strains, S77A did not palmitoylated lipid A and treating the cells with EDTA did not affect the palmitoylation (fig. 14 and 15). Therefore, S77A can be used in the gene expression experiment, as a catalytically inactive construct, to identify the relationship between palmitoylation and signaling event perform by different domains of PagP.
Figure 14. Lipid A analysis of NR754 strains. $^{32}$P labeled lipid A, with and without the addition of EDTA, was isolated from all the NR754 strains and analyzed in TLC plates. Percentage of lipid A palmitoylation increased up to 49% and 43% after the addition of EDTA in pagP and Y87F, whereas the percentage of palmitoylation did not get affected even after the addition of EDTA in S77A and Bad18. The plate shown is representative of 3 independent experiments.
Figure 15. Lipid A analysis of NR760 strains. $^{32}$P labeled lipid A, with and without the addition of EDTA, was isolated from all the NR760 strains and analyzed in TLC plates. 45% and 42% of lipid A is palmitoylated even before the addition of EDTA in pagP and Y87F respectively, which increases up to 55% and 52% after the addition of EDTA. Percentage of palmitoylation did not get affected even after the addition of EDTA in S77A and Bad18. The plate shown is representative of 3 independent experiments.
3.4 Correction and validation of strains

The pagP deletion strain from the Keio Collection [79] was used to convert NR754 (E. coli MC4100, ara⁺), and NR760 (E. coli MC4100, ara⁺,imp4213) to NR754ΔpagP and NR760ΔpagP. Plasmid suitable for using in the λ Inch system for making chromosomal mutants were generated by sub cloning pagP from pACPagP, pACS77A, and pACY87F into pBad18. Finally, these clones were moved by λInCh vector to the λatt site of NR754ΔpagP and NR760ΔpagP to generate the desire strains for this project (Smith unpublished data) [83].

All the strains designed for this project were obtained from previous undergraduate student from Dr. Bishop’s lab. Efforts were made to verify the deletion of pagP from original locus and insertion of correct mutants of pagP (pagP, Y87F and S77A) at the λatt site of both control (NR754) and experimental (NR760) strains. Polymerase chain reaction (PCR) method was used to check the pagP deletion in 14.13 minutes of E. coli chromosome. Extracted chromosomes from these strains were amplified with the primers that hybridize flanking pagP. For all the mutants (both control and experimental), gel showed two bands; one corresponding to the kanamycin cassette which was used to delete pagP and the other corresponding to pagP (data not shown). This result implies that pagP, which was supposed to be deleted, is still present in the chromosome. Multiple repetition of this experiment to check the deleted pagP at the original locus was showing similar results.
Since the mutants were constructed in the pagP deleted strains from the Keio Collection, ΔpagP strain from the Keio collection library were tested. Chromosome of ΔpagP strain was amplified by the primers that hybridize flanking pagP and got similar pattern of two bands. Keio collection is a set of single-gene deletion mutants of E. coli K-12 and all the deletion mutants are analyzed by high throughput technologies [79]. Therefore, the continuous appearance of pagP band in pagP deleted strain seems to be an artifact or contamination present in the reagents. Therefore, instead of relying on the strains given to me, I decided to go back to the original stock of these mutants.

Surprisingly, while amplifying the original stocks of all the mutants by the same set of primers that hybridize flanking pagP, single band corresponding to excised kanamycin cassette appears for control (NR754) strains and a single band corresponding to kanamycin cassette was present for experimental (NR760) strains (fig. 16 (a) and 17 (a)). Kanamycin cassette was excised with helper plasmid pCP20 (fig. 17 (b)). Analysis of the mutants that were inserted at the λatt site was done by using pagP terminal primers and got the single band corresponding to wild-type pagP (fig. 16 (b) and 17(c)). Sequencing of these bands showed the presence of desire sequences in all mutants. Finally, all the strains necessary to carry forward the gene expression experiments were obtained.
Figure 16. **PCR verification of NR754 strains.** Extracted chromosomes from all the NR754ΔpagP strains were amplified by different primers and visualized in an agarose gel. A) Primers that hybridize by flanking pagP at the original locus were used to amplify the pagP region and the entire ΔpagP strains show lower band than wild-type. B) pagP and its mutants (S77A and Y87F) inserted at the λatt site were amplified with the primers specific to pagP, and the entire construct (except Bad18, negative control) shows similar band size.
Figure 17. **PCR verification of NR760 strains.** Extracted chromosomes from all the NR760ΔpagP strains were amplified and visualized in agarose gel. A) Primers that hybridize by flanking pagP at the original locus were used to amplify the pagP region and all the ΔpagP strains show higher band than wild-type equivalent to the presence of kanamycin. B) After exercising the kanamycin cassette, same primers were used to amplify the pagP region and show a lower band than wild-type. C) pagP and its mutants (S77A and Y87F) inserted at the λatt site were amplified with pagP specific primers and the entire construct (except Bad18, negative control) shows the similar band size.
3.5 PagP activation does not affect the transcription of WaaG glucosyl transferase

In *E. coli* O157:H7, activation of PagP in the OM affects the addition of first outer core resulting truncated LPS. In these strains, deficiency of MsbB enzyme, which transfers the myristate chain to the distal glucosamine unit of lipid A is sufficient to breach the outer permeability barrier and activate PagP [71]. This experiment is shifted to *E. coli* K-12, where MsbB deficiency does not activate PagP. Instead, *imp4213* allele has been used, which attenuates the assembly of LPS in the OM [82], and activates PagP. Glucosyl transferase encoded by *waaG* is responsible for the addition of first outer core glucose residue. Therefore, assessing the transcription of *waaG* in all the mutants, in both control and experimental strains, will give us the clear overview of the effects of the OMP PagP over the transcription of *waaG*.

*waaG* transcription, using the quantitative real time PCR, was analyzed in Wild-type, catalytically inactive (S77A) and signalling deficient (Y87F) mutants of PagP. These mutants were inserted in strains where PagP remained in the dormant state (NR754) and activated state (NR760). Results show similar level of transcription for wild-type, catalytically inactive (S77A) and signalling deficient (Y87F) in both control (NR754) and experimental (NR760) strains (fig. 18). The transcription of *waaG* in wild-type and signalling deficient mutant, Y87F, is also similar in NR754 and NR760 strains. The stable mRNA levels of *waaG* in all the mutants suggested that activation of PagP which resides on the OM does not affect the transcription of *waaG*, which encodes cytoplasmic enzyme of first outer core biosynthesis (WaaG).
In *E. coli* O157:H7, truncation of core occurs at the first outer core glucose which is added by WaaG glucosyl transferase, but the expression of WaaG from recombinant plasmid did not correct the core truncation. Instead, replenishing the substrate of WaaG, UDP-Glucose, corrects the core truncation [71]. Similarly, transcription of *waaG* is also unaffected by the activation of PagP in *E. coli* K-12.
Figure 18. Transcription of *waaG* in different mutants of *pagP*. Transcription of *waaG* in different mutants of *pagP* in PagP inactivated (NR754) and activated (NR760) strains were analyzed and the result shows the similar level for each mutant in both NR754 and NR760 strains.
3.6 PagP controls transcription of slyB

PagP has a unique interior with the hydrophobic upper half and hydrophilic lower half lined with 9 molecules of water [63]. Since the putative catalytic triad is present at the interface of the OM and the periplasm, water molecules needed for the catalytic reaction can be easily provided [84]. To transduce the signal from PagP the catalytic triad needs a substrate to act upon and SlyB, which is a putative lipoprotein present in the inner leaflet of the OM [46], might act as a potential substrate. SlyB is the only lipoprotein present in the PhoPQ regulon of both Salmonella enterica and E. coli [48] and has the probability of interacting with PagP. We propose when a permeability defect in the OM causes PagP to become activated [67], then Asp61, His67, Tyr87 residues on the periplasmic side will become exposed through an allosteric transition so as to cleave the N-terminal lipid anchor of SlyB and release it into the periplasm.

Decrease in transcription of slyB by four fold in a strain containing pagP than in the negative control suggested that PagP inhibits the transcription of slyB. This transcription pattern of slyB is similar in permeability intact (NR754) and permeability defective strains (NR760). The highest transcription of slyB is present in catalytically inactive mutants for palmitoylation (S77A) and shows the similar pattern in both NR754 and NR60 strains. Signalling deficient mutant (Y87F) in NR745 strain has two fold increases in the transcription of slyB than wild-type, which increases up to four fold in a strain that has constitutively activated PagP (fig. 19). This shows the signal to terminate transcription of slyB is transducing through the putative catalytic triad of PagP.
Therefore, \textit{pagP} has the inhibitory effect towards \textit{slyB} transcription. Presence of
PagP, although in inactive state, seems to be sufficient to inhibit the transcription of \textit{slyB}.
Higher transcription of \textit{slyB} in one of the mutants of catalytic triad (Y87F) than the wild-type
supports the role of putative catalytic triad, present in the periplasmic side of PagP, in the
inhibition of \textit{slyB} transcription. Therefore, when PagP, present in the OM gets activated, the
transcription of \textit{slyB} gets inhibited probably through PhoPQ system.
Figure 19. Transcription of slyB in different mutants of pagP. qPCR approach was used to analyze the transcription of slyB in different mutants of pagP. Transcription of slyB is high in negative control than in the strain with pagP in both the types of strain. Y87F mutant has higher slyB transcription in NR760 than in NR754 strain.
3.7 PagP and stress response regulator (σE)

PagP is activated as a result of defect in permeability barrier of the OM and transduce its signal across the OM which finally reaches to cytoplasm. In the cytoplasm, this signal leads to truncate the LPS at the first outer core by limiting the substrate, UDP-glucose, of its enzyme, WaaG. σE is also predicated to upregulate the transcription of membrane enhancing substances like colonic acid, [85] which use up UDP-glucose, [86] and resulting in its depletion in the cytoplasm. Since both, PagP and σE, are known to deplete the level of UDP-glucose in the cytoplasm, the relationship between them is plausible.

Production of atypical lipid A by the mutation of msbB or htrB, which transfer myristoyl and lauryl residues respectively to the distal glucosamine of lipid A, also has been shown to increase the expression of σE regulon [57]. PagP also produces the atypical lipid A by palmitoylating lipid A thereby converting regular hexa-acylated lipid A into hepta-acylated lipid A [58]. It has also been shown that this palmitoylated lipid A also activates σE expression but when cells are grown at 42º [57]. These evidences show another way of link between the activation of PagP and extra cytoplasmic factor E (σE).

qPCR specific to rpoE was done to analyze its transcription pattern among the different mutants of pagP in permeability intact (NR754) and defective (NR760) strains. The transcription level of negative control and wild-type was similar in both NR754 and NR60 strains. Therefore, PagP does not seem to affect the transcription of stress response regulator, σE. Catalytically inactive (S77A) and signalling deficient mutant (Y87F) of
PagP also showed the similar level of transcription in NR754 and NR760 strains. Therefore, \( rpoE \) transcription is independent of PagP.

**Figure 20. Transcription of \( rpoE \) in different mutants of \( pagP \).** Result shows the normalized expression of \( rpoE \) in various mutants of \( pagP \) in NR754 and NR760. All the mutants show similar level of transcription in the strain containing pagP in dormant state (NR754) and active state (NR760).
Chapter 4: Discussion

PagP is an OM protein of *E. coli* designed to cope with the OM stress. PagP responds to a breach in the permeability barrier and repairs it by palmitoylating the lipid A. PagP is normally a latent enzyme but gets activated whenever lipid asymmetry is perturbed. In *E. coli* O157:H7, deficiency in the cytoplasmic lipid A biosynthesis enzyme, MsbB, causes the activation of PagP, which in turn affects the cytoplasmic events of core biosynthesis. This finding has revealed the new role for PagP as a signal transducer [71].

The multifunctional OMP OmpA, which is involved in various functions like adhesion, invasion and immune evasion, is already known [87]. Similarly, OMPs involved in signalling have already been characterized as well. FecA can perform dual role as a transporter protein and also as a signalling protein. FecA transports ferric citrate across the OM and binding of ferric citrate also initiates a signaling cascade resulting in transcription of the *fecABCDE* iron transport machinery [88].

*E. coli* O157:H7 is a pathogenic strain and is subjected to harsh environmental conditions. During the process of adjustment to survive in such environment, it might undergo numerous modifications. It is possible that signalling of PagP also occurs during this process and might not be true in all strains. Therefore, to test whether signalling is a regular phenomenon in PagP or is present particularly to these pathogenic strains, the model organism has been shifted to laboratory strain, *E. coli* K-12. A deficiency of *msbB*
does not activate PagP in *E. coli* k-12 [71] and hence, alternative approach should be used to activate PagP in this strain.

PagP remains in the inactive state unless the permeability barrier of the OM is breached. EDTA can breach the permeability barrier by stripping the Mg$^{2+}$ ions that are used to bridge the adjacent LPS in the outer leaflet of the OM, but prolonged treatment of bacteria with EDTA inhibits the RNA synthesis and compromises cell viability [67]. An *imp4213* mutant of *E. coli* is known to permeablize the OM [82]. Imp is an OM protein involved in transporting LPS to the OM outer leaflet [76]. *imp* is essential, but the *imp4213* mutant is viable although it possesses severe permeability defects [82]. This work has confirmed the permeability defects in the strains containing *imp4213* allele and also established that permeability defect causes by *imp4213* results in the constitutive activation of PagP.

PagP has well established role as a palmitoyltransferase but this activity, occurring in the outer leaflet side of the OM, does not affect its signalling ability [71]. Thus, PagP is communicating with the cytoplasm through a separate mechanism, likely via a different domain of PagP. PagP’s inactive conformation reveals three amino acid residues (Asp61, His67, and Tyr87) at the periplasmic side, which are nearly superimposable with the catalytic triad of chymotrypsin. This hydrolytic triad, present at the periplasmic side of PagP, has the probability of playing role in signal transduction, separated from the palmitoylation of lipid A, in response to the OM stress.
As PagP’s palmitoylation of lipid A impacts the integrity of the OM, and, therefore, the Gram-negative response to OM stress, it is crucial that any mutant of PagP used as a signaling-deficient construct in the impending investigation of PagP’s ability to affect gene expression should palmitoylate lipid A to the same degree as the wild-type enzyme. Y87F fulfill these characteristics and can be comparable to wild-type in gene expression experiments to identify the members of PagP mediated signalling.

A qPCR approach was taken to analyze the differences in gene expression of some candidate genes of PagP mediated signalling. qPCR is the PCR based technology that monitors the amplification of specific target sequences during the course of the reaction with the help of fluorescence dyes or probes. The amount of fluorescence produced is proportional to the amount of product formed, and the number of amplification cycles required to obtain a particular amount of DNA is registered. Assuming a certain amplification efficiency, which typically is close to a doubling of the number of molecules per amplification cycle, it is possible to calculate the concentration of DNA initially present in the sample [89].

While analyzing the phenotypic trait (structure of LPS), activated PagP was shown to affect the addition of first outer core glucose added by WaaG glucosyl transferase. Substrate UDP-glucose rather than the enzyme WaaG glucosyl transferase was shown to be limiting, which leads to the truncation of the first outer core [71]. Using qPCR method, the effect of PagP activation to the waaG transcription was analyzed and found that waaG transcription is unaffected by PagP activation.
PagP is activated by the stress in the OM and plays a role in mitigating this stress. Similarly, Gram-negative bacteria consist of various stress response regulators, which respond to stress in the OM. Extracytoplasmic factor, σE, is also one of the stress response regulators that is activated by stress in the OM. σE has been shown to respond to the presence of atypical lipid A, and activation of PagP has also been shown to increase the expression of σE, but only when cells are grown at 42°C [57]. This work has established that rpoE transcription is unaffected by the activation of PagP.

In *E. coli*, 90% of more than 100 lipoproteins are found in the inner leaflet of the OM. Most of them function in synthesizing and maintaining the components of the OM [42]. Due to their location, they are in a position to interact with OMPs to perform these functions. LptE (RlpB), a lipoprotein present at the inner leaflet of the OM, works together with integral OMP, LptD (Imp), to flip the LPS from the inner leaflet to the outer leaflet of the OM [31].

The proteolytic triad of PagP is present at the periplasmic side, which can cleave the lipoprotein, present in the inner leaflet of the OM, to transduce the signal. Of the various lipoproteins present in the inner leaflet, a PhoPQ regulated lipoprotein called SlyB has the higher possibility to link with PagP because it is the only lipoprotein present in the PhoPQ regulon of both *Salmonella enterica* and *E. coli* [48]. This work has established that PagP controls transcription of slyB, and that a signal must be transduced across the cell envelope through the putative catalytic triad present on the periplasmic face of PagP.
PagP and SlyB both maintain the integrity of the OM of the Gram-negative cell envelope [43, 58]. In our model, membrane perturbing agents like CAMPs breach the OM permeability barrier, to activate PagP. Activated PagP palmitoylates the lipid A and restores the permeability barrier of the OM. In addition, a signal somehow reaches the IM and influences the PhoQ sensor kinase to autophosphorylate. The PhoQ response regulator then negatively regulates the transcription of *slyB*. When PagP is absent in the OM to maintain the permeability barrier, the PhoPQ signal transduction system activates transcription of *slyB*. In this manner, SlyB might compensate for the absence of PagP (fig. 21).
Figure 21: Model for *pagP* control of *slyB* transcription. A breach in the permeability barrier of the OM activates PagP, which restores OM function by palmitoylating lipid A. The PhoPQ system, in addition to sensing CAMPs, also receives a signal from activated PagP and terminates activation of *slyB* transcription by phosphorylated PhoP. In contrast, when PagP fails to send its signal, the PhoPQ system activates transcription of *slyB*. 
Chapter 5: Future Directions

Traditional qPCR can solve many biological questions by assessing the expressions of genes involved in various pathways, but only one gene at a time. This time consuming and labor intensive method is rapidly being replaced by data sets generated by using new high throughput technologies. Recent advances in RNA high-throughput sequencing (RNA-seq) overcome these limitations and provide high resolution for characterizing the transcriptome and differential gene expression [90]. Therefore, RNA-seq will be a useful method to see the changes in gene expression at the global level. The overall process of RNA-seq analysis includes the following steps - mRNA isolation, cDNA library construction, next generation Illumina sequencing, and bioinformatics and statistical analyses to identify differentially expressed transcripts [91].

A mutant of PagP, Y87F, can be compared with the wild-type by RNA-seq to reveal the downstream targets. Both, wild-type and Y87F, has been transduced into the strains having imp and imp4213 background and are labeled as NR754 and NR760. NR754 can be used as control strain because it has intact OM asymmetry and PagP is in a dormant condition. The NR760 strain with wild-type and signal deficient mutant (Y87F) can be used as experimental strains for this experiment. Wild-type and Y87F mutant palmitoylate lipid A to a similar extent in NR760 strain. As a result, both mutants have the same OM composition; only differing in their signalling ability. Gene expression of these two strains can be analyzed and compared. Those genes that are differentially
expressed in wild-type and Y87F will give us an idea of the genes that might be involved in PagP mediated signalling.

PagP is continuously activated in the strains having the \textit{imp4213} allele and can take up both substrates (phospholipid and lipid A), which are available in the outer leaflet to perform the acyl transferase reaction. PagP-mediated signalling also has been shown to be initiated when PagP is activated. Therefore, these two events, which are occurring in different parts of PagP, might be related. In order to find the link between these two events, the catalytically inactive S77A mutant can be use with negative control. The difference between the wild-type and S77A mutant in the \textit{imp4213} background is that, wild-type PagP can add an extra acyl chain to lipid A but S77A is unable to do so. The presence of extra acyl chain and the difference in the OM structure, as a result, can lead to the differential gene expression. Again, these mutants in NR754 strains can be use as control. Through RNA-seq, the differentially expressed genes between the wild-type and catalytically inactive mutant can be analyzed, and the relationship between catalysis and signalling will be identified.

The signal transduced by activated PagP leads to decrease in the cytoplasmic pool of UDP-glucose which is manifested as the truncated core [71]. UDP-glucose is the key regulatory step in bacterial metabolism and the depletion of UDP-glucose pool might be due to the metabolic effects. Metabolic effects can be achieved by allosteric control mechanism. Although, most regulatory control points in bacteria are exerted at the level of transcription, the regulation through allosteric enzyme activity cannot be overlooked. Therefore, if the gene expression experiment did not give any convincing results,
allosteric regulation can be the alternative way to elucidate the pathway of PagP mediated signalling.
References


49. Firoved, A.M., J.C. Boucher, and V. Deretic, *Global genomic analysis of AlgU (sigma(E))- dependent promoters (sigmulon) in Pseudomonas aeruginosa and *


