EFFECTS OF PROTAMINE ON *PSEUDOMONAS AERUGINOSA* CELL ENVELOPE COMPONENTS: SURFACE REMODELLING

by

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DALHOUSIE UNIVERSITY

DEPARTMENT OF PROCESS ENGINEERING AND APPLIED SCIENCE

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Abstract

The main objective of the study was to understand the mode of interaction of protamine (Ptm), a cationic antibacterial peptide from fish milt on the Gram negative bacterial envelope. The present study was designed to resolve the question of Ptm translocation across the seemingly impermeable Gram negative cell envelope. The Gram negative pathogen *Pseudomonas aeruginosa* was studied as an example of a microorganism that is Ptm-sensitive but doesn't lyse even at bactericidal concentrations. Acquired resistance to Ptm was induced in *P. aeruginosa* by continuous sub-culturing in nutrient rich media containing increasing concentrations of Ptm. Alterations in bacterial surface charge, LPS composition, cell morphology and Ptm localisation on acquiring resistance were also examined. Expression of outer membrane proteins significantly decreased as *P. aeruginosa* acquired resistance to Ptm. OprF, the major porin in *P. aeruginosa* was found to be stably expressed in control, revertant (Ptm-Rev) and resistant (Ptm-Res) groups. No change in expression of efflux proteins was observed as a result of induced Ptm resistance, indicating that efflux is not among the Ptm resistance mechanisms at least in *P. aeruginosa*. OprM, which is part of the major efflux system (MexAB-OprM) in *P. aeruginosa,* was found to be down-regulated in Ptm-resistant *P. aeruginosa*. Another outer membrane protein down-regulated in Ptm-resistant *P. aeruginosa* was found to be petidyl-prolyl *cis trans* isomerase (PPIase) which plays a major role in proper folding and maturation of channel proteins in the outer membrane. Among the sarcosinate soluble proteins, DNA dependent RNA polymerase β and β' subunits were found to be down-regulated in Ptm-resistant group indicating lower transcription levels in them. Lipopolysaccharide (LPS) from the three groups of *P. aeruginosa* under study was isolated and separated by SDS-PAGE. LPS composition of Ptm-Res *P. aeruginosa* was found to be significantly different from that of the control and Ptm-Rev but was found to be similar with that of LPS from O-antigenic mutant (A+B-, which possessed only A band structures). Comparison of the zetapotential of control, Ptm-Rev and Ptm-Res *P. aeruginosa,* proved that electrostatic shielding was coincidental in acquired resistance to Ptm in *P. aeruginosa*. The MIC of the parent strain of *P. aeruginosa* (A+B+) and the O-antigenic mutants (A+B-, A-B+ and A-B-) were found to be the same which may be indicating that alterations in O-antigenic components alone cannot contribute to Ptm resistance. Effects of Ptm treatment on morphologies of *E. coli*, *S. typhimurium* and *P. aeruginosa* whole cells and spheroplasts were also studied using transmission immuno-electron microscopy. Condensation of cytoplasmic contents was observed when whole cells and spheroplasts were treated with Ptm. Also, Ptm-treated cells and spheroplasts were stained with colloidal gold-labelled antibodies against Ptm to determine distribution within the target cells. It was quite evident that Ptm internalised in whole cells and spheroplasts without lysis and was found to be concentrated in the cytoplasm. Morphological changes observed in Ptm-Rev *P. aeruginosa* when exposed to Ptm were comparable with that of the control. Condensation of cytoplasmic contents was not observed in Ptm-Res *P. aeruginosa* when challenged with Ptm. Most of the Ptm was localized at or near the outer membrane of Ptm-treated Ptm-Res *P. aeruginosa,* indicating decreased outer membrane permeability. Results obtained from these experiments confirm that the resistance to Ptm observed in *P. aeruginosa* is at the very least, coincidental with the pleiotropic mutations involving change in outer surface including change in LPS composition, loss of porins and or alterations of porin size in OprF.

List of Abbreviations and Symbols

ACN	Acetonitrile
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAPs	Cationic antimicrobial peptides
CF	Cystic fibrosis
cfu	Colony forming unit
Cntl	Control
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
g	Centrifugal force
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
IEF	Iso-electric focusing
IgG	Immunoglobulin G
KDO	Keto-deoxy-octulonate
KLH	Keyhole Limpet Hemocyanin
LC	Liquid chromatography
LPS	Lipopolysaccharide
m	Mass
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
mV	milli volt
NPH	Neutral protamine Hagedorn
NPN	1-N-phenylnapthylamine
nS	nano siemens
OM	Outer membrane
OMP	Outer membrane proteins

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
pI	Isoelectric pH
PI	Putative type I secretion subfamily
PII	Putative type II secretion channel
PIII	Putative type III secretion channel
POE	Polyoxyethylene
PPIase	petidyl-prolyl *cis trans* isomerase
ppm	Parts per million
Ptm	Protamine
PZI	Protamine zinc insulin
Res	Resistant
Rev	Revertant
RNA	Ribonucleic acid
RNAP	Ribonucleic acid polymerase
SDS	Sodium dodecyl sulphate
SP	Specific porin
SQ	Subcutaneous
TBS	Tris Buffered saline
TEM	Transmission electron microscopy
Tris	tris(hydroxymethyl)aminomethane
TSA	Tryptic soy agar
z	Charge

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Chapter 1.

Introduction

Antibiotic resistance has been a great concern in recent years due to the extensive clinical use of classical antibiotics (Wu *et al*., 1999). The development of new families of antibiotics that can overcome the resistance problem has become important because of the emergence of more and more antibiotic-resistant pathogens worldwide (Boman, 2000). Among the possible candidates, the cationic antimicrobial peptides (CAPs) have attracted increasing research and clinical interest.

The antimicrobial peptides often display a broad spectrum of activity. Many antibacterial peptides are cytotoxic, fungicidal (Kieffer *et al*., 2003; Fehlbaum *et al*., 1996), anti-protozoan (Arrighi *et al*., 2002), anti-carcinogenic (Lindholm *et al*., 2002) and anti-viral (Tamamura *et al*., 1998; Robinson *et al*., 1998). Some antimicrobial peptides can kill bacteria *in vitro* at the concentrations of 0.25 to 4 μg/mL. Their ability to kill target cells rapidly, their unusually broad activity spectra, and their activity against some of the serious antibiotic resistant pathogens are definite advantages of these peptides (Hancock and Lehrer, 1998).

The current trend to limit the use of chemical food preservatives has also led to the increased interest in the application of CAPs to control bacterial pathogens as well as food spoilage microorganisms (Ueno *et al*., 1987; Epand and Vogel, 1999). Ptm, a CAP isolated from the spermatic cells of Pacific herring was identified as a potential novel-antibacterial agent and has applications as a food preservative (Ueno *et al*., 1987 and Ueno *et al*., 1989). Mechanisms of action of Ptm are still debated. The possibility of Ptm

internalising Gram-negative bacteria through the porins is currently being studied by our group.

1.1. Background and Objectives

Most of the CAPs studied to date exhibit antimicrobial properties depending on cell disruption and/or internalization by poration of the bacterial cell envelope. The mechanism by which Ptm inhibits bacterial growth is still not clear and the possible role of bacterial porins in Ptm internalization is suspected. Although Ptm has been shown in some cases to porate and lyse Gram-positive and some Gram-negative cells (Johansen *et al*., 1996; Islam *et al*., 1987), it has also been shown that at least in the case of *E. coli* and *S. typhimurium*, that Ptm can internalize without poration and lysis (Tolong, 2004). Our hypothesis is that Ptm may have more than one mechanism for internalization, particularly since computer simulation studies have suggested that there is effectively zero probability that Ptm can pass through the external leaflet of a Gram-negative cell (Pink *et al*., 2003).

We hypothesize instead in the case of some Gram-negative bacteria, that perhaps Ptm internalises through a particular porin (most likely a cation-specific porin) and therefore one may expect that such bacteria will down-regulate the expression of that particular porin in order to acquire resistance. The long-term objective of the study was to understand the mechanism by which Ptm internalises in *P. aeruginosa* and other Gram-negative bacteria. Elucidation of resistance mechanisms may allow or enhance our understanding of specific modes of action of Ptm. The short term specific objectives of the study were:

- To follow the surface remodelling taking place in *P. aeruginosa* as it acquires resistance to protamine.
- To understand the change in bacterial proteome and surface electrokinetics in Ptm resistant *P. aeruginosa* when compared to the revertant and control *P. aeruginosa*.
- To examine LPS-mediated surface changes in *P. aeruginosa* resistant to Ptm.
- To study the translocation of Ptm during internalization as well as changes in bacterial morphology in response to different concentrations of Ptm ranging from sub-inhibitory to bactericidal concentrations.
- To understand the changes in cell morphology and localisation of Ptm when Ptm resistant, revertant and control *P. aeruginosa* are treated with Ptm.

Chapter 2

Literature Review

2.1. Cationic antimicrobial peptides (CAPs)

CAPs are the products of single genes and most can be classified based on structure into four categories: helical, β-sheet, loops with tails, and extended structures with a predominance of a single amino acid (usually Lys, Pro, Arg and His) (Hancock, 1997). Another common characteristic of CAPs is their tendency to fold into amphipathic structures. Amphipathicity could be defined as the ability of the CAP to cross bacterial membranes. This is accomplished by folding such that one side of the molecule is hydrophilic and the other is hydrophobic. Amphipathicity of the peptide confers an ability to disrupt or traverse a phospholipid membrane (Deslouches, 2005). The cationic nature of CAPs is of great importance since the cell envelopes of susceptible bacteria are generally anionic and the initial stages of interaction are governed by electrostatics (Dathe *et al*., 1997).

Most of the CAPs have hydrophobicity in the range of 50% and is considered for some peptides to be an essential feature of antimicrobial peptide- membrane interactions, as it influences the extent to which a peptide can partition into the lipid bilayer (Yeaman and Yount, 2003). Increasing levels of peptide hydrophobicity can result in mammalian cell toxicity and loss of antimicrobial activity (Wieprecht *et al*., 1997). Polar angle, which is a measurement of relative proportion of polar versus non-polar facets of a peptide conformed to an amphipathic helix, is another factor, which has a profound influence on

the properties and activities of CAPs. In a number of studies using native and synthetic peptides, a small polar angle (as a result greater hydrophobic surface) was found to be associated with increased capacity to permeabilise bacterial membranes (Uematsu and Matsuzaki, 2000; Wieprecht *et al*., 1997; Dathe *et al*., 1997).

Fundamental composition and amino acid sequence influences not only the biochemical properties of the peptide (e.g. charge, amphipathicity and hydrophobicity), but also govern their three dimensional configuration (e.g. conformation, polar angle and overall stereo-geometry). Therefore changes in composition, sequence, and intra-molecular bonds may profoundly affect the structure-activity relationships of antimicrobial peptides in solution, upon binding to target membranes or as they may undergo conformational phase transition to activated states. Moreover, these features may be specific for distinct peptides as they interact with specific pathogens or in specific physiologic microenvironments. Therefore optimal microbial peptide efficacy lies in the relevant coordination of these relationships as they relate to microbial target versus host cells in a particular context of infection (Yeaman and Yount, 2003).

There are a variety of sequences that have been shown to confer antimicrobial function. Some of the research on mammalian CAPs shows that they are sometimes synthesized as a part of a protein with no innate antimicrobial function. The active form of peptide is released after a protease-specific digestion takes place as reported in the case of magainins, cecropins and lactoferricin B (Murakami *et al*., 2004; Shinnar *et al*., 2003; Valore and Ganz, 1992). Most of the CAPs are believed to exist in relatively unstructured or extended conformations prior to interaction with the target microbial cells. Some

others are believed to be held in specific conformations by intra-molecular bonds (Yeaman and Yount, 2003).

Tam and others (2000) recently reported on the influence of conformation on membranolytic selectivity of conformationally restricted cyclic and non-cyclic analogs of protegrin-1, an 18 amino acid cationic peptide exhibiting broad-spectrum antimicrobial activity. Antimicrobial assays carried out in low and high salt concentrations revealed cyclic protegrins exert differential antimicrobial profiles against Gram-positive and Gram-negative bacteria, fungi and HIV-1. As compared to protegrin-1, the more conformationally constrained analog (ccPG-3) displayed a 10-fold increase in haemolytic propensity to human cells and a thirty-fold increase in membranolytic selectivity against various bacteria, fungi and virus particles.

2.2. Protamine

Ptm is a low molecular weight cationic peptide (approx. 4000 Da) associated with DNA of spermatozoan nuclei of mammals, fish and birds. Unlike most other CAPs reported to date, its natural function is not antibacterial but compacts eukaryotic DNA and delivers it to the nucleus of the egg after fertilization (Suzuki and Ando, 1972). Sperm nuclei contain 36-40% Ptm by weight (Suzuki & Ando, 1972). It is highly cationic in nature with a pI of 11 – 13 due to its unusually high level of arginine content (≈66% on a molar basis).

According to the reports by Ando *et al*., (1973), Ptm in most aqueous solutions exists in the form of an extended or random coil rather than an α-helical configuration. It was also

found by Toniolo (1980) that clupeines can partially adopt an α-helical form in structure-supporting organic solvents. However, another study by Ottensmeyer and others (1975) which employed high resolution dark-field electron microscopy found that clupeine Y1 and the majority of the other fish Ptms consist of a fairly loose, irregular coil with arginine groups splayed away from each other in a loop or turn of the coil (Fig. 2.1).

The antimicrobial activity of Ptm was first reported by McClean (1930). He reported that Ptm could inhibit the *Vaccinia* virus and *B. typhosus*. Studies conducted later indicated that clupeine, in bactericidal concentrations, prevented root nodule bacteria (*Rhizobium leguminosarum*) and its bacteriophage from combining and also interrupted the normal course of phage development (Kleczkowski and Kleczkowski, 1956).

Studies on clinical applications of Ptm showed that it could inhibit the growth of tumours (Garvie, 1965; Hughes, 1964; O’Meara and O'Halloran, 1963). Ptm was also found to inhibit angiogenesis, which may be useful in treating various disease conditions common in ophthalmology, dermatology, rheumatology and certain cancers. A study by Taylor and Folkman (1982) showed that Ptm was toxic when administered systemically in doses large enough to reach the appropriate tissue concentration at the tumour site.

Currently, Ptm is used to reverse heparin-induced blood anticoagulation in patients undergoing certain procedures such as cardiac or vascular surgery and dialysis; and as a complexing agent in subcutaneous insulin preparations, such as NPH (neutral protamine Hagedorn) and PZI (protamine zinc insulin), to delay the absorption of insulin and thus prolong its effects (Gottschlich *et al*., 1988; Horrow, 1985).

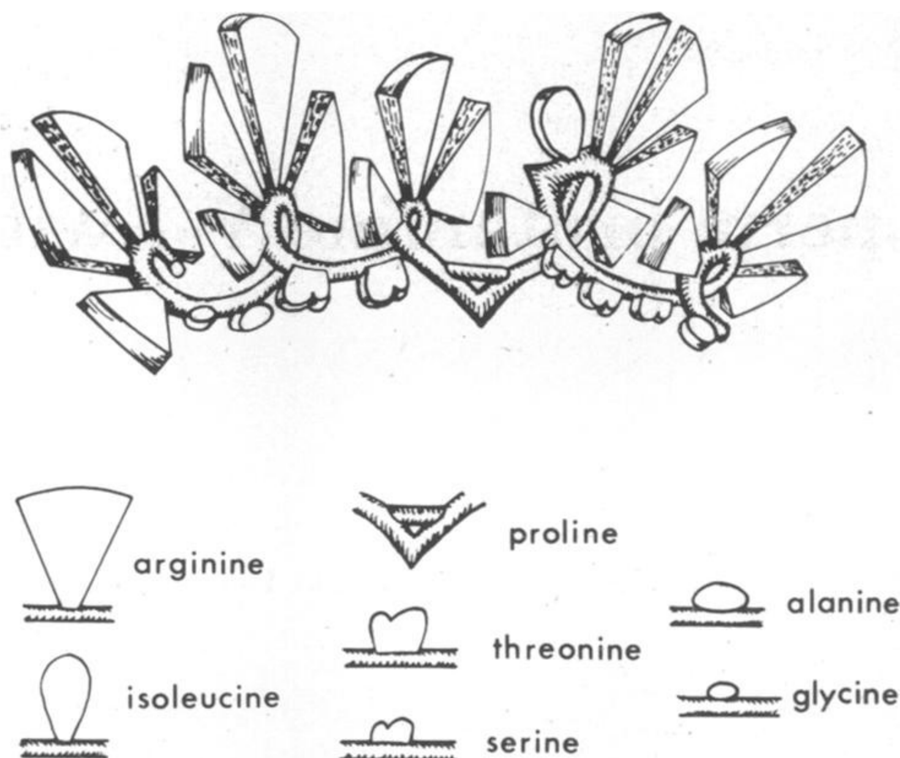


Figure 2.1 Schematic diagram of protamine from herring (Ottensmeyer *et al*., 1975)

Ptm can exist in the forms of mono-Ptm, di-Ptm or tri-Ptm. Mono-Ptm like clupeine and salmine contain only one kind of basic amino acid (arginine), while di-Ptm contain two kinds (arginine and lysine or histidine). Tri-Ptm contain all the three basic amino acids (Briskin *et al*., 1978).

Ptm is odourless, colorless, and almost tasteless, has an antibacterial and antifungal property which makes it an attractive choice as a natural food preservative (Islam *et al*., 1987). Another advantage of Ptm as a food additive is that it is highly heat stable (Brock, 1958) and can be cleaved by digestive enzymes like trypsin and chymotrypsin (Kleczkowski and Kleczkowski, 1956). A study by Yanagimoto *et al*., (1992) determined that browned salmine (prepared by heating salmine with xylose at 100ºC (pH 7.0) for 10 h) at a level of 1% could significantly lengthen the shelf-life of walleye pollack surimi.

Salmine conjugated with galactomannan was reported to have better emulsifying properties and at the same time maintained its antibacterial properties against *E. coli* and *B. cereus* even after a heat treatment at 90ºC for 5 minutes (Matsudomi *et al*., 1994).

2.3. CAPs - membrane permeabilisation models

The action of antimicrobial peptides in some cases induces membrane defects such as phase separation or membrane thinning, pore formation, promotion of non-lamellar lipid structure or bilayer disruption (in Gram negatives), depending on the molecular properties of both peptide and lipid (Lohner and Prenner, 1999). Several mechanisms have been proposed to describe the process of phospholipid membrane permeation by membrane- active peptides.

Yang *et al*., (2001) suggested that when the ratio of peptide / lipid is low, the peptides are bound parallel to a lipid bilayer. When this ratio increases, the peptides were found to align perpendicular to the lipid bilayer and insert into the bilayer resulting in trans-membrane pores referred to as the "I" state. Recently Lee and his coworkers (2004) reported that the 'I' state peptide / lipid ratio varies with different peptides and lipid composition. Barrel stave, carpet and the aggregate models are among the most prominent put forward by various researchers (Figure 2.2).

2.3.1. Barrel-stave model:

In the case of the barrel-stave model, peptide helices form a bundle in the membrane with a central lumen, much like a barrel composed of helical peptides as the staves forming a water filled pore (Yang *et al*., 2000; Montville and Chen, 1998; Ehrenstein and Lecar,

1977). Initial binding to the bilayer is reported to be by electrostatic interaction alone. The non-polar face of the peptide faces the hydrophobic fatty acid tails at the inside of the phospholipid bilayer and hydrophilic side chains orient into the lumen of the pore (Boheim, 1974). Incorporation of more peptides into the pore leads to the increase in pore size and as a result, leakage of intracellular components occurs and leads to cell death. This type of mechanism was observed in the case of alamethicin. Oriented circular dichroism (Lee *et al*., 2004), neutron scattering (Yang *et al*., 2001) and synchrotron based X-ray scattering (Spaar *et al*., 2004) studies showed that alamethicin adopts an α-helical configuration, attaches to, aggregates and inserts into oriented bilayers that are hydrated with water vapour. The alamethicin-induced pores can contain 3-11 parallel helical molecules, and the inner and outer diameters have been estimated at approximately 1.8 nm and 4.0 nm respectively (Spaar *et al*., 2004).

2.3.2. Carpet model:

The carpet model was initially used to describe the mode of action of dermaseptin (Shai and Oren, 2001) and ovispirin (Yamaguchi *et al*., 2001), which orient parallel to the membrane surface. In this model (Fig. 2.2), peptides accumulate on the bacterial surface (Pouny *et al*., 1992). At high peptide concentrations, surface oriented peptides are thought to disrupt the bilayer like a detergent (Brodgen, 2005). The formation of so-called "wormholes" or torroidal pores was proposed to describe the mode of action of dermaseptin (Mor and Nicolas, 1994), magainin (Ludtke *et al*., 1996; Yang *et al*., 2000),

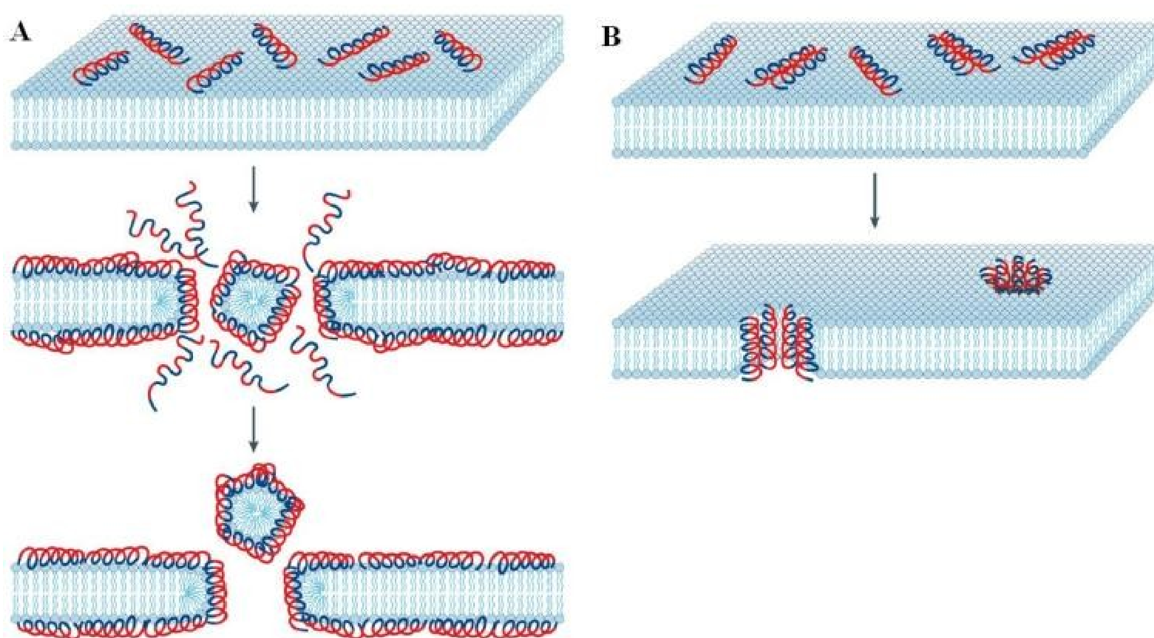


Figure 2.2. Diagram showing the sequence of steps involved in beta-barrel stave (B) and carpet model (A) of antimicrobial peptide induced killing. (Source: Brodgen, 2005).

protegrin (Heller *et al*., 1998), and melittin (Yang *et al*., 2001). Neutral in-plane scattering data showed that pores of magainin molecules are almost twice as large as the alamethicin pores and suggested that the lipid layer bends back on itself like the inside of a torus (Ludtke *et al*., 1996).

2.3.3. Aggregate channel model:

Studies by Yeaman *et al*., (1998) and Friedrich *et al*., (2000) showed that permeabilisation alone may not be enough to explain the antimicrobial activity. This model was proposed by Hancock and Chapple (1999) to explain the mechanism of action of bactenecin and gramicidin S. Bactenecin induced membrane depolarization at its minimum inhibitory concentration (MIC) but gramicidin S could induce maximum membrane depolarization well below its MIC which shows that membrane depolarization

was not the only crucial step involved in the bactericidal activity. According to the aggregate channel model, after the peptide binds to the phospholipid head groups, they insert into the membrane then cluster into unstructured aggregates that span the membrane as proposed by Wu *et al*., (1999). They also proposed that gramicidin S has associated water molecules providing channels for leakage of internal bacterial ions and possibly larger molecules through the membrane. In this model, the pores formed are short-lived and they allow the peptides to cross the membrane without causing significant membrane depolarization (Wu *et al*., 1999).

The cell envelope structures of Gram-negative and Gram-positive bacteria play a major role in their sensitivity towards antibacterial compounds (Nikaido and Vaara, 1985). The cell envelope of Gram-negative bacteria consists of an inner membrane or cytoplasmic membrane (approx. 60-80Å), the periplasmic space, peptidoglycan layer and the outer membrane (Brooks *et al*., 1998).

The inner membrane consists of a phospholipid bilayer with integrated membrane transport proteins. The peptidoglycan layer which helps to maintain the three dimensional shape of the cell is 30-80Å thick and constitutes $\leq$ 5% of total cell wall mass. Gram-negative bacteria are particularly resistant to a large number of antibiotics because of the effective permeability barrier function of their outer membrane. A number of reviews are available that discuss this aspect (Nikaido, 1989; Nikaido, 1990; Nakai, 1985).

Tolong (2004) compared membrane permeabilisation of Ptm, polymyxin B and nisin and concluded that there is no generalized mechanism for the bactericidal effect of CAPs on

bacteria. That is, no cell lysis or leakage of cytoplasmic components was observed when cultures of *S. typhimurium* or *E. coli* were challenged with inhibitory or lethal concentrations of Ptm or polymyxin B, the latter being a well-characterized peptide antibiotic produced by the bacterium *Bacillus polymyxa* (Storm *et al*., 1977). This was indeed surprising since a number of previous studies suggested that for many CAPs, including Ptm, poration and cell lysis were the primary modes of antimicrobial activity (Brogden, 2005). For example, Johansen *et al*., (1996) reported that the cells of *Sh. putrefaciens* clumped together and large holes were formed when treated with Ptm. As early as 1992, Vaara postulated that the mode of action of Pxn comprised of two steps, the binding to and the permeabilization of the outer membrane of Gram-negatives and the induction of lethal leakages from the cytoplasmic membrane for cytoplasmic components. This difference in observations can perhaps be attributed to the differences in membrane composition and/or structures among cultures studied or perhaps due to metabolic differences among strains of bacteria. It is perhaps interesting to note that an increase in turbidity of cells and spheroplasts were noticed on exposure to Ptm and this may be due to the increase in cell sizes which in turn contribute to the increase in turbidity (Tolong, 2004).

Among CAPs, there is a separate class which have a non-membranolytic mechanism of action and as a result won't fit into any of the models described above.

2.4. CAPs- non-lytic mechanisms

Unlike other CAPs, short arginine- and proline-rich peptides like pyrrhocoricin, drosocin, PR-39, bactenecin, and apidaecin were found to have a non-lytic mechanism of action

(Otvos *et al*., 2000). Bactenecin 7 and PR-39 share similarly high contents of proline (47 and 49 % respectively) and arginine (23 and 26% respectively) (Shi *et al*., 1996). Ptm has a very high concentration of arginine (65%) but a very low concentration of proline. The primary structures of Ptm, PR-39 and bactenecin 7 is compared in Table 2.1.

The bacterial heat shock protein DnaK was found to be the target for short proline–rich antibacterial peptides pyrrhocoricin, drosocin, and apidaecin (Otvos *et al*., 2000). The activity of DnaK as a molecular chaperone is inhibited by such peptides. DnaK activity involves binding to unfolded or partially folded proteins, thus is essential for bacterial survival (Kragol *et al*., 2001). Apidaecin binds to an outer membrane component in *E. coli* followed by internalisation into the cytoplasm where it binds with its target which may be the protein synthesis machinery (Otvos, 2002).

PR-39 also seems to have a similar mechanism of action where the internalised peptide kills the bacteria by stopping protein and DNA synthesis by degradation of the cytoplasmic components. A study by Cabiaux *et al*., (1994) on the secondary structure and membrane interaction of PR-39 showed that its secondary structure was not altered upon incubation of the peptide with negatively charged vesicles and that nearly all of the added peptide was membrane bound. This was later supported by the work of Shi *et al*., (1996). Boman *et al*., (1993) reported that PR-39 required a lag period of about 8 min to penetrate the outer membrane of wild-type *E. coli*. Subsequent killing is by a mechanism that stops protein and DNA synthesis and results in degradation of these components which is quite fast. Pyrrhocoricin was found to inhibit chaperone assisted protein folding and block the inherent ATPase activity of DnaK (Podda *et al*., 2006).

Table 2.1 Primary sequence of protamine (Ottensmeyer *et al*., 1975), bactenecin 7 (Benincasa *et al*., 2003), apidaecin (Casteels *et al*., 1989) and PR-39 (Agerbeth *et al*., 1991)

CAP	**Amino acid sequence**
Protamine (Clupeine Y-1)	Ala-**Arg**-**Arg**-**Arg**-**Arg**--Ser-Ser-Ser-**Arg**-Pro-Ile-Pro-**Arg**-**Arg**-**Arg**-**Arg**-**Pro**-**Arg**-**Arg**-**Arg**-Thr-Thr-**Arg**-**Arg**-**Arg**-**Arg**-Ala-Gly-**Arg**-**Arg**-**Arg**-**Arg**
Bactenecin 7	**Arg**-**Arg**-Ile-**Arg**-Pro-**Arg**-Pro-Pro-**Arg**-Leu-Pro-**Arg**-Pro-**Arg**-(Pro-**Arg**-Pro-Leu-Pro-Phe-Pro-**Arg**-Pro-Gly-Pro-**Arg**-Pro-Ileu)$_3$-Pro-**Arg**-Pro-Leu
PR-39	Pro-Phe-**Arg**-Pro-Pro-Phe-**Arg**-Pro-Pro-Phe-Gly-Pro-Pro-Ile-**Arg**-Pro-Pro-Leu-**Arg**-Pro-Pro-Phe-Phe-Pro-Pro-Pro-**Arg**-Pro-**Arg**-Pro-Leu-Tyr-Pro-Pro-**Arg**-Pro-**Arg**-**Arg**-**Arg**
Apidaecin	Gly-Asn-Asn-**Arg**-Pro-Val-Tyr-Ile-Pro-Gln-Pro-**Arg**-Pro-Pro-His-Pro-**Arg**-Ile

Bac7, a linear 60 residue proline-rich antimicrobial peptide effective against most of the Gram-negative bacteria has a non-membranolytic mechanism of cell internalization (Otvos, 2002). It has an Arg-rich cationic N-terminal region followed by three hydrophobic tandem repeats of 14 residues (Frank *et al*., 1990). A unique feature of this peptide is the absence of any measurable activity of the all-D enantiomers (Casteels and Tempst, 1994). Bac7 (1-35 amino acids) has the same antimicrobial activity as the intact parent molecule. The D-enantiomers of Bac7 (1-35) were completely excluded from the cells indicating the possible role of a peptide carrier on the membrane which specifically permits the transfer of L-forms (Podda *et al*., 2006). This suggested that Bac7 at concentrations near the MIC may have a stereo-specificity-dependent uptake to the bacterial cell that is likely followed by its binding to an intracellular target. At concentrations several times higher than the MIC, Bac7 (1-35) had a non-stereo-selective, membranolytic mechanism.

There was a remarkable decrease in Bac7 (1-35) internalisation in the presence of the metabolic uncouplers like 2,4-dinitrophenol (DNP) and carbonyl cyanide m-chlorophenylhydrazone (CCCP), which dissipate the proton-motive force suggesting the existence of a transport mechanism that likely requires a membrane potential or a source of expendable cellular energy (Otvos *et al*., 2000).

Ptm is another CAP which was found to have a non-lytic mechanism at least in the case of Gram-negative bacteria like *E. coli* and *P. aeruginosa.* Details regarding the mechanism of Ptm action can be found in Section 2.5.

2.5. Ptm mediated bacterial inhibition

The antimicrobial characteristics of Ptm have been widely studied and reported. Johansen *et al*., (1996) recorded cell clumping and formation of large holes in cell envelopes when *L. monocytogenes* and *Shewanella putrifaciens* were treated with Ptm. Condensation of cytoplasmic contents into electron-dense inclusions were observed with immune-labelling of Ptm. Another study by Johansen et al., (1997) showed that the addition of Ptm to *E. coli, L. monocytogenes* or *S. putrefaciens* resulted in inhibition of oxygen consumption after less than one minute and loss of internalized carboxyfluorescein and ATP within 2-5 minutes. It was also concluded that the disruption of both Gram-positive and Gram-negative cell envelopes was a generalized phenomenon causing a rapid and non-specific efflux of cellular components.

Localization studies of Ptm within *Bacillus* spp. showed the rapid and marked poration of the cell membranes causing leakage of the A_{260} absorbing material, inorganic phosphate

and pentose from the Ptm-treated cells (Islam *et al*., 1987). Also a non-lytic mechanism of Ptm inhibition was reported by Aspedon and Groisman (1996) for *S. typhimurium* and was concomitant with a reduction in cellular ATP and protein synthesis. The antibacterial action of Ptm was apparently due to the disruption of the energy transduction and loss of nutrient uptake functions of the cytoplasmic membrane.

Islam *et al*., (1984) and Johansen *et al*., (1995) reported that Gram-positives are more vulnerable than Gram-negatives. Later it was found that Gram reaction had nothing to do with sensitivity to Ptm (Johansen *et al*., 1997; Truelstrup-Hansen and Gill, 2000).

The protein content in the medium of testing was found to have a dramatic effect on Ptm action. A study by Truelstrup-Hansen and Gill (2000) demonstrated that the addition of Ptm to TSB made the media turbid and probably due to the formation of a Ptm-protein precipitate. Truelstrup-Hansen and Gill (2000) also reported that 90% or more Ptm was precipitated with the proteinaceous components of the tryptic soy broth at pH's between 6.5 and 8.0. This was also observed by Uyttendaele and Debevere (1994).

Ptm action was also dependent upon pH (Kamal and Motohiro, 1986). Efficacy of Ptm was reported to be highest at neutral to alkaline pHs and low ionic strengths in model systems (Islam *et al*., 1985). Antibacterial activity of Ptm was found to decrease at acidic pH. Another study by Pink *et al*., (2003) showed that increasing concentrations of divalent calcium and magnesium ions decreased the antibacterial activity of Ptm on *E. coli* and *P. aeruginosa* PAO1.

Potter *et al.* (2005) carried out bacterial inhibition assays using modified Ptms, (*ie.* Ptms with guanido groups blocked using 1, 2 cyclohexandione), thus reducing positive charge

and increasing hydrophobicity. Microbicidal efficacy of modified Ptms increased slightly for modest levels of derivatization (i.e. resulting in modification of $\leq$ 26% of the guanido groups), whereas more extensive modification resulted in an overall decrease in antimicrobial efficacy. Reduced electrostatic affinity for the bacterial cells and / or the increased size of Ptm due to the guanido groups may have contributed to the decreased efficacy.

Ptm has been shown to cause aggregation of bacterial cells (Johansen *et al*., 1995) making it difficult or impossible to rely on traditional methods such as plate counts and absorbance measurements for enumeration and determination of MICs and minimum bactericidal concentrations (MBCs) (Truelstrup-Hansen *et al*., 2001). The Alamar blue assay is therefore commonly used for assessing the MIC for Ptm (Tolong, 2004; Truelstrup-Hansen and Gill, 2000). Alamar blue (resazurin) is a redox dye that undergoes a change in colour (from blue to pink) when it gets reduced. Aerobic microbiological activity as a result of bacterial growth will reduce the media and dye. In a study by Johansen *et al*., (1995), impedimetric measurements were also used to assay the antibacterial effect of Ptm.

2.6. LPSs

LPSs are the major components of the outer membrane of Gram-negative bacteria (Alexander and Rietschel, 2001; Raetz and Whitfield, 2002) (Fig. 2.3). They play a major role in bacterial virulence (Crysz *et al*., 1984) and the structure and function of the outer membrane (Krospinski *et al*., 1985). They play a major role in eliciting both chronic and acute conditions in burn victims, immune-compromised patients and individuals

suffering from cystic fibrosis. They are also believed to play a key role in processes that involve metal binding, surface adhesion and microbe-mediated redox reactions (Soares and Straatsma*,* 2008). LPS consists of a lipid component, termed lipid A, covalently linked to a polysaccharide. The polysaccharide is composed of three separate domains: an inner-core, an outer core, and an O-specific chain or O-polysaccharide. Each domain has distinct structural and functional properties. LPS is essential for most of the Gram-negative bacteria and represents one of the most conserved microbial structures involved in the activation of the innate immune system (Reynolds *et al*., 2006). Bacterial LPS has been extensively reviewed (Alexander and Rietschel, 2001; Trent *et al*., 2006).

LPS plays a major role in the impermeability of the outer membrane to hydrophobic molecules like antibiotics and detergents. The highly charged (anionic) nature of the O-antigen and the cross-bridging of the core region via phosphate groups and divalent cations (Wiese *et al*., 1999) also contribute to the low permeability of the LPS (Kumar and Schweizer, 2005).

2.6.1. Lipid A

The lipid A anchors the LPS in the outer membrane, is the primary immuno-stimulatory centre of LPS (Trent *et al*., 2006) and is required for bacterial growth as it is essential to maintain the integrity of the outer membrane barrier (Vaara, 1992). Lipid A can stimulate overproduction of tissue factors that may lead to septic shock (Alexander and Rietschel, 2001) in bacterially infected individuals.

The structure of *P. aeruginosa* lipid A has been studied extensively (Ernst *et al*., 1999; Ernst *et al*., 2003; Kulshin *et al*., 1991; Bedoux *et al,* 2004; Karunaratne *et al*., 1992).

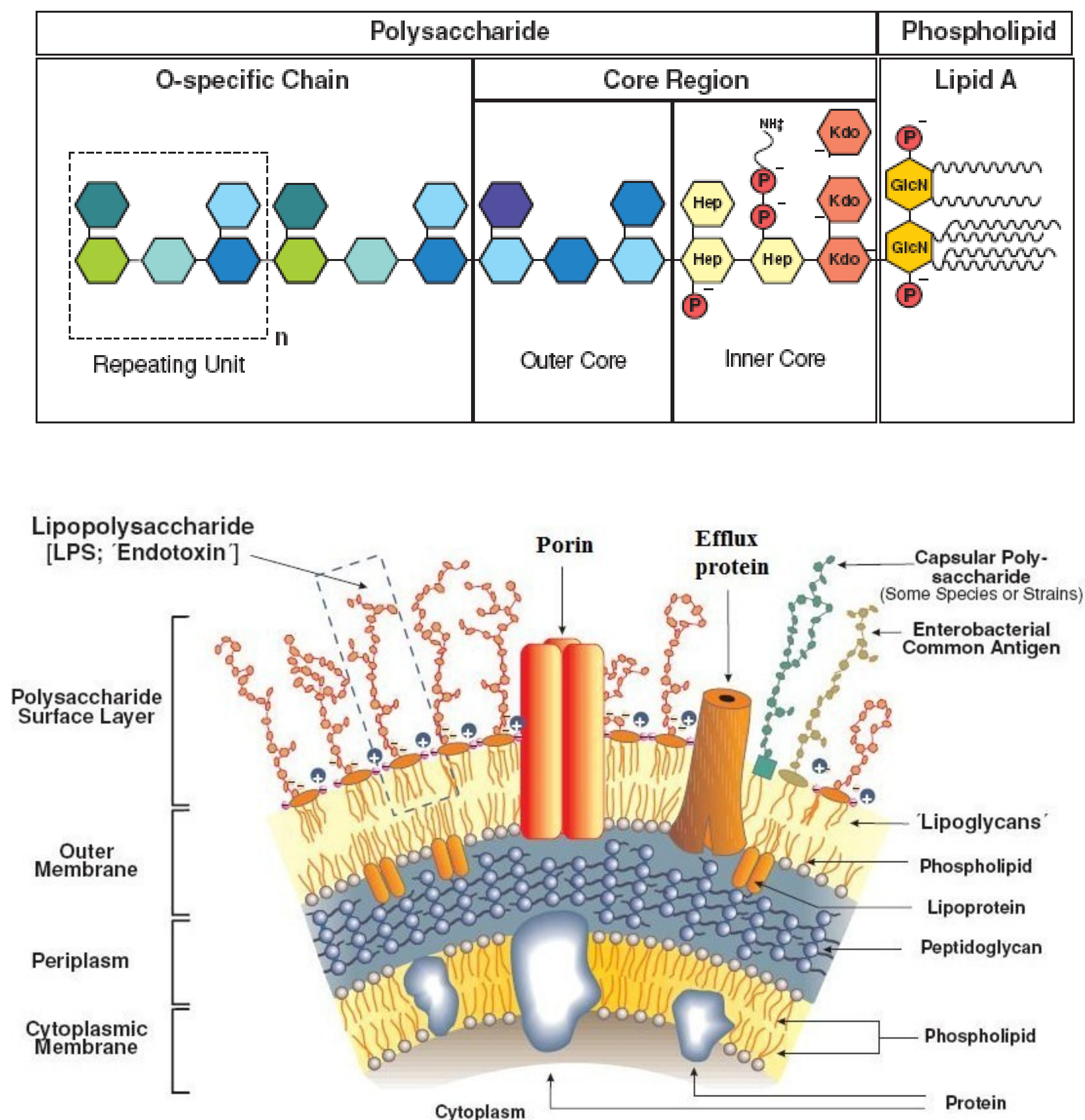


Figure 2.3. Cell wall architecture of Gram-negative bacteria and general chemical structure of LPSs. (Source: Alexander and Rietschel, 2001)

Lipid A consists consists of an N- and O-acylated diglucosamine bisphosphate backbone [4–P–β–D– GlcpNII–(1→6)–α–D–GlcpNI–(1→P]. The general acylation patterns are conserved within different strains of the species but heterogeneity is observed in respect to the number of primary acyl groups and the number, nature and position of the secondary acyl groups. Negative charges contributed by phosphate groups are critical in

strengthening the LPS monolayer *via* ionic bridges with divalent cations (Vaara, 1999; Alakomi, 2007). Although variations in lipid A have been observed in various organisms, the biochemical synthesis of this part of the LPS is understood as a highly conserved process (Reynold *et al*., 2006; Alakomi, 2007). Recent reports show that *S. typhimurium* can respond to various environmental stresses by producing certain enzymes that can change the membrane fluidity (Venter *et al*., 2006; Alakomi, 2007). Alterations in the lipid A region were also observed earlier for survival in environments with low concentrations of divalent cations, in providing resistance to CAPs, during growth in low temperatures and in virulence (Guo *et al*., 1998; Frirdich and Whitfield, 2005; Gutsmann *et al*., 2005; Reynolds *et al*., 2006). There are reports that L-Ara4N moieties protect bacteria against polymyxins and certain other antimicrobial peptides (Gunn, 2001).

Addition of L-Ara4N creates a positively charged LPS and may help in reducing the binding of CAPs to the lipid A (Gunn *et al*., 2000). *P. aeruginosa* isolated from patients with cystic fibrosis (CF) had aminoarabinose and palmitate in their lipid A which may lead to resistance to host innate immune defences mediated by cationic antimicrobial peptides (Ernst *et al*., 1999). Lipid A modification mediated by aminoarabinose was observed in *P. aeruginosa* resistant to colistin, a specific isoform of the CAP polymyxin commonly used to treat CF lung infections (Moskowitz *et al*., 2004).

2.6.2. Core oligosaccharides

The hetero-polysaccharide region is covalently linked to position 6’ of lipid A (Gronow and Brade, 2001). The polysaccharide region is composed of three separate domains; an

inner and outer core (core oligosaccharides) and O-specific chain or O-polysaccharide. Each domain has distinct structural and functional properties.

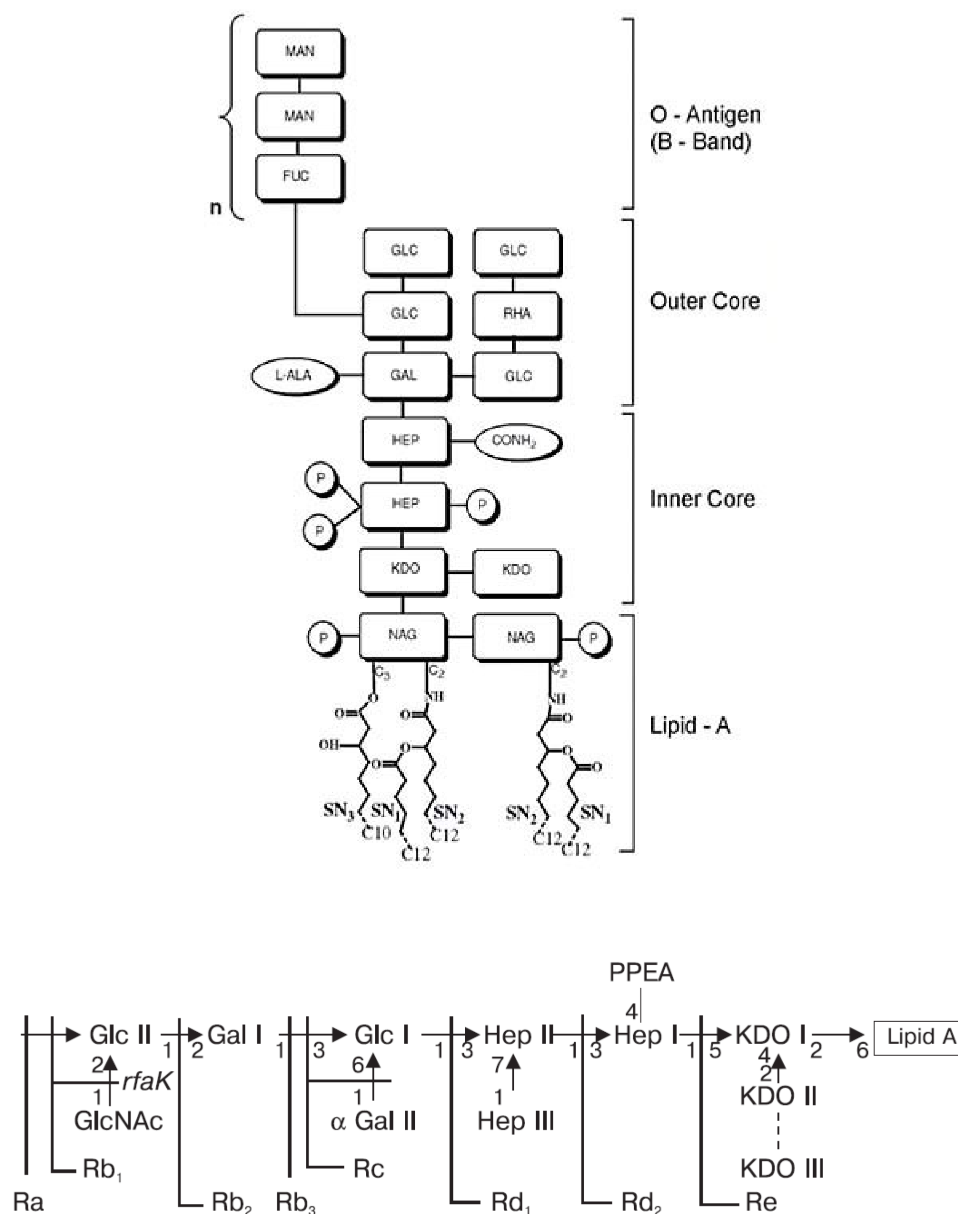


Figure 2.4. Schematic representation of LPS unit (NAG: N-acetyl-D-glucosamine; P: phosphatidyl group; KDO: 3-Deoxy-D-manno-octulosonic Acid; Hep: heptose; GAL: D-galactose; GLC: D-glucose; L-ALA: L-alanine; RHA: D-rhamnose; FUC: D-fucose; MAN: D-manose). Acyl lipid chains SN1, SN2 and SN3 are labelled. (Source: Soares & Straatsma, 2008). LPS R core structures; from Ra to Re (Source: Zubova & Prokhorenko, 2006)

The core oligosaccharide is composed of a short series of sugars (Fig. 2.4). It is expressed by all Gram-negative bacteria and is a functionally important part of the LPS. The core region is divided into two domains, the O-polysaccharide-proximal outer core and the lipid A proximal inner core. The core region usually demonstrates little structural variability within a bacterial species (Alakomi, 2007). The outer core is more variable than the inner core region. Sugars present in the outer and inner core were found to be different. The outer core is made of hexose sugars like glucose, galactose, N-acetyl galactosamine and N-acetyl glucosamine while inner core is made up of 3-deoxy α-D-manno-oct-2-ulopyranosonic acid, KDO) and two or more residues of L-glycero-α-D-manno-heptopyranose (L, D-Hep) (Frirdrich and Whitfield, 2005). Mutants with a complete R core (Ra LPS) or with a core deficient only in the galactose and N-acetyl glucosamine moities (Rb LPS) (Fig. 2.4) were found to have an unaltered permeability barrier (Nikaido, 2003).

Gram-negative bacteria require an LPS consisting at least of Lipid A and KDO with a negatively charged substituent to sustain growth and viability (Healander *et al*., 1998). Vaara (1992) reported that the negative charges provided by phosphate residues in the Hep (L-glycero-α-D-manno-heptopyranose) region play a major role in maintaining the barrier function by providing sites for cross-linking of adjacent LPS molecules with divalent cations or polyamines.

According to Walsh *et al*., (2000), the inner core phosphates mostly mediate intrinsic drug resistance mechanisms in *P. aeruginosa*. A study by Knirel *et al*., (2006) reported that the presence of ethanolamine in lipid A plays a major role in the resistance to CAPs in *P. aeruginosa* (Alakomi, 2007).

2.6.3. O-antigens

A study by Koval and Meadow (1977) proposed that there might be four types of LPS in a single bacterial strain: a rough LPS with an un-substituted core polysaccharide, a smooth-rough LPS, and two long-chain LPSs each with a different side chain. The polysaccharide part of the LPS consists of a core oligosaccharide of different lengths and a polysaccharide chain formed of repeating tetrasaccharide units known as O-antigen (Fig. 2.4). Organisms lacking O-antigen chains are termed ***rough*** LPS whereas the chemo-type associated with the presence of the O-antigen is known as ***smooth*** LPS according to the appearance of the colonies on artificial culture media (Burrows and Lam, 1999). Two types of O-antigen chains were identified in wild type *P. aeruginosa*, A-band and B-band (Riviera *et al*., 1988). The A-band O-side chain is electro-neutral at physiological pH, while the B-band O-side chain contains numerous negatively charged sites due to the presence of uronic acid residues in the repeat unit structure. The A-band, a conserved O-polysacharide region, consists of D-rhamnose (homopolymer) while the B-band is a heteropolymer, which varies among the twenty O-serotypes. Genes that are organised in two separate clusters in different chromosomal locations (Rocchetta *et al*., 1999) code the enzymes that direct the synthesis of the two O-antigens.

Loss of O-antigenic structures, which are key targets for the action of host antibody and complement, may help these bacteria evade host defence mechanisms. Modifications of the polysaccharide portion of the LPS have a profound effect on the physicochemical properties of the outer membrane and thus on microbial physiology and susceptibility to diverse antibiotics (Soares and Straatsma, 2008). The O-antigen is involved in the resistance to various cationic antimicrobial peptides of the innate immune system

(Nicolas & Mor, 1995). The O-antigen is supposed to hinder the internalization of CAPs to inner LPS targets (Ugalde *et al*., 2000; Vemulapalli *et al*., 2000; McCoy *et al*., 2001). Computer simulations published for the rough LPS membrane suggest that the inclusion of the B-band lowers the electrostatic potential, surface charge and promotes membrane expansion (Soares and Straatsma, 2008; Lins and Straatsma, 2001).

The type of O-antigens expressed on the surface was found to have an effect on transport of ions through channel proteins on the bacterial outer membrane (Langley and Beveridge, 1999). Outer membrane permeability to hydrophobic antibiotics of bacteria with rough LPS is higher since a rough LPS is more hydrophobic (Tsujimoto *et al*., 2003).

2.7. Porins

Porin monomers cross the outer lipid bilayer of Gram-negative bacteria as β-barrel or a series of sixteen-β-strands. These strands were found to be tilted to 30° to 60° with respect to barrel axis as determined by Fourier transform infrared spectroscopy (Nabedryk *et al*., 1988) (Fig. 2.5a). The axial tilt of the barrel results in an increase in the diameter of the channel (Schulz, 2000) (Fig. 2.6). Monomers of the porins are stabilized by hydrophobic and polar interactions and loop 2 extends to the wall of the other porin monomer contributing to the total stability (Nikaido, 2003). Three such monomers form stable trimers (Fig. 2.5b). Transmembrane pores formed in the monomers are constricted by an internal loop that folds inward and is attached to the inner side of the barrel wall forming the eyelet (Schirmer, 1998).

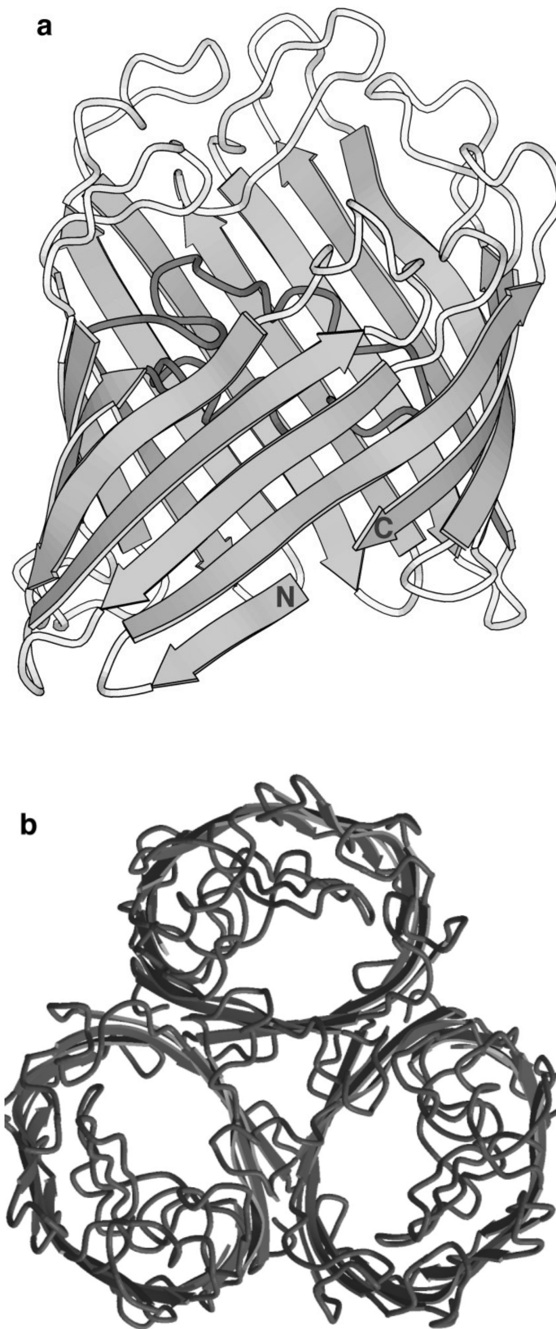


Figure 2.5. (a) Ribbon diagram of a porin monomer (OmpF porin from *E. coli;* Cowan *et al*.,1992). (b) Schematic representation of the OmpF trimer. The view is from the extracellular space along the molecular threefold symmetry axis (Source: Schirmer, 1998).

Pink *et al.* (2003) have shown that from a theoretical standpoint, there is a very small probability that Ptm can even reach the base of the O-side chains of the outer membrane of Gram-negative bacteria. Since Ptm is apparently able to internalise into the cells of *E. coli*, *S. typhimurium* and *P. aeruginosa* without any lysis or membrane damage there appears to be a paradox: translocation through both outer and inner membranes although from a thermodynamic point of view, this should be impossible. However, since Pink's computer model did not include the presence of porins or other membrane proteins, it may be that porins are in some way responsible for Ptm transport through the outer envelope of susceptible bacteria. If this were true, it may be logical to assume that cation-conducting porins may be particularly competent in Ptm transport. Even though the molecular weight of Ptm is well above the proposed permeability limit of most of the cationic porins, the peculiar charge distribution on the Ptm molecule may help in internalisation through cationic porins.

Studies on *Pseudomonas* showed that it has very low outer membrane permeability (Nikaido and Hancock, 1986) when compared to *E. coli*, therefore substrates needed for its growth have to take specialized pathways for uptake (Hancock and Worobec, 1998). A review on *Pseudomonas* porins by Hancock and Brinkman (2002) provides a list of all the porins of the outer membrane in this particular organism.

The functional significance of a transverse electrical field that is formed by charged amino acid residues within the constriction zones of PhoE and OmpF porins from *E.coli* was investigated by van Gleder and others (1997). It was reported that the substitution of positive and negative charges in the constriction zone of OmpF resulted in increased and decreased trans-membrane voltage sensitivity, respectively which clearly suggest that

opposite charges, i.e. positive charges in anion-selective and negative charges in cation-selective porins, act as sensors for voltage gating (van Gelder *et al*., 1997). According to the studies conducted by Nikaido and Rosenberg (1983) the additional negative charge of the solute molecules retarded the diffusion rate through *E.coli* OmpF and OmpC considerably while incorporation of more positive charges accelerated the diffusion rate (van Gleder *et al*., 1997).

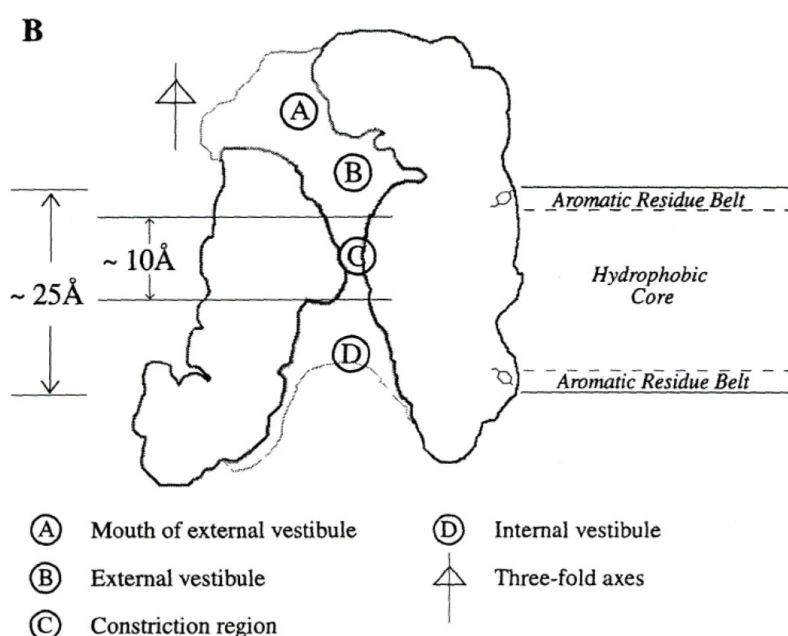


Figure 2.6. Schematic diagram of PhoE porin cross-section indicating the major structural features (Source: Jap and Walian, 1996)

OprF is a multifunctional non-specific porin in *P. aerginiosa* that is bound to the peptidoglycan layer and is required for cell growth in low osmolarity media and for maintenance of cell shape (Hancock and Brinkman, 2002). It resembles *E.coli* OmpA in structure and function and it is included in the OmpA family of proteins. A 16 β-stranded membrane topology model was proposed which includes an N terminal domain (initial 160 amino acids), a loop region which is made up of 161 to 209 amino acids containing

poly proline-alanine repeat regions and two disulfide bonds, and finally a highly conserved C-terminal region (210 to 326 amino acids) (Hancock and Worobec, 1998). The functional studies of OprF showed that they are mainly non-specific in nature although weakly cation-selective.

Table: 2.2. Features of major porins in *E. coli*, *S. typhimurium* and *P. aeruginosa* (Benz *et al*., 1985)

Porin and bacterial source	Molecular weight (kDa)	Pore diameter (nm)	Ion selectivity
E. coli			
OmpF	38	1.2	cation
OmpC	37	1.1	cation
PhoE	36	1.2	anion
S. typhimurium			
OmpD	38	1.2	cation
OmpF	39	1.4	cation
OmpC	40	1.4	cation
P. aeruginosa			
OprF	32	2.2	cation
OprD	48	0.6	cation
P	48	0.6	anion

Surface exposed peptidoglycan binding regions were observed on the C-terminal regions of OprF, pointing to the fact that the OprF protein forms more than one conformation varying in both structure and channel formation. This characteristic was also observed in *P. fluorescens* (El Hamel *et al*., 2000). OprF and *E. coli* OmpA showed decreased mobility in SDS-PAGE when the samples were heated at 100ºC, a property called “heat modifiability”.

OprG in *P. aeruginosa* forms large cation-selective channels and was observed to have a single channel conductance of 500 pS in 1M KCl ((McPhee *et al*., 2009). Yates *et al*., (1989) showed that OprG in *P. aeruginosa* was found to be expressed under iron-rich conditions and was originally believed to function as a low affinity iron transporter. However this was later ruled out by McPhee *et al*., (2009). Expression of OprG was found to contribute to the cytotoxicity of *P. aeruginosa* during the early stages of infection (Blumer & Haas, 2000; McPhee *et al*., 2009). Down-regulation of OprG was reported to be associated with antibiotic resistance to norfloxacin, kanamycin, and/or tetracycline in *P. aeruginosa* (Chamberland *et al*., 1989; Peng *et al*., 2005).

OprD is also a sixteen stranded β-barrel porin (45.9 kD) which strongly resembles the *E. coli* non-specific porin OmpF in structure (Huang *et al*., 1995). OprD mutants with deletions in loops 2 and 3 demonstrated a decreased ability to bind imipenum and mediate imipenum susceptibility (Ochs *et al*., 1999a). Imipenum is a beta-lactam broad spectrum antibiotic with a molecular weight of about 300 Da often used to treat *Pseudomonas*-mediated lung infections. According to the studies by Koebnik *et al*., (2000) loop 2 is responsible for substrate binding and deletion variants of loops 5, 7 and 8 of OprD showed increased susceptibility to multiple antibiotics and produced larger channels (Huang *et al*., 1995). OprD was found to be moderately expressed but regulated by multiple systems. OprD was found to be activated by arginine / arginine R and a variety of other amino acids as carbon and nitrogen sources (Ochs *et al*., 1999b) and was repressed by salycilate and the over-expression of the MexT gene (Ochs *et al*., 1999a).

OprH is a *P. aeruginosa* outer membrane protein that is up-regulated upon Mg^{2+} starvation (Macfarlane *et al*., 1999). Results of insertion and deletion mutagenesis studies

suggest that it's an eight stranded β-barrel protein and while devoid of porin activity, the deletion of loop 4 resulted in channel formation (Hancock and Brinkman, 2002). It was reported by Bell *et al*., (1991) that over-production of OprH leads to resistance of cells to EDTA and polycationic antibiotics, such as polymyxins and aminoglycosides.

Active efflux is the major contributor to intrinsic multiple antibiotic resistance in pathogens like *P. aeruginosa* and there are 18 outer membrane proteins specifically involved in efflux, thereby contributing to antibiotic resistance (Table 2). **OprM** is the major efflux protein in *P. aeruginosa* and the deletion of its gene was shown to contribute to a 10-1000 fold increase in antibiotic susceptibility. It's a trimer that comprises a single channel tunnel spanning the outer membrane and periplasm. The trimer forms a 12 stranded β-barrel (4 strands in each monomer) that lodges in the outer membrane and sits atop a coiled 12 helix α-helical barrel that spans the periplasm and is presumed to contact the MexB pmp/MexA-linker complex in the cytoplasmic membrane (Hancock and Brinkman, 2002).

OprJ and **OprN** are the other two efflux proteins which are normally silent but on mutation are highly expressed which could lead to multi-drug resistance in *P. aeruginosa* (Poole, 2001).

Nikaido and Rosenberg (1983) showed that the porin pores are not simply water-filled channels but most exhibit certain selectivity for cations or anions. The work was done using artificial lipid bilayers, a technique that is now an accepted and valuable tool for the study porin trimers as this technique allows access to both sides of a membrane, and the pore properties can be studied as a function of changes of pH and salt concentration

(Benz *et al*., 1985). The measurement of zero-current membrane potentials yields precise information on the selectivity of the pores under all conditions. Furthermore, the lipid bilayer technique allows the study of a single unit, which is not possible by other methods (Benz *et al*., 1985).

Bacterial porins do not behave as static, permanently open pores but display a much more complex and dynamic behaviour. Research on porin channel activity has proved that the proteins have the capability to switch between short-lived open and closed conformations (gating activity). They also have the capacity to remain in an inactivated, non-ion conducting state for prolonged periods of time (Delcour, 1997). This indicates that the porins are not just molecular filters, but they control outer membrane permeability through the regulation of their channel activity. A typical Gram-negative bacterium was estimated to possess approximately 10^5 porins in its outer membrane (Beuchner *et al*., 1990). The regulation of channels of such a large number of porins on bacterial surfaces permits the bacteria to respond quickly to environmental challenges. A large number of porins with an open conformation may be necessary only when the cell is growing in a low nutrient media (Bavoil *et al*., 1977). In rich media, bacteria may have only 1% of its total porins in the open conformation. The potential of the cells to regulate the activity of porins in a reversible way helps them survive harsh exterior conditions (Delcour, 1997).

Electrophysiological studies have demonstrated that both gating and inactivation, of numerous porin channels are modulated by a variety of physical and chemical parameters. This cooperativity helps to amplify porin’s sensitivity to modulation by external or internal factors (Delcour, 1997). By dictating permeability properties to the

outer membrane, porins play a major role in the antibiotic susceptibility and survival of the bacteria in various environmental conditions (Delcour, 1997).

It is quite evident that transport through ion channels is influenced by membrane composition, and porins appear to require the presence of LPSs for function (Delcour, 1997; Schindler and Rosenbusch, 1981). LPS is known to be strongly associated with the bacterial porins of *P. aeruginosa* (Woodruff and Hancock, 1989) and is also known to be involved in the insertion (Sen and Nikaido, 1991; Ried *et al*., 1990), trimerisation (Sen and Nikaido, 1991; Bolla *et al*., 1988), and channel activity of some porins (Ishi and Nakae, 1993; Buehler *et al*., 1991).

OprF, the major porin from *P. aeruginosa* was found to have the capability to change the channel permeability based on the environmental temperature (De *et al*., 1997). More research (El Hamel *et al*., 2000) indicated that the difference in channel diameter is influenced by the interaction of the C-terminal region of the porin with the LPS bilayer. As environmental temperature changes, the level of phosphorylation varies and this in-turn affects the interaction with C-terminal region of OprF. Ionic composition of the surrounding regions of the porin was also found to have profound influence on the properties of the OprF channel. Lower pH values promote stabilisation of the closed state and shifts threshold voltage to lower levels in the case of OprF. (Delcour, 1997; Buehler, 1991; Xu, 1986).

In the case of Gram-negative pathogens like *P. aeruginosa,* the barrier function of the OM is mainly regulated by the LPS in the outer leaflet of the membrane. LPS together with multi-drug efflux pumps contribute to antibiotic resistance in most Gram-negative

organisms (Nizet, 2006). Continued exposure to sub-inhibitory antibiotherapy selects for step-by-step porin-expression modifications, resulting in further reduced influx at each stage (Pagès, 2004). Loss of porins or mutated porins can result in severe loss of bacterial fitness owing to restricted entry of nutrients, but can enable survival in the face of intensive and continuous antibiotherapy (Davin-Regli *et al*., 2008, Pages *et al*., 2008).

Weak acids such as salycilates were found to suppress porin synthesis in certain Gram-negative bacteria like *Pseudomonas cepacia* thereby inducing antibiotic resistance (Burns and Clark, 1992). Loss of porins which contribute to antibiotic resistance was also seen in *E. coli, Klebsiella pneumoniae* and *Serratia marcescens* (Sawai *et al*., 1987). Hancock and Wong (1984) reported that *P. aeruginosa* grown in the presence of 1.2 mM acetylsalicylate increased the outer membrane permeability of the organism to the β-lactam, nitrocefin, but not to lysozyme or the hydrophobic fluorescent probe NPN (1-N-phenylnapthylamine). Enhancement of permeability was inhibited by the addition of 1 mM Mg^{2+} indicating that acetyl salicylate may be a weak chelator.

Chapter 3.

Acquired Resistance in *Pseudomonas aeruginosa* to Ptm: Changes in Bacterial Proteome and Electrokinetics

3.1. Introduction

Microbes have a multitude of resistance mechanisms that limit the effectiveness of antimicrobial peptides (Sugimura and Nishihara, 1988; Friedrich *et al*., 1999; Yeaman and Yount, 2003) and conventional antibiotics (Benveniste and Davies, 1973; Spratt, 1994; Stewart and Costerton, 2001). Intrinsic, acquired and genetic resistance are the major known classes of antibiotic resistance. Intrinsic antibiotic resistance involves mechanisms that are found in a given bacterium irrespective of antibiotic exposure. Acquired antibiotic resistance involves induction of unstable resistance without any observable change in genotype. This type of resistance develops because of exposure to a set of inducing conditions that can include antibiotic exposure and can fully revert when the inducing conditions are removed (Hancock, 1998).

Most of the earlier works on antimicrobial peptide resistance focussed on changes in Gram-negative bacterial LPS and changes in surface properties of the bacteria (Gunn, 2001; Yeaman and Yount, 2003). The present study was designed to examine the change in surface proteins, surface charge (electrokinetics) and bacterial morphology on developing Ptm resistance. The mechanism by which Ptm inhibits bacterial growth is still not clear and the possible role of bacterial porins has never before been considered in the literature. Our hypothesis is that there is more than one mechanism for Ptm internalization and although Ptm treatment results in poration and cell lysis in some

Gram-negatives, this is clearly not the case for *P. aeruginosa*. Also, Tolong (2004) has suggested that Ptm can penetrate the cell envelopes of at least some Gram-negative bacteria including *E. coli* and *S. typhimurium* without cell clumping, lysis or any visible sign of membrane damage. Furthermore, Pink *et al*., (2003) have shown that from a theoretical standpoint, there is only a very small probability that Ptm can even reach the base of the O-side chains of the outer membrane of Gram-negative bacteria. Instead, we hypothesize that perhaps Ptm internalises through a particular porin and therefore one may expect that bacteria will inhibit the expression of that particular porin in order to acquire resistance.

3.2 Objectives

The main objective of the study was to understand the change in bacterial proteome and in particular the outer membrane proteins when *P. aeruginosa* PAO1 acquires resistance to Ptm. It was anticipated that such changes may reveal critical information on the inhibitory action of Ptm. The role of bacterial surface charge in Ptm resistance was also studied to see if acquired resistance to Ptm results from an acquired reduction in surface charge (more neutral, less electronegative).

3.3. Materials and Methods

3.3.1. Bacterial strains and culture conditions

P. aeruginosa PAO1 was cultured in tryptic soy broth (TSB) (Oxoid, Hampshire, England). Cultures were incubated overnight in a shaker at 120 rpm and a temperature of

37ºC. Ptm sulphate, Grade III (Sigma-Aldrich, St. Louis, US) from herring was used in this experiment.

3.3.2. Induction of Ptm resistance

Resistance to Ptm was induced in *P. aeruginosa* by continuous sub-culturing in TSB containing increasing concentrations of Ptm. Serial passage was initiated from overnight cultures grown to exponential growth phase and then inoculated into Ptm-containing TSB (final bacterial concentration of approximately 1.0×10^6 CFU/mL). The bacteria were incubated overnight at 37ºC and an aliquot was transferred to fresh media with a higher concentration of Ptm. Concentrations of Ptm used in the thirteen passages were 100, 300, 600, 1200, 1750, 2400, 3000, 3750, 4250, 4750, 5500, 6000 and 6500 ppm respectively. After the 12th passage, an aliquot of the culture was transferred to fresh medium without any Ptm to check for revertants (Pa-Ptm revertants) and another aliquot to TSB with 6500 ppm of Ptm (Pa-Ptm resistant). When the revertants are re-innoculated into TSB with 6500 ppm Ptm no growth is observed. A control culture was maintained by sub-culturing the *P. aeruginosa* PAO1 in TSB devoid of Ptm (Pa-Ptm control).

3.3.3. Estimation on Minimum Inhibitory Concentration (MIC)

The MIC for Ptm was estimated by using the resazurin dye (0.01% wt/vol in distilled water and filter sterilised; Accumed International Sensititre, Westlake, OH, USA) microtitre plate dilution method (Baker *et al.*, 1994). A stock solution 100,000 ppm of Ptm was prepared by dissolving 100 mg of Ptm in 1 mL of distilled water. A second stock solution of 7.2 mg/mL was prepared (0.72 mL of the stock solution and 9.28 mL of

TSB) to obtain a maximum concentration of 5000 ppm in the assay (180 μL per well). The bacterial culture was added at a concentration of 10^3-10^4 colony forming units (cfu/mL). The resazurin dye was added and the plates were then incubated aerobically at 30ºC and the readings were done after 12-24 h. The MIC was defined as the minimum concentration of the peptide that inhibited the growth of the target organism and therefore did not result in colour change (blue to red) after the overnight incubation period.

3.3.4. Turbidity studies

Upon challenging control, revertant and Ptm-resistant *P. aeruginosa* with Ptm, A_{650} readings were recorded to monitor aggregation or any change in cell size or shape. *P. aeruginosa* cultures in TSB were harvested by centrifugation at 2000 *x* g for 10 min and pellets suspended and washed twice with 50 mM HEPES pH 7.5 and then brought to a final absorbance of 1.0 at 600 nm. Serial dilutions of Ptm were made in 96-well microtitre plates to give a concentration range of 5000 to 5 ppm using a 2-fold dilution between adjacent wells. Diluted cell suspensions were added to each well and the A_{650} recorded after 20 min using an automatic plate reader (ThermoMax, Molecular Devices Corporation, Sunnyvale, CA, USA). A control lane with no Ptm was also maintained.

3.3.5. Solubilisation of bacterial membrane proteins

Solubilisation of outer membrane proteins was done according to the method of Nikaido *et al*., (1991). The cultures obtained from resistance induction experiments were harvested by centrifugation (Sorvall RC-3, Sorvall Instruments Div., Dupont Co., Newtown, CT) at 2000 *x* g. washed three times with 25 mM Tris buffer pH 7.5 containing 1 mM $MgCl_2$. The harvested cells were suspended in a breaking buffer

containing 10 mM Tris-HCl, 5% sucrose, 0.1 M NaCl, 3 mM sodium azide, 0.1 mg/mL DNase (Sigma Chemical Co., St. Louis, MO) and protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) and lysed using a French press at 14000 psi. Cells not lysed after the French press treatment were separated by centrifugation (Universal 32 R, Hettich, Germany) at 2000 *x g* for 10 min. Sodium lauryl sarcosinate (1%) was added to the resultant supernatant to selectively extract cytoplasmic membrane proteins (Fillip *et al*., 1973). Outer membranes were pelleted from the suspension by centrifugation at 75,000 *x* g for 90 min using an ultra-centrifuge equipped with a SW-27 rotor (Model L2-65B, Beckman Instruments, California, USA). This pellet was suspended in a washing buffer containing 10 mM Tris-HCl, 20 mM magnesium sulphate, 0.1 mg/mL DNase, 0.1 mg/mL RNase and protease inhibitors at 4ºC for two h and finally the crude membranes were separated by centrifugation at 75,000 *x* g for 90 min (Nikaido *et al*., 1991). Crude cell envelopes obtained were further washed overnight at 4ºC with 10 mM Tris-HCl (pH 8), containing 0.5% octyl-polyoxy ethylene (Octyl-POE, Alexis Biochemicals, San Diego, CA) to remove contaminating proteins and other cell debris (Nikaido, 1986). Outer membrane envelopes were pelleted by centrifugation and then suspended at 4ºC in 10 mM Tris-HCl (pH 8) with 100 mM NaCl, 5 mM EDTA and 3% octyl-POE to preferentially solubilise the porin proteins (Nikaido *et al*., 1991). These were later centrifuged at 75,000 *x* g for 90 min at 4ºC to remove the non-soluble components.

3.3.6. Protein assay

The concentration of protein was assayed using a bicinchoninic acid (BCA) Quantipro protein assay kit (QPBCA-1KT, Sigma, St. Louis, MO) using the bovine serum albumin as a standard and the protocol provided with the kit.

3.3.7. SDS-PAGE

Gradient (5-15%) SDS-polyacrylamide gels, prepared according to the discontinuous system of Laemmli (1970) were used to separate the extracted cytoplasmic and outer membrane proteins. SDS-PAGE was carried out using an LKB 2001 Vertical Electrophoresis unit (LKB Produkter; Bromma, Sweden). Broad range molecular mass protein markers (Bio-Rad, Mississauga ON) (6.6 to 200 kDa) were used to estimate apparent molecular weights of the dissociated proteins. LC-MS/MS compatible silver staining of the gels was carried out according to the modified procedure of Blum *et al*., (1987).

The concentration of the extracted proteins was estimated using the BCA assay as described earlier and 20 µg of proteins were loaded in each lane. Earlier studies (Bunai and Yamane, 2005; Ong and Pandey, 2001) involving separation of membrane proteins using 2-dimensional electrophoresis indicated a possible bias against very large and hydrophobic proteins, which may be difficult to solubilise, and very basic or acidic proteins, which are not adequately resolved during the IEF step. So in this study, a 1-dimensional gradient SDS-PAGE approach was used followed by nano LC-MS/MS as this method resolves both basic and acidic proteins without bias and also is likely to show very little bias against large and/or hydrophobic proteins. Although gradient SDS-

PAGE does not resolve all of the outer membrane proteins, subsequent nano LC-MS/MS has the capacity to identify many proteins within a single gel slice. A study by Gorg *et al*., (2004) recommends this method for membrane proteins.

3.3.8. Mass spectrometry (LC-MS/MS) protein identification

The bands of interest were excised carefully from the silver-stained SDS-electrophoresis gels and digested with trypsin according to Ebanks *et al*., (2004). Digests were analyzed using an LC-MS/MS system (Q-TRAP® Applied Biosystems MDS SCIEX, CA, USA). A linear gradient, run at 5 µL/min, from 15% B to 50% B over a period of 20 min was employed for the peptide separation (A: 5% acetonitrile (ACN), 0.5% formic acid, B: 90% ACN, 0.5% formic acid). The HPLC peaks were introduced to the MS *via* a nanoelectrospray ionization source. Mass to charge ratios (m/z) of the tryptic peptides were measured in the scan mode and used to generate a peak list of peptides for MS/MS analysis. The tandem MS spectra were submitted to the database search program MASCOT (Matrix Science, London, UK) to search against NCBInr and SwissProt databases.

3.3.9. Zeta potential measurement

Zeta potential distributions of Pa-Ptm control, Pa-Ptm revertant and Pa-Ptm resistant groups were determined by micro-electrophoresis (Hiemenz, 1991). The cultures were harvested by centrifugation (6500 *x* g, 10 min, 18ºC) and washed twice with 1 mM potassium phosphate, pH 7.0. Zetapotential distributions were measured in 1 mM potassium phosphate, pH 7.0 and pH 4.0 (pH adjusted using HCl or KOH) with a Lazer Zee Meter 501 (PenKem, Bedford Hills, NY, USA), equipped with image analysis

options for zeta sizing. The micro-electrophoresis chamber was filled with a cell suspension with a density of 10^7–10^8 cells mL^{-1} and a voltage difference of 150 V was applied over the chamber. The velocity of each individual cell was determined by image sequence analysis and from this; its zeta potential was calculated, assuming the Helmholtz-Smoluchowski equation holds. Zeta potential distributions were measured in triplicate for separate cultures.

3.4. Results and Discussion

3.4.1. Estimation of Minimum Inhibitory concentration (MIC)

The Alamar blue assay was carried out for *P. aeruginosa* in TSB medium before inducing Ptm resistance and yielded a Ptm MIC of 2500 µg/mL. As a result of the acquired resistance Pa-Ptm-Res cells could survive in media containing 6500 µg/mL Ptm. The MIC of the Pa-Ptm-Rev culture was found to be 2500 µg/mL at the end of the study. Since the assay was carried out in TSB medium (pH~7.3), much of the Ptm (up to nearly 90%) may be bound to the proteinaceous broth (Truelstrup Hansen and Gill, 2000) and therefore the available concentration of Ptm for bacterial inhibition will be much less than what was actually added to the wells.

3.4.2. Turbidity studies

After the 13th passage in the presence of 6500 ppm Ptm in TSB to recover Pa-Ptm resistants, all three cultures (Pa-Ptm-ctl, Pa-Ptm-rev and Pa-Ptm-res) were exposed to different concentrations of Ptm ranging from 5 to 5000 ppm in 96 well microtitre plates for twenty minutes. A blank control lane with Ptm and no bacteria was run

simultaneously. Turbidity measured as A_{650} was recorded using an automatic plate reader equipped with mechanical agitation. The turbidity was plotted against concentration of Ptm in each well (Fig 3.1). The Pa-Ptm-ctl and Pa-Ptm-rev demonstrated a marked increase in turbidity with respect to Ptm concentration to a maximum at 156 ppm and then stabilised at 313 ppm and higher. However, in the case of Pa-Ptm-res, absorbance increased slightly up to the 10 ppm level and then decreased and remained more or less stable till the maximum concentration of 5000 ppm. The turbidity of the Pa-Ptm-rev was almost double that of the Pa-Ptm-res for Ptm concentrations of 156 ppm and above (Fig. 3.1), while the Pa-Ptm-ctl had absorbance values higher than that of Pa-Ptm-rev. This may be an indication that the Pa-Ptm-rev is in the process of repairing a disrupted cell surface. No clumping was observed when the cells were observed under microscope suggesting that perhaps the increases in absorbance were due to an increase in cell size or surface topography of control and revertant cells as a result of increasing levels of Ptm. Similar results were observed in the case of *E. coli* and *S. typhimurium* cells when exposed to Ptm (*i.e.* no apparent cell clumping, Tolong, 2004). In Tolong's original study, a mathematical approach was used to prove that a small increase in the cell diameter would lead to a big change in turbidity by using a set of equations developed by Kasireddy and Taweel, (1990).

Brock (1958) also reported that an increase in turbidity occurred when a variety of both Gram-negative and Gram-positive bacteria were treated with Ptm (1000 ppm) and this was attributed to the higher permeability of the cells to water causing an increase in the size of these cells and an increase in turbidity. Formation of blebs, which are small membrane blisters or protrusions on bacterial surfaces, were observed when polymyxin

(a cationic peptide antibiotic) treated *S. typhimurium* cells were freeze etched and observed under electron microscopy (Schindler and Teuber, 1975). The formation of blebs contributed to an increase in apparent cell size of 11 to 44% as a result of Ptm treatment (Schindler and Teuber, 1975).

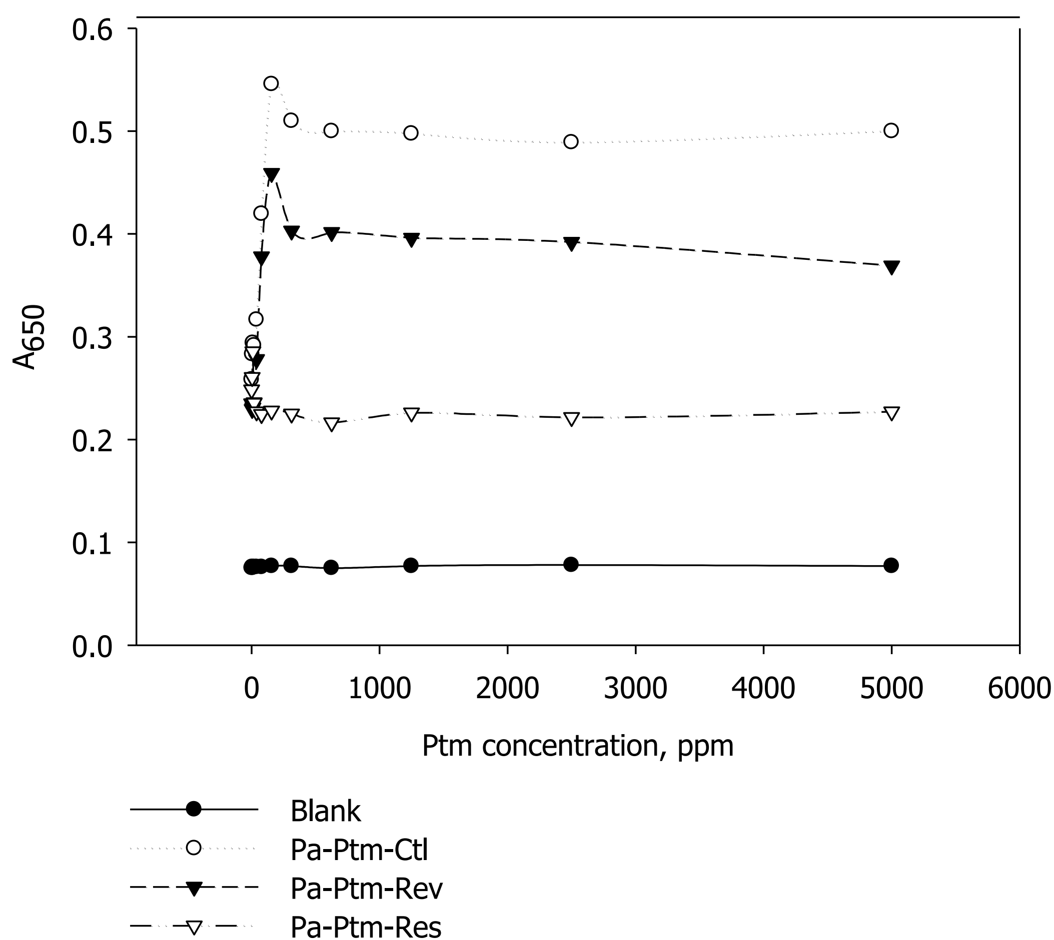


Figure 3.1. Optical density (650 nm) of Pa-Ptm control (Ctl), Pa-Ptm revertant (Rev) and Pa-Ptm resistant (Res) on exposure to various concentrations of Ptm (5 to 5000ppm). Each value is expressed as mean ± standard deviation (n=3).

These types of blebs were also reported in the case of *E. coli* and *S. typhimurium* exposed to Ptm (Storm *et al.*, 1977; Vaara and Vaara, 1983; Johansen *et al.*, 1996). In the present

study, formation of blebs or an increased permeability of the Ptm-susceptible cells to water may be contributing to the increased absorbance in the case of the control and Pa-Ptm revertant cells but this must be confirmed by microscopic examination (Please see Chapter 4).

3.4.3. Zeta potential measurements

In the present study, the zeta potential of the Pa-Ptm-res bacterium had changed dramatically as compared to controls and revertants and found to be close to neutral (Table 3.1). There were no significant differences in the zeta potentials of the control and the revertant groups.

Table: 3.1. Electrophoretic mobility and zetapotential values of Pa-Ptm control (Cntl), Pa-Ptm revertant (Rev) and Pa-Ptm resistant (Res) in 1mM potassium phosphate

	Electrophoretic mobility $\mu ms^{-1}/Vcm^{-1}$	Zetapotential, mV
Pa-Ptm Cntl	-2.56 ± 0.29[a]	-33.88 ± 3.88[a]
Pa-Ptm Rev	-2.58 ± 0.26[a]	-34.08 ± 3.40[a]
Pa-Ptm Res	-0.10 ± 0.16[b]	-1.39 ± 2.06[b]

Bacteria are able to change their surface properties based on interactions with each other and their environment. Bacteria invest a major portion of their metabolic energy in the synthesis and maintenance of macromolecular components on the cell surface (Wilson *et al*., 2001). Bacterial cell-surface interactions are influenced by the bacterial surface charge, pH, and ionic strength of the suspending medium. Since direct measurement of the surface charge is difficult, these interactions are often characterized by the zeta

potential, which can be deduced from electrophoretic mobility measurements (Karniadakis *et al*., 2005).

There are a number of possibilities for bacteria to alter surface charge. Components of the cell envelope contributing to an overall net negative surface charge in Gram negative bacteria include: membrane lipids (relative proportions of phosphatidyl glycerol, cardiolipin and phosphatidyl serine; all of which are negatively charged); as well as negatively charged phosphate groups of the lipid A portion of the outer leaflet. Viable Gram negative bacteria maintain a negative membrane potential of -130 to -140 mV (Yeaman and Yount, 2003) giving the entire cell a net negative charge by actively eliminating protons from the cytoplasm. Reduction in metabolic activity naturally results in a reduction in the rate in which protons may be expelled from the cytoplasm. Also, virulent strains of *P. aeruginosa* have been shown to be able to promote electrostatic shielding of their outer envelope by secreting anionic alginic acid (Friedrich *et al*., 1999).

A single bacterial strain can produce multiple forms of LPS continuously during balanced growth and discontinuously as a result of genetic changes such as phase variation, or in response to physiological signals such as temperature, culture density and nutrition. Most of the *Pseudomonas* strains produce a large proportion of LPS termed the S (smooth) form which has three parts: lipid A; a complex core oligosacharide which is attached at its reducing end to the lipid *via* an eight carbon sugar 3-keto-3-deoxy-D-mannooctonic acid (ketodeoxyoctonic acid, KDO); and finally a polysaccharide called the O-antigen (Gerhardt, 1994). Binding of CAPs is enhanced by electrostatic interactions between LPS and negatively charged phosphate and carboxylic groups of LPS localized in the lipid A core (Yermak and Davydova, 2008). Electrostatic attraction

increases the deposition of the CAPs onto the negatively charged microbial surfaces and thereby promoting their effectiveness (Ganz, 2001). It is interesting that based on a computer model of thermodynamic interaction, the probability for Ptm interacting with the base of the LPS was close to zero (Pink *et al*., 2003). This discrepancy may be because Ptm is substantially different from conventional CAPs or that Pink's model did not include the presence of exposed membrane proteins.

From electrophoretic mobility measurements previously reported for various bacteria, Bayer and Sloyer (1990) concluded that bacterial cells are negatively charged under standard culture conditions. The bacterial surface charge, measured as electrophoretic mobility, was found to be important for the antimicrobial efficacy of Ptm (Potter *et al*., 2005) for both Gram positive and Gram negative bacteria. With few exceptions, bacteria found to be more resistant to Ptm had surface charges approaching neutrality, while highly negatively charged bacteria tended to be more susceptible. Thus, the results of the present study on Ptm-resistant *P. aeruginosa* appear to agree with the earlier study and perhaps the loss of negative surface charge can be attributed to changes in response to the Ptm in the growth media. Perhaps a detailed study of the LPS and other chemical structures on the Ptm resistant *P. aeruginosa* will be able to explain this resistance mechanism in a greater depth. Some of the earlier studies indicate that the largest change in surface charge density as a result of antibiotic stress was generated by capsular antigens and to a lesser extent by lipopolysacharides (Bayer and Sloyer, 1990).

Earlier studies showed that *P. fluorescens'* resistance to CAPs was related to the availability of phosphate in the growth medium and this could be due to the loss of negative surface charges when grown in phosphate-deficient media (Dorrer and Teuber,

1977). Because of restricted access to phosphate in the growth medium, anionic phospholipids on bacterial membranes were largely replaced by positively charged ornithine-modified lipids and the bacteria became completely resistant to polymyxin. Another study involving the bacterial resistance to polymyxin and cecropin found that the resistance is mediated by an increased content of aminoarabinose and decreased anionic charge of lipopolysacharide (Vaara and Vaara, 1994; Shafer *et al*, 1984). Alterations in lipids in the outer membrane, which resulted in antibiotic resistance, were also noted in Gram-positive organisms as well and these changes most likely due to loss of teichoic acid from the membranes of organisms with acquired resistance (Yeaman and Yount, 2003).

3.4.4. SDS-PAGE of extracted proteins and identification by LC-MS/MS

As stated earlier, the main objective of this study was to understand the change in protein expression in *P. aeruginosa* as it acquires resistance to Ptm at the end of passage 13. At the end of the Ptm passage experiments, the sarcosinate-soluble (cytoplasmic) proteins as well as the Octyl POE-soluble (outer membrane) proteins were fractionated and separately examined by gradient SDS-PAGE (Fig. 3.2).

Higher molecular weight cytoplasmic proteins (above 116 kD) were found in both the control and revertant groups but found to be absent in the Ptm-resistant group. Major high molecular weight cytoplasmic proteins missing from the Ptm-resistant cells (Fig. 3.2, bands 1 and 2) were excised and tentatively identified using LC-MS-MS as DNA-directed RNA polymerase β subunit with a molecular weight of 154.2 kD and DNA-directed RNA polymerase β’ subunit with molecular weight of 150.7 kD (Table: 3.2).

RNA polymerase (RNAP) is the enzyme responsible for recognizing appropriate genes under specific environmental conditions, and for creating the mRNA transcripts that can be translated into new proteins (Hurwitz, 2005). DNA-dependent RNAP catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates. The RNAP catalytic core consists of two α, one β, one β' and one Ω subunit (Hurwitz, 2005). When a sigma factor is associated with the core, the holoenzyme is formed, which can initiate transcription (Borukhov and Nudler, 2003).

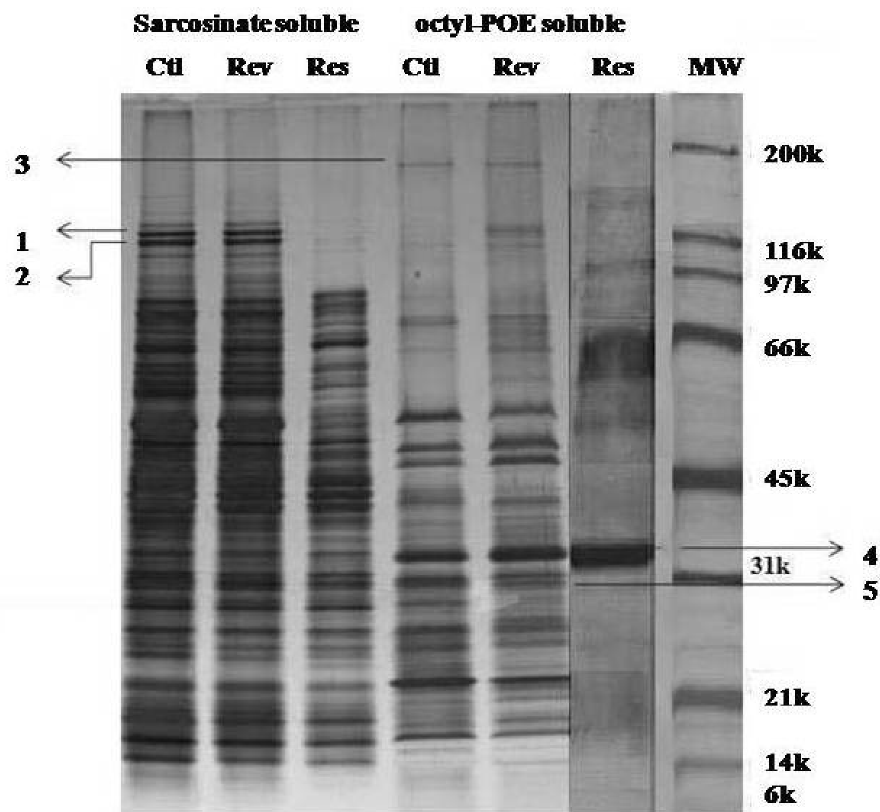


Figure 3.2. Gradient SDS-PAGE of sarcosinate-soluble and Octyl-POE-soluble proteins from Pa-Ptm controls, Pa-Ptm revertants and Pa-Ptm resistants. Bands marked and numbered 1 to 5 were tentatively identified as DNA directed RNA polymerase subunit β', DNA directed RNA polymerase subunit β, OprM, OprF and putative peptidyl-prolyl *cis trans* isomerase respectively.

Lower levels of RNAP in Pa-Ptm resistants may indicate lower transcription levels as a result of antibiotic stress. This in turn could possibly be interpreted as a signal to down-regulate the entire metabolism in response to the Ptm challenge.

Octyl-POE is a non-ionic detergent that selectively solubilises the porin proteins and has been used effectively as a first step in purification of a variety of porins from Gram negative cell envelopes (Luckey, 2008). Outer membrane protein expression of *P. aeruginosa* octyl-POE-soluble proteins as shown in Fig, 3.2 indicates that the Pa-Ptm resistant mutant was different from the control and revertants. Expressions of most octyl-POE- soluble outer membrane proteins except OprF (band 4) appeared to be down-regulated including OprM (band 5). Functional OprM is a typical triple barrelled trimeric protein, which exhibits long periplasmic extensions capable of spanning the periplasm (see Fig.3.3; from Nakajima *et al*., 2000).

Table 3.2. Proteins identified using peptide finger-printing followed by LC-MS/MS

Band #	Protein identified	Theoretical mass	Theoretical pI	Score	% sequence coverage
1	DNA directed RNA polymerase subunit β’	154287	6.65	1443	28%
2	DNA directed RNA polymerase subunit β	150700	5.64	1194	20%
3	OprM, *P. aeruginosa* PAO1	52566	5.52	114	24%
4	OprF, *P. aeruginosa* PAO1	37616	4.98	263	21%
5	Putative peptidyl-prolyl *cis trans* isomerase *P. aeruginosa* PAO1	26829	5.09	379	26%

Although OprM is a member of the MexAB-OprM xenobiotic-antibiotic transporter whose subunits are assumed to function as a conduit for antibiotic discharge across the

inner membrane and periplasmic space (Nakajima *et al*., 2000). It is apparently down-regulated in the presence of Ptm. OprM is important in the final stage of antibiotic ejection across the periplasm (Nakajima *et al*., 2000). The MexAB-OprM system of *P. aeruginosa* contributes to the antimicrobial resistance of wild type strains and has the broadest substrate range of all *P. aeruginosa* efflux pumps characterised to date. Increased expression of the MexAB-OprM protein system was observed in clinically isolated antibiotic resistant *P. aeruginosa*. This finding suggests that perhaps the efflux action of MexAB-OprM was not involved in Ptm resistance or that it serves another yet

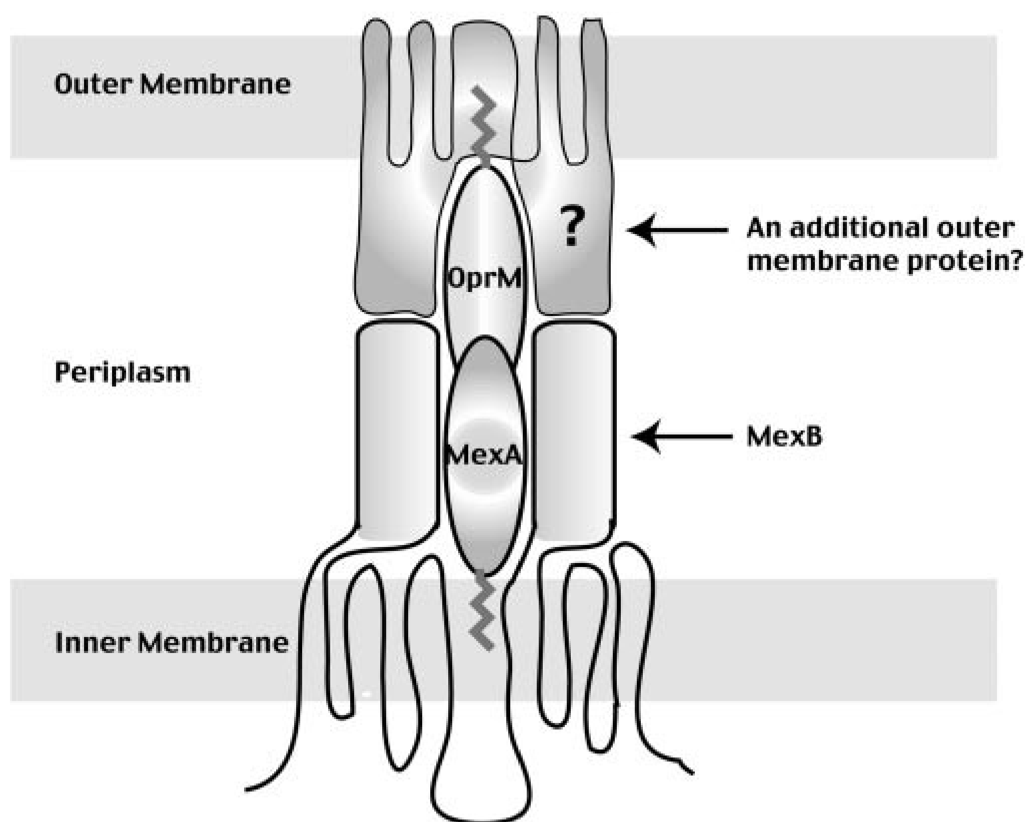


Figure 3.3. Schematic diagram of MexAB-OprMM efflux pump (Nakajima *et al*., 2000).

unknown function with regard to antibacterial susceptibility in *P. aeruginosa*. In view of the high electrostatic affinity of Ptm for most proteins, perhaps it is not unreasonable to

expect that most bacteria would experience extreme difficulty in excretion once Ptm had come in contact with cytoplasmic proteins. OprM in *P. aeruginosa* remains a trimer on heating, even in the presence of a detergent like SDS (Broutin *et al*., 2005; Yoshihara and Eda, 2005). OprM monomers have a molecular weight around 52 kD but they were found to be heat-modifiable (Gotoh *et al*., 1994; Masuda *et al*., 1995) resulting in varied mobility on SDS-PAGE as observed in the current study (Fig. 3.2).

Another membrane protein of interest (band 5, MW 27 kD) with varied expression levels in the Pa-Ptm control, revertants and resistants was identified as putative petidyl-prolyl *cis trans* isomerase (PPIase). PPIase is clearly down-regulated in the presence of Ptm but fully expressed in non-exposed control *P. aeruginosa* as well as the revertant strain. These enzymes are ubiquitous in both prokaryotes and eukaryotes (Maruyama and Furutani, 2000; Schiene and Fischer, 2000) in which they accelerate the rate of conformational inter-conversions around proline residues in polypeptides *in vitro* (Bang *et al*., 2000; Fischer *et al*., 1998). They are in fact periplasmic chaperonins that specifically assist in folding newly manufactured porins during their translocation to their permanent homes in the outer membrane of Gram negative bacteria. Some chaperonins are in fact isomerases that help proper protein folding by increasing the rate of transition of proline residues between the *cis* and *trans* states. Experimental data published so far suggests at least in *E. coli* s that PPIases are involved in modification of outer membrane porins and channels, components of secretion systems, and secreted proteins. It is reported that a “SurA” type of PPIase is involved in the maturation of outer membrane porins (Lazar and Kolter, 1996; Missikas *et al*., 1996). Lower expression of *Sur*A in *E. coli* was shown to interfere with an early folding step in LamB maturation, the

conversion of unfolded to folded monomer, and negatively affects expression of the major trimeric porins OmpC, OmpF and LamB, as well as of OmpA (Behrens *et al*., 2001).

Bacterial porins play a major role in the permeability of the outer membranes of Gram-negative bacteria to hydrophilic molecules (Benz and Bauer, 1988). Earlier studies have indicated the role played by these outer membrane proteins in antibacterial resistance (Yoneyama *et al*., 1995). Alterations in porin size or loss of porins are implicated in the reduced uptake of antibiotics (Achouak *et al*., 2001; Yoneyama *et al*., 1995). Deficient OprF and OprD porins were involved in antibiotic resistance in *P. aeruginosa* (Dubois *et al*., 2001; Gimeno *et al*., 1996; Quinn *et al*., 1988). The multidrug antibiotic resistance in *Enterobacter aerogenes* was also found to be associated with a decreased outer membrane permeability associated with change in porins. In most cases when the antibiotic stress was removed, expression of porin proteins started to increase (Denyer and Maillard, 2002). However to date, molecules as large as Ptm have never been thought to internalize through porin channels because of size constraints.

Decreased permeability of the outer membrane to cations may be the result of a higher level of interaction between LPSs, the outer membrane proteins and divalent cations (Beherens *et al*., 2001) in Gram-negative bacteria. The LPS's of Gram-negative bacteria are in part responsible for the net negative surface charge and the proteins of the outer membrane participate in a strong network held together with divalent cations (particularly calcium) to create an impermeable barrier except of course for the internalization of nutrients and elimination of end-products of metabolism. Any alteration in the amount of either of these components may result in an impaired barrier function

making it crucial for the cells to maintain the normal complement of porins in the outer membrane (Beherens *et al*., 2001).

Perhaps surprising was the fact that the general function and partially cation specific OprF porin (Table 2.2), the 37 kD protein (band 4) was equally expressed in all the three groups (Pa-Ptm controls, Pa-Ptm revertants and Pa-Ptm resistants). One would probably predict that Ptm resistance would in some way involve the down-regulation of OprF. OprF is the major outer membrane protein required for cell growth in a low-osmolarity medium and for the maintenance of cell shape (Woodruff and Hancock, 1988; 1989; Woodruff *et al*., 1986; Rawling *et al*., 1998). *P. aeruginosa* OprF forms 0.36-nS channels (average single channel conductance) and, rarely, 2- to 5-nS channels in lipid bilayer membranes. OprF in *P. aeruginosa* has been studied extensively due to its proposed utility as a vaccine component, its role in antimicrobial drug resistance, and its porin function (Benz and Hancock, 1981; Knapp *et al*, 1999; Piddock *et al*, 1992; Woodruff and Hancock, 1988; Brinkman *et al*., 2000). Perhaps one explanation to this apparent paradox is that OprF channels have the capacity to change channel size. The modulation of pore size may in fact account for more or less OprF conductance and this may be why higher conductance is not always associated with the production of more porins. This paradox was reported earlier by Malhotra *et al*., (2000) suggesting that regulation of OprF may involve post-transcriptional modifications. The asymmetric structure of bacterial LPS introduces a potential gradient across the membrane (with a negative surface charge density on LPS side and positive charge density on periplasmic side of the membrane), which plays a crucial role in membrane related processes like transport through porins (Soares and Straatsma, 2007). The decreased surface electro-negativity

observed in Ptm-Res *P. aeruginosa* may have resulted from narrowing of OprF channels when compared to that of control *P. aeruginosa* (Lins and Straatsma, 2001; Shroll and Straatsma, 2002).

3.5. Conclusions

Results obtained from these experiments confirm that the acquired resistance to Ptm observed in *P. aeruginosa* is coincidental with down-regulation of a number of porin proteins and this may imply that porins are in some way responsible with the resistance or susceptibility to Ptm. One possible explanation for these observations could be that a number of porins are responsible for the internalization of Ptm in *P. aeruginosa*. Another explanation could be that on Ptm exposure, *P. aeruginosa* growth rate diminishes as a result of general down-regulation of porins. Perhaps surprising was the fact that OprF, a well characterized general-function porin was not down-regulated as a result of prolonged exposure to Ptm. It may be that since this particular porin can alter the channel diameter, possibly as a function of LPS phosphorylation. One could perhaps speculate that the presence of Ptm in the environment results in a loss of phosphate groups from OprF-associated LPS.

Of particular interest was the fact that two well characterized periplasmic proteins were also down regulated: OprM whose activity has been associated with antibiotic export across the inner membrane and periplasmic space, and PPIase, a protein believed to be involved in folding newly synthesized porins. No significant increase in the expression of efflux proteins was observed in Ptm resistant *P. aeruginosa,* indicating that efflux of Ptm is probably not involved in Ptm resistance at least in the case of *P. aeruginosa*. This is

not entirely surprising since the current evidence suggests that once internalized, Ptm becomes electrostatically bound to the bacterial DNA and proteins and therefore there is no advantage in developing a mutational strategy for its elimination. Higher expression of OprF when the expressions of all the other porins were inhibited deserves a detailed study.

Loss of surface charge and/or electrostatic shielding observed as a result of Ptm resistance clearly confirms previous studies showing that the negative surface charge on bacteria plays a major role in Ptm susceptibility. Morphological examination of cell envelope components may help in understanding the mechanism of action of Ptm particularly when there was no increase in absorbance noted in the Pa-Ptm resistant group when compared to the control and the revertants.

Chapter 4.

LPS mediated surface remodelling in protamine resistant *Pseudomonas aeruginosa*

4.1. Introduction

The O-polysaccharide side chains of Gram-negative bacterial LPS plays a major role in structural versatility and adaptability. Studies have proven that the loss of the O-polysaccharide chain can alter the overall surface charge and hydrophobic character of Gram-negative cell surfaces (Williams *et al*., 1986; Palomar *et al*., 1995). LPS carrying O-antigenic structures were found to have the ability to prevent access of colicins, antibodies and phages to their outer membrane protein receptors (Van Der Ley *et al*., 1986). Shielding of deeper, more conserved layers of the bacterial cell surface from noxious agents is another important function of the O-antigenic part of the LPS. They also play a major role in maintaining the structure and function of outer membrane proteins (Van Der Ley *et al*., 1986).

P. aeruginosa produces two chemically distinct types of LPSs, termed A and B-band LPS. The A-band O-side chain is electro-neutral at physiological pH, while the B-band O-side chain contains numerous negatively charged sites due to the presence of uronic acid residues in the repeat unit structure. The use of chemical mutagenesis and LPS-specific phages has generated isogenic *P. aeruginosa* strains in which the A^+B^+ phenotype has been altered to A^+B^- , A^-B^+ and A^-B^- attributes (Lightfoot & Lam, 1991). Strain PAO1 (A^+B^+) and three isogenic LPS mutants (A^-B^- , A^-B^+, and A^+B^-) were obtained from the T. Beveridge Lab at the University of Guelph and used for this study.

The core region of PAO1 LPSs is composed primarily of neutral sugars but do contain some negatively charged sites (e.g., on the 2-keto-3-deoxyoctulosonic acid residues, as well as several phosphate groups in the inner core).

Surface electrokinetic studies and electrophoretic analyses of outer membrane proteins of Ptm-resistant *P. aeruginosa* indicated that Ptm perturbs membrane components and it would appear that this bacterium behaves much like *S. typhimurium* and *E. coli* in that no lysis or poration seems to take place (Tolong, 2004) despite an altered outer membrane. Most of the antibiotic resistance observed in bacteria is mediated by the alterations in exposed LPS on the surface. To see the difference in LPS expression as a result of Ptm resistance, LPS from control, revertants and resistant bacteria were extracted and compared with other isogenic mutants of *P. aeruginosa* using SDS-PAGE.

The Gram negative bacterial outer membranes, especially the negatively charged LPS are the first line of defence against deleterious factors of the extracellular milieu; therefore it is perhaps reasonable to expect that Ptm-resistant strains could have mutated LPS resulting in altered surface charge. Porins are synthesised as lipoproteins and are then transported across the cytoplasm (Driessen *et al*., 1998). Porins are incorporated into the outer membrane in close association with LPS. Some of the earlier studies (Ried *et al*., 1990; de Cock and Thommassen, 1996; de Cock *et al*., 1999; de Cock *et al*., 2001) indicate that LPS mutations resulting in altered surface charge can also cause changes in outer membrane protein expression.

Down-regulation of porins and loss of electronegative surface charge were found to be concomitant with acquired resistance to Ptm in *P. aeruginosa* cells exposed to successive

increases in environmental Ptm levels (Chapter 3). Since in previous studies, Ptm has been shown to cause cell envelope damage in the case of susceptible bacteria like *L. monocytogens* and *S. putrefaciens* (Johansen *et al*., 1996), it was desirable to see if induced resistance to Ptm would result in any ultra-structural changes to cell morphology. Previous work in our lab (Chapter 3; and Tolong, 2004) demonstrated that Ptm exposure can result in an increase in turbidity as measured by absorbance in broth cultures of *P. aeruginosa, E. coli* and *S. typhimurium.* In order to study this phenomenon more closely, the ultrastructures of Ptm-resistant (Ptm-Res) and revertant (Ptm-Rev) cells with cells that had never been exposed to Ptm were compared. In order to accomplish this, colloidal gold labels attached to anti-Ptm polyclonal antibodies were used.

4.2. Objectives

The main objective of this work was to electrophoretically examine the LPS expressed in the control, revertant and Ptm resistant *P. aeruginosa* (see Section 3.2.2) and compare the results with LPS mutants obtained with modified A and B O-side chains. Another objective of this work was to see if the presence or absence of A- and B-band structures on *P. aeruginosa* PAO1 had any effect on outer membrane protein expression and resistance to Ptm. The cellular distribution of Ptm was examined in cells that were highly resistant (Ptm-Res) to this peptide (6500 ppm) and the results were compared with those produced from cells that had never been exposed to Ptm (Controls) and to cells that had developed acquired resistance but had lost it (Ptm-Rev).

4.3. Materials and Methods

4.3.1. Bacterial strains and culture conditions

The *P. aeruginosa* strains used are listed in Table 4.1. All strains were cultured in TSB (Oxoid, Hampshire, England). Cultures were incubated overnight in a shaker at 120 rpm and a temperature of 37ºC.

Table 4.1. LPS mutant strains used in this study

Strain	Surface characteristics	Reference
P. aeruginosa PAO1 (Wild type)	Produces A-band LPS and B-band LPS (A+B+)	Rivera *et al*., (1988)
P. aeruginosa AK1401 (mutant)	Produces only A-band LPS (A+ B-)	Berry & Kropinski., (1986)
P. aeruginosa dps89 (mutant)	Produces only B-band LPS (A- B+)	Kadurugamuwa *et al.*, (1993)
P. aeruginosa rd7513 (mutant)	Produces neither A-band LPS nor B-band LPS (A-B-)	Lightfoot & Lam., (1991)

4.3.2. Induction of Ptm resistance

Resistance to Ptm was induced in *P. aeruginosa* by continuous sub-culturing in TSB containing increasing concentrations of Ptm. Serial passage was initiated from overnight cultures grown to exponential growth phase and then inoculated into Ptm-containing TSB (final bacterial concentration of approximately 1.0×10^6 CFU/mL).

The bacteria were incubated overnight at 37ºC and an aliquot was transferred to fresh media with a higher concentration of Ptm. Concentrations of Ptm used in the thirteen passages were 100, 300, 600, 1200, 1750, 2400, 3000, 3750, 4250, 4750, 5500, 6000 and 6500 ppm respectively. After the 13th passage, an aliquot of the culture was transferred to fresh medium without any Ptm to check for revertants (Pa-Ptm revertants) and another aliquot to TSB with 6500 ppm of Ptm (Pa-Ptm resistant). A control culture was maintained by sub-culturing the *P. aeruginosa* PAO1 in TSB devoid of Ptm (Pa-Ptm control). Fresh cells were used in all the experiments.

4.3.3. LPS extraction

LPS was extracted using the procedure of Hitchcock and Brown (1983). Overnight cultures of control, Ptm-Res, Ptm-Rev and LPS mutants of *P. aeruginosa* (A+B-, A-B+ and A-B-) were harvested by centrifugation at 1000 *x g*. Harvested cells were washed with 0.9% saline and then resuspended in lysis buffer consisting of 2% SDS, 10% glycerol and 4% β-mercaptoethanol in 1M Tris pH 6.8. A 5 µL aliquot of 20 mg/mL proteinase-K (Sigma Chemical Co., St. Louis, Mo) was added to the mixture and incubated at 65ºC for one h. The resultant extract was separated on SDS-PAGE.

4.3.4. SDS-PAGE

LPS was fractionated on an SDS-polyacrylamide gel (Laemmli, 1970) containing 4% and 15% acrylamide in the stacking and separating gels, respectively. Electrophoresis was done at 30 mA in the stacking gel and 40 mA in the separating gel until the tracking dye had reached the bottom of the gel.

4.3.5. Silver staining

Silver staining of LPS was carried out according to the procedure of Fomsgaard *et al.* (1990). LPS was oxidized in the gel with 0.7% periodic acid in 40% ethanol-5% acetic acid at 22ºC for 20 min without prior fixation. The gel was then washed three times with distilled water for 5 min. The gel was stained for 10 min with a freshly prepared staining solution as follows. A 4-mL volume of concentrated ammonium hydroxide was added to 56 mL of 0.1 M sodium hydroxide. After the addition of about 200 mL of water, 10 mL of 20% (w/v) silver nitrate was added dropwise with stirring. The final volume was adjusted to 300 mL with water. The gel was washed three times with distilled water for 5 min. The color was developed in 200 mL water containing 10 mg citric acid and 0.1 mL of 37% formaldehyde. The reaction was stopped by exposure to 10% acetic acid for 1 min followed by repeated washings in distilled water.

4.3.6. Membrane protein extraction from LPS mutants

P. aeruginosa LPS mutants were cultured overnight at 37ºC in TSB. The cells were harvested by centrifugation at 2000 *x g* (Universal 32 R, Hettich, Germany). Harvested cells were further washed with 10 mM Tris-HCl pH 8. The pellet obtained was suspended in breaking buffer (10 mM Tris-HCl, 5% sucrose, 0.1 M NaCl, 3 mM NaN_3, 0.1 mg/mL DNase and protease inhibitors (Roche Diagnostics, Mannheim, Germany), (Nikaido *et al*., 1991). Cells were passed through a French press cell twice at 14,000 psi. Unbroken cells were pelleted out by centrifugation at 1000 *x* g for 10 min at 4ºC. Sodium lauryl sarcosinate (1%) was added to the resultant supernatant to selectively extract cytoplasmic membrane proteins (Fillip *et al*., 1973). Outer membranes were pelleted

from the suspension by centrifugation at 75,000 *x* g for 90 min using an ultra-centrifuge (Model L2-65B, Beckman Instruments, California, USA) equipped with a SW-27 rotor. The pellet containing the crude outer membranes was resuspended in washing buffer which consisted of 10 mM Tris-HCl, 3 mM NaN_3, 20 mM $MgSO_4$, 0.1 mg/mL DNase, 0.1 mg/mL RNase and protease inhibitors (Roche Diagnostics, Mannheim, Germany) (Nikaido *et al*., 1991). This was allowed to stand for 2 h at room temperature and later centrifuged at 75,000 *x* g for 90 min. The pellet obtained was suspended in 10 mM Tris-HCl, 0.2 M NaCl, 3% octyl- POE at pH 8 and was allowed to stand overnight at 4ºC. This was centrifuged at 75,000 *x g* for 90 min. The resultant supernatant contained porin proteins.

4.3.7. Membrane Protein Analysis by SDS-PAGE

Gradient (5-15%) SDS-polyacrylamide gels, prepared according to the discontinuous buffer system of Laemmli (1970) were used to separate the extracted cytoplasmic and the outer membrane proteins. The concentration of the extracted proteins was estimated using BCA assay as described earlier (Chapter 3) and 20 µg of protein were loaded on each lane. SDS-PAGE was carried out using an LKB 2001 Vertical Electrophoresis unit (LKB Produkter; Bromma, Sweden). Broad range (6.6 to 200 kDa) molecular mass markers (Bio-Rad, Mississauga ON) were loaded in one of the lanes for reference. LC-MS/MS compatible silver staining of the gels was carried out according to the modified procedure of Blum *et al.,* (1987).

4.3.8. Estimation of Minimum Inhibitory Concentration (MIC)

MIC of Ptm against wild-type and LPS mutants of *P. aeruginosa* were estimated using the Alamar blue assay as described earlier (Potter *et al*., 2005) with slight modifications in the case of the A-B+ mutant which had a very slow growth rate. Based on the difference in growth rate, higher numbers of cells (100x) were incubated for this particular mutant and the samples were incubated for a longer time (36 h) with Ptm. The MIC was defined as the minimum concentration of the peptide that inhibited the growth of the target organism and therefore did not result in colour change (blue to red) after the overnight incubation period.

4.3.9. Zeta Potential Measurements

Zeta potential distributions of wild and LPS mutant *P. aeruginosa* were determined by micro-electrophoresis (Hiemenz, 1991) as described in section 3.3.9.

4.3.10. Ptm Treatment

Control, Ptm-Rev and Ptm-Res *P. aeruginosa* in TSB were exposed to 3000 ppm Ptm for 30 minutes at 37°C. The control, Ptm-Res and Ptm-Rev *P. aeruginosa* after the Ptm treatment were harvested by centrifugation at 1000 *x* g and then washed in 50 mM HEPES (pH 8) buffer before treatment with Ptm.

4.3.11. Transmission Electron Microscopy

After Ptm treatment, the cells were washed with excess 50 mM HEPES buffer (pH 8) to remove any unbound Ptm. The cells were harvested by centrifugation at 1000 *x* g for 5

min and bacterial pellets fixed in ice-cold 0.1% (v/v) glutaraldehyde with 2% formaldehyde for 45 min using 50 mM HEPES (pH 8) as fixation buffer. Fixed cells were further washed three times in HEPES buffer and enrobed in 2% Noble Agar tempered to 60ºC. These were later dehydrated in a graduated ethanol series. The dehydrated blocks were infiltrated with LR White resin (Marivac; Halifax, NS, Canada) and embedded in gelatine capsules which provided an oxygen-free environment for LR White polymerisation at 60ºC for one h. They were later cooled to room temperature. One hundred nm thin sections were cut using an ultramicrotome (LKB-Huxley Ultramicrotome, LKB Instruments, Inc., Rockville, MD) equipped with a diamond knife. Thin sections obtained were placed on 300 mesh formvar-carbon-coated copper grids and stained with 2% uranyl acetate for 10 min followed with a distilled water wash. The sections were counter stained with 2% lead citrate (Reynolds, 1963) for 4 min and then rinsed with distilled water. The grids were then air dried before being viewed using a JEM 1230 transmission electron microscope at 80 kV (JEOL Ltd. Musashino Akishima, Tokyo, Japan). Images were captured using a Hamamatsu ORCA-HR digital camera (Hamamatsu, Hamamatsu City, Japan).

4.3.12. Immunolelectron Microscopy

Anti-Ptm polyclonal antibodies produced according to the following procedure were obtained from Open Biosystems (Huntsville, AL) and were used in immuno-electron microscopy to understand Ptm localization. The polyclonal anti-Ptm serum was produced by immunizing two New Zealand white rabbits using Ptm sulphate salt from herring (Sigma-Aldrich, St. Louis, MO, USA), conjugated with Keyhole Limpet Hemocyanin (KLH) and emulsified with Freund's complete adjuvant (Day 1). Control serum was

collected before primary immunization (Day 0). After the primary immunization (0.50 mg antigen at 10 subcutaneous (SQ) sites), three booster doses (0.25 mg of Ptm in 4 SQ sites) were administered on days 14, 42 and 56. Serum was collected on days 28, 56 and 70. Anti-Ptm antibodies were purified by affinity chromatography and the titre value assessed using indirect ELISA.

Localisation of Ptm in *P. aeruginosa* immuno-labelling with 10 nm colloidal gold was carried out on 100 nm thin sections on 300 mesh nickel grids. Sections were washed with 1 mg/mL sodium borohydride, then with 30 mM glycine in 0.1 M borate buffer (pH 9.6). The sections were then incubated in a blocking solution consisting of TBS buffer with 1% skim milk and 1% BSA for 45 min then washed with TBS buffer consisting of 10 mM Tris, 0.3 M NaCl and 0.1% bovine serum albumin (BSA) at pH 8.1. The sections were then exposed overnight to diluted primary antibody (1:100) (Open Biosystems, Huntsville, AL) to Ptm. The sections were washed three times with 10mM Tris pH 8.1 with 0.3 M NaCl and 0.1% BSA to remove any unbound primary antibody. The sections on grids were later allowed to react with Anti-rabbit IgG (whole molecule) developed in goat, conjugated with 10 nm colloidal gold (Sigma-Aldrich, St. Louis, MO, USA), then washed to remove any unbound secondary antibody. The specimens were fixed by floating the grids on drops of 2.5% (vol/vol) glutaraldehyde in PBS for 10 min (Garduno *et al*., 1998). The sections were again washed with distilled water before staining the grids by placing on drops of 2% aqueous uranyl acetate for 10 min. The stained sections were further washed with distilled water and counter stained with lead citrate solution. Sections were blot dried before being viewed. Control samples untreated with primary antibodies were maintained for each group of specimens.

4.4. Results and Discussion

4.4.1. LPS extraction and SDS-PAGE

LPS extracted from control, Ptm revertant and Ptm resistant *P. aeruginosa* was compared by electrophoresis (Fig. 4.1. lanes 1, 2 and 3 respectively). The Ptm resistant *P. aeruginosa* had a much lower concentration of O-antigenic components when compared to the control and revertants. LPS from the *P. aeruginosa* wild type (A+B+) and LPS mutants A+B-, A-B+ and A-B- are shown in lanes 4, 5, 6 and 7, respectively.

The electrophoretogram of mutant LPS from *P. aeruginosa* (control and A+B+) was similar to that recorded earlier for *P. aeruginosa* (Fomsgaard *et al*., 1990; Lam *et al*., 1989). O-antigens in the LPS of the Ptm resistant group (Fig. 4.1., Lane 3) were found to be similar to those of O-antigens on A+B- mutant (Fig. 4.1., lane 5) indicating a possible resistance mechanism involving the loss of B-bands. The B-band O-side chain contains numerous negatively charged sites due to the presence of uronic acid residues in the repeat unit structure, but the loss of B-band components resulted in a more electro-negative surface on the A+B- mutants (See section 4.4.4.). This was earlier reported by Makin and Beveridge (1996). Computer simulations published for the rough LPS membrane suggest that the inclusion of B-band components lowers the electrostatic potential, surface charge and promotes membrane expansion (Soares and Straatsma, 2008; Lins and Straatsma, 2001). These types of alterations in O-antigenic structure and lipid A were also reported by Goebel *et al*., (2008) as part of a resistance mechanism to complement and other cationic immune factors (Ernst *et al*., 2001). As part of the resistance to cationic antibiotics, bacteria tend to neutralise the charge distribution at the

core and lipid A. The negative surface charge of the bacteria is partially due to phosphate

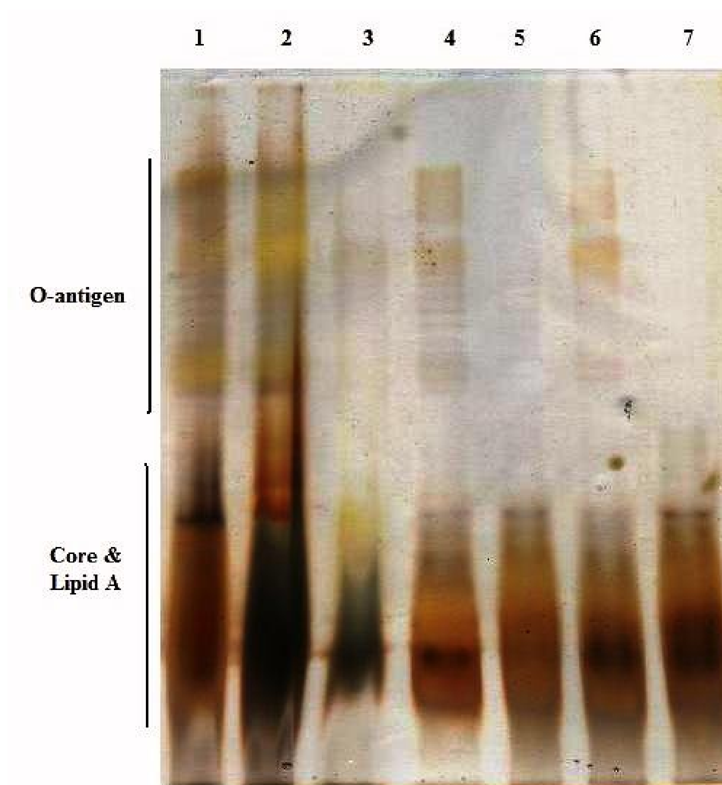


Figure 4.1 SDS-PAGE of LPS extracted from Ptm control, revertant and resistant *P. aeruginosa* PAO1 in lanes 1, 2 and 3 respectively. LPS from the *P. aeruginosa* wild type (A+B+) and LPS mutants A+B-, A-B+ and A-B- in lanes 4, 5, 6 and 7, respectively. Each lane was loaded with 20 µL of sample.

residues in the heptose region, which play a major role in maintaining their barrier function by providing sites for cross-linking of adjacent LPS molecules with divalent cations or polyamines (Vaara, 1992; Fridrich and Whitfield, 2005). These negative charges help in creating interactions between LPS and positive charges of outer membrane proteins. Some of the cationic antibiotics and other positively charged host defence peptides and proteins may target this part of the LPS (Alexander and Rietschel, 2001).

4.4.2. Membrane and Cytoplasmic Protein Extractions and SDS-PAGE

Bacteria have large numbers of porins on their surfaces (10^4 - 10^6 copies per cell; Koebnik *et al*., 2000). The outer membrane protein (OMP) pattern of the Ptm-resistant mutants was significantly different from revertant and the control groups (Chapter 3). It has been reported (Ried *et al*., 1990; de Cock and Thommassen, 1996; de Cock *et al*., 1999; de Cock *et al*., 2001) that changes in LPS structures and membrane fluidity can bring about changes in OMP expression and therefore transport characteristics through these proteins. In order to see if a change in O-antigenic structures can alter the porin expression, octyl-POE was used to extract OMPs which were subsequently examined by SDS-PAGE on 15% acrylamide gels. No change in OMP pattern was observed (Fig. 4.2) in any of the LPS mutants (A^+B^-, A^-B^+ and A^-B^-) when compared to the wild type control which had both A and B-band structures (A^+B^+).

In addition, there were no major differences in the banding patterns when comparing electrophoretograms of sodium lauryl sarcosinate-soluble cytoplasmic proteins from wild and LPS mutants of *P. aeruginosa* (Fig. 4.3). The results indicate that these particular mutations of O-antigenic structures in *P. aeruginosa* do not appear to make any difference in the porin expression pattern despite the fact that early research on porin functions reported that the channels are influenced by membrane composition, and porins require the presence of LPS for proper function (Schindler & Rosenbusch, 1981). LPS mutations did not affect the MIC values for Ptm as determined by the Alamar Blue assay. The asymmetric nature of the outer membranes introduces a potential gradient across the membrane with a net negative surface charge density on the LPS (external) side and a

positive charge density on the periplasmic side of the membrane (Soares and Straatsma, 2007). This potential plays a crucial role in many membrane-related processes,

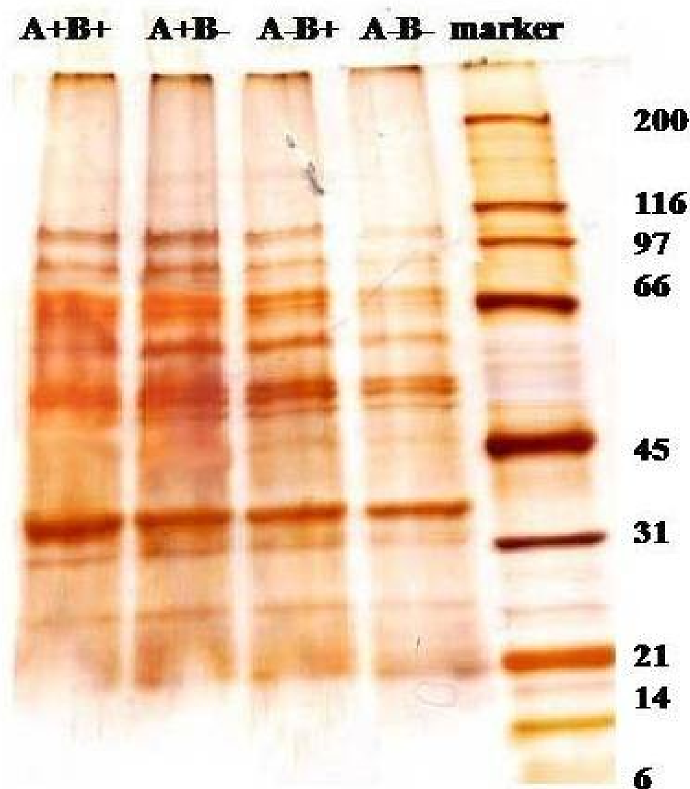


Figure 4.2. Electrophoretogram of 3% octyl-POE extracted outer membrane proteins (Lanes 2 to 5) from the wild type *P. aeruginosa* PAO1 (A^+B^+) and the three LPS mutants (A^+B^-, A^-B^+ and A^-B^-) respectively.

particularly binding of charged species and voltage-dependent gating of porin channels (Straatsma and McCammon, 2001; Shroll and Straatsma, 2002).

Alterations in the ionic composition of the surrounding milieu can also have an impact on behaviour of bacterial porin channels. Earlier research proved that at acidic pH, the closed state of porins are stabilised and threshold gating voltage lowered (Saint *et al.*,

1996; Todt *et al*., 1992; Delcour, 1997). Highly cationic polyamines like cadaverine, putrescine and spermidine can associate with the LPS in the vicinity of porins and modulate transport through the porin channels *in vivo* (Koski and Vaara, 1991).

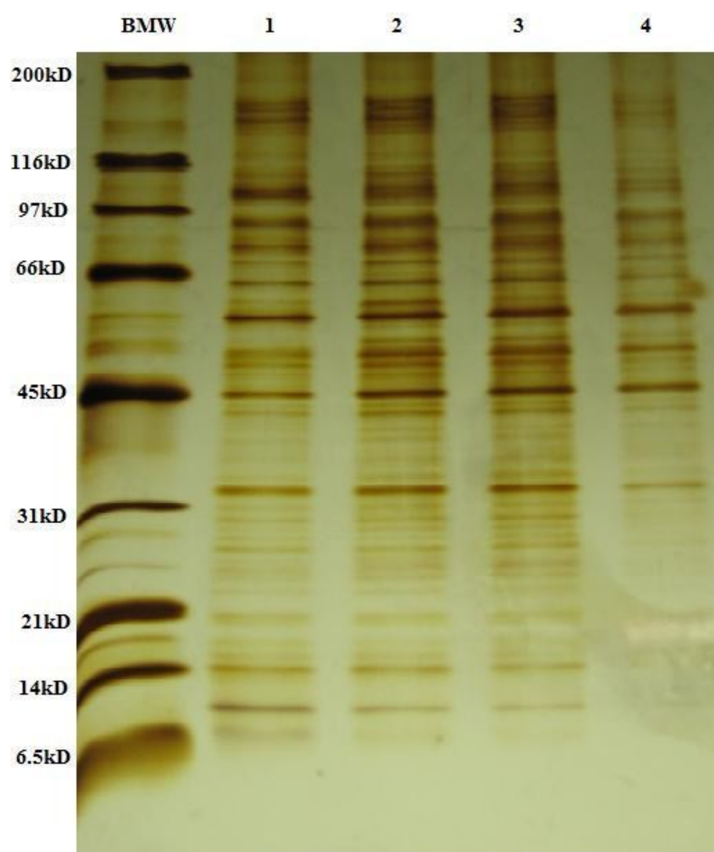


Figure 4.3. Electrophoretogram of sodium lauryl sarcosinate-soluble proteins from the wild *P. aeruginosa* PAO1 (A^+B^+) and the three isogenic mutants (A^-B^- , A^-B^+, and A^+B^-) in lanes 1, 2, 3, and 4, respectively. The first lane is loaded with broad range molecular weight marker.

In the current study, decreased expression of porins (all except OprF) may have reduced the internalisation of Ptm (Section 3.3.4.)

4.4.3. Estimation of Minimum Inhibitory Concentration (MIC)

The MIC of Ptm to the wild-type and LPS mutants of *P. aeruginosa* was determined to see the effect of O-antigenic composition on Ptm action. The MIC of Ptm to the wild type *P. aeruginosa* (2,500 ppm) was found to be the same as that of LPS mutants. The results clearly indicate that the presence or absence of O-antigenic structures alone cannot contribute to resistance to Ptm. The loss of porins and or the modification of core LPS may have contributed to Ptm resistance in *P. aeruginosa*.

4.4.4. Zeta Potential Measurements

Table 4.2 shows the electrophoretic mobilities and zeta potentials of wild type and LPS mutants and compares them with those of Ptm resistant, revertant and control *P. aeruginosa*. Considering the LPS mutants alone, surface electronegativity was found to be highest in A-B- mutant and the lowest in A-B+ mutant. Zeta potentials of A+B+ and A-B+ mutants were comparable with those of revertant and control *P. aeruginosa*. The close to neutral surface charge observed in Ptm-resistant *P. aeruginosa* may have been contributed by the altered LPS structures and porin distribution in the outer membrane. A detailed study is needed to examine the changes taking place in *P. aeruginosa* LPS resulting in Ptm resistance.

Perhaps surprising was the fact that for the LPS mutants, removal of B-band component resulted in an overall increase in electronegativity, rather than a neutralization of surface charge. One explanation for this paradox could be that the removal of B-band structures may have exposed the electronegative groups in the core-lipid A region of the LPS (Makin and Beveridge, 1996). Voltage-dependent porin channels are sensitive to the

local electric field and neutralization of surface charge is expected to cause changes in channel properties. The importance of LPS in addition to other factors for voltage sensing in porins, was reported earlier (Lakey and Pattus, 1989; Brunen and Engelhardt, 1993). In the current study, Ptm resistance may have been mediated by change in surface charge as a result of modifications in LPS and or decreased expression of porins may have influenced the membrane potential of the cell and there by affecting the internalisation of peptides.

Table 4.2. Electrophoretic mobility of LPS mutants, Ptm resistant, revertant and control *P. aeruginosa* in 1 mM potassium phosphate at pH 4

	Electrophoretic mobility, μms^{-1}/Vcm^{-1}	**zeta potential, mV**
A^+B^+ (wild)	-2.56 ± 0.29^{a}	-36.30 ± 5.80^{a}
A^-B^- (LPS mutant)	-4.65 ± 0.40^{b}	-63.63 ± 6.36^{b}
A^+B^- (LPS mutant)	-3.90 ± 0.46^{c}	-53.86 ± 0.29^{c}
A^-B^+ (LPS mutant)	-2.58 ± 0.32^{a}	-35.50 ± 4.08^{a}
Pa-Ptm Cntl	-2.56 ± 0.29^{a}	-33.88 ± 3.88^{a}
Pa-Ptm Rev	-2.58 ± 0.26^{a}	-34.08 ± 3.40^{a}
Pa-Ptm Res	-0.10 ± 0.16^{d}	-1.39 ± 2.06^{d}

The changes in surface exposed proteins, and surface charge observed in Pa-Ptm Res cells indicates the possible role of bacterial surface electro-negativity in initial binding of Ptm and subsequent internalisation.

4.4.5. Transmission electron microscopy

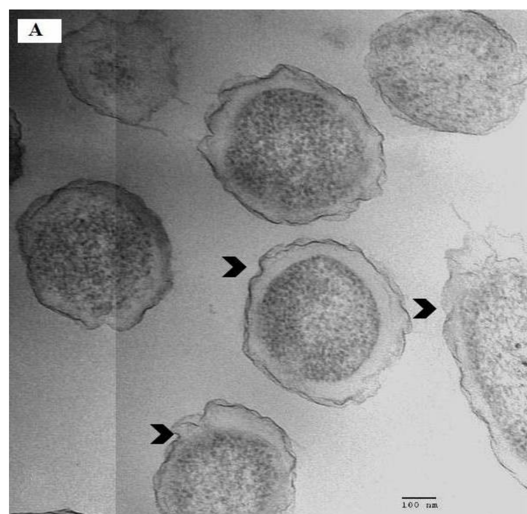
Bulges were observed on the outer envelopes of control *P. aeruginosa* cells exposed to 3000 ppm Ptm for 30 min (Fig. 4.4A). Formation of the bulges may have contributed to the increase in turbidity as a result of Ptm action observed earlier (Chapter 3) and also reported by Tolong (2004). Fewer bulges were observed in the case of Ptm-revertant *P. aeruginosa* (Fig. 4.4B). Formation of bulges was not observed in Ptm-resistant *P. aeruginosa* when challenged with Ptm (Fig. 4.4C). By the same token, these cells yielded no increase in turbidity in the presence of Ptm (Chapter 3). The formation of bulges may be the result of interaction of the Ptm with bacterial LPS or binding to exposed membrane proteins. According to Arsenault *et al*., (1991) and Li *et al*., (1996), bleb formation is perhaps less likely to occur with A-band LPS because it is more electrically neutral than the B-bands and contains smaller O-side chains consisting of α 1-2- and α 1-3-linked D-rhamnose and little phosphate. Perhaps a more detailed study is required to understand the role played by O-antigenic structures in bulging as a result of Ptm treatment. Thammasirirak *et al*., (2010) found that surface blebs were observed when *Vibrio cholerae* cells were treated with peptides derived from goose egg lysozyme. The number of blebs increased with increase in concentration of the peptide and exposure time. In other studies reported earlier, melittin (Oren *et al*., 1997), human antimicrobial peptide LL-37 (Oren *et al*., 1999) and D, L-amino acid containing peptides (Papo *et al*., 2002; Shai & Oren, 1996) were observed to lead to the formation of blebs and eventual cell lysis in *E. coli* and other bacterial strains. Formation of blebs was also recorded in the case of *S. typhimurium* when treated with polymyxin and Ptm (Storm *et al*., 1977; Vaara and Vaara, 1983; Johansen *et al*., 1996). The interaction of polymyxin with the

LPS may have contributed to bleb formation in various Gram-negative bacteria (Weber *et al*., 1979). Although Tolong (2004) did not observe blebs on Ptm-treated *E. coli* and *S. typhimurium*, he did observe increases in turbidity but did not conduct an ultrastructural examination.

4.4.6. Immuno-electron Microscopy

The Ptm treated control and Ptm-Rev *P. aeruginosa* cells, appeared to have intact outer membranes while the cytoplasmic components appeared to be aggregated (Fig. 4.4A and B). Immuno-gold labelling was carried out to understand the distribution of Ptm. In the case of Ptm-treated controls, all the Ptm appeared to internalize and localize in the condensed cytoplasm. There was little evidence of Ptm near the surface of the outer membrane. In the case of Ptm-Rev cells, Ptm was found to be internalized but with a lower degree of cytoplasmic condensation (Fig. 4.4E).

In the case of Ptm-Res *P. aeruginosa*, condensation of cytoplasm was not observed. The distribution of Ptm in the resistant group was found to be quite different from that of control and Ptm-Rev cells. Ptm was found to be localised more at or near the surface of the cell envelope (Fig. 4.4F), possibly indicating lower outer membrane permeability as a result of acquired resistance to Ptm. Loss of porins or impaired porin channels observed in Ptm-Res *P. aeruginosa* (Chapter 3) may have affected the permeability of Ptm to the interior of the cell.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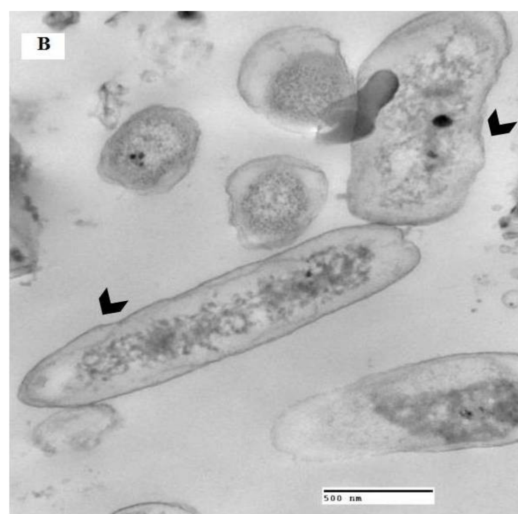
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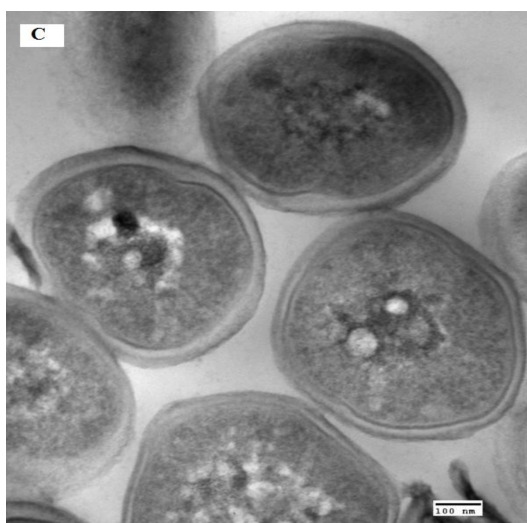
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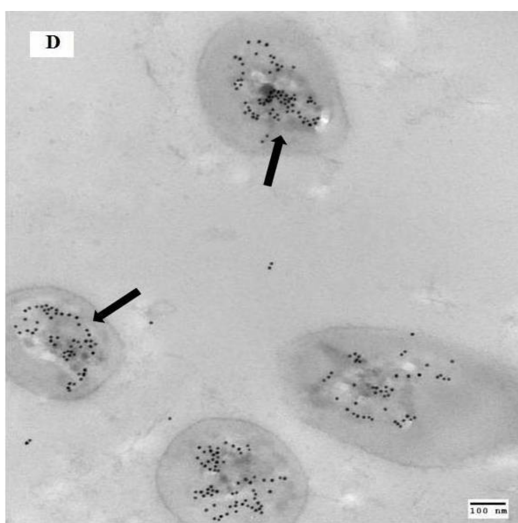
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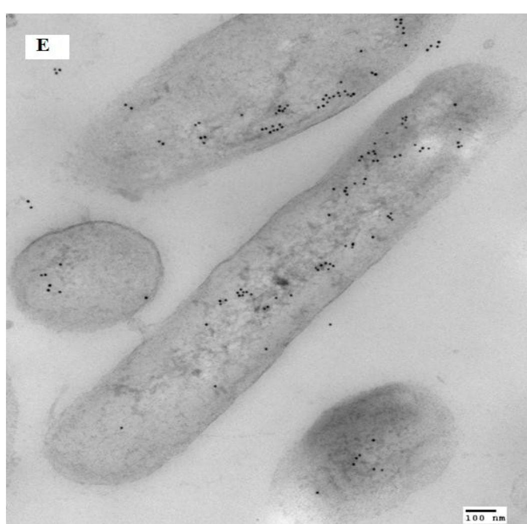
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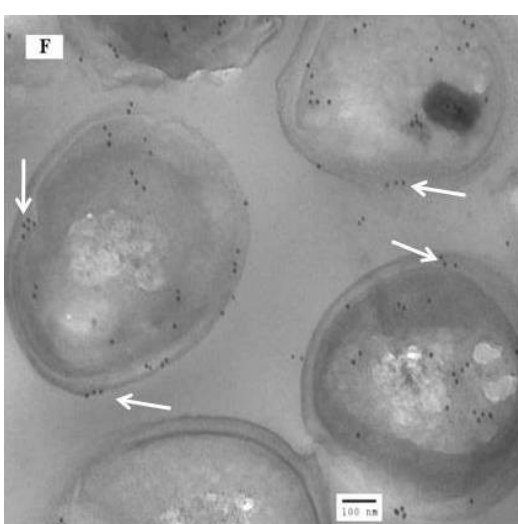
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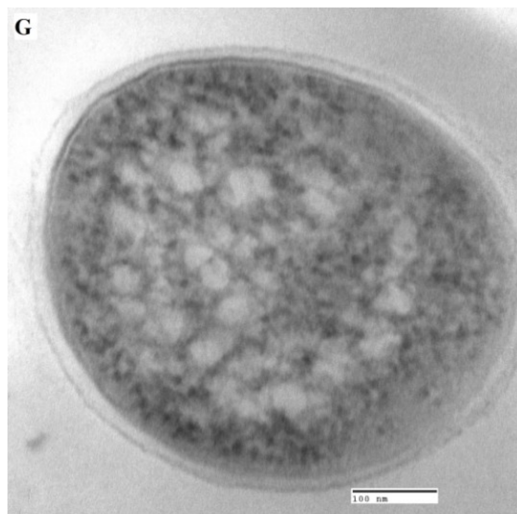


Figure. 4.4. TEM images of control (A), revertant (B) and resistant (C) *P. aeruginosa* treated with 3000 ppm Ptm. Immuno-TEM images of control (D), revertant (E) and resistant (F) *P. aeruginosa* treated with 3000 ppm Ptm. Black arrows (in Fig. 4.4D) show Ptm localised in the condensed cytoplasm. White arrows show Ptm localised at or near the outer membrane of Ptm-resistant *P. aeruginosa.* Bulges on control and revertant *P. aeruginosa* are shown using arrow heads in tiles A and B. Controls were performed in which the primary antibody was eliminated (Fig. 4.4G).

Results reported in Chapter 3 showed that one of the important characteristics observed in Ptm-Res *P. aeruginosa* was a change in surface charge as measured by electrophoretic mobility; the Ptm-Res cells were more electrically neutral than non-resistant (Control) cells and Ptm-Rev cells. The zetapotentials at pH 7.0 were -33.55±4.40, -35.86±4.33 and -14.42±4.27, mV for the Control, Ptm-Rev and Ptm-Res cells, respectively. It therefore appears that in this particular culture, electrostatic shielding was at least one of the acquired resistance mechanisms.

Immuno-gold labelling of Ptm-Res *P. aeruginosa* challenged with Ptm (Fig. 4.4F) clearly shows that the majority of the Ptm molecules bound at or near the cell surface. It may be possible that the Ptm may be binding to the porin proteins still expressed on Ptm-Res *P. aeruginosa*.

4.5. Conclusion

Electrostatic shielding in the Ptm resistant *P. aeruginosa* may have altered the channel properties of expressed porins resulting in impaired transport of Ptm through them resulting in resistance. Acquired Ptm resistance in *P. aeruginosa* observed in the current study may have been contributed by altered LPS that resulted in electrostatic shielding together with loss of porins and or impaired transport through OprF. It is quite evident that alterations in O-antigenic structures alone cannot contribute to Ptm resistance. Loss of porins and or closed conformation of OprF may be the main mechanism that contributed to Ptm resistance in *P. aeruginosa*. A more detailed study is essential to understand the role of OprF and LPS components in acquired resistance to Ptm in *P. aeruginosa*. Bulges were observed in Ptm-Rev and control *P. aeruginosa* when exposed to Ptm. No such bulging was observed in Ptm-Rev cells. Condensation of cytoplasmic contents was not observed in Ptm-Res *P. aeruginosa* when challenged with Ptm. Localisation of the majority of Ptm on the near-neutral bacterial membrane in the case of Ptm-Rev *P. aeruginosa* may be due to the binding of the peptide to the porin proteins present on the OM. The possibility of an increased residence time for Ptm in the constricted OprF channels may have a role in Ptm resistance observed in this study. The role of OprF in resistance to Ptm needs to be studied in depth.

Chapter 5.

Ptm action on *Pseudomonas aeruginosa, Escherichia coli and Salmonella typhimurium* cells and spheroplasts: morphology and Ptm localisation

5.1. Introduction

The mechanisms by which Ptm inhibits bacterial growth are still not clear and the role of bacterial porins if any in Ptm action is poorly understood. Earlier studies by Johansen *et al*., (1996) suggested that for most bacteria (including both Gram positive and Gram negative cells) Ptm may aggregate bacteria, causing poration and/or lysis, concomitant with loss of proton-motive force. This was demonstrated in the Gram positive bacterium *Listeria monoctyogenes* and the Gram negative bacterium *Shewanella putrefaciens* and it was assumed that poration and lysis were generalized mechanisms for the Ptm-induced destruction of most susceptible bacteria. However in 2004, a study by Tolong demonstrated that Ptm does not apparently aggregate, porate or lyse the Gram-negative bacteria *E. coli* and *S. typhimurium*. Furthermore, Tolong clearly demonstrated that Ptm was unable to lyse Gram negative spheroplasts prepared from *E. coli* and *S. typhimurium* even at concentrations that were bactericidal to intact cells. Furthermore, computer simulation studies carried out by Pink *et al*., (2003) revealed that the probability for Ptm to penetrate the asymmetrical outer membrane of a Gram negative cell was effectively zero.

Since the mathematical model provided by Pink *et al.* (2003) did not consider the presence of membrane proteins in the Gram negative envelope, the paradox of Ptm

internalization without lysis or poration has led to the hypothesis that translocation across inner and outer membranes may in some cases be facilitated by porin proteins despite the fact that to date the largest molecule shown to be internalized by porins has a mass around 644 Da (Satake *et al*., 1990; Yoshimura and Nikaido, 1985). It is estimated that 50% of the mass of the Gram-negative outer membrane is composed of a mixture of proteins including general porins (eg. OmpF in *E. coli* and OprF in *P. aeruginosa*) and specific porins (eg. scrY in *S. typhimurium*), enzymes like OmpT protease (Mangel *et al*., 1994) and phospholipase A (Dekker, 2000), protein export systems referred to as autotransporters (Pugsley, 1993), proteins involved in biogenesis of flagella and pilli (Macnab, 1999; Soto and Hultgren, 1999). Among these, porins are expressed at high levels and are believed to be the major membrane proteins of the outer leaflet (Koebnik *et al*., 2000). Thus, any model of membrane transport of hydrophilic peptides such as Ptm should consider the possibility of the involvement of porins.

5.2. Objectives

The main objective of the present study was to determine if there was conclusive experimental evidence that Ptm is actually internalised without cell lysis. Additional objectives were to monitor the morphological changes taking place when treated with various concentrations of Ptm. Immunogold labelling of Ptm followed by transmission electron microscopy (TEM) was used in order to examine the localisation and distribution of Ptm in *P. aeruginosa, E.coli and S. typhimurium.* TEM-immunolabelling of Ptm treated spheroplasts of *E.coli, S. typhimurium* and *P. aeruginosa* were also carried

out to understand relative importance of inner and outer membranes with regard to Ptm internalization.

5.3. Materials and Methods

5.3.1. Preparation of spheroplasts

P. aeruginosa. Overnight cultures of *P. aeruginosa* in tryptic soy broth (TSB) were harvested by centrifugation at 1000 *x* g for 10 min (Sorvall superspeed RC-2 automatic refrigerated centrifuge, Norwalk, CT, USA). The resulting pellet was re-suspended and washed twice with 20 mL of 35 mM Tris HCl at pH 8. Cells were harvested each time by centrifugation at 1000 *x* g for 10 min. The washed pellet was treated with 3 mM EDTA and 100 mg lysozyme per mL in the presence of 0.5 M KCl and 25 mM Tris-HCl at pH 8.0. After incubation at 37ºC for 20 min, the suspension was centrifuged at 1000 *x* g for 10 min and the pellet was gently suspended in 35 mM Tris-HCl with 10% sucrose. The efficacy of recovery of spheroplasts was judged subjectively by phase contrast microscopy at 1000-fold magnification.

E. coli* and *S. typhimurium. Overnight cultures of *E. coli* and *S. typhimurium* grown in TSB were used for the preparation of spheroplasts. Fifty mL of the overnight cultures were centrifuged at 1000 x g for 10 min and washed twice with 50 mM Tris-HCl, pH 8.0. The pellets were then resuspended in 2.5 mL of digestion buffer (10% sucrose solution in 50 mM Tris-HCl buffer, pH 8.0) and then incubated for 5 min in crushed ice. After the incubation, 0.5 mL and 0.6 mL of lysozyme was added (Sigma, St. Louis, Mo) (5.0 mg/mL in 50 mM Tris-HCl buffer, pH 8.0) for *E. coli* and *S. typhimurium*, respectively.

The suspension was then incubated for 5 min in crushed ice and then 1 mL of 25 mM potassium ethylene-diaminetetraacetic acid (EDTA, Sigma, St. Louis, Mo) in 50 mM Tris-HCl pH 8.0 was added and mixed. Incubation was continued for further 5 min while gently shaking. At this point, almost all the rod-shaped cells had been converted to spheroplasts as observed using phase contrast microscopy. The spheroplasts were then washed twice with a 10% sucrose solution in 50 mM Tris-HCl buffer (pH 8.0) and centrifuged at 2000 x g for 10 minutes to remove the outer cell envelope debris. The pellet was resuspended in the 10% sucrose solution in 50 mM Tris-HCl buffer (pH 8.0) for further use.

5.3.2. Ptm treatment

Exponential phase *P. aeruginosa* cells in TSB were treated with sub-inhibitory (500 ppm) and lethal (3000 ppm) concentrations of Ptm for 30 min at 37°C. Spheroplasts were treated with 100 ppm Ptm. A control was maintained in both the cases in which cells / spheroplasts were not exposed to Ptm.

5.3.3. Transmission Electron Microscopy

Ultrastructural studies without immunogold labelling. After the Ptm treatment, the cells were washed with excess 50 mM HEPES buffer (pH 8) to remove any unbound Ptm. The cells were harvested by centrifugation at 1000 *x* g for 5 min and the bacterial pellets fixed in ice-cold 0.1% (v/v) glutaraldehyde with 2% formaldehyde for 45 min using HEPES (pH 7.4) as fixation buffer. Fixed cells were further washed three times in HEPES buffer and enrobed in 2% Noble Agar tempered to 60ºC. These were later dehydrated in graduated ethanol series. The dehydrated blocks were infiltrated with LR White resin

(Marivac, Halifax, NS, Canada) and embedded in gelatine capsules which provided an oxygen-free environment for LR White polymerisation at 60ºC for one h. They were later cooled to room temperature.

One hundred nm thin sections were cut using an ultramicrotome (LKB-Huxley Ultramicrotome, LKB Instruments, Inc., Rockville, MD) equipped with a diamond knife. Thin sections obtained were placed on 300 mesh copper grids and stained with 2% uranyl acetate for 10 min followed with a distilled water wash. The sections were counter stained with 2% lead citrate (Reynolds, 1963) for 4 min and then rinsed with distilled water. The grids were then air dried before being viewed using a JEM 1230 transmission electron microscope at 80 kV (JEOL Ltd. Musashino, Akishima, Tokyo, Japan). Images were captured using a Hamamatsu ORCA-HR digital camera (Hamamatsu, Hamamatsu City, Japan).

5.3.4. Immunolelectron Microscopy

Anti-Ptm polyclonal antibodies produced in rabbits were obtained from Open Biosystems (Huntsville, AL) and were used in immunoelectron microscopy to understand Ptm localization. The polyclonal anti-Ptm serum was produced by immunizing two New Zealand white rabbits using Ptm sulfate salt from herring (Sigma-Aldrich, St. Louis, MO, USA), conjugated with keyhole limpet hemocyanin (KLH) and emulsified with Freund's complete adjuvant (Day 1). Control serum was collected before primary immunization (Day 0). After the primary immunization (0.50 mg antigen at 10 subcutaneous (SQ) sites), three booster doses (0.25mg of Ptm in 4 SQ sites) were administered on days 14,

42 and 56. Serum was collected on days 28, 56 and 70. Anti-Ptm antibodies were purified by affinity chromatography and the titre value assessed using indirect ELISA.

Localisation of Ptm in cells was carried out by immuno-labelling Ptm with 10 nm colloidal gold labelled antibodies on 100 nm thin sections on 300 mesh nickel grids. Sections were washed with 1mg/mL sodium borohydride, then with 30 mM glycine in 0.1 M borate buffer (pH 9.6). The sections were then incubated in a blocking solution consisting of TBS buffer with 1% skim milk and 1% BSA for 45 min then washed with TBS buffer consisting of 10mM Tris, 0.3 M NaCl and 0.1% bovine serum albumin (BSA) at pH 8.1. The sections were then exposed overnight to diluted primary antibody (1:100). The sections were washed three times with 10 mM Tris pH 8.1 with 0.3 M NaCl and 0.1% BSA to remove any unbound primary antibody. The sections on grids were later allowed to react with Anti-rabbit IgG (whole molecule) developed in goat conjugated with 10 nm colloidal gold (Sigma-Aldrich, St. Louis, MO, USA), then washed to remove any unbound secondary antibody. The specimens were fixed by floating the grids on drops of 2.5% (vol/vol) glutaraldehyde in PBS for 10 min (Garduno *et al*., 1998). The sections were again washed with distilled water before staining the grids by placing on drops of 2% aqueous uranyl acetate for 10 min then further washed with distilled water and counter stained with 2% lead citrate solution. Sections were blot dried before viewing. Control samples untreated with primary antibodies were maintained for each group of specimens.

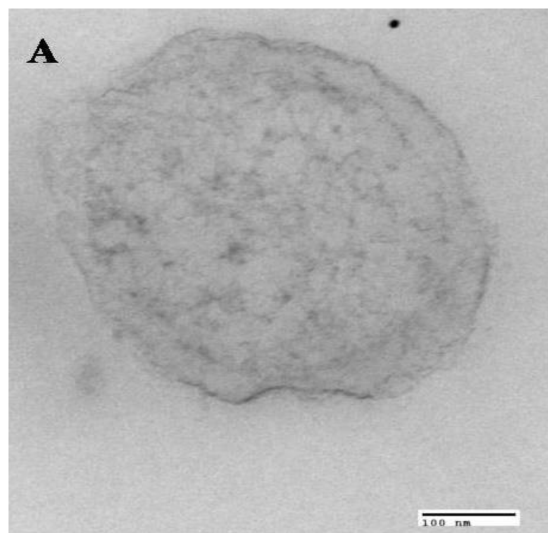
5.4. Results & Discussion

5.4.1. Transmission immuno-electron microscopy

Whole Cells. TEM images of *P. aeruginosa, E. coli and S. typhimurium* exposed to no Ptm, sub-inhibitory (500 ppm) and lethal concentrations (3000 ppm) of Ptm were compared. Cells receiving no Ptm exposure showed intact membranes more or less homogeneous distribution of cytoplasmic components and no sign of 10 nm immune-gold labels (Figs. 5.1 A, D and G).

Treatment for 30 min at sub-inhibitory concentrations (Figs. 5.1 B, E and H) showed that Ptm had internalized and there was clear evidence of partial condensation of the cytoplasmic material observed as electron dense areas within the central portion of the cytoplasm. The colloidal gold labels suggest that Ptm becomes bound to these condensed components, probably consisting of proteins and DNA. In these cells, there appeared to be no evidence of membrane disruption or blebbing.

After 30 min exposure to inhibitory (3000 ppm) concentrations of Ptm, there was still no evidence of morphological changes to the envelope or cell shape (Figs. 5.1 C, F and I). Many of the cells treated with such a high dose would have most certainly been dead or unable to respire normally. In no case did we observe complete membrane breakdown and streaming cytoplasm as reported by Johansen *et al.* (1996) for both Gram negatives and Gram positives. Furthermore, the immuno-gold labels showed that most of the Ptm had passed through both outer and inner membranes as well as the periplasm and completely internalized. Essentially none of the Ptm localized within the cell envelope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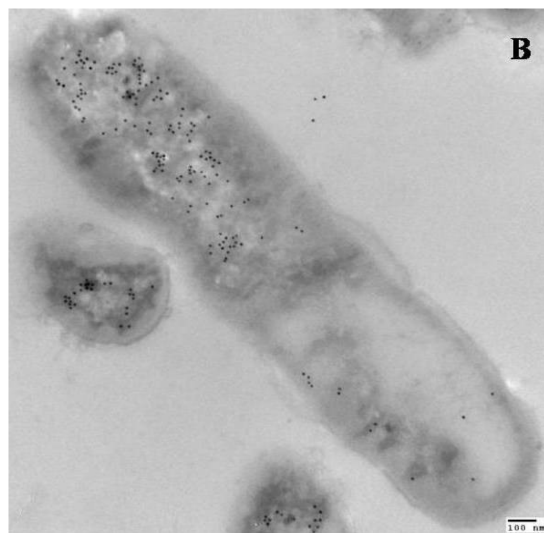
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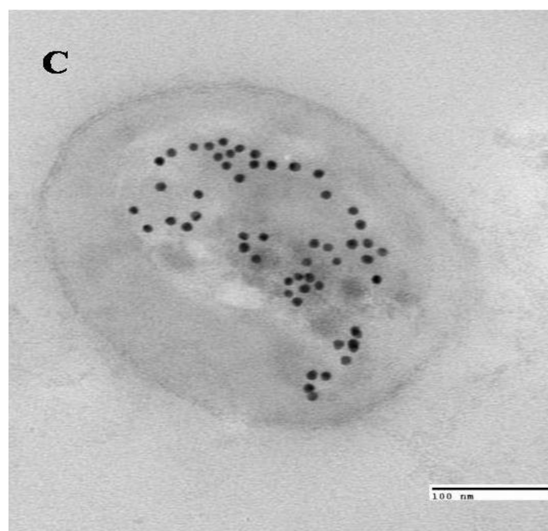
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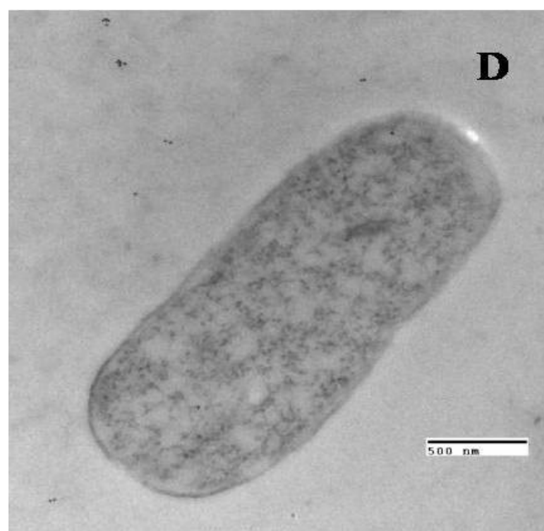
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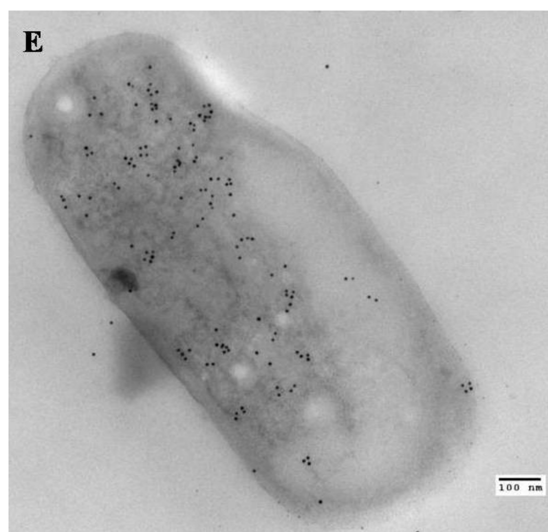
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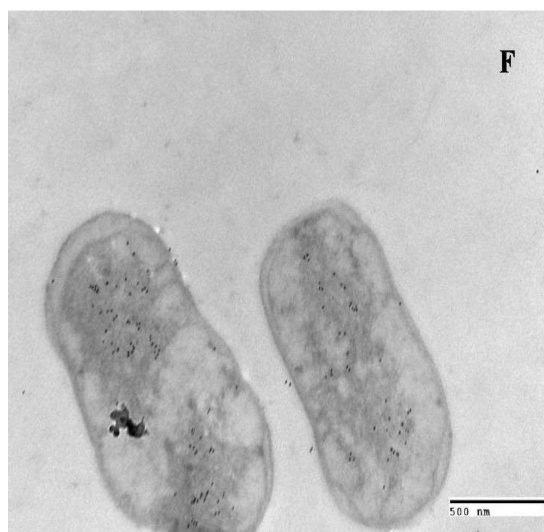
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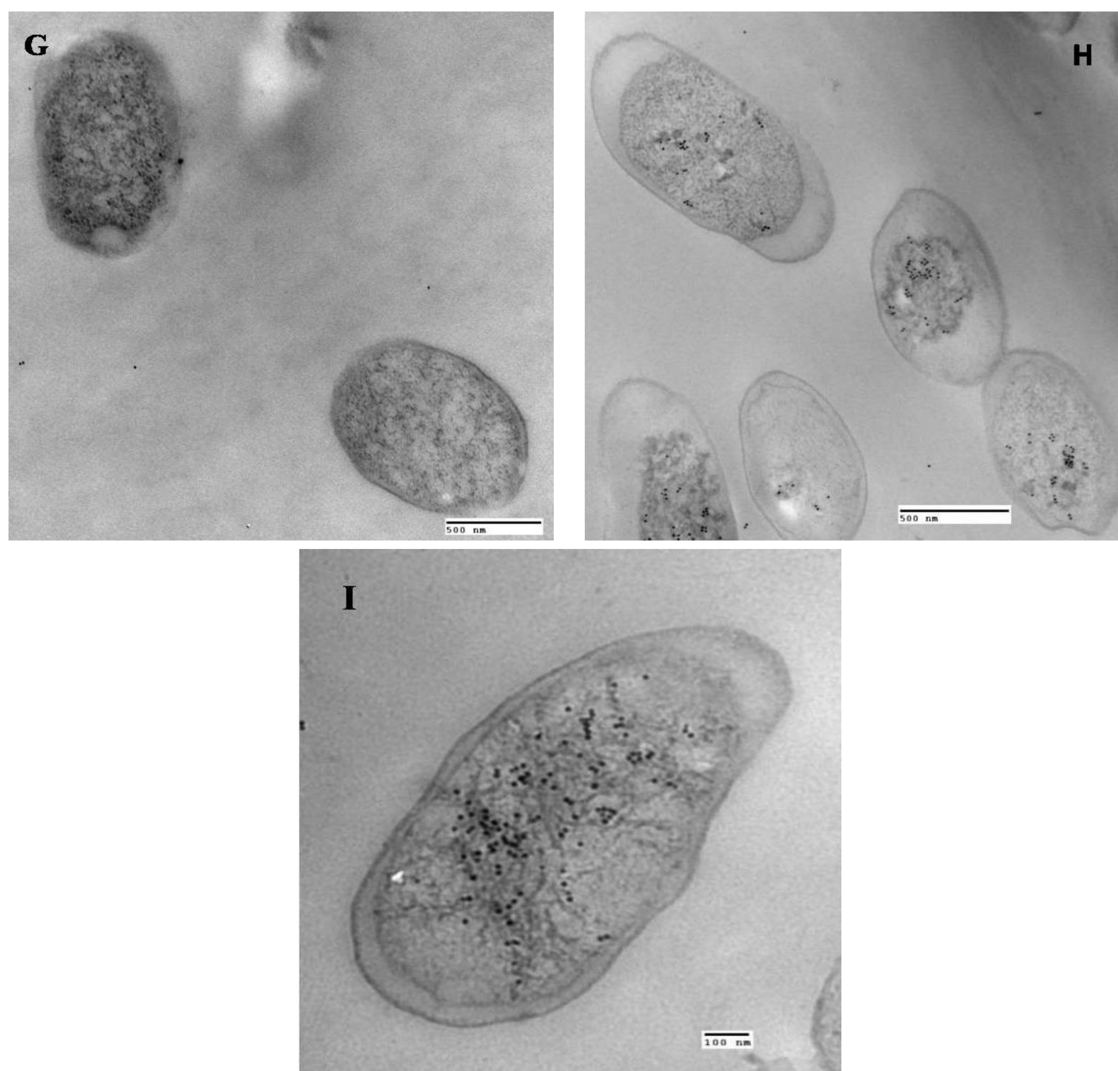

Fig. 5.1. TEM images of *P. aeruginosa, E. coli* and *S. typhimurium* cells treated with 0 ppm (A, D, G), 500 ppm (B, E, H) and 3000 ppm Ptm (C, F, I) respectively. Ptm is visualised with 10 nm gold labels. Cell lysis or membrane damage was not observed in any of the organisms. Ptm was found to be concentrated in the cytoplasm when the organisms were treated with Ptm (B, C, E, F, H and I).

constitutents. Like Ptm, PR-39 apparently kills Gram negative bacteria without cell lysis (Aspedon and Groisman, 1996). The present study clearly shows that at least in the case of *P. aeruginosa*, *E. coli* and *S. typhimurium,* cell lysis was never observed although it is possible that some perturbation or even transient poration of the outer envelope was possible as a result of treatment with Ptm. The Ptm MIC levels were ~2500 ppm Ptm for all three Gram negatives as determined by the Alamar Blue assay of Baker (1994).

According to an earlier study on *Salmonella* (Aspedon and Groisman, 1996), protein synthesis and amino acid uptake ceased within 20 minutes of Ptm action. Formation of ghost cells (cells with leaky cell membranes and as a result without cytoplasm) were reported in earlier studies when *E. coli* was treated with antimicrobial peptides like temporin L (Mangoni *et al*., 2004) and MDpep9 (Tang *et al*., 2009). Ghost cells were not observed when *E. coli*, *P. aeruginosa* or *S. typhimurium* were treated with lethal and sub-inhibitory concentrations of Ptm indicating that leakage of cytoplasmic components is not the mechanism involved in Ptm mediated cell killing. From the TEM images, it seems that the condensation of cytoplasmic components by Ptm may have precipitated the genetic materials thereby inhibiting vital intracellular processes like transcription and translation.

Spheroplasts. Spheroplasts of all the three organisms under study were treated with Ptm to understand the relative importance of inner and outer membranes with regard to Ptm internalization. Spheroplasts were clearly permeable to Ptm, indicating that even at sub-inhibitory concentrations, lysis of the cytoplasmic membrane does not occur (Figs. 5.2 B, D and F) when compared to the non-exposed control specimens (Figs. 5.2 A, C and E). When treated with Ptm, cytoplasmic constituents appeared to be electron dense. These observations are in good agreement with Tolong's (2004) phase contrast microscopic observations. Also in agreement was the presence of low density inclusions that were apparently transparent to the electron beam. This was also observed in the case of whole cells treated with Ptm. Brightening of cytoplasm was observed under

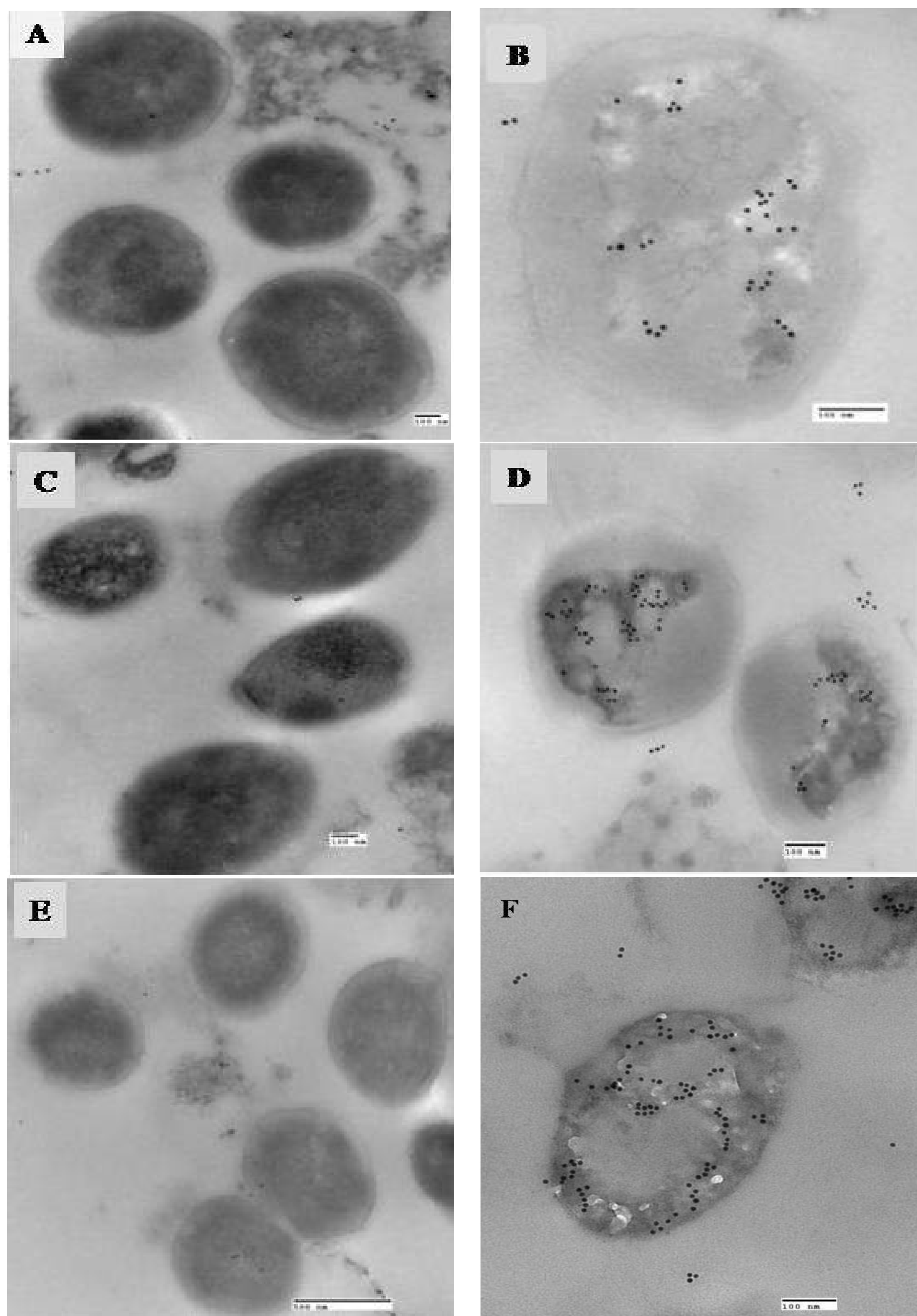

Fig. 5.2. TEM images of *P. aeruginosa, E. coli* and *S. typhimurium* spheroplasts treated with 0 ppm (A, C, E), and 100 ppm (B, D, F) Ptm respectively. Ptm is visualised with 10

nm gold labels. Lysis or membrane damage was not observed in any of the organisms. Ptm was found to be concentrated in the cytoplasm (B, D and F).

phase contrast microscope when spheroplasts of *E. coli* and *S. typhimurium* were treated with Ptm (Tolong, 2004).

5.5. Conclusions

The results obtained from this study with both spheroplasts and intact cells show that *P. aeruginosa, E. coli and S. typhimurium* could not be lysed by Ptm. Both inner and outer membranes of these three Gram negative bacteria were readily permeable to Ptm even at sub-inhibitory concentrations. Since computer models (Pink *et al*., 2003) of this phenomenon based upon lipid bilayers predict that at the very least, the outer membrane should be impermeable to Ptm, we conclude that other mechanisms are at work in the translocation process. It is also reasonable to conclude that membrane proteins are involved since these constituents were not present in the original computer models and it is known that bacterial membrane proteins represent 50% of the mass of the membrane. The present results also demonstrate that this peptide inactivates target bacteria with a mechanism essentially based on a non-lytic mechanism mediated by the translocation of the peptide into the cells, where it likely inhibits vital intracellular processes most probably related to the condensation of cytoplasmic material. This suggests the possibility of Ptm internalising through membrane channel proteins like porins.

Chapter 6.

Conclusion

In conclusion, experiments confirm that the resistance to Ptm observed in *P. aeruginosa* is at the very least, coincidental with the pleiotropic mutations involving loss of porins and or may even be related to porin channel size; however speculations on channel constriction in response to Ptm attack are beyond the scope of this thesis. From the results obtained it was quite evident that Ptm internalised both whole cells and spheroplasts without lysis and was found to be concentrated in the cytoplasm. Bulges were found on *P. aeruginosa* cell surfaces when treated with Ptm and seemed to be dependent on the concentration of the Ptm. The bulging observed on the cell surfaces may be the reason behind the increase in cell turbidity recorded on exposure to Ptm. This increase in cell turbidity was not observed when Ptm-Res *P. aeruginosa* cells were exposed. Immuno-electron microscopic images showed that Ptm localised on the outer envelope or possibly in the periplasmic space, indicating exclusion of the peptides from the cytoplasm. The mechanism for the induced resistance is currently not known, but the results indicate that the resistance mechanisms may be as diverse as that of the mode of action of Ptm.

A more detailed study is essential to understand the role of various outer membrane components in Ptm internalisation and resistance. Channel properties of OprF in *P. aeruginosa* and internalisation of Ptm through the cytoplasmic membrane needs to be studied. The possibility of using Ptm together with conventional antibiotics deserves detailed study as this may help in better treatment of bacterial infections. The potential of

using Ptm to transfer drug molecules, which are membrane impermeable, needs to be examined.

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