CLONING AND EXPRESSION OF *PSEUDOMONAS AERUGINOSA* MAJOR OUTER MEMBRANE PORIN PROTEIN F (OprF) AND SPECIFIC POLYCLONAL ANTIBODY PRODUCTION

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (MICROBIOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2013

Thesis
entitled

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was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Microbiology)
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ACKNOWLEDGEMENTS

The success of this thesis is able to accomplish by the attentive support from my major advisor, Asst. Prof. Chanwit Tribuddharat who gave me invaluable suggestions. I wish to express my sincere appreciation for his patient instruction, criticism, and encouragement continuously throughout my study.

My gratitude is also expressed to my co-advisor Prof. Somporn Srifuengfung, Assoc. Prof. Chertsak Dhiraputra, and Lecturer Dr. Iyarit Thaipisuttikul for useful comments and suggestions.

I would like to special acknowledge the assistance providing by Assoc. Prof. Pintip Pongpech and Asst. Prof. Penphun Naenna from Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

I would like to express my appreciation to Lecturer Dr. Duangjit Kanistanon from Department of Immunology and Prof. Pilaipan Puthavatana from Department of Microbiology, Faculty of Medicine Siriraj Hospital for their kind support.

I would like to express my sincere thanks to Dr. Pirom Noisumdaeng, Mr. Chaiyaporn Chaisatit, Mr. Don Changsom and Miss Areeya Disratthakit for providing me with their help and guidance.

Most of all, my deepest gratitude goes to my family for their understanding and forever encouragement.

I also thank all persons who have directly or indirectly helped me in completing my project.

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ABSTRACT

Carbapenems are effective antibiotics for the treatment of infections caused by multidrug-resistant (MDR) *Pseudomonas aeruginosa*. However, resistance to these antibiotics, such as imipenem, has increased steadily in recent years. The major mechanism of imipenem resistance in *P. aeruginosa* is a loss of OprD protein on the outer membrane that usually forms specific channels for uptake of basic amino acids and imipenem into the cells. From OprD-mediated resistance, an OprD detection technique was developed for imipenem susceptibility testing. Species confirmation of the pathogen should be considered as an internal control of the immunological test, since this resistance mechanism may be shared also by other pseudomonads. In this study, a major outer membrane porin protein F (OprF) is interesting in view of its species specificity for *P. aeruginosa*. The whole *oprF* gene of *P. aeruginosa* PA10 clinical strain was cloned into pET-28b plasmid and expressed for a $(His)_6$-tagged OprF fusion protein in *E. coli* BL21. The fusion protein in form of an insoluble inclusion body was purified by HisTrap HP column chromatography, confirmed its identity by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry, and prepared for specific polyclonal antibody production in a New Zealand White rabbit. The anti-OprF polyclonal antibody was used to determine the specificity in Western blot analysis, and it showed a specific band of OprF protein from wild type *P. aeruginosa* and 6 clinical isolates. It showed no cross-reaction in other *Pseudomonas* species, such as *P. alcaligenes*, *P. fluorescens*, *P. mendocina*, *P. putida*, *P. stutzeri*, and *P. syringae*. There was no specific banding pattern in *E. coli* ATCC 25922 and BL21. These results suggested that OprF protein may be used in characterization of *P. aeruginosa* at the species level.

KEY WORDS: OprD / OprF / $(HIS)_6$-TAGGED OprF / MALDI-TOF

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การโคลนนิ่งและการสร้าง MAJOR OUTER MEMBRANE PORIN PROTEIN F (OprF) ของเชื้อ *PSEUDOMONAS AERUGINOSA* และการผลิต POLYCLONAL ANTIBODY ที่จำเพาะ

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บทคัดย่อ

ยากลุ่ม carbapenem นับเป็นยาต้านจุลชีพที่มีประสิทธิภาพสำหรับการรักษาการติดเชื้อ *Pseudomonas aeruginosa* ที่ดื้อยาหลายขนาน แต่ในปัจจุบันพบว่าเชื้อดื้อต่อยาในกลุ่มนี้เช่น ยา imipenem เพิ่มขึ้นอย่างต่อเนื่อง โดยกลไกหลักที่เชื้อใช้ในการดื้อยา imipenem คือ การสูญหายของโปรตีน OprD บนเยื่อหุ้มเซลล์ชั้นนอก ซึ่งปกติมีหน้าที่ในการนำกรดอะมิโนที่เป็นเบสเข้าสู่เซลล์และยังเป็นช่องทางสำหรับนำเข้ายา imipenem จากความรู้เรื่องการดื้อยาจากการเปลี่ยนแปลงของโปรตีน OprD ได้มีการนำเทคนิคการตรวจหาโปรตีน OprD มาใช้กับชุดตรวจหาความไวต่อยา imipenem ของเชื้อ *P. aeruginosa* ซึ่งในอนาคตจะมีการพัฒนาต่อไป แต่การพิสูจน์ยืนยันเชื้อ *P. aeruginosa* นับเป็นเรื่องสำคัญในแง่ของการเป็น internal control ของชุดทดสอบ เนื่องจากกลไกการดื้อยานี้อาจพบได้ในเชื้อ pseudomonads อื่นๆ ในการทดลองนี้จึงได้ศึกษาโปรตีน OprF ซึ่งเป็นโปรตีนส่วนใหญ่บนเยื่อหุ้มเซลล์ชั้นนอกของเชื้อ *P. aeruginosa* ในแง่ของความจำเพาะ เพื่อบอกว่าเชื้อที่ทดสอบเป็นเชื้อ *P. aeruginosa* หรือไม่ การทดลองเริ่มจากการนำยีน *oprF* ของเชื้อ *P. aeruginosa* PA10 เข้า pET-28b expression vector และทำให้เกิดการสร้าง $(His)_6$-tagged OprF ใน *E. coli* BL21 โปรตีนนี้อยู่ในรูป inclusion body และถูกนำมาทำให้บริสุทธิ์โดยคอลัมน์โครมาโตกราฟฟี HisTrap HP หลังจากนั้นทำการวิเคราะห์โปรตีนเพื่อยืนยันโดย MALDI-TOF mass spectrometry ก่อนจะนำไปเตรียมเพื่อทำการผลิต polyclonal antibody จากกระต่าย ซึ่งแอนติบอดี้ที่ได้จะมีความจำเพาะต่อโปรตีน OprF ในการวิเคราะห์หาความจำเพาะของโปรตีน OprF ต่อเชื้อ *P. aeruginosa* ใช้วิธีวิเคราะห์โดย Western blot ซึ่งผลการทดลองพบว่า โปรตีน OprF มีความจำเพาะต่อเชื้อ *P. aeruginosa* ไม่พบ cross-reaction กับเชื้อ *Pseudomonas* สปีชีส์อื่น ได้แก่ *P. alcaligenes*, *P. fluorescens*, *P. mendocina*, *P. putida*, *P. stutzeri*, *P. syringae* และไม่พบว่ามีความจำเพาะต่อโปรตีนจาก *E. coli* BL21 และ *E. coli* ATCC 25922.

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LIST OF ABBREVIATIONS

Abbreviation	Term
ATCC	American Type Culture Collection
DMST	Culture Collection for Medical Microorganism Department of Medical Sciences Thailand
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
PCR	Polymerase chain reaction
bp	Base pair
MW	Molecular Weight
kDa	Kilodalton
ºC	Degree Celsius
×g	Gravitational force
mM	Millimolar (10^{-3} molar (M); M: mole/liter)
µm	Micrometer (10^{-6} meter)
µl	Microliter (10^{-6} liter)
ng	Nanogram (10^{-9} gram)
Abs600	Absorbance at 600 nm
v/v	Volume/volume
w/v	Weight/volume
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
IPTG	Isopropyl-β-D-thiogalactopyranoside
A	Adenine
T	Thymine
C	Cytosine
G	Guanine

LIST OF ABBREVIATIONS (cont.)

Abbreviation	Term
Ala/A	Alanine
Arg/R	Arginine
Asn/N	Asparagine
Asp/D	Aspartic acid (Aspartate)
Cys/C	Cysteine
Gln/Q	Glutamine
Glu/E	Glutamic acid (Glutamate)
Gly/G	Glycine
His/H	Histidine
Ile/I	Isoleucine
Leu/L	Leucine
Lys/K	Lysine
Met/M	Methionine
Phe/F	Phenylalanine
Pro/P	Proline
Ser/S	Serine
Thr/T	Threonine
Trp/W	Tryptophan
Tyr/Y	Tyrosine
Val/V	Valine

CHAPTER I
INTRODUCTION

Pseudomonas aeruginosa is considered an opportunistic pathogen reported to be a major cause of nosocomial infections, such as chronic lung infections in cystic fibrosis (CF) patients, urinary tract infections, bacteremia, and wound infections (1, 2). Resistance to multiple antimicrobials in Gram-negative bacilli has become a worrisome issue that renders increased mortality and increased hospital length of stay (3). Multidrug-resistant (MDR) *P. aeruginosa* has been increasing worldwide, and been a clinical problem due to limited therapeutic options. Intrinsic antibiotic resistance accompanied with acquisition of resistance genes on mobile genetic elements encoding antibiotic-hydrolyzing enzymes is a reason for a variety of antibiotic resistances in this pathogen (4).

Carbapenems are effective antibiotics for treatment of the infections caused by MDR *P. aeruginosa* that remains susceptible to carbapenems. In the absence of carbapenem-hydrolyzing enzymes, the main mechanism for imipenem resistance is a loss of outer membrane protein OprD, whereas the main mechanism for meropenem resistance is from overexpression of efflux pumps together with the loss of OprD (5, 6). MexAB-OprM is associated with meropenem resistance but not actually imipenem resistance. The other types of efflux pump, such as MexXY-OprM and MexCD-OprJ may also affect to meropenem resistance (7). Moreover, MexAB-OprM relates with resistance to fluoroquinolones, tetracycline, chloramphenicol, macrolides, and most β-lactams in this organism (8). The interplay between endogenous efflux genes and the low outer membrane permeability of *P. aeruginosa* contributes to the intrinsic resistance (9). Although efflux systems are considered to play role in low-to-moderate level of resistance, they are able to limit available antibiotics and cooperate with other mechanisms allowing higher levels of resistance (10).

OprD, an outer membrane porin protein of *P. aeruginosa*, has a role in an uptake of basic amino acids, some small peptides, and certain carbapenems. The structure of OprD contains 18-stranded transmembrane β-barrel that forms an entrance of imipenem (11). The decreased OprD production caused by mutation in *oprD* gene or reduction in protein translation implicates resistance to imipenem in this organism (12). The study of Pirom Noisumdaeng et al. found that 98% (54 of 55 isolates) of imipenem-and meropenem-resistant *P. aeruginosa* clinical isolates did not produce OprD. It is expected that the strains which produce OprD should be susceptible to imipenem (13). Other studies supported that the absence or weak expression of OprD was predominant in imipenem-resistant *P. aeruginosa* isolates in Thailand and other countries such as France and Spain (6, 14, 15). However, an acquired high level carbapenem resistance should be considered since metallo-β-lactamases (IMP, VIM, SPM, GIM types), and the most notable carbapenem-hydrolyzing enzymes, can be transmitted through plasmids or transposons associated with integron element (16).

From the knowledge in OprD-mediated resistance, OprD detection technique was developed for imipenem susceptibility testing. Species confirmation of *P. aeruginosa* is important, since this specific resistance mechanism although belongs to *P. aeruginosa*, there may be shared by other pseudomonads. In this study, OprF, a species specific major outer membrane porin protein of *P. aeruginosa*, was investigated. OprF serves as a porin channel and a structural protein also. As a structural protein, OprF maintains cell shape and enables cells to grow in low-osmolarity medium. For another role of this protein, it forms small water-filled channel through the outer membrane (17, 18). The porin channels allow slow diffusion of nonspecific molecules such as oligosaccharides. The structure of OprF is divided into two conformers, a majority is two-domain closed-channel conformer and a minority is one-domain open-channel conformer that exists <5% of the protein population. The closed-channel majority conformer causes a higher level of antibiotic resistance owing to the difficulty to penetrate into the bacterial cells. This two-domain protein consists of the N-terminal half folding into an eight-stranded β-barrel spanning the outer membrane bilayer and the C-terminal half folding into a globular domain located in the periplasm (19, 20).

OprF and the orthologous *E. coli* outer membrane protein (OmpA) share only 15% amino acid identity in N-terminal domain (21). The outer membrane proteins of Gram negative bacteria are available for recognition by antibodies. In 1996, the study of L. Kragelund et al. indicated that polyclonal antibody specific to a 37-kDa OprF-like protein of *P. fluorescens* DF57 reacted with OprF proteins from members of *Pseudomonas* rRNA homology group I (including *P. aeruginosa*) but not with the proteins from non-pseudomonads (22). OprF is an attractive target for diagnosis of *P. aeruginosa* infection. Study of G.W. Counts et al., 1988, an immunofluorescent-antibody test was developed for rapid detection of *P. aeruginosa* in blood cultures. Anti-OprF monoclonal antibody used in that study was specific to *P. aeruginosa*, but not other *Pseudomonas* sp. or other Gram-negative (23).

As described above, the specificity of OprF protein to *P. aeruginosa* was evaluated by Western blot analysis. Anti-OprF polyclonal antibody was used to detected OprF from *P. aeruginosa* wide type and clinical isolates, and other pseudomonads (*P. alcaligenes*, *P. fluorescens*, *P. mendocina*, *P. putida*, *P. stutzeri*, and *P. syringae*), and *E. coli*. From this study, the information may be applied in a production of an immunological test using combination detections of *P. aeruginosa*. The OprF and OprD proteins for the test of imipenem susceptibility of MDR *P. aeruginosa* obtained from critical infections that need rapid turnaround time of species identification and antibiotic susceptibility result.

CHAPTER II
OBJECTIVES

1. To clone and express the major outer membrane porin protein F (OprF) of *Pseudomonas aeruginosa*.
2. To produce anti-OprF polyclonal antibody from a New Zealand White rabbit. The anti-OprF polyclonal antibody will be used further for determining *P. aeruginosa* in Thailand and may be developed to be an immunological test combining with imipenem susceptibility testing.
3. To evaluate specificity of OprF protein for *P. aeruginosa* and other related species, by Western blot hybridization. The anti-OprF polyclonal antibody was used to determine specific binding between the antibody and the OprF proteins from *P. aeruginosa* wild type and clinical isolates, and other related Gram-negative bacteria.

CHAPTER III
LITERATURE REVIEW

Members of the genus *Pseudomonas* (also called pseudomonads) containing over 100 species have been characterized in a group of aerobic, non-fermentative, Gram-negative bacilli. They are ubiquitous organisms found in soil, water, decaying organic matter, and others moist environments. Most of *Pseudomonas* spp. is saprophytes but some are pathogenic for plants, insects, and animals. The ability to survive including their simple growth requirements or nutritional versatility is a key factor that pseudomonads, particularly *P. aeruginosa*, become a major problem in hospitals. *P. aeruginosa* has been found in a variety of aqueous solutions and surfaces in the hospital such as disinfectants, irrigation fluids, dialysis fluids, medical equipments, sinks, and toilets. There is an uncommon colonization of *P. aeruginosa* in healthy persons that gastrointestinal tract is the most frequent site of colonization. Otherwise, it can be found in, throat, nasal mucosa, and moist skin surfaces such as the axillae and perineum. Patients who have been hospitalized for extended time and/or have received a broad-spectrum antimicrobial therapy or chemotherapy have been found at higher rate of colonization and especially in the respiratory tract. Among the species of *Pseudomonas, P. aeruginosa* is the most clinically significant associated with human diseases, since it is a major cause of nosocomial infections and also occasionally causes community-acquired infections. Several other species such as *P. putida*, *P. fluorescens*, and *P. stutzeri* are occasionally isolated from human clinical specimens as opportunistic pathogens (24-26).

3.1 Morphology and identification of *P. aeruginosa*

P. aeruginosa is a motile and rod-shaped, measuring about 0.6 × 2 µm. It is non-fermenting Gram-negative, and sometimes produces grape-like odor. The bacterium is described as an aerobe, but it can utilize nitrate as a terminal electron acceptor that associated with the biofilm production in the absence of oxygen. Most isolates of *P. aeruginosa* exhibit β-hemolytic and flat spreading colonies with metallic sheen on blood agar. It often produces the yellow-green fluorescent pigment pyoverdin and the nonfluorescent bluish pigment pyocyanin. A few strains produce pyorubrin (red) or pyomelanin (dark brown). The water-soluble, diffusible pigment pyocyanin is produced by *P. aeruginosa*, however about 4% of clinical strains do not show this pigment. No other fluorescent *Pseudomonas* (i.e., *P. fluorescens* and *P. putida*) and including non-fermentative Gram-negative bacilli produce pyocyanin. In addition, some mucoid strains of *P. aeruginosa* from cystic fibrosis patients may not produce this pigment. Besides pyocyanin, the culturing temperature at 42 ºC is helpful in differentiation of *P. aeruginosa* from other pseudomonads in the fluorescent group. *P. aeruginosa* is oxidase positive and unable to utilize lactose (27-32).

Table 3.1 Crucial characteristics of the fluorescent pseudomonad

Test	*P. aeruginosa*	*P. fluorescens*	*P. putida*
Pyoverdin	+	+	+
Pyocyanin	+	-	-
Growth at 42 ºC	+	-	-
NO_3 reduction	V (74%)	V (19%)	-
Acetamide	V	-	-
Gelatin hydrolysis	V (46%)	+	-

+, ≥90% of strains positive; -, ≥90% of strains negative; V, 11-89% of strains positive Data from reference (27)

3.2 Virulence factors of *P. aeruginosa*

P. aeruginosa possesses many virulence factors comprising of structural components, enzymes, and toxins that drive effective colonization and infection, and make the organism to be the clinically significant bacteria among non-fermenters (27).

3.2.1 Pili and nonpilus adhesins

For bacterial cell surface virulence factors, pili and nonpilus adhesins such as carbohydrate-binding proteins (lectins) play a role in adherence to host cells. Pili are crucial colonization factors involved in binding to epithelial cells. This organism also produces neuraminidase (sialidase), an enzyme that cleaves α-2, 3-linked sialic acids from sialylated gangliosides, thereby receiving asialo-gangliosides M1 (asialo-GM1). The asialo-GM1 is a main pili receptor, thus enhancing attachment to the respiratory tract (24, 33).

3.2.2 Alginate and biofilm

Alginate, a mucoid exopolysaccharide of *P. aeruginosa*, is a repeating polymer composed of mannuronic acid and glucuronic acid. It is a matrix of biofilm that functions in adhesion facilitation to epithelial cells and tracheobronchial mucin. Biofilm formation, which has commonly found in cystic fibrosis (CF) patients enables the bacteria to evade from lymphocytes, phagocytosis, and ciliary action of the respiratory tract. Otherwise, this, biofilm can protect the bacteria from activities of disinfectants and antibiotics such as aminoglycosides. It obstructs penetration of antibiotics, therefore the community of the bacteria in the biofilm possess distinct antibiotic resistance mechanisms and other various antimicrobial resistance mechanisms (33, 34).

3.2.3 LPS

Lipopolysaccharide (LPS) is a hydrophobic domain of most Gram-negative bacteria that employs endotoxin activity. The LPS structure is compose of lipid A, core polysaccharide, and O-specific polysaccharide. The lipid A stimulates many pathways of pro-inflammation. The development of the bacteria during acute phase to chronic phase shows a modification of acylation pattern that renders the

lower immunogenicity. In cystic fibrosis patients, lipid A modification due to mutation affects resistance to host antimicrobial peptides (34, 35).

3.2.4 Pyocyanin

For extracellular virulence factor, pyocyanin is a blue pigment produced by *P. aeruginosa* associated with invasion through production of hydrogen peroxide and superoxide to damage tissue. This pigment also increases interleukin-8 (IL-8) and induces apoptosis in neutrophils. CF patients with chronic lung infection were found to have high concentration of pyocyanin in their sputa, suggested that it is a significant factor to damage pulmonary tissue (24, 33, 34).

3.2.5 ExoA toxin

This unique toxin, exotoxin A (ExoA), is one of the most considerable virulence factors found in pathogenic strains of *P. aeruginosa*. It inhibits protein synthesis of eukaryotic cells through blocking peptide chain elongation, thereby leading to cell death. ExoA elicits tissue damage such as dermatonecrosis in burn wounds, and corneal damage in ocular infections (24, 34).

3.2.6 ExoS, ExoT, ExoY, and ExoU toxins

Type III secretion system (TTSS) plays a role in translocation of extracellular toxins such as ExoS, ExoT, ExoY, and ExoU to host cells. Exotoxin S (ExoS) interferes eukaryotic DNA synthesis, causes cytotoxicity, and disrupts actin cytoskeletal. It has functions similar to ExoA, but doesn’t play a key role in cytotoxicity. ExoT inhibits wound healing. ExoY is an adenylate cyclase, which increases intracellular cAMP levels of host cells, leading to increase intercellular gap formation of pulmonary microvascular cells and increase lung permeability. The fourth type III secretion toxin, ExoU, is the major toxin secreted via the TTSS. The toxin has more virulence (about 100-time) than ExoS in its cytotoxicity and is implicated in severe cases (33, 34).

3.2.7 Elastases

Elastases are also major virulence factors of *P. aeruginosa* in acute infection, since elastin is a component of lung tissue and blood vessels. LasA (serine protease) and LasB (metalloprotease) elastases work synergistically to destroy elastin. The enzymes are capable of inhibiting neutrophil chemotaxis and degrading complement components, resulting in dissemination of the pathogen at acute phase of infection (24, 33).

Alkaline Protease is a fibrin-degrading enzyme that is transported out of the bacterial cells by type I secretion system. The combined activation of alkaline protease and LasB inactivates the human cytokines gamma-interferon and tumor necrosis factor alpha (33, 34).

3.2.8 Phospholipase C and rhamnolipid

Phospholipase C, a heat-labile hemolysin, degrades eukaryotic membrane phospholipids. It is implicated in acute lung injury and inflammation by *P. aeruginosa*. Other hemolysin, rhamnolipid, is a heat-stable hemolysin, which damages lecithin-containing tissues. It is also implicated in inhibition of pulmonary ciliary activity (24, 34).

3.3 Pathogenesis of *P. aeruginosa* infections

The pathogenesis of *P. aeruginosa* infections is complicate to exactly indicate due to numbers and dissimilarity of virulence determinants held by the bacterium. The various virulence determinant compositions are suggested to be associated with particular diseases. The general pathogenesis of *P. aeruginosa* allows three stages to accomplish the infections, comprising of (1) bacterial adherence and colonization, (2) local invasion, (3) disseminated systemic disease (2). Pili are responsible for adherence toward epithelial surface that also play a critical role in initial colonization. Four known exotoxins: ExoS, ExoT, ExoY, and ExoU secreted via type III secretion system show a major role in cytotoxicity allowing invasion and

dissemination of *P. aeruginosa*. Biofilm participates in bacterial protection from host defenses (34).

3.4 Antimicrobial drugs resistance

Compared with most other Gram-negative bacteria, *P. aeruginosa* exhibits high intrinsic resistance that the important mechanism belongs to low permeability of its outer membrane (37). Several antibiotics including many β-lactams, tetracyclines, macrolides, co-trimoxazole (trimethoprim/sulfamethoxazole), and most fluoroquinolones are involved in the intrinsic resistance of *P. aeruginosa* (38). Its high intrinsic resistance supports secondary adaptive resistance mechanisms including efflux pump overexpression and enzymatic inactivation of antibiotics (e.g. β-lactamase). Besides these resistances, *P. aeruginosa* has a remarkable ability to acquire additional resistance mechanisms enabling it often resistant to many antibiotics (37). The following antipseudomonal drugs: β-lactams, aminoglycosides and fluoroquinolones are the most frequently used for treatment the infections (39).

3.4.1 Mechanisms of resistance to β-lactams

In *P. aeruginosa* many mechanisms are implicated in β-lactam antibiotics resistance such as β-lactamase production, efflux system, impermeability, and target alteration (PBPs). These mechanisms may work simultaneously or with different combinations (39).

β-lactamases are greatly importance to resistance in Gram-negative bacteria including *P. aeruginosa* since they cleave the amide bond of β-lactam ring and lead to antimicrobial ineffective. β-lactamases were classified into four groups (A to D) by Ambler classification: class A, C, and D (active site serine) and class B (Zn^{2+} requirement) (40).

With regards to endogenous β-lactamases, *P. aeruginosa* carries an inducible chromosome-encoded AmpC β-lactamase (cephalosporinase) which belongs to class C β-lactamase (Ambler classification). Normally, the enzyme is produced in low basal level resulting in the bacteria still susceptible to third generation

cephalosporins. β-lactamase inhibitors such as clavulanic acid, sulbactam cannot inhibit this enzyme. AmpC β-lactamase can be induced by exposure to β-lactams especially imipenem. In addition, AmpC overproduction combined with additional mechanisms including loss of OprD, efflux pump overexpression, and/or production of carbapenemase plays a role in resistance to carbapenems. In *P. aeruginosa* extended-spectrum AmpC possessing amino acids alteration near their active sites confers reduced susceptibility to cefepime, ceftazidime, and imipenem via increased hydrolytic activity (38, 39, 41, 42).

With regards to acquired β-lactamases, *P. aeruginosa* is able to produce class A carbenicillin hydrolyzing β-lactamases: PSE-1 (CARB-2), PSE-4 (CARB-1), CARB-3, and CARB-4 which these carbenicillinase do not inactivate ceftazidime and carbapenems. Unlike carbenicillinase, class A extended-spectrum β-lactamase (class A ESBL) which renders resistance not only to carboxypenicillins and ureidopenicillins, but also to expended-spectrum cephalosporins and aztreonam has been reported in this pathogen. In addition to TEM and SHV types that are also well known in *Enterobacteriaceae*, PER (mostly in Turkish clinical isolates), VEB (France, Bulgaria, and Southeast Asia), GES/IBC (France, Greece, and South Africa), and BEL types (Belgium) have been characterized in *P. aeruginosa*. The TEM- and SHV-types ESBLs in this species are likely originated from *Enterobacteriaceae* and then were transmitted by gene transfer (39, 43). SHV variants including SHV-2a, SHV-5, SHV-12, and recently SHV-1 were reported in *P. aeruginosa* (39, 44, 45). TEM ESBLs found in *P. aeruginosa* (e.g. TEM-4, TEM-21, TEM-24, TEM-42, and TEM-116) are similar to classical ESBLs in *Enterobacteriaceae* that hydrolyze narrow-spectrum penicillins, extended-spectrum cephalosporins, and aztreonam (39, 43). VEB-1 is another ESBL found in *P. aeruginosa*. In 1998, the first isolation of this β-lactamase was in France. Later, a high prevalence of $bla_{\text{VEB-1}}$-containing class I integron in ceftazidime-resistant nosocomial *P. aeruginosa* isolates occurred in the University Hospital in Thailand. VEB-1a, VEB-1b, and VEB-2 were also reported in *P. aeruginosa* (46). PER-1 was first characterized in *P. aeruginosa* and then subsequently found in *Acinetobacter* spp. (47). GES-1, GES-2, GES-5, GES-9, and IBC-2 (GES-8) have been described in *P. aeruginosa* (39). The GES-2 and GES-5 also demonstrate catalytic activity against carbapenems (40). BEL-1, a novel ESBL

was identified in *P. aeruginosa* from a hospital in Belgium. The BEL-1 activity was inhibited by clavulanic acid, tazobactam, cefoxitin, and imipenem. This chromosome-encoded ESBL gene was also located in class I integron (48).

Regarding class D β-lactamase (oxacillinases), the classical OXA enzymes such as OXA-1, OXA-2, and OXA-10 hydrolyze carboxypenicillins and ureidopenicillins but not ceftazidime. However, several derivatives serve as ESBL and some exhibit carbapenem-hydrolysing activities (39). Many extended-spectrum oxacillinases have been identified in *P. aeruginosa* clinical isolates such as OXA-17, OXA-18, OXA-141, and OXA-145 (49-52). OXA-type carbapenemase, OXA-50, are suspected to be naturally occurring enzyme in this organism (53, 54).

Another enzymatic resistance, class B metallo-β-lactamases (MBLs) are considered to be clinically significant carbapenemases. The carbapenem-hydrolysing enzymes can degrade all of β-lactams except monobactams. MBLs are not inhibited by clavulanic acid or tazobactam. In addition, bivalent ionic chelators such as EDTA can suppress the enzymes. IMP, VIM, SPM, and GIM-type are recognized in *P. aeruginosa* (16, 39). In the carbapenems-resistant bacteria including *P. aeruginosa*, IMP and VIM enzymes are predominant MBLs (40). IMP variants including IMP-1, IMP-7, IMP-9, IMP-13, IMP-16, IMP-18, and recently IMP-6 were identified in *P. aeruginosa* (39, 55). VIM-1 (Verona imipenemase) was first characterized from nosocomial *P. aeruginosa* strain at Verona University Hospital, Italy in 1997 (16). VIM-family occurred mostly in *P. aeruginosa* (e.g. VIM-1 to VIM-5, VIM-7, VIM-8, VIM-11, VIM-13, VIM-15, and VIM-16) (39, 56). SPM-1 has been appeared predominantly in Brazil which is an origin of this MBL (40). In 2002, GIM-1, a new MBL, was first identified from multidrug-resistant *P. aeruginosa* isolates in Germany (39). Recently, it was reported in *Serratia marcescens* and *Enterobacter cloacae* isolates from Germany (57).

In addition to β-lactamases production, altered outer membrane permeability is an important resistance mechanism. Alteration in outer membrane permeability in β-lactams resistance is specific for imipenem. OprD is an outer membrane protein that promotes the entry of basic amino acids and carbapenems (imipenem and meropenem). OprD deficiency has been found in imipenem-resistant *P. aeruginosa* clinical isolates (39, 58).

In *P. aeruginosa*, target alteration is an uncommon mechanism of resistance to β-lactams. Modification in penicillin-binding proteins (PBPs) as an instance of overproduced PBP-3s showed decrease susceptibility to β-lactams in *P. aeruginosa* strains (39).

3.4.2 Mechanisms of resistance to aminoglycosides

Aminoglycoside resistance consists of many mechanisms such as production of modifying enzymes, impermeability, efflux system, and target modification. Aminoglycosides inactivation though their modification have shown as a major mechanism in resistant *P. aeruginosa*. There are three modifying enzymes that are classified into aminoglycoside phosphoryltransferase (APH), aminoglycoside acetyltransferase (AAC), and aminoglycoside adenyltransferase (AAN). The enzymes carry phosphate, acetyl, or adenyl radical to the drug molecule, thereby decreasing affinity between the antibiotic and the bacterial ribosome target. Aminoglycoside-modifying enzymes have been found frequently encoded by mobile genetic elements that also contain other resistance determinants.

Impermeability has been found sometimes in aminoglycoside resistance that is characterized for all aminoglycosides resistance. Reduced outer membrane permeability has been appeared as a most common resistance mechanism in CF patients.

Active efflux is relatively uncommon in this drug resistance mechanism. MexXY-OprM is an effective system and another outer membrane proteins such as OprG, OprH, or OprI are suggested to be involved (39, 59).

3.4.3 Mechanisms of resistance to fluoroquinolones

There are two major fluoroquinolone resistance mechanisms in *P. aeruginosa*: target-site alterations and efflux-mediated resistance. As an antimicrobial agent, fluoroquinolones inhibit DNA synthesis by blocking two essential bacterial enzymes: DNA gyrase (also known as topoisomerase II) which is considered as a major target of Gram-negative bacteria, and the homologous topoisomerase IV. These bacterial enzymes are tetrameric with pairs of two different subunits. Effective mutations in *gyrA* and/or *gyrB* resulting in GyrA and/or GyrB subunits alteration are

causative of low binding affinity between fluoroquinolones and the modified DNA gyrase. As same as mutations in *parC* and/or *parE* may lead to deficient binding between the drugs and topoisomerse IV (39, 60).

Efflux pump systems encourage high-level fluoroquinolones resistance in *P. aeruginosa* by interplaying with target modification in DNA gyrase and topoisomerase IV. MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM, and a new identified MexVW-OprM have been shown to transport fluoroquinolones out of the cells (61).

3.5 Epidemiology of *P. aeruginosa*

P. aeruginosa is an often cause of nosocomial infections especially in intensive care unit (ICUs) patients. This organism has found to be a common cause of hospital-acquired pneumonia and ventilator-related pneumonia. In burn patients, *P. aeru ginosa* is a considerable pathogen that causes wound infections, bacteremia, and ventilator-related pneumonia. In patients with bone marrow transplantation, the appearance of *P. aeruginosa* bacteremia is higher than general population in the hospital. In view of resistance profiles, the National Nosocomial Infections Surveillance (NNIS) of the United States reported that *P. aeruginosa* isolated from ICUs in 2003 displayed increase resistance rate to fluoroquinolones, third-generation cephalosporins, and imipenem (38). The National Antimicrobial Resistance Surveillance Thailand (NARST) revealed a high ceftazidime resistance rate (24.6-27.4%) among *P. aeruginosa* clinical isolates in Thailand from 2000 to 2005. The data also revealed a high susceptibility rate to carbapenems such as imipenem (84.6-87.2%) and meropenem (84.5%) (62). However, in recent year, the increasing of carbapenems resistance is a problematic concern. The resistance to this compound via carbapenem hydrolysing metallo-β-lactamases (MBLs) has been widely reported, thereby affecting the limit of antibiotics treatment. In 2008, a hospital in Tunisia found nosocomial outbreak of *P. aeruginosa* carrying VIM-2 MBL gene in a kidney transplantation unit. Outbreaks due to VIM β-lactamase producing *P. aeruginosa* have been found in other

countries such as Italy, Greece, and Kenya. As well as IMP-type MBLs in *P. aeruginosa*, they have been also reported worldwide (63).

3.6 Other *Pseudomonas* Species

Pseudomonas spp. outside *P. aeruginosa* are actually rare cause of infections because of their low virulence. The infections due to these species mostly relate to contamination in solutions, medicines, blood, and catheters. Most published cases of *P. putida* bacteremia have been neonatal infections and outbreak infections through transfusion of contaminated blood or fluid. In addition, *P. putida* has been reported to cause catheter-associated bacteremia in cancer patients and other immunosuppressive diseases. Besides the device implicating with the bacteremia, patients who exist in immunocompromise state have a higher risk to be infected with this organism. The abilities in adherence of medical devices and production of biofilm have been supposed to be their pathogenicity (26, 64). Multidrug resistance and also carbapenem resistance caused by *P. putida* have been found occasionally for nosocomial infections in immunocompromised patients or severely ill. The emergence of metallo-β-lactamases (MBLs) in this microorganism, such as IMP-1, IMP-12, VIM-1, VIM-2, and VIM-5 has been problematic in selection of available therapeutic drugs. Furthermore, metallo-β-lactamase produced by *P. putida* could be a reservoir of multidrug resistance elements that will be transferred to *P. aeruginosa*. Since the VIM-2 integron was settled in the same transposon in both *P. aeruginosa* and *P. putida* that isolated in Spanish hospital during 2005 to 2008 (65, 66). For *P. fluorescens,* this species can grow at 4 °C and can be found on the skin of blood donors, so the organism has caused occasionally transfusion-associated septicemia in the recipient. This unusual organism has been implicated in pseudobacteremia. The false-positive blood cultures bring diagnostic confusion, wasted time in repeating laboratory examination, and inappropriate therapy such as unnecessary antibiotic treatment, or prolonged hospital admission. A clinical research revealed the investigation of pseudobacteremia outbreak due to *P. fluorescens,* contaminated lithium heparin tubes were found to be a source of the outbreak since they were filled

prior to the filling of blood culture bottles (26, 67). It also has been reported to cause catheter-associated bloodstream infection among patients with cancer. In USA during December 2004- March 2006, 6 states were occurred outbreak of *P. fluorescens* bacteremia that the patients were traced to contaminated compounded pharmaceutical as a source of the outbreak. After the exposure to contaminated heparinized saline flush, thirty three (41%) of 80 cases had potential for delayed infection onset (84-421 days) (68). For *P. stutzeri*, it is also an uncommon cause of nosocomial infection. This organism has been revealed to be an agent of bacteremia in immunocompromised individuals, meningitis in human immunodeficiency virus-infected man, endophthalmitis subsequent cataract surgery, pneumonia in alcoholics, and brain abscess subsequent subdural grid implantation (26, 69). Other pseudomonad are moreover less be found in human infection. *P. mendocina* has been reported to cause endocarditis. *P. alcaligenes* caused catheter-associated endocarditis in a person with bone marrow transplantation (26).

3.7 Outer membrane proteins (OMPs) of Gram-negative bacteria

The outer membrane of Gram-negative bacteria acts as a protective barrier against the harsh environments with still controlling the influx and efflux of materials required for life existence. It forms asymmetric bilayer consisting of phospholipid and lipopolysaccharides (LPS). The LPS exclusively exists in the outer leaflet with three compositions: lipid A, core oligosaccharide, and O-chain. The core oligosaccharide and O-chain are variable portions: the core portion influences bacterial viability whereas the O-antigen provides a high antigenic variation within species. For the inner leaflet, the structure consists of phospholipids as same as cytoplasmic membrane (70, 71).

Outer membrane proteins (OMPs) are found in the outer membrane of Gram-negative bacteria and certain organelles (i.e. mitochondria and chloroplasts) of eukaryote. The OMPs of Gram-negative bacteria fold into transmembrane antiparallel β-barrels and have diverse functions including passive diffusion (i.e. porins and efflux channels), active transport, iron-siderophore uptake, pathogenesis (e.g. bacterial

adhesion), and catalysis (72, 73). With regard to folding process, OMPs with N-terminal signal peptide are initially secreted across the cytoplasmic membrane into the periplasm via Sec translocation machinery. The periplasmic chaperones such as SurA and Skp have been proposed to support folding and insertion of the β-barrels OMPs by holding in an unfolded form and non-aggregation in the periplasm (74-77). Later on, the OMPs are delivered to the β-barrel assembly machinery (BAM) complex in the outer membrane. The multi-subunit Bam complex has been suggested to be involved in the assembly of β-barrels into the outer membrane of Gram-negative bacteria (78-80).

3.8 *P. aeruginosa* outer membrane proteins

The outer membrane proteins (OMPs) are suggested to have importance in view of the cell-surface exposure, antibiotics transport, secretion of extracellular virulence factors, and anchoring contribute to cells adhesion and motility. The complete genome sequence of *P. aeruginosa* PAO1 revealed approximately 150 genes predicted encoding OMPs. Among these, three large families are recognized: the OprD-specific porin family, the OprM efflux family, and the TonB-dependent gated porin family (81).

3.8.1 General porins

Non-specific or general porins form water-filled channels across the outer membrane which their permeability is depend on rate of solutes diffusion. Certainly, these porins lack specific ligand-binding sites (82) that consequently allow the passage of ions and small hydrophilic solutes (83).

OprF is the major outer membrane protein of *P. aeruginosa* that is involved in non-specific porin. (Characteristics and the specificity to *P. aeruginosa* are described below.)

3.8.2 Specific porins

The well-known specific porin is *E. coli* LamB, which particularly uptakes maltose and maltodextrins. The LamB, also known as maltoporin, exists in trimeric form that each monomer consists of 18-stranded β-barrel. The ligand recognition and binding of this substrate-specific channel are mediated by aromatic residues which so-called “greasy slide” region (84). In *P. aeruginosa*, the penetration via specific classes of ligands is found in many outer membrane porins, including OprB (glucose porin), OprO (pyrophosphate porin), OprP (phosphate porin), and OprD (basic amino acid porin) (85).

OprB is the homologous protein of *E. coli* LamB (86) and serves as glucose-selective porin in *P. aeruginosa*. In *E. coli*, small substrates such as glucose or glycerol are believed that they can also permeate through nonspecific porins. In contrast, the low outer membrane permeability of *P. aeruginosa* contributes to limit the penetration of even small molecules in the absence of substrate-specific porin. OprB is considered to be a major component for carbohydrate diffusion when it also facilitates the diffusion of several other sugars including fructose, xylose, mannitol, and glycerol (87).

OprP plays a role in the high-affinity uptake of phosphate ions that is induced under phosphate starvation condition. The structure reveals trimeric form that each monomer consists of 16-stranded β-barrel. At the central binding sites, arginine 133 (R133) is considered to be responsible for ion selectivity and transport properties due to its positive charge, H-bond donor capacity, and large side chain (88).

OprO serves as a polyphosphate-specific porin and shares 76% amino acid identity with the homologous protein OprP. Under phosphate limitation situation, *oprO* gene expression is induced when the cells exist in the stationary growth phase (89).

OprD serves as a basic amino acid porin. In *P. aeruginosa*, 19 outer membrane proteins belong to OprD family and share 46-57% amino acids identity with OprD protein. There are eight homologs closely related to OprD, which these protein have a function in amino acid or peptide uptake. The remaining homologs are similar to *P. putida* PhaK, the porin that is required for growth on phenyl acetic acid. Among the rest of homologs, they have a function in organic carbon sources uptake

(86). (More characteristics and the impact on imipenem susceptibility are described below.)

3.8.3 Gated porins

Iron is essential for bacterial growth, thus bacteria have evolved strategies for iron acquisition, including synthesis of iron-chelating compounds called siderophores (86). Gram-negative bacteria possess outer membrane proteins (TonB-dependent receptors) involving in the acquisition of iron-siderophores complexes. TonB-dependent gated porins show high affinity and specificity for metal chelates. The porins consist of 22-stranded β-barrel spanning the outer membrane and "plug" domain folded into the barrel interior. The plug domain binds a specific siderophores (also colicins) at the outside of the closed conformer and interacts with inner membrane protein TonB complex (in conjunction with ExbB and ExbD) to initiate transport. Since the gates derive energy in form of protonmotive force from TonB complex, their conformation is changed to open and let the iron-siderophores access to the cells (90, 91). In *P. aeruginosa*, FpvA acts as pyoverdine transporter that seems to be a predominant siderophore that chelating iron from transferrin or serum. Despite pyoverdine are produced by many pseudomonad, there is chemical heterogeneity corresponding its own siderophore uptake in each species. The other ferric-pyoverdine receptors are *P. putida* PupA and PubB which are homologous to FpvA (86).

OprC forms a small channel that is involved in copper utilization. It shows the highest similarity with *P. stuzeri* NosA (65% amino acid identity), an outer membrane required for the supply of Cu^{2+} to nitrate reductase (92).

3.8.4 Efflux porins

Active efflux has an important role in intrinsic resistance of *P. aeruginosa*. The most efflux systems in this organism belong to the resistance-nodulation-division (RND) family. This family is active transporters which derived the energy from protonmotive force. RND pumps form a tripartite protein complex composed of a cytoplasmic membrane transporter, an outer membrane factor, and a periplasmic membrane fusion protein.

In *P. aeruginosa*, the outer membrane protein OprM, OprN, and OprJ form tripartite systems that contribute to multiple antibiotic resistance. OprM is a predominant antibiotic efflux porin that can be constituted MexAB-OprM and MexXY-OprM (42, 86). The efflux systems extrude a large number of antimicrobial agents, for example, MexAB-OprM can extrude macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin, quinolones, and β-lactams except imipenem (93).

Many pumps in this family were described in other Gram-negative bacteria such as *Escherichia coli*, *Acinetobacter baummanii*, *Klebsiella pneumoniae*, and *Stenotrophomonas maltophilia*. The AdeABC (RND family) efflux pump is widespread in *A. baummanii* and has much more antibiotic substrates (i.e. aminoglicosides, fluoroquinolones, some β-lactams, tetracyclines, chloramphenicol, erythromycin, trimethoprim, and tigecycline) (94).

Besides antibiotic efflux, *P. aeruginosa* produces the other efflux porins involved in toxins and virulence factors secretion. For instance, AprF is a constituent of a three-component type I secretion system AprDEF, which secretes alkaline protease (86, 95).

3.9 Characteristics of OprD and the impact on imipenem susceptibility

An outer membrane protein OprD was first characterized in the incidence of *P. aeruginosa* clinical isolates appeared resistant to imipenem (96). This broad-spectrum β-lactam has been used over two decades, it demonstrates high potent to treat severe or difficult infections including from multiple drug-resistant pathogens (97). However, the emergence of imipenem resistance due to alteration of OprD protein in *P. aeruginosa* was followed simultaneously with this antibiotic use (96, 98). The structure of imipenem resembles a dipeptide containing a basic charged residue. Therefore, it can be penetrated through the OprD, a specific porin binding with basic amino acids and peptides containing these amino acids (86, 99). The OprD structure from X-ray crystallization shows a monomeric 18-stranded β-barrel, which contrasts

with the previous prediction that has 16 strands. According the crystal structure, two short β-strands S5 and S6 were discovered (86, 100).

In *P. aeruginosa*, the main mechanism of imipenem resistance is noted due to downregulation of the gene encoding OprD porin (101). Although the loss and decreased levels of OprD is predominant, efflux pumps and metallo-β-lactamases (MBLs) are involved in the resistance against imipenem (102). Furthermore, the AmpC hyperproduction in conjunction with diminished OprD production and several efflux systems have been found to be responsible for carbapenems resistance (103). Nevertheless, Pirom Noisumdaeng et al. found that 98% (54 of 55 isolates) of imipenem-and meropenem-resistant *P. aeruginosa* clinical isolates did not produce OprD (13). Other studies supporting that the absence or weak expression of OprD is a major mechanism of imipenem resistance and was found predominant in imipenem-resistant *P. aeruginosa* isolates in Thailand and other countries including France and Spain (6, 12, 14, 15).

3.10 Characteristics of OprF and the specificity to *P. aeruginosa*

The outer membrane protein OprF is a major channel-forming protein in the outer membrane of *P. aeruginosa*. The protein functions as nonspecific porin allowing low diffusion of solutes. OprF shows the existence of two distinct structures, with the majority folding into two-domain closed-channel and only about 5% of the protein population being single domain open-channel. The closed-majority conformer contains N-terminal eight-stranded β-barrel domain (transmembrane region) and C-terminal α-helices, globular domain (periplasmic region) (Fig. 3.1). This structure lacks porin function to permeate even small molecules such as glycine, whereas the open-minority structure consisting of 16 (to 18)-stranded β-barrel provides the passage of large solutes like oligosaccharides. Indeed, the closed channel plays a crucial role in morphology stabilization by holding outer membrane with peptidoglycan. The end of β-barrel domain contains four cysteine residues that might form disulfide bridges. The prevention of the disulfide bridges was found to be increasing the single domain open-channel OprF (19, 20, 104-106).

This major outer membrane protein is predicted to involve in the intrinsic resistance of *P. aeruginosa* owing to its low permeability that presumably limits the entrance of antimicrobial drugs. Nevertheless, OprF deficient was considered to have less effect on antibiotic resistance. Therefore, it has been proposed to raise the resistant level when combine with secondary adaptive resistance mechanisms such as efflux pumps overexpression and β-lactamase (1).

Regarding conservation of OprF, there have been previously demonstrated that the OprF protein is conserved among the 17 different serotype strains, clinical and environmental isolates of *P. aeruginosa* (107, 108). It contains certain well-conserved surface epitopes. Mutharia LM et al. illustrated conservation of the OprF epitopes on *P. aeruginosa* and *P. putida*, which this closely related specie had cross-reactivity with OprF-specific monoclonal antibody MA4-4 (109, 110). The protein seems to be distributed in *Pseudomonas* rRNA homology group I (e.g. *P. aeruginosa*, *P. putida*, *P. fluorescens*, *P. syringae*). The study of Kragelund L et al. demonstrated that the polyclonal antibody specific to OprF-like protein from *P. fluorescens* DF57 could react with OprF proteins from members of homology group I pseudomonads but no reactivity against nonpseudomonads (22, 111). From the NCBI database, OprF proteins of *P. fluorescens*, *P. syringae*, and *P. putida* share 68.9, 67.1, and 66.9% amino acid identity, respectively, to *P. aeruginosa* OprF. Besides that, the OprF of *P. aeruginosa* and the orthologous protein OmpA from *E. coli* share 25-26% overall amino acid identity. Interestingly, there is only 15% identity in the β-barrel domain that some certain parts are surface-exposed epitopes (21).

In addition to *E. coli*, there are many bacteria that use OprF homologs as a ubiquitous porin including *Acinetobacter baumannii* and *Salmonella* spp. The low permeability of the OmpA-family proteins in Gram-negative bacteria surviving in environments is considered to protect the bacteria from antimicrobial compounds produced by other microbes. Moreover, the major protein of *A. baumannii* OmpAB serves as a low-permeability porin like the OprF of *P. aeruginosa* and also implicates the high intrinsic resistance, especially when combination with the constitutive β-lactamases and multidrug efflux pumps (19, 112-114).

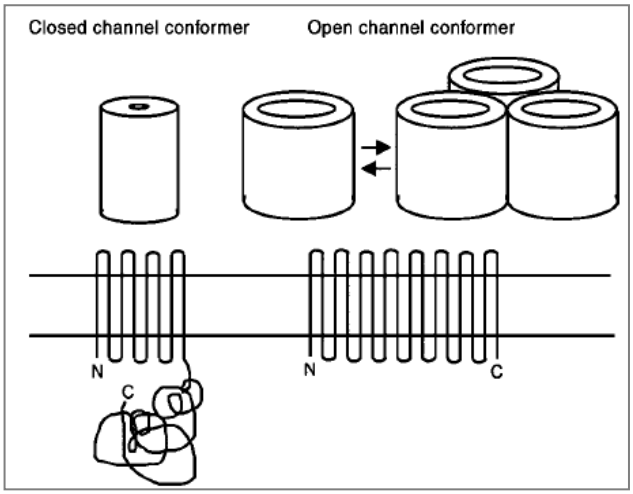


Figure 3.1 Models of OprF protein in two distinct conformers: the closed-majority conformer (left) and the open-minority conformer (right) (19).

Table 3.2 Known the outer membrane proteins of *P. aeruginosa*

Gene	Protein function and name	No. of amino acids	Known porin (class)
algE	Alginate production protein AlgE	490	P
aprF	Alkaline protease secretion protein AprF	481	EP, PI
fliF	Flagella M-ring protein	598	
fptA	Fe(III)-pyochelin receptor	720	GP
fpvA	Ferripyoverdine receptor	815	GP
hasR	Heme uptake receptor HasR	891	GP
icmP	Insulin-cleaving metalloproteinase; ICMP	446	
lppL	Lipopeptide LppL	46	
omlA	Lipoprotein OmlA	176	
oprB	Glucose/carbohydrate porin OprB; protein D1	454	SP
oprC	Putative copper transport porin OprC	723	GP
oprD	Basic amino acid, basic peptide and imipenem porin OprD; also named Porin D, Protein D2	443	SP, P
oprE	Anaerobically induced porin OprE; Porin E1	460	SP
oprF	Major porin and structural porin OprF; Porin F	350	P
oprG	Outer-membrane protein OprG	232	
oprH	PhoP/Q and low Mg^{2+}-inducible outer-membrane protein H1	200	GP
oprI	Outer-membrane lipoprotein OprI	83	
oprJ	Multidrug efflux protein OprJ	479	EP
oprL	Peptidoglycan-associated lipoprotein OprL	168	
oprM	Major intrinsic multiple antibiotic resistance efflux protein OprM	485	EP
oprO	Pyrophosphate-specific porin OprO	438	SP
oprP	Phosphate-specific porin OprP; protein P	440	SP
pfeA	Ferric enterobactin receptor PfeA	746	GP

Table 3.2 Known the outer membrane proteins of *P. aeruginosa* (continued)

Gene	Protein function and name	No. of amino acids	Known porin (class)
phuR	Heme/Hemoglobin uptake receptor PhuR	764	GP
pilQ	Type 4 fimbrial biogenesis protein PilQ	714	
popD	Translocator protein PopD; PepD	295	
popN	Type III secretion protein PopN	288	
pscC	Type III secretion protein PscC	600	PIII
xcpQ	General secretion pathway protein D	658	PII
xcpU	General secretion pathway protein H; PilD-dependent protein PddB	172	
xqhA	Secretion protein XqhA	776	PII

P, general porin; EP, OprM family member of efflux and protein secretion porins

SP, specific porin; GP, putative gated porin

PI, putative type I secretion subfamily; PII, type II secretion channel; PIII, type III secretion channel (modified from (86))

CHAPTER IV
MATERIALS AND METHODS

4.1 Bacterial strains and plasmids

4.1.1 Bacterial strains for gene detection and protein analysis

P. aeruginosa ATCC 27853

P. aeruginosa PAO1

P. alcaligenes (DMST 21133)

P. fluorescens ATCC 13525

P. mendocina ATCC 25411

P. putida ATCC 12633

P. stutzeri (DMST 12562)

P. syringae ATCC 19310

E. coli ATCC 25922

E. coli BL21 (DE3)

P. aeruginosa clinical isolates: PA10, PA65, PA70, PA80, PA100, PA102, and PA103

P. putida clinical isolate

The clinical isolates were collected from patients admitted at Siriraj Hospital during September 2001 to February 2002. Bacterial identification of these isolates was performed at the Clinical Bacteriology Laboratory, Department of Microbiology, Faculty of Medicine Siriraj Hospital, according to the Clinical and Laboratory Standard Institute (CLSI) guidelines. The isolates were stored at -70 °C in LB broth supplemented with 20% glycerol until use.

There were six species of pseudomonads including *P. alcaligenes*, *P. fluorescens*, *P. mendocina*, *P. putida*, *P. stutzeri*, and *P. syringae* that received from National Antimicrobial Resistance Surveillance Center, Department of Medical Sciences, Ministry of Public Health.

4.1.2 Bacterial strains and plasmid for cloning and protein expression

E. coli TOP10 competent cells (Invitrogen): cloning strain, which provides high-efficiency cloning and plasmid propagation including maintenance for the plasmids

E. coli BL21 (DE3) competent cells (Invitrogen): expression strain, which provides protein expression under IPTG induction of T7 polymerase from *lac*UV5 promoter

pET-28b (Novagen): cloning and expression vector

4.2 Animal

The four-month old female New Zealand White rabbit was acquired from Faculty of Veterinary Science, Chulalongkorn University. The rabbit was used in the experiment of antibody production supplied by Animal Facility Center at Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

4.3 Equipments and special chemicals

1) Orbital incubator shaker, Gyromax (Amerex)
2) Centrifuge, Sorvall Biofuge Primo R
3) PCR Thermal cycler, Esco Healthcare Swift MaxPro
4) Digital dry bath (Labnet)
5) Incubator (Memmert)
6) Protein electrophoresis (Bio-Rad)
7) Gel documentation (Syngene)
8) Spectrophotometer (Bio-Active)
9) Semi-dry electrophoretic transfer cell (Bio-Rad)
10) DyNAzyme^{TM}II DNA Polymerase (Finnzymes)
11) 1 Kb Plus DNA Ladder (Invitrogen)
12) Amicon Ultra-4 10K Centrifugal Filter Device (Millipore Corporation)

13) HisTrap HP, 1 ml affinity column (GE Healthcare)
14) Bradford reagent (Bio-Rad)
15) Bovine serum albumin (BSA) standard (Thermo Scientific)
16) Hybond-P PVDF membrane (Amersham)
17) Anti-rabbit IgG (Goat), HRP-Labeled, 1mg at 1mg/ml (PerkinElmer)
18) Pre-stained Protein Ladder (Fermentas)

4.4 Extraction of genomic DNA

The genomic DNA of *P. aeruginosa* strain PAO1, ATCC 27853, and clinical isolates (PA10, PA70, PA80, and PA100) were extracted by using Gentra Puregene Yeast/Bact. Kit according to manufacturer's instruction. Briefly, a single colony of the strain was inoculated into 5 ml of LB broth and shaken at 250 rpm, 37 °C for overnight (18 hours). The cultured bacterial cells (500 µl) were sedimented by centrifugation at 12,000 × g for 1 minute. The cells were lysed with Cell Lysis Solution, mixed by vortex, and incubated at 80 °C for 5 minutes. RNase A Solution was added, mixed by inverting, and incubated at 37 °C for 40 minutes. The sample was incubated for 1 minute on ice to quickly cool, added with Protein Precipitation Solution, vortexed, and centrifuged at 14,000 × g for 3 minutes. Isopropanol (300 µl) was used for DNA precipitation (the DNA showed as a small white pellet). The next step, 300 µl of 70% ethanol was used to wash the DNA pellet. The suspension was centrifuged at 13,000 × g for 1 minute, then discarded the supernatant, drained the tube on a clean absorbent paper, and placed to dry for 15 minutes. Finally, DNA hydration was performed as following: add DNA Hydration Solution, mix by vortex, andincubate at 65 °C for 1 hour to dissolve the DNA, then place at room temperature for overnight and lastly storage the purified genomic DNA at -20 °C.

4.5 Detection of *oprF* gene in *P. aeruginosa*

P. aeruginosa 6 strains: ATCC 27853, PAO1, and clinical strains PA10, PA70, PA80, and PA100 were examined the existence of *oprF* gene by polymerase chain reaction (PCR) method. The forward primer was 5´-CACCATGAAACTGAAG AACACC-3´. The reverse primer was 5´-TTACTTGGCTTCAGCTTCTACTTC-3´. PCR was done with a 50-µl reaction mixture containing 42 µl of sterile Milli-Q (MQ) water, 5 µl of 10x DyNAzyme^{TM} buffer, 1 µl of 10 mM dNTPs (200 µM each), 0.25 µl of 100 µM *oprF*-forward primer (0.5 µM), 0.25 µl of 100 µM *oprF*-reverse primer (0.5 µM), 0.5 µl of DyNAzyme^{TM} DNA polymerase, and 1 µl of DNA template (1:200 dilution).

Table 4.1 Thermal cycler program for *oprF* amplification

Cycle step		Temp.	Time
Initial denaturation		94 °C	5 min
35 cycles	Denaturation	94 °C	1 min
	Annealing	55 °C	1 min
	Extension	72 °C	1 min 20 s
Final extension		72 °C	10 min

4.6 Determination of PCR product

The PCR products amplified from many strains of *P. aeruginosa* were determined restriction endonuclease analysis to ensure that they were *oprF* gene. *Sma*I (TaKaRa) was used in this experiment and gave a band pattern. The reaction mixture was composed of 1 µl of restriction enzyme (*Sma*I), 2 µl of buffer (10x T Buffer for *Sma*I), 2 µl of 0.1% BSA, ≤ 1 µg of substrate DNA, and sterilized distilled water up to 20 µl. The reactions were incubated 1 hour at 30 °C for *Sma*I. Inactivate the reaction (*Sma*I: 65 °C, 20 min) and apply on agarose gel electrophoresis.

4.7 Amplification of a whole *oprF* gene for using in cloning and expression experiments

The complete open reading frame of *P. aeruginosa oprF* gene encoding OprF porin protein was amplified from chromosomal DNA of *P. aeruginosa* PA10 strain. The forward primer (5´-AGGATCCGATGAAACTGAAGAACAC-3´) included the DNA sequence corresponding to the initiation methionine and an additional *BamH*I site (GGATCC) flanking with 1 base for well binding of this restriction enzyme at 5´ end. The reverse primer (5´-CTAAGCTTTTACTTGGC TTCAGCTTC-3´) included the DNA sequence corresponding to the TAA stop codon and an additional *Hind*III site (AAGCTT) flanking with 2 bases for well binding of this restriction enzyme at 3´ end.

PCR was done with a 25-µl reaction mixture containing 11.75 µl of sterile Milli-Q (MQ) water, 2.5 µl of 10x DyNAzyme^{TM} buffer, 5 µl of 5M Betaine (1 M), 1.25 µl of DMSO (5% v/v), 0.5 µl of 10 mM dNTPs (200 µM each), 1.25 µl of 10 µM *oprF*-forward primer (0.5 µM) , 1.25 µl of 10 µM *oprF*-reverse primer (0.5 µM), 0.5 µl of DyNAzyme^{TM} DNA polymerase, and 1 µl of DNA template. The two-step PCR was performed in this experiment.

Table 4.2 Thermal cycler program of two-step PCR for the whole *oprF* amplification

Cycle step		Temp.	Time
Initial denaturation		94 °C	5 min
5 cycles	Denaturation	94 °C	1 min
	Annealing (Ta_1)	41 °C	1 min
	Extension	72 °C	1 min 20 s
25 cycles	Denaturation	94 °C	1 min
	Annealing (Ta_2)	58 °C	1 min
	Extension	72 °C	1 min 20 s
Final extension		72 °C	10 min

4.8 Analysis of *oprF* gene amplified from *P. aeruginosa* PA10

The PCR products were determined in 1% (w/v) agarose gel-horizontal electrophoresis. The electrophoresis was set at 100 volt for 30 minutes to separate DNA fragments in 1x TBE buffer. Then, the gel was stained for 8 minutes with ethidium bromide (2 µg/ml) and destained for 5-10 minutes with water. The PCR products were visualized on UV transilluminator. The expected size should be about 1,070 basepairs (bp) which covers the size of *oprF* gene (1,053 bp) and that of sequences encoding *BamH*I and *Hind*III site.

4.9 Cloning

4.9.1 Ligation of the amplified *oprF* gene and pET-28b plasmid

The *oprF* gene- PCR product was purified by using Gel Extraction Kit (Geneaid) according to manufacturer's instruction. Briefly, the agarose gel slice containing the *oprF* gene- PCR product was excised and transferred to an eppendorf tube. The gel was suspended in DF Buffer. The suspension was transferred to DF column and centrifuged at 14,000 × g for 30 seconds for DNA binding. The repeat of DNA binding step may lose a partial quantity of DNA. The bound DNA into DF column was washed with W1 Buffer and Wash Buffer (ethanol added). The purified DNA was eluted with Elution Buffer or MQ.

The *oprF* gene- PCR product and the pET-28b plasmid were ligated with T4 DNA ligase (TaKaRa) which the procedure was modified from manufacturer's instruction. Initially, the *oprF* gene- PCR product and the pET-28b plasmid were cut separately with 2 restriction enzymes (*BamH*I and *Hind*III of TaKaRa). The reaction mixture contained 1 µl of *BamH*I, 1 µl of *Hind*III, 2 µl of 10x K Buffer, 90 ng of *oprF*- PCR product, and distilled water up to 20 µl. The another reaction tube contained 1 µl of *BamH*I, 1 µl of *Hind*III, 2 µl of 10x K Buffer, 375 ng of pET-28b plasmid, and distilled water up to 20 µl. The restriction reaction was incubated at 37 °C for 1 hour 30 minutes. Then, it was inactivated at 70 °C for 15 minutes.

For ligation between vector and inserted DNA, the reaction mixture contained 1 µl of T4 ligase, 2 µl of 10x Ligation Buffer, 50 ng of *oprF*- PCR product cutting with *BamH*I and *Hind*III (from previous step), 50 ng of pET- 28b plasmid cutting with *BamH*I and *Hind*III, and distilled water up to 20 µl. The reaction mixture was incubated at 16 °C for 5 hours.

4.9.2 Tranformation of the recombinant plasmid into *E. coli* TOP10

The recombinant plasmid was transformed into chemically competent *E. coli* TOP10 cells (Invitrogen) by using the heat shock method. The competent cells were thawed rapidly on ice. The 15 µl of ligation mixture was added into 100 µl of thawed competent cells and placed on ice for 10 minutes. The cells were treated by heat-shock at 42 °C for 45 seconds and immediately transferred to ice. The transformation mixture was pipette into15 ml tube containing 1 ml of LB medium and shaked at 37 °C for 1 hour. The 300 µl of transformation mixture was spread on a pre-warmed LB/ kanamycin plate and incubated overnight at 37 °C.

After transformation of the recombinant plasmid into *E. coli* TOP10, the *E. coli* cells were cultured overnight in LB broth supplemented with kanamycin (total concentration = 50 µg/ml), due to pET-28b contains kanamycin resistant gene as a selective marker. The recombinant plasmid was extracted from the *E. coli* cells by Geneaid High-Speed Plasmid Mini Kit according to manufacturer's instruction. Briefly, the 1.4 ml of cultured bacterial cells were transferred to a microcentrifuge tube, then centrifuged at 12,000 × g for 1 minute, and discarded the supernatant. Repeat the harvesting step 2-3 times for appropriate concentration. The cell pellets were resuspended with PD1 Buffer (RNaseA added), and mixed by vortex. For the step of lysis, PD2 Buffer was added and mixed gently by inverting the tube 6 times to avoid shearing the genomic DNA, and let stand 2 minutes at room temperature. The sample was added with PD3 Buffer for neutralization, mixed immediately by inverting the tube 6 times, and centrifuged at 16,000 × g for 3 minutes. The supernatant was transferred to PD column and centrifuged at 14,000 × g for 30 seconds for DNA binding. For the step of wash, W1 Buffer was filled into the PD Column, centrifuged at 14,000 × g for 30 seconds, and discarded the flow-through. Thereafter, wash again with Wash Buffer (ethanol added) by following the previous step. Next, the column

matrix was centrifuged at 14,000 × g for 4 minutes to dry. The final step: DNA elution, the dried PD Column was transferred to a new microcentrifuge tube, 50 µl of Elution buffer or MQ was added into the center of the column matrix, and let stand 2 minutes for absorption of matrix. Finally the tube was centrifuged at 16,000 × g for 2 minutes to elude the DNA.

4.10 Analysis of recombinant plasmid

4.10.1 Restriction endonuclease analysis

The extracted recombinant plasmids were analyzed by restriction endonuclease analysis. *Sma*I was used to confirm the presence and correct orientation of the *oprF* gene insert. This restriction enzyme cut once in the pET-28b plasmid and once in the *oprF* gene. The reaction mixture contained 1 µl of *Sma*I (TaKaRa), 2 µl of 10x T Buffer, 2 µl of 0.1 % BSA, 5 µl of plasmids, and distilled water up to 20 µl. The restriction reaction was incubated at 37 °C for 1 hour 30 minutes. Then, it was inactivated at 60 °C for 15 minutes. The obtained DNA fragments were separated by using 1% agarose gel electrophoresis. The digestion pattern of recombinant plasmid containing the correct orientation of insert should appear 2 fragments (1,988 and 4,414 bp). The absence of insert will appear only one band at 5,355 bp on the agarose gel.

4.10.2 Sequencing

The expected recombinant plasmid containing the correct orientation of insert was sequenced by using T7 promoter/ terminator primers and *oprF* forward/ reverse walking primers. The forward walking primer was 5´-CTGGAGAAGCGTGA CAACG-3´ and the reverse walking primer was 5´-ACCGTACTGGCCGTCGAG-3´. The sequencing result will include the DNA sequence corresponding to an N-terminal histidine tag and whole *oprF* gene. The *oprF* gene was compared with *P. aeruginosa* PAO1 *oprF* gene.

4.11 Expression of the *oprF* gene

4.11.1 Tranformation of the recombinant plasmid into *E. coli* BL21

The recombinant plasmid was transformed into *E. coli* BL21 by electroporation. Electrocompetent cells were prepared as the following. *E. coli* BL21 cells (Invitrogen) were cultured at 37 °C with shaking for overnight. The bacterial cells culture were inoculated in 1 liter of L-broth minus salt and grown at 37 °C with shaking to an ABS_{600} of 0.5 to 1. The cells were centrifuged at 5,000 rpm, 4 °C for 10 minutes and discarded the supernatant. The cell pellets were resuspended with sterile, cold, 10% glycerol and centrifuged (at 5,000 rpm, 4 °C for 10 minutes) three times (200 ml of the glycerol solution for the first time, 100 ml, and 8 ml for the second and the third time respectively). Finally, the cell pellets were resuspended again with 0.6 ml of sterile, cold, 10% glycerol and freezed at -70 °C. The procedure of electroporation was performed as the following. The competent cells (50 µl) were thawed on ice , placed in sterile, cold electroporation cuvette, mixed with 1 µl of the recombinant plasmid (eluted with distilled water), electroporated at 25 µF, 2.5 kV, 200Ω, and immediately resuspended with 1 ml of LB broth (with salt). The mixture was incubated at 37 °C with shaking for 1 hour, spread (300 µl) on a LB/ kanamycin plate and incubated overnight. The colonies on the plate were picked up to analyze the correct orientation of insert as the previous procedure. Thereafter, the colony which has the correct recombinant plasmid was cultured and collected stock at -70 °C.

4.11.2 Expressing the *oprF* gene (a pilot scale)

The appropriate condition of *oprF* expression was studied in a pilot scale expression and then a large scale expression was performed. The large scale expression was needed for a large amount of recombinant protein production. The pilot scale expression was done as the following. The overnight culture of transformant cells from previous step (500 µl) were inoculated in 10 ml of LB containing 50 µg/ml kanamycin and grown 2 hours at 37 °C with shaking (OD_{600} should be about 0.5-0.8). The culture was split into two tubes at the volume of 5 ml. One tube was induced protein expression by isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 1 mM. Another one was not induced with IPTG (un-induced

control). Each culture was grown and collected every hour for 4 hours in a volume of 500 µl. The collected samples were centrifuged, discarded the supernatant, and frozen the cell pellets at -20 °C. The samples were analyzed total cell proteins by 12% SDS-PAGE electrophoresis. The polyacrylamide gel was stained with Coomassie blue.

4.11.3 Analyzing soluble/ insoluble protein

A large scale expression was performed for expressing the recombinant OprF protein and analyzing whether it is produced in soluble or insoluble form. The transformant cells were cultured for overnight at 37 °C. The 500 µl of cell culture was inoculated into 1 L of LB broth containing 50 µg/ml of kanamycin. The bacterial cells were grown at 37 °C with shaking at 200 rpm for 18 hours. The 0.8 M IPTG was added into the culture to be a final concentration of 1 mM. The culture was continuous incubated for 3 hours to induce protein expression. A large scale expression (1 L) was centrifuged at 8,000 rpm, 4 °C for 10 minutes. The cell pellets were washed with sterile distilled water (centrifuge at 3,000 rpm, 4 °C for 20 minutes) and resuspended with 20 ml of sodium phosphate buffer pH 7.4. The cell solution was added with 2 ml of 10 mg/ml lysozyme and incubated on ice for 2 hours to lyse cells that was together with freeze-thaw method. The cell solution was freezed at -20 °C for 15-30 minutes until hard, thawed at 37 °C until dissolved, and vortexed. The bacteria were performed 3-4 times of freeze/thaw for efficient lysis. Afterwards, 1 ml of the solution was transferred into a microcentrifuge tube and centrifuged at 13,000 rpm, 4 °C for 20 minutes. The supernatant and pellet were collected for soluble and insoluble form, respectively. For the insoluble protein (OprF should be appeared in this form), the pellet was resuspended with 5 ml of solubilization buffer containing 6 M urea and incubated at room temperature for 1 hour with shaking to dissolve the proteins. The sample were centrifuged at 13,000 rpm, 4 °C for 15 minutes to collect the supernatant and analyzed on 12% SDS-PAGE.

4.12 Purification of $(His)_6$- tagged OprF fusion protein

The N-terminal polyhistidine (6xHis) tag was utilized for protein purification by allowing purification of the recombinant fusion protein with a metal-chelating resin. The chromatography HisTrap HP 1 ml column (GE Healthcare) was used for purification according to the manufacturer's instruction. The purified protein was analyzed by using MALDI time-of-flight mass spectrometry and the result was taken to compare with *oprF* gene of *P. aeruginosa* from the database.

4.12.1 Optimization protocol for purification

For protein preparation, the pellet (from approximately 50 ml of induced culture) as described above was used because the OprF fusion protein was found in the insoluble form. The pellet was resuspended in 5 ml solubilization buffer and incubated for 1 hour at room temperature with shaking. The solution was centrifuged at 13,000 rpm, 4 °C for 15 minutes to collect the supernatant. The solubilized $(His)_6$-tagged OprF fusion protein was passed through 0.45 µm filter and then used for purification. Stepwise gradient elution was performed for an optimum purity. The HisTrap column was washed according to Column preparation. Afterwards, the column was equilibrated with 10 ml binding buffer (1x phosphate buffer, 5 mM imidazole, pH 7.4). The sample (extracted OprF protein) from previous step was applied into the column and collected the flow-through fraction. The column was washed with 10 ml binding buffer and collected the wash fraction. The elution was started with 5 ml 1x Phosphate buffer containing 20 mM imidazole and proceeded with the buffers of increasing imidazole concentration, as 40, 60, 100, 300, and 500 mM of imidazole. The eluate was collected in 1 ml fractions. The different fractions were checked by SDS-PAGE.

4.12.2 Protein determination by MALDI-TOF

After destaining, the expected band of OprF fusion protein was cut from polyacrylamide gel, dried by Centrifugal Vacuum Concentrator, and sent to Proteomics International, Australia for analysis. The protein sample was trypsin digested and peptides extracted according to standard techniques (Bringans et al. Proteomic 2008). Peptides were analyzed by MALDITOF-TOF mass spectrometer

using a 4800 Proteomics Analyzer. Mascot sequence matching software was used for analyzed spectra to identify protein of interest.

4.12.3 Desalting (buffer exchange)

The purified OprF fusion protein was exchanged buffer by using Amicon Ultra-4 10K Centrifugal Filter Device according to the manufacturer's instruction. Initially, the protein solution was diluted with MQ water to have a concentration of 100 mM imidazole (following the chemical compatibility of this device). The filter was pre-rinsed with MQ water, filled with 3.5 ml of the protein solution, and centrifuged at 7,500 × g for 15 minutes. The flow-through was discarded and the left protein solution was filled in the filter and centrifuged again. The flow-through was discarded while the concentrated solute was filled with MQ water (3.5 ml) and centrifuged at the same condition, this step was repeated until the concentration of salt and imidazole was very low. The final volume of concentrated solute was sterilized through 0.45 µm membrane filter and measured protein concentration.

4.13 Measurement of protein concentration

$(His)_6$-tagged OprF fusion protein was determined concentration by using Bio-Rad Protein Assay, based on the method of Bradford. Dry reagent was prepared by diluting 1 part Dye Reagent Concentrate with 4 parts deionized water. The protein standard was prepared in a linear range of 10 to 50 (µg/ml). For the sample, 10 µl of OprF fusion protein was pipette into an eppendorf with 975 µl dry reagent and 15 µl deionized water. The mixture of each tube was vortexed, incubated at room temperature for 10 minutes directly, and measured absorbance at 595 nm by spectrophotometer.

Table 4.3 Preparation of standard curve for Bio-Rad Protein Assay

2 mg/ml BSA (µl)	Water (µl)	Bio-Rad reagent 1:5 dilute in water (µl)	Concentration (µg/ml)
0	25	975	0
5	20	975	10
10	15	975	20
15	10	975	30
20	5	975	40
25	0	975	50

4.14 Generation of anti-OprF polyclonal antibody

The purified protein was desalted and sterilized through 0.45 µm membrane filter before used as an antigen. The 200 µl of antigen (0.4 mg/ml concentration) and 200 µl of Incomplete Freund's Adjuvant were injected intramuscularly into each hind legs of a New Zealand white rabbit (200 µl of mixture per site) and given booster immunization in intervals of 7 and 14 days (at day 7 and day 21 after blood collections). The blood was drawn from the marginal ear vain by syringe on days 0 (pre-immunization), 7 (for 1st immunization test), 21 (for 2nd immunization test), and 28 (for 3th immunization test). For day 0, 7, and 21, the bloods were collected in the volume of 1 ml, while the final blood collection was collected in 5 ml. In this study, no need to collect blood on day 14, because there were two boosters (the second booster was on day 21). The animal will not be disturbed unnecessarily on day 14. The collected blood was placed at 37 °C for 2 hours to inactivate complement. The clotted blood was centrifuged at 4,000 rpm, 4 °C for 10 minutes, placed at 4 °C for overnight, and centrifuged again to separate serum. Then, the serum was stored at -20 °C until use.

Table 4.4 Schedule of immunization and blood collection

Day	Procedure	Blood volume
0	Blood collection (pre-immunization) and 1st immunization	1 ml
7	Blood collection and 2nd immunization	1 ml
21	Blood collection and 3th immunization	1 ml
28	Final blood collection	5 ml

4.15 Western blot analysis

Many strains of *P. aeruginosa* such as ATCC 27853, PAO1, and strains from clinical isolates (PA65, PA70, PA80, PA100, PA102, and PA103) including others species of *Pseudomonas* (*P. alcaligenes, P. fluorescens, P. mendocina, P. putida, P. stutzeri, P. syringae*) and *E. coli* (ATCC 25922 and BL21) were used in Western blot analysis to determine specificity of OprF protein to *P. aeruginosa*. For sample preparation, the bacterial cells were cultured in 5 ml LB broth for overnight with shaking. *P. aeruginosa* and *E. coli* were grown at 37 °C whereas the other species of *Pseudomonas* were grown at 30 ± 2 °C. The cell cultures were adjusted concentration to 9 McFarland and 500 µl of the cultures were centrifuged at 8,000 rpm for 1 minute to precipitate cells. The supernatants were discarded and the cell pellets were resuspended (by vortex) in 100 µl of 1x SDS-PAGE sample buffer and heated at 95 °C for 5 minutes. Total cell protein samples (2 µl) were loaded in SDS-PAGE with 12% acrylamide in running gel. The power supply was set to voltage of 70 V which could be adjusted to 100 V when the proteins flowed to 1 ml of separating gel. The proteins were run on SDS-PAGE for 150 minutes.

Before blotting, the stacking gel was cut off and trashed. The resolving gel was rinsed with distilled water and soaked in transfer buffer for 15 minutes. PVDF (polyvinyl difluoride) membrane was soaked in methanol for a few second and equilibrated in transfer buffer for 15 minutes. With semi-dry transfer, the blotting sandwich in the following order: Whatman papers (3-4 sheets), gel, membrane,

Whatman papers was assembled. The semi-dry transfer cell (Bio-Rad) was set to constant current (10 mA) for 90 minutes. The blotted membrane was rinsed with distilled water and then incubated for 1 hour with shaking at low speed in blocking buffer (3% BSA in 0.1% (v/v) Tween-20 in PBS). After blocking, the blot was incubated at room temperature in 20 ml diluted primary antibody (1:300,000 diluted in PBS-T with 1% BSA) for 1 hour with shaking at low speed. The membrane was washed 4-5 times with PBS-T for 2 hours. For indirect detection, the membrane was incubated in 20 ml of secondary antibody for 2 hour with shaking at room temperature. Anti-rabbit IgG, HRP-labeled was diluted into 1:6,500 in PBS-T with 1% BSA. Next step, the membrane was washed 4-5 times with PBS-T for 30 minutes. The membrane was detected chromogenically with DAB (diaminobenzidine) substrate. The DAB peroxidase substrate solution (3-5 ml) was poured to cover the membrane, incubated with a few second (until the interested protein band appear) and the reaction was stopped by rinsing with distilled water.

CHAPTER V
RESULTS

5.1 Detection of *oprF* gene in *P. aeruginosa* and determination of the PCR product

P. aeruginosa 6 strains: ATCC 27853, PAO1, and clinical strains PA10, PA70, PA80, and PA100 were examined the existence of *oprF* gene by polymerase chain reaction (PCR) procedure. The PCR products were produced as a DNA fragment of 1,057 bp with forward primer: 5´-CACCATGAAACTGAAGAACACC-3´ and reverse primer: 5´-TTACTTGGCTTCAGCTTCTACTTC-3´ (Figure5.1). The specificity of the amplification was confirmed by restriction endonuclease analysis. DNA cleavage with *Sma*I restriction endonuclease produced two fragments of 742 bp and 315 bp (Figure 5.2).

5.2 Amplification of *oprF* gene

The whole *oprF* gene was amplified from genomic DNA of *P. aeruginosa* clinical strain PA10 for using in cloning and expression. The PCR products were produced as a DNA fragment of 1,069 bp with forward primer: 5´-AGGATCCG ATGAAACTGAAGAACAC-3´ and reverse primer: 5´-CTAAGCTTTTACTTG GCTTCAGCTTC-3´ (Figure 5.3). The primers were designed to have *BamH*I and *Hind*III sites in forward and reverse primers, respectively that facilitated cloning into the pET-28b vector.

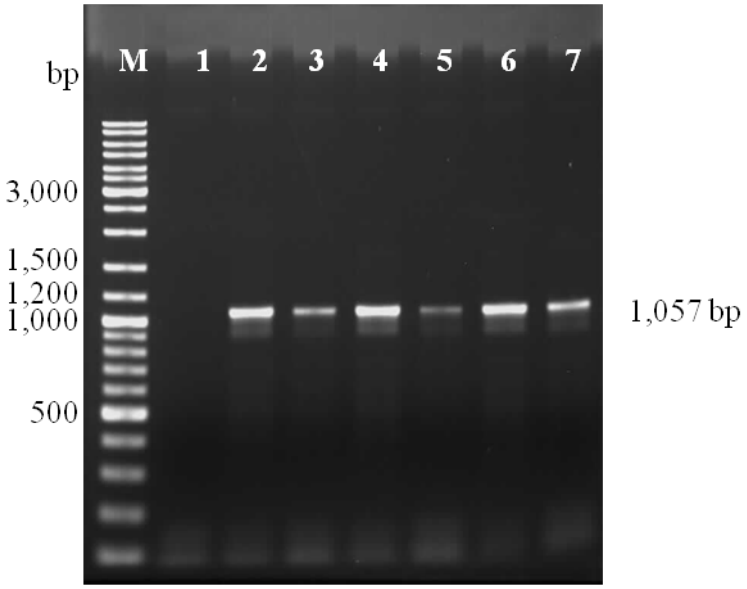


Figure 5.1 Agarose gel electrophoresis of PCR products obtained with forward primer: 5´-CACCATGAAACTGAAGAACACC-3´ and reverse primer: 5´-TTACTTG GCTTCAGCTTCTACTTC-3´ targeting the whole *oprF* gene from *P. aeruginosa* ATCC 27853, PAO1, and 4 clinical isolates.

Lane M: Thermo Scientific GeneRuler DNA Ladder (100-10,000 bp)
Lane 1: Negative control without template
Lane 2: *P. aeruginosa* ATCC 27853
Lane 3: *P. aeruginosa* PAO1
Lane 4: *P. aeruginosa* clinical strain PA10
Lane 5: *P. aeruginosa* clinical strain PA70
Lane 6: *P. aeruginosa* clinical strain PA80
Lane 7: *P. aeruginosa* clinical strain PA100

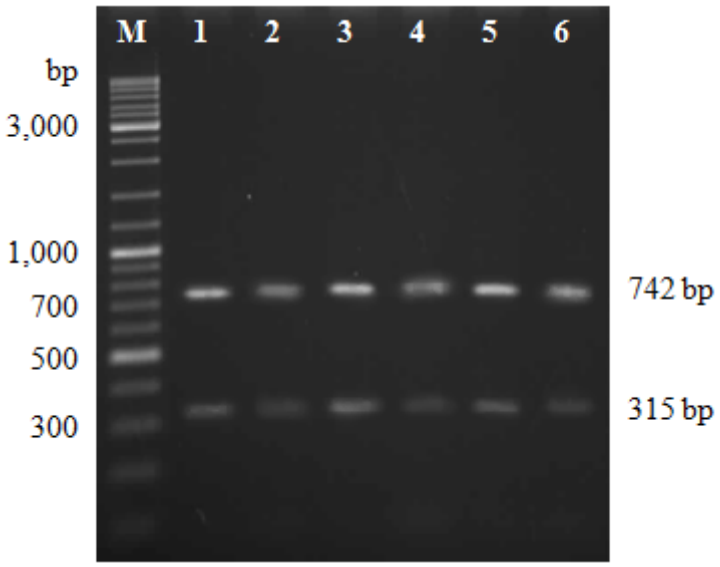


Figure 5.2 Restriction enzyme analysis of 1,057 bp PCR products from *P. aeruginosa* ATCC 27853, PAO1, and 4 clinical isolates using *Sma*I. The digestion resulted in generation of two smaller sized products (742 bp and 315 bp).

Lane M: Thermo Scientific GeneRuler DNA Ladder (100-10,000 bp)

Lane 1: *P. aeruginosa* ATCC 27853

Lane 2: *P. aeruginosa* PAO1

Lane 3: *P. aeruginosa* clinical strain PA10

Lane 4: *P. aeruginosa* clinical strain PA70

Lane 5: *P. aeruginosa* clinical strain PA80

Lane 6: *P. aeruginosa* clinical strain PA100

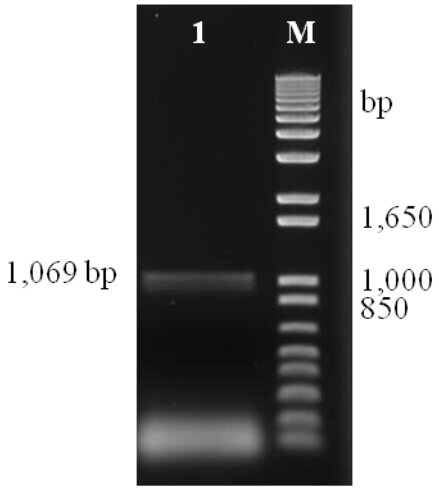


Figure 5.3 Agarose gel electrophoresis of PCR products obtained with forward primer: 5´-AGGATCCGATGAAACTGAAGAACAC-3´ and reverse primer: 5´-CTAAGCTTTTACTTGGCTTCAGCTTC-3´ targeting the whole *oprF* gene from *P. aeruginosa* clinical strain PA10.

Lane M: 1 kb Plus DNA Ladder (Invitrogen)

Lane 1: *P. aeruginosa* PA10

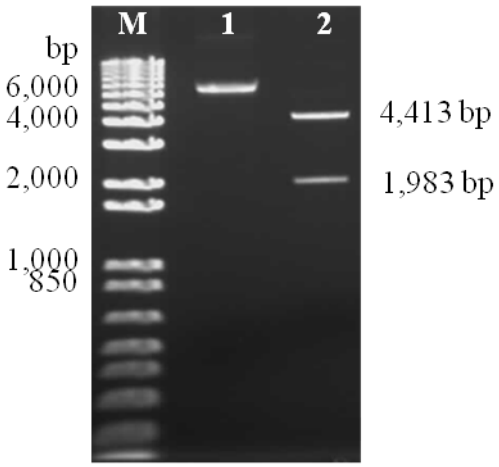


Figure 5.4 Agarose gel electrophoresis of the recombinant plasmid determined by restriction endonuclease (*Sma*I) analysis

Lane M: 1 kb Plus DNA Ladder (Invitrogen)

Lane 1: Undigested recombinant plasmid

Lane 2: *Sma*I digested recombinant plasmid

5.3 Cloning of *P. aeruginosa oprF* gene

The amplicon size of 1,069 bp containing the *oprF* gene amplified from *P. aeruginosa* PA10 was cloned into the pET-28b plamid and transformed into *E. coli* TOP10 cells successfully. Restriction endonuclease analysis, *Sma*I was used to confirm the presence and correct orientation of the *oprF* gene. This restriction enzyme cut once in the pET-28b and once in the *oprF* gene. The digested recombinant plasmid containing correct orientation of the *oprF* gene appeared 2 bands of 1,983 and 4,413 bp (Figure 5.4). DNA sequencing showed that the recombinant plasmid pET 28b-*oprF* had an in-frame translation of the inserted *oprF* gene (Figure 5.5). There was six bases in the *oprF* sequence dissimilar with the *oprF* gene of *P. aeruginosa* PAO1 (Figure 5.6).

5.4 Expression of $(His)_6$-tagged OprF fusion protein

$(His)_6$-tagged OprF fusion protein was expressed in *E. coli* BL21 (DE3). The predicted 48 kDa fusion protein was produced as the form of inclusion bodies under IPTG induction. No protein production was observed in *E. coli* BL21 without IPTG induction. From the pilot scale expression, 12% SDS-PAGE exhibited $(His)_6$-tagged OprF fusion protein after 1 mM IPTG induction for 1, 2, 3, and 4 hours (Figure 5.7). This high-level expression protein in inclusion bodies could be solubilized in the solubilization buffer containing 6 M urea (Figure 5.8).

```
1   ATG GGC AGC AGC CAT CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG   45
1   Met Gly Ser Ser His His His His His His Ser Ser Gly Leu Val   15

46  CCG CGC GGC AGC CAT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG   90
16  Pro Arg Gly Ser His Met Ala Ser Met Thr Gly Gly Gln Gln Met   30

91  GGT CGG GAT CCG ATG AAA CTG AAG AAC ACC TTA GGC GTT GTC ATC   135
31  Gly Arg Asp Pro Met Lys Leu Lys Asn Thr Leu Gly Val Val Ile   45

136 GGC TCG CTG GTT GCC GCT TCG GCA ATG AAC GCC TTT GCC CAG GGC   180
46  Gly Ser Leu Val Ala Ala Ser Ala Met Asn Ala Phe Ala Gln Gly   60

181 CAG AAC TCG GTA GAG ATC GAA GCC TTC GGC AAG CGC TAC TTC ACC   225
61  Gln Asn Ser Val Glu Ile Glu Ala Phe Gly Lys Arg Tyr Phe Thr   75

226 GAC AGC GTT CGC AAC ATG AAG AAC GCG GAC CCG TAC GGC GGC TCG   270
76  Asp Ser Val Arg Asn Met Lys Asn Ala Asp Pro Tyr Gly Gly Ser   90

271 ATC GGT TAC TTC CTG ACC GAC GAC GTC GAG CTG GCG CTG TCC TAC   315
91  Ile Gly Tyr Phe Leu Thr Asp Asp Val Glu Leu Ala Leu Ser Tyr   105

316 GGT GAG CAC CAT GAC GTT CGT GGC ACC TAC GAA ACC GGC AAC AAG   360
106 Gly Glu His His Asp Val Arg Gly Thr Tyr Glu Thr Gly Asn Lys   120

361 AAG GTC CAC GGC AAC CTG ACC TCT CTG GAC GCC ATC TAC CAC TTC   405
121 Lys Val His Gly Asn Leu Thr Ser Leu Asp Ala Ile Tyr His Phe   135

406 GGT ACC CCG GGC GTA GGT CTG CGT CCG TAC GTG TCG GCT GGT CTG   450
136 Gly Thr Pro Gly Val Gly Leu Arg Pro Tyr Val Ser Ala Gly Leu   150

451 GCT CAC CAG AAC ATC ACC AAC ATC AAC AGC GAC AGC CAA GGC CGT   495
151 Ala His Gln Asn Ile Thr Asn Ile Asn Ser Asp Ser Gln Gly Arg   165

496 CAG CAG ATG ACC ATG GCC AAC ATC GGC GCT GGT CTG AAG TAC TAC   540
166 Gln Gln Met Thr Met Ala Asn Ile Gly Ala Gly Leu Lys Tyr Tyr   180

541 TAC ACC GAG AAC TTC TTC GCC AAG GCC AGC CTC GAC GGC CAG TAT   585
181 Tyr Thr Glu Asn Phe Phe Ala Lys Ala Ser Leu Asp Gly Gln Tyr   195

586 GGT CTG GAG AAG CGT GAC AAC GGT CAC CAG GGC GAG TGG ATG GCT   630
196 Gly Leu Glu Lys Arg Asp Asn Gly His Gln Gly Glu Trp Met Ala   210
```

Figure 5.5 Amino acids translated from DNA sequencing of the recombinant *oprF* gene. The sequence started with start codon and followed with 6-histidine tag. The whole *oprF* gene initiated at position 35 and stopped at TAA (stop codon) which totally had 350 amino acids. The result indicating that the recombinant plasmid pET 28b- *oprF* had an in-frame translation of the inserted *oprF* gene.

631 GGC CTG GGC GTC GGC TTC AAC TTC GGT GGT TCG AAA GCC GCT CCG 675
211 Gly Leu Gly Val Gly Phe Asn Phe Gly Gly Ser Lys Ala Ala Pro 225

676 GCT CCG GAA CCG GTT GCC GAC GTT TGC TCC GAC TCC GAC AAC GAC 720
226 Ala Pro Glu Pro Val Ala Asp Val Cys Ser Asp Ser Asp Asn Asp 240

721 GGC GTT TGC GAC AAC GTC GAC AAG TGC CCG GAT ACC CCG GCC AAC 765
241 Gly Val Cys Asp Asn Val Asp Lys Cys Pro Asp Thr Pro Ala Asn 255

766 GTC ACC GTT GAC GCC AAC GGC TGC CCG GCT GTC GCC GAA GTC GTA 810
256 Val Thr Val Asp Ala Asn Gly Cys Pro Ala Val Ala Glu Val Val 270

811 CGC GTA CAG CTG GAC GTG AAG TTC GAC TTC GAC AAG TCC AAG GTC 855
271 Arg Val Gln Leu Asp Val Lys Phe Asp Phe Asp Lys Ser Lys Val 285

856 AAA GAG AAC AGC TAC GCT GAC ATC AAG AAC CTG GCT GAC TTC ATG 900
286 Lys Glu Asn Ser Tyr Ala Asp Ile Lys Asn Leu Ala Asp Phe Met 300

901 AAG CAG TAC CCG TCC ACT TCC ACC ACC GTT GAA GGT CAC ACC GAC 945
301 Lys Gln Tyr Pro Ser Thr Ser Thr Thr Val Glu Gly His Thr Asp 315

946 TCC GTC GGC ACC GAC GCT TAC AAC CAG AAG CTG TCC GAG CGT CGT 990
316 Ser Val Gly Thr Asp Ala Tyr Asn Gln Lys Leu Ser Glu Arg Arg 330

991 GCC AAC GCC GTT CGT GAC GTA CTG GTC AAC GAG TAC GGC GTA GAA 1035
331 Ala Asn Ala Val Arg Asp Val Leu Val Asn Glu Tyr Gly Val Glu 345

1036 GGT GGT CGC GTG AAC GCT GTT GGT TAC GGC GAG TCC CGC CCG GTT 1080
346 Gly Gly Arg Val Asn Ala Val Gly Tyr Gly Glu Ser Arg Pro Val 360

1081 GCC GAC AAC GCC ACC GCT GAA GGC CGC GCT ATC AAC CGT CGC GTT 1125
361 Ala Asp Asn Ala Thr Ala Glu Gly Arg Ala Ile Asn Arg Arg Val 375

1126 GAA GCC GAA GTA GAA GCT GAA GCC AAG TAA 1155
376 Glu Ala Glu Val Glu Ala Glu Ala Lys End 385

Figure 5.5 Amino acids translated from DNA sequencing of the recombinant *oprF* gene. The sequence started with start codon and followed with 6-histidine tag. The whole *oprF* gene initiated at position 35 and stopped at TAA (stop codon) which totally had 350 amino acids. The result indicating that the recombinant plasmid pET 28b- *oprF* had an in-frame translation of the inserted *oprF* gene (continued).

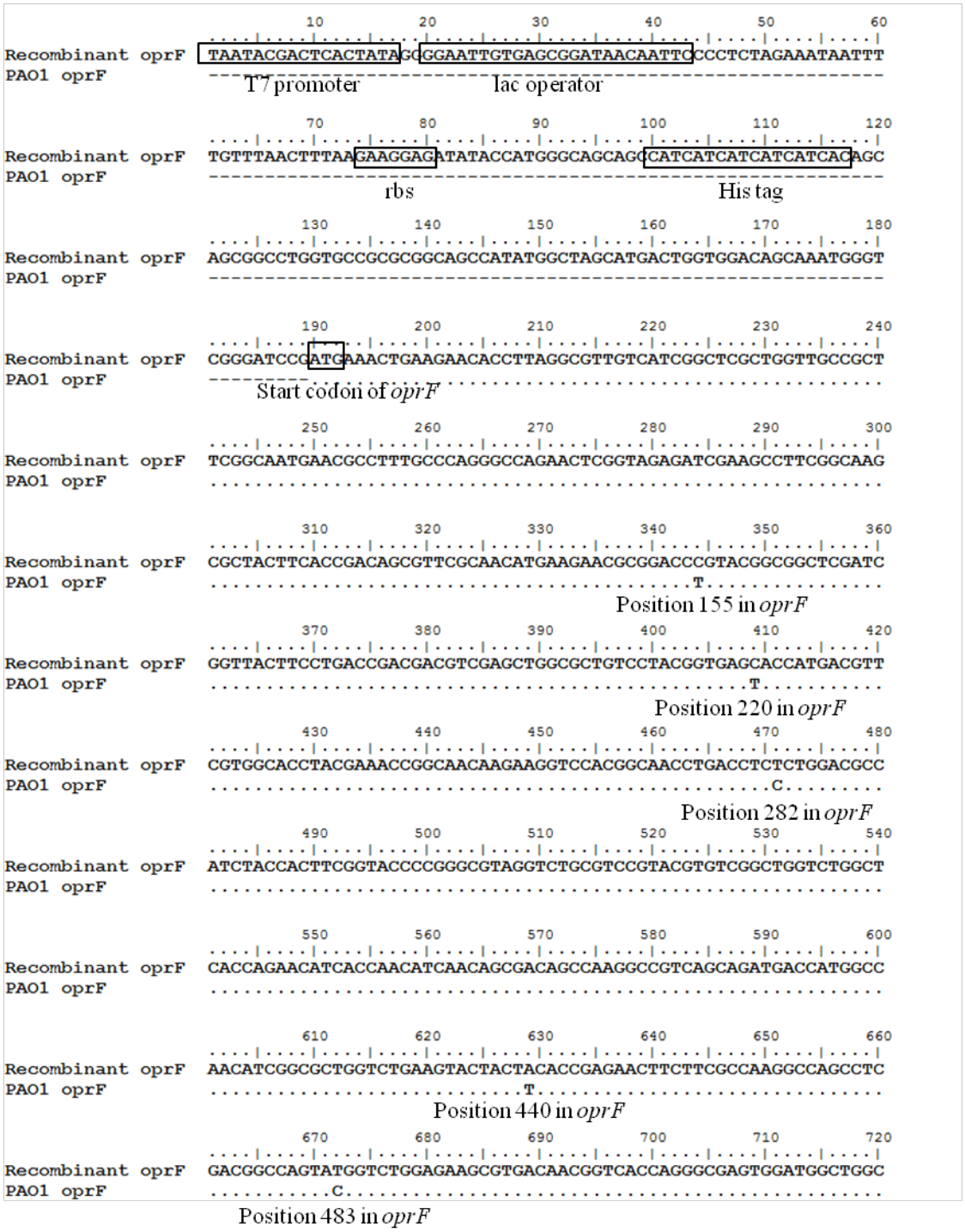


Figure 5.6 DNA sequencing of the recombinant plasmid pET 28b- *oprF* obtained with T7 promoter primer, T7 terminator primer, and the walking primers. The sequence was compared with *oprF* gene of *P. aeruginosa* PAO1, the bottom dots meant bases of *P. aeruginosa* PAO1 similar to the recombinant *oprF*. There was 6 bases at positions 155, 220, 282, 440, 483, and 927 different from *oprF* of PAO1.

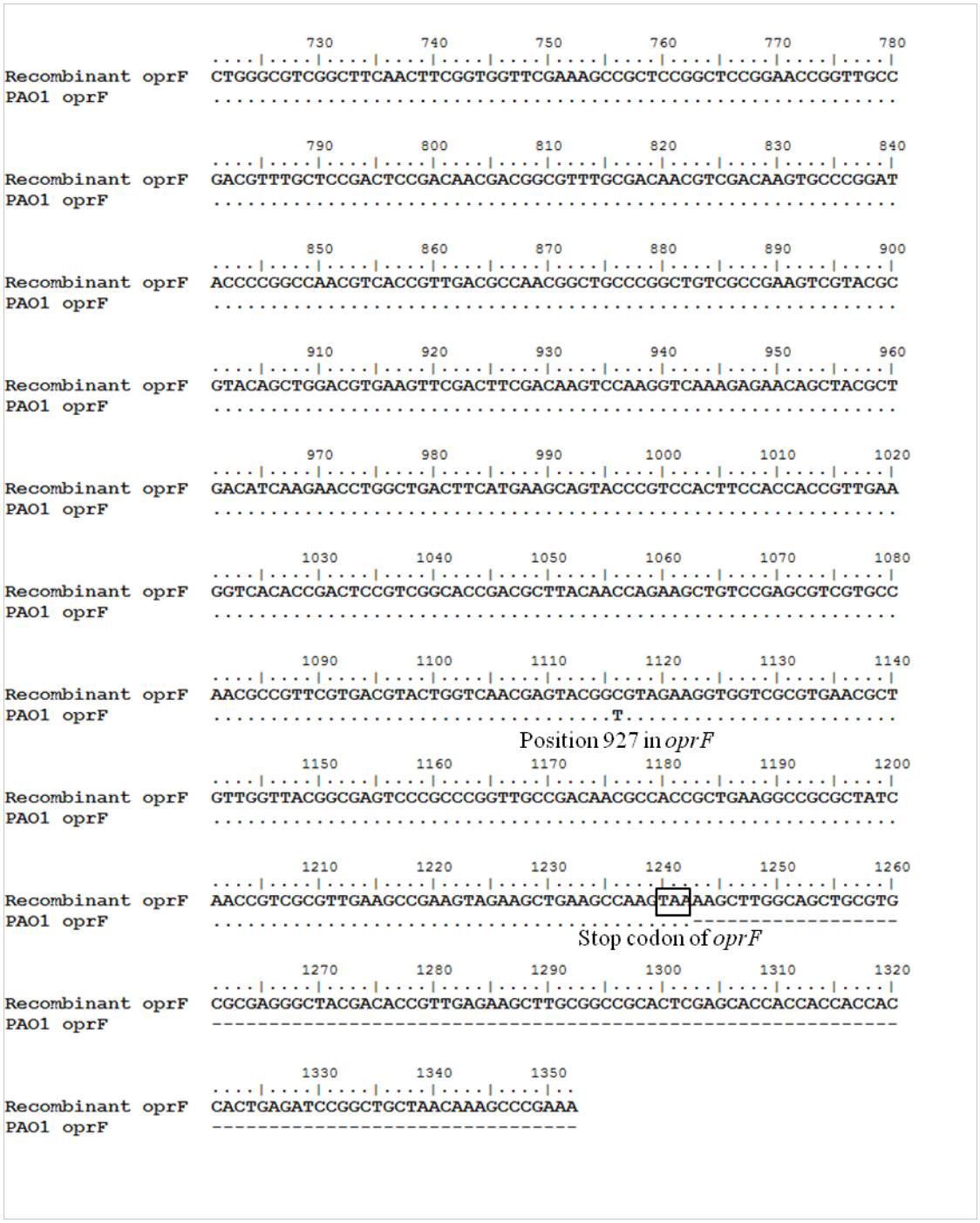


Figure 5.6 DNA sequencing of the recombinant plasmid pET 28b- *oprF* obtained with T7 promoter primer, T7 terminator primer, and the walking primers. The sequence was compared with *oprF* gene of *P. aeruginosa* PAO1, the bottom dots meant bases of *P. aeruginosa* PAO1 similar to the recombinant *oprF*. There was 6 bases at positions 155, 220, 282, 440, 483, and 927 different from *oprF* of PAO1 (continued).

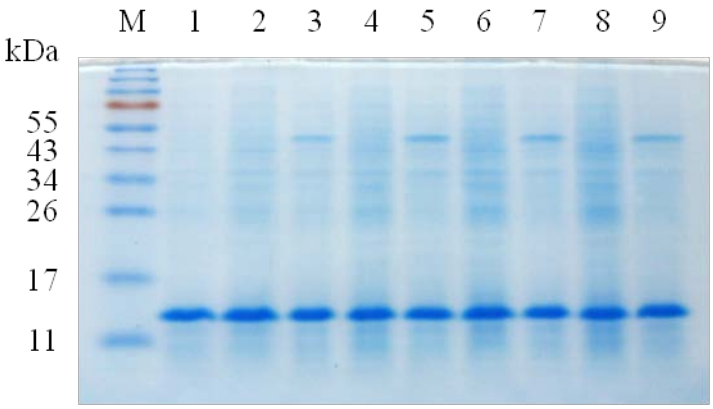


Figure 5.7 Expression of $(His)_6$-tagged OprF fusion protein in *E. coli* BL21. Lane 1-9 showed total protein of the *E. coli* cells with/without IPTG induction. Lane M: Prestained Protein Ladder (Fermentas), lane 1: uninduced cells at 0 h, lane 2: uninduced cells at 1 h, lane 3: induced cells at 1 h, lane 4: uninduced cells at 2 h, lane 5: induced cells at 2 h, lane 6: uninduced cells at 3 h, lane 7: induced cells at 3 h, lane 8: uninduced cells at 4 h, and lane 9: induced cells at 4 h.

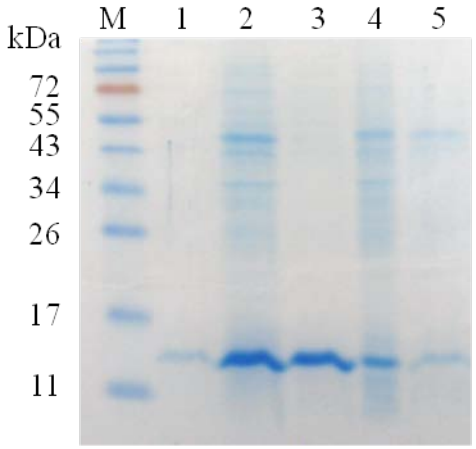


Figure 5.8 Analysis of soluble/insoluble form of $(His)_6$-tagged OprF fusion protein. Lane M: Prestained Protein Ladder, lane 1: total protein of uninduced cells, lane 2: total protein of induced cells (3 h), lane 3: protein from supernatant after cell lysis, lane 4: protein from pellet after cell lysis, and lane 5: protein from the pellet after solubilization

5.5 Purification of $(His)_6$-tagged OprF fusion protein

$(His)_6$-tagged OprF fusion protein was purified using HisTrap HP column based on an affinity chromatographic method. The column pre-charged with Ni^{2+} ions was bound strongly by His-tagged protein and eluted with buffers containing imidazole. When optimum purity was required, the optimization protocol for stepwise gradient elution was performed. The stepwise elution contained a constant concentration of phosphate buffer and increasing concentration of imidazole. An appropriate concentration of imidazole was needed to prevent non-specific binding of contaminants. After optimization, the elution buffers with 300 mM and 500 mM imidazole were used for purification. Protein fraction eluted by phosphate buffer containing 500 mM imidazole was used in the further experiment and expected to be more pure than the fractions from elution with 300 mM imidazole. The fusion protein analyzed by 12% SDS-PAGE showed a band of approximately 48 kDa (Figure 5.9). The eluted protein band was cut from the gel and analyzed by using MALDI-TOF mass spectrometry in order to confirm OprF protein. The Mascot search results showed Outer membrane porin F of *Pseudomonas* sp. 2_1_26 was the top protein hit with high protein score. However, Outer membrane porin F Tax_Id=208964 *P. aeruginosa* was protein matching the same set of peptides and its amino acids sequence was totally similar to Outer membrane porin F of *Pseudomonas* sp. 2_1_26. The purified peptide matched the outer membrane porin F precursor of *P. aeruginosa* with 32% sequence coverage (Figure 5.11).

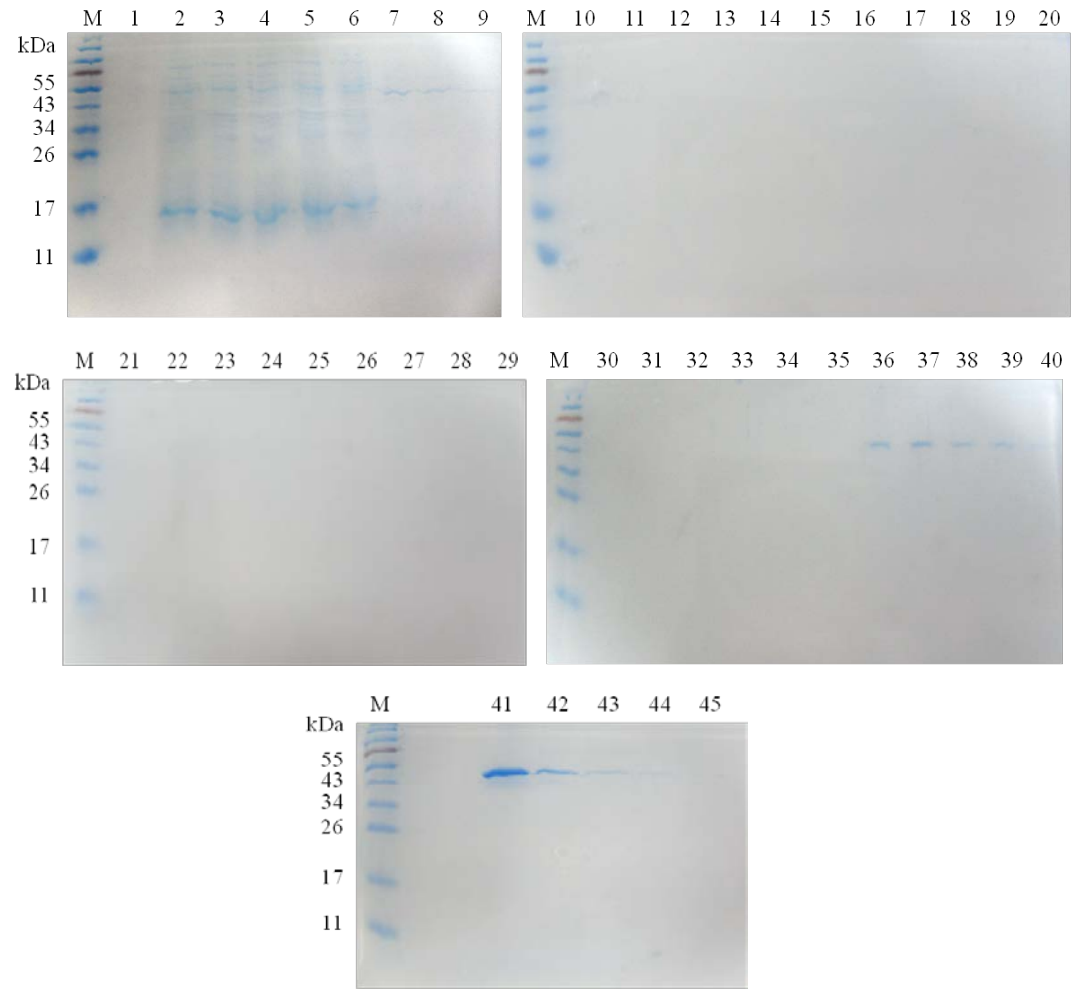


Figure 5.9 Purification of $(His)_6$-tagged OprF fusion protein using HisTrap column. For imidazole gradient elution, the column was loaded with 5 ml of each elution buffer and collected the eluate in 1 ml fractions. The fusion protein was eluted at 300 and 500 mM imidazole concentration and showed an approximately 48 kDa band.

Lane M: Prestained Protein Ladder

Lane 1-5: Flow-through fractions (applied with 5 ml of the protein sample)

Lane 6-15: Wash fraction 1-10 (washed with 10 ml of 5 mM imidazole elution buffer)

Lane 16-20: Elution fraction 1-5 (eluted with 20 mM imidazole elution buffer)

Lane 21-25: Elution fraction 6-10 (eluted with 40 mM imidazole elution buffer)

Lane 26-30: Elution fraction 11-15 (eluted with 60 mM imidazole elution buffer)

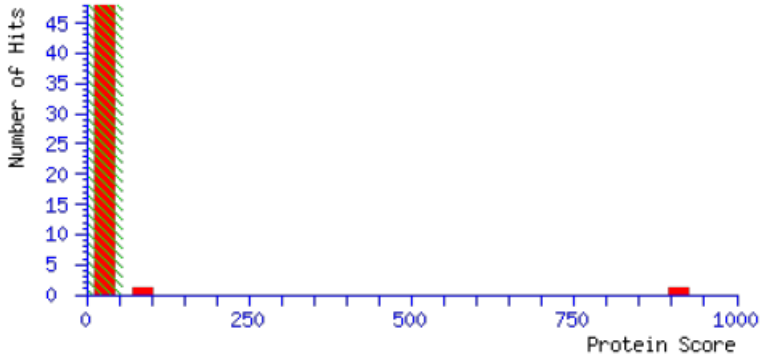
Lane 31-35: Elution fraction 16-20 (eluted with 100 mM imidazole elution buffer)

Lane 36-40: Elution fraction 21-25 (eluted with 300 mM imidazole elution buffer)

Lane 41-45: Elution fraction 26-30 (eluted with 500 mM imidazole elution buffer)

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 53 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. G5FTC9 **Mass:** 37616 **Score:** 909 **Matches:** 12(8) **Sequences:** 12(8)
tr|G5FTC9|Outer membrane porin F Tax_Id=665948 [Pseudomonas sp. 2_1_26]
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
4	838.4130	837.4057	837.4055	0.0003	0	39	1.8	3	U	K.NLADFMK.Q
6	887.4415	886.4343	886.4185	0.0158	0	45	0.59	1	U	R.YFTDSVR.N
8	1043.5302	1042.5229	1042.5196	0.0033	1	37	3.1	1	U	K.RYFTDSVR.N
10	1180.5856	1179.5783	1179.5771	0.0012	0	65	0.0041	1	U	K.ASLDGQVGLEK.R
12	1230.6490	1229.6417	1229.6251	0.0166	1	97	2.7e-06	1	U	R.RVEAEVEAEAK.-
14	1336.7058	1335.6985	1335.6783	0.0203	1	84	5.3e-05	1	U	K.ASLDGQVGLEKR.D
18	1362.6799	1361.6726	1361.6795	-0.0069	0	78	0.0002	1	U	R.QQMTMANIGAGLK.Y
20	1406.7111	1405.7038	1405.6838	0.0201	0	102	8.3e-07	1	U	R.DVLVNEYGVEGGR.V
28	1917.9929	1916.9856	1916.9704	0.0152	1	79	0.00014	1	U	R.ANAVRDVLVNEYGVEGGR.V
34	2133.0457	2132.0384	2132.0246	0.0138	0	110	1e-07	1	U	R.VNAVGYGESRPVADNATAEGR.A
38	2586.2222	2585.2149	2585.1518	0.0631	0	152	4.2e-12	1	U	K.QYPSTSTTVEGHTDSVGTDAVNQK.L
40	3520.7432	3519.7359	3519.6978	0.0381	1	20	43	1	U	R.DVLVNEYGVEGGRVNAVGYGESRPVADNATAEGR.A

Proteins matching the same set of peptides:
P13794 **Mass:** 37616 **Score:** 909 **Matches:** 12(8) **Sequences:** 12(8)
sp|P13794|Outer membrane porin F Tax_Id=208964 [Pseudomonas aeruginosa]

2. H0J8Y8 **Mass:** 37337 **Score:** 94 **Matches:** 1(1) **Sequences:** 1(1)
tr|H0J8Y8|Major porin and structural outer membrane porin OprF Tax_Id=1112217 [Pseudomonas psychrotolerans L19]
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
34	2133.0457	2132.0384	2132.0246	0.0138	0	94	3.3e-06	2	U	R.VNAVGYGESRPVADNATEAGR.A

Figure 5.10 Mascot search results of the digested 48 kDa-OprF fusion protein. The results showed that the protein score of the His-tagged OprF was 909 and 94 (the scores more than 53 indicate identity or extensive homology, p<0.05). The higher score showed the better identification, so the fusion protein had much higher sequence coverage to Outer membrane porin F of *Pseudomonas* sp. 2_1_26 and *P. aeruginosa*.

MASCOT Search Results

Protein View: P13794

sp|P13794|Outer membrane porin F Tax_Id=208964 [Pseudomonas aeruginosa]

Database:	LudwigNR
Score:	909
Nominal mass (M_r):	37616
Calculated pI:	4.98
Taxonomy:	Unknown species

Sequence similarity is available as an NCBI BLAST search of P13794 against nr.

Search parameters

MS data file:	C:\Program Files\applied biosystems\Proteomics\120802\ppw_M13_134749844200.txt
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Variable modifications:	Oxidation (M)

Protein sequence coverage: 32%

Matched peptides shown in *bold red*.

```
  1 MKLKNTLGVV IGSLVAASAM NAFAQGQNSV EIEAFGKRYF TDSVRNMKNA
 51 DLYGGSIGYF LTDDVELALS YGEYHDVRGT YETGNKKVHG NLTSLDAIYH
101 FGTPGVGLRP YVSAGLAHQN ITNINSDSQG RQQMTMANIG AGLKYYFTEN
151 FFAKASLDGQ YGLEKRDNGH QGEWMAGLGV GFNFGGSKAA PAPEPVADVC
201 SDSDNDGVCD NVDKCPDTPA NVTVDANGCP AVAEVVRVQL DVKFDFDKSK
251 VKENSYADIK NLADFMKQYP STSTTVEGHT DSVGTDAYNQ KLSERRANAV
301 RDVLVNEYGV EGGRVNAVGY GESRPVADNA TAEGRAINRR VEANVEAEAK
```

Unformatted sequence string: 350 residues (for pasting into other applications).

Figure 5.11 Mascot search results showed protein sequence coverage of $(His)_6$-tagged OprF fusion protein. The results indicating that the peptides matched an outer membrane porin F of *P. aeruginosa* with 32% protein sequence coverage.

5.6 Measurement of protein concentration

After desalting and sterilizing, $(His)_6$-tagged OprF fusion protein was determined concentration by Bio-Rad Protein Assay prior use for injection. In the Bio-Rad Protein Assay, a Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding with protein and the colour change responds to protein concentration. The protein sample was prepared and measured absorbance at 595 nm. Protein standard curve from bovine serum albumin (BSA) provided a relative measurement of protein concentration. The fusion protein was diluted 1:100 in the dye reagent and the average absorbance (for duplicate) was equal to 0.108. The fusion protein's concentration was calculated using an equation of standard curve: $y = 0.027x$ and the resulting concentration of $(His)_6$-tagged OprF fusion protein was 0.40 mg/ml (Figure 5.12).

The sterile fusion protein was used for polyclonal antibody production in the amount of approximately 80 µg per each immunization. Western blot was carried out to determine specificity of OprF protein to *P. aeruginosa* by using the obtained anti-OprF polyclonal antibody as primary antibody.

5.7 Western blot analysis

Total cell proteins of *P. aeruginosa* ATCC 27853, PAO1, and 6 clinical strains, *P. alcaligenes*, *P. fluorescens*, *P. mendocina*, *P. putida*, *P. stutzeri*, *P. syringae*, and *E. coli* ATCC 25922, BL21 were determined the appearance of OprF protein in these organisms by Western blot analysis using the antibody against the recombinant OprF. A 38-kDa band corresponding to the native form of OprF protein was clearly detected in *P. aeruginosa* ATCC 27853, PAO1, and 6 clinical strains (PA65, PA70, PA80, PA100, PA102, and PA103). The specific band for OprF did not appear in other *Pseudomonas* species and *E. coli*. Although the immunoblot showed cross-reaction in other *Pseudomonas* species with a pale band at a size less than 38 kDa, no specific banding pattern in *E. coli* ATCC 25922 and BL21 (Figure 5.13).

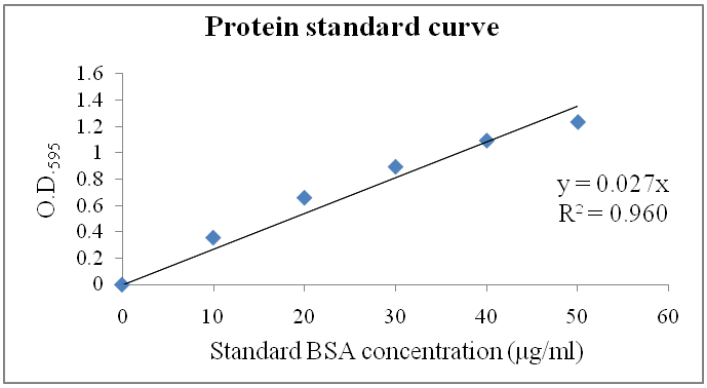


Figure 5.12 Protein standard curve for Bio-Rad Protein Assay. The absorbance of $(His)_6$-tagged OprF fusion protein diluted 1:100 was 0.108, therefore the fusion protein had a concentration of 0.40 mg/ml.

Figure 5.13 Western blot analysis of total cell proteins probed with anti-OprF polyclonal antibody. A native OprF protein of *P. aeruginosa* ATCC 27853, PAO1, and clinical strains were detected as a clear band at a size of 38 kDa.

Lane M: Prestained Protein Ladder (Fermentas)
Lane 1: *P. aeruginosa* ATCC 27853
Lane 2: *P. aeruginosa* PAO1
Lane 3: *P. aeruginosa* PA100
Lane 4: *P. alcaligenes* (DMST 21133)
Lane 5: *P. fluorescens* ATCC 13525
Lane 6: *P. mendocina* ATCC 25411
Lane 7: *P. putida* ATCC 12633
Lane 8: *P. stutzeri* (DMST 12562)
Lane 9: *P. syringae* ATCC 19310
Lane 10: *E. coli* ATCC 25922
Lane 11: *P. aeruginosa* ATCC 27853
Lane 12: *P. aeruginosa* PA65
Lane 13: *P. aeruginosa* PA70
Lane 14: *P. aeruginosa* PA80
Lane 15: *P. aeruginosa* PA102
Lane 16: *P. aeruginosa* PA103
Lane 17: *P. putida* (clinical isolate)
Lane 18: *E. coli* BL21
Lane 19: *E. coli* ATCC 25922

CHAPTER VI
DISCUSSION

P. aeruginosa is an important opportunistic pathogen that causes nosocomial infections of the respiratory and urinary tracts, bloodstream, wounds, and central nervous system. The infections by this organism are associated with morbidity and mortality, especially in immunocompromised patients that are often severe and life-threatening (1, 2, 26). *P. aeruginosa* has been reported that resistance to several antimicrobial drugs and limitation of therapeutic options subsequently (4). Resistance to carbapenems, which are the effective antibiotics for treatment of the infections caused by MDR *P. aeruginosa*, has increased steadily in recent years (6). Among these antibiotics, imipenem and meropenem have been used widely in Gram-negative bacteria including *P. aeruginosa* because of their safety and efficacy. However, the resistances are increasing common in *P. aeruginosa*. A development of resistance to imipenem is acquired through carbapenemases/metallo-β-lactamases (MBLs), down-regulation of OprD porin, and/or alteration of critical PBPs. About the mechanism of meropenem resistance, it is similar to that of imipenem, but only OprD loss is not enough for meropenem resistance. Opposite to the meropenem, downregulation of the OprD is a main mechanism for imipenem resistance (115). The OprD protein was used to generate polyclonal antibody for characterization of imipenem susceptibility in *P. aeruginosa* by using Western blot analysis. The result indicated that 98% of imipenem-and meropenem-resistant *P. aeruginosa* clinical isolates did not produce OprD (13). Other studies supported that the absence or weak expression of OprD was found predominantly in imipenem-resistant *P. aeruginosa* isolates in Thailand and other countries including France and Spain (6, 12, 14, 15).

The rapid and accurate method for diagnosis of *P. aeruginosa* infection is necessary for control the infections in time. Regarding to the OprD-mediated resistance, OprD detection technique was developed for imipenem susceptibility testing. Nevertheless, various Gram-negative bacteria other than *P. aeruginosa* (e.g.,

Enterobacter spp. and *Klebsiella pneumoniae*) also have OprD or OprD homologues (115). In addition, an OprD homologue in *A. baumannii* (43 kDa) was found to be involved in carbapenem resistance like *P. aeruginosa* (116). Hence, species confirmation of the pathogen should be considered as an internal control of the immunological test.

In the present study, the entire *oprF* gene from *P. aeruginosa* ATCC 27853, PAO1, and clinical strains of PA10, PA70, PA80, and PA100 were determined by PCR and showed the similar length corresponding *oprF* gene of *P. aeruginosa* PAO1 deposited in the NCBI databases. The *oprF* amplification was confirmed by restriction endonuclease analysis in which *Sma*I cut only one site (CCC|GGG) within the *oprF* gene at position 311. As the previous studies showed, the *oprF* is conserved among the 17 different serotype strains, clinical and environmental isolates of *P. aeruginosa* (107, 108). The nucleotide BLAST result at the *Pseudomonas* Genome Database demonstrated sequence identity of *oprF* gene among *P. aeruginosa* ranging from 99% to 100% (117). Moreover, OprF has been involved in *P. aeruginosa* virulence such as cellular adhesion and the production of quorum-sensing-dependent virulence factors exotoxin A, pyocyanin, and elastase (118). Therefore, the gene from *P. aeruginosa* clinical strain PA10 was chosen randomly as the representative of the *oprF* gene for cloning and expression experiments in order to produce anti-OprF polyclonal antibody.

OprF protein was produced as a recombinant protein using pET-28b plamid that gives advantages in a high-level protein expression, isolation and purification. To prepare the *oprF* amplicon for cloning into the pET-28b, primers were designed to incorporate *BamH*I at 5′ end and *Hind*III at 3′ end of the amplicon. However, the PCR amplicon was unable to amplify efficiently due to primer-dimer and hairpins formations, which influenced the palindromic restriction sites. Therefore, many PCR reactions were performed and then concentrated for ligation with the plasmid in a molar ratio of at least 1:1. At this ratio, one colony from six colonies that grew on the LB/kanamycin plate showed successful insertion. Therefore, the higher ratio of the insert to the vector could increase the achievement of recombinant plasmids. *Sma*I restriction endonuclease digested the recombinant plasmid at the *Sma*I sites in the pET-28b and the *oprF* gene and demonstrated 1,983 and 4,413 bp

fragments implying that the *oprF* gene inserted in correct orientation. The DNA sequencing with T7 forward and reverse primers showed an in-frame translation of the inserted *oprF* gene. However, there were six bases not identical to the *oprF* gene of *P. aeruginosa* PAO1: i.e., T155C, T220C, C282T, T440A, C483T, and T927C, resulted in 3 amino acids changes (Leu52Pro, Tyr74His, and Phe147Tyr). These amino acids were found at the positions not involved in structural change (19) implying that the *oprF* gene of *P. aeruginosa* clinical strain PA10 would have the protein structure and function similar to that of *P. aeruginosa* PAO1.

Regarding a cloning and expression vector, pET-28b plasmid carries T7*lac* promoter and a *lac* operator (*lac*O) sequence just downstream of the promoter. The *lac* operator serves as transcriptional regulator coordinating with *lac* repressor encoded by *lac*I gene. The target gene under control of the T7*lac* promoter is negatively regulated when the repressor bind with the *lac* operator. In this study, the full coding sequence of *oprF* gene was inserted in a multiple cloning site (*BamH*I/*Hind*III) and located downstream of His tag coding region of the pET-28b. His-tagged OprF protein was produced in *E. coli* BL21 (DE3). The *E. coli* strain contains λDE3 lysogen encoding T7 RNA polymerase under the control of IPTG-inducible *lac*UV5 promoter. For protein induction with IPTG, the IPTG binds to the *lac* repressor and causes a conformational change in the repressor that subsequent reduces affinity for the *lac* operator. Without the repressor bound to the *lac* operator, *E. coli* RNA polymerase is able to bind with the *lac*UV5 promoter and transcribe T7 RNA polymerase in the host chromosome. On the pET-28b vector, T7 RNA polymerase can bind with the T7*lac* promoter in the absence of the *lac* repressor thereby enables a six-histidine coding sequence and the *oprF* gene to be transcribed (119,120).

$(His)_6$-tagged OprF protein was highly expressed as the insoluble inclusion body form and recovered by lysozyme and freeze-thaw treatment. The fusion protein had molecular weight approximately 48 kDa calculated from types and number of amino acids that were transcribed. The predicted weight corresponded with the weight on 12% SDS-PAGE. The fusion protein was produced as aggregated insoluble protein in cytoplasm. The outer membrane protein produced with this expression system was supposed to be inactive due to the OprF folding into two-domain closed channels (a major conformer) in the outer membrane and is associated with Skp periplasmic

chaperone (19). However, the inclusion bodies have advantages in purification and protection from cellular protease (120). The fusion protein could be dissolved in solubilization buffer containing 6 M urea and 1 mM 2-mercaptoethanol. The solubilization was expected to bring monomolecular dispersion and minimum non-native intra-or inter-chain interactions (121).

The fusion protein was purified by using HisTrap HP column based on an affinity chromatography. A protein tagged with histidine residues can bind with Ni^{2+} ions immobilized in the column. For the stepwise gradient elution, $(His)_6$-tagged OprF protein was eluted by elution buffers containing 300 and 500 mM imidazole. However, at 300 mM imidazole, a host protein contaminant was found and the fusion protein couldn't be eluted completely. Hence, the column was eluted again at 500 mM imidazole resulted in the eluted protein had a high purity. As the concentration of imidazole needed to prevent non-specific binding, the binding buffer or solubilization buffer could contain a higher concentration of imidazole to 10 mM. The purified protein was determined by MALDI-TOF mass spectrometry in order to confirm OprF protein. The purpose of protein analysis was not to sequence the entire protein but instead to identify protein from peptide fragments compared with protein database searches (122). Mascot search results indicated that the peptides matched an outer membrane porin F of *P. aeruginosa* with 32% protein sequence coverage.

The identified protein was desalted by using Amicon® Ultra-4 Centrifugal Filter Device prior to rabbit immunization. In this step, toxic substances such as urea and imidazole were diluted to a safe level. The protein could be refolded by reducing concentration of urea. The desalted protein solution was sterilized through 0.45 µm membrane filter and measured the decreased concentration due to protein aggregation in the refolding. The total amount of OprF protein in three time intramuscular immunization was 240 µg, which ranged in the recommendation for immunizing rabbit. Incomplete Freund's Adjuvant (IFA) was mixed with the antigen at ratio of 1:1 in order to induce a high titer of the specific antibody (123). This emulsion based adjuvant is different from Complete Freund's Adjuvant (CFA), in that CFA is composed of killed *M. tuberculosis* that attributes to cellular immune response. However, IFA was suitable for this experiment, because it had significantly less side effects than CFA and could strongly enhance the production of antigen-specific

immunoglobulin G (IgG) (124). Polyclonal antibody against OprF protein was produced by a New Zealand White rabbit for characterization of *P. aeruginosa*. Rabbit is most commonly used for polyclonal antibody production in a small volume (<100 ml) (125). The young rabbit (16 weeks of age) was used, because the ages of 10-16 weeks have undetectable maternal IgG antibodies and potential immune system responsible to new antigens (123). Polyclonal antibodies are generated by antibody-producing plasma cells that differentiate from multiple B-cell clones. They recognize more than one epitope on the target molecule, which are opposite to monoclonal antibodies that recognize a single epitope (125). With these characteristics, monoclonal antibodies may not bind proteins in a denatured state or conformational change. The protein structure can be altered by many factors, including temperature, salt concentration, and pH. However, it's less concerned when using polyclonal antibodies. Furthermore, polyclonal antibodies are enough to screen the target protein from the other pseudomonads with rather high OprF homology (126). With these reasons, polyclonal antibody was used in Western blot analysis in this study.

The anti-OprF polyclonal antibody could bind specifically and significantly to OprF protein of *P. aeruginosa* ATCC 27853, PAO1, and 6 clinical strains. It showed high specificity in Western blot analysis when no cross-reactivity occurred in the other proteins from *E. coli*. In addition, the anti-OprF polyclonal antibody did not recognize the expected proteins from *P. alcaligenes*, *P. fluorescens*, *P. mendocina*, *P. putida*, *P. stutzeri*, and *P. syringae*. Regarding protein homology, OprF-like protein of these *Pseudomonas* species has high percentage of protein sequence identity. The BLAST information from NCBI database demonstrates 69% identity in *P. fluorescens*, 67% in *P. putida*, 64% in *P. stutzeri*, and 63% in *P. mendocina* comparing with OprF of *P. aeruginosa* PAO1. Moreover, OprF shares 25-26% protein identity with the orthologous outer membrane protein A (OmpA) from *E. coli*, *A. baumannii*, and *B. pseudomallei*. The result could suggest that OprF protein may be used in characterization of *P. aeruginosa* at the species level. The study of G.W. Counts et al. supported that the OprF was an interesting target for rapid detection of *P. aeruginosa* in blood cultures. They developed an immunofluorescent-antibody test using a specific monoclonal antibody. A previous work also indicated that certain epitopes on the OprF may be present on only *P. aeruginosa* but not on

other Gram-negative bacteria (23). Besides antibody-based diagnostic assay, real-time PCR has been used to identify *P. aeruginosa* especially from patients with cystic fibrosis (CF). This organism from CF patients has phenotypic diversity and may be difficult to identify in microbiological laboratory. An outer-membrane lipoprotein (*oprI*) gene and gyrase B (*gyrB*) gene were effective targets for identification of atypical *P. aeruginosa* and for non-*P. aeruginosa* Gram-negative bacteria. Although, these genes showed high accurate to characterize *P. aeruginosa*, but their proteins seemed to be unsuitable for an immunological test (127). Blast searches from NCBI database, *P. aeruginosa* GyrB shares 89% amino acid identity with *P. mendocina* GyrB, and *P. aeruginosa* OprI shares 95% identity with *P. fluorescens* OprI. The OprF protein of *P. aeruginosa* is highly conserved when compares with the high heterogeneity of O-specific polysaccharide and the flagella molecule. Therefore, OprF is a valuable antigen for the production of antibodies that recognize *P. aeruginosa* (128).

CHAPTER VII
CONCLUSION

The nucleotides sequence encoding OprF protein was cloned into pET-28b expression vector at the *Bam*HI and *Hin*dIII restriction sites and then transformed into *E. coli* TOP10. The clone was identified by restriction endonuclease analysis to determine the presence and correct orientation of the *oprF* gene. Then, a positive clone was sequenced and the result confirmed the gene in frame with N-terminal histidine tag in the recombinant plasmid pET-28b-*oprF*. The confirmed recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells and satisfactorily expressed $(His)_6$-tagged OprF fusion protein with IPTG induction. The approximately 48 kDa-fusion protein was formed as an insoluble inclusion body that could be dissolved in solubilization buffer containing 6 M urea and 1 mM 2-mercaptoethanol. It was purified using HisTrap HP column based on metal chelation chromatography. The histidine residues bound to immobilized Ni^{2+} ions and His-tagged protein was recovered by elution with 300 mM and 500 mM imidazole, respectively. Subsequently, the purified fusion protein was examined by MALDI-TOF mass spectrometry and the result revealed the peptides matched on OprF protein of *P. aeruginosa*. After desalting with Amicon Ultra-4 10K Centrifugal Filter and sterilizing of the purified protein, it was used for raising rabbit polyclonal antibody. A New Zealand White rabbit was intramuscularly immunized three times with approximately 160 µg of the purified $(His)_6$-tagged OprF fusion protein emulsified in an equal volume of Incomplete Freund's adjuvant. The anti-OprF polyclonal antibody as the primary antibody was used for analysis of Western blot using non-tagged OprF protein from total cell proteins extraction as an antigen on transfer membranes. The polyclonal antibody showed great specificity and sensitivity against the OprF protein of *P. aeruginosa*. A strong band was observed at a position corresponding to OprF protein (≈38 kDa) from total cell proteins of *P. aeruginosa* ATCC 27853, PAO1, and clinical strains (PA65, PA70, PA80, PA100, PA102, and PA103). The OprF-specific band did

not appear in other *Pseudomonas* species and *E. coli* ATCC 25922 and BL21. However, the Western blot showed a mild non-specific band at a size less than 38 kDa in other *Pseudomonas* species such as *P. alcaligenes*, *P. fluorescens*, *P. mendocina*, *P. putida*, *P. stutzeri*, and *P. syringae*. When compared amino acid identity, *P. aeruginosa* OprF and *P. fluorescens* OprF shared the highest amino acid identity among these studied species at 68.9%. With this identity value, *P. aeruginosa* could be differentiated from other *Pseudomonas* species and certainly from *E. coli* as shown in Western blot analysis.

These results suggested that OprF protein probably has capability to characterize *P. aeruginosa*, however anti-OprF monoclonal antibody may have to be developed to be produced at high amount with no cross-reaction. This study is valuable for application of immunological tests in the future. The rapid diagnostic tests will combine a detection of *P. aeruginosa* by the OprF and the imipenem susceptibility by the presence of the OprD protein.

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APPENDICES

APPENDIX A
REAGENTS FOR MOLECULAR BIOLOGY

1) 10x TBE buffer

Tris base	108	g
Boric acid	55	g
0.5 M EDTA, pH 8.0	40	ml
Distilled water to make a final volume of	1000	ml

Mix the following components to make 1 liter of 10x TBE buffer. The buffer was stored at room temperature.

2) 0.5 M EDTA (pH 8.0)

0.5 M EDTA (ethylenediaminetetraacetic acid), pH 8.0 was prepared by dissolving 186.1 g of $Na_2EDTA.2H_2O$ in 700 ml of distilled water and adjusting pH to 8.0 with 10 M NaOH (approximately 50 ml). Add distilled water to make a final volume of 1 liter. Sterilize by autoclaving and stored at room temperature.

APPENDIX B
REAGENTS FOR SDS-PAGE, PROTEIN PURIFICATION AND WESTERN BLOT ANALYSIS

1) Isopropyl-β-D-thiogalactoside (IPTG) 20% (w/v, 0.8 M)

Stock solution of IPTG 20% (w/v) 3 ml was prepared by dissolving 0.6 g of IPTG in 1 ml of distilled water. Then, bring the solution to a final volume of 3 ml with distilled water and sterilize with 0.22 µm syringe-driven filter. The solution was stored in 1 ml aliquots at -20 °C.

2) Lysozyme (10 mg/ml)

Dissolve solid lysozyme at a concentration of 10 mg/ml in 10 mM Tris-Cl (pH 8.0). The solution should be prepared freshly before use. The Tris solution should have pH 8.0 because the lower pH than this, lysozyme will not work effectively.

3) 30% Acrylamide/0.8% Bisacrylamide solution

Acrylamide PAGE (PlusOne), M.W. 71.08	30	g
Methylenebisacrylamide (PlusOne), M.W. 154.17	0.8	g
Distilled water to make a final volume of	100	ml

The solution was filtered through 0.45 µm filter and stored at 4 °C up to 1 month in dark bottle (or aluminum foil covered bottle).

4) 1.5 M Tris-HCL, pH 8.8

Dissolve 36.3 g of Tris (M.W. 121.14) in 100-120 ml of distilled water. Adjust the pH to 8.8 with concentrated hydrochloric acid (HCL, approximately 1 ml) and 1 N HCL (when the pH closed to 8.8). Fill distilled water to a final volume of 200 ml and sterilize using autoclave. The Tris buffer can be kept at room temperature.

5) 1 M Tris-HCL, pH 6.8

Dissolve 9.7 g of Tris (M.W. 121.14) in 50 ml of distilled water. Adjust the pH to 6.8 with concentrated hydrochloric acid (HCL, approximately 2 ml) and 1 N HCL (when the pH closed to 6.8). Fill distilled water to a final volume of 80 ml and sterilize using autoclave. The Tris buffer can be kept at room temperature.

6) 2x SDS-PAGE sample buffer

1 M Tris-HCL, pH 6.8	1.25	ml
87% Glycerol	2.3	ml
SDS (sodium dodecyl sulfate)	0.4	g
Bromophenol blue	0.02	g
2-Mercaptoethanol	0.4	ml

Adjust the volume to 10 ml with sterile distilled water. The solution was stored in 1 ml aliquots at -20 °C.

7) 1x SDS-PAGE sample buffer

1 M Tris-HCL, pH 6.8	0.625	ml
87% Glycerol	1.15	ml
SDS (sodium dodecyl sulfate)	0.2	g
Bromophenol blue	0.01	g
2-Mercaptoethanol	0.2	ml

Adjust the volume to 10 ml with sterile distilled water. The solution was stored in 1 ml aliquots at -20 °C.

8) 10% (w/v) Ammonium persulfate

Weight 0.1 g of ammonium persulfate in Eppendorf tube and dissolve with 1 ml of sterile distilled water. The solution was stored at -20 °C up to 1 month.

9) 5x SDS electrophoresis buffer

Glycine	72.0	g
Tris base	15.1	g
SDS	5.0	g
Distilled water to make a final volume of	1	L

These components were dissolved thoroughly by stirring and stored at 4 °C.

10) Coomassie brilliant blue G-250 staining solution

Coomassie brilliant blue G-250	0.15	g
Methanol	150	ml
Acetic acid	30	ml
Distilled water	120	ml

Add coomassie brilliant blue in methanol and dissolve by swirling. Add distilled water and acetic acid, afterward mix together again. The reagent was stored at room temperature.

11) Destaining solution (for quick destaining)

Methanol	225.0	ml
Acetic acid	50.0	ml
Distilled water	225.0	ml

These components were mix together and kept at room temperature.

12) 8x Phosphate buffer, pH 7.4

$Na_2HPO_4.2H_2O$	1.42	g
$NaH_2PO_4.H_2O$	1.11	g
NaCl	23.38	g
Distilled water to make a final volume of	100.0	ml

Dissolve $Na_2HPO_4.2H_2O$, $NaH_2PO_4.H_2O$, and NaCl in 80 ml of distilled water. Adjust pH to 7.4 and add distilled water to 100 ml. The buffer was filtered through a 0.45 µm filter and stored at 4 °C.

13) 2 M Imidazole, pH 7.4

Dissolve 13.62 g of imidazole in 80 ml of distilled water. Adjust pH to 7.4 with conc. HCL and make a total volume to 100 ml with distilled water. The reagent was filtered through a 0.45 µm filter and stored at 4 °C.

14) Solubilization buffer (or binding buffer) and elution buffers

Imidazole concentration	8x Phosphate buffer, pH 7.4	2 M Imidazole, pH 7.4	Distilled water
5 mM	15.0 ml	0.3 ml	to 120 ml
20 mM	5.0 ml	0.4 ml	to 40 ml
40 mM	5.0 ml	0.8 ml	to 40 ml
60 mM	5.0 ml	1.2 ml	to 40 ml
100 mM	5.0 ml	2.0 ml	to 40 ml
300 mM	5.0 ml	6.0 ml	to 40 ml
500 mM	5.0 ml	10.0 ml	to 40 ml

These buffers had various concentrations of imidazole but all of these contained similar concentration of phosphate and NaCl (20 mM phosphate and 0.5 M NaCl). The solubilization buffer contained 20 mM phosphate, 0.5 M NaCl, and 5 mM imidazole. In this study, elution buffer with 500 mM imidazole was appropriate for protein elution.

The buffers were filtered through a 0.45 µm filter and stored at 4 °C.

15) 10x Phosphate-buffered saline (PBS), pH 7.4

NaCl	16.0	g
KCl	0.4	g
Na_2HPO_4	2.3	g
KH_2PO_4	0.4	g
Distilled water to make a final volume of	200.0	ml

Dissolve NaCl, KCl, Na_2HPO_4, and KH_2PO_4 in 170 ml of distilled water. Adjust pH to 7.4 with NaOH and fill distilled water to 200.0 ml (total volume).

The buffer was sterilized by autoclaving at 121 °C for 20 minutes and stored at room temperature.

16) PBS-T

For 1x PBS-T (phosphate-buffered saline with Tween-20), 100 ml of 10x PBS buffer was diluted 1:10 in 900 ml of distilled water. Sterilize using autoclave and add 1 ml of Tween-20 (0.1% v/v) in the buffer (Tween-20 is heat sensitive). Store at room temperature

17) Blocking buffer

BSA (bovine serum albumin) was used as a blocking reagent. Dissolve BSA at a concentration of 3% (w/v) in PBS-T.

18) Transfer buffer

Tris base	1.52	g
Glycine	7.21	g
Methanol	75	ml
Distilled water to make a final volume of	1	L

Dissolve Tris base and glycine together in 700 ml of distilled water. Add methanol and mix again. The solution was filled with distilled water to make 1 L (total volume) and stored at room temperature.

19) DAB solution

DAB (3, 3'-diaminobenzidine) is a chromogenic substrate for peroxidase (HRP) detections. Dissolve 0.03 g of DAB in 50 ml of PBS, pH 7.4. The solution was aliquoted, wrapped in foil, and stored at -20 °C.

20) DAB peroxidase substrate solution

The substrate solution was prepared freshly before used. Mix 5 ml of DAB solution with 30 µl of 8% $NiCl_2$ and 100 µl of 3% H_2O_2**.** Keep the solution protected from light. DAB and $NiCl_2$ are carcinogenic so should avoid contact with skin.

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