EFFECT OF BENZALKONIUM CHLORIDE AND CHLORHEXIDINE EXPOSURE TO THE EXPRESSION OF *MEXAB-OPRM* AND THE FUNCTION OF MULTIDRUG EFFLUX PUMP OF *PSEUDOMONAS AERUGINOSA*

BY

THITINAN PHUTTHILERTMETHAWEE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE
(BIOCHEMISTRY AND MOLECULAR BIOLOGY)
FACULTY OF MEDICINE
THAMMASAT UNIVERSITY
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was approved as partial fulfillment of the requirements for
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Thesis Title	EFFECT OF BENZALKONIUM CHLORIDE AND CHLORHEXIDINE EXPOSURE TO THE EXPRESSION OF *MEXAB-OPRM* AND THE FUNCTION OF MULTIDRUG EFFLUX PUMP OF *PSEUDOMONAS AERUGINOSA*
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ABSTRACT

Pseudomonas aeruginosa is a multidrug-resistant pathogen commonly found in the hospital environment. One strategy for controlling the spread of this pathogenic bacteria in hospitals is the use of biocides (disinfectants and antiseptics). However, ongoing debates continue regarding the possible impact of using biocides including benzalkonium chloride (BKC) and chlorhexidine (CHX) on the emergence of antibiotic resistance. Therefore, the aims of this study are to investigate the impact of BKC and CHX on efflux pump gene expression and activity in various strains of *P. aeruginosa* and their effects on antibiotic susceptibility.

P. aeruginosa PAO1 (ATCC27853), K767 (wild type), K1523 (*MexB*-deletion), K1455 (*MexAB-OprM* overexpression) and 15 clinical isolates were tested against BKC and CHX to determine the minimal inhibitory concentration (MIC). Bacteria were then exposed to subinhibitory concentrations (SIC) of the biocides for five consecutive passages. MICs for ciprofloxacin, ceftazidime and gentamicin, as well as the

susceptibility status, the transcripts of *MexAB* and *OprM* genes and the activity of efflux pump were determined and compared before and after exposure to BKC and to CHX.

Most *P. aeruginosa* strains retained their antibiotic MICs after exposure to BKC and CHX. A few strains had MIC exceeding twice the pre-exposure MICs of the corresponding antibiotics, but these changes were not statistically significant. No changes in antibiotic susceptibility status were observed after exposure to BKC and CHX. Upon exposure to BKC, *MexA, MexB*, and *OprM* genes exhibited increased expression in K767, K1455, and clinical isolates (P10 and SP2) though not statistically significant. The *MexB* deletion strain K1523 showed a significant reduction in *MexA* expression. Despite some gene upregulation, efflux pump activity remained largely unchanged, except in a clinical isolate P10, which exhibited increased activity. BKC exposure led to an increase in ciprofloxacin, ceftazidime, and gentamicin MICs. However, these changes did not alter the susceptibility status of the isolates. CHX exposure resulted in both decreased and increased efflux pump gene expression and led to variable changes in MICs of ciprofloxacin, ceftazidime, and gentamicin. K1523 showed significant *MexA* reduction, while K767, K1455, clinical isolates (P10 and SP2) demonstrated increased *MexA* expression, with K767 exhibiting a more than sevenfold increase. P10 also showed significant upregulation of *MexB* and *OprM*. Efflux pump activity remained unchanged in most strains, except for increased activity in P10.

The findings highlight the strain-specific response of *P. aeruginosa* to biocides, impacting gene expression, efflux pump activity, and antibiotic susceptibility. Some *P. aeruginosa* strains could employ efflux pump to exclude biocides from their cytoplasm. Despite transcriptional changes, efflux pump activity and resistance phenotypes remained largely unaffected. Exposure to SICs of BKC and CHX minimally alters the antibiotic MICs of *P. aeruginosa*. There was no observed alteration in the susceptibility status to antibiotics. This research provides valuable data for the use of biocides in controlling infections both in healthcare settings and in the general environment, emphasizing the need for understanding biocide effects on bacterial resistance mechanisms.

Keywords: Benzalkonium chloride, Chlorhexidine, Multidrug efflux pump, Antibiotic Resistance, *Pseudomonas aeruginosa*

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Thitinan Phutthilertmethawee

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
%	Percentage
/	Per
<	Less than
=	Equivalent
>	Greater than
±	Plus-minus
≥	Greater than or equal to
x	Multiplication
℃	Degree Celsius
α	Alpha
β	Beta
µg	Microgram
µl	Microlitre
µmol	Micromole
A	Absorbance
bp	Base pair
BKC	Benzalkonium chloride
cm	Centimetre
CHX	Chlorhexidine
DNA	Deoxyriobonucleic acid
DNase	Deoxyriobonucleaes
et al.	Et alii, and colleagues
etc.	Et cetera
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide

g	Gram
mg	Milligram
ml	Millilitre
mm	Millimeter
MIC	Minimal inhibitory concentration
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
nm	Nanometre
O.D.	Optical density
rpm	Revolutions per minute
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
SIC	Sub inhibitory concentration
TSB	Tryptone soy broth
U	Unit(s)

CHAPTER 1
INTRODUCTION

1.1 Background and rationale

Nosocomial infection (Healthcare associated infection) is one of the top problems worldwide. The impacts of this problem are not just individual health status or public health but also the economy and even national security. The problem of infections occurring in the hospital which is often caused by antibiotic-resistant infections is on the rise, and it needs large sums of money to control this problem. Despite the continued research, discovery and development of effective antimicrobial drugs, infections by antimicrobial-resistant pathogens remain the top cause of death. This is because many pathogens especially nosocomial pathogens has the ability to develop or adjust itself to be able to resist multiple antibiotics and to transfer this ability to other pathogens or strains through genetic processes, causing the problem of healthcare-associated infections widely spread. One of the most common hospital pathogens is *Pseudomonas aeruginosa*. This pathogen is a common causative agent of bloodstream, lungs and urinary tract infections, especially in patients with low immunity. This notorious bacterium is known for its multiple antimicrobial resistant nature. It has many mechanisms responsible for the drug resistance, such as the excretion of antibiotics from cells via a multidrug efflux pump.

To control infection problems in hospitals either from *P. aeruginosa* or other pathogens, one of the strategies is the use of disinfectants for cleaning hospital environment as well as antiseptic for decontaminating human tissue. Benzalkonium chloride (BKC) and Chlorhexidine (CHX) are among substances used for this purpose. However, the continued usage of these biocides may cause the development of disinfectant-resistant strains, and they may change the properties of pathogens to become more resistant to antibiotics. For this reason, the project wants to study the effect of using these biocides (BKC and CHX) on the antibiotic resistance of *P.*

aeruginosa, with a focus on the expression and function of the multidrug efflux pump which is the mechanism responsible for various antibiotic resistance.

1.2 Specific objectives

1.2.1 To study the effect of BKC and CHX on the expression of genes that control the multidrug efflux pump of *P. aeruginosa*.

1.2.2 To study the effect of BKC and CHX on the function of the multidrug efflux pump of *P. aeruginosa*.

1.2.3 To study the effect of BKC and CHX on antibiotic resistance of *P. aeruginosa*.

CHAPTER 2
REVIEW OF LITERATURE

2.1 *Pseudomonas aeruginosa*

2.1.1 Characteristics and epidemiology

Pseudomonas aeruginosa is a saprophytic bacterium. While some strains are plant pathogens, several strains are opportunistic pathogens of humans and animals. They have broad antibiotic resistance and are responsible for several infections in many organs. They are also one of the most common pathogens responsible for nosocomial infections (infections occurring in patients during their time being in a hospital).

2.1.1.1 Microbiology characteristics

P. aeruginosa is an aerobic non-fermentative gram-negative bacillus, belonging to the Family Pseudomonadaceae. It has a genome size range of 5.2 – 7.0 Mbp (Stover et al., 2000). *P. aeruginosa* has an oxidase and catalase positive and is motile by its polar flagella. It is capable of growing in a wide variety of substrates, as well as a wide range of temperature (ranging from 6°C - 42°C with the optimum at 37°C)(Excellence, 2022). It produces a characteristic grape-like odor. *P. aeruginosa* has the largest genome among all known bacteria, and its 6.3 million base pairs genome contains 5,570 predicted genes.

2.1.1.2 Epidemiology

P. aeruginosa is ubiquitous in natural environment ranging from soil, swimming pools and flowers; it is also commonly found in healthcare settings such as dialysis machines, contact lens solution, respirators, humidifiers, floor mops, sinks, sponges, toilets, and even disinfectant solutions (Santajit & Indrawattana, 2016). Therefore, it is listed as one of the important pathogens causing healthcare-associated infections. It often infects immunocompromised patients or patients with chronic diseases who need to be hospitalized.

2.1.2 Virulence factors

P. aeruginosa can produce many virulence factors, resulting in a variety of diseases. Those virulence factors can be divided into “cell-associated virulence factors” and “secreted virulence factors” as shown in Figure 2.1.

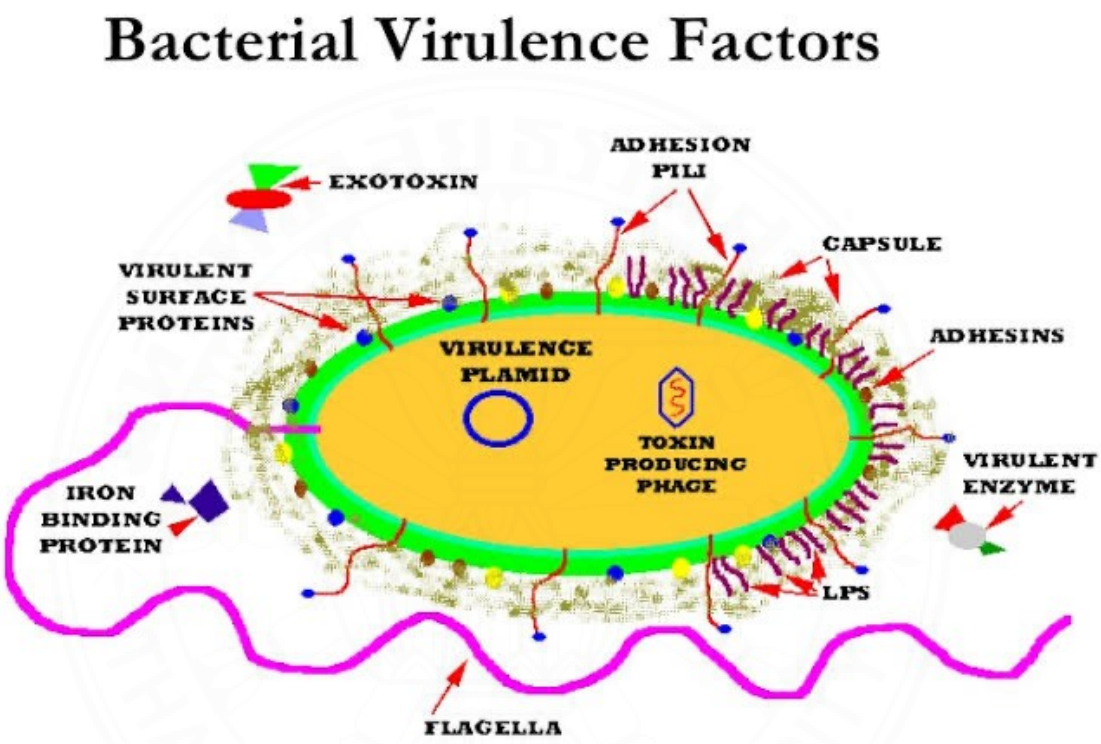


Figure 2.1: Bacterial virulence factors

2.1.2.1 Cell-associated virulence factors

Cell-associated virulence factors are cellular components or structures that are important in pathogenesis. These include adhesins, capsules and endotoxin (or lipopolysaccharide).

Adhesins are molecules found on pili and cell surfaces that help the attachment of bacterial cells to the epithelial cells. The important receptor for the binding of adhesins is N-acetylneuraminic acid (or sialic acid) which is found widely distributed in host tissues.

Capsule is the substance containing polysaccharide or alginate, creating a sticky mucous layer surrounding cells. In addition to helping

bacterial cells to adhere to the epithelial cells, it also helps to resist the phagocytosis from white blood cells as well as the effect of antibacterial drugs. *P. aeruginosa* strains with capsule productions can form a mucoid-phenotype colony. These strains are often associated with severe and/or chronic pathologies with a poor prognosis.

Lipopolysaccharide (LPS), also called endotoxin, is the major component of the outer membrane of the bacterium. It consists of a hydrophobic domain, Lipid A, which embeds into the phospholipid bilayer of the outer membrane, and a hydrophilic tail composed of core polysaccharide or O polysaccharide. LPS is a strong pyrogen. It can cause fever, shock and disseminated intravascular coagulation (DIC).

2.1.2.2 Secreted virulence factors

P. aeruginosa produces many extracellular enzymes and toxins that possess the effect of tissue destruction, such as elastase, phospholipase C, rhamnolipid, exotoxin A, pyocyanin and alkaline protease. These secreted virulence factors can cause the bacteria to spread easily after entering the human body.

Among the numerous extracellular virulence factors produced by *P. aeruginosa*, elastase is considered to be a major contributor in its pathogenesis. Elastase has the effect of destroying antibodies and inhibiting the movement of neutrophil cells to the infected site. It also has the effect of destroying the epithelial cells of the blood vessels, causing an invasion of pathogens into the bloodstream leading to infectious niches in various systems.

Phospholipase C is an enzyme that can damage tissue by hydrolysis of fat. Secreted phospholipase C of *P. aeruginosa* has been found to decrease neutrophil activities, rendering to the chronic persistent infection by this pathogen (Terada et al., 1999).

Rhamnolipid is a surfactant secreted by bacteria including *P. aeruginosa*. It has been shown to aid bacterial colonization and spread by disrupting tight junction of epithelia. It also inhibits the cilia function on the respiratory lining.

Exotoxin A is another important toxin of *P. aeruginosa*. It is found in most pathogenic strains. It can interfere with the protein production of host

cells by inhibiting the elongation factor-2 (EF-2) function in the protein production process, resulting in the death of cells and tissues at the infection sites.

Pyocyanin is the blue pigment produced by *P. aeruginosa*. It has several roles in the pathogenesis including: an ability to cause transferrin to release iron which is a crucial requirement for growth (Cox, 1986); inducing an apoptosis of neutrophils as shown (Allen et al., 2005); and stopping ciliary beating by decreasing intracellular cAMP and ATP of the respiratory epithelial cells (K. Kanthakumar & Wilson, 1993). It can also destroy superoxide, hydrogen peroxide and other free oxygen radicals which may be harmful to bacterial cells.

Siderophores are bacterial products mediating bacterial iron uptake systems (Crosa, 1997). Siderophores compete for iron by converting the transferrin-bound iron into a soluble form which P. aeruginosa can utilize (Sriyosachati & Cox, 1986).

Alkaline protease is another virulence protein important in the pathogenesis of *P. aeruginosa*. It can suppress host immunity by inhibiting the production of interferon-γ from T cells (Parmely, 1988) as well as by inhibiting neutrophil function, particularly chemotaxis. Alkaline protease is thus responsible for an evasion of this pathogen from the phagocytic defense system (Kharazmi et al., 1984).

Biofilms, by definition, are a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Costerton et al., 1999). In nature, bacteria including *P. aeruginosa* thrive in the biofilm mode of growth in order to survive in a hostile environment. Biofilm formation offers protection from various environmental challenges ranging from heavy metal toxicity (Teitzel & Parsek, 2003) to host immune response (Leid et al., 2002) and antimicrobial agents (Stewart & Costerton, 2001) This is because the bacteria residing in the biofilm are covered with an extracellular mucus, which can protect them from antibacterial drugs and the immune system. *P. aeruginosa* growing on biofilms have been increasingly recognized as an important cause of several diseases.

2.1.3 Diseases and clinical Manifestations

Infections by *P. aeruginosa* do not usually occur in people with generally good health but often in people with impaired immune systems(Hirsch & Tam, 2014). This pathogen is also common among patients admitted in the hospital especially those who receive chemotherapy or who have catheterization (Jayanetra P, 1999). Even being described as an opportunistic pathogen, *P. aeruginosa* still can cause variety of diseases in several body systems (Klevens R, 2007).

2.1.3.1 Skin and soft tissue infection

P. aeruginosa can infect skin or mucous membranes. This is often associated with exposure to contaminated environments such as swimming pool, natural pool, hot tub or onsen. Conditions such as folliculitis are reported in onsen visitors, hence the name hot tub folliculitis as shown in Figure 2.2.

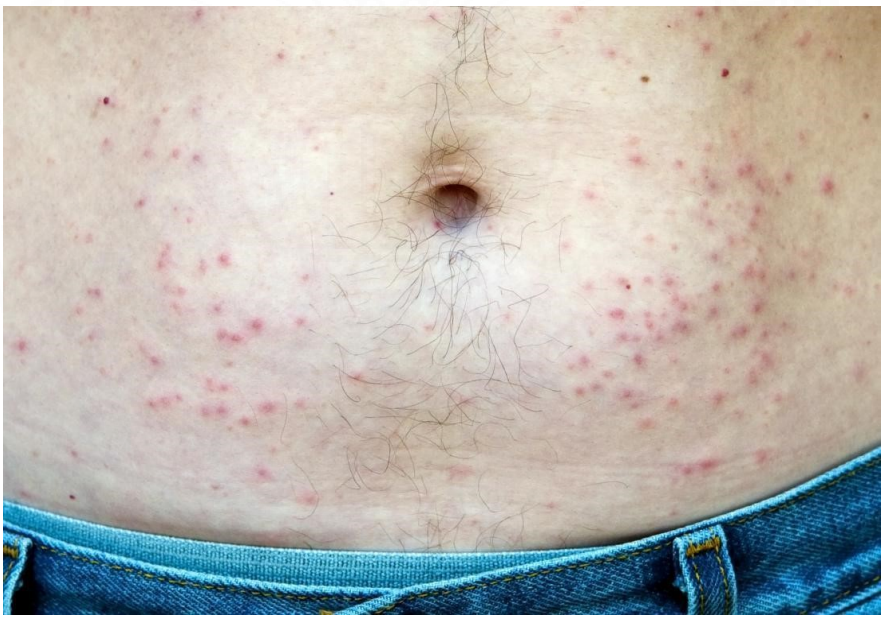


Figure 2.2: Hot tub folliculitis

Apart from folliculitis, *P. aeruginosa* is a common pathogen in patients suffering from burns. It has been reported that exudate from burn wounds can readily support the proliferation as well as virulence factor production of *P. aeruginosa* (Gonzalez Manuel et al., 2016) as shown in Figure 2.3.

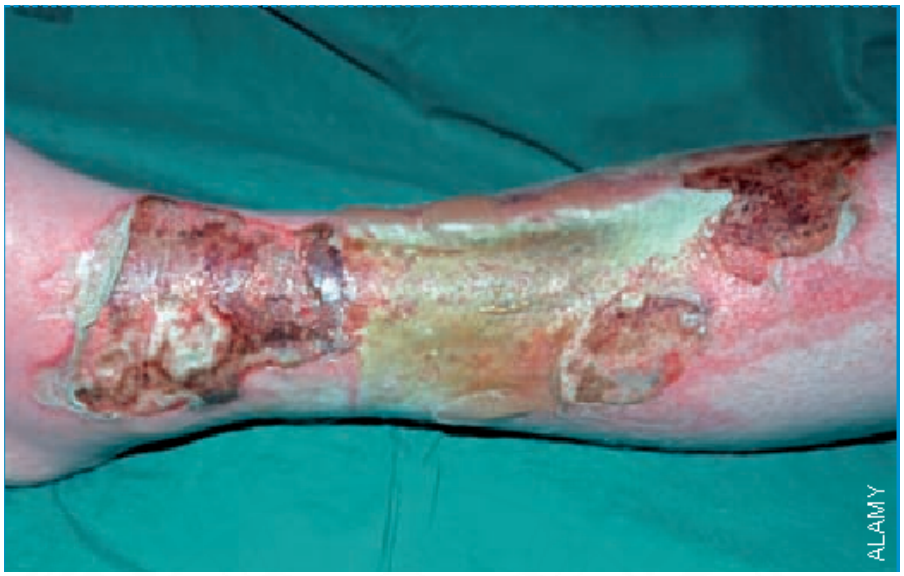


Figure 2.3: Wound infection by *P. aeruginosa*

2.1.3.2 Pulmonary infection

Pulmonary infections by *P. aeruginosa* are often found in people with immune deficiency as well as people with chronic lung disease such as cystic fibrosis (CF). *P. aeruginosa* pneumonia is often severe and has a high mortality rate. In CF, a chronic hereditary disease causing thick sputum buildup in the lungs, *P. aeruginosa* can grow within the mucus and produce biofilms, causing chronic or persistent infections (Giwercman B, 1992). Lung infections by this pathogen is the leading cause of death in CF patients (Bergmans et al., 1998).

2.1.3.3 Ear infection

An infection of the outer ear or ear canal (otitis externa) is often found in swimmers or people who play water sports, and hence called swimmer's ear disease.

Otitis externa (or swimmer's ear disease) is common in people of all ages, especially in adolescence as shown in Figure 2.4. This disease can be more severe in people with diabetes, AIDS, or other immunocompromised conditions. *P. aeruginosa* is a common causative agent of the otitis externa, and this infection is quite severe and, if left untreated, can result in serious complications.

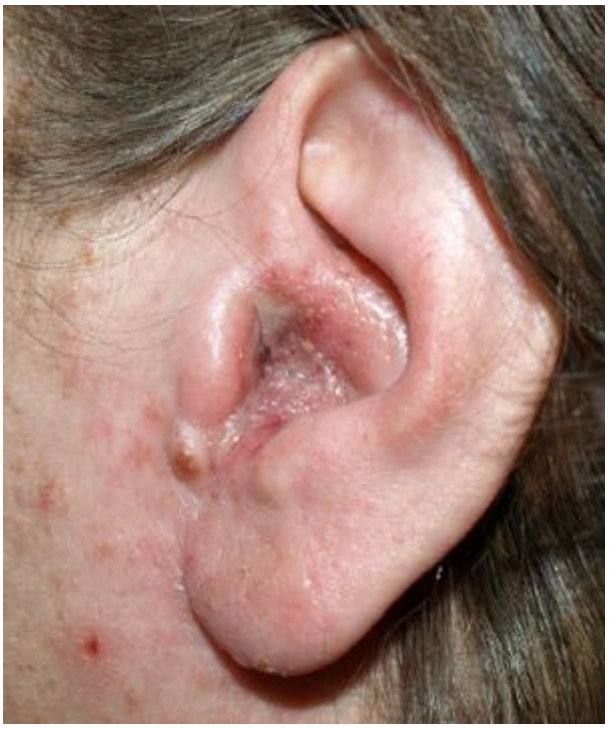


Figure 2.4: Otitis externa

2.1.3.4 Bloodstream infection

Septicemia or bloodstream infection by *P. aeruginosa* is often seen in people with immunodeficiency (Hilmar Wisplinghoff & Edmond, 2004). These include cancer patients, people with low white blood cells, patients with severe burns, diabetic patients, organ transplant recipients, and AIDS patients. The infection often occurs while patients are being admitted in the healthcare facilities and results in a high mortality rate.

2.1.3.5 Endocarditis

Endocarditis, an infection of the inner lining of the heart, is a rare condition. It is often found in intravenous drug abusers. Moreover, patients with long-term intravenous catheterization (such as end-stage renal disease patients who require hemodialysis) are at risk of this infection. Similar to the bloodstream infection, infectious endocarditis by *P. aeruginosa* is associated with high mortality, and in many cases, treatment with antibiotic alone cannot eliminate the source of infections. Combined surgical treatment may need to achieve the cure.

2.1.3.6 Urinary tract infections

Urinary tract infections by *P. aeruginosa* often occur in the hospital especially in those who have a urinary catheter (Shigemura et al., 2015). Urinary tract infections can be caused by many bacterial pathogens including *Escherichia coli* and *P. aeruginosa*. As these pathogens is adept at forming surface-associated biofilms, colonization of these pathogens on indwelling urinary catheter thus causes a catheter-associated urinary tract infection or CAUTI. CAUTI is a serious condition which can lead to a more severe condition such as sepsis.

2.1.4 Treatment and antibiotics

As *P. aeruginosa* naturally resists several antibiotics, treatment of *P. aeruginosa* infections can be difficult. Usually, the treatment follows an antibiotic susceptibility result of each isolate, and combination of more than one antibiotics is often required (Lambert, 2002), (Jarvis, 2001). Antibiotics with an activity against *P. aeruginosa* are called “antipseudomonal antibiotics”. These include:

A. aminoglycosides including gentamicin, amikacin and tobramycin (but not kanamycin)
B. quinolones including ciprofloxacin and levofloxacin (but not moxifloxacin)
C. cephalosporins including ceftazidime, cefepime, cefoperazone, cefpirome and ceftobiprole (but not cefuroxime, cefotaxime, or ceftriaxone)
D. antipseudomonal penicillins including carboxypenicillins (such as carbenicillin and ticarcillin) and ureidopenicillins (such as mezlocillin, azlocillin, and piperacillin)
E. carbapenems including meropenem, imipenem, doripenem (but not ertapenem)
F. polymyxins including polymyxin B and colistin
G. monobactams such as aztreonam

2.1.5 Mechanisms of antibiotic resistance

Several pathogenic microbes (bacteria, fungi, viruses, and protozoa) have the ability to resist to the treatment with antimicrobial agents (Nikaido, 1998). This protective ability can occur naturally or evolve when the microbes expose to the antimicrobials. For pathogenic bacteria, there are five main mechanisms which are responsible for the resistance (Woodford et al., 1998). As shown in Figure 2.5, these antibiotic resistant mechanisms include:

1. Inhibition of drug uptake (or decrease permeability)
2. Alteration of target sites
3. Production of drug-inactivating enzymes
4. Bypass of the pathway inhibited by drugs
5. Efflux pump

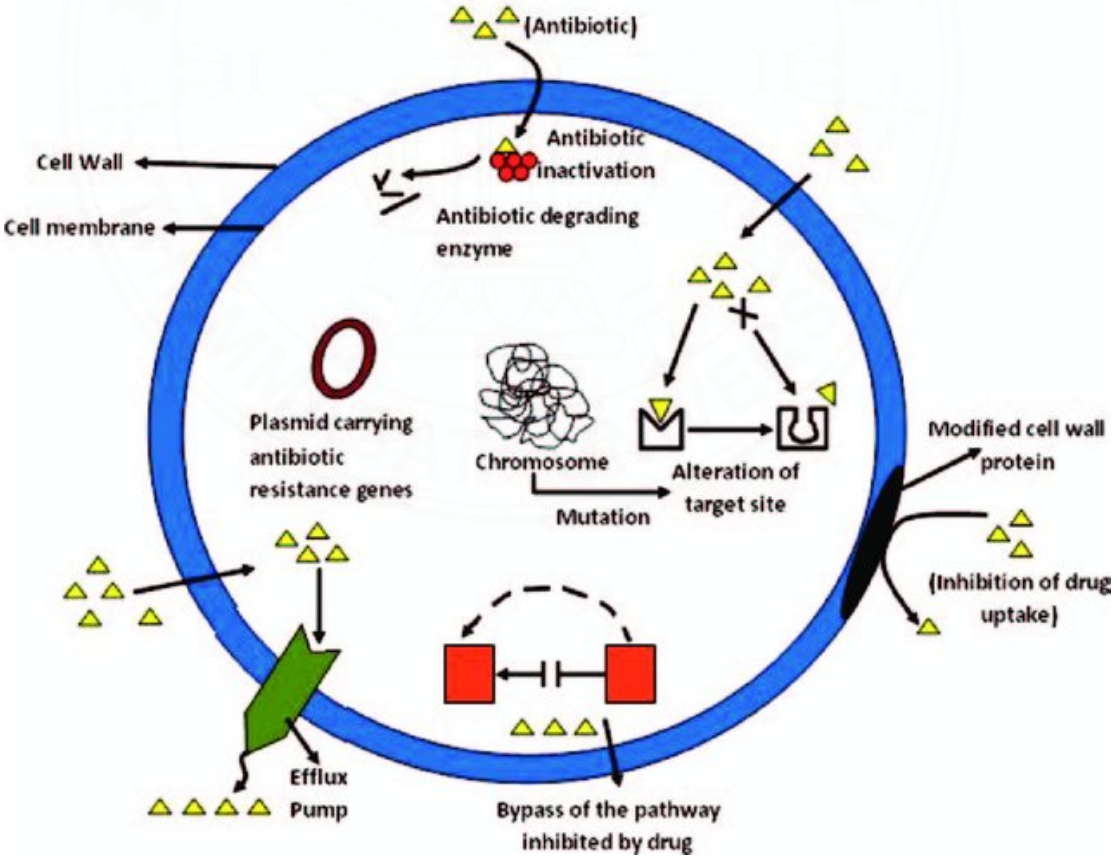


Figure 2.5: Mechanisms of antimicrobial resistance

2.1.6 Mechanism of antibiotic resistance of *Pseudomonas aeruginosa*

P. aeruginosa is notoriously known for its difficulty to be eradicated. It employs four main mechanisms which are responsible for its remarkable capacity to resist antibiotics as follow:

1. Changing the structure of the cell wall causing a decrease of drug passing into the cell less and cause the drug to naturally resistant to many drugs (intrinsic resistance), the creation of and structural changes of penicillin-binding protein (PBP) causes most bacteria resistant to β-lactams
2. Producing enzymes that destroy the drugs i.e. β-lactamase enzymes and aminoglycoside modifying enzymes
3. Modifying targets of the drugs i.e. mutation of the DNA gyrase causing resistance to quinolones (Akasaka et al., 2001).
4. Employing efflux system that pumps or expels the antibiotics out of the cells (Figure 2.6). Currently, there are four efflux pump systems known to play roles in resistant property of P. aeruginosa. These include *MexAB-OprM*, *MexEF-OprN*, *MexCD-OprJ* and *MexXY-OprM* (Aeschlimann, 2012).

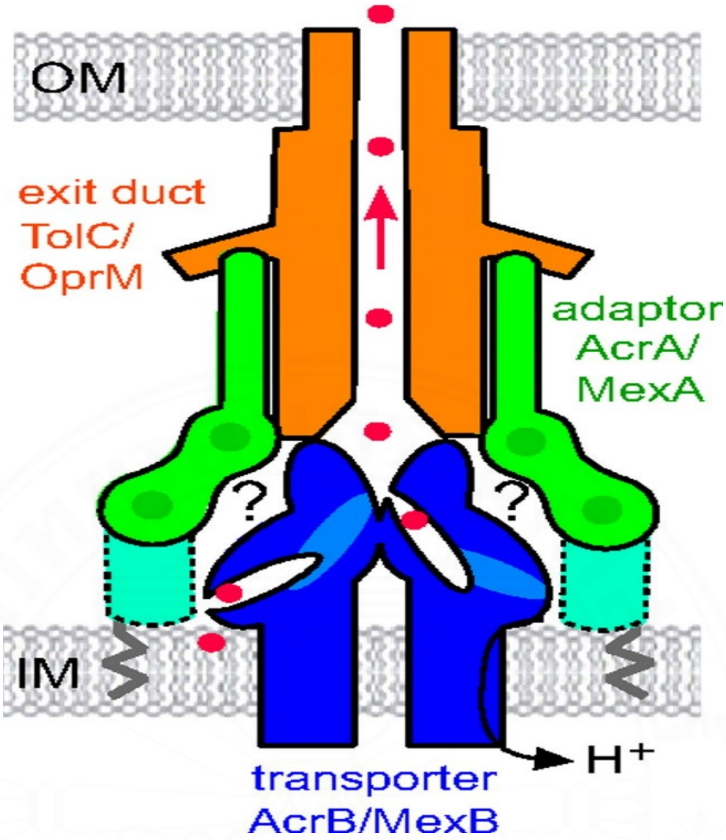


Figure 2.6: *P. aeruginosa* efflux pump

2.2 Benzalkonium chloride (BKC)

BKC is classified as a quaternary ammonium compound. It can be used as both a disinfectant and an antiseptic. It is often included as a disinfectant component in hand sanitizers, soaps, shampoos, mouthwash (Merchel Piovesan Pereira et al., 2019). It is also an ingredient in various surface cleaners. Its mechanism of action is through the disruption of bacterial cell membrane lipid bilayers, leading to increasing cell permeability and subsequently leakage of cellular content and death. BKC is more active against gram-positive bacteria than gram-negative bacteria (Nagai et al., 2013), with properties that can be both bactericidal and bacteriostatic according to the concentration of the substance used.

2.3 Chlorhexidine (CHX)

CHX is classified as a biguanide compound. Similar to BKC, CHX is both a disinfectant and an antiseptic. It can be used on the skin (body and hands) for cleansing, on the wound, as a mixture of mouthwash, and on medical equipment. At the most used concentrations of 2-4% (Horner et al., 2012), it is highly effective against most microbes with the exception of the destruction of spores (James et al., 2017). CHX acts by releasing positively charged cation which interacts with the negatively charged bacterial cell wall. This leads to the disruption of cellular membrane, ultimately resulting in cell death.

2.4 BKC and CHX resistance mechanisms

For the natural or intrinsic resistance to BKC and CHX as found in Mycobacterium or bacterial spores, the mechanism relies on the overly thicken cell wall of those microbes that both biocides cannot penetrate to destroy the inner cellular component (Horner et al., 2012), (Yoshikazu Sakagami, 1989). However, for acquired resistance, it can be caused by the transformation of cell membranes into neutral charged molecules to reduce the binding to positively charged ion of both substances (Loughlin, Jones Mv Fau - Lambert, et al., 2002), (Maillard, 2022) or by increasing in the amount of phospholipid, which is a component of cell membranes (Cieplik et al., 2019). Moreover, resistance to BKC can be a result of the multidrug efflux pump, as found in the BKC-resistant *Klebsiella pneumoniae* (Ahmed Abdelaziz 2019) and *Pseudomonas aeruginosa* (Nagai et al., 2013).

2.5 Cross resistance between antibiotics and biocides

Biocide used for surface disinfection is one of the causes of antibiotic resistance. A well-known example is the use of triclosan, a biocide that has been used as an ingredient in soaps for a long time because of its low cost and high effectiveness.

Nevertheless, triclosan-containing cleaning products were banned in the United States in 2016 because there are several reports of an association of risks causing antibiotic resistance from the use of this substance in cleaning products. The use of triclosan puts pressure on pathogens in the environment, resulting in the evolvement of pathogens to become resist to not only triclosan but also antibiotics.

For the antibiotic resistance which is crossed from the resistance of biocides (both antiseptic and disinfectant) of *P. aeruginosa,* there is no definitive conclusion. There have been reports of exposure to BKC disinfectants can induce cross resistance against penicillin G, tetracycline and ciprofloxacin (Vickery et al., 2009), (Tandukar et al., 2013), and polymyxin (Minjae Kim, 2018). However, a study by Loughlin et al. found no association of cross resistance in *P. aeruginosa* to the antibiotics including ceftazidime, imipenem, ciprofloxacin, tobramycin (Loughlin, Jones Mv Fau - Lambert, et al., 2002). Because of these discrepancy results, the U.S. Food and Drug Administration classified BKC as Category III antiseptic active ingredient, which means a substance that has insufficient data to determine the safety and efficacy of the substance. It is recommended to conduct more tests to obtain more information about this agent, and one of the data needed is to assess the potential of this substance to induce antibiotic resistance (Merchel Piovesan Pereira et al., 2019).

2.6 Efflux pump

Efflux pump is a mechanism of excretion of substances outside the cell (Du et al., 2018). It was first identified in the 1980s in tetracycline-resistant bacteria, and it is now known as one of the key mechanisms in bacterial drug resistance. Efflux pump or efflux protein is a transmembrane protein that penetrates the cell membrane and can expel certain antibiotics or toxins from cells. It can be found in both eukaryotic and prokaryotic cells (both Gram-positive and Gram-negative bacteria) (Du et al., 2015). By transporting substances from the intracellular to the extracellular space of bacteria, the accumulation of intracellular toxic substances, such as anti-bacterial agents, is

limited. Moreover, the efflux pump can expel a wide variety of drugs with different structures, making it one of the effective contributors for the resistance of bacteria.

2.6.1 Efflux pump structure

The efflux pump is a tripartite form as shown in Figure 2.7, consisting of three parts: the inner membrane protein (or efflux transporter), the outer membrane protein (or outer membrane channel), and the membrane fusion protein (or periplasmic adapter protein). The inner membrane protein (IMP) or the efflux transporter is located in the cytoplasmic membrane, and the outer membrane protein (OMP) is located in the outer membrane of the gram negative bacteria as shown in Figure 2.8. The membrane fusion protein (MFP) is in the periplasm and links between the IMP and OMP. Overall, this complex tripartite structure spans across bacterial membrane, facilitating the transportation of substances from intracellular to extracellular environment.

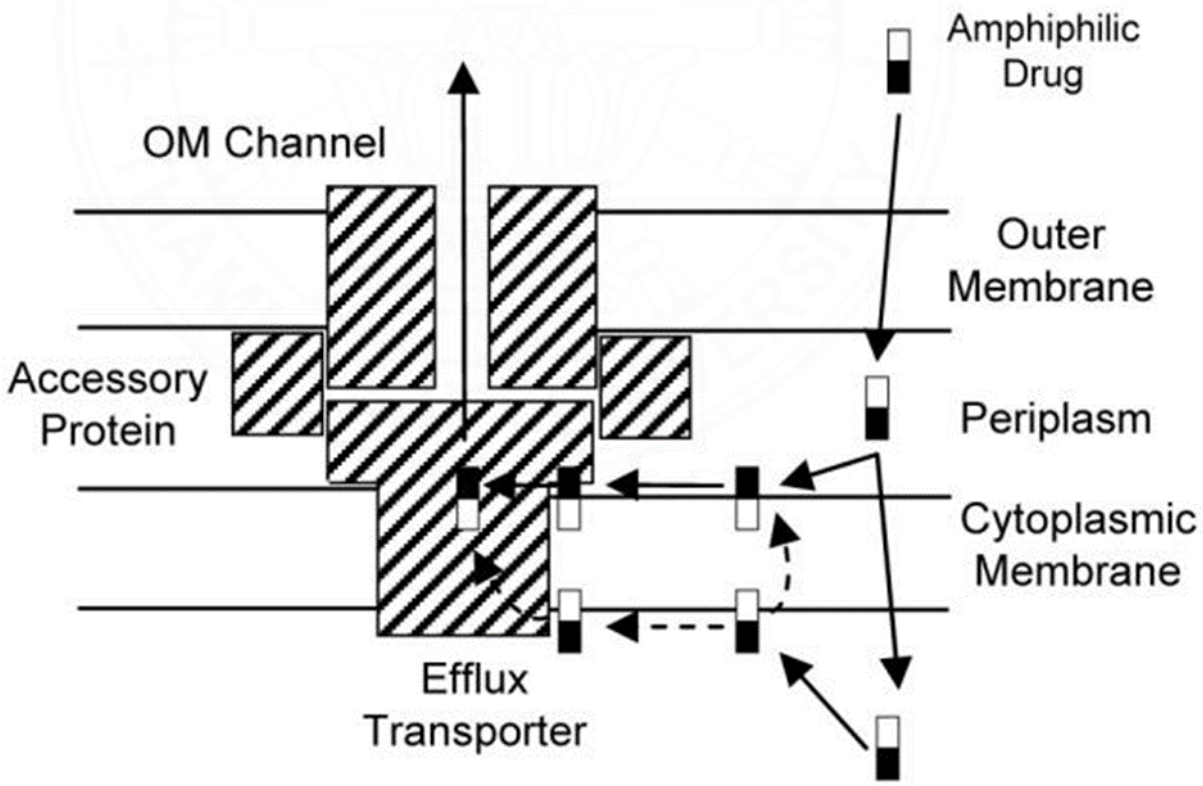


Figure 2.7: Efflux pump tripartite structure

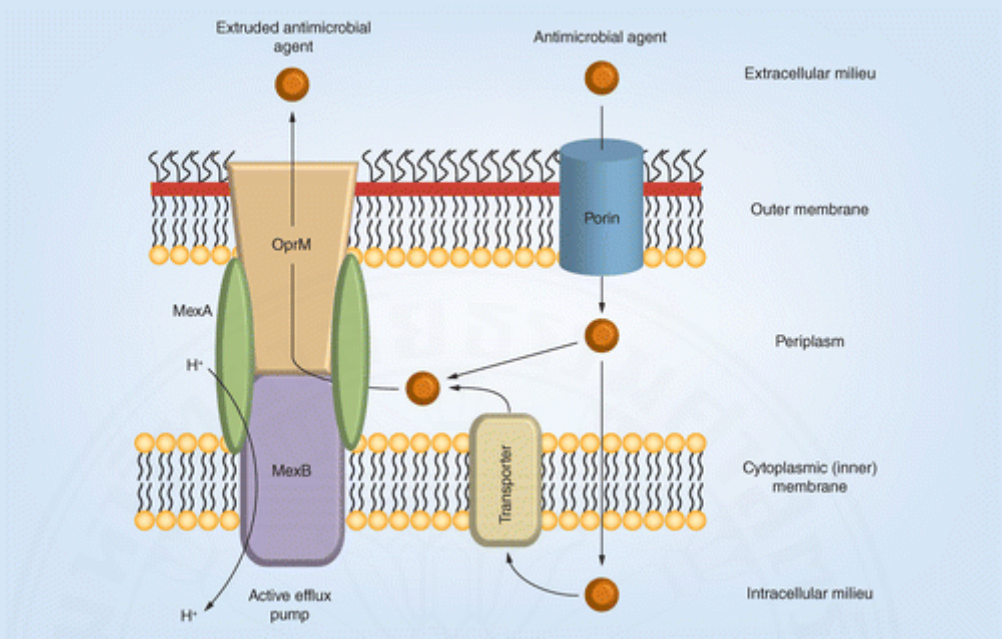


Figure 2.8: Mechanisms of Efflux Pumps of *P. aeruginosa*

2.6.2 Efflux pump classification

Bacterial efflux pumps can be classified into two main groups according to their energy sources, substrates and compositions:

2.6.2.1 Primary active transporter

Primary active transporter utilizes ATP as an energy source. It is also known as ATP-binding cassette (ABC) transporter. It is a transporter located on the surface of a typical cell membrane. ABC transporter consists of two distinct protein domains: membrane protein domain (TMD) and nucleotide binding domain (NBD) (or cytosolic domain). The transport of substances occurs by interaction between the reactants and TMD. Once reactants react with TMD, they cause alteration of TMD structure, and then initiate NBD to degrade ATP. It is unclear how many ATP molecules from ATP hydrolysis involve in the transportation of extracellular substances. This transporter is able to excrete various antibiotics such as tetracycline, fluoroquinolones, macrolides, rifampin, chloramphenicol, aminoglycosides as well as certain substances that are components of the bacterial cell (e.g. capsular polysaccharide).

2.6.2.2 Secondary active transporters

These can be divided into 4 families: as shown in Figure 2.9

2.6.2.2.1 Small multidrug resistance protein (SMR) is a protein that transports substances into and out of the cell with a structure of 4 α-helices transmembrane, which is the binding domain for the ligand and contains approximately 100-140 amino acids SMR required proton (H+) as an energy source. It has been shown to transport anionic and neutral compounds, quaternary ammonium compounds, as well as a range of antibiotics including tetracycline, erythromycin, sulfadiazine.

2.6.2.2.2 Multidrug and toxic compound extrusion (MATE) is a transmembrane protein consisting of 12 α-helices. MATE excretes drugs by transporting them against sodium or protons gradients. MATE is found in various organisms, including archaea, bacteria, and eukaryotes. For example, MATE efflux pump in *Vibrio parahaemolyticus* is known as *NorM* efflux protein, which has a function to facilitate the efflux of drugs such as norfloxacin.

2.6.2.2.3 Resistance nodulation division (RND) efflux pumps are frequently seen in gram-negative bacilli. They play a crucial role in the efflux of antibiotics and other chemicals. The RND efflux pump is a tripartite protein complex, with its components located in the outer membrane and the periplasm. For example, in *Escherichia coli*, the efflux pump consists of the outer membrane proteins such as *TolC* and the periplasmic proteins such as *AcrA*. Similarly, in *P. aeruginosa*, the efflux pump outer membrane proteins such as *OprM* and the periplasmic proteins such as *MexA*. It is important that all three protein components are required for the proper functioning of the drug efflux process. This RND transporter can expel a variety of antibiotics, including β-lactams, tetracycline, fluoroquinolones, erythromycin, rifampin, chloramphenicol, aminoglycosides, and fusidic acid.

2.6.2.2.4 Major facilitator superfamily (MFS) is the largest group of transport proteins. It can transport a wide range of substrates, including sugars, metabolic products, and antibiotics. MFS has two proteins comprising 12-14 parts of transmembrane segments. MFS can employ various mechanisms, such as uniport, symport or antiport, depending on their specific function. MFS may utilize energy from

ATP or from the proton motive force. MFS transporters can expel various antibiotics, including tetracycline, fluoroquinolones, erythromycin, rifampin, chloramphenicol, aminoglycosides, lincosamide, and pristinamycin.

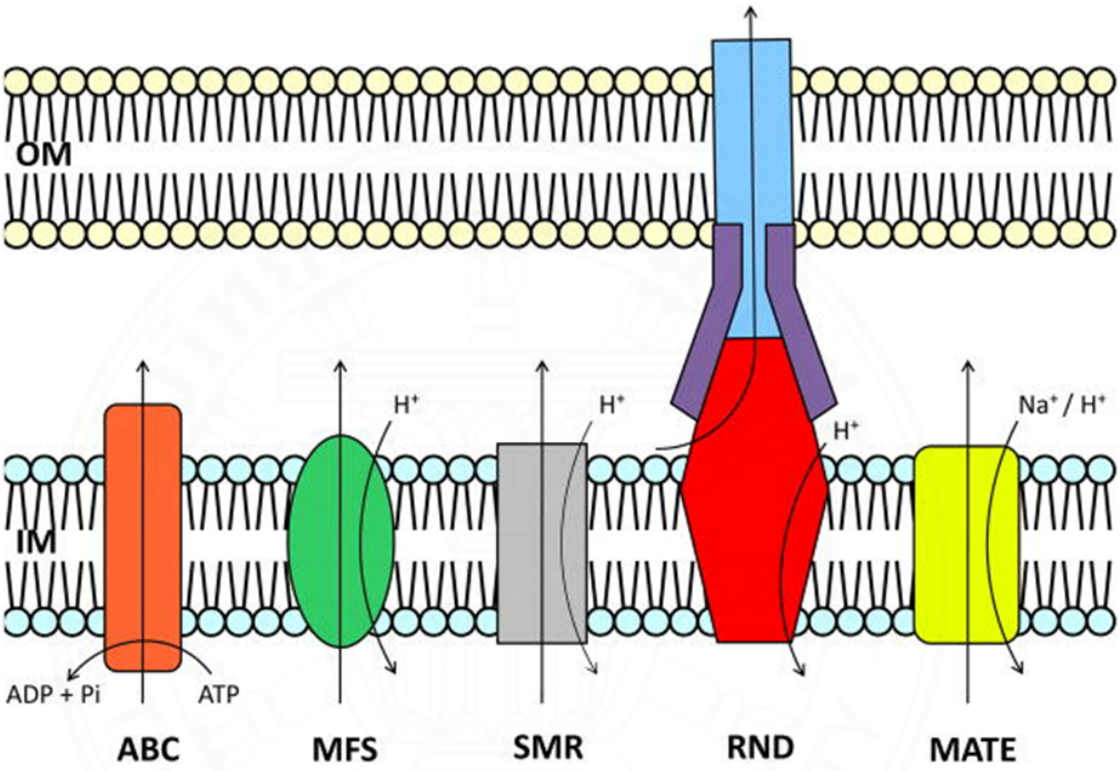


Figure 2.9: Types of efflux pumps.

The ABC transporter proteins (on the left) rely on ATP to transport a wide range of antimicrobials across the cellular inner membrane. SMR, MFS, and RND proteins operate through an H+-substrate antiport mechanism. MATE transporter proteins use both H+ and Na+ as energy sources. Notably, RND transporters have multi-protein structures that span the inner and outer membranes.

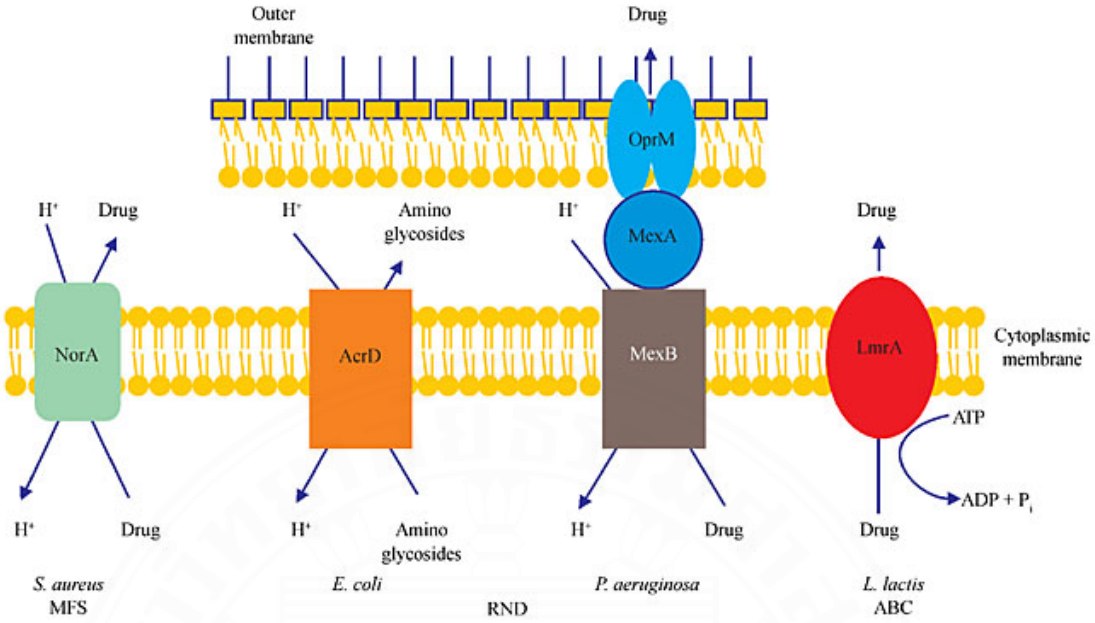


Figure 2.10: Examples of various efflux pumps in bacteria (Schweizer, 2003)

Drug efflux pumps are found in both Gram-negative and Gram-positive bacteria. However, the pumps found in Gram-negative bacteria are more complex due to the structure of the cell envelope Figure 2.10 demonstrates the examples of efflux pumps in *S. aureus, E. coli, P. aeruginosa*, and *L. lactis* (Schweizer, 2003).

2.6.3 Efflux pump in gram-negative bacteria

Gram-negative bacteria exhibit rapid drug resistance due to the complex structure of their cell membrane, which consists of two membranes: the outer membrane and the inner membrane, separated by the periplasm. The structure is responsible for the high degree of antibiotic resistance found in Gram-negative bacteria, making them a leading cause of antibiotic-resistant infections (Sahar Razavi, 2020).

The cell membrane of Gram-negative bacteria is composed of a variety of proteins that provide channels for the transport of nutrients and various toxic compounds. The transport of these substances depends on energy differences in the

efflux pump process. This process allows them to be excreted from the periplasm or cytoplasm to the outside of the cell.

The most common efflux pump in Gram-negative bacteria is the RND family (Srikumar R, 1999). This family of efflux pumps plays a crucial role in the development of drug-resistant Gram-negative bacteria. RND proteins are located within the periplasm. They form a tripartite structure in conjunction with outer membrane channel proteins and periplasm binding proteins, which are integral components of the efflux pump system. The loss of any of the proteins involved in this tripartite structure disrupts the entire efflux pump process.

Structure of the RND efflux systems

The RND efflux pump system consists of three key components:

1. Transporter Protein (Efflux Protein): This component, often referred to as the efflux protein, is located in the inner membrane of the bacterial cell. It recognizes and binds to the specific substances to be expelled.
2. Periplasmic Accessory Protein (or Membrane Fusion Protein - MFP): This protein is located in the periplasm, which is the space between the inner and outer membranes of Gram-negative bacteria. It helps connect the transporter protein to the outer membrane protein.
3. Outer Membrane Protein (OMP): This protein is situated in the outer membrane of the bacterial cell and forms a channel through which the expelled substances are released into the external environment.

Two well-known RND efflux systems that have been found earlier including *AcrAB-TolC* from *E. coli* and *MexAB-OprM* structural system from *P. aeruginosa* (Figure 2.11).

AcrAB-TolC System: This system was first identified in *E. coli*, and is a major contributor to antibiotic resistance in *E. coli* and other related bacteria. It includes the *AcrB* transporter protein in the inner membrane, the *AcrA* periplasmic accessory protein (or MFP), and the *TolC* outer membrane protein. This system is capable of expelling a wide range of antibiotics and other toxic compounds from the bacterial cell.

MexAB-OprM System: This system was initially discovered in *P. aeruginosa*, and is a key factor in its multidrug resistant characteristic. It consists of the *MexB* transporter protein, the *MexA* periplasmic accessory protein (MFP), and the *OprM* outer membrane protein. This system, similar to *AcrAB-TolC*, can effectively pump out various antibiotics. *P. aeruginosa* can also harbor other efflux pump systems, such as *MexXY* and *MexEF-OprN*, which contribute to its multidrug resistance and make treatment of infections caused by this bacterium more complex. *P. aeruginosa* can transport two efflux pumps simultaneously: *MexAB-OprM* with *MexXY* or *MexAB-OprM* with *MexEF-OprN*.

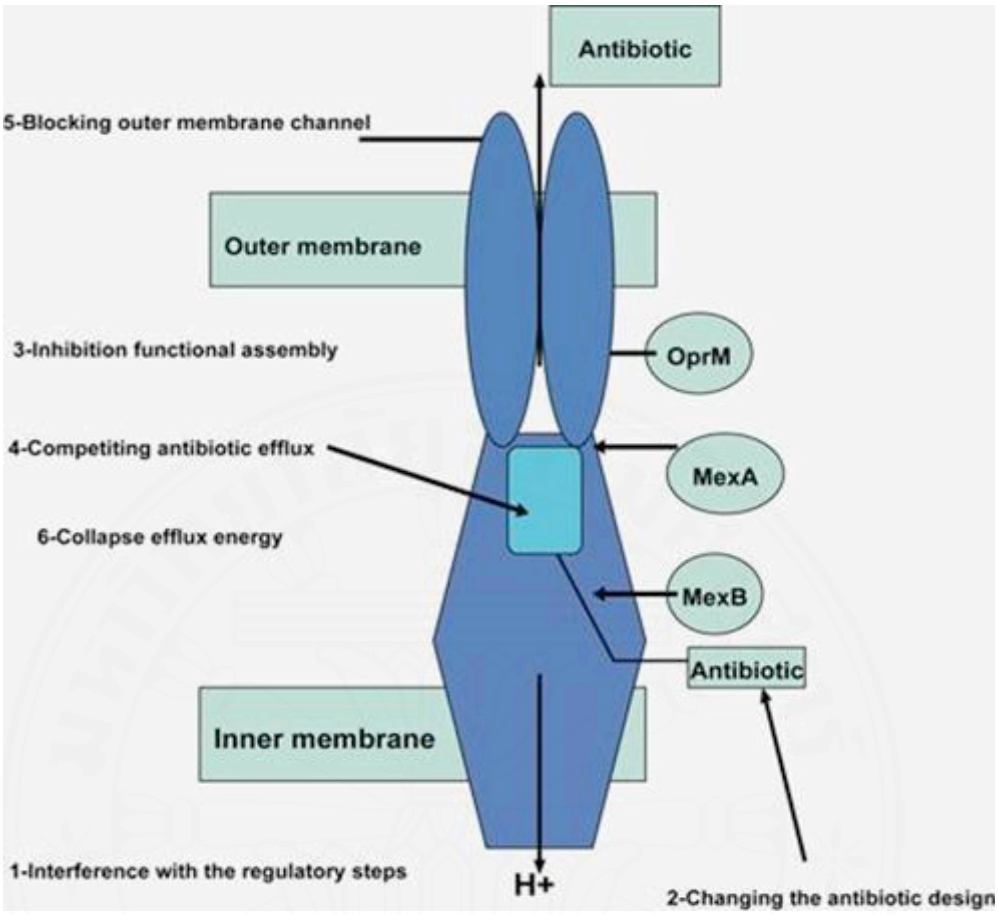


Figure 2.11: Structure of the *MexAB-OprM* pump of *P. aeruginosa*

The transport of drugs is driven by the difference in electrochemical potential across cell membranes, also known as the transmembrane electrochemical gradient of protons. The RND family utilizes the proton gradient as a driving force to expel drugs from the cell in exchange between one H+ ion per drug molecule.

2.6.4 Efflux pump systems in *P. aeruginosa*

Efflux pump systems in *P. aeruginosa* are a critical component of its multidrug resistance. These systems are responsible for pumping out a wide range of toxic compounds, including antibiotics. Efflux pump systems in *P. aeruginosa* can be classified into several major families including:

1. *MexAB-OprM*: This tripartite efflux pump system consists of *MexA*, *MexB*, and *OprM* proteins. *MexB* is located in the inner membrane, *MexA* in the periplasm, and *OprM* in the outer membrane. *MexAB-OprM* is known for its

role in expelling a variety of antibiotics, including beta-lactams and fluoroquinolones (Figure 2.12).

2. *MexCD-OprJ*: Similar to *MexAB-OprM*, *MexCD-OprJ* is another important efflux pump system in *P. aeruginosa*. It is responsible for expelling antibiotics like aminoglycosides, fluoroquinolones, and chloramphenicol.
3. *MexEF-OprN*: This efflux pump system is associated with resistance to fluoroquinolones and various other antimicrobial agents. It is involved in the resistance of *P. aeruginosa* to multiple classes of antibiotics.
4. *MexXY*: *MexXY* efflux pump system is responsible for expelling aminoglycosides, which are a class of antibiotics. It also plays a role in resistance to other toxic compounds and is associated with acquired resistance.
5. Other Efflux Pumps: *P. aeruginosa* may possess additional efflux pump systems, such as *MexGHI-OpmD* and others. These pumps contribute to the bacterium's multidrug resistance by expelling a range of antibiotics and toxic substances.

MexAB-OprM efflux pump is one of the most well-studied efflux pumps in *P. aeruginosa*. It is primarily controlled by a set of regulatory genes that govern its expression and function. The key regulatory genes associated with *MexAB-OprM* include:

1. *mexR*: The *mexR* gene codes for a transcriptional repressor protein, *MexR*. MexR inhibits the expression of the *MexAB-OprM* efflux pump. Mutations in the *mexR* gene can lead to overexpression of *MexAB-OprM*, resulting in increased antibiotic resistance in *P. aeruginosa*.
2. *mexZ*: This gene encodes the transcriptional repressor protein *MexZ*, which negatively regulates *mexXY* and *oprA* genes. The *MexXY-OprA* system is another efflux pump in *P. aeruginosa* that contributes to multidrug resistance.

3. *mexT*: *MexT* is a transcriptional activator that can enhance the expression of the *MexAB-OprM* pump. It is often induced in response to certain environmental conditions or stressors.
4. *nalC*: *NalC* is a transcriptional regulator that controls the *MexAB-OprM* system. Mutations in *nalC* can also lead to overexpression of the efflux pump.

These regulatory genes, particularly mexR (Figure 2.12), play a crucial role in modulating the expression of the *MexAB-OprM* efflux pump in response to environmental factors and the presence of antibiotics. Mutations or alterations in these regulatory genes can contribute to antibiotic resistance by leading to increased expression of the efflux pump. Efflux pump systems in *P. aeruginosa* are often inducible, meaning their expression is upregulated in response to the presence of specific substrates, particularly antibiotics. This adaptive response allows the bacterium to survive in hostile environments and evade the effects of antimicrobial agents.

Figure 2.12: The operon of the *MexAB-OprM* Efflux Pump in *P. aeruginosa*

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Bacterial strains and cultivation

The *P. aeruginosa* strains used in this study included a standard strain PAO1 ATCC27853, K767 (wild-type), K1523 (*MexB* deletion), K1455 (*MexAB-OprM* overexpressed), and 15 strains separated from the clinical samples (Table 3.1). *P. aeruginosa* strains were cultured on Blood agar at 37°C for 18 hours or inoculated into Tryptic Soy Broth (TSB), at 37°C for 18 hours. Stock cultures were prepared by transferring the isolated colonies grown on blood agar into 15% glycerol in 2-mL TSB and stored at -80°C. For each experiment, subculturing was performed directly from the stock culture to ensure consistent characteristics of the strains in each experiment.

Table 3.1 *P. aeruginosa* isolates used in this study

Strains	Source	Characteristic
PAO1 ATCC 27853	Purchased from ATCC	Standard strain
PAO1 K767	Gifted from Assoc. Prof. Srimanote (Siriyong et al., 2017)	Wild-type strain
K1523	Gifted from Assoc. Prof. Srimanote (Siriyong et al., 2017)	*MexB* deletion strain
K1455	Gifted from Assoc. Prof. Srimanote (Siriyong et al., 2017)	*MexAB-OprM* overexpression strain
H2173	Clinical isolate from TUH	Isolated from hemoculture
P4	Clinical isolate from TUH	Isolated from pus sample
P10	Clinical isolate from TUH	Isolated from pus sample
P327	Clinical isolate from TUH	Isolated from pus sample
P330	Clinical isolate from TUH	Isolated from pus sample
SP2	Clinical isolate from TUH	Isolated from sputum sample
SP21	Clinical isolate from TUH	Isolated from sputum sample
SP30	Clinical isolate from TUH	Isolated from sputum sample
SP48	Clinical isolate from TUH	Isolated from sputum sample
SP119	Clinical isolate from TUH	Isolated from sputum sample
SP275	Clinical isolate from TUH	Isolated from sputum sample
SP539	Clinical isolate from TUH	Isolated from sputum sample
SP541	Clinical isolate from TUH	Isolated from sputum sample
SP546	Clinical isolate from TUH	Isolated from sputum sample
SP556	Clinical isolate from TUH	Isolated from sputum sample

TUH = Thammasat University Hospital

3.2 Determination of the minimum inhibitory concentration (MIC) of benzalkonium chloride (BKC) and chlorhexidine (CHX)

All the MIC of BKC and CHX was determined using the broth microdilution method, adapted from the Clinical Laboratory Standards Institute (CLSI) guidelines for antimicrobial susceptibility testing (James S. Lewis & Sandra S. Richter, 2022). *P. aeruginosa* strains were incubated in Mueller Hinton Agar (MHA) at 37°C for 18 hours. After the incubation period, the bacterial cultures were adjusted to a turbidity equivalent to 0.5 McFarland standard and diluted 1:100 with MHB and tested with BKC at an initial concentration of 2% v/v and CHX at an initial concentration of 2% v/v in a 96-well plate. Each well contained 50 microliters of the biocides, followed by a 2-fold serial dilution with MHB. Then, 50 microliters of the adjusted *P. aeruginosa* culture were added to each well (final volume of 100 microliters). Positive control wells contained *P. aeruginosa* PAO1 ATCC27853 in MHB, while negative control wells contained only MHB at a volume of 100 microliters. The inhibition of bacterial growth was recorded, and the MIC was defined as the lowest concentration of the antimicrobial agent that inhibited bacterial growth.

3.3 Cultivation in the subinhibitory concentration (SIC) of BKC and CHX

Subinhibitory concentrations (SIC) was calculated as half of the MIC values. *P. aeruginosa* isolates were inoculated into Mueller Hinton Broth (MHB) containing the SIC of BKC and the SIC of CHX and incubated at 37°C for 18 hours. Subsequently, the cultures were transferred onto Mueller Hinton Agar (MHA) plates containing the same concentration of biocides and incubated again at 37°C for 18 hours. This process was repeated four more times. After completion, the remaining cultured bacteria were then subjected to further testing.

3.4 Determination of the MIC for ciprofloxacin, ceftazidime and gentamicin using broth microdilution test

The MIC values of ciprofloxacin, ceftazidime, and gentamicin for each *P. aeruginosa* strain were obtained through the broth microdilution test method as recommended in the CLSI guidelines for antimicrobial susceptibility testing M100-S32 (James S. Lewis & Sandra S. Richter, 2022). The MIC testing was conducted for all three types of antibiotics before the bacteria were exposed to the antimicrobial agents and again after culturing the bacteria under the subinhibitory concentration of BKC and CHX. Each test was repeated three times, and the results were reported as the average MIC values obtained for all tests. Additionally, the interpretation of antibiotic susceptibility status was performed according to the criteria defined by CLSI, where "S" indicated susceptibility to the antibiotic, "I" indicated intermediate susceptibility, and "R" indicated resistance to the antibiotic (as shown in Table 3.2).

Table 3.2 CLSI guidelines for antimicrobial susceptibility testing M100-S32 for *P. aeruginosa*

Antimicrobial Agent	Interpretive Categories and MIC Breakpoints, μg/mL		
	Susceptible (S)	Intermediate (I)	Resistance (R)
Ciprofloxacin	≤0.5	1	≥2
Ceftazidime	≤8	16	≥32
Gentamicin	≤4	8	≥16

3.5 RNA extraction and determination of RNA quality

P. aeruginosa strains including PAO1 ATCC27853, strain K767 (wild-type), K1523 (MexB deletion), K1455 (MexAB-OprM overexpression), and two clinical isolates (P10 and SP2) were chosen for the gene expression study. These six isolates, both from the baseline culture and the final biocide-exposed culture, underwent extraction using

the PureLink™ RNA MiNi Kit. The extracted RNA was then treated with RNase-free DNase I for 30 minutes to eliminate contaminating DNA. The resulting purified RNA was stored at -80℃ for future use.

Determination of RNA purity

The purity and concentration of the RNA was evaluated by the Nanodrop™ 2000/2000c spectrophotometry at 260 and 280 nm. RNA solution with an A_{260}/A_{280} ratio about 1.8-2.0 was accepted for its purity.

Determination of RNA quality

The RNA quality was checked by agarose gel electrophoresis. 1.0%agarose gel was prepared by dissolving 0.35 g of agarose in 35 ml TBE buffer. Then, 4 µL of 50 mg/ml ethidium bromide was added into the suspension and melted the gel by a microwave oven. After that, the gel solution was poured into the gel cassette. After the gel was thoroughly set, it was transferred into an electrophoresis chamber containing TBE buffer. The RNA was mixed with formamide loading dye and the mixture was pipetted into a well of the agarose gel. The electrophoresis was set at 100 volts for 70 minutes. The RNA band was visualized by the gel documentation system under the UV light.

3.6 Gene expression study

The extracted RNA was used to assess the expression levels of *MexA, MexB, OprM*, and the housekeeping gene *rpsL* through real-time PCR (Thermo Fisher, QuantStudio3). The Brilliant II SYBR® Green QRT-PCR Master Mix Kit, 1-Step, was employed for this analysis, with specific primers detailed in Table 3.2. The temperature and setting conditions for real-time PCR are provided in Table 3.3.

Table 3.3 qPCR Primers of tested genes

Genes	Primers (5'-3')	Product (bp)	Function
MexA	F: 5'- CTCGACCC GATCTACGTC -3' R: 5'- GTCTTCACCTCGACACCC -3'	503	Efflux pump (*MexA*)
MexB	F: 5'- TTGATAAGGCCCATTTTCGCGT -3' R: 5'- TCTGCTGCTCGATCACCTGGA -3'	189	Efflux pump (*MexB*)
OprM	F:5'-GATCCCCGACTACCAGCGCCCCG -3' R:5'-ATGCGGTACTGCGCCCGGAAGGC-3'	247	Efflux pump (*OprM*)
rpsL	F: 5'- GCAAGCGCATGGTCGACAAGA -3' R:5'-CGCTGTGCTCTTGCAGGTTGTGA -3'	201	Housekeeping control gene

Table 3.4 Setting conditions for real-time PCR

Setting	*MexA*	*MexB*	*OprM*	*rpsL*
RNA (100 ng/µL) (µL)	0.5	0.5	0.5	0.5
Primer (µL)	0.5	0.5	0.5	0.5
SYBR® GREEN (µL)	6.25	6.25	6.25	6.25
Denaturing Temp (°C)	95	95	95	95
Annealing Temp (°C)	57	57	57	57
Extension Temp (°C)	72	72	72	72

3.7 Assessment of efflux pump activity

P. aeruginosa strains, including PAO1 ATCC27853, K767, K1523, K1455, P10 and SP2, from the gene expression study were further tested for efflux pump activity. To assess the efflux pump activity, ethidium bromide (EtBr)-agar cartwheel method as described by Martins (Martins et al., 2011), was employed. If the bacteria have efflux pump activity, the bacteria will expel EtBr from the overnight-cultured colony, making it invisible under the UV illuminator due to the lack of fluorescent color. If it fluoresces, it means there is no efflux activity. The higher the concentration of EtBr required to produce fluorescence indicates a higher presence of efflux pumps. Overnight cultures of *P. aeruginosa* isolates were streaked onto the Mueller Hinton agar (MHA) containing serial EtBr concentration ranging from 0 to 7 mg/L, and then incubated at 37°C for 16 hours. After incubation, EtBr-containing MHA plates were examined under a UV transilluminator to record the minimum concentration of EtBr that produced fluorescence in the bacterial streak.

3.8 Statistic analysis

Statistical analyses were performed using GraphPad Prism version 10.3.0 for Windows. Statistical analysis of the data involved comparing the MIC values of antimicrobial drugs, expression of the efflux pump genes, and the efflux pump activity before and after exposure to the BKC or CHX. This was performed using either the paired *t*-test or the Wilcoxon signed rank test. A *p*-value less than 0.05 was considered statistically significant.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 MIC of biocides

The MIC values of BKC and CHX, which were the minimal concentrations of biocides that inhibited the growth of *P. aeruginosa*, and the SIC values, which were half of the MIC, are shown in Table 4.1

In this experiment, the MIC values of both BKC and CHX were found to be lower than the recommended concentrations of both biocides. The typical concentration for microbial eradication of BKC ranges from 0.1% to 1%. However, this study demonstrated that concentrations as low as 5 to 50 times lesser than the recommended range still successfully inhibited the growth of *P. aeruginosa*. Similarly, for CHX, which is commonly used at a concentration of 0.5% to 4% for skin disinfection and for surgical site preparation, this study found that a minimum concentration of 0.001% was sufficient to inhibit microbial growth.

The findings illustrate that the utilization of both BKC and CHX at their recommended concentrations exhibits potent bactericidal efficacy due to their MIC values being significantly below the recommended concentrations.

Table 4.1 Minimal inhibitory concentrations of biocides

Strains	Benzalkonium Chloride			Chlorhexidine		
	MIC (%)	SIC (%)	Chosen concentration for culture (%)	MIC (%)	SIC (%)	Chosen concentration for culture (%)
PAO1	0.023	0.0117	0.008	0.001	0.0005	0.0005
K767	0.012	0.0050	0.004	0.001	0.0005	0.0005
K1523	0.008	0.0039	0.004	0.001	0.0005	0.0005
K1455	0.023	0.0117	0.008	0.001	0.0005	0.0005
H2173	0.008	0.0039	0.004	0.001	0.0005	0.0005
P4	0.008	0.0039	0.004	0.001	0.0005	0.0005
P10	0.016	0.0078	0.008	0.001	0.0005	0.0005
P327	0.002	0.0010	0.004	0.001	0.0005	0.0005
P330	0.008	0.0039	0.004	0.001	0.0005	0.0005
SP2	0.016	0.0078	0.008	0.001	0.0005	0.0005
SP21	0.047	0.0234	0.008	0.001	0.0005	0.0005
SP30	0.016	0.0078	0.008	0.001	0.0005	0.0005
SP48	0.016	0.0078	0.008	0.001	0.0005	0.0005
SP119	0.016	0.0078	0.008	0.001	0.0005	0.0005
SP275	0.016	0.0078	0.008	0.001	0.0005	0.0005
SP539	0.016	0.0078	0.008	0.001	0.0005	0.0005
SP541	0.008	0.0039	0.004	0.001	0.0005	0.0005
SP546	0.016	0.0078	0.008	0.001	0.0005	0.0005
SP556	0.016	0.0078	0.008	0.001	0.0005	0.0005

Abbreviations: MIC, Minimal inhibitory concentration; SIC, Subinhibitory concentration; BKC, Benzalkonium chloride; CHX, Chlorhexidine

4.2 Culturing of *P. aeruginosa* in media containing SIC of BKC and CHX

For BKC, we initially chose concentrations based on the SIC levels of each strain. However, it was observed that when culturing *P. aeruginosa* isolates in media containing BKC at those concentrations for 18 hours, strains PAO1 ATCC27853 and K1455 failed to grow. Therefore, the final concentration used for culturing strains 27853 and K1455 was reduced to 0.008% alongside other strains, and a concentration of 0.004% for the other strains shown in (Table 4.1).

For CHX, it was found that the MIC values for all strains were the same, at 0.001%. This resulted in a calculated SIC value of 0.0005%. When culturing *P. aeruginosa* with this concentration, all strains were able to grow. Therefore, we selected a concentration of 0.0005% for CHX for evaluating its effect on *P. aeruginosa*.

4.3 Effect of BKC on the susceptibility of *P. aeruginosa* to antibiotics

Table 4.2 shows the MICs and antibiotic resistance levels of *P. aeruginosa* strains to ciprofloxacin, ceftazidime, and gentamicin, comparing pre- and post-culturing with sublethal concentrations of BKC. Overall, most strains exhibited only minor changes, and these changes were not statistically significant. There was no strain that their antibiotic susceptibility status had changed. For the response to ciprofloxacin, strain K767, P330, SP30, SP48 and SP275 showed an increase in MIC to ciprofloxacin by more than 2-fold, but this increase was not statistically significant and did not alter the strain's resistance level (remaining sensitive to the ciprofloxacin after exposure to BKC). Strain P10 showed a statistically significant decrease in MIC to ciprofloxacin ($P < 0.0001$). For the response to ceftazidime, strains PAO1, K767, K1455, P330, SP2, SP119, SP275 and SP546 showed an increase in MIC to ceftazidime by more than 2-fold. Notably, strain K1455 had a statistically significant 4.0-fold increase in MIC to ceftazidime after BKC exposure ($P < 0.0001$). However, there was no change in the sensitivity level to ceftazidime in these three strains. As for gentamycin, all strains exhibited slight alterations of their MICs, strain P330, SP30, SP48, SP119, SP539, SP541 and SP546

showed an increase in MIC to gentamycin by 2-fold and above, but this increase was not statistically significant and did not alter the strain's resistance level (remaining sensitive to the gentamycin after exposure to BKC). With strain K767 showing a significant decrease in MIC to gentamycin. There was no change in gentamicin sensitivity levels when comparing before and after cultivation under the influence of BKC.

Table 4.2 Comparison of MIC and susceptibility status of *P. aeruginosa* to ciprofloxacin, ceftazidime and gentamicin before and after exposure to BKC

Strains	Ciprofloxacin (µg/ml)			Ceftazidime (µg/ml)			Gentamicin (µg/ml)		
	Baseline	BKC	Fold Change	Baseline	BKC	Fold Change	Baseline	BKC	Fold Change
	Susceptibility level			Susceptibility level			Susceptibility level		
PAO1	0.094	0.125	1.3	0.50	2.0	4.0	1.0	1.0	1.0
	S --> S			S --> S			S --> S		
K767	0.047	0.125	2.7	0.5	1.0	2.0	1.5	1.0	0.6*
	S --> S			S --> S			S --> S		
K1523	0.023	0.016	0.7	0.5	0.25	0.5	1.0	1.0	1.0
	S --> S			S --> S			S --> S		
K1455	0.190	0.250	1.3	1.0	4.0	4.0*	1.0	1.0	1.0
	S --> S			S --> S			S --> S		
H2173	0.063	0.063	1.0	2.0	2.0	1.0	0.25	0.25	1.0
	S --> S			S --> S			S --> S		
P4	0.063	0.063	1.0	1.5	1.0	0.7	2.0	1.0	0.5
	S --> S			S --> S			S --> S		
P10	0.094	0.016	0.2*	1.0	1.0	1.0	1.0	1.0	1.0
	S --> S			S --> S			S --> S		
P327	8.0	8.0	1.0	>256	>256	N/A	32.0	32.0	1.0
	R --> R			R --> R			R --> R		
P330	0.063	0.125	2.0	1.0	4.0	4.0	1.0	2.0	2.0
	S --> S			S --> S			S --> S		

Table 4.2 Comparison of MIC and susceptibility status of *P. aeruginosa* to ciprofloxacin, ceftazidime and gentamicin before and after exposure to BKC (Cont.)

SP2	0.047	0.063	1.3	1.5	4.0	2.7	1.0	1.0	1.0
	S --> S			S --> S			S --> S		
SP21	32.0	32.0	1.0	8.0	4.0	0.5	256	>256	N/A
	R --> R			S --> S			R --> R		
SP30	0.031	0.063	2.0	2.0	2.0	1.0	1.0	2.0	2.0
	S --> S			S --> S			S --> S		
SP48	0.063	0.125	2.0	6.0	6.0	1.0	1.0	4.0	4.0
	S --> S			S --> S			S --> S		
SP119	0.063	0.063	1.0	1.0	4.0	4.0	1.0	2.0	2.0
	S --> S			S --> S			S --> S		
SP275	0.063	0.250	4.0	2.0	4.0	2.0	0.75	1.0	1.3
	S --> S			S --> S			S --> S		
SP539	0.125	0.125	1.0	1.5	2.0	1.3	2.0	4.0	2.0
	S --> S			S --> S			S --> S		
SP541	0.125	0.125	1.0	4.0	4.0	1.0	1.0	2.0	2.0
	S --> S			S --> S			S --> S		
SP546	0.063	0.063	1.0	1.5	8.0	5.3	1.0	2.0	2.0
	S --> S			S --> S			S --> S		
SP556	16.0	16.0	1.0	>256	>256	N/A	256	>256	N/A
	R --> R			R --> R			R --> R		

Abbreviations: MIC, Minimal inhibitory concentration; BKC, Benzalkonium chloride; CHX, Chlorhexidine; S, Susceptible; R, Resistance; *, $p < 0.05$

4.4 Effect of CHX on the susceptibility of *P. aeruginosa* to antibiotics

The effect of CHX exposure on antibiotic susceptibility is shown in Table 4.3. Overall, significant changes of MICs that increased more than twofold were found in five strains (K1455, H2173, P10, SP2, SP48), while the remaining other strains showed

only minor or changes. As for ciprofloxacin, SP2, SP30, SP48 and SP556 showed an increase in MIC by more than 2-fold. However, this increase was not statistically significant and did not alter the resistance level. Additionally, K1523 and P10 showed statistically significant decreases in MIC to ciprofloxacin (P = 0.0184 and P = 0.0157, respectively), but these changes also did not affect the resistance levels. Six strains including PAO1, K1455, P10, P330, SP2 and SP119 showed increases in MIC to ceftazidime by more than 2-fold, with statistically significant changes observed specially in the K1455 and P10 strains. However, these changes did not affect the sensitivity levels to ceftazidime in any of the six strains. Strain H2173, P10, P327, P330, SP30, SP541 and SP546 showed more than a twofold increase in MIC to gentamicin, but this change was not statistically significant and did not lead to a change in susceptibility status to gentamicin.

Table 4.3 Comparison of MIC and susceptibility status of *P. aeruginosa* to ciprofloxacin, ceftazidime and gentamicin before and after exposure to CHX

Strains	Ciprofloxacin (µg/ml)			Ceftazidime (µg/ml)			Gentamicin (µg/ml)		
	Baseline	CHX	Fold Change	Baseline	CHX	Fold Change	Baseline	CHX	Fold Change
	Susceptibility level			Susceptibility level			Susceptibility level		
PAO1	0.094	0.125	1.3	0.50	1.0	2.0	1.0	1.0	1.0
	S --> S			S --> S			S --> S		
K767	0.047	0.063	1.3	0.5	0.5	1.0	1.5	1.0	0.7
	S --> S			S --> S			S --> S		
K1523	0.023	0.016	0.7*	0.5	0.25	0.5	1.0	1.0	1.0
	S --> S			S --> S			S --> S		
K1455	0.190	0.250	1.3	1.0	2.0	2.0*	1.0	1.0	1.0
	S --> S			S --> S			S --> S		
H2173	0.063	0.063	1.0	2.0	2.0	1.0	0.25	1.0	4.0
	S --> S			S --> S			S --> S		
P4	0.063	0.063	1.0	1.5	2.0	1.3	2.0	2.0	1.0
	S --> S			S --> S			S --> S		

Table 4.3 Comparison of MIC and susceptibility status of *P. aeruginosa* to ciprofloxacin, ceftazidime and gentamicin before and after exposure to CHX (Cont.)

P10	0.094	0.063	0.7*	1.0	2.0	2.0*	1.0	2.0	2.0
	S --> S			S --> S			S --> S		
P327	8.0	8.0	1.0	>256	>256	N/A	32.0	64.0	2.0
	R --> R			R --> R			R --> R		
P330	0.063	0.063	1.0	1.0	2.0	2.0	1.0	2.0	2.0
	S --> S			S --> S			S --> S		
SP2	0.047	0.125	2.7	1.5	4.0	2.7	1.0	1.0	1.0
	S --> S			S --> S			S --> S		
SP21	32.0	32.0	1.0	8.0	4.0	0.5	256	>256	N/A
	R --> R			S --> S			R --> R		
SP30	0.031	0.063	2.0	2.0	2.0	1.0	1.0	2.0	2.0
	S --> S			S --> S			S --> S		
SP48	0.063	0.500	8.0	6.0	8.0	1.3	1.0	1.0	1.0
	S --> S			S --> S			S --> S		
SP119	0.063	0.031	0.5	1.0	2.0	2.0	1.0	1.0	1.0
	S --> S			S --> S			S --> S		
SP275	0.063	0.063	1.0	2.0	2.0	1.0	0.75	1.0	1.3
	S --> S			S --> S			S --> S		
SP539	0.125	0.094	0.8	1.5	2.0	1.3	2.0	2.0	1.0
	S --> S			S --> S			S --> S		
SP541	0.125	0.125	1.0	4.0	4.0	1.0	1.0	2.0	2.0
	S --> S			S --> S			S --> S		
SP546	0.063	0.063	1.0	1.5	2.0	1.3	1.0	2.0	2.0
	S --> S			S --> S			S --> S		
SP556	16	32	2.0	>256	>256	N/A	256	>256	N/A
	R --> R			R --> R			R --> R		

Abbreviations: MIC, Minimal inhibitory concentration; BKC, Benzalkonium chloride; CHX, Chlorhexidine; S, Susceptible; R, Resistance; *, $p < 0.05$

4.5 RNA isolation

Total RNAs of *P. aeruginosa* before and after biocide exposure were isolated from the bacterial cell pellets using the PureLink™ RNA MiNi Kit. The concentration of the RNA samples, determined by 260 nm spectrophotometrically absorption, was satisfactory. The purity of the RNA samples was good enough for our purpose (A260/A280 ratio within range 1.8-2.0) as measured by a nanodrop™ spectrophotometer. The quality of the extracted RNAs was acceptable, as determined by a gel electrophoresis (Figure 4.1).

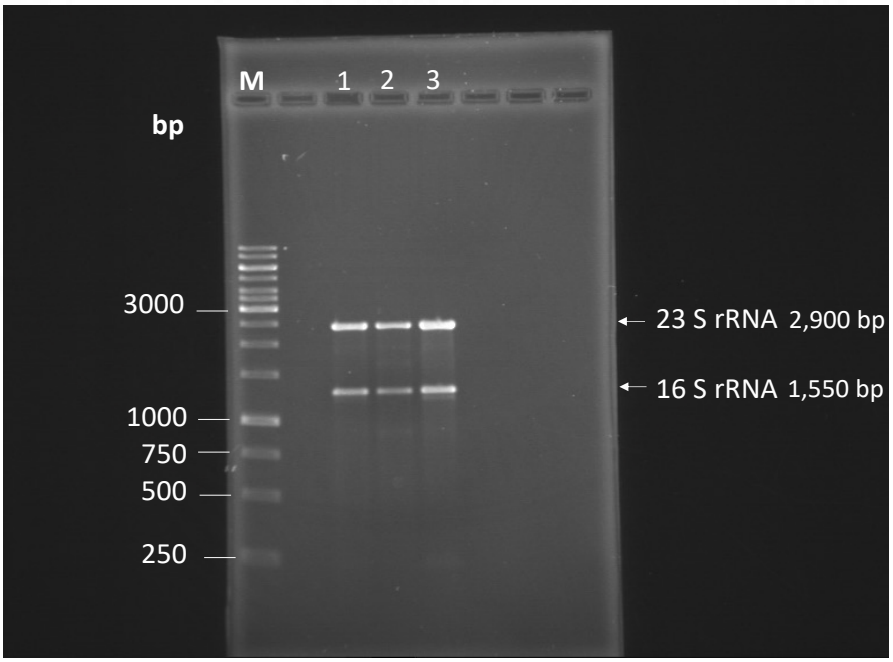


Figure 4.1: Agarose gel electrophoresis of extracted total RNAs of *P. aeruginosa*

Lane M: 1 kp DNA ladder

Lane 1: *P. aeruginosa* before biocide exposure

Lane 2: *P. aeruginosa* after BKC SIC exposure

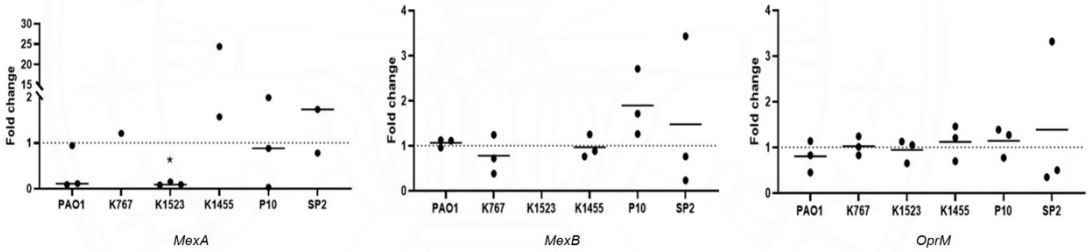
Lane 3: *P. aeruginosa* after CHX SIC exposure

Table 4.4 Total RNAs of *P. aeruginosa* before and after biocide exposure as measured by a nanodrop™ spectrophotometer

Sample	Nano drop Conc. (ng/µl)	A260	A280	A260/280	A260/230	Sample Type
27853	143.8	3.594	1.893	1.90	1.90	RNA
27853-BKC	181.0	4.526	2.338	1.94	0.83	RNA
27853-CHX	144.6	3.614	1.872	1.93	0.55	RNA
K1523	249.9	6.248	3.342	1.84	0.80	RNA
K1523-BKC	220.1	5.503	2.877	1.91	0.87	RNA
K1523-CHX	182.2	4.556	2.358	1.93	0.94	RNA
K767	256.0	6.400	3.330	1.92	1.06	RNA
K767-BKC	213.4	5.334	2.691	1.98	0.42	RNA
K-767-CHX	196.1	4.903	2.580	1.90	0.50	RNA
K1455	308.9	7.723	3.959	1.95	0.75	RNA
K1455-BKC	204.9	5.123	2.582	1.98	0.56	RNA
K1455-CHX	257.0	6.426	3.302	1.95	0.71	RNA
P10	123.0	3.075	1.625	1.89	0.32	RNA
P10-BKC	192.8	4.821	2.596	1.86	0.88	RNA
P10-CHX	123.8	3.095	1.662	1.86	0.33	RNA
SP2	391.4	9.784	4.883	2.00	1.24	RNA
SP2-BKC	358.3	8.957	4.311	2.00	1.46	RNA
SP2-CHX	271.3	6.783	3.415	1.99	0.94	RNA

4.6 Expression of efflux pumps gene after biocide exposure

When comparing the expression of efflux pump genes before and after exposure to sublethal concentrations of BKC, it was observed that overall, the gene expression of most tested *P. aeruginosa* strains did not change significantly, not exceeding two-fold from the baseline. Moreover, most of these changes were not statistically significant (Figure 4.2). Notably, the strain K1523 showed a statistically significant decrease in expression of *MexA* after BKC exposure. For *MexB* and *OprM*, it was found that no strain exhibited an increase in gene expression of more than two-fold, and there was no statistical significance observed when comparing before and after culturing under the influence of BKC.



- ● represents fold changes
- ─ represents the mean of fold changes
- * represents statistical significance

Figure 4.2: Fold changes in the expression of *MexA, MexB*, and *OprM* before and after exposure sublethal concentrations of BKC

In examining the expression of efflux pump genes under the influence of CHX, it was found that strains K767, K1455, and P10 showed an increase in *MexA* gene expression, surpassing a two-fold threshold (Figure 4.3). Particularly, the fold changes in *MexA* expression within strain K767 was statistically significant (P = 0.0157). It is

noteworthy that strain K1523 exhibited a statistically significant decrease in *MexA* gene expression (*P* = 0.0233). Regarding *MexB* gene expression, the majority of strains showed nearly unchanged expression levels, with no statistically significant increase exceeding two-fold, except for strain P10, which demonstrated an approximately two-fold increase (*P* = 0.0234). As for the *OprM* gene expression, most strains maintained expression levels comparable to baseline. Nonetheless, strain P10 exhibited a statistically significant approximately 1.5-fold increase in gene expression (*P* = 0.0263) when comparing before and after culturing under the influence of CHX.

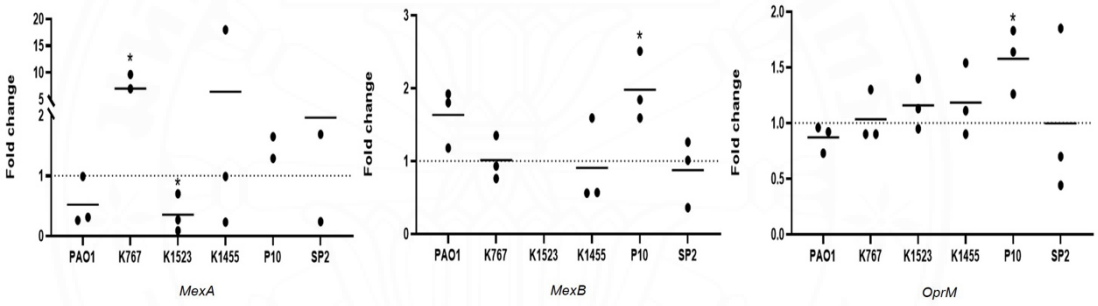


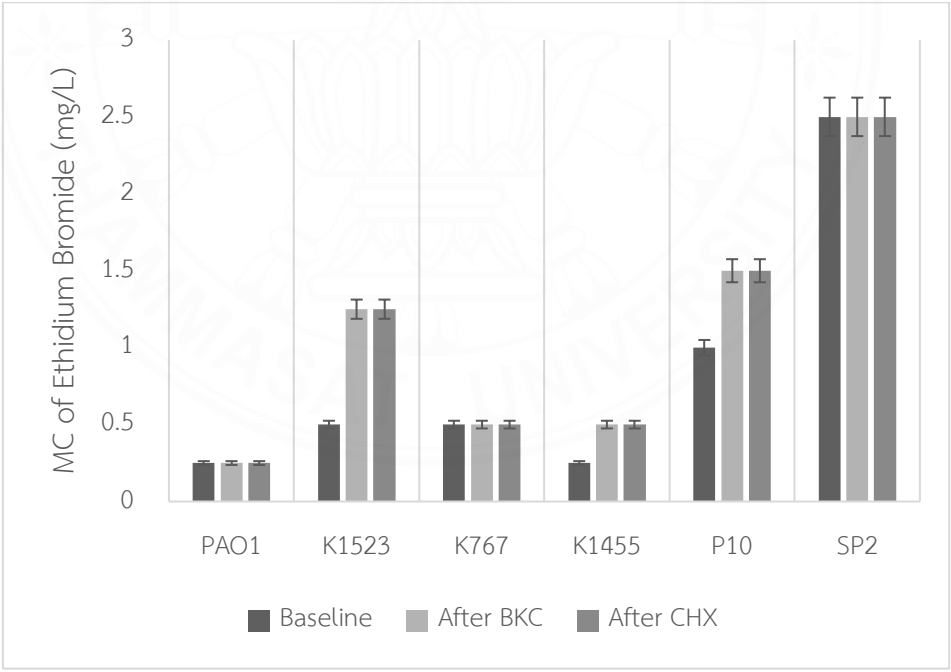
Figure 4.3: Fold changes in the expression of *MexA, MexB*, and *OprM* before and after exposure sublethal concentrations of CHX

4.7 Efflux pump activity after biocide exposure

Overall, the efflux pump activity of the majority of strains remained unchanged. However, strains K1523 and P10 exhibited an increase in efflux pump activities after exposure to both BKC and CHX as shown in Table 4.5 and Figure 4.4. Nevertheless, these changes were not statistically significant.

Table 4.5 Minimal concentration of EtBr indicating the efflux pump activities before and after biocide exposure

Strains	Minimal Concentration of Ethidium Bromide (mg/L)				
	Baseline	BKC	Fold change	CHX	Fold change
PAO1	0.25	0.25	1	0.25	1
K1523	0.5	1.25	2.5	1.25	2.5
K767	0.5	0.5	1	0.5	1
K1455	0.25	0.5	2	0.5	2
P10	1	1.5	1.5	1.5	1.5
SP2	2.5	2.5	1	2.5	1



MC: Minimal Concentration

Figure 4.4: Comparison of efflux pump activities before and after biocide exposure

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

Healthcare-associated infections become a significant issue in global public health. One of the common pathogens responsible for hospital-acquired infections is *P. aeruginosa*. This notorious pathogen plays a crucial role in causing infections in various systems of the body. It is often found in patients with reduced immune levels, those undergoing chemotherapy, and hospitalized patients. Additionally, *P. aeruginosa* commonly exhibits resistance to antibiotics, making it necessary for World Health Organizations to prioritize it as one of the pathogens of critical concern that urgently required research and development of new antibiotics (Reitzel et al., 2020). Therefore, it is important to investigate factors that may contribute to the drug resistance of this pathogen.

One method to control hospital-acquired infection is the use of biocides for cleaning purposes. Biocides are substances that are used to kill or inhibit the growth of microorganisms, including bacteria, viruses, and fungi (Jones & Joshi, 2021). They are commonly employed in hospitals and healthcare facilities to disinfect surfaces and equipment, thereby reducing the risk of transmission of pathogens and preventing infections.

However, despite their widespread use, there is ongoing debate and controversy surrounding the potential effects of biocides on antibiotic resistance (Kampf, 2018; Rozman et al., 2021). Some studies suggest that the use of biocides may contribute to the development of antibiotic resistance in bacteria (Reitzel et al., 2020). This could occur through mechanisms such as cross-resistance, where exposure to biocides leads to the selection of bacteria that are also resistant to antibiotics (Basiry et al., 2022).

Biocides, including disinfectants and antiseptics such as benzalkonium chloride (BKC) and chlorhexidine (CHX), are widely used in healthcare and domestic settings to control microbial growth and prevent infections. However, the widely use of these chemical agents has raised significant concerns about their potential role in

promoting antibiotic resistance. The interaction between biocides and bacterial resistance mechanisms, particularly efflux pumps, is of interest. Efflux pumps are integral membrane proteins that actively expel a variety of toxic substances, including antibiotics and biocides, out of bacterial cells. Their overexpression can lead to multidrug resistance, complicating treatment strategies. Understanding how exposure to biocides influences the expression and activity of efflux pumps in pathogens like *P. aeruginosa* is critical, as this knowledge could inform more effective infection control practices. This study thus aimed to elucidate the impact of BKC and CHX on the gene expression and activity of efflux pumps in various strains of *P. aeruginosa* and their subsequent effects on antibiotic susceptibility. The findings across different strains have revealed some insights into the interaction between these biocides and the bacterial resistance mechanisms.

Previous studies have found that the use of biocides such as BKC and CHX supports antibiotic resistance in various pathogens (Kim et al., 2018; Merchel Piovesan Pereira & Tagkopoulos, 2019). *Salmonella enterica* and *Escherichia coli* have been shown to exhibit resistance to biocides and demonstrate cross-resistance to antibiotics (Braoudaki & Hilton, 2004). Reports also indicate a correlation between *Acinetobacter* spp. with high MIC values for biocides and increased antibiotic resistance (Kawamura-Sato et al., 2010). Two *P. aeruginosa* strains, PAO1 and OO14, expressed cross-resistance between BKC and antibiotics, including chloramphenicol and polymyxin B (Loughlin, Jones, et al., 2002). Considering the mechanisms of drug resistance, *P. aeruginosa* utilizes similar mechanisms for both antibiotic and biocide resistance. This involves reducing the permeability of the cell membrane, as well as employing active efflux to pump substances out, thereby conferring resistance to BKC, CHX, and other biocides (Russell, 2003). This mechanism is similar to that used for antibiotic resistance. Therefore, it is speculated that biocide stimulation may increase the activation of the bacterial drug resistance mechanism, leading to the development of more drug-resistant strains.

On the other hand, other research suggests that the relationship between biocide use and antibiotic resistance is complex and may not always lead to increased resistance (Thomas et al., 2005). Factors such as the specific biocide used, the concentration and frequency of use, and the genetic makeup of the bacteria being targeted can all influence the outcome.

This research project was therefore conducted in order to elucidate the effect of using the biocides including BKC and CHX on the antibiotic resistance of *P. aeruginosa*, with a focus on the expression and function of the multidrug efflux pump which is the mechanism responsible for various antibiotic resistance. Our specific objectives include:

- To study the effect of BKC and CHX on antibiotic resistance of *P. aeruginosa*.
- To study the effect of BKC and CHX on the expression of genes that control the multidrug efflux pump of *P. aeruginosa*.
- To study the effect of BKC and CHX on the activity of the efflux pump of *P. aeruginosa*.

5.1 Effect of BKC and CHX on antibiotic resistance of *P. aeruginosa*

The current study compared MIC values and antibiotic susceptibility levels of *P. aeruginosa* before and after exposure to the subinhibitory concentrations of BKC and CHX. Across both experimental conditions, the majority of tested strains did not show significant changes in MIC values, with most alterations being statistically insignificant. Moreover, there was also no strains of *P. aeruginosa* demonstrating shifts in their antibiotic susceptibility status following exposure to the subinhibitory concentrations of BKC and CHX. Notably, one strain, P10, exhibited a statistically significant increase in MIC to ceftazidime by more than twofold (2.0-fold) after exposed to CHX. This singular observation suggests a possible inducement of resistance to ceftazidime due to CHX exposure. We also observed that exposure to BKC and CHX caused strain P10 to exhibit a significant decrease in MIC to ciprofloxacin by 0.2-fold and 0.7-fold, respectively. However, it is important to note that this observation was

limited to a single isolate out of the 19 tested strains. Particularly, the *MexAB*-overexpressing strain K1455 displayed a significant increase of more than twofold in the MIC of ceftazidime, raising concerns about potential cross-resistance between BKC and ceftazidime. This observation is consistent with previous reports suggesting antibiotic and BKC cross-resistance mediated by efflux pumps (Amsalu, 2020), and it did not result in a change in the susceptibility status to antibiotics of this particular strain.

Our findings demonstrate that the level of antibiotic resistance of *P. aeruginosa* after being cultured in subinhibitory concentration did not differ compared to the baseline antibiotic resistance level of the strain. These experimental results do not align with our anticipation that when bacteria adapt to become resistant to biocides, they will also exhibit increased resistance to antibiotics.

While no significant changes were observed, they are consistent with the findings in various reports. Thomas *et al*. conducted experiments using *P. aeruginosa* NCIMB 10421, wherein they induced resistance to chlorhexidine diacetate and subsequently tested for antibiotic resistance. They found no cross-resistance between biocides and antibiotics (Thomas et al., 2000; Thomas et al., 2005). This aligns with the findings of Cole *et al*., who studied cross-resistance in over 1200 strains of various pathogens collected from patients and household settings, dividing them into two groups: those exposed to biocides and those not exposed (Cole et al., 2003). They concluded that there was no correlation between antibiotic resistance and biocide exposure (Cole et al., 2003).

Regarding with the conflicting findings in these reports, it should be cautioned when applying laboratory findings to clinical practice. The discovery of a relationship between biocide tolerance and antibiotic resistance is often tested in laboratory settings, and its application to practical medical contexts should be approached with caution (Russell, 2003).

One important point that should not be missed was despite the contradictory conclusions from the aforementioned reports, stated that the risk of developing biocide-resistant bacteria is minimal (Meyer & Cookson, 2010). This is

because the actual biocides used have concentrations much higher than those required to inhibit bacterial growth by several times, which aligns with the findings of this study (Table 4.1).

Overall, these results underscore the complex interplay between antimicrobial agents and bacterial resistance mechanisms. While some strains exhibited changes in MIC values, the overall susceptibility profile remained largely unaffected. These findings emphasize the need for further research to elucidate the underlying mechanisms driving antimicrobial resistance in *P. aeruginosa*, aiding in the development of effective intervention strategies to combat hospital-acquired infections (van Dijk et al., 2022).

5.2 Effect of BKC and CHX on the expression of *MexA, MexB, OprM* genes and the efflux pump activity of *P. aeruginosa*

Upon exposure to BKC, it was observed that the *MexA*, *MexB*, and *OprM* genes, which are integral components of the efflux pump, exhibited expression levels above their baseline, albeit not reaching statistical significance (Table 5.1). The wild-type strain K767, the *MexAB*-overexpressing strain K1455, and two clinical isolates (P10 and SP2) demonstrated upregulation in the expression of these efflux pump genes, indicating the possibility of the induced expression from the BKC exposure. However, a significant reduction in *MexA* gene expression was observed in the *MexB* deletion strain K1523. This reduction is likely attributable to the deletion of *MexB* in this strain, given that *MexA* and *MexB* interact within the efflux pump tripartite structure (Nehme, 2005). Despite the observed upregulation of *MexAB-OprM* genes in PAO1, K767, K1455, and SP2, this did not translate into heightened efflux pump activity, as assessed by the efflux of ethidium bromide. The clinical isolate P10 was the sole strain to exhibit increased efflux pump activity corresponding with the elevated expression of its efflux pump genes.

Notably, exposure to BKC resulted in an increase in the MICs of ciprofloxacin and ceftazidime of PAO1, K767, K1455 and SP2. However, the MICs of these drugs were lower in the K1523 strain, and in particular the strain P10 where the decrease was statistically significant (Table 5.1). The MexAB-overexpressing strain K1455 displayed a significant increase of more than twofold in the MIC of ceftazidime, correlating with the increasing gene expression and the efflux pump activity, raising concerns about potential cross-resistance between BKC and ceftazidime. This observation is consistent with previous reports suggesting antibiotic and BKC cross-resistance mediated by efflux pumps (Amsalu, 2020). However, despite the observed increase in antibiotic MICs following BKC exposure, none of the isolates exhibited a change in their susceptibility status to the tested antibiotics. All isolates remained sensitive to ciprofloxacin, ceftazidime, and gentamicin.

Table 5.1 Summarized effect of BKC on *MexA, MexB, OprM* genes expression, the efflux pump activity, MICs and sensitivity status of antibiotics

Strain	Effect of BKC exposure									
	Relative gene expression (Fold change)			Efflux pump activity	MIC fold change			Sensitivity status		
	MexA	*MexB*	*OprM*		CIP	CAZ	GM	CIP	CAZ	GM
PAO1	↓	↑	↓	↔	↑	↑↑	↔	↔ S	↔ S	↔ S
K767 (Wild Type)	↑↑	↓	↑	↔	↑↑	↑↑	↓* 0.6*	↔ S	↔ S	↔ S
K1523 (*MexB* deletion)	↓* 0.11*	-	↓	↑	↓	↓	↔	↔ S	↔ S	↔ S
K1455 (*MexAB* overexpression)	↑↑	↓	↑	↑	↑	↑↑ 4.0*	↔	↔ S	↔ S	↔ S
P10 (Clinical isolate)	↓	↑	↑	↑	↓* 0.2*	↔	↔	↔ S	↔ S	↔ S
SP2 (Clinical isolate)	↑↑	↑	↑	↔	↑	↑↑	↑	↔ S	↔ S	↔ S

Symbol: ↔; no change (1-fold)

↑; increase > 1-fold

↑↑; increase ≥ 2 folds

↓; decrease < 1-fold

S: sensitivity

*, $p < 0.05$ and data was labeled where significance was detected.

In contrast to BKC, exposure to CHX resulted in both decreased and increased expression of efflux pump genes (Table 5.2). Notably, a significant reduction in MexA gene expression was observed in the K1523 strain, which is characterized by a MexB deletion. Conversely, strains K767, K1455, P10, and SP2 demonstrated increased MexA expression, with K767 exhibiting a particularly notable increase of more than sevenfold. Additionally, significant upregulation of MexB and OprM was detected in the clinical isolate P10. Efflux pump activity, as assessed by the expulsion of ethidium bromide, remained unchanged among PAO1, K767 and SP2 strains. However, K1523, K1455 and P10 isolates exhibited increased efflux activity, correlating with elevated gene expression. CHX exposure led to variable changes in the minimum inhibitory concentrations (MICs) of the antibiotics tested. Specifically, ciprofloxacin MICs increased from baseline in PAO1, K767, K1455, and SP2 strains following CHX exposure, whereas K1523 and P10 displayed significantly decreased MICs. For ceftazidime, PAO1, SP2, and particularly K1455 and P10, showed significantly elevated MICs compared to baseline values. Despite these increases in MICs, the susceptibility levels to these antibiotics remained unchanged.

Table 5.2 Summarized effect of CHX on *MexA, MexB, OprM* genes expression, the efflux pump activity, MICs and sensitivity status of antibiotics

Strain	Effect of CHX exposure									
	Relative gene expression (Fold change)			Efflux pump activity	MIC fold change			Sensitivity status		
	MexA	*MexB*	*OprM*		CIP	CAZ	GM	CIP	CAZ	GM
PAO1	↓	↑	↓	↔	↑	↑↑	↔	↔ S	↔ S	↔ S
K767 (Wild Type)	↑↑* 7.03*	↑	↑	↔	↑	↔	↓	↔ S	↔ S	↔ S
K1523 (*MexB* deletion)	↓* 0.35*	-	↑	↑	↓* 0.7*	↓	↔	↔ S	↔ S	↔ S
K1455 (*MexAB* overexpression)	↑↑	↓	↑	↑	↑	↑↑ 2.0*	↔	↔ S	↔ S	↔ S
P10 (Clinical isolate)	↑↑	↑* 1.98*	↑* 1.58*	↑	↓* 0.7*	↑↑ 2.0*	↑↑	↔ S	↔ S	↔ S
SP2 (Clinical isolate)	↑	↓	↔	↔	↑↑	↑↑	↔	↔ S	↔ S	↔ S

Symbol: ↔; no change (1-fold)

↑; increase > 1-fold

↑↑; increase ≥ 2 folds

↓; decrease < 1-fold

S: sensitivity

*, $p < 0.05$ and data was labeled where significance was detected.

5.3 Conclusion and Recommendation

Biocide exposure influenced the MIC values and the efflux pump gene expression and the efflux pump activity of various *P. aeruginosa* strains to varying extents. From these findings, it can be suggested that:

BKC predominantly impacts *MexA* gene expression, with some effects on *MexB* and *OprM* in certain strains and shows some increase in efflux pump activity. Despite the findings that MIC changes were often not statistically significant or not resulting in the change of susceptibility status, the decreases of antibiotic susceptibility were apparent.

CHX influences both MIC and gene expression more distinctly in certain strains, with significant changes in *MexA, MexB,* and *OprM* gene expression. However, the efflux pump activity remained mostly unchanged. MIC values increased in certain strains but did not alter the susceptibility status. Nevertheless, the decreases of antibiotic susceptibility were also observed.

Overall, these observations suggest that while biocides can induce some biological responses, they might not significantly alter the antibiotic resistance mechanisms in the tested strains. The results highlight the strain-specific nature of *P. aeruginosa*'s response to biocides, with varying impacts on gene expression, efflux pump activity, and antibiotic susceptibility. The lack of consistent changes in efflux pump activity despite alterations in gene expression suggests that transcriptional changes alone do not dictate efflux pump function and resistance phenotypes. Additionally, the differential responses observed between laboratory strains and clinical isolates underscore the complexity of resistance mechanisms in natural environments compared to controlled laboratory settings.

These findings emphasize the need for an understanding of how biocides affect bacterial physiology and resistance, which is crucial for developing strategies to mitigate resistance development in clinical and environmental contexts. Further research should investigate the underlying molecular mechanisms driving these strain-

specific responses and explore the potential role of other resistance determinants beyond the *MexAB-OprM* efflux pump system

In conclusion, while biocides play a crucial role in infection control, it is important to continue studying their effects on antibiotic resistance to ensure that their use does not inadvertently contribute to the problem. This requires ongoing research and monitoring of both biocide and antibiotic resistance patterns to inform best practices for infection prevention and control in healthcare settings.

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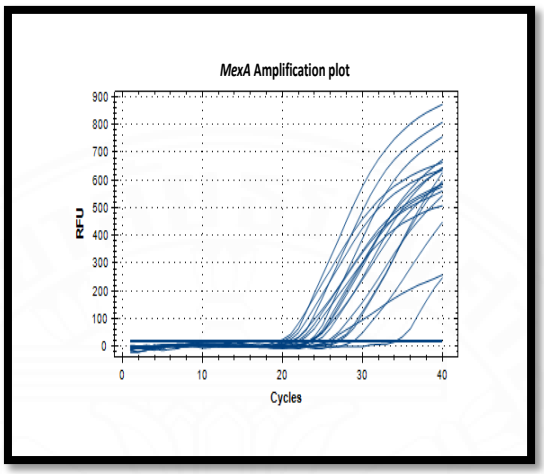
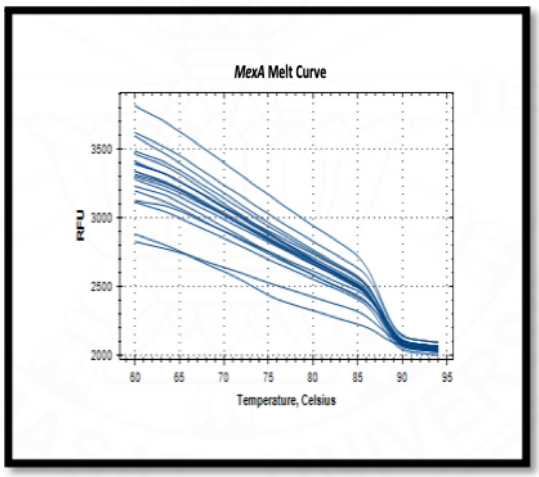
APPENDICES

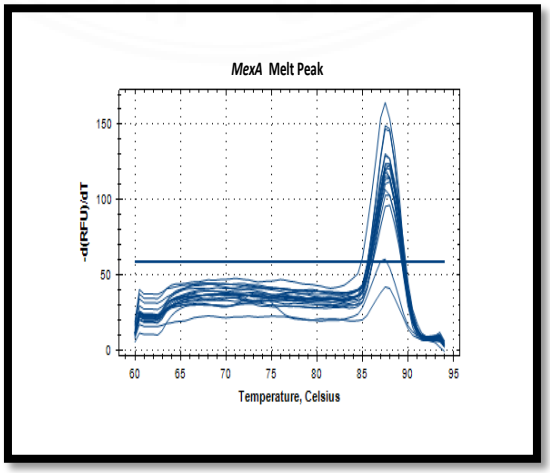
APPENDIX A

Amplification plot, melt curve and melt peak analysis:

MexA gene expression of *P. aeruginosa* after biocide exposure



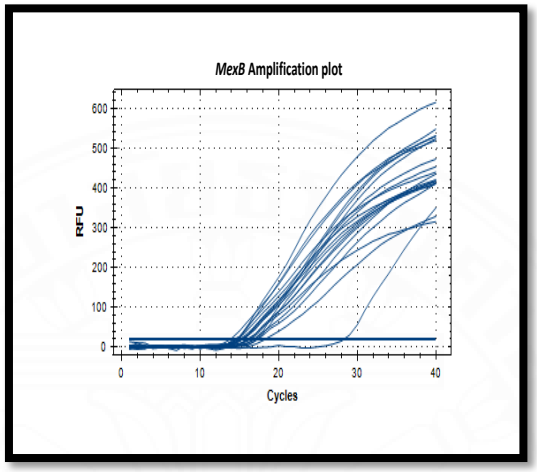


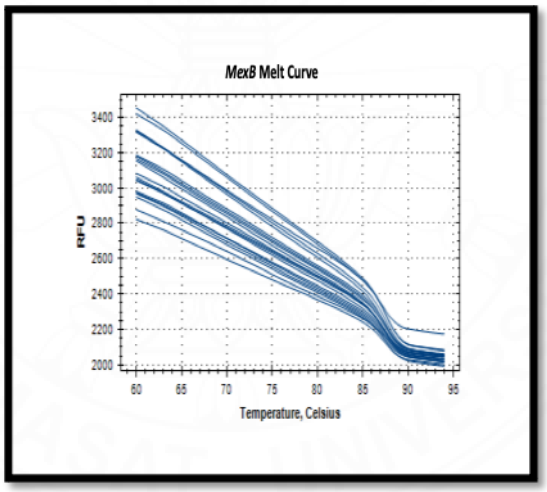


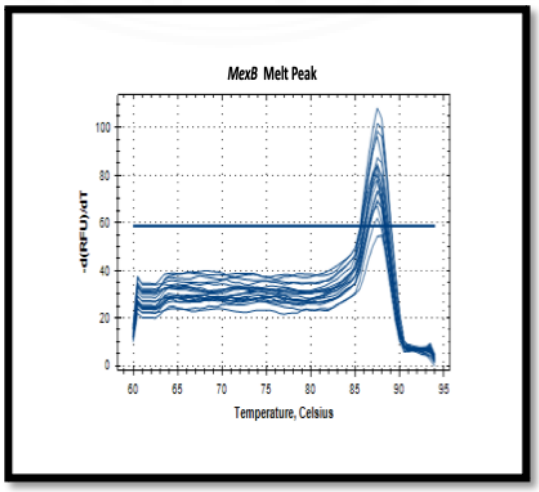
APPENDIX A

Amplification plot, melt curve and melt peak analysis:

MexB gene expression of *P. aeruginosa* after biocide exposure (Cont.)



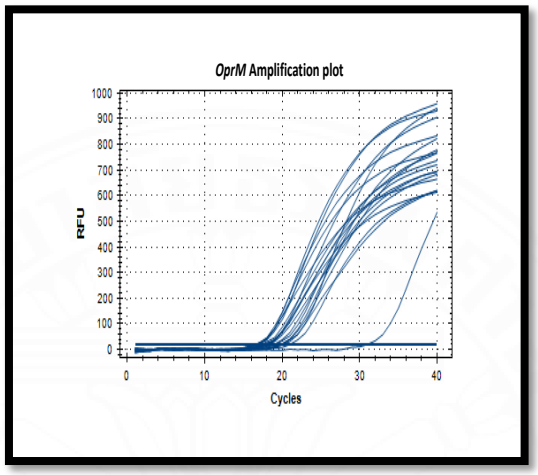


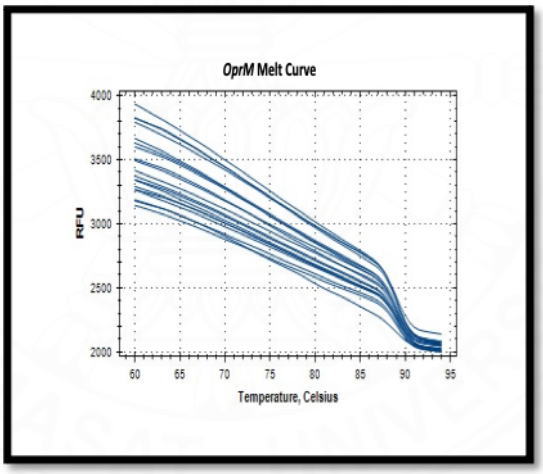


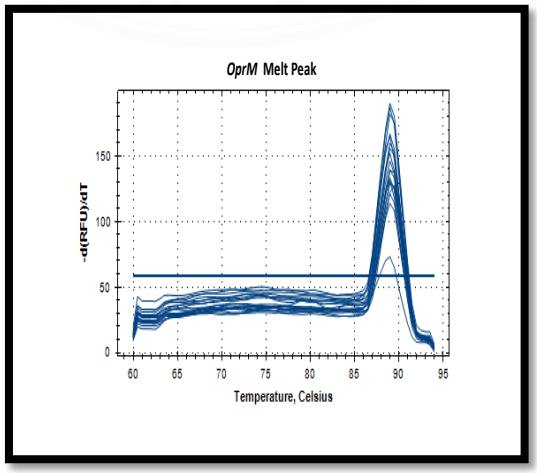
APPENDIX A

Amplification plot, melt curve and melt peak analysis:

OprM gene expression of *P. aeruginosa* after biocide exposure (Cont.)



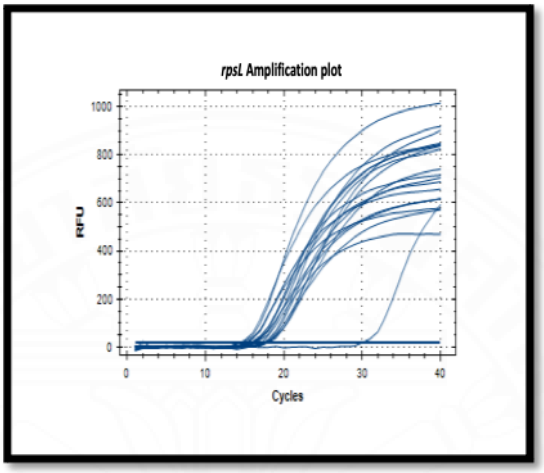


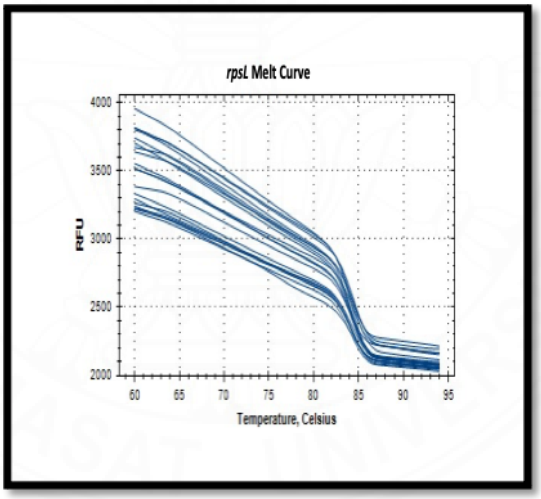


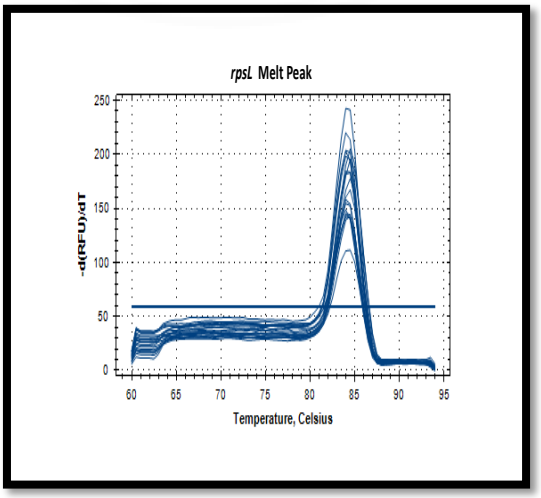
APPENDIX A

Amplification plot, melt curve and melt peak analysis:

rpsL gene expression of *P. aeruginosa* after biocide exposure (Cont.)







APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
PAO1	Baseline	rpsL	15.76			
PAO1	Baseline	MexA	23.54	7.78		
PAO1	BKC exposure	rpsL	17.78			
PAO1	BKC exposure	MexA	28.96	11.18	3.40	0.09
PAO1	CHX exposure	rpsL	17.72			
PAO1	CHX exposure	MexA	27.19	9.47	1.69	0.31
PAO1	Baseline	rpsL	15.76			
PAO1	Baseline	MexB	16.23	0.47		
PAO1	BKC exposure	rpsL	17.78			
PAO1	BKC exposure	MexB	18.31	0.53	0.06	0.96
PAO1	CHX exposure	rpsL	17.72			
PAO1	CHX exposure	MexB	17.34	-0.38	-0.85	1.80
PAO1	Baseline	rpsL	15.76			
PAO1	Baseline	OprM	18.07	2.31		
PAO1	BKC exposure	rpsL	17.78			
PAO1	BKC exposure	OprM	19.90	2.12	-0.19	1.14
PAO1	CHX exposure	rpsL	17.72			
PAO1	CHX exposure	OprM	20.15	2.43	0.12	0.92

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
K767	Baseline	rpsL	16.42			
K767	Baseline	MexA	25.66	9.24		
K767	BKC exposure	rpsL	15.29			
K767	BKC exposure	MexA	21.42	6.13	-3.11	8.63
K767	CHX exposure	rpsL	15.84			
K767	CHX exposure	MexA	22.28	6.44	-2.80	6.96
K767	Baseline	rpsL	16.42			
K767	Baseline	MexB	15.31	-1.11		
K767	BKC exposure	rpsL	15.29			
K767	BKC exposure	MexB	13.87	-1.42	-0.31	1.24
K767	CHX exposure	rpsL	15.84			
K767	CHX exposure	MexB	15.12	-0.72	0.39	0.76
K767	Baseline	rpsL	16.42			
K767	Baseline	OprM	18.16	1.74		
K767	BKC exposure	rpsL	15.29			
K767	BKC exposure	OprM	17.02	1.73	-0.01	1.01
K767	CHX exposure	rpsL	15.84			
K767	CHX exposure	OprM	17.73	1.89	0.15	0.90

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
K1523	Baseline	rpsL	14.60			
K1523	Baseline	MexA	19.62	5.02		
K1523	BKC exposure	rpsL	17.97			
K1523	BKC exposure	MexA	26.70	8.73	3.71	0.08
K1523	CHX exposure	rpsL	15.82			
K1523	CHX exposure	MexA	24.27	8.45	3.43	0.09
K1523	Baseline	rpsL				
K1523	Baseline	MexB				
K1523	BKC exposure	rpsL				
K1523	BKC exposure	MexB				
K1523	CHX exposure	rpsL				
K1523	CHX exposure	MexB				
K1523	Baseline	rpsL	14.60			
K1523	Baseline	OprM	16.60	2.00		
K1523	BKC exposure	rpsL	17.97			
K1523	BKC exposure	OprM	19.79	1.82	-0.18	1.13
K1523	CHX exposure	rpsL	15.82			
K1523	CHX exposure	OprM	17.90	2.08	0.08	0.95

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
K1455	Baseline	rpsL	17.94			
K1455	Baseline	MexA	23.43	5.49		
K1455	BKC exposure	rpsL	17.16			
K1455	BKC exposure	MexA	22.00	4.84	-0.65	1.57
K1455	CHX exposure	rpsL	16.12			
K1455	CHX exposure	MexA	23.70	7.58	2.09	0.23
K1455	Baseline	rpsL	17.94			
K1455	Baseline	MexB	16.40	-1.54		
K1455	BKC exposure	rpsL	17.16			
K1455	BKC exposure	MexB	16.01	-1.15	0.39	0.76
K1455	CHX exposure	rpsL	16.12			
K1455	CHX exposure	MexB	15.38	-0.74	0.80	0.57
K1455	Baseline	rpsL	17.94			
K1455	Baseline	OprM	19.02	1.08		
K1455	BKC exposure	rpsL	17.16			
K1455	BKC exposure	OprM	17.69	0.53	-0.55	1.46
K1455	CHX exposure	rpsL	16.12			
K1455	CHX exposure	OprM	17.36	1.24	0.16	0.90

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
P10	Baseline	rpsL	14.44			
P10	Baseline	MexA	20.24	5.80		
P10	BKC exposure	rpsL	15.26			
P10	BKC exposure	MexA	26.14	10.88	5.08	0.03
P10	CHX exposure	rpsL	15.26			
P10	CHX exposure	MexA	20.69	5.43	-0.37	1.29
P10	Baseline	rpsL	14.44			
P10	Baseline	MexB	14.71	0.27		
P10	BKC exposure	rpsL	15.26			
P10	BKC exposure	MexB	14.76	-0.50	-0.77	1.71
P10	CHX exposure	rpsL	15.26			
P10	CHX exposure	MexB	14.65	-0.61	-0.88	1.84
P10	Baseline	rpsL	14.44			
P10	Baseline	OprM	16.60	2.16		
P10	BKC exposure	rpsL	15.26			
P10	BKC exposure	OprM	17.07	1.81	-0.35	1.27
P10	CHX exposure	rpsL	15.26			
P10	CHX exposure	OprM	16.55	1.29	-0.87	1.83

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
SP2	Baseline	rpsL	17.49			
SP2	Baseline	MexA	23.36	5.87		
SP2	BKC exposure	rpsL	17.41			
SP2	BKC exposure	MexA	23.64	6.23	0.36	0.78
SP2	CHX exposure	rpsL	16.11			
SP2	CHX exposure	MexA	21.22	5.11	-0.76	1.69
SP2	Baseline	rpsL	17.49			
SP2	Baseline	MexB	16.89	-0.60		
SP2	BKC exposure	rpsL	17.41			
SP2	BKC exposure	MexB	17.20	-0.21	0.39	0.76
SP2	CHX exposure	rpsL	16.11			
SP2	CHX exposure	MexB	15.18	-0.93	-0.33	1.26
SP2	Baseline	rpsL	17.49			
SP2	Baseline	OprM	20.08	2.59		
SP2	BKC exposure	rpsL	17.41			
SP2	BKC exposure	OprM	21.01	3.60	1.01	0.50
SP2	CHX exposure	rpsL	16.11			
SP2	CHX exposure	OprM	19.21	3.10	0.51	0.70

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
PAO1	Baseline	rpsL	16.11			
PAO1	Baseline	MexA	23.31	7.20		
PAO1	BKC exposure	rpsL	17.84			
PAO1	BKC exposure	MexA	28.27	10.43	3.23	0.11
PAO1	CHX exposure	rpsL	18.05			
PAO1	CHX exposure	MexA	27.20	9.15	1.95	0.26
PAO1	Baseline	rpsL	16.11			
PAO1	Baseline	MexB	16.65	0.54		
PAO1	BKC exposure	rpsL	17.84			
PAO1	BKC exposure	MexB	18.22	0.38	-0.16	1.12
PAO1	CHX exposure	rpsL	18.05			
PAO1	CHX exposure	MexB	17.65	-0.40	-0.94	1.92
PAO1	Baseline	rpsL	16.11			
PAO1	Baseline	OprM	17.68	1.57		
PAO1	BKC exposure	rpsL	17.84			
PAO1	BKC exposure	OprM	20.55	2.71	1.14	0.45
PAO1	CHX exposure	rpsL	18.05			
PAO1	CHX exposure	OprM	20.07	2.02	0.45	0.73

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
K767	Baseline	rpsL	17.05			
K767	Baseline	MexA	24.18	7.13		
K767	BKC exposure	rpsL	15.15			
K767	BKC exposure	MexA	22.01	6.86	-0.27	1.21
K767	CHX exposure	rpsL	17.23			
K767	CHX exposure	MexA	22.20	4.97	-2.16	4.47
K767	Baseline	rpsL	17.05			
K767	Baseline	MexB	15.67	-1.38		
K767	BKC exposure	rpsL	15.15			
K767	BKC exposure	MexB	15.16	0.01	1.39	0.38
K767	CHX exposure	rpsL	17.23			
K767	CHX exposure	MexB	15.96	-1.27	0.11	0.93
K767	Baseline	rpsL	17.05			
K767	Baseline	OprM	18.25	1.20		
K767	BKC exposure	rpsL	15.15			
K767	BKC exposure	OprM	16.62	1.47	0.27	0.83
K767	CHX exposure	rpsL	17.23			
K767	CHX exposure	OprM	18.58	1.35	0.15	0.90

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
K1523	Baseline	rpsL	15.13			
K1523	Baseline	MexA	21.04	5.91		
K1523	BKC exposure	rpsL	17.93			
K1523	BKC exposure	MexA	27.26	9.33	3.42	0.09
K1523	CHX exposure	rpsL	16.28			
K1523	CHX exposure	MexA	24.06	7.78	1.87	0.27
K1523	Baseline	rpsL	15.13			
K1523	Baseline	MexB				
K1523	BKC exposure	rpsL				
K1523	BKC exposure	MexB				
K1523	CHX exposure	rpsL				
K1523	CHX exposure	MexB				
K1523	Baseline	rpsL	15.13			
K1523	Baseline	OprM	17.39	2.26		
K1523	BKC exposure	rpsL	17.93			
K1523	BKC exposure	OprM	20.12	2.19	-0.07	1.05
K1523	CHX exposure	rpsL	16.28			
K1523	CHX exposure	OprM	18.36	2.08	-0.18	1.13

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
K1455	Baseline	rpsL	16.78			
K1455	Baseline	MexA	24.40	7.62		
K1455	BKC exposure	rpsL	16.26			
K1455	BKC exposure	MexA	22.03	5.77	-1.85	3.61
K1455	CHX exposure	rpsL	16.44			
K1455	CHX exposure	MexA	24.07	7.63	0.01	0.99
K1455	Baseline	rpsL	16.78			
K1455	Baseline	MexB	16.69	-0.09		
K1455	BKC exposure	rpsL	16.26			
K1455	BKC exposure	MexB	15.85	-0.41	-0.32	1.25
K1455	CHX exposure	rpsL	16.44			
K1455	CHX exposure	MexB	17.18	0.74	0.83	0.56
K1455	Baseline	rpsL	16.78			
K1455	Baseline	OprM	18.95	2.17		
K1455	BKC exposure	rpsL	16.26			
K1455	BKC exposure	OprM	18.15	1.89	-0.28	1.21
K1455	CHX exposure	rpsL	16.44			
K1455	CHX exposure	OprM	17.99	1.55	-0.62	1.54

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
P10	Baseline	rpsL	14.53			
P10	Baseline	MexA	20.44	5.91		
P10	BKC exposure	rpsL	15.37			
P10	BKC exposure	MexA	21.47	6.10	0.19	0.88
P10	CHX exposure	rpsL	15.60			
P10	CHX exposure	MexA	20.79	5.19	-0.72	1.65
P10	Baseline	rpsL	14.53			
P10	Baseline	MexB	14.72	0.19		
P10	BKC exposure	rpsL	15.37			
P10	BKC exposure	MexB	15.23	-0.14	-0.33	1.26
P10	CHX exposure	rpsL	15.60			
P10	CHX exposure	MexB	15.12	-0.48	-0.67	1.59
P10	Baseline	rpsL	14.53			
P10	Baseline	OprM	16.13	1.60		
P10	BKC exposure	rpsL	15.37			
P10	BKC exposure	OprM	17.34	1.97	0.37	0.77
P10	CHX exposure	rpsL	15.60			
P10	CHX exposure	OprM	16.87	1.27	-0.33	1.26

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
SP2	Baseline	rpsL	17.61			
SP2	Baseline	MexA	22.66	5.05		
SP2	BKC exposure	rpsL	19.50			
SP2	BKC exposure	MexA	23.76	4.26	-0.79	1.73
SP2	CHX exposure	rpsL	16.43			
SP2	CHX exposure	MexA	23.55	7.12	2.07	0.24
SP2	Baseline	rpsL	17.61			
SP2	Baseline	MexB	17.51	-0.10		
SP2	BKC exposure	rpsL	19.50			
SP2	BKC exposure	MexB	17.62	-1.88	-1.78	3.43
SP2	CHX exposure	rpsL	16.43			
SP2	CHX exposure	MexB	16.32	-0.11	-0.01	1.01
SP2	Baseline	rpsL	17.61			
SP2	Baseline	OprM	20.83	3.22		
SP2	BKC exposure	rpsL	19.50			
SP2	BKC exposure	OprM	20.99	1.49	-1.73	3.32
SP2	CHX exposure	rpsL	16.43			
SP2	CHX exposure	OprM	18.76	2.33	-0.89	1.85

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
PAO1	Baseline	rpsL	16.99			
PAO1	Baseline	MexA	28.13	11.14		
PAO1	BKC exposure	rpsL	18.51			
PAO1	BKC exposure	MexA	29.74	11.23	0.09	0.94
PAO1	CHX exposure	rpsL	18.40			
PAO1	CHX exposure	MexA	29.56	11.16	0.02	0.99
PAO1	Baseline	rpsL	16.99			
PAO1	Baseline	MexB	17.54	0.55		
PAO1	BKC exposure	rpsL	18.51			
PAO1	BKC exposure	MexB	18.91	0.40	-0.15	1.11
PAO1	CHX exposure	rpsL	18.40			
PAO1	CHX exposure	MexB	18.71	0.31	-0.24	1.18
PAO1	Baseline	rpsL	16.99			
PAO1	Baseline	OprM	19.25	2.26		
PAO1	BKC exposure	rpsL	18.51			
PAO1	BKC exposure	OprM	21.04	2.53	0.27	0.83
PAO1	CHX exposure	rpsL	18.40			
PAO1	CHX exposure	OprM	20.72	2.32	0.06	0.96

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
K767	Baseline	rpsL	16.79			
K767	Baseline	MexA	27.36	10.57		
K767	BKC exposure	rpsL	15.76			
K767	BKC exposure	MexA	25.08	9.32	-1.25	2.38
K767	CHX exposure	rpsL	17.10			
K767	CHX exposure	MexA	24.40	7.30	-3.27	9.65
K767	Baseline	rpsL	16.79			
K767	Baseline	MexB	16.39	-0.40		
K767	BKC exposure	rpsL	15.76			
K767	BKC exposure	MexB	15.84	0.08	0.48	0.72
K767	CHX exposure	rpsL	17.10			
K767	CHX exposure	MexB	16.27	-0.83	-0.43	1.35
K767	Baseline	rpsL	16.79			
K767	Baseline	OprM	18.59	1.80		
K767	BKC exposure	rpsL	15.76			
K767	BKC exposure	OprM	17.25	1.49	-0.31	1.24
K767	CHX exposure	rpsL	17.10			
K767	CHX exposure	OprM	18.52	1.42	-0.38	1.30

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
K1523	Baseline	rpsL	15.83			
K1523	Baseline	MexA	22.98	7.15		
K1523	BKC exposure	rpsL	17.82			
K1523	BKC exposure	MexA	27.66	9.84	2.69	0.15
K1523	CHX exposure	rpsL	16.80			
K1523	CHX exposure	MexA	24.46	7.66	0.51	0.70
K1523	Baseline	rpsL	15.83			
K1523	Baseline	MexB				
K1523	BKC exposure	rpsL				
K1523	BKC exposure	MexB				
K1523	CHX exposure	rpsL				
K1523	CHX exposure	MexB				
K1523	Baseline	rpsL	15.83			
K1523	Baseline	OprM	18.20	2.37		
K1523	BKC exposure	rpsL	17.82			
K1523	BKC exposure	OprM	20.81	2.99	0.62	0.65
K1523	CHX exposure	rpsL	16.80			
K1523	CHX exposure	OprM	18.68	1.88	-0.49	1.40

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
K1455	Baseline	rpsL	17.23			
K1455	Baseline	MexA	28.43	11.20		
K1455	BKC exposure	rpsL	16.09			
K1455	BKC exposure	MexA	22.68	6.59	-4.61	24.42
K1455	CHX exposure	rpsL	17.02			
K1455	CHX exposure	MexA	24.05	7.03	-4.17	18.00
K1455	Baseline	rpsL	17.23			
K1455	Baseline	MexB	17.01	-0.22		
K1455	BKC exposure	rpsL	16.09			
K1455	BKC exposure	MexB	16.06	-0.03	0.19	0.88
K1455	CHX exposure	rpsL	17.02			
K1455	CHX exposure	MexB	16.13	-0.89	-0.67	1.59
K1455	Baseline	rpsL	17.23			
K1455	Baseline	OprM	18.68	1.45		
K1455	BKC exposure	rpsL	16.09			
K1455	BKC exposure	OprM	18.05	1.96	0.51	0.70
K1455	CHX exposure	rpsL	17.02			
K1455	CHX exposure	OprM	18.32	1.30	-0.15	1.11

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
P10	Baseline	rpsL	14.65			
P10	Baseline	MexA	22.00	7.35		
P10	BKC exposure	rpsL	15.61			
P10	BKC exposure	MexA	21.97	6.36	-0.99	1.99
P10	CHX exposure	rpsL	15.80			
P10	CHX exposure	MexA	20.96	5.16	-2.19	4.56
P10	Baseline	rpsL	14.65			
P10	Baseline	MexB	15.40	0.75		
P10	BKC exposure	rpsL	15.61			
P10	BKC exposure	MexB	14.92	-0.69	-1.44	2.71
P10	CHX exposure	rpsL	15.80			
P10	CHX exposure	MexB	15.22	-0.58	-1.33	2.51
P10	Baseline	rpsL	14.65			
P10	Baseline	OprM	16.80	2.15		
P10	BKC exposure	rpsL	15.61			
P10	BKC exposure	OprM	17.28	1.67	-0.48	1.39
P10	CHX exposure	rpsL	15.80			
P10	CHX exposure	OprM	17.24	1.44	-0.71	1.64

APPENDIX A

Data analysis in the expression of efflux pumps gene after biocide exposure (Cont.)

Strains	Experiment status	Genes	Ct	Δ Ct	ΔΔ Ct	$2^{-\Delta\Delta Ct}$
SP2	Baseline	rpsL	18.59			
SP2	Baseline	MexA	27.11	8.52		
SP2	BKC exposure	rpsL	17.49			
SP2	BKC exposure	MexA	24.07	6.58	-1.94	3.84
SP2	CHX exposure	rpsL	16.42			
SP2	CHX exposure	MexA	22.95	6.53	-1.99	3.97
SP2	Baseline	rpsL	18.59			
SP2	Baseline	MexB	17.84	-0.75		
SP2	BKC exposure	rpsL	17.49			
SP2	BKC exposure	MexB	18.86	1.37	2.12	0.23
SP2	CHX exposure	rpsL	16.42			
SP2	CHX exposure	MexB	17.13	0.71	1.46	0.36
SP2	Baseline	rpsL	18.59			
SP2	Baseline	OprM	20.22	1.63		
SP2	BKC exposure	rpsL	17.49			
SP2	BKC exposure	OprM	20.62	3.13	1.50	0.35
SP2	CHX exposure	rpsL	16.42			
SP2	CHX exposure	OprM	19.25	2.83	1.20	0.44

APPENDIX B

Ethidium bromide cartwheel agar tested for efflux pump activity

	Strain	Ethidium Bromide Concentration (mg/L)															
		0	0.25	0.5	0.75	1	1.25	1.5	1.75	2	2.25	2.5	2.75	3	4	5	6	7
Baseline	PAO1	-	-/+	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	PAO1	-	-/+	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	PAO1	-	-/+	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
BKC	PAO1	-	-/+	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	PAO1	-	-/+	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	PAO1	-	-/+	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
CHX	PAO1	-	-/+	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	PAO1	-	-/+	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	PAO1	-	-/+	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+

APPENDIX B

Ethidium bromide cartwheel agar tested for efflux pump activity (Cont.)

	Strain	Ethidium Bromide Concentration (mg/L)															
		0	0.25	0.5	0.75	1	1.25	1.5	1.75	2	2.25	2.5	2.75	3	4	5	6	7
Baseline	K1523	-	-	-/+	-/+	-/+	-/+	+	+	+	Not done	Not done	Not done	+	+	+	+	+
	K1523	-	-	-/+	-/+	-/+	-/+	+	+	+	Not done	Not done	Not done	+	+	+	+	+
	K1523	-	-	-	-/+	-/+	-/+	+	+	+	Not done	Not done	Not done	+	+	+	+	+
BKC	K1523	-	-	-	-	-	-/+	+	+	+	Not done	Not done	Not done	+	+	+	+	+
	K1523	-	-	-/+	-	-	-/+	+	+	+	Not done	Not done	Not done	+	+	+	+	+
	K1523	-	-	-	-	-	-/+	+	+	+	Not done	Not done	Not done	+	+	+	+	+
CHX	K1523	-	-	-	-	-	-/+	-/+	+	+	Not done	Not done	Not done	+	+	+	+	+
	K1523	-	-	-	-	-	-/+	+	+	+	Not done	Not done	Not done	+	+	+	+	+
	K1523	-	-	-	-	-	-/+	+	+	+	Not done	Not done	Not done	+	+	+	+	+

APPENDIX B

Ethidium bromide cartwheel agar tested for efflux pump activity (Cont.)

	Strain	Ethidium Bromide Concentration (mg/L)															
		0	0.25	0.5	0.75	1	1.25	1.5	1.75	2	2.25	2.5	2.75	3	4	5	6	7
Baseline	K767	-	-	-/+	-/+	-/+	-/+	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	K767	-	-	-/+	-/+	-/+	-/+	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	K767	-	-	-/+	-/+	-/+	-/+	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
BKC	K767	-	-	-/+	-/+	-/+	-/+	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	K767	-	-	-/+	-/+	-/+	-/+	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	K767	-	-	-/+	-/+	-/+	-/+	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
CHX	K767	-	-	-/+	-/+	-	-/+	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	K767	-	-	-/+	-/+	-/+	-/+	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	K767	-	-	-/+	-	-/+	-/+	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+

APPENDIX B

Ethidium bromide cartwheel agar tested for efflux pump activity (Cont.)

	Strain	Ethidium Bromide Concentration (mg/L)															
		0	0.25	0.5	0.75	1	1.25	1.5	1.75	2	2.25	2.5	2.75	3	4	5	6	7
Baseline	K1455	-	-/+	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	K1455	-	-/+	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	K1455	-	+	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
BKC	K1455	-	-	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	K1455	-	-	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	K1455	-	-	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
CHX	K1455	-	-	+	-/+	-/+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	K1455	-	-	+	+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+
	K1455	-	-	+	-/+	+	Not done	Not done	Not done	+	Not done	Not done	Not done	+	+	+	+	+

APPENDIX B

Ethidium bromide cartwheel agar tested for efflux pump activity (Cont.)

	Strain	Ethidium Bromide Concentration (mg/L)																
		0	0.25	0.5	0.75	1	1.25	1.5	1.75	2	2.25	2.5	2.75	3	4	5	6	7
Baseline	P10	-	-	-	-	+	+	+	+	+	Not done	Not done	Not done	+	+	+	+	+
	P10	-	-	-	-	+	+	+	+	+	Not done	Not done	Not done	+	+	+	+	+
	P10	-	-	-	-	+	+	+	+	+	Not done	Not done	Not done	+	+	+	+	+
BKC	P10	-	-	-	-	-	-	+	+	+	Not done	Not done	Not done	+	+	+	+	+
	P10	-	-	-	-	-	-	+	+	+	Not done	Not done	Not done	+	+	+	+	+
	P10	-	-	-	-	-	-	+	+	+	Not done	Not done	Not done	+	+	+	+	+
CHX	P10	-	-	-	-	-	-	+	+	+	Not done	Not done	Not done	+	+	+	+	+
	P10	-	-	-	-	-	-	+	+	+	Not done	Not done	Not done	+	+	+	+	+
	P10	-	-	-	-	-	-	+	+	+	Not done	Not done	Not done	+	+	+	+	+

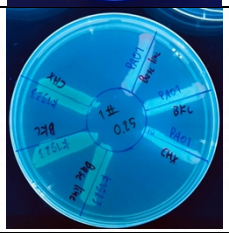
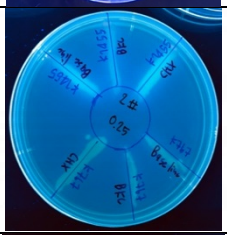
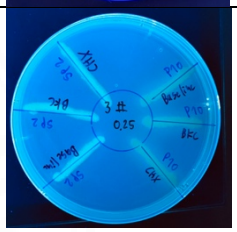
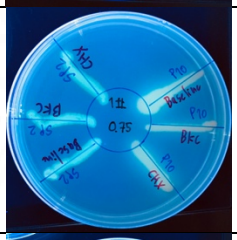
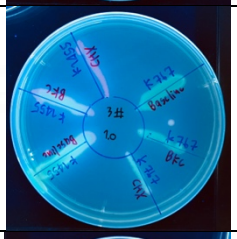
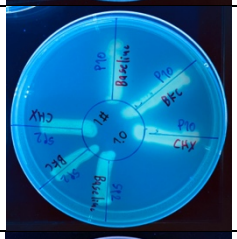
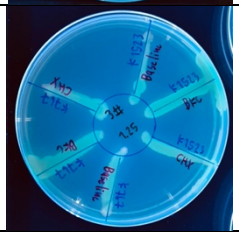
APPENDIX B

Ethidium bromide cartwheel agar tested for efflux pump activity (Cont.)

	Strain	Ethidium Bromide Concentration (mg/L)																
		0	0.25	0.5	0.75	1	1.25	1.5	1.75	2	2.25	2.5	2.75	3	4	5	6	7
Baseline	SP2	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
	SP2	-	-	-	-	-	-	-	-	-	-	-/+	+	+	+	+	+	+
	SP2	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
BKC	SP2	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
	SP2	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
	SP2	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
CHX	SP2	-	-	-	-	-	-	-	-	-	-	-/+	+	+	+	+	+	+
	SP2	-	-	-	-	-	-	-	-	-	-	-/+	+	+	+	+	+	+
	SP2	-	-	-	-	-	-	-	-	-	-	-/+	+	+	+	+	+	+

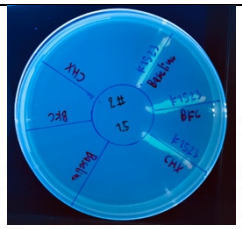
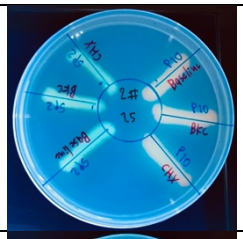
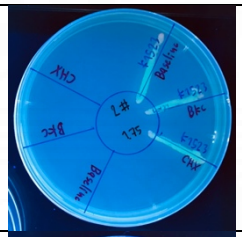
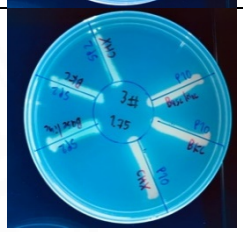
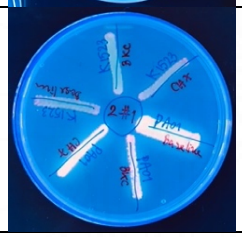
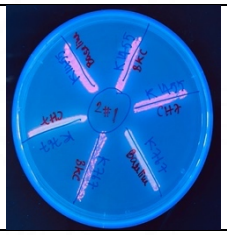
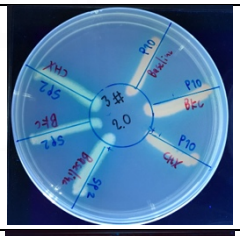
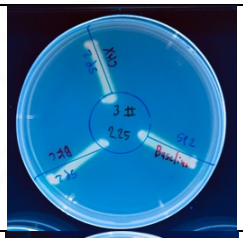
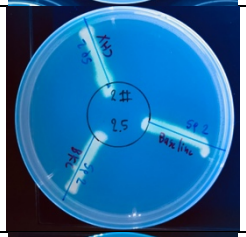
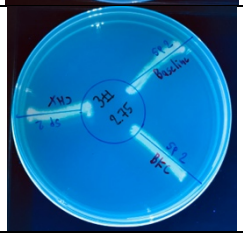
APPENDIX B

Figures of ethidium bromide cartwheel agar tested for efflux pump activity

Et Br 0 mg/L			
Et Br 0.25 mg/L			
Et Br 0.5 mg/L			
Et Br 0.75 mg/L			
Et Br 1 mg/L			
Et Br 1.25 mg/L	Not done		

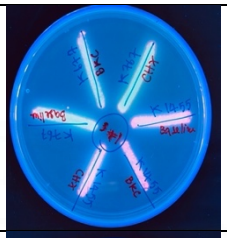
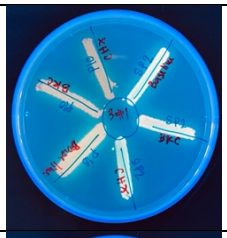
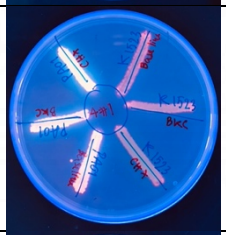
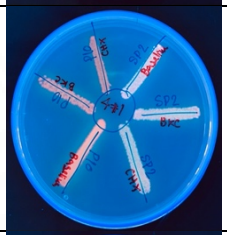
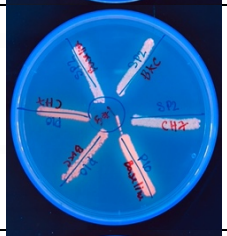
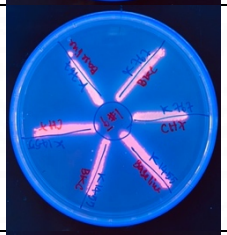
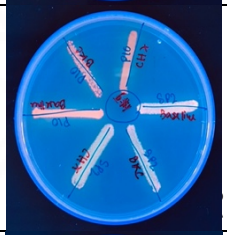
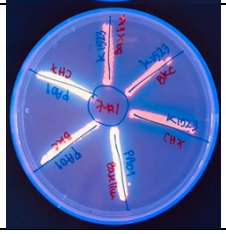
APPENDIX B

Figures of ethidium bromide cartwheel agar tested for efflux pump activity (Cont.)

Et Br 1.5 mg/L		Not done	
Et Br 1.75 mg/L		Not done	
Et Br 2 mg/L			
Et Br 2.25 mg/L	Not done	Not done	
Et Br 2.5 mg/L	Not done	Not done	
Et Br 2.75 mg/L	Not done	Not done	

APPENDIX B

Figures of ethidium bromide cartwheel agar tested for efflux pump activity (Cont.)

Et Br 3 mg/L			
Et Br 4 mg/L			
Et Br 5 mg/L			
Et Br 6 mg/L			
Et Br 7 mg/L			

APPENDIX C

Reagent preparation

Culture media

1. Mueller Hinton Agar

For 1000 mL preparation;

Mueller Hinton Agar powder	38 g
Distilled water	1000 mL

Procedure;

I. Resuspend Mueller Hinton Agar powder in distilled water in a bottle.
II. Autoclave the mixture at 121°C for 15 minutes.
III. Cool the media to 50 °C.
IV. Pour the media into sterile Petri dishes.
V. Cool down the MHA at room temperature.
VI. Store the media at 4 °C until use.

2. Mueller Hinton Broth

For 1000 mL preparation;

Mueller Hinton broth powder	21 g
Distilled water	1000 mL

Procedure;

I. Resuspend Mueller Hinton Broth powder in distilled water in a bottle.
II. Autoclave the mixture at 121°C for 15 minutes.
III. Cool down the MHB at room temperature.

Store the media at 4 °C until use.

APPENDIX C

Reagent preparation (Cont.)

Solutions for gel electrophoresis

1. Tris/Borate/EDTA (TBE) buffer

For 500 ml of 5x TBE buffer preparation;

Tris base	26 g
Boric acid	13.75 g
EDTA	2.325 g
Distilled water	500 mL

Procedure;

I. Dissolve 26 g Tris base and 13.75 g boric acid in 400 ml distilled water in a bottle.

II. Add 13.75 g of EDTA.

III. Adjust pH to 8.3 and adjust volume to 500 L.

IV. Store at room temperature.

2. Stock solution of 0.5M EDTA (pH 8.0)

For 200 ml preparation;

Disodium EDTA · $2H_2O$	37.2 g
Distilled water	160 mL

Procedure;

I. Dissolve EDTA in 160 ml distilled water in a bottle.

II. Add approximately 4 g NaOH pellet until solution becomes pH 8.0. Adjust the volume to 200 ml with distilled water.

3. 10% (w/v) SDS

For 100 ml preparation;

Sodium dodecyl sulfate 10 g

Adjust volume to 100 mL with distilled water

BIOGRAPHY

Name	Thitinan Phutthilertmethawee
Educational Attainment	Academic Year 2010: Bachelor of science (Medical technology)
Scholarship	2021-2024: Teaching Assistant Scholarship of Thammasat University

Publications

1. Phutthilertmethawee T, Srimanote P, Tingpej P. Effect of Benzalkonium Chloride and Chlorhexidine on the Antibiotic Susceptibility of *Pseudomonas aeruginosa*: Biocide Effect on *P. aeruginosa* Susceptibility. Asian Medical Journal and Alternative Medicine. 2024;24(3):20-9.
2. Phutthilertmethawee, T., Tiengtip, R., Archanachan, B., Srimanote, P., Tingpej, P., *MexAB-OprM* expression, efflux pump activity and antibiotic susceptibility of *Pseudomonas aeruginosa* after exposure to benzalkonium chloride and to chlorhexidine, (Manuscript in preparation)