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A NEW HIGH-THROUGHPUT ASSAY FOR QUANTIFICATION OF ANTIBIOTIC PENETRATION IN GRAM-NEGATIVE BACTERIAL CELLS

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Abstract

Antibiotic resistance has been on the rise and poses major threats to public health. Bacteria develop multidrug resistance through various mechanisms such as mutated or modified antibiotic target site, modification of antibiotic molecules, reduction of antibiotic penetration through bacterial cell membranes and extrusion of drug molecules by efflux pumps. Increasing evidence indicates that antibiotic permeability is a major barrier hindering microbial control. However, a robust and rapid screening method to quantitatively determine the penetration of candidate agents through bacterial membranes is still missing. To address this challenge, we recently developed a high throughput method for quantifying membrane penetration of Gram-negative bacteria by optimizing treatment conditions to lyse the inner and outer membranes separately. In this study, we further improved the assay throughput and evaluated the penetration of ciprofloxacin (CIP) and tobramycin (TOB) into *Escherichia coli* MG1665 and *Pseudomonas aeruginosa* PAO1. With optimized experimental conditions, it was found that cytoplasmic membrane is a critical barrier to CIP and TOB penetration in both species tested. The results were corroborated by *P. aeruginosa* PAO1 efflux pump mutants. This new method can be used in future studies to identify novel antimicrobials and inhibitors of bacterial efflux pumps.

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by

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CHAPTER 1

INTRODUCTION

1.1 The rising problem of antibiotic resistance

Antibiotic resistance has become one of the most significant global threats to human health. In the United States alone, more than 2 million people suffer from bacterial infections caused by antibiotic-resistant strains each year (Blair, Webber, Baylay, Ogbolu, & Piddock, 2015). *P. aeruginosa* is one of the typical strains that can adapt abilities against antibiotic treatment rapidly through mutation. It is highly acceptable to different environments so often can be found in vegetables, foods, as well as drinking water. Infections associated with *P. aeruginosa* are often life-threatening and difficult to cure due to the limitation of susceptible antimicrobial agents (Carmeli, Troillet, Eliopoulos, & Samore, 1999) (Garner, Jarvis, Emori, Horan, & Hughes, 1988). In 2013, Multi-drug resistant Gram-negative bacteria, including *Pseudomonas*, were identified as major pathogens by U.S. Centers for Diseases Control and Prevention (Prevention, 2013). In 2016, the first death on the U.S. due to superbug infection was reported (Christensen & Goldschmidt, 2016), where a patient showed a rare case of *E. coli* infection, which showed resistance to all 26 tested antibiotics. Multiple factors have been found contributing to antibiotic resistance, including the overuse of antibiotics, as well as failed patient compliance of antibiotic treatment. Besides, antibiotics kill bacteria by targeting growth-related targets and thus have intrinsic disadvantages in killing dormant or slow-growing bacterial cells such as those in biofilms, presisters, and viable but non-culturable cells (Ayrapetyan, Williams, & Oliver, 2018). Therefore, it is urgent to develop new molecules to better control infections.

1.2 Significances of Gram-negative Bacteria

The emergence and spread of multidrug resistance in Gram-negative bacteria are threatening public health. Currently, more than 80% of severe bacterial clinical infections are caused by multidrug-resistant Gram-negative bacteria (Chopra, Hawkey, & Hinton, 1992; Du et al., 2018; Fraimow & Tsigrelis, 2011; Nikaido, 1989; Viale, Giannella, Tedeschi, & Lewis, 2015). Different from Gram-positive bacteria, Gram-negative species such as *E. coli*, *P. aeruginosa* and *Salmonella spp.*, are characterized by the presence of an outer membrane containing lipopolysaccharide and a periplasm space. The complex structure of Gram-negative bacteria often protects bacteria from damaging substances and outside environment. One of the unique features of Gram-negative bacteria is the presence of an outer membrane, which renders Gram-negative bacteria more resistant to lipophilic and amphiphilic inhibitors, such as detergents, antibiotics, and chemotherapeutic agents compare to Gram-positive bacteria (Nikaido, 1996). As a result, antibiotics are generally facing more challenges to penetrate Gram-negative membranes. To address this challenge, we developed a new method, which allows the study of drug accumulation levels between different layers of Gram-negative species to enable high throughput screening of drug penetration to Gram-negative cells.

1.3 Platforms of antibiotic discovery

Antibiotics available to date are either natural compounds produced by microbes or synthetic compounds based on the modification of existing antimicrobials. Most of the currently used antibiotics were discovered between 1940s and 1960s. The first antibiotic, penicillin was discovered from an accidental experiment. The Sottish scientist Alexander Fleming accidentally left a petri dish containing Staphylococci open, and the petri dish was contaminated by blue-green molds. He observed an inhibited growth around the area that has been contaminated by the mold so that he discovered penicillin. Later on, Selman Waksman and his students estabilshed a screening method called the 'Waksman platform'. The 'Waksman platform' adapted the idea from the discovery process of penicillin by Alexander Fleming (Fleming, 1929) but more schematically and simply. In this platform, soil-derived streptomycetes were tested for antimicrobial activity by the disk diffusion test on an overlay plate (Schatz, Bugie, & Waksman, 2005). The screening of streptomycetes led to the discovery of the first aminoglycoside, streptomycin. The 'Waksman platform' earned Selman Waksman a Nobel prize. In the following 20 years, the method has been widely adopted in pharmaceutical companies led to mass production of major classes of antibiotics. Meanwhile, fluoroquinolones were discovered by the modification of existing compounds. However, after the 1970s, there was no new class of broad-spectrum agents discovered (Lewis, 2013).

In the 1990s, multiple platforms were developed aiming to discover new sources of antibiotics, such as using 'high-throughput screening' to identify lead compounds for development and studying the three-dimensional structure of existing compounds for

'rational drug design' (Lewis, 2013). Unfortunately, the efforts failed due to the difficulties of targeting compounds that can efficiently penetrate the cell membrane. Thus, it is urgent to develop a capability to identify compounds that can effectively penetrate bacterial membranes, especially those of Gram-negative species.

1.4 Antibiotic killing mechanisms

Conventional antibiotics kill bacteria by inhibiting a wide range of targets (Walsh, 2003). Based on the outcome of treatments, antibiotics can be divided into two categories: bactericidal drugs that kill cells and bacteriostatic drugs that inhibit cell growth. (Pankey & Sabath, 2004). There are multiple significant antimicrobial drug-target interactions such as DNA replication, DNA repair, protein synthesis as well as cell wall synthesis (Kohanski, Dwyer, Hayete, Lawrence, & Collins, 2007; Walsh, 2000). However, we still have limited understanding of certain primarily antimicrobial drug-target responses and interactions (Davis, 1987; Drlica & Zhao, 1997; Kohanski et al., 2007; Lewis, 2000). This project aims to optimize and validate a new screening method developed in our lab for identifying structural elements that promote membrane penetration. Various antimicrobials are tested in this study, including ciprofloxacin, tobramycin, and tetracycline.

Ciprofloxacin is a bactericidal drug that belongs to the fluoroquinolone family. Similar to other fluoroquinolones, it inhibits DNA gyrase (LeBel, 1988). DNA gyrase is a bacterial enzyme essential to DNA coiling (Reece & Maxwell, 1991). By binding to DNA gyrase, ciprofloxacin can result in DNA double-strand breakage, which eventually leads to cell death. Tobramycin is also a bactericidal antibiotic. It binds to the ribosome of bacterial

cells. As a result, the 70S complex cannot be formed so that the translation cannot occur (G. Yang, Trylska, Tor, & McCammon, 2006). Tetracycline is a bacteriostatic antibiotic. It passively diffuses through hydrophilic pores into the outer membrane, and then by an energy-dependent active transport to pass the inner membrane into the cytoplasmic space (Chopra et al., 1992; Piédrola Angulo, 2001; Roberts, 1996). By using efflux pump mutants of *P. aeruginosa* PAO1, we examined factors that can affect drug penetration and accumulation.

1.5 Importance of Gram-negative bacteria membrane barriers

As mentioned in the previous section, different from Gram-positive bacteria, Gram-negative bacteria contain two cell membranes: an inner membrane or cytoplasmic membrane and outer membrane. Previous studies have found that the outer membrane is directly involved in the intrinsic resistance of Gram-negative bacteria to antimicrobial agents (Hancock, 1987, 1997; Nikaido, 2003). The outer-membrane barrier has narrow pores, which act as matrixes to slow down or prevent drug penetration (Nikaido & Vaara, 1985). The low fluidity of the lipoprotein reduces the rate of transmembrane diffusion of lipophilic solutes (Plésiat & Nikaido, 1992). Thus, the efficiency of antibiotic treatment is reduced dramatically in the presence of outer membranes.

Besides the double-layer structure, efflux systems are well documented for in the intrinsic antibiotic resistance. (Lewis, 1994; Ma, Cook, Hearst, & Nikaido, 1994). As demonstrated by Nikaido et al. (Nikaido, 1989), due to the large surface-to-volume ratio of bacteria cells, antibiotic penetration through the outer membrane often achieves the equilibrium rapidly.

For example, the periplasmic layer can reach equilibrium of its external concentration within 10 to 30 seconds in *P. aeruginosa* and even more instant in *E. coli* species (Nikaido, 1989). Therefore, additional mechanisms are involved in the intrinsic antibiotic resistance of Gram-negative mechanism. With a better understanding of resistance mechanisms, more effective platforms for antibiotic discovery can be developed.

1.6 Efflux assays

Currently, efflux assays rely on the measurement of fluorescence intensity either by monitoring the penetration of fluorescent dyes or by characterizing intracellular conversion of fluorescent products (Figure 1). One type of efflux assay is to introduce a traceable non-fluorescent component into living cells, which converted to a fluorescent product after entering the cell (Figure1-A) (Dreier & Ruggerone, 2015). For example, Alamar Bule® is a blue colored, non-fluorescent resazurin dye. The present of oxidoreductase in bacterial cells reduces resazurin to red-fluorescent resorufin, which can be detected by microscope (Gonzalez & Tarloff, 2001; Rampersad, 2012). Since oxidoreductase commonly utilizes NADH, NADPH, and FADH as cofactor, it is commonly used to label live cells. This method also has been used to monitor bacterial growth. However, this dye does not work well in *P. aeruginosa* since it is a substrate of MexB (Vidal-Aroca, Meng, Minz, Page, & Dreier, 2009). Fluorescein-di-β-D-galactopyranoside (FDG) is another good candidate for tracing non-fluorescent components. FDG could be hydrolyzed by β-galactosidase to produce fluorescein, which is a common fluorescent tracer (Fieldler & Hinz, 1994; Russo-Marie, Roederer, Sager, Herzenberg, & Kaiser, 1993;

N. C. Yang & Hu, 2004). However, current research only indicated that FDG can be hydrolyzed by *E. coli* strains, not *P. aeruginosa*, limiting its application.

The second type of efflux assay adapted the idea of using fluorescent dye as a probe to characterize bacterial membrane potential and membrane integrity (Figure 1-B). From the physicochemical properties of fluorescent dye, the information of the efflux pump mechanism in terms of substrate recognition could be understood (Dreier & Ruggerone, 2015). For example, N-phenyl-1-naphthylamine (NPN) is a substrate of MexB in *P. aeruginosa* and AcrB in *E. coli* (Lomovskaya et al., 1999; Ocaktan, Yoneyama, & Nakae, 1997; Sedgwick & Bragg, 1996); since NPN can penetrate the phospholipid membrane, the fluorescence signal can be detected when the outer membrane structure is disturbed (Loh, Grant, & Hancock, 1984; Lomovskaya et al., 1999; Vaara, 1992). Therefore, the efflux activity can be detected by monitoring the flux of fluorescent dyes.

In addition, liquid chromatography-mass spectrometry (LC-MS) or high-performance liquid chromatography (HPLC) can be applied to cell lysates to quantify molecule penetration and thus efflux activity. However, it requires separations of intracellular components from extracellular substances; and, thus, can be applied to high throughput assays. In comparison, we developed a new method that allows quantification of the concentration of candidate molecules in the periplasm and cytoplasm spaces of Gram-negative bacteria in a high throughput manner.

In the past decays, increasing evidence showed that the active efflux by Gram-negative bacteria significantly contributes to antibiotic resistance (Levy, 1992; Nikaido, 1994). Considering the critical role of efflux mechanism in antibiotic resistance in *P. aeruginosa*, it is necessary to study the role of efflux pump as well as each part of the efflux pump that controls the drug accumulation during antibiotic treatments. By studying the specific efflux systems, this assay can provide new information to guide a rational design of the next-generation antimicrobials.

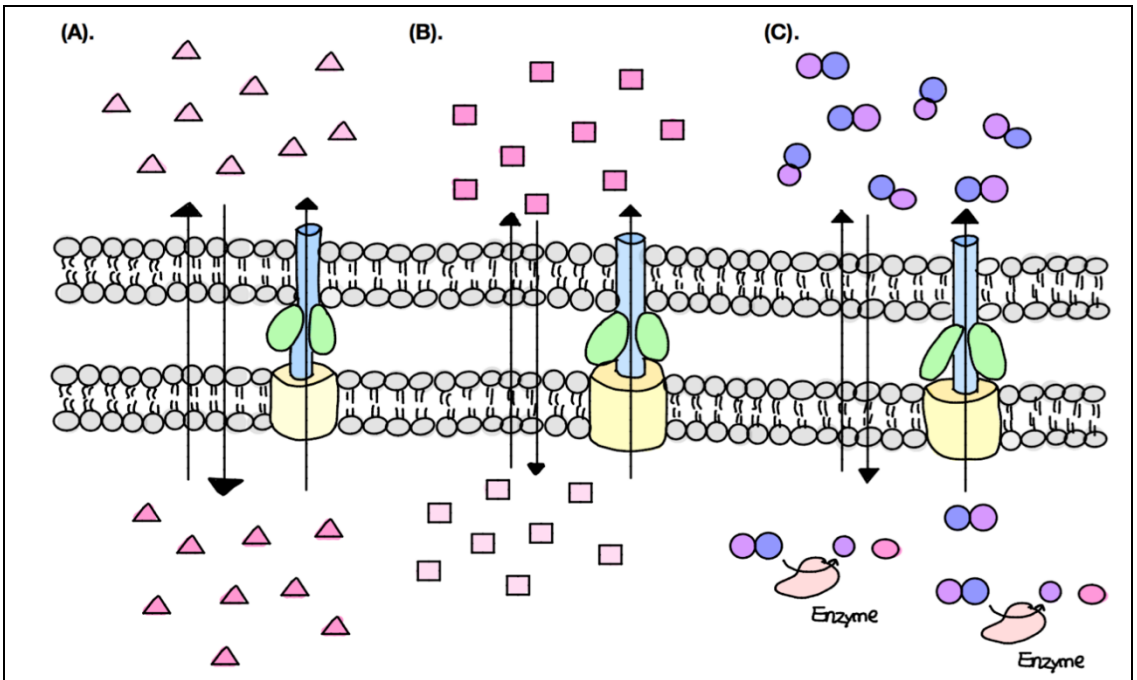


Figure 1. Schematic representation of efflux assays. (A) Introducing non-fluorescence, traceable product into living cells. The non-florescent product (light pink) increases becomes florescent upon internalization and interaction with cellular products (dark pink), efflux of the probe (double arrows) decreases internal florescence intensity and efflux inhibition increases the internal florescence intensity. (B) Introducing florescence probe into live cells. The florescence probe (dark pink) decreases its florescence intensity upon interaction with cellular products. Efflux of the probe (double arrows) increases internal florescence intensity and efflux inhibition decreases the internal florescence intensity. (C) Introducing intracellular convertible probe into live cells. The product can be enzymatically converted into a florescent product. Efflux of the intact probe (double arrows) slows down the production, so the internal florescence intensity decreases, and efflux inhibition increases the internal florescence intensity by increasing the rate of product formation.

1.7 P. aeruginosa Efflux Pumps

The last few decays have witnessed a rapid increase in antibiotic resistance, which is now a well-recognized global threat to public health. *P. aeruginosa* is a major Gram-negative bacterium frequently involved in infections such as urinary tract infections, bloodstream infections, and surgical site infections (Dreier & Ruggerone, 2015; Jones, Stilwell, Rhomberg, & Sader, 2009; Zhanel et al., 2010). One major factor that contributes to *P. aeruginosa* multi-drug resistances is the low membrane permeability (Nakae, Nakajima, Ono, Saito, & Yoneyama, 1999). Molecular genetic evidence also suggests that the efflux pumps in Gram-negative bacteria are strategies developed through evaluation for survival in harsh environments (Du et al., 2018). To date, six families of bacterial efflux pumps have been identified (Figure 2), including: **1**. ATP-Binding Cassette (ABC) family; **2**. Major Facilitator Superfamily (MFS); **3**. Small Multidrug Resistant (SMR) family; **4**. Multidrug and Toxin Extrusion (MATE) family; **5**. Resistance-Nodulation-Cell Division (RND) superfamily; and **6**. Proteobacterial Antimicrobial Compound Efflux (PACE) family. This study is mainly focused on the RND efflux pump family. The RND pump contains three components: the outer membrane channel, assembled by three monomers, located in the inner membrane and overhung into the periplasm space (Dreier & Ruggerone, 2015; Murakami, Nakashima, Yamashita, Matsumoto, & Yamaguchi, 2006; Murakami, Nakashima, Yamashita, & Yamaguchi, 2002). The outer membrane channel contains three monomers, connecting the periplasmic space with the outside (Du et al., 2018). The adaptor protein completes the efflux system spanning across the inner membrane, periplasmic space, and outer membrane (Dreier & Ruggerone, 2015). *P.*

aeruginosa efflux pumps known to date, and their corresponding antibiotic substrates are summarized in Table 1. In this study, we adapted our method to further investigated the drug accumulation level for each efflux components mutant from *P. aeruginosa* PA14 *mexB oprM with ciprofloxacin* treatment, *P. aeruginosa* PAO1 *mexC,* mexD, *oprJ* with Tetracycline as well as *P. aeruginosa* PAO1 *mexY* and *oprM* with tobramycin.

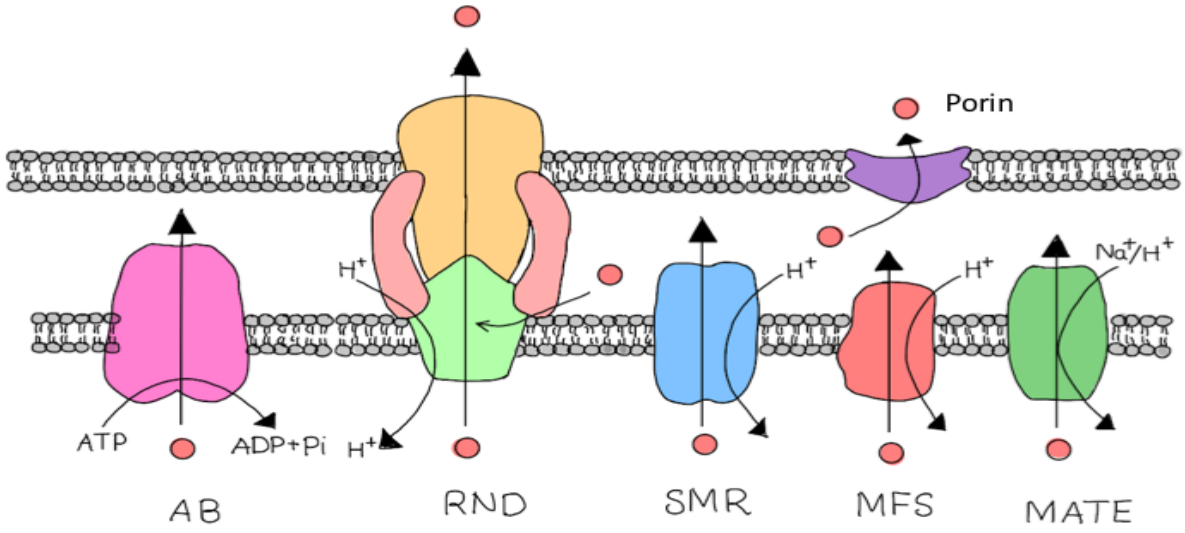


Figure 2. Schematic of Gram-negative bacterial efflux pumps.

Table 1. Currently known *P. aeruginosa* efflux pumps and associated substrates.

Efflux System			Antibiotic and Other components	Reference
Periplasmic Membrane Fusion Protein (MFP)	Cytoplasmic Membrane Transporter (RND)	Outer Membrane Factor (OMF)		
MexA	MexB	OprM	Aminoglycoside; amphenicols; Beta-lactams; Chloramphenicol; Ciprofloxacin; Fluoroquinolo; Macrolides; Novobiocin; suflonamides; Tetracycline; Thiolactomycin; Tigacycline; Trimethoprim; Quinolones; Sulphamethoxazole; Biocides/detergents/dyes/HSL/aromatic hydrocarbons	(X. Z. Li, Nikaido, & Poole, 1995)
MexC	MexD	OpJ	Trimethoprim; Fluoroquinolo; Cefpirome; Cefozopran; Tetracycline; Chloramphenicol; Quinolones; Macrolide; Ethromycin; Biocides/detergents/dyes/HSL/aromatic hydrocarbons	(Gotoh et al., 1998)
MexE	MexF	OprN	Trimethoprim; Chloramphenicol; Quinolones; Imipenem; Fluoroquinolones; Biocides/detergents/dyes/HSL/aromatic hydrocarbons	(Maseda, Yoneyama, & Nakae, 2000)
MexX	MexY	OprM	Fluoroquinolones; Beta lactams; Tetracycline; Aminoglycosides; Macrolides; chloramphenicol; Tobramycin	(Morita, Tomida, & Kawamura, 2012)
MexJ	MexK	OprM/OpmH	Tetracycline; Ethromycin; Biocides	(Chuanchuen, Murata, Gotoh, & Schweizer, 2005)
(MexG) MexH	MexI	OpmD	Fluoroquinolones; Vanadium	(Aendekerk et al., 2005)
MexV	MexW	OprM	Fluoroquinolones; Tetracycline; Chloramphenicol; Macrolide	(Y. Li et al., 2003)
MexM	MexN	OprM	Chloramphenicol; Thiamphenicol	(Mima, Sekiya, Mizushima, Kuroda, & Tsuchiya, 2005)
TriA TriB	TriC	OpmH	Triclosan	(Ntreh, Weeks, Nickels, & Zgurskaya, 2016)

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strains and Growth Medium

Planktonic *E. coli MG1665* and *P. aeruginosa PAO1* as well as *P. aeruginosa PAO1* efflux mutants (Stover et al., 2000) (Table 2) were routinely grown Luria-Bertani (LB) medium (NaCl (10g/L);tryptone (10g/L); yeast Extra (5g/L) dissolve in dH_2O then autoclave) (Sambrook & Russel, 2001). Overnight cultures of both strains were inoculated from streaked plate made from glycerol stocks. Planktonic *E. coli* MG1665 was used in this study because antibiotic accumulation level in this strain has been studied (Richter et al., 2017) , which provides a positive control for our study.

Table 2. Bacteria strains used in this study

Microorganism	**Source**
E. coli MG1665	(Parkinson & Houts, 1982)
P. aeruginosa PAO1	(Tseng et al., 2013)
P. aeruginosa PAO1 *oprJ* mutant	University of Washington Manoil Lab (Stover et al., 2000)
P. aeruginosa PAO1 *mexD* mutant	
P. aeruginosa PAO1 *mexC* mutant	
P. aeruginosa PAO1 *oprM* mutant	
P. aeruginosa PAO1 *mexY* mutant	

2.2 Sample preparation

Our method has been validated on antibiotic penetration to stationary phase cells, we especially interested in the stationary phase cell since stationary phase cells are more tolerance to antibiotic so we can introduce our test efficiently without damage the cell membrane. For small amounts of cells (sample volume less than 50 mL), overnight bacterial (Table 2) cultures were diluted to OD_{600} of 0.5 in 1mL of phosphate buffered saline (PBS) (NaCl(8g/L); KCl (0.2g/L); Na_2HPO_4 (1.44g/L); KH_2PO_4 (0.24g/L) dissolve in dH_2O then adjust to pH=7.4, autoclave) in micro-centrifuge tubes; and then pelleted at 16.1 relative centrifugal force (rcf) for 3 minutes at room temperature. After the supernatant was discarded, the pellets were washed three times with 1X PBS (NaCl(8g/L); KCl (0.2g/L); Na_2HPO_4 (1.44g/L); KH_2PO_4 (0.24g/L) dissolve in dH_2O then adjust to pH=7.4, autoclave) and re-suspended in equal volume of 1X PBS. Two hundred ug/mL selected antibiotic was added to each sample. The samples treated with antibiotic were then incubated at 37°C with shaking at 200 rpm for 15 minutes. Untreated cells were washed and incubated under the same condition as controls. Immediately after incubation, cells were pelleted at 16.1 rcf for 3 minutes and washed once with 1X PBS. Vortex the pellets until the smear of pellets appeared on the bottom of the micro-centrifuge tube. At this point, the cells were ready for lysis. For large amounts of cells (sample volume is greater than 50 mL), cell (Table 2) overnight culture has been diluted to OD_{600} of 0.5 in 50mL Falcon™ tubes then pelleted at 9600 rcf for 5 minutes at room temperature and the supernatant was discarded. The pellet was washed three times with 1X PBS and re-suspended in 1X PBS. One mL washed cells were transferred into a micro centrifuge tube for antibiotic treatment. Same treatment condition described above was followed.

2.3 Preparation of cell lysates

Our new method was inspired by Gram Staining, which is distinguishes Gram-positive and Gram-negative bacteria. The decolonization step of Gram Stain uses alcohol/acetone as to wash away the crystal violet from Gram-negative cells by lysing the outer membranes. The inner membrane remains intact in this treatment due to the presence of peptidoglycan (Coico, 2001). In our new penetration assay, we use acetone: ethanol (vol:vol=1:1) solution lyse the outer membrane and release drug molecules from the periplasm for quantification. Meanwhile, the whole cell was lysed using chloroform to quantify the total concentration. The difference between the two will inform the concentration in the cytoplasm. This process is summarized in Figure 3.

To lyse the outer membrane without damaging the inner membrane, the treatment time needs to be optimized. One hundred uL acetone/ethanol (1:1=vol:vol) solution was added to a micro-centrifuge tube (or 100 uL in a well of 96 well plate, see section 2.9) that contained cell pellet. The sample was mixed by pipetting up and down for 10 seconds. Then the cell debris was removed by filtering the mixture through 96-well filter plates using vacuum manifold (0.2um in diameter; Pall® Corporation, New York, USA) and collecting the filtered solution with a microfuge tube.

To lyse the whole cell, 100 uL chloroform was added to each microfuge tube containing re-suspended cell pellet. After gentle mixing of the solution, the sample was then centrifuged at 5000rpm for 5 minutes. The supernatant was collected using a and vacuum dried overnight (or ≥12 hours).

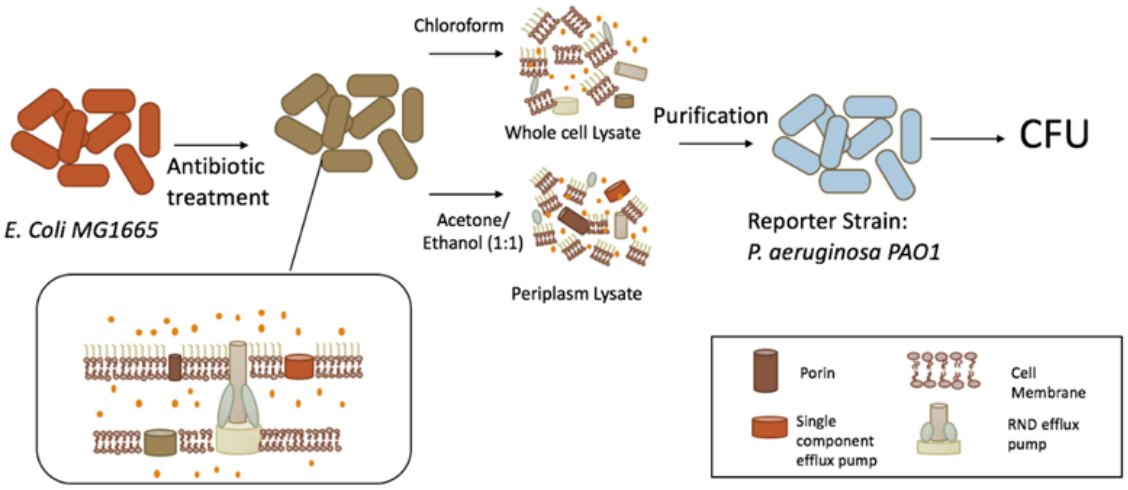


Figure 3. Schematic illustration of antibiotic penetration and cell lysing procedures

2.4 Determining antibiotic concentration using reporter strains.

All cell lysates containing antibiotics released from the periplasm fraction and whole cell were analyzed to determine the antibiotic concentration using planktonic *P. aeruginosa* PAO1 in exponential phase as reporter. Briefly, 1 mL of an overnight culture of *P. aeruginosa* PAO1 was diluted with 100ml fresh LB broth and grown at 37°C with shaking at 200rpm to an OD_{600} of 0.3. The bacterial cells were then concentrated to OD600 of 0.5 and pelleted at 16.1 rcf for 3 minutes at room temperature. The pellet was washed three times with 1X PBS and re-suspended in 1X PBS. The lysate was suspended in 100 ul 1X PBS by vortexing for 5 minutes and added to 1 mL of reporter strains. The samples were then incubated at 37°C with shaking at 200 rpm for 1 hour, followed by pelleting at 13.2 rpm for 3 minutes and washing twice with 1X PBS to remove the antibiotic that did not penetrated the cells. Finally, the pellets were re-suspended into 1XPBS. Viable cells were quantified using the drop plate method (Herigstad, Hamilton, & Heersink, 2001). CFU were counted after overnight incubation at 37°C. The killing was determined based on CFU results, which was used to calculate the concentration of antibiotic by fitting a standard curve (established by treating reporter cells with known concentrations of antibiotic spike in cell lysates).

2.5 Optimizing experimental conditions

Temperature Increased experimental temperature may alter the metabolic activity of the cells which raise the concern that if the experimental temperature would affect the drug uptake by gram-negative bacteria. As a comparison to the original protocol conditions (treated in room temperature), planktonic *E. coli* cells were treated and lysed followed the same steps described in section 2.1-2.4 in 37°C incubation room.

Vacuum filtration force. The vacuum force was varied as -5kPa and -10kPa. One drop (~10 µL) of food color (McCormic® Neon Assorted Food Color) was added to the acetone/ethanol (1:1=vol: vol) solution to ease visualization during this test. The penetration of color was followed as an indicator of filter efficiency.

Filtration of cell lysates. The periplasm samples were filtered by applying a vacuum to 96 well filter plates (Pall® Corporation). The results were compared with those of centrifuge to identify the best assay condition. Planktonic *E. coli* MG1665 cells were treated and lysed following the same steps described in section 2.1-2.4. For centrifugation filtration, cells were centrifuged for one minute at 16100 rcf after lysis with acetone/ethanol (1:1=vol: vol)

Acetone/ethanol (1:1=vol: vol) concentration and treatment duration. To verify that lysing target cells' outer membrane with acetone/ethanol (1:1=vol: vol) only lysed the outer membrane and released the drug molecules from the periplasm space without disrupting the inner membrane, different lysis times were tested. Planktonic E. coli

MG1665 cells were treated and lysed following the same steps described in section 2.1-2.4. A series of dilution was also tested (95%, 75%, and 50%) along with different cell lysis time (10, 20, and 30 s). To corroborate the results, treated samples were also stained with live/dead staining for 15 minutes and imaged using an Axio Imager M1 Fluorescence microscope with a camera (Orca-Flash 4.0 LT; Hamamatsu Photonics, Hamamatsu City, Japan) by Dr. Huan Gu.

Throughput. To achieve high throughput screening, it is important to conduct the assays in 96-well plates. In this test, we compared the results using microcentrifuge tubes and 96-well plates.

CHAPTER 3

RESULTS

3.1 CIP penetration.

This assay is designed to control the lysis of the two layers of Gram-negative bacterial membranes separately and to quantify the concentration of drug molecules in the periplasm and cytoplasm. Dr. Huan Gu in the Ren lab has obtained preliminary results demonstrating the feasibility of this approach. Experimental group cells were treated with ethanol: acetone (1:1=vol: vol) solution, both with and without Ciprofloxacin. Then cells were both stained with Propidium Iodine (PI) and Syto® 9. PI is a typical, red-fluorescent, nuclear and chromosome counterstain dye used to detect dead cells in a population because it only penetrates compromised membranes and impermeable to live cells. Ethanol: acetone (1:1=vol: vol) treatments the outer membrane of the cells, so the PI dye could easily penetrate the cells' outer membrane. Syto® 9 is a green-fluorescent, nuclear and chromosome counterstain which can penetrate intact membranes to bind to DNA. Thus, it can stain all the cells. As showed in Figure 4, the control groups, untreated cells and cells treated only with CIP are showed green color, which indicated that the cell membranes remained intact. However, for experimental groups, cell treated with ethanol: acetone (1:1=vol: vol) solution, the majority of cells showed orange/ orange-red color, which is the indication that both Syto® 9 and PI got into the cells. Thus, the outer membrane is disrupted by the treatment solution. This result not only directly proved that the ethanol: acetone (1:1=vol: vol) treatment successfully damaged the cell's outer membrane, but also demonstrated treating cells with ethanol; acetone solution only dissolved the outer membrane, not disrupting the whole cell structure. Besides, since the majority of CIP treated cells still showed green fluorescence, it is confirmed that the CIP treatment time and concentration were not enough to damage the cell membrane or

disrupt the cell structure. In addition, analysis with transmission electron microscopy (TEM) (Figure 4) showed that only the outer membrane was damaged by ethanol: acetone (1:1=vol: vol) treatment, whereas the inner membranes and cytoplasm space stayed intact. Thus, the preliminary data confirmed this method could successfully lyse the cell membranes and allowed the penetration to be evaluated by membrane lysis with ethanol: acetone (1:1=vol: vol) treatment with the tested antimicrobial.

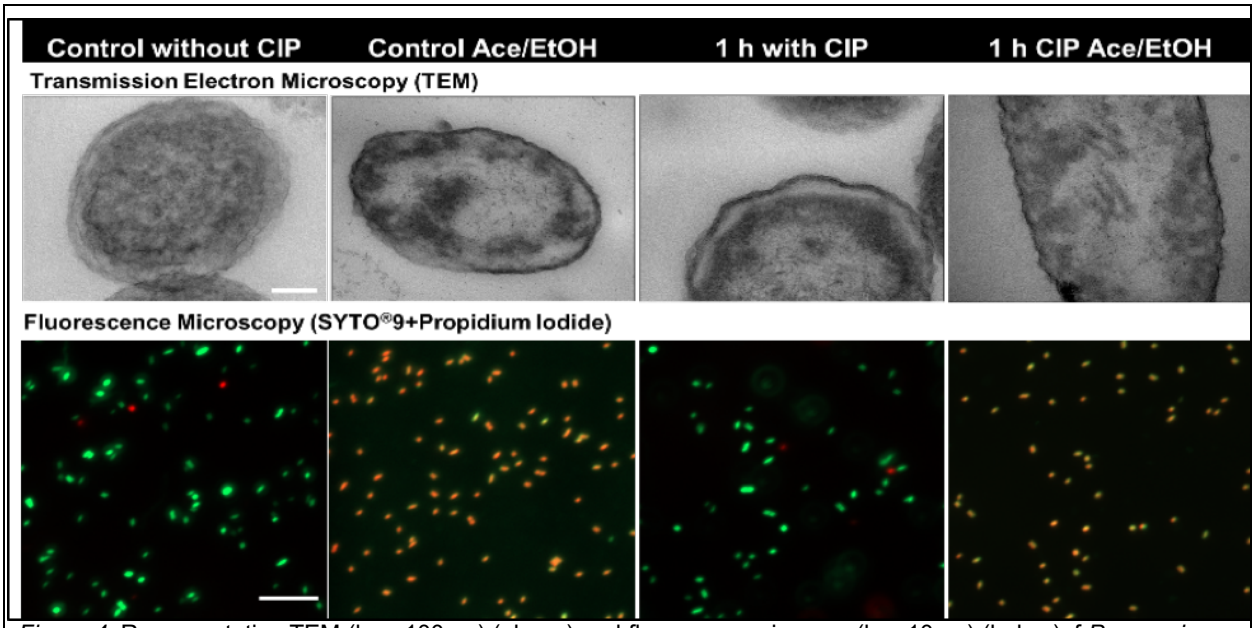


Figure 4. Representative TEM (bar=100nm) (above) and fluorescence images (bar-10um) (below)of *P. aeruginosa* PAO1 planktonic cells (with or without 1h CIP treatment) before and after Ethanol: acetone (1:1=vol:vol) treatment.

*Data from Dr. Huan Gu

3.2 Confirmation of experimental parameters

3.2.1 Effects of temperature on CIP penetration in *E. coli* MG1665 cells.

It has been shown by Hajdu et al. *(Hajdu et al., 2010)* that an increase in temperature can significantly alter several antimicrobial activities, including tigecycline, daptomycin, fosfomycin, and cefamandole, in *Staphylococcus aureus*. The majority of microorganisms have preferred growth temperature in the range between 25°C to 40 °C (Murray et al., 2003). In this study, to eliminate artifacts that may affect experimental results, a temperature control experiment has been performed. Our results indicated that performing an experiment at different temperatures had impact on the experimental results (Figure 5). Therefore, it is crucial to perform a screening assay under a controlled environment. Thus, our future analysis has been performed under room temperature (23-25 °C)

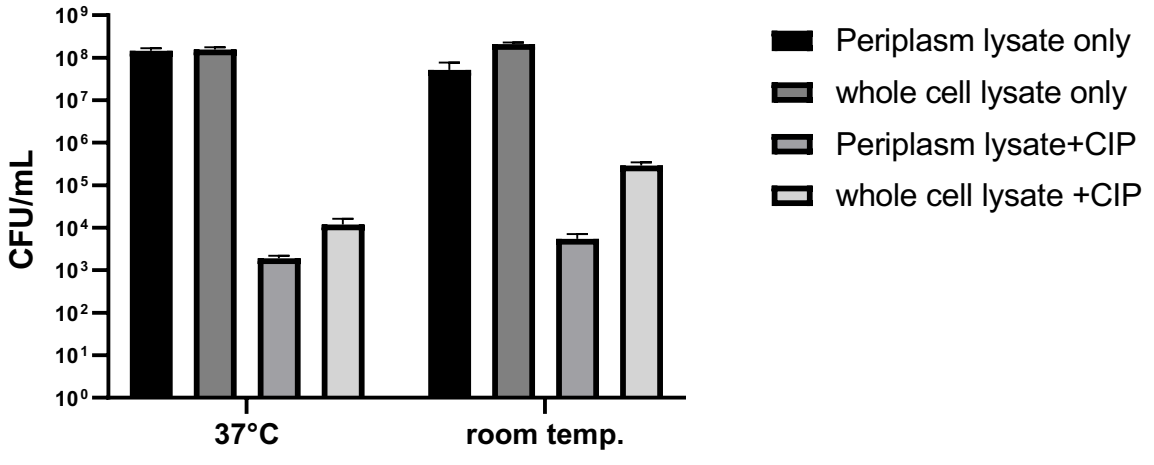


Figure 5. Temperature dependent CIP treatment - *E. coli MG1665*

3.2.2 Optimizing the filtration.

To ensure the lysis of outer membrane does not affect the integrity of inner membrane, it is important to remove acetone and ethanol quickly after the treatment. From a visual inspection of the colored solutions, higher filtration force acetone: ethanol (vol: vol=1:1) mixture went through the filter quicker (Figure 6). Thus, the higher vacuum force was preferred with the purpose of control the cell lysing time. Also, we observed that the diluted treatment solution required a more extended time pass through the filter. Therefore, to optimize the periplasmic membrane lysing time, it is necessary to use the undiluted solution for treatment.

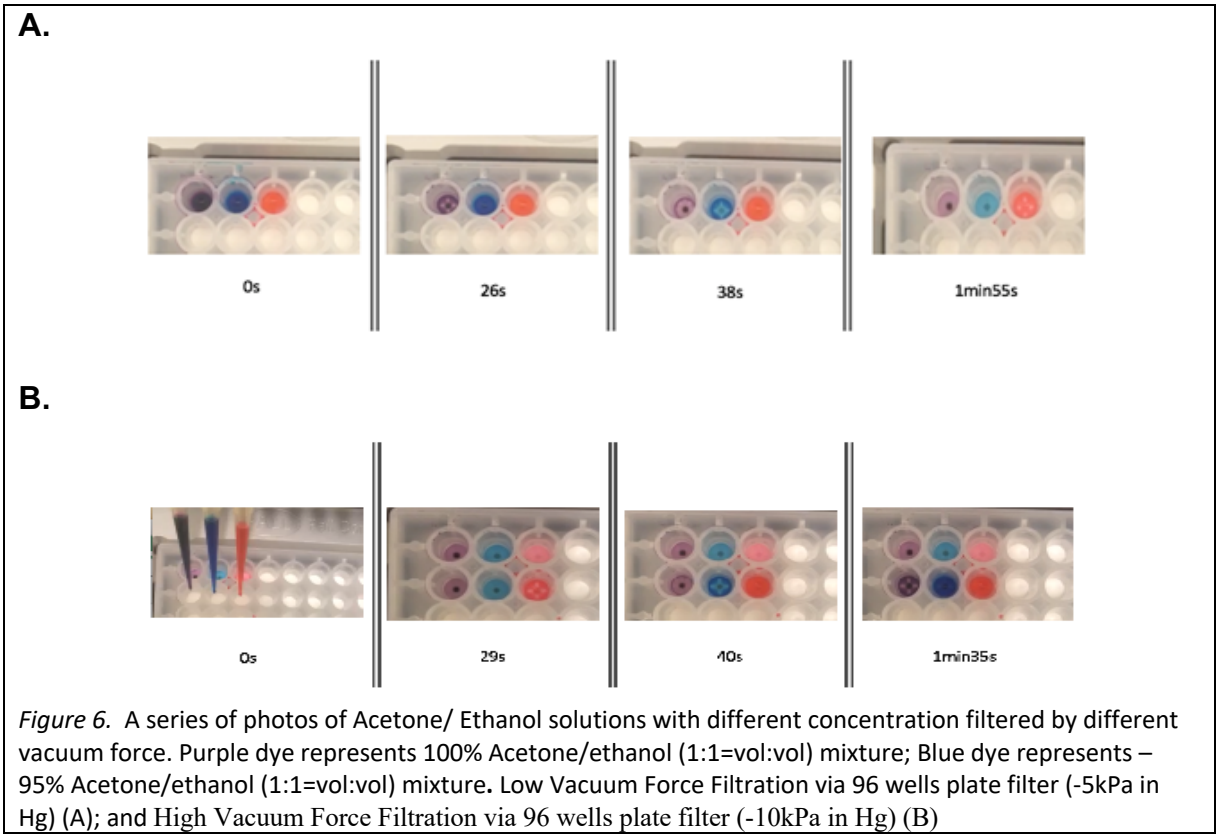


Figure 6. A series of photos of Acetone/ Ethanol solutions with different concentration filtered by different vacuum force. Purple dye represents 100% Acetone/ethanol (1:1=vol:vol) mixture; Blue dye represents – 95% Acetone/ethanol (1:1=vol:vol) mixture**.** Low Vacuum Force Filtration via 96 wells plate filter (-5kPa in Hg) (A); and High Vacuum Force Filtration via 96 wells plate filter (-10kPa in Hg) (B)

3.2.3 96 well filter plate is the best purification method to prepare periplasmic lysate.

As described in the previous section, extended outer membrane lysis time may result in a disruption of the inner membrane structure. Different separation methods (filtration vs. centrifugation) were tested to optimize the protocol. For the cell lysate after acetone: ethanol treatment, periplasm lysate collected by centrifugation contained more released antibiotic compared to the one filtered through the filter plate. This is expected since the cells remained in contact with the lysing agents during centrifugation and thus may have partial lysis of inner membranes. In comparison, 96 wells filter plates provided a more rapid separation, which was more suitable for a high-throughput screening system (Figure 7).

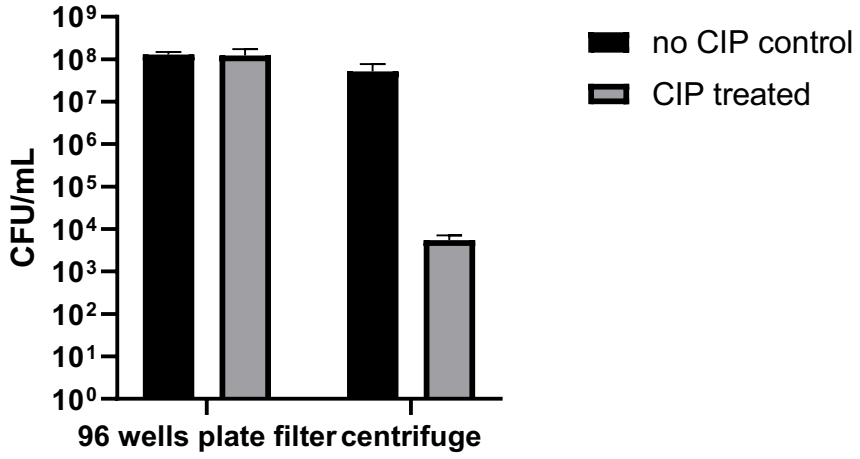


Figure 7. Relative antibiotic activities of cell lysates collected by centrifugation and filtration.

3.2.3 Effects of lysis time.

Another parameter that needs to be optimized is the lysis time for releasing antibiotic molecules in the periplasm space. Figure 8 indicates, more cell was killed by cell lysates collected from 20 seconds and 30 seconds lysing groups, thus,10-second cell-lysing time is effective without damaging the inner membrane. Besides, compared the 30- and 20-seconds' lysate to whole cell lysate, the viable cell number validated the inner cell membrane was being disrupted with more than 20 seconds' cell lysing time. Thus, 10 seconds was chosen as the lysis time for incubation with 100% acetone: ethanol (1:1=vol: vol) solution.

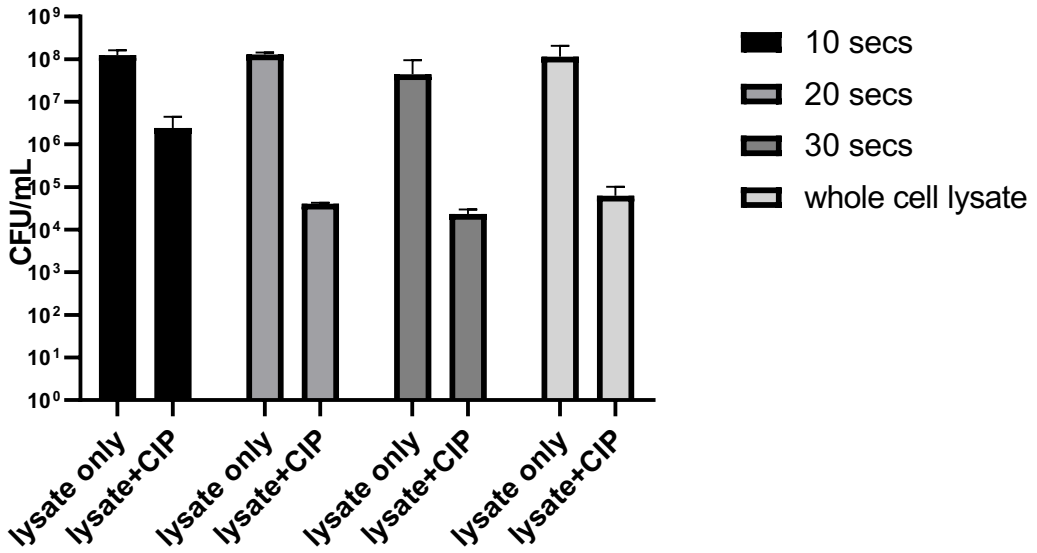


Figure 8. Effects of lysis time on antibiotic release

In addition, to enable high-throughput screening, we aimed to lyse cells' outer membranes in 96 well plates. Figure 9 indicated that using 96 well plate for acetone: ethanol (1:1=vol: vol) treatment has no significant difference (less than 1% variation in cell surviving rate)

compared to treat cells in microfuge tubes. Lysing cells in 96 well plates are more scalable to allow screening and tests with more repeats.

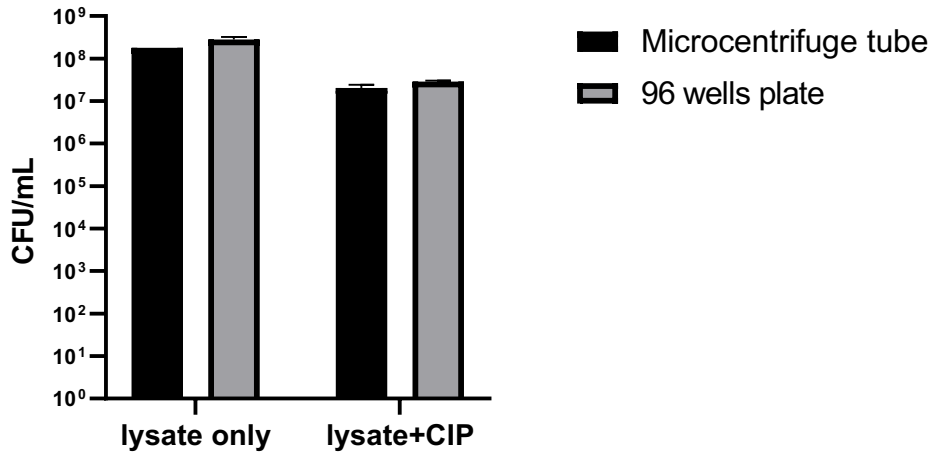


Figure 9. The lysis protocol can be adapted to assays using 96-well plates.

3.4 Qualification of antibiotic concentrations in cytoplasm and periplasm

3.4.1 The method to determine antibiotic concentration in the periplasm or cytoplasm

The concentration of antibiotics can be determined by fitting the CFU of reporter strain with standard curves of the same strained treated with known concentrations of antibiotics. The volumes of periplasm and cytoplasm can be estimated based on the average size of Gram-negative bacteria cells; the average length of bacterial cells (l_w) is 3μm; the radium of bacteria cells (r_w) is 1μm, and the thickness of the peptidoglycan layer (r_w-r_c) is 20nm (Figure 10).

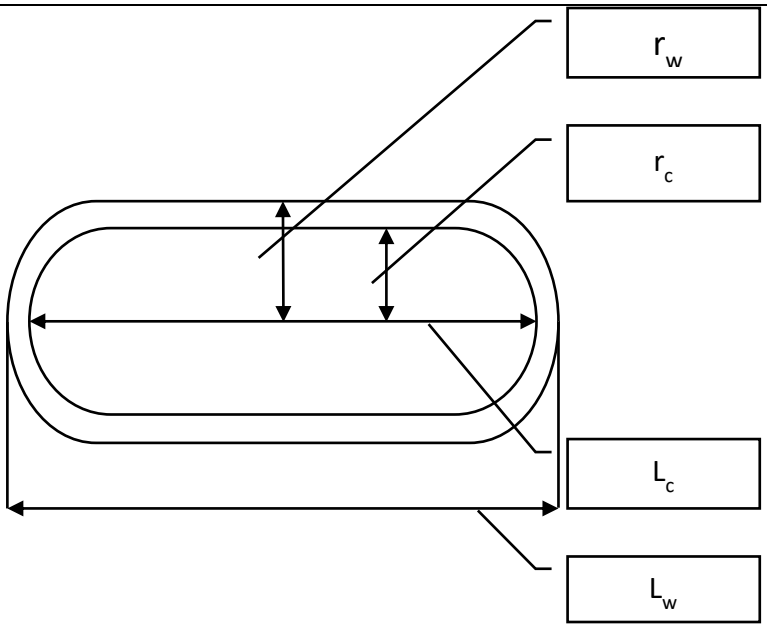


Figure 10. Dimension of Gram-negative cells used in calculation.

Therefore, the following equation was used to estimate the volume of each cell fractions, where V_w represents the whole cell volume; V_c represents the volume of the cytoplasmic space, and V_p is the volume of the periplasm space.

$$V_w = \pi * r_w^2 * L_w \quad \text{(Equation 1)}$$

$$V_c = \pi * r_c^2 * L_c \text{ (Equation 2)}$$

$$V_P = V_w - V_c \text{ (Equation 3)}$$

Thus, the concentration of antibiotic accumulated in the periplasm space [P] of the cell can be calculated as:

$$[P] = \frac{Xp*1ml}{CFU_{CTL}*4.5ml*Vp} \text{ (Equation 4)}$$

Where X_p represents the concentration of antibiotic isolated from 10ml of the periplasm lysate.

Followed the same idea, the concentration of antibiotic accumulated in the whole cell [W] can be calculated as:

$$[W] = \frac{Xw*1ml}{CFU_{CTL}*4.5ml*Vw} \text{ (Equation 5)}$$

Where X_w is the concentration of antibiotic isolated from 10ml of the whole cell lysate. The concentration of antibiotic accumulated in the cytomplasmic space can be calculated by substracting [P] from [W].

Since we can easily obtain a linear regression from a developed standard curve with lysate controls, we can calcuate X_w and X_p from the linear regression.

$$Y_w = a_w * \exp(b_w * X) \Rightarrow [X_w] \text{ in ug/mL (Equation 6)}$$

$$Y_p = a_p * \exp(b_p * X) \Rightarrow [X_p] \text{ in ug/ml (Equation 7)}$$

$$[X_c] = [X_W] - [X_P] \text{ (Equation 8)}$$

Based on the standard curve, the concentration of CIP in *P. aeruginosa* PAO1 periplasm space were estimated to be 107 μg/ml and 0.32μg/ml in cytoplasm space (Table 5). The stepwise lysis protocol was validate using a reporter assay and LC-MS analysis. LC-MS is an analytical allows to separation and quantification of compounds in a mixed sample. Based on Dr. Gu's preliminary result using reporter strain, there was 92.9ug/ml CIP in the

periplasm space and 0.2ug/ml CIP in the cytoplasmic space (Courtesy Dr. Huan Gu), which show consistent result of CIP penetration in *P. aeruginosa*.

3.4.2 High level of CIP is accumulated in *E. coli* MG1665 cells

We also validated our approach by comparing with the LC-MS data of CIP accumulation in *E. coli*, as reported by Richter et al. (Richter et al., 2017), Standard curves for ciprofloxacin quantification were constructed using *P. aeruginosa* PAO1 as reporter with the addition of whole cell lysates of *E. coli* MG1655 (Figure 11). By fitting the standard curves, we estimated that CIP accumulated in the periplasm and cytoplasm of *E. coli* MG1665 cells (Table 4, Figure 13) was 2.78 μg/mL and 2.04μg/ml, respectively. This first set of validation results were constructed from the average of 10 biological replicates to demonstrate the repeatability of this assay. In addition, we tested the effect of treating P. aeruginosa PAO1 cells *E. coli* MG1665 lysate to ensure there was no artificial effect (Figure 12).

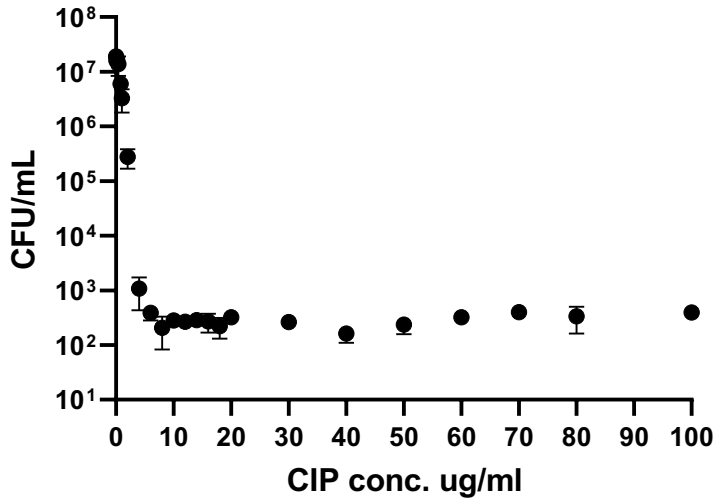


Figure 11. Standard killing curve of the reporter strain *P. aeruginosa* PAO1 by ciprofloxacin. The reporter cells were harvested from exponential cultures and the treatment was conducted in PBS.

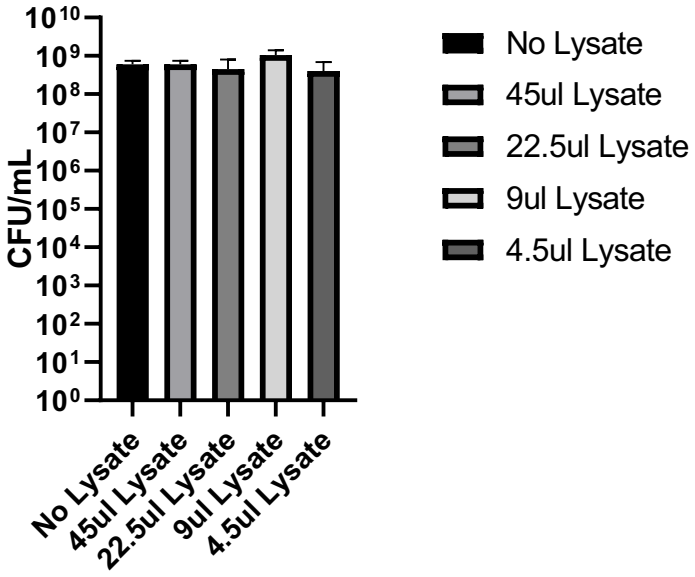
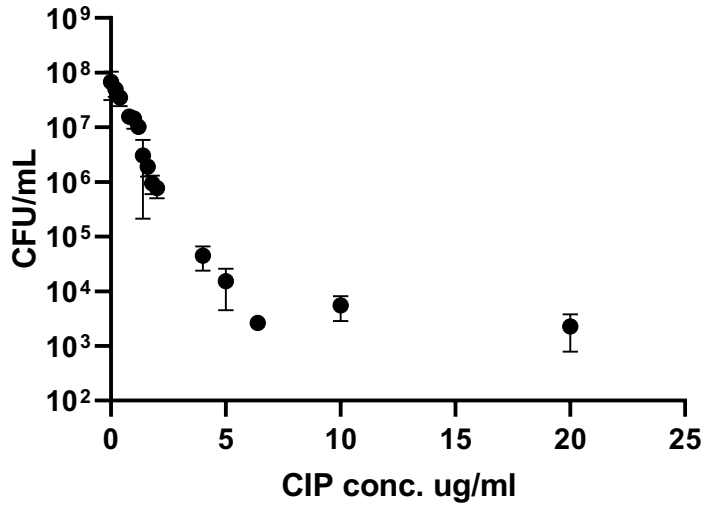


Figure 12. Reporter strain treated with *E. coli* MG1665 lysate. Exponential phase *P. aeruginosa* PAO1 cells were treated with *E. coli* MG1665 lysate for 1 hours at 37℃.

A.



B.

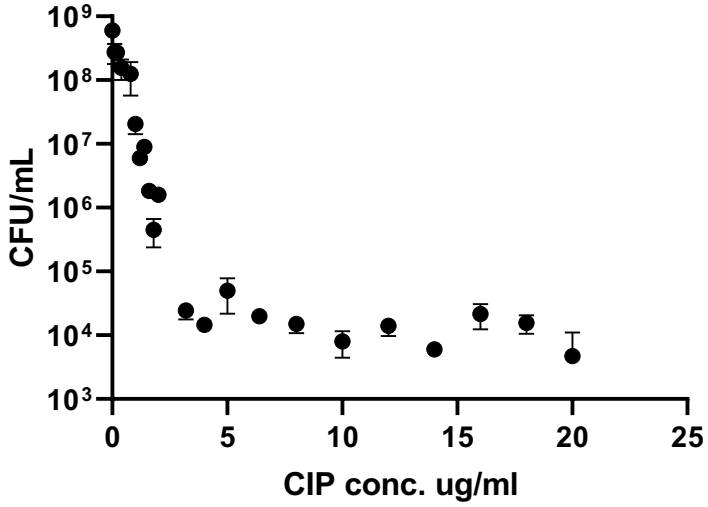


Figure 13. Standard killing curve the reporter strain *P. aeruginosa* PAO1 treated by released ciprofloxacin from *E. coli* MG1665 periplasm lysate (A) and whole cell lysate (B). The reporter cells were harvested from exponential cultures and the treatment was conducted in PBS.

Table 3. Quantified concentration of drug accumulation in the periplasm and cytoplasm spaces of *E. coli* MG1665, *P. aeruginosa* PAO1, *P. aeruginosa* PAO1 efflux pump mutants and *P. aeruginosa* PA14 efflux pump mutants based on standard curve established using reporter strain (exponential phase *P. aeruginosa* PAO1).

Microorganism	Antibiotic Used	Periplasm	Cytoplasm
E. coli MG1665	CIP	2.04μg/mL	2.78μg/mL
P. aeruginosa PAO1	CIP	107μg/mL *	0.32μg/mL *
P. aeruginosa PA14 *mexB* mutant	CIP	77.6μg/mL *	7.28μg/mL *
P. aeruginosa PA14 *oprM* mutant	CIP	131.9μg/mL *	0.02μg/mL *
P. aeruginosa PAO1	TCN	0.78μg/mL	0.38μg/mL
P. aeruginosa PAO1 *oprJ* mutant	TCN	0.62μg/mL	0.45μg/mL
P. aeruginosa PAO1 *mexD* mutant	TCN	0.54μg/mL	0.62μg/mL
P. aeruginosa PAO1 *mexC* mutant	TCN	0.80μg/mL	0.39μg/mL
P. aeruginosa PAO1	TOB	84μg/mL	2μg/mL
P. aeruginosa PAO1 *oprM* mutant	TOB	169μg/mL	11μg/mL
P. aeruginosa PAO1 *mexY* mutant	TOB	29μg/mL	261μg/mL

*Data from Dr. Huan Gu

3.4.3 Cytoplasmic membrane is the major barrier of antibiotic penetration into *P. aeruginosa* cells.

Our new assay provides an effective method for understanding bacterial antibiotic resistance. Periplasm and whole cell lysates from *P. aeruginosa* PAO1 treated with 200μg/mL ciprofloxacin were obtained and used to treat exponential phase *P. aeruginosa* PAO1 reporter strain. Based on the quantified concentration of ciprofloxacin accumulation in the periplasm and cytoplasm spaces of *P. aeruginosa* PAO1 cells, the periplasm layer was found to accumulate more ciprofloxacin (107μg/mL) compared than the cytoplasmic space (0.32μg/mL) (data from Dr. Huan Gu). To understand if this is limited to CIP, we also conducted a similar experiment to compare the penetration of tobramycin. From Tobramycin-*P. aeruginosa* PAO1 killing curve (Figure 14), we narrowed the linear killing range with tobramycin concentration up to 100ug/mL. The standard curve for the wild-

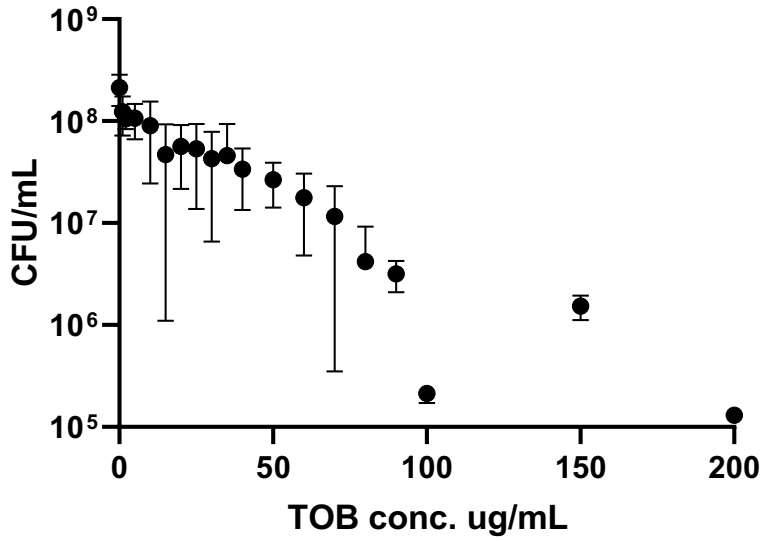
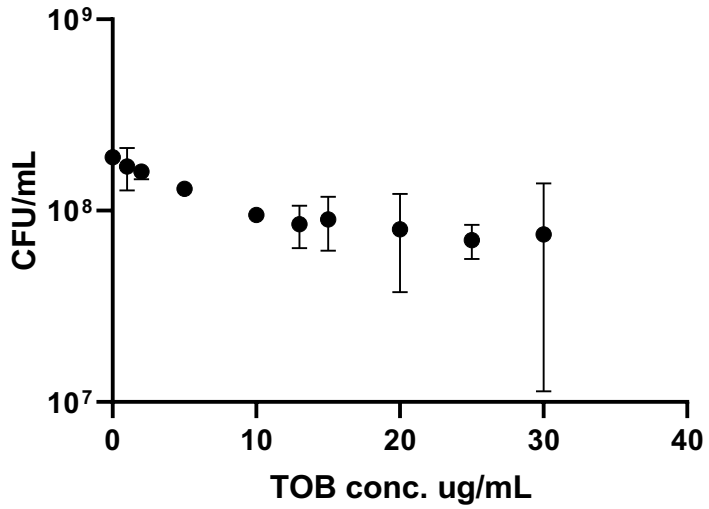


Figure 14. Standard killing curve of the reporter strain *P. aeruginosa* PAO1 by tobramycin. The reporter cells were harvested from exponential cultures and the treatment was conducted in PBS.

type *P. aeruginosa* PAO1 and its efflux pump mutants with lysate control were tested (Figure 15).

A.



B.

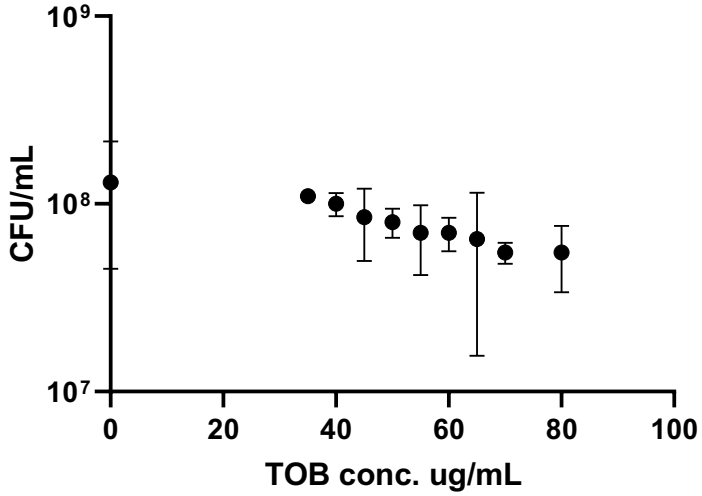


Figure15. Standard killing curve the reporter strain *P.* aeruginosa PAO1 treated by released tobramycin from *P. aeruginosa* PAO1 periplasm lysate (A) and whole cell lysate (B). The reporter cells were harvested from exponential cultures and the treatment was conducted in PBS.

The lysates from *P. aeruginosa* PAO1 treated with 200μg /mL of tobramycin were obtained and then used to treat exponential-phase *P. aeruginosa* PAO1 reporter cells.

Our data (Table 5 and Figure 15) showed that more tobramycin accumulated in the periplasm space of *P. aeruginosa* PAO1 (84μg/mL) compared to the cytoplasmic space (2μg/mL). This result suggests that the inner membrane of *P. aeruginosa* PAO1 plays a critical role in antibiotic resistance.

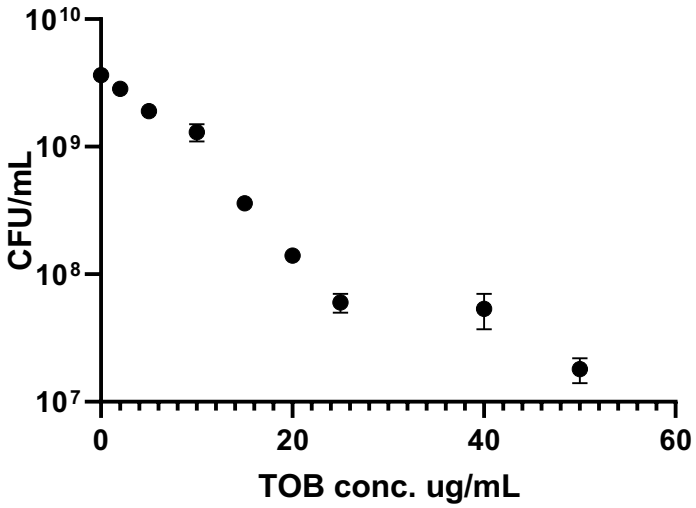
3.4.4 Antibiotic penetration in efflux pump mutants

Efflux mutants were used in this study to confirm if the inner membrane acts as the main barrier for drug penetration into *P. aeruginosa* cells, which also enables us to compare the roles of each efflux pump system. In this project, we studied *P. aeruginosa* PAO1 *mexY* and *oprM* mutants. MexXY-OprM is an active efflux system known has the distinctive function to export aminoglycosides, including gentamicin, amikacin, tobramycin, streptomycin, and neomycin (Hocquet et al., 2003). MexX and MexY together form tripartite efflux machinery with the outer membrane protein OprM. Therefore, we investigated the *mexX* and *oprM* mutants to test the antibiotic accumulation levels. We hypothesized that the *mexX* mutant would have more drugs accumulated in the cytoplasmic space since *mexX* is the transporter protein located on the cytoplasmic membrane. The *oprM* mutant would have drug accumulated level in the periplasmic membrane would be impacted since OprM is the outer membrane protein that forms connections from the periplasmic space to the outside environment.

We first performed the test on wild type P. aeruginosa PAO1 cell. The lysates obtained from *P. aeruginosa* PAO1 wild type, *P. aeruginosa* PAO1 *mexX* mutant, and *P. aeruginosa* PAO1 *oprM* mutant were treated with 200μg/mL of tobramycin and used to

treat exponential phase *P. aeruginosa* PAO1 wild type population. According to the standard curves (Figure 16 and 17), our data (Table 5) showed that a higher concentration of tobramycin accumulated in wild type *P. aeruginosa* PAO1 periplasm space (84μg/mL) compared to the cytoplasmic space (2μg/mL). Then, we further test the methods as well as our hypothesis with mutants. When the outer membrane protein channel (*oprM*) was mutated, higher concentration of tobramycin was accumulated in the periplasmic space (169μg/mL) compare to the wild type, which indicated that malfunctioned outer membrane protein channel could potentially cause in membrane leakage, thus result in a higher drug accumulation level within the cytoplasmic membrane. Followed by testing the strain has transport protein (*mexY*) mutation, the TOB concentration in the cytoplasmic space (261μg/mL) increased significantly compared to the wild type. Our result demonstrated that the efflux system has a significant effect on ciprofloxacin and tobramycin penetration. It also further confirmed that our method could effectively detect the drug penetration in cytoplasm and periplasm of Gram-negative cells.

A.



B.

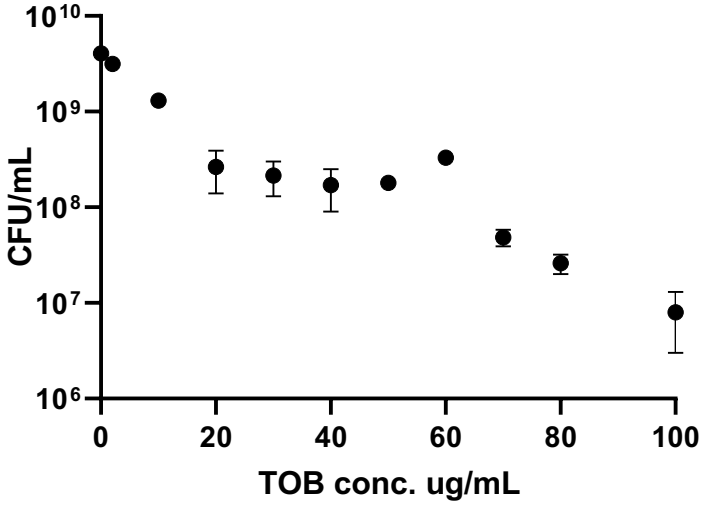
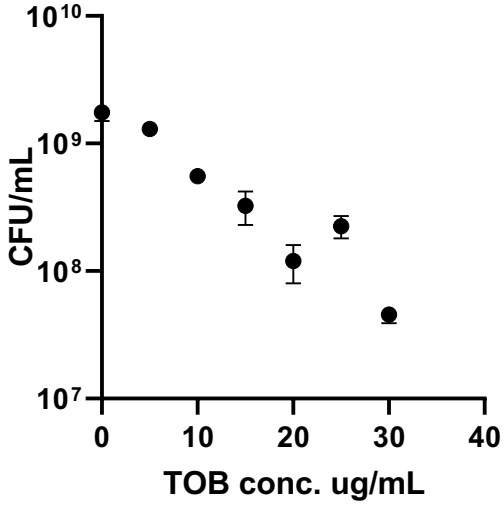


Figure 16. Standard killing curve the reporter strain *P. aeruginosa* PAO1 treated by released tobramycin from *P. aeruginosa* PAO1 *oprM* mutant periplasm lysate (A) and whole cell lysate (B). The reporter cells were harvested from exponential cultures and the treatment was conducted in PBS.

A.



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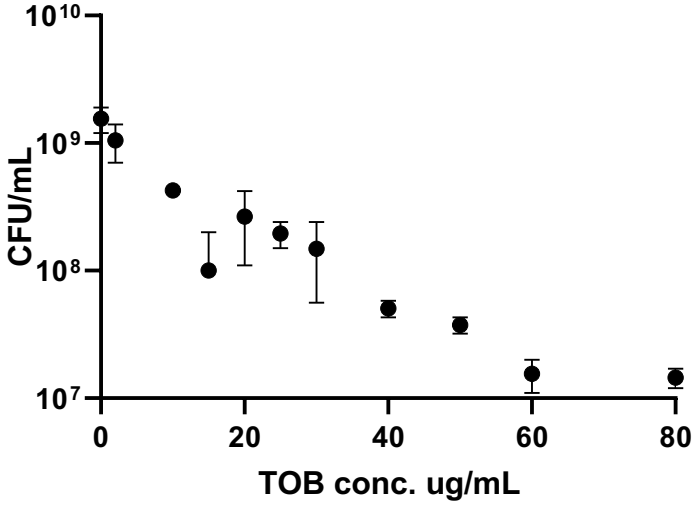


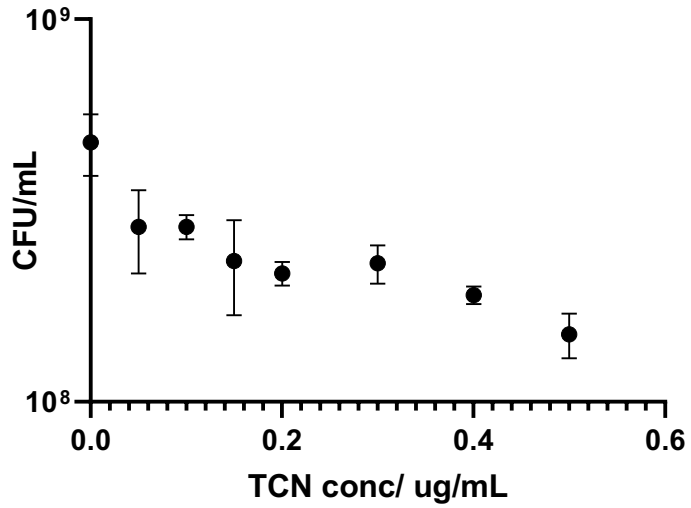
Figure 17. Standard killing curve the reporter strain *P. aeruginosa* PAO1 treated by released tobramycin from *P. aeruginosa* PAO1 *mexY mutant* periplasm lysate (A) and whole cell lysate (B). The reporter cells were harvested from exponential cultures and the treatment was conducted in PBS.

3.4.5 Tetracycline (TCN) has low membrane permeability against *P. aeruginosa* PAO1 cells

As described in the previous sections, we have demonstrated that our high throughput assay can selectively lyse the outer membrane or the whole cell to extract the lysates with accumulated antibiotics within *P. aeruginosa*. Consistently, we have shown that the cytoplasmic membrane is also a major barrier for CIP and TOB penetration. It is well known that *P. aeruginosa* PAO1 is less susceptible to TCN treatment in PBS solution (X. Z. Li et al., 1995) with MIC around 20 μg/mL. Therefore this strain has been chosen as a negative control to validate this assay. We tested *that P. aeruginosa* PAO1 is sensitive to tetracycline when the treatment is performed in LB medium. Therefore, we used exponential phase P. aeruginosa PAO1 in LB medium as the reporter strain. We hypothesize that tetracycline has low accumulation in *P. aeruginosa* cells when the cells were treated in PBS. To test this hypothesis, the penetration of TCN lysate has been tested on exponential phase *P. aeruginosa* PAO1 cells. The killing effect of the reporter strains was converted to calculate the amount of tetracycline accumulation in the cell based on the standard curves (Figure 18). With a treatment concentration of 200 μg/mL of tetracycline, 0.78μg/mL tetracycline was found accumulated in the periplasm space, and 0.38μg/mL was found in the cytoplasmic space of *P. aeruginosa* PAO1. In comparison, 0.62μg/mL and 0.45μg/mL were found accumulated in the periplasm and cytoplasm of the *oprJ* mutant, respectively; 0.54μg/ml accumulated in the periplasm space and 0.62 μg/mL accumulated in the cytoplasmic space of *P. aeruginosa* PAO1 *mexD* mutant; and 0.80μg/mL accumulated in the periplasm space and 0.39μg/mL accumulated in the cytoplasmic space of *P. aeruginosa* PAO1 *mexC* mutants (Table 5).

Compared to the ciprofloxacin results, tetracycline accumulation in both periplasmic and cytoplasmic space has been significantly reduced. This is consistent with tetracycline resistance of *P. aeruginosa*.

A.



B.

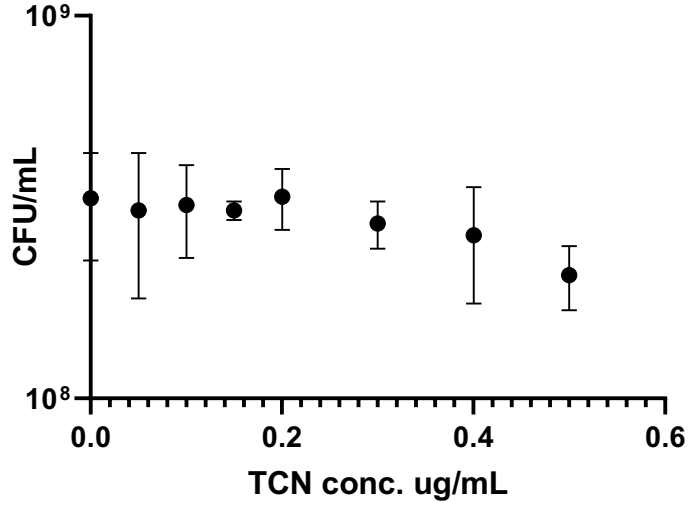


Figure 18. Standard killing curve the reporter strain *P. aeruginosa* PAO1 treated by released tetracycline from *P. aeruginosa* PAO1 mutant periplasm lysate (A) and whole cell lysate (B). The reporter cells were harvested from exponential cultures and the treatment was conducted in PBS.

CHAPTER 4

Discussion

Multidrug-resistant infection is a significant challenge to human health. (Kraemer, Ramachandran, & Perron, 2019; Neu, 1992; Wu, Moser, Wang, Høiby, & Song, 2015). After the discovery of major classes of antibiotics using the 'Waksman platform', the pool of naturally existing antibiotics has become exhausted (Ribeiro da Cunha, Fonseca, & Calado, 2019). There have only been a few semi-synthesized, and fully synthesized antibiotics developed based on those naturally occurred drugs. The 'Waksman platform' selects lead compounds based on killing activities and does not address penetration. This platform led to an antimicrobial boom in the 1960s-1980s (Ribeiro da Cunha et al., 2019). However, this method only provides limited information regarding the mechanism behind the antibiotic activity. For example, many natural compounds have low efficiency in penetrate bacterial membranes, especially those of Gram-negative species that have double-membrane structures. Therefore, it is necessary to develop a method to enable screening for membrane penetration.

This capability is also needed because the success rate of finding a compound through screening against *P. aeruginosa* is 100-fold lower than against Gram-positive species (Silver, 2011). The permeability of *P. aeruginosa* outer membranes is approximately only 1-8% of *E. coli (Tamber & Hancock, 2004)*. With the low membrane permeability barriers and insufficient compound diversity of the screening library to probe this barrier, the discovery of new antibiotics became extremely challenging (Zgurskaya, Löpez, & Gnanakaran, 2015). It remains unclear if the extensive studies of *E. coli* would also apply to other Gram-negative species. Therefore, our study used *P. aeruginosa* PAO1 as a model organism. We demonstrate that not only the outer membrane but also the inner

membrane of *P. aeruginosa* plays a critical role in protecting cells from antimicrobial attack. Based on our result, there were significantly more CIP accumulated in the periplasmic space of *P. aeruginosa* PAO1 cells (Table 5). Whereas for the wild-type *E. coli* MG1665 cells, a comparable amount of CIP accumulated in the periplasmic and cytoplasmic space. This observation confirmed that the difference in the membrane between those two species.

All current studied Gram-negative bacteria have at least one multidrug efflux pump responsible for antimicrobial resistance (Chang et al., 2015; Nikaido, 2009; Zgurskaya et al., 2015). The efflux pump, AcrAB-TolC in *E. coli,* is the best-characterized efflux system. Numerous studies, including structural and functional analysis of the AcrAB-TolC efflux pump, has been performed. As indicated by multiple groups, AcrB, a proton-motive force driven transporter, with the help of AcrA, periplasmic membrane fusion protein, capture compounds from cytoplasmic and periplasmic spaces and expels them through TolC, the outer membrane channel(Love, Bhandari, Dobson, & Billington, 2018; Lowy et al., 2010; Schmelcher, Donovan, & Loessner, 2012). However, there are fewer studies performed on *P. aeruginosa* efflux system, especially there are still uncertainties regarding the mechanism that was corresponding to the functionality of the three-component complex system.

Our method is able to quantitatively compare the concertation of candidate compounds in the periplasm and cytoplasm of Gram-negative bacteria separately. Interestingly, *P. aeruginosa* PAO1 efflux pump mutants have significantly different drug accumulation

levels compared to the wild-type strain. To our best knowledge, this has not been studied before. In this project, we have tested multiple antibiotics with their corresponding efflux pump component mutants. Under our experimental conditions, we demonstrate that the efflux transporter protein plays important role in the extrusion of antimicrobial agents. With the mutation of efflux transporter protein, the accumulation of CIP increased by 20 times, and TOB accumulation increased 100 times. The result of this study can lead to the rational design of new molecules with better penetration efficiency.

To achieve high throughput screening, we optimized the protocol by adjusting assay conditions including temperature, vacuum force, lysing time, and lysate separation. We take consideration of major environmental factors and performed control experiments alongside the process of method development. In summary, by optimizing the assay and comparing different antimicrobials, this study revealed that the inner membrane of Gram-negative bacteria is an essential barrier for the penetration of ciprofloxacin and tobramycin; While each of the antibiotic tested here has a different target, we have observed a higher antibiotic accumulation in the cytoplasmic space of efflux mutants. These observations are consistent with the critical role of efflux in antibiotic resistance of Gram-negative bacteria and further validated our new assay method. With all the validated parameters, this study has been further improved to a high-throughput screening method for molecules library screening.

CHAPTER 5

Conclusion and Future Works

This high throughput assay provides a new capacity to compare the penetration of selected compounds into Gram-negative cells. The results demonstrated that our high-throughput assay could efficiently quantify the drug penetration in both cytoplasmic and periplasmic space of various Gram-negative bacteria, including *E. coli* MG1665, *E. coli* K12, *P. aeruginosa* PAO1, and *P. aeruginosa* PA14. This assay can be used in future studies to help design new molecules that can better penetrate the bacterial membranes. This approach can start with screening of bioactive compound libraries. Currently, more than 9,000 bioactive compounds with certain antimicrobial and antiviral activities have been identified (Bérdy, 1984), and some major biomedical companies has commercial products that offer a broad range of antimicrobials for screening purposes. Such libraries can pre-screen for killing activities against appropriate reporter strains. After the identification of reporter strain, the selected library will be tested for compounds that have better penetration capabilities and identify the important structural elements. Thus, by using this high throughput assay to screen compound libraries, we can identify more effective antimicrobial molecules and enable the rational design of next-generation control agents.

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EDUCATION

Syracuse University, Syracuse, NY

Dec. 2019 **M.S. in *Chemical Engineering***, College of Engineering and Computer Science

Thesis: A New High-Throughput Assay for Quantification of Antibiotic Penetration in Gram-Negative Bacterial Cells

May 2017 **B.S. in *Biochemistry***, College of Arts and Sciences

- **Capstone: Retrogene function in *Drosophila melanogaster* spermatogenesis**
- Renee Crown Honors Program;

RESEARCH AND WORKING EXPERIENCE

Aug 2019 - Present **Yan Biofilm Lab, P.I. Professor Jing Yan, *Lab Manager & Postgraduate research fellow,*** Yale University**,** Department of Molecular, Cellular and Developmental Biology

- Using cloning and imaging techniques to study the function of a cell surface adhesion protein from *Vibrio Cholerae*
- Prepare or supervises preparation of samples for analysis or testing
- Maintain laboratory equipment performance by developing operation and troubleshooting procedures; as well as laboratory supply inventory by checking stock, placing and expediting orders for supplies

Jan. 2019 - July 2019 **Bristol-Myers Squibb, *Associate Scientist***, Syracuse, NY

- Proficient in sterile techniques including maintenance of Chinese hamster ovary (CHO) cell cultures that include vial thaw, passages and sampling
- Assist study lead on troubleshooting process related problems observed at manufacturing scale through the effective design of scale-down studies and the evaluation of data from manufacturing lots
- The operation, cleaning and maintenance of laboratory equipment, the preparation of media and regents with SOP
- Documentation of experimental data and results and data verification

Mar 2017 – Dec. 2018 **Biofilm Engineering Lab, PI: Dr. Dacheng Ren**, ***Graduate Student Researcher***, Syracuse University, College of Engineering, Department of Biomedical and Chemical Engineering

- Optimized a new robust assay to quantify the amount of antibiotic penetrated in gram negative bacteria cells including *Escherichia coli* and *Pseudomonas aeruginosa*
- Prepared buffers and medium as well as bacteria cultures. Proficient in a variety of laboratory equipment including fluorescence microscope imaging, counting chamber, spectrophotometer, centrifuges and plate reader.

Mar 2017 – Dec. 2018 **Flow Core and Cell Sorting Core Facility**, ***Operator***, Syracuse University

- Facilitated the facility by collaborating with researchers to obtain the best data possible on either the Accuri C6 Flow Cytometer or the Aria II Cell Sorter with daily maintenance.

- Managed in house cost of billing, record keeping, website maintenance and schedule arrangements

Sep. 2014 – May 2017 **Center for Reproductive Evolution, PI: Dr. Steven Doris**, ***Undergraduate Researcher***, Syracuse University, College of Arts and Sciences

- Employed RNAi and an array of sperm phenotype and function to assess the contribution of newly created genes to mitochondrial function during *Drosophila* spermatogenesis

PUBLICATION

- Gu, H., Lee, S., Carnicelli, J., **Jiang, Z.**, and Ren, D. **Antibiotic susceptibility of *Escherichia coli* cells during early-stage biofilm formation.** Journal of Bacteriology.