EFFECTS OF VARIOUS LEVELS OF ERYTHROMYCIN ON THE DEVELOPMENT OF ANTIBIOTIC RESISTANCE IN *ESCHERICHIA COLI*

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NATIONAL UNIVERSITY OF SINGAPORE

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Wang Tianren

07 November 2014

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TABLE OF CONTENTS

SUMMARY

Erythromycin is a macrolide antibiotic and has been widely used as human and veterinary medicine. This study evaluated microbial fitness under selective pressure of various concentrations of erythromycin and the development of erythromycin resistance genes in an *Escherichia coli* (*E. coli*) strain. Eight concentrations of erythromycin were applied to test microbial regrowth in an erythromycin resistant *E. coli* strain isolated from soil samples. The development of erythromycin resistance genes and genes expressions were evaluated with one conventional culture-based method, plate counting method (PCM), and two molecular microbiology techniques, fluorescence *in situ* hybridization (FISH) analysis and quantitative polymerase chain reaction (qPCR). PCM was used to test cultivable *E. coli* under selective pressure of erythromycin. FISH analysis was used to measure methylation of 23S rRNA resulted from gene expression of erythromycin resistance methylase (*erm*) genes by quantifying the total numbers of bacterial cells and MSL_B resistant cells in total microbial communities. qPCR was used to quantify 16S rRNA genes and erythromycin resistance genes. In addition, high-performance liquid chromatography (HPLC) and LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits were used to measure erythromycin persistence and bacterial viability, respectively. The results indicated that bacterial regrowth under exposure of erythromycin levels could be explained by bacterial fitness, and *E. coli* cells adapted to different erythromycin resistance levels under selective pressure in the presence of erythromycin. Furthermore, the concentration at one minimum inhibitory concentration (MIC) was most effective to select for antibiotic

resistance. The biological costs associated with fitness led to different regrowth rates for different concentrations and even viable but nonculturable (VBNC) cells during cultivation. In summary, the erythromycin resistant *E. coli* strain well adapted to the liquid cultures with selective pressure of erythromycin by acquiring and proliferating resistance genes.

NOMENCLATURE

ASTM	American Society for Testing and Materials
CFU	Colony forming units
$cMLS_B$	Constitutive MLS_B
DAPI	4’,6-diamidino-2-phenylindole
DD-water	Distilled deionized water
Ere	Erythromycin esterase
erm	Erythromycin ribosome methylase
E. coli	*Escherichia coli*
FCM	Fuzzy *c*-means
FISH	Fluorescence *in situ* Hybridization
HPLC	High-performance liquid chromatography
$iMLS_B$	Inducible MLS_B
LB	Luria-Bertani/Lysogeny Broth
mef	Macrolide efflux
MIC	Minimum inhibitory concentration
MLS_B	Macroilde-lincosamide-streptogramin B
mph	Macrolide phosphotransferase
msr	Macrolide-streptogramin resistant
PBS	Phosphate buffer solution
PCM	Plate counting method
qPCR	Quantitative polymerase chain reaction
PFA	Paraformaldehyde
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate

VBNC	Viable but nonculturable

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1 INTRODUCTION

When an environmental stress, such as antibiotic, is introduced into the culture, the bacterial growth curve may be delayed or inhibited, and bacterial cell density, viability, cultivability, and gene expressions could be affected. These changes are related with bacterial fitness to environmental stress, and could be associated with serious human health concerns if antibiotic resistant bacteria or antibiotic resistance genes are selected.

The selective pressure of various levels of erythromycin on the development of antibiotic resistance was evaluated in this study. Erythromycin and *E. coli* were used as a representative antibiotic and a representative microorganism, respectively. Erythromycin is a commonly used macrolide antibiotic in humans and animals, and could be transferred to the environment as they are usually poorly absorbed. Wastewater treatment plants (WWTPs) are usually not effective to removal trace levels of antibiotics, and therefore natural soils and surface waters could be substantial reservoirs for erythromycin, which may pose a selective pressure to select for erythromycin resistant microorganisms and lead to health concerns. Hence, erythromycin was selected as a model antibiotic in this study. *E. coli* is a gram-negative bacterium and widely exists in natural environments. It is one of most extensively studied microorganisms. Different types of erythromycin resistant genes have been detected, such as *erm* genes. In addition, *E. coli* is also intrinsically resistant to erythromycin because of its membrane permeability. Hence, an *E. coli* strain isolated from soil samples was used as a model microorganism in this study.

The result of this study can provide useful information on the effects of antibiotics on microbial cultivability, viability, and gene expression, as well as the potential health risks of trace levels of antibiotics in natural environments.

1.1 Objectives

The main purpose of this study was to evaluate selective pressure of erythromycin on the growth of an erythromycin resistant *E. coli* strain. In addition, the study was aimed to explore potential effects of bacterial fitness on bacterial viability, cultivability, and genes expression. The results could provide useful information for environmental risk assessment of antibiotics and antibiotic resistant bacteria in natural environments.

1.2 Scopes

The scope of this study was to isolate an erythromycin resistant *E. coli* strain from soil samples. Then, its MIC value to erythromycin was determined by broth macrodilution method. After that, HPLC-UV detection method was employed to test the persistence of erythromycin in *E. coli* suspensions. *E. coli*'s viability tests under different erythromycin concentrations were evaluated. Finally PCM, FISH, and qPCR were used to test *E. coli*'s cultivability and gene expression in liquid culture under eight levels of erythromycin concentration during a seven days' incubation.

CHAPTER 2 LITERATURE REVIEW

2.1 Health risks of antibiotics

Antibiotics are widely used in animal livestock production for therapeutic treatment of disease and at sub-therapeutic levels for growth promotion and improvement of feed efficiency (Chee-Sanford et al., 2009). It was reported that about 13,216 tons of antibiotics were used in European Union in 1999, and 65% of them was applied in human medicine (Kümmerer, 2009). In the U.S., a report estimated that US livestock producers would use approximately 11,200 metric tons of antimicrobials for non-therapeutic purposes primarily to promote the growth of livestock in 2001 (Kümmerer, 2009). In addition, antibiotics are used to control certain bacterial diseases of high-value fruits, vegetables, and ornamental plants (Kümmerer, 2009). But it is estimated that about 75% of antibiotics are not absorbed by human and animals and excreted in waste (Chee-Sanford et al., 2009). Thus, antibiotics may transfer and contaminate natural environments such as soils, surface water, and ground water, because of extensively usage of antibiotics and low absorption of antibiotics. These antibiotics could confer antibiotic resistance genes and promote antibiotic resistance levels for environmental microbial communities. Moreover, many microorganisms are antimicrobial producers in nature (Colomer-Lluch, Jofre, & Muniesa, 2011). These antibiotic-producing organisms are naturally resistant to the antibiotics they produce (Colomer-Lluch et al., 2011). In addition, both induced antibiotic resistant genes and naturally conferred antibiotic resistant genes can be transferred from resistant organisms

to non-resistant organisms through horizontal gene transfer. Consequentially, the increasing antibiotic resistance and resistance genes can increase the morbidity and mortality of bacterial infections and cost of treating infection diseases (Colomer-Lluch et al., 2011), finally pose an emerging threat to public and environmental health in the future (Knapp, Dolfing, Ehlert, & Graham, 2009). And the greater mobility of population and industrialization may exacerbate this threat (Colomer-Lluch et al., 2011).

2.2 Microbial growth

The growth curve of a bacterial culture is consisted of a succession of phases characterized by variable growth rates: lag phase, exponential phase/log phase, stationary phase and death phase (Monod, 1949). At lag phase, bacterial amount is stable, but their metabolic activity is high. At log phase, the growth rate of bacteria reaches optimal. At stationary phase, the cell growth and death achieve equilibrium. At death phase, the death rate is overwhelming the growth rate. Figure 1 illustrates a typical bacterial growth curve.

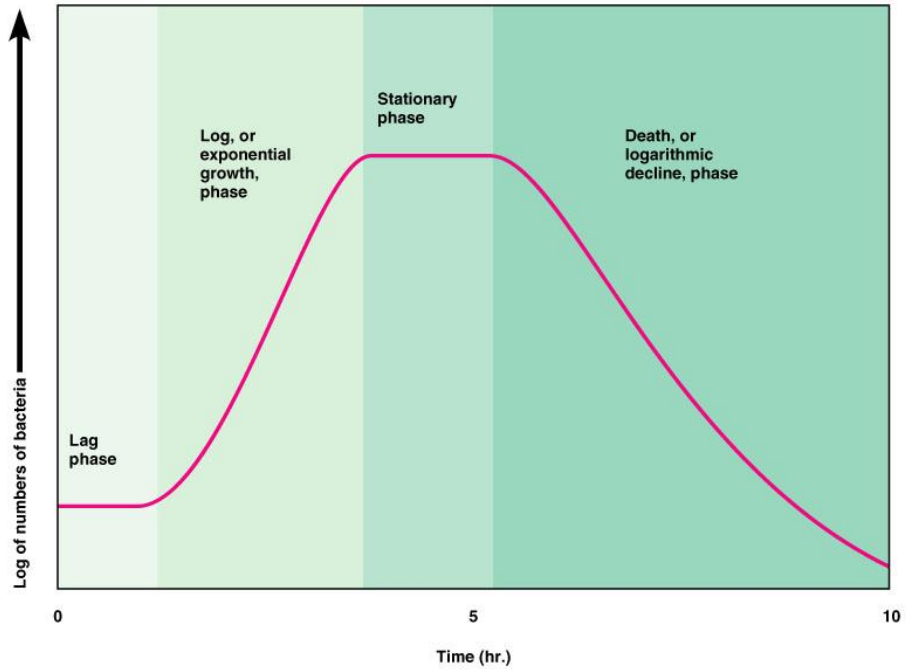


Figure 1: Bacterial growth curve (Bauman, 2004)

Various factors can affect microbial growth, such as availability of nutrients in liquid media, pH, temperature, salinity (Gibson, Bratchell, & Roberts, 1988; Nannipieri, Johnson, & Paul, 1978). Among them, one of the crucial factors is availability of nutrients in liquid media. It is because the nutrients provide the essential substrates and energy for bacterial growth. Other factors are can change bacterial growth rates and yields. Antibiotics can affect bacterial growth as well. They can inhibit bacterial growth or even kill them, and their inhibition/killing effects are associated with concentrations. In this study, the effects on antibiotic concentrations on microbial growth were studied. The change of microbial viability, cultivability, and gene expressions were monitored and their correlations were investigated as well.

2.3 Erythromycin

Erythromycin is the first discovered macrolide antibiotic and has been used since 1950s for the treatment of acute upper and lower respiratory tract and skin and soft tissue infections caused by gram-positive bacteria, especially in the "penicillin-allergic patient" (Marilyn C. Roberts, 2008; Zuckerman, 2004). It is effective for the treatment of infections caused by some intracellular pathogens, including species of *Legionella*, *Mycoplasma*, and *Chlamydia* (Zuckerman, 2004). Besides, it is applied in livestock and poultry production to promote animal's growth, improve feed efficiency, and prevent disease (Jessick, Moorman, & Coats, 2011).

Erythromycin is a crystalline, colorless compound which is slightly soluble in water but dissolves easily in most of the common organic solvent (Flynn, Sigal Jr, Wiley, & Gerzon, 1954). Erythromycin consists of a 14-member macrocyclic lactone ring attached to two sugar moieties: D-desosamine and L-cladinose (Omura, 2002). Figure 2 shows the structure of an erythromycin molecular. D-desosamine generates a basic character to erythromycin ($pK_a = 8.8$), and make it unstable under acidic condition (Kanfer, Skinner, & Walker, 1998).

Erythromycin has a moderate spectrum and is effective against gram-positive and some gram-negative bacteria (Marilyn C. Roberts, 2008). Erythromycin exerts its antibacterial effect by inhibiting RNA-dependent protein synthesis by reversibly binding to the 50S ribosomal subunit of susceptible microorganisms (Zuckerman, 2004). Its inhibition effect to bacterial growth is expressed by dissociation of peptidyl-tRNA from the ribosome during

the elongation phase (Omura, 2002). Figure 3 shows the inhibiting mechanism of erythromycin.

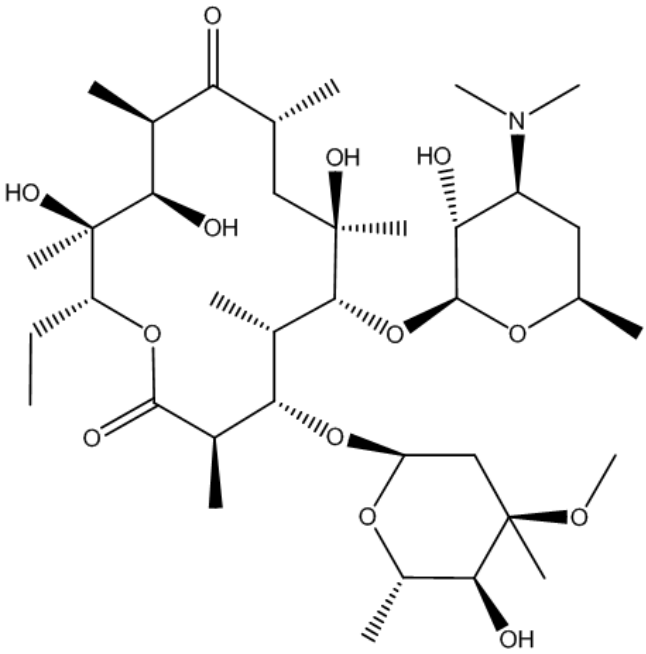


Figure 2: Structure of erythromycin

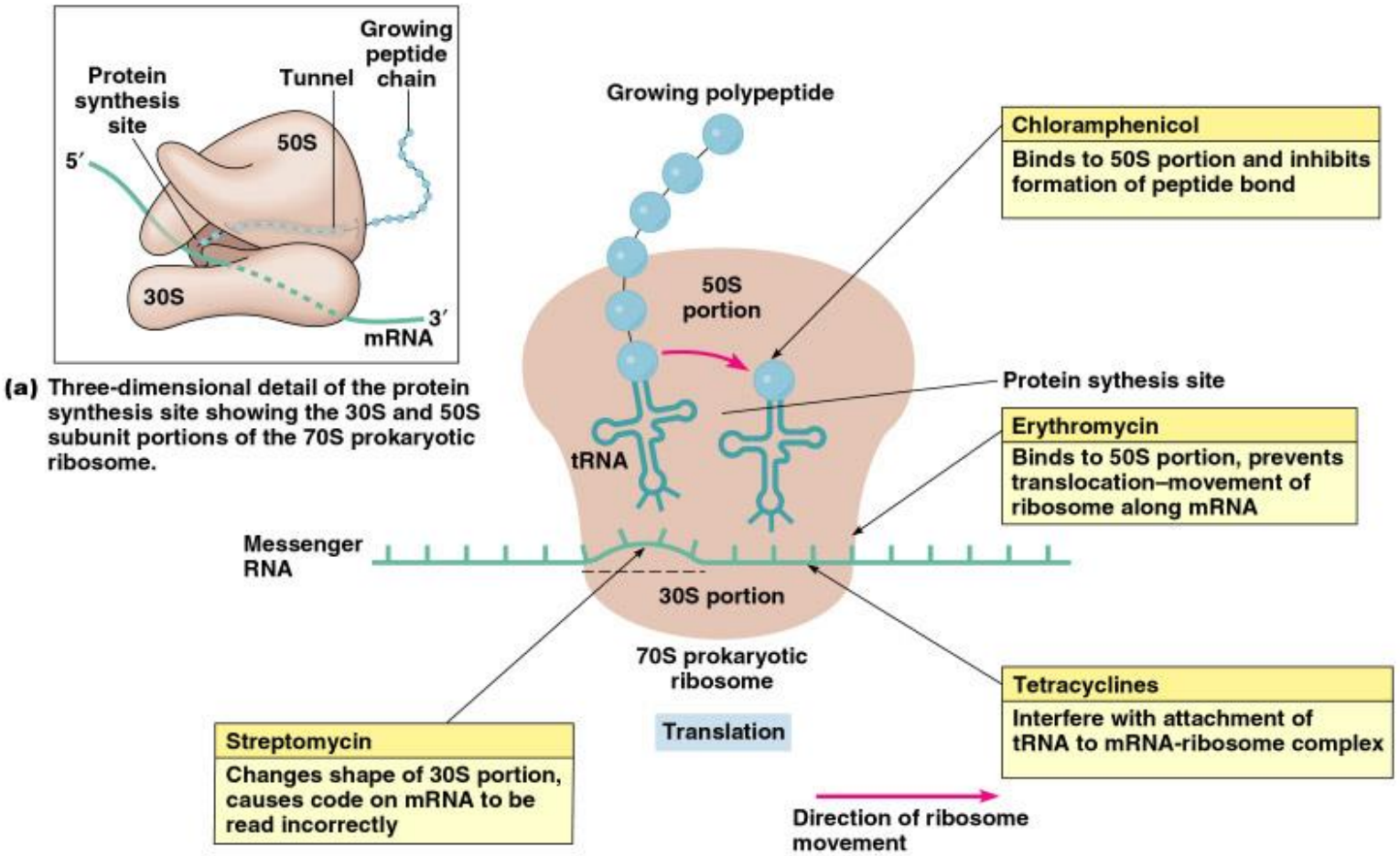


Figure 3: Inhibiting mechanism of erythromycin (Bauman, 2004)

2.4 Mechanisms of erythromycin resistance

Bacterial resistance to erythromycin can be active or passive. Active resistance is generated by a specific evolutionary pressure to adapt to a defensive mechanism against antibiotics, and passive resistance is led by "general adaptive processes that are not necessarily linked to a given class of antibiotics" (G. D. Wright, 2005), such as gram-negative bacteria are "intrinsically resistant to low levels of erythromycin" because of impermeability of the cellular outer membrane (Arthur, Andremont, & Courvalin, 1987; Leclercq & Courvalin, 1991). Bacteria can achieve active resistance to erythromycin by three widely recognized mechanisms: rRNA methylation, efflux-mediated resistance, and macrolide inactivation. Resistant genes, proteins, and genera resistant bacteria are listed in Table 1.

Table 1: Erythromycin resistant genes and bacteria (Marilyn C. Roberts, 2008; Marilyn C Roberts et al., 1999)

Class	Protein name	Gene name	Gene(s) included	Genus/genera of resistant bacteria
rRNA methylases				
A	Erm (A)	*erm* (A)	*erm* (A) & *erm* (TR)	*Aggregatibacter, Bacteriodes, Enterococcuse, Haemophilusr, Peptostreptococcusa,Prevotellaa, Staphylococcus, Streptococcus, Helcococcus*
B	Erm (B)	*erm* (B)	*erm* (AM), *erm* (B), *erm* (AMR), *erm* (BC), *erm* (P), *erm* (BP), *erm* (IP), *erm* (Z), *erm* (BZ1), *erm* (BZ2), *erm* , *erm* (2) & *erm* (80)	*Aggregatibacter, Acinetobacter, Aerococcus, Arcanobacterium, Bacillus, Bacteriodes, Citrobacter, Corynebacterium, Clostridium, Enterobacter, Escherichia, Eubacterium, Enterococcus, Fusobacterium, Gemella, Haemophilus, Klebsiella, Lactobacillus, Micrococcus, Neisseria, Pantoeae, Pediococcus, Peptostreptococcus, Porphyromonas, Proteus, Pseudomonas, Rothia, Ruminococcus, Serratia,*

Class	Protein name	Gene name	Gene(s) included	Genus/genera of resistant bacteria
				Staphylococcus, Streptococcus, UreaplasmaO, Wolinella, Treponema
C	Erm (C)	*erm* (C)	*erm* (C), *erm* (IM) & *erm* (M)	*Aeromonas,Aggregatibacter, Actinomyces, Arcanobacterium,Bacillus, Bacteriodes, Clostridium, Corynebacterium, Escherichia, Eubacterium, Enterococcus Haemophilus, Lactobacillus, Macrococcus, Micrococcus,Neisseria, Prevotella, Peptostreptococcus, Pseudomonas, Rhizobiu ,Staphylococcus, Streptococcus, Wolinella*
D	Erm (D)	*erm* (D)	*erm* (D), *erm* (J) & *erm* (K)	*Bacillus, Salmonella*
E	Erm (E)	*erm* (E)	*erm* (E) & *erm* (E2)	*Bacteroides, Eubacterium, Fusobacterium, Ruminococcus, Saccharopolyspora, Shigella, Streptomyces*

Class	Protein name	Gene name	Gene(s) included	Genus/genera of resistant bacteria
F	Erm (F)	*erm* (F)	*erm* (F), *erm* (FS) & *erm* (FU)	*Aggregatibacter, Actinomyces, Bacteroides, Capnocytophaga,Clostridium, Corynebacterium,Eubacterium, Enterococcus, Fusobacterium, Gardnerella, Haemophilus,Lactobacillus, Mobiluncus, Neisseria, Porphyromonas, Prevotella,Peptostreptococcus, Ruminococcus, Shigella, Selenomonas, Staphylococcus, Streptococcus,Treponema, Veillonella, Wolinella*
G	Erm (G)	*erm* (G)	*erm* (G)	*Bacillus, Bacteroides, Catenibacterium, Lactobacillus, Prevotella, Porphyromonas, Staphylococcus*
H	Erm (H)	*erm* (H)	*car* (B)	*Streptomyces*
I	Erm (I)	*erm* (I)	*mdm* (A)	*Streptomyces*
N	Erm (N)	*erm* (N)	*tlr* (D)	*Streptomyces*
O	Erm (O)	*erm* (O)	*lrm* & *srm* (A)	*Streptomyces*

Class	Protein name	Gene name	Gene(s) included	Genus/genera of resistant bacteria
Q	Erm (Q)	*erm* (Q)	*erm* (Q)	*Aggregatibacter, Bacteroides, Clostridium, Staphylococcus, Streptococcus, Wolinella*
R	Erm (R)	*erm* (R)	*erm* (R)	*Arthrobacter, Aeromicrobium*
S	Erm (S)	*erm* (S)	*erm* (SF) & *tlr* (A)	*Streptomyces*
T	Erm (T)	*erm* (T)	*erm* (GT)	*Enterococcus, Lactobacillus, Streptococcus, Staphylococcus*
U	Erm (U)	*erm* (U)	*lmr* (B)	*Streptomyces*
V	Erm (V)	*erm* (V)	*erm* (SV)	*Brevundimonas, Chryseomonas, Eubacterium, Fusobacterium, Leifsonia, Mesorhizobium, Paenibacillus, Pseudomonas, Rhizobiu, Shewanella, Streptomyces*
W	Erm (W)	*erm* (W)	*myr* (B)	*Micromonospora*
X	Erm (X)	*erm* (X)	*erm* (CD), *erm* (A), *erm* (Y)	*Acintobaculum, Arcanobacterium, Bifidobacterium, Burkholderia, Brevundimonas, Corynebacterium, Leifsonia, Paenibacillus, Propionibacterium, Pseudomonas,*

Class	Protein name	Gene name	Gene(s) included	Genus/genera of resistant bacteria
				Rhizobium, Shewanella, Sphingomonas, Stenotrophomans, Streptomyces
Y	Erm (Y)	*erm* (Y)	*erm* (GM)	*Staphylococcus*
Z	Erm (Z)	*erm* (Z)	*srm* (D)	*Streptomyces*
30	Erm (30)	*erm* (30)	*pikR1*	*Streptomyces*
31	Erm (31)	*erm* (31)	*pikR2*	*Streptomyces*
32	*Erm* (32)	*erm* (32)	*tlr* (B)	*Streptomyces*
33	Erm (33)	*erm* (33)		*Staphylococcus*
34	Erm (34)	*erm* (34)		*Bacillus*
35	Erm (35)	*erm* (35)		*Bacteriodes*
36	Erm (36)	*erm* (36)	*erm* (MT)	*Micrococcus*
37	Erm (37)	*erm* (37)		*Mycobacterium*
38	Erm (38)	*erm* (38)		*Mycobacterium*
39	Erm (39)	*erm* (39)		*Mycobacterium*
40	Erm (40)	*erm (*40)		*Mycobacterium*
41	Erm (41)	*erm* (41)		*Mycobacterium*
42	Erm (42)	*erm* (42)	*erm* (MI)	*Mannheimia, Pasteurella, Photobacterium*
43	Erm (43)	*erm* (43)		*Staphylococcus*

Class	Protein name	Gene name	Gene(s) included	Genus/genera of resistant bacteria
ATP-binding Transporters				
	Msr (A)	*msr* (A)	*msr* (A), *msr* (SA), *msr* (B)	*Brevundimonas, Burkholderia, Chryseomonas Corynebacterium, Enterobacter, Enterococcus, Gemella, Lysinibacillus, Photobacterium, Pseudomonas, Shewanella, Staphylococcus, Streptococcus, Streptomyces, Ureaplasma*
	Msr (C)	*msr* (C)	*msr* (C)	*Enterococcus*
	Msr (D)	*msr* (D)	*mel*	*Acinetobacter, Bacteroides , Citrobacter, Clostridium, Corynebacterium, Enterococcus, Enterobacter, Escherichia, Gemella, Fusobacterium, Klebsiella, Morganella, Neisseria, Proteus, Providencia, Pseudomonas, Ralstonia, Staphylococcus, Streptococcus, Serratia, Stenotrophomonas, Ureaplasma*
	Msr (E)	*msr* (E)	*mel*	*Acinetobacter, Citrobacter, Escherichia, Klebsiella, Pasteurella, Serratia*

Class	Protein name	Gene name	Gene(s) included	Genus/genera of resistant bacteria
Major Facilitators				
	Mef *(*A)	*mef* (A)	*mef* (A), *mef* (E)	*Acinetobacter, Bacteroides, Citrobacter, Clostridiuma, Corynebacterium, Enterococcus, Enterobacter, Escherichia, Fusobacterium, Gemella, Haemophilusr Klebsiella, Lactobacillus, Micrococcus, Morganella, Neisseria, Pantoeae, Pediococcus, Providencia, Proteus, Ralstonia, Rothia, Pseudomonas, Salmonella, Serratia, Staphylococcus, Streptococcus, Stenotrophomonas, Ureaplasma, Weissella*
	Mef *(*B)	*mef* (B)	*mef* (B)	*Escherichia*
Esterases				
	Ere *(*A)	*ere* (A)	*ere* (A), *ere* (A_2), *ere* (C)	*Achromobacter, Aermonas, Citrobacter, Enterobacter, Escherichia, Klebsiella, Laribacter, Pantoeae, Providencia, Pseudomonas, Salmonella, Serratia, Staphylococcus, Stenotrophomonas, Vibrio*

Class	Protein name	Gene name	Gene(s) included	Genus/genera of resistant bacteria
	Ere *(*B)	*ere* (B)	*ere* (B)	*Acinetobacter, Citrobacter, Enterobacter, Escherichia, Klebsiella, Proteus, Pseudomonas, Staphylococcus*
Phosphorylases				
	Mph *(*A)	*mph* (A)	*mph* (A), *mph (*K*)*	*Aeromonas, Escherichia, Citrobacter, Enterobacter, Klebsiella, Pantoeae, Pseudomonas, Proteus, Serratia, Shigella, Stenotrophomonas*
	Mph *(*B)	*mph* (B)	*mph* (B)	*Escherichia, Enterobacter, Pseudomonas, Proteus*
	Mph *(*C)	*mph* (C)	*mph* (BM), *mph (*C*)*	*Staphylococcus, Stenotrophomonas*
	Mph *(*D)	*mph* (D)	*mph* (D)	*Escherichia, Klebsiella, Pantoeae, Proteus, Pseudomonas, Stenotrophomonas*
	Mph *(*E)	*mph* (E)	*mph, mph1, mph2*	*Acinetobacter, Citrobacter, Escherichia, Klebsiella, Pasteurella, Serratia*
	Mph *(*F)	*mph* (F)	*mph* (F)	*Pseudomonas*

2.4.1 rRNA methylation

rRNA methylation is the most widespread mechanism of macrolide resistance. It involves the adenine-N^6 methyltransferase to cause posttranscriptional modification of 23S rRNA by adding one or two methyl groups to a single adenine (A2058) in the 23S rRNA moiety or one of the adjacent residues in the peptidyl transferase region (A2057 or A2059) (Marilyn C. Roberts, 2008; Marilyn C Roberts et al., 1999). Through this modification, these enzymes prevent binding of antibiotics to its ribosomal target and confer resistance to erythromycin (Aktas, Aridogan, Kayacan, & Aydin, 2007). The genes encoding these methylases are named as *erm* (erythromycin ribosome methylation) genes (Marilyn C Roberts et al., 1999). These genes can not only generate resistance to macrolides, and lincosamides, but lead to cross-resistance between macrolides, lincosamides, and streptogramin B, which are well known as MLS_B phenotype (Aktas et al., 2007).

From 1970s to the date, a great amount of *erm* genes have been detected and isolated from a variety of bacteria in both gram-negative and gram-positive species (Marilyn C Roberts et al., 1999). A previous study has summarized more than 30 different types of *erm* genes (Marilyn C. Roberts, 2008). The differences between different *erm* genes are related to the regulation of their phenotype expressions, which is inducible or constitutive (Marilyn C Roberts et al., 1999). In inducible phenotype, mRNA is active to encode methylase only after exposure to a macrolide inducer (Aktas et al., 2007), erythromycin is a good inducer in most species (Marilyn C Roberts et al., 1999). Inducible *erm* genes normally are controlled by translational attenuation of the mRNA leader

sequence or rho factor-independent termination (Marilyn C Roberts et al., 1999). The strains with inducible *erm* genes are resistant to inducers and remain susceptible to non-inducer MLS_B antibiotics (Aktas et al., 2007). In constitutive phenotype, the mRNA is active even in the absence of inducers and generating the high level cross-resistance to MLS_B antibiotics (Leclercq, 2002). Constitutive *erm* genes are regulated by structural alterations in the *erm* translational attenuator, such as deletions, duplications, and point mutations (Marilyn C Roberts et al., 1999).

2.4.2 Efflux-mediated resistance

Efflux proteins are associated with low-level resistance to MLS_B antibiotics (only 14- and 15-membered macrolides) (Varaldo, Montanari, & Giovanetti, 2009) by pumping out the antibiotics of "the cell or cellular membrane, keeping intracellular concentrations low and ribosome free from antibiotics" (Marilyn C Roberts et al., 1999). Currently, there are 14 different genes coded for efflux proteins (Marilyn C. Roberts, 2008). Active efflux proteins are encoded by *mef*-class genes, and *msr*-class genes encoded ABC transporter superfamily (Marilyn C Roberts et al., 1999; Varaldo et al., 2009).

2.4.3 Macrolide inactivation

The mechanism for macrolide inactivation is via "the synthesis of modifying enzymes that selectively target and destroy the activity" of macrolide antibiotics (G. D. Wright, 2005). There are three types of enzymes responsible

for macrolide inactivation (esterase, phosphotransferase, and glycosyltransferases) (G. D. Wright, 2005) among a total of 6 inactivation enzymes (Marilyn C. Roberts, 2008).

Macrolides are cyclized via an ester bond, which can be targeted by macrolideesterase (G. D. Wright, 2005). These estereases may "cleave the macrocycle ester, which is followed by non-enzymatic intramolecular hemiketal formation, followed by a second internal cyclization event via intramolecular condensation, followed by dehydration" and destroy the activity of macrolides (G. D. Wright, 2005). Esterases are encoded by *ere* (erythromycin esterification) genes, including *ere* (A) and *ere* (B) (Marilyn C Roberts et al., 1999). These *ere* genes first detected from *E. coli* isolates, and they will lead to very high levels of resistance to erythromycin (MIC ≥ 1600 μg/ml) (G. D. Wright, 2005). Additionally *ere* genes are located on mobile genetic elements, which make them have the potential to be widespread in microbial communities (G. D. Wright, 2005). A previous study has confirmed their potential, i.e., ere genes were identified in seven Gram-positive and five new Gram-negative genera (Marilyn C. Roberts, 2008)

Phosphotransferases (MPHs) can block macrolides by phosphorylation to the free hydroxyl of the desosamine sugar of macrolides that react with the 23S rRNA and generate resistance (G. D. Wright, 2005). These enzymes are encoded by *mph* (macrolide phosphotransferase) genes, and they were first detected from *E.coli* isolated as well (Marilyn C Roberts et al., 1999) and resulted in very high MIC values (> 2000 mg/ml) for macrolides (G. D. Wright, 2005).

Glycosyltransferases is associated with self-protection in antibiotic-producing organisms and can catalyze glucosylation at the desosmine sugar of macrolides to confer resistance(G. D. Wright, 2005).

2.5 *Escherichia coli*

Escherichia coli (*E. coli*) is a gram-negative, facultative anaerobic, and rod-shaped bacterium. It is one of the most frequently used indicator organisms for fecal pollution in environmental waters (Anderson, Whitlock, & Harwood, 2006). *E. coli* abundantly exists in the intestine of humans and warm-blooded animals (Donnenberg, 2002). Through deposition of fecal material, it can be released to environments, such as surface waters, sediments, and soils (Ibekwe, Murinda, & Graves, 2011).

E.coli has diverse subtypes and varied genotypic and phenotypic characteristics. Some of them are pathogenic, and cause diseases such as diarrhea, dysentery to the hosts (Donnenberg, 2002). But most of the strains are nonpathogenic, "coexisting in harmony with their hosts" (Donnenberg, 2002). *E.coli* is crucial not only in natural environments, but also in the fields of laboratory studies because of its fast growth rate and low cost of culturing. It is one of most studied prokaryotic model organisms in microbiology. For example, many of antibiotic resistant genes were identified and sequenced from *E.coli* isolates, such as *ere* A and *ere* B.

In summary, excessive usage of antibiotics could lead to a risk to human health and environment. Antibiotics may promote the development of antibiotic resistance genes in natural microbial communities and resistant genes could

transfer from non-pathogens to pathogens. However, knowledge on the development of antibiotic resistance under selective pressure of antibiotics is still limited. This study aimed to evaluate the development of antibiotic resistance during microbial growth of an *E.coli* strain under selective pressure of various erythromycin concentrations.

Chapter 3 ISOLATION OF ERYTHROMYCIN RESISTANT *ESCHERIA COLI*

3.1 Introduction

As mentioned previously, *E. coli* is abundant in intestinal systems of humans and warm-blooded animals, and widely exists in soil environments and plays an important role in soil. MacConkey medium is commonly used to select or culture *E. coli* isolates from soils. MacConkey medium is used for isolation of gram-negative enteric bacteria and differentiation of lactose fermenting from lactose fermenting from lactose non-fermenting gram-negative bacteria, particularly members of the family *Enterobacteriaceae* and the genus *Pseudomonas*. Crystal violet and bile salts in the medium inhibit the growth of gram-positive enteric bacteria. And gram-negative bacteria growing on the medium are differentiated by their ability to ferment lactose. Bacteria that ferment lactose are shown in pink or red colonies on MacConkey agar. Conversely, bacteria that do not ferment lactose are colorless. *E. coli* shows in pink or red color in the MacConkey agar plate between 37 to 42 ^{o}C, which other gram-negative bacteria species that ferment lactose cannot grow. Hence, MacConkey medium is a good option for selection of erythromycin resistant *E. coli* isolates from natural soils. Previously 10 μg/ml erythromycin was used in MacConkey agar plates to select erythromycin resistant *E. coli* strains.

LB medium stands for “Lubria broth”, “Lennox broth”, “Luria-Bertani”, or “Lysogeny Broth” medium, and was invented by Giuseppe Bertani (Bertani, 2004). It is widely used to support growth for many species because its rich

nutrient can support bacterial fast growth and good growth yields (Sezonov, Joseleau-Petit, & D'Ari, 2007). LB medium is commonly used to support *E. coli* growth to an optical density at 600nm (OD_{600}) of 7 under 37°C, and the doubling time of *E. coli* is roughly 20 minutes in LB broth (Sezonov et al., 2007). The ingredients of LB broth are tryptone (10g/l), yeast extract (5g/l) and sodium chloride (10g/l), and the carbon sources for the growth of *E. coli* in LB broth are catabolizable amino acids, instead of sugars. Hence, LB medium is a good choice for enumeration of the selected *E. coli* isolates and for the growth experiment.

Minimum inhibitory concentration (MIC) is defined as "the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation", and it is a "gold standard" for determining the susceptibility of organisms to antimicrobials (Andrews, 2001). There are many methods to determine MICs, such as agar dilution method, broth macrodilution method, and broth microdilution method. Considered the requirements of growth experiment, the broth macrodilution method is an appropriate option to determine MIC for the resistant *E. coli* isolate. MICs of *E. coli* to erythromycin have a wide range depends on different strains and resistant genes, normally their values are less than 500 μg/ml, but some highly resistant strains' MICs are higher than 2000 μg/ml to erythromycin (Andremont, Gerbaud, & Courvalin, 1986).

3.2 Materials and methods

3.2.1 Incubation of erythromycin resistant *Escherichia coli* colonies from soil samples

Soil samples were collected from 20 Toh Guan Road in December, 2012. 10 grams of soil were weighed and homogenously mixed with 95ml 1 × PBS buffer (0.137M NaCl (Merck, Germany), 2.7 mM KCl (Applichem, USA), 10 mM $Na_2HPO_4 \cdot 2H_2O$ (Merck, Germany), 2 mM KH_2PO_4 (Applichem, USA), pH 7.4) to prepare 10^{-1} soil suspension. Then, 10^{-1} soil suspension was diluted 100 times in LB broth (tryptone 10g/L (Fluka, USA), yeast extract 5g/L (Sigma-Adrich, USA), sodium chloride 10g/L, pH 7.2) to 10^{-3} soil suspension. 100µl of diluted soil suspension was evenly spread onto the MacConkey (Fluka, USA) agar plate with 10µg/ml erythromycin (Sigma, USA) and incubated at 37ºC for 20 hours. One red colony was randomly selected from the plate to the target erythromycin resistant *E. coli* strain.

3.2.2 Isolation and enumeration of pure cultured *E. coli* strain

In order to get pure culture strain, the target erythromycin resistant *E. coli* colony was streaked twice on fresh MacConkey plates with 10µg/ml erythromycin, and incubated for 20 hours. One isolated red colony was selected from the streaked plate and transferred to LB broth to prepare pure culture of erythromycin resistant *E. coli,* and streaked to another erythromycin

MacConkey plate to store its biomass. The liquid culture was mixed well and incubated at 37ºC overnight.

3.2.3 Macrodilution method to test MIC

The first step was to prepare antibiotic stock solution. The target antibiotic in this study was erythromycin. The concentration of erythromycin stock solution was 10,000 μg/ml. It was prepared by erythromycin powder and absolute ethanol (Sigma-Aldrich, USA) using the following formula:

$$1000/P \times V \times C = W$$

where P is potency given by the manufacturer (μg/mg), for erythromycin was 850 μg/mg in this study;

V is the volume of stock required (ml), like 1ml;

C is the final concentration of stock solution (10000μg/ml);

W is the weight of antibiotic to be solved in ethanol, like 11.76 mg.

The second step was to prepare microbial inoculum. 100 μl of original *E. coli* culture was transferred to 10 ml fresh LB broth and incubated overnight at 37ºC with a shaking speed of 150 rpm. In the next morning, 100 μl of overnight liquid culture was transferred to fresh LB broth and incubated about 2 hours for sub-culture to maintain *E. coli* cells at log phase.

The third step was to prepare MIC test suspensions. These suspensions were mixtures of *E. coli* inoculum and different volumes of erythromycin stock

solution. They were used to test MIC of isolated *E. coli* strain to erythromycin. The target inoculum size was 10^6cells/ml in this experiment. OD_{600} was used to measure *E. coli* cell density, at the wavelength of 600 nm using a spectrophotometer. Normally, OD_{600} value of one means that there are roughly 10^9 cells in each ml liquid culture, the OD values are linearly correlated with culture density. OD_{600} is more accurate in the range of 0.5 to 0.1, which can be converted to cell densities of 5×10^8 cells/ml to 10^8cells/ml. Hence, in this experiment, the *E. coli* sub-culture was measured by spectrophotometer and diluted by fresh LB broth until its OD_{600} reached 0.1 to 0.5, and its OD_{600} was recorded as 0.171, which was corresponding to 1.7×10^8 cells/ml. The dilution factor for this suspension from 1.7×10^8 cells/ml to the target inoculum 10^6 cells/ml was 170. Eight erythromycin concentrations were diluted from erythromycin stock solution and used for MIC testing: 0, 20, 40, 60, 80, 100, 120, and 150 μg/ml. The final inoculum volume for MIC test was 8 ml, the volumes distribution of erythromycin stock solution, LB broth, and *E. coli* suspension (1.7×10^8 cells/ml) were shown in Table 2.

Table 2: Scheme for preparation MIC broth macrodilution test

Label	Erythromycin concentration (µg/ml)	Volume of erythromycin stock solution (µl)	Volume of *E. coli* suspension (µl)	Volume of LB broth (ml)	Final volume (ml)	Cell density (cells/ml)
Control	0	0	47	7.953	8	10^6
1	20	16	47	7.937	8	10^6
2	40	32	47	7.921	8	10^6
3	60	48	47	7.905	8	10^6
4	80	64	47	7.889	8	10^6
5	100	80	47	7.873	8	10^6
6	120	96	47	7.857	8	10^6
7	150	120	47	7.833	8	10^6

The final step was to record the OD_{600} values of the prepared test suspensions before incubation. Then these suspensions were incubated for 24 hours under 37^{o}C with a shaking speed of 150 rpm. After incubation, OD_{600} values of the incubated suspensions were recorded and compared with the original OD_{600} to find the MIC value.

3.3 Results and discussion

The comparison between OD_{600} values of before incubation and after incubation was shown in Figure 4.

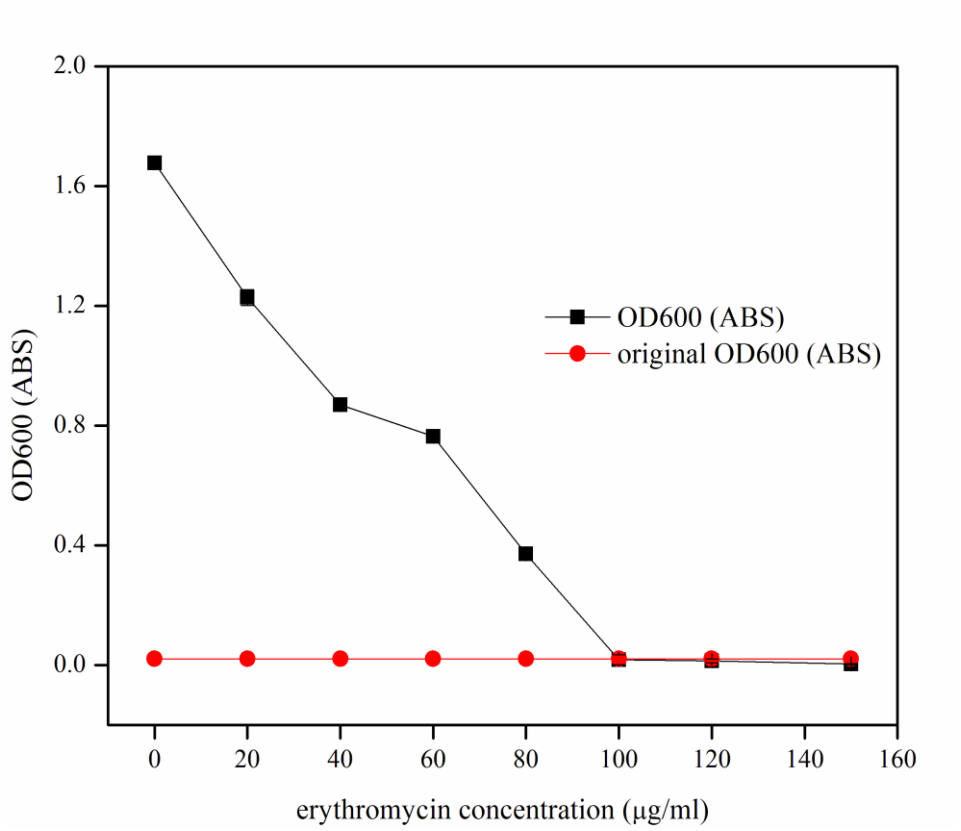


Figure 4: Broth macrodilution method to determine MIC for *Escherichia coli*

The results in Figure 4 showed that OD_{600} values were similar to the original OD_{600} values at 100 µg/ml of erythromycin with an inoculum size of 10^6 ells/ml after 24 hrs of incubation. Thus, the resistant *E. coli* isolate's MIC value through broth macrodilution was identified as 100 µg/ml.

CHAPTER 4 PERSISTENCE OF ERYTHROMYCIN

4.1 Introduction

In this study, two possible factors may affect *E. coli's* growth: one is the persistence of erythromycin, and the other one is the erythromycin resistance. The persistence of erythromycin can be regarded as "removal of inhibitor" to *E. coli* culture through the reduction of erythromycin concentration. The acquisition and proliferation of erythromycin resistant genes can be regarded as "self-defence" of *E. coli* to overcome the inhibition effect of erythromycin. Many factors may affect the persistence of erythromycin, such as pH. Erythromycin is extremely acid sensitive, and converted rapidly under aqueous acid conditions to inactive metabolites destroying the antibiotic activity (Hassanzadeh, Barber, Morris, & Gorry, 2007). Under alkaline conditions, erythromycin can be degraded to pseudoerythromycin A enol ether through base-catalyzed hydrolysis and dehydration reactions (Kim, Heinze, Beger, Pothuluri, & Cerniglia, 2004).

Different techniques for erythromycin determinations have been utilized, such as LC-MS and HPLC-UV, HPLC-MS and so on. In this study, reverse phase HPLC with UV detector was used to measure the persistence of erythromycin. HPLC is a chromatographic technique to separate a mixture of compounds and quantify individual compounds of the mixture. It is widely used to detect erythromycin concentration and persistence for biological matrices (Gebeyehu, 2012), soil and water matrices (Jessick et al., 2011). In HPLC-UV detection, the extensively applied UV wavelength ranges from 210 nm to 215

nm, they are the optimal wavelengths to monitor erythromycin and its related compounds because of its low molar absorptivity (Gebeyehu, 2012).

This chapter aimed at testing erythromycin persistence through HPLC. The following chapters illustrated the results of erythromycin resistance on *E. coli*'s growth through both culture-based method and molecular method.

4.2 Materials and methods

4.2.1 Mobile Phases

The HPLC consisted of two mobile phases: mobile phase A with organic solvent, Acetonitrile (HPLC grade, Fisher, USA), and mobile phase B with inorganic solvent, 10 mM ammonium formate (HPLC grade, Sigma-Aldrich, USA). All the solvents were filtered through PTFE filter (0.2 µm pore-size, SMI-LabHub Incorp., UK) before operation. The mixing ratio for these two mobile phases was 50%-50%.

4.2.2 Calibration samples

Erythromycin stock solution (10,000 µg/ml) and tylosin (Sigma-Aldrich, USA) stock solution (1000 µg/ml) were prepared followed the procedures mentioned in Chapter 3. But the solvent for tylosin was distilled deionized water (DD-water), instead of ethanol. Erythromycin stock solution was dissolved by filtered DD-water to eight concentration levels: 0 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml, and 800 µg/ml.

These erythromycin solutions were used to measure the calibration curve of erythromycin, the target antibiotic. Tylosin stock solution was dissolved by DD-water to six concentration levels: 0 μg/ml, 21 μg/ml, 35 μg/ml, 49 μg/ml, 63 μg/ml, and 70 μg/ml. These tylosin solutions were used to measure the calibration curve of tylosin, which was used as an internal standard in HPLC test. To avoid potential sample degradation, the calibration samples were freshly prepared before measurements.

4.2.3 HPLC

Separations were performed using a reverse column, Poroshell 120 EC-C_{18} (100 × 2.1 mm, particle size 2.7 μm; Agilent, USA) with the Poroshell 120 EC-C_{18} guard column (particle size 4.6mml; Agilent, USA) at 30°C. The key feature of this column is its superficially porous microparticulate column packing. This design can make a good performance with high efficiency and high resolution. The flow rate was 0.7 ml per minute and running time was 5 minutes. The wavelength was 210 nm and the injection volume was 2 μl. The HPLC system was Agilent 1260 Infinity Binary LC system (Agilent, USA).

4.2.4 Sample collection and preparation

The first step was to prepare eight *E. coli* suspensions (10^6 cells/ml) under various erythromycin concentrations: 0 MIC (0 μg/ml), 0.125 MIC (12.5 μg/ml), 0.25MIC (25 μg/ml), 0.5 MIC (50 μg/ml), 1 MIC (100 μg/ml), 2 MIC (200 μg/ml), 4 MIC (400 μg/ml), and 8 MIC (800 μg/ml). The volume for each

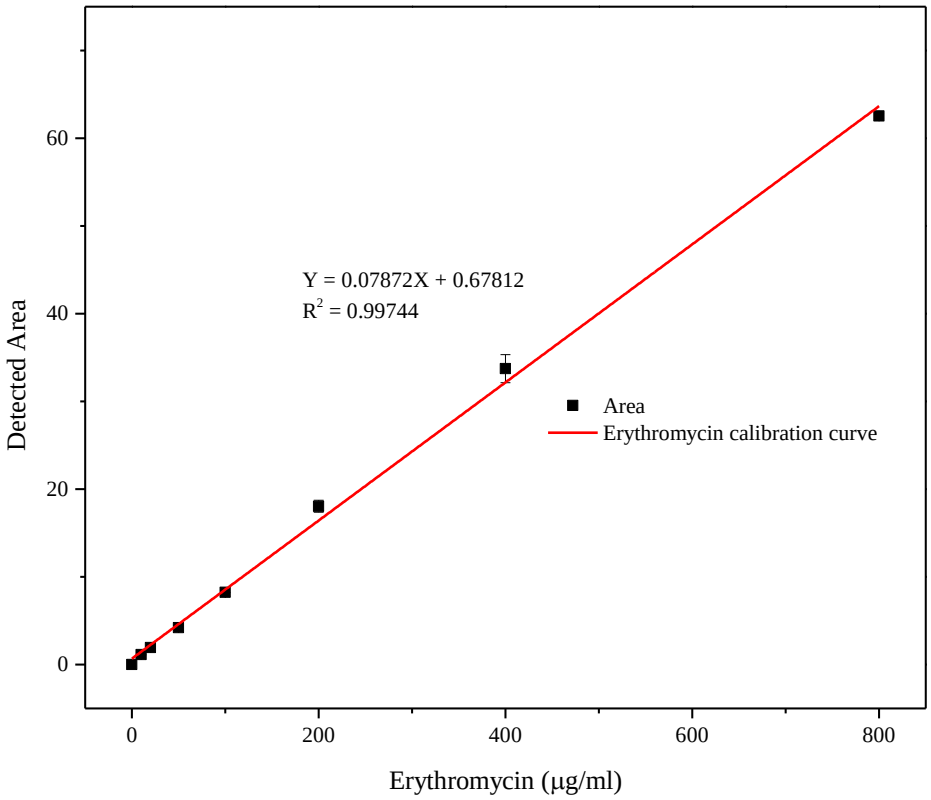
suspension was 16 ml in with LB broth. These *E. coli* suspensions were incubated at 37^{o}C with shaking for 7 days. 1ml sample was collected from each *E. coli* suspension in every incubation day. The collected samples were used for HPLC analysis.

The second step was to prepare samples for HPLC analysis. Since the collected samples were relatively clean, the procedures for sample preparation were relatively simple. The major task for sample preparation was to remove the cells from samples. Internal standard, tylosin stock solution, was added to the collected samples with a tylosin concentration of 70 μg/ml, and the samples were mixed homogeneously. Then they were centrifuged for 5 minutes at 13,000 rpm. 0.5ml supernatant was filtered in a 0.2 μm PTFE filter and transferred to a 2 ml amber vial (Agilent, USA).

4.3 Results and discussion

4.3.1 The calibration curve of erythromycin and tylosin

The detection time was 1.9 minutes and 2.34 minutes for erythromycin and tylosin, respectively. The calibration curves of erythromycin and tylosin and correlation coefficient (R^2) between detected area and chemical concentrations were listed in Figure 5. Both correlation coefficients were above 0.99, suggesting the HPLC system can accurately measure the concentrations of erythromycin and tylosin.



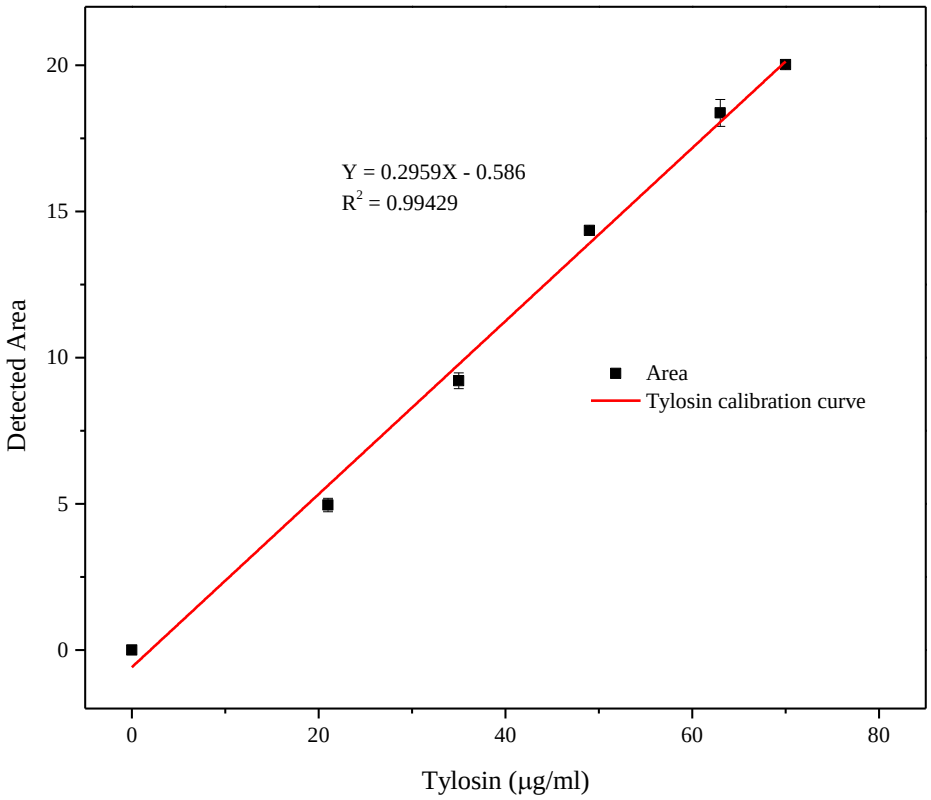


Figure 5: Calibration curves of erythromycin and tylosin

4.3.2 Results for erythromycin persistence

Figure 6 showed the results from HPLC detection for various erythromycin concentrations within 7 days' incubation. The black solid lines stand for detected erythromycin concentrations in control experiments which no microbe was cultivated. The red solid lines stand for detected erythromycin concentrations of the samples with cultivation of *E.coli*. The green dash lines stand for erythromycin concentrations which were aimed to be prepared. The concentration differences between control experiments and samples were due to impact of microbes.

Under 0.125MIC, the concentrations of control experiments and samples were fluctuated around 12.5 μg/ml. The erythromycin concentrations of control experiment were changed within 1.1 μg/ml (9.32%) compared to their initial erythromycin concentrations, which were not significant. And the concentrations of samples were fluctuated within 1.29 μg/ml (10.93%) during incubation period, which were not significant. Under 0.25MIC, the concentrations of control experiments and samples were slightly lower than 25 μg/ml. The erythromycin concentrations of control experiment were varied within 2.23 μg/ml (8.92%) compared to their initial value, which were not significant. And the concentrations of samples were varied within 1.47 μg/ml (5.88%) during incubation period, which were not significant. Under 0.5MIC, the concentrations of control experiments and samples were detected around 50 μg/ml. The concentrations of control experiments were slightly larger than the samples'. The erythromycin concentrations of control experiments were changed within 3.26 μg/ml (6.52%) compared to their initial value, which was

not significant. And the concentrations of samples were varied within 4.12 μg/ml (8.24%) during incubation period, which was not significant. Under 1MIC, the concentrations of control experiments and samples were detected slightly lower than 100 μg/ml. The concentrations of control experiments were slightly larger. The erythromycin concentrations of control experiments were changed within 11.93 μg/ml (11.93%) compared to their initial value. And the concentrations of samples were varied within 7.84 μg/ml (7.84%) during incubation period, which was not significant. Under 2MIC, the concentrations of control experiments and samples were detected slightly lower than 200 μg/ml. The concentrations of control experiments were slightly larger than the ones of samples. The erythromycin concentrations of control experiments were changed within 13.37 μg/ml (6.69%) compared to their initial value, and the concentrations of samples were varied within 22.92 μg/ml (11.46%) during incubation period, which were not significant. Under 4MIC, the concentrations of control experiments and samples were detected around 400 μg/ml. The concentrations of control experiments were slightly larger than the ones of samples. The erythromycin concentrations of control experiments were changed within 66.43 μg/ml (16.6%) compared to their initial value; and the concentrations of samples were fluctuated within 37.3 μg/ml (9.33%) during incubation period, which were not significant. Under 8MIC, the concentrations of control experiments and samples were detected slightly lower than 800 μg/ml. The concentrations of control experiments were slightly larger than the ones of samples. The erythromycin concentrations of control experiments were changed within 72.2 μg/ml (9%) during incubation; and the concentrations of

samples were fluctuated within 100.63 μg/ml (12.58%) during incubation period, which were not significant.

In conclusion, erythromycin was persistent within 7 days' incubation. And existence or growth of microbes may have a slight impact on erythromycin degradation. The impact was positively correlated with erythromycin concentrations, larger the concentration, stronger the impact. However, the impact of microbes was not significant.

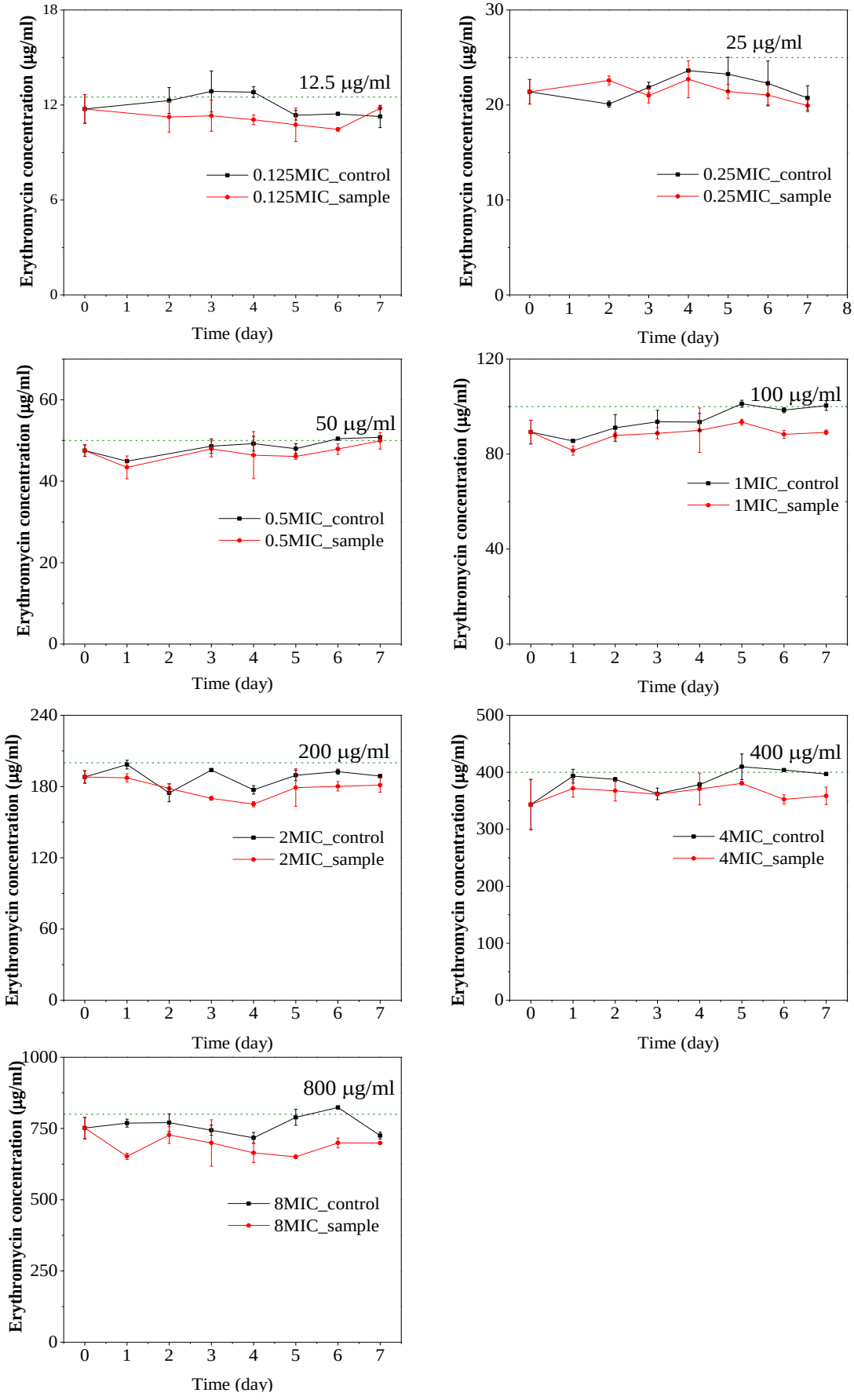


Figure 6: Persistence of erythromycin after seven days of incubation

CHAPTER 5 VIABILITY TEST OF *ESCHERICHIA COLI*

5.1 Introduction

Viable but nonculturable (VBNC) state means that bacteria fail to grow on "the routine bacteriological media on which they would normally grow and develop into colonies, but keep alive and capable of renewed metabolic activity" (Oliver, 2005). The characters of VBNC cells are "very low levels of metabolic activity but on resuscitation are again culutrabe" (Oliver, 2005), whereas their metabolic processes are reduced to baseline (Trevors, 2011). Due to these characters, VBNC cells pose a challenge to detection and raise a health concern on VBNC pathogens cause infections in human and animals when transported to water and food where they may grow (Trevors, 2011). The VBNC state is different from the starvation survival state. Cells in starvation survival state are still "full culturable even though they experienced dramatic deceases in metabolism" (Oliver, 2005). While for VBNC state, the cells are non-culturable with baseline levels of metabolic processes. Figure 7 shows an example of relationship between total cell counts, cultural counts, and viable counts. In culturable curve, because of environmental stresses, culturable cells declined in colony forming units. But in the same time, the total cell counts remains stable. Because of VBNC cells, the viable counts are slightly decreased and relatively stable during the incubation period.

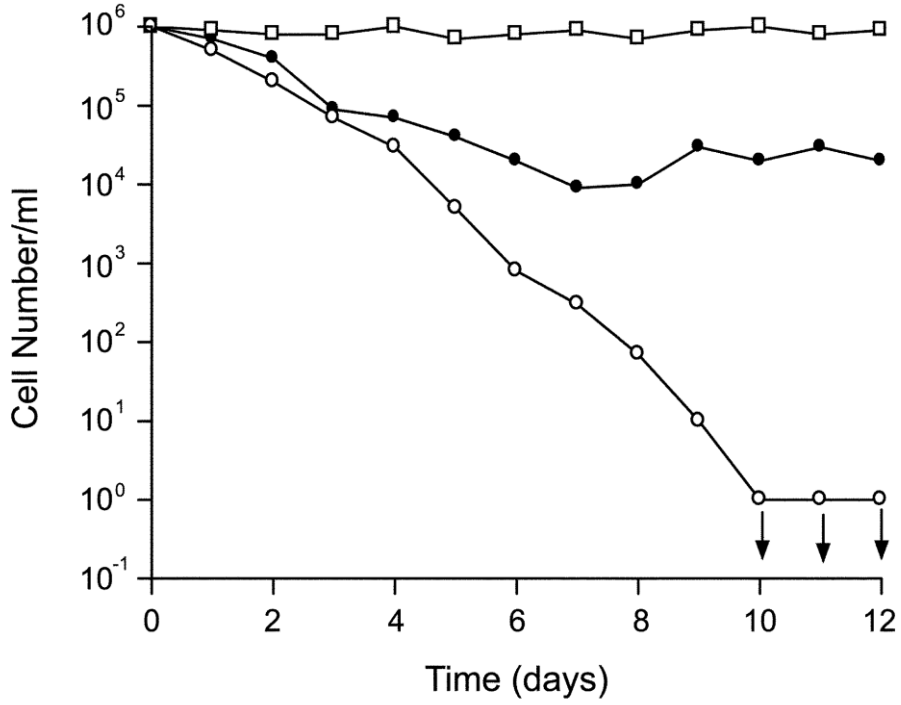


Figure 7: An example of relationship between total cells counts (□), cultural counts (○) and viable counts (●) (Oliver, 2005)

VBNC state is resulted from environmental stresses, such as soil texture, extremes of temperature, pH, redox of the environment, soil water, water type (fresh, salt, or brackish), organic matter, nutrient and nutrient gradients, any limiting nutrients, gene transfer and evolution, agro-chemical, pollutants, antibiotics an disinfectants, soil animals, plant residues and rhizosphere, other microorganisms and predation, soil atmosphere, light, planktonic or biofilm mode of growth, different rates at which the cells enter the VBNC state, presence of other living tissue such as plant or the gut of an earth worm, composition of the surface that the biofilm is attached to and climate changes (Trevors, 2011). In this study, two main factors may lead to VBNC state are nutrient starvation and erythromycin concentrations.

Large number of bacterial species can enter the VBNC state, such as *Aeromonas salmonicida, Enterococcus faecalis, Streptococcus faecalis, Klebsiella aerogenes* (Oliver, 2005). And among them, *E. coli* was the targeted specie for this study.

At VBNC state, bacteria may become smaller and even undergo a morphological transition from a rod to a more spherical morphology, and their DNA become compressed and surrounded by dense cytoplasm (Trevors, 2011). At VBNC state, many changes may happen in metabolic processes, like reductions in nutrient transport, respiration rates, and macromolecular synthesis (Oliver, 2005). Biosynthesis does not cease; plasmids are retained and ATP levels and membrane potential remain high; and continued amino acid uptake and incorporation has been detected (Oliver, 2010). Even though VBNC cells demonstrate low metabolic activity, they become more resistant to antibiotics (Oliver, 2010). Moreover, gene expression by cells in the VBNC state doesn't terminate, while many genes, such as *mobA, rfbE, stxI* and 16S rRNA synthesis genes, can be expressed in VBNC cells of *E. coli* (Oliver, 2005). However, the resistant gene expression of VBNC cells has not been well studied. This study may provide information on resistant gene expression for VBNC cells, especially MLS_B resistant genes.

Viability count is the key to determine whether cells are dead, or alive but in a VBNC state. Several assays were used to conduct the bacterial viability test by utilizing VBNC cells' characters, such as metabolic activity or of cellular integrity (Oliver, 2005). Among these methods, LIVE/DEAD *Bac*Light Bacterial Viability Kits (Invitrogen, Singapore) were chosen to test the viability of *E. coli* cells in liquid culture under different erythromycin concentrations in

this study. These kits are fluorescence-based assays to measure bacterial cell viability (Molecular Probes, 2001). Conventional direct-count assays of bacterial viability are based on metabolic characteristics or membrane integrity. However, those methods are easily affected by sensitivity on growth and staining conditions, thus they only work on a limited subset of bacterial groups and have high levels of background fluorescence (Molecular Probes, 2001). LIVE/DEAD *Bac*Light Bacterial viability kits are easily, reliably and quantitative distinguish live and dead bacteria quickly, even in a mixed population containing range of bacteria types. They utilize mixtures of SYTO® 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. The SYTO® 9 stain labels both live and dead cells in a population- those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates bacteria with damaged membranes, causing a reduction in the SYTO® 9 stain fluorescence when both dyes are present. Thus, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red. Live and dead cells can be viewed separately or simultaneously by fluorescence microscopy with suitable optical filter sets. The excitation/emission maxima for these two dyes are about 480nm/500nm for STYPO 9 stain and 490nm/635nm for propidium iodide. Thus FITC filter was used in this experiment with exposure time 40 milliseconds. These kits was used to estimation of viable and total cell counts in drinking water (Boulos, Prevost, Barbeau, Coallier, & Desjardins, 1999), pure culture strains, detection of extremophilic archaea in environmental hypersaline samples (Leuko, Legat, Fendrihan, & Stan-Lotter, 2004). The kit was applied in this experiment is LIVE/DEAD BacLight Bacterial Viability

Kits L-7012. It was very flexible because it provides separate solutions of SYTO 9 and propidium iodide stains. Its setting facilitates the calibration of bacterial fluorescence for quantitative procedures.

5.2 Materials and methods

The first step was to prepare *E. coli* suspensions with four erythromycin concentrations: 0MIC (0 μg/ml), 0.25MIC (25 μg/ml), 1MIC (100 μg/ml) and 4MIC (400 μg/ml). For each concentration level, the volume of prepared *E. coli* suspension was 8 ml. The preparing procedures for bacterial suspension were the same as the ones mentioned in Chapter 3, except that LB broth and deionized water used in viability test were filtered through 0.2 μm pore-size filter (SMI-LabHut Ltd, UK) to remove particular matters.

After that, those suspensions were incubated at 37ºC with shaking for 7 days. And 0.5ml sample for each suspension was collected for viability test in successive days during incubation. The collected samples were centrifuged at 10,000 g for 15 minutes to settle down the biomass. After centrifugation, the supernatant for these samples were removed and the pellets were suspended in 0.5 ml filtered deionized water. The washing step was repeated once, and suspended the washed pellets in 0.5 ml filtered deionized water as the test suspension for staining. For 0 MIC and 0.25 MIC, the cell density may be very large. In order to get optimal performance, the suspensions were diluted 10 times or 100 times by filtered DI-water for staining. For early samples from 1 MIC and 4 MIC, their cell densities were low. In order to keep the cell counts, the washing step was removed; the original samples were used for staining.

The third step was to stain cells in the prepared suspensions. In L-7012 kit, SYTO 9 dye is labeled as Component A, and dissolved in 300µL DMSO with concentration of 3.34 mM; and propidium iodide is labeled as Component B, and dissolved in 300µL DMSO with concentration of 20 mM. The staining stock solution was prepared by well mixing of 50µl of component A and 50µl of component B with 0.9ml filtered DI-water. And the preparation of stock solution needs avoid light and stored in -20ºC. For staining purpose, 15 µl staining stock solution was mixed with 0.5 ml prepared suspension, and incubated in dark, room temperate for 10minutes.

The final step was visualization of staining cells through fluorescence microscope. 5µl of stained *E. coli* suspension was transferred to slide, evenly spread and covered with cover slips. Then the slide was viewed under the fluorescence microscope and the cells were located under the 20 × magnification through the FITC filter with an exposure time of 40 milliseconds. Dead cells were shown in red color and live cells were shown in green color. Images of evenly distributed cells were captured for cell counting.

5.3 Results and discussion

Figure 8 shows the results of *E. coli*'s viability under four erythromycin concentrations within 7 days through DEAD/LIVE *Bac*Light kit. For the erythromycin control level (0 MIC) and sub-MIC level (0.25 MIC), *E. coli*'s viability was stable (within 60%) before the 4^{th} day of incubation, but after it, their viability sharply declined to less than 10%, even though their viable cell counts were still very large. For relatively high erythromycin concentrations,

such as 1 MIC and 4 MIC, their viability was relatively stable within 7 days, slightly decreased from 100% to 60%. However, their total cell counts were much less than 0 MIC and 0.25 MIC's.

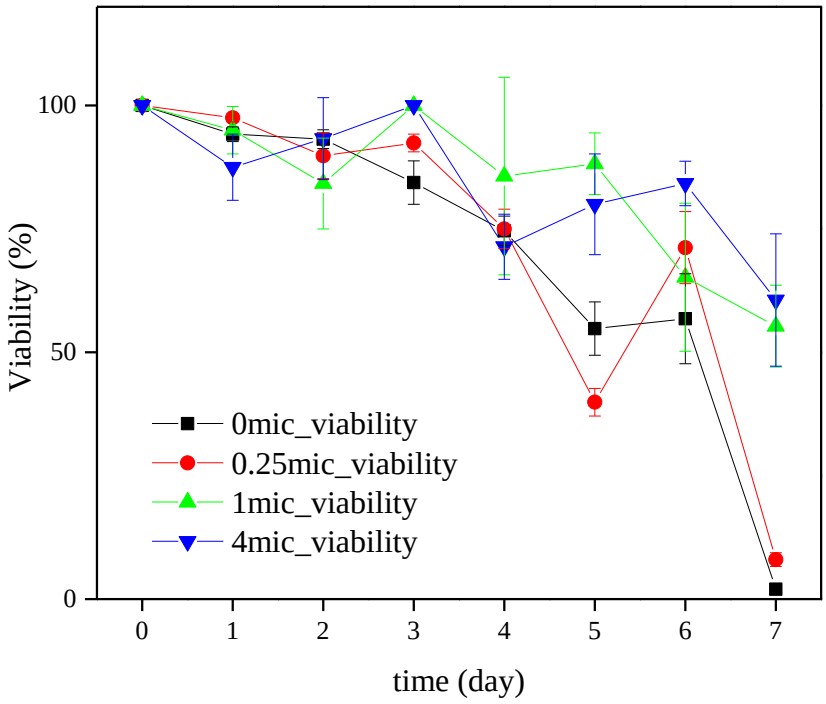


Figure 8: Viability results under exposure of erythromycin

Another observation was from fluorescence images of the stained cells. Cells detected in 0 MIC and 0.25 MIC had varied cell shapes, such rod shape and spherical shape. It indicated that under these two concentration levels, both dead cells, alive and culturable cells and VBNC cells were coexisted. With increasing incubation period, the percentages of dead cells increased sharply. On the contrary, cells detected in 4MIC and 1MIC in early incubation period, most of them, were small spherical shape. It indicated that under these situations, the VBNC cells may be in the large portion of detected cells and is reason for the stable viability. These findings were compared with the results of microbial growth, especially resistant genes expression, and discussed in the next chapter.

CHAPTER 6 *ESCHERICHIA COLI* GROWTH UNDER SELECTIVE PRESSURE OF ERYTHROMYCIN

6.1 Introduction

Bacterial growth in liquid culture may be significantly affected, if an antibiotic is added to the culture. Whether bacterial growth is delayed or inhibited depends on antibiotic concentrations and bacterial antibiotic resistance levels. The viability and cultivability of cells in bacterial suspension may be affected as well. In this study, one culture-based method, plate-counting method (PCM), and two molecular microbiology methods, fluorescence *in situ* hybridization (FISH) and quantitative polymerase chain reaction (qPCR), were applied to test the microbial growth of the *E. coli* under different erythromycin concentrations.

PCM utilizes agar plates to grow and represent the viable and culturable cell counts in the solid surface of media. VBNC cells cannot be tested through PCM. The amount of colonies formed on each agar plate should be kept in range of 30 to 300 CFUs for accurate counting. In this study, PCM was applied to measure both the total and erythromycin resistant viable and culturable *E. coli* cells. The LB agar plates were used to test total colony forming units (CFU) counts, and LB agar plates with 100 μg/ml erythromycin were used to measure erythromycin resistant CFU counts.

FISH utilizes fluorescently labeled oligonucleotide probes through *in situ* hybridization of specific RNA or DNA sequences to detect target bacteria (Zhou, Pons, Raskin, & Zilles, 2007). FISH images can be viewed and captured in a fluorescence microscope, and these images have been automatically analyzed using in an automated image analysis program to quantify erythromycin resistance (Zhou et al., 2007).

Quantitative polymerase chain reaction (qPCR) combines PCR amplification and detection into a single step and enables the quantification of target product (Life Technologies, 2012), and can be used to detect erythromycin-resistant determinants. With qPCR, fluorescence dyes are used to label PCR products during thermal cycling (Life Technologies, 2012). While qPCR measures the accumulation of fluorescent signal during the exponential phase of the reaction (Life Technologies, 2012). There are two kinds of strategies to label the qPCR products fluorescently: TaqMan® fluorogenic probes and SYBR® Green dye.

Resistance gene *erm* K is an inducible *erm* genes under *erm* D class and originated from *Bacillus,* and erythromycin is a good inducer for *erm* K (Marilyn C Roberts et al., 1999). The *erm K* methylase expression is regulated by transcriptional attenuation in contrast other inducible *erm* genes, which are regulated translationally. The *erm*K leader peptide contains two rho-factor independent transcriptional terminators: T1 and T2 (Kwon et al., 2006). In absence of inducer, truncated transcription products only are synthesized and the full length transcription product is not detected. But the stalling of erythromycin-bound ribosome in the *erm* K leader peptide disrupts terminator structures, allowing rapid induction of *erm* K methylase transcription (Kwon et

al., 2006). There several mutant plasmids for *erm* K: pECMT1 (T1 mutant), pECMT2 (T2 mutant), and pECMT3 (T1 & T2 double mutant), pECMT109 (methylase SD region mutant), and pECMT309 (T1 plus T2 plus methylase SD region mutant) (Choi, Kim, Oh, & Choi, 1997), and these mutants are related to antibiotic concentrations (Kwon et al., 2006).

6.2 Materials and methods

6.2.1 Preparation of *E. coli* suspensions

The isolated *E. coli* strain was cultured in LB broth and erythromycin stock solution was prepared for preparation of *E. coli* suspensions under eight levels of concentrations: 0 MIC (0 μg/ml), 0.125 MIC (12.5 μg/ml), 0.25 MIC (25 μg/ml), 0.5 MIC (50 μg/ml), 1 MIC(100 μg/ml), 2 MIC (200 μg/ml), 4 MIC (400 μg/ml), and 8 MIC (800 μg/ml). The initial cell density of *E.coli* was kept around 10^6 cells/ml. The scheme of *E. coli* suspensions was shown in Table 3. Growth suspensions were incubated at 37ºC with shaking for seven days. Within the incubation period, samples were collected for PCM, FISH and qPCR analysis. The sampling time points were 0 day, 6^{th} hour, 12^{th} hour, 1^{st} day, 1.5^{th} day, 2^{nd} day, 3^{rd} day, 4^{th} day, 5^{th} day, 6^{th} day and 7^{th} day.

Table 3: Scheme of E. coli suspensions for growth experiment

Erythromycin concentration levels	LB broth with *E. coli* culture (ml)	Erythromycin stock solution (µl)	Final erythromycin concentration (µg/ml)	Final inoculum size (cells/ml)	Final suspension volume (ml)
0 MIC (control)	120	0	0	10^6	120
0.125 MIC	119.85	150	12.5	10^6	120
0.25 MIC	119.7	300	25	10^6	120
0.5 MIC	119.4	600	50	10^6	120
1 MIC	118.8	1200	100	10^6	120
2 MIC	117.6	2400	200	10^6	120
4 MIC	115.2	4800	400	10^6	120
8 MIC	110.4	9600	800	10^6	120

6.2.2 Plate counting method for microbial growth

LB plates and LB plates with 100 µg/ml erythromycin were prepared to measure the total CFU counts and erythromycin resistant CFU counts under different erythromycin levels. Collected liquid samples were evenly spread on LB plates and LB resistant plates. If the cell densities were too high, the liquid samples were diluted by fresh LB broth several times, and then spread on plates. After spreading of plates, they were incubated for 24 hours at 37°C for CFU counting.

In the PCM method, if no colony was formed, the result could be reported as 1 CFU , with the recommendation from ASTM (Sutton, 2011). In this PCM experiment, 100 µl of suspension was spread on the surface of agar plate, and therefore the estimated CFU density would be recorded as 10 CFU/ml if there was no colony forming on the agar plate. Therefore 10 CFU/ml was the lower detection limit for total cell density and resistant cell density in the PCM method.

6.2.3 FISH analysis for microbial growth

In this study, dual labeling hybridization (Zhou, Raskin, & Zilles, 2009) was conducted to test the changes of total cell density and resistant cell density of the isolated *E. coli* strain. Two fluorescently labeled probes, which are Bact338 probe and MLS_B probe, were used to target total *E. coli* cells and MLS_B sensitive unmethylated *E. coli* cells, respectively. The MLS_B resistance can be indirectly quantified from the difference between the MLS_B sensitive cells and the total cells. The details of these two primers are listed in Table 4.

FISH consists of four major steps: sample fixation, slide preparation, probe hybridization, and image analysis.

The first step was sample fixation. 0.5ml of growth suspension was fixed for FISH analysis under each erythromycin level. The collected sample was mixed with1.5 ml 4% paraformaldehyde and incubated 30 minutes at room temperature. Then the mixture was centrifuged at 10,000 rpm, 3 minutes, and decanted the suspended liquid, suspended in 1ml 1×PBS buffer twice. After

third spin, the sample was suspended in 0.5 ml PBS: Ethanol solution and stored at -20^{o}C for next step.

The next step was slide preparation. Gelatin coated slides were prepared. The microscope slides with 6 wells were soaked in ethanolic KOH (20g KOH (Sigma-Aldrich, USA) and 200ml ethanol) for 1 hour. Then the slides were rinsed by distilled deionized water and air dried. Then the slides were dipped in heated gelation solution (0.1g gelatin (Sigma-Aldrich, USA), 0.01 g chromium potassium sulfate (Sigma-Aldrich, USA), 100 ml DD-water, heated to 60^{o}C) and dried vertically. Then 5 µl fixed sample was transferred to a gelatin-coated slide and dried in oven for 10 minutes. The cells of the fixed samples were fully dispersed. If the fixed sample contained too many cells, dilution was conducted for several times before transferring. Then, the prepared slides were sequentially dipped in 50%, 80%, and 95% ethanol for 3 minutes to wash off impurities and dehydrate embedded cells.

Table 4: Oligonucleotide probes used for quantification of total *E. coli* cells and MLS_B sensitive *E. coli* cells (Amann et al., 1990; Zhou et al., 2009)

Probe	Systematic name	Label (5')	Target organism	Target molecule	Formamide (%)	Specificity (%)	Coverage (%)	Probe sequence (5' -3')
MLS_B	L-*-MLSS-2053-a-S-13	5' Alexa488	MLS_B sensitive *E. coli*	23S rRNA	12.5	99	97.5	GGG TCT TTC CGT C
Bact338	S-D-Bact-0338-a-A-18	5'Cy3	*E. coli*	16S rRNA	20	100	90.6	GCT GCC TCC CGT AGG AGT

The third step was dual-labeling hybridization. The compositions of hybridization buffers and washing buffers for Bact 338 probes and MLS_B probes were listed in Tables 5 and 6. For each well, 15 µl hybridization buffer and 1 µl probe solution were mixed and applied on slides. Then dual-labeling hybridization was conducted under dark condition since the light may decrease the fluorescence signals. The experimental procedure consisted of two times probe hybridizations and washing-offs.

Table 5: Compositions of hybridization buffers

	Bact338 Hybridization Buffer	**MLS_B Hybridization Buffer**
1.8M NaCl	1 ml	1 ml
1M Tris pH 7.2	40 µl	40 µl
10% SDS	2 µl	2 µl
Deionized H_2O	0.558 ml	0.708 ml
Formamide	0.4 ml	0.25 ml
Final volume	2 ml	2 ml
Final formamide concentration (v/v)	20%	12.50%

Table 6: Compositions of washing buffers

	Bact338 Wash Buffer	**MLS_B Wash Buffer**
1.8M NaCl	3.68 ml	6.95 ml
1M Tris pH 8	0.8 ml	0.8 ml
10% SDS	40 µl	40 µl
Deionized H_2O	35.48 ml	32.21 ml
Final volume	40 ml	40 ml

Hybridization buffer and Bact338 probe were added to slides first, and then the sample slides were placed into incubator at 46 ºC for 2 hours. Then slides were rinsed with 1 ml preheated (48 ºC) Bact338 washing buffer twice and placed into the remaining preheated wash buffer and incubated for 20 minutes at 48ºC under dark. After 20 minutes, the slides were rinsed with 1 ml deionized water for about two times and air dried. Second hybridization was for MLS_B probe, 15 µl of the MLS_B hybridization solution and 1 µl probe solution were aliquoted onto each well, and the slides were incubated for 2 hours at 37ºC under dark. Then, the hybridized slides were rinsed with 1 ml preheated MLS_B washing buffer at 37 ºC twice and incubated in the remaining preheated MLS_B washing buffer for another 20 minutes at 37ºC. Slides were rinsed with 1ml of deionized water and air dried. Finally sample slides were stained by 10 µg /ml 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA) for 5 minutes, rinsed twice with 1 ml distilled deionized water and air dried. 3 µl citifluor (Marivac Limited, Canada) was added to each well to prevent the degradation of DAPI and slowly covered the slides with cover slips and sealed with nail polish. The prepared FISH slides were stored in dark at 4ºC.

The final step was to capture and analyze the FISH images. Sample slides were viewed under a fluorescence microscope and the cells are located under the 20 × magnification. The image of a particular position on the well through the FITC (for MLS_B probe) filter was taken with the exposure time of 666 milliseconds. Subsequently at the same position, two other images were captured using the Cy3 filter (Bact338) and DAPI filter under exposure times of 500 milliseconds and 50 milliseconds, respectively. This procedure was repeated for several more random locations on the well and images were taken

under the same exposure timings. The captured FISH images were analyzed via a program Visilog. In Visilog, individual cells in the image were detected with the DAPI image used as a DAPI mask to determine the discrete locations of cells. In addition, the fluorescence intensities (maximum and mean) were measured following a procedure reported previously (Zhou et al., 2007). The statistical data from Visilog was analyzed by another program called FuzzMe to use fuzzy c-means clustering (FCM) analysis (10 clusters) to classify all data points. The clusters were classified as either positive (target cells) or negative (non-target cells) by comparison of the cluster centroids with the mean values for maximum intensity obtained from the negative control. The positive percentage of target cells is therefore the number of cells in positive clusters divided by the total number of cells (Zhou et al., 2007) and through this percentage the percentage of MLS_B resistant cells were calculated.

6.2.4 qPCR analysis for microbial growth

In this experiment, SYBR® GreenER™ dye was selected to label the qPCR products. SYBR® Select Master Mix for CFX (Applied Biosystems, USA) was used as master mix to enable the labeling. This type of master mix can minimize primer-dimmer and non-specific amplification and provide maximum brightness for fluorescence signal detection. The Eub 338 and Eub 518 primers (AIT Biotech, Singapore) were used to detect and quantity total cell gene copies. The *erm*K primers (AIT Biotech, Singapore) were used to detect and quantify MLS_B resistant gene levels, because *erm*K was the predominant

MLS_B resistant gene for the selected *E. coli* strain through previous qPCR test. Table 7 shows the information of two fluorescently labeled probes.

Table 7: qPCR primers used for this study

Target	Primer	Sequence (5'-> 3')	Amplicon size (bp)	Annealing Temperature (^{O}C)	Reference
16S rRNA	Eub-338Fw	ACT CCT ACG GGA GGC AGC AG	180	63	(M. S. Wright et al., 2008)
	Eub-518Rv	ATT ACC GCG GCT GCT GG			
erm K	*erm*K-fw	GTT TGA TAT TGG CAT TGT CAG AGA AA	75	60	(Zhu et al., 2013)
	*erm*K-rv	ACC ATT GCC GAG TCC ACT TT			

5 ml growth suspension was collected in each sampling time for each erythromycin concentration. DNA extraction for these samples was operated by Mo-Bio UltraClean Microbial DNA Isolation kit (Mo-Bio Laboratories, USA). Nanodrop was used to measure the extracted DNA concentrations and purities. The extracted DNA samples were stored in -20 ^{o}C. The details of qPCR reaction solutions for 16S rRNA and *erm*K were shown in Table 8. The DNA

concentrations of DNA templates were within 50 ng/µl. The reactions were conducted by StepOnePlus™ Real-Time PCR System and software (v2.3; Applied Biosystems, USA). Reaction conditions were shown in Table 9.

Table 8: Recipes of qPCR reaction solutions

Chemicals/ reaction	**16S rRNA**	***erm* K**
SYBR Green Master Mix for CFX	10 µl	10 µl
Primer solution (forward primer + reverse primer; 10 µM)	0.4 µl	0.4 µl
DD-water	7.6 µl	6.6 µl
DNA template	2 µl	3 µl
Final volume	20 µl	20 µl

Table 9: qPCR reaction conditions

Step	**Temperature**	**Duration**	**Cycles**
UDG Activation	50°C	2 min	Hold
AmpliTaq DNA Polymerase, UP activation	95°C	2 min	Hold
Denature	95°C	15 sec	40
Anneal	60°C	1 min	40

6.3 Results

6.3.1 Plate-counting method (PCM) results

Figure 9 shows the results of total viable and culturable cell densities detected by PCM. For *E. coli* incubated under erythromycin concentrations above 1 MIC (such as 2 MIC, 4 MIC and 8 MIC), there was no obvious bacterial growth observed within 7 days' incubation for total culturable cells. Their densities sharply decreased to below detection limit within the first 2 days' of incubation, and after that, no colony was formed on the LB agar plates for these concentrations. Moreover, the order of total culturable cell density's decreasing rates was 8 MIC > 4 MIC > 2 MIC.

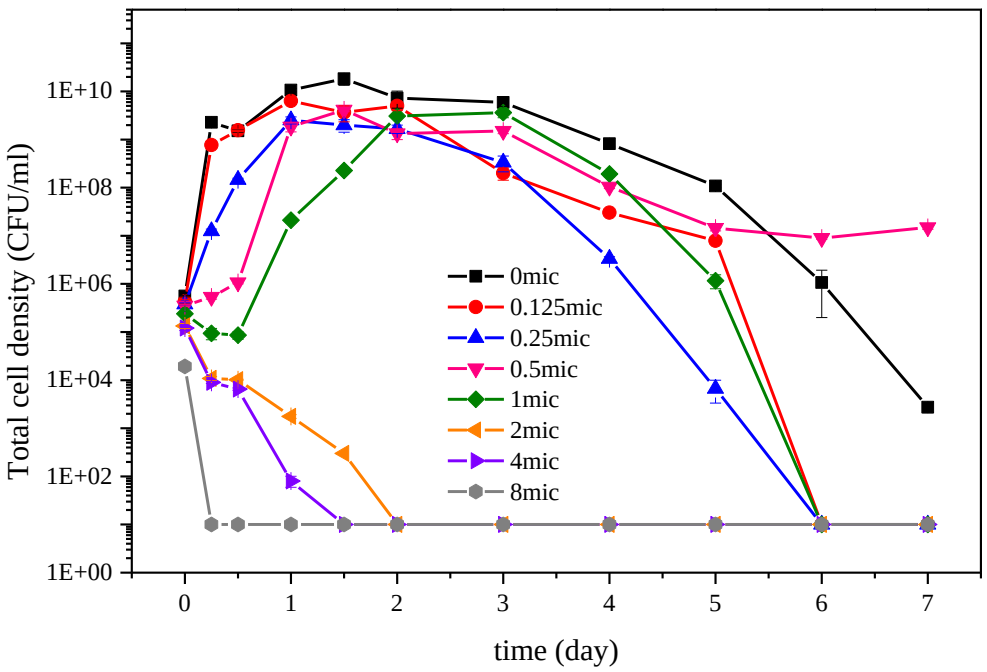


Figure 9: Total cell density detected by PCM

For the culturable cells of *E. coli* incubated under the selective pressure of 1 MIC and 0.5 MIC of erythromycin, microbial growth was observed within first day of incubation. The growth rate of *E. coli* under 0.5 MIC was higher than that under 1 MIC. After 6 days, no colony was observed under 1 MIC, but the CFU counts under 0.5 MIC remained stable. These results indicated that the cells under 1 MIC lost their cultivability or entered decay phase.

For the culturable cells of *E. coli* incubated under selective pressures of 0 MIC, 0.125 MIC, and 0.25 MIC of erythromycin, there was no decline in CFU counts during the first two days. The difference in erythromycin concentrations affected their culturable bacterial growth rates and a negative relationship between erythromycin concentration and bacterial growth rates was observed, i.e., low growth rates were observed at high erythromycin concentrations, and the order of growth rates followed the following order: 0 MIC > 0.125 MIC > 0.25 MIC. Erythromycin concentration affected the bacterial decay rates as well, but the relationship was positive, i.e., high decay rates were observed at high erythromycin concentrations.

In conclusion, erythromycin concentration was an important factor for bacterial cultivability. A negative relationship between bacterial growth rate and erythromycin concentration was observed, and a positive relationship between bacterial decay rate and erythromycin concentration was observed. Under high antibiotic concentrations above 1 MIC, bacteria may lose their cultivability or enter to VBNC state. Among the different antibiotic concentrations, it seems that 1 MIC was a threshold to separate microbial cultivability.

Figure 10 shows the results of erythromycin resistant culturable cell densities detected by PCM. There was no resistant colony formed under the selective pressure of erythromycin above 1MIC (such as 2 MIC, 4 MIC and 8 MIC) along the incubation period. The results indicate that most resistant cells lost their cultivability under exposure of erythromycin.

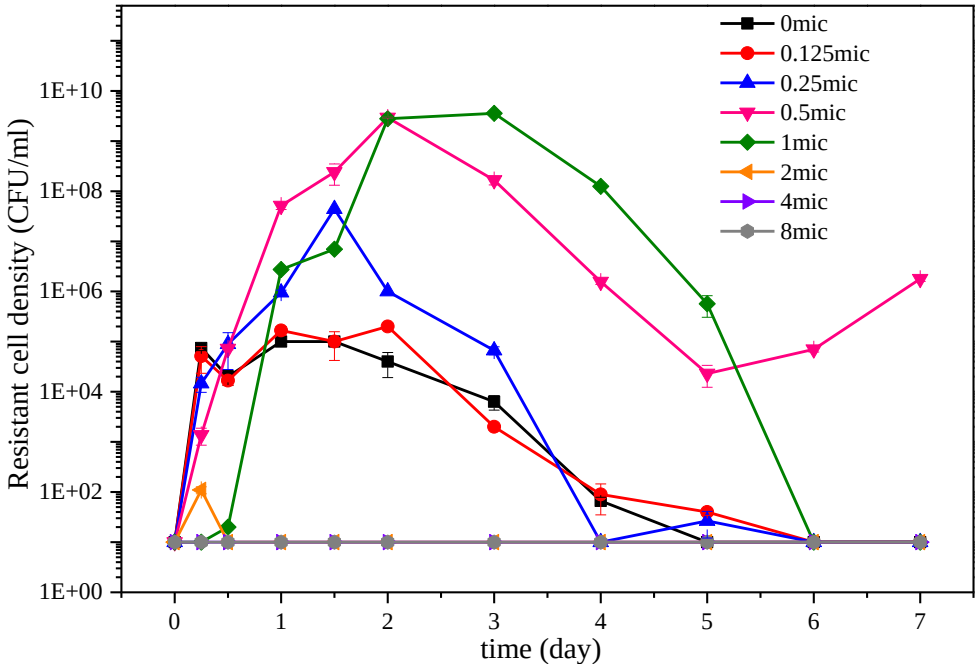


Figure 10: Erythromycin resistant cell density detected by PCM

Under 0.5 MIC of erythromycin, resistant culturable colonies formed after 6 hours of incubation. Under 1 MIC of erythromycin, the culturable resistant colonies delayed for an additional 6 hours than the exposure under 0.5 MIC. Within first two days, resistant colonies of 0.5 MIC were larger than 1 MIC, but their growth rates were similar after two days incubation. After that, cell densities of *E. coli* under 1 MIC were larger than those under 0.5 MIC until the 5th day of incubation. Finally, there was no resistant colony formed for 1 MIC. But cell densities of *E. coli* under 0.5 MIC increased slightly after day 5,

suggesting additional change, such as mutation, may happen during the cultivation.

Under selective pressure of 0 MIC, 0.125 MIC, and 0.25 MIC of erythromycin, resistant *E. coli* colonies increased during the first day or one and half days, the reduced below the detection limit. Their cell densities were positively related with erythromycin concentrations and the order of resistant cell densities was: 0.25 MIC > 0.125 MIC> 0 MIC. Resistant cells without exposure of erythromycin lost their cultivability in the 5^{th} day, but resistant cells under exposure of 0.25 MIC and 0.125 MIC of erythromycin lost their cultivability in the 6^{th} day of incubation.

In conclusion, under high erythromycin concentrations, resistant cells lost their cultivability because of strong inhibition effect. Under erythromycin concentrations less than 1 MIC, the resistant bacterial lost their cultivability gradually with the incubation period because of consistent antimicrobial effect. Among them, 1 MIC is the threshold to differentiate the power of antimicrobial effect. The order of resistant cell densities was 1MIC ≈ 0.5MIC > 0.25 MIC > 0.125 MIC ≈IC MIC > 2 MIC, 4 MIC, 8 MIC. In addition, microbial growth of resistant cells was promoted by exposure of erythromycin in MIC and sub-MIC levels.

Figure 11 showed the resistant levels for eight erythromycin levels which were as the ratios of total cell density (Figure 8) and resistant cell density (Figure 9). Only under 0.5 MIC and 1 MIC, resistant levels increased significantly, while resistance under other concentrations were relatively low (<1%). Resistant levels under 1 MIC were much larger than those under 0.5

MIC. Thus, the results suggest that that 1 MIC is the crucial concentration to select antibiotic resistance.

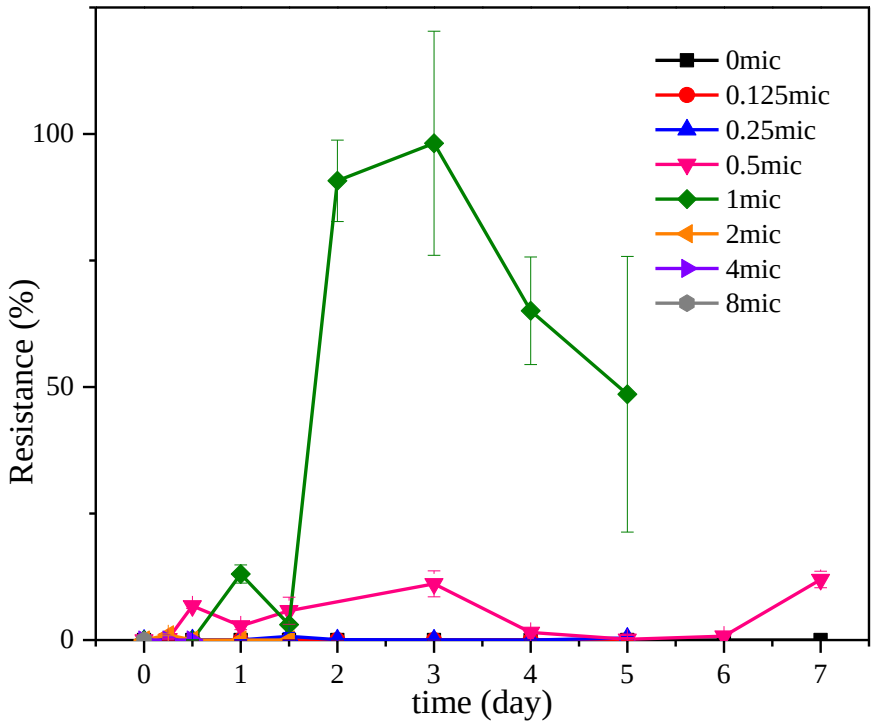


Figure 11: Erythromycin resistance calculated from resistant and total cell densities

Figure 9, 10 and 11 represented the cultivability variations of microbial growth under selective pressure of erythromycin. They illustrated the effect of different antibiotic concentrations on bacterial cultivation via culture-based technique. Under erythromycin levels above 1 MIC, both total and resistant cells quickly lost their cultivability. In contrast, under sub-MIC levels, cultivability of both total and resistant cells was gradually increased and decreased. The result indicated that microbial growth was significantly affected by antibiotic concentrations, and the highest resistant levels were achieved under the exposure of 1 MIC erythromycin.

6.3.2 FISH results

Figure 12 to Figure 19 present densities of total cells and MLS_B resistant cells and MLS_B resistances under selective pressure of erythromycin concentrations which were detected by FISH within 7 days' incubation.

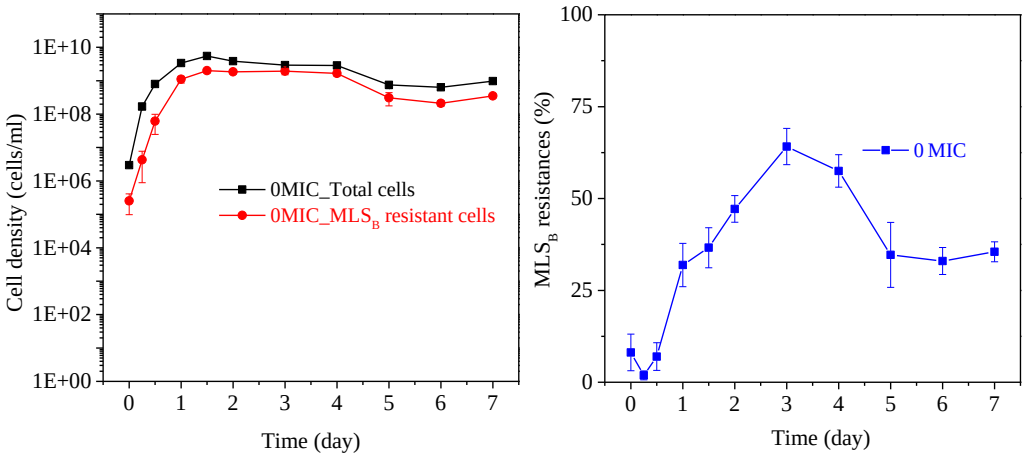


Figure 12: FISH results under 0MIC of erythromycin

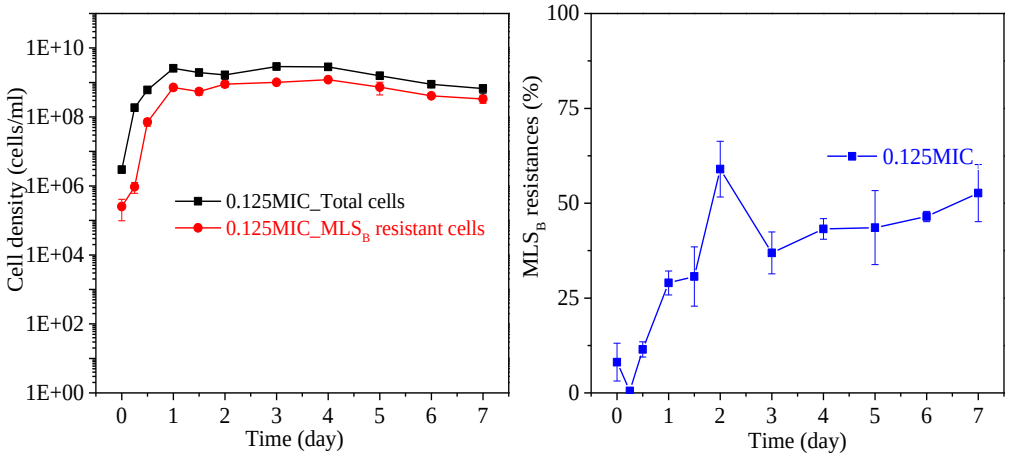


Figure 13: FISH results under 0.125MIC of erythromycin

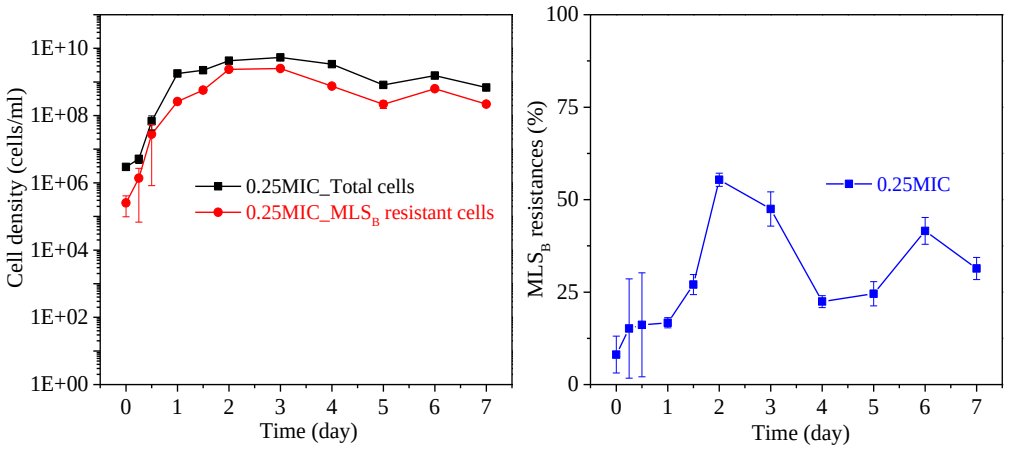


Figure 14: FISH results under 0.25MIC of erythromycin

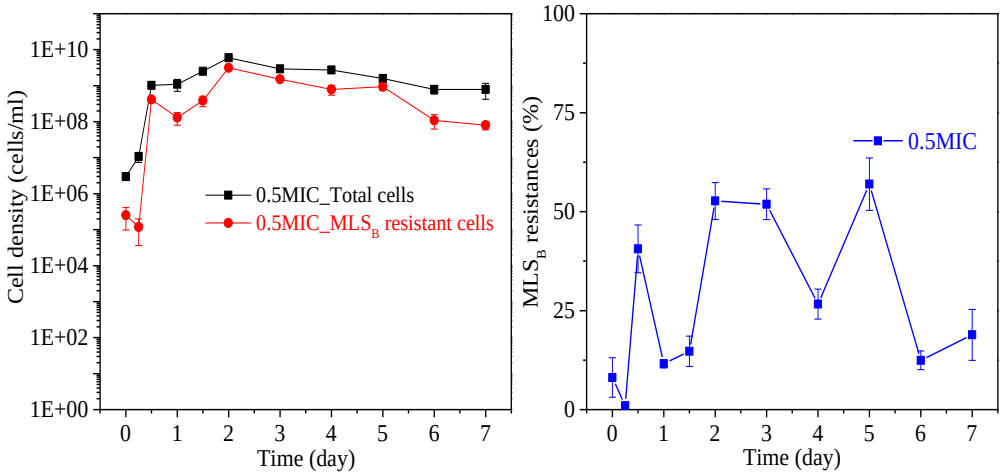


Figure 15: FISH results under 0.5MIC of erythromycin

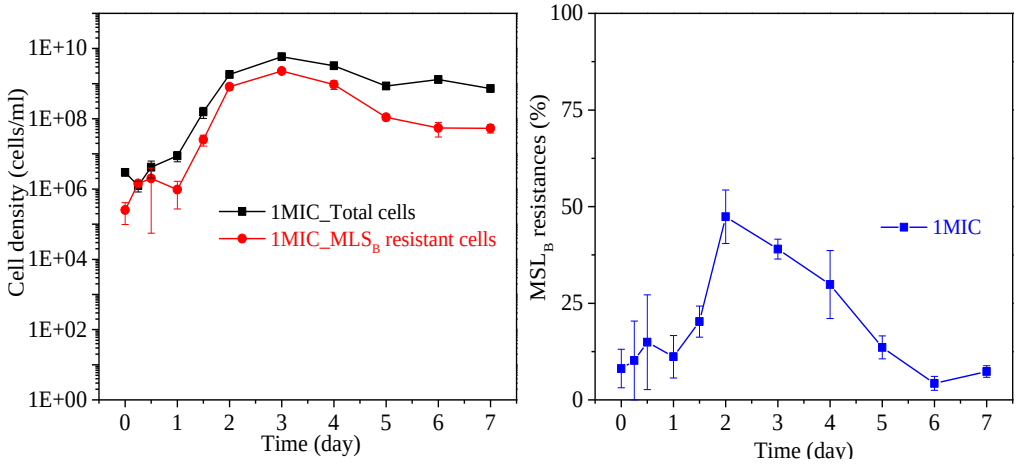


Figure 16: FISH results under 1MIC of erythromycin

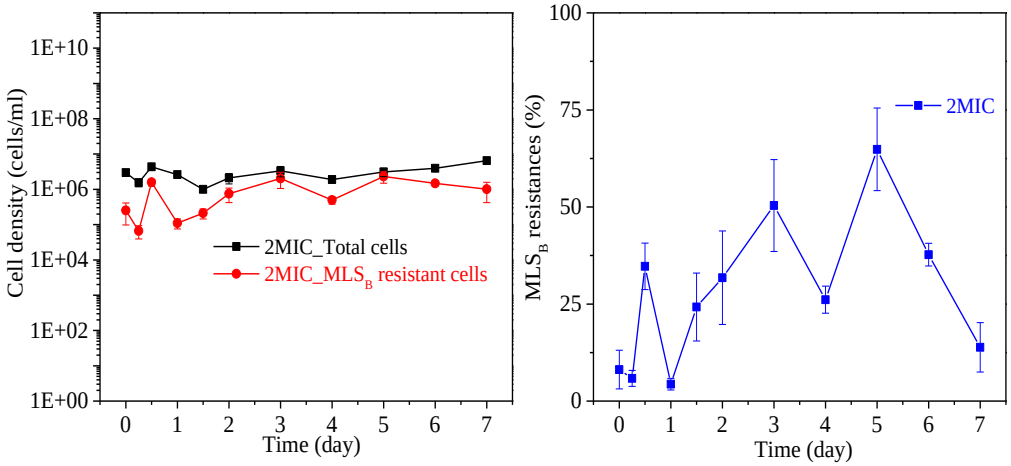


Figure 17: FISH results under 2MIC of erythromycin

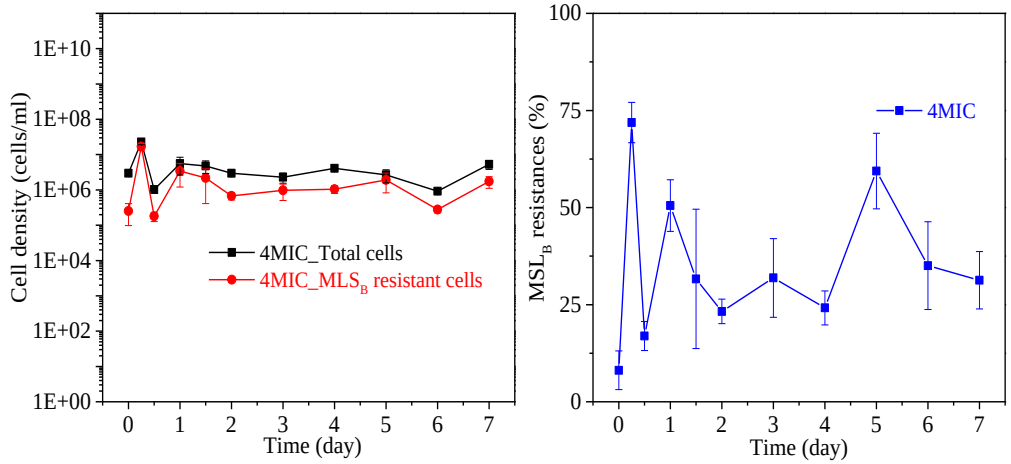


Figure 18: FISH results under 4MIC of erythromycin

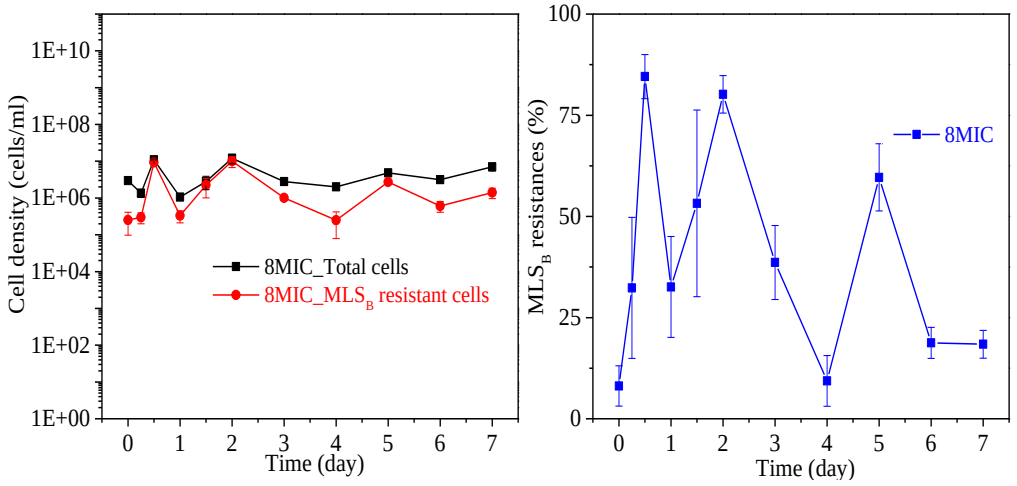


Figure 19: FISH results under 8MIC of erythromycin

Total cell densities targeted by Bact338 probe under selective pressure of erythromycin above 1 MIC (such as 2 MIC, 4 MIC and 8 MIC) and the results showed that total cell densities were relatively stable. These results were different from the results of culturable *E. coli* shown in Figure 9, which showed that no colony was formed under erythromycin concentrations above 1 MIC. As shown in Figure 8, viable *E. coli* under 1 MIC and 4 MIC ranged from 100% to 60% within 7 days. All the results showed that most detected bacterial cells in FISH were in viable but VBNC state under erythromycin concentrations above 1 MIC. Under selective pressures of 0.25 MIC, 0.5 MIC, and 1 MIC erythromycin, there was a delay on total cell growth within the first day. The longest delay was observed in cells under 1 MIC, which was about one day. Under 0.5 MIC and 0.25 MIC, cell growth delayed for about 6 hours. The results showed that higher antibiotic concentrations could cause longer delay in bacterial growth.

No delay on bacterial growth was observed under 0 MIC and 0.125 MIC. Furthermore, total cell densities and cells' growth rates were similar and inhibition effect of antibiotic was not obvious.

In conclusion, there was a strong relationship between total cell growth and antibiotic concentrations when they were within certain ranges, such as from 0.25 MIC to 1 MIC. Beyond these ranges, this relationship became less obvious. Most bacterial cells were in VBNC state under exposure of erythromycin levels of 2 MIC, 4 MIC and 8 MIC, and there was almost no difference between inhibition effects on cell growth rates because of extremely high erythromycin concentrations. Under erythromycin levels of 0 MIC and 0.125 MIC, erythromycin concentrations were too low to affect cell growth rates.

Under selective pressure of erythromycin above 1 MIC (such as 2 MIC, 4 MIC and 8 MIC), MLS_B resistant cell densities were roughly stable within the incubation period. During the first three days, MLS_B resistant cell densities were higher under 8 MIC and 4 MIC rather than those under 2 MIC. After the third day, resistant cell densities were relatively stable under these three erythromycin levels. These result showed that even cells were in VBNC state, erythromycin resistant genes were expressed, which were detected by the FISH method.

Under selective pressure of other erythromycin concentrations (such as 0 MIC, 0.125 MIC, 0.25 MIC, 0.5 MIC, and 1 MIC), MLS_B resistant cell densities were much higher than those under erythromycin concentrations above 1 MIC. Resistant cell growth rates were related with erythromycin concentrations as well. Under erythromycin concentration of 1 MIC, resistant bacterial regrowth delayed for one day, but no delay was detected under other concentration levels. Resistant bacterial growth rates of 0.5 MIC and 1 MIC were highest within the first three days. After three days, resistant cell densities under erythromycin concentrations below 1 MIC were similar.

MLS_B resistant levels under different erythromycin concentrations were different from the results detected by PCM (Figure 11). By PCM, significant resistance levels were observed only under exposure of 0.5 MIC and 1 MIC erythromycin. But by FISH, resistance levels were significant under all erythromycin concentrations. Relatively high MLS_B resistant levels (up to 80%) were observed under erythromycin levels of 1 MIC, 2 MIC, 4 MIC, and 8 MIC because of VBNC cells. These cells could not be cultivated, but were still able to express resistance genes.

In summary, FISH results and viability test results indicated that bacteria under high antibiotic concentrations may lose cultivability and enter the VBNC state. However, they may still be able to express resistance genes. Hence, the potential health risks associated with pathogens or antibiotic resistant genes may be underestimated via conventional culture-base techniques. In addition, a relationship between antibiotic concentrations and expression of bacterial genotypes were detected.

6.3.3 qPCR results

Figure 20 represented the results of total cell counts detected by primer16S rRNA. The 16S rRNA gene copies were relatively stable under selective pressure of erythromycin levels of 2 MIC, 4 MIC, and 8 MIC. Because cells were in VBNC state, the expression of 16S rRNA gene did not cease under high erythromycin concentrations. Moreover, the lowest values of 16S RNA gene copies were observed under 8 MIC. These results and were consistent with FISH results.

16S rRNA gene copies under other erythromycin concentrations were much higher than those under high erythromycin concentrations. Similarly, gene copies were related to erythromycin concentrations. 1 MIC had the lowest 16S RNA gene copies in the first 3 days and their gene copies' increasing rates were slowest. The order of 16S rRNA gene copies and their increasing rates during the first three days were 0 MIC ≈ 0.125 MIC > 0.25 MIC > 0.5 MIC > 1 MIC. After three days, their values were stable and almost the same.

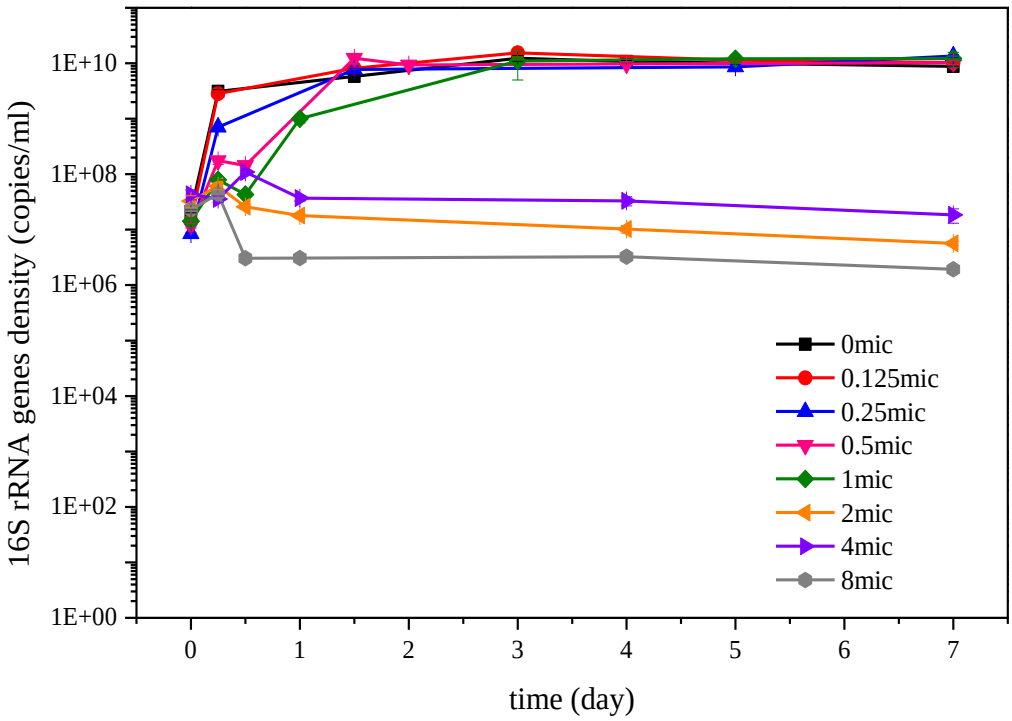


Figure 20: 16S rRNA gene copies detected by qPCR

In summary, 16S rRNA gene copies under various selective pressure of erythromycin were consistent with the results of PCM and FISH experiments. One was that their gene expression of 16S rRNA was related with antibiotic concentrations; and 1 MIC was the threshold to differentiate various growth rates.

Figure 21 expressed the results of erythromycin resistant cell counts detected by primer *erm*K. Among the *erm* genes detected by the previous experiments, *erm*K was most abundant resistant gene, and therefore was chosen as the target resistance genes in qPCR experiments.

The results showed that gene copies of *erm*K under sub-MIC levels and control level were lower than 1 MIC and high erythromycin concentrations. 1 MIC played as a threshold to separate low *erm*K gene copies and high ermK gene copies. And most variations in values of *erm*K gene copies happened

within the first day of incubation. Within this period, *erm*K gene copies were sharply increased up to 10^8 copies per ml under erythromycin concentrations above 1 MIC. But under sub-MIC levels and control level of erythromycin, *erm*K resistant gene copies remained low. After the first day, gene copies of *erm*K under all erythromycin level did not change significantly. Under high antibiotic concentrations, gene expression of *erm*K for VBNC cells did not cease, and high erythromycin concentrations could even promote the activity of *erm*K's expression.

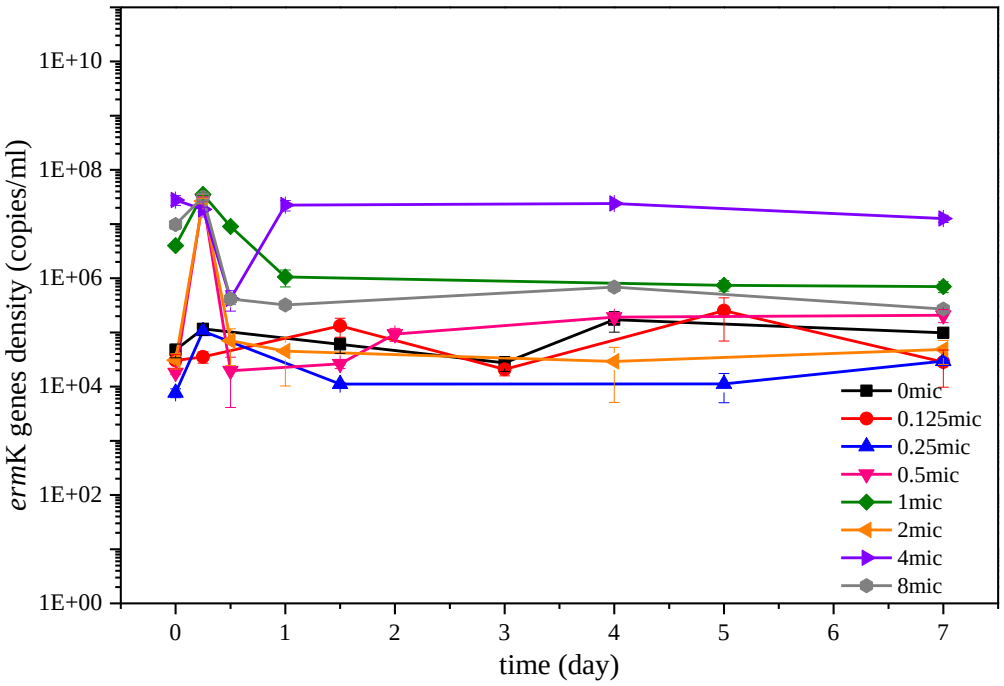


Figure 21: *erm* K gene copies detected by qPCR

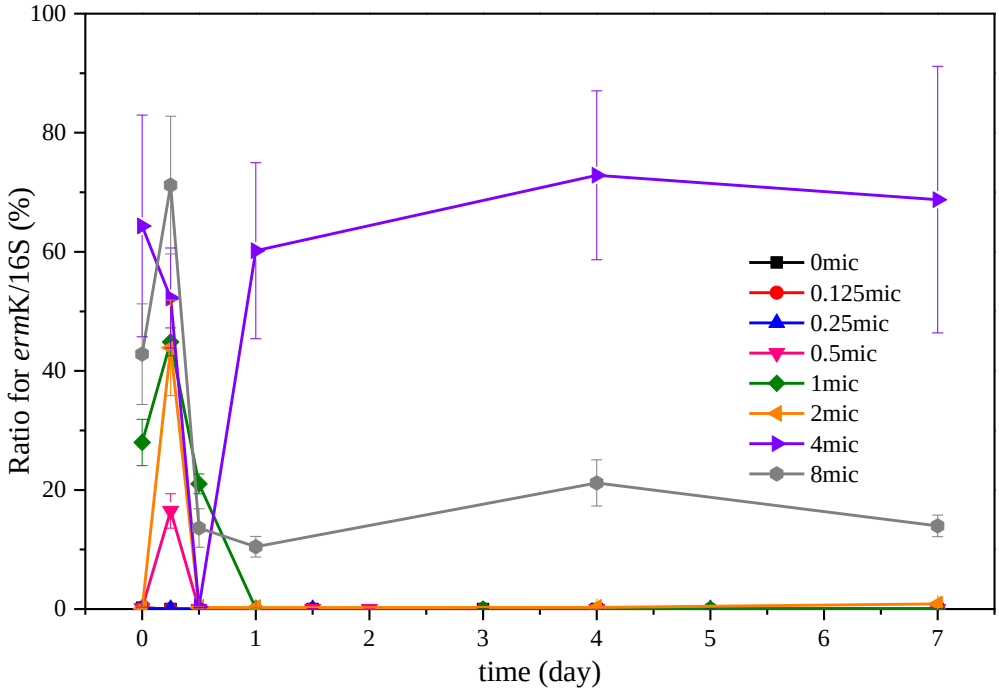


Figure 22: ratios between *erm*K to 16S rRNA gene copies

Figure 22 showed the results of relative abundance of *erm*K. Resistant levels under erythromycin concentrations of 8 MIC, 4 MIC, 2 MIC, 1 MIC, and 0.5 MIC had significant values; and among them, resistant levels under the exposure of 4 MIC and 8 MIC were relatively with resistance levels up to 70%, which could be the result of low gene copies in their 16S rRNA genes.

The results indicated that both 16S r RNA gene and *erm*K kept their activities under exposure of erythromycin, and the expression of *erm*K could even be promoted by high erythromycin concentrations. Most of the cells under high erythromycin levels were VBNC cells. This finding reveals potential health risks caused by excessive usage of antibiotics. And since *erm*K is located in plasmids, its mobility should also be considered for environmental risk assessment.

6.4 Discussion

6.4.1 Antibiotic concentration-dependent selection of bacterial fitness or adaption

The results in this study can be explained with the concept of fitness or adaption, which means the selection process that help microorganisms survive under selective pressures. Selection is the amplifying mechanism for organisms with mutations and leading to an increase in fitness (Baquero, Negri, Morosini, & Blázquez, 1998). Both bacterial survival and proliferation are essential for bacterial fitness (Baquero et al., 1998). The term "selective pressure" was defined as environmental conditions (Tenover & McGowan Jr, 1996), and it was used to describe the factors that create an "environmental landscape and allow organisms with novel mutations or newly acquired characteristics" to survive and proliferate (Baquero et al., 1998). It induces the expression of differences in fitness.

In this study, selective pressure was erythromycin. Under this selective pressure, the isolated *E. coli* strain promoted the growth of erythromycin resistant genes to increase the fitness for the antibiotic stressed environments. As shown in the results from PCM, the growth for both total cells and erythromycin resistant cells under exposure of 0 MIC to 1 MIC reflected that the isolated *E. coli* strain could well adapt to stressed environments. In addition, erythromycin resistant cell densities were corresponding to erythromycin concentrations. The results indicated that the fitness conferred by this system was antibiotic concentration dependent selection. However, under pressure

above 1 MIC erythromycin, no colony formed during incubation by PCM, which may be caused by the inhibition effect of excess antibiotic dosages. In order to survive, the cells lost their cultivability and entered VBNC state to keep their viability.

The antibiotic concentration driven fitness of microorganisms was proven by the FISH results of the isolated *E. coli* strain. Under 2 MIC to 8 MIC of erythromycin, MLS_B resistance levels were significant even though their total and resistant cell densities were relatively low and stable within incubation period. It was caused by the cells in VBNC state, which was one of the adaptive methods of bacteria. For the rest concentrations, the bacterial growth rates or regrowth rates were negatively correlated with antibiotic concentrations. These findings reflected concentration-dependent fitness.

And the results from qPCR gave similar findings on fitness study. As mentioned previously, the fitness involved in this experiment was antibiotic concentration dependent selection. Under 1 MIC, 2 MIC, 4 MIC and 8 MIC, their *erm*K ratios were quite significant during incubation, especially within first day of incubation. The results indicated that 1 MIC antibiotic was an important threshold for bacterial fitness and antibiotic resistance selection. In fact, the study conducted by Hermsen (Hermsen, Deris, & Hwa, 2012) described a term "selective window" for fitness study. It described as the antibiotic or drug concentrations in narrow range near 1 MIC of microorganisms. Under the selective window, the microorganisms experience an effective selection of antibiotic resistance. If the antibiotic concentration is much larger than selective window, both sensitive and resistant organisms may be inhibited; whereas if it is too low, the sensitive organisms may out-compete the resistant ones. The

environment under selective window is called resistant-selective environment, which favored the evolution of antibiotic resistance (Hermsen et al., 2012). Hence, the interesting results around 1 MIC can be explained through above theories. Additionally, the relatively stable cell densities detected by FISH and qPCR under 2 MIC and above can be partially explained by the "selective window", in which their cells' growth was inhibited and entered VBNC state.

6.4.2 Fitness cost and amelioration of fitness costs by compensatory evolution involved in microbial regrowth

The difference between growth rates under varied erythromycin levels and stable cell densities and high erythromycin resistant levels between 2 MIC to 8 MIC of erythromycin cannot be simply explained by biological fitness or nature selection ideas. Another term, biological fitness cost, could be introduced. Most antibiotic resistance mechanisms confer a fitness cost and lead to a declined bacterial growth rate (Andersson & Hughes, 2010). The magnitude of fitness cost can influence antibiotic resistant development rate and stability of resistance (Andersson & Hughes, 2010). The differences in growth rates and stabilized total cell and MLS_B resistant cell densities can be well understood. For example, the fitness cost was proved and detected in erythromycin resistant *Campylobacter jejuni* (*C. jejuni*) with 23S rRNA gene mutation (Almofti, Dai, Sun, Haihong, & Yuan, 2011). The growth rate of ery-resistant *C. jejuni* was slower than susceptible *C. jejuni (Almofti et al., 2011)*. It was because mutations in 23S rRNA were associated with the general translational ability of the bacteria, thus, this mutation might have effect on "rate of the synthesis and the

activity of multiprotein complexes" of the resistant strain (Almofti et al., 2011). However, there are limited studies on fitness cost of MSL_B resistant phenotype and *erm* genotype under exposure of erythromycin. But biological cost could be used to explain the results in this study. Ribosomes are the translational centers of the cell, and erythromycin has an effect on bacterial ribosomes. Microorganisms acquired mutations in their ribosomes (such as *erm* genes) tend to decline in the efficacy of antibiotics, because it could be possible to see a decrease in their growth rate, due to less efficient translation and production of needed proteins (Dodgen, 2008). Moreover, a previous study showed that fitness costs for mutations in the ribosome that lead to antibiotic resistance tended to be high (Dodgen, 2008). Moreover, the decreased viability of cells in the lateral incubation period may be caused by delaying the multiplication of bacteria or synthesis of some proteins required for sustainable survival of the 23S rRNA gene mutation (Almofti et al., 2011). The fitness cost can be used to explain the results from PCM, FISH and qPCR. In addition, *erm*K was located in plasmids, which are inducible resistant genes (Kwon et al., 2006; Marilyn C Roberts et al., 1999). A number of studies have reported that fitness cost is associated with the carriage of resistance-encoding plasmids (Björkman & Andersson, 2000).

As mentioned previously, acquisition of resistance entails a fitness cost. Thus, within a long incubation period, especially in absence of the antibiotic selection pressure, the antibiotic resistant strains can be out-competed by susceptible strains and antibiotic resistance may decline or disappear. However, the results did not support this hypothesis. Under 0 MIC, there were erythromycin resistant colonies formed during incubation. MLS_B resistance

levels and *erm*K gene copies were relatively high within incubation. These observations were caused by compensatory evolution.

Fitness costs conferred by resistance mutations can be partly or fully ameliorated by compensatory evolution without loss of resistance (Andersson, 2003). With these amelioration mutations, the antibiotic resistance could be maintained or even increased (Andersson & Hughes, 2010). For example, streptomycin resistant *rps*L mutants in *S.typhimurium* could be compensated by a variety of mutations in the ribosomal proteins S4, S5 and L19 (Andersson, 2003). And, fusidic acid resistant *fus*A mutants in *S. typhimurium* and *S. aureus* and rifampicin resistant *rpo*B mutants in *E. coli* could be ameliorated by a number of different intragenic mutations (Andersson, 2003). Streptomycin resistant mutant *rps*L in *E. coli* could be compensated by *rps*D and *rps*E (Andersson & Hughes, 2010).

Even though there are limited studies on amelioration of MLS_B resistant mutants in *E. coli* strains, the existence of such compensation regulation can be predicted based on the observed results. The persistence of erythromycin resistance detected in microbial regrowth experiment could be explained. Compensation evolution may lead to stabilization of resistant bacteria.

6.4.3 VBNC cells

Based on the results of microbial cultivability, VBNC cells may play an important role in gene expressions. Above 2 MIC erythromycin, cells were in VBNC state and lost cultivability and activity of some metabolic processes. However, their ability and activity of gene expressions were not ceased,

especially antibiotic resistant gene expressions was not terminated. Moreover, under high antibiotic concentrations, their ability to express resistant genes was better than the ones under low erythromycin concentrations. Thus, antibiotic concentrations could promote resistant gene expression for VBNC cells.

These results also raise the concerns on human health of VBNC cells. Although VBNC cells lose their cultivability and are hardly detected by conventional detection techniques, their viability is still maintained and their antibiotic resistant genes' expression is promoted, thus the risks of antibiotic resistant VBNC cells may be underestimated. In addition, mobility of antibiotic resistant genes makes the concerns more serious.

6.4.4 Microevolution in microbial growth experiments

Microevolution is described as the evolution occurs below the species level, and can result in subspecies (Dodgen, 2008). It is driven by "natural selection, gene flow, random genetic drift, and mutation" (Nadler, 1995). Microevolution normally involves the recombination and short generation time within populations (Stearns, 1986). Microevolution is important on "processes that maintain genetic heterogeneity within populations", because "heritable variability is a prerequisite for effective selection" (Stearns, 1986). The population of *E. coli* cells used in this study was controlled to eliminate the possibility of acquiring antibiotic resistance genes from other microorganisms and allow the cells to be tested for the hypothesis that natural selection and mutations can result in favorable genetic changes. The results of MLS_B resistant levels and gene copies did prove that natural selection and mutations could lead

to favorable genetic changes for effective selection. Thus, the microbial growth under selective pressure of varied erythromycin concentrations can be considered as a microevolution for the isolated *E. coli* strain.

CHAPTER 7 CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

There were some interesting results obtained from these experiments in this study, as listed in the following list:

- Erythromycin is persistent in bacterial liquid culture under neutral condition within seven days' incubation. Under concentrations between 0 to 800 μg/ml, erythromycin only degraded within 20%. Thus, erythromycin degradation in liquid culture can be neglected, and its inhibiting effect did not decline along the incubation.
- Under high antibiotic dosages, bacterial in liquid culture could enter VBNC state to maintain their viability. Since these VBNC cells cannot be detected by conventional culture method, they may pose a potential health risk to humans.
- Microbial regrowth under varied erythromycin concentrations can be regarded as a microevolution for the isolated *E. coli* strain under selective pressure. Through proliferation and spread of erythromycin resistant genes, this strain survived in the antibiotic stressed environment. The selection was antibiotic concentration dependent.
- Antibiotic concentration around 1MIC is "selective window", which is the most effective antibiotic resistance can be selected under this range.
- Both fitness cost and amelioration of fitness costs were involved in microbial growth experiment or microevolution.

- Under extreme conditions, the cells in VBNC can persistently express genes, even antibiotic resistant genes. Moreover, the excessive amount of antibiotic may promote the growth of antibiotic resistant genes and raise antibiotic resistant levels. Hence, the existence of VBNC cells indicates high potential health risks to humans and animals.

7.2 Future study

The following areas need further investigation:

- Insufficient types of erythromycin resistant genes were detected. In this study, only *erm*K gene was detected by qPCR. They only presented the data under one type of antibiotic resistance mechanisms. The experimental results were representative but not comprehensive. Other erythromycin resistance genes, such as *ere* genes, can be quantified.
- Insufficient types of bacteria were detected. In this study, only one type of bacterium, E.coli, a gram-negative bacterium, was selected as the target microorganism. The experimental results were representative but not comprehensive. In order to remedy this imperfection, a gram-positive bacterium, such as Enterococcus, can be included as another target bacterium in this study. The results of E.coli and Enterococcus can be compared. In this way, the understanding of impact of various erythromycin levels to the development of antibiotic resistance can be more in-depth and comprehensive.
- This study could be designed to simulate natural environments. To simply the design of this study, it was conducted in laboratory conditions

instead of environmental conditions, only nutrient gradients and antibiotic concentrations were selected as the parameters. Other interesting factors could be tested, such as horizontal gene transfer in microbial communities under various antibiotic concentrations, and effects of soil particles.

- Insufficient study on fitness cost and correlated compensation mechanisms for MLS_B resistance. Further studies on the types, magnitudes, and associated regulator of fitness cost and compensation evolution are necessary.

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