RNA POLYMERASE INHIBITOR ACTIVITY AGAINST *STAPHYLOCOCCUS EPIDERMIDIS* BIOFILMS

By

SEANGHUOY HO

A thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Microbial Biology

written under the direction of

Dr. Richard H. Ebright

and approved by

_____

_____

_____

New Brunswick, New Jersey

May 2014

ABSTRACT OF THE THESIS

RNA Polymerase Inhibitor Activity Against *Staphylococcus epidermidis* Biofilms

By SEANGHUOY HO

Thesis Director:

Dr. Richard H. Ebright

Biofilms were defined as the structural phenotype of microbial communities enclosed in the self-produced polymeric matrix mainly composed of extracellular polysaccharides (EPS). *S.epidermidis* is associated with chronic diseases involving implant medical devices due to its strong ability to form biofilms and they are difficult to eradicate. Rifampin, one of the most active antibiotics used to treat biofilm-associated infections, is a bacterial RNA polymerase (RNAP) inhibitor; however strong bacterial resistance to this drug leads to the need to find more active compounds acting at the same target: bacterial RNAP. In this study, I test whether other bacterial RNAP inhibitors are also active against *S.epidermidis* biofilms by determining their Minimum Biofilm Eradication Concentration (MBEC) as compared to those of antibiotics functioning through target other than bacterial RNAP (reference antibiotics). Two biofilm assays were used: MBEC P&G assay using Calgary Biofilm device (peg assay) and glass biofilm assay. In the peg assay, biofilm density of 6 $\log_{10}$ of cfu/peg was obtained and used for biofilm susceptibility testing. In addition to Rifampin, three novel synthesized bacterial RNAP inhibitors were found to be more effective in completely eradicate *S.epidermidis* biofilms *in vitro*: 3RHTK27, 3RHTK44 and OMTK13 with MBEC value

of 25µg/ml, 12.5µg/ml and 50µg/ml, respectively. None of the other antibiotics were able to eradicate *S.epidermidis* biofilms (MBEC > 400µg/ml). These three RNAP inhibitors were also found to be effective in glass biofilm assay having MBEC values of 6.25µg/ml, 3.125µg/ml and 25µg/ml, respectively. These three compounds are interesting candidates for further development as antibacterial and anti-biofilm agents. Not all RNAP inhibitors which are active against planktonic cells are active against *S.epidermidis* biofilms. Nevertheless, seeing the same pattern of result from two different assays that reference antibiotics tested were not capable of eradicating biofilms while three out of eleven bacterial RNAP inhibitors tested were active is encouraging and suggesting that RNAP makes a good target for finding drugs to treat biofilm-related diseases.

Acknowledgements

I would like to express my sincere thanks to Dr. Richard H.Ebright for giving me the opportunity to work in his wonderful lab. I surely appreciate his great guidance and patience during the past years of my graduate studies. With his support and encouragement, I am able to learn new things and tackle challenges in scientific research. I also want to extend my deep thanks to Dr. Michael Chikindas and Dr. Bryce Nickels for being as my thesis committee members and giving excellent advice.

I would like to express my sincere thanks to Dr. Gerben Zylstra, my program director who has been really supportive during my graduate study here and helped guide me to be well-prepared for school work.

I would like to offer my sincere thanks to all lab members who are always helpful in giving good advices and assisting in any technical problems occurred during my research. I want to express my special thanks to David Degen, Soma Mandal, and Shuang (Carol) Liu for their assistance and advice.

I want to express my thanks to my parents for their unconditional love and support. I am inspired by their encouragement to be able to move forward for a better future. Their motivation is what gives me strengths and energy to fulfill my goal.

Last but not least, I would like to express my profound thanks to my husband for always being here for me and help me go through this difficult time. I want to thank him for being proud of me and showing me that everything can be achieved as long as we have strong commitment. His role is beyond a husband; he is a father, a brother and a close friend to me. I feel blessed to have him in my life.

Table of contents

List of Tables

List of Figures

Chapter I INTRODUCTION

Traditionally, microorganisms have been identified and characterized as single cells or in a planktonic form; however studies on sessile microbe communities from various environmental sources have demonstrated that the planktonic form is not the common form of many microorganisms; instead about 99% of them survive as "biofilm"[1]. Biofilms were first described in 1978 as a structured phenotype of a microbial community enclosed in self-produced polymeric matrix mainly composed of extracellular polysaccharides (EPS) and other components including nucleic acids, proteins and lipids [1, 2]. To form this cell aggregation, freely-floating cells initially require attachment to both biotic and abiotic surfaces- involving various alterations physiologically and genetically during the transition from planktonic state to surface-attached community [2]. Biofilm-related infections have now become a big concern in medical applications because they are very difficult to eradicate by both conventional antibiotic therapy and host immunity [2]. Biofilms can form on many implant medical devices such as catheters, artificial hips, contact lens, artificial heart valves and artificial joint [3]. Removal of the implant devices sometimes is the only way to treat persistent biofilm infections that do not respond to antibiotic therapy [3]. According to the announcement made by National Institute of Health, more than 60% of all infections are caused by biofilms [4]. Approximately 65% of nosocomial infections are also associated with biofilm which cost the US government about $1 billion annually. [3].

Staphylococcus epidermidis is an opportunistic pathogen and is the species that is most commonly found to be associated with hospital-acquired infections due to its strong

ability to attach to surfaces and form biofilm on implanted medical devices [5]. Traditionally-available antibiotic therapy is often not sufficient in combating the *S.epidermidis* infection. Thus, there is an urgent need for new agents and new strategies that can be combined with current drugs to effectively treat these biofilms. To fulfill this urgent need, many research groups are now being attracted to find better agents against bacterial biofilms. New generation of antibiotics such as tigecycline, daptomycin, linezolid, arylomycins, etc are becoming increasingly important therapeutic alternatives or can be used in combination with current treatment if having synergistic effects [5]. Beside antibiotics, researchers also have much interest in using natural substances that can be used against *S. epidermidis* including alcohol, aminoacid N-acetylsysteine (NAC) and several other essential natural oils [5]. Based on the compositions that make up the biofilms, others have recently also described novel treatment strategies have recently been described, involving using biofilm-disrupting enzymes such as amylase, lyase, lactonase, etc. to disperse the biofilms [6].

Although there are several alternatives approaches which can be used against biofilms, antibiotic treatment is still one of the most important options for the patients with chronic biofilm infections especially for those who are unable to endure the surgical procedures necessary to remove the infected implanted devices [7]. Some typical examples of antibiotics which are used against both actively-dividing cells and dormant cells include DNA, RNA and protein synthesis inhibitors. Notably, RNA polymerase (RNAP) inhibitor such as Rifampin (Rif) is one of the most effective therapies to treat biofilm-related infections, especially when used in combination with other drugs [7]. However, prolong use of rifampin has been shown to result in the development of

bacterial resistance to the drugs [7]. Thus, it is very important to perform further investigation on other molecule inhibitors that act against the same cellular target, i.e bacterial RNAP.

We hypothesize that other bacterial RNAP inhibitors would also eradicate *S. epidermidis* biofilms. To test this hypothesis, we used two *in vitro* biofilm-forming systems developed by previous researchers: one of which is a new technology from MBEC P&G Calgary Biofilm Devices (peg assay) and the other one is a system known as glass biofilm assay system (glass biofilm assay).

The first objective of this thesis is to determine the Minimum Biofilm Eradication concentrations (MBECs) of bacterial RNAP inhibitors and some other reference compounds against *in vitro S. epidermidis* biofilms using the commercial peg assay. The second objective is to determine MBECs of biofilms from the same organism against the same compounds in glass biofilm assay.

The significance of this work is to hopefully identify several other potential lead compounds which can be further investigated in *in vivo* system for the treatment of *S.epidermidis* biofilm-related diseases. More studies on drug combinations starting from these compounds with other common known drugs can be made possible by this study.

Chapter II LITERATURE REVIEW

II.A. Global biofilm-associated infections in healthcare system

Nosocomial-associated disease is the fourth leading cause of death in the US with 2 million cases every year at the cost of more than $5 billion [8]. Approximately 60-70% of nosocomial infections are related to some sorts of indwelling medical devices such as prosthetic heart valves, orthopedic implants, intravascular catheters, artificial heart, urinary catheters, cardiac pacemaker, contact lens, etc. In the US, it is estimated that over 5 million such implant devices are used annually and microbial infections (specifically biofilms) were found to be associated with most of those devices [8]. Besides these implanted devices, tissue engineering constructs, biomedical devices or biomaterials are also susceptible to microbial infections [8]. According to the 2007 report by Center for Disease Control and Prevention (CDC), 1.7 million infections and 99,000 associated deaths annually in the American hospitals are healthcare-associated (or nosocomial-associated). Among these infections, 32% are urinary tract infections, 22% are surgical site infections, and 15% are lung infections. The European Center for Disease Prevention and Control (ECDC) also reported in 2007 that annually 3 million people in European countries have healthcare-associated infections, consequently leading to 50,000 estimated deaths. [8].

From these statistical data, it is obvious that biofilm-related infection is a serious problem in health care settings which not only claim many human lives worldwide but also cost a lot of money. Thus, there is an urgent need to seek new approaches to treat biofilm infection and, to do so, it is very important to understand biofilm properties.

Since the study of early infections was limited to only planktonic cells and since it is obvious that biofilm form is more common in clinical environment [8], researchers are now beginning to thoroughly investigate biofilm properties to shed some light on how to improve the treatment of biofilm infections.

II .B. Mechanism of biofilm formation

Life cycle of biofilm development involves mainly three important stages: attachment, maturation and detachment (Figure 1) [6]. (Some researchers divide the biofilm formation mechanism as early, intermediate and mature [9]; however, the concept is similar). The attachment step starts with initial attachment of the planktonic cells to the biotic or abiotic surfaces with strong adhesion [2, 6, 9]. Usually, the cells can either use their flagella movements or they are passively transferred to the surfaces by bodily fluids [6]. In medical devices, this step usually occurs 1-2 hours after implantation [2]. This initial attachment stage is reversible and associated with several forces such as van der Waal, gravitational, hydrogen bonds or hydrophobic interactions [2]. It should be pointed out that in this early attachment the bacterial cells are still susceptible to antibiotic treatment, which is important to successfully treat the infection [6]. The maturation stage involves irreversible attachment of the cells to the surfaces where they multiply and form a microcolonies producing a large amount of polymer matrix primarily polysaccharides (approximately 50-90% of the total organic matter in biofilm) [6]. In this step, the cells continue to proliferate inside the EPS matrix and mature as mushroom-shaped microcolonies (or other shapes) containing more complex materials such as polysachharides, proteins, lipids, nucleic acids and many other cell components [6]. Other essential adhesion factors are also involved in this step. The matured biofilms are

characterized by a very large number of non-growing or slow-growing cells positioned in the center of the matured biofilms. In the detachment step of the biofilm life cycle, the dissemination of microbial cells on the top of the matured biofilm occurs, transitioning from surface-attached to the planktonic state. Disruptive factors such as Phenol-soluble modulins (PSMs), proteases and nucleases facilitate in detachment step [9,10]. Detached cells are then liberated to spread and attach to other surfaces as they travel through the blood stream, causing expansion of the infections [2, 6].

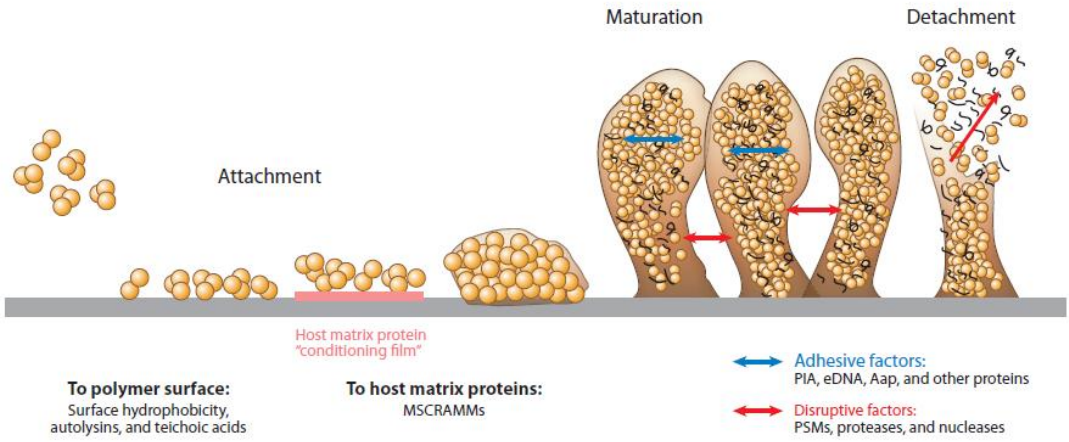


Figure 1: Phase of staphylococcal biofilm development: Attachment, Maturation and Detachment steps with necessary required factors at each step [10] (reproduced with permission from the author).

II. C Driving factors for bacteria to form biofilm

The clinical relevance of bacterial biofilms was initially proposed about four decades ago by Dr. R.J Gibbon who demonstrated the important role of polysaccharides formation on teeth by the bacteria *Streptococcus mutans* [11]. Following that observation, bacterial biofilms became increasingly important and many studies were conducted in order to understand why bacteria switch from a planktonic state into a biofilm. Using the

available data from those researchers, K.K. Jefferson reviewed several significant factors suggesting the reasons why bacteria switch from planktonic to biofilm form [11]. Taking Darwin' theory of evolution into consideration, he pointed out that reproductive fitness is the only real driving force for any action of the microorganism to switch from planktonic state to biofilm. Four models regarded as advantages for biofilm were discussed in his review article: defense, favorable habitat, community, and default mode.

Although a complete understanding of biofilm resistance is not yet achieved, we now know that bacteria form biofilms as one of the defense mechanisms against external environment and host immune response [12]. Biofilms are resistant to lack of nutrients, pH changes, oxygen radicals, disinfectants and antibiotics. They are also resistant to host immunity's actions including the washing force of saliva, blood flow, and phagocytosis [11]. This stress response of biofilm is mediated by the formation of EPS matrix which plays an important role in protecting the biofilm in the harsh environments and dehydrating conditions [1, 11]. EPS also assists in cell aggregation and gives the architectural structure of the biofilm matrix [1].

One of the other reasons which suggest why bacteria switch from planktonic into a biofilm form is to remain in its favorable niche. The possible explanation given for this factor is due to the idea that bacteria are continuously trying to invade human and animal bodies where oxygen availability, nutrients and preferred temperature can be found. When the situation is compromised between the host and microorganism, bacteria tend to remain fixed on the bodies as their mode of growth [11]. A number of bacterial surface proteins known as MSCRAMMs (microbial surface component recognizing adhesion matrix molecules) have been identified among various bacteria species such as

Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus pyogenes,etc and these proteins are known to play an important role in initial attachment of bacteria to the surface within the host by binding to host surface proteins such as fibrinogen, fibronectin and elastin [11].

A third advantage for the biofilm mode of growth is existence as community. It is widely accepted now that in nature bacteria mostly are found in the biofilm form. Biofilms are regarded as complex, heterogeneous and interactive communities between both the same or different microorganism species [11, 12] and the complexity in the community give biofilms advantages, including division of metabolic burden, gene transfer and selfless behavior [11]. Division of metabolic burden refers to the different growth requirements of different bacteria species within the same biofilm. Usually, oxygen availability is not evenly distributed throughout the biofilm (more oxygen on top and less as deeper into biofilm), thus the bottom of a biofilm provides good condition for anaerobic bacteria while the upper part is good for aerobic bacteria [11]. Another important concept of being as biofilm is the advantage of gene transfer. Vertical gene transfer (from parents to daughter cell) is not common in biofilm since they are not actively dividing cells, but biofilms are great environment for horizontal gene transfer which is a genetic exchange between two microorganisms. The process of conjugation (exchange genetic material between bacterial cells) requires close proximity between cells and biofilm communities are ideal conditions for that process. Toxin-encoding or antibiotic-resistance genes are some of those typically transferred on plasmids between bacterial cells within biofilm [11]. Lastly, there is some evidence from mathematical modeling that suggests that biofilm communities are actually altruistic or selfless

environments. The beneficial symbiotic relationships results in greater fitness for the bacteria [11].

The last hypothesis presented by K.K. Jefferson suggests that bacteria form biofilm as their default mode of growth and only a solid surface is necessary and sufficient for bacteria to attach and form a biofilm. A vast amount of evidence on gene regulation in biofilms was obtained which explain the signaling system bacteria use to sense the surface and the gene expression as response to those environmental signals [11]. We will discuss some of the main genetic regulations observed in biofilm in the next section.

II. D. Signaling systems in bacterial biofilm

Switching from planktonic state to biofilm state is highly governed by regulatory networks which integrate environmental signals through bacterial sensing systems [13]. There are two important signaling systems used by bacteria to sense external environmental stimuli that are associated with biofilm structure formation: the two-component system (TCS) and extracytoplasmic function (ECF) signaling pathway [13]. TCS contains two proteins, sensor protein histidine kinase (HK) and the response regulator (RR) [13, 14]. After an external signal is sensed (such as nutrients, temperature, ions, etc), HK is activated by autophosphorylation in conserved histidine residue followed by phosphoryl group transfer to the RR on a conserved aspartate residue [14]. Activated RR acts as transcriptional regulator. One of the typical examples of TCS in *P.aeruginosa* is GacS (HK)/GacA (RR), which controls biofilm formation in this bacterium. Upon activation by signals, GacS/GacA TCS turn on transcription of *rsm*

genes which encode a couple of small-non coding RNA (sRNA): RsmY and RsmZ, both of which are responsible for controlling the switch between a planktonic and a biofilm phenotype [13]. Biofilm formation is observed when these two genes are highly expressed while lower expression results in biofilm impairment [13]. TCS-dependent regulation of biofilms is also found among many other bacteria such as Staphylococcal biofilms (like *S.aureus*) which involve the GraS (HK)/GraR (RR) system [13] and represents an example of polysachharide-independent biofilm formation [15].

The second important signaling system is the ECF signaling system which involves sigma and anti-sigma factor. Sigma factor (σ) is an initiation factor responsible for specific promoter DNA recognition during bacterial transcription [16]. It binds to RNAP core enzyme to make RNAP holoenzyme [16]. There are different sigma factors required for recognition of different promoter sequence during transcription as a response to different signals; for example transcription in *E.coli* requires σ^{70} for controlling most genes and σ^{32} for controlling heat shock genes (the superscript on sigma indicates the protein size) [17]. Anti-sigma factor inhibits sigma factor and several other periplasmic or outer membrane proteins [13]. Upon signal detection, anti-sigma factor is degraded which results in release of the sigma factor. Sigma factors can then promote transcription of specific genes associated with EPS formation [13]. For example, AlgU sigma factor control EPS production in *P.aeruginosa* [13]. AlgU sigma factor functions with the anti-sigma MucA whose C-terminal can be cleaved by AlgW/AlgP proteases [13]. The activation of AlgW/AlgP results in anti-sigma factor MucA cleavage from sigma factor AlgU. The released sigma factor AlgU can then activate the transcription of *alg UmucABCD* operon involving in EPS alginate production in this bacterium [13].

Besides these two signaling systems, there are other systems which are thought to be important in biofilm formation such as the quorum sensing (QS) system. QS plays an important role in bacteria' communication via autoinducer production, which enhance bacteria' population density upon reaching a concentration threshold. QS is known to regulate transcription of genes related to virulence and pathogenicity [13]. One of the important signaling molecules in biofilm formation is c-di-GMP (cyclic diguanylic acid). Higher c-di-GMP level promotes biofilm formation by either increasing EPS production or adhesive organelles and decreasing bacteria motility [13]. c-di-GMP is highly controlled by different enzymatic activities i.e activity of diguaylate cyclases (DGC) carrying GGDEF domain result in higher amount of c-di-AMP while activity of phosphoesterase (PDE) carrying EAL domain results in lower amount of this messenger molecule [13, 18].

Although many signaling systems have been investigated, the phenomena in biofilm lifestyle is far more complicated and this requires further extensive research on biofilm genetics in order to obtain a comprehensive picture of how bacteria integrate all these system to form a multicellular community [13].

II.E Biofilm resistance mechanism to antibiotics

Biofilms pose a lot of challenges for scientists to successfully develop effective therapeutic strategies in order to treat biofilm-related infection. The difficulty is due to the fact that this phenotypic structure of pathogenic microbes is more resistant to host immune response and antibiotics which are normally active against planktonic cells. Some studies have suggested that the concentration needed to eradicate a biofilm is a

thousand times higher than that needed to kill or inhibit the same organism in its planktonic state [3]. In order to develop effective therapeutic treatment, it is critical to understand how a biofilm becomes resistant to antibiotics. It is obvious to researchers that multiple resistance mechanisms exist in this community [3]. Recent studies have revealed several explanations and hypotheses which help us to understand how the biofilm communities confer resistance to antibiotics. It is believed that the familiar resistance mechanisms such as efflux pump, target mutation and modified enzymes are unlikely to be responsible for biofilm resistance [19]. Nevertheless, there are several other biofilm-specific resistance mechanisms discussed in many articles: failure of antibiotics to fully penetrate the biofilm, decreased growth rate in biofilm and general stress response [3, 4, 20] .

1. **Antibiotic penetration within the biofilm**

One of the hypotheses about biofilm resistance is that the self-produced EPS matrix in biofilms acts as the diffusion barrier to antibiotics by not allowing the antibacterial compounds to fully penetrate inside the biofilm. Small positively-charged aminoglycoside antibiotics are retarded by the negatively-charged exopolysaccharides [4, 20]. Some studies have found that limiting drug penetration assists in postponing cell death by providing more time for bacterial to produce necessary enzymes to degrade the antiobiotics, resulting in biofilm resistance [4]. The synergistic effect between antibiotic retardation and degradation provides an effective resistance mechanism observed in β-lactamase-producing *Pseudomonas aeroginosa* biofilms against β-lactam antibiotic [21].

Nevertheless, several other groups of researchers have addressed the same question and found different results. Z. Zheng. and P.S. Stewart measured the Rif

penetration through *S.epidermidis* biofilm and were able to show that Rif uniformly penetrated into the biofilm especially near the air interface [20]. However, Rif failed to kill the biofilm which led to the conclusion that biofilm protection is not due to the lack of penetration but rather there are other alternative resistance mechanisms[22]. Another similar finding about drug penetration through *Klebsiella pneumoniae* biofilm was described by Anderl et al. The study found that ampicillin could not penetrate the biofilm producing β-lactamase but could penetrate in mutant lacking β-lactamase, suggesting that β-lactamase is responsible for the penetration limitation [21]. Interestingly, mutants lacking β-lactamase grown in biofilms are still resistance to ampicillin. A similar result was also observed with ciprofloxacin which was able to penetrate the biofilm but could not eradicate the biofilm [23].

According to these results, it is obvious that even though EPS was shown to slow down drug penetration through biofilm, the diffusion barrier property of EPS may play only partial role in biofilm resistance against antimicrobial agents; this is because in some results drugs could not kill the biofilm even though they can penetrate into biofilm. Therefore, there must be other alternative mechanism exists such as reduced growth rate [3].

2. Reduced growth rate in biofilm

Slow growth is observed in biofilm and it has been claimed to be associated with their antibiotic resistance [24]. As we discussed earlier, one of the possible explanations why bacteria transit from planktonic into biofilm form is for survival. In its dormant stage, bacteria can be more resistant to antibiotics which normally are most effective against actively dividing cells. Many antibiotics require that bacterial cells be rapidly

growing in order to be effective; for example ampicillin and penicillin, which target cell wall synthesis, work only against actively dividing cell and are unable to kill non-growing or slow-growing cells [4]. Changes in the microenvironment within a biofilm play a significant role in leading the bacteria to turn into its non-growing form. Oxygen and substrate availability, for example, vary throughout the biofilm, where more oxygen and substrate can be found on the surface and are depleted deeper into the bottom. This variation can possibly reduce the growth rate, slow down protein synthesis and other metabolic activities especially cells at the bottom [19, 25]. In addition, acidic waste products might lower the pH, which subsequently affect antibacterial compound activity [19].

3. Biofilm stress response

Recent studies have proposed that nutrient limitation does not cause slow growth rates within biofilm *per se*, but instead initiates stress responses, leading to reduced growth rates [3]. Stress response results in enhanced mutability and physiological changes that protect biofilm against environmental stresses [3, 25]. Biofilm-based bacteria have been shown to have increased mutation frequencies which is likely related to the increased horizontal gene transfer within the biofilm community [25, 26]. Genetic mutations involved in DNA repair systems such as the mismatch repair system (*mutS, mutL*, and *uvrD*) and the DNA oxidative lesions repair system (*mutT, mutY* and *mutM*) from biofilm cell isolates have additionally been described to determine antibiotic resistance, especially in multidrug-efflux pumps expressing cells [25]. Increased generation of reactive oxygen species (ROS) leads to oxidative stress, which ultimately assists biofilm in acquiring genetic adaptation and evolutionary change [25]. Bole and

Singh have demonstrated that endogenous oxidative stress stimulates antibiotic resistance of *Pseudomonas aeruginosa* biofilms [25, 27].

In summary, there are multiple possible sources of biofilm resistance to antibiotics. As discussed above, all of these could possibly lead to increased levels of resistance. Besides the three hypotheses discussed here, mechanism such as quorum-sensing, biofilm-specific resistance genes, persister cells and heterogeneity of the biofilm itself should also be taken into consideration as sources of antibiotic resistance of biofilm.

II. F. *Staphylococcus epidermidis* biofilm and chronic infection

Staphylococcus epidermidis is a Gram positive opportunistic pathogen that predominantly causes infections in immune-compromised individuals such as AIDs patients, IV drug users, and newborns [9]. *S.epidermidis* is the most common coagulase-negative staphylococci (CNS), accounting for 60-90% of all staphylococci recovered from blood [28]. *S.epidermidis* is normally found over the surface of human body, where it makes up the majority of commensal bacteria microflora [29]. Although previously it was regarded as non-pathogenic and as a contaminant when found from blood samples, *S. epidermidis* is now widely known to cause infective complication in patients receiving surgical vascular grafts or medical device implants [9, 29]. Furthermore, 50-70% of catheter-related infections have been shown to be associated with this microorganism. Methicillin-resistant and vancomycin-resistant *S. epidermidis* infections have occurred, resulting in treatment difficulty [29]. Emergence of infection caused by this microorganism is associated with the bacteria's ability to adhere to and form biofilm on both abiotic and biotic inert surface such as the prosthetic medical devices and host tissues. [5, 7, 9, 29]. Patients with *S. epidermidis* biofilm-related infections can develop

diseases such as septicemia and endocarditis [9]. Some other *S. epidermidis*-specific infection include bacteremia, central nervous system shunt infection, urinary tract infection and endophthalmitis [30].

Trends in medical practices are regarded as the major cause leading to more occurrences of hospital-acquired infection related to *S. epidermidis*. These trends include the increase use of medical device as the supportive care for hospitalized patients in the last 2-3 decades, wide use of broad-spectrum antibiotics to treat infections in hospitalized individuals (resulting in emergence of drug-resistance *S .epidermidis* strains) and the growing number of immune-compromised patients who become vulnerable to *S. epidermidis* infections [28].

Due to its strong ability to form biofilms and cause chronic infections associated with implanted medical devices, *S. epidermidis* is now one of the well-studied biofilm-related microorganisms [9]. The genome of two *S.epidermidis* isolates (ATCC12228 and ATCC35984 or RP62A) has been fully sequenced [9, 31]. *S.epidermidis* ATCC 12228 is a non-biofilm forming strain isolated in the US and usually is used as a reference strain in biofilm study as compared to the *S.epidermidis* ATCC 35984 which is a biofilm-forming strain originally isolated in Mumphis, Tennessee during 1979-1980 outbreak of intravascular catheter-associated sepsis [32-35]. Studies on *S.epidermidis* biofilm formation has revealed an important gene cluster *ica* which contains all genes required for production of polysaccharide intercellular adhesion (PIA), one of the major polysaccharides found in *S.epidermidis* biofilm [29]. The *ica* locus consists of an operon *icaADBC* for PIA synthesis. IcaA is a transmembrane proteins; IcaB and IcaC funtions are not yet clear but IcaB is proposed to be secreted while IcaC is predicted to be integral

membrane protein; IcaD was shown to be involved in N-acetylglucosaminyltransferase activity [29]. Upstream of the operon, there is an *icaR* gene, whose product may functions as the transcriptional regulatory of *ica* structural genes [29, 31]. It should also be noted that *S. epidermidis* isolated from biomaterial infections encoded the *icaADBC* genes, while other *S. epidermidis* isolated from different sites like skin of healthy individual do not contain these genes; however, according to the studies, *S.epidermidis* mutants lacking icaADBC gene outcompete its wild type when colonize on skin. This suggested that PIA synthesis causes fitness cost when colonize on skin [31]. Besides PIA, recent studies have revealed several important proteins involving in cell agglomeration in PIA-independent *S.epidermidis* biofilm [10, 36]. These proteins include accumulation-associated protein (Aap) and extracellular matrix binding protein (Embp) [36]. Although PIA-, Aap- and Embp-mediated biofilm exhibit distinct morphological differences, all are effective in protecting the biofilm against phagocytosis [36].

To better understand biofilm physiology, Yao et al. has conducted a genomewide microarray analysis of gene expression in *Staphylococcus epidermidis* biofilms and found a number of genes that are up-regulated and down- regulated as compared to the planktonic cells [37]. *S.epidermidis* biofilms have lower expression of several genes involved in aerobic metabolism, several ribosomal proteins and lower expression of proteins RNA polymerase. This indicates that *S.epidermidis* biofilms have undergone a reduction in normal cellular processes such as energy production, translation and transcription respectively as compared to planktonic cells [37]. In contrast, *S.epidermidis* biofilms show increased transcription of several genes related to resistance (antibiotic resistance determinant Drp35, and zinc resistance proteins Czrb and CapC, part of poly-γ-

glutamte PGA biosynthesis complex), chaperones and stress factors (Hsp33 heat-shock proteins, Rec DNA repair proteins) and osmoprotection factors [37]. In addition, there is increased expression of genes involved in fermentation, reflecting the metabolic shift from aerobic respiration into fermentation which can be the result of low oxygen availability throughout the biofilm. Expression of pro-inflammatory peptides such as phenol-soluble modulins (PSM) was suppressed, which can enable cells to stick together and avoid host defense [37]. With all these findings, two mechanisms have been proposed for biofilm physiological alterations including decrease susceptibility to antibiotics and harmful molecules and a switch to nonaggressive state by reducing inflammation and attraction of immune cells to infected site [37].

II. G. Approaches for *S.epidermidis* biofilm treatment

S.epidermidis-related infection treatment becomes more troublesome not only because it is easily contaminated in the clinical setting but also because there is increased resistance of biofilm to various antibiotics and host defense [7]. Treatment of biofilm-related infection with indwelling medical devices often does not show improvement unless the devices are surgically removed [7, 29].

However, there are a number of studies attempting to find a better way to treat biofilm at least in the laboratory by either prevent biofilm to form or eradicate the formed biofilm. Together with traditional antibiotic treatment, other alternative approaches have been discussed. Those include enzymatic treatment (dispersin B, DNases and Lysostaphin), quorum-sensing system (*agr* system), natural substances (Farnesol, essential oils), and some other small molecules [5, 7]. Interestingly, one approach

involving using antimicrobial agents together with passage of low voltage electrical current has been shown to be effective in killing surface-attached bacteria through the generation of protons, hydroxyl ions, reactive oxygen intermediates, hydrochlorous acid, etc [29, 38, 39].

To overcome antibiotic resistance, several new generation antibiotics have been developed including linezolid, daptomycin, tigecycline, quinupristin/dalfopristin, and dalbavancin. Together with rifampicin, an RNA polymerase inhibitor, these drugs can be combined to have better treatment [5].

II. H. RNA Polymerase: also an attractive drug target in biofilm?

Most antibiotics targeting actively dividing cells such as penicillin or ampicillin are not effective against *S. epidermidis* biofilms. So far, there are only a few antibiotics which are active against *S .epidermidis* biofilms, as stated earlier, one of which is bacterial transcription inhibitor rifampin [40]. However, since the introduction of Rif, there has been a remarkable increase in the development of Rif resistances [40, 41]. For this reason, Rif is usually prescribed in combination with another antibiotics to treat biofilm-related infections and overcome antibiotic-resistance [40, 41].

RNA polymerase (RNAP) is the enzyme that catalyzes the synthesis of RNA from DNA template. There are 5 subunits in the bacterial RNA polymerase core enzyme including β', β, αI, αII and ω which are coded by *rpoC, rpoB, rpoA* and *rpoZ* respectively (*rpoA* codes for both αI and αII) [42, 43]. β' and β are the two biggest subunits in the enzyme. Bacterial RNAP is an attractive and proven target for antibiotics due to several reasons: (I) It is an essential enzyme in gene expression and regulation, thus it is

important for bacteria grow (efficacy), (II) it is highly conserved among bacteria, thus RNAP inhibitors can act against a broad-spectrum of bacteria species (broad-spectrum activity) and (III) it is different from eukaryotic RNAP (therapeutic selectivity) [44, 45].

Rifampin (or rifampicin), a semisynthetic derivative of Rifamycin group produced by *Streptomyces mediterranei*, which was reclassified as *Nocardia mediterranei*, was isolated in 1957 [46]. In many studies aiming to eradicate *S.epidermidis* biofilms, Rif has been shown to remain one of the most effective antibacterial agents usually at low concentration and relatively short exposure times [47, 48]. Rifamycins inhibit bacterial RNA synthesis by binding to a site adjacent to bacterial RNAP active center , blocking RNA extension beyond 2-3 nucleotides in length and resulting in a bactericidal effect [45, 49]. Rifamycins are also effective in treatment of Gram-positive and gram-negative pathogens and are used as the first-line antituberculosis agent with rapid activity in clearing the infection and preventing relapse [45]. However, at higher concentration, rifampin-resistance usually followed and for staphylococci, mutation in the gene *rpoB* usually lead to rifampin-resistance; even single point mutation can confer high resistance [7] . This mutation rate is at frequency of 10^{-6} [49]. Many studies, therefore, tested combination therapies by including Rif with other drugs. Some drugs exhibit rapid synergistic effects with Rif, especially the cell-wall targeting antibiotics (such as cloxacillin, cephalothin, cefazolin, and cefamandole) while others have little synergy (vancomycin, tetracycline, and amikacin) [50]. In contrast, some drugs were shown to be antagonistic to Rif including gentamicin while others did not have any influence such as tobramycin, erythromycin, clindamycin, and fusidic acid [50].

Rif is very effective in staphylococcal biofilm treatment; however, the reasons why it is so effective are still not yet well understood [7] . Rif has excellent pharmacokinetic properties, readily penetrates into biological membrane such as biofilms, and is effective on dormant cells or slow-growing bacteria [49]. In addition, Rif is still bactericidal in the acidic environment found in some microenvironment in mature biofilms (increasing acidic metabolites lowers the pH). However, as we discussed earlier in some studies, good drug penetration alone does not necessarily eradicate a biofilm. So there must be another mechanism involved, especially since RNA synthesis plays an important in biofilms. Feldon et al. has described the increasing roles of small RNAs which regulate a number of bacterial cell processes, especially in response to stress or environmental changes and virulence [51], so one proposal for the effectiveness of Rif against biofilms is that this drug's action would inhibit the production of those small RNAs, leading to the loss of ability to generate these important regulatory molecules and ultimately kill biofilm [7].

To further address whether the efficacy of Rif is due to the structural properties of rifampin or the favorable target of RNAP, Villain-Guillot et al. investigated the behaviors of several additional bacterial RNAP inhibitors against *in vitro S. epidermidis* biofilms [52]. Seven structurally-different RNAP inhibitor families were tested. Those include Rifamycins (rifampin and two recently commercialized analogs rifampentine, and rifaximin), streptolydigin, lipiarmycin , pyrrothines family (thiolutin and holomycin), synthetic molecules (CBR703 and CBR64), SB2 and ureidothiophene. The conclusion from this study is that the physiochemical properties (hydrophobicity or size) are not the only critical factor to explain why Rif is better than other drugs. Most of the tested

compounds are active against biofilms at the concentration close to their MICs; however, at higher concentration (16X MIC), those drugs could not fully eradicate biofilms. Another important conclusion for this study is that all antibiotics that directly target RNA polymerase (rifampin, rifampentine, rifaximin, streptolydigin, lipiarmycin and CBRs) are active against *S. epidermidis* biofilms, suggesting that RNAP is possibly an attractive target which can be used to generate the drugs against bacterial biofilms [52].

Gualtieri et al. also studied the *in vitro* activity of a new bacterial transcription inhibitor known as SB13 against *S.epidermidis* biofilm [53]. The result showed that SB13 activity against biofilms is as efficient as Rif at a concentration close to its MICs and the concentration that is often used in biofilms. This result further suggests that antibiotics targeting transcription have a similar efficacy against biofilms [53] i.e suggesting RNAP as a good target for biofilm eradication.

II.I. Characteristics of tested compounds:

In this study, we attempt to further test more bacterial RNAP inhibitors, in search of more compounds that are effective against *S.epidermidis* biofilms. Beside Rif, we test ten bacterial RNAP inhibitors: two of which were commercially available including streptolydigin and lipiarmycin A3; four compounds are in collaboration with other labs including myxopyronin B (PY3a) (also synthesized in Ebright's lab), ripostatin A (naturally produced), APY61, and APY116; four other compounds were directly synthesized in Ebright's lab including PY63 (Y.E and R.H.E, unpublished), 3RHTK27, 3RHTK44 and OMTK13 (J.Shen and R.H.E, unpublished). The reasons why these compounds were selected for this study fall into one of the following: 1. They are all

bacterial RNAP inhibitors either confirmed by genetic and structural analysis or transcription assay; 2. They exhibit little or no cross resistance to Rif; 3. They have never been tested against *S.epidermidis* biofilms except streptolydigin and lipiarmycin A3 which have been tested anywhere else using different biofilm assay [52].The following describes the origin of these compounds, their antibacterial activities and mechanism of actions against bacterial RNAP.

Bacterial transcription reactions involve three main important steps: initiation, elongation and termination [16]. Transcription initiation is a multiple-step process [43, 45]. 1. The binding of RNAP to the promoter to make RNAP-promoter closed complex (RP_c) in which the DNA template is in its double-stranded form and is outside the active center cleft. 2. The insertion of position -11 to +15 of promoter DNA by RNAP into its active center cleft to make RNAP-promoter intermediate complex (RP_i). 3. Unwinding the position -11 to +2 of promoter DNA by RNAP to yield RNAP-promoter opened complex (RP_o) in which 14 nucleotides of the DNA is now unwound as the single-stranded DNA in the active center cleft. 4. Synthesis of first 10-15nt of the RNA product to make RNAP-promoter-initial transcribing complex (RP_{itc}) through scrunching mechanism [54] in which the RNAP remains anchor on the promoter, pulling and unwinding the downstream DNA into its active center [54]. 5. Transcription elongation proceeds by breakage of RNAP from the promoter to yield RNAP-DNA elongation complex through a process called “stepping mechanism” in which RNAP translocates in each nucleotide addition step [55]. 6. Transcription termination occurs as RNAP reaches termination signal on the DNA template (factor-independent termination) or with the facilitation of termination factor such as Rho (factor-dependent termination) [56].

Streptolydigin (Stl, MW = 600.70 Da), a broad spectrum antibiotics originally produced by *Streptomyces lydicus* is a bacterial RNAP inhibitor at elongation step and it does not inhibit eukaryotic RNAP [57, 58]. Stl can also be synthesized as a derivative of 3-acetyl-tetramic acid [58]. Stl has only partial cross-resistance with Rif and other RNAP inhibitors such as microcin J25 [58, 59], CBR703 [58, 60] and sorangicin [58, 61]. Crystal structure of Stl-*Thermus thermophilus* core RNAP complex demonstrated that Stl's binding involves both β and β' subunit of RNAP active center, specifically binds along the bridge helix (BH) at the distance of approximately 20 A^o from the catalytic Mg^{2+} ion [58]. Recent study shows that non-catalytic Mg^{2+} ion is also required for Stl binding [58]. The ability of Stl to inhibit all RNAP catalytic activities can be explained by the structural and biochemical analysis which showed that Stl affects the conformation of the trigger loop (TL), a flexible domain in the active center that is essential for catalysis.[58].

Lipiarmycin or Lpm (or also known as fidaxcimicn, clostomicin, tiacumicin, diffimicin, PAR 101, OPT 80 and dificid, MW = 1058 Da) is an 18-membered macrocyclic-lactone antibiotics produced by Actinomycetes species such as *Actinoplanes deccanesis, Micromonospora echinospora* ,and *Dactylosporangium aurantiacum hamdenensis* [44, 45, 62]. The different names of Lpm are applied to the strains it is produced from. In particular, Lpm that produced from *A. deccanesis* and *D. aurantiacum* have identical major component Lpm A3 [44]. Lpm exhibits potent activity against bacterial RNAP of both gram-positive (especially *Clostridium difficile,* a gastrointestinal tract pathogen causing diarrhea) and some gram-negative bacteria (such as *E.coli*) [44]. Identification of sequencing of spontaneous Lpm-resistant mutants of

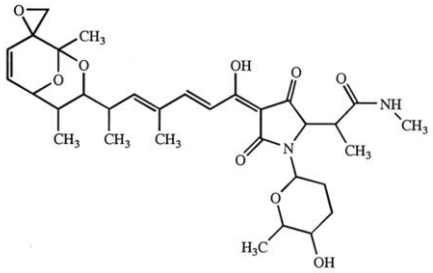
Bacullus subtilis, Staphylococcus areus, Enterococcus faecalis and *Mycobacterium tuberculosis* indicated that the substitutions conferring Lpm- resistances was obtained in both β and β' subunits of RNAP [44]. Lpm target does not overlap with Rif, which makes Lpm does not exhibit cross-resistance with Rif [44]. Lpm is one of the antibiotics determined to inhibit the "switch region" of bacterial RNAP (other compounds that also inhibits switch region includes Myxopyronin, Corallopyronin and Ripostatin) [44]. Switch region, a new drug target, is a structural element of RNAP located at the base of RNAP clamp and mediates the opening and closing of the clamp, necessary for loading and retaining the DNA [44, 45].

Myxopyronin (Myx A, MW = 417; Myx B,MW = 432 Da) is an α-pyrone antibiotic originally produced by Myxobacterium *Myxococcus fulvus* Mxf50 [44, 63, 64]. Myx inhibits bacterial RNAP of a broad-spectrum species of both gram-positive and gram-negative bacteria including *Mycobacterium tuberculosis, Staphylococcus aureus, Bacillus anthracis, Enterococcus faecium, Enterobacter cloacae, Pseudomonas aeruginosa,* and *E.coli DH21tolC* [44, 45]. Myx does not inhibit eukaryotic RNAP II [44, 45]. Isolation of Myx-resistance strains by random mutagenesis of genes encoding β and β' subunit of *E.coli* RNAP was conducted to determine the Myx-target. The result revealed the substitutions of residues of both β and β' subunits [44, 45]. Further structural analysis of Myx target showed that those substitutions conferring resistance to Myx are tightly located at the switch region, leading to the conclusion that Myx target is located in the "switch region" [44, 45]. In addition, Myx target does not overlap with Rif target and so it does not exhibit cross-resistance to Rif [44, 45]. Supperadditive or

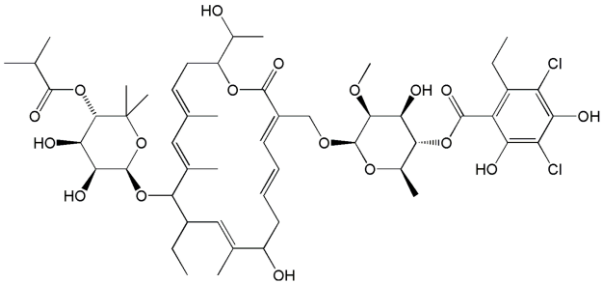
synergistic antibacterial activity was observed when Myx is co-administered with Rif (A.S and R.H.E, unpublished).

Ripostatin A (Rip, MW = 494 Da) is a 14-membered macrocyclic-lactone antibiotic produced by Myxobacterium *Sorangium cellulosum* So ce377 [44, 65]. Rip is an bacterial RNAP inhibitor and has narrow spectrum antibacterial activity and potency as compared to Myx; however, the frequency of rif-resistance, spontaneous resistance and resistant fitness cost characteristics are similar to that of Myx [44]. Rip does not inhibit eukaryotic RNAP II and is structurally unrelated to Myx or Cor [44, 45]. Mechanism of action of Rip is similar or identical to Myx and Cor by interfering the opening and closing of the RNAP clamp (switch region target), interfering interaction between RNAP and promoter position -11 to +15 and inhibiting isomerization of RNAP-closed complex (RPc) to RNAP-opened complex (RPo) [44, 45].

APY61, APY116, PY63, 3RHTK27, 3RHTK44 and OMTK13 are tested in Ebright's lab and most of them have shown to inhibit bacterial transcription by RNAP of *E.coli, M.tuberculosis* and *S.aureus* in ribogreen transcription assays at the concentration that is similar to that of Myx B (J.Shen, S.Liu and R.H.E, unpublished). Synthesis of these compounds is originated from Myx and Cor as well as from other natural products known as lupulones (from hops, [66]) and leptospermones (from manuka oil [67]) (J.Shen and R.H.E, unpublished).

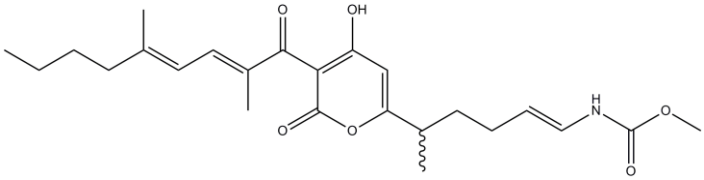


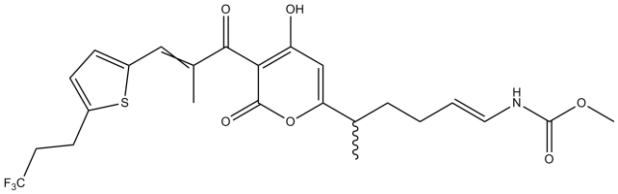
Streptolydigin



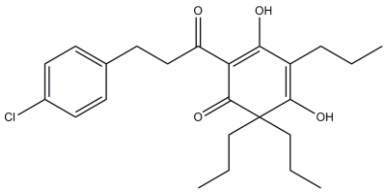
Lipiarmycin A3

PY3a

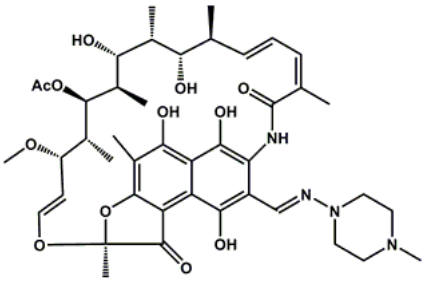




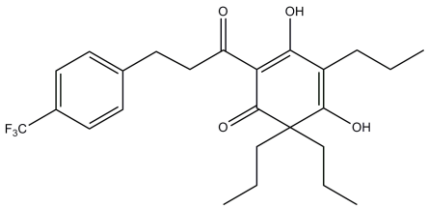
APY61



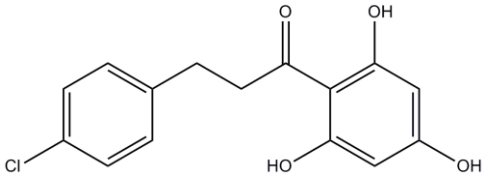
3RHTK27



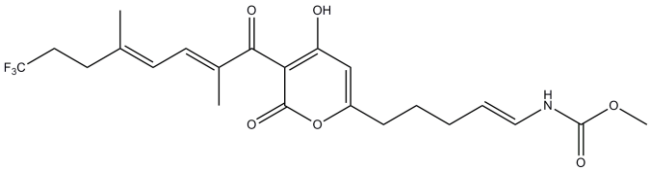
Rifampin



3RHTK44



OMTK13



PY63

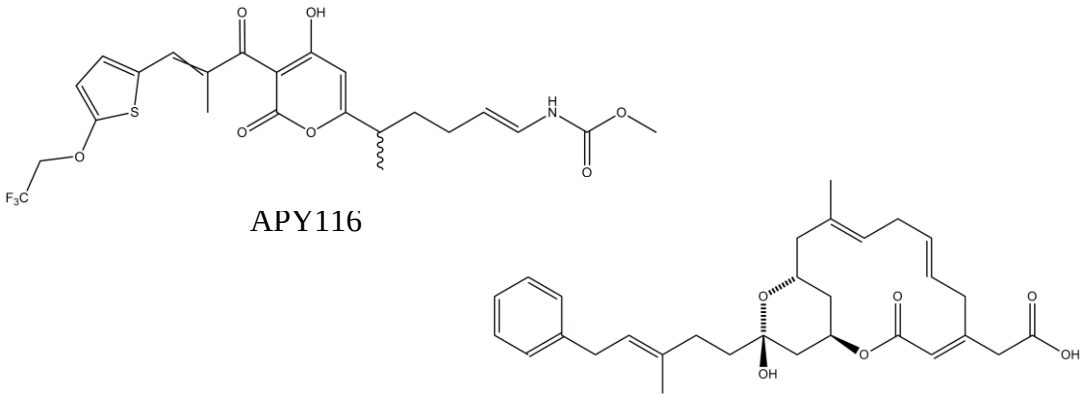


Figure 2: Structure of RNA polymerase inhibitors

For comparison of the efficacy of bacterial RNAP inhibitors against *S.epidermidis* biofilms, seven different reference antibiotics functioning through bacterial target other than RNAP and are commonly used in clinical setting have been selected for testing. These compounds include ciprofloxacin (DNA gyrase inhibitor), tetracycline (Ribosome inhibitor), gentamicin (Ribosome inhibitor), quinupristin-dalfopristin complex (Ribosome inhibitor), nafcillin (Cell wall inhibitor), vancomycin (Cell wall inhibitor) and linezolid (Ribosome inhibitor) (Drug Bank Database). Tested in other biofilm assay systems, some of these reference antibiotics have been shown to be effective in *in vitro* *S.epidermidis* biofilms when used in combination with Rif (Rif-Vancomycin and Rif-tetracycline) but decrease efficacy against biofilm when used alone [50, 68].

Chapter III HYPOTHESIS AND OBJECTIVES

III.A Hypothesis

As seen in the above discussion, Rif, an RNAP inhibitor, is one of the most effective drugs used to treat-biofilm related infection specifically *S.epidermidis*-related diseases. Excellent pharmacokinetic properties, the ability to remain active in the acidic environment which is found in the biofilms and the inhibitory activity of small regulatory RNA synthesis essential for biofilm survival are some of the important reasons why Rif is very effective in biofilm treatment. Since Rif target RNAP, and with the purpose of solving the problem of Rif-resistant strains in biofilm treatment, we hypothesize that other RNAP inhibitors may also be effective in eradicating *S.epidermidis* biofilms in *vitro*.

III. B. Objectives

1. To determine the Minimum Biofilm Eradication Concentration (MBEC) of various bacterial RNAP inhibitors and reference compounds against *S.epidermidis* ATCC 35984 biofilms using commercial MBEC^{TM} P&G assay using Calgary Biofilm device (peg assay).
2. To determine the Minimum Biofilm Eradication Concentration (MBEC) of various bacterial RNAP inhibitors and reference compounds against *S.epidermidis* ATCC 35984 biofilms using glass biofilm eradication assay (glass biofilm assay).

Chapter IV MATERIALS AND METHODS

IV.A. MBEC™ peg assay

To obtain the MBEC of the test compounds, the peg assay using the commercial Calgary Biofilm device (CBD) was done (specifically the MBEC ™ Physiology and Genetics P&G assay kit). The peg assay is commercially available from Innovotech MBEC biofilm technology Ltd., Calgary Alberta, Canada. The instructions from the manufacture were followed as described in the attached manual as well as described in Ceri. H. et al [69] with some modifications. The assay condition was optimized for the organisms tested to get the desired biofilm densities. This test was done for triplicate for each compound. Assays were performed as follows:

1. **Sub-culture preparation:**

Staphylococcus epidermidis ATCC 35984 (RP62) was streaked onto BBL™ Mueller Hinton II agar plate (MHII) (Becton Dickinson & Co.Spark, MD) and incubated at 37^{o}C overnight (first sub-culture). The second-subculture was made by streaking the first sub-culture onto a second MHII agar plate and incubating at 37^{o}C overnight. This particular bacteria strain was used due to the its strong ability to colonize solid support such as plastic culture dish, and catheters and it is the strain that was originally isolated from patients with catheter-associated sepsis [35, 52, 70].

2. **Biofilm formation**

The biofilm was grown by using CBD for peg assay. CBD is a two-part reaction vessel containing a top and a bottom part. The top is a lid containing 96 identical pegs on

which the biofilm is formed. The lid is designed in such a way that it fits into the bottom part which is a standard 96-well plate and a place where the cell suspension in media is put and serve as the channel for the media flow (shear force) [69, 71].

After the second-subculture of *S.epidermidis*, the bacteria were transferred by a sterile cotton swab into 1.5ml of BBL^{TM} Mueller Hinton II cation adjusted broth (Beckton Dickinson & Co.Spark, MD). The optical density was measured at wavelength of 600nm. The goal was to get the cell density of $1.5x10^8$ cfu/ml (OD_{600} = 0.2). The cell suspension was diluted 30- folds with MHII broth to achieve the cell density of $5x10^6$ cfu/ml. 150µl of the diluted cell suspension was put into each well of a new 96-well plate (bottom part of CBD). The top 96 peg lid was submerged into the cell suspension. The device was then put in a humidified container and incubated at 37^{o}C with shaking at 150 rpm for 48 hours. The starting inoculum was serially diluted and spotted on MHII agar to verify the cfu count. After 48h, biofilm formation was determined by obtaining viable bacteria count on MHII agar plate following the biofilm disruption by sonication. The satisfied biofilm should be around 10^6 to 10^7 cfu/peg.

3. Antibiotic preparation and biofilm susceptibility testing

Eight different antibiotics were tested at a time with a concentration range from 400 µg/ml to 0.78 µg/ml with 2-fold interval from one concentration to another. Each compound was made to a 10mg/ml stock concentration and stored at -20^{o}C, and freshly prepared to the desired concentration by appropriate dilution in MHII broth before the experiment. The antibiotic challenge plate was made in the 96-well plate as described by the manufacture. The control was made by antibiotic-free Mueller Hinton broth. Below is the table of all tested compounds against *S.epidermidis* ATCC 35984 biofilms.

No	Compound	Bacterial Target	Source	Molecular weight (g/mol)	Tested conc. (µg/ml)
Reference antibiotics					
1	Ciprofloxacin	DNA gyrase	Sigma-aldrich	385.82	400-0.78
2	Tetracycline	Ribosome	MP, Biomedicals	480.90	400-0.78
3	Gentamicin	Ribosome	Sigma-aldrich	477	400-0.78
4	Quinupristin-dalfopristin	Ribosome	BioAustralis	1022; 690.85	400-0.78
5	Nafcillin	Cell wall	Sigma-aldrich	454.48	400-0.78
6	Vancomycin	Cell wall	Sigma-aldrich	1485.71	400-0.78
7	Linezolid	Ribosome	Sigma-aldrich	337.35	400-0.78
Bacterial RNA Polymerase inhibitors					
1	Rifampin	RNAP	Sigma-aldrich	822.94	400-0.78
2	Streptolidygin	RNAP	Sourcon-Padena	600.70	400-0.78
3	LipiramycinA3 (Fidaxomicin)	RNAP	BioAustralis	1058	400-0.78
4	Myxopyronin B (PY3a)	RNAP	Synthesized as in [72]	432	400-0.78
5	APY61	RNAP	(Provid and RHE unpublished)	514	400-0.78
6	3RHTK27	RNAP	(J.Shen and R.H.E, unpublished)	419	400-0.78
7	3RHTK44	RNAP	(J.Shen and R.H.E, unpublished)	453	400-0.78
8	OMTK13	RNAP	(J.Shen and R.H.E, unpublished)	293	400-0.78
9	PY63	RNAP	(Y.E and R.H.E unpublished)	457	400-0.78
10	APY116	RNAP	(Provid and R.H.E unpublished)	516	400-0.78
11	Ripostatin A	RNAP	Prepared as in [65]	494.6	400-0.78

Table 1 : List of all tested compounds and the concentration tested against *S.epidermidis* ATCC 35984 biofilms by MBEC assays.

After 48h, peg lids were removed from the incubator and washed with 1X phosphate buffer saline (PBS). The lid was then submerged into a 96-well plate containing the prepared test compounds at different concentration as described earlier. Four individual peg were also broken off from the lid prior to challenging. The biofilm was removed from the pegs by sonication for 5mn (Sonicator brand FS60). The resulting cell were serially diluted and plated on MHII agar plate to quantify the biofilm cfu count. A planktonic cfu count was also done at this time by diluting the bacterial culture from the 96-well plate (bottom part of the device). The challenge plate was incubated at 37^0C with shaking at 150 rpm for 24 hours.

4. Biofilm recovery and MBEC determination

After a 24 hour challenge, the biofilm peg lid was taken out, rinsed twice with 1XPBS, and submerged into a new 96-well plate containing 200µl of recovery media. Recovery media is MHII broth supplemented with saponin (10g/L), tween-80 and universal neutralizer as described by manufacture. The pH of the recovery media was adjusted with NaOH to 7.0 ± 0.2. The purpose of the recovery media is to neutralize the possible carry-over test compounds after challenging. The recovery plate containing the after-challenged biofilm peg lid was then sonicated on high for 5mn, followed by serial dilution and plating on MHII agar plates to count the remaining CFUs. Minimal Biofilm Eradication Concentration (MBEC) was defined as the minimal concentration of the test compound required to eradicate the biofilm, i.e zero CFU count [69, 71, 73].

5. Statistical Analysis

Microsoft excel was used to calculate standard deviation (SD). Standard Error of Mean (SEM) was calculated according to this formula:

$$SEM = \frac{SD}{\sqrt{n}} \ where\ n\ is\ the\ number\ of\ replicate$$

IV.B. MIC Assay: Broth Dilution Susceptibility test

In parallel to the peg assay, broth microdilution MIC assays were also performed (in duplicate) for each compound to determine their Minimum Inhibitory Concentration (MIC) against *S.epidermidis* ATCC 35984. The assays were performed following the CLSI (Clinical and Laboratory Standard Institute) guidelines [74] .Briefly, single colony of strain *S.epidermidis* ATCC 35984 was inoculated in 5ml MHII broth and incubated for 3-4 hours at 37^{o}C with shaking at 150rpm. 50µl of diluted cell suspension of 4x10^{6} cfu/ml (concentration determined using OD_{600}=1 for 10^{9} cfu/ml) was put on 50µl of MHII broth containing 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25 and 50µg/ml of test compounds and incubated at 37^{o}C for 16hr with shaking at 150rpm. The MIC was defined as the lowest concentration of an antimicrobial agent that prevent visible growth of a microorganism in an agar or broth dilution susceptibility test [74]. MIC is a standard test to quantified the susceptibility of the bacteria in its planktonic form to the antimicrobial compounds [75].

IV.C. Glass biofilm assay

In addition to the peg assays, antibacterial compound efficacy against *S.epidermidis* 35984 biofilm was also assessed on glass biofilm assay as described in Richard G.K. et.

al [76, 77]. This test was done in triplicate for each compound. These assays were performed as follows:

1. **Glass slide preparation**

Standard laboratory glass microscopic slides (75mm x 25 mm, Premiere brand) were cut into 6mm x 25mm with a glass cutter. The glass pieces were marked on one side for clarity as the uppermost for which biofilm is formed and autoclaved at 121 ^{o}C for 15mn.

2. **Bacteria culture preparation**

2-3 well isolated colonies of *S.epidermidis* ATCC 35984 from nutrient agar plate (Difco ^{TM} Nutrient Agar, Becton Dickinson & Spark Co, MD) were inoculated in 5ml of nutrient broth consisting of mixture of 75% BBL^{TM} Trypticase soy broth (Soybean-casein digest broth, Becton Dickinson) and 25% of Bacto ^{TM} peptone water (enzymatic digest of protein, BD). The inoculum was vigorously shaken at 37^{o}C at 250 rpm to obtain a log-phase culture (OD $_{600}$= 0.6-0.8).

3. **Biofilm preparation on glass surface**

The log-phase culture of bacteria was measured by spectrophotometer to obtain optical density at 600nm wavelength and diluted with nutrient broth to get the final density of OD_{600} = 0.001 (approximately 10^6 cfu/ml). 20 ml of the diluted cell culture was placed onto a sterile petri dish containing the cut glass slides with their marked “face up” (maximum 20 glasses each petri dish), followed by the incubation without shaking at 37^0C for 18 hours.

4. Biofilm exposure

Test compounds were prepared by diluting stock concentration (10mg/ml) to the desired concentration with peptone water. Peptone water is a medium that does not have fermentable carbohydrate. The glass slides carrying grown biofilms were washed (3 dips in 3 eppendorf tube containing 1XPBS) and placed in 6-well plate. Each glass slide was covered with 3.5ml of the test compound medium and incubated at 37^0C for 8 hours. A positive control (glass slide with biofilm in antibiotic-free peptone water) and a negative control (glass slide with biofilm in 4% formal saline consisting of 37% formaldehyde and NaCl) were also performed with every batch of experiment.

5. Residual viability determination

After 8 hours of exposure to test compounds, the glass slides were washed with PBS (3 dips in 6 tubes), and put onto the indicator agar plate with the biofilm surfaces facing down onto the plates. Indicator agar plates were made by adding 1% 2,3,5 triphenyltetrazolium chloride (TTC) into nutrient agar (0.5ml of 1%TTC in every 100ml of nutrient agar). The TTC agar plates were then incubated at 37^0C for 18 hours before determining the MBEC. *S.epidermidis* biofilm eradication by antibacterial agents was observed by the loss of red color from the glass slides. In metabolically active cells, yellow dye TTC, acts as an electron acceptor and is reduced by electron donors such as dehydrogenase or NADH to make insoluble red formazan [78, 79], coloring the glass slides red. Dead, metabolically inactive, cells will not produce this red color change.

Chapter V RESULTS

V.A. MBEC™ peg assay

1. Biofilm formation on CBD

Matured *S.epidermidis* biofilms were formed after 48h of incubation. A satisfactory biofilm density was obtained by counting the cfu/peg of the bacteria formed on the CBD device before challenged with antibiotics. The goal was to have a biofilm density of approximately 6-7 $\log_{10}$ cfu/peg count. Table 2 showed starting culture and the biofilm density obtained from six different experiments in $\log_{10}$ of cfu/ml and cfu/peg respectively.

Exp.No.	$\log_{10}$ cfu/ml starting culture± SEM	$\log_{10}$ biofilm density cfu/peg ±SEM
1	6.70±0.12	7.52±0.25
2	6.75±0.04	7.16±0.08
3	6.73±0.15	7.55±0.33
4	6.85±0.10	7.03±0.04
5	6.65±0.02	6.78±0.18
6	6.69±0.05	6.54±0.05

Table 2: Log_{10} of cfu/ml of starting culture and *S.epidermidis* biofilm density before challenged with test compounds

2. Antibiotic susceptibility: MICs and MBEC

For MICs assay, *S.epidermidis* was grown for 3-4hr and the cell count on MHII agar plate of $4\text{x}10^6$ cfu/ml on average was used for broth dilution susceptibility test. Similar results of MICs were obtained from at least twice for each compound. In this study, MICs were determined as the lowest concentration which inhibits 99% of visible

growth in the broth microdilution susceptibility test (Table 3). All tested compounds are active against planktonic cells.

The Minimum Biofilm Eradication Concentration (MBEC) was obtained by challenging biofilms with the test compounds at the desired concentrations for 24h. Eight different compounds were tested at a time. The remaining surviving cells after the challenge were removed from the peg by sonication and quantified by spotting on MHII agar. The MBEC was determined as the lowest concentration at which zero cfu count was observed. The Near Biofilm Eradication Concentration (NBEC) was also determined as the lowest concentration at which a cfu count of 1000 or fewer was obtained (table 3). The effect of all tested compounds (reference antibiotics and RNAP inhibitors) was shown in table 4.

In peg assay, none of the reference antibiotics were effective in fully eradicating the biofilm even at concentration up to 400µg/ml (ciprofloxacin, tetracycline, gentamicin, quinupristin-dalfopristin, nafcillin, vancomycin and linezolid). However, some of these compounds were generally effective in reducing biofilm count by 2-3 $\log_{10}$ cfu/peg. While ciprofloxacin, and quinupristin-dalfopristin decreased 2-3 $\log_{10}$CFU/peg of biofilms at the lowest concentration tested 0.78µg/ml, tetracycline, gentamicin, nafcillin, vancomycin and linezolid could reduce the same biofilm count at concentration of 1.56, 12.5, 100, 12.5, and 6.25µg/ml respectively.

Unlike the reference compounds, four out of eleven bacterial RNAP inhibitors tested were able to completely sterilize *S.epidermidis* biofilms; As expected, Rifampin is one of these compounds. While biofilms could be completely cleared by Rif at 200µg/ml,

we found three additional RNAP inhibitors 3RHTK27, 3RHTK44 and OMTK13 that also fully eradicated the biofilms at the concentration of 25µg/ml, 12.5µg/ml and 50µg/ml, respectively. Complete eradication of biofilm is really essential to ensure that there is no reformation of the biofilm from the remaining cells. Other bacterial RNAP inhibitors were not active against the biofilms, having MBEC values over 400µg/ml. Although only certain bacterial RNAP inhibitors are active against biofilm, this result supports the potential advantages of bacterial RNAP inhibitors over other antibiotics in treating biofilms. Notably, there is a clear difference between MICs and MBECs value which support the fact that *S.epidermidis* is difficult to eradicate once they form as biofilms.

No	Antibiotics	MIC (µg/ml)	MBEC (µg/ml)	NBEC (µg/ml)
Reference antibiotics				
1	Ciprofloxacin	0.048	>400	200
2	Tetracycline	0.19	>400	400
3	Gentamicin	25	>400	400
4	Quinupristin-dalfopristin	0.048	>400	200
5	Nafcillin	0.78	>400	400
6	Vancomycin	1.56	>400	400
7	Linezolid	1.56	>400	>400
RNA polymerase inhibitors				
1	Rifampin	0.003	200	50
2	Streptolydigin	50	>400	>400
3	Lipiarmycin A3	1.56	>400	200
4	Myxopyronin B (PY3a)	1.56	>400	200
5	APY61	1.56	>400	>400
6	3RHTK27	0.78	25	12.5
7	3RHTK44	1.56	12.5	6.25
8	OMTK13	12.5	50	50
9	PY63	6.25	>400	400
10	APY116	6.25	>400	400
11	Ripostatin A	>50	>400	>400

Table 3 : MICs, MBECs and NBEC of all tested compounds against *S.epidermidis*.

Con (µg/ml)	400	200	100	50	25	12.5	6.25	3.125	1.56	0.78	Control
Reference antibiotics											
Ciprofloxacin	2.09±0.25	2.15±0.15	1.56±0.14	2.42±0.02	2.16±0.04	2.19±0.1	2.21±0.3	2.46±0.16	2.67±0.24	2.56±0.36	6.5±0.07
Tetracycline	2.45±0.03	2.81±0.11	2.71±0.22	2.55±0.16	3.07±0.1	3.31±0.16	4.34±0.26	4.43±0.11	4.99±0.29	5.11±0.39	6.55±0.11
Gentamicin	3.38±0.75	3.53±0.62	4.12±0.43	4.77±0.28	4.91±0.05	5.59±0.33	6.35±0.33	6.46±0.05	6.17±0.38	6.52±0.066	6.73±0.10
Quinupristin-dalfopristin	2.68±0.39	2.79±0.3	2.59±0.3	2.36±0.43	2.97±0.54	3.08±0.27	3.28±0.43	3.59±0.51	3.61±0.56	3.73±0.63	6.73±0.10
Nafcillin	2.94±0.27	4.04±0.01	5.06±0.14	5.76±0.34	5.97±0.65	6.24±0.54	5.75±0.45	6.11±0.66	6.17±0.27	6.55±0.57	6.68±0.13
Vancomycin	2.54±0.07	2.85±0.38	2.84±0.28	3.14±0.15	3.98±0.98	5.61±0.53	6.1±0.23	6.61±0.036	7.04±0.04	7.05±0.164	6.68±0.1
Linezolid	3.18±0.2	3.17±0.18	3.12±0.26	3.29±0.35	3.34±0.08	3.86±0.19	4.82±0.35	5.78±0.26	6.29±0.27	6.49±0.35	6.41±0.22
RNA polymerase inhibitors											
Rifampin	0.00	0.00	2.63±0.29	3.1±0.89	3.26±0.87	4.3±0.9	3.42±0.92	3.85±1.04	3.56±0.83	4.09±0.66	6.67±0.09
Streptolydigin	3.11±0.01	3.79±0.78	5.38±0.39	5.12±0.57	5.39±0.15	5.93±0.04	6.49±0.1	6.51±0.12	6.56±0.1	6.67±0.11	6.73±0.05
Lipiarmycin A3	2.34±0.16	1.78±0	3.08±0.3	3.62±0.82	3.09±0.43	4.57±0.39	4.7±0.11	5.79±0.19	6.78±0.3	6.44±0.2	6.46±0.12
Myx B (PY3a)	2.64±0.15	2.63±0.3	2.53±0.17	3.02±0.23	3.78±0.38	3.91±0.63	4.6±0.27	5.64±0.3	6.22±0.35	6.44±0.52	6.70±0.15
APY61	3.63±0.28	3.68±0.13	3.31±0.07	3.16±0.04	2.99±0.06	2.79±0.1	3.27±0.33	3.76±0.23	5.92±0.26	6.27±0.39	6.66±0.19
3RHTK27	0.00	0.00	0.00	0.00	0.00	2.58±0.16	3.2±0.14	3.51±0.08	4.66±0.49	6.18±0.39	6.69±0.07
3RHTK44	0.00	0.00	0.00	0.00	0.00	0.00	2.21±0.11	2.87±0.22	4.61±0.86	5.45±0.8	6.56±0.36
OMTK13	0.00	0.00	0.00	0.00	6.01±0.67	6.75±0.13	6.68±0.52	6.69±0.44	7.12±0.29	7.36±0.09	6.98±0.23
PY63	3±0.27	2.83±0.08	3.23±0.16	4.36±0.18	5.42±0.44	5.95±0.65	5.48±0.07	6.5±0.01	6.57±0.12	6.92±0.04	6.61±0.21
APY116	3.14±0.42	3.22±0.35	4.39±0.81	3.33±0.13	3.91±0.28	4.46±0.07	6.01±0.12	6.86±0.35	6.96±0.37	6.91±0.27	6.71±0.08

Table 4: Effects of bacterial RNA polymerase inhibitors and reference antibiotics incubated for 24h with an *S.epidermidis* ATCC 35984 biofilm

[a]Mean value of $\log_{10}$ remaining cfu/peg ± standard error of mean . The zero here means there was no single colony observed.

V.B. Glass biofilm assay

The end-point metabolic cessation of biofilm formed on glass was observed to determine the MBEC of the same test compounds after 8h of exposure to antibiotics. The MBEC was determined as the lowest concentration, at which there was no red precipitation of tetrazolium formazan, which is an evidence of cell death. This was determined by eye. The treated glass slides were also compared to positive control (glass biofilm in antibiotic-free peptone water) and negative control (glass biofilms in 4% formal saline solution).

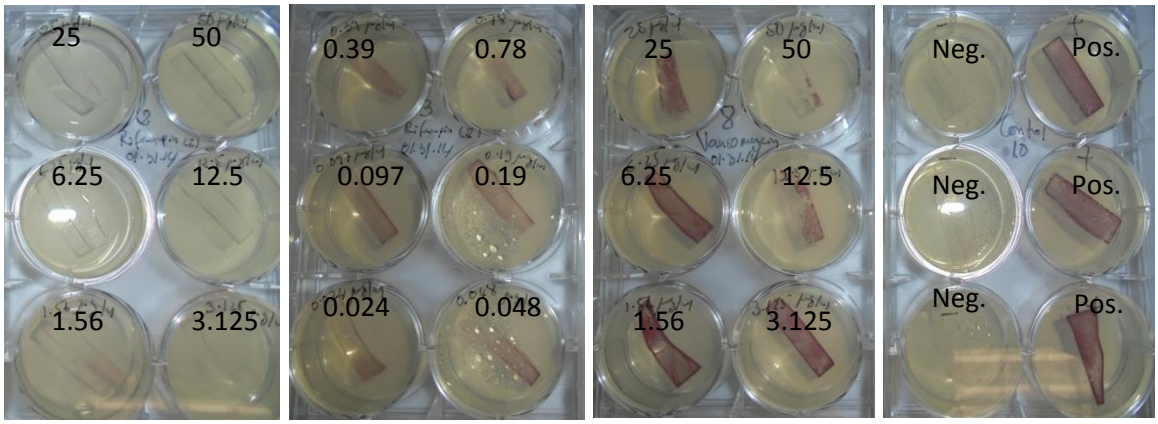
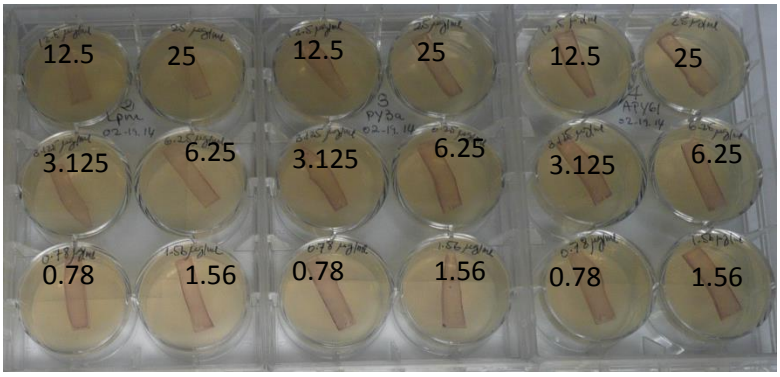


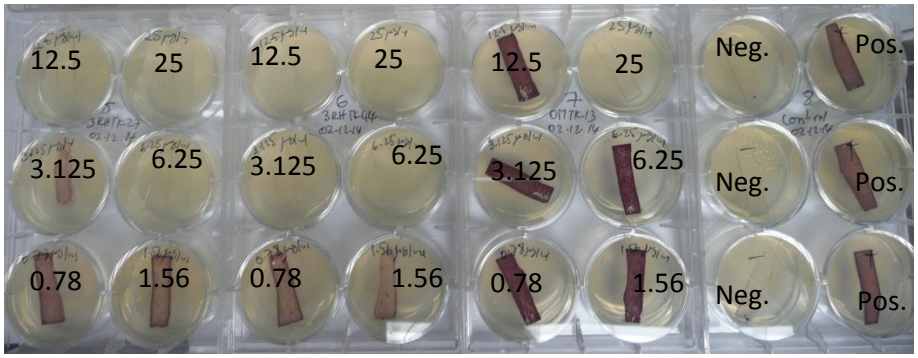
Figure 3. Rifampin and vancomycin effect on glass biofilm as compared to the control. Cell death is shown by permanent loss of ability to reduce TTC to form red formazan. (a) and (b) Rifampin tested at concentration range of 50 to 0.024µg/ml; (c) vancomycin tested at concentration range 50-1.56µg/ml; (d) negative and positive control.

Similar to the peg assay, all reference antibiotics failed to completely eradicate the *S.epidermidis* biofilms on glass slide even at the highest concentrations we tested (50µg/ml) (is shown for vancomycin in panel above). In contrast, four bacterial RNAP

inhibitors tested against glass biofilm were active against biofilms and as expected Rif is one of them. Beside Rifampin, three other RNAP inhibitors fully eradicated the biofilm at the tested concentration. These three compounds were the same as those identified in the peg assay: 3RHTK27, 3RHTK44 and OMTK13. While OMTK13 and 3RHTK27 have higher MBEC value than that of Rif (MBEC of 25µg/ml and 6.25µg/ml respectively), 3RHTK44 exhibited similar activity to Rif in the glass biofilm assay with an MBEC of 3.125µg/ml. As in the peg assay, other bacterial RNAP inhibitors such as Lipiarmycin A3, PY3a and APY61 did not completely eradicate biofilm up to the highest concentration tested (Figure 4).



(a) Lipiarmycin A3 (b) PY3a (c) APY61



(d) 3RHTK27 (e) 3RHTK44 (f) OMTK13 (g) Control

Figure 4: Bacterial RNAP inhibitors effect on *S.epidermidis* glass biofilms. All compounds were tested at concentration range of 25µg/ml to 0.78µg/ml.

N0	Compound	MBEC (µg/ml)
Reference antibiotics		
1	Ciprofloxacin	>50
2	Tetracycline	>50
3	Gentamicin	>50
4	Quinupristin-dalfopristin	>50
5	Nafcillin	>50
6	Vancomycin	>50
7	Linezolid	>50
RNA polymerase inhibitors		
1	Rifampin	3.125
2	Lipiarmycin A3	>25
3	Myxopyronin B (PY3a)	>25
4	APY61	>25
5	3RHTK27	6.25
6	3RHTK44	3.125
7	OMTK13	25

Table 5: MBEC of tested compounds in glass biofilm assay. MBEC is determined as the lowest concentration which gives complete cell death shown by loss of red color resulting in *S.epidermidis* biofilm cell death.

Chapter VI DISCUSSION

VI.A MBEC peg assay

The activity of seven reference compounds (non-RNA polymerase inhibitors) and eleven RNA polymerase inhibitors against in *vitro S.epidermidis* biofilms were tested by MBEC P&G assay on Calgary Biofilm device. The MBEC results from this assay showed that none of the reference antibiotics could eradicate biofilms at the concentration tested while four out of eleven tested bacterial RNAP inhibitors were able to eradicate the biofilms. Rifampin, one of the most effective drugs used to treat biofilm could eradicate biofilms but only at a concentration that is very high compared to its MIC. This may be due to high bacterial resistance to this drug. Several studies on biofilm susceptibility testing were done using bacterial RNAP inhibitors and reference compounds, but they showed different MBEC values because they used different biofilm testing methods and/or different bacteria strains [52, 53]. However, some of those studies revealed the advantages of bacterial RNAP inhibitors over reference compounds in killing bacterial biofilms.

Remarkably, in this study we also identified three other bacterial RNAP inhibitors, synthesized by J.Shen in the Ebright lab, which seem to be even more effective against *S.epidermidis* biofilms: 3RHTK27, 3RHTK44 and OMTK13. These compounds can eradicate the biofilms at the concentration of 25, 12.5 and 50μg/ml, respectively. In contrast, the other tested bacterial RNAP inhibitors (Streptolydigin, Lipiarmycin A3, Myxopyronin B, APY61, PY63, APY116 and Ripostatin A) were not capable of completely killing the biofilms even though they were all active against

planktonic cells. Seeing that not all bacterial RNAP inhibitors tested are active against biofilms suggests that considering target only bacterial RNAP may not be sufficient to treat biofilm but properties (size, hydrophobicity, pharmacokinetic) of the compounds itself should also be taken into consideration. Nevertheless, being able to find a few active bacterial RNAP inhibitors against biofilm while all reference antibiotics are not active, is promising and encouraging; this still suggests that bacterial RNAP is still a good target although not sufficiently active as a group. The three compounds identified here are interesting candidate for further development in the treatment of *S.epidermidis* biofilm infections.

VI.B. Glass biofilm assay

The glass biofilm assay permits a simple and direct method of assessing biofilm antibacterial activity. Although this technique is more qualitative than quantitative, it involves simple procedures and did not need any specialized equipment. The same bacterial RNAP inhibitors and reference compounds tested in the peg assay also were tested in the glass biofilm assay. The same compounds that were active against biofilms in the peg assay were also active against biofilms in the glass biofilm assay although the MBEC values from both assays were different. As stated earlier this assay is more qualitative than quantitative. In addition, the biofilm system is also different i.e plastic biofilm vs. glass biofilm. The biofilm thickness is also different between the two. However, the fact that the same bacterial RNAP inhibitors were effective in two completely different biofilm susceptibility testing systems supports that these compounds may be useful in the treatment of biofilm infections.

VI.C Suggestion for future studies

According to these results, it is clear that *S.epidermidis* is very difficult to eradicate once it forms a biofilm, as seen by the big differences between planktonic MIC values and biofilm MBEC values. Selecting effective antibacterial compounds to treat-biofilm related infections is very important. Finding a few effective bacterial RNAP inhibitors against *S.epidermidis* biofilms encourages us to look for more RNAP-targeting compounds that are active against biofilms. Combination drug test using these compounds should also be conducted to see what compounds could work synergistically with RNAP inhibitors to treat biofilms. In addition to antibiotic treatment, further development on new strategies using natural substances, enzymes or physical approaches as discussed earlier can also be taken into consideration to effectively treat biofilm-related diseases.

Kokai-Kun et al. demonstrated the efficacy of staphylocidal enzyme Lysostaphin against *S.aureus* biofilms in animal models [80]. The result revealed that the administration of Lysostaphin at 15mg/kg in combination with nafcillin at 50mg/kg three times per day for a period of 4 days eradicated the generated biofilms from the implanted catheter in mice [80]. In addition, pre-treatment of catheter with 10mg/kg Lysostaphin completely protected catheterized mice from *S.aureus* biofilm infection [80]. While the efficacy of antibacterial compounds against bacterial biofilms in animal model in this study required sacrifice of animal and time-consuming colony count procedure, Kadurugmuwa et al. described a new direct, rapid and real-time monitoring biofilm infection in mouse model, which can be used in determining biofilm infection on medical devices nondestructively as well as monitoring the effect of antibacterial compounds

against bacterial biofilm in mouse model [81]. In this *in vivo* system, the catheter-associated biofilm infection in mice was monitored by the production of bioluminescence produced by the bacteria biofilm (such as *P.aeruginosa* and *S.aureus*) which were engineered with *lux* operon (encoding bioluminescent enzyme luciferase) within their chromosome [81]. This *in vivo* system can be possibly used in the future studies for evaluation of the efficacy of bacterial RNAP inhibitors against foreign body-associated biofilm infections in the animal model.

Chapter VII REFERENCES CITED

1. Vu, B., et al., Bacterial Extracellular Polysaccharides Involved in Biofilm Formation. *Molecules 2009.* 14: p. 2535-2554.

2. Seema, B. and G.A. Krishna, Biofilms: A Challenge to Medical Science. *Clinical and Diagnostic Research,* 2011. 5(1): p. 127-130.

3. Mah, T.-F.C. and G.A. O'Toole, Mechanisms of biofilm resistance to antimicrobial agents *Trends in Microbiology,* 2001. 9(1): p. 34-39.

4. Lewis, K., Riddle of Biofilm resistance. *Antimicrobial agent and Chemotherapy* 2001. 45(4): p. 999-1007.

5. Gomes, F., et al., Strategies to control Staphylococcus epidermidis biofilms. Science against microbial pathogens: communicating current research and technological advances 2011: p. 843-852.

6. Taraszkiewicz, A., et al., Innovative Strategies to Overcome Biofilm Resistance. *Biomed Research International,* 2013: p. 1-13.

7. Kiedrowski. M.R, and Horswil. A.R, New approaches for treating staphylococcal biofilm interactions. *Ann. N. Y. Acad. Sci.* , 2011. 1241(1): p. 104-121.

8. Bryers, J.D., Medical Biofilms. *Biotechnol Bioeng* 2008. 100(1): p. 1-18.

9. Otto, M., Staphylococcal Biofilms. *Curr Top Microbiol Immunol* 2008. 322: p. 207-228.

10. Otto, M., Staphylococcal Infections: Mechanisms of Biofilm Maturation and Detachment as Critical Determinants of Pathogenicity. *Annu.Rev.Med* 2013. 64: p. 175-188.

11. Jefferson, K.K., What drives bacteria to produce a biofilm? *FEMS Microbiology letter,* 2004. 236: p. 163-173.

12. B. Prakash, B.M.V.and.G.K., Biofilm: A survival strategy of bacteria. *Current Science,* 2003. 85(9): p. 1299-1307.

13. Bordi, C. and S.D. Bentzmann, Hacking into bacterial biofilms: a new therapeutic challenge. *Annals of Intensive Care* 2011. 1(19): p. 1-8.

14. Stock, A.M., V.L. Robinson, and P.N. Goudreau, Two-component signal transduction. *Annual review of Biochemistry,* 2000. 69: p. 183-215.

15. Boles, B.R., et al., Identification of Genes Involved in Polysaccharide-Independent Staphylococcus aureus Biofilm Formation. *PLoS ONE*, 2010. 5(4): p. 1-9.

16. Vassylyev, D.G., et al., Crystal structure of a bacterial RNA polymerase holoenzyme at 2.6 A˚resolution. *Nature*, 2002. 417: p. 712-719.

17. Madigan, M.T. and J.M. Martinko, Brock Biology of Microorganism, eleventh edition. 2006.

18. Cotter, P.A. and S. Stibitz, c-di-GMP-mediated regulation of virulence and biofilm formation. *Current Opinion in Microbiology*, 2007. 10: p. 17-23.

19. S.Stewart, P. and J.W. Costerton, Antibiotic resistance of bacteria in biofilms. *Lancet*, 2001. 358: p. 135-138.

20. Fux, C.A., et al., Bacterial Biofilms: A diagnostic and therapeutic challenge. *Expert Rev Anti-infect Ther*, 2003. 1(4): p. 667-683.

21. Giwercman, B., et al., Induction of beta-Lactamase Production in Pseudomonas aeruginosa Biofilm. *Antimicrobial agent and chemotherapy*, 1991. 35(5): p. 1008-1010.

22. Zheng, Z. and P.S. Stewart, Penetration of Rifampin through Staphylococcus epidermidis biofilms. *Antimicrobial agents and chemotherapy*, 2002. 46(3): p. 900-903.

23. Anderl, J.N., M.J. Franklin, and P. S.Stewart, Role of Antibiotic Penetration Limitation in Klebsiella pneumoniae Biofilm Resistance to Ampicillin and Ciprofloxacin. *Antimicrobial agents and chemotherapy*, 2000. 44(7): p. 1818-1824.

24. Brown, M.R.W., D.G. Allison, and P. Gilbert, Resistance of bacterial bionlms to antibiotics: a growth-rate related effect? . *Antimicrobial chemotherapy*, 1988. 22: p. 777-783.

25. Hoiby, N., et al., Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial agents*, 2010. 35: p. 322-332.

26. Molin. S., and Tolker-Nielson T., Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Curr Opin Biotechnol*, 2003. 14(3): p. 255-261.

27. Boles, B.R. and P.K. Singh, Endogenous oxidative stress produces diversity and adaptability in biofilm communities. *PNAS*, 2008. 105(34): p. 12503-12508.

28. Raad, I., A. Alrahwan, and K. Rolston, Staphylococcus epidermidis: Emerging Resistance and Need for Alternative Agents. *Clinical infectious diseases* 1998. 26: p. 1182-1187.

29. O'Gara, J.P. and H. Humphreys, Staphylococcus epidermidis biofilms: importance and implications. *J.Med. Microbiol* 2001. 50: p. 582-587.

30. Huebner, J. and D.A. Goldmann, Coagulase-negative staphylococci: Role as Pathogens. *Annu. Rev. Med.*, 1999. 50: p. 223-236.

31. Fey, P.D. and M.E. Olson, Current concepts in biofilm formation of Staphylococcus epidermidis. *Future Microbiol* 2010. 5(6): p. 917-933.

32. D.Christensen, G., L. M.Baddour, and W.A. Simson, Phenotypic Variation of Staphylococcus epidermidis Slime Production In Vitro and In Vivo. *Infection and Immunity*, 1987. 55(12): p. 2870-2877.

33. Christensen, G.D., et al., Adherence of Slime-Producing Strains of Staphylococcus epidermidis to Smooth Surfaces. *Infection and Immunity,* 1982. 37(1): p. 318-326.

34. Christensen, G.D., et al., Adherence of Coagulase-Negative Staphylococci to Plastic Tissue Culture Plates: a Quantitative Model for the Adherence of Staphylococci to Medical Devices. *Clinical Microbiology*, 1985. 22(6): p. 996-1006.

35. Gill, S.R., et al., Insights on Evolution of Virulence and Resistance from the Complete Genome Analysis of an Early Methicillin-Resistant Staphylococcus aureus Strain and a Biofilm-Producing Methicillin-Resistant Staphylococcus epidermidis Strain. *Journal of Bacteriology*, 2005. 187(7): p. 2426-2438.

36. Schommer, N.N., et al., Staphylococcus epidermidis Uses Distinct Mechanisms of Biofilm Formation To Interfere with Phagocytosis and Activation of Mouse Macrophage-Like cells 774A.1. *American Society for Microbiology*, 2011. 79(6): p. 2267-2276.

37. Yao, Y., D.E. Sturdevant, and M. Otto, Genomewide anaylysis of gene expression in Staphylococcus epidermidis biofilms: Insights into the pathophysiology of S.epidermidis biofilms and the role of phenol-soluble modulins in formation of biofilms. *Journal of infectious disease,* 2005. 191: p. 289-298.

38. Rabinovitch, C. and P.S. Stewart, Removal and Inactivation of Staphylococcus epidermidis biofilms by electrolysis. *Applied and Environmental Microbiology*, 2006. 72(9): p. 6364-6366.

39. Costerton, J.W., et al., Mechanism of Electrical Enhancement of Efficacy of Antibiotics in Killing Biofilm Bacteria. *Antimicrobial agents and chemotherapy,* 1994. 38(12): p. 2803-2809.

40. Gomes, F., et al., Evaluation of antimicrobial activity of certain combinations of antibiotics against in vitro Staphylococcus epidermidis biofilms. *Indian J Med Res*, 2012. 135: p. 542-547.

41. Svensson, E., et al., Factors affecting development of rifampicin resistance in biofilm-producing Staphylococcus epidermidis. *Antimicrobial Chemotherapy*, 1997. 39: p. 817-820.

42. Zhang, G., et al., Crystal Structure of Thermus aquaticus Core RNA Polymerase at 3.3 A°Resolution. *Cell*, 1999. 98: p. 811-824.

43. Mekler, V., et al., Structural Organization of Bacterial RNA Polymerase Holoenzyme and the RNA Polymerase-Promoter Open Complex. *Cell*, 2002. 108: p. 599-614.

44. Srivastava, A., et al., New Target for Inhibition of bacterial RNA polymerase: "Switch Region". *Curr.Opin.Microbiol*, 2011. 14(5): p. 532-543.

45. Mukhopadhyay, J., et al., The RNA Polymerase ‘‘Switch Region’’ Is a Target for Inhibitors. *Cell* 2008. 135: p. 295-307.

46. P.Sensi, History of the Development of Rifampin. *Review of Infectious diseases*, 1983. 5: p. 402-406.

47. Garrison, J.R., et al., In vitro and In vivo effects of rifampin on Staphylococcus epidermidis graft infections. *ASAIO journal* 1997: p. 8-12.

48. Obst, G., et al., The activity of rifampin and analogs against Staphylococcus epidermidis biofilms in a CAPD environment model. *Am J Nephrol* 1989. 9: p. 414-420.

49. Coiffier, G., et al., Optimizing combination rifampin therapy for staphylococcal osteoarticular infections. *Joint Bone Spine*, 2013. 80: p. 11-17.

50. Gagnon. RF,, Richard. GK, and Kostiner. GB, Time-kill efficacy of antibiotics in combination with rifampin against Staphylococcus epidermidis biofilms. *Adv Perit Dial*., 1994. 10: p. 189-192.

51. Felden1, B., et al., The Staphylococcus aureus RNome and Its Commitment to Virulence. *PloS Pathogens*, 2011. 7(3): p. 1-10.

52. Villain-Guillot, P., et al., In vitro activities of different inhibitors of bacterial transcription against Staphylococcus epidermidis biofilm. *Antimicrob Agents Chemother*, 2007. 51(9): p. 3117-21.

53. Gualtieri, M., et al., In vitro activity of a new antibacterial rhodanine derivative against Staphylococcus epidermidis biofilms. *J Antimicrob Chemother*, 2006. 58(4): p. 778-83.

54. Kapanidis, A.N., et al., Initial Transcription by RNA polymerase proceeds through a DNA-Scrunching Mechanism. *Science*, 2006. 314(5802): p. 1144-1147.

55. Busby, S. and R. Ebright, Promoter structure, promoter recognition, and transcription activation in prokaryotes. *Cell*, 1994. 79: p. 743-746.

56. Lewin, B., Genes, VII edition. 2002: Oxford University press.

57. Pronin.SV and Kozmin. SA, Synthesis of Streptolydigin, A potent bacteria RNA Polymerase Inhibitor. *Am.Chem.Soc*, 2010. 132(41): p. 14394-14396.

58. Zorov, S., et al., Antibiotic Streptolydigin Requires Non catalytic Mg2+ for Binding to RNA Polymerase. *Antimicrobial agent and Chemotherapy*, 2014. 58(3): p. 1420-1424.

59. Yuzenkova, J., et al., Mutations of Bacterial RNA Polymerase Leading to Resistance to Microcin J25. *Biological Chemistry*, 2002. 277(52): p. 50867-50875.

60. Artsimonvitch, Chu C., Lynch. AS, and Landick. R., A new class of bacterial RNA polymerase inhibitor affects nucleotide addition. *Science*, 2003. 302(5645): p. 650-654.

61. Campbell EA., et al., Structural, functional, and genetic analysis of sorangicin inhibition of bacterial RNA polymerase. *EMBO*, 2005. 24(4): p. 674-682.

62. Parenti F, Pagani H., Beretta G., Lipiarmycin, a new antibiotic from Actinoplanes.I. Describtion of the producer strain and fermentation studies. J*ournal of antibiotics*, 1975: p. 247-252.

63. Irschik H., et al., The Myxopyronins, new inhibitors of bacterial RNA synthesis from Myxococcus fulvus (Myxobacterales). *J. of Antibiotics*, 1983. 36(12): p. 1651-1658.

64. Doundoulakis, T., et al., Myxopyronin B Analogs as inhibitors of RNA polymerase, synthesis and biological evaluation. *Bioorganic and Medicinal Chemistry Letters*, 2004: p. 5667-5672.

65. Irschik, H., et al., The ripostatins, novel inhibitors of eubacterial RNA polymerase isolated from myxobacteria. *Journal of Antibiotics*, 1995. 48(8): p. 787-792.

66. Almeida NE., Nascimento ES., Cardoso DR., On the Reaction of Lupulones, Hops β‑Acids, with 1‑Hydroxyethyl Radical. *Agricultural and Food Chemistry*, 2012. 60: p. 10649-10656.

67. Franck, D., et al., Manuka oil, a natural herbicide with preemergence activity. *Weed Science*, 2011. 59: p. 464-469.

68. Monzon, M., et al., Synergy of different antibiotics combination in biofilms of Staphylococcus epidermidis. *Antimicrobial Chemotherapy*, 2001. 48: p. 793-801.

69. Ceri, H., et al., The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms. *Clinical Microbiology* 1999. 37(6): p. 1771-1776.

70. ATCC, Methicillin-Resistant and Methicillin-sensitive Reseach Materials. American Type Cultur Collection, 2010.

71. Ceri, H., et al., The MBEC Assay System: Multiple Equivalent Biofilms for Antibiotic and Biocide Susceptibility Testing. *Methods in Enzymology*, 2001. 337: p. 377-385.

72. Ebright, R.H. and Y.W. Ebright, Antibacterial agents: high-potency myxopyronin derivatives. 2013: U.S.

73. Ceri, H., et al., Minimal Biofilm Eradication Concentration (MBEC) Assay: Susceptibility Testing for Biofilms. *Biofilms, infection and Antimicrobial Therapy* 2006: p. 257-269.

74. CLSI, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; *Approved Standard.* 2012. 32(2).

75. PDI, MIC Versus MBEC to Determine theAntibiotic Sensitivity of Staphylococcusaureus in Peritoneal Dialysis Peritonitis. *Perit Dial Int*, 2010. 30(6): p. 652-656.

76. Richards, G.K. and R.F. Gagnon, An assay of Staphylococcus epidermidis biofilm responses to therapeutic agents. *Int J Artif Organs*, 1993. 16(11): p. 777-88.

77. Richards, G.K., R.F. Gagnon, and R.J. Morcos, An assay to measure antibiotic efficacy against Staphylococcus epidermidis biofilms on implant surfaces. *ASAIO journa*l, 1994: p. M570-M575.

78. Tarafdar, P.-K.J.C., 2,3,5-Triphenyltetrazolium chloride (TTC) as electron acceptorof culturable soil bacteria, fungi and actinomycetes. *Bio Fertil Soils* 2003. 38: p. 186-189.

79. Mohammadzadeh, A., et al., Rapid and low-cost colorimetric method using2,3,5-triphenyltetrazolium chloride for detection ofmultidrug-resistant Mycobacterium tuberculosis. *Medical Microbiology* 2005. 55: p. 1657-1659.

80. Kokai-kun, J.F., T. Chanturiya, and J. J.Mond, Lysostaphin eradicates established Staphylococcus aureus biofilms in jugular vein catherized mice. *J. Antimicrobial Chemotherapy*, 2009. 64: p. 94-100.

81. Kadurugamuwa, J.L., et al., Direct continuous method for monitoring biofilm infection in a mouse model. *Infection and Immunity*, 2003. 71(2): p. 882-890.