Investigation of Staphylococcus epidermidis biofilm resistance to rifampin
by Zhilan Zheng

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering
Montana State University

Abstract:
Biofilms of Staphylococcus epidermidis ATCC 35984 were used to simulate the antibiotic resistant infections that sometimes develop on biomedical implants. Biofilms were grown in continuous flow reactors and challenged with 0.1 mg/1 rifampin. Bacteria in the biofilm resisted killing by this antibiotic. It took 4 hours to achieve a 4.48 +0.14 log reduction of planktonic bacteria with this agent, while treatment of the biofilm realized only a 0.62 + 0.14 log reduction after 48 hours. The goal of this project was to investigate two hypothesized mechanisms of resistance of S. epidermidis biofilms to rifampin: 1) incomplete penetration of the antibiotic through the biofilm, and 2) physiological limitation of antibiotic efficacy due to slow-growing or non-growing bacteria in the biofilm. Rifampin was shown to completely penetrate colony biofilms of S. epidermidis within 12 hours, while failing to kill bacteria much in the same period. Transmission electron micrographs of antibiotic-treated colony biofilms confirmed that the antibiotic was able to penetrate through the colony and affect bacteria at the opposite side of the colony. These results show that biofilm resistance to killing by the antibiotic was not due to failure of the agent to penetrate the biofilm. Experiments comparing growth and susceptibility of exponential phase planktonic cells, stationary phase planktonic cells, intact biofilm, and resuspended biofilm cells indicated that susceptibility decreased as the growth rate decreased. Biofilms were more effectively killed when the antibiotic was applied in medium in which dissolved nutrients were concentrated. On the other hand, treatment of biofilms in gaseous atmospheres ranging from pure nitrogen to air to pure oxygen showed little difference in susceptibility suggesting that oxygen did not play an important role in mediating biofilm resistance. Resistance of S. epidermidis biofilms to rifampin probably stems from the existence of some bacteria in the biofilm in a slow-growing or non-growing state.

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APPROVAL

of a thesis submitted by

Zhilan Zheng

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the college of Graduate Studies.

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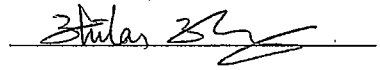
Signature 

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ABSTRACT

Biofilms of *Staphylococcus epidermidis* ATCC 35984 were used to simulate the antibiotic resistant infections that sometimes develop on biomedical implants. Biofilms were grown in continuous flow reactors and challenged with 0.1 mg/l rifampin. Bacteria in the biofilm resisted killing by this antibiotic. It took 4 hours to achieve a 4.48 ± 0.14 log reduction of planktonic bacteria with this agent, while treatment of the biofilm realized only a 0.62 ± 0.14 log reduction after 48 hours. The goal of this project was to investigate two hypothesized mechanisms of resistance of *S. epidermidis* biofilms to rifampin: 1) incomplete penetration of the antibiotic through the biofilm, and 2) physiological limitation of antibiotic efficacy due to slow-growing or non-growing bacteria in the biofilm. Rifampin was shown to completely penetrate colony biofilms of S. epidermidis within 12 hours, while failing to kill bacteria much in the same period. Transmission electron micrographs of antibiotic-treated colony biofilms confirmed that the antibiotic was able to penetrate through the colony and affect bacteria at the opposite side of the colony. These results show that biofilm resistance to killing by the antibiotic was not due to failure of the agent to penetrate the biofilm. Experiments comparing growth and susceptibility of exponential phase planktonic cells, stationary phase planktonic cells, intact biofilm, and resuspended biofilm cells indicated that susceptibility decreased as the growth rate decreased. Biofilms were more effectively killed when the antibiotic was applied in medium in which dissolved nutrients were concentrated. On the other hand, treatment of biofilms in gaseous atmospheres ranging from pure nitrogen to air to pure oxygen showed little difference in susceptibility suggesting that oxygen did not play an important role in mediating biofilm resistance. Resistance of *S. epidermidis* biofilms to rifampin probably stems from the existence of some bacteria in the biofilm in a slow-growing or non-growing state.

INTRODUCTION

Biofilm and Biofilm Resistance

Biofilms are aggregates of living microorganisms and their products attached to an inert surface or the substratum. The matrix contains extracellular polymeric substances such as polysaccharides, glycoproteins, and proteins (Geesey 1982; Christensen 1990 and Costerton 1995). Microorganisms existing in biofilms are found in all aquatic systems. It is thought that by colonizing surfaces and forming biofilm, microorganisms optimize their survival.

There are numerous industrial problems caused by biofilms, such as reduction of heat transfer capacity in cooling water towers and reducing drinking water quality (Characklis et al., 1990). Biofilms are recognized as medical problems as well. They are responsible for dental plaque and persistent infections on medical implants. A major cause of implant failure is the formation of biofilm. For instance, *Staphylococcus epidermidis* has been recognized as a significant pathogen associated with implants, particularly prosthetic heart valves, intravascular catheters, cardiac pacemakers, artificial hearts and prosthetic joints (Wilson et al., 1973; Masur and Johnson 1980; Wilson et al., 1982; Sugarman and Young, 1984; Linares et al., 1985; Gristina et al., 1987; Khardori and Yassien 1995). Figure 1 shows an example of *Staphylococci* on a pacemaker lead. Such infection by biofilm often limits the lifetime of indwelling medical devices. Once biofilm forms on these devices the bacteria become resistant to antimicrobial agents, and the only way to

resolve the infection is to remove the device. This results in additional trauma and expense to the patient (Costerton, et al. 1999).

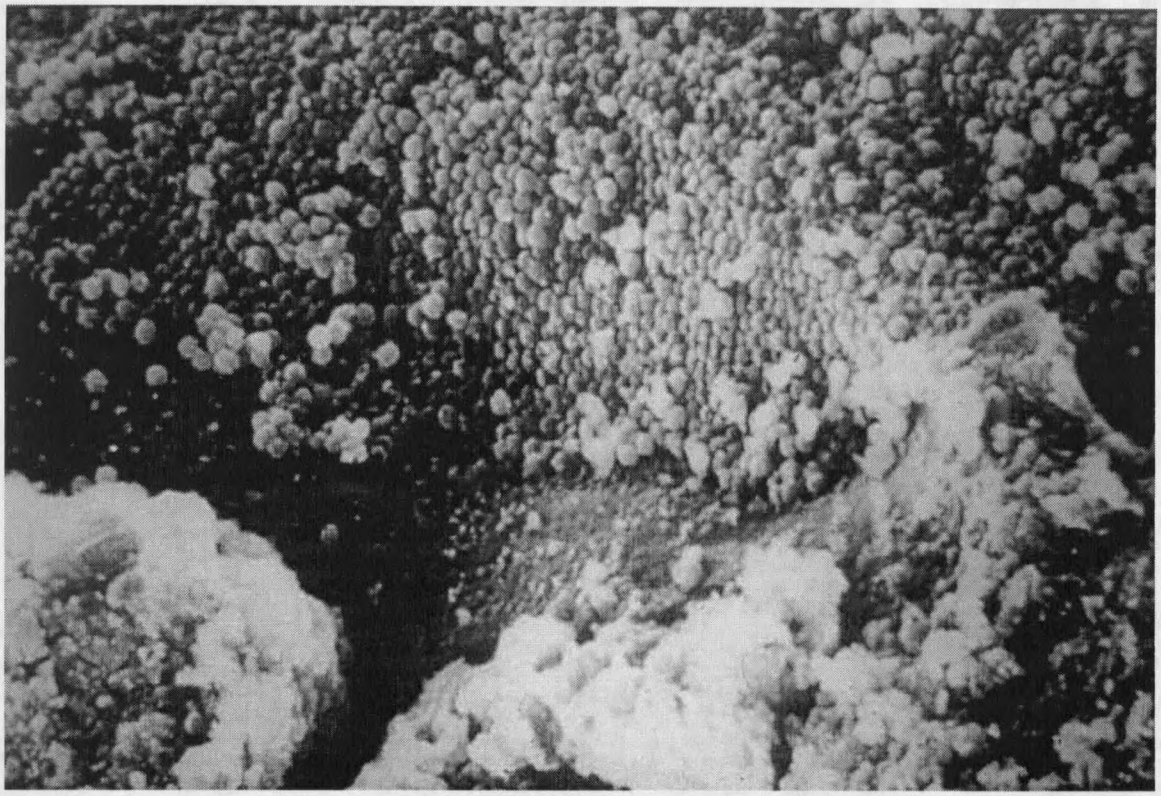

Figure 1. Staphylococci on a pacemaker lead (Marrie et al., 1982)

Microorganisms in biofilm are almost always found to be much less susceptible to antibiotics than their free floating or planktonic counterparts (Anwar et al. 1992; Darouiche et al. 1994; Dunne et al. 1993; Hoyle et al. 1992; Nichols et al. 1989; Vrany et al. 1997). Planktonic microorganisms can be treated effectively with traditional concentrations of antibiotics, but the biofilm population is left virtually unaffected

(Ruseska et al., 1982; LeChevallier et al., 1988). In fact, bacteria in biofilm can become 10-1000 times more resistant to the effects of antimicrobial agents.

Biofilm Resistance Mechanisms

In order to effectively control the biofilm, biofilm resistance and resistance mechanisms must be understood. Interest in the study of microbial biofilms has increased greatly in recent years. However, the development of resistance to antimicrobial agents is still not well understood. A variety of model systems have been used to determine why and how biofilm are so resistant to antibiotics. There are two primary resistance mechanisms suggested by researchers.

One of the proposed mechanisms of biofilm resistance is failure of the antibiotic to penetrate the biofilm. This mechanism has been demonstrated for reactive oxidants like hypochlorite and hydrogen peroxide (de Beer et al., 1994; Xu et al., 1996; Liu et al., 1998). These antibicrobials fail to fully penetrate to all cells within the biofilm because they are neutralized by reaction or adsorption with the constituents of the biofilm faster than they diffuse. Anderl et al. (2000) found that the failure of ampicillin to penetrate *Klebsiella pneumoniae* biofilm was due to its deactivation in the surface layer of the biofilm faster than it could diffuse in. Penetration was restored by deleting β-lactamase activity. Some reports demonstrate that some antibiotics readily diffuse into biofilm, yet kill it incompletely (Dunne 1993; Vrany et al. 1997; Anderl et al. 2000). Inhibition of diffusion cannot explain this resistance. The resistance of biofilm to antibiotics must have multiple mechanisms.

The other hypothesis to explain reduced biofilm susceptibility to antibiotics is physiological limitation. This could be caused by bacteria existing in a slow-glowing or starved state. When a bacterial cell becomes starved for a particular nutrient, it slows its growth. Many studies have examined the influence of growth rate and susceptibility and suggested that the growth rates protect the cells from antimicrobial action (Gilbert et al., 1990; Ashby et al. 1994; Schierholz et al.1998). Bacterial susceptibility increased for planktonic culture, intact biofilm and resuspended biofilm as the growth rate was increased (Duguid et al 1992). Biofilms have been shown to exhibit spatial heterogeneity in pattern of metabolic activity and protein synthesis. For example, spatial pattern of alkaline phosphatase expression was observed with biofilms, which was in response to phosphate starvation of the *Pseudomonas aeruginosa* biofilm cells (Huang et al. 1998). Oxygen plays a very important role in aerobic bacteria. Oxygen availability limits the region of protein synthetic activity in *P. aeruginosa* biofilm (Xu et al 1999). Oxygen limitation also was demonstrated to contribute to enhanced antibiotic resistance of agar-entrapped *E. coli* (Tresse et al. 1995)

Implant-associated infections caused by *S. epidermidis* are often resistant to antibiotic therapy. Rifampin, the most efficient drug to eradicate *S. epidermidis* infections, is structurally related to the macrolides and inhibits the synthesis of mRNA. It has the ability to penetrate tissue and reach therapeutic levels. Widmer (1990) found rifampin failed in curing those device-related infections. Does this failure also correlate with its low efficacy on organisms with a slow growth rate?

Thesis Goal

The goal of this work was to investigate the resistance mechanisms of *Staphylococcus epidermidis* Biofilm to rifampin. The roles of slow growth in the biofilm, as determined by nutrient and oxygen availability, and of permeation of the antibiotic through the biofilm were studied.

MATERIALS AND METHODS

Microorganism, Media and Antibiotic

The microorganism used in this study was *Staphylococcus epidermidis* ATCC 35984, which was grown in pure culture. One-tenth strength Tryptic Soy Broth (TSB) medium was used throughout the study in both planktonic and biofilm experiments (Table 1). Phosphate-buffered water (PBW) with a pH of 7.2 ± 0.5 was used to dilute bacterial culture (Table 2).

Table 1. Composition of 1/10 TSB medium.

Component	Concentration (g/L)
Pancreatic Digest of Casein	1.70
Papaic Digest of Soybean Meal	0.30
Dextrose	0.25
Sodium Chloride	0.55
Dipotassium Phosphate	0.25

Table 2. Composition of PBW.

Component	Concentration (g/L)
KH_2PO_4	0.0425
$MgCl_2 6H_2O$	0.4055

Powdered rifampin (Sigma Chemical Co. St. Louis, Mo) was dissolved in methanol at a concentration of 2 mg/L. This antibiotic stock solution was added to culture broth for planktonic and biofilm treatment experiments or was added to molten Tryptic Soy Agar (TSA) to create antibiotic-containing agar plates for colony biofilm experiments.

Biofilm Culture Procedure

Drip flow reactor

A drip flow reactor was designed to cultivate biofilm (Figure 2). The reactor had four chambers. Biofilm grew on the surface of stainless steel slides, which rested on the bottom of each chamber. Sterile 1/10 TSB was pumped into the reactor and flowed dropwise over the slides at a constant flow rate of 50 ml/h. The air vent on the top of each chamber maintained an aerobic environment for the biofilm growth. Biofilm were allowed to develop for 48 hours prior to treatment.

Stainless steel slides pre-treatment

A cleaning process was used to maintain the same surface characteristics of steel slides for each experiment. Slides were dipped in acetone to remove grease and allowed to air dry. The slides were transferred to a fresh 1: 50 RBS 35 working solution (a surface active agent for cleaning and radioactive decontamination of laboratory glassware, surgical and pharmaceutical instruments. Pierce, Rockford, Illinios) and heated to 50°C in a water bath for 5 minutes. After being sonicated for 5 minutes, the slides were rinsed with nanopure water until they no longer foamed and then sonicated again for 5 minutes.

Slides were rinsed three more times with nanopure water and allowed to air dry. Slides were stored in containers at room temperature. Acid treatment of the slides was applied after cleaning to obtain hydrophilicity. Each clean slide was soaked in a tube with 35 ml of 2.0 M HCl solution for 2 hours. The acid-treated slides were rinsed thoroughly with de-ionized water then nanopure water. After air-drying, slides were ready to use.

Operation of drip flow reactor

Reactor sterilization. Tubing and reactor components were washed and air-dried before using. The treated slides were placed into the reactor and fixed with a small piece of autoclave tape. Reactor chambers were covered with lids, but the lids were left unscrewed. The reactor and all open ends of tubing were then wrapped with aluminum foil. Reactor, tubing and empty carboys (for waste) were autoclaved for 25 minutes on gravity cycle. After autoclaving, the reactor was allowed to cool to room temperature.

Inoculation of slides. A 50 ml sterile tube holding 20 ml of 1/10 TSB was inoculated with *S. epidermidis*. This culture was incubated at 37°C in a shaking incubator for 18 hours. Working inside the biological hood, the effluent tubing was attached to the reactor and clamped. Fifteen ml of sterile 1/10 TSB was added to each chamber of the reactor. One ml of overnight culture was then added to each chamber. The reactor rested horizontally in a 37°C incubator with no flow for 5 hours.

Biofilm formation. The reactor was inclined 10° from horizontal by placing a plastic wedge underneath the reactor. The inoculation culture was drained by unclamping the effluent tubing, which was connected to the waste reservoir. The influent tubing was

connected to a carboy containing sterile 1/10 TSB, which had been autoclaved for at least 2 hours. Sterile needles were attached to the end of pump tubing and pierced into the top of the lids. Biofilm was fed with 1/10 TSB at 37°C for 48 hours by pumping the medium into the reactor at a constant rate of 50 ml/hr, which was 5.6 ml/cm^2·h.

Biofilm treatment and sampling. After a biofilm reached its desired age, the following procedure was implemented. The bacterial air vent of the reactor was either connected to pure oxygen (50 ml/min), pure nitrogen (50 ml/min) or exposed to ambient air to create different gaseous environments. At the same time the biofilm was fed with medium amended with 0.1 mg/L rifampin or with medium lacking antibiotic for an untreated control. After determined time was reached, the pump was turned off and medium flow to the reactor was stopped. Each slide was then transferred with sterile forceps to a 100 ml beaker holding 50 ml PBW. Small plastic scrapers (rubber policeman) were used to scrape the slides and remove the biofilm into the beaker. The sample was homogenized for 30 seconds with a Ultra-Turrax T25 homogenizer (Janker & Kunkel Co.) and was then ready for viable cell enumeration.