Abstract:
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INVESTIGATION OF *Staphylococcus epidermidis* BIOFILM RESISTANCE TO RIFAMPIN

By

Zhilan Zheng

A thesis submitted in partial fulfillment
of the requirements for the degree

of

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in

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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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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).

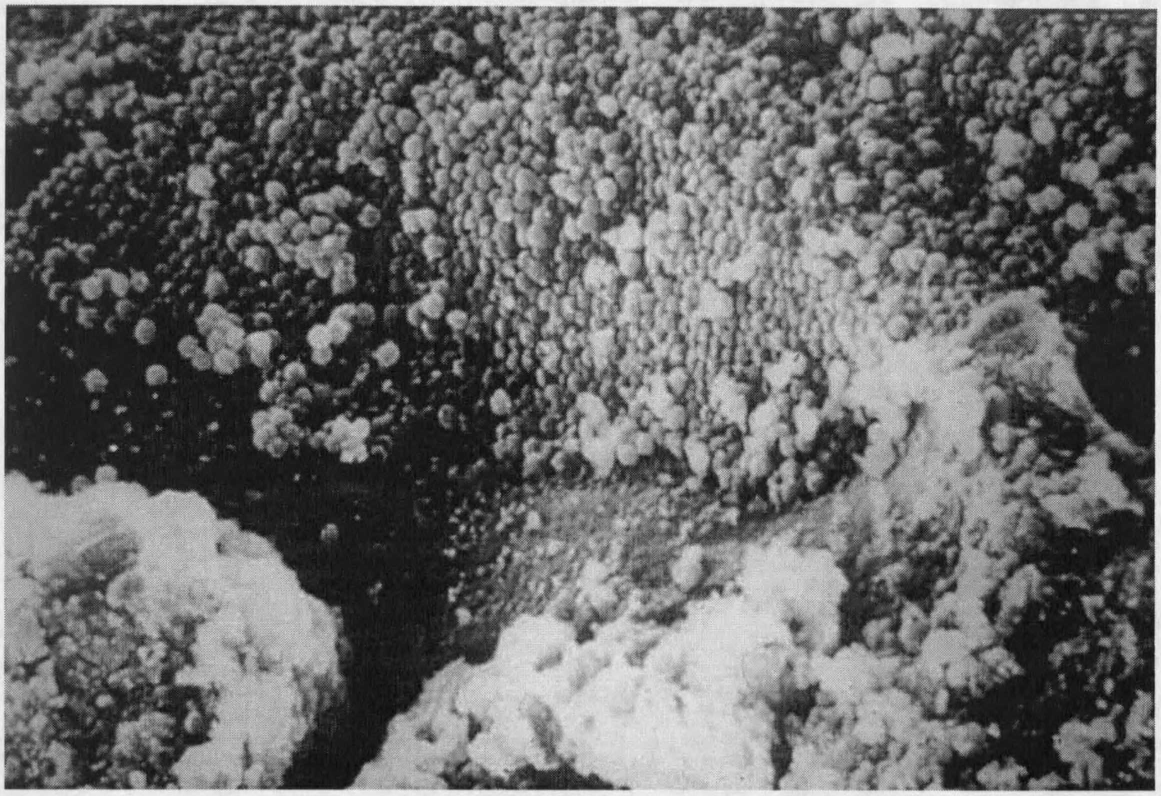


Figure 1. Staphylococci on a pacemaker lead (Marrie et al., 1982)

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
$MgCl_2 \cdot 6H_2O$	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 $ml/cm^2 \cdot h$.

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.

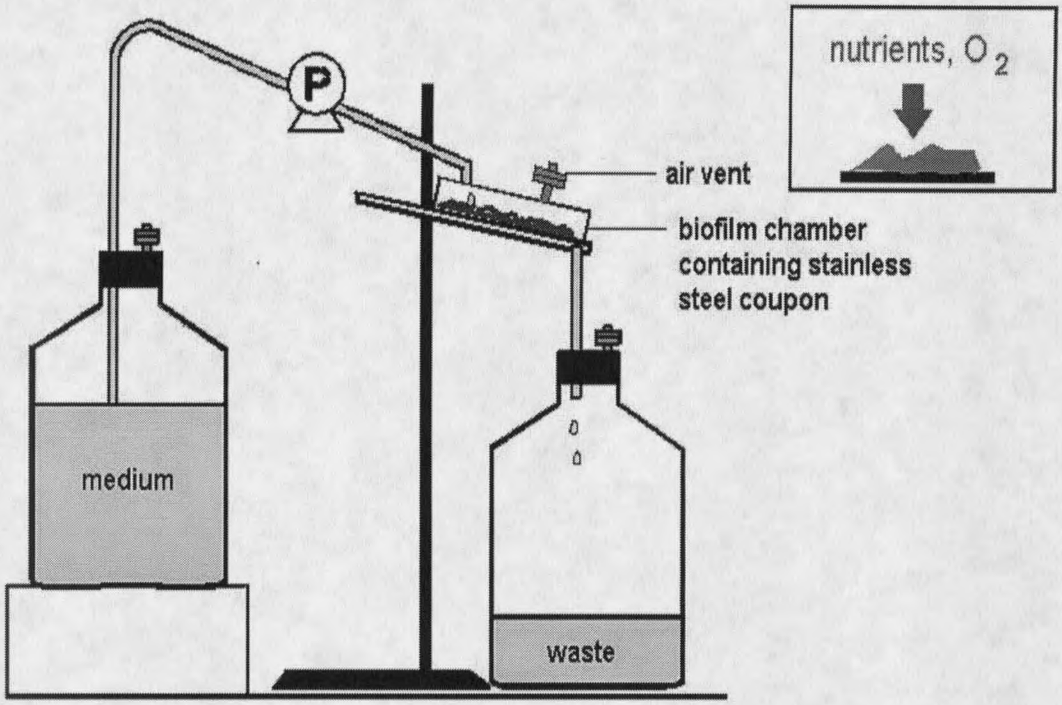


Figure 2. Schematic diagram of drip-flow biofilm reactor.

Planktonic Culture Procedure

A 100 µl aliquot of a *S. epidermidis* overnight culture was inoculated to a 250 ml flask holding 100 ml of 1/10 TSB medium. This culture was incubated in a shaking incubator for 6 hours for log phase planktonic experiment and 30 hours for stationary phase planktonic experiment. Both types of planktonic cultures were diluted with 1/10 TSB to an optical density of 0.02 prior to treatment. Antibiotic was added to the culture broth for treatment experiments.

Resuspended Biofilm Procedure

48-hour-old biofilm was grown up in a drip flow rector as described above. Biofilm was scraped into a beaker holding 50 ml 1/10 TSB and then homogenized. The resuspended biofilm was diluted with 1/10 TSB medium to an optical density of 0.02. Antibiotic was then added to the cell suspension.

Viable Cell Enumeration

A 1 ml volume of the sample culture was used to make serial 10-fold dilutions. TSA plates were prepared for the drop-plate method by dividing each plate into four quadrants, allowing one quadrant per dilution. An automatic pipettor was set to pick up 100 μl volume and to expel 10 μl with each push of the button. Cell dilution tubes were vortexed and expelled in 5 evenly spaced 10 μl drops per dilution on duplicate plates. Once the drops dried, the plates were inverted and incubated at 37°C for 17-20 hours. Colonies on the agar were counted. Each colony was taken to represent one colony forming unit (CFU).

Average Specific Growth Rates Measurement

The average specific growth rate of exponential phase planktonic, stationary phase planktonic and resuspended biofilm, assumed exponential growth, were calculated using the equation (1):

$$\ln(X/Xo)=\mu t \qquad (1)$$

X (CFU/cm^3): cell density in the culture at time t

Xo (CFU/cm^3): cell density in the culture at time t=0

μ (1/hr): average specific growth rate

The average specific growth rate of intact biofilm was calculated using mass balance (2), assuming biofilm was at steady state:

$$\mu XaA - QXe = \mathrm{d}(XaA)/dt \qquad (2)$$

μ (1/hr): average specific growth rate

Xa (CFU/cm^2): cell density in the biofilm

Q (ml/hr): nutrient flow rate

Xe (CFU/cm^2): cell density in the effluent

A (cm^2): area of biofilm

Staining, Cryoembedding and Cryosectioning Procedures

CTC (5-cyano-2,3-ditolyl tetrazolium chloride, Polyscience Inc. Warrington, PA) was used as an indicator of bacterial respiratory activity. The DNA stain DAPI (4',6-diamidino-2-phenylindole, Sigma Chemical Co. Louis MO) was used to indicate cellular biomass independent of activity.

Biofilm samples were stained and cryoembedded as described by Yu et al. (1994). The prepared biofilm samples were collected by withdrawing slides from the reactor and placing them in a staining container with the biofilm side up. A 0.16% CTC solution was added to stain the specimens. After incubation at 37°C for 2 hours, the CTC solution was removed and the biofilms were subsequently cryoembeded and cryosectioned. Cryoembedding was performed with Tissue-Tek OCT compound (Miles Inc., Elkhart, IN) by dispensing a thick layer of OCT of the top of the biofilm, and immediately placing the slide on top of a dry ice slab. The biofilm was allowed to rapidly freeze until the specimen turned opaque white. The specimen was then separated from the substratum by gently bending the slide to remove the frozen sample. The embedded biofilm was then turned over to coat the substratum side. The whole process was performed on the dry ice block to prevent the samples from thawing. After embedding, the biofilm was completely surrounded by OCT. The sample was then wrapped with aluminum foil and stored in a -70°C freezer till it could be sectioned.

Frozen biofilm samples were sectioned with a Leica CM 1800 cryostat (Leica Inc., Deerfield, IL) at –19°C. The sample was bisected for mounting on a precooled (-19°C) sample chuck. A small amount of OCT was poured on the chuck and the sample was pressed into the OCT. When the OCT solidified, the sample was fixed on the chuck and ready for cutting. Each 5 μm thick frozen section was collected on Superfrost Plus Microscopic Slides (Fisher Scientific, Pittsburgh, PA). The frozen biofilm sections were counterstained with 10 μg/ml DAPI for 3 minutes.

Microscopy and Image Analysis

Biofilm sections stained with CTC/DAPI were visualized under a Nikon Microscope Eclipse E800 (Nikon Inc. Melville, NY) with epifluorescence illumination. After staining, activity respiring cells exhibited red fluorescence with a TRITC epifluorescence filter, whereas all bacteria appeared blue through a DAPI epifluorescence filter.

Images captured at the same spot by different filters were digitized by a cooled CCD camera (Optronics, Coleta, CA) and saved as 8-bit TIFF files. The fluorescence intensity was determined by Image Pro Plus image analysis software. An image of a ruler was first taken to calibrate the distance. The fluorescence intensity along a transect was measured with the built-in "line profile" function. A line was drawn across the biofilm section and the "line profile" function gave the fluorescence intensity along the line. The result was opened in Microsoft Excel and graph of fluorescence intensity versus position was generated.

Total Cells/Active Cells Count

Intact biofilms were stained with mixture of 0.16% CTC and 10 μg/ml DAPI with the method described before for CTC staining. Stained biofilm was then scraped into a 100ml beaker holding 50 ml PBW. The sample was homogenized for 30 seconds and then diluted as appropriate for cells counting.

Resuspended biofilm bacteria were also stained with CTC. Untreated biofilm was scraped into a 100 ml beaker holding 50 ml PBW and homogenized for 30 seconds. Resuspended biofilm was centrifuged at 10,000 rpm in a Micro14 microcentrifuge (Fish Science, San Francisco, CA) for 5 minutes. The cells were washed twice with water and then diluted to the designed density of approximate 10^5 cells/ml. Cells were stained with a mixture of 0.16% CTC and 10 μg/ml DAPI for 2 hours at 37°C.

Planktonic cells were stained as well. A 100 μl *S. epidermidis* overnight culture was inoculated to a 250 ml flask holding 100 ml of 1/10 TSB medium and was incubated in a shaking incubator for 6 hours for log phase planktonic cells. The culture was washed with water and stained with CTC/DAPI by using the same procedure as described above for resuspended biofilm

The same procedure was used to make all sample slides. A 1 ml volume of diluted sample was dropped slowly onto the top of a polycarbonate membrane filter on a filtration apparatus stage. Vacuum was applied to the filter for 1 minute. A small drop of immersion oil was placed on a labeled glass microscope slide. The filter membrane was put on top of the immersion oil (cell side up). Another drop of immersion oil was placed on top of the membrane. A glass cover slip was then placed over the membrane.

Cells counts were made with a Nikon Microscope Eclipse E800 with DAPI and TRITC filters. Twenty random fields were chosen to count the cells. Total cells were visualized by DAPI filter. At the same spot, red cells visualized with the TRITC filter represented active cells.

Colony Biofilm Model

In a biological hood, black polycarbonate membrane filters (diameter, 25 mm, pore size, 0.2 μm, Poretics, Corp. Livermore, Calif.) were placed on the bottom of petri dishes. Both sides of the membranes were sterilized by UV light exposure for 15 minutes. Sterile membrane filters were placed on TSA plates with shiny side up. A 40 μl volume of overnight culture of *S. epidermidis* was dropped onto the membrane for inoculation. The plates were inverted and incubated at 37°C for 48 hours. The membrane-supported biofilms were transferred to fresh TSA plates every 12 hours. The colony biofilm system is diagrammed in Figure 3.

Colony Biofilm Susceptibility

Colony biofilms were transferred to antibiotic-containing agar plates for treatment experiments and the agar plates were incubated at 37°C. The biofilms were sampled every 1 hour for 4 hours, or every 2 hours for 12 hours, or every 4 hours for 48 hours. When sampled, each membrane supported biofilm was transferred to 9 ml of PBW, and the mixture was vortexed with Maxi Mix II Vortex mixter (Barnstead /Thermolyne, Dubuque, IW) at highly speed for 1 minute. The resulting cell suspension was then ready for viable cell enumeration.

Antibiotic Penetration

Standard curve

Rifampin bioassay standard curve was made to get the relationship between rifampin concentration absorbed in the disks and the inhibition zones in bacteria lawn caused by these disks. Concentration disks (blank paper disc, 6 mm diameter, catalog no. 1599-33-6; Difco Laboratories) were moistened with 25 μl of a series of rifampin concentration ranging from 0.01 mg/L to 0.1 mg/L, respectively. The moistened disks were placed on TSA plates spread with 100 μl of planktonic *S. epidermidis.* The *S. epidermidis* was grown in 1/10 TSB at 37°C and the culture was diluted with the same medium to an optical density of 0.050 prior to plating. The plates were incubated in the 37°C incubator for 24 hours. The zone of inhibition reflected the concentration of active rifampin in the disk.

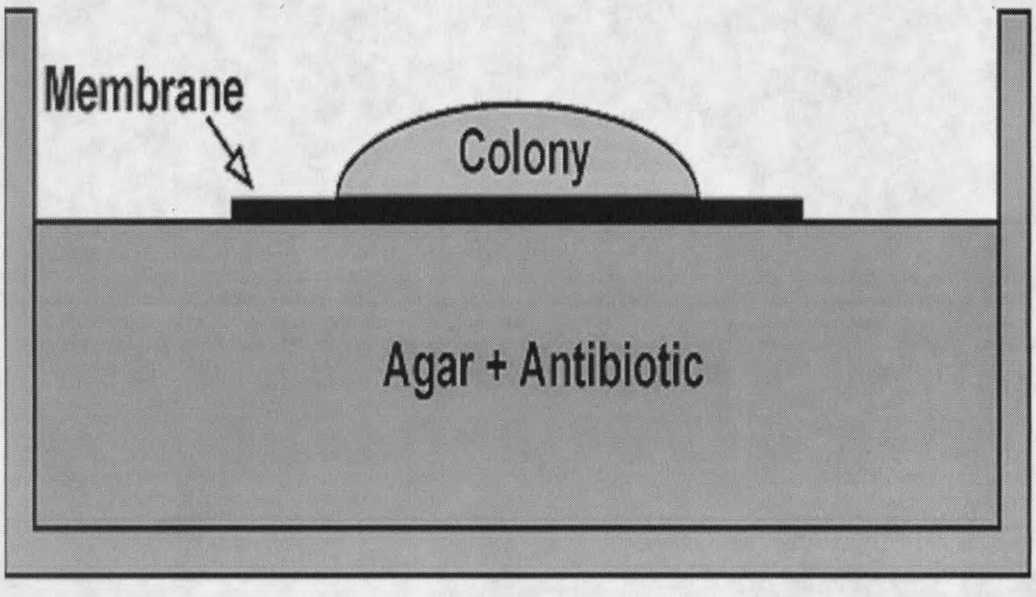


Figure 3. Schematic depiction of the colony biofilm.

Antibiotic penetration measurement

The experimental system and procedure used to measure antibiotic penetration through colony biofilms of *S epidermidis* is shown in Figure 4 and Figure 5. In two membranes control system, a black polycarbonate membrane filter (diameter, 25 mm, pore size, 0.2 μm, Poretics, Corp. Livermore, Calif.) was placed on the TSA plate, and followed by the other black polycarbonate membrane filter (diameter, 13 mm, pore size, 0.2 μm, Poretics, Corp. Livermore, Calif.). A concentration disk (blank paper disc, 6mm diameter, catalog no. 1599-33-6; Difco Laboratories) was moistened with 25 μl of 1/10 TSB culture broth, and then placed on top of the 13-mm-diameter membrane. Two membranes with the moistened disk were transferred to 0.1 mg/L rifampin-containing TSA plate. The disk was removed after specified exposure time and wrapped with parafilm (American Matinal Can, Chicago, III.) to prevent it from drying out. All disks were stored at 4°C. After collecting all the samples, the disks were placed on the TSA plates spread with bacteria as described above in the standard curve subsection. The zone of inhibition was measured to determine the equilibrium concentration of active rifampin in the disc. The effective rifampin concentration was estimated by averaging the equilibrated concentration observed in the disks.

To measure antibiotic penetrated through a colony biofilm, 13mm-diameter black polycarbonate membrane filters were placed on the top of 48-hour-old *S. epidermidis* colony biofilms. A concentration disk moistened with 25 μl of 1/10 TSB culture broth was placed on the top of the 13-mm-diameter membranes. The entire assembly was transferred to TSA plates containing 0.1 mg/L rifampin. After a determined time, the disk was removed. The concentration of rifampin in the disk was measure as described before. The concentration that penetrated through the biofilms was divided by the effective rifampin concentration in the agar to compute relative concentration curve.

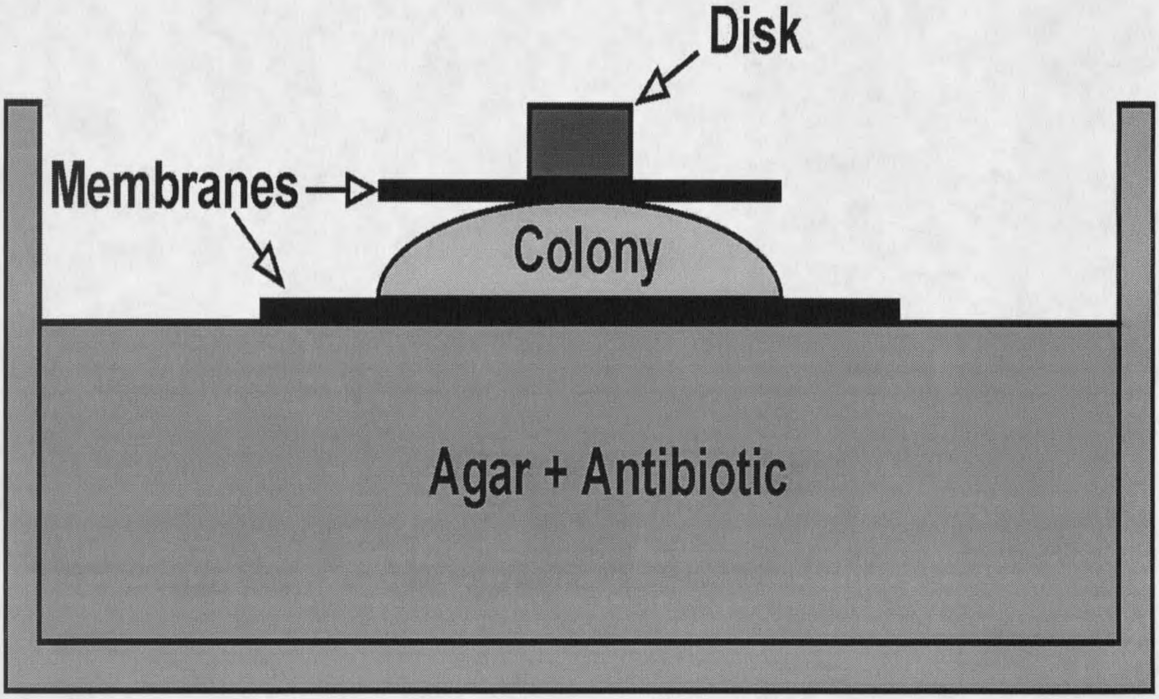


Figure 4. Schematic depiction of the experimental system used to track the penetration of antibiotic through the biofilm.

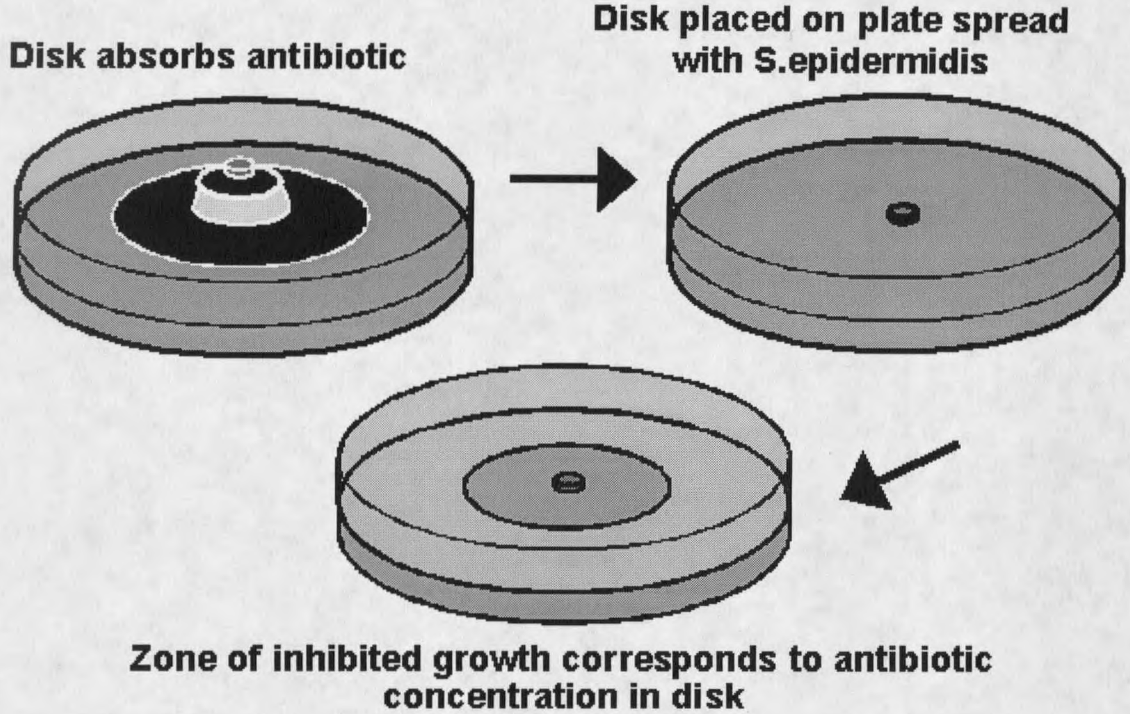


Figure 5. Experimental approach for measuring antibiotic penetration using a zone-of-inhibition bioassay to determine the presence of the antibiotic.

Colony Biofilm Crosections/TEM

48-hour-old *S. epidermidis* colony biofilms samples were prepared as described above. Samples were fixed in 5% glutaraldehyde in PBW. Two membranes were transferred to a petri dish containing filter paper holding 5% glutaraldehyde for 24 hours. After fixation the membranes and colony biofilms were transferred to another petri dish containing filter paper soaked with PBW and stored at 4°C. These samples were considered time zero control. Other membranes were transferred to TSA plates and TSA plates containing 0.1mg/L rifampin for another 48 hours. These colony biofilms were removed and fixed in glutaraldehyde for 24 hours. The samples were then washed in 0.1 M Millonigs phosphate buffer at 4°C three times for 15 minutes each time. Next, the samples were fixed in 1% OsO_4 for 1 hour. The samples were again washed in 0.1 M Millonigs phosphate buffer at 4°C three times for 15 minutes each time. The samples then underwent a series of dehydration staining and resin embedding steps: 50% ethanol 15 minutes; 70% ethanol 15 minutes; 1% uranyl acetate / 1% phosphotungstic acid in 70% ethanol 1 hour; 95% ethanol 15 minutes; 100% ethanol 15 minutes each for 3 times; 100% ethanol (2 part) and Spurrs (1 part, Ernest F. Fullam, Inc) 1 hour; 100% ethanol (1 part) and Spurrs (1 part) 1 hour; Spurrs epoxy resin 8 hours or overnight. The resin was polymerized at 68°C for 14 hours. After polymerization, samples were stained with Reynolds lead acetate and washed in distilled water. Ultrathin sections were cut and examined using a Jeol EM-100 CX electron microscope.

RESULTS

This chapter reports the results of measurements of influences of slow-growing bacteria and transport limitation of rifampin on resistance of *S. epidermidis* biofilm to this antibiotic. The measurements include growth rates of bacteria in planktonic culture and biofilm, nutrient and oxygen limitation in biofilm, as well as penetration of rifampin through colony biofilm.

Planktonic, Biofilm, and Resuspended Biofilm Susceptibilities

The observed MIC of rifampin for planktonic *S. epidermidis* was 0.01 mg/L. Exponential phase planktonic cells and biofilm were challenged with 10 times the MIC of this antibiotic. Planktonic bacteria were rapidly killed by this treatment. The log reduction in the number of CFU was 4.48 ± 0.34 after 4 hours treatment. However, the log reduction in the number of CFU in biofilm challenged with rifampin for 4 hours was only 0.62 ± 0.13. This reduction was statistically different from that of 4-hour planktonic treatment (P=0.002). Even when the challenge time was extended to 48 hours, the log reduction of biofilm (3.14 log reduction) was still less than 4-hour treated planktonic cells. This indicated that biofilm cells were clearly less susceptible than planktonic cells to the same rifampin treatment (Figure 6).

Susceptibilities of exponential phase planktonic, stationary phase planktonic and resuspended biofilm to 4 hours rifampin exposure were investigated (Table 3 and Figure 7). The extent of growth in untreated controls observed in stationary phase planktonic and resuspended biofilm cells were less than and statistically different (P=0.008 and P=0.05, respectively) from the extent of growth measured for exponential phase planktonic cells. The log reductions of treated cells in stationary phase planktonic and resuspended biofilm cells were also less than and statistically different (P=0.00001 and P=0.00003, respectively) from the log reduction in treated exponential phase planktonic cells.

In order to further characterize the effect of growth rate on bacterial susceptibility, the dependence of log reduction on average specific growth rates were examined for exponential phase planktonic, stationary phase planktonic, intact biofilm and resuspended biofilm (Table 4 and Figure 8). It is clear that the fast the bacteria grow, the more susceptible they are.

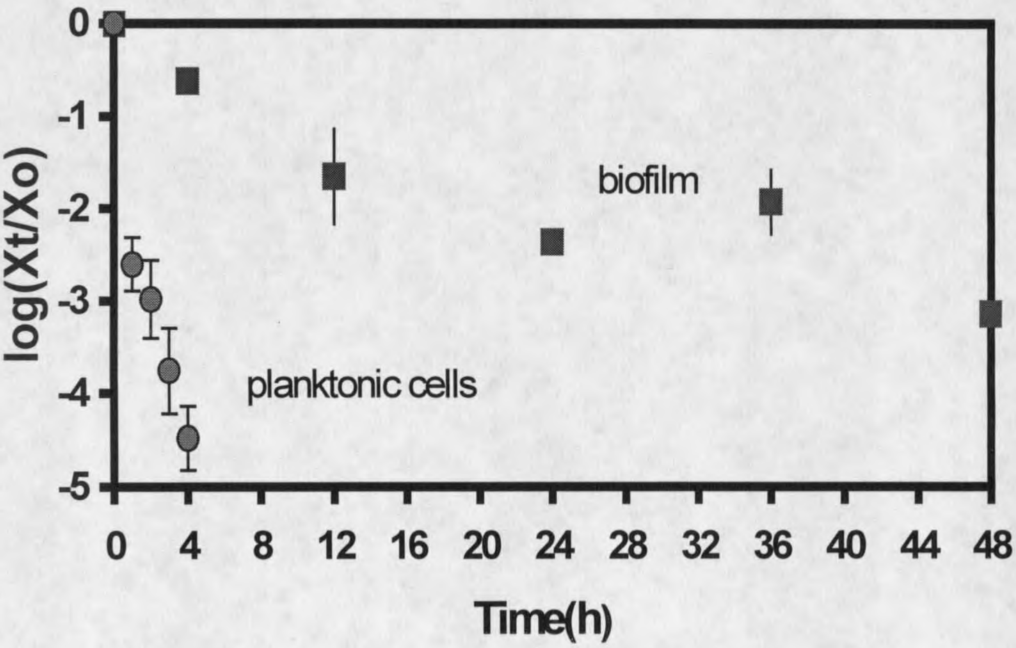


Figure 6. Comparison of susceptibility of planktonic and 48-hour *S. epidermidis* biofilm exposed to 0.1 mg/L rifampin. Error bars reflect ± 1 standard error of the mean.

Table 3. Comparison of susceptibilities of exponential phase planktonic, stationary phase planktonic and resuspended biofilm in 4 hours.

Source of Bacteria	-Log (X/X_o)	
	Untreated Control	Treated
Exponential phase planktonic	-1.84 ± 0.54	4.48 ± 0.34
Stationary phase planktonic	-0.29 ± 0.11	1.11 ± .0.05
Resuspended biofilm	-0.93 ± 0.17	1.53 ± 0.06

Table 4. Summary of susceptibilities of exponential phase planktonic, stationary phase planktonic, resuspended biofilm and their average growth rate.

Source of Bacteria	Growth Rate (h^{-1})	-Log (X_t/X_o)
Exponential phase planktonic	0.82±0.34	4.48 ± 0.34
Stationary phase planktonic	0.15±0.06	1.11 ± .0.05
Resuspended biofilm	0.51±0.03	1.53 ± 0.06
Intact Biofilm	0.08±0.02	0.62 ± 0.13

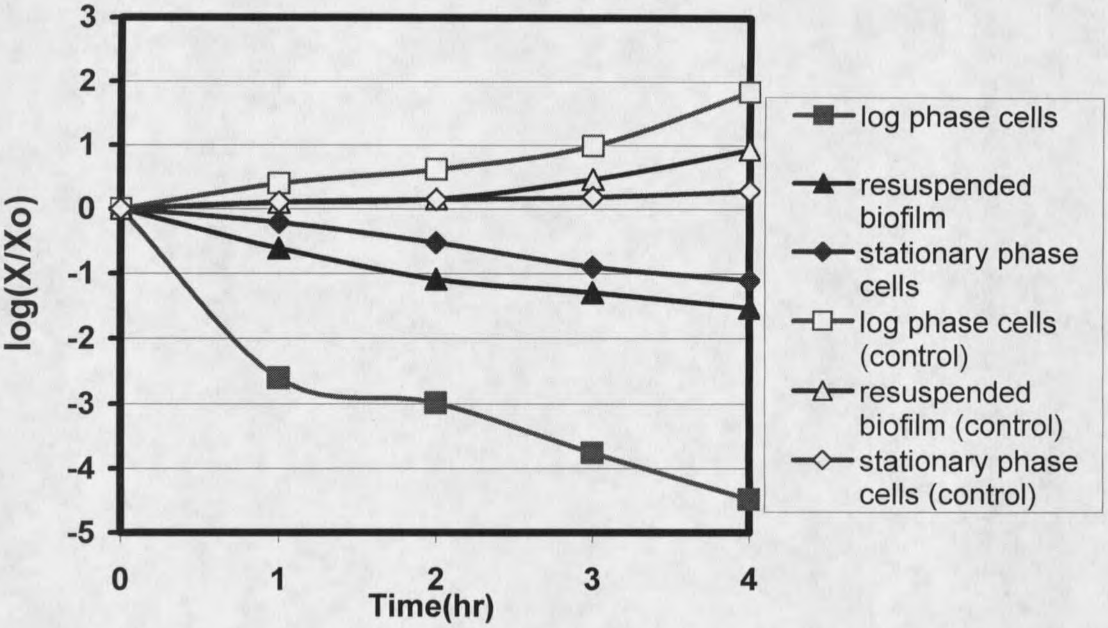


Figure 7. Comparison of susceptibilities of exponential phase planktonic cells, stationary phase planktonic cells and resuspended biofilm cells of *S. epidermidis* to 0.1 mg/L rifampin over a period of 4 hours.

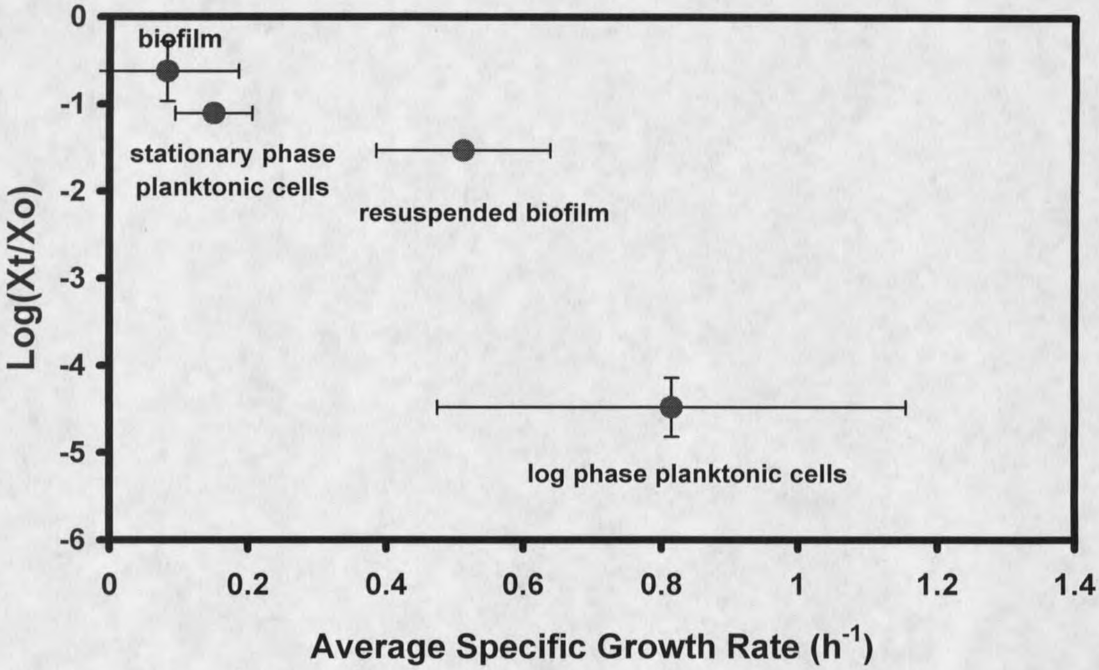


Figure 8. Dependence of susceptibility of exponential phase planktonic cells, stationary phase planktonic cells, resuspended biofilm cells and intact biofilm cells of *S. epidermidis* on their average specific growth rate. Error bars reflect ± 1 standard error of the mean.

Effect of Oxygen on Biofilm Susceptibility

Biofilms were treated with 0.1 mg/L rifampin while exposed to different gaseous environments: ambient air, pure oxygen and pure nitrogen. The results are shown in Figure 9. It is clear that reduction of *S. epidermidis* biofilm does not change much with changes in the oxygen partial pressure. The log reductions observed in the numbers of CFU were 0.62 ± 0.14, 0.33 ± 0.05 and 0.95 ± 0.23 after 4 hours in ambient air, pure nitrogen and pure oxygen, respectively. Increasing the treatment time to 12 hours still did not result in much difference in log reduction, which were 1.98 ± 0.49, 1.68 ± 0.14 and 2.09 ± 0.22 for ambient air, pure nitrogen and pure oxygen, respectively. Statistical analysis of these two groups of data revealed no significant difference of pure nitrogen or pure oxygen from those of ambient air experiments (Table 5).

Table 5. Comparisons of log killings of biofilms in different air condition for 4 hours and 12 hours. The P-value refers to the comparison with the ambient air result.

Air condition	4 hours treatment		12 hours treatment	
	-Log (X_t/X_c)	P-value	-Log (X_t/X_c)	P-value
Ambient air	0.62 ± 0.14	–	1.98 ± 0.49	–
Pure nitrogen	0.33 ± 0.05	0.03	1.67 ± 0.14	0.35
Pure oxygen	0.95 ± 0.22	0.08	2.09 ± 0.22	0.83

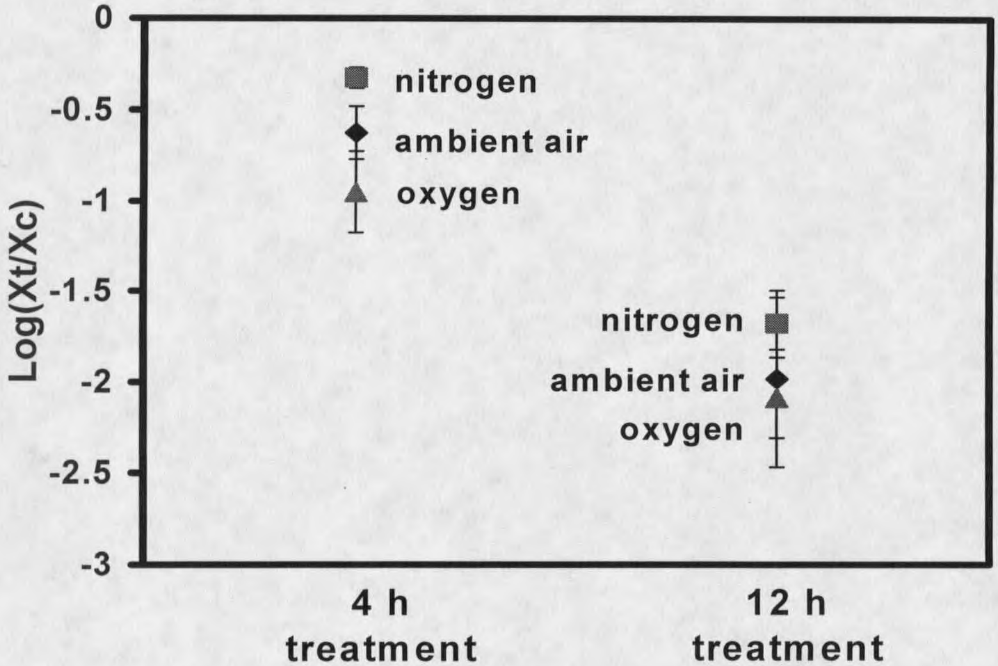


Figure 9. *S. epidermidis* biofilm susceptibilities under ambient air, pure nitrogen and pure oxygen in response to 0.1 mg/L rifampin. Error bars reflect ± 1 standard error of the mean.

Effect of Nutrient Concentration of Biofilm Susceptibility

To investigate the relationship between the physiological state of the cells and their antibiotic susceptibility, antibiotic was applied in media with different nutrient concentrations (Table 6). Nutrient strengths of 1/100 TSB, 1/50 TSB, 1/10 TSB, 1/5 TSB and full strength TSB were chosen to apply in the system for 4 hours. On a plot of $\log_{10}$ of survival fraction versus nutrient strength, the extent of biofilm log killing was not linear (Figure 10). The log reductions were statistically different from the log reduction when treated biofilm with 1/10 TSB in 4 hours. When 1/100 TSB, 1/10 TSB and full strength TSB were applied in the system for 12 hours, the biofilm log killing was proportional to the nutrient concentration (Figure 11). The log reduction in 1/100 TSB and full strength TSB were significant different from that in 1/10 TSB for 12 hours.

Table 6. Effect of nutrient on *S. epidermidis* biofilm susceptibility in 4 hours and 12 hours. The P-value refers to the comparison with the 1/10 TSB result.

	4 hours		12 hours	
Medium (fraction of TSB)	-Log(X_t/X_c)	P-value	-Log(X_t/X_c)	P-value
1/100	0.23 ± 0.14	0.006	0.87 ± 0.15	0.015
1/50	0.41 ± 0.13	0.034	–	–
1/10	0.62 ± 0.14	–	1.59 ± 0.31	–
1/5	0.33 ± 0.02	0.013	–	–
1	0.39 ± 0.13	0.028	2.66 ± 0.46	0.014

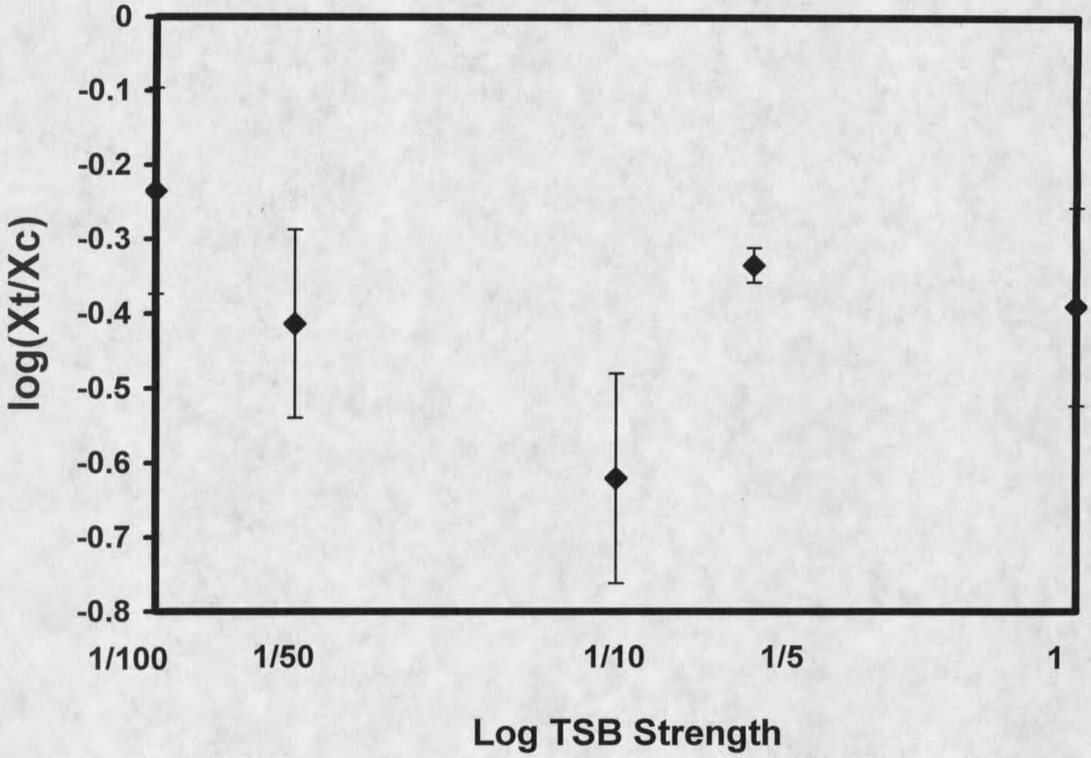


Figure 10. Killing of 48-hour biofilm by 0.1 mg/L rifampin in 1/100, 1/50, 1/10, 1/5 and full strength TSB over a period of 4 hours. Error bars reflect ± 1 standard error of the mean.

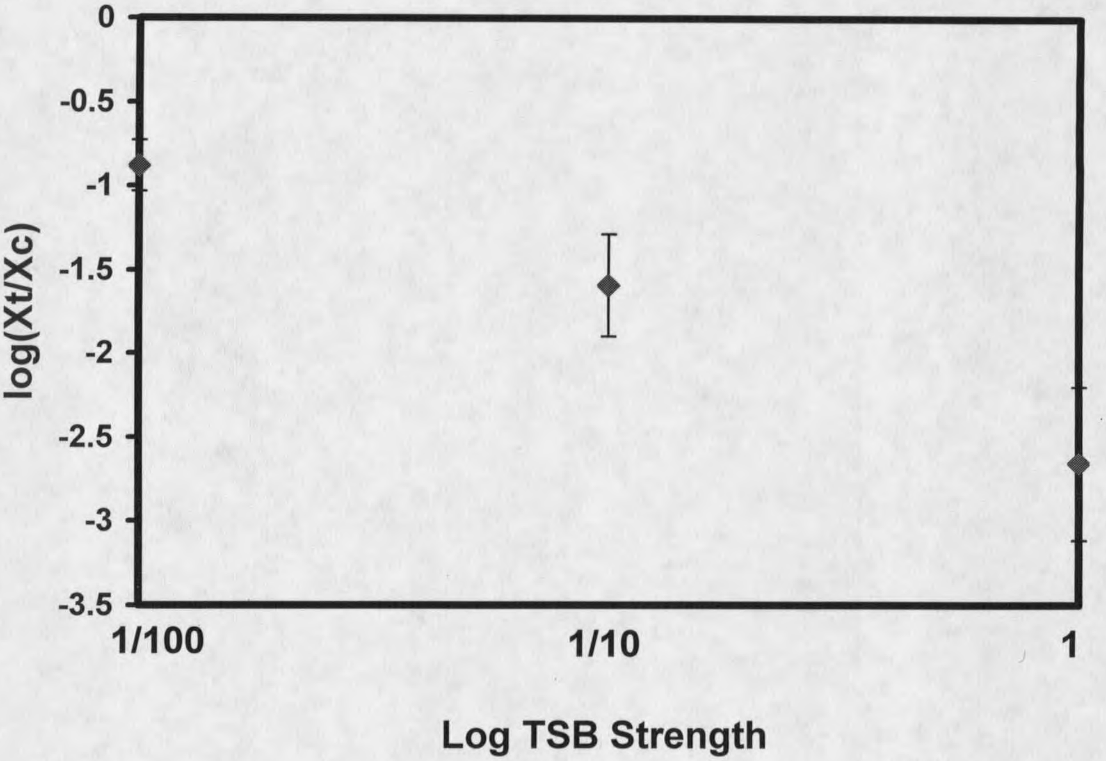


Figure 11. Killing of 48-hour biofilm by 0.1 mg/L rifampin in 1/100, 1/10, and full strength TSB over a period of 12 hours. Error bars reflect ± 1 standard error of the mean.

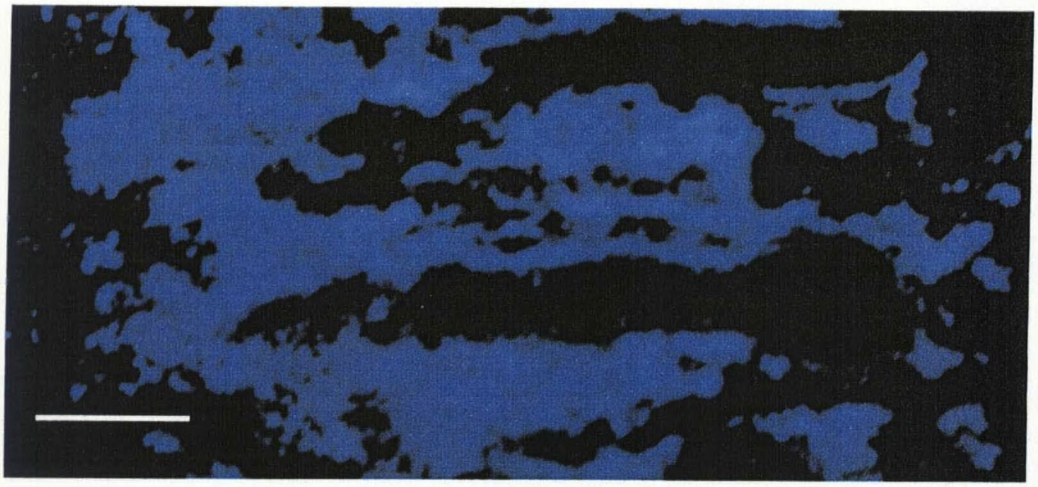
Structure and Spatial Pattern of Respiratory Activity in Biofilms

Distinct patterns of respiratory activity, as indicated by CTC staining, were observed in control and treated biofilm specimens. In untreated control biofilm samples, an intense layer of red CTC-formazan formed at the biofilm-bulk liquid interface (Figure 12B), with less intense staining near the substratum. DAPI counterstained of the same section revealed a uniform blue intensity throughout the biofilm (Figure 12A). Image analysis was used to quantify the zone of higher respiratory activity. A representative image analysis result is shown in Figure 13. In control biofilm samples, the inactive zone near the substratum was about one-third of the overall thickness (Table 7).

In treated biofilm, the staining results were different from those seen in control biofilm. A layer of weak red CTC-formazan staining appeared at the biofilm-bulk liquid interface (Figure 14B), with more intense red staining throughout the rest of the biofilm section. DAPI still revealed a relatively uniform blue intensity throughout the biofilm (Figure 14A). Image analysis was again used to quantify zone of activity and inactivity. A representative image analysis result for a treated biofilm is shown in Figure 15. The thickness of treated biofilm was less than that of control biofilm (Table 7).

Table 7. Summary of image analysis results of CTC/DAPI staining in control and treated biofilms.

Sample Type	Overall thickness (μm)	Inactive zone near substratum (μm)	Inactive zone near biofilm-bulk liquid interface (μm)
Control biofilm	636 ± 64	203 ± 28	0
Treated biofilm	466 ± 13	0	91 ± 13



(A)

(B)

Figure 12. Photographs of a cross-section of S. *epidermidis* untreated control biofilm stained with CTC/DAPI. (A) DAPI staining visualized with DAPI filter. (B) CTC staining visualized with TRITC filter. The substratum is on the right side of each photograph. Bars represent 100 μm.

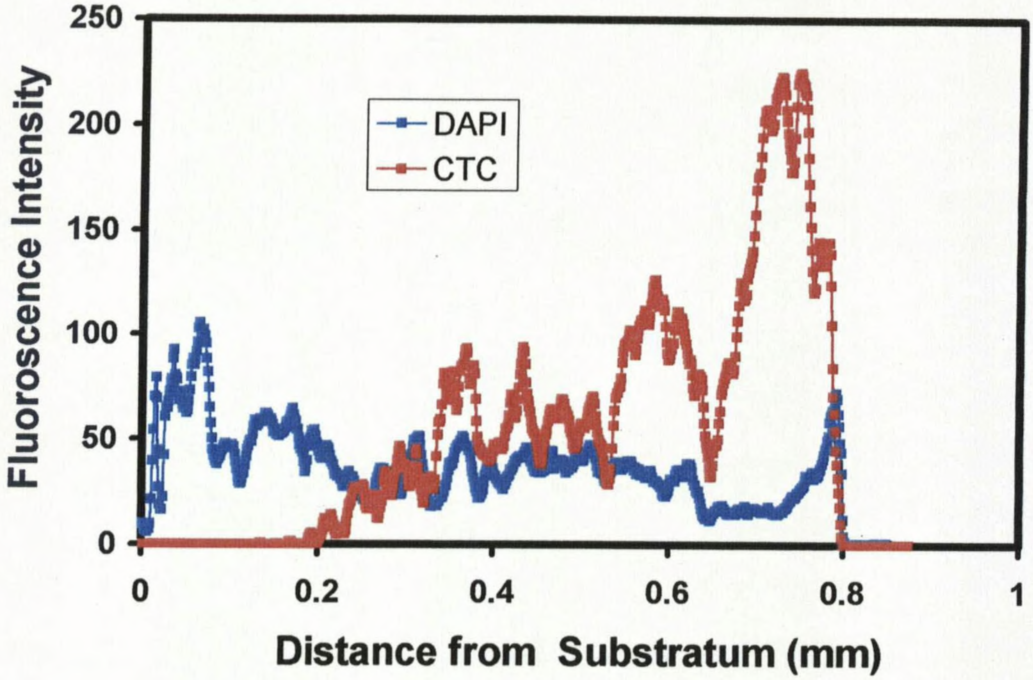
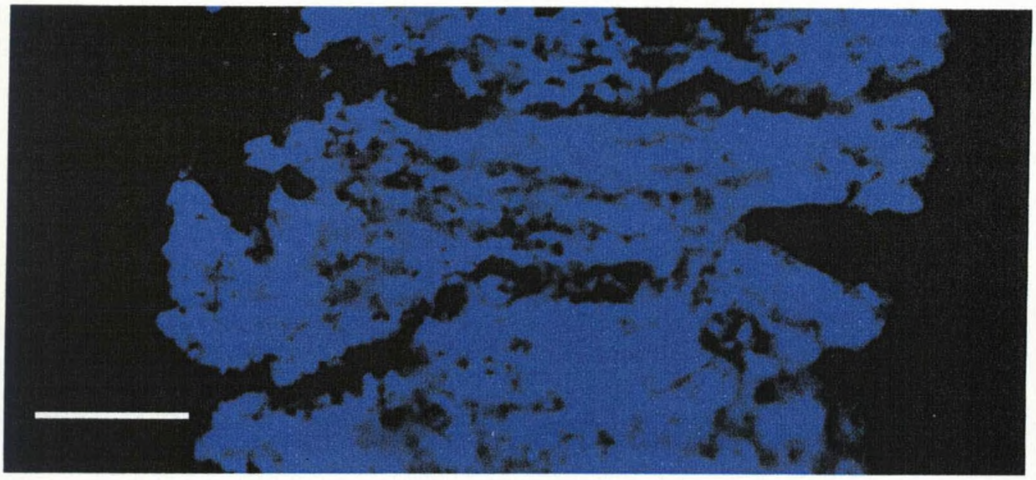
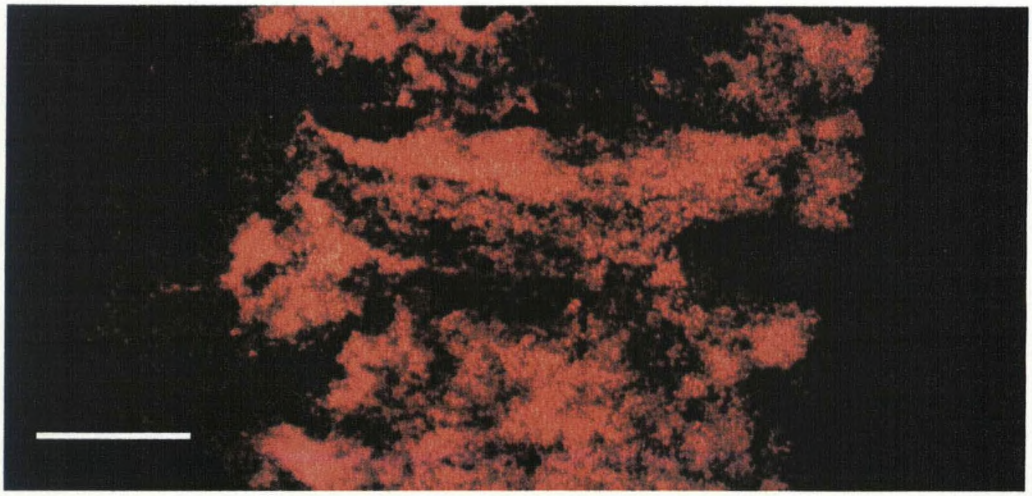


Figure 13. A representation image analysis result of inactive zone in *S. epidermidis* untreated control biofilm.



(A)



(B)

Figure 14. Photographs of a cross-section of S. *epidermidis* 4-hour treated biofilm stained with CTC/DAPI. (A) DAPI staining visualized with DAPI filter. (B) CTC staining visualized with TRITC filter. The substratum is on the right side of each photograph. Bars represent 100 μm.