Antibiotic penetration through Pseudomonas aeruginosa colony biofilms
by Marshall Charles Walters, III

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

Abstract:
P. aeruginosa has been implicated in infections pertaining to nearly every organ system. Despite numerous treatment methods total eradication of infections has proved to be impossible in most cases. Heightened resistance to intensive chemotherapy has lead to the hypothesis that P. aeruginosa infections involve a biofilm state of growth. The exact mechanism(s) providing this resistance within biofilms is unknown. The failure of antibiotics to fully penetrate through the biofilm is one hypothesis which was experimentally investigated in this work. The penetration of tobramycin and ciprofloxacin through biofilms formed by a. mucoid clinical isolate of P. aeruginosa was measured. Colony biofilms of strain FRD-1 were formed by inoculation of microporous membranes resting on trypic soy agar plates. Such colony biofilms formed a primitive model of the biofilm that may form in the airways of people with cystic fibrosis. Bacteria in biofilms were profoundly resistant to killing by tobramycin. When biofilms were challenged with 10 μg/mL of tobramycin for up to 100 h there was no decrease in the number of viable cells. Treatment with this concentration of tobramycin for 4 h was sufficient to kill (greater then 4 log reduction) planktonic cells or bacteria resuspended from colony biofilms. Tobramycin failed to penetrate colony biofilms at detectable levels in 12 h exposure. When challenged with 1 μg/mL ciprofloxacin, biofilms again exhibited resistance while planktonic cells or resuspended bacteria were extremely sensitive. In contrast, ciprofloxacin fully penetrated colony biofilms within a few hours. These results show that there is not a generic barrier to antibiotic transport through such biofilms, but that some mechanism does hinder the delivery of tobramycin through P. aeruginosa biofilm.

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ABSTRACT

P. aeruginosa has been implicated in infections pertaining to nearly every organ system. Despite numerous treatment methods total eradication of infections has proved to be impossible in most cases. Heightened resistance to intensive chemotherapy has lead to the hypothesis that *P. aeruginosa* infections involve a biofilm state of growth. The exact mechanism(s) providing this resistance within biofilms is unknown. The failure of antibiotics to fully penetrate through the biofilm is one hypothesis which was experimentally investigated in this work. The penetration of tobramycin and ciprofloxacin through biofilms formed by a mucoid clinical isolate of *P. aeruginosa* was measured. Colony biofilms of strain FRD-1 were formed by inoculation of microporous membranes resting on trypic soy agar plates. Such colony biofilms formed a primitive model of the biofilm that may form in the airways of people with cystic fibrosis. Bacteria in biofilms were profoundly resistant to killing by tobramycin. When biofilms were challenged with 10 μg/mL of tobramycin for up to 100 h there was no decrease in the number of viable cells. Treatment with this concentration of tobramycin for 4 h was sufficient to kill (greater then 4 log reduction) planktonic cells or bacteria resuspended from colony biofilms. Tobramycin failed to penetrate colony biofilms at detectable levels in 12 h exposure. When challenged with 1 μg/mL ciprofloxacin, biofilms again exhibited resistance while planktonic cells or resuspended bacteria were extremely sensitive. In contrast, ciprofloxacin fully penetrated colony biofilms within a few hours. These results show that there is not a generic barrier to antibiotic transport through such biofilms, but that some mechanism does hinder the delivery of tobramycin through *P. aeruginosa* biofilm.

CHAPTER 1

INTRODUCTION

Overview of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a nutritional versatile rod-shaped, gram-negative bacterium. Classic strains of P. *aeruginosa* typically exhibit a non-mucoid phenotype, are motile and express smooth lipopolysaccharides (LPSs) (23). This bacterium can colonize both inanimate and host plant or animal environments. Due to this ability, *P. aeruginosa* are often referred to as "ubiquitous" microorganisms (43). A unique phenotypic change of these bacteria is a conversion to an unstable mucoid form. This mucoid variant is typically sessile, has a slower growth rate, possesses rough LPS, and most characteristically, overproduces the exopolysaccharide alginate (23). Alginate is a linear copolymer of D-mannuronic and L-guluronic acids (23). The exact role of alginate is unknown but may include: a mechanism for increased adherence (16, 48), a barrier against phagocytosis (3), or a mechanism to protect against oxygen radicals (35). There are no known environmental niches of the mucoid phenotype. This mucoid phenotype is isolated from certain infections.

P. aeruginosa rarely infects hosts with intact immune defenses. Infections become a concern though, for patients with compromised immune systems. *P. aeruginosa* is the second most frequent gram-negative bacterium to cause infections in hospitalized patients (43). *P. aeruginosa* has been implicated in an array of infections pertaining to nearly every organ system. Serious infections associated with non-mucoid

P. aeruginosa include ear and eye infections and burn wounds (29, 47), while mucoid strains have been linked to urinary tract infections (39, 55), medical implants fouling (15, 43, 44), and respiratory infections of CF patients (28, 33, 58). Antibiotic treatment has improved patient prognosis, but total eradication of infections has proved to be impossible in most cases. Heightened resistance to intensive chemotherapy has lead to the hypothesis that *P. aeruginosa* infections involve a biofilm state of growth.

P. aeruginosa in Cystic Fibrosis

CF is an autosomal recessive disease caused by a mutation in the CF transmembrane conductance regulator (CFTR) gene. The disease results in respiratory failure and death (10). The basic defect involves a disruption in chloride and sodium ion transport across cells. *P. aeruginosa* infections have been implicated directly as the main factor of mortality and morbidity in CF cases (27). The reason this condition causes the victim to become susceptible to recurrent, and ultimately chronic respiratory infection, is unknown (45). Antibiotic chemotherapy has dramatically increased the survival rate of CF patients, though total eradication is still impossible (19, 22).

Initial bacterial infection of CF patients occurs in the upper respiratory tract, predominately by nonmucoid strains of *P. aeruginosa*. Research has shown that early and aggressive antibiotic treatment is capable of eliminating this early colonization (21). Over time a conversion occurs within the bacterial population, to the mucoid phenotype. The mucoid infection can no longer be eliminated from the lungs, even after aggressive antibiotic treatment (21).

P. aeruginosa in the CF lung are believed to grow as a biofilm. The possible factors to initiate this phenotypic switch from nonmucoid to mucoid include: the dehydrated environment (5), the patient's inflammatory response to colonization (45), antibiotic presence (46), or nutrient limitation (51).

The exact role of alginate within the colony remains a question. Research has been conducted on the susceptibility of both mucoid and nonmucoid isolates. Data has suggested the mucoid phenotype is more resistant to antibiotic treatment (4, 25, 56), while conflicting data has suggested the opposite is true (17, 26, 49, 57). Alginate appears to be vital for the formation of the bacteria biofilm (36), but its role as a virulence factor in a biofilm remains as topic of debate.

Biofilm Resistance to Antibiotics

It has been shown through numerous works that bacterial biofilms are nearly impossible to eradicate (12), and are more resistant to antibiotic chemotherapy than their planktonic counterparts (2, 11, 35, 41). There are multiple hypotheses proposed to explain the increased resistance of biofilms to antibiotic treatment. One hypothesis is poor penetration of antibiotics through the biofilm (11, 30, 31, 49, 52, 53, 54). A possible source for this poor penetration is modifying enzymes which would neutralize the effects of antibiotics (37, 38). The biofilms ability to survive would be a reflection of rate of neutralization of antibiotics. Another mechanism could be electrostatic interaction between the polyanionic exopolysaccharide of *P. aeruginosa* and positively charged antibiotic. Incomplete penetration of an antibiotic into a biofilm would cause

only the outer layer exposed to the antibiotic. The remaining portion of the biofilm would either receive no antibiotic, or a concentration below the bactericidal threshold. A second hypothesis is the physiological difference between cells within the biofilm due to nutrient limitation (8, 59) or other physiological factors. Nutrient limitation leads to regions of slow growth rates and regions of faster growth rates. Work has shown that slow or nongrowing cells are less susceptible to antibiotics (16), which would lead to reduced susceptibility of cells within areas of the biofilm (8, 24). In all likelihood, there is not a sole mechanism, but a combination of two or more of the previously proposed mechanisms that operate in concert.

Goals and Objectives

The goal of this study was to test the hypothesis that incomplete antibiotic penetration was the sole resistance mechanism for increased resistance of bacterial biofilms. An *in vitro* colony biofilm, grown on a polycarbonate membrane, was used to simulate an *in vivo* biofilm of a typical infected patient. The hypothesis was judged by measuring bacterial susceptibility and biofilm penetration of two bactericidal antibiotics against a mucoid strain of *P. aeruginosa*.

The first objective was to study the susceptibility mucoid *P. aeruginosa* against two clinically relevant antibiotics. The mucoid strain was FRD-1, which had been previously isolated from a CF patient, was utilized. The first of the two antibiotics was the aminoglycoside tobramycin, which binds to the 30S subunit of the ribosome and inhibits protein synthesis. The second was the fluoroquinolone ciprofloxacin, which

inhibits DNA gyrase activity and blocks the supercoiling of DNA. Both antibiotics were utilized at a concentration of 10 times the minimum inhibitory concentration (MIC). These antibiotics were used to demonstrate the bactericidal activity of typically employed antibiotics against mucoid *P. aeruginosa.* Tests were conducted to establish possible differences between varied forms of growth. The *P. aeruginosa* was studied in planktonic, colony biofilm and resuspended colony biofilms forms. Comparisons of planktonic and intact colony biofilms determined if classic *in vivo* biofilm susceptibility/resistance characteristics were present in the *in vitro* model. Identical tests were conducted on resuspended biofilms. This was done to test whether biofilm bacteria, when disaggregated, would return to a susceptible state. If the penetration hypothesis was the sole mechanism of defense, the disrupted biofilm would expose cells to the antibiotic, and susceptibility would to treatment would be seen. If the dispersed bacteria remained resistant to killing, the penetration hypothesis would appear to be disproved.

The second objective was to study the penetration of ciprofloxacin and tobramycin through mucoid biofilms using a bioassay technique. The test used biofilm systems identical to those used in susceptibility studies. Differences between the two antibiotics in penetration was studied. The role of penetration, as a resistance mechanism, was investigated by measuring the penetration of these antibiotics through biofilms over various periods of time. The amount of antibiotic which penetrated would be presented as a ratio between the concentration at a particular time versus the equilibrium concentration.

CHAPTER 2

MATERIALS AND METHODS

Bacteria

Pure cultures of *Pseudomonas aeruginosa* FRD-1, kindly provided by Michael Franklin of the Department of Microbiology at M.S.U.- Bozeman, were used in this study. FRD-1 is an alginate over-producing (mucoid) strain isolated from a CF patient named Ferdinand. One experiment was performed with *P. aeruginosa* strain FRD-2 which is a non-mucoid variant. *Escherichia coli*, (ATCC 25922), was used as an antibiotic sensitive microorganism, in zone inhibition bioassay experiments. It was obtained from frozen stock cultures at the Center of Biofilm Engineering. Frozen stock of microorganisms were prepared and stored at –70°C in a solution of 20% glycerol and 2% peptone.

Media

P. aeruginosa cultures were grown in trypic soy broth (TSB), (Difco Laboratories, Detroit, Mich.). *E. coli* cultures were grown in LB Broth (Difco Laboratories, Detroit, Mich.). Trypic soy agar (TSA)(Difco Laboratories, Detroit, Mich.) plates were used to grow colony biofilms and were augmented with antibiotics for susceptibility experiments. Mueller Hinton (Difco Laboratories, Detroit, Mich.) plates were poured particularly thin (0.3 cm +/- 0.02 cm) to accentuate zones of inhibition of

these plates. Plates thinner than 0.3 cm had a tendency to tear during the spreading of *E.coli* (ATCC 25922) lawns.

Antibiotics

Tobramycin sulfate salt (C = 648 μg/mg) was purchased from Sigma Chemical Company (St. Louis, MO.). Ciprfloxacin hydrochloride salt (C = 848 μg/mg) was a gift of the Bayer Corporation (Leverkusen, Germany). Antibiotics were dissolved in filtered nanopure water. Antibiotic stock solutions were added to previously autoclaved culture media once the media was allowed to cool to near 50°C. Antibiotic plates were stored at 4°C until needed.

Colony biofilm preparation

FRD-1 frozen stock solutions were used to inoculate 25 mL of TSB in a 50 mL disposable flask , and allowed to grow overnight (18-20 h) at 37°C within an orbital shaker. These cultures were diluted with fresh TSB to achieve an optical density of 0.13 at 600 nm. One 5 μL drop of the diluted culture was placed on each sterile polycarbonate membrane filter (25 mm diameter, 0.2 um, Poretics Corporation: Livermore, CA), which had been placed on a TSA plate. Membranes were sterilized, prior to inoculation by exposing each side to UV light in a Bioguard hood for 10 minutes. Six membranes were placed on each TSA plate. Plates were then incubated at 37°C for 48 h, colony biofilms were removed and transferred to fresh TSA plates every 24 h (Figure 1).

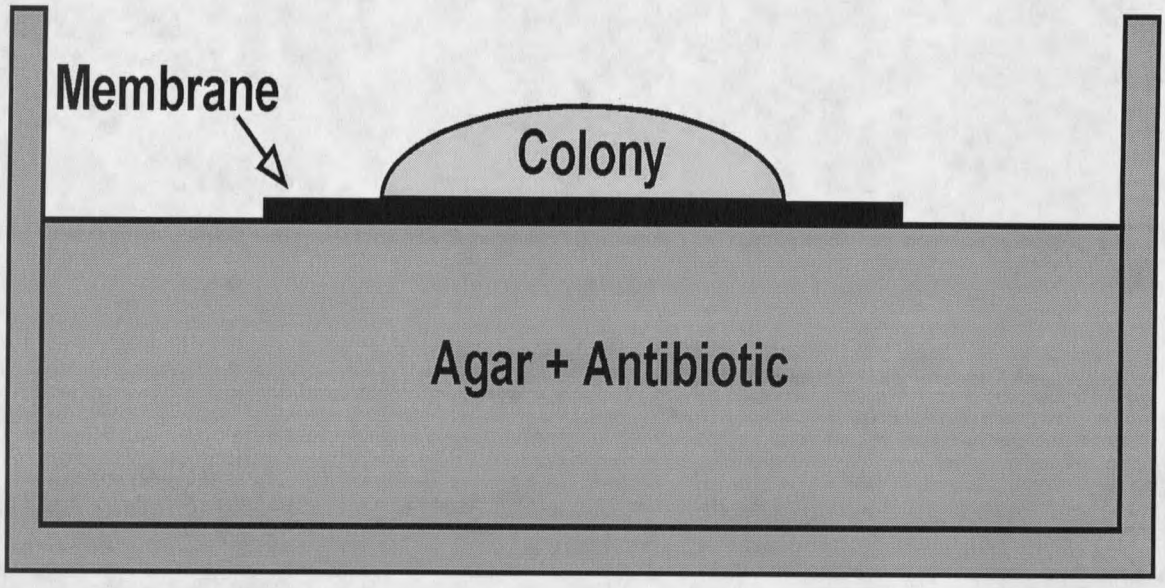

Figure 1. Illustration of the experimental system used to grow *P. aeruginosa* colony biofilms. The biofilm grew on top of a 25.0 mm diameter polycarbonate membrane, which rested on trypic soy agar (TSA) medium.

Colony biofilm growth curve

Membrane filters were inoculated with diluted FRD-1 culture as described above. Zero time points were taken immediately and the remaining colonies were incubated at 37°C. Samples were taken at various time points over the 48 h development period. Multiple samples were taken at each time point to enable determination of standard deviations. Colony biofilms were sampled by removing the colony from the TSA plate and were placing it into 9.0mL of phosphate buffered water (PBW). The biofilms were assumed to be resuspended, after vortexing the tube for 1 minute with a Maxi Mix II

Vortex (Barnstead / Thermolyne: Dubuque, IA). The viable bacteria were then determined by serial dilution and plating.

Colony biofilm cryosections / TEM

Developed biofilms were removed from TSA plates after 48 h. Two membranes were immediately transferred to a petri dish containing a paper towel moistened with 5% gluteraldehyde, for 12 h. These samples were considered time zero "pretreatment" samples. Additional membranes were transferred to plates of TSA, TSA with 1 μg/mL ciprofloxacin and TSA with 10 μg/mL tobramycin. After determined times were reached, membranes were removed and placed in similar glutaraldehyde-containing petri dishes. Samples for transmission electron microscopy (TEM) samples were immediately delivered to Andy Blixt at the Veterinary and Molecular Biology Laboratories at Montana Sate University.

Biofilm samples were fixed by Andy Blixt, who used the following procedure. The biofilms were first washed in 0.1 M Millonigs PO_4 buffer at 4°C three times for 15 minutes each time. Samples were then fixed in 1% OsO_4 for 1-h. The next step was to wash each sample in 0.1 M Millonigs PO_4 buffer again three times for 15 minutes each time. Samples were each then washed first in 50% and then in 70% ethanol (ETOH) for 15 minutes each. Samples were then prestained in 1% uranyl acetate (UA), 1% phosphotungstic acid (PTA) in 70% ETOH for 1-h. Each sample was then washed for 15 minutes in 95% ETOH, followed by three washes for 15 minutes in 100% ETOH. Samples were then soaked in 2 parts ETOH and 1 part Spurrs for 1-h, then 1 part ETOH

and 1 part Spurrs for an additional hour. The samples were soaked in pure Spurrs for 8 h. The resin was allowed to polymerize at 68 °C for 14 h. After polymerization samples were stained with Reynolds lead acetate, and washed in distilled H_2O.

The samples were then examined / photographed using the JEOL 100CX TEM. Photographs were taken randomly within the samples at three generalized locations: next to the membrane, near the middle of the sample, and at the air-biofilm interface. One representative photograph of each biofilm, from each location were presented in this study. Visual inspection was done on each sample, and the thickness of the biofilms was also determined.

Untreated biofilms were also prepared for investigation by microscopy by staining with 4', 6-diamidino-2-phenylindole (DAPI) as described by Huang *et al* (32).

Colony biofilm susceptibility

After 48 h, colony biofilms were removed from the TSA plates, and transferred to TSA plates containing antibiotic. The plates were incubated at 37°C, with samples taken at various time points. Extended antibiotic treatments were also performed. Samples were taken up to 24 h from the original plates. At 24 h the membranes were transferred to new TSA-antibiotic plates. Every 24 h the membranes were transferred to a fresh plate. Samples were taken at various times up to 100 h. When sampled, a membrane was removed from the TSA-antibiotic plates and placed in 9.0 mL of PBW. Bacteria were resuspended using a Maxi Mix II Vortex (Barnstead / Thermolyne: Dubuque, IA) for 1 minute. The viable bacteria were determined by serial dilutions and plating. To test for

possible antibiotic degradation due to enzymes produced by the bacteria, plates were spread with *E.coli* (ATCC 25922) after the membranes were removed (data not shown). The presence of *E. coli* growth indicated antibiotics within the agar, were compromised by a product of the biofilms.

Planktonic and resuspended biofilm preparation and susceptibility

Planktonic susceptibility testing began with 25 mL of TSB inoculated with FRD-1 frozen stock and grown overnight (18-20 h) at 37°C within an orbital shaker. Then 15 mL of the overnight culture and 5 mL of TSB were combined, and a zero time point sample was taken. The culture was then divided into equal parts. Antibiotic was added to one tube to achieve the desired concentration and the same volume of PBW added to the other tube. Both cultures were placed in an orbital shaker at 37°C and sampled every hour for 4 h. Each sample was washed through centrifugation followed by resuspension to remove residual antibiotic. Surviving bacteria were enumerated by colony formation. A 1.5 mL sample from the culture was removed and placed in a microcentrifuge tube (Fisher Scientific: San Francisco, CA). The tube was spun in a Micro14 microcentrifuge (Fisher Scientific: San Francisco, CA) for 10 minutes at 10,000 rpm to form a bacterial pellet. The tube was removed and the supernatant was decanted. 1.5 mL of PBW was used to wash the pellet by resuspending the bacteria. The bacteria were repelleted a second time. A second washing was done on the pellet. After a final spin, 1.5 mL of PBW was added to resuspended the bacteria. One mL of this resuspension was added to 9.0 mL PBW. Viable bacteria were determined by serial dilutions and plating.

Bacteria resuspended from biofilms were also examined for their antibiotic susceptibility. Developed colony biofilms were removed from TSA plates and placed in 9.0 mL PBW. The biofilms were dispersed by vortexing the tube for 2 minutes. The resuspended biofilms were combined with TSB or TSB with antibiotic to achieve the desired final concentrations. The resuspended cultures were then placed in an orbital shaker at 37°C, and samples were taken at various times over 4 h. Sampling procedures for the resuspended biofilms were the same as for planktonic samples.

Enumeration of viable microorganisms

Serial dilutions and colony forming units (CFUs) of each sample were made to determine viable bacteria present after treatment methods. One mL of test culture was added to 9.0 mL of PBW, and this tenfold diluting would continue until the necessary dilution factor was achieved. The drop plate technique (Hoben, H. J.,1982) was utilized for determination of CFUs and plates were incubated for 16-18 h at 35°C. The concentration of viable bacteria were expressed as colony forming units per mL (CFU/mL). The extent of bacterial killing was expressed by taking the common logarithm of the ratio of viable cell numbers in a particular sample to the number of viable cells initially. A positive value of log (X/X_o) would indicate an increase in viable bacteria over time and a negative value would represent a decrease. Error bars indicate the standard error of multiple samples at the same time point.