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
© Copyright by Marshall Charles Walters, III (2001)

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.

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
Bozeman, Montana

January 2001

Archives
N378
W1712

APPROVAL

of a thesis submitted by

Marshall Charles Walters III

This thesis has been read by each member of the thesis committee and has 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.

Dr. Philip Stewart Philip A. Stewart Jan. 30, 2001
(Signature) Date

Approved for the Department of Chemical Engineering

Dr. John Sears John T. Sears Jan 30, 2001
(Signature) Date

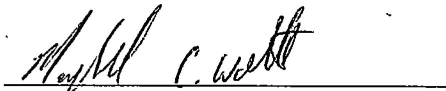
Approved for the College of Graduate Studies

Dr. Bruce McLeod Bruce R. McLeod 2-1-01
(Signature) Date

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library.

If I have indicated my intention to copyright this thesis by including a copyright notice page, copying is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for permission for the extended quotation from or reproduction of this thesis in whole or in parts may be granted only by the copyright holder.

Signature 

Date 1-30-01

TABLE OF CONTENTS

1. INTRODUCTION 1
    - Overview of *Pseudomonas* aeruginosa 1
    - *P. aeruginosa* in Cystic Fibrosis 2
    - Biofilm Resistance to Antibiotics 3
    - Goals and Objectives 4

2. MATERIALS AND METHODS 6
    - Bacteria 6
    - Media 6
    - Antibiotics 7
    - Colony biofilm preparation 7
    - Colony biofilm growth curve 8
    - Colony biofilm cryosections / TEM 9
    - Colony biofilm susceptibility 10
    - Planktonic and resuspended biofilm preparation and susceptibility 11
    - Enumeration of viable microorganisms 12
    - Antibiotic penetration 13

3. RESULTS 16
    - Visual Observations 16
    - Biofilm thickness 17
    - Colony biofilm accumulation 17
    - Colony Reversion 19
    - Microscopy 19
    - 4 hr Tobramycin susceptibility 20
    - 4 hr Ciprofloxacin susceptibility 26
    - Extended antibiotic susceptibility 28
    - Tobramycin penetration 30
    - Elevated tobramycin concentration penetration 34
    - Ciprofloxacin penetration 36

4. DISCUSSION 40
    - Colony Biofilm Resistance 40
    - Antibiotic Penetration 42
    - Resistance Mechanism(s) and Future Work 43
    - Colony Biofilm Model System 46
    - Conclusion 46

REFERENCES CITED 48

APPENDICES .... (Attached Disk)

LIST OF TABLES

Table | Page

1. Colony biofilm specific growth rates.. 18

2. Colony biofilm mucoid to non-mucoid reversion percentages............... 19

LIST OF FIGURES

Figure Page

1. Illustration of the experimental system used to grow *P. aeruginosa* colony biofilms........ 8
2. Illustration of the experimental system used to measure antibiotic penetration through *P. aeruginosa* colony biofilms........ 13
3. Illustration of the experimental procedure for penetration measurement... 15
4. *P. aeruginosa* FRD-1 biofilm accumulation curve........ 18
5. Electron micrograph of *P. aeruginosa* colony biofilm, 0 h control........ 21
6. Electron micrograph of *P. aeruginosa* colony biofilm, 12 h control....... 22
7. Electron micrograph of *P. aeruginosa* colony biofilm, 12 h ciprofloxacin........ 23
8. Electron micrograph of *P. aeruginosa* colony biofilm, 12 h tobramycin........ 24
9. Electron micrograph of *P. aeruginosa* colony biofilm, 36 h tobramycin........ 25
10. A. Susceptibility of planktonic *P. aeruginosa* to antibiotic treatment for 4 h........ 20
10. B. Susceptibility of *P. aeruginosa* colony biofilm to antibiotic treatment for 4 h........ 26
10. C. Susceptibility of resuspended *P. aeruginosa* colony biofilm to antibiotic treatment for 4 h........ 27
11. Susceptibility of *P. aeruginosa* colony biofilm FRD-1 to antibiotic treatment for 12 h........ 29
12. Susceptibility of *P. aeruginosa* colony biofilm FRD-1 to antibiotic treatment for 100 h........ 29
13. Standard curve for tobramycin concentration........ 30

14. Bioassay of 10 ug/mL tobramycin penetration: Zone of inhibition data... 32

15. Bioassay of 10 ug/mL tobramycin penetration: Calculated antibiotic concentration data........ 32

16. Bioassay of 10 ug/mL tobramycin penetration: Normalized antibiotic concentration data........ 33

17. Extended bioassay of 10 ug/mL tobramycin penetration: Normalized antibiotic concentration data........ 33

18. Standard curve for elevated tobramycin concentration........ 34

19. Elevated bioassay of tobramycin penetration: Normalized antibiotic concentration data........ 35

20. Standard curve for ciprofloxacin concentration........ 36

21. Bioassay of 1 ug/mL ciprofloxacin penetration: Zone of inhibition data.. 37

22. Bioassay of 1 ug/mL ciprofloxacin penetration: Calculated antibiotic concentration data........ 38

23. Bioassay of 1 ug/mL ciprofloxacin penetration: Normalized antibiotic concentration data........ 39

24. Susceptibility of *P. aeruginosa* to 5 ug/mL tobramycin treatment........ 40

25. Susceptibility of *P. aeruginosa* to 10 ug/mL tobramycin treatment...... 41

26. Susceptibility of *P. aeruginosa* to 1 ug/mL ciprofloxacin treatment...... 41

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).

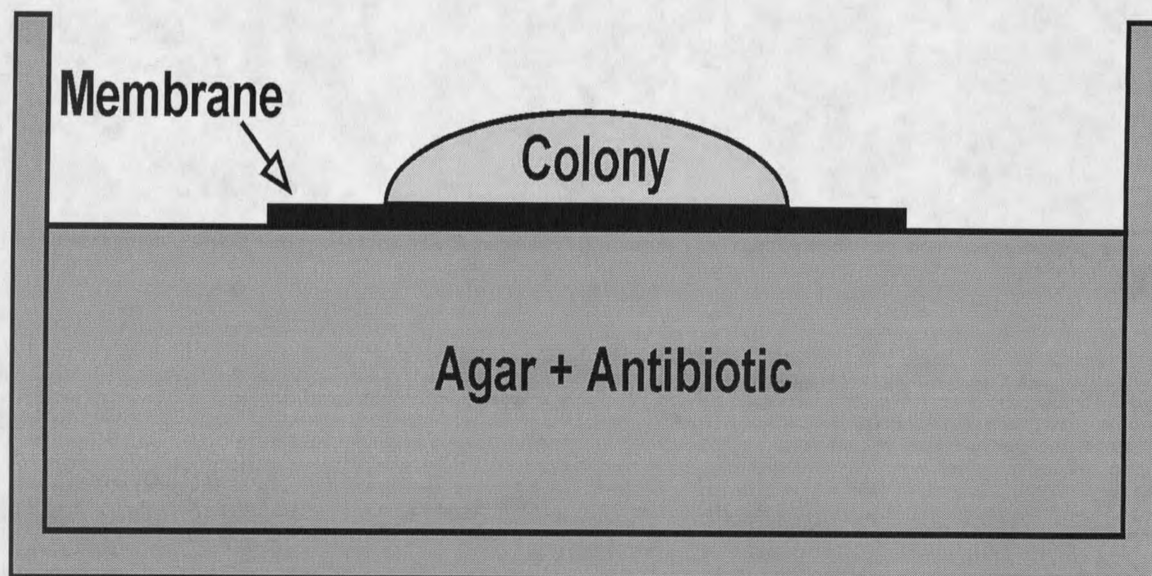


Figure 1. Illustration of the experimental system used to grow *P. aeruginosa* colony biofilms. The biofilm grew on top of a 25.0 mm diameter polycarbonate membrane, which rested on trypic soy agar (TSA) medium.

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_0) 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.

Antibiotic penetration

FRD-1 biofilms were grown for 48 h. A 13 mm diameter, 0.2 μm pore polycarbonate membrane (Porectics Corporation: Livermore, CA) was placed on top of the colony biofilm in its center (Figure 7). Twenty five μL of PBW was placed on a concentration disk (Difco Laboratories: De0troit, MI), this was then placed on top of the 13 mm membrane. This assembly, in which the biofilm was sandwiched between two microporous membranes, was then moved to an antibiotic-containing agar plate (Figure 2).

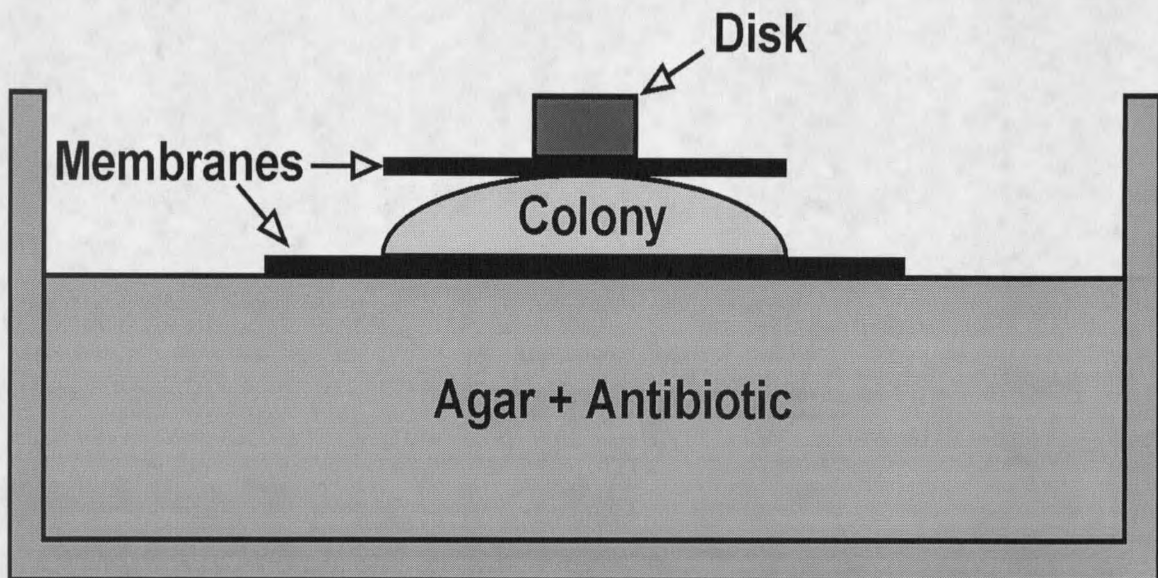


Figure 2. Illustration of the experimental system used to measure antibiotic penetration through *P. aeruginosa* colony biofilms. After the 48-h growth period, a 13 mm polycarbonate membrane was placed on top of the biofilm (A). A moistened 0.6 cm concentration disk (B) was placed on the top of the small membrane. Complete assemblies were then transferred to an antibiotic-containing agar plate with forceps.

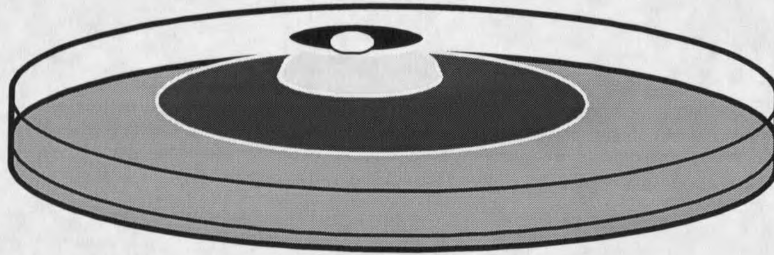
At desired time points a concentration disk was removed, sealed tightly in parafilm, and then placed in storage at 4°C. Near the final sample time, thin Mueller-

Hinton (Difco Laboratories: Detroit, MI) plates were spread with 100 μL of *E. coli* to form a lawn of bacteria. The *E. coli* were grown for 16-18 h in LB broth, and then diluted with additional fresh LB broth to achieve an optical density of 0.05 at 600 nm before being plated. Four concentration disks from varied sample times were placed on each spread plate, and the plates were incubated for 8 h at 37°C. The zone of inhibited growth (zone of inhibition) was measured as the diameter across the center of each disk (Figure 3).

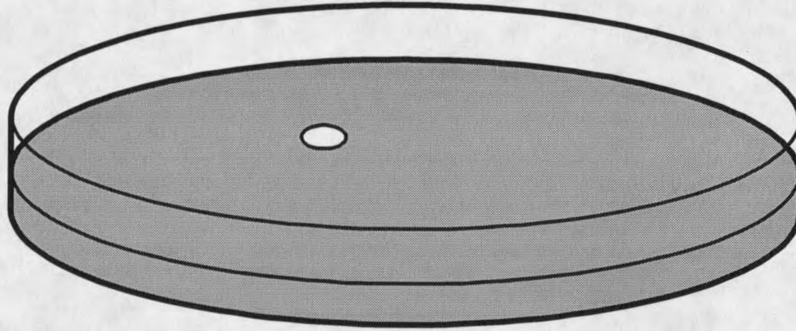
Parallel experiments were performed with sterile control assemblies. These consisted of the two membranes and concentration disk without bacteria.

Standard curves for both antibiotics were made by adding 25 μL of specific antibiotic concentration to multiple disks, which were then placed on *E. coli* lawns as before. From these standard concentration curves, a regression curve was fitted to each zone of inhibition curve. Experimental zone values were then converted to calculated concentration value from the equation of the regression curve. Calculated values of the equilibrium concentration (C_0) were used to form ratios with the calculated colony biofilm values (C), to provide the percentage of total penetration which occurred.

A.



B.



C.

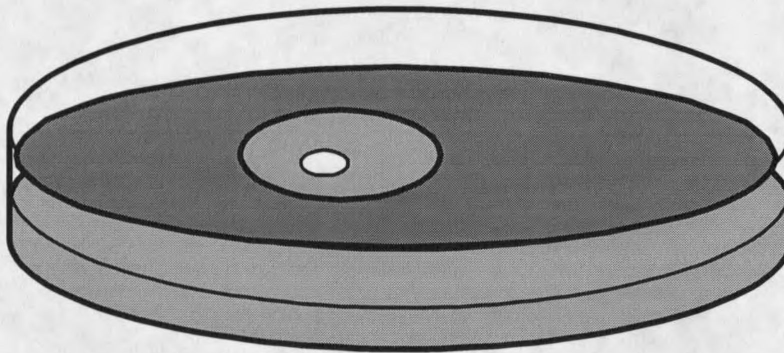


Figure 3. Illustration of the experimental procedure for penetration measurement. The concentration disks were removed from the penetration system (A), were transferred to a plate spread with antibiotic sensitive *E. coli* (B). After incubation a lawn of bacteria became visible and zones of inhibition were measured. The detection limit was 0.7 cm, which was the diameter of the disks. Zones of inhibition were measured for systems of biofilms and membranes, and systems of membranes alone.

CHAPTER 3

RESULTS

Visual Observations

Membranes were inoculated with a 5 μL drop of bacterial suspension. The drop was clear, with a diameter of 0.5 ± 0.1 cm. The drop was absorbed within 10 minutes leaving a residual stain on the membrane the size of the initial drop. After 10 h the biofilm was nearly the same size in diameter, but had began to thicken and turn slightly cloudy yellow in color. After 24 h the diameter of the biofilm had increased to 0.75 ± 0.1 cm, and the yellowish creamy color was more apparent. There was also the emergence of a region surrounding the original drop spot, which was thinner and not as yellow in color. The membranes were transferred to fresh TSA plates, leaving a dark residual ring on the old plate. This footprint was the same size of the biofilm colony. At 48 h the biofilm had increased slightly in diameter and thickness, while the thin surrounding band increased in diameter to form a 0.5 cm border which also became more yellowish in color.

Untreated control biofilms increased in diameter until 65-70 h when they began to grow past the edge of the 2.5 cm diameter membrane and onto the agar. Biofilms transferred to antibiotic-containing agar after 48 h increased only slightly in diameter over treatment periods up to 100 h. After 24 h of treatment the biofilms began to express dark circular regions. These circular regions became more numerous, and increased in size to eventually reach 0.1 ± 0.05 cm in diameter. Over time, biofilms treated with

either antibiotic visually appeared to decreased in thickness to nearly a third of their initial thickness, also becoming more brownish yellow in color.

Biofilm thickness

Biofilm thickness was measured for each treatment case from both TEMs and from micrographs of DAPI-stained frozen sections. TEM measurements represent a single point in each biofilm. The individual thickness measurements were: 142 um for untreated control (0 h), 146 μm for untreated control (12 h), 246 μm for 1 μg/mLciprofloxacin (12 h), 152 μm for 10 μg/mL tobramycin (12 h), and 146 μm for 10 μg/mL tobramycin (36 h). The DAPI-stained untreated control (12 h) was measured along multiple points on the biofilm with an average thickness of 233 μm ± 26. The thickness of these colony biofilms ranged from approximately 150 to 250 μm.

Colony biofilm accumulation

The accumulation of bacteria in colony biofilms was determined over a 48 h period in triplicate experiments (Figure 4). The initial cell numbers were 9.0 x10^{6}. CFU/mem and increased to 5.5 x 10^{10} over 48 h, each trial exhibited an exponential growth phase in the first seven hours, and a stationary phase from hours approximately 24-48 h. The exponential and stationary phase specific growth rates of each trial are summarized in Table 1.

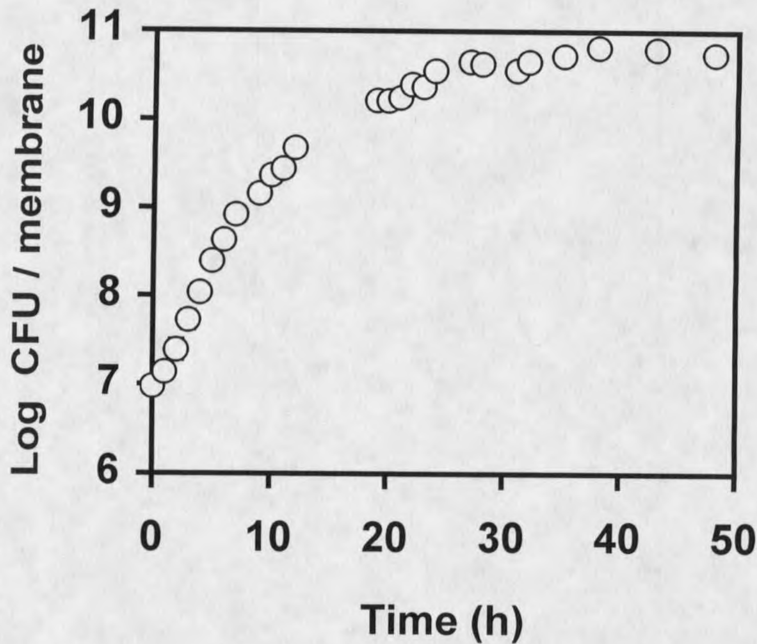


Figure 4. *P. aeruginosa* FRD-1 biofilm accumulation curve. Error bars reflect the standard error of triplicate trials.

Table 1. Colony biofilm specific growth rates. The error is the standard deviation

Trial	μ - exponential (hr^{-1})	Error	μ - stationary (hr^{-1})	Error
One	0.704	0.023	0.003	0.005
Two	0.827	0.021	0.026	0.005
Three	0.623	0.026	0.012	0

The average exponential specific growth rate for the three trials was 0.72 ± .10. The average specific stationary growth rate was an order of magnitude smaller at 0.01 ± 0.01.

Colony Reversion

To test the frequency of reversion from the unstable mucoid phenotype to the nonmucoid form, 48 h biofilms were resuspended and spread as a bacterial lawn. The mucoid and nonmucoid colonies were counted and percentage of cells converted to nonmucoid was determined, which is shown in Table 2 below.

Table 2. Colony biofilm mucoid to non-mucoid reversion percentages.

Sample	Mucoid Colonies	Nonmucoid Colonies	Percent Converted
One	600	1	0.17
Two	555	5	0.89
Three	360	4	1.08
Total	1515	10	0.66

The frequency of reversion was less than one percent and probably had a negligible influence on the measurement of antibiotic penetration or susceptibility.

Microscopy

Images in Figures 5-9 are transmission electron microscopic (TEM) photographs of biofilms at three general positions; next to the membrane (a), in the middle of the biofilm (b), and at the air interface (c). Figure 5 a-c are an untreated biofilm after the 48 h growth period (0 h). Figure 6 a-c are a 48 h old biofilm, which has been transferred to an antibiotic-free TSA plate for 12-h (12-h untreated control). Figures 7 a-c and 8 a-c are a 48 h biofilm, which has been transferred to TSA plates containing 1 μg/mL ciprofloxacin for 12 h and 10 μg/mL tobramycin for 12 h respectively. Figure 9 a-c are a

48 h biofilm which has been transferred to TSA plates containing 10 μg/mL tobramycin for 36 h.

4 hr Tobramycin susceptibility

FRD-1 planktonic, colony biofilms, and resuspended colony biofilms were challenged with 10 times the MIC of planktonic culture, 10 μg/mL tobramycin, for a 4 h time period. Results were plotted for controls and antibiotic treatments (Figures 10 A, 10 B and 10 C). Control planktonic cultures exhibited a log increase of 0.67 ± 0.12. Tobramycin treated planktonic cultures were extremely susceptible to treatment and expressed a log decrease of –5.2 ± 0.30 (Figure 10 A).

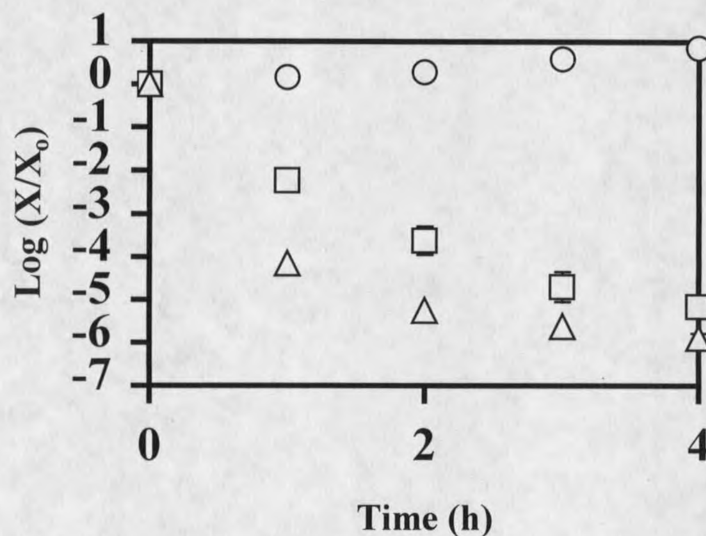
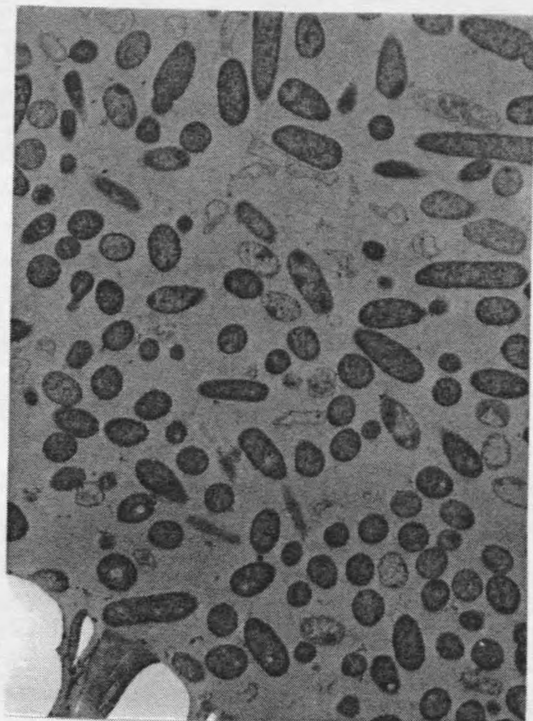
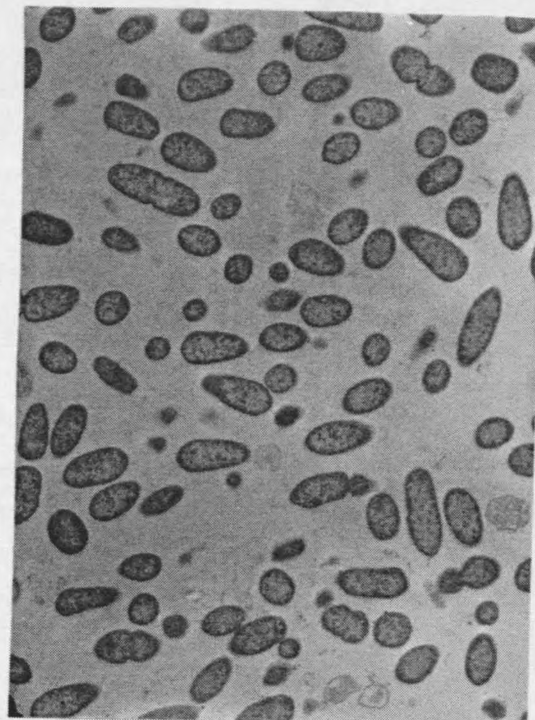


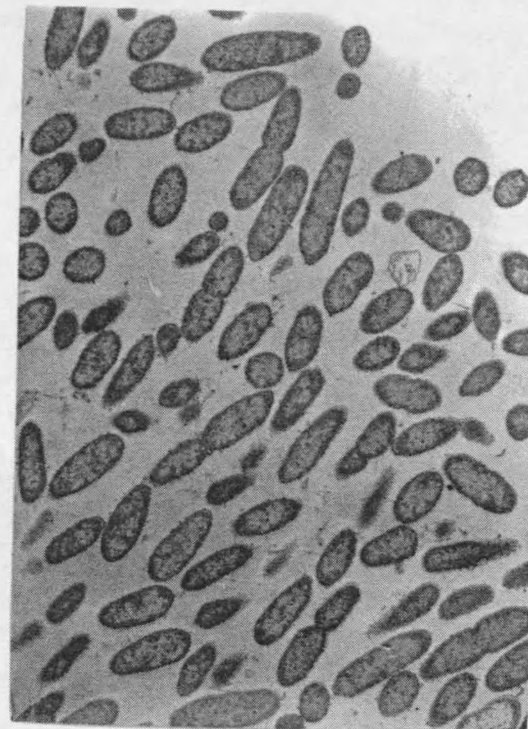
Figure 10 A. Susceptibility of planktonic *P. aeruginosa* to antibiotic treatment for 4 h. Data represented by: (○) no antibiotic, (□) 10 μg/mL tobramycin, (Δ) 1 μg/mL ciprofloxacin. Error bars reflect the standard error of triplicate experiments.



(A)

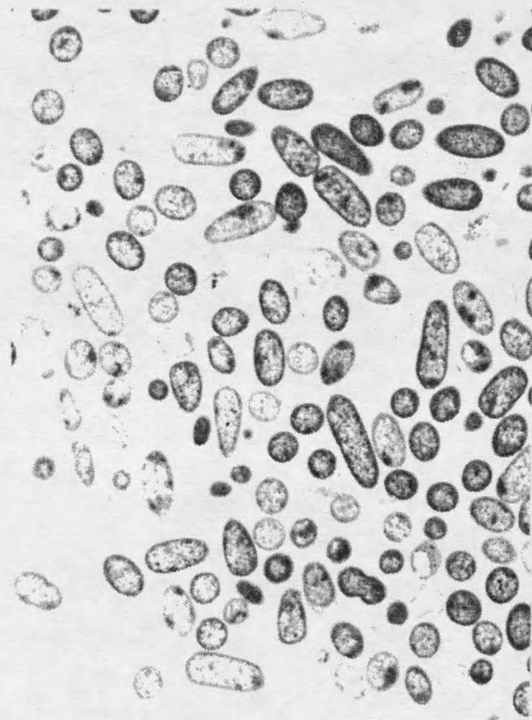


(B)

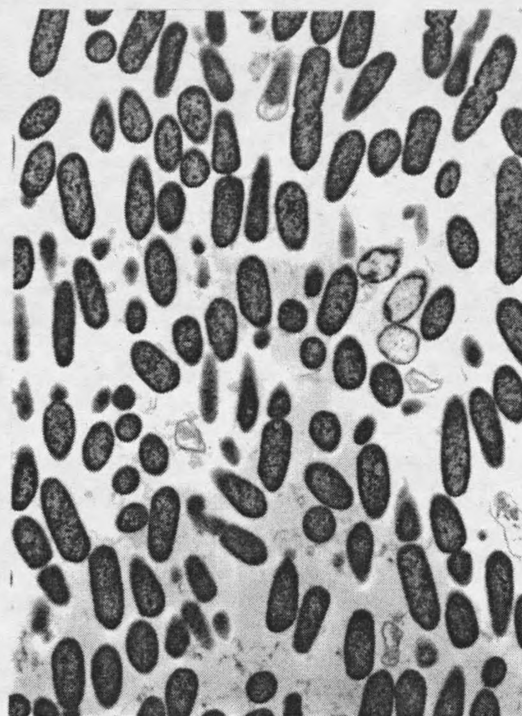


(C)

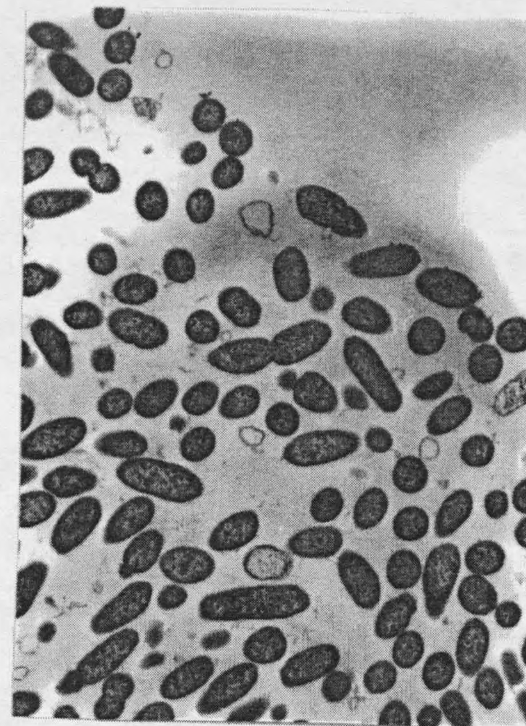
Figure 5. Electron micrograph of P. aeruginosa colony biofilm , 0 h control: (a) near the membrane, (B) middle of the biofilm, (c) air-interface (x19,800).



(A)

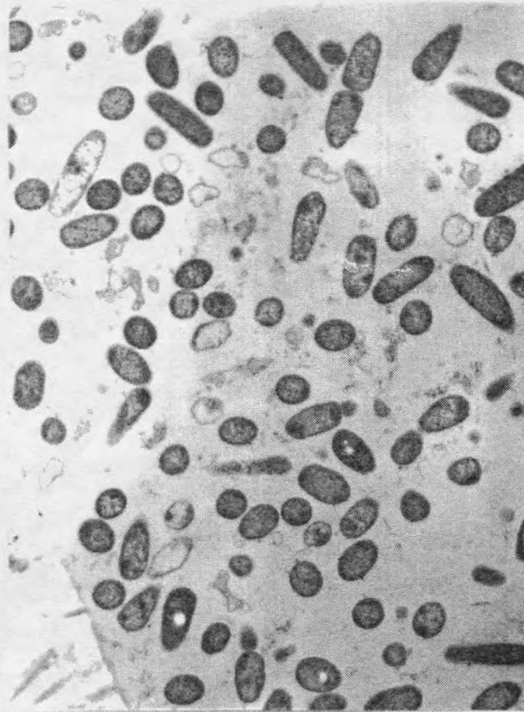


(B)

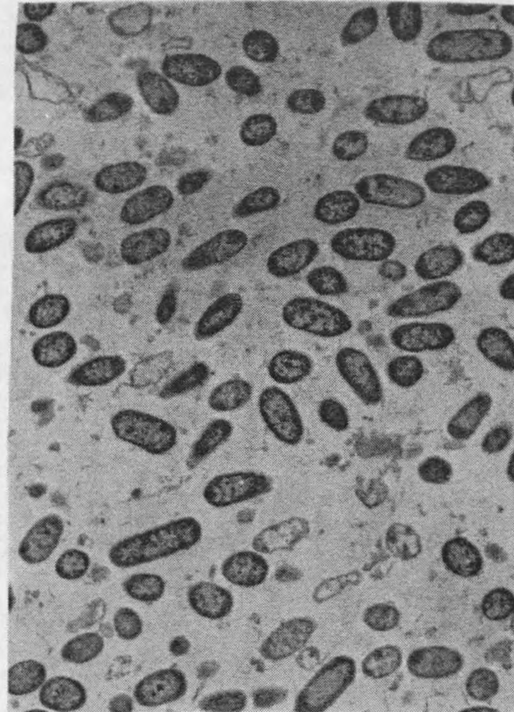


(C)

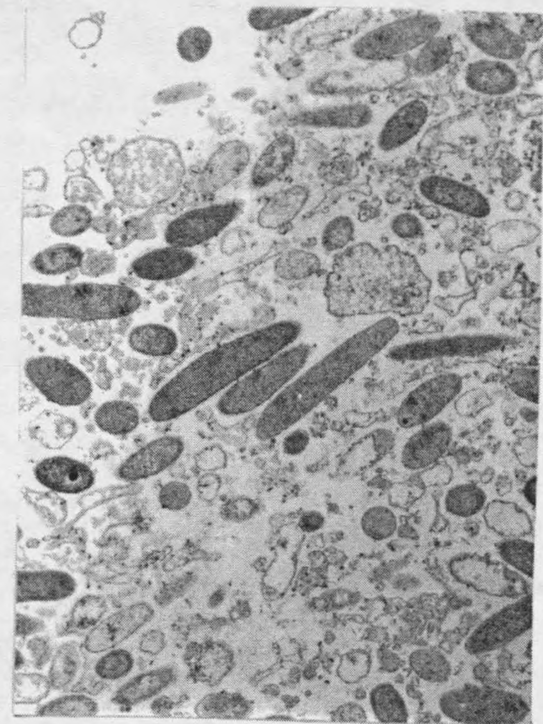
Figure 6. Electron micrograph of P. aeruginosa colony biofilm , 12 h control: (a) near the membrane, (B) middle of the biofilm, (c) air-interface (x19,800).



(A)

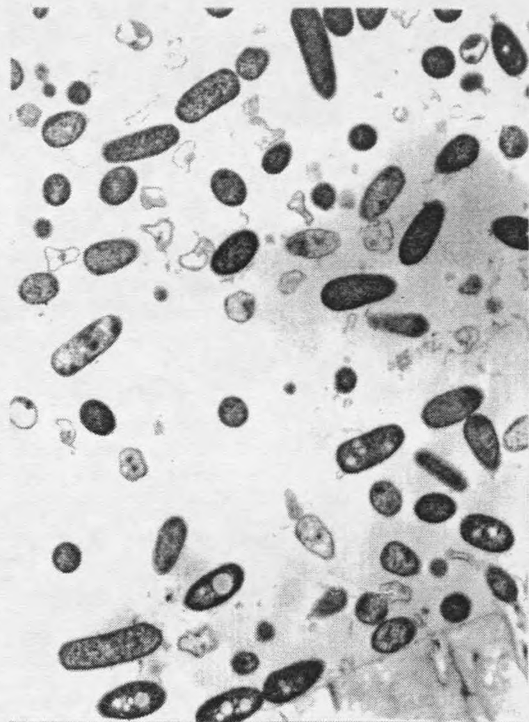


(B)

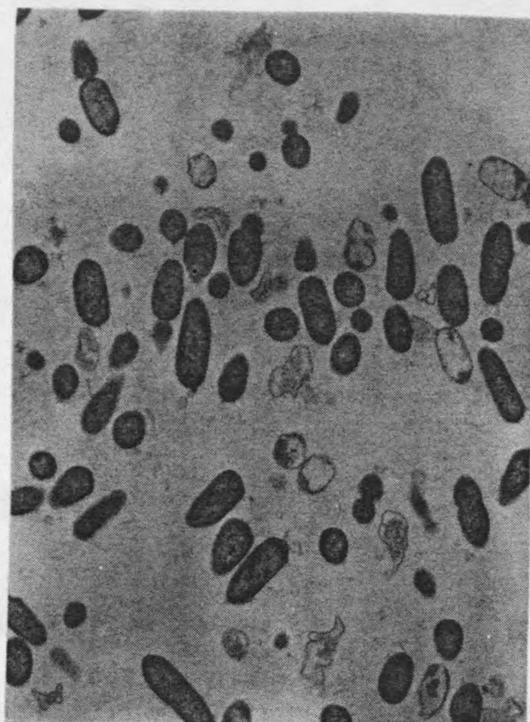


(C)

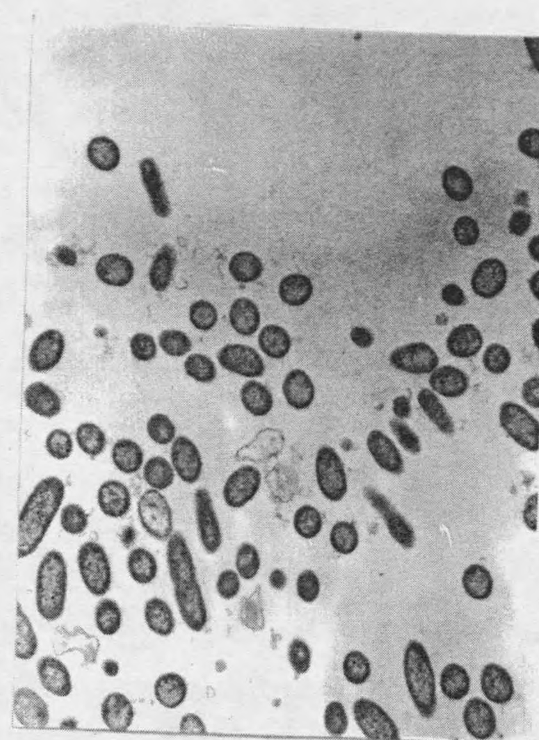
Figure 7. Electron micrograph of P. aeruginosa colony biofilm , 12 h ciprofloxacin: (a) near the membrane, (B) middle of the biofilm, (c) air-interface (x19,800).



(A)

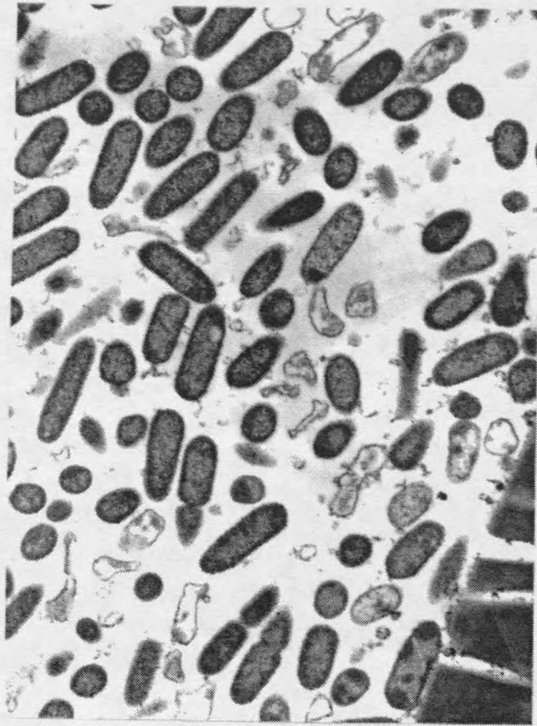


(B)

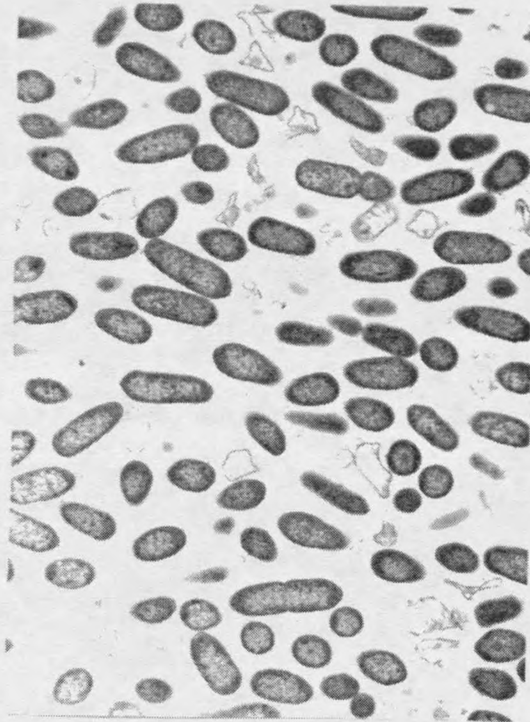


(C)

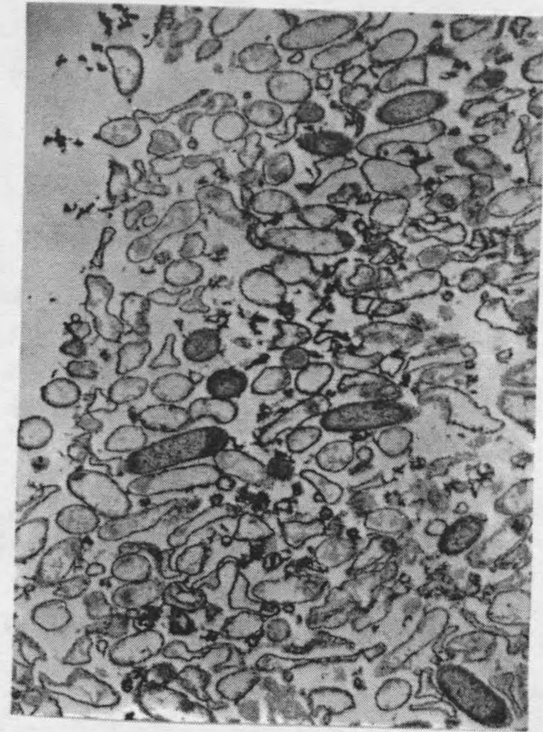
Figure 8. Electron micrograph of P. aeruginosa colony biofilm , 12 h tobramycin: (a) near the membrane, (B) middle of the biofilm, (c) air-interface (x19,800).



(A)



(B)



(C)

Figure 9. Electron micrograph of P. aeruginosa colony biofilm , 36 h tobramycin: (a) near the membrane, (B) middle of the biofilm, (c) air-interface (x19,800).

Colony biofilms exhibited characteristic reduced antibiotic susceptibility in comparison to suspension cultures. Control and antibiotic treated colony biofilms both experienced log increases of 0.27 ± 0.04 and 0.04 ± 0.15, respectively (Figure 10B).

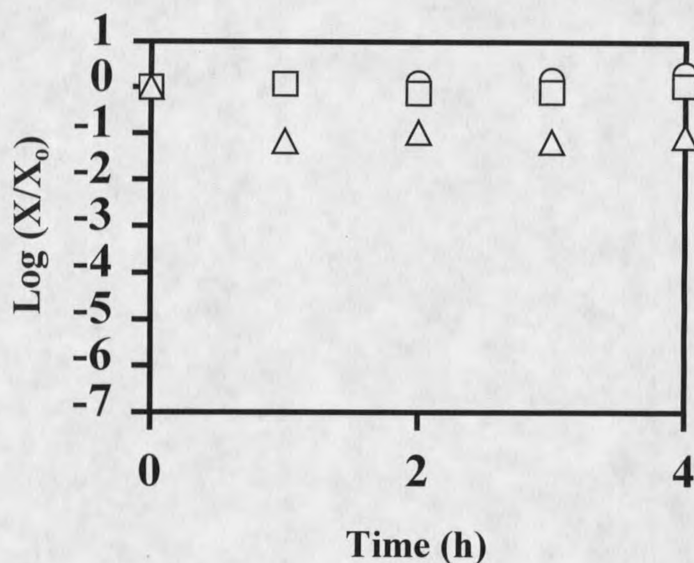


Figure 10 B. Susceptibility of *P. aeruginosa* colony biofilm to antibiotic treatment for 4 h. Data represented by: (○) no antibiotic, (□) 10 μg/mL tobramycin, (Δ) 1 μg/mL ciprofloxacin. Error bars reflect the standard error of triplicate experiments.

Resuspended colony biofilms expressed similar susceptibility to planktonic cultures. Resuspended biofilm controls exhibited a log increase of 0.77 ± 0.01 while the antibiotic treatment showed a reduction of –4.9 ± 0.25.

4 hr Ciprofloxacin susceptibility

FRD-1 planktonic, colony biofilms, and resuspended colony biofilms were challenged with 10 times the MIC of planktonic culture, 1 μg/mL ciprofloxacin, for a 4 h time period. Results were plotted for controls and antibiotic treatments (Figure 10 A, 10 B and 10 C).

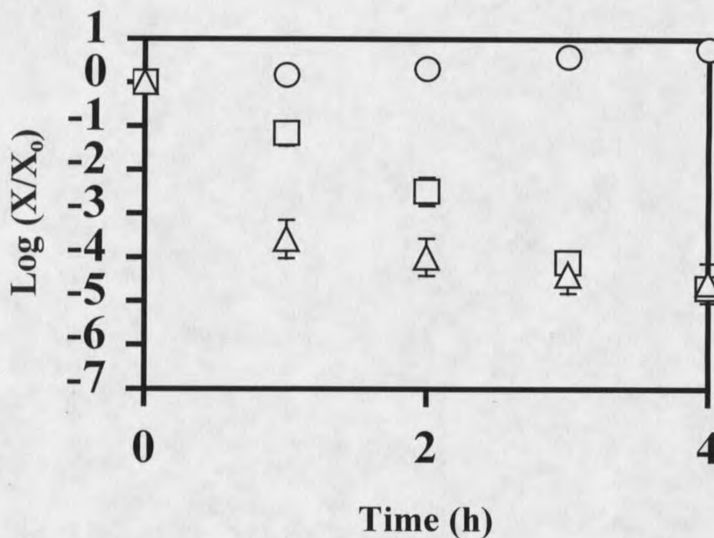


Figure 10 C. Susceptibility of resuspended *P. aeruginosa* colony biofilm to antibiotic treatment for 4 h. Data represented by: (○) no antibiotic, (□) 10 μg/mL tobramycin, (Δ) 1 μg/mL ciprofloxacin. Error bars reflect the standard error of triplicate experiments.

Log (X/X_o) values for untreated control planktonic, colony biofilm and resuspended colony biofilm cultures were identical to those in the tobramycin experiments.

Ciprofloxacin treated planktonic cultures were extremely susceptible to antibiotic treatment and expressed a log decrease of -5.89 ± 0.19. Colony biofilms were mildly susceptible to ciprofloxacin. Antibiotic treated colony biofilms experienced a log decrease of -1.05 ± 0.18. Resuspended colony biofilms exhibited susceptibility to

ciprofloxacin treatment, with similar results to planktonic cultures. Ciprofloxacin treatment produced a log decrease of –4.57 ± 0.76. One distinctive difference between the two antibiotic cases was in the susceptibility of colony biofilms to each antibiotic. A slight 1-log decrease occurred with exposure to ciprofloxacin, whereas tobramycin treatment caused little variation after treatment.

Extended antibiotic susceptibility

Colony biofilms were treated with 5 μg/mL, 10 μg/mL tobramycin, and 1 μg/mL ciprofloxacin, but with extended exposure times. One set of treatments were conducted over 12 h (Figure 11), another over 100 h (Figure 12). Control colony biofilms again expressed a log increase over time. The log (X/X_o) value for the 12 h and 100 h periods were 0.53 ± 0.05 and 1.26 ± 0.02 respectively.

Colony biofilms showed only slight susceptibility to either antibiotic treatment over both periods. Colony biofilms treated with 10 μg/mL tobramycin experienced a log decrease for 12 and 100 h of -0.19 ± 0.17 and -0.49 ± 0.26, respectively. Ciprofloxacin treated biofilms responded with slightly higher killing with log (X/X_o) values of -0.99 ± 0.04 over 12 hours and -1.42 ± 0.19 over 100 h.

Colony biofilms treated with 5 μg/mL tobramycin experienced a slight log increase of 1.5 over the 100 h (data not shown).

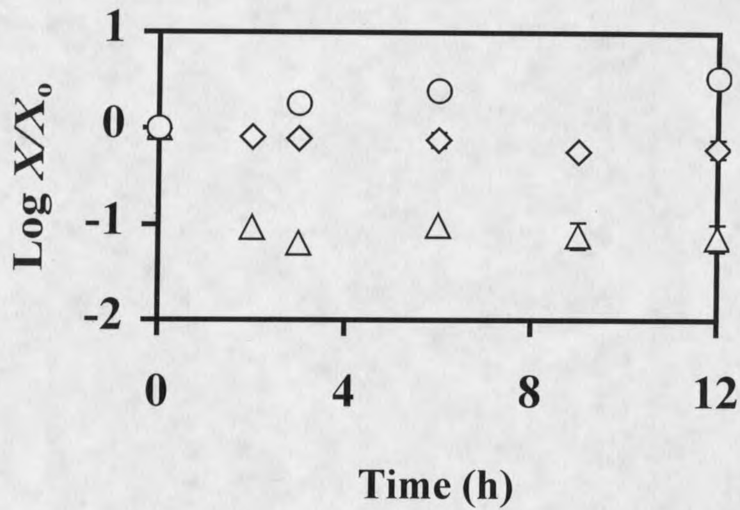


Figure 11. Susceptibility of *P. aeruginosa* colony biofilm FRD-1 to antibiotic treatment for 12 h. Data represented by: (○) no antibiotic, (□) 10 μg/mL tobramycin, (Δ) 1 μg/mL ciprofloxacin. Error bars reflect the standard error of triplicate experiments.

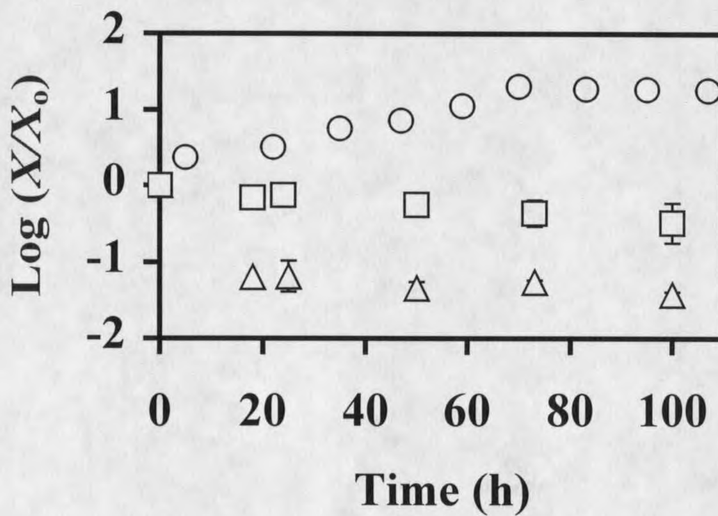


Figure 12. Susceptibility of *P. aeruginosa* colony biofilm FRD-1 to antibiotic treatment for 100 h. Data represented by: (○) no antibiotic, (□) 10 μg/mL tobramycin, (Δ) 1 μg/mL ciprofloxacin. Error bars reflect the standard error of triplicate experiments.

Tobramycin penetration

A standard curve for determination of tobramycin concentrations was produced from zone of inhibition bioassay data (Figure 13).

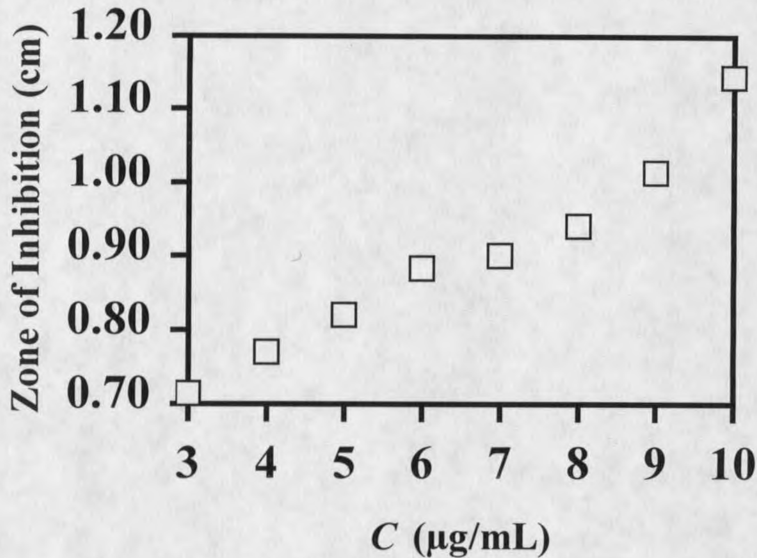


Figure 13. Standard curve for tobramycin concentration. Concentrations from 0-10 ug/mL tobramycin were fit with a regression line to determine *C*.

The standard curve for tobramycin increased by 1.0 µg/mL increments, from 0.0 to 10 µg/mL. A regression line was fit to the inhibition data curve. The equation of the curve was $y = -130 x^3 + 350 x^2 - 280 x + 75$ with an R^2 of 0.99, where y is the zone of inhibition in cm and x is the tobramycin concentration in µg/mL. This curve was used to convert zone of inhibition data, to calculated concentrations. The size of the concentration disk 0.7 cm, was the detection limit for inhibited zone measurements. Parallel tests were run with assemblies placed on antibiotic free agar. This was done to

test for biological byproducts that could lead to inhibited zones, even when no antibiotic was present. Zones for these control samples were below detection limits (data not shown).

Penetration assemblies (Figure 2) were made with biofilms colonies on membranes ("biofilms") and with membranes without biofilms ("membranes"). Both biofilms and membranes were exposed to antibiotics for 12 h in some experiments and 36 hours in others.

Zone of inhibition data were determined for both biofilms and membranes over 12 h (Figure 14).

Inhibition zones could not be detected for concentrations below 3.0 μg/mL tobramycin. Observable inhibition zones were converted to calculated concentrations (C) (Figure 15) by use of the regression curve equation.

An equilibrium control (C_o) was determined as the average of the final four calculated concentrations from membrane assemblies. A ratio between the C_o and C was then used to express the fraction of the bulk agar tobramycin concentration that penetrated over 12 h (Figure 16) and then for 36 h (Figure 17).

Tobramycin penetrated through membrane assemblies more quickly than it did through biofilm assemblies. Inhibition zones were detected after 1 h for membranes alone, while tobramycin penetration through biofilms was undetectable until 24 h. In membrane experiments a tobramycin concentration slightly less than bulk concentration was attained within 7 h, while the tobramycin concentration measured in biofilm experiments were less than 50% of the bulk concentration after 36 h.

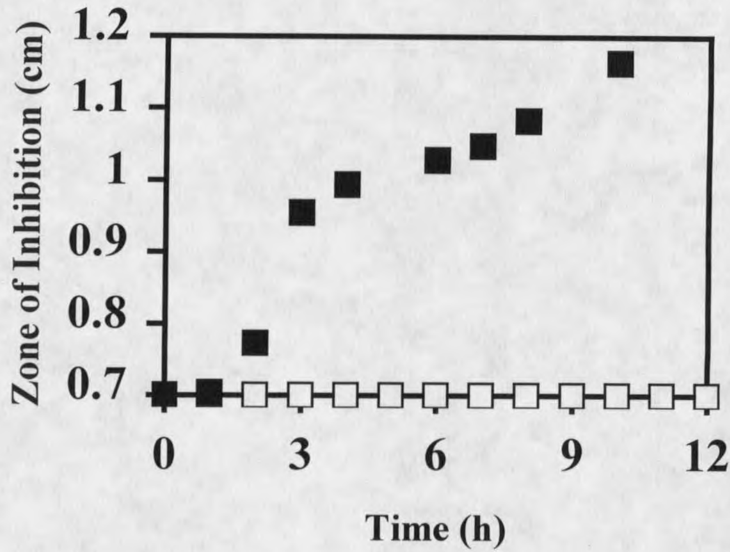


Figure 14. Bioassay of 10 μg/mL tobramycin penetration: Zone of inhibition data. Data represented by (■) membranes, (□) biofilm colony and membranes.

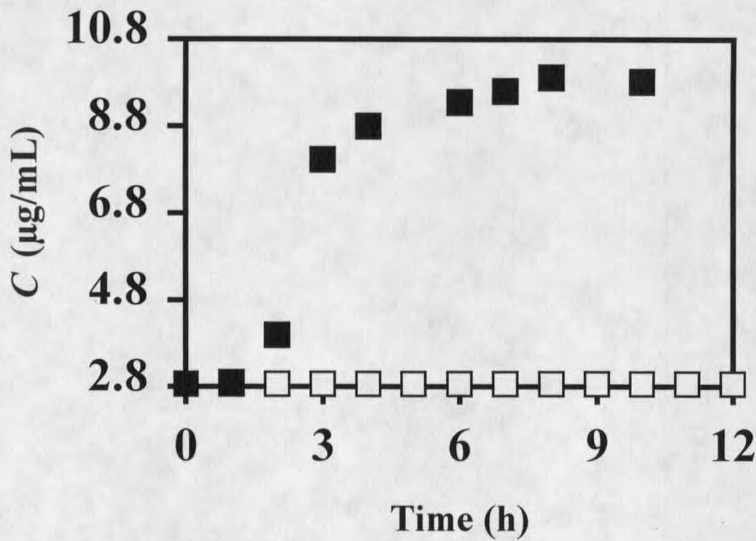


Figure 15. Bioassay of 10 μg/mL tobramycin penetration: Calculated antibiotic concentration data. Data represented by (■) membranes, (□) biofilm colony and membranes.

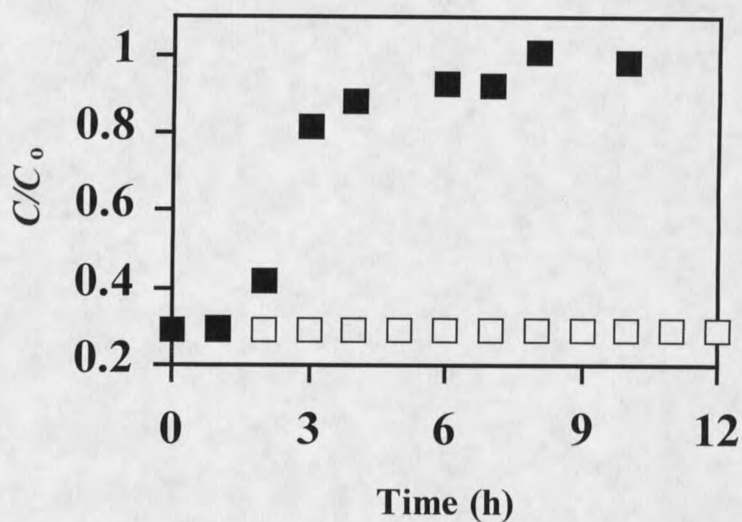


Figure 16. Bioassay of 10 μg/mL tobramycin penetration: Normalized antibiotic concentration data. Data represented by (■) membranes, (□) biofilm colony and membranes.

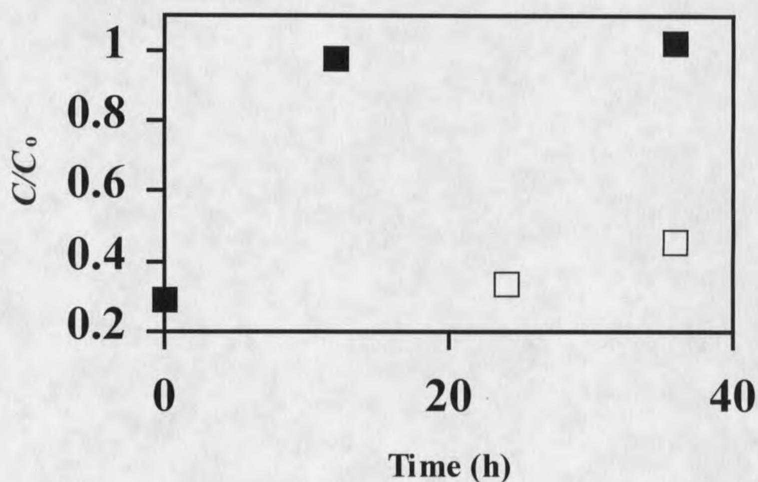


Figure 17. Elevated concentration bioassay of tobramycin penetration: Normalized antibiotic concentration data. Data represented by (■) membranes, (□) biofilm colony and membranes.

The C/C_o ratio of membranes was equal to 1.0 at 8 h, whereas in biofilms the maximum C/C_o ratio measured was 0.45 after 36 h (Figure 17).

Nonmucoid *P. aeruginosa* FRD-2 biofilms were also used in parallel penetration experiments over a 12 hour time period. Zones of inhibition were not detectable over 12 h (data not shown).

Elevated tobramycin concentration penetration

Some penetration experiments were run at elevated tobramycin concentrations. A bioassay standard curve for determination of tobramycin concentrations in the range of 0 to 100 ug/mL, was produced from zone of inhibition data (Figure 18).

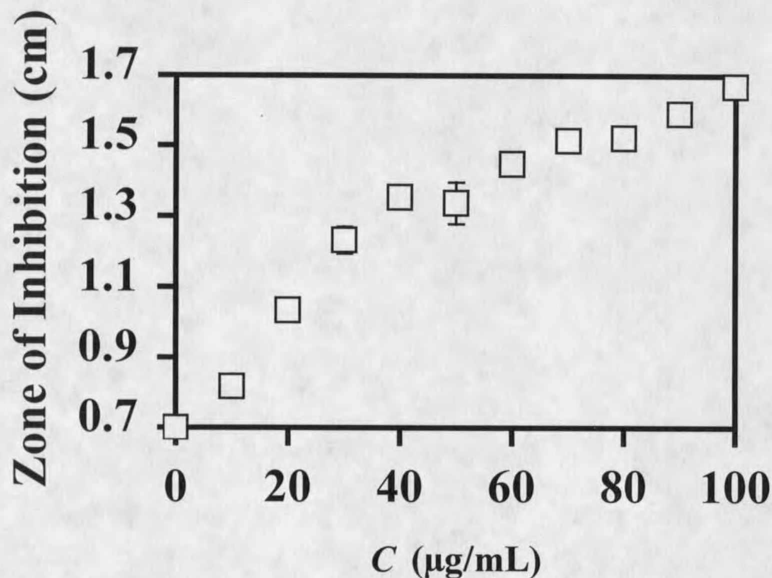


Figure 18. Standard curve for elevated tobramycin concentration. Concentrations from 0-100 µg/mL tobramycin were fit with a regression line to determine *C*. Error bars reflect the standard error of triplicate experiments.