Electrical enhancement of antibiotic killing against bacterial biofilms
by Wanida Wattanakaroon

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

Abstract:
The electrical enhancement of antibiotic efficacy against biofilms of Pseudomonas aeruginosa and Streptococcus gordonii was investigated. Three day-old P. aeruginosa biofilms or 6 day-old S. gordonii biofilms were challenged with 5 µg/ml tobramycin and 2 µg/ml gentamicin, respectively. An untreated P. aeruginosa biofilms attained a mean areal cell density of 7.8x 10^7 cfu/cm^2 and S. gordonii biofilms attained a mean density of 2.4 x 10^8 cfu/cm2. Tobramycin alone at a concentration of 5 pg/ml caused a 2.88 log reduction in biofilm viable cell numbers compared to the untreated positive control in P. aeruginosa biofilm while a 0.84 log reduction was measured in S. gordonii biofilm treated with 2 pg/ml gentamicin. Electrical current alone had only a slight detrimental effect on both biofilms. The effect of antibiotics in both cases was significantly enhanced in the presence of a 2 mA electrical current (log reductions of 5.58 and 4.3 against biofilms of P. aeruginosa and S. gordonii, respectively). The possible mechanism of the bioelectric effect in the P. aeruginosa model system was proposed to involve the role of electrolysis products including protons, hydroxyl ions, reactive oxygen intermediates, oxygen, hydrogen, or heat. This study showed that of these only electrolytically generated oxygen could possibly explain the mechanism of action. When gaseous oxygen was bubbled into the treatment chamber during exposure to tobramycin without electric current, a 1.8 log enhancement of killing resulted. Gentamicin was less effective against S. gordonii planktonic cells under anaerobic conditions than it was under aerobic conditions. When treated with 2 µg/ml gentamicin for 24 h, a S. gordonii planktonic culture exhibited a 4.7 log reduction whereas it was only 1.2 Iog when an oxygen-scavenging enzyme was added to the medium. It is suggested that oxygen generated by electrolysis may also play a role in mediating the bioelectric effect in that system. The results of this work point to an important role of oxygen in mediating reduced susceptibility of the biofilm.

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APPROVAL

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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

The electrical enhancement of antibiotic efficacy against biofilms of *Pseudomonas aeruginosa* and *Streptococcus gordonii* was investigated. Three day-old *P. aeruginosa* biofilms or 6 day-old *S. gordonii* biofilms were challenged with 5 μg/ml tobramycin and 2 μg/ml gentamicin, respectively. An untreated *P. aeruginosa* biofilms attained a mean areal cell density of 7.8×10^7 cfu/cm^2 and *S. gordonii* biofilms attained a mean density of 2.4×10^8 cfu/cm^2. Tobramycin alone at a concentration of 5 μg/ml caused a 2.88 log reduction in biofilm viable cell numbers compared to the untreated positive control in *P. aeruginosa* biofilm while a 0.84 log reduction was measured in *S. gordonii* biofilm treated with 2 μg/ml gentamicin. Electrical current alone had only a slight detrimental effect on both biofilms. The effect of antibiotics in both cases was significantly enhanced in the presence of a 2 mA electrical current (log reductions of 5.58 and 4.3 against biofilms of *P. aeruginosa* and *S. gordonii*, respectively). The possible mechanism of the bioelectric effect in the *P. aeruginosa* model system was proposed to involve the role of electrolysis products including protons, hydroxyl ions, reactive oxygen intermediates, oxygen, hydrogen, or heat. This study showed that of these only electrolytically generated oxygen could possibly explain the mechanism of action. When gaseous oxygen was bubbled into the treatment chamber during exposure to tobramycin without electric current, a 1.8 log enhancement of killing resulted. Gentamicin was less effective against *S. gordonii* planktonic cells under anaerobic conditions than it was under aerobic conditions. When treated with 2 μg/ml gentamicin for 24 h, a *S. gordonii* planktonic culture exhibited a 4.7 log reduction whereas it was only 1.2 log when an oxygen-scavenging enzyme was added to the medium. It is suggested that oxygen generated by electrolysis may also play a role in mediating the bioelectric effect in that system. The results of this work point to an important role of oxygen in mediating reduced susceptibility of the biofilm.

INTRODUCTION

Problem Overview

Biofilm-forming bacteria which grow as a thin organic layer on implanted biomedical materials or immune-compromised tissues cause a variety of medical problems. Implanted prosthetic devices, transdermal devices and associated medical equipment may become contaminated by bacterial biofilms (Nickel et al, 1985). Antimicrobial treatment fails to eradicate these biofilms because of their increased resistance to a wide range of biocides and antibiotics. Often chronic-infected medical implants require surgical removal. The bioelectric effect, the combined use of antimicrobial agents and weak electrical currents, developed by Dr. Costerton and his coworkers was proposed as a potential antimicrobial strategy in the treatment of medical device-related infections and in the sterilization of medical instruments.

Goal and Objectives

The goal of the research described in this thesis was to elucidate the mechanism of the electrical enhancement of antibiotic efficacy against biofilm bacteria. Another important goal was to determine if this phenomenon might be applicable in a dental context. Specific objectives of this project were to: a) investigate the role of electrolysis products in mediating the bioelectric effect in *Pseudomonas aeruginosa* biofilms challenged with tobramycin, and b) evaluate whether the bioelectric effect occurred when biofilms of *Streptococcus gordonii*, a characteristic oral plaque bacterium, were challenged with gentamicin.

LITERATURE REVIEW

Bioelectric Effect and Mechanism

The study of the enhancement of antimicrobial efficacy by the application of a weak direct current against microbial biofilm was first done by Dr. Costerton's group. They used a modified Robbins device (MRD) incorporating a platinum wire electrode at the bottom of the flow chamber as one electrode and the stainless steel sample studs as the other electrode. Biofilm was established on the inner surface of those studs. A direct current electric field, 1.5 V/cm with a current density of 15 $\mu A/cm^2$, was applied with reversal of polarity every 64 sec. Tobramycin was used against clinical isolate strains of *P. aeruginosa*, *E. coli* and *S. epidermidis* while ciprofloxacin and cycloheximide were used to treat *P. aeruginosa* and *C. albicans*, respectively. They reported a complete kill of *P. aeruginosa* in the presence of 8 MIC of tobramycin and a DC current after 12 hours of exposure. Their work also showed that the bioelectric effect can be applied to a variety of agents/microorganisms (Khoury et al, 1992). A second paper from the same team described experiments in a similar system (12 V/cm of a low-strength electric field and a current density of 2.1 mA/cm^2) showing enhancement of biofilm killing by kathon, a quaternary ammonium compound and glutaraldehyde against an environmental isolate of *P. aeruginosa*. They reported that 10 ppm of quaternary ammonium compound gave a total kill (6 log reduction compared with no treatment) and approximately a 4 log reduction using 5 ppm of glutaraldehyde when applied with DC current at 24 hours (Blenkinsopp et al, 1992).

To study the mechanism of electrical enhancement of antibiotic efficacy,

Costerton et al (1994) developed a flow cell device composed of three stainless steel electrodes and two inserts placed between the electrodes. This allowed them to ascertain whether the bioelectric effect was applicable to both electrode surfaces and to materials within the electric field that were not a part of the electrical circuit. The electrodes conducted a DC current generating a field strength of 5 V/cm and a current density of 1.7 mA/cm^2. To minimize the concentration of charged species, they alternated the polarity of the electrodes every 64 sec. In this work, *P. aeruginosa* UR-21 biofilm was almost completely killed by 5 time the MIC of tobramycin (5mg/l) in the presence of the electric field at 48 hours on all areas of the electrodes and on the conductive elements. They proposed that the bioelectric effect may be caused by electrophoresis and electrochemically generated ions/agents. Stoodley et al (1997) hypothesized that the bioelectric mechanism may be caused by the structural changes of the biofilm and increased convective transport due to electrostatic influences between charged groups in the biofilm and charges on the wire that cause biofilm expansion and contraction when a voltage was applied with oscillating polarity. Also shifting pH may play an important role in the enhancement of antibiotic efficacy. Jass et al (1995) developed an electrical colonization cell (ECC) based on two parallel stainless steel electrode plates with a biofilm formed onto a dialysis membrane surface suspended in parallel between those two electrodes in order to avoid electrochemical and mechanical disturbances. A direct current was applied and the polarity changed every 32 sec. They studied different doses of tobramycin in several electrical current densities with *P. aeruginosa* biofilm formed over 12, 24 and 48

hours, and suggested that the metabolic activity and growth rate of cells within the biofilms may also play an important role in determining the bioelectric effect.

Jass and Lappin-Scott (1996) confirmed this phenomenon by using an ECC at the current density of 9 mA/cm^2 to enhance the activities of ciprofloxacin, polymyxin B and piperacillin at 10 times their MIC against *P. aeruginosa* for 12 hours. They showed that only ciprofloxacin and polymyxin B are effectively enhanced by the electrical current used in their system. Wellman et al (1996) reported 7-8 log increase in killing by using 5 mg/l of tobramycin in the presence of 1 mA of DC current compared with treatment by the same amount of tobramycin alone against *P. aeruginosa* ERC1 biofilm grown on a polycarbonate coupon for 24 hours of exposure.

Oral Biofilm and Its Antimicrobial Susceptibility

The initiation and progression of oral diseases result from the formation of biofilms on the surfaces of the oral cavity. The changes in the balance of the natural resident plaque microflora and metabolism following to ecological factors lead to the development of pathological oral biofilms. The oral streptococci are the early colonizers on a clean tooth surface and represent between 60 and 80 % of the cultivable cells during the first 4 hours after professional teeth cleaning (Nyvad and Kilian, 1990). Abbe et al (1991) reported that streptococci play a predominant part in oral species because they have several metabolism pathways depending on sugars and oxygen to adapt themselves to the changing environment of the human oral cavity.

Methods to kill dental plaque are of great interest. Cummins (1991) proposed different routes to control dental plaque. Use of antimicrobial agents which inhibit

growth and metabolism is the most successful approach. Wilson (1996) summarized the data concerning the susceptibility of oral bacterial biofilms to antimicrobial agents. Pacini et al (1997) evaluated the activity of twenty antimicrobials on mixed bacterial plaque of subjects with periodontal diseases under anaerobiosis. They reported all plaque specimens were susceptible to penicillins and cephalosporins, whereas colistin, gentamicin, kanamycin and nalidixic acid showed no activity.

Because plaque is a biofilm, its susceptibility is generally lower than cells in the planktonic state. Millward and Wilson (1989) compared the bactericidal effect of chlorhexidine on *S. sanguis* biofilm and suspended cells. The MIC value for the biofilm was found to be higher than for the suspension. They also recorded that the age of the biofilm, exposure time to effective chlorhexidine concentration and addition of blood to the chlorhexidine-containing medium mimicking the crevicular fluid each had marked effects on susceptibility. Another possible explanation of the reduced antimicrobial susceptibility in plaque is the result of metabolic factor. A report from Tack and Sabath (1985) shows that aminoglycoside activity decreased under anaerobic conditions due to the lack of oxidative metabolism, but no decrease in efficacy was seen against streptococci that have no true oxidative mechanisms to which an active-transport sugar or aminoglycoside system can be coupled.

In order to study the ecology and biochemistry of the oral biofilms including the effect of antimicrobial agents, the design of a predictive in vitro model simulating oral condition for culturing plaque-forming oral bacteria has been investigated. Zampatti et al (1994) used a model based on the continuous irrigation of bovine teeth, which

have enamel structures similar to human teeth, with artificial saliva to study the colonization of *S. mutans* plaque and the treatment of antiplaque toothpaste containing 0.004% chlorhexidine. Herles et al (1994) developed chemostat flow cell system consisting of a chemostat, a source of mixed culture of five oral bacteria, and flow cells inserted with two types of hydroxyapatite (HA) and germanium surfaces to allow plaque formation to assess the potential efficacy of a placebo mouthrinse and an antiplaque mouthrinse containing 0.03% triclosan. Both test systems represent rapid tools for predicting antimicrobial formulations. Recently Bradshaw et al (1996) investigated the effect of oxygen on a ten species mixed-population biofilm including aerobes, facultative anaerobes and obligate anaerobes grown on HA discs in a two-stage chemostat model. Their study indicated that obligately anaerobic species were able to reproduce and predominate in older biofilms under an aerated environment as a result of the presence of aerobic or facultative species and the capability of being organized spatially within the community. This explanation invoked an organized and interactive consortium in those mixed-culture oral biofilms. Kinniment et al (1996) used the constant depth film fermenter (CDFF) model system to investigate steady state biofilms comprising nine species of dental plaque microorganisms found in health and disease. The system is set up by using two steady state devices coupled together, the chemostat reactor operated anaerobically as an inoculum and the CDFF suffused with air for biofilm formation. Also the first description of the application of CDFF system to evaluate efficacy of antimicrobial agents for use in clinical dentistry was reported by the same team (Kinniment et al, 1996). Chlorhexidine, an antiseptic agent formulated

into detifrices and mouthwashes, was pulsed at two different concentrations every 12 hours. According to their model, increasing the chlorhexidine concentration to 10 times MIC significantly disrupted the ecology in a mixed culture biofilm community. *A. naeslundii* and obligately anaerobic gram negative bacteria were the most sensitive species.

Treatment of dental infections by antibiotic therapy, in most situations, is preferred based on culture and sensitivity testing (Jeske, 1997). Unfortunately, in cases of antibiotic allergy, or the disadvantages of using high doses of antibiotics including rapid development of microbial resistance to the drug, and gastrointestinal side effects, the possibility of other therapies was introduced as an alternative means of treatments of plaque-related diseases. Dobson and Wilson (1992) reported that biofilms of *S. sanguis* and the periodontopathogenic species could be killed by exposure to light from a 7.3 mW Helium/Neon low-power laser in combination with toluidine blue O and methylene blue for 30 sec.

MATERIALS AND METHODS

This section presents experimental procedures used for two different microbial systems, *P. aeruginosa* and *S. gordonii*. Both procedures include biofilm growth, antimicrobial agent/electric current treatment of the biofilm, and microbial susceptibility to antibiotic.

Part I. *P. aeruginosa* System

Microorganism, Medium and Growth Reactor Set-up

P. aeruginosa ERC1 was used throughout in pure culture. A 1-liter growth reactor containing eight polycarbonate coupons (1.7 by 7.2 cm each) was connected to 32-fold concentrated medium-comprising solution reservoirs of phosphate buffer and glucose-mineral and dilution water stored in a plastic garbage can. Glucose-mineral solution was magnetically stirred and dilution water was suffused with air by using fish aquarium oxygen pump during operation. The medium composition is given in Table 1. Two reservoirs of phosphate buffer solution and mineral solution and glucose were autoclaved separately, then glucose was added after autoclaving. Dilution water was sterilized by using two 0.2 μm mini-capsule filters (Gelman Sciences, Ann Arbor, MI) connected in series. The reactor was set up at room temperature (18-20°C). To generate biofilms, the concentrated solutions were pumped through the reactor with a peristaltic pump (Cole-Parmer, Chicago, IL) to reach the working volume of 500 ml and 1 ml of frozen stock culture was then inoculated. The reactor was operated in batch mode for 24 hours with magnetic stirring. After this period of batch growth, concentrated solutions and dilution water

Table 1. Composition of *P. aeruginosa* culture medium.

Phosphate Buffer		
Na_2HPO_4	426	mg/l
KH_2PO_4	205	mg/l
Glucose-Mineral Solution		
KNO_3	13.6	mg/l
$MgSO_4$	1.0	mg/l
$CaCO_3$	1.0	mg/l
nitrilotriacetic acid	200	µg/l
$FeSO_4$	159	µg/l
$ZnSO_4$	142	µg/l
$MnSO_4$	11.4	µg/l
$CuSO_4$	2.8	µg/l
$Co(NO_3)_2$	2.3	µg/l
$Na_2B_4O_7$	1.4	µg/l
ammonium molybdate	1.4	µg/l
glucose	20	mg/l

were fed continuously into the culture at 1 ml/min and 30 ml/min, respectively to give a dilution rate of 3.84 hr^{-1}. Biofilms were allowed to grow for 72 hours.

Treatment Chamber Design

The treatment chamber was milled from a polycarbonate rectangular block (1.6 x 7.1 x 3.3 cm of internal dimension) covered with the polycarbonate lid as shown in Figure 1. The chamber consisted of two connectors inserted into either end of the block to create a fluid flow through the chamber in a net horizontal direction. The connector near the bottom of the chamber was for incoming nutrient, and the connector near the top of the chamber was for the effluent. A seal was made between the lid and the chamber with a thin sheet of soft rubber by placing this gasket under the entire bottom side of the lid. Two 22-gauge stainless steel wires were placed through the lid and a rubber gasket at opposite ends of the long axis of the chamber and extended down into near the bottom of the chamber in order to form the electrical contact to the liquid and allow for the passage of an electrical current during the experiments. The electrode on the left side (influent) of the chamber was connected to an ammeter which was connected, via a current controller for accurate delivery of the current, to the positive (+) side of the direct current power supply, whereas the electrode on the right side (effluent) of the chamber was connected to the negative (-) side of the power supply. A voltmeter was connected across the electrodes.

Antimicrobial Agent/Electric Current Challenge

The growth medium listed in Table 1 was used to support the biofilm during the experiment. The mineral-phosphate buffer was autoclaved in an Erlenmeyer flask, then

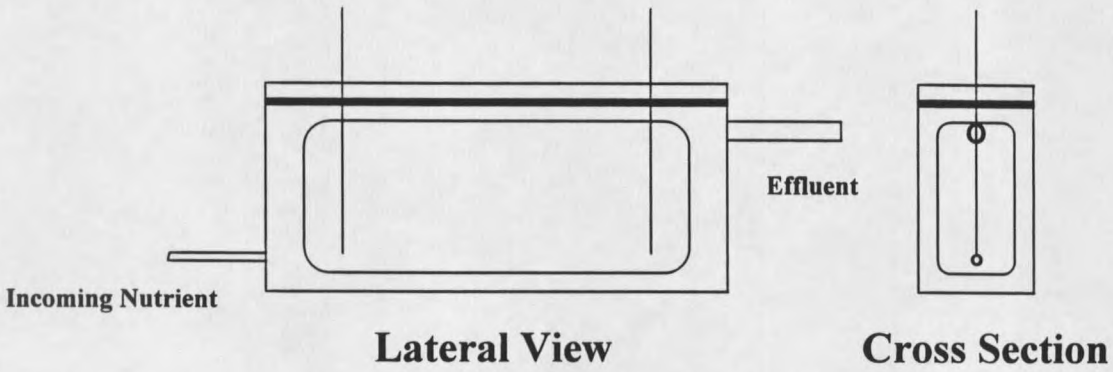

Figure 1. Treatment chamber apparatus.

glucose and stock salt ($MgSO_4$, $CaCO_3$ and KNO_3) solutions were added to a final concentration using 0.2 µm syringe filter (Acrodisc, Gelman Sciences, Ann Arbor, MI) after autoclaving. A volume 75 µl of tobramycin (A vial contains a concentration of 40 mg/ml, Apothecon, A Bristol-Myers Squibb Co., Princeton, NJ) was added directly to a 600-ml nutrient flask to give a final concentration of 5 µg/ml for experiments incorporating antibiotic. Masterflex silicone tubings size 13 and 16 were used to connect a flask to the treatment chamber and the effluent connector to the waste reservoir. These tubes were autoclaved prior to use. Each chamber was sterilized with 70% ethanol followed by sterile distilled water prior to use. The entire system was connected together as shown in Figure 2. To begin an experiment, the treatment chambers were filled with growth medium by using peristaltic pumps to reach the working fluid volume of approximately 30 ml. The medium was collected from the effluent of each chamber to measure the initial pH. The nutrient flow rate was then adjusted to approximately 2.8 ml/hr and the system allowed at least two hours to reach equilibrium prior to transferring the biofilm coupons. Biofilms developed on polycarbonate coupons were transferred aseptically from the reactor to their desired continuous flow treatment chambers. Biofilm grown on one slide, referred to as the original count, were scraped into a sterile beaker contained 9 ml of phosphate buffer using a stainless steel scraper. The detached biofilm was collected into a test tube. 1 ml of buffer was added to the beaker to rinse the biofilm left in the beaker and then added to a test tube to 10 ml of total volume. The resuspended biofilm was vortexed, serially diluted in phosphate buffer and 10 µl drops were plated at the appropriate

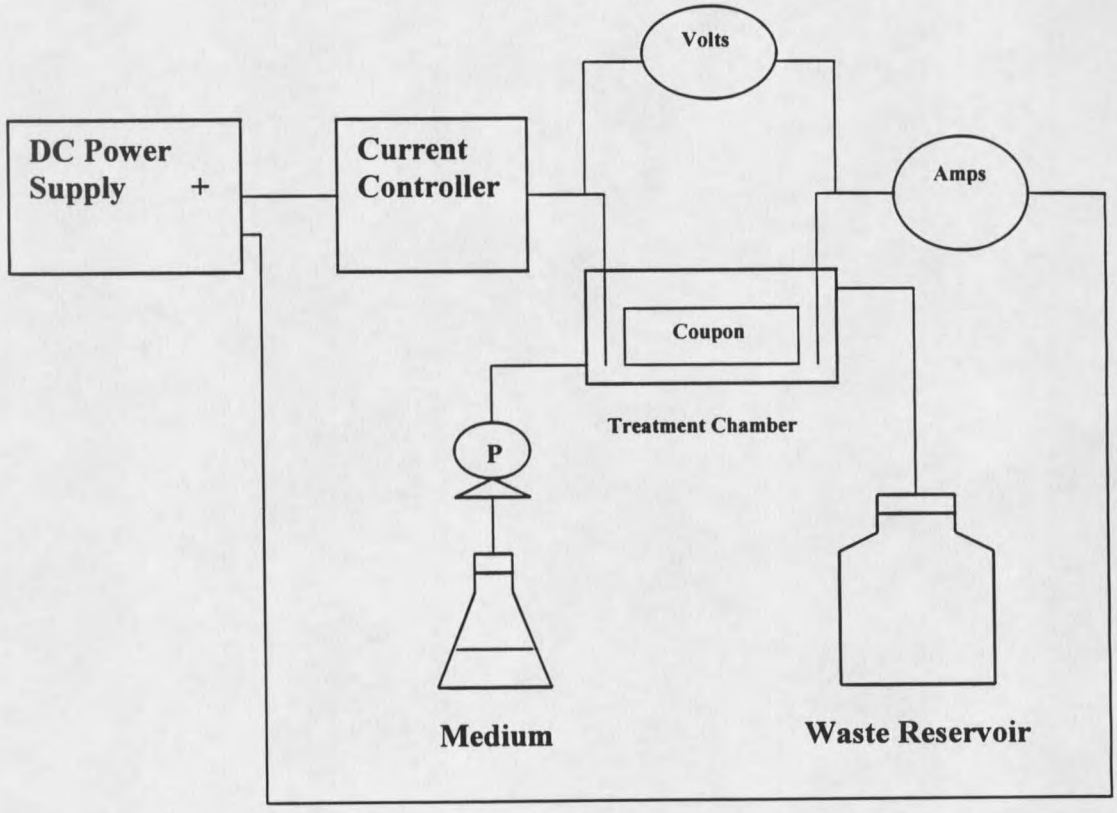

Figure 2. Diagram of the entire system.

dilution onto R2A agar (Difco, Detroit, MI). Colony forming units (cfu) were counted after incubating at 35°C for 18 hours. Biofilm areal cell density (cfu/cm^2) was calculated by dividing the total number of viable bacteria on the sample slide by the surface area of the slide. The standard treatments included were an untreated positive control (PC), control with antibiotic alone (C), field control with electric current alone (FC) and experiment treated with antibiotic plus electric current (E). DC current used in this system was set at 2 mA by a current controlling circuit. This corresponded to a current density of 4×10^{-4} amp/cm^2. The applied potential to achieve this current was 9 to11 volts. Treatment experiments lasted 24 hours. Slides were then removed to process for viable cell enumeration using the procedure described previously.

To further investigate the possible mechanisms of the biofilm being killed by the bioelectric effect, following experiments were done in a series of seven groups. All the procedures described previously were kept constant. The variation in each set is the one noted for the group. These groups are:

i) E-field exposure: The electrical field was obtained by placing the electrodes outside the treatment chamber ends and held in place with scotch tape. A potential was applied to approximately 10-11 volts with no current.

ii) Buffer concentration: The phosphate buffer (Na_2HPO_4 and KH_2PO_4) composition was varied as note for each experiment.

iii) Forced pH buffer: The phosphate buffer was formulated to have a pH of 5.0 by altering the relative proportions of two buffer constituents with the same total phosphate concentration. Buffer calculation is shown in the Appendix A. The

experiments were done without electric current for field control with electric current alone and experiment treated with antibiotic plus electric current.

iv) Active oxygen intermediates: A stock sodium thiosulfate solution was added to the culture medium to final concentrations of 1and 10 mg/ml using 0.2 µm syringe filter after autoclaving. Thiosulfate neutralization calculation is shown in the Appendix A.

v) Salts omitted: In order to eliminate ions, a version of the medium containing only phosphate buffer and glucose was used in this series.

vi) Electrolytically generated oxygen and hydrogen: Oxygen and hydrogen were sparged at the upstream end of the treatment chamber using the syringe needle (PrecisionGlide 22G1½, Becton Dickinson, Franklin Lakes, NJ) at the flow rate of 3 and 10 ml/min, respectively without electric current.

vii) Heat: The experiments, an untreated positive control and field control with electric current alone, were conducted without the biofilm. Temperature in the treatment chamber was measured at 0 and 24 hours by a digital thermometer.

Antibiotic Susceptibility Testing of Resuspended Biofilm

A 112 µl volume of tobramycin solution prepared from 40 mg/ml of tobramycin and sterile distilled water in volume ratios of 0.1:9.9 was added to 9 ml of the original resuspended biofilm to make final concentration of 5 µg/ml and placed in an incubator shaker at 35°C for 24 hours. The surviving cells were enumerated by serially diluting and drop-plating onto R2A agar plates and incubating at 35°C for 18 hours.

Part II. *S. gordonii* System

Microorganism and Culture Method

S. gordonii DL1 was used throughout in pure culture. The strain was stored in glycerol peptone solution as a frozen culture at -70°C. A 500-ml volume of a trypticase peptone broth medium (TPB medium) with an oxygen-scavenging enzyme formulation, Oxyrase (Oxyrase, Mansfield, OH), in volume ratios of 50:0.25 was added to the growth reactor containing eight polycarbonate coupons. The TPB medium composition is given in Table 2. The reactor was then placed in an incubator (Imperial III, Lab-Line Instruments, Melrose Park, IL) at 37°C and allowed to stand for at least 30 minutes to produce an anaerobic environment. A volume of 1 ml of frozen stock culture was inoculated into the reactor and grown in batch mode for 24 hours with magnetic stirring.

Reactor Set-up and Chemostat Biofilm Growth Method

The reactor was switched to continuous mode after batch growth period. A high salt version of TPB medium containing 22 g/l of NaCl, which was magnetically stirred, and dilution water were fed into the culture at 1 ml/min and 10 ml/min, respectively to give a dilution rate of 1.32 hr^{-1}. To investigate the growth of the biofilm, polycarbonate sample slides were removed at 24-hour intervals from the reactor after continuous flow initiation. Biofilm was scraped into a sterile beaker using a stainless steel scraper. Serial dilution and drop-plate onto brain heart infusion agar (Difco, Detroit, MI) were performed. Colony forming units were counted after incubating plates in an anaerobic jar at 37°C for 24 hours.

Table 2. Composition of Trypticase Peptone Broth (TPB) medium.

trypticase peptone	20	g/l
yeast extract	5	g/l
K_2HPO_4	4	g/l
KH_2PO_4	1	g/l
NaCl	2	g/l
dextrose	5	g/l

*Trypticase peptone and yeast extract were dissolved in 200 ml first, then salts and dextrose with water were added to make a final volume of 1 liter.

Cryosectioning and Microscopy

The protocol for embedding and sectioning has been described in detail elsewhere (Wentland et al, 1996). Biofilm frozen sections were fixed for 10 min at 4 °C in a fixative consisting of 10 % formaldehyde, 5 % glacial acetic acid, and 85% ethanol. Slides were then rinsed with two changes of 85 % ethanol and allowed to air dry. Fixed sections were stored under refrigeration. A stock solution of 200 mg/l acridine orange (Sigma Chemical, St.Louis, MO) in phosphate buffer (pH 7.2) was formulated and incubated at 35 °C overnight. The stock solution was then filtered through a 0.2 µm syringe filter to remove any undissolved particulates. From the stock solution, a fresh solution of 4 mg/l acridine orange in phosphate buffer was prepared for colony staining. Colonies were stained by locating the cross section on the glass slide and placing 3 µl drops in succession along the length of the cross section. Sections were stained for 5 min, then excess staining solution was blotted from the slide and dried (Wentland et al, 1996). Images captured digitially were analyzed using Image Tool Software. Thickness was measured using this software at multiple points along a section approximately 1 cm in length. A Nikon Eclipse E800 microscope fitted with a G-1B filter was used to view the biofilm cryosection. Photomicrographs were taken using Kodak Tmax 400 CN black & white film.

Antimicrobial Agent/Electric Current Challenge

After 6 days of continuous growth, biofilms formed on polycarbonate coupons were transferred to their individual treatment chambers which were in an incubator at 37°C. The apparatus and protocol for biofilm treatment were identical to described

above for those *P. aeruginosa* ERC1 unless otherwise indicated. The one tenth strength TPB medium lacking NaCl or medium containing 2 g/l of NaCl were used to support the biofilms during the experiments. A stock solution of gentamicin (gentamicin sulfate, Sigma Chemical, St.Louis, MO) was prepared in sterile distilled water and was filter-sterilized prior to adding it to a nutrient flask to give a final concentration of 2 µg/ml for experiments indicated. The applied potentials required to achieve an electrical current of 2 mA in the medium lacking and containing NaCl were approximately 6 to 8 volts and 1 to 2 volts, respectively. For the determination of viable cell counts, the brain heart infusion agar plates were used and incubated for 24 hours at 37°C in an anaerobic jar. Dissolved oxygen concentrations in the bulk fluid at the end of the 24 h treatment period were measured using a Hach (Loveland, CO) model 50175 meter.

Antibiotic Susceptibility Testing of Intact and Resuspended Biofilms

The sensitivity of intact biofilm to gentamicin was established by transferring 6 day-old biofilms to their treatment chambers filled with one tenth strength NaCl-free TPB medium containing 0 and 2 µg/ml of gentamicin. Two biofilm coupons from untreated and antibiotic treatment chambers were removed at 4, 8 and 18 hours. The efficacy of antibiotic against intact biofilm was calculated as the ratio of the number of viable cells after treatment with antibiotic to the number of viable cells in the untreated positive control. Six day-old biofilm developed on the slide referred to as original count was scraped and resuspended into 10 ml of phosphate buffer. The suspension was vortexed, serially diluted and plated onto brain heart infusion agar plates for viable cell

counts. Gentamicin stock solution was then added to 9 ml of the original resuspended biofilm to give a final concentration of 2 µg/ml and incubated in a stationary incubator at 37°C. At 4, 8, 18 and 24 hours, 1 ml was withdrawn and the surviving bacteria were enumerated using the procedure described previously.

Antibiotic Susceptibility Testing of Planktonic Bacteria

S. gordonii DL1 was recovered from a frozen stock culture by growth on brain heart infusion agar plate. After overnight incubation at 37°C in an anaerobic jar, one colony from the plate was inoculated into each of 50 ml of TPB medium in a centrifuge tube supplemented with either no or 0.25 ml of Oxyrase and incubated for 24 hours at 37°C. Both tubes were incubated at 37°C for at least 30 minutes before inoculation for allowing Oxyrase to remove dissolved oxygen in a broth medium. To determine the original number of planktonic cells, a 1.5 ml of each culture was added in a 2-ml microcentrifuge tube. The cells were centrifuged at 10,000 rpm for 7.5 min and resuspended in one tenth strength NaCl-free TPB medium. Colony forming units were determined. A 0.4 ml of each original suspension was inoculated into 9.6 ml of one tenth strength NaCl-free TPB medium with or without Oxyrase in volume ratios of 50:0.25 to a final cell concentration of 10^7 cfu/ml. Gentamicin stock solution was then added to give a final concentration of 2 µg/ml and incubated at 37°C. A 1.5 ml culture sample was taken at 4, 8, 12 and 24 hours, microcentrifuged and resuspended in phosphate buffer to remove the antibiotic. The cells were vortexed for 1 min, and the counting of colony forming units was determined.

ELECTROLYTIC GENERATION OF OXYGEN PARTIALLY EXPLAINS THE ELECTRICAL ENHANCEMENT OF TOBRAMYCIN EFFICACY AGAINST *PSEUDOMONAS AERUGINOSA* BIOFILM

Introduction

The striking enhancement of antibiotic efficacy against microbial biofilm by application of a weak direct electric current was first reported by Costerton and co-workers (Blenkinsopp et al, 1992; Khoury, et al, 1992) who termed this phenomenon the "bioelectric effect." Subsequent research has confirmed this effect over a range of conditions (Khoury et al, 1992; Blenkinsopp et al, 1992; Costerton et al, 1994; Jass et al, 1995; Whitham, 1995; Jass and Lappin-Scott, 1996; Wellman et al, 1996). The significance of the bioelectric effect is that it affords a means to overcome the nearly universally observed reduced susceptiblity of microorganisms when growing in the biofilm state compared to their susceptibility in suspension cultures (Brown and Gilbert, 1993).

The mechanism of electrical enhancement of antibiotic action remains unclear but is interesting for at least two reasons. First, knowledge of the mechanism will facilitate design of technological applications of electrical enhancement of biofilm killing. Second, data on the mechanism of the bioelectric effect may shed light on the still obscure mechanisms by which biofilms resist antimicrobial challenge. Some of the mechanisms of electrical enhancement of biofilm killing that have been postulated include electrophoretic augmentation of antimicrobial transport (Khoury et al, 1992), membrane permeabilization (Khoury et al, 1992), reduction of biofilm capacity for

binding the antimicrobial agent (Blenkinsopp et al, 1992), electrochemical generation of potentiating oxidants (Armstrong, 1993; Costerton et al, 1994), increased bacterial growth - and hence increased antibiotic susceptibility - due to electrolytic oxygen generation (Jass et al, 1995), increased convective transport due to contraction and expansion of the biofilm (Stoodley et al, 1997), and increased antimicrobial efficacy due to pH changes resulting from electrolysis reactions (Stoodley et al, 1997). Other potential mechanisms of the bioelectric effect include increased transport through electroosmosis (Chang et al, 1995), physical removal of biofilm by electrolytically generated gas bubbles, and increased susceptibility due to a temperature increase arising from resistive heating.

The multiple hypotheses on this daunting list are not easily discriminated experimentally. Costerton et al (1994) argued against the electrochemical generation of antimicrobial molecules or ions based on the absence of antimicrobial activity immediately downstream of an electrified chamber. This interpretation is consistent with reports that electric current alone does not result in discernable killing (Blenkinsopp et al, 1992; Costerton et al, 1994; Jass et al, 1995; Jass and Lappin-Scott, 1996). In the experimental system used in the work reported in this article, a slight deleterious effect of the current alone was detected (McLeod et al, 1998). Jass et al (1995) measured a plateau in the electrical enhancement versus current dose response and suggested that this implied a mechanism other than enhanced transport, which they postulated would behave linearly with current. Stoodley et al (1997) have shown by direct microscopic examination the remarkable expansion and contraction

of biofilm growing on a wire electrode when subjected to current polarity reversal. Antimicrobial susceptibility was not measured.

The purpose of the work reported in this article was to investigate the role of electrolysis products in mediating the bioelectric effect. Electrolysis of aqueous solutions leads to the generation of molecular oxygen, molecular hydrogen, hydrogen cations, hydroxyl anions, other reactive oxygen species, and heat. The first few of these effects can be seen by examining the principal cathodic and anodic reactions:

$$2H_2O \longrightarrow O_2 + 4H^+ + 4e^-$$

$$4H_2O + 4e^- \longrightarrow 2H_2 + 4OH^-$$

The net reaction in a closed system is

$$2H_2O \longrightarrow O_2 + 2H_2$$

Since the test systems used to study the bioelectric effect are all continuous flow devices, the pH in the system can fall out of balance if one of the electrodes is closer to the reactor effluent than the other. Additional reactions can lead to the formation of reactive oxygen intermediates such as superoxide anion, peroxide, and hydroxyl radicals:

$$O_2 + e^- \longrightarrow O_2^-$$

$$O_2^- + e^- \longrightarrow O_2^{=}$$

$$H_2O_2 + H^+ + e^- \longrightarrow H_2O + OH\cdot$$

Another product of electrolysis is heat. Energy dissipated by resistive heating could raise the temperature of the fluid bathing the biofilm. Because disinfection and growth rates are highly dependent on temperature, it is possible that relatively small increases

in temperature could account for part or all of the bioelectric effect. The experiments reported in this paper were designed to test the specific roles of oxygen, hydrogen, pH, active oxygen intermediates, and heat in contributing to the electrical enhancement of antibiotic efficacy.

Materials and Methods

Biofilm Development

Pseudomonas aeruginosa strain ERC1, an environmental isolate maintained in the Center for Biofilm Engineering culture collection, was used in pure culture throughout. Biofilms were grown as described previously (McLeod et al, 1998). The growth medium contained per liter: 20 mg glucose, 426 mg Na_2HPO_4, 205 mg KH_2PO_4, 13.6 mg KNO_3, 1.0 mg $MgSO_4$, 1.0 mg $CaCO_3$, 200 µg nitrilotriacetic acid, 159 µg $FeSO_4$, 142 µg $ZnSO_4$, 11.4 µg $MnSO_4$, 2.8 µg $CuSO_4$, 2.3 µg $Co(NO_3)_2$, 1.4 µg $Na_2B_4O_7$, 1.4 µg ammonium molybdate. Experiments were conducted at ambient temperature which was 18-20°C. A continuous flow stirred reactor containing eight polycarbonate coupons (1.7 by 7.2 cm each) was filled with 32-fold concentrated medium and inoculated with 1 ml of frozen stock culture. The reactor was operated in batch mode for 24 hours with magnetic stirring. After this period of batch growth, continuous flow of regular strength medium was initiated at a dilution rate of 3.84 hr^{-1}. Biofilms were allowed to develop for 72 hours in the continuous flow mode.

Antimicrobial/Electric Current Challenge

The apparatus and protocol for biofilm treatment has been described in detail

elsewhere (McLeod et al, 1998). Biofilms developed on polycarbonate slides were transferred aseptically to rectangular treatment chambers with a working fluid volume of approximately 30 ml. The treatment chamber was filled with nutrient medium, amended where indicated with 5 µg/ml of tobramycin, then a slow continuous flow, approximately 2.8 ml/hr, of this same solution was initiated through the chamber. Where indicated, an electric current of 2 mA was delivered through the chamber by means of a circuit containing a current controller and two stainless steel wires at opposite ends of the long axis of the treatment chamber. Electric current flowed approximately parallel to the substratum to which the biofilm was attached at a current density of 4×10^{-4} amp/cm^2. The potential required to establish this current was approximately 9 to 11 volts. The treatment (either untreated control, antibiotic alone, electric current alone, or antibiotic plus electric current) lasted 24 hours.

Analytical Methods

At the end of the treatment period, biofilm sample slides were removed from their individual treatment chambers and immediately processed. Biofilm was scraped into a sterile beaker using a stainless steel scraper. The biofilm was resuspended in 10 ml of phosphate buffer and serial dilutions were drop-plated (Reed and Reed, 1948; Hoben and Somasegaran, 1948) onto R2A agar (Difco, Detroit, MI). Colony forming units were counted after incubating plates at 35°C for 18 hours. Biofilm areal cell density (cfu cm^{-2}) was calculated by dividing the total number of viable bacteria on the sample slide by the surface area of the slide.

Results

Biofilm viable cell densities after no treatment (which we denote by PC for positive control), treatment with antibiotic alone (C, control), electric current alone (FC, field control), and the combination of antibiotic and electric current (E) are summarized in Figure 3. The untreated positive control exhibited a mean cell density of 7.80×10^7 cfu/cm^2. Treatment with antibiotic alone resulted in a mean log reduction of 2.88 ± 0.66 compared to the untreated positive control and this reduction was statistically significant ($p < 10^{-4}$). Treatment of planktonic bacteria at an initial cell density of approximately 10^9 cfu/mL with the same antibiotic concentration for 24 h resulted in a log reduction of 4.9 ± 1.4. A significant reduction in viable cell numbers (log reduction of 0.65 ± 0.42) was measured when biofilm was exposed to an electric current alone compared to the untreated positive control ($p = 0.0016$). The electrical enhancement of antibiotic efficacy was calculated by comparing the combined treatment against the treatment with antibiotic alone (log{E/C}). The mean log reduction of combined treatment compared to antibiotic alone was 2.75 ± 0.95 and this reduction was statistically significant ($p < 10^{-4}$).

Four experiments were performed in which the electrodes were placed outside the treatment chamber and a potential was applied. This established an electric field similar to that developed in the normal experiment but there was no current flow. No enhancement of bacterial killing was measured in these experiments (Table 3).

When oxygen was sparged into a treatment chamber receiving antibiotic (but no electrical current), there was a significant ($p = 0.027$) enhancement of the antibiotic

efficacy (Table 3). The enhancement was about 1.8 log in these experiments, which was approximately two-thirds of the enhancement realized by 2 mA of direct current. No enhancement was detected when hydrogen was sparged during antibiotic challenge (Table 3).

Striking changes in pH occurred when an electric current was applied in this experimental system. The average pH in positive control, control, field control, and experimental conditions was 7.16, 7.18, 4.52, and 4.74, respectively. The pH drop observed in experiments with current was statistically significant ($p = 0.028$).

To test whether the pH decrease was responsible for the enhancement of antibiotic efficacy, a series of experiments was performed in which the buffer strength was increased. Increasing the buffer strength reduced the pH change when current was applied, but also reduced the antibiotic efficacy (Figure 4). At three times the normal buffer strength, the mean pH in experiments with 2 mA of current was 6.7 whereas in regular buffer the mean pH in experiments with current flow was 4.7. Increasing the buffer strength did not diminish the electrical enhancement of antibiotic action (Figure 4D). With three times the normal buffer strength the mean log reduction observed in comparing the effect of current and antibiotic with antibiotic alone was slightly less than for the standard experiment, but was not significantly different (Table 3).

A further test of the role of pH was undertaken by artificially forcing a pH change by altering the relative proportions of the two buffer constituents. The phosphate buffer was formulated to have a pH of 5.0 with the same total phosphate concentration. This

forced reduction in pH actually reduced antibiotic efficacy rather than enhanced it (Table 3).

To test the possibility that active oxygen intermediates, such as peroxide, were responsible for potentiating antibiotic efficacy, sodium thiosulfate was added to the medium. Thiosulfate at 1 mg/mL did not abolish the bioelectric effect (Table 3) nor did affect efficacy of the antibiotic alone. Thiosulfate at 10 mg/mL reduced the efficacy of the antibiotic alone, but the electrical enhancement of killing was even more dramatic than in the standard experiment (Table 3). Thiosulfate did not potentiate the killing effect of the electric current alone (Table 4). When a skeletal medium consisting only of glucose and the two phosphate buffer components was used the electrical enhancement also remained the same (Table 3).

The measured temperature increase brought about by the delivery of 2 mA for 24 h, compared to an identical treatment chamber not receiving current, was 0.18 ± 0.05 °C.

A definitive experiment to preclude the intrusion of electrolysis products into the experimental treatment chamber without eliminating current flow was attempted. This was done by replacing each wire electrode with a salt bridge, in this case a flexible tube filled with agar containing sufficient potassium chloride to conduct 2 mA. These experiments were unsuccessful because the salt leached from the agar bridge interfered strongly with antibiotic action.