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Biofilms

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menter[13] can be tested using a protocol originally devised by Evans *et al.*[22] The method compares intact biofilms, suspended biofilm cells, and daughter cells newly budded from the biofilm. When the steady state has been reached in either model system, the filter is removed from the apparatus and cut in half (longitudinally for the cylindrical filter). One-half of the filter is immersed in drug solution (10 ml) for 1 hr at 37° and the adherent cells are then suspended. For the cylindrical filter, this is done by breaking up the filter with a sterile aluminum rod, followed by vortexing for 1 min; the flat cellulose acetate filter is shaken vigorously for 10 min. Cells on the other halves of the filters are first suspended in sterile water (10 ml) and then incubated with the drug at 37° for 1 hr. Samples of perfusate (1 ml) containing newly formed daughter cells are also incubated similarly at an identical final drug concentration. Viable counts are made for all samples by serial dilution and plating in triplicate on Sabouraud's dextrose agar. Values for percentage survival are calculated by using counts for untreated control samples processed similarly. Colony counts for control samples before and after the 1-hr incubation period show only very small increases in cell numbers.[13,14]

Acknowledgments

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[49] Enhanced Bacterial Biofilm Control Using Electromagnetic Fields in Combination with Antibiotics

By B. R. McLeod, S. Fortun, J. W. Costerton, and P. S. Stewart

Bacterial Biofilms

Bacterial biofilms, two rather innocent looking words, describe an entity that has become the focus of a very large field of investigation involving researchers around the world. Even a casual glance at the titles of the articles of this volume conveys both the breadth and the depth of the studies being carried out on this persistent, opportunistic form of bacterial growth. Over the past few years, practitioners in the medical field have become aware of the extent of clinical infection problems that are caused or sustained by bacterial biofilms and how relatively ineffective antibiotics are in controlling them.

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Many of the research efforts are directed toward studying the physiology of the bacteria in a "biofilm environment," identifying the methods by which biofilms form, or studying the polysaccharide matrix in which bacteria reside. This article will, however, focus on a unique means of killing the bacteria in a biofilm and on establishing the physical parameters that produce "total kill."

Electromagnetic Fields and Biological Systems

There has been a certain amount of interest in using electromagnetic fields to produce observable changes in biological systems since about 1774 when Galvoni used short pulses of current to make an isolated frog leg twitch. Although controversy still abounds when this subject is discussed, at least one area has become established clinically in which electromagnetic fields play an important role in obtaining patient recovery. That area is the healing of problem bone fractures (more specifically, nonunion bone fractures). A large body of literature exists concerning this clinical practice.[1] As this clinical method of electromagnetic-enhanced bone healing has emerged, it is not surprising that results from experiments involving bacterial biofilms and electromagnetic fields also began to appear in the literature. In a paper published in 1992, Blenkinsopp *et al.*[2] coined the name "bioelectric effect" to describe data that small dc electrical currents could be used to enhance the efficacy of biocides against *Pseudomonas aeruginosa* biofilms. Today, one can access a number of other papers that describe work involving the bioelectric effect.[3–7]

The papers cited earlier[2–7] each reported varying degrees of success in enhancing the kill achieved with an antibiotic when a dc current was added as part of the biofilm treatment, but each laboratory used an exposure system, bacteria, antibiotic, and biofilm growth system that was designed to address their particular interest. Most of this work concentrated on holding the electrical parameter (e.g., the dc current) fixed and studying

[1] A. A. Pilla, *in* "Electricity and Magnetism in Biology and Medicine" (M. Blank, ed.), p. 17. San Francisco Press, San Francisco, 1993.
[2] S. A. Blenkinsopp, A. E. Khoury, and J. W. Costerton, *Appl. Environ. Microbiol.* **58,** 3370 (1992).
[3] J. Jass, J. W. Costerton, and H. M. Lappin-Scott, *J. Indust. Microbiol.* **15,** 234 (1995).
[4] N. Wellman, S. M. Fortun, and B. R. McLeod, *Antimicrob. Agents Chemother.* **40,** 2012 (1995).
[5] J. W. Costerton, B. Ellis, K. Lam, F. Johnson, and A. E. Khoury, *Antimicrob. Agents Chemother.* **38,** 2803 (1994).
[6] A. E. Khoury, K. Lam, B. Ellis, and J. W. Costerton, *ASAIO Trans.* **38,** M174 (1992).
[7] P. Stoodley, D. de Beer, and M. M. Lappin-Scott, *Antimicrob. Agents Chemother.* **41,** 1876 (1997).

the result of varying the biology (such as the level of antibiotic). The following methods and results resulted from work designed to hold the biology constant and vary the current. We wanted to establish a dc current versus an increased bacterial biofilm killing dose–response curve and to develop an experiment and an experimental protocol that could be reproduced readily in other laboratories. The dose–response curve is presented later. Also, a sufficiently high level of confidence in experimental repeatability was achieved for a current density of about 360 $\mu A/mm^2$ in the experimental chamber to allow the exploration of several paths that could lead to an explanation of the mechanism of the bioelectric effect. A summary of these results is also presented.

Experimental Approach

Biofilm Development

A strain of *P. aeruginosa* (maintained in the Montana State University-Bozeman (MSU) Center for Biofilm Research as ERC-1) is used for these experiments. The biofilm is grown in a growth chamber (described later) that is connected to two peristaltic pumps (MasterFlex Model 6-600 rpm and 1-100 rpm, Cole-Parmer Instrument Co., Chicago, IL) with peristaltic pump heads (MasterFlex Model, Cole-Parmer Instrument Co.). The growth chamber is stirred magnetically and is connected to the substrate, the buffer, and the dilution water by MasterFlex tubing. Dilution water is stored in a plastic container (34 gal) that is suffused continuously with air using an aquarium pump. The air pump is a Model Elite 802 (Rolf C. Hagen Corp., Mansfield, MA 02048). The growth chamber, along with pumps, support containers, and flexible tubing, is shown in Fig. 1.

The chamber itself is a cylindrical, straight-sided glass beaker that is modified slightly in the glass-blowing shop at Montana State University. The modification is the addition of a glass overflow pipe that is set to be just above the level of the growth suspension in which the biofilms are formed. A second, thin-walled polycarbonate cylinder with a slightly smaller diameter is made that slips into the glass beaker but whose diameter is chosen so it has a snug fit to the inner wall of the glass beaker. Slots are cut into the polycarbonate cylinder to support the slides (see Fig. 1) on which the biofilms are grown. Eight such slides can be inserted in the growth chamber in this arrangement, which gives sufficient biofilms to have four controls and four "experimentals" with each experimental run. To grow the biofilms, the sterile growth chamber (Fig. 1) is filled with the following buffer and substrate–mineral solutions at 30 times the concentrations listed,

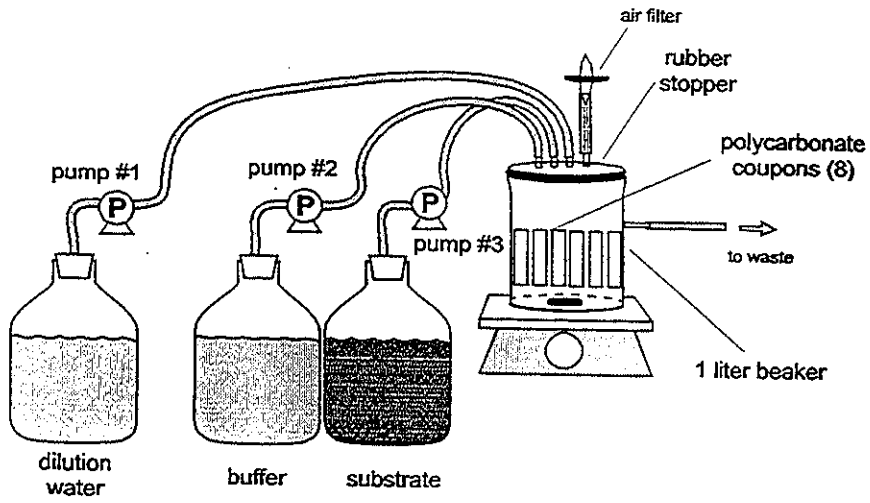

FIG. 1. Growth chamber used to grow the biofilms. The chamber consists of a straight-sided glass beaker with a glass overflow pipe added just above the level of the biofilm slides (the overflow pipe is marked "to waste" in the figure and the biofilm slides are marked as "polycarbonate coupons"). The polycarbonate slides are held close to but not touching the glass wall of the cylinder by a second, thin-walled (0.41 cm thick) polycarbonate cylinder in which slots have been cut. The biofilm slides slip into these slots and are held in position as the biofilm is being grown.

and 1 ml of the frozen *P. aeruginosa* culture is thawed and inoculated into the chamber. The buffer solution consists of (per liter) NaH_2PO_4, 454 mg and KH_2PO_4, 219 mg. The substrate solution consists of (per liter) KNO_3, 14.5 mg.; $MgSO_4 \cdot 7H_2O$, 1 mg.; $CaCO_3$, 1 mg.; $N(CH_2COOH)_3$, 427 μg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.5 μg; $ZnSO_4 \cdot 7H_2O$, 151 μg; $MnSO_4 \cdot H_2O$, 12.2 μg; $CuSO_4 \cdot 5H_2O$, 3 μg; $Na_4B_4O_7 \cdot 10H_2O$, 1.5 μg; $Co(NO_3)_2 \cdot 6H_2O$, 1.79 μg; $FeSO_4 \cdot 7H_2O$, 170 μg; and glucose, 21.3 mg. This "batch phase" continues for 24 hr with constant stirring; 24 hr after inoculation, buffer, substrate, and dilution water are fed continuously into the reactor. The dilution water flow rate (the water is sterilized using two filters in series) is 30 ml/min and the substrate flow rate is 1 ml/min. After approximately 72 hr of growth, the polycarbonate slides, with the attached bacterial biofilm, are transferred aseptically to the experimental chambers for the dc electrical current experiments. All the growth and the experiments are carried out at room temperature (22° ± 2°). Our experience indicates that temperature is not critical in this protocol.

Experimental Chamber

An experimental chamber is machined from a solid, rectangular block of polycarbonate with outside dimensions of 30 × 40 × 84 mm. An end mill is used to remove the material in a volume measuring 16 × 34 × 70.5 mm (approximately 38 cm^3) as shown in Fig. 2. The fluid flow connectors are also shown in Fig. 2. A 34 × 88 mm, rectangular piece of polycarbonate, 5.7 mm thick, is used to form the lid for each experimental chamber. The lid is made thick in order to give solid support to the 22-gauge stainless steel wires (type 316, McMaster-Carr Supply Co., Santa Fe Springs, CA) that form the electrical contacts to the liquid in the chamber. Two holes are drilled through the lid, one at each end, such that the 22-gauge wire pushes easily through the holes and extends down into the exposure chamber. The diameter of the holes is chosen so that the wires fit snugly in the holes. To further prevent leakage around the electrodes, a thin sheet of sterilized soft rubber is placed under the entire bottom side of the lid (e.g., a rubber sealing gasket) and the electrodes are pushed through the rubber at the start of each experiment. The holes for the electrodes are positioned so that the electrodes extend down the distal end of the grooves holding the biofilm substrate centered and upright in the experimental chamber (see Fig. 2). The wire electrodes are cut to a length that exposes 32 mm of electrode in the chamber (e.g., extending from the lid to near the bottom of the chamber), plus approximately 3 cm of wire extending outside the box for the purpose of connecting the electrode to the power supply circuit.

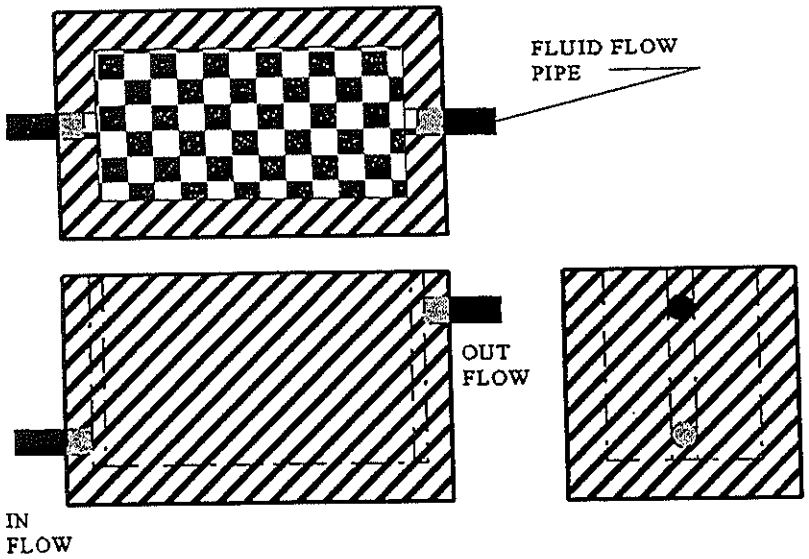

FIG. 2. Top, side, and end view of a polycarbonate exposure chamber. Note the small grooves at each end of the exposure chamber with which the biofilm support substrate (a 0.72-mm-thick, 18 × 73.5-mm strip of polycarbonate) is held upright and centered in the chamber. The 22-gauge stainless steel electrodes also fit into these grooves and are cut to clear the bottom of the chamber by about 1 mm. The chamber inside dimensions are 70.5 × 16 × 34 mm deep. Each end groove is 2 mm deep. The fluid input and outflow ports are discussed in Fig. 3. A flat cover of polycarbonate measuring 88 × 34 × 5.7 mm thick and fitted with a soft rubber gasket is held on the exposure chamber by six screws (three on each long side) during each experiment. There are two holes drilled in the cover through which the electrodes are pushed in order to make the electrical connections to the chamber. The holes provide a snug fit for the 22-gauge wire, and the gasket acts as a septum through which the electrodes are pushed. This provides a seal against liquid leakage during the experiment.

The lid and sealing gasket are then fastened to the chamber with six screws spaced equally along the long sides of the chamber (three on each side). With the lid mounted to the chamber in this way, the slowly flowing support liquid fills the entire chamber during each experiment, the fluid level remains constant, and there are no leaks from the experimental chamber. It should be pointed out that no data have been taken that indicate that the exact size or shape of the exposure chamber is important. It is considered important to keep the level of the support medium constant in order to have a constant cross section of conducting medium through which the current is flowing (e.g., to have a known and constant current density in the chamber).

Treatment of Biofilm

The antibiotic used for all of the experiments is tobramycin (Apothecon, Bristol-Myers Squibb Co., Princeton, NJ) at a concentration of 5 mg/liter [five times the minimum inhibitory concentration (MIC) of tobramycin for planktonic cells of this strain of *P. aeruginosa*]. The antibiotic is obtained in 2-ml vials (concentration 40 mg/ml) stored, when not in use, as per the manufacturer's directions and used well before the label expiration date. As a further check on the efficacy of the antibiotic, a planktonic control is run with each experiment, i.e., tobramycin is tested against planktonic bacteria at the just-mentioned concentration to be sure that the drug is still potent. The equipment used for this part of the experiment is shown in Fig. 3, and a nutrient support medium (pH 6.8 to 7), consisting of the buffer and support solution (without the salts) in the concentrations listed earlier, is prepared in 1000-ml flasks and autoclaved along with all of the experimental chambers, tubing, and connectors (anything that comes in contact with the biofilm is heat or filter sterilized). All material is left overnight to cool and then salts, glucose, and the antibiotic are added to the growth medium flask in a sterile procedure using sterile syringes, nee-

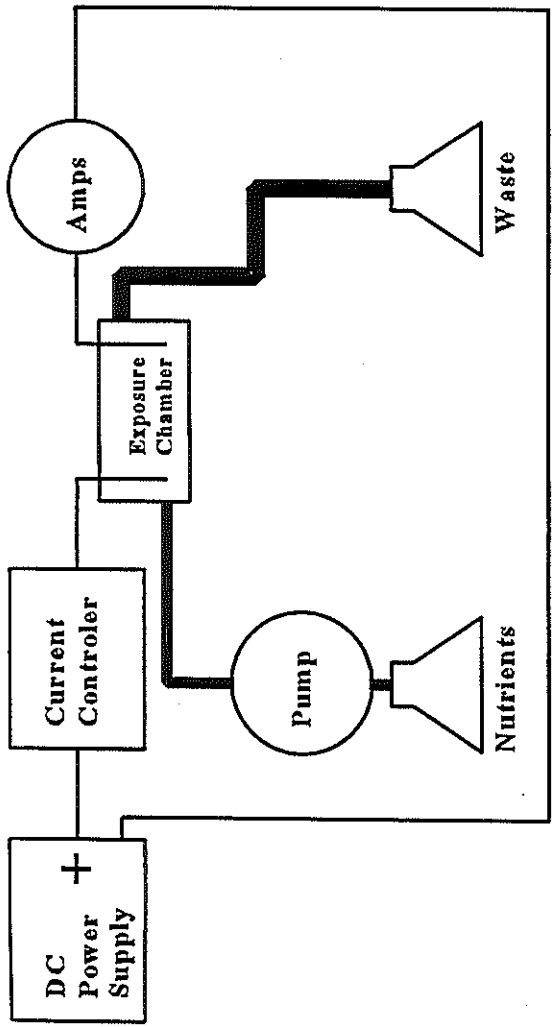

FIG. 3. A block diagram of the electrical circuit used in the experiments. Also shown is the fluid flow circuit. Note that the inflow and the outflow pipes are of different size. The outflow pipe was made larger simply to allow the fluid to exit freely. The input stainless pipe has dimensions of 1.7 mm o.d. and 1.21 mm i.d., and the outflow pipe has dimensions of 4.77 mm o.d. and 3.21 mm i.d. Both pipes are push fitted tightly into the experimental chamber.

dles, and syringe filters. The tubing is connected to the flasks and to the experimental chambers through peristaltic pumps, and the nutrients are allowed to flow to the chambers for about 1 hr in order to fill them. Once the experimental chambers are full, the transfer of the slides is completed using aseptic techniques, and the nutrient flow rate is continued at 2.7 ml/hr. Currents are set at the desired value in each individual chamber and the experiment is carried out for 24 hr. The original count (the cell density in the biofilm on one of the polycarbonate slides immediately after the slides are removed from the growth reactor and immediately before an experiment is initiated) is obtained using the same procedure used to obtain the experimental counts (described later). Tobramycin, at a concentration of 5 mg/liter, is then added to the original biofilm suspension, which is placed in a stirred water bath for 24 hr, serially diluted, and plated. This part of the protocol produces data about the original density of cells as well as information about the level of killing of the planktonic (free floating) bacteria by the antibiotic.

After 24 hr, each slide is removed from its experimental chamber, and the biofilm is scraped, using sterile techniques, into a sterile beaker from the polycarbonate slide using tweezers and a stainless steel spatula. Nine milliliters of sterile buffer solution is added to the beaker. An additional 1 ml of sterile buffer is added to the beaker after the first 9 ml containing the biofilm has been poured back into a sterile test tube. This extra 1 ml of buffer solution rinses out any biofilm left in the beaker and is then added to the 9 ml in the test tube. Eight serial dilutions are performed on the suspended cells using a Rainin 100-ml electronic digital pipette (edp 2 by Rainin), and the planktonic culture with antibiotic is also diluted and plated. The petri dishes are incubated at 35° and, after 17 to 18 hr, colonies are counted and data are recorded.

Electric Circuit

Because one of the goals of the work reported in this article is to develop a current versus killing dose response, it is important to be able to set the current at the desired value and know that the set value does not vary during the 24 hr during which the experiment is performed. Therefore, a current controller commonly used to control current to laser diodes (Model LDD200-1M, Wavelength Electronics, Bozeman, MT), is placed in the circuit as shown in Fig. 3. It is placed between the experimental chamber and the dc power supply in order to be sure that the set value of current is flowing to the chamber. The dc ammeter (shown in Fig. 3 in the circle labeled "Amps") is placed in the circuit to measure the amount of current flowing through the chamber. The controller has the capability of producing

up to a constant 200 mA of current (when used in conjunction with a 12-V dc source). It should be pointed out that having exactly the same electronics in every circuit is not important as long as the current is controlled and measured carefully. There are five identical circuits used in each experimental run so that the desired current level can be set and maintained in individual chambers. The desired current in each chamber is set at the initiation of each experiment and the current controller keeps that value constant (desired value ± 5%) independent of any resistance changes in the chamber.

Evaluation of Results

As stated earlier, the initial work was designed to produce a dose–response curve for the current needed to produce increasing levels of killing of the bacteria in the biofilm. As the work progressed, the scope was enlarged to investigate the role of electrolysis products in mediating the bioelectric effect. Table I shows a summary of the general design of the experiment as well as the names attached to the various sets of data.

Because the biofilm growth reactor was designed to allow a biofilm to grow on eight polycarbonate slides, each experiment was designed to make use of all eight of the slides. One slide was removed from the reactor, the biofilm was scraped into a sterile beaker using a stainless steel scraper, and suspended in 10 ml of phosphate buffer. Antibiotic at the concentration used in these experiments (5 mg/liter) was then added and the suspension was placed in a stirred water bath for 24 hr. At the end of this period, eight serial dilutions were drop-plated into R2A agar and incubated for 18 hr at 35°, and then the plates were counted. These data indicated that the efficacy of the tobramycin at 5 mg/liter against the planktonic form of the bacteria was a mean log reduction of 4.27 ± 1.3.

Three of the slides for each experiment were used for the sham control (SC), the antibiotic control (C), and the current control (CC) as explained in Table I. The remaining four slides were exposed in the experimental

TABLE I
GENERAL EXPERIMENTAL DESIGN

Current	Antibiotic	Data name
No	No	Sham control (SC)
No	Yes	Antibiotic control (C)
Yes	No	Current control (CC)
Yes	Yes	Experiment (E)

chambers to set levels of current flow (recall that the antibiotic was present in the slowly flowing support medium as described in "Treatment of the Biofilm") with data being recorded as "experiment" (E). The (E) data from each experiment and from different experiments were treated as independent data sets because each circuit consisted of a complete set of independent equipment. All seven of these slides were treated in identical experimental boxes. The untreated sham control data exhibited a mean cell count of 8.7×10^8 cfu with a mean cell density of 7.8×10^7 cfu/cm^2.

When the biofilm was treated with antibiotic alone [log(*C/SC*)], the mean log reduction was 2.88 ± 0.66 ($p < 10^4$), and treatment with current alone [log(*CC/SC*) with the current level set at 2 mA], the log reduction was 0.65 ± 0.42 ($p = 0.0016$). At the 2 mA current level, the electrical enhancement of the antibiotic efficacy was calculated by comparing the combined effect of current and antibiotic with antibiotic alone [log(*E/C*) with the result being a mean log reduction of 2.75 ± 0.95]. If the combined current (2 mA) and antibiotic treatment was compared to the untreated biofilm [log(*E/SC*)], the result was a mean log reduction of 5.77 ± 0.26. Dose–response data shown in Fig. 4 is plotted as current plus antibiotic in the exposure chamber versus treatment with antibiotic alone [log(*E/C*)] and as current plus antibiotic in the chamber versus no treatment [log(*E/SC*] in order to illustrate both the increase in effectiveness of the antibiotic as the current flow is increased and also to allow one to observe the enhancement due to the bioelectric effect.

In addition to these results, data were also obtained from a series of experiments that were designed to investigate hypotheses concerning the mechanism by which the current enhances the antibiotic efficacy. In four experiments, the electrodes were placed outside the treatment chamber and a potential was applied across the electrodes. The electric field established in the chamber was adjusted to be the same as that developed in the normal experiment but, of course, there was no current flow. There was no enhancement of bacterial killing. In another series of experiments, oxygen was sparged into a treatment chamber that was receiving antibiotic but no current. There was a significant ($p = 0.027$) enhancement of the efficacy of the antibiotic of about 1.8 log in these experiments. For the same experiments with hydrogen replacing oxygen, there was no enhancement.

It was observed that a large change in pH occurred when the electric current was applied to the suspension in the experimental system. For example, pH values in the SC, the C, the CC, and the E at 2 mA were 7.16, 7.18, 4.52, and 4.74, respectively. A series of experiments was then performed in which the buffer strength was increased. This reduced the pH change that occurred when the current was flowing in the chamber, but the increased buffer strength also reduced the antibiotic efficacy. A

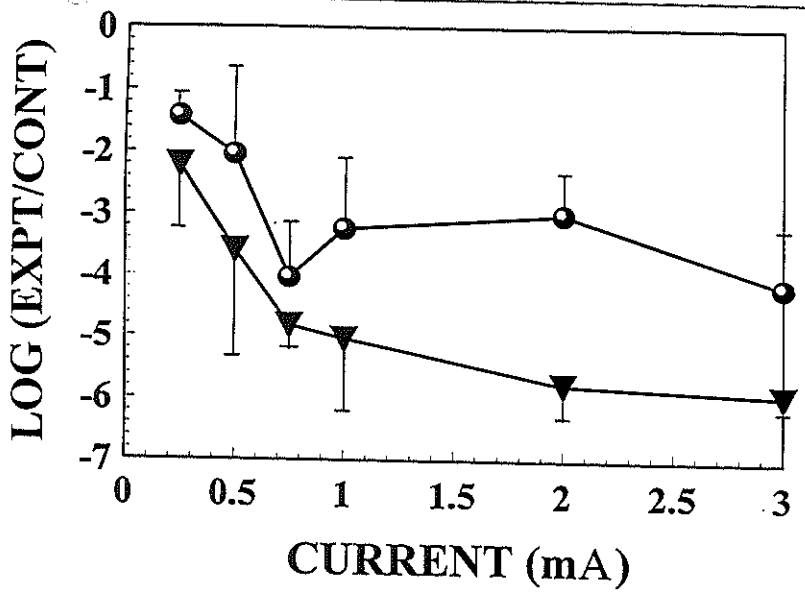

FIG. 4. Data from Table III plotted to show the results of antibiotic plus current compared to antibiotic with no current (log[*E/C*], ●) and for antibiotic plus current compared to the sham control (log[*E/SC*], ▼). Recall that control data are obtained with the experimental chambers using antibiotic but no current in the support medium. The sham control is obtained with no antibiotic and no current in the support medium. Therefore, in the upper curve (●) one can see the effect of adding current to an antibiotic treatment for a biofilm. The lower curve (▼) indicates the combined effect of adding both current and antibiotic to treat a biofilm. One-half of the error bars are shown upward for (●) data and downward for (▼) data. The ANOVA analysis of the data points is given in Table III.

further test of the role of pH in the bioelectric effect was conducted by artificially forcing a pH change by altering the relative proportions of the two buffer constituents. The phosphate buffer was reformulated to have a pH of 5.0 with the same total phosphate concentration. When this buffer was used in the experiments (with no current flow), the forced reduction in pH reduced the antibiotic efficacy instead of enhancing it.

Other possible mechanisms were investigated, such as a temperature change in the suspension in the experimental chamber, the possibility that active oxygen intermediates such as peroxide were being generated, and that ions from the salts in the support medium were involved in causing the bioelectric effect. The temperature increase with 2 mA flowing in the experimental chamber for 24 hr was measured and found to be 0.18 ± 0.05°, which is not sufficient to account for the effect. To test the possibility that oxygen intermediates were responsible for increasing the efficacy of

the antibiotic, sodium thiosulfate was added to the medium, first at 1 g/liter then at 10 g/liter. At the lower concentration, the thiosulfate did not abolish the bioelectric effect nor did it affect the efficacy of the antibiotic alone. At the higher concentration, the efficacy of the antibiotic alone decreased, but the electrical enhancement of killing of the biofilm increased. When a skeletal medium consisting of only glucose and the two phosphate buffer compounds was used (e.g., no salts), the electrical enhancement remained the same. A summary of the various treatments of the biofilm is given in Table II.

Discussion of Bioelectric Effect on Biofilms

The material contained in this article has focused on the bioelectric effect and the electrical enhancement of antibiotic efficacy against a biofilm, which, in this case, was a model system of *P. aeruginosa* and tobramycin. Data indicate that close to a 3 log reduction in colony-forming units (when the treatment of current plus antibiotic is compared to the treatment of antibiotic alone) can be achieved when the current through the experimental chamber is 2 mA. This corresponds to a current density of 3.7×10^{-6} A/mm^2. If the enhancement of current plus antibiotic is compared to a biofilm with no treatment (the sham control), the result is more than a 5.5 log reduction in the colony-forming unit. This level of current has been established in the experimental system described in this article as the amount of current that is required to give a nearly complete kill of the

TABLE II
COMPARISON OF BIOELECTRIC ENHANCEMENT OF ANTIBIOTIC EFFICACY WITH OTHER TREATMENTS[a]

Treatment	Mean log(*E/C*)	*n*	*p*	
			Mean = 0	Mean = 2 mA
2 mA	−2.75	15	<0.0001	na
E field, no current	0.33	4	0.082	<0.0001
2 mA, 3× buffer	−2.3	3	0.077	0.27
No current, pH 5.0	2.41	3	0.008	<0.0001
2 mA, 1 g/liter, thiosulfate	−2.67	3	0.074	0.93
2 mA, 10 g/liter, thiosulfate	−5.56	3	<0.0001	<0.0001
2 mA, salts omitted	−2.87	3	0.044	0.87
No current, oxygen	−1.83	3	0.027	0.07
No current, hydrogen	0.51	3	0.11	<0.0001

[a] *E* denotes the viable cell count in the presence of both antibiotic and electric current and *C* denotes the viable cell count when treated with antibiotic alone.

biofilm. More sensitive tests are now available that could be used to establish 9 or 12 log reductions in the number of viable colonies, but that was not the point of this work. The two curves presented in Fig. 4 are quite suggestive of dose–response curves, even though the error bars are rather wide. ANOVA analysis (see Table III) indicated that the data points on the two curves were significantly different at 1 and 2 mA and close to being significantly different at 3 mA.

The mechanism by which the electric current augments the efficacy of the antibiotic has not been established. Because of this, a number of experiments were run using the exposure chambers and the 2-mA current level to eliminate some of the possible explanations for the bioelectric effect. It has been established that current must flow in the chamber containing the biofilm for the effect to be operable, i.e., a non-time varying (direct current or dc) electric field by itself does not increase the killing of the biofilm.

TABLE III
ANOVA ANALYSIS[a]

Condition	Data points			
	p	F	Critical F	Significant at 95%?
Antibiotic + I/current control				
2 to 0.5 mA	0.0693	3.79	4.494	Near
2 to 1 mA	0.5629	0.3461	4.3512	No
2 to 3 mA	0.052	4.4545	4.5431	Near
E/sham control				
2 to 0.5 mA	0.001	15.94	4.49	Yes
2 to 1 mA	0.0563	4.1053	4.3512	Near
2 to 3 mA	0.6162	0.262	4.5431	No
Between curves (E/SC to E/CC)				
0.5 mA SC to 0.5 mA CC	0.276	1.4355	5.9814	No
1 mA SC to 1 mA CC	0.0143	7.8074	4.6001	Yes
2 mA SC to 2 mA CC	1E-11	131.37	4.222	Yes
3 mA SC to 3 mA CC	0.0651	6.3708	7.7086	Near
Current value				Number of data points
0.5 mA				4
1 mA				8
2 mA				14
3 mA				3

[a] Data plotted in Fig. 4 are examined to determine if points on the same curve are significantly different at the 95% confidence level (E/CC or E/SC). Then the same analysis is used to determine if points at the same current level but on different curves are significantly different (E/SC to E/CC).

Data presented here also indicate that electrolytically generated changes in pH, an increase in antibiotic efficacy due to a current-mediated rise in the temperature of the medium, and the generation of reactive oxygen intermediates can be ruled out as mechanisms. We established that virtually the same electrical enhancement of the killing could be obtained with the current flowing in either direction (but not reversing during any individual experiment) in the exposure chamber, which eliminates enhanced convective transport via electrically driven contraction and expansion of the biofilm[7] as a mechanism. When all of the salts were removed from the medium, leaving only two phosphate buffer components, the bioelectric effect persisted. This suggests that the electrochemical generation of an inhibitory ion, such as nitrite from nitrate, is not likely to be the explanation for the electrical enhancement of antibiotic efficiency.

One possible explanation for the bioelectric effect did emerge from these experiments. It is possible that there is increased delivery of oxygen to the biofilm due to oxygen generation by *in situ* electrolysis. Calculations indicate that the flow of current at the levels established in these experiments was sufficient to saturate the aqueous medium with oxygen. When gaseous oxygen was bubbled into the treatment chamber during exposure to tobramycin but in the absence of current, a 1.8 log enhancement of the killing of the biofilm was recorded. We also noted that oxygen applied without antibiotic decreased the biofilm accumulation compared to the sham control (no current, no antibiotic) by about 0.47 log, which mimicked the effect(s) of direct current alone (which resulted in a reduction of about 0.65 log). The "bioelectric effect mechanism" for this set of experiments was linked to oxygen because a similar set of experiments with hydrogen replacing oxygen being applied to the biofilm resulted in no enhancement of killing of the biofilm. This line of reasoning is currently being further investigated, focusing on two hypotheses. It is possible that when oxygen reaches toxic levels in the biofilm, it weakens the bacterial cells and they may become more susceptible to the antibiotic. Another hypothesis is that the increased delivery of oxygen could enhance the growth in the biofilm, which would negate the reduced susceptibility of the bacteria in the biofilm associated with slow growth.[8] It has been shown that *P. aeruginosa* biofilms are readily oxygen limited, which leads to zones of slow or no growth within the depths of the biofilm.[9,10] It would follow then that if biofilm resistance

[8] P. Gilbert and M. R. Brown, *in* "Microbial Biofilms" (H. M. Lappin-Scott and J. W. Costerton, eds.), p. 118. Cambridge Univ. Press, Cambridge, 1995.
[9] C. T. Huang, K. D. Xu, G. A. McFeters, and P. S. Stewart, *Appl. Environ. Microbiol.* **64**, 1525 (1998).
[10] K. D. Xu, P. S. Stewart, F. Xia, C. T. Huang, and G. A. McFeters, *Appl. Environ. Microbiol.* **64**(10), 4035 (1998).

to antibiotics is due to slow growth in the biofilm, the augmenting of the concentration of the limiting nutrient could actually make the biofilm more susceptible.

Conclusions

This work has established a protocol and a test system for investigating the result of adding a dc current flow in conjunction with the antibiotic tobramycin to sharply increase the kill of the bacterium *P. aeruginosa* that is growing in a biofilm. The dose–response curves contained in Fig. 4 and the design of the experimental exposure chambers allow one to estimate the current densities that are needed to reach a desired level of killing. This has several implications with respect to the design of devices that would accomplish the sterilization of medical devices. Data that were developed to help define the mechanism of interaction are of equal importance as once again, if one desires to develop a device that will translate these results from the laboratory to a clinical application, the design of the device will depend on an understanding of the means by which the antibiotic efficacy is augmented. There appears to be no doubt that the bioelectric effect can be used to strikingly increase the ability of tobramycin to be effective against *P. aeruginosa* when the concentration of the antibiotic is about 5 MIC.