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# Bacterial Biofilms and the Bioelectric Effect

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**Bacterial biofilms are acknowledged to be a major factor in problems of ineffective sterilization often encountered in clinics, hospitals, and industrial processes. There have been indications that the addition of a relatively small direct current electric field with the sterilant used to combat the biofilm greatly increases the efficacy of the sterilization process. The results of the experiments reported in this paper support the concept of the "bioelectric effect" as reported by J. W. Costerton, B. Ellis, K. Lam, F. Johnson, and A. E. Khoury (Antimicrob. Agents Chemother. 38:2803–2809, 1994). With a current of 1 mA flowing through the chamber containing the biofilm, an increase in the killing of the bacteria of about 8 log orders was observed at the end of 24 h (compared with the control with the same amount of antibacterial agent but no current). We also confirmed that the current alone does not affect the biofilm and that there appear to be optimum levels of both the current and the sterilant that are needed to obtain the maximum effect.**

Biofilm-forming bacteria are surface-adhering bacteria that form colonies characterized by the production of an exopolysaccharide matrix in which they reside. Bacteria that have formed into biofilms on medical devices present major problems, since these biofilms show greatly increased resistance to antimicrobial chemotherapy, the primary treatment for internal, medical device-related infections. Previous research indicates that in order for sterilants and antibiotics to be effective against biofilm bacteria, concentrations from 500 to 5,000 times greater than those required for killing planktonic (floating) strains of the same bacterial species are necessary (8). At these extremely high antibiotic levels, it becomes impossible to safely treat patients with internal medical device infections with antibiotics alone. Often medical implants must be removed to effect sufficient sterilization, and this, of course, implies additional surgical trauma and increased risk of externally introduced infection.

A number of research efforts have been concentrated on the problem of the resistance of biofilm bacteria to antimicrobial agents (1–3, 5, 6). The focus of the study reported in this paper is a process referred to as the "bioelectric effect." In research done by Costerton et al. (4), the efficacy of antibiotics was shown to be increased through the application of weak electric fields. With the combined application of direct current electric fields of about 1.5 to 20 V/cm (current densities of about $15 \times 10^{-6}$ to $2.1 \times 10^{-3}$ A/cm$^2$) and antibiotics, the concentrations of antibiotics needed to be effective against biofilm bacteria fell to only 1.5 to 4.0 times those necessary for planktonic bacteria (4). The use of an electric field of the same field strength alone had only a small effect on the biofilm (4), which may suggest that the effect is a result of a synergistic interaction of the field and the antibiotic. As yet, however, no definitive explanation of the phenomenon exists.

The research reported in this paper was undertaken, using the work of Costerton et al. (4) as a guide, to establish an experimental protocol that would result in a consistent baseline direct current experiment and to develop some of the data that could be used to construct a model of the interaction between the biofilm, the electric field, and the antibiotic. As yet, it is not clear which of the electric parameters, such as the electric field strength, the current density, the direction of flow of the current, or the time of application of the field, is the key parameter to be used to maximize the effect. Further data are also needed to confirm the optimum level of antibiotic necessary to produce the bioelectric effect.

## MATERIALS AND METHODS

**Biofilm growth.** A mixed-culture biofilm consisting of cells of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (maintained in the cell collection in the Center for Biofilm Engineering at Montana State University and designated cell line ERC-1 and cell line KP-1 for the *P. aeruginosa* and *K. pneumoniae* cells, respectively) were used. The initial cell densities and ratio of species in the mixed culture were not controlled in these initial experiments. The growth chamber was inoculated on day 1, the growth was continued for 7 days (the amount of time that we determined would result in a steady state in the biofilm), and species-selective growth agar was used at the completion of the treatment to compare treated growth with control growth. The bacteria were grown as a biofilm on a polycarbonate "coupon" (2 by 31 cm) by using a RotoTorque reactor (Bio Surface Technology Corporation, Bozeman, Mont.). The RotoTorque was autoclaved with the coupons already inserted into the grooves. Three reservoirs, containing dilution water, substrate-mineral solution, and phosphate buffer solution, respectively, were connected to the RotoTorque. A flow rate of 1 ml/min was used for both the substrate-mineral solution and the phosphate buffer solution, and a flow rate of 30 ml/min was used for the dilution water. The resulting influent (pH 7.2) consisted of (per liter) glucose, 21.3 mg; $KNO_3$, 14.5 mg; $MgSO_4$, 1.0 mg; $CaCO_3$, 1.0 mg; $N(CH_2COOH)_3$, 213 μg; $(NH_4)_6Mo_7O_{24}$, 1.5 μg; $ZnSO_4$, 151 μg; $MnSO_4$, 12.2 μg; $CuSO_4$, 3 μg; $Na_2B_4O_7$, 1.5 μg; $Co(NO_3)_2$, 2.5 μg; $FeSO_4$, 170 μg; $Na_2HPO_4$, 454 mg; and $KH_2PO_4$, 219 mg. One milliliter of the frozen mixed culture was thawed and then inoculated into the RotoTorque, which was filled with influent at 30 times the concentrations listed above. After 24 h, the dilution water was started (at 30 ml/min) and the influent was diluted until it had the concentrations listed above. The biofilm was allowed to grow for 7 days until it reached a steady state. The substrate-mineral solution and the phosphate buffer solution had been autoclaved prior to being connected to the RotoTorque system. The dilution water was sterilized using two filters in series connected to the RotoTorque.

**Experimental chamber.** The reaction chambers were built from FisherBrand five-slide, fifty-gauge polypropylene slide transporter boxes (3 by 1 in. [ca. 8 by 3 cm]; Fisher Scientific) (Fig. 1). To create a pathway for the nutrient flow through the chamber in a left-to-right horizontal direction, holes were drilled on either end of the box with a 1/8-in. (ca. 0.3175 cm) drill. The hole on the left end of the box (influent) was drilled near the bottom of the chamber in the center, and the hole on the right end of the box (effluent) was drilled near the top of the chamber in the center. The larger ends of two connectors (1/16 by 3/32 in. [ca. 0.16 by 0.238 cm]) were cut at the groove, inserted into the holes previously drilled, and

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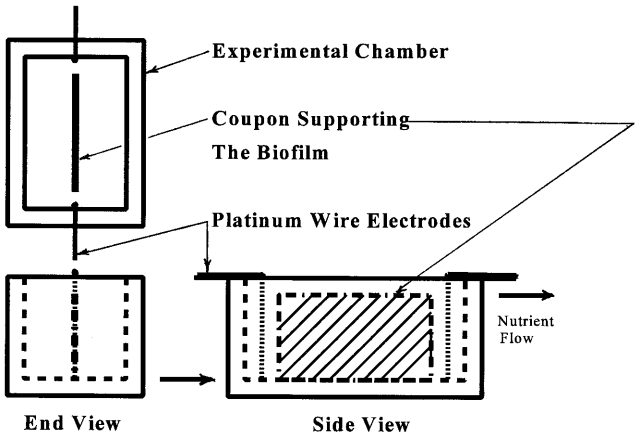


FIG. 1. Top, end, and side views of the experimental chamber. The biofilm was grown on the coupon (crosshatched in the side view), which, in turn, was centered in the chamber during the experiments. Thus, the electric field and the current density vectors were pointed along the length of the biofilm. The electrodes were 0.63-mm (ca. 0.025 in.)-diameter platinum wire. The nutrient flow rate was 30 ml/h.

fastened in place with silicone adhesive. The positive electrode was always at the influent (left) end of the chamber.

Number 22-gauge, platinum wire electrodes were placed at either end of the chamber (inside the chamber). Notches were cut in the end of the chamber, allowing the electrodes to project from the chamber. A small strip of scotch tape was placed on the top edge of the chamber to help stabilize the electrodes. The electrode on the left (influent) side of the chamber was connected to an ammeter, which, in turn, was connected to the positive (+) side of the direct current power supply. The electrode on the right (effluent) side of the chamber was connected to the negative (−) side of the power supply. A voltmeter was connected across the electrodes. The ammeter was used throughout the experiment to set and monitor the current flow through the experimental chamber, and the voltmeter was used to monitor the voltage across the experimental chamber.

**Support medium.** A nutrient solution (pH 6.8 to 7) which consisted of (per liter) glucose, 20 mg; $Na_2HPO_4 \cdot 7H_2O$, 804.6 mg; $KH_2PO_4$, 205 mg; and 0.1563 ml of trace element solution [which consisted of (per liter) $N(CH_2COOH)_3$, 1.28 g; $(NH_4)_6Mo_7O_{24}$, 8.96 mg; $ZnSO_4$, 0.9088 g; $MnSO_4$, 72.96 mg; $CuSO_4$, 17.92 mg; $Na_2B_4O_7$, 8.96 mg; $Co(NO_3)_2$, 14.7 mg; and $FeSO_4$, 1.018 g) was made to support the biofilm during the experiment. The nutrient solution was autoclaved in an Erlenmeyer flask, and glucose was added after autoclaving. Tubing to connect the flask to the reaction chamber was also autoclaved. A Cole-Palmer peristaltic pump was used to create the nutrient flow with a flow rate of 30 ml/h. Each chamber was sterilized with an ethanol wash followed by distilled water. The entire system was connected and turned on for approximately 2 h prior to the transport and installation of the biofilm coupon to allow the nutrient flow to reach equilibrium in the experimental chambers.

**Treatment of the biofilm.** In order to maintain sterile conditions, the biofilm was transported from the RotoTorque to the experimental chambers in sterile boxes that were exactly like the experimental chambers except that they had no electrodes or holes for nutrient flow. The boxes were filled with the nutrient solution described above. The polycarbonate coupons on which the biofilms were grown were removed from the RotoTorque and cut to the length of the experimental chambers with flame-sterilized scissors. The cut coupons were then placed inside the transportation chambers and subsequently inserted into the experimental system with flame-sterilized forceps. The treatments used included the following: (i) a control with antibiotic but no electric field (the cell count from the biofilm exposed to the electric field was compared with the cell count from this control); (ii) an experimental treatment with antibiotic and with an electric current; (iii) a positive control that had no nutrient flow or electrodes, which was configured as an identical chamber and coupon and filled with nutrient solution which contained no antibiotic (this control was used simply to indicate that the bacteria were still healthy in the biofilm at the end of the 24-h exposure period); and (iv) an experimental treatment with an electric field but with no antibiotic. The antibiotic used was tobramycin (Apothecon; Bristol-Myers Squibb Co., Princeton, N.J.) at concentrations of 5 or 1 mg/liter (5.0 and 1.0 times the MIC of tobramycin for this strain of *P. aeruginosa*, respectively, and approximately the same for the *K. pneumoniae* strain), which was added directly to the nutrient solution.

The experiments were run for a 24-h period (the data of Costerton et al. showed about a 6-log increase in killing after 24 h of exposure to the direct current [4]), and as yet, no other exposure times have been tried. The coupons were then scraped with a razor blade, and the detached biofilm was vortexed, serially diluted with a phosphate buffer, and plated. The dilutions were plated on two types of agar, R2A and *P. aeruginosa* isolation agar (Fisher Scientific), to help distinguish between colonies of *P. aeruginosa* and *K. pneumoniae*. Each dilution was plated as 5 10-μl drops with four different dilutions sectioned off onto each plate, and each plate was done in duplicate. The R2A agar was kept at room temperature and counted at 24 h after plating. The *P. aeruginosa* isolation agar was incubated at 35°C and counted at 12 h after plating. The 12-h elapsed time in the incubator was chosen for convenience, and other incubation times have not been used.

## RESULTS

As can be seen in Table 1, for either of the bacteria, we obtained about an 8-log increase in killing (in experiment 1 [compare row 3 with row 2] and experiment 1A [compare row 6 with row 5]). The antibiotic alone (Table 1, rows 2 and 5) produced little increased killing. We consistently got approximately a 1-log increase in the kill with the current alone in the experimental cell. In experiment 2, and with *P. aeruginosa* as the bacterium, different current levels were used, with 1 mA plus antibiotic being ineffective and 5 mA plus antibiotic showing about a 7-log-increased kill. In experiment 3, 0 and 10 mA were used with *K. pneumoniae*, with neither current level showing a significant increase in the killing. There was about a 1-log-order difference between the two counts, but this is within the noise level of the counting of the CFU. It is noted for experiment 3 that the CFU count for zero current (Table 1, row 11) was 3 log orders below that obtained in experiment 1A (Table 1, row 5). Thus, it is possible that the 10-mA current level was effective but that the control (antibiotic with no current) count was in error. Subsequent experiments suggest that this is not the case, but a full-current dose-response curve is one of the experimental objectives in our present work.

## DISCUSSION

In the earlier studies of Costerton et al. (4), approximately a 6-log increase in killing was observed after a 24-h exposure (see Fig. 2 in reference 4). Our experiments consistently show 6- to 8-log increases in killing. This could be experimental variation, or it is possible that since the electrode configuration was different in the two experiments, the killing effectiveness could be dependent on the electric field strength at the surface of the biofilm. This point was partially addressed in a recent paper by Jass et al. (7), since their exposure chamber was designed to orient the electric field perpendicular to the surface of the

TABLE 1. Results from three experiments[a]

| Expt | Bacterium | Antibiotic concn (mg/liter) | Current ($10^3$ A) | No. of CFU[b] | SD[c] |
|---|---|---|---|---|---|
| 1 | *P. aeruginosa* | 0 | 0 | 1.63 E9 | 0.65 |
| 1 | *P. aeruginosa* | 5 | 0 | 3.62 E10 | 6.7 |
| 1 | *P. aeruginosa* | 5 | 1 | 3.0 E2 | 6.7 |
| 1A | *K. pneumoniae* | 0 | 0 | 1.18 E8 | 0.29 |
| 1A | *K. pneumoniae* | 5 | 0 | 1.97 E8 | 0.26 |
| 1A | *K. pneumoniae* | 5 | 1 | 0 | 0 |
| 2 | *P. aeruginosa* | 1 | 0 | 1.11 E8 | 0.74 |
| 2 | *P. aeruginosa* | 1 | 1 | 8.63 E7 | 5.4 |
| 2 | *P. aeruginosa* | 5 | 0 | 2.67 E7 | 1.0 |
| 2 | *P. aeruginosa* | 5 | 1 | 0 | 0 |
| 3 | *K. pneumoniae* | 5 | 0 | 4.2 E5 | 1.6 |
| 3 | *K. pneumoniae* | 5 | 10 | 1.5 E4 | 0.9 |

[a] Results for experiments 1 and 1A are reported separately, but since these were mixed cultures, experiments 1 and 1A were the same experiment with the counts being done on selective agar. Experiments 2 and 3 were monocultures.

[b] Values are averages for 8 to 10 fields counted times the appropriate dilution multiplier (e.g., 1.63 E9 = $1.63 \times 10^9$).

[c] Standard deviation in the average field count.

biofilm (from top to bottom in their words) while the exposure chamber of Costerton et al. (4) had the electric field across the biofilm. In the work reported in the present paper, the electric field was primarily across the biofilm (similar to that of Costerton et al.), but the wire electrodes would allow a small portion of the field to be at an angle with the biofilm. A new exposure chamber that will be used in future experiments that will allow the exposure to be done with a well-known electric field has been designed. It is interesting that in the study of Jass et al. (7) the levels of both current and antibiotic were considerably higher than the levels in this work or that of Costerton et al. (4) but their level of killing increased only about 2 log orders. The exposure time in the study of Jass et al. (7) was 12 h rather than 24, however.

In the present work, the total current through the exposure chambers was used as the key electrical parameter, with the voltage across the chamber being the second parameter monitored. In the experiments in which the current flow was 1 mA, the voltage across the chambers was between 4 and 7 V. These parameters are not reported as current density (current per area) or electric field strength (volts per length) since the electric field configuration in the exposure chambers has not yet been established. Modeling the electric field configuration is part of the work that will be done as this project continues. For those who want to replicate the experiments, however, it is easy to set the current flow through the chamber at the 1- or 5-mA level.

The data indicate that a dose response may exist for the level of antibiotic plus electric field, since enhanced killing was seen at 5 mg of tobramycin per liter and 1 mA of current (Table 1, row 3) but no enhanced killing was obtained at 1 mg/liter and 1 mA of current (Table 1, row 8). A similar statement can be made with respect to the level of current flowing through the exposure box (Table 1, rows 9 and 10). It is interesting that experiment 3 suggests that there may be a level of current above which the bioelectric effect ceases, as was mentioned above. The bioelectric effect is considerably smaller with zero current flowing in the chamber (Fig. 2 in reference 4 shows a difference of about 1 log with no current flowing, and our data agree with this), and so the "current dose-response" curve may turn out to be a "window," that is, the curve may go from no enhanced killing through a maximum and back to no enhanced killing as the current is increased monotonically from zero. Experiments are currently under way to establish the dose-response curves for both the antibiotic and the current flow through the exposure chamber. As mentioned above, electric field modeling in the chamber is also being done in order to develop a better understanding of how the electric field, the biofilm, and the antibiotic are interacting.

Costerton et al. reported that "the current polarity was alternated every 64 seconds to help prevent the accretion of ions on the stainless steel surfaces" (4). We chose not to reverse the direction of the field in our experiments, since our electrodes were much smaller than those in the previous work. The question of whether voltage-generated ion species may be the cause of the increased killing in these experiments may arise. Previous experiments have indicated that this is probably not the case (4), but we are also conducting experiments to support or refute this possibility.

This set of initial experiments supports the data previously reported (4) and has allowed us to establish a protocol for a baseline experiment. We have repeated the work with the *P. aeruginosa* strain, 5 mg of tobramycin per liter, and 1 mA of current a number of times with consistent results each time. These data have allowed us to develop sufficient confidence in the experiment that it has become a "control." If less than a 6-log increase in killing is obtained in the box with these parameters, the entire experiment is considered to be suspect and the data are not used. This, then, has allowed the construction of an "experimental question matrix" wherein a systematic set of experiments have been planned to answer specific questions. This work is currently under way.

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