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A Radio Frequency Electric Current Enhances Antibiotic Efficacy against Bacterial Biofilms

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Bacterial biofilms are notably resistant to antibiotic prophylaxis. The concentration of antibiotic necessary to significantly reduce the number of bacteria in the biofilm matrix can be several hundred times the MIC for the same bacteria in a planktonic phase. It has been observed that the addition of a weak continuous direct electric current to the liquid surrounding the biofilm can dramatically increase the efficacy of the antibiotic. This phenomenon, known as the bioelectric effect, has only been partially elucidated, and it is not certain that the electrical parameters are optimal. We confirm here the bioelectric effect for *Escherichia coli* biofilms treated with gentamicin and with oxytetracycline, and we report a new bioelectric effect with a radio frequency alternating electric current (10 MHz) instead of the usual direct current. None of the proposed explanations (transport of ions within the biofilm, production of additional biocides by electrolysis, etc.) of the direct current bioelectric effect are applicable to the radio frequency bioelectric effect. We suggest that this new phenomenon may be due to a specific action of the radio frequency electromagnetic field upon the polar parts of the molecules forming the biofilm matrix.

Biofilms are constituted by surface-adhering bacteria that form microcolonies characterized by the production of an exopolymer matrix in which they reside. The extracellular polymeric substances that form the matrix are predominantly composed of polymeric sugars, but proteins, nucleic acids, and lipids may also be present. Biofilm bacteria have an increased resistance to traditional antimicrobial agents due to protection afforded by these substances and to the fact that they have modified metabolic states that make them less susceptible to antibiotic action (1, 5, 7). For example, it has been observed (12, 15, 25) that the concentration of antibiotic necessary to kill a mature (7-day-old) biofilm can be several hundred times the concentration necessary to kill the same quantity of bacteria in a planktonic phase. This increased resistance is responsible for certain nosocomial device-related infections due to the formation of microbial biofilms upon medical implants (prostheses, catheters, etc.). There are similar decontamination problems encountered in the food industry and in public water distribution systems, where biofilms formed by strains of *Salmonella*, *Listeria*, *Legionella*, etc., are a frequent health hazard.

The bioelectric effect, first described in references 2 and 12, suggests a possible line of attack. The phenomenon is a synergy between a relatively weak continuous electric current in the liquid surrounding the biofilm and the antimicrobial substance (such as an antibiotic) used to eradicate the biofilm bacteria. A typical example of this synergy would be a log 2 reduction in the number of CFU/ml with an antibiotic alone, a log 1 reduction in the number of CFU/ml with the DC current alone, but a log 6 reduction with the antibiotic and the electric current together. This phenomenon has been reproduced in several biofilm systems: *Pseudomonas aeruginosa* treated with tobramycin (2, 4, 10, 11, 14, 21, 23), *Staphylococcus epidermis* treated with tobramycin (12), *Staphylococcus gordonii* treated with gentamicin (24), *Candida albicans* treated with cycloheximide (12), and *Klebsiella pneumoniae* treated with tobramycin (24). There is neither a clearcut complete explanation of the effect nor the certainty that the electrical parameters are optimal. The proposed explanations (21–23) of the direct current (DC) bioelectric effect are based upon the transport of ions between the electrodes, modification of the pH, or the production of additional biocides by electrolysis.

We first sought to replicate the bioelectric effect before modifying the electrical parameters, so our experimental setup (biofilm growth reactor, treatment cells, and experimental protocol) followed very closely that initiated by the Montana University workers (described in reference 14). Here, we first confirm the DC bioelectric effect with *Escherichia coli* biofilms treated with either gentamicin or with oxytetracycline. After this confirmation, our experimental innovation was to replace the DC by a radio frequency current (RFC) at 10 MHz with the same effective intensity as the DC and keep the rest of the experimental protocol exactly the same. At this frequency there is no transport of ions between the electrodes, no creation of new ions, and no electrolysis, however, we did observe a "radio frequency bioelectric effect."

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MATERIALS AND METHODS

Bacteria. *Escherichia coli* (CIP 54127). The strain was stored in glycerol peptone medium as a frozen culture at −80°C. In each experiment we used freshly thawed aliquots.

Substrate solution. We used M56 (8). Per 0.9 liter of distilled water, the solution contained 4.35 g of Na_2HPO_4, 2.69 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.01 g of $MgSO_4 \cdot 7H_2O$, 0.001 g of $Ca(NO_3)_2 \cdot 4H_2O$, and 0.00184 g of $FeSO_4 \cdot 7H_20$ (pH 7). The solution was then sterilized for 20 min at 120°C. Then, 50 ml of an 18% glucose solution and 50 ml of a 0.132% L-leucine solution, sterilized at 105°C for 30 min, were added.

Antibiotics. Gentamicin and oxytetracycline (Sigma) were used. The MICs for these antibiotics against the planktonic form of our *E. coli* strain were determined both by a spiral colony counting method (Whitley Automatic Spiral Plater; Don Whitney Scientific, Shipley, United Kingdom) and by an impedance technique (R.A.B.I.T.; Don Whitley Scientific). In our experiments we used concentrations five times the corresponding MIC, namely, 5 mg/liter for gentamicin and 50 mg/liter for oxytetracycline.

Biofilm growth reactor and protocol. The reactor was inspired by the system described in reference 14. It holds eight glass biofilm supports (2.5 by 9 cm). The biofilm supports are immersed vertically in the culture medium. Provision was made for magnetic stirring and circulation via peristaltic pumps. Then, 10 ml of *E. coli* overnight culture (10^8 CFU/ml) was added to 500 ml of M56, which was then used to fill the reactor containing the biofilm supports. For the next 24 h, we passed a continuous flow of M56 through the reactor at 1 ml/min. The supports were then removed from the reactor under sterile conditions and dipped in M56 to remove free bacteria from the surface. Four slides were put into each treatment chamber, and the chambers were filled with M56.

Biofilm treatment chamber and protocol. The dimensions of the chamber are 14 by 8 by 5 cm. The stainless steel electrodes (7.5 by 4 cm) are 12 cm apart. The biofilm supports are placed perpendicular to the electrodes and are held in place by Teflon stubs. For the next 24 h, a continuous flow (1 ml/min) of M56 (or M56 plus antibiotic), depending upon the experiment, was assured by a peristaltic pump. At the same time, in one of the chambers, we passed an electric current (either a DC or an RFC, depending upon the experiment). The other treatment chamber served as a control. The DC was provided by a standard constant current generator (KEPCO Power Supply; MB Electronique, Paris, France). We passed a current of 200 mA for a voltage drop of ~20 V, which for the treatment chambers corresponds to a current density of 6 mA/cm^2. This current density is a median value of those used in the published reports of the DC bioelectric effect. The RFC was produced by a generator that was custom-built by two of the authors (J.M.M. and W.J.E.). (The generator has a frequency range of 5 to 20 MHz and an output power range of 0 to 160 W. A circuit diagram is available upon request.) For the RFC experiments, we used a RMS current of 150 mA at frequency of 10 MHz and with a power output of 5 W.

After 24 h of treatment, the biofilm supports were removed from the treatment chambers under sterile conditions and put into individual test tubes containing 40 ml of maximum recovery diluant (Difco). The tubes were ultrasonicated for 5 min (Deltasonic type 011C T; Aerosec Industrie, Fécamp, France), and the numbers of viable resuspended bacteria (in CFU/ml) were counted by using the spiral plater method.

RESULTS

Using the reactor and experimental protocol described above, *E. coli* biofilms were grown on eight glass supports per reactor. We first verified that the density of biofilm bacteria on each of the supports was a reproducible quantity. There were slight differences in the mean CFU/ml counts between runs (with typical values being 1.4×10^7, 7.3×10^6, etc.), but for a given run there were no significant differences between the CFU/ml counts for each of the eight supports (the standard deviations about the mean were always <10% of the mean value). Thus, we were justified in comparing the CFU/ml counts between the four supports used for controls and the four supports that were treated. The experimental run combinations—gentamicin versus control, oxytetracycline versus control, DC versus control, DC plus gentamicin versus control, DC plus oxytetracycline versus control, RFC versus control, RFC plus gentamicin versus control, and RFC plus oxytetracycline versus control—were each repeated five times. The effect of the treatment was measured by using the logarithmic reduction factor (LRF) in the numbers of CFU/ml, i.e., the log[(mean CFU/ml of control supports)/(mean CFU/ml of treated supports)].

TABLE 1. Mean LRFs in DC experiments

Expt	Mean LRF	SD
DC alone	0.91	0.15
Gentamicin alone	2.11	0.05
Oxytetracycline alone	1.90	0.40
DC + gentamicin	4.27	0.30
DC + oxytetracycline	>5.15	0.30

For the DC experiments, the mean LRFs and their standard deviations are shown in Table 1, and the results of the RFC experiments are shown in Table 2. The differences between the treated group and the control group in both sets of experiments are significant ($P < 0.05$ as determined by using the nonparametric Wilcoxon test). The DC results are substantially the same as those reported in other studies of the DC bioelectric effect. In the RFC experiments we also observe a synergy effect between the RFC and the antibiotics. The phenomenon, which is less pronounced than that observed in the DC experiments, is interesting because the proposed explanations of the DC bioelectric effect do not seem to be applicable.

DISCUSSION

The general opinion is that the DC bioelectric effect is due to either modifications in the pH (22) or the production and transport of additional biocide ions into the biofilm by an electrophoretic process (12) or the production of free oxygen (21) by electrolysis. However, a radio frequency electric current: (i) does not transport any of the existing ions in the surrounding liquid (the frequency is such that any charged particle will only vibrate about a mean position); (ii) does not create any new ions in the liquid (at a frequency of 10 MHz and at the low effective intensity [6 mA/cm^2], the resulting electromagnetic fields are nonionizing); (iii) does not produce any electroporation effects (the electric fields necessary to produce electroporation are ca. 1,000 V/cm, which is much higher than the 2 V/cm we used); (iv) does not produce free oxygen or other electrolytic substances; and (v) does not produce a major heating effect (we noted a temperature rise of <1°C in the treatment cell, which was the same as that observed by us and other workers in the DC experiments).

Mathematical models of biofilms are still rather rudimentary, with many simplifying assumptions. One model (20) describes the diffusion of an antibiotic within a biofilm, and a more recent model (3) attempts to describe the influence of an electric field upon a multispecies biofilm. It does not seem easy to use these models either to interpret our experimental data

TABLE 2. Mean LRFs in RFC experiments

Expt	Mean LRF	SD
RFC alone	0.50	0.30
Gentamicin alone	2.11	0.05
Oxytetracycline alone	1.90	0.40
RFC + gentamicin	3.43	0.27
RFC + oxytetracycline	2.80	0.31

or to predict the DC and RFC bioelectric effects. Consequently, discussions of possible mechanisms of the DC and RFC bioelectric effects must remain largely qualitative.

A target for the action of the RFC is the exopolysaccharide (EPS) matrix produced by the bacteria, which contains many types of charged particles and molecular chains with polar subsystems (2, 7, 9, 13). Such a structure is susceptible to the influence of electromagnetic fields. A striking example is reported in reference 22. A biofilm grown upon a wire electrode expanded or contracted when a voltage was applied with an oscillating polarity.

A RFC will vibrate polar molecules, charged particles, and polar parts of large molecular chains. A molecular structure that is subject to an imposed vibration can have its fluidity increased and its structure weakened. This could increase the exchanges between the bacterial cells in the biofilm and the surrounding liquid.

The possibility that the RFC could produce a mechanical effect upon the EPS matrix should be compared to the fact that the use of ultrasound at frequencies between 70 kHz and 10 MHz to vibrate a biofilm gives rise to a synergy phenomenon with antibiotics that is very similar to the bioelectric effect (16–19). The proposed explanation was that the phenomenon was due to an increased "fluidity" of the matrix, which allowed a better penetration of the antibiotic. If the action of the RFC is uniquely via a physical effect upon the EPS matrix, this could explain the fact that the synergy between the RFC and the antibiotic is less than the synergy between the constant current and the antibiotic, because the DC produces additional biocides (6, 21).

We used an RFC with a frequency of 10 MHz. There is no reason to believe that the phenomenon is optimal at this frequency. In order to target likely frequencies it is necessary to carry out a dielectric spectroscopic analysis of the EPS matrix over the range 0 to 10 MHz and search for relaxation frequencies. This work is now in progress in one of our laboratories (Physique des Interactions Ondes-Matière) and will be reported upon later.

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