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Influence of Electric Fields and pH on Biofilm Structure as Related to the Bioelectric Effect

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Mixed species biofilms of *Klebsiella pneumoniae*, *Pseudomonas fluorescens*, and *Pseudomonas aeruginosa* were grown in a flow cell fitted with two platinum wire electrodes. The biofilm growing on the wires reached a thickness of approximately 50 μm after 3 days. When a voltage was applied with oscillating polarity, the biofilm attached to the wire expanded and contracted. The biofilm expanded by approximately 4% when the wire was cathodic but was reduced to 74% of the original thickness when the wire was anodic. The phenomenon was reproduced by alternately flushing the flow cell with media adjusted to pH 3 and pH 10 with no electric current. At pH 10 the biofilm was unaltered, but it became compacted to 69% of the original thickness at pH 3. We explained these phenomena in terms of the molecular interactions between charged acidic groups in the biofilm slime and the bacterial cell walls. Contraction of the biofilm under acidic conditions may be caused by (i) the elimination of electrostatic repulsion from neutralization of negatively charged carboxylate groups through protonation and (ii) subsequent hydrogen bonding between the carboxylic acids and oxygen atoms in the sugars. Electrostatic interactions between negatively charged groups in the biofilm and the charged wire may also be expected to cause biofilm expansion when the wire was cathodic and contraction when the wire was anodic. The consequences of the explanation of the increased susceptibility of biofilm cells to antibiotics in an electric field, the "bioelectric effect," are discussed.

Recently, it has been reported that the efficacies of antibiotics against bacterial biofilms may be increased if the antibiotics are applied in the presence of an electric current (8). It is hoped that the "bioelectric effect" will prove useful in the control of biofilm infections on indwelling medical devices. Jass et al. (9) showed that a tobramycin concentration of 10 μg/ml alone did not reduce the numbers of viable biofilm cells in biofilms grown for 24 and 48 h, but in the presence of an electric current (9 mA/cm^2 for 12 h) there were 0.8 and 1.6 log reductions in the numbers of CFU, respectively. The use of the same electric field with no antibiotic had little effect. Similar results were reported by Wellman et al. (14), who found that tobramycin (1 and 5 μg/ml) alone and a direct-current electric field (1 mA) alone had little effect on viable biofilm cells but that the combined treatments caused an 8-log reduction in the numbers of CFU. Costerton et al. (3) hypothesized that the bioelectric effect may be caused by electrolysis and electromigration effects. To minimize the concentration of charged species, they alternated the polarity of the electrodes every 64 s. The mechanism of the bioelectric effect is still not understood, but it has been postulated that it is a form of electrophoresis in which charged antibiotics are driven into the biofilm by the electric current (3, 9).

Although there have been a number of studies on the bioelectric effect, no one, to our knowledge, has observed a live biofilm at the microscopic scale in the presence of an electric field. We hypothesized that the electric field may cause structural changes to the biofilm which may help provide an explanation of the bioelectric mechanism. It was the goal of this work to observe a live biofilm in real time in the influence of an electrical field and quantify any structural changes.

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MATERIALS AND METHODS

Biofilm flow cell, medium, and inoculum. The flow-cell system consisted of a polycarbonate closed-channel (0.5 cm wide, 1.0 cm deep) flow cell in a recycle loop which also included an aerated mixing chamber and recirculation pumps. The flow cell is shown in detail in Fig. 1. Two platinum wires (diameter, 100 μm) were positioned across the top of the channel, with about 5 mm of wire left exposed on either side of the flow cell. A seal was made between the coverslip and polycarbonate with a butyl rubber gasket and nonconductive high-vacuum grease (Dow Corning).

The wires were separated by a distance of 2 mm and were connected to a voltage supply and ammeter (Hewlett-Packard 4140B multimeter). The voltage was ±1.3 V of direct current just below the point at which gas bubbles were produced at the electrodes. The current was approximately 50 μA, with a corresponding current density of 3.1 mA/cm^2. The polarity was alternated in a square wave function at various frequencies from 0.016 to 20 Hz.

The system was initially filled with a minimal salts growth medium [2.2 mM KH_2PO_4, 4 mM K_2HPO_4, 0.76 mM $(NH_4)_2SO_4$, 4.1×10^{-2} mM $MgSO_4$, 2.2 mM glucose] and was inoculated with 1-ml stock cultures of *Klebsiella pneumoniae* (7×10^{10} CFU/ml), *Pseudomonas fluorescens* (5×10^{10} CFU/ml), and *Pseudomonas aeruginosa* (8×10^9 CFU/ml) (13). The pH of the growth medium was 7.2. The reactor was operated in batch mode for 6 h to allow colonization of the flow cell surfaces and was subsequently switched to continuous culture with a liquid residence time of 26 min and a recycle flow rate of 4.5 ml/s. The biofilm was allowed to grow for 3 days, after which the experiments were performed.

Initially, the biofilm was exposed to an electric field of alternating polarity. Then the biofilm was alternately flushed with media that had been adjusted to pH 3 with 1 M HCl and pH 10 with 1 M NaOH with no electric field. Biofilm growth and subsequent experiments were performed at 20 ± 1°C.

pH indicators. Two pH indicators were used to monitor changes in pH during application of the electric field. Fluorescein (0.1 mM), which is colorless below pH 4 but which fluoresces green above pH 4.5, was added during confocal scanning laser microscopy monitoring by using an excitation wavelength (Ex) of 488 nm (1, 11). Bromthymol blue (0.03 g/liter) in sterile medium (with no biofilm on the electrodes) was used to make macroscopic observations. The indicator was calibrated against a pH meter and was green in the normal medium (pH 7.2), but it turned yellow when the pH was below 5.5 and blue when the pH was above 8.

Microscopy. A biofilm growing on the platinum wire was observed by confocal scanning laser microscopy (Bio-Rad MRC600 attached to an Olympus BH2

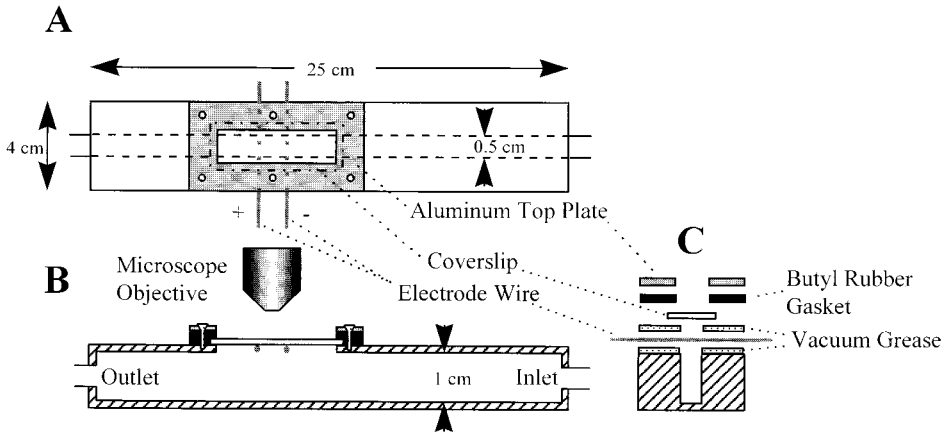

FIG. 1. Schematic of biofilm flow cell showing the plan view (A), a lateral cross section through the center of the flow cell (B), and a transverse section through the viewing port (C). The section through the viewing port is drawn in expanded form to show how the wire electrodes were sealed across the top of the flow cell channel.

microscope) by (i) transmission microscopy by using Ex's of 488, 568, and 647 nm and (ii) fluorescence staining with fluorescent latex spheres (density at 20°C, 1,055 kg/m^3; Ex, 568; diameter, 0.282 μm; Molecular Probes, Eugene, Oreg.) (5, 13). The scan rate of a full screen was 0.945 frames/s, and the scan rate of a single line (line scan) was 484 lines/s. These scan rates were used to estimate the response time of the biofilm structure and pH changes to electrical currents.

Biofilm thickness measurements image analysis. The thickness of the biofilm growing on the wire was measured by image analysis by using the public domain NIH-Image, version 1.59, program (developed at the National Institutes of Health and available from the Internet by anonymous file transfer protocol from zippy.nimh.nih.gov or on a floppy disk from the National Technical Information Service, Springfield, Va. [part number PB95-500195GEI]) on a Macintosh 7200/90 computer. Length measurements were calibrated by using a 1-mm graticule with 10-μm divisions (reference no. CS990; Graticules Ltd., Tonbridge, United Kingdom).

A side view of the biofilm was obtained by focusing on the edge of the wire perpendicular to the observation angle. The biofilm thickness was measured at specific locations along the wire by finding the distance between the edge of the wire and the outside edge of the biofilm. The thicknesses of two biofilm clusters were measured at 20 locations along the wire when the wire was uncharged. These measurements were used to calculate an average thickness and the associated standard error. The thickness of the biofilm was measured again at the same 20 locations when the wire was anodic and again when the wire was cathodic. Similarly, the biofilm thickness was measured at 10 different locations in the pH 3 and pH 10 solutions.

Statistical analysis. The two-way analysis of variance (ANOVA) procedure was performed by using Quattro Pro, version 6.0 (Novell Inc.), to evaluate if there was a significant difference in biofilm thickness when (i) the wire was either uncharged, anodic, or cathodic and (ii) when the biofilm was exposed to pH 3 or pH 10 medium. Two-way ANOVAs were also used to determine if there was a significant difference between the percent change in biofilm thickness caused by electric current and pH.

RESULTS

Influence of electric current. A patchy biofilm grew on the wire electrodes and consisted of clusters of cells separated by interstitial channels; similar biofilms are described in more detail elsewhere (5, 13). The thickest parts of the biofilm were approximately 50 μm (Fig. 2). When the electrical potential was applied the biofilm contracted and expanded at the same frequency at which the polarity was alternated. When the wire was cathodic the thickness of the monitored biofilm cluster increased by 4%, from 44.7 μm (standard error [SE], 3.3 μm; $n = 20$) to 46.4 μm (SE, 3.3 μm; $n = 20$) (Fig. 2A and B). However, the thickness was reduced by 26%, to 33.2 μm (SE, 2.7 μm; $n = 20$), when the wire was cathodic (Fig. 2C). The response time of the biofilm to the polarity changes was on the order of seconds. At frequencies greater than 5 Hz the biofilm could not respond quickly enough to reach full contraction or expansion, and at 20 Hz the biofilm appeared to "fibrillate." There was no biofilm sloughing (removal of large pieces of biofilm), and after 6 h of experimentation the biofilm in the relaxed state was structurally the same as that at the start.

When the biofilm was imaged with fluorescein, the fluorescence intensity alternated synchronously as the electrode polarity was alternated. The area in the immediate vicinity of the electrodes (ca. 200 μm) became more acidic when the electrode was anodic and more basic when the electrode was cathodic. It took 0.031 s (SE, 0.001 s; $n = 3$) for the fluorescence intensity to go from bright to dark (indicating a pH drop below 4.5) and 0.021 s (SE, 0; $n = 3$) to go from dark to bright at a distance of 10 μm from the wire. Bromthymol blue in sterile medium turned yellow near the anode and blue near the cathode, indicating that the pH values in these regions were less than 5.5 and greater than 8, respectively.

Influence of pH with no electric field. When the flow cell was flushed with the alkali solution, the thickness of the monitored biofilm cluster on the wire was unchanged at 30.2 μm (SE, 3.3 μm; $n = 10$), but it contracted by 31% to 20.9 μm (SE, 2.7 μm; $n = 10$) when it was flushed with the acidic solution (Fig. 3). The response was on the order of seconds. The expansions and contractions were reversible upon an exchange of the solutions.

ANOVA. The results from the two-way ANOVA are presented in Table 1. The results indicate that the contraction and expansion of the biofilm when the wire was anodic and cathodic or exposed to pH 3 or pH 10 medium, respectively, were all significant to the 5% level. However, the change in biofilm thickness caused by the electric current was not significantly different from that caused by the pH change.

DISCUSSION

The application of the electric current caused significant changes to the structure of the biofilm attached to the wire but not to the structure of the biofilm elsewhere in the flow cell, indicating that the phenomenon was a localized electrochemical effect. The biofilm on the wire contracted and became more compact when the wire was anodic. The pH in the immediate vicinity of the wire was acidic, as indicated by the fluorescein and the bromthymol blue. The response time for the pH shift was very rapid, on the order of a few hundredths of a second, before steady-state conditions were reached 10 μm from the surface of the wire. Acid may be produced by the anodic oxidation of water: $2H_2O \rightarrow 4H^+ + O_2(g) + 4e^-$ (equation 1). When the wire is cathodic hydroxyl ions would be

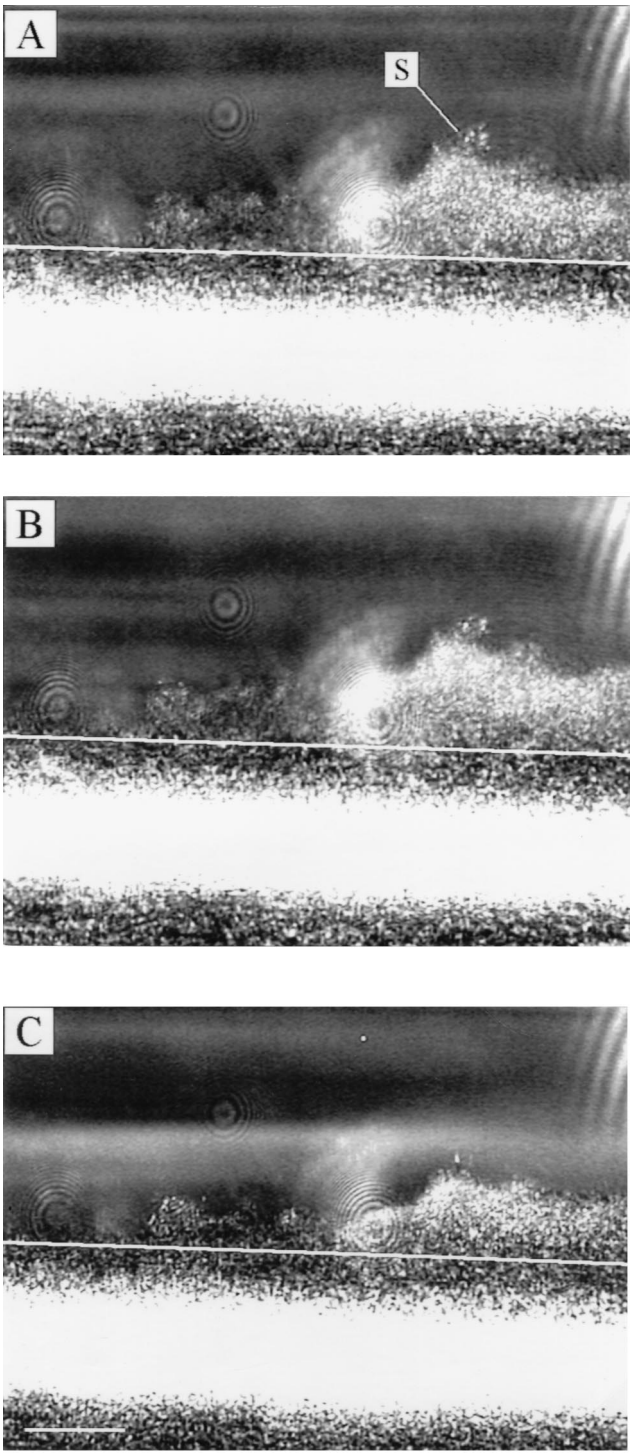

FIG. 2. Side view of the biofilm growing on the platinum wire (the edge of the wire is indicated by the thin white line) which was used to measure biofilm thickness. The surface (S) of the biofilm is indicated. The normal biofilm on an uncharged wire (A) expanded slightly when the wire was cathodic (B) and contracted markedly when the wire was anodic (C). Scale bar (lower left corner of panel C), 50 μm. Bright concentric circles are out-of-focus artifacts.

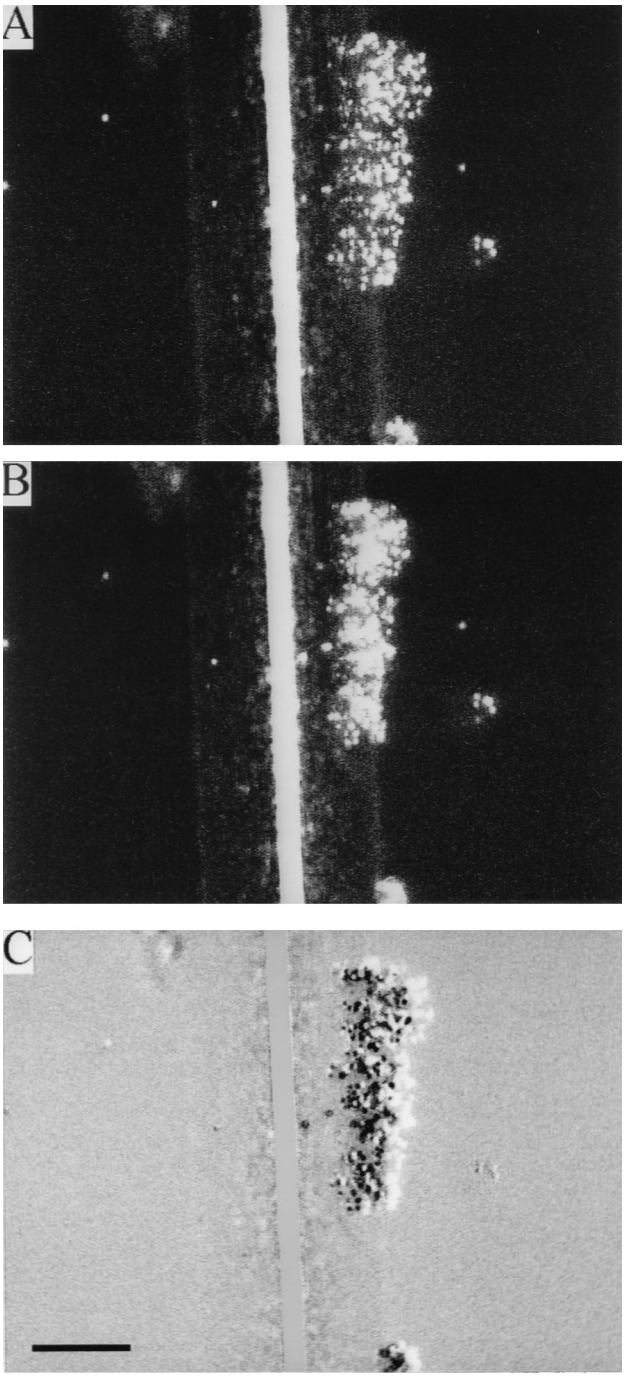

FIG. 3. Biofilm structure was significantly influenced by pH. Fluorescent latex spheres were used to stain the biofilm (bright dots). The biofilm was significantly thicker in the pH 10 medium (A) than in the pH 3 medium (B). The difference in structure was clearly demonstrated by subtracting image B from A by image analysis (on a Macintosh 7200/90 computer with the public domain NIH-Image, version 1.59, software). The biofilm at pH 3 (black dots) was approximately two-thirds of the thickness of the biofilm at pH 10 (white dots) (C). Scale bar, 50 μm.

produced raising the pH: $2H_2O + 2e^- \rightarrow H_2(g) + 2OH^-$ (equation 2). This is only one possible electrochemical activity in a complex chemical environment, and other reactions may be involved in the resulting pH changes. In research on enhanced microbial killing with electric currents, Davis et al. (4) found that the pH of a saline solution increased from 7 to 10.6 due to iontophoresis, in which sodium hydroxide and chlorine were produced. However, this occurred over a 24-h period. We did not have added chlorides in the medium.

TABLE 1. *P* values for four two-way ANOVAs between biofilm thickness and differences in biofilm thickness under various conditions (factors 1 and 2)

Factor 1	Factor 2	*n*	*P*
Wire anodic	Wire uncharged	20	3.6×10^{-10}
Wire cathodic	Wire uncharged	20	0.031
pH 3	pH 10	10	1.4×10^{-6}
Difference between anodic and cathodic wire	Difference between pH 3 and pH 10	10	0.204

The structural changes of the biofilm may be explained in terms of electrostatic influences between charged groups in the biofilm, pH influences, and charges on the wire. The extracellular polymeric substances (EPSs) that form the matrix of the biofilm are predominantly composed of polymeric sugars, but proteins, nucleic acids, and lipids may also be present (10). Uronic acids, such as mannuronic acid and guluronic acid, are commonly found in the EPSs of gram-negative bacteria (7). Charged carboxylate groups in uronic acids can play an important role in the determination of polymer structure (2). It has been shown that divalent cations such as calcium have a gelling effect when added to *P. aeruginosa* EPSs, presumably because of the cross-linkages of the cations with the carboxylate groups (2). In our medium there was a very low concentration of divalent cations (4.1×10^{-2} mM $MgSO_4$), and the structure of the EPSs may be most influenced by electrostatic repulsion of negatively charged carboxylate groups. At low pH such carboxylate groups may become neutralized through protonation, thus eliminating electrostatic repulsion. Protonated carboxylate groups in the EPS may also form hydrogen bonds with oxygen atoms in the sugars. It is to be expected that these processes would cause the biofilm to contract, causing a reduction in thickness.

Another possible influence on the structure is the electrostatic interaction between charged groups in the biofilm and charges on the wire. In addition to negatively charged groups in the EPS, bacterial walls themselves are often negatively charged. In gram-negative bacteria negative charges can be caused by phospholipids in the plasma membrane and charged sugars and phosphates in the outer-membrane lipopolysaccharides. In gram-positive bacteria negative charges can be caused by anionic polymers such as teichoic and teichuronic acids (6). When the wire was cathodic it would also become negatively charged and the biofilm would be expected to be repulsed, causing an expansion and an increase in thickness. The reverse would be true when the wire was cathodic.

ANOVA showed that there was no significant difference between the change in biofilm thickness caused by the electric current and that caused by pH, indicating that structural changes in the biofilm may possibly be caused by pH alone. However, we did not directly measure the pH near the electrodes with biofilm. The influence of electrostatic interactions between the electrodes and the biofilm alone could be further studied by conducting similar experiments at a constant pH, possibly in a heavily buffered solution.

Both structural pulsing and pH shifts may play important roles in the bioelectric effect. Structural pulsing may behave like a pumping mechanism and increase the mass transport of antibiotics into the biofilm. Shifting acidic and basic conditions may increase the effectiveness of antibiotics against biofilm cells, even though controls suggest that the bioelectric effect alone does not influence cell viability. Although recent work by Stewart (12) concluded that transport limitation (into the biofilm) was probably not the governing factor in the reduced susceptibility of biofilms to antibiotics, increased transport convective mixing and shifting pH may combine with other processes such as electrophoresis to cause the bioelectric effect.

The cited studies on the bioelectric effect have used viable cell counts from biofilms growing on nonconductive coupons deliberately positioned away from the electrodes to try and avoid localized electrochemical effects. However, those studies did not measure pH or changes in biofilm structure in the sampling areas, parameters that should be monitored as controls in further studies.

To date most of the research into the bioelectric effect has been directed toward medical applications. It may also be possible to exploit the phenomenon reported here to control biofilms growing in industrial systems under certain limited circumstances. Structural pulsing could be initiated during the application of antimicrobial agents to increase effectiveness. However, such an application would be limited to biofilms growing on or near conductive surfaces in which a counterelectrode is available.

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REFERENCES

1. **Caldwell, D. E., D. R. Korber, and J. R. Lawrence.** 1992. Imaging of bacterial cells by fluorescence exclusion using scanning confocal laser microscopy. J. Microbiol. Methods **15:**249–261.
2. **Christensen, B. E., and W. G. Characklis.** 1980. Physical and chemical properties of biofilms, p. 93–130. *In* W. G. Characklis and K. C. Marshall (ed.), Biofilms. John Wiley & Sons, Inc., New York, N.Y.
3. **Costerton, J. W., B. Ellis, K. Lam, F. Johnson, and A. E. Khoury.** 1994. Mechanism of electrical enhancement of efficacy of antibiotics in killing biofilm bacteria. Antimicrob. Agents Chemother. **38:**2803–2809.
4. **Davis, C. P., M. E. Shirtliff, N. M. Trieffe, S. L. Hoskins, and M. M. Warren.** 1994. Quantification, qualification, and microbial killing efficiencies of antimicrobial chlorine-based substances produced by iontophoresis. Antimicrob. Agents Chemother. **38:**2768–2774.
5. **de Beer, D., P. Stoodley, F. Roe, and Z. Lewandowski.** 1994. Effects of biofilm structures on oxygen distribution and mass transfer. Biotechnol. Bioeng. **43:**1131–1138.
6. **Ellwood, D. C., C. W. Keevil, P. D. Marsh, C. M. Brown, and J. N. Wardell.** 1982. Surface-associated growth. Phil. Trans. R. Soc. London Ser. B **297:** 517–532.
7. **Hughes, K. A.** 1995. Bacterial polysaccharides from industrial environments, p. 107–108. *In* J. W. T. Wimpenny, P. S. Handley, P. Gilbert, and H. M. Lappin-Scott (ed.), The life and death of biofilm. BioLine, Cardiff, United Kingdom.
8. **Jass, J., and H. M. Lappin-Scott.** 1996. The efficacy of antibiotics enhanced by electrical currents against *Pseudomonas aeruginosa* biofilms. J. Antimicrob. Chemother. **38:**987–1000.
9. **Jass, J., J. W. Costerton, and H. M. Lappin-Scott.** 1995. The effect of electrical currents and tobramycin on *Pseudomonas aeruginosa* biofilms. J. Ind. Microbiol. **15:**234–242.
10. **Lazarova, V., and J. Manem.** 1995. Biofilm characterization and activity analysis in water and wastewater treatment. Water Res. **29:**2227–2245.
11. **Robinson, R. A.** 1978. Buffer solutions—operational definitions of pH, p. D136–D139. *In* R. C. Weast (ed.), CRC handbook of chemistry and physics, 58th ed. CRC Press, Inc., West Palm Beach, Fla.
12. **Stewart, P. S.** 1996. Theoretical aspects of antibiotic diffusion into microbial biofilms. Antimicrob. Agents Chemother. **40:**2517–2522.
13. **Stoodley, P., D. deBeer, and Z. Lewandowski.** 1994. Liquid flow in biofilm systems. Appl. Environ. Microbiol. **60:**2711–2716.
14. **Wellman, N., S. M. Fortun, and B. R. McLeod.** 1996. Bacterial biofilms and the bioelectric effect. Antimicrob. Agents Chemother. **40:**2012–2014.