PERGAMON

Archives of Oral Biology 45 (2000) 167–171

Archives of Oral Biology

www.elsevier.com/locate/archoralbio

Short communication

Electrical enhancement of *Streptococcus gordonii* biofilm killing by gentamicin

W. Wattanakaroon, P.S. Stewart*

Center for Biofilm Engineering and Department of Chemical Engineering, Montana State University — Bozeman, Bozeman, Montana 59717-3980, USA

Received 20 May 1999; accepted 29 September 1999

Abstract

This electrical enhancement was demonstrated in an in vitro model. *Streptococcus gordonii* biofilms were grown for 6 days in continuous-flow reactors on one-tenth strength trypticase peptone broth. The biofilms attained a mean areal cell density of 2.4×10^8 c.f.u./cm^2 and a thickness of approx. 19 μm. Biofilms exhibited characteristic resistance to killing by an antibiotic. When treated with 2 μg/ml gentamicin for 24 h, they exhibited a 0.84 log reduction in viable cell numbers; a 4.7 log reduction was measured in a planktonic culture. Killing of planktonic bacteria by this treatment was reduced to 1.2 log when an oxygen-scavenging enzyme was added to the medium. When a 2-mA direct current was applied during antibiotic treatment, biofilm killing increased to a 4.3 log reduction. Electrical current alone caused a 1.9 log reduction in biofilm cell counts. It is suggested that gentamicin was less effective against *Strep. gordonii* under anaerobic conditions than it was under aerobic conditions and that this can explain both the reduced susceptibility of the biofilm (due to oxygen depletion) and electrical enhancement of efficacy (due to oxygen generation by electrolysis). © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Biofilm; Antibiotic; Bioelectric; Gentamicin; Resistance; *Streptococcus*; Oxygen

The enhancement of antibiotic efficacy against microbial biofilms by application of a weak direct electric current, termed the 'bioelectric effect' by its inventors (Blenkinsopp et al., 1992; Khoury et al., 1992), is interesting because it affords a means to overcome the characteristic antimicrobial resistance exhibited by microorganisms in biofilms (Brown and Gilbert, 1993; Costerton et al., 1987; Wilson, 1996). Electrically enhanced biofilm susceptibility has been reproduced in several systems using *Pseudomonas aeruginosa* (Khoury et al., 1992; Costerton et al., 1994; Jass et al., 1995; Jass and Lappin-Scott, 1996; Wellman et al., 1996; Stewart et al., 1999), and has been reported in one instance with *Staphylococcus epidermidis* (Khoury et al., 1992). Now that dental plaque is widely recognized as a classic biofilm phenomenon (Marsh and Bradshaw, 1995; Costerton and Lewandowski, 1997; Darveau et al., 1997), it is of interest to evaluate whether the bioelectric effect could have application in oral health care. Our purpose here was to test whether an electric current enhanced the efficacy of an antibiotic, gentamicin, against a selected oral microorganism, *Streptococcus gordonii*, in an in vitro model.

Strep. gordonii DL1 was used in pure culture throughout. The strain was stored in glycerol peptone

Abbreviations: c.f.u, colony-forming units; TPB, trypticase peptone broth.

* Corresponding author. Tel.: +1-406-994-2890; fax: +1-406-994-2890.

E-mail address: phil_s@erc.montana.edu (P.S. Stewart).

0003-9969/00/$ - see front matter © 2000 Elsevier Science Ltd. All rights reserved.
PII: S0003-9969(99)00132-6

solution as a frozen culture at −70°C. A 500 ml volume of a TPB medium with an oxygen-scavenging enzyme formulation (Oxyrase, Mansfield, OH) in volume ratios of 50:0.25 was added to the growth reactor containing eight polycarbonate coupons (1.7 × 7.2 cm each). The apparatus of the growth reactor has been described elsewhere (McLeod et al., 1999). The TPB medium contained per litre: 20 g trypticase peptone, 5 g yeast extract, 4 g K_2HPO_4, 1 g KH_2PO_4, 2 g NaCl, 5 g dextrose. The reactor was placed in an incubator at 37°C and allowed to stand for at least 30 min 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 h with magnetic stirring. After this batch growth, continuous flow of one-tenth strength TPB medium containing 2 g/l of NaCl was begun at a dilution rate of 1.32/h. To investigate the growth of the biofilm, polycarbonate sample slides were removed at 24-h intervals from the reactor after the start of continuous flow. Biofilm was scraped into a sterile beaker with a stainless-steel scraper. It was resuspended in 10 ml of phosphate buffer and serial dilutions were drop-plated on to brain–heart infusion agar (Difco, Detroit, MI). Colony-forming units were counted after incubating plates in an anaerobic jar at 37°C for 24 h.

A cryoembedding technique was used to preserve the structure of bacterial biofilms for sectioning (Yu et al., 1994). The protocol for embedding and sectioning has been described in detail elsewhere (Wentland et al., 1996). Biofilm frozen sections were fixed and stained with acridine orange (Wentland et al., 1996). Images captured digitially were analysed using ImageTool software. Thickness was measured using this software at multiple points along a section approx. 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-and-white film.

The apparatus and protocol for biofilm treatment have been described in detail elsewhere (McLeod et al., 1999). Biofilms grown for 6 days on polycarbonate slides were transferred aseptically to rectangular treatment chambers, which were in an incubator at 37°C, with a working fluid volume of approx. 30 ml. The treatment chamber was filled with one-tenth strength TPB medium lacking NaCl or medium containing the usual 2 μg/l of NaCl in some experiments. The solution was also amended where indicated with 2 μg/ml of gentamicin (gentamicin sulphate; Sigma Chemical, St. Louis, MO). Once filled, a slow continuous flow, approx. 2.8 ml/h, of this same solution was begun through the chamber. Where indicated, a direct 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} A/cm^2. The potentials required to establish this current in the medium lacking and containing NaCl were approx. 6 to 8 V and 1 to 2 V, respectively. The treatment (either untreated control, antibiotic alone, electric current alone, or antibiotic plus electric current) lasted 24 h. At the end of the treatment period, biofilm sample slides were removed from their individual treatment chambers and immediately processed as described previously. 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.

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 h. 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 a sample slide was scraped and resuspended in 10 ml of phosphate buffer. The suspension was vortexed, serially diluted and plated on to brain–heart infusion agar 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 h, 1 ml was withdrawn and the surviving bacteria were counted as described previously. Intact biofilm experiments were performed in triplicate and resuspended biofilm experiments in duplicate.

Strep. 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 h at 37°C. Both tubes were incubated at 37°C for at least 30 min before inoculation allowing Oxyrase to remove dissolved oxygen in a broth medium. To determine the original number of planktonic cells, 1.5 ml of each culture was added to a 2 ml microcentrifuge tube. The cells were centrifuged at 10,000 rev/min for 7.5 min and resuspended in one-tenth strength NaCl-free TPB medium. A portion (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 c.f.u./ml. Gentamicin solution was then added to give a final

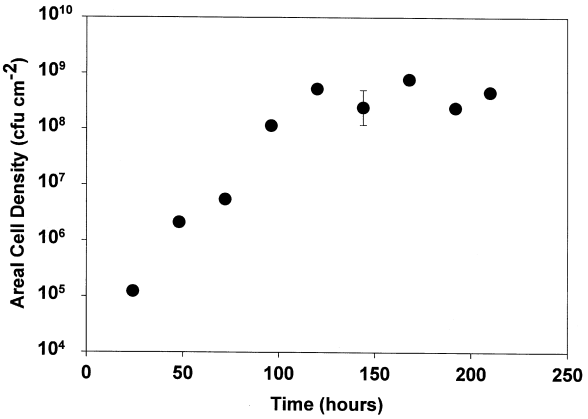

Fig. 1. Accumulation of *Strep. gordonii* biofilms. The representative error bar is the SD of the log-transformed values for 10 replicates.

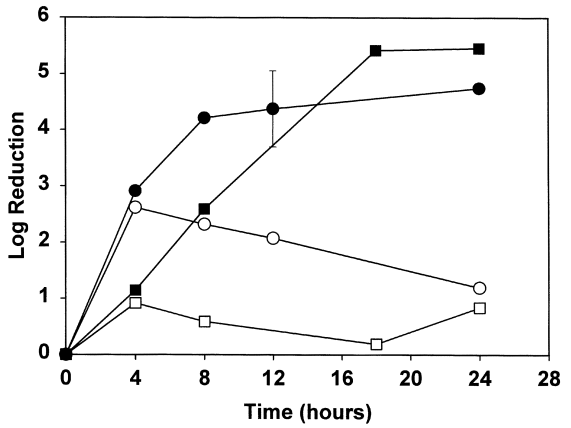

Fig. 3. Comparison of *Strep. gordonii* planktonic (○), intact biofilm (□) and resuspended biofilm (■) sensitivity to gentamicin. Planktonic experiments were performed in the presence (○) or absence (●) of an oxygen-scavenging enzyme formulation. The representative error bar is the SD of the log-transformed values for three replicates.

concentration of 2 μg/ml and incubated at 37°C. A 1.5-ml culture sample was taken at 4, 8, 12 and 24 h, microcentrifuged and resuspended in phosphate buffer to remove the antibiotic. The cells were vortexed for 1 min, and the colony-forming units counted.

A two-sample, two-sided *t*-test was used to compare log reductions in viable counts under different conditions.

Strep. gordonii biofilms accumulated steadily in the growth reactor, reaching a stable level after approx. 5 days (Fig. 1). Six-day-old biofilms, which were used in all antimicrobial efficacy tests, exhibited a mean areal cell density of 2.4×10^8 c.f.u./cm^2. Six-day-old biofilms were locally as thin as 2.8 and as thick as 72 μm (Fig. 2). The mean thickness and SD of 102 measurements was 19 ± 15 μm.

When challenged with 2 μg/ml gentamicin for 24 h, biofilms exhibited a mean log reduction (compared to an untreated control) in viable cell areal density of 0.84 in medium lacking NaCl. Greater antibiotic efficacy was measured for the same treatment in suspended cultures, though the extent of killing depended on whether an oxygen-scavenging enzyme formulation

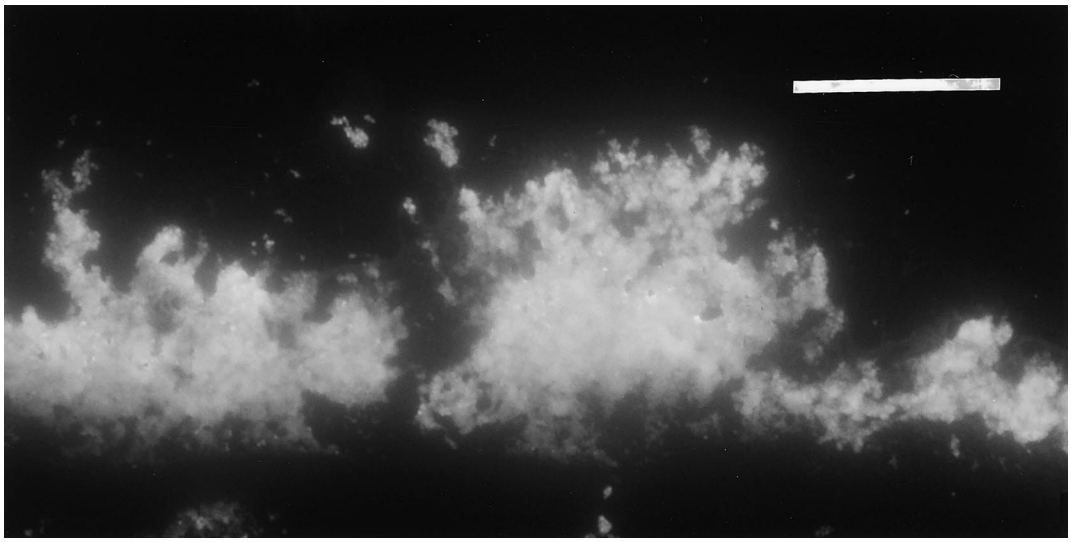

Fig. 2. Microscopic image of a *Strep. gordonii* biofilm cross-section. A frozen section was post-stained with acridine orange. The substratum was at the bottom and the nutrient medium was at the top. This represents one of the thickest spots of the biofilm. Bar = 50 μm.

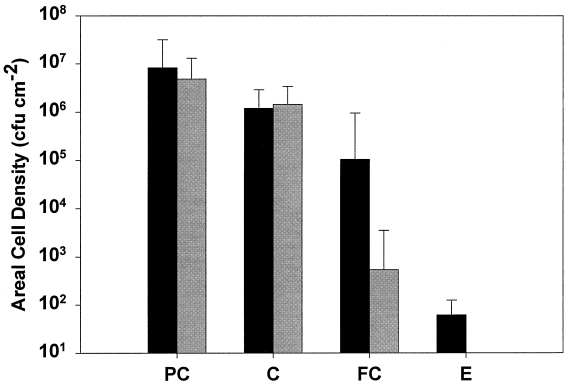

Fig. 4. Effect of electric current and antibiotic on biofilm. Dark shading indicates experiments performed in NaCl-free medium and light shading indicates experiments performed in medium containing NaCl. Error bars indicate SDs. PC, positive control; C, control; FC, field control; E, combined antibiotic and electric current.

(Oxyrase) was included in the medium (Fig. 3). Without Oxyrase the mean log reduction in a planktonic culture after 24-h treatment was 4.74, whereas it was only 1.20 when Oxyrase was included in the medium. The difference in efficacy between the biofilm and planktonic systems was statistically significant for the biofilm–planktonic without Oxyrase comparison ($P = 0.015$) but was not statistically significant for the biofilm–planktonic with Oxyrase comparison ($P = 0.69$). When biofilm was dispersed and resuspended before exposure to gentamicin, its susceptibility was similar to that of planktonic cells (Fig. 3). Resuspended biofilm challenged with gentamicin (without Oxyrase) exhibited a mean log reduction of 5.46, which was not statistically significantly different from the comparable planktonic kill ($P = 0.59$).

A direct electric current enhanced biofilm killing by gentamicin. Biofilm viable cell densities after no treatment (positive control), treatment with antibiotic alone (C, control), electric current alone (field control), and the combination of antibiotic and electric current (E) are summarized in Fig. 4. The untreated positive control exhibited a mean cell density of 8.3×10^6 c.f.u./cm^2. Treatment with antibiotic alone resulted in a mean log reduction of 0.84 ± 0.13 compared to the untreated positive control and this reduction was almost statistically significant ($P = 0.021$). A reduction in viable cell numbers (log reduction of 1.90 ± 0.54) was measured when biofilm was exposed to an electric current alone compared to the untreated positive control, although this reduction was not statistically significant ($P = 0.072$). 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 4.3 ± 0.14 and this reduction was statistically significant ($P = 0.0011$). Because these three t-test results are interpreted simultaneously, the P-value should be less than $0.05/3 = 0.0167$ before one claims statistical significance. This conservative procedure provides assurance that the chance is less than 0.05 for one or more falsely significant results among the three t-tests (Bonferroni inequality method; Neter et al., 1985).

Some biofilm experiments were repeated in medium containing added NaCl (Fig. 4). The antibiotic alone caused only a 0.52 ± 0.10 log reduction in this case. Increased killing by the current alone was measured in this case compared to the experiment in medium lacking NaCl; the mean log reduction was 3.96 ± 0.71. Extensive corrosion of the electrodes was noted when the medium contained NaCl and a current was applied.

Strep. gordonii biofilms were less susceptible to gentamicin than were planktonic cultures of the same organism grown under conditions that did not eliminate all oxygen. When oxygen was removed from a planktonic *Strep. gordonii* culture, the bacteria displayed a reduced susceptibility to gentamicin that was comparable to the biofilm susceptibility. This behaviour is consistent with the general observation that aminoglycoside antibiotics are less effective in anaerobic than aerobic conditions (Verklin and Mandell, 1977; Daniels, 1982; Tack and Sabath, 1985). On the other hand, the only literature data concerning *Streptococcus* spp. and gentamicin in particular found no difference between the minimum inhibitory concentration in aerobic and anaerobic conditions (Tack and Sabath, 1985). It is suggested that the reduced susceptibility of *Strep. gordonii* biofilms was due to localized oxygen depletion within the biofilm (de Beer et al., 1994; Zhang et al., 1995; Xu et al., 1998). The enhancement of biofilm killing by an electrical current would then be explained by the increased levels of oxygen present in the system due to electrolysis of water (Stewart et al., 1999). If this interpretation is correct, then this work lends support to the hypothesis that the nutrient status of bacteria within biofilms is critical to understanding their relative resistance to antibiotic chemotherapy (Gilbert and Brown, 1995). It would be interesting to test this concept further in a more realistic, multiple species, dental plaque model.

Acknowledgements

This work was supported by a grant from the Murdock Charitable Trust. It was made possible by facilities in the Center for Biofilm Engineering which is supported by cooperative agreement EEC-8907039 between the National Science Foundation and

Montana State University and by contributions of the Center's industrial associates. We thank Wendy Cochran for assistance with microscopy and image analysis and Martin Hamilton for advice on statistical analyses.

References

Blenkinsopp, S.A., Khoury, A.E., Costerton, J.W., 1992. Electrical enhancement of biocide efficacy against *Pseudomonas aeruginosa* biofilms. Appl. Environ. Microbiol. 58, 3770–3773.

Brown, M.R.W., Gilbert, P., 1993. Sensitivity of biofilms to antimicrobial agents. J. Appl. Bacteriol. Symp. Suppl. 74, 87S–97S.

Costerton, J.W., Cheng, K.-J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., Marrie, T.J., 1987. Bacterial biofilms in nature and disease. Ann. Rev. Microbiol. 41, 435–464.

Costerton, J.W., Ellis, B., Lam, K., Johnson, F., Khoury, A.E., 1994. Mechanism of electrical enhancement of efficacy of antibiotics in killing biofilm bacteria. Antimicrob. Agents Chemother. 38, 2803–2809.

Costerton, J.W., Lewandowski, Z., 1997. The biofilm lifestyle. Adv. Dent. Res. 11, 192–195.

Daniels, P.J.L., 1982. Aminoglycosides. In: Grayson, M. (Ed.), Antibiotics, Chemotherapeutics, and Antibacterial Agents for Disease Control. John Wiley & Sons, New York, pp. 38–70.

Darveau, R.P., Tanner, A., Page, R.C., 1997. The microbial challenge in periodontitis. Periodontol. 2000 14, 12–32.

de Beer, D., Stoodley, P., Roe, F., Lewandowski, Z., 1994. Effects of biofilm structure on oxygen distribution and mass transport. Biotechnol. Bioeng. 43, 1131–1138.

Gilbert, P., Brown, M.R.W., 1995. Mechanisms of the protection of bacterial biofilms from antimicrobial agents. In: Lappin-Scott, H.M., Costerton, J.W. (Eds.), Microbial Biofilms. Cambridge University Press, Cambridge, UK, pp. 118–130.

Jass, J., Costerton, J.W., Lappin-Scott, H.M., 1995. The effect of electrical currents and tobramycin on *Pseudomonas aeruginosa* biofilms. J. Indust. Microbiol. 15, 234–242.

Jass, J., Lappin-Scott, H.M., 1996. The efficacy of antibiotics enhanced by electrical currents against *Pseudomonas aeruginosa* biofilms. J. Antimicrob. Chemother. 38, 987–1000.

Khoury, A.E., Lam, K., Ellis, B., Costerton, J.W., 1992. Prevention and control of bacterial infections associated with medical devices. ASAIO J. 38, M174–M178.

Marsh, P.D., Bradshaw, D.J., 1995. Dental plaque as a biofilm. J. Indust. Microbiol. 15, 169–175.

McLeod, B.R., Fortun, S.M., Costerton, J.W., Stewart, P.S., 1999 Enhanced bacterial biofilm control using electromagnetic fields in combination with antibiotics, Meth. Enzymol. 310, 656–670.

Neter, J., Wasserman, W., Kutner, M.H., 1985. Applied linear statistical models, 2nd ed. Richard D. Irwiin, Inc, Homewood, IL, p. 582.

Stewart, P.S., Wattanakaroon, W., Goodrum, L., Fortun, S.M., McLeod, B.R., 1999. Electrolytic generation of oxygen partially explains electrical enhancement of tobramycin efficacy against *Pseudomonas aeruginosa* biofilm. Antimicrob. Agents Chemother. 43, 292–296.

Tack, K.J., Sabath, L.D., 1985. Increased minimum inhibitory concentration with anaerobiasis for tobramycin, gentamicin, and amikacin, compared to latamoxef, piperacillin, chloramphenicol, and clindamycin. Chemother. 31, 204–210.

Verklin, R.M., Mandell, G.L., 1977. Alteration of effectiveness of antibiotics by anaerobiosis. J. Lab. Clin. Med. 89, 65–71.

Wellman, N., Fortun, S.M., McLeod, B.R., 1996. Bacterial biofilms and the bioelectric effect. Antimicrob. Agents Chemother. 40, 2012–2014.

Wentland, E., Stewart, P.S., Huang, C.-T., McFeters, G.A., 1996. Spatial variations in growth rate within *Klebsiella pneumoniae* colonies and biofilm. Biotechnol. Prog. 12, 316–321.

Wilson, M., 1996. Susceptibility of oral bacterial biofilms to antimicrobial agents. J. Med. Microbiol. 44, 79–87.

Xu, K.D., Stewart, P.S., Xia, F., Huang, C.-T., McFeters, G.A., 1998. Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. Appl. Environ. Microbiol. 64, 4035–4039.

Yu, F.P., Callis, G.M., Stewart, P.S., Griebe, T., McFeters, G.A., 1994. Cryosectioning of biofilms for microscopic examination. Biofouling 8, 85–91.

Zhang, T.C., Fu, Y.-C., Bishop, P.L., 1995. Competition for substrate and space in biofilms. Wat. Environ. Res. 67, 992–1003.