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The antibacterial effect of photodynamic therapy in dental plaque-derived biofilms

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Background and Objective: Photodynamic therapy has been advocated as an alternative to antimicrobial agents to suppress subgingival species and to treat periodontitis. Bacteria located within dense biofilms, such as those encountered in dental plaque, have been found to be relatively resistant to antimicrobial therapy. In the present study, we investigated the ability of photodynamic therapy to reduce the number of bacteria in biofilms by comparing the photodynamic effects of methylene blue on human dental plaque microorganisms in the planktonic phase and in biofilms.

Material and Methods: Dental plaque samples were obtained from 10 subjects with chronic periodontitis. Suspensions of plaque microorganisms from five subjects were sensitized with methylene blue (25 μg/mL) for 5 min then exposed to red light. Multispecies microbial biofilms developed from the same plaque samples were also exposed to methylene blue (25 μg/mL) and the same light conditions as their planktonic counterparts. In a second set of experiments, biofilms were developed with plaque bacteria from five subjects, sensitized with 25 or 50 μg/mL of methylene blue and then exposed to red light. After photodynamic therapy, survival fractions were calculated by counting the number of colony-forming units.

Results: Photodynamic therapy killed approximately 63% of bacteria present in suspension. By contrast, in biofilms, photodynamic therapy had much less of an effect on the viability of bacteria (32% maximal killing).

Conclusion: Oral bacteria in biofilms are affected less by photodynamic therapy than bacteria in the planktonic phase. The antibacterial effect of photodynamic therapy is reduced in biofilm bacteria but not to the same degree as has been reported for treatment with antibiotics under similar conditions.

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Bacteria growing in biofilms adhere to a solid surface where they multiply and form microcolonies embedded in an extracellular polymeric matrix, which includes water and nutrient channels (1). Biofilms that colonize tooth surfaces and epithelial cells lining the periodontal pocket/gingival sulcus (subgingival dental plaques) are among the most varied and complex biofilms that exist in nature. These biofilms may include a subset of selected species

from more than 700 bacterial species or phylotypes (2–4) and can lead to periodontal diseases. Mechanical removal of the periodontal biofilms is currently the most frequently used method of periodontal disease treatment. Antimicrobial agents are also used, but biofilm species exhibit several antibiotic-resistance mechanisms (5–7). In addition, disruption of the oral microflora and the difficulty of maintaining therapeutic concentrations of antimicrobials in the oral cavity are also problems associated with the use of these agents (8).

Photodynamic therapy has been suggested as an alternative to chemical antimicrobial agents to eliminate subgingival species and to treat periodontitis (9). Photodynamic therapy is based on the concept that nontoxic photosensitizers can be preferentially localized in certain tissues and subsequently activated by light of the appropriate wavelength to generate singlet oxygen and free radicals that are cytotoxic to cells of the target tissue (10). Several studies have shown that oral bacteria in planktonic cultures (9,11,12) and in plaque scrapings (9,13,14) are susceptible to photodynamic therapy. Moreover, recent studies have reported that photodynamic therapy-induced bacterial cell killing reduced bacterial numbers by more than 10-fold in *Streptococcus mutans*, *Streptococcus sobrinus* and *Streptococcus sanguinis* (15–18) biofilms when toluidine blue O or erythrosine was used as the photosensitizer. Data produced in our laboratory, however, have shown that eradication of oral bacteria is incomplete in *Actinomyces naeslundii* biofilms (19,20) and in multispecies biofilms produced from human saliva as inoculum (21) following biofilm sensitization with methylene blue and exposure to red light.

In this study, we investigated the effects of photodynamic therapy on bacteria derived from human natural dental plaque under planktonic or biofilm conditions *in vitro*. The goal of our research was to compare the susceptibility of dental plaque bacteria in suspension or in biofilms to photodynamic therapy after sensitization with certain concentrations of methylene blue and exposure to red light at 665 nm.

Material and methods

Subjects and plaque samples

Samples of dental plaque were taken from 10 subjects. Permission to collect dental plaque samples was authorized by Institutional Review Board-approved informant consent. All the subjects were diagnosed as having chronic periodontitis with probing depths greater than 5 mm. None of the subjects had used antibiotics or had undergone periodontal treatment during the 3 mo prior to sampling. Dental plaque samples were taken from supragingival and subgingival mesiobuccal aspects of premolars or molars in each subject (four to eight samples per subject) using individual sterile Gracey curettes. After removal, all plaque samples from each subject were placed immediately into one vial containing 4.5 mL of prereduced, anaerobically sterilized Ringer's solution (Anaerobe Systems, Morgan Hill, CA, USA). The microorganisms from the plaque samples were dispersed in the prereduced, anaerobically sterilized Ringer's solution by sonication and repeated passage through Pasteur pipettes. Aliquots of the dispersed bacteria were transferred to 1-mL cuvettes and the optical density of the bacterial suspensions was measured in a spectrophotometer (one optical density unit was considered as approximately 10^9 cells/mL at 600 nm). Then, each sample from five subjects (subjects 1–5) was divided into two parts. The first part, in suspension, was exposed to photodynamic therapy. The second part was used for the development of biofilms, which were also exposed to photodynamic therapy 1 wk later. In the first group, we compared the photodynamic effects of the same methylene blue concentration (25 μg/mL) on both planktonic and biofilm bacteria. The samples from the other five subjects (subjects 6–10) were used only for the development of biofilms that were also exposed to photodynamic therapy. In the second group, we compared the photodynamic effects of two different methylene blue concentrations (25 and 50 μg/mL) on biofilm species.

Preparation of blood agar culture plates

An enriched agar medium was prepared containing 20 g/L of trypticase soy agar (BBL, Cockeysville, MD, USA), 26 g/L of brain–heart infusion agar (Difco Laboratories, Detroit, MI, USA), 10 g/L of yeast extract (BBL) and 5 mg/L of hemin (Sigma Chemical Co., St Louis, MO, USA). The medium was autoclaved and cooled to 50°C. Then, 5% defibrinated sheep blood (Northeast Laboratory Services, Waterville, ME, USA), 5 mg/mL of menadione (Sigma Chemical Co.) and 10 mg/mL of *N*-acetylmuramic acid (Sigma Chemical Co.) were added under aseptic conditions. Aliquots of 150 μL of the agar mixture were dispensed into wells of 96-well microtiter plates at a volume of 150 μL per well (NUNC, Rochester, NY, USA) and allowed to dry.

Development of plaque-derived biofilms

The dental plaque samples collected from each subject were placed into one vial containing prereduced, anaerobically sterilized Ringer's solution. Under anaerobic conditions, the entire sample was dispersed and added to brain–heart infusion broth (Beckton, Dickinson & Company, Sparks, MD, USA). For biofilm development, the plaque/brain–heart infusion broth inoculum contained approximately 10^7 cells/mL. One-hundred and fifty microlitres of this inoculum (approximately 1.5×10^6 bacteria) was carefully pipetted to fill four blood agar wells in each 96-well plate. The plates were then incubated anaerobically (80% N_2, 10% H_2 and 10% CO_2) at 35°C for 7 d. After an initial incubation period of 48 h, the liquid medium was carefully aspirated from each well and the biofilms were replenished with fresh brain–heart infusion broth. Then, fresh brain–heart infusion broth was added daily into each well, very slowly, to avoid disruption of the biofilm.

Biofilm characterization

Counts of biofilm microorganisms — On day 7 of their development, biofilms were gently scraped from blood agar in each well using a sterile bacteriological loop to remove the entire visible biomass. Then, spectroscopy was performed to determine the total bacterial load.

Confocal scanning laser microscopy — A Leica SP2 confocal scanning fluorescence microscope (Leica Inc., Malvern, PA, USA), with a 40× or a 100× water-dipping objective lens, was used to observe the distribution of dead/live microorganisms in biofilms. Biofilms were grown on agar in 24-well plates (to accommodate the confocal microscope objective) as described above. For optimum biofilm development, the plaque : brain–heart infusion inoculum contained 10^9 cells/mL. Live and dead biofilm bacteria were simultaneously viewed using the reagents SYTO 9 stain and propidium iodide in the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer's instructions. Biofilms were stained in the dark at room temperature (20–24°C) for 15 min. An argon laser (476 nm) was used as the excitation source for the reagents, and the fluorescence light emitted was collected by two separate emission filters at 500 nm (SYTO 9) and 635 nm (propidium iodide). Sections were collected at 20-μm intervals and analyzed using image-processing techniques to assess the distribution of dead/live bacteria within the biofilm matrices.

Microbial analysis — The microbial composition of biofilms was assayed using whole genomic probes to 40 oral microorganisms, as described below.

Composition of pooled dental plaque and biofilms

Part of each dental plaque sample (4×10^8 bacteria) obtained from subjects 6–10 was split into four tubes containing brain–heart infusion broth (10^8 bacteria/ mL). The bacterial solutions were serially diluted and 100-μL aliquots were spread over the surfaces of blood agar plates, which were then incubated anaerobically for 7 d. Then, the microbial composition was assayed using a whole genomic probe assay, as described previously (22). In biofilms that were developed from the same subjects and were not exposed to light and/or methylene blue (L^- MB^-), the composition was assayed 7 d after photodynamic therapy. Briefly, Tris–EDTA buffer (1.5 mL) was added to the plates and the bacterial colonies were scraped off the surface using sterile L-shaped glass rods. The suspensions were placed into individual Eppendorf tubes and sonicated for 10 s to break up clumps. The optical density of each suspension was adjusted to a final optical density of 1.0, which corresponded to approximately 10^9 cells. Ten microlitres of the suspension (10^7 cells) were removed and placed in another Eppendorf tube containing 140 μL of Tris–EDTA buffer and 150 μL of 0.5 M NaOH. The samples were lysed and the DNA was placed in lanes on positively charged nylon membrane using a Minislot device (Immunetics, Cambridge, MA, USA). After fixation of the DNA to the membrane, the membrane was placed in the Miniblotter 45 (Immunetics) with the lanes of DNA perpendicular to the lanes of the device. Digoxigenin-labeled whole genomic DNA probes to 40 bacterial species were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA probes were detected using antibody to digoxigenin, conjugated with alkaline phosphatase for chemifluorescence detection. Signals were detected using AttoPhos substrate (Amersham Life Science, Arlington Heights, IL, USA) and were scanned using a Storm Fluorimager (Molecular Dynamics, Sunnyvale, CA, USA). Computer-generated images were analyzed to determine the fluorescence intensity associated with each sample and probe. Two lanes in each membrane contained DNA standards with 1 ng (10^5 bacteria) and 10 ng (10^6 bacteria) of each species, respectively. The sensitivity of the assay was adjusted to permit detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. The measured fluorescence intensities were converted to absolute counts by comparison with the standards on the same membrane. Failure to detect a signal was recorded as zero.

Photosensitizer

Methylene blue (Sigma) was dissolved in brain–heart infusion broth to give solutions at concentrations of 25 and 50 μg/mL before use. The ultraviolet-visible absorption spectra of methylene blue in brain–heart infusion broth were recorded from 300 to 700 nm using quartz cuvettes with a 1-cm path length on a diode-array spectrophotometer (model 335907P-000; ThermoSpectronic, Rochester, NY, USA), and were characterized by a long-wavelength maximum at 665 nm.

Light source

A diode laser (BWTEK Inc., Newark, DE, USA), with an output power of 1 W and a central wavelength of 665 nm, was used. The system was coupled to a 1 mm optical fiber that delivered light into a lens, which formed a uniform circular spot, 2 cm in diameter, on the base of the 24- or 96-well plate. This spot of light was able to irradiate, each time, either one well in a 24-well plate or a group of four wells in a 96-well plate. The power density of incident radiation was measured using a power meter (Ophir Optronics Ltd, Danvers, MA, USA). The distance between the lens and the illuminated plates was adjusted to create a spot of light, 2 cm in diameter, with a fixed power density of 100 mW/cm^2.

Photodynamic treatment

The light parameters used in this study for bacterial suspensions and biofilms were 100 mW/cm^2 (power density) and 30 J/cm^2 (energy fluence). The methylene blue concentration of 25 μg/mL

was applied on both suspensions and biofilms that were derived from samples obtained from subjects 1–5 (Table 1). Methylene blue concentrations of 25 and 50 μg/mL were applied on biofilms developed using plaque samples from subjects 6–10 (Table 2). The following groups were used: (i) $L^- MB^-$ (no light, no methylene blue), (ii) $L- MB^+$ (treated only with methylene blue), (iii) $L^+ MB^-$ (treated only with light), and (iv) $L^+ MB^+$ (treated with methylene blue and light; photodynamic therapy group). Groups 1 and 2 were kept in plates at room temperature and covered with aluminum foil during irradiation.

Plaque samples — Suspensions of bacteria (10^8/mL) were incubated with methylene blue (25 μg/mL) for 5 min in the dark at room temperature in tetraplicate. Following incubation, bacterial suspensions were placed in the wells of 24-well plates and exposed to light of 665 nm from above for 5 min in the dark at room temperature. Two neighboring wells with bacterial suspensions were separated by at least two empty wells to avoid any overlapping exposure of wells. During photodynamic therapy, 24-well plates remained covered with a lid, and special care was taken not to disturb the plates. After illumination of the appropriate wells, bacterial suspensions underwent serial dilutions in brain–heart infusion broth and 100-μL aliquots were plated on blood agar plates and then incubated under anaerobic conditions for 7 d.

Biofilms — Four wells of 96-well plates containing the biofilms were exposed to methylene blue (25 or 50 μg/mL) for 5 min. These wells were then simultaneously irradiated with red light. There was only one group of four wells with biofilms in each 96-well plate, thereby avoiding any exposure of adjacent wells. During photodynamic therapy, 96-well plates remained covered with a lid and were not disturbed. After illumination, adherent bacteria were gently scraped from blood agar in each well using a sterile bacteriological loop to remove the biofilm and dispersed in brain–heart infusion broth. The same experienced researcher removed all of the biofilms to ensure that the scrapings collected the entire biofilm and did not add variability to the results. Aliquots were measured in a spectrophotometer in 1 mL cuvettes. Then, serial dilutions were prepared and 100-μL aliquots were spread over the surface of blood agar plates. The plates were incubated anaerobically at 35°C for 7 d.

Data analysis

The multiple comparisons of 40 individual species in suspensions and biofilms were evaluated against a Bonferroni-adjusted *p*-value (with overall alpha = 0.10). Survival fractions in each group ($L^+ MB^+$, $L^- MB^+$, $L^+ MB^-$) were calculated by dividing the mean number of colony-forming units with the number of colony-forming units from dark controls ($L^- MB^-$), planktonic suspensions or biofilm as appropriate, from the same subject. Survival fractions in Tables 1 and 2 were evaluated using repeated-measures analysis of variance to compare treatment groups while controlling variation across subjects. Pairwise comparisons were performed using least significant difference tests.

Results

Characterization of biofilms

The average number of microorganisms obtained from 50 independent biofilms was approximately 10^9. Confocal images (*X*–*Y*) that were obtained from dental plaque-derived biofilms on day 7 of growth showed a mixture of dead and live microorganisms extending to a depth of 180–200 μm (Fig. 1). No fluorescent signal was observed below 200 μm.

Microbial analysis

DNA probe analysis of plaque samples and biofilms demonstrated that the composition of each was similar. Although there were, in general, slightly more bacteria in the suspensions (5.9×10^6/sample) than in the biofilms (4.6×10^6/sample), these differences were not statistically significant. No significant differences in species levels were found in statistical comparisons between suspensions and biofilms after applying Bonferroni criteria (with overall alpha = 0.10) to adjust for multiple comparisons. Although counts were consistently somewhat

Table 1. Phototoxicity mediated by methylene blue in planktonic dental plaque bacteria and in plaque-derived biofilms[a]

	Planktonic bacteria			Biofilm bacteria		
Subject no.	$L^+ MB^-$	$L^- MB^+$	$L^+ MB^+$	$L^+ MB^-$	$L^- MB^+$	$L^+ MB^+$
1	91	90.6	40	112.8	135.5	85.1
2	112	49.8	28.9	111.6	94.5	38.3
3	52.8	103.8	23.7	81.5	79.6	79.2
4	75.2	59.2	34.7	91.9	75.3	75.3
5	88.5	61.9	58.6	127.6	66.9	66.9
Mean survival fraction	83.9	73.1	37.2	105.1	101.9	69
SEM	9.8	10.3	6	8.2	9.8	8.2

[a]The percentage survival of bacteria was assayed using the colony-forming assay following 5 min of treatment with 25 μg/mL of methylene blue and exposure to light (30 J/cm^2) at 665 nm. Surviving bacteria were expressed as a percentage of dark controls ($L^- MB^-$). Each value represents the mean survival fraction from three or four independent experiments.
$L^- MB^-$, no light, no methylene blue; $L- MB^+$, treated only with methylene blue; $L^+ MB^-$, treated only with light; and $L^+ MB^+$, treated with both methylene blue and light, photodynamic therapy group.

Table 2. Phototoxicity mediated by methylene blue in plaque-derived biofilms[a]

Subject no.	L^+ MB^-	L^- MB^+ (25 μg/mL)	L^- MB^+ (50 μg/mL)	L^+ MB^+ (25 μg/mL)	L^+ MB^+ (50 μg/mL)
6	98.6	96.2	76.5	96.3	45.5
7	91.2	98.3	101.8	92.1	83.6
8	95	83.4	69.7	84.7	64.4
9	115.4	95.9	95.5	104.8	88.1
10	106	106	97.5	80.5	58.3
Mean survival fraction	103.2	95.9	88.2	91.6	67.9
SEM	5.2	3.6	6.3	4.2	7.9

[a]The percentage survival of bacteria was assayed using the colony-forming assay after incubation with 25 or 50 μg/mL of methylene blue for 5 min followed by exposure to light (30 J/cm^2) at 665 nm. Surviving bacteria were expressed as a percentage of dark controls (L^- MB^-). Each value represents the mean survival fraction from three or four independent experiments.
L^- MB^-, no light, no methylene blue; L– MB^+, treated only with methylene blue; L^+ MB^-, treated only with light; and L^+ MB^+, treated with both methylene blue and light, photodynamic therapy group.

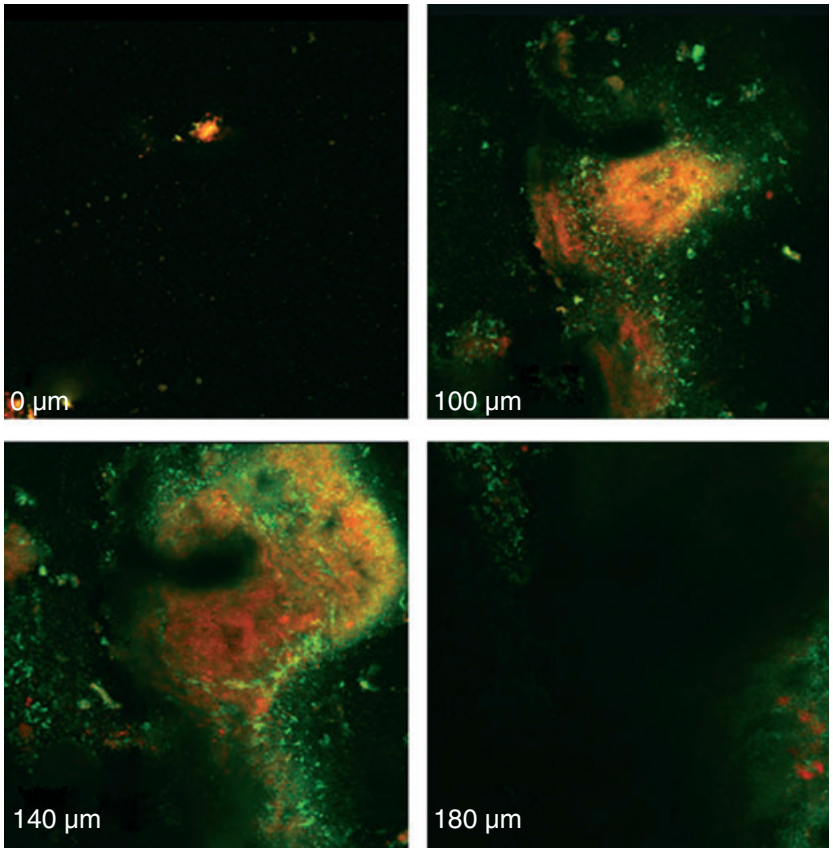

Fig. 1. Confocal images (horizontal *X–Y* sections) obtained of dental plaque-derived biofilms grown on agar in 24-well plates. Live bacteria with intact membranes were stained fluorescent green using the SYTO 9 stain, while dead bacteria with damaged membranes were stained fluorescent orange using propidium iodide. The fluorescent signals were obtained to a depth of 180 μm.

lower in biofilms, species profiles were generally similar for biofilms and suspensions.

Photodynamic treatment of planktonic bacteria vs. biofilms

The effects of light with/without methylene blue (25 μg/mL) were evaluated on dental plaque bacteria, isolated from five subjects, in planktonic vs. biofilm phases (Table 1). Pairwise comparisons using least significant difference tests indicated significant differences ($p < 0.05$) between L^+ MB^+ relative to methylene blue alone (L^- MB^+) and to light alone (L^+ MB^-) in both planktonic and biofilm states. The synergism of light and methylene blue did not fully kill plaque microorganisms. The survival fractions for the photodynamic therapy groups were approximately 37% and 69% in planktonic and biofilm cultures, respectively, compared with dark controls (L^- MB^-). Samples for all five subjects had higher survival

fractions for L^+ MB^+ in the biofilms relative to the corresponding planktonic values.

Photodynamic treatment of biofilm bacteria

The effects of light with/without methylene blue (either 25 or 50 μg/mL) were evaluated on biofilms from subgingival plaque samples of five additional subjects (Table 2). Least significant difference tests indicated that the light + methylene blue (50 μg/mL) treatment group (L^+ MB^+) contained a significantly lower number of bacteria ($p < 0.05$) than any other group. Differences among mean survival fractions for the other treatment groups were quite modest.

Discussion

Several studies have reported that oral microorganisms in planktonic cultures (11–13), plaque scrapings (14) and biofilms (17,18,23) are susceptible to photodynamic therapy. Recently, it was reported that photodynamic therapy induced bacterial cell killing to a level of > 1 $\log_{10}$ in oral monospecies biofilms using erythrosine (15,16), which is currently used clinically as a dental plaque-disclosing agent. However, other studies have demonstrated incomplete destruction of oral pathogens in plaque scrapings (20,24), monospecies biofilms (19,20) and multispecies biofilms derived from human saliva (21). In the present study, we investigated the photodynamic effects of methylene blue on human dental plaque microorganisms in the planktonic phase vs. the biofilm phase. Methylene blue, whose intravenous administration is approved by the Food and Drug Administration for the treatment of methemoglobinemia, has been tested as a promising candidate for photodynamic therapy of cancer (25) and has also been used in photodynamic therapy for targeting various gram-positive and gram-negative oral bacteria (26). The hydrophilicity of methylene blue (27), its low molecular weight and its positive charge allow passage across the porin-protein channels in the outer membrane of gram-negative bacteria. Methylene blue interacts predominantly with the anionic macromolecule lipopolysaccharide, resulting in the generation of methylene blue dimers (28), which participate in the photosensitization process (28,29).

In our study, photodynamic therapy killed approximately 63% of bacteria in the planktonic phase (Table 1), whereas in biofilms derived from the same plaque samples the effect of light resulted in much lower reductions of microorganisms (31% were killed) (Table 1). Although photodynamic therapy was less effective in the treatment of bacteria within dense biofilms formed by dental bacteria than in planktonic culture, the difference was only twofold, whereas antibiotics have been reported to be approximately 250-fold less effective under these conditions (30). In comparing biofilm with planktonic effects, a degree of reduced efficacy would be expected of any penetrant molecular species. Incomplete bacterial killing by photodynamic therapy is not limited to methylene blue. In a previous study (20), a conjugate between the photosensitizer $chlorin_{e6}$ and poly-L-lysine failed to eradicate microorganisms completely in dental plaque scrapings. Recently, incomplete elimination of microorganisms in subgingival scrapings was reported after their sensitization with toluidine blue, a phenothiazinium-based photosensitizer such as methylene blue, and their subsequent exposure to red light at 635 nm (24). There are several explanations for the lowered photodynamic therapy effect in dental plaque microorganisms. First, the reduced susceptibility to photodynamic therapy may be related to the distinct and protected phenotypes expressed by dental plaque microorganisms once they attach to the tooth (31). These phenotypic changes, which are critical for the development of dental biofilm resistance (32), are still carried by dental plaque bacteria in suspension. Second, the photodynamic effects of methylene blue on dental plaque bacteria were probably affected by the presence of serum proteins in brain–heart infusion broth (20,33,34). In the present study, methylene blue was dissolved in brain–heart infusion broth because proteins from both saliva and gingival crevicular fluid would also reduce the effect of methylene blue in the hypothetical case of its *in vivo* application (34). Third, it has been shown that phenothiazinium-based photosensitizers, including methylene blue, toluidine blue O and 1,9-dimethylmethylene blue, are substrates of multidrug resistance pumps in bacteria (35).

The microcosm biofilm model that was employed in this study originates directly from the whole-mixed natural dental plaque, is technically simple to prepare and maintain, and, possibly, reflects the complexity of dental plaque. Microbial analysis (Fig. 2) showed the establishment of a mixed microflora, whereas confocal scanning laser microscopy (Fig. 1) showed a biofilm structure which resembled that of natural dental plaque. The growth of microorganisms from pooled human dental plaque on blood agar has been demonstrated by other investigators (36,37). Plaque microcosms are functional models used to study drug delivery and targeting (38). The characterization of the biofilm model used in the present study has been reported previously (39), whereas its validity has been demonstrated using novel drug-delivery and therapeutic procedures (21).

Biofilm bacteria showed resistance to photodynamic therapy, with killing not exceeding 32% compared with dark controls (Table 2). Although differences in the photodynamic sensitivity of biofilms at 25 μg/mL of methylene blue, as illustrated in Tables 1 and 2, appear substantial (91.6% vs. 69% reduction in colony-forming unit numbers, $p = 0.05$ by *t*-test), these differences would not be considered significant if corrected for multiple testing. Biofilms were developed using dental plaque obtained from different donors, and therefore biofilm variability may reflect differences in responses to photodynamic therapy. Recently, Müller *et al.* (40) reported less than 1 $\log_{10}$ destruction of bacteria in six-species oral biofilms developed on bovine-enamel discs after their sensitization with methylene blue

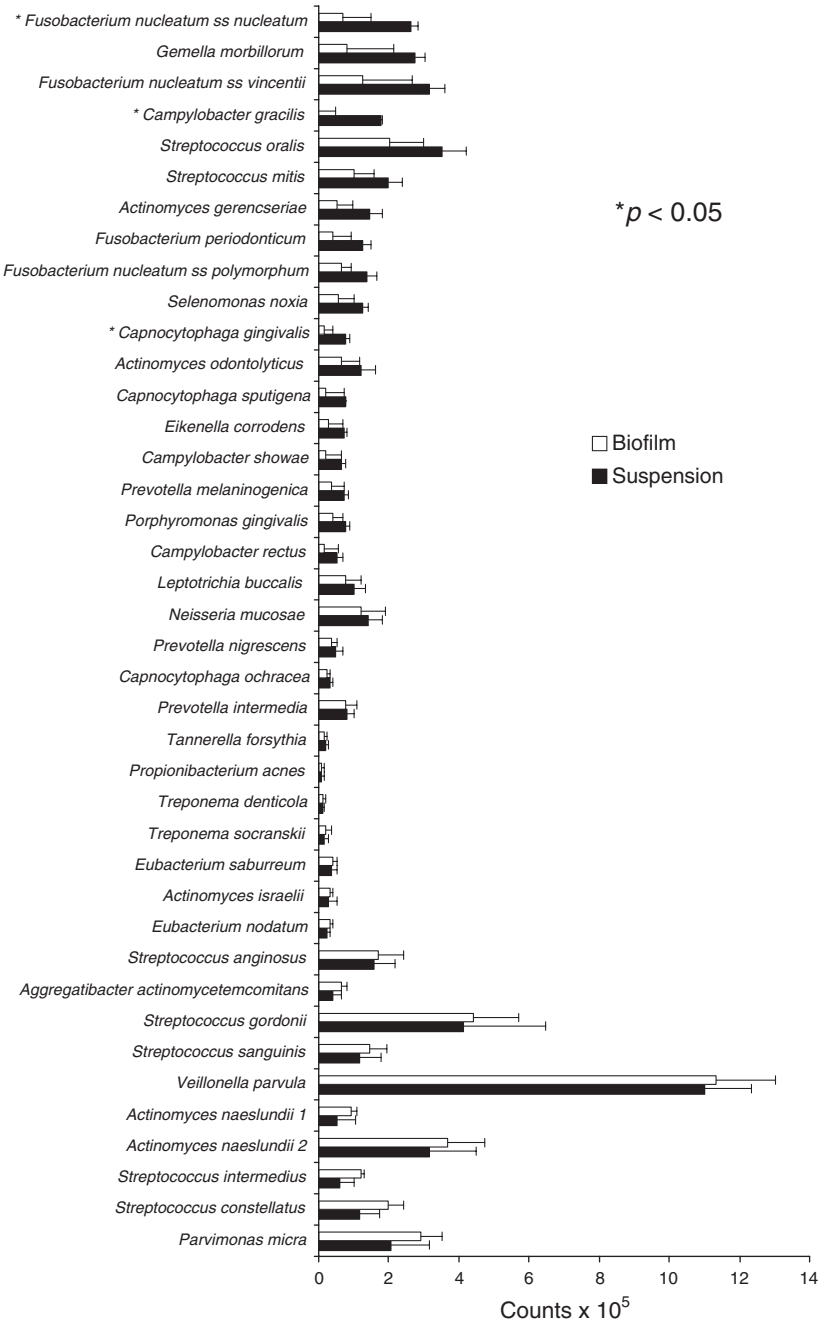

Fig. 2. Profiles of mean DNA counts of 40 microorganisms in dental plaque samples (suspensions) and in plaque-derived biofilms. Each bar represents the mean DNA count ($\times 10^5$) of values obtained from five subjects (nos 6–10) with chronic periodontitis (data from each subject were representative of three or four independent suspensions or biofilms). Error bars denote the standard error of the mean. No significant differences in species levels were found in statistical comparisons between suspensions and biofilms after applying Bonferroni criteria (with overall alpha = 0.10) to adjust for multiple comparisons.

followed by irradiation with red light at 665 nm. Incomplete destruction of bacteria has been reported to occur after sensitization with methylene blue and exposure to red light; these studies were carried out using *A. naeslundii* biofilms (19,20) as well as microcosm laboratory biofilms developed on agar in the wells of 24-well plates using human saliva as inoculum (21). In these studies, the reduced susceptibility of biofilms to photodynamic therapy was attributed to reduced penetration of methylene blue, as revealed by confocal scanning laser microscopy, an explanation that has been introduced previously (41). Similar findings were obtained by O'Neill *et al.* (42). In their study, confocal scanning laser microscopy images of saliva-derived biofilms revealed that photodestruction

occurred predominantly in the outer layers of biofilm clusters after exposure to toluidine blue O and light. It has been suggested that water channels can carry solutes into or out of the depths of a biofilm, but they do not guarantee access to the interior of the cell clusters (43), whose diameter may range from 20 to 600 μm (44). The mechanism responsible for the reduced susceptibility of biofilms to photodynamic therapy may also be related to the inactivation of methylene blue (45), the existence of biofilm bacteria in a slow-growing or starved state (46), and to distinct and protected phenotypes expressed by biofilm species when they attach to the agar surface (32). Although the optimal photodynamic therapy parameters for eradication of microorganisms in oral microcosm biofilms remain to be determined, preliminary results obtained in our laboratory using 50 μg/mL of methylene blue and light with energy fluence of 60 J/cm^2 (twofold greater fluence than that used in this study) produced incomplete (40%) killing of bacteria in biofilms developed using human dental plaque as the inoculum (47). Despite the reduced efficacy of photodynamic therapy, however, the effect was much greater than seen with antibiotic therapy and is amenable to modifications that could increase efficacy. In addition, in photodynamic therapy one is able to use smaller, more permeant molecular species, which are more capable of negotiating the water channels of established biofilm structure than larger, less permeant species. A recent *in vivo* study showed that scaling and root planing combined with photodynamic therapy using methylene blue led to significant improvements of the investigated clinical parameters over the use of scaling and root planing alone (48). The role of photodynamic therapy in the clinical treatment of periodontal disease, either alone or in combination with traditional methods of periodontal care, warrants further investigation. Novel delivery and targeting approaches may need to be developed to overcome the reduced susceptibility of complex dental biofilms to antimicrobial therapy.

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