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Influence of Photosensitizer Solvent on the Mechanisms of Photoactivated Killing of *Enterococcus faecalis*

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ABSTRACT

This study evaluated the mechanisms involved and the influence of photosensitizer solvent in the killing of *Enterococcus faecalis* using photodynamic therapy (PDT). *Enterococcus faecalis* cells incubated with 100 μM methylene blue dissolved in water and in MIX (a mixture of glycerol:ethanol:water) were irradiated with 664 nm diode laser (63.69 J cm^{-2}). The effect of PDT on the viability of bacteria, and the functional integrity of cell wall, chromosomal DNA and membrane proteins were analyzed. The bactericidal action of PDT was significantly higher when a MIX-based photosensitizer solvent was used ($P < 0.001$). Fluorimetric and fluorescence microscopy-based analysis showed the functional impairment of *E. faecalis* cell wall which was significantly higher when a MIX-based photosensitizer solvent was used ($P < 0.001$). PDT with MIX-based photosensitizer solvent showed extensive damage to chromosomal DNA. However, both PDT conditions showed similar trend in the degradation of membrane proteins, although cross-linked proteins were evident only in PDT conducted with MIX-based photosensitizer solvent. The findings from our study showed that PDT destroyed the functional integrity of cell wall, DNA and membrane proteins of *E. faecalis*. The degrees of damage on these targets were influenced by the photosensitizer solvent used during PDT.

INTRODUCTION

The emergence of antibiotic-resistant clinical strains of infectious bacteria necessitates an effective alternate treatment strategy (1). Lethal photosensitization/photodynamic therapy (PDT) of bacteria has been recognized as a promising alternative to conventional antibacterial strategies based on antibiotics (2–4). Unlike antibiotics, PDT acts on multiple targets in bacterial cells such as membrane lipids, genomic DNA, proteins and enzymes that reduce the chance of bacteria acquiring resistance to the treatment (2,3,5). In addition, a cumulative assault on bacterial cell insures instantaneous killing of bacteria. In principle, the photoactivated killing of bacteria involves the activation of a photosensitizer by irradiation with a visible light of appropriate wavelength. The activated photosensitizer can either interact with oxygen to produce singlet oxygen (Type 2 reaction) or can directly react with molecules present in the immediate vicinity (Type 1 reaction) (6). Although the bacterial killing can be mediated by either of the abovementioned reactions, singlet oxygen is the predominant chemical entity causing cell death. The short half-life and limited diffusion length of singlet oxygen necessitate the close association of photosensitizers with the target site (7). Further, the effectiveness of photoactivation and subsequent free radical generation are greatly influenced by factors such as (1) the interaction of photosensitizer molecules, (2) the physicochemical environment at the site of application, (3) half-life of the free radicals generated, and (4) oxygen availability at the site of application (6,7). All these factors may be influenced by the solvent in which the photosensitizer is dispersed.

Earlier studies have shown that the photophysical properties of photosensitizers can be influenced by the polarity and viscosity of the solvent (8). The half-life of singlet oxygen which influences the antibacterial effect is extremely dependent on the nature of the solvent used (9). Although a considerable amount of work has been done in achieving bacterial kill using the photodynamic approach, only a few studies describe the mechanism of action on bacterial cells. Many researchers have looked at the feasibility of targeting photosensitizers to specific bacteria to minimize adverse side effects. However, only very few attempts have been made to enhance the efficacy of PDT by modifying the photosensitizer solvent.

Enterococcus faecalis has been associated with a wide range of human infections comprising endocarditis, urinary tract infections, persistent endodontic infection and biomaterial-centered infections in humans (10,11). This bacterium produces biofilm on anatomic sites which are highly resistant to conventional treatment strategies (12–14). PDT has been suggested as a possible alternative to conventional antibiotic treatment in combating biofilm-mediated infections (1,15). Previous research has shown that the susceptibility of *E. faecalis* to PDT varied in different photosensitizer solvents of methylene blue (MB) (16). MB dissolved in MIX (ethanol:glycerol:water 20:30:50) photosensitizer solvents enhanced disinfection potential of PDT on biofilm-infected root canal of tooth (16). MIX-based photosensitization solvents prevented MB aggregation, and enhanced the photo-oxidation potential of MB during irradiation, contributing toward the effective inactivation of biofilm bacteria (16). The use of such a solvent system to improve the photophysical property of indocyanine green was also reported earlier (17). However, the effect of photosensitizer solvents on the mechanisms of bacterial killing has not yet been established. This study aimed

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at understanding the mechanisms of photoinactivation of *E. faecalis* as well as the influence of photosensitizer solvents on the mechanisms of photoinactivation of *E. faecalis*.

MATERIALS AND METHODS

Photosensitizer and laser. All the chemicals used in the study were of analytical grade and were purchased from Sigma Aldrich (St. Louis, MO) unless mentioned otherwise. MB dissolved in water (deionized water) or in a mixture of glycerol:ethanol:water (30:20:50) (MIX) was used as the photosensitizer. Earlier studies from our laboratory have shown that the photophysical and photochemical performance of MB is superior when dissolved in MIX solvent system at the specified concentration of each component (16). MIX containing glycerol and ethanol which are used in biocompatible drug formulations (17) are expected to enhance the tissue penetration and uptake of MB by bacteria. Moreover, ethanol in MIX is expected to enhance the antibacterial potential of PDT, as the half-life of singlet oxygen is considerably longer in ethanol (20 μs) than in water (4 μs) (9). Diode laser of wavelength 664 nm with an output power of 30 mW was used as the light source. The wavelength of the light source corresponded with the excitation wavelength of MB. The laser light was delivered using an optical fiber of 400 μm outer diameter (LDCU/6130; Power Technology, Inc., Little Rock, AR).

Fluorescence characteristics of MB in water and MIX. The effect of photosensitizer solvent on the photophysical characteristics of MB at different concentrations was evaluated by measuring the fluorescence intensity (FI). The FI of MB at 1, 5, 10, 15, 20 and 25 μM were measured using an LS50B luminescence spectrometer (Perkin–Elmer, Beaconsfield, UK; 1 cm path length cuvette) at room temperature and the average of three independent readings was plotted (excitation wavelength 650 nm, emission wavelength 686 nm, excitation slit 10 nm, emission slit 15 nm). The fluorescence spectral changes of the 10 μM MB in water and MB in MIX, with bacterial cells (0.1 optical density [OD]) were also recorded at room temperature. The shift in the fluorescence spectra of MB dissolved in water and MIX was used as an indication of photosensitizer binding to the bacterial cell wall (18).

Bacterial culturing and harvesting. A single colony of *E. faecalis* (ATCC 29212) maintained in TBX agar was transferred to 50 mL of All Culture Media (AC). Bacterial cells were allowed to grow overnight (14 h) at 37°C in an orbital incubator (120 r.p.m.). The cells were harvested by centrifugation for 10 min at 3000 ***g*** at 4°C and were washed twice with DI water. OD of cell suspension was made 1 at 600 nm (UV-VISIBLE Spectrophotometer; Shimadzu, Japan), that corresponded to 10^9 cells mL^{-1}. Cell suspension of 1 mL each was transferred to 1.5 mL microcentrifuge tubes and cells harvested by centrifugation were treated accordingly.

Bactericidal action of PDT on E. faecalis. Stationary phase *E. faecalis* cells collected by centrifugation were dispersed in 1 mL of 100 μM of MB in water and in MIX. The final cell concentration was 10^9 cells mL^{-1}. The cell suspension taken in a microcentrifuge tube was subjected to irradiation using 664 nm diode laser (fluence 63.69 J cm^{-2}). Cell suspensions treated with either irradiation or photosensitizer were also included in the study to assess their individual contribution to bactericidal effect. After the specified treatment, the cells were harvested by centrifugation and were assayed for (1) functional integrity of cell wall; (2) chromosomal DNA damage; and (3) degradation of total membrane proteins.

Effect of PDT on functional integrity of cell wall. One set of bacterial cells under each group were subjected to LIVE/DEAD BacLight bacterial viability stain (Molecular Probes, Eugene, OR) that contains syto9 (and propidium iodide, an indicator of cell wall integrity) (19). The integrity of the cell wall and the viability were assessed depending on the fluorescence emitted by the cells upon observation under a fluorescence microscope (Nikon Eclipse 80i, Tokyo, Japan). The viable cells having intact, functional cell wall permitted the entry of syto9 alone and emitted green fluorescence, while the functionally compromised cell wall which was permeable to both dyes emitted red fluorescence. BacLight solution was prepared according to the manufacturer's instruction. Further, fluorimetric evaluations were also carried out to assess the extent of cell wall damage caused by PDT (LS 55 Luminescence Spectrometer; Perkin–Elmer). For this assay, the stained bacterial suspension was diluted in PBS and the FI was monitored by measuring 470 nm-excited fluorescence emission spectrum (490–700 nm). The FI at 530 nm (due to syto 9) and 630 nm (due to propidium iodide) were taken as indicators of intact and damaged cell wall, respectively. The ratio of FI at 530–630 nm was calculated, for treatment with and without irradiation. The experiment was repeated three times with triplicate samples in each group (total $n = 9$). Paired *t*-test was conducted to study statistical significance.

Effect of PDT on bacterial DNA. *E. faecalis* cells subjected to PDT using MB in water and MB in MIX-based photosensitization solvent as in the preceding experiment were analyzed for chromosomal DNA damage. After the specified treatment, bacterial DNA was isolated using the ZR fungal/bacterial DNA kit™ (Zymo Research Corp.) according to the manufacturer's instructions. The isolated DNA was analyzed using agarose gel electrophoresis stained with ethidium bromide (1% agarose gel, 50 mV for 2.5 h). The DNA bands were visualized under UV light.

Effect of PDT on total membrane proteins. Equal number of *E. faecalis* cells (10^9 cells mL^{-1}) subjected to PDT as in previous experiments were collected by centrifugation. The outer membrane proteins were isolated as detailed elsewhere (20). Briefly, the cells collected were suspended in HEPES buffer (pH 7.4) and cells were disrupted with a sonicator at 15 kHz output (Sonoplus, Bandelin, Germany). During sonication, the cell suspension was kept in an ice bath to avoid excessive heating. Four bursts of 20 s each were given. Whole cells and cell debris were removed by centrifugation at 2500 ***g*** for 20 min and the total membrane fraction was pelleted by centrifugation at 100 000 ***g*** for 60 min.

SDS-PAGE. The outer membrane fractions were solubilized by boiling for 5 min in sample loading buffer containing 2% SDS, 5% mercaptoethanol, 10% glycerol and 0.003% bromophenol blue in tris-hydrochloride buffer. Equal quantity of samples was loaded in discontinuous SDS-PAGE with 3.8% stacking gel and 10% separating gel (21). Protein bands were visualized by staining with Coomassie brilliant blue. Electrophoresis reagents were purchased from Bio-Rad (CA).

RESULTS

Fluorescence characteristics of MB in water and MIX

Figure 1A shows the FI of MB in water-based and MIX-based photosensitizer solvents. The emission maximum was seen at 686 nm. The FI was found to increase initially and gradually decreased as the concentration of MB increased. However, the FI declined faster when MB was dissolved in water, implying the internal quenching of fluorescence because of aggregation of MB molecules. The fluorescence emission spectra of suspensions of *E. faecalis* and MB strongly depended on the composition of the photosensitizer solvent used (Fig. 1B). The emission spectra of MB in water with bacterial cells indicated association of MB with the bacterial cell wall.

Bactericidal action of PDT on *E. faecalis*

The effect of PDT on the viability of *E. faecalis* is summarized in Fig. 2. Viability of bacteria was not significantly affected by irradiation alone or by MB in water without irradiation. However, viability of *E. faecalis* cells was considerably lowered when cells were subjected to MB dissolved in MIX even without irradiation (Fig. 2). There was a significant decrease in the number of viable cells when irradiated after photosensitization with MB in water and MB in MIX compared to the control group (no treatment). Nevertheless, the bactericidal action was significantly higher when the MIX-based photosensitizer solvent was used for PDT compared with photosensitization with MB dissolved in water ($P < 0.001$).

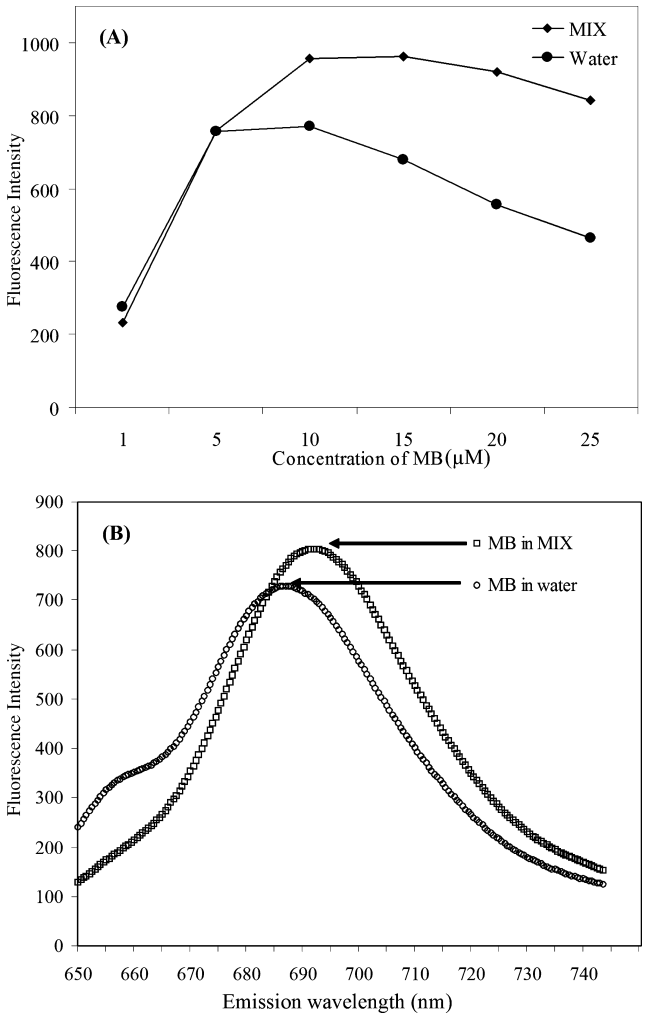

Figure 1. (A) The fluorescent intensity of increasing concentrations of methylene blue (MB) in water and glycerol:ethanol:water (MIX). Above 5 μM, the fluorescent intensity was significantly lower when MB was dissolved in water compared with MB dissolved in MIX ($P < 0.001$). (B) Fluorescence emission spectra of 10 μM of MB in water and MIX, when added with *Enterococcus faecalis* cells. The redshift of emission maxima of MB in MIX when added with *E. faecalis* cells indicated the localization of MB in a hydrophilic microenvironment.

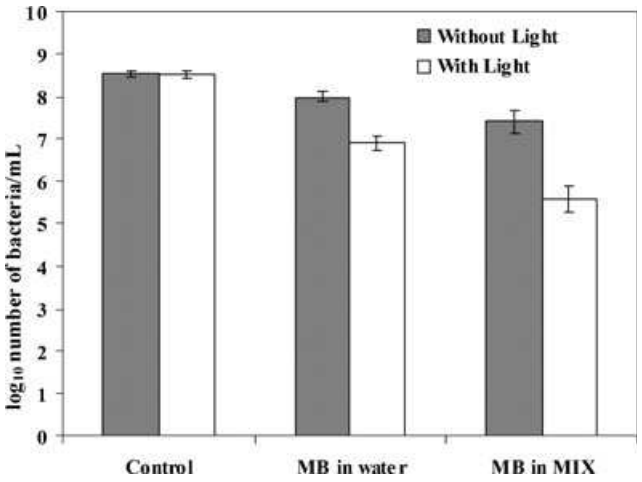

Figure 2. The $\log_{10}$ number of *Enterococcus faecalis* surviving photodynamic therapy treatment when performed with methylene blue (MB) dissolved in different media. MB dissolved in glycerol:ethanol:water (MIX) produced significantly higher bacterial killing compared to MB dissolved in water ($P < 0.001$).

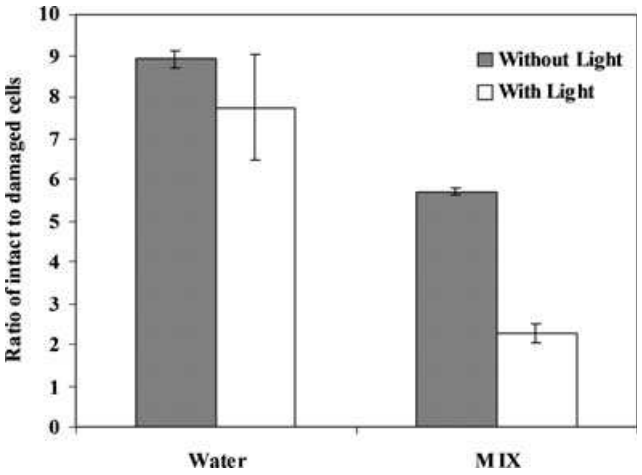

Figure 3. The ratio of fluorescence intensity at 530 nm/630 nm measured as an index of cell wall damage. The difference between the ratio of the nonirradiated and the irradiated group was significant only in the glycerol:ethanol:water (MIX)-based methylene blue (MB) solvent system ($P < 0.001$).

Effect of PDT on functional integrity of cell wall

The ratio of intact to damaged cells obtained from the fluorescent intensities at 530 nm/630 nm is shown in Fig. 3. There was a significant reduction in this ratio when cells were subjected to PDT with MB in MIX compared with the control group ($P < 0.001$). However, there was no significant reduction in the ratio once the cells were irradiated with MB dissolved in water. The fluorescence microscopic images of *E. faecalis* cells subjected to different treatments are shown in Fig. 4. The conspicuous increase in the red fluorescence (630 nm) of bacterial cells indicative of the damaged cell wall when subjected to PDT with MB dissolved in MIX is evident in Fig. 4F.

Effect of PDT on bacterial DNA

The agarose gel electrophoresis of chromosomal DNA from *E. faecalis* subjected to PDT is shown in Fig. 5. The chromosomal DNA was found to degrade on PDT with MB dissolved in different photosensitizer solvents. The intensity of the DNA band was reduced upon treatment with MB dissolved in MIX even without irradiation. The extensive DNA damage by irradiation is evident in lane 6, which displayed a faint DNA band.

Effect of PDT on total membrane proteins

The effect of PDT on the total outer membrane protein is shown in Fig. 6. The protein profile of bacterial cells subjected to light alone (lane 3) or photosensitizer solvent without irradiation (lanes 4 and 5), were similar to that of the control cells that received no treatment (lane 2). However, when subjected to PDT, the protein profile was different from that of

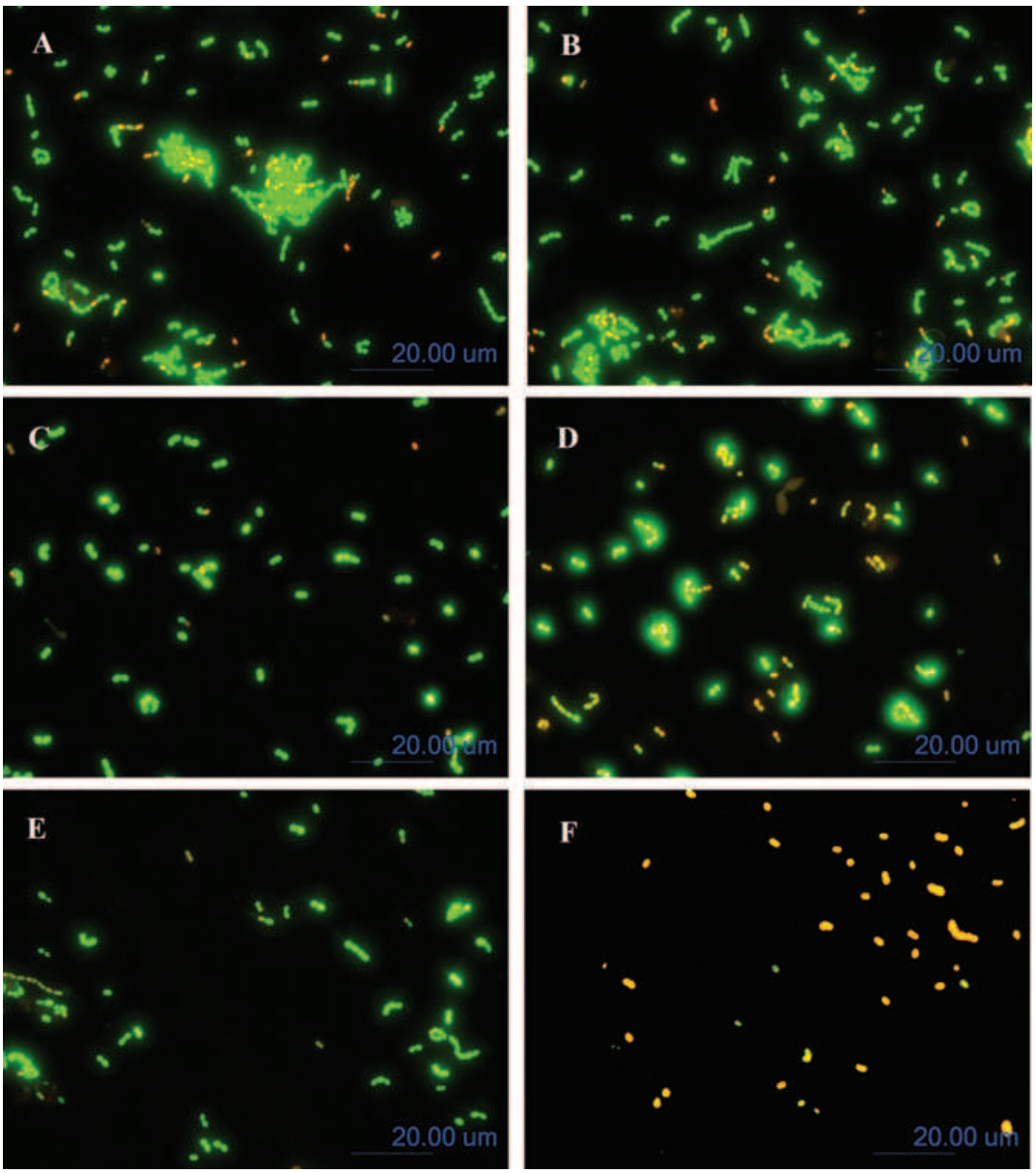

Figure 4. The fluorescence microscopic images of *Enterococcus faecalis* cells subjected to BacLight staining after different treatment. (A) Control group receiving no treatment; (B) irradiation alone; (C) cells treated with MB in water alone; (D) cells treated with methylene blue (MB) in glycerol:ethanol:water (MIX) alone; (E) cells treated with MB in water and irradiated; (F) cells treated with MB in MIX and irradiated. The red fluorescence due to propidium iodide uptake which is an indicator of damaged cell wall is evident in cells irradiated with the MIX-based photosensitizer solvent system.

the control, with some of the protein bands disappearing altogether (lanes 6 and 7). High molecular weight proteins (50–250 kDa) were completely degraded, under both conditions of PDT. Other protein bands that were completely absent from the control group included those with molecular weights 45, 30, 27 and 17 kDa (indicated by arrows in Fig. 6). Unlike PDT with MB in water, MB dissolved in MIX showed the presence of high molecular weight cross-linked proteins (circled in lane 7).

DISCUSSION

The effectiveness of PDT depends upon the simultaneous action of adequate amounts of photosensitizer and light in the presence of sufficient oxygen (6). Therefore, it is important to insure adequate intake of photosensitizer within the bacterial cell in order to achieve proper bacterial killing. Earlier studies conducted with bacterial uptake of MB had shown that the association of MB with *E. faecalis* cells was higher when applied in water. However, during photoactivation, MB in water showed lesser bacterial killing compared with other MB solvents (16). Subsequent studies conducted to understand the mechanism of MB uptake by *E. faecalis* suggested the absence of specific protein transporters (S. George and A. Kishen, unpublished). The association is thought to be mediated by electrostatic interaction between the positively charged MB molecules and negatively charged bacterial surface molecules. Bhatti

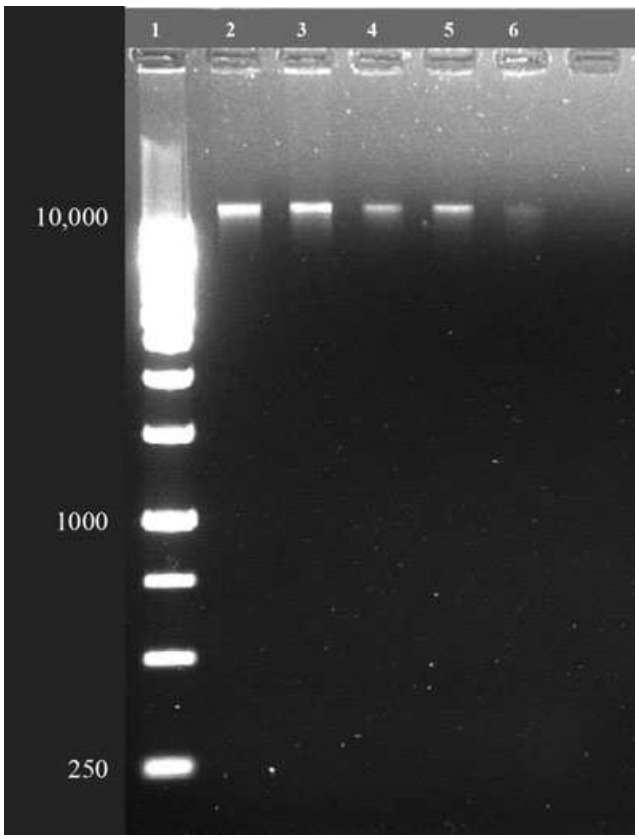

Figure 5. Agarose gel electrophoresis of chromosomal DNA from *Enterococcus faecalis* subjected to photodynamic therapy using methylene blue (MB) dissolved in different photosensitizer solvents. The intensity of the DNA band was reduced upon treatment with MB dissolved in the glycerol:ethanol:water (MIX)-based solvent system even without irradiation (lane 4). The extensive DNA damage on irradiation is evident from lane 6 showing a faint band. Lane 1 = marker; lane 2 = control; lane 3 = MB dissolved in water; lane 4 = MB dissolved in MIX; lane 5 = MB in water irradiated; lane 6 = MB in MIX irradiated.

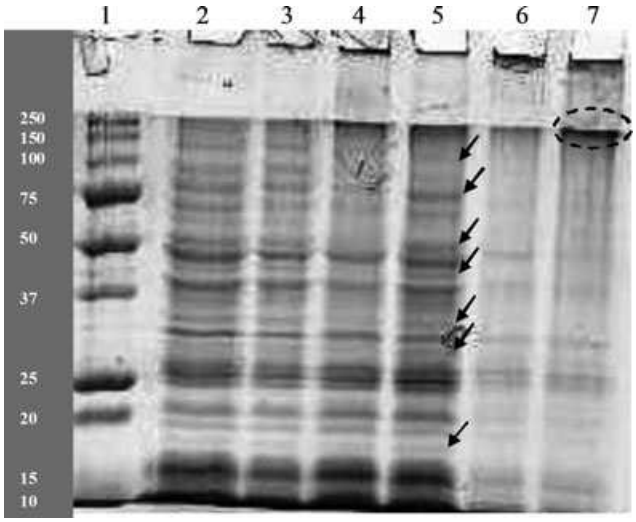

Figure 6. The total membrane protein profile of *Enterococcus faecalis* subjected to photodynamic therapy (PDT) using methylene blue (MB) dissolved in different photosensitizing solvent systems. The intensity of the protein band was reduced upon treatment with MB dissolved in both water and glycerol:ethanol:water (MIX) solvent systems. Arrows show the protein bands which are totally degraded by PDT. The circle in lane 6 shows the high molecular weight cross-linked proteins formed during PDT using MB in MIX. Lane 1 = marker; lane 2 = control; lane 3 = light alone; lane 4 = MB dissolved in water; lane 5 = MB dissolved in MIX; lane 6 = MB in water irradiated; lane 7 = MB in MIX irradiated.

et al. showed that when a water-based photosensitizer solvent is used to sensitize bacteria, a major portion of the photosensitizer binds to the outer membrane of bacteria (5). Hence a solvent that disrupts the bacterial cell wall and enhances the half-life of singlet oxygen was thought to enhance the bactericidal action of PDT using MB. In this regard, the present study aimed at understanding the mechanisms involved in the photoinactivation of *E. faecalis* and the influence of photosensitizer solvents on the mechanisms of action of PDT.

The viability of bacteria was not affected by light alone or water-based MB solution. Likewise, bacteria exposed to light treatment or water-based MB solution had no significant effect on the functional integrity of the cell wall. However, there was a slight reduction in the viability when bacteria were subjected to sensitization with a MIX-based photosensitizer solvent. This finding corresponded with the bacterial cell wall damage observed from the fluorimetric assay. During light activation of sensitized bacteria, extensive damage to the cell wall was evident in those cells sensitized with a MIX-based MB solution. Under this condition, there was a significant reduction in the FI ratio when compared with the nonirradiated group ($P < 0.001$). There was no significant reduction in the FI ratio of bacteria irradiated with MB in water. The fluorescence microscopic images showed obvious difference between PDT with MB in water and MB in MIX. The accumulation and aggregation of MB molecules at the cell wall interface of *E. faecalis* is evident from the fluorescence spectra of MB dissolved in water (detailed below). Therefore, the reduced cell wall damage caused by PDT using MB dissolved in water could be due to the reduced singlet oxygen yield because of aggregated MB molecules (22). However, in the case of MB dissolved in MIX, the cell wall damage caused in the absence of irradiation indicated the cell wall-damaging effect of components such as glycerol and ethanol. Further, as evident from fluorescence studies, the photosensitizer solvent based on MIX prevented the aggregation of MB. The disruptive action of ethanol and glycerol added with the improved photo-oxidation potential of MB, cumulatively, result in the extensive cell wall damage upon irradiating *E. faecalis* cells in MIX-based photosensitizer solvent. The above observations also indicate that sensitizing the bacterial cells with a MIX-based photosensitizer solvent could achieve MB penetration across the cell wall to reach intracellular targets like DNA and bacterial enzymes. In addition to our observation, a photosensitizer solvent system, similar to MIX, increased the diffusion of the photosensitizer through the skin, implying its clinical application (4,17).

Data from the DNA damage study indicated that PDT using MB dissolved in MIX could cause extensive damage to chromosomal DNA of *E. faecalis* cells. As a close association of photosensitizer with the target is required to cause the deleterious effect, the data indicated that MB

molecules penetrated across the cell wall, causing damage to chromosomal DNA. However, when applied in water, the DNA damage was considerably lower. This could be due to the accumulation of MB at the cell surface interface. Once MB molecules penetrate the cell wall of bacteria, its cationic nature may facilitate binding to DNA (23). Thus, the DNA damage observed could be due to MB molecules present in the cytoplasm and that bind to the DNA. The degradation of DNA is thought to be mediated by chain break. In addition, the oxidative degradation of bases such as thymine and guanine of DNA is unavoidable as the damage is mediated by singlet oxygen (5).

The membrane proteine of *E. faecalis* was also found to be degraded by the photoactivation of MB. Although MB dissolved in water and MIX showed a similar trend in protein degradation, protein cross-linking was more evident in a MIX-based photosensitizer solvent. The singlet oxygen generated during PDT could interact with oxidizable amino acid residues such as His, Cys, Trp and Tyr of adjacent protein molecules allowing them to interact and cross-link *via* covalent bonds (24). In some cases, photochemically generated free radicals other than singlet oxygen may also be involved in the cross-linking of proteins (23,24).

Studies based on the FI of MB in different solvents enable us to correlate the observed difference in the photobiological properties of MB with water structural effects (entropy and enthalpy contributions) (22). The decrease in the FI with an increase in the concentration of MB in water indicated aggregation of MB molecules. The aggregated dye molecules are less efficient in trapping energy and lead to a decrease in FI because of internal quenching (22). The shift in the fluorescence emission spectra of MB in water in the presence of bacterial cells showed characteristics of a hydrophobic environment, indicating binding of the photosensitizers to the bacterial membrane (18). Combining the observation from the FI study, the data suggested the accumulation and aggregation of MB at the cell wall surface when applied in a water-based photosensitizer solvent, similar to observations by Usacheva *et al*. (25). If the photosensitizer solvent promotes MB binding to the outer surface of bacteria, it could result in a localized increase in the photosensitizer concentration as suggested by Gabrielli *et al*. (26). Subsequently, the aggregated MB would lower the quantum yield of singlet oxygen resulting in decreased bacterial killing. This could be the possible reason for the decreased bacterial killing observed with MB in water. In conclusion, the results from this study showed that PDT destroys the functional integrity of cell wall, DNA and membrane proteins of *E. faecalis* cells. However, the extent of damage at these target sites is highly influenced by the photosensitizer solvent. The MIX-based MB solution used in this study amplified the deleterious effect of PDT on *E. faecalis* cells.

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