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Photoinactivation of *Acinetobacter baumannii* and *Escherichia coli B* by a Cationic Hydrophilic Porphyrin at Various Light Wavelengths

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Abstract. Photodynamic treatment by the cationic TMPyP photosensitizer was undertaken on the multiple antibiotic-resistant bacteria *Acinetobacter baumannii* and *Escherichia coli*. Total eradication of the bacterial cultures was determined immediately after initiation of illumination when these bacteria were treated with 5, 10, 15, 20-tetra (4-N methylpyridyl)porphine (TMPyP) at a concentration of 29.4 μmol/L and illuminated by blue, green, or red light. Total eradication of both bacteria was obtained also after treatment of bacterial cultures with 3.7 μmol/L TMPyP and illumination with blue light (400–450 nm). On the other hand, an 8- or 16- to 20-fold higher light intensity, respectively, was required for total eradication upon illumination with green (480–550 nm) or red light (600–700 nm). A 407-nm blue light only 7 and 9 joules/cm^2, respectively, was needed for total eradication of both bacteria even at a concentration of 3.7 μmol/L TMPyP. X-ray-linked microanalysis demonstrated loss of potassium and a flood of sodium and chloride into the cells, indicating serious damage to the cytoplasmic membrane. Transmission electron microscopy (TEM) revealed structural changes and damage to the membrane of treated *E. coli*. In *A. baumannii*-treated cells, mesosomes and black dots that resemble aggregation of polyphosphate polymers could be seen. DNA breakage appeared only after a long period of illumination, when the bacterial cell was no longer viable. It can be concluded that cytoplasmic membrane damage and not DNA breakage is the major cause for bacterial death upon photosensitization.

Photodynamic treatment by photosensitizers was established for photoinactivation of bacteria, in an attempt to overcome the problem of bacterial multidrug resistance. Gram-positive bacteria are susceptible to photosensitized porphyrin-induced antibacterial activity [21, 23, 24]. The efficient and non-recovering antimicrobial killing effects of phototherapy are independent of the antibiotic susceptibility spectrum of the treated pathogen [5, 6, 14, 23, 26]. Gram-negative bacteria have been found to exhibit induced damage with light only when the small non-toxic peptide derived from polymyxin B nonapeptide (PMNP) is used, which stimulates the translocation of porphyrins through the membranes of Gram-negative bacteria and allows photodynamic damage [25]. Cationic porphyrins have been shown to photoinduce direct inactivation of Gram-negative bacteria without the presence of an additional permeabilization agent [17–19]. The high efficiency of photoinactivation by these photosensitizers depends on a low-protein content and on the type of protein existing in the bacterial environment [26].

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Members of the genus *Acinetobacter*, particularly *Acinetobacter baumannii*, are known to be multiresistant. These strains are implicated in a wide spectrum of nosocomial infections, including bacteremia, secondary meningitis, urinary tract infections, and pneumonia in intensive care units [3, 4, 7, 11, 12, 32]. These bacteria usually infect patients who are on antibiotic or immunosuppressive therapy, who have malignancies, and those under tracheal or intravascular intervention. Infections by these bacteria are a therapeutic challenge, partially because of the significant increase in incidence and partly because of a spread of multiple resistance to antimicrobial agents [27]. Strains of *Acinetobacter baumannii* have been shown to exhibit resistance to penicillins including imipenem, all classes of cephalosporins [9, 29, 31], quinolones [36], aminoglycosides, and other non-related antimicrobial agents [35]. Novel combinations of azithromycin, rifampin, doxycycline, or trova-

floxacin with sulbactam have generally been additive or indifferent against multiresistant *A. baumannii* isolated in intensive care units [1, 10]. Biofilm formation is these bacteria's natural strategy for colonization on solid surfaces and under dry conditions [37]. This ability increases the virulence of *Acinetobacter* in the hospital environment and protects it from antibacterial agents [34].

In the present study, we examined the photoinactivation of the Gram-negative bacteria *Acinetobacter baumannii* and *Escherichia coli B* by the cationic hydrophilic 5, 10, 15, 20-tetra (4-N-methylpyridyl) porphine (TMPyP). Photoinactivation was achieved by illumination of the cultures with different light sources at different wavelengths. The main purpose of this study was to compare the photoinactivation efficiencies of this photosensitizer when illuminated by these various intense lights at different wavelengths. By finding the most efficient illumination conditions, we also sought to obtain more insight into the exact mechanism causing the bacterial cell death.

Materials and Methods

Bacterial strains. The following strains were used in this study: a.) *Acinetobacter baumannii* biotype 9, recovered from clinical material which was submitted to the clinical bacteriology laboratory at Meir Hospital, Kfar Saba, Israel. This strain was found to be multi-resistant to the following antibiotics: ampicillin, mezlocillin, piperacillin, cefoxitin, ceftriaxone, aztreonam, tetracycline, chloramphenicol, gentamycin, amikacin, ciprofloxacin and norfloxacin. It was sensitive to colistin, imipenem, and polymyxin. b.) *Escherichia coli* serotype $O_{111}B_4$. This strain was multi-resistant to the following antibiotics: tetracycline, ampicillin, chloramphenicol and sulfamethoxazol-trimethoprim.

Bacterial growth and photosensitization procedure. Overnight cultures of *Acinetobacter baumannii* were grown for 18h on Brain-Heart agar plates (Difco, Detroit, MI). These cultures were transferred into Nutrient Broth (Difco) at a final volume of 25 ml and an initial optical density of 0.1 at 660 nm and allowed to grow at 37°C with aeration. The cationic photosensitizer TMPyP was added to the culture medium at the beginning of the logarithmic phase, when the culture reached an optical density of 0.3 at 660 nm. Cultures were illuminated by different wavelengths of light as described below. Viable bacteria were monitored and their number calculated by counting the number of colony forming units after appropriate dilution on agar plates. Bacterial cultures grown under the same conditions and light exposures, but without addition of any photosensitizer, served as controls.

Methods of illumination. In this study several methods of light illumination were used: A. Illumination by 407 nm blue light. B. Illumination by 400–450 nm blue light. C. Illumination by 480–550 nm green light. D. Illumination by 600–700 nm red light. In method A a CureLight system was used for illumination and in methods B, C and D a Universal ARC Lamp (Ealing, MA USA) was used.

Photosensitizer solution. The photosensitizer 5, 10, 15, 20 tetra (4-methylpyridyl) porphine tetratosylate (TMPyP) was dissolved to a 2.5 mg/ml concentration in sterile distilled water. Each stock solution was stored in the dark at 4°C for a maximum of 1 week. The porphyrin was purchased from Porphyrin Products (Logan UT).

Transmission electron microscopy. *A. baumannii* and *E. coli* cultures were treated with TMPyP and each was photosensitized by blue light. Untreated cultures were used as controls. Samples were centrifuged, fixed in 2.5% glutaraldehyde/paraformaldehyde at room temperature for 1 hour, washed with veronal-acetate buffer and postfixed in 1% osmium tetraoxide and uranyl acetate. The cells were dehydrated with ethanol and embedded in Epon. Thin-sectioned samples were prepared using an LKB ultratome III and examined using a Jeol 1200EX transmission electron microscope.

X-ray microanalysis (XRMA). X-ray microanalysis combined with scanning electron microscopy provides a tool for elemental analysis of individual cells whose content has been fixed by deep freezing. The method for bacterial cell analysis has been described previously [21]. *A. baumannii* or *E. coli* treated by TMPyP and illuminated with blue light were washed twice with 0.1M ammonium acetate and resuspended in 20 μl ammonium acetate. Each suspension (20 μl) was attached to an aluminum grid, air-dried at room temperature for at least 24 h and then coated with a layer of carbon. X-ray microanalysis was performed using an X-ray system of the type eXL Linx system attached to a Jeol 840 SEM. Each spectrum was an average determination of approximately one million cells. The background level was the same during all measurements.

Genomic DNA purification. Bacterial cultures of *A. baumannii* and *E. coli* were grown, treated and illuminated as described above. The following method was used for purification of the genomic DNA [38]. Samples of the bacterial cultures were (1.5 ml each) spun in a microcentrifuge for 2 minutes. Pellets were resuspended in 567 μl (TE) Tris acetate EDTA buffer by repeated pipetting and 30 μl of 10% SDS and 3 μl of 20 mg/ml proteinase K were added to give a final concentration of 100 μg/ml proteinase K in 0.5% SDS. All components were mixed thoroughly. After incubation for 1 hr at 37°C 100 μl of 5 M NaCl were added and mixed. DNA was extracted with an equal volume (0.7–0.8 ml) of chloroform:isoamylalcohol (24:1 v/v, respectively). After centrifugation for 5 minutes in the microcentrifuge, the aqueous, viscous supernatant was removed to a fresh microcentrifuge tube, leaving the interface behind. An equal volume of phenol:chloroform:isoamylalcohol solution (25:24:1 v/v/v, respectively) was added. The extract was spun in a microcentrifuge for 5 minutes. The supernatant was transferred to another tube and isopropanol at an amount of 0.6 volumes of the supernatant was added to precipitate the nucleic acids. The precipitate was washed with 70% ethanol. The supernatant was carefully removed and the pellet was dried and redissolved in 100 μl TE buffer. DNA samples were examined by 0.8% agarose gel electrophoresis.

Statistical analysis. Linear regressions were used for analyzing the data of log CFU/ml as a function of light intensities. R^2 values were found to be ≥ 0.85 in all cases. Covariance analysis demonstrated that there was no significant difference between the tetraplicates. One experiment of each treatment was therefore chosen as a representative experiment for each treatment

Results and Discussion

In previous studies, TMPyP was shown to have bactericidal effects upon illumination [17, 18, 21, 26, 33]. The results of these studies clearly indicated that TMPyP can photosensitize Gram-positive bacteria such as *Enterococcus seriolicida* [17] or *Deinococcus radiodurams*

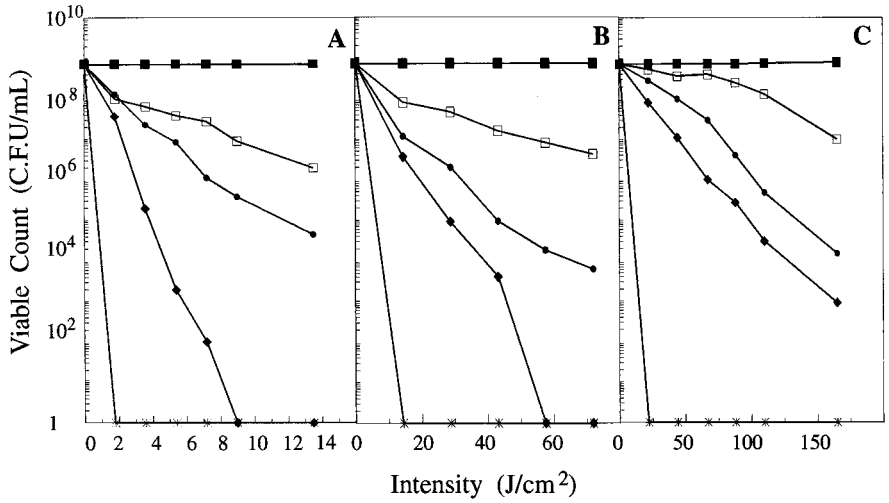

Fig. 1. Viability of *A. baumannii* after illumination by intense blue (400–450 nm), green (480–550 nm), or red light (600–700 nm) in the presence of TMPyP (A, B, and C, respectively). Bacterial cultures were treated with TMPyP at concentrations of 29.4 μmol/L (*), 3.7 μmol/L (◆), 1.83 μmol/L (●), and 0.73 μmol/L (□). The control culture was not treated with TMPyP but was illuminated (■). Viable count was monitored and plotted as a function of light fluence (J/cm^2).

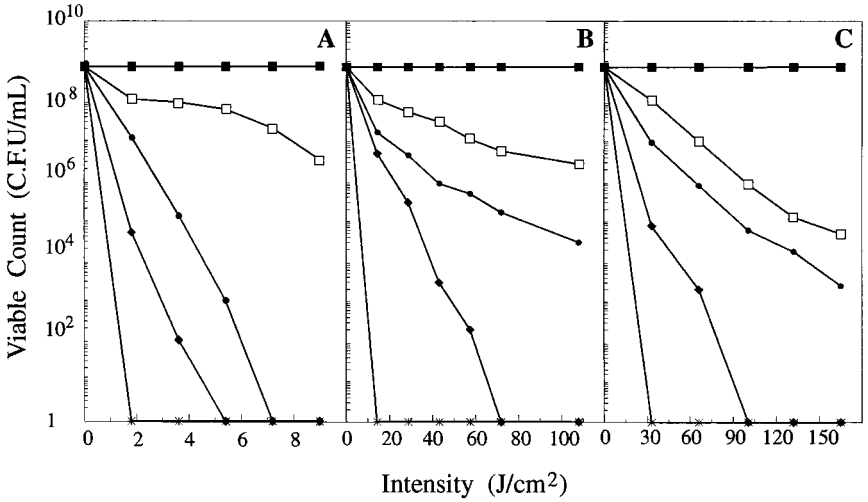

Fig. 2. Viability of *E. coli B* after illumination by intense blue (400–450 nm), green (480–550 nm), or red (600–700 nm) light in the presence of TMPyP (A, B, and C, respectively). Bacterial cultures were treated with TMPyP at concentrations of 29.4 μmol/L (*), 3.7 μmol/L (◆), 1.83 μmol/L (●), and 0.73 μmol/L (□). The control culture was not treated with TMPyP but was illuminated (■). Viable count was monitored and plotted as a function of light fluence (J/cm^2).

[21] very efficiently. Since TMPyP is a cationic porphyrin, it can cause efficient photoinactivation of Gram-negative bacteria, such as *E. coli, Vibrio angullarum* [17, 18], or *Acinetobacter baumannii* [26] upon illumination. The photoinactivation of Gram-negative bacteria was found to occur even in the absence of a membrane-permeabilizing peptide, polymyxin nonapeptide (PMNP), which has been used for the photoinactivation of Gram-negative bacteria by non-charged porphyrins [25]. In all previous studies, illumination was carried out by white light at a fluence rate of 140–150 mW/cm^2. In the present study, photoinactivation of *A. baumannii* and *E. coli* by TMPyP was undertaken. In an attempt to improve the efficiency of the treatment, the photoinactivation ability of TMPyP as a photosensitizer was examined upon illumination with intense light at different wavelengths. The results revealed that photosensitized TMPyP has strong photokilling activity on the tested Gram-negative bacteria. The best photodynamic effects were obtained when *A. baumannii* and *E. coli B* were treated with TMPyP as the photosensitizer at a concentration of 29.4 μmol/L and illuminated by each of the three different lights (Figs. 1 and 2, respectively). Under these conditions, total eradication of the bacterial viability of both bacteria was obtained by illumination with an intensity of approximately 2 joules/cm^2 of blue light. Illumination with green light requires approximately 15 joules/cm^2. Approximately 24 joules/cm^2 were required for eradication of *E. coli* by red light, and 32 joules/cm^2 were needed for the same eradication of *A. baumannii*. An eightfold lower photosensitizer concentration was also found to have efficient photoinactivation activity. When *A. baumannii* was treated with blue light (400–450 nm) and 3.7 μmoles/L TMPyP (Fig. 1A), cultures were found to be sterile after illumination with an intensity of only 9 joules/cm^2. When *E. coli B* were treated

with the same concentration of TMPyP and illuminated with blue light (Fig. 2A), complete eradication was achieved after illumination with 5.5 joules/cm^2. With a culture of *A. baumannii* treated with 3.7 μmoles/L TMPyP and illuminated with green (480–550 nm) or red (600–700 nm) light, approximately 60 and 150 joules/cm^2, respectively, were required for a decrease of 8 or 5 orders of magnitude in viability, respectively (Fig. 1B and 1C). For total eradication of *E. coli* at an identical photosensitizer concentration and illumination with green or red light, 70 and 100 joules/cm^2 were required, respectively (Fig. 2B and 2C). Lower TMPyP concentrations were found to have some antibacterial effects on both bacteria. However, significantly more light fluence was required with blue light for total eradication of the bacteria from the culture.

The survival patterns of the tested bacteria indicate that for total eradication of these bacteria at a given concentration of TMPyP, approximately an eightfold higher light energy is needed with green intense light than with blue light, and approximately a 16- to 20-fold higher light energy with red than with blue light. *E. coli B* is apparently also more susceptible to photoinactivation than *A. baumannii*. However, the significance of this finding is not clear. The efficacy of therapy apparently decreases with increase in illumination light wavelengths. Blue light was always the most efficacious [21, 22], since at blue light porphyrins have maximal light absorbency at these wavelengths. This blue-light illumination (400–450) is not phototoxic compared with the shorter wavelength blue light. Red-light illumination might be preferable for clinical treatment, since red light penetration into tissues is better. However, blue light is more efficient in photokilling bacteria, and shorter illumination periods are required for treatment.

Since the best light for photoinactivation of bacteria is blue light, we used an intense light system with a wavelength of 407 nm. This lamp system was designed for curing acne caused by the bacterium *Propionobacterium acne*. The *P. acne* bacterium is known to produce endogenous porphyrins and is photoinactivated by using this lamp [2], although blue light does not penetrate tissues as well as red light.

Cultures of *E. coli B* or *A. baumannii* become sterile after illumination with the specific 407-nm wavelength blue light at an intensity of 7 and 9 joules/cm^2 respectively, and a concentration of 3.7 μmol/L TMPyP. At a concentration of 0.73 μmol/L, 12 and 20 joules/cm^2 respectively are required for obtaining the same result (Fig 3).

One of the most important advantages in the clinical use of photoinactivation of bacteria is the fact that, shortly after the initiation of light exposure, almost all

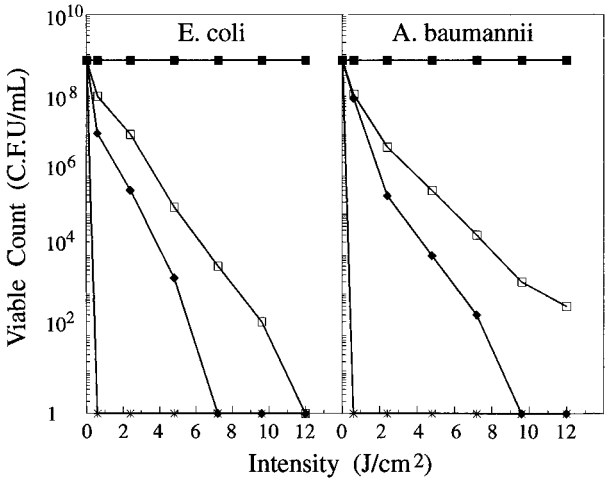

Fig. 3. Viability of *A. baumannii* and *E. coli B* after illumination by intense blue light (407 nm) in the presence of TMPyP. Bacterial cultures were treated with TMPyP at concentrations of 29.4 μmol/L (∗), 3.7 μmol/L (◆), and 0.73 μmol/L (□). The control culture was not treated with TMPyP but was illuminated (■). Viability counts were monitored and plotted as a function of light fluence (J/cm^2).

bacterial cells exhibit serious and irreversible damage. This damage does not allow the cells to create or operate any kind of antidrug mechanisms. Photodynamic therapy is therefore always very effective, and up until now no photosensitization-resistant mutants have been found. From the easy inactivation of *A. baumannii*, which is multidrug resistant, it seems very clear that photoinactivation is independent of the antibiotic resistance status of the bacteria.

Membrane damage was determined by X-ray microanalysis immediately after initiation of the phototreatment. Treatment of *A. baumannii* cells with 3.7 μmol/L TMPyP and illumination with 407 nm blue light at an intensity of only 1.2 joules/cm^2 resulted in damage to the sodium-potassium pump, which was expressed by increased levels of intracellular sodium (Fig. 4A). After illumination with 6 joules/cm^2, the membrane damage was even more significant and was demonstrated by a drastic influx of sodium and chloride and a parallel efflux of potassium ions from the cells (Fig. 4B). These results can be interpreted as consequences of bacterial membrane damage, probably owing to the different bacterial ion pumps as demonstrated in previous studies [8, 16]. Untreated *A. baumannii* exhibited normal XRMA spectra with a low sodium peak and a very low chloride peak.

Treatment of TMPyP and illumination of *E. coli B* may also cause leakage of the cell content. This effect was found in this study (not shown), as well as in a previous study [33] where complete loss of the enzymes lactate dehydrogenase and NADH dehydrogenase was demonstrated.

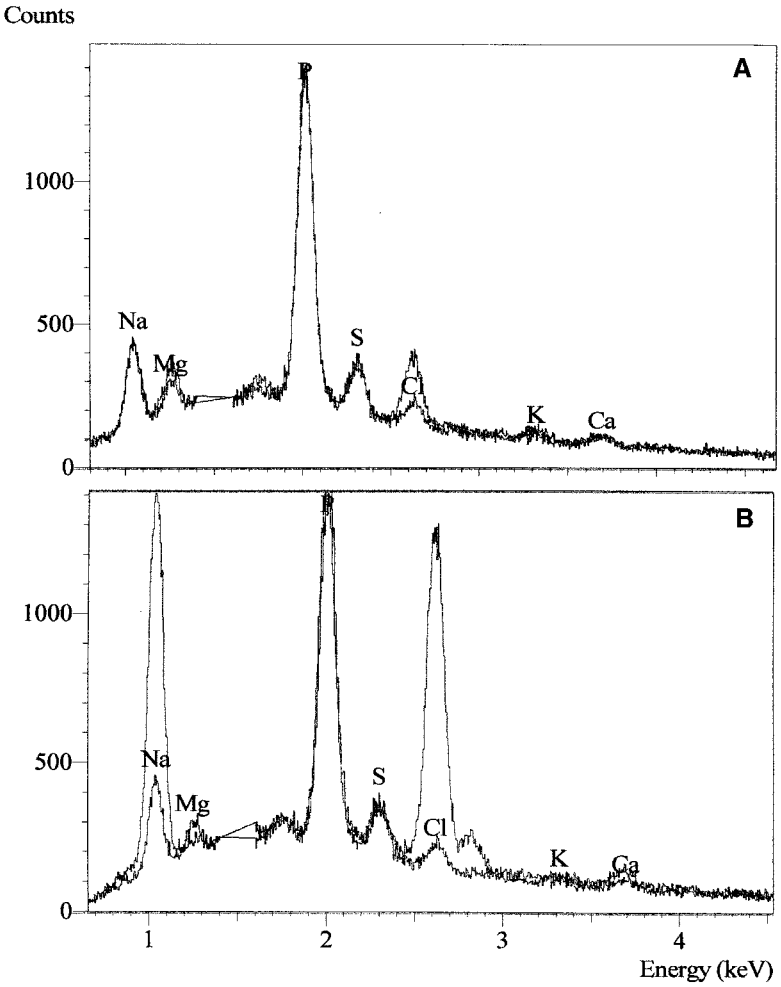

Fig. 4. X-ray elemental spectra of *A. baumannii* treated with 3.7 μmol/L TMPyP and illuminated with blue light at 407 nm. (A) The elemental concentrations in bacterial cells treated with 1.2 joules/cm^2 (upper line) were determined compared with untreated cells (lower line). (B) The elemental concentrations in bacterial cells treated with 6.0 joules/cm^2 (upper line) were determined compared with untreated cells (lower line). These preparations were examined by X-ray microanalysis of air-dried cells.

Ultrastructural changes in the bacterial cells are depicted by transmission electron microscopy (Fig 5.). After treatment of *E. coli B* with TMPyP and illumination by blue light (407 nm), the ultrastructural picture was found to be identical to that found with deuteroporphyrin + PMNP and white light [25]. Figure 5A depicts elongated cells connected to each without separation of the daughter cells and the appearance of a low-density area in the middle of the cells. Alteration and irregularities in the cell barriers are observed along the bacterium. The latter finding strengthened the findings of the X-ray microanalysis (XRMA) spectra on membrane damage of the *E. coli* cells.

Multilamellar membrane structures, which look like mesosomes, appeared after photodynamic treatment. These mesosome-like structures are directly connected to the bacterial membrane (Fig. 5B and D). This might indicate membrane damage in cells that have just begun the dividing process and with the membrane folded into a multilamellar form instead of septation between daughter cells.

Large electron-dense black dots were observed in the cytoplasm of the treated bacteria (Fig. 5B, C, and D). These deposits may represent aggregation of polyphosphate inclusions that are heavily stained by uranyl acetate. This statement on polyphosphate inclusion has not yet been proven by a specific stain. It is peculiar that these dense black spots appear just upon photosensitization at very short light intensities. They do not appear at all in the untreated cells or in untreated but illuminated cells. It seems that the photodynamic process initiates a process of formation of polyphosphate. The ability to produce polyphosphate inclusions in *Acinetobacter spp.* when incubated in inorganic phosphate-rich medium is already known [13, 28, 30]. It is assumed that, with the membrane damage by the singlet oxygen, the system of phosphatases is destroyed or leaked out and the polyphosphate synthase is free to produce the polyphosphate. This point and the connection to membrane damage by singlet oxygen have to be proven and are under investigation.

With this short illumination time, damage of the

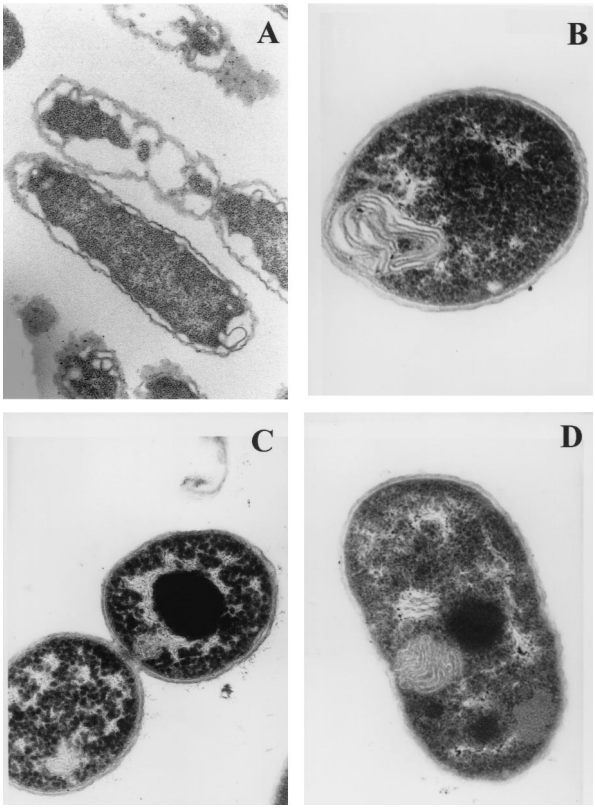

Fig. 5. Effect of TMPyP and illumination by blue light on the ultrastructure of *E. coli B* (panel A) and of *A. baumannii* (panels B, C, and D) cells. Transmission electron micrograph of TMPyP (3.7 μmol/L) treated cells after illumination with 2.4 joules/cm^2 blue light at 407 nm. Magnification was 20,000 for panel A, 60,000 for panel B, 30,000 for panel C, and 40,000 for panel D.

chromosomal DNA could not be pinpointed, as shown in Fig. 6. In previous studies [14, 15, 20] we assumed that cell death is initiated by DNA damage and breakage. This assumption was based on the fact that DNA synthesis was the first to be reduced upon interaction with photosensitized porphyrins [14]. Furthermore, disappearance of the plasmidial supercoiled fraction and changes in the chromosomal DNA pattern were also observed in bacteria after photosensitization [20]. In this study, intense light for very short periods (about 2–5 min) was required for total eradication of the bacteria. These short illumination periods of a few minutes resulted in the death of all the bacteria in the culture, while the chromosomal DNA remained intact. This phenomenon can be seen in Fig. 6 and in a current work on *E. coli* strains [33]. On the other hand, damage to the cytoplasmic membrane was observed concomitantly to bacterial death. In former studies with *E. coli* [33] or with *Deinococcus radiodurans* [21], it was also concluded that DNA does not represent a critical target for photosensitization.

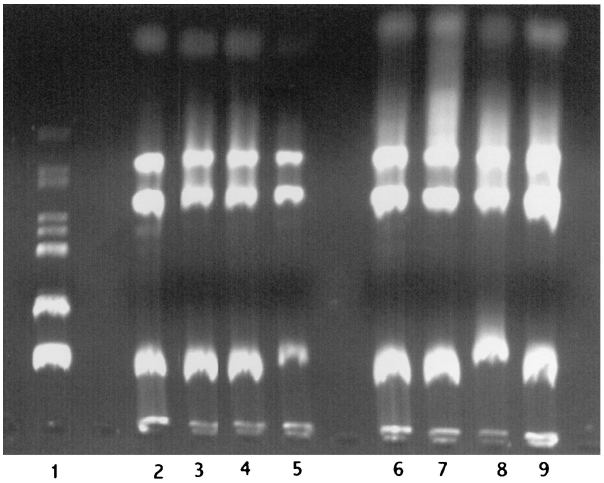

Fig. 6. Electrophoretic analysis of genomic DNA samples prepared from *A. baumannii* or *E. coli B* cells. Samples were prepared after the cells were treated with TMPyP and illuminated by blue light at 407 nm. *A. baumannii* DNA prepared from cells after treatment with 3.7 μmol/L TMPyP and illuminated for 1, 2, and 5 min (lines 3–5, respectively). DNA from untreated *A. baumannii* (line 2) served as control. *E. coli B* DNA prepared from cells treated with 3.7 μmol/L TMPyP and illuminated for 1, 2, and 5 min (lines 7–9, respectively). DNA from untreated *E. coli B* (line 6) served as control. λ DNA restricted for *Eco*RI and *Hin*dIII was run in line 1.

The present study is in agreement with previous studies and confirms that the structural damage to the membrane of Gram-negative bacteria is induced by the singlet oxygen produced upon photosensitization when the photosensitizers reach the inner membrane. This damage plays a major role in bacterial photoinactivation. This inactivation depends on the penetration capabilities of the photosensitizer into the bacterial cells and on the wavelength of illumination and not on its resistance pattern to antibiotics.

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