

Eradication of *Propionibacterium acnes* by its endogenic porphyrins after illumination with high intensity blue light

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Abstract

Propionibacterium acnes is a Gram-positive, microaerophilic bacterium that causes skin wounds. It is known to naturally produce high amounts of intracellular porphyrins. The results of the present study confirm that the investigated strain of *P. acnes* is capable of producing endogenic porphyrins with no need for any trigger molecules. Extracts from growing cultures have demonstrated emission peaks around 612 nm when excited at 405 nm, which are characteristic for porphyrins. Endogenic porphyrins were determined and quantified after their extraction from the bacterial cells by fluorescence intensity and by elution retention time on high-performance liquid chromatography (HPLC). The porphyrins produced by *P. acnes* are mostly coproporphyrin, as shown by the HPLC elution patterns. Addition of δ -aminolevulinic acid (ALA) enhanced intracellular porphyrin synthesis and higher amounts of coproporphyrin have been found. Eradication of *P. acnes* by its endogenic porphyrins was examined after illumination with intense blue light at 407–420 nm. The viability of 24 h cultures grown anaerobically in liquid medium was reduced by less than two orders of magnitude when illuminated once with a light dose of 75 J cm^{-2} . Better photodynamic effects were obtained when cultures were illuminated twice or three times consecutively with a light dose of 75 J cm^{-2} and an interval of 24 h between illuminations. The viability of the culture under these conditions decreased by four orders of magnitude after two illuminations and by five orders of magnitude after three illuminations. When ALA-triggered cultures were illuminated with intense blue light at a light dose of 75 J cm^{-2} the viability of the treated cultures decreased by seven orders of magnitude. This decrease in viability can occur even after a single exposure of illumination for the indicated light intensity. X-ray microanalysis and transmission electron microscopy revealed structural damages to membranes in the illuminated *P. acnes*. Illumination of the endogenous coproporphyrin with blue light (407–420 nm) apparently plays a major role in *P. acnes* photoinactivation. A treatment protocol with a series of several illuminations or illumination after application of ALA may be suitable for curing acne. Treatment by both pathways may overcome the resistance of *P. acnes* to antibiotic treatment.

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1. Introduction

Propionibacterium acnes is a Gram-positive non-sporulating bacterium which normally inhabits human sebaceous glands. These bacteria are also the major cause of acne, which affects more than 80% of young adults. Extensive research has shown that more than 40% of *P. acnes* is resistant to commonly used topical and oral anti-acne antibiotics [1–4]. Other anti-acne therapies are of limited appeal due to their side effects. Acquired resistance to

erythromycin, clindamycin and tetracycline has been reported in strains from diverse geographical areas [5]. Resistance to novel oligosaccharide antimicrobial agents was also found [6]. The prevalence of resistance correlates with antibiotic usage patterns in different countries [7,8]. Carriage of resistant strains results in the therapeutic failure of most antibiotic regimes [9]. *P. acnes* is also known to naturally produce high amounts of intracellular metal-free porphyrins [10–14].

Since bacterial resistance to antibiotics is becoming an increasing problem, research is being directed towards photodynamic therapy as an alternative method for killing bacteria [15]. Photoinactivation of various Gram-positive bacteria, including *P. acnes*, has been demonstrated using exogenous photosensitizers [10,16–20]. The efficient and

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non-recovering antimicrobial killing effects are independent of the antibiotic resistance spectrum of the treated bacteria [16,18,21,22]. A new method for photosensitizing cells is by enhancing endogenous porphyrin production with δ -aminolevulinic acid (ALA), which is a naturally occurring metabolite in the synthesis pathway of cellular heme production [23]. In bacteria, addition of ALA may also induce porphyrin synthesis. ALA induction leads to an increase in the synthesis of uroporphyrin, coproporphyrin and protoporphyrin IX, which are the immediate precursors of heme [24,25]. It has also been shown that when illuminated with blue light, porphyrins damage the cells very efficiently [24–26]. The emission peak of the best light source is at 407–420 nm, which is the most efficient porphyrin photodestructive wavelength range. In contrast to ultraviolet light, this visible blue light irradiation is not phototoxic to human cells. The aim of the present study was to determine the sensitivity of *P. acnes* to photokilling by its endogenously produced porphyrins when illumination was produced by an intense narrow band of UV-free blue light and to propose practical possibilities of photoeradication of *P. acnes* using solely illumination or after application of ALA. Elucidation of the ultrastructural changes and intracellular mechanisms of bacterial photodestruction in *P. acnes* were also investigated.

2. Materials and methods

2.1. Bacterial strain

The strain used in this study was *P. acnes* ATCC 6919 which was obtained from the American Type Culture Collection at Rockville, MD, USA.

2.2. Growth media

P. acnes was grown on reinforced clostridial agar from Oxoid (Basingstoke, Hampshire, UK) at pH = 6–6.2. Illumination tests were carried out when bacteria were grown in reinforced clostridial broth which was prepared from the same ingredients, except the agar, at pH = 6–6.2.

2.3. Bacterial growth

P. acnes was transferred from the bacterial stock into reinforced clostridial agar plates. Bacteria were streaked on the plates for isolation of single colonies by the 'clock plate technique'. These plates were called 'start plates' and were incubated for three days under anaerobic conditions in an anaerobic jar. The jar contained Aero Gen sachets from Oxoid to maintain anaerobic conditions suitable for *P. acnes*. Single colonies were transferred from the 'start plates' into reinforced clostridial broth in the presence or absence of ALA. Each broth culture was equally distrib-

uted into test tubes with screwed caps. Bacteria were then allowed to grow anaerobically in the dark for 24, 48, 72 or 96 h. Monitoring the growth of bacteria was performed by counting the colony-forming units at the indicated times after appropriate dilution in saline and cultivation on the reinforced clostridial agar plates under anaerobic conditions for 3 days. Since *P. acnes* are microaerophilic bacteria, long periods of incubations were needed for the experiments and for counting the viable bacteria.

2.4. Photosensitization procedure

Cultures in the test tubes were transferred to illumination by placing the test tubes horizontally in order to obtain maximal exposure to the blue light. Some cultures were illuminated again after 24 h and some were even illuminated three times, after an additional 24 h as indicated in the results. A sample was taken out from the culture after each illumination and viable bacteria were counted. The colony-forming units of the survivals were calculated per ml. Unilluminated cultures served as controls.

2.5. Illumination method

Illumination was carried out using a CL-420-1 acne therapy system which utilized high intensity narrow band blue light between 407 and 420 nm with total UV cutoff. The Lab model system contained a metal halide lamp which produced 20 mW cm^{-2} homogeneous illumination at the tube surface. (Lab prototype of ClearLight[®], by CureLight, distributed by Lumenis). The lamp has a square $17 \times 15 \text{ cm}$ shape and is located 10 cm above the horizontal test tubes. Two ventilators are also located near the lamp on both sides in order to prevent any heating of the illuminated samples. Light fluence was calibrated with a light power meter (OPHIR model PD2-A, Israel). It was calculated that it takes 1 min to get 1.25 J cm^{-2} .

2.6. High-performance liquid chromatography (HPLC) analysis

P. acnes cultures grown as above were extracted by a 0.1 M NH_4OH acetone solution (1:9 v/v). Porphyrins from the extracts were identified and quantified by an HPLC system (Merck Hitachi D-7000) using a C-18 modified silica column and a reversed phase system. The system was equipped with a LaChrom fluorescence detector L-7480, where the excitation wavelength was at 407 nm and emission at 612 nm. Elution was performed using a gradient consisting of 10% acetonitrile in 1 M ammonium acetate pH = 5.1 (solvent A) and 10% acetonitrile in methanol (solvent B). Porphyrin chromatographic marker kit (Porphyrin Product, Logan, UT, USA) was used as a standard kit for evaluation and quantification of the porphyrins produced. The amount of each porphyrin in the kit

was 10 nmol ml⁻¹ and from the fluorescence intensities the amounts of the related porphyrins in the tested sample could be calculated.

2.7. Fluorescence measurements

P. acnes cultures were extracted as above and underwent fluorescence spectral determination. This was performed using a Perkin-Elmer model LS-50 spectrofluorometer interfaced to a data station 7500 computer. The excitation wavelength was 405 nm and emission spectra were recorded in the 550–750 nm range. Extractions from cultures at zero time, which were taken from the ‘start plates’, were used as controls.

2.8. ALA solution

Stock solutions of ALA were prepared by dissolving ALA to a concentration of 5 mg ml⁻¹ in sterile distilled water. Each stock solution was kept in the dark at 4°C for a maximum of 24 h.

2.9. Transmission electron microscopy

Treated and untreated control *P. acnes* cultures were centrifuged and fixed in 2.5% glutaraldehyde/paraformaldehyde in phosphate buffer at room temperature for 1 h. Samples were washed with phosphate buffer and postfixed in both 1% osmium tetroxide 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 with a Jeol 1200 EX transmission electron microscope.

2.10. X-ray microanalysis (XMRA)

XMRA combined with scanning electron microscopy was used for elemental analysis of individual cells whose content was fixed by deep-freezing. The method for bacterial cell analysis has been described previously [26,27]. In brief, *P. acnes* cultures were washed twice with 0.1 M 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. XMRA was per-

formed using an X-ray system of the eXL Link type attached to a Jeol 840 scanning electron microscope. Each spectrum was an average determination of approximately 10⁶ cells. The background level was the same during all measurements.

3. Results

P. acnes cultures grown in reinforced clostridial broth, in the absence or presence of ALA, for 0, 24, 48, 72 and 96 h have demonstrated increase in the viable number of bacteria from 1.4 × 10⁸ to 7.1 × 10⁸ CFU ml⁻¹ after 4 days of growth. Growth in the presence of ALA was not significantly different from the pattern without ALA and the viable count increased from 1.4 × 10⁸ to 6.7 × 10⁸ CFU ml⁻¹ (Table 1). Fluorescence spectral examinations of extractions from these samples revealed emission peaks at 612 nm when excited at 405 nm (data not shown). This emission peak is characteristic for porphyrins. These porphyrins are endogenous porphyrins produced and accumulated in the growing bacteria.

Examination of *P. acnes* porphyrin content by HPLC after extraction of the endogenous porphyrins from culture grown anaerobically in broth for 24 h revealed that the porphyrin produced by *P. acnes* is mostly coproporphyrin (Fig. 1, upper panel). The retention time for the porphyrin produced in the bacteria was 12 min exactly as the retention time of coproporphyrin in the standard kit. The first peak, presented in both panels of Fig. 1, has a retention time of 2 min and is of unidentified materials that contain fluorescent molecules found in the cytoplasm of the bacterial cells and are not porphyrins. Addition of 100 µg ml⁻¹ ALA, an inducer of endogenous porphyrin synthesis, demonstrated that this inducer causes a significant increase in coproporphyrin production in the 24 h incubated culture (Fig. 1, lower panel). The porphyrins in both cases were extracted from the same number of bacteria.

Porphyrin amounts increase as a function of growth time as seen in Fig. 2. Addition of ALA (100 µg ml⁻¹) enhances this increase and the amounts of coproporphyrin produced were significantly higher in the presence of ALA. Almost zero amounts of porphyrins were detected at the beginning of the experiment in the cells resuspended in the broth medium which were transferred from the ‘start plates’.

Photoinactivation of *P. acnes* was obtained when cultures that grew anaerobically for 24 h in liquid medium were illuminated by a blue light dose of 75 J cm⁻¹. Under these conditions the viable count of the culture decreased between one and two orders of magnitude. In cultures that grew for 48 h and were illuminated once the viability is decreased by almost two orders of magnitude. Two consecutive illuminations at an interval of 24 h between the treatments (illumination at 24 h and 48 h of growth) caused a decrease in the viable count of the culture by

Table 1
Growth of *P. acnes* anaerobically in the absence or presence of ALA (100 µg ml⁻¹)

Incubation time (h)	Growth (CFU ml ⁻¹)	
	-ALA	+ALA
0	1.4 × 10 ⁸	1.4 × 10 ⁸
24	2.2 × 10 ⁸	1.9 × 10 ⁸
48	4.5 × 10 ⁸	4.1 × 10 ⁸
72	5.6 × 10 ⁸	5.5 × 10 ⁸
96	7.1 × 10 ⁸	6.7 × 10 ⁸

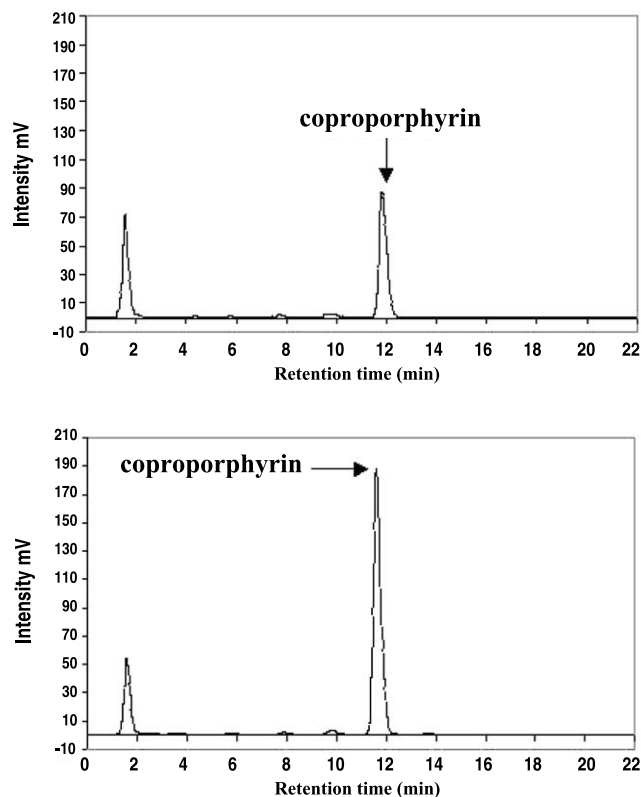


Fig. 1. HPLC of porphyrins extracted from *P. acnes* (upper panel) and from cultures incubated with $100 \mu\text{g ml}^{-1}$ ALA (lower panel). Cultures were incubated anaerobically for 24 h, then extracted and submitted to HPLC analysis.

four orders of magnitude. Three consecutive illuminations (at 24, 48 and 72 h) resulted in a decrease in viability of five orders of magnitude (Fig. 3).

ALA-triggered cultures of *P. acnes*, where endogenous porphyrin production was enhanced, exhibited a better inactivation pattern upon illumination by intense 407–

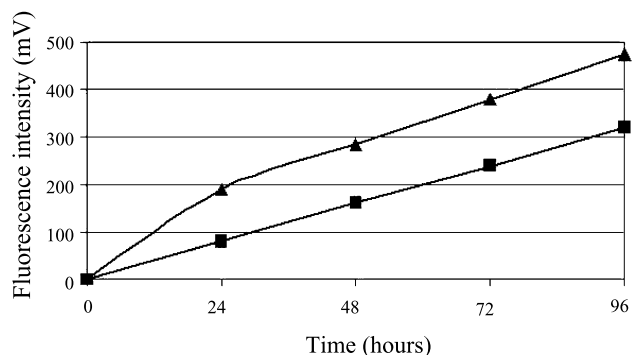


Fig. 2. Fluorescence intensities of coproporphyrin from *P. acnes* cultures at various stages of growth. Cultures were grown in liquid medium without (■) or with (▲) ALA ($100 \mu\text{g ml}^{-1}$) for 24, 48, 72 and 96 h. Samples of each incubation time were extracted and transferred to HPLC analysis. Fluorescence intensities of coproporphyrin were recorded for extracts taken from the same number of cells.

420 nm blue light (Fig. 4). The viability of cultures grown with ALA for 24 h and illuminated with a light dose of 75 J cm^{-2} decreased by two orders of magnitude and when illuminated by 100 J cm^{-2} the viability decreased by three orders of magnitude only (the last result is out of the light fluence scale in Fig. 4). Cultures grown with ALA for 48 h exhibited reduced viability by seven orders of magnitude with a light dose of 75 J cm^{-2} . Cultures grown with ALA for 72 h exhibited a decrease in viability by seven orders of magnitude even with a light dose of 50 J cm^{-2} . The decrease in viability of the cultures grown with ALA and illuminated with blue light was far more significant than that of cultures grown without ALA. One illumination only was sufficient for such photoinactivation after incubation with ALA.

Fast ionic fluxes which were determined by XMRA revealed that *P. acnes* cultures that have been incubated with ALA ($100 \mu\text{g ml}^{-1}$) for 24 h and illuminated with 100 J cm^{-2} showed a loss of 90% of potassium by calculation (Fig. 5, upper panel). Furthermore, bacteria incubated with the same concentration of ALA for 72 h and illuminated with 75 J cm^{-2} demonstrated the same leakage of potassium (96%) but also a 40% decrease in phosphorus content (Fig. 5 lower panel). The above results can be interpreted as consequences of bacterial membrane damages. Observations by transmission electron microscopy demonstrates the antibacterial effects of the photosensitization process of endogenous porphyrins. The appearance of low density areas in the middle of the cells can be interpreted as leakage of intracellular components (Fig. 6B,C). Asymmetrical septation of treated cells was observed (Fig. 6D). Undivided elongated cells connected one to the other without separation of the daughter cells was commonly detected (Fig. 6B,C). As a result of these structural alterations in the treated bacteria, a lytic process can be developed leading to bacterial cell death.

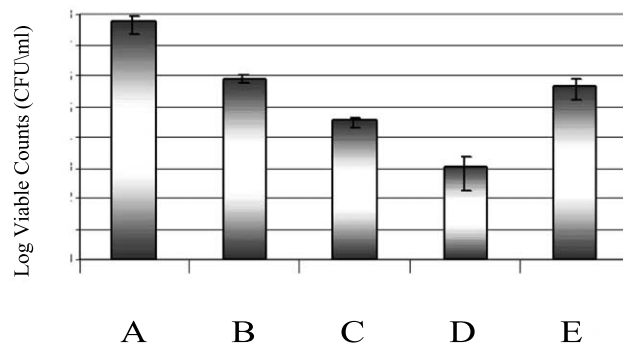


Fig. 3. Survival of *P. acnes* after illumination with intense blue light. Cultures were grown anaerobically in liquid medium. Illuminations were performed using 407–420 nm blue light with a light fluence of 75 J cm^{-2} . A: Control culture with no illumination. B: Cultures illuminated once after 24 h. C: Cultures illuminated twice, after 24 and 48 h. D: Cultures illuminated three times, after 24, 48 and 72 h. E: Cultures illuminated once after 48 h. Viable counts per ml were monitored. Each point is the mean \pm S.D. of five experiments, $P < 0.005$ for each experiment vs. control.

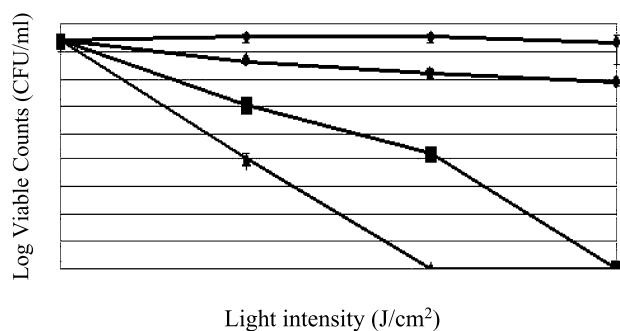


Fig. 4. Viable counts of *P. acnes* grown with ALA and illuminated with blue light. *P. acnes* cultures were incubated with ALA ($100 \mu\text{g ml}^{-1}$) for 24 (●), 48 (■) and 72 h (▲) and illuminated at various light fluences with 407–420 nm blue light. Cultures incubated with ALA but not illuminated served as controls (◆). Viable counts per ml were monitored. Each point is the mean \pm S.D. of five experiments, $P < 0.005$ for each experiment vs. control.

4. Discussion

Photoinactivation of bacteria by endogenously produced porphyrins is of growing interest in the field of photodynamic therapy [24,25,28,29] as well as in the field of acne treatment [13,30–35]. The knowledge that *P. acnes* produces porphyrins naturally and accumulates them in the bacterial cell became known before the start of photodynamic therapy [36]. Since the early days of photodynamic treatment, investigators have tried to treat acne using visible light at various wavelengths [10,11,13,20,30,31,33,37–39]. In the present study we demonstrate the possibility of treating this infection by taking advantage of the fact that *P. acnes* produces porphyrins which can be photosensitized and the bacteria will be killed by their own products. We used an intense blue light lamp that produces blue light at a narrow band between 407 and 420 nm. This light is free of all UV light and is not toxic to mammalian cells or to intact bacterial cells that do not contain a photosensitizer. In the case of *P. acnes* or other bacterial cells that produce porphyrins, the blue light may photoinactivate the intact bacterial cells as a consequence of the photosensitizer molecules produced and stored within the bacterial cells. Some investigators have used white light or red light for photokilling of *P. acnes*, with little success [30,32,34,38,39]. Red light is more penetratable into the depth of the skin, but a high light intensity is necessary for eradication of the bacteria by red light. On the other hand, blue light is less penetratable into the skin, but low intensities of light are needed for eradication of the bacteria [30,37]. The yield of bacterial photoeradication was a factor for the protocol developed using phototreatment of *P. acnes*.

Photoinactivation of *P. acnes* by its endogenous porphyrins can be caused by coproporphyrin, the predominant porphyrin found to be produced by this bacterium in this study. In a recent study [40], protoporphyrin and traces of coproporphyrin were found after 7 days of in-

cubation. An increase in these porphyrins was also found after 14 days of incubation. On the other hand, other works have analyzed predominance of coproporphyrin and lower amounts of protoporphyrin [14,27,41]. The amounts of porphyrin may vary with the pH of the growth medium and the length of the incubation time [42]. Our results are in agreement with the latter studies and demonstrate that coproporphyrin is the main product and increases as a function of incubation time up to 96 h (4 days).

A similar phenomenon was observed when ordinary bacterial strains were incubated with ALA, which is the precursor for porphyrin synthesis. In bacterial strains that naturally do not produce porphyrins in high amounts they produce high amounts of porphyrins only upon induction by ALA. Uroporphyrin was found [25] to be the main porphyrin produced in the Gram-positive bacterium *Staphylococcus aureus*. The predominant porphyrin produced in the Gram-negative *Escherichia coli* is also uroporphyrin, but protoporphyrin IX production is also significant. In another strain of *E. coli* it was found [43] that ALA, after a very short incubation time, mainly induces the formation of protoporphyrin IX followed by mesoporphyrin IX and minute amounts of coproporphyrin. In this case, photoinactivation occurring upon illumination by blue light causes photokilling of the bacteria [24,25,28,29]. This phenomenon of porphyrin induction by ALA has been demonstrated in *P. acnes* like in the other bac-

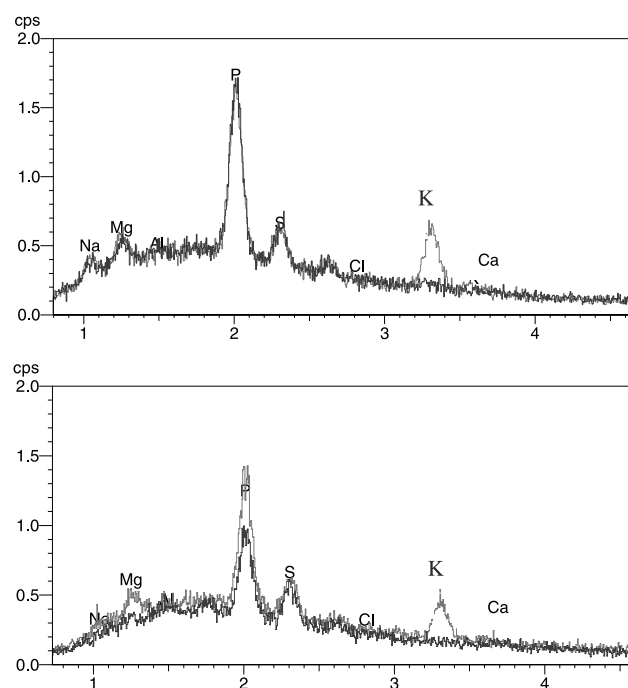


Fig. 5. X-ray elemental spectra of *P. acnes* treated with ALA. Bacteria were incubated with ALA ($100 \mu\text{g ml}^{-1}$) for 24 (upper panel) or 72 h (lower panel). Cultures were illuminated with blue light at a light fluence of 100 or 75 J cm^{-2} , respectively. Cultures incubated with ALA but not illuminated served as controls.

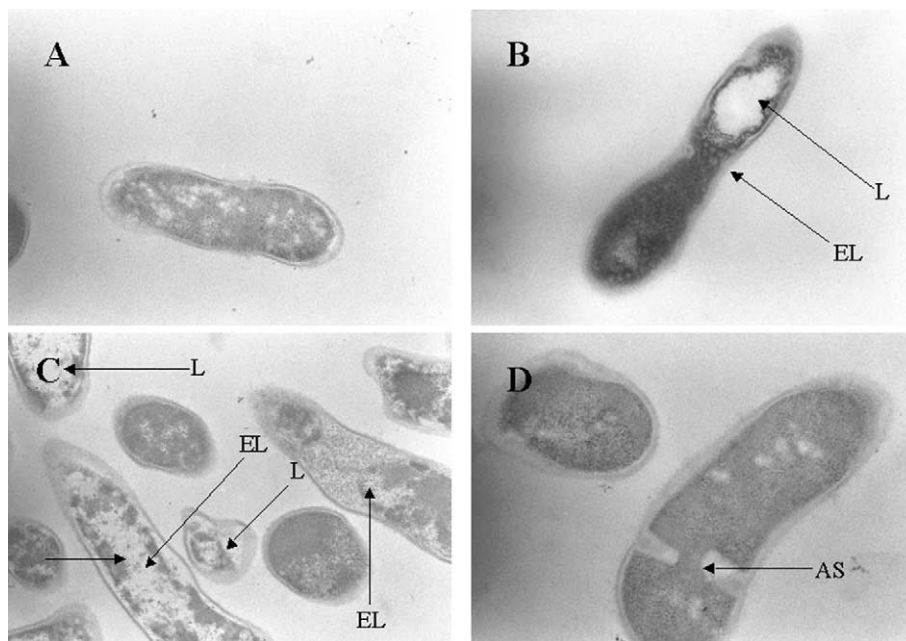


Fig. 6. Effects of illumination by blue light on the ultrastructure of *P. acnes*. Transmission electron micrograph of *P. acnes* cells incubated with ALA ($100 \mu\text{g ml}^{-1}$) for 24 h and illuminated with 407–420 nm intense blue light at a fluence of 100 J cm^{-2} (B,C,D). Arrows are pointing at: asymmetric septation (AS), elongated cells (EL) or leakage (L). Intact cells with no illumination after 24 h of incubation served as controls (A). Magnification was 40 000 for panel A, 30 000 for panels B and C and 50 000 for panel D.

teria that naturally do not accumulate porphyrins. ALA by itself is not toxic and can easily be used for topical treatment of acne [32].

The increase in the amounts of porphyrins in *P. acnes* as a result of ALA induction was significantly above the natural production of this bacterium. From the results shown in this study it is indicated that *P. acnes* can produce and accumulate more porphyrin than it produces naturally. It seems that the maximal natural production is not really the maximal amount that can be found in the bacterium. Furthermore, the same porphyrin (coproporphyrin) was shown to be produced with or without the inducer. From the point of photoinactivation it seems that the greater the intracellular amount of the porphyrin the better are the eradication results.

The efficiency of bacterial photokilling by naturally produced endogenous porphyrins is relatively low and in order to obtain a sharp decrease in viability of *P. acnes* two or even three consecutive illuminations must be given in order to reduce viability by four or five orders of magnitude (respectively). We postulated that since the amount of porphyrins is relatively low, photosensitization may further reduce this amount by destruction of the photosensitizer molecules. The survivors now produce new porphyrin molecules and the second or third illumination will photoinactivate the bacteria by additional orders of magnitude. The above assumption may be proven from the fact that when the bacteria produce more coproporphyrin, as observed when ALA enhances their synthesis, the bacteria are readily photoinactivated and viability decreases by seven orders of magnitude at the same light intensity that causes a decrease of only two orders of magnitude in the

non-ALA-induced *P. acnes*. Furthermore, the amount of porphyrins increases in the bacteria as a function of incubation time. It was demonstrated with ALA-induced *P. acnes* that the older cultures are readily photoinactivated. For example, the viability of cultures incubated with ALA for 24 h and illuminated with 75 J cm^{-2} is decreased by less than two orders of magnitude whereas 48 h cultures decreased by seven orders of magnitude with the same light intensity. Photokilling at seven orders of magnitude was obtained for 72 h cultures even with a 50 J cm^{-2} light intensity (66% less intensity). This leads to the conclusion that the amount of endogenous porphyrin plays a role in maintaining a successful phototreatment of *P. acnes*. Successive illuminations by 407–420 nm blue light exposure may result in a significant cure of patients with acne. In this case no exogenous material is added. It must be mentioned that the number of bacteria in an infected acne wound is several orders of magnitude lower than that in the initial steps of the in vitro experiments. This fact may lead to better possible results upon in vivo trials. Fully cured acne wounds of patients were shown by dermatologists using the successive illumination regimen. Another regimen of trials is by applying ALA on the acne wounds for a certain period and then illuminating. Clinical trials using this regimen are also under investigation, with good preliminary results.

The mechanism by which the photoinactivation of *P. acnes* bacteria occurs is suggested by the results obtained from XMRA and transmission electron microscopy. Illumination of the naturally produced endogenous coproporphyrin or the coproporphyrin induced by ALA resulted in an efflux of potassium from the cells and a

significant loss of phosphate. These results can be interpreted as consequences of bacterial membrane damage, probably to the various *P. acnes* ionic pumps. The free radicals evolved by light-activated porphyrin play a major role in membrane cross-linking disturbance and alteration and may be responsible for diminishing K^+ and ATPase activity and cell death.

Alterations in *P. acnes* cell ultrastructure as a result of the photodynamic effect are consistent with our previous results with other Gram-positive bacteria photosensitized by endogenous [25] or exogenous [44] porphyrins. Ultrastructure alteration may indicate that the perturbations occurring in the photosensitized cell, especially by photosensitizers that are readily available in the bacterial cytoplasm, are conceivably related to the general cytotoxic phenomenon of bacterial cell death.

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