Proton Magic Angle Spinning NMR Reveals New Features in Photodynamically Treated Bacteria

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The bacterium *Propionibacterium acnes* is light-sensitive due to porphyrin-induced photosensitization. The light sensitivity increases with incubation of 5-aminolevulinic acid, ALA. For the first time, ^{1}H magic angle spinning NMR spectroscopy is used to describe the photo-induced changes in the bacterium after ALA incubation. Successful photosensitization was performed with light-emitting diodes in the blue and red regions (430 and 654 nm, respectively). The irradiation setup, suitable for irradiation of bacaterium suspensions in petri dishes is described. For NMR studies blue light diodes with about 90 μmol/m^{2}s were chosen. After blue light irradiation, the endogenous glycine betaine, proline, glutamate and choline levels in *P. acnes* decreased with increasing irradiation time. For sublethal light doses (50% survival fraction), the endogenous glycine betaine level decreased 80% on average. The corresponding percentages for proline, choline and glutamate were about 40, 25 and 10, respectively. It is hypothesized that the irradiation, inducing porphyrin photosensitization amplified by ALA incubation, leads to elimination of the osmolyte glycine betaine and possibly also proline by so-called regulatory volume decrease (RVD) mechanisms. These mechanisms are known to be active in several prokaryotic and eukaryotic cells when exposed to hypotonic stress. They are also known to be present in several eukaryotic cells during photodynamic therapy (PDT) exposure leading to hypotonoc stress. The findings contribute to the knowledge of the inactivation mechanisms of *P. acnes* in photosensitization, and could therefore be of interest in the efforts to use PDT as treatment of the acne disease.

Key words: Light Emitting Diodes, 5-Aminolevulinic Acid, *Propionibacterium acnes*

Introduction

Propionibacterium acnes, *P. acnes*, is closely connected with the skin disease acne vulgaris (Cunliffe, 1989; Cunliffe and Goulden, 2000). However, details of its participation in the acne mechanisms still need to be elucidated. The Gram-positive bacteria produce several porphyrins (Romiti *et al.*, 2000; Johnsson *et al.*, 1987; Kjeldstad *et al.*, 1984) and these are accumulated to a concentration which makes the bacteria fluorescent when irradiated with broad-band near UV-light (so-called Woods light). The porphyrins, furthermore, make the bacteria light-sensitive and several investigations point to light destruction mechanisms that are mediated via excited porphyrins and subsequent singlet oxygen reactions (Kjeldstad *et al.*, 1984; Kjeldstad and Johnsson, 1986; Melø and Reisæther, 1986; Arakane *et al.*, 1996; Hirobe, 1996).

Much interest has been focused on the detailed mechanisms starting when *P. acnes* is irradiated with visible light as well as with near-UV and UV-light. The cellular reactions upon light irradiation seem to be complex, involving pH changes across the membrane, calcium transport and changes in cellular calcium concentration etc. (Futsæther *et al.*, 1995; Ramstad *et al.*, 1997). A ^{31}P NMR study (Kjeldstad and Johnsson, 1987) showed that the bacteria were fairly easy to investigate with this technique and that light-induced changes in cellular pH and polyphosphate concentration could be monitored.

One rate-limiting step in the porphyrin production in cell systems is controlled by 5-aminolevulinic acid synthetase, ALA synthetase. This has been shown in eukaryotes and is the basis for the so-called ALA-based photodynamic therapy (ALA-PDT) of cancer (Henderson and Dougherty, 1992). Since light and ALA can easily be

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applied to the skin, PDT is of particular interest in dermatology (Kalka *et al.*, 2000). ALA is also taken up by *P. acnes* (Ramstad *et al.*, 1997). The light sensitivity of the bacateria increases correspondingly and there is a current interest in the possibilities to use light in the treatment of the acne disease, with or without ALA administered to the skin (Hongcharu *et al.*, 2000; Cunliffe and Goulden, 2000; Papageorgiou *et al.*, 2000; Itoh *et al.*, 2000; Zeina *et al.*, 2001).

We wanted to extend our studies of the light reactions in the *P. acnes* bacteria and its relation to uptake of ALA by using ^{1}H NMR magic angle spinning (MAS) spectroscopy. MAS can increase the signal to noise ratio in ^{1}H NMR spectra where this ratio is low due to anisotropic dipolar interactions (Andrew, 1995). The application of this method in ^{1}H NMR spectroscopy of intact cells is fairly new (Weybright *et al.*, 1998) and never performed on *Propionibacteria* before, as far as the authors know. We, therefore, wanted to use this technique to investigate light-induced reactions in *P. acnes* and cellular mechanisms initiated by ALA-induced porphyrins. This was done by studying ^{1}H NMR spectra of *P. acnes* exposed to increasing doses of PDT. Finally, we describe an efficient and handy light source based on light emitting diodes (LEDs) that was used in the light treatment of the bacteria.

Materials and Methods

Culturing and collection of P. acnes

P. acnes (American Type Culture Collection, ATCC 6919) used in the present experiments were incubated on blood agar, and regularly replated every week. The blood agar plates were stored at 37 °C in dark, anaerobic environment.

The replated stock culture was about 4 to 5 days old when bacteria were transferred to petri dishes with bactoagar (Merck Darmstadt, Germany) enriched with ALA (1.8 mM). The petri dishes were then incubated in dark, anaerobic environment at 37 °C for ca. 70 h. The bacteria were collected by adding 2 ml sterile PIPES (1,4-Piperazinediethanesulfonic acid) buffer to each petri dish, and a sterile glass rod was used to scrape off the bacteria. The bacteria were then centrifuged at $2600 \times g$ for 5 min, and the pellet resuspended in PIPES buffer to O. D. 1.5. Finally 20 ml of the suspension was transferred to a petri dish with a diameter of 5 cm for irradiation.

The light exposure device

A light source was constructed using arrays of LEDs. LEDs constitute an interesting light source in PDT treatment etc. due to its advantages compared with several other light sources etc. (Drollette, 2000). Light weight, sturdiness, cheapness and often high radiation output are important features as well as the wavelength ranges nowadays available, from blue light to IR.

The irradiation of the *P. acnes* bacteria was done *in vitro* in petri dishes and represented an efficient and simple way to irradiate a suspension of cells. The irradiation system consisted of a LED array, four fans (2.2 W when operated at 12 V), a petri dish holder under a DC-motor platform rotating at 5 rpm, and a plexiglas chassis. The distance between the LED array and the bottom of the petri dish was 1.8 cm. The fans kept the LED array and the petri dish at the controlled room temperature. A polished aluminum mirror (97% reflection) was placed above the petri dish to increase the irradiance. Rotating the petri dish holder ensured that all bacteria were exposed to the same average level of light. The chassis rested on rubber pads to reduce any vibrations transported from the fan system.

Blue and red light arrays were used and had dimensions of 7.8 cm × 7.2 cm, each comprising 14×12 LEDs. The LEDs had peak emission at 430 nm (EL383UB/H2), Everlight, Cary, NC) or at 654 nm (HLMP-C124, Agilent Technologies, Palo Alto, CA), and bandwidths of about 65 nm and 30 nm, respectively. The LEDs were driven at recommended maximum forward currents of 50 mA and 20 mA, respectively. The light emitted from these arrays were measured in a plane at a distance of 1.8 cm from the array. Light measurements were performed with a quantum sensor (Q 24466 from LI-COR, Lincoln, NE) and the quantum flux of the blue light array is presented in Fig. 1. The arrays were fairly homogeneous and the intensitites in the center region varied with ca. 10% in both arrays.

Temperature was measured in a test suspension of O. D. 1.5 before and after light exposure, confirming that the temperature of the suspensions

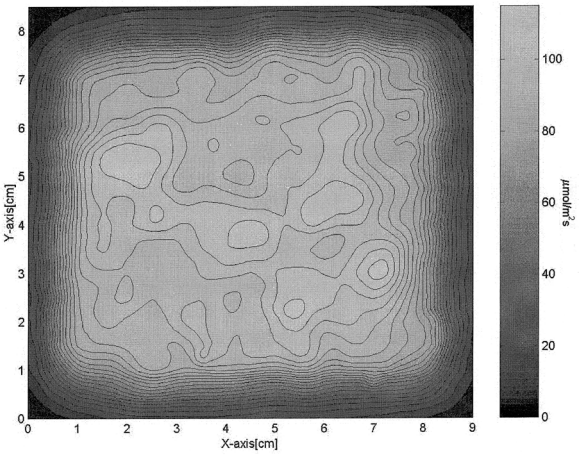

Fig. 1. Irradiation contours of the blue light LED array used in the experiments. The irradiance from the array was measured (in μmol/m^{2}s) at the height of the petri dishes and plotted in a contour map. The scale to the right indicates the irradiation levels. The contour increment is 5 μmol/m^{2}s. The irradiation level in the centre area (with a diameter of about 5 cm) is fairly homogeneous, and rotation of the dish averages out the remaining variations.

remained fixed at room temperature through the test session. by use of an IR camera (ThermaCAM PM 595, FLIR Systems Inc, North Billerica, MA) it was also documented that the petri dishes transmitted no IR radiation.

Irradiation contours of blue light LED array used in the experiments

The irradiance from the array of blue light LED's used in the experiments was measured (in μmol/m^{2}s) at the height of the petri dishes and plotted in a contour map. The scale to the right indicates the irradiation levels. The contour increment is 5 μmol/m^{2}s. The irradiation level in the centre area with a diameter of about 5 cm is fairly homogeneous and rotation of the dish averages out the remaining variations.

Blue and red light inactivation of P. acnes

Counting of bacterium colonies was performed to determine the proper light dose to be used in the NMR experiments. The bacteria were grown on bactoagar containing 1.8 mM ALA. The intensity of the light arrays was kept constant and the duration of the light exposure was varied to change the dose.

The irradiance of the blue and red light at 1.8 cm distance were approx 100 μmol/m^{2}s and 1800 μmol/m^{2}s, respectively. Compensating for absorption in the petri dish, the respective irradiances falling onto the bacteria suspensions directly from the LED arrays were about 90 μmol/m^{2}s and 1640 μmol/m^{2}s. There was also a contribution to the total irradiance from the light transmitted in the suspension and then reflected by the mirror.

Bacteria for NMR spectroscopy were irradiated with blue light for 0, 1.5, 3, 5 or 10 min. For light exposures shorter than 10 min, the bacteria were left unexposed to a total time of 10 min before transfer to the NMR tubes. This was done to keep the time from collection of bacteria to acquisition of NMR spectra constant, and avoid effects from changes in metabolism due to transition from anaerobic to aerobic conditions. After irradiation the bacteria were washed once with PBS (5% D_2O) and transferred to 4 mm ZrO_2 NMR MAS tubes with spherical inserts.

^{1}H MAS NMR spectroscopy

The spectrometer used was a Bruker DMX-600 Avance NMR spectrometer equipped with a high resolution MAS probe. A MAS spin rate of 4300 Hz was used. 1D spectra were acquired using 45° pulses (5 μsec), 1.32 sec acquisition time, 3 sec relaxation time and 128 scans. 1 Hz exponential line broadening was applied before Fourier transform. Homonuclear chemical shift correlated spectroscopy (COSY) and total correlation spectroscopy (TOCSY) spectra were acquired with 350 F1 increments, 16 scans, an acquisition time of 128 msec, a relaxation time of 2 sec and a spectral width of 13 ppm. In COSY recordings, shaped gradient pulses were used for coherence selection. In the phase-sensitive (TPPI) TOCSY experiments a Hartman Hahn transfer with a MLEV 17 mixing sequence was used (Bax and Davis, 1985). Sine und qsine window functions were applied in COSY and TOCSY, respectively. Water presaturation was applied in all recordings. The acquisition sequence for the 1D spectra were about 10 min, and the length of the COSY and TOCSY recordings was 185 and 207 min, respectively. The temperature during the experiments was about 21 °C.

Results and Discussion

The purpose of this study was to identify changes in ^{1}H NMR resonance signals of *P. acnes* treated with ALA and irradiated with light from light-emitting diodes. Most of the identification work on the NMR spectra is described in Johansen *et al.* (in preparation), but an overview is presented.

The survival curves for the ALA-incubated bacteria, irradiated with blue and red LED light, are given in Fig. 2. The irradiation levels chosen, based on recommended LED currents, produced increased cell destruction for both 430 nm and 654 nm light. A survival fraction of 10% was achieved after about 4 and 25 min, respectively (40% after about 2 and 5–6 min, respectively). The blue LED array was used in the NMR experiments in order to keep irradiation time short, yet achieving clear photosensitization effects.

We did not make an effort to find red LED's with an optimum wavelength in the red part of the spectrum, since the detailed absorption spectrum of the photosensitizers (probably mainly protoporphyrin IX (Kjeldstad *et al.*, 1984)) in an *in vivo* situation is not known. We, therefore, demonstrated the NMR effects by choosing a blue light LED that was efficient in producing spectral changes. Corresponding changes were also found in the red light experiments, although optimum wavelengths were not pinpointed (635 nm is used in some studies).

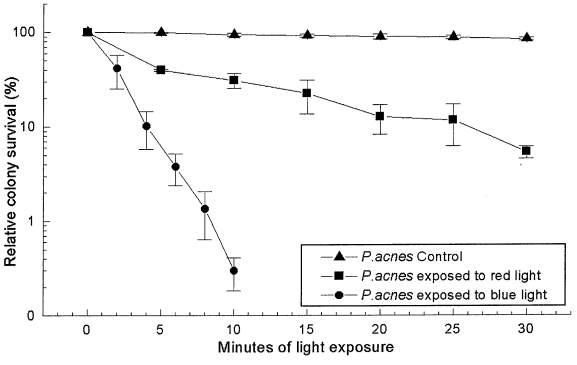

Fig. 2. Survival of *Propionibacterium acnes* after irradiation with blue and red LED light. The curves show percentage of surviving cells on non-irradiated controls (triangles), of cells treated with blue light (430 nm, 90 μmol/m^{2}s; circles) and of cells treated with red LED light (654 nm, 1640 μmol/m^{2}s; squares). Means ± S. E. M. of n = 3 repetitions are plotted.

The experiments showed that the *P. acnes* cells could be successfully studied in ^{1}H MAS NMR experiments. Spinning the cells at 4.3 kHz for at least 2 h was not detrimental and the MAS spectra that were acquired were possible to interpret and identify. The spectra were calibrated by the singlet resonance peak of succinate, at chemical shift 2.39 ppm (Willker *et al.*, 1996; Fan, 1996). All spectra were automatically base line corrected. Identification of metabolites were performed with the aid of published spectra (Willker *et al.*, 1996; Fan, 1996; Ferretti *et al.*, 1999; Weybright *et al.*, 1998) and 2D COSY and TOCSY NMR spectra of bacteria spun at 4.3 or 10 kHz (not shown; Johansen *et al.*, in preparation). The pH of the NMR buffer was 7.4, assumed to give a weakly acidic environment in the bacteria (Futsæther *et al.*, 1993). Fig. 3, upper curve, shows a 1D ^{1}H MAS NMR spectrum of the bacteria, with identification of some of the peaks. Metabolites identified in ^{1}H MAS NMR spectra are listed in Table I.

P. acnes incubated on ALA enriched bactoagar was exposed to blue light for different periods of time. Consistent changes in the spectra, varying with the exposure dose were achieved. Three sets of light exposure experiments were performed, each set consisting of five samples of ALA-incubated *P. acnes* that had been given different doses of light treatment.

Since no external standard could be inserted into the MAS sample holder, only relative integ-

Table I. Resonances identified in ^{1}H MAS NMR spectra of *P. acnes*.

Metabolite	ppm
Acetate	1.92
Succinate	2.39
Propionate	1.05, 2.17
Glycine betaine	3.25, 3.91
Choline (mainly)	3.23
Alanine	1.45, 3.75
Aspartate	3.94, 2.74, 2.82
Glutamate	2.33, 2.07, 3.76
Isoleucine	1.03, 2.01
Leucine	0.97, 1.71, 1.75
Lysine	1.65, 3.02
Proline[a]	3.33, 3.40, 4.14
Valine	0.95, 1.01, 2.23
Polyamines	~ 1.70, ~ 3.00 (1.73, 3.05)

[a] Possible overlap with taurine at 3.33 and 3.40 ppm.

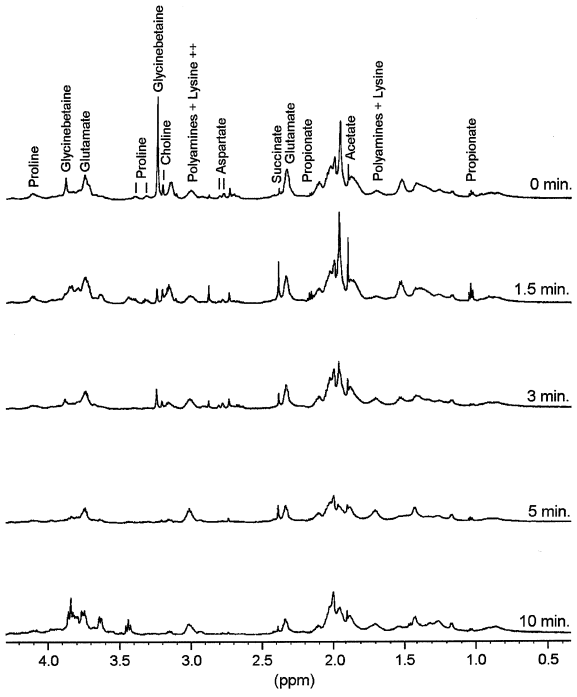

Fig. 3. ^{1}H MAS NMR spectra, before and after irradiation of ALA-treated *P. acnes*. the uppermost curve shows the MAS NMR spectrum of non-irradiated but ALA-incubated *P. acnes* cells (controls). Peaks are identified. Corresponding spectra from cells irradiated (blue LED array) for 1.5, 3, 5 and 10 min are shown below the control curve. The peaks of glycine betaine (3.25 ppm), proline (3.33 ppm), glutamate (2.33 ppm) and choline (3.23 ppm) were integrated and normalized in order to quantify the systematic decrease, cf. Fig. 4. The signals emerging at 3.4–3.9 ppm after 10 min of irradiation were not identified. Resonances in this area sometimes appeared after 3 or 5 min of irradiation as well, and did not seem to be related to the irradiation time.

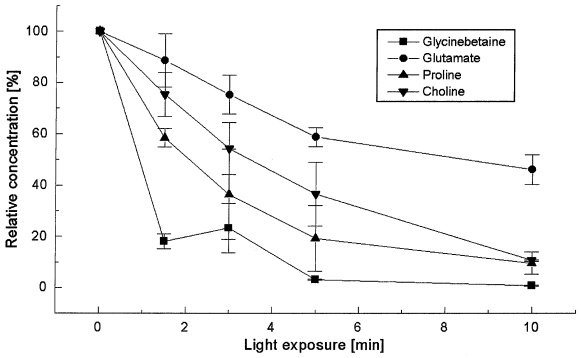

Fig. 4. Decay curves of *P. acnes* metabolites after blue light LED treatment. The light inactivation of *P. acnes* are accompanied with changes in the MAS NMR peaks. Light exposure (430 nm) for times indicated reduces the peaks of glycine betaine, glutamate, proline and choline as indicated. Means +-S. E. M. of n = 3 repetitions are plotted.

rals of spectral peaks could be calculated. The signals were normalized to the integral of the spectral region 4.5 to 0 ppm (Fig. 4). It should be noted, that as the total spectrum signal decreased with increasing light exposure, the relative integrals will indicate erroneously high compound levels. Therefore, the relative compound levels plotted in Fig. 4 are even less than indicated. This figure shows that the concentration of glycine betaine (3.25 ppm), glutamate (2.33 ppm), proline (3.33 ppm) and choline (3.23 ppm) decrease rapidly with increased irradiation time. The same tendency is also seen in Fig. 3 for several other resonance signals as well (*e. g.* 2.82 and 2.74 ppm). Glycine betaine and proline seem to respond fastest and to the largest extent. This is interesting, as both proline and glycine betaine have been reported to be effective osmolytes (Rudulier *et al.*, 1984). After 1.5 min of blue light exposure, glycine betaine was reduced to about 18%. After 5 or 10 min of light exposure it was almost absent.

Control spectra were acquired to ensure that the changes seen in the spectra of ALA-incubated *P. acnes* when exposed to light, truly originated from the photosensitization effects. Spectra were acquired from *P. acnes* incubated on plain bactoagar before and after 30 min of blue light exposure. From each prepared *P. acnes* sample a 1D ^{1}H NMR, a COSY and a TOCSY spectrum were acquired. Only small changes were seen in the 2D spectra, but in the 1D spectra, a 50% decrease in the intensity of the glycine betaine signal was detected in response to irradiation (data not presented). As discussed in the literature (cf. Introduction) the amount of endogenous porphyrins is fairly high in *P. acnes*, and photosensitization occurs after irradiation even if ALA is not administrated (Kjeldstad and Johnsson, 1986). Still, a dose of 30 min irradiation at 90 μmol/m^{2}s of blue light was necessary to produce this change when ALA was not supplied to the *P. acnes* cells. Hence, it is likely that the changes seen in ALA-incubated cells are caused by PDT effects.

In the ^{1}H MAS spectra of *P. acnes*, reduced concentrations of glycine betaine, proline, choline and glutamate were found after ALA-induced photo-

sensitization. Some leakage from the cells may have occurred. However, at low light doses, corresponding to 50% inactivation, there was an 80% reduction in the glycine betaine signal, which cannot be explained purely by leakage. This points to a rapid and active mechanism for the removal of glycine betaine.

Glycine betaine and proline are known osmolytes (Boyaval *et al.*, 1999; Rudulier *et al.*, 1984). In particular glycine betaine has been extensively studied in *E. coli* and been proven to have osmoprotective abilities (Rudulier *et al.*, 1984). Recent results (Johansen *et al.*, in preparation) indicate that this is true for *P. acnes* as well. It is, therefore, not unlikely that the glycine betaine and proline effects seen in the present pohotoinactivation experiments are consequences of rapid intracellular regulation of osmotic pressure.

Since *P. acnes* eliminates glycine betaine and proline, it seems as if photoinduced porphyrin sensitization can induce hypotonic stress (swelling) in *P. acnes*. Malik *et al.* (1993) postulated that photochemical reactions release protein-bound K^+ and that the concentration of free K^+ ions increases as photosensitization proceeds. This hypothesis was based on *i. a.* measurements showing K^+ efflux in PDT treated bacteria, and erythocytes. K^+ efflux during photoinduced porphyrin sensitization has also been reported for many other cell types and photosensitizers (Murphy, 1988; Paardekooper *et al.*, 1995), and K^+ efflux is a typical regulatory volume decrease (RVD) mechanism.

In the *P. acnes* experiments, the amino acids vanished in a slow manner as compared to glycine betaine, and their decrease might be an effect of other mechanisms than RVD processes. Some bacteria metabolize amino acids (Madigan *et al.*, 2000) with fatty acids and putrescine as end products. Both of these compounds were identified in *P. acnes*. Photooxidation of the amino acids could also be an explanation, but such reactions have not been reported to be particularly effective for the amino acids discussed (Spikes, 1989).

Osmotic stress thus adds to other photo induced inactivation mechanisms of the cells. Photo induced production of singlet oxygen and subsequent reaction sequences (as for instance membrane damages), are likely to be the main cause for inactivation (Arakane *et al.*, 1996; Romiti *et al.*, 2000; Kjeldstad and Johnsson, 1986, and references mentioned in the Introduction). However, even if the cell does not undergo lysis, irreversible K^+ loss may be considered as the initial step toward cell death (Malik *et al.*, 1993). Therefore the photoinduced hypotonic stress should be further investigated to unveil its part in the total picture of cellular inactivation. This will also be of importance from clinical aspects if photo induced treatment of acne will be introduced in the future.

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