Photodynamic destruction of Haemophilus parainfluenzae by endogenouslys produced porphyrins

van der Meulen, F.W.; Ibrahim, K.; Sterenborg, H.J.C.M.; van Alphen, A.J.W.; Maikoe, A.; Dankert, J.

DOI
10.1016/S1011-1344(97)00057-2

Publication date
1997

Published in
Journal of Photochemistry and Photobiology B-Biology

ELSEVIER

Journal of Photochemistry and Photobiology B: Biology 40 (1997) 204–208

Journal of PHOTOCHEMISTRY AND PHOTOBIOLOGY B:BIOLOGY

Photodynamic destruction of *Haemophilus parainfluenzae* by endogenously produced porphyrins

F.W. van der Meulen [a,c,*], K. Ibrahim [a,b], H.J.C.M. Sterenborg [a], L.V. Alphen [b], A. Maikoe [b], J. Dankert [b]

[a] *Laser Centre, Academic Medical Centre at the University of Amsterdam, Meibergdreef 9, NL-1105 AZ Amsterdam, Netherlands*
[b] *Department of Medical Microbiology, Academic Medical Centre at the University of Amsterdam, Amsterdam, Netherlands*
[c] *Department of Otolaryngology, Academic Medical Centre at the University of Amsterdam, Amsterdam, Netherlands*

Received 13 May 1997; accepted 29 May 1997

Abstract

Bacterial resistance against antibiotic treatment is becoming an increasing problem in medicine. Therefore methods to destroy microorganisms by other means are being investigated, one of which is photodynamic therapy (PDT).

It has already been shown that a variety of Gram-positive and Gram-negative bacteria can be killed in vitro by PDT using exogenous sensitizers. An alternative method of photosensitizing cells is to stimulate the production of endogenous sensitizers. The purpose of this study was to investigate the bactericidal efficacy of PDT for *Haemophilus parainfluenzae* with endogenously produced porphyrins, synthesized in the presence of δ-aminolaevulinic acid (δ-ALA). *H. parainfluenzae* incubated with increasing amounts of δ-ALA showed decreased survival after illumination with 630 nm light. No photodynamic effect on the bacterial viability was found when *H. parainfluenzae* was grown without added δ-ALA. *H. influenzae*, grown in the presence of δ-ALA, but not capable of synthesizing porphyrins from δ-ALA, was not affected by PDT. Of the range of incident wavelengths, 617 nm appeared to be the most efficient in killing the bacteria. Spectrophotometry of the bacterial porphyrins demonstrated that the maximum fluorescence occurred at approximately 617 nm, with a much lower peak around 680 nm. We conclude that a substantial killing of *H. parainfluenzae* by PDT in vitro after endogenous sensitization with δ-ALA can be achieved.

Keywords: δ-Aminolaevulinic acid; *Haemophilus parainfluenzae*; Photodynamic therapy; Porphyrin

1. Introduction

Since bacterial resistance against antibiotic treatment is becoming an increasing problem, research is being directed towards other approaches to achieve bacterial killing, including photodynamic therapy (PDT) [1]. PDT involves the activation of a photosensitive drug by (laser) light, which in turn induces cytotoxic events, leading to cell death [2]. PDT is clinically mainly applied in oncology, but its use in benign diseases is increasing.

In vitro, it has been shown that various Gram-positive bacteria can be killed by PDT with exogenous sensitizers, mainly porphyrins [3–5]. Until recently, Gram-negative bacteria appeared to be insensitive to the lethal action of PDT with exogenously supplied porphyrins, because, hindered by the presence of the bacterial outer membrane, these sensitizers could not be transported into the cells [4,5]. However, it has been demonstrated that *Escherichia coli* sensitized by phthalocyanines, after pre-treatment with Tris–EDTA or by the induction of competence to induce bacterial outer membrane alterations, can be rendered susceptible to photodynamic inactivation [6]. Moreover, it has been shown that, after the enhancement of the permeabilization of the outer membrane by exposure to polymyxin B nonapeptide (PMBN), exogenous sensitizers can enter bacteria. After subsequent illumination, such bacteria are killed [7]. In addition, certain Gram-negative bacteria can be photosensitized effectively without any pre-treatment by cationic water-soluble dyes, such as meso-substituted porphyrins and zinc phthalocyanine [8–10].

In addition to using exogenous sensitizers, cells can be photosensitized by enhancing their endogenous production of porphyrins by adding δ-aminolaevulinic acid (δ-ALA), a naturally occurring metabolite in the synthesis of cellular haem. The addition of δ-ALA leads to an increase in the

* Corresponding author. Tel.: 00-31-20-5664386; fax: 00-31-20-6975594.

1011-1344/97/$17.00
PII S1011-1344(97)00057-2

synthesis of uroporphyrin, coproporphyrin and protoporphyrin IX (PpIX), the immediate precursor of haem. PpIX produced in this way appears to be clinically useful in the photodynamic treatment of certain skin cancers [11].

In contrast with (exogenously administered) hydrophobic porphyrins, δ-ALA is a water-soluble substance and may therefore enter the intracellular compartment of Gram-negative bacteria through hydrophilic pores in the outer membrane. Some bacterial species, including *Haemophilus parainfluenzae*, can synthesize porphyrins from δ-ALA [12]. The lack of enzymes in *H. influenzae* is used to discriminate between these *Haemophilus* species [13].

H. influenzae causes upper and lower airway infections, systemic infections (epiglottitis, meningitis, cellulitis and arthritis) and conjunctivitis. *H. parainfluenzae* is less pathogenic, but sometimes causes infections of the respiratory tract and bacterial endocarditis.

This study was undertaken to determine whether endogenously produced porphyrins would render *H. parainfluenzae* susceptible to PDT. This Gram-negative microorganism was taken as a model since, in a recent study, the production of porphyrins by this bacterium in the presence of an excess of δ-ALA has been well characterized [14].

2. Materials and methods

2.1. Bacterial strains and growth

Two strains of *H. parainfluenzae* (strain A 920055 and A 900103) and one *H. influenzae* strain (strain D 1) were kept frozen at −70 °C, and, after thawing, were plated onto chocolate agar and incubated for 24 h at 37 °C in a CO_2 incubator. Subsequently, bacteria were suspended in sodium phosphate buffer containing 0.15 M NaCl (pH 6.9) (PBS) at a density of 10^8 colony forming units (CFUs) per millilitre. The number of CFUs was determined by counting the number of colonies on plates inoculated with aliquots from tenfold serial bacterial dilutions after overnight incubation.

2.2. Incubation with δ-ALA

δ-ALA (Lamers and Pleuger's, Hertogenbosch, Netherlands) was used at various concentrations (Fig. 1). The final concentration was 2 mM in 10 ml 10 mM PBS. During incubation for 4 h, the culture flasks were kept at 37 °C with shaking at 180 rev min^{-1}. To avoid any precocious photodynamic activation, the flasks were wrapped in aluminium foil and all samples were kept in the dark.

2.3. Fluorescence measurements

After incubation for 4 h, the fluorescence spectrum of the porphyrins produced was determined using a fluorescence spectrophotometer (Perkin–Elmer 650-40). The excitation wavelength used was 405 nm. *H. parainfluenzae* incubated in PBS without δ-ALA and *H. influenzae* incubated in PBS with δ-ALA served as controls. In one experiment, the bacterial fraction was separated from the surrounding medium by centrifugation at 5000 rev min^{-1} for 10 min. After the supernatant had been removed, the bacterial pellet was resuspended in PBS and the fluorescence spectra of both components were obtained.

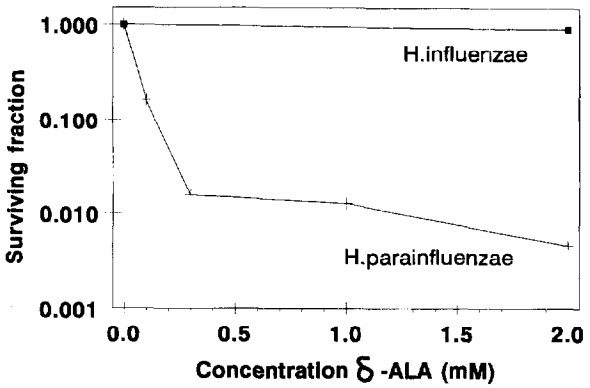

Fig. 1. Surviving fraction of *H. parainfluenzae* after incubation with various concentrations of δ-ALA in PBS for 4 h and subsequent illumination. The light dose was 50 J cm^{-2} at a wavelength of 630 nm. *H. influenzae* was taken as control.

2.4. Photoactivation

After incubation, aliquots (1 ml) of the samples and controls were transferred to clear polystyrene cuvettes and subsequently illuminated with a beam from an argon dye laser (Spectra Physics 2040 argon laser pumping a model 375 dye laser) set at 630 nm. The power density used was 150 mW cm^{-2}; the light dose delivered and wavelength varied according to the experimental design. Tubes were wrapped in aluminium foil to avoid unintentional exposure to light. *H. parainfluenzae* suspensions without δ-ALA and *H. influenzae* suspensions with δ-ALA were used as controls and exposed to 500 J cm^{-2} of light.

Bacterial survival after photoactivation was determined by comparing the number of CFUs before and after photoactivation. Survival was calculated as follows: N_1/N_0, where N_0 is the number of CFUs per millilitre in samples kept in the dark and N_1 is the number of CFUs per millilitre in the illuminated samples. All experiments were performed in triplicate.

3. Results

3.1. Bacterial survival after photoactivation

In order to determine whether excitation of the porphyrins produced could generate a lethal photodynamic effect on *H. parainfluenzae*, bacterial suspensions in PBS with various concentrations (up to 2 mM) of δ-ALA were illuminated with a standard dose of 50 J cm^{-2} at 630 nm after 4 h of incubation. Subcultures showed that the number of CFUs decreased with increasing concentration of δ-ALA above 0.3

mM (Fig. 1). No detrimental influence of light on the viability of *H. parainfluenzae* incubated in PBS without δ-ALA was noted. *H. influenzae* incubated in PBS with 2 mM δ-ALA and subsequently illuminated did not show a decreased survival. Bacteria incubated in PBS without δ-ALA and subsequently exposed to 500 J cm^{-2} did not show a decreased survival.

3.2. Effect of the incident wavelength on bacterial killing

Subsequently, the influence of the incident wavelength on bacterial killing was studied. A light dose of 10 J cm^{-2} was chosen as a suboptimal dose for comparison of the effect of various wavelengths on bacterial killing. As shown in Fig. 2, the maximum efficacy of killing of *H. parainfluenzae* was found to be around 615 nm. Irradiation with an argon laser beam (514 nm) was less efficient in killing the microorganisms, and wavelengths above 640 nm were not effective in this respect. Illumination at 630 nm, the wavelength commonly applied to excite porphyrins, appeared to be relatively inefficient for killing *H. parainfluenzae*.

In order to study the influence of the wavelength on the bactericidal activity more quantitatively, the survival of *H. parainfluenzae* after illumination with two different wavelengths, i.e. 617 nm and 630 nm, was compared (Fig. 3). An

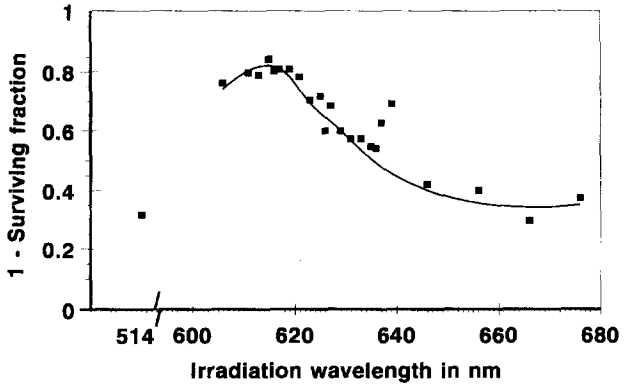

Fig. 2. Effect of the variation in wavelength on the killing of *H. parainfluenzae* after incubation with δ-ALA (2 mM). Effectiveness is depicted as 1 − surviving fraction (left scale). The light dose applied was 10 J cm^{-2}.

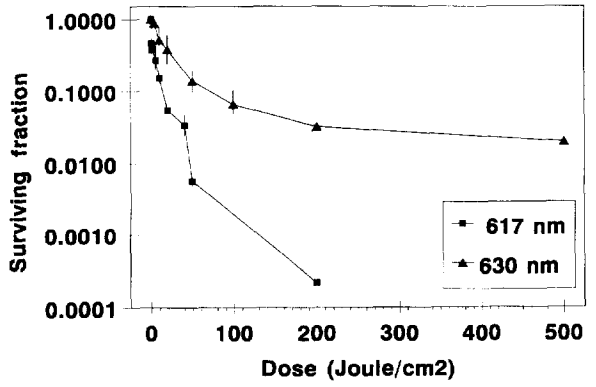

Fig. 3. Surviving fraction of *H. parainfluenzae* illuminated with various light doses at two different wavelengths. Bacteria were incubated with δ-ALA (2 mM) for 4 h prior to illumination.

increase in the light dose of either wavelength led to a progressive loss of viability of the microorganisms. Irradiation at 617 nm yielded the most pronounced effect. With a dose of 200 J cm^{-2} of 617 nm light more than 99.9% of the exposed *H. parainfluenzae* were killed, whereas irradiation with 630 nm led to the killing of less than 96% of the exposed inoculum.

3.3. Fluorescence spectrum of synthesized porphyrins

In addition, the fluorescence spectrum of the porphyrins synthesized by *H. parainfluenzae* in PBS with δ-ALA (2 mM) after incubation for 4 h at pH 6.9 was determined. The spectrum demonstrated a high fluorescence intensity around 617 nm, accompanied by a lower peak around 680 nm (Fig. 4). Furthermore, fluorescence measurements were performed during the course of the illumination of a suspension of *H. parainfluenzae* prepared as mentioned above. Fig. 5 shows a gradual decrease in the fluorescence with increasing light doses of 50, 100 and 200 J cm^{-2}.

In order to determine whether the porphyrins, released into the medium, differ from those present in or bound to the bacteria, fluorescence measurements of the medium and of

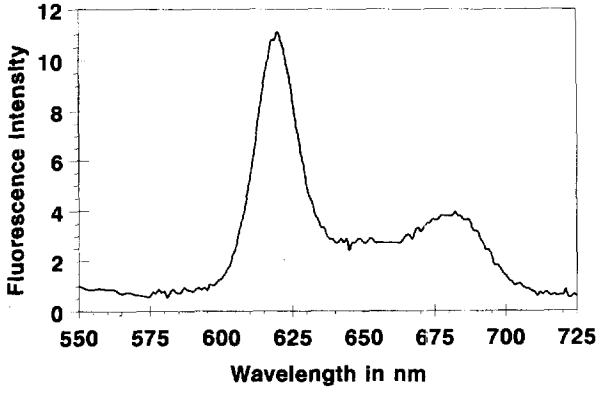

Fig. 4. Fluorescence spectrum of *H. parainfluenzae* after 4 h of incubation with δ-ALA (2 mM) in PBS at pH 6.9. Emission is presented in arbitrary units (a.u.).

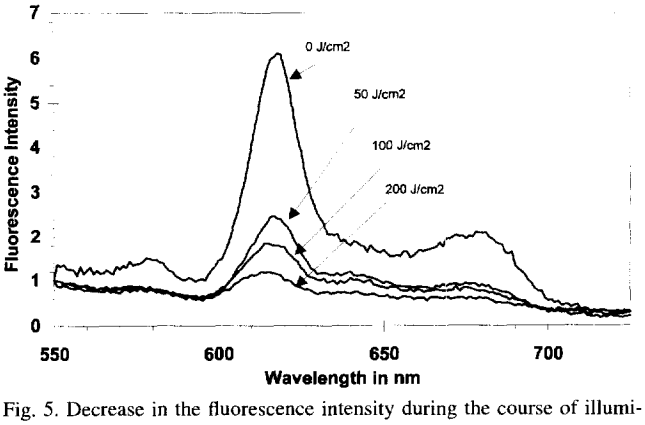

Fig. 5. Decrease in the fluorescence intensity during the course of illumination of *H. parainfluenzae*, incubated for 4 h with 2 mM δ-ALA in PBS at pH 6.9. The fluorescence intensity is shown after illumination with 0, 50, 100 and 200 J cm^{-2}. The emission is presented in arbitrary units.

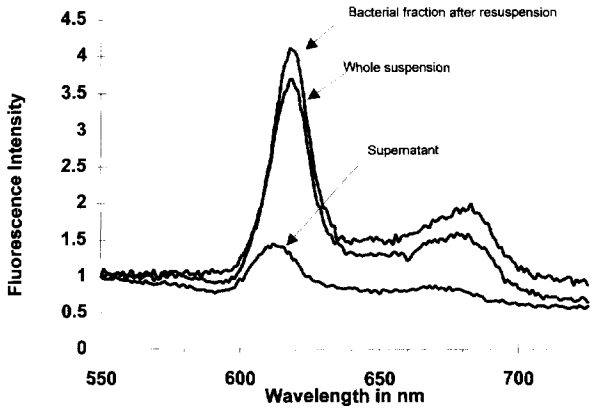

Fig. 6. Fluorescence spectra of the bacterial suspension, bacterial pellet and supernatant of *H. parainfluenzae* after 4 h of incubation with δ-ALA (2 mM) in PBS at pH 6.9. The emission is presented in arbitrary units.

the bacterial fraction were performed separately. For this purpose, a suspension of endogenously sensitized *H. parainfluenzae* was centrifuged, and the fluorescence spectra of the supernatant and the bacterial pellet, after resuspension in PBS, were determined. From Fig. 6, it appears that the maximum fluorescence is detectable in the bacteria compared with the surrounding medium. Furthermore, the spectrum of the resuspended bacterial pellet demonstrates the same peaks as the suspension as a whole. The spectrum of the supernatant shows a slightly different peak around 613 nm.

No fluorescence of *H. parainfluenzae* incubated in PBS without δ-ALA and *H. influenzae* incubated in PBS with δ-ALA (2 mM) was noted, indicating that porphyrin production was absent.

4. Discussion

The synthesis of porphyrins by *Haemophilus* species was initially reported in 1963 [15]. Thirty years later, Luppa et al. [14] analysed and quantified the porphyrin production more accurately using high-performance liquid chromatography. The type of porphyrin synthesized by *H. parainfluenzae* in the presence of excess δ-ALA appears to be strongly pH dependent. At pH 6.9, uroporphyrins and coproporphyrins constitute the majority of the metabolites produced, whereas hardly any protoporphyrins are observed [14]. Since we used the same experimental conditions as Luppa et al. [14], the production of mainly uroporphyrins and coproporphyrins was expected. The outcome of the fluorescence measurements (Fig. 4) is in accordance with these findings: uroporphyrins in PBS show peaks at 618 and 680 nm, and coproporphyrins display maximum fluorescence at 617 and 680 nm. Furthermore, after centrifugation, the bacterial fraction shows the same fluorescence spectrum as the suspension as a whole. If the rather lipophilic PpIX were present within the bacteria, we would expect the pellet to display a fluorescence peak at 635 nm, which was not detectable (Fig. 6). Illumination of *H. parainfluenzae* incubated with δ-ALA (2 mM) leads to bacterial cell death of over 99% of the population (Fig. 1). This photodynamic effect is similar to that demonstrated in experiments with *Staphylococcus aureus* and *Streptococcus faecalis*, sensitized with exogenously supplied porphyrins [3–5]. Gram-negative bacteria can be killed by PDT through this "exogenous" route after pre-treatment to alter the outer membrane composition [6,7], or by bacterial sensitization with cationic water-soluble dyes, as recently shown in Refs. [8–10]. In this study, we have demonstrated the feasibility of the killing of a Gram-negative bacterium by "endogenous sensitization". For those Gram-negative bacteria, such as *Pseudomonas aeruginosa*, *E. coli* and *Klebsiella pneumoniae* [12], capable of producing porphyrins in the presence of δ-ALA, this opens up a route for the sensitization of bacteria to photodynamic inactivation.

Further PDT experiments were carried out using the same concentration of ALA (2 mM) as Luppa et al. [14]. In this way, the composition of the porphyrins produced by *H. parainfluenzae* was assumed to be known. The choice of a suboptimal light dose of 10 J cm^{-2} allowed a comparison of the effectiveness of the various wavelengths. A wavelength of 617 nm appeared to be the most efficient in decreasing the number of CFUs of *H. parainfluenzae* (Fig. 2). In comparison with 617 nm, 630 nm light, mostly used for PDT, was less efficient.

From Fig. 3, it appears that the killing effectiveness (i.e. the slope of the survival curve) decreases with increasing light dose. This phenomenon is suggestive of photobleaching. The decrease in fluorescence occurring during illumination (Fig. 5) supports this hypothesis.

The killing efficiency of 3–4 log measured here is less than that found by other investigators who obtained a 5–6 log bacterial photoinactivation using other photosensitizers [6,8,9]. However, in the present experiments, we did not investigate the circumstances leading to maximum kill; moreover, this limited extent of destruction may be related to the photobleaching of the endogenous porphyrins. Our results concerning the killing effect of this procedure on *H. parainfluenzae* in the presence of δ-ALA are similar to those described by Tuveson and Sammartano [16] on *E. coli* incubated with δ-ALA. In addition, Peak et al. [17] demonstrated photodynamically induced membrane damage, illustrated by increased rubidium leakage from the cell. This leakage was enhanced after incubation of the cells with increasing amounts of δ-ALA and light exposure [17]. This is in accordance with our findings that, in the presence of increasing concentrations of δ-ALA, the bacterial viability decreases following illumination (Fig. 1).

In conclusion, a substantial reduction in the number of CFUs of *H. parainfluenzae*, endogenously sensitized with δ-ALA, is obtained by PDT in vitro.

Acknowledgements

The authors thank M.L. Langenberg for her support in realizing this study and for her critical appraisal of the manuscript.

References

[1] Z. Malik, J. Hanania, Y. Nitzan, New trends in photobiology (invited review). Bactericidal effects of photoactivated porphyrins—an alternative approach to antimicrobial drugs, J. Photochem. Photobiol. B: Biol., 5 (1990) 281–293.

[2] A.M.R. Fisher, A.L. Murphree, C.J. Gomer, Clinical and preclinical photodynamic therapy, Review series article, Lasers Surg. Med., 17 (1995) 2–31.

[3] Y. Nitzan, S. Goshansky, Z. Malik, Effect of photoactivated hematoporphyrin derivative on the viability of *Staphylococcus aureus*, Curr. Microbiol., 8 (1983) 279–284.

[4] Y. Nitzan, B. Shainberg, Z. Malik, Photodynamic effects of deuteroporphyrin on Gram positive bacteria, Curr. Microbiol., 15 (1987) 251–258.

[5] G. Bertoloni, B. Salvato, M. Dall'Acqua, M. Vazzoler, G. Jori, Hematoporphyrin-sensitized photoinactivation of *Streptococcus faecalis*, Photochem. Photobiol., 39 (1984) 811–816.

[6] G. Bertoloni, F. Rossi, G. Valduga, G. Jori, J. van Lier, Photosensitizing activity of water- and lipid-soluble phthalocyanines on *Escherichia coli*, FEMS Microbiol. Lett., 71 (1990) 149–156.

[7] Z. Malik, H. Ladan, B. Ehrenberg, Y. Nitzan, Bacterial and viral photodynamic inactivation, in: B.W. Henderson, T.J. Dougherty (Eds.), Photodynamic Therapy, Marcel Dekker, New York, 1992, pp. 97–113.

[8] M. Merchat, G. Bertoloni, P. Giacomini, A. Villanueva, G. Jori, Meso-substituted cationic porphyrins as efficient photosensitizers of Gram-positive and Gram-negative bacteria, J. Photochem. Photobiol. B: Biol., 32 (1996) 153–157.

[9] A. Minnock, D.I. Vernon, J. Schofield, J. Griffiths, J.H. Parish, S.B. Brown, Photoinactivation of bacteria. Use of a cationic water-soluble zinc phthalocyanine to photoinactivate both Gram-negative and Gram-positive bacteria, J. Photochem. Photobiol. B: Biol., 32 (1996) 159–164.

[10] M. Merchat, J.D. Spikes, G. Bertoloni, G. Jori, Studies on the mechanism of bacteria photosensitization by meso-substituted cationic porphyrins, J. Photochem. Photobiol. B: Biol., 35 (1996) 149–157.

[11] J.C. Kennedy, R.H. Pottier, Endogenous protoporphyrin IX, a clinically useful photosensitizer for photodynamic therapy, J. Photochem. Photobiol. B: Biol., 14 (1992) 275–292.

[12] W.K. Philipp-Dormston, M. Doss, Comparison of porphyrin and heme biosynthesis in various heterotrophic bacteria, Enzyme, 16 (1973) 57–64.

[13] M. Kilian, A rapid method for the differentiation of *Haemophilus* strains. The porphyrin test, Acta Path. Microbiol., Scand. Sect. B, 82 (1974) 835–842.

[14] P. Luppa, K. Jacob, W. Ehret, The production of porphyrins from delta-aminolaevulinic acid by *Haemophilus parainfluenzae*, J. Med. Microbiol., 39 (1993) 262–267.

[15] E.L. Biberstein, P.D. Mini, M.G. Gills, Action of *Haemophilus* cultures on delta-aminolevulinic acid, J. Bacteriol., 86 (1963) 814–819.

[16] R.W. Tuveson, L.J. Sammartano, Sensitivity of hemA mutant *Escherichia coli* cells to inactivation by near-ultraviolet light depends on the level of supplementation with delta-aminolevulinic acid, Photochem. Photobiol., 43 (1986) 621–626.

[17] M.J. Peak, J.S. Johnson, R.W. Tuveson, J.G. Peak, Inactivation by monochromatic near-UV radiation of an *Escherichia coli hemA8* mutant grown with and without delta-aminolevulinic acid: the role of DNA vs. membrane damage, Photochem. Photobiol., 45 (1987) 473–478.