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ARTICLE TYPE

Protein profiles of *Escherichia coli* and *Staphylococcus warneri* are altered by photosensitization with cationic porphyrins

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Oxidative stress induced by photodynamic treatment of microbial cells causes irreversible damages on vital cellular components such as proteins. Photodynamic inactivation (PDI) of bacteria, a promising therapeutic approach for the treatment of superficial and localized skin and oral infections, can be achieved by exciting a photosensitizing agent with visible light in an oxygenated environment. Although some studies have addressed the oxidative alterations of PDI in bacterial proteins, the present study is the first to compare the electrophoretic profile of proteins of Gram-positive and Gram-negative bacteria, with two structurally different porphyrins, with different kinetics of photoinactivation. The cationic porphyrins 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide (Tri-Py^{+}-Me-PF) and 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^{+}-Me) were used to photosensitize *Escherichia coli* and *Staphylococcus warneri* upon white light irradiation at an irradiance of 4.0 mW cm^{-2}. After different photosensitization periods, proteins were extracted from bacteria and analyzed by one-dimensional SDS-PAGE. Apparent molecular weights and band intensities were determined after an irradiation period corresponding to a reduction of 4 $\log_{10}$ in cell viability. After photodynamic treatment, there was a general loss of bacterial proteins, assigned to large scale protein degradation. Protein loss was more pronounced after PDI with Tri-Py^{+}-Me-PF in both bacteria. There was also an increase in the concentration of some proteins as well as an increase in the molecular weight of other proteins. We show that proteins of *E. coli* and *S. warneri* are important targets of PDI. Although there is an attempt of cellular response to the PDI-induced damage by overexpression of a limited number of proteins, the damage is lethal. Our results show that changes occurring in the protein pattern during photodynamic treatment are different with the two photosensitizers, which helps to explain the different inactivation kinetics of the two bacteria. SDS-PAGE is a rational approach to assign the type of cellular response to stress that is being induced in the cells.

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Introduction

Reactive oxygen species (ROS) cause oxidative damage to biological molecules, such as lipids, nucleic acids, carbohydrates and proteins.[1] These ROS (singlet oxygen, $^{1}O_2$, and free radicals) have a very short lifespan in biological systems (nano to milliseconds) and, therefore, a limited diffusion radius (some micrometers).[2,3] ROS can be produced by photodynamic action, *i.e.*, through the concerted action of molecular oxygen, a photosensitizer (PS) and a light source of a wavelength corresponding to the maximum absorption peak of the PS.[4]

The generation of both $^{1}O_2$ and free radicals depends on the PS energetic and chemical features,[2] but usually one of the forms is

predominant.[5] In porphyrin-mediated photodynamic inactivation (PDI), $^1O_2$ is considered the main damaging species.[6] PS with an overall cationic charge can permeabilize the intricate outer membrane of the cell wall of Gram-negative bacteria, and so, lead to an efficient elimination of these type of bacteria.[7] The efficient inactivation of Gram-positive bacteria by cationic, and also by neutral and anionic PS, is due to their relatively simple and porous cell wall.[8] However, the effect of a PS depends not only on its physicochemical properties but also on the particular site where its bind and acts.[9]

The main targets of photodynamic action are the lipids and proteins of external structures, cytoplasmic membrane and cell wall.[9] In bacteria, the cytoplasmic membrane consists of a phospholipid bilayer, some minor lipids, and proteins. Since bacteria are devoid of intracellular organelles, inner membrane proteins play vital functions, such as energy production, lipid biosynthesis, protein secretion and transport.[10] The cell wall of Gram-negative and Gram-positive bacteria is distinct. In Gram-negative bacteria, beside the thin peptidoglycan layer, the presence of an intricate outer membrane creates an impermeable barrier to antimicrobial agents. The outer membrane consists of glycolipids in the outer leaflet, mainly lipopolysaccharides (LPS), lipoproteins and ß-barrel proteins, lipoteichoic acids, a phospholipid bilayer in the inner leaflet which anchors these constituents and the peptidoglycan (2-7 nm).[9] In Gram-positive bacteria, formed by only one thick peptidoglycan layer, surface proteins (fibronectin, fibrinogen, elastin) are attached to peptidoglycan, to teichoic acids (adhesins) or to stem peptides within the peptidoglycan layers. There are other proteins involved in immune system evasion, internalization and phage binding.[9]

Although the bacterial membrane is likely to be the main target of photodynamic inactivation, information concerning this topic is scarce because the identification and characterization of photodynamic damages on lipids is quite complex. However, the first lipidomic studies on the phospholipid oxidation of Gram-positive and Gram-negative bacteria after PDI with a cationic *meso*-substituted porphyrin (Tri-Py$^+$-Me-PF) revealed formation of new oxidized molecular species and changes in the relative amounts of the different phospholipid classes.[11] For instance, in the Gram-positive *Staphylococcus warneri*, there was an increase in the amount of phosphatidylglycerols (PG), and a decrease of cardiolipins and other phospholipids. Also, hydroxyl and hydroperoxy derivatives from unsaturated fatty acyl chains of cardiolipins were identified.[12] In *E. coli*, hydroxyl and hydroperoxy derivatives were also identified as oxidized molecular species from unsaturated fatty acyl chains of phosphatidylethanolamines, the major phospholipid component.[11]

The LPS, of the cell wall of the Gram-negative bacteria, play a critical role in the cell defense against antimicrobial agents. Molecules of LPS can avidly join, particularly if divalent cations such as Mg^{2+} and Ca^{2+} are present in the cell surface, stabilizing the negative charge of the phosphate groups in the molecules.[9] The electrostatic forces generated between the positively charged PS and the constituents of the Gram-negative cell wall promote the destabilization of the wall native organization, allowing the binding and eventual penetration of the PS into the cell.[9] For this reason, the cationic PS have broader spectrum of action.

Relatively to proteins, theirs damage by PDI has already been shown by several authors. Proteins are considered major cellular targets of photodynamic oxidation not only because of their essential functions but also because they are highly abundant, may have endogenous chromophores, can bind to exogenous chromophoric materials and can rapidly react with other excited state species.[2,13] Protein oxidation is the most important damage after PDI. Protein oxidation is, by definition, its covalent modification induced by ROS or by reaction with by-products of oxidative stress.[1] The processes involving oxidation by $^1O_2$ can induce deep changes in the proteins' structure and function.[14] Those $^1O_2$-mediated damages start, in general, at electron rich side-chains of amino acid residues (due to double bonds or sulphur moieties) such as cysteine, cystin, histidine, tyrosine, methionine and tryptophan residues.[15] Reaction with these residues originates new reactive species that damage other targets, leading to a cascade of deleterious events.[14] These new targets can be other proteins,[14] and also lipids or DNA.[16,17] The major consequences of $^1O_2$-mediated protein oxidation are enzyme inactivation, protein peroxides and carbonyls formation, side-chain product formation, backbone fragmentation, formation of cross-links and aggregates, and protein unfolding.[2,13]

Several *in vitro* studies have reported the photodynamic effect of PS in human proteins such as serum albumin,[18,19] or have identified the major protein damages in bacteria under oxidative stress.[20] *In vitro* studies of bacterial PDI using porphyrins are few and use sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis, enzymatic assays, and quantification of total carbonyls to characterise protein damage. SDS-PAGE of membrane proteins normally reveals on the electrophoretic pattern of the irradiated samples, namely attenuation or disappearance of some proteins and increased concentration of high molecular weight products corresponding to cross-linked material.[9,21-23] Inactivation or loss of enzyme function has been reported for lactate dehydrogenase, NADH dehydrogenase, ATPase and also for succinate dehydrogenase.[23] These modifications in outer membrane proteins and enzymes are time-dependent and concomitant with a decrease in cell survival. The amount of protein carbonyls also increases with irradiation time.[24] Oxidative damage to membrane proteins and bacterial enzymes have also been demonstrated using other PS such as phthalocyanines,[25] and phenothiazinium dyes, as methylene blue[26,27] or toluidine blue O.[28-30]

A single report describing the molecular targets of bacterial PDI by proteomics has shown that most of the altered proteins of *Staphylococcus aureus* by porphyrin treatment are involved in metabolic activities such as the response to oxidative stress, cell division and sugar uptake.[22] It has also been suggested that the damages induced by PDI are specific and are likely to be dependent on the location of the PS in the bacteria.[22] Despite these contributions there are, as far as it is known, no reports comparing the photo-oxidative effects of structurally different porphyrin derivatives on the protein profiles of the two types of bacteria. *S. warneri* is a skin commensal, also involved in skin diseases, is a potentially opportunist etiological agent.[31-35] Being a non-virulent strain, it is an easy and suitable model to use in laboratory studies. *Escherichia coli* is commonly chosen as a representative biological model for Gram-negative bacteria, which possess a structurally far more complex cell wall structure

than that of Gram-positive bacteria.[36]

The aim of this study was to evaluate and to compare the photo-oxidative effect of two efficient but structurally different cationic porphyrins (Fig. 1), which have different photoinactivation kinetics, on the Gram-positive and Gram-negative bacteria proteins. Although some studies have addressed the oxidative alterations of PDI in bacterial proteins, the present study is the first to compare the electrophoretic profile of proteins of Gram-positive and Gram-negative bacteria, with two structurally different porphyrins, with different kinetics of photoinactivation.

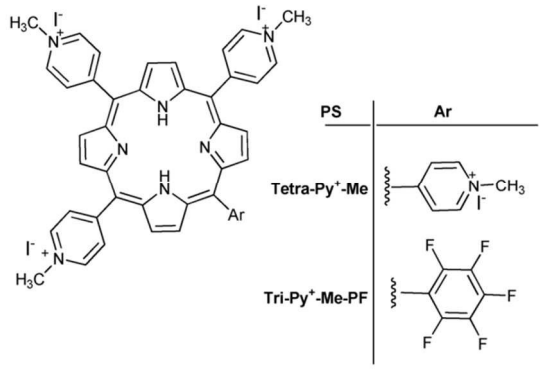

Fig. 1 Structure of the cationic porphyrin derivatives used as photosensitizers.

Experimental

Photosensitizers

The two cationic porphyrin derivatives selected for this study, 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py$^+$-Me) and 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide (Tri-Py$^+$-Me-PF) have already been described.[37-39] The former is a reference compound, widely used in PDI experiments, and the later is a patented compound from our research group[40] with a large spectrum of activity.[37,41-45] Porphyrins purity was confirmed by thin layer chromatography and by ^{1}H NMR spectroscopy. Stock solutions (500 μM) of each porphyrin derivative were prepared in dimethyl sulfoxide and sonicated for 30 min before use. Tetra-Py$^+$-Me in DMSO: λ_{max} (log ε) 425 (5.46), 516 (4.30), 550 (3.78), 588 (3.86), 644 (3.34) nm; Tri-Py$^+$-Me-PF in DMSO: λ_{max} (log ε) 422 (5.48), 485 (3.85), 513 (4.30), 545 (3.70), 640 (3.14) nm.

Bacterial strains and growth conditions

E. coli ATCC® 25922™ (American Type Culture Collection, VA, USA) and *S. warneri*, isolated in our laboratory,[12] from fresh cultured plates, were inoculated in tryptic soy (TS) broth (Merck) and grew aerobically at 37 °C under 100 rpm overnight. Afterwards, an aliquot was transferred into fresh TS broth at the same growth conditions to reach the early stationary phase. For *E. coli*, an optical density at 600 nm (OD_{600}) of 1.6 ± 0.1 corresponded to ≈ 10^8 colony forming units (CFU) mL^{-1}. For *S. warneri*, an OD_{600} of 1.9 ± 0.1 corresponded to 10^8 CFU mL^{-1}.

Photosensitization procedure and cell viability assays

Bacterial suspensions (≈ 10^8 CFU mL^{-1}) were prepared, in phosphate buffered saline (PBS: 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per litre; pH 7.4), from the early stationary phase cultures, distributed in 100 mL beakers (final volume of 10 mL per beaker), incubated in the dark with porphyrin for 10 min at 25 °C under 100 rpm stirring to promote the porphyrin binding to the cells, and then irradiated by a light system, consisting in 13 parallel OSRAM 2' 18 W/840 lamps with an irradiance of 4.0 mW cm^{-2}, emitting in the range of 380–700 nm.

Bacterial suspensions of *E. coli*, with 5.0 μM of PS, were irradiated up to 270 min (total light dose of 64.8 J cm^{-2}) and sub-samples of 1.0 mL were collected before irradiation and after 15, 30, 60, 90, 180 and 270 min of light exposure. Bacterial suspensions of *S. warneri*, with 0.5 μM of each PS, were irradiated up to 40 min (total light dose of 9.6 J cm^{-2}) and sub-samples of 1.0 mL were collected before irradiation and after 5, 10, 15, 20, 30 and 40 min of light exposure. After each photosensitization interval, the suspensions were serially diluted in PBS, plated in TS agar and incubated at 37 °C for 24–36 h for viability monitoring. The cell viability was determined by counting the CFU on the most appropriate dilution on the agar plates.

Light and dark controls were carried out simultaneously to the PDI procedure: light control (LC) comprised a bacterial suspension exposed to light; and dark control (DC) comprised a bacterial suspension incubated with PS at the studied concentrations but protected from light. Three independent experiments were performed and, for each, two replicates were plated.

Protein extraction and quantification

The photosensitization procedure for protein experiments was the same to that used in the cell viability assays, with some modifications. Before irradiation, bacterial pellets were obtained by centrifugation (18 mL of early stationary phase cell culture), washed twice with PBS and re-suspended in PBS in glass beakers (final volume of 60 mL). The centrifugation conditions were 10 min at 13,000 x *g*, 20 °C, in 50 mL tubes for Avanti® J-25 (Beckman Coulter, Inc.). After photosensitization (0 - 270 min for *E. coli* and 0 - 40 min for *S. warneri*), bacterial pellets were obtained by centrifugation in the conditions described above, based on a protocol developed earlier.[46]

Cells pellets were carefully suspended in an urea solution [8 M urea, 100 mM Tris, 100 mM bicine, and 2% SDS (w/v)] and disrupted with a sonicator (U200S control, IKA Labortechnik Janke & Kunkel GmbH & CO, Staufen, Germany) at 50% maximum output. Cells were sonicated with bursts (2 sec each) alternating with cooling in an ice bath (3 sec), for a total of 120 sec. Cell debris was pelleted by centrifugation at 17,000 x *g* for 10 min and supernatants were kept at -80 °C until analysis.

Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay kit (Pierce™, Rockford, USA) according to the manufacturer's instructions. Each sample was quantified in triplicate and compared to a bovine serum albumin calibration curve prepared in the same urea solution.

Protein electrophoresis (SDS-PAGE)

Twenty five μg of *E. coli* proteins and 5 μg of *S. warneri* proteins

were reduced (2% β-mercaptoethanol), denaturated (5 min at 100 °C) and separated by SDS–PAGE.[46,47] The separation was performed in the Mini-PROTEAN 3 (Bio-Rad) with lab casted SDS polyacrylamide gels (15%). Gels ran for 2 h, at 120 V and at 4 °C. The apparent molecular weight of the proteins was determined using a molecular weight calibration kit as marker, consisting of a mixture of proteins with 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa (Precision Plus Protein Standards All Blue, Bio-Rad). Proteins were visualized by colloidal Coomassie staining.[48] Each gel image was acquired using the GS-800 calibrated imaging densitometer (Bio-Rad).

Apparent molecular weights and band intensities were determined using the Quantity One v4.6.9 software (Bio-Rad). Band optical density (OD) was determined, subtracted for background and corrected for OD differences between gels, as described earlier.[49,50] All samples were analyzed in triplicate.

Protein assignment

The assignment of proteins was based on the determined molecular weights of proteins, according to specific literature for each bacterial strain.[51,52] The search considered representative protein bands that were new, increased or disappearing after PDI. The molecular weights found (within a range of ± 0.4 kDa) were assigned to possible proteins and to their respective accession number, and afterwards, this information was confronted with the information of UniProt database.

Results

The reduction in cell viability as well as the kinetics of photoinactivation of *E. coli* and *S. warneri* with Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me depended on the porphyrin and on the light dose used (Fig. 2), as shown and discussed in a previous work.[54] The kinetics of bacterial PDI was significantly different between the two PS.[53]

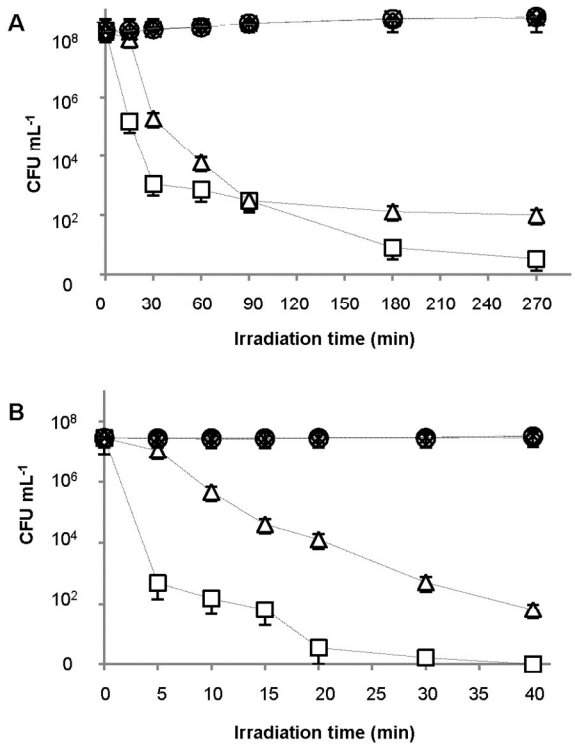

Fig. 2 Photodynamic inactivation of (**A**) *E. coli* incubated with 5.0 µM of Tri-Py$^+$-Me-PF (empty square) and 5.0 µM of Tetra-Py$^+$-Me (empty triangle) and (**B**) *S. warneri* incubated with 0.5 µM of Tri-Py$^+$-Me-PF (empty square) and 0.5 µM of Tetra-Py$^+$-Me (empty triangle), exposed to artificial white light (4.0 mW cm^{-2}) with different light doses. Light control (cross), dark control of Tri-Py$^+$-Me-PF (empty circle) and dark control of Tetra-Py$^+$-Me (filled circle). Values represent the mean of three independent experiments with two replicates each; error bars indicate the standard deviation.

In *E. coli* (Fig. 2A), after 15 min of irradiation (light dose of 3.6 J cm^{-2}), there was a reduction of 3.1 $\log_{10}$ CFU mL^{-1} with 5.0 µM of Tri-Py$^+$-Me-PF and 0.2 $\log_{10}$ CFU mL^{-1} with 5.0 µM of Tetra-Py$^+$-Me, and after 30 min (7.2 J cm^{-2}), the log reductions were 5.2 *versus* 2.9 respectively.

In *S. warneri* (Fig. 2B), after photosensitization with 0.5 µM of Tri-Py$^+$-Me-PF, there was a steep decrease of survival (4.8 $\log_{10}$ CFU mL^{-1}) after 5 min of irradiation (1.2 J cm^{-2}), contrasting with a minor reduction (0.4 log CFU_{10} mL^{-1}) with Tetra-Py$^+$-Me. With this porphyrin, the detection limit of viable cells was not reached after 40 min of irradiation, even though a reduction of 5.6 $\log_{10}$ CFU mL^{-1} was achieved.

Bacterial viability was neither affected by irradiation *per se* nor by the PS in the dark, as shown by the light control and dark control profiles, respectively.

The protein profile of the photosensitized bacteria was also affected by the porphyrin and the light dose, as revealed by SDS-PAGE shown in Fig. 3 and 4 for *E. coli* and *S. warneri*, respectively.

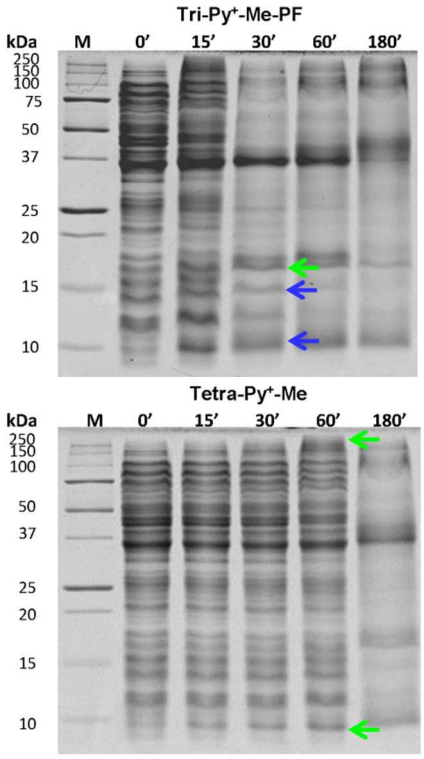

Fig. 3 SDS-PAGE of *E. coli* proteins after photosensitization. Cells were incubated for 10 min in the dark with 5.0 µM of photosensitizer and irradiated with visible light (4.0 mW cm^{-2}) for different irradiation times. M: molecular weight marker Blue arrows represent new bands. Green arrows indicate representative protein bands with increased intensity after treatment.

In the light and dark controls (non-photosensitized bacteria), there were no changes in the protein concentration (see Fig. S1 in Supporting Information), nor at 0 min of irradiation (bacteria incubated 10 min in the dark with porphyrin but not irradiated, lanes 0' in Fig. 3 and 4). With irradiation and in the presence of the PS, a general disappearance of bacterial proteins was observed, assigned to large scale protein degradation. However, for the same light doses, the two PS acted differently: protein degradation was more pronounced in the case of photo-treatment with Tri-Py^+-Me-PF (Fig. 3 and 4).

In order to point out possible differences in the effects of the two porphyrins on the protein profiles of each bacterium, gel analysis was carried out at the time corresponding to a reduction of cell viability of about 4 $\log_{10}$ CFU mL^{-1}, considering an initial bacterial concentration of 8 $\log_{10}$ CFU mL^{-1} (*cf.* Fig. 2).

For *E. coli*, this reduction was observed after 30 min with Tri-Py^+-Me-PF and after 60 min with Tetra-Py^+-Me. For *S. warneri*, the 4 $\log_{10}$ CFU mL^{-1} reduction in survival was observed after 5 min with Tri-Py^+-Me-PF and after 20 min with Tetra-Py^+-Me.

By comparison with the controls, there was a clear alteration on the protein profile of *E. coli* after 30 min of irradiation with Tri-Py^+-Me-PF (Fig. 3). There was an increase in the intensity of the 16.8 kDa protein and bands with 14.9 kDa and 10.7 kDa were detected (Fig. 3).

On the other hand, the protein profile of *E. coli* after 60 min of irradiation with 5.0 µM of Tetra-Py^+-Me was quite different and not as dramatically modified as with Tri-Py^+-Me-PF in relation to the initial profile (Fig. 3). There was an intensity increase of some bands (proteins with 263.2 kDa, 201.8 kDa and 9.8 kDa). Also, some proteins disappeared completely after 60 min of treatment (94.6 kDa, 87.3 kDa, 60.6 kDa, 59 kDa and 12 kDa).

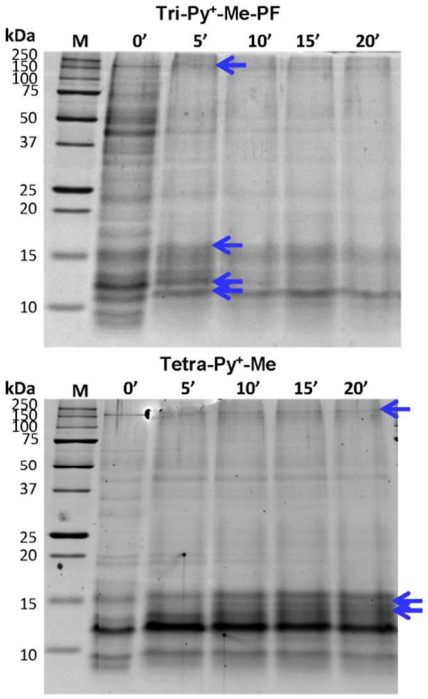

Fig. 4 SDS-PAGE of *S. warneri* proteins after photosensitization. Cells were incubated in the dark with 0.5 µM of photosensitizer and irradiated with visible light (4.0 mW cm^{-2}) for different irradiation times. M: molecular weight marker. Blue arrows represent new bands that appear after treatment.

The photosensitization of *S. warneri* with both porphyrinic PS induced changes in the protein profile after very short irradiation times (Fig. 4). The induced changes were much more pronounced with Tri-Py^+-Me-PF than with Tetra-Py^+-Me as it was observed for *E. coli*. In the presence of Tetra-Py^+-Me, after 20 min of irradiation, a protein with 15.7 kDa showed increased intensity. New bands with 200.2 kDa, 14.6 kDa and 14.0 kDa were detected (Fig. 4). Also the comparison of the densitometric superimposed profiles of samples (data not shown) revealed an increase in the molecular weight of some proteins.

The protein pattern of *S. warneri* was drastically changed after 5 min of irradiation with Tri-Py^+-Me-PF, with a marked decrease in the intensity of the majority of the proteins detected (Fig. 4). We also detected the appearance of new bands with molecular weights of 196.3 kDa, 15.9 kDa and 11.3 kDa.

Discussion

This study aimed to provide a new insight into the photo-oxidative effects of two cationic porphyrin derivatives on the protein profile of Gram-negative and Gram-positive bacteria, by SDS-PAGE.

As showed by Alves et al[11,12] lipids of these Gram-positive and Gram-negative bacteria, using the same PDI protocol, are differently affected during PDI by the two porphyrins. The results of this study show, by the first time, that as lipids, proteins of the

two bacteria are also differently affected by the two PS. In general, the widespread disappearance of proteins over time suggests a large scale degradation process. In some cases, we detected an increase in the intensity of some bands. This increase may be caused by an overexpression of these proteins or by aggregation phenomena. On the other hand, there is a slight increase in the molecular weight of some proteins (1 - 5 kDa) indicative of changes possibly associated with protein oxidation, and also the appearance of aggregates of high molecular weight (> 100 kDa), probably related with formation of cross-linked complexes. These evidences agree with the literature concerning the effects of photoinactivation on bacterial proteins.[21-23]

We have made and attempt to make protein assignments according to the molecular weights determined, using the information available in the literature and in appropriate data basis.[51,52] The information concerning presumable proteins from *E. coli* is summarized in Table 1 and from *S. warneri* in Table 2. This should be considered preliminary data and further studies should be conducted in order to confirm the identity of these proteins by methods such as mass spectrometry.

The protein profile of *E. coli* after photosensitization is different for the two tested porphyrins. Apart from the degradation of most proteins, after 30 min of photosensitization with Tri-Py^{+}-Me-PF there is an increase in the intensity of a protein (16.8 kDa) putatively corresponding to Dps, a protein responsible for DNA protection during protein starvation (Table 1). This protein binds nonspecifically to DNA, protecting cells from toxicity, as shown by *E. coli* exposure to hydrogen peroxide.[54]

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Table 1 Presumable proteins of *E. coli* modified after photodynamic treatment with Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me. Possible assignments were based on the literature[52] and on UniProt database.

		Determined molecular weight (kDa)	Possible assignments	Access no.	Gene id.	Protein name	Biological process
E. coli treated with Tri-Py^{+}-Me-PF	Upregulated	**16.8**	16.8	P0ABT2	Dps	DNA protection during starvation protein	DNA condensation; Iron storage
	New	**14.9**	15.0	P0C0L2	OsmC	Peroxiredoxin OsmC	Response to oxidative stress
			15.0	P02413	RplO	50S ribosomal protein L15	Translation
			15.1	P0A6E6	AtpC	ATP synthase epsilon chain	Plasma membrane ATP synthesis coupled proton transport
		10.7	11.0	P0A8P3	YggX	Probable Fe^{2+}-trafficking protein	Response to oxidative stress
			10.6	P39177	UspG	Universal stress protein G	Response to stress
			10.7	P0AES9	HdeA	Acid stress chaperone HdeA	Cellular response to acidity
E. coli treated with Tetra-Py^{+}-Me	Upregulated	**9.8**	9.9	P0ADU2	YgiN	Probable quinol monooxygenase YgiN	Response to oxidative stress
			9.8	P68066	GrcA	Autonomous glycyl radical cofactor	Response to stress
	Undetectable after PDI	**94.6**	94.0	P0A9M0	Lon	Lon protease	Cellular response to stress; misfolded or incompletely synthesized protein catabolic process; response to heat
		87.3	87.4	P33136	MdoG	Glucans biosynthesis protein G	Glucan biosynthetic process; oligosaccharide biosynthetic process; response to osmotic stress
			87.4	P07395	PheT	Phenylalanine-tRNA ligase beta subunit	Phenylalanyl-tRNA aminoacylation; tRNA processing
			87.4	P23538	PpsA	Phosphoenolpyruvate synthase	Gluconeogenesis; pyruvate metabolic process
		60.6	60.7	P77306	YqiK	Inner membrane protein YqiK	Unknown
			60.4	P11875	ArgS	Arginine-tRNA ligase	Protein biosynthesis
			60.6	P0A6Y8	DnaK	Chaperone protein DnaK	DNA replication; Stress response
			60.8	P0A6F5	GroL	60 kDa chaperonin	Cell cycle; cell division; protein folding; protein refolding; response to heat
		59.0	59.4	P02942	Tsr	Methyl-accepting chemotaxis protein I	Chemotaxis
		12.0	12.2	P0A8Q6	ClpS	ATP-dependent Clp protease adapter protein ClpS	Proteolysis involved in cellular protein catabolic process
			12.3	P0A7K2	RplL	50S ribosomal protein L7/L12	Translation

With Tetra-Py^{+}-Me, the process of *E. coli* protein degradation is slower, which is in agreement with the slowest photoinactivation rate of these bacteria with this porphyrin. There is an increase in the intensity of a band (with 9.8 kDa)

corresponding presumably to stress response proteins (YgiN or GrcA). At the same time, several other bands disappear (87.3 and 60.6 kDa). Overexpression of proteins with identical molecular weights (*e.g.* GroL) has been reported in *Streptococcus mutans* photosensitized with rose Bengal, without DNA degradation.[55] In *E. coli* photosensitized with toluidine blue O, there is also up-regulation of GroL and DnaK.[56] According to these evidences, the induction of heat shock proteins has been suggested as a possible mechanism of development of resistance.[56] However, PDI of *S. aureus* by Tetra-Py$^+$-Me leads to a decrease of enzymes involved in direct and indirect response to ROS,[22] as also suggested by the observations from this study. These contradictory conclusions might be due to the use of different PS types (porphyrin *versus* phenothiazinium derivatives) and PDI protocols (*e.g.*, irradiation conditions).

In the case of *S. warneri*, there is also a large degradation of the majority of proteins, being more evident the difference caused by porphyrin Tri-Py$^+$-Me-PF in the beginning of the treatment. The new bands detected, namely some of high molecular weight, are probably associated with the formation of cross-linked complexes. Furthermore, protein degradation due to photo-oxidation is likely to originate low molecular weight products as protein decomposition products.[57]

The results of this study show clearly that changes occurring in the protein pattern during photodynamic treatment are different with the two photosensitizers. Knowing that PDI efficiency depends on the PS nature and on its ability to generate ROS like $^1O_2$, it is important to identify which PS characteristics increase the photocytotoxic activity. These features will allow to improve the design of the PS and to decide the best conditions for bacterial photoinactivation. The photocytotoxic activity of these two PS is mainly via Type II mechanism ($^1O_2$ production), however, Tri-Py$^+$-Me-PF is more efficient to produce $^1O_2$ than Tetra-Py$^+$-Me[6,41] which can explain the better results of Tri-Py$^+$-Me-PF. Aditionally, it is well known that the increase in the number of charges improves the amphiphilic character of the porphyrins, and consequently, the PS affinity for bacteria which contributes to a better binding to the cells accompanied by an increase in the photocytotoxic activity.[6,9,12,41,53] In fact, considering that Tri-Py$^+$-Me-PF caused, in general, a stronger protein pattern change than Tetra-Py$^+$-Me, the lipophilic character of the *meso*-substituent groups may be a relevant factor in the outcome of the photosensitization process. The presence of a lipophilic pentafluorophenyl group in one of the *meso* positions of the tetrapyrrolic macrocycle of Tri-Py$^+$-Me-PF plays an important role in PDI, as already observed.[6,9,41] The distribution of the charges in the porphyrin structure also affects the PDI efficiency.[6,9,41] It has been observed that PS with adjacent charges cause molecular distortion due to electrostatic repulsion caused by the asymmetric cationic molecule. This can explain the fact that, in this study, Tri-Py$^+$-Me-PF causes a higher impact on proteins than Tetra-Py$^+$-Me. The PS Tri-Py$^+$-Me-PF, which has three charges and one pentafluorophenyl group, is asymmetric, but the Tetra-Py$^+$-Me, which has four charges, is symmetric.

The results show that proteins are very rapidly degraded by photoinactivation induced by both porphyrins; Tetra-Py$^+$-Me is extremely effective degrading most bacterial proteins in just few minutes. This may explain the effectiveness of antimicrobial PDI,, reducing the likelihood of developing resistance mechanisms, which otherwise have not yet been identified or are not clearly established.[9]

Conclusion

We have confirmed that bacterial proteins are important targets of photosensitization with cationic porphyrins Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me. The changes that occur in the protein pattern during photodynamic treatment are different with the two photosensitizers, which help to explain the inactivation kinetics of the two bacteria. It is important to stress that protein identification is the next natural step of this investigation. Using the information gathered here, coupling mass spectrometry technologies to shorter inactivation times will allow identifying the initial protein targets of phtotoinactivation. This may lead to improved strategies of antimicrobial photosensitization.

Table 2 Presumable proteins of *S. warneri* modified after photodynamic treatment with Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me. Possible assignments were based on the literature[51] and on UniProt database.

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		Determined molecular weight (kDa)	Possible assignments	Access no.	Gene id.	Protein name	Biological process
S. warneri treated with Tri-Py$^+$-Me-PF	New	**196.3**	Cross-links				
		15.9	15.7	L7WVR6		Putative Holliday junction resolvase	DNA recombination; DNA repair; nucleic acid phosphodiester bond hydrolysis
			15.8	L7WY50		Glycerol-3-phosphate cytidylyltransferase	Teichoic acid biosynthetic process
		11.3	11.2	L7WYE7	GatC	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C	Translation
S. warneri treated with Tetra-Py$^+$-Me	Upregulated	**15.7**	15.8	L7WY50		Glycerol-3-phosphate cytidylyltransferase	Teichoic acid biosynthetic process
			15.6	L7WXX9	RplO	50S ribosomal protein L15	Translation
			15.6	L7WUN7	LacA	Galactose-6-phosphate isomerase subunit lacA	Lactose catabolic process
			15.7	L7WVR6		Putative Holliday junction resolvase	DNA recombination; DNA repair; nucleic acid phosphodiester bond hydrolysis
	New	**200.2**	Cross-links				
		14.6	14.6	L7WUN1	RpsI	30S ribosomal protein S9	Translation
		14.0	14.0	L7WXY2	RpsM	30S ribosomal protein S13	Translation

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