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ORIGINAL PAPER

Photodynamic inactivation of recombinant bioluminescent *Escherichia coli* by cationic porphyrins under artificial and solar irradiation

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Abstract A faster and simpler method to monitor the photoinactivation process of *Escherichia coli* involving the use of recombinant bioluminescent bacteria is described here. *Escherichia coli* cells were transformed with *luxCDABE* genes from the marine bioluminescent bacterium *Vibrio fischeri* and the recombinant bioluminescent indicator strain was used to assess, in real time, the effect of three cationic *meso*-substituted porphyrin derivatives on their metabolic activity, under artificial (40 W m^{-2}) and solar irradiation (≈620 W m^{-2}). The photoinactivation of bioluminescent *E. coli* is effective (>4 log bioluminescence decrease) with the three porphyrins used, the tricationic porphyrin Tri-Py^{+}-Me-PF being the most efficient compound. The photoinactivation process is efficient both with solar and artificial light, for the three porphyrins tested. The results show that bioluminescence analysis is an efficient and sensitive approach being, in addition, more affordable, faster, cheaper and much less laborious than conventional methods. This approach can be used as a screening method for bacterial photoinactivation studies in vitro and also for the monitoring of the efficiency of novel photosensitizer molecules. As far as we know, this is the first study involving the use of bioluminescent bacteria to monitor the antibacterial activity of porphyrins under environmental conditions.

Keywords Cationic porphyrins · Photodynamic antimicrobial therapy · Bioluminescence · *Escherichia coli* · Solar irradiation

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Introduction

The growing reduction of water resources due to environmental pollution has become a major public health concern. Thus, wastewater treatment and disinfection is even more necessary at large scale. The inactivation of pathogenic microorganisms, in the last stage of wastewater treatment, can be achieved by several techniques such as chlorination, ozonation and ultraviolet radiation. To overcome the high costs and the difficulty in implementing these techniques, alternative physico-chemical methods have been studied, namely the photodynamic antimicrobial therapies [1–3]. This somewhat new technique uses a light source (sunlight or artificial light), an oxidizing agent (molecular oxygen dissolved in water) and an intermediary agent [named photosensitizer (PS)], able to absorb and transfer the energy of the light source to molecular oxygen leading to the formation of highly cytotoxic reactive oxygen species ($^1O_2$, $OH^{\bullet}$, O_2^{-}, H_2O_2) [4]. Such species are able to irreversibly alter the cells' vital constituents resulting in oxidative lethal damage [5]. The potential use of this approach for water disinfection has already been studied, showing that photoinactivation (PI) of bacteria in drinking [3] and residual waters [1, 6] is possible under artificial or solar irradiation. In those studies, different experimental conditions, namely

the light source and fluence rate were used. Also, several microorganisms and the PS (methylene blue, Rose Bengal and porphyrins) were tested [3, 7]. The results were promising, namely with porphyrins. Porphyrins can be transformed into cationic entities through the insertion of positively charged substituents in the peripheral *meso*-positions of the tetrapyrrole macrocycle which may largely affect the kinetics and extent of binding to microbial cells [8]. Cationic *meso*-substituted porphyrins are known to efficiently destroy Gram-negative and Gram-positive bacteria [9–14]. The combination of hydrophobic and hydrophilic substituents in the PS structure results in an intramolecular polarity axis, which can facilitate membrane penetration and produce a better accumulation in subcellular compartments, enhancing the effective photosensitization [15].

To monitor the bacterial PI process, faster methods are required instead of the laborious conventional methods of plating, overnight incubation and time-consuming counting of colony-forming units (CFU) [16, 17]. New approaches to study potential PS in vitro are essential to accelerate the development of photodynamic antimicrobial therapy in drinking and residual water treatment. To this end, the bacterial bioluminescence method, when applied in others areas, is considered to be a rapid [18], sensitive [19] and cost-effective option [17]. It also allows only living or viable cells to be detected and does not need exogenous administration of substrates [20] to obtain light emission. Bioluminescence refers to the process of visible light emission by living organisms [21] and this emission is directly dependent on the metabolic activity of the organism [17]. In that way, the inhibition of cellular activity results in a decrease of the bioluminescence rate. The light-emitting reaction in bacteria involves the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long chain fatty aldehyde with the emission of blue–green light [21, 22]. In both marine and terrestrial bioluminescent bacteria, a five-gene operon (*luxCDABE*) encodes the luciferase and biosynthetic enzymes (for the synthesis of the aldehyde substrate) necessary for light production. *luxA* and *luxB* genes encode the α and β subunits of the luciferase, and *luxC*, *luxD* and *luxE* encode proteins for aldehyde production [23]. Although a number of additional *lux* genes in bioluminescent bacteria have been identified, only *luxCDABE* are essential for the biosynthetic production of light [21, 24].

Nearly 2 decades ago, the isolation of the genes responsible for bioluminescence in bacteria and the ability to transfer these genes into prokaryotic and eukaryotic organisms have greatly extended the capacity and potential uses of bacterial bioluminescence [21]. Amongst the applications of these recombinant bacteria, the clinical [20, 25–28], environmental [29–33] and biotechnology research [34, 35] are the most promising ones. In the clinical studies, this methodology is applied to laboratory animal models inoculated with genetically engineered bioluminescent bacteria in order to assess in vivo and in real time the progress of infectious diseases by optical detection through sensitive imaging cameras [18, 19, 26, 27, 36, 37]. The light output from these bioluminescent bacteria is a highly sensitive reporter of their metabolic activity [20, 38]. Furthermore, in experimental systems in which a strong correlation between bioluminescence and viable counts can be demonstrated, measurement of bioluminescence offers a rapid and alternative method for monitoring bacterial viability [20, 39]. Light output is noncumulative, reflecting actual metabolic rate, and can be measured directly, continuously and nondestructively in high-throughput screening or continuous-culture models [40]. Thus, the transformation of pathogenic bacteria into indicator bioluminescent strains allows using a rapid, sensitive and cost-effective methodology to evaluate the efficiency of PI [17, 18, 40–42].

In this study, we proposed to develop a rapid method to assess the antibacterial effect of *meso*-substituted porphyrins based on the metabolic activity of recombinant bioluminescent *Escherichia coli* (*E. coli*) under artificial and solar irradiation. As *E. coli* is an indicator of faecal pollution and is used to evaluate the quality of drinking, recreational and residual waters, it can be considered an adequate bacterium model to test the applicability of the bioluminescent method. Moreover, *E. coli* is a Gram-negative bacterium and it is much more resistant to photoinactivation than Gram-positive bacteria [9, 11, 43] and consequently when *E. coli* cells are inactivated, it is likely that other vegetative cells of Gram-positive bacteria are also inactivated [14, 44].

As far as we know, the use of bioluminescent bacteria with the purpose to monitor the antibacterial activity of porphyrins under environmental conditions has not yet been reported.

Materials and methods

Photosensitizers

The photosensitizers 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^+-Me), 5-(pentafluorophenyl)-10,15,20-*tris*(1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py^+-Me-PF) and 5-(4-methoxycarbonyl-10,15,20-*tris*(1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py^+-Me-CO_2Me) (Fig. 1) used in this work were prepared in two steps according to the literature [45, 46]. First, the neutral porphyrins were obtained from the Rothemund and crossed Rothemund reactions using pyrrole and the adequate aldehydes (pyridine-4-carbaldehyde and pentafluorobenzaldehyde or methyl 4-formylbenzoate) at reflux in acetic acid and nitrobenzene [45, 46]. These reagents were

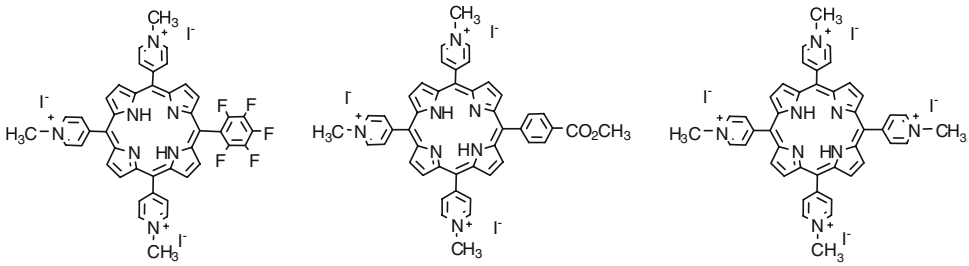

Fig. 1 Structure of the three porphyrin derivatives used for the photoinactivation of bioluminescent *E. coli*

purchased from Sigma-Aldrich (Madrid, Spain). After purification of the resulting porphyrins by column chromatography (silica), their pyridyl groups were quaternized by reaction with methyl iodide. The cationic porphyrins were purified by crystallization from chloroform/methanol/petroleum ether and their purities were confirmed by thin layer chromatography and by ^{1}H NMR spectroscopy. The spectroscopic date was in accordance with the literature [45, 46]. Stock solutions (500 μM) of each porphyrin in dimethyl sulfoxide were prepared by dissolving the adequate amount of the desired porphyrin in a known volume. The absorption spectral features of the PS were the following: [porphyrin] λ_{max} nm (log ε); [Tetra-Py^{+}-Me] in DMSO 425 (5.43), 516 (4.29), 549 (3.77), 588 (3.84), 642 (3.30); [Tri-Py^{+}-Me-PF] in DMSO 422 (5.48), 485 (3.85), 513 (4.30), 545 (3.70), 640 (3.14); [Tri-Py^{+}-Me-CO_2Me] in H_2O 420 (5.54), 518 (4.12), 556 (3.74), 583 (3.78), 640 (3.27).

Bacterial strain, plasmid constructs and *E. coli* transformation

In this study, two plasmids (pHK724 and pHK555) were inserted into *E. coli* Top10 (Invitrogen, USA). These plasmids contain the *lux* operon from the bioluminescent marine bacterium *Vibrio fischeri,* required to produce light. The plasmid pHK724 contains a ColE1 replicon, an ampicillin resistance marker and *luxR* gene whose gene product is a transcription regulatory protein. The plasmid pHK555 contains a P15A replicon, a chloramphenicol resistance marker and a functional *luxCDABE* operon. The *luxR* gene of pHK555 is inactive because of the insertion of phage DNA. When pHK724 is inserted into *E. coli* containing pHK555, the resultant colonies grow on selective media and are bioluminescent [47, 48].

Chemically competent cells of *E. coli* Top 10 with plasmid pHK555 were prepared in the laboratory, and were further transformed with plasmid pHK724, as described earlier [49], resulting in a bioluminescent strain.

Bacterial growth conditions

Bioluminescent *E. coli* were grown on Luria Bertani agar (LB, Merck) supplemented with 50 mg mL^{-1} of ampicillin (Amp) and with 34 mg mL^{-1} of chloramphenicol (Cm). A stock culture was stored at −80 °C in 10% glycerol. Before each PI assay, one colony of bioluminescent bacteria was aseptically inoculated into 30 mL of triptic soy broth (TSB, Merck) supplemented with both antibiotics (150 μL Amp/100 mL TSB and 60 μL Cm/100 mL TSB) and were grown for one day, at room temperature, at 100 rpm stirring. Then an aliquot of this culture was subcultured in 30 mL of fresh TSB with both antibiotics and was grown overnight, at room temperature, at 100 rpm stirring, to reach stationary growth phase ($OD_{600} \approx 1.3$).

Bioluminescence versus CFU of an overnight culture

To assess the correlation between the colony-forming units (CFU) number and the bioluminescent signal of our indicator strain, two assays were carried out with and without porphyrin, in dark conditions. An overnight culture of bioluminescent *E. coli* (10^{7} CFU mL^{-1}) was used that was serially diluted (10^{-1}–10^{-7}) in fresh phosphate buffered saline (PBS), pH 7.4. The non-diluted (10^{0}) and diluted aliquots were read on a luminometer (TD-20/20 Luminometer, Turner Designs, Inc., USA) and simultaneously 1 mL of each dilution was pour plated in TSA medium. When the porphyrin (5 μM) was added, a dark incubation was performed during 4 h at 25 °C under stirring, before serially dilution and plating. Both experiments were done in duplicate and the results were averaged.

Photosensitization procedure

Experimental setup

Bacterial cultures grown overnight were tenfold diluted in PBS to a final concentration of 10^{6} CFU mL^{-1}. This bacterial suspension was equally distributed in 100 mL sterilized and acid-washed glass beakers. Then, appropriate quantities of the three porphyrins under study were added to achieve final concentrations of 0.5, 1.0 and 5.0 μM (total volume was 10 mL per beaker). The samples were protected from light with aluminium foil and incubated for 10 min under 100 rpm stirring, at 25–30 °C, to promote the

porphyrin binding to *E. coli* cells. Light and dark controls were carried out during the experiments. In the light control no porphyrin was added, but the beaker was exposed to the same irradiation protocol. In the dark control, the photosensitizer at the highest concentration (5.0 μM) was added to the beaker and it was covered with aluminium foil.

Irradiation conditions

Following the pre-irradiation incubation period, the samples were exposed to two different light sources, until 270 min, under 100 rpm stirring.

In laboratorial experiments with artificial light, white light (PAR radiation, 13 OSRAM 21 lamps of 18 W each, 380–700 nm) was used with a fluence rate of 40 W m^{-2} (measured with a radiometer LI-COR Model LI-250). Samples were placed on a tray with clamps and the bottom of the tray was covered with water in order to maintain the samples at constant temperature (25 °C), as bioluminescent *V. fischeri lux* genes, emit light preferentially at temperatures below 30 °C [50].

The experiments with solar irradiation were carried out outside the laboratory. Samples were exposed to solar PAR light on sunny summer days, in the Littoral Centre of Portugal, where the averaged PAR light fluence rate was 620 W m^{-2} (measured with a radiometer LI-COR Model LI-250). To filter the ultraviolet radiation, samples were covered with a glass petri plate. Only the PAR radiation of the solar spectrum was used in order to avoid ultraviolet inactivation of the bacteria and thus to allow comparing of these results with those obtained with artificial light. Samples were also placed on a tray with clamps and the bottom was covered with water. Water temperature was monitored and maintained at 25 °C.

Bioluminescence monitoring

In both irradiation experiments, aliquots of treated and control samples were collected at time 0 and after 15, 30, 60, 90, 180 and 270 min of light exposure and the bioluminescence was measured in the luminometer.

Statistical analysis

All experiments for the three porphyrins were done in duplicate. Statistical analysis was performed by using SPSS (SPSS 15.0 for Windows, SPSS Inc., USA). Normal distributions were assessed by the Kolmogorov–Smirnov test. The significance of both light conditions and porphyrin derivatives on bacterial inactivation was assessed by two-way univariate analysis of variance (ANOVA) model with the Bonferroni post hoc test. A value of $P < 0.05$ was considered significant.

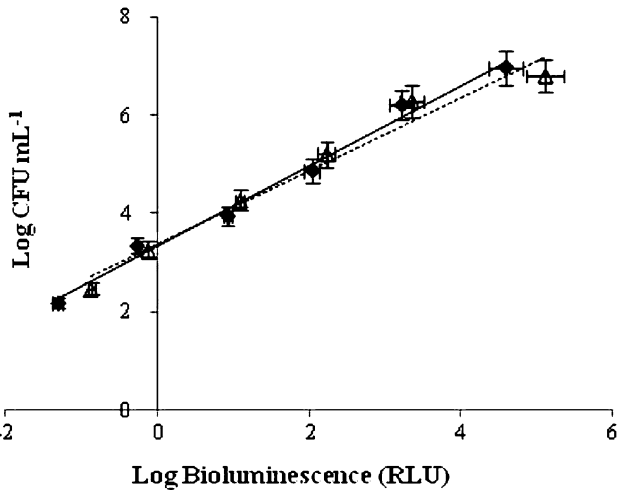

Fig. 2 Relationship between bioluminescence and viable counts of overnight cultures of recombinant *E. coli* (≈10^7 CFU mL^{-1}) serially diluted in PBS. Bioluminescence is expressed in relative light units (RLU) and viable counts in CFU mL^{-1}. The values are expressed as the means of two independent experiments; *error bars* indicate the standard deviation. (*filled diamond E. coli* suspension in the absence of PS, *open triangle E. coli* suspension with 5.0 μM of Tri-Py^+-Me-PF incubated 4 h in the dark at 25 °C)

Results

Bioluminescence versus CFU of an overnight culture

The linear relationship between the bioluminescence and viable counts of a growing culture of recombinant *E. coli* is presented in Fig. 2. The bioluminescence results reflect the viable bacterial abundance.

Artificial light experiments

Comparing the bioluminescence values obtained in the experiments carried out under the artificial light conditions (Figs. 3a, 4a, 5a), a clear difference in the PI patterns of the three porphyrins is observed. For the lower concentrations used (0.5 and 1.0 μM), the tricationic porphyrins (Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me) are more efficient than the tetracationic one (Tetra-Py^+-Me) ($P < 0.05$, ANOVA). They caused more than 4 log decrease in bioluminescence (reaching the limits of detection) after 270 min of irradiation, while at these concentrations and after the same period, the tetracationic porphyrin (Tetra-Py^+-Me) shows only a 0.50 and a 0.65 log decrease in bioluminescence (Fig. 5a). With 5.0 μM, the PI pattern is not significantly different amongst the three porphyrins ($P > 0.05$, ANOVA) all causing decreases in the bioluminescence higher than 4.2 log.

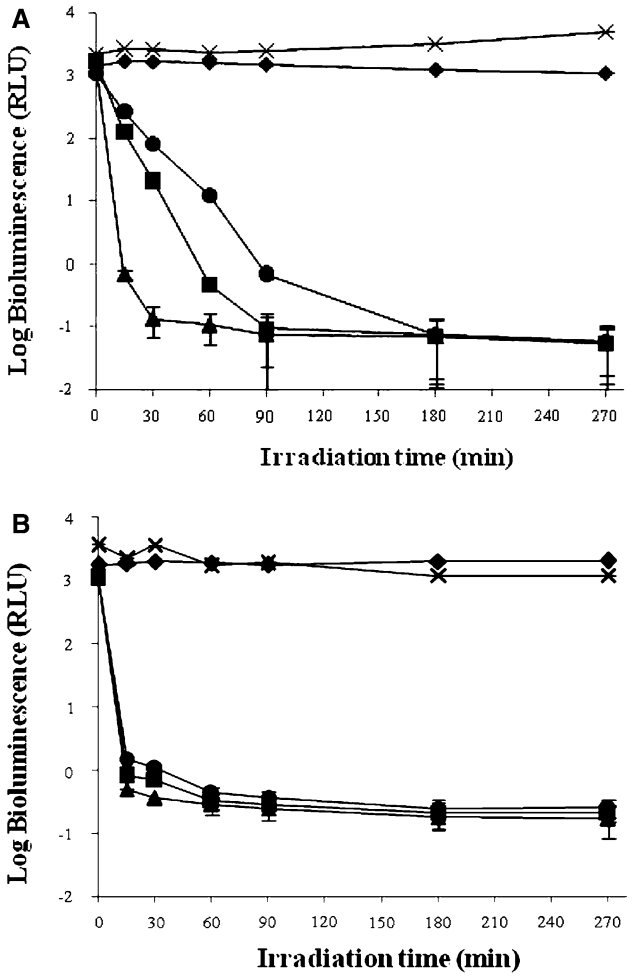

Fig. 3 Bioluminescence monitoring of *E. coli* treated with Tri-Py$^+$-Me-PF. **a** Irradiation with artificial light (40 W m^{-2}). **b** Irradiation with solar PAR light (620 W m^{-2}). The values are expressed as the means of two independent experiments; *error bars* indicate the standard deviation; *multisign* light control, *filled diamond* dark control, *filled circle* 0.5 μM, *filled square* 1.0 μM, *filled triangle* 5.0 μM

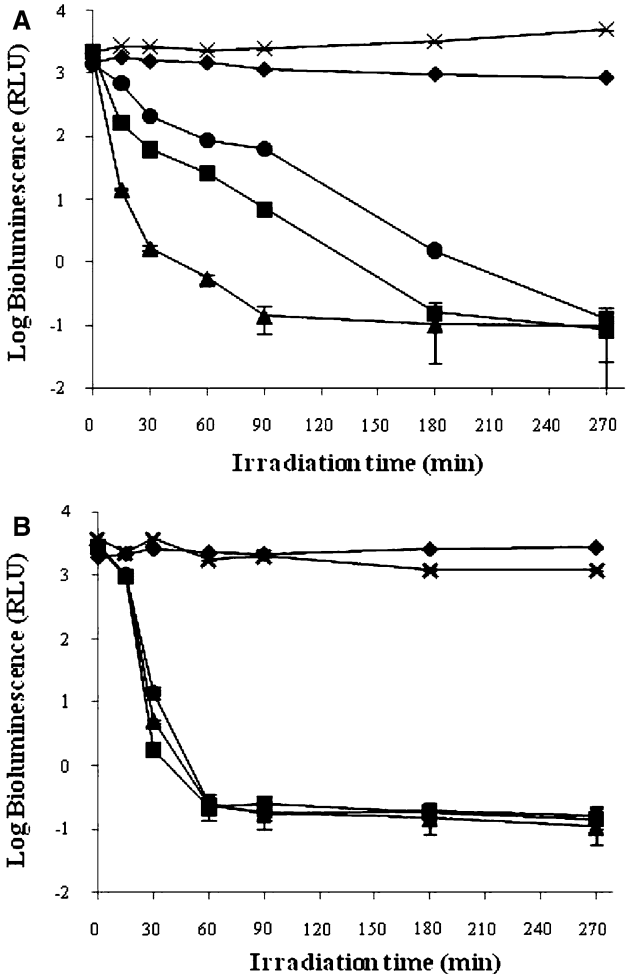

Fig. 4 Bioluminescence monitoring of *E. coli* treated with Tri-Py$^+$-Me-CO_2Me. **a** Irradiation with artificial light (40 W m^{-2}). **b** Irradiation with solar PAR light (620 W m^{-2}). The values are expressed as the means of two independent experiments; *error bars* indicate the standard deviation; *multisign* light control, *filled diamond* dark control, *filled circle* 0.5 μM, *filled square* 1.0 μM, *filled triangle* 5.0 μM

Solar light experiments

The results of the experiments carried out under solar PAR light conditions (≈620 W m^{-2}) show that the three porphyrins studied are able to cause a decrease in the bioluminescence signal to the limit of detection (more than 4 log decrease) after 270 min of irradiation even with the lowest concentration tested (Figs. 3b, 4b, 5b). However, for the lower concentration values (0.5 and 1.0 μM), with Tri-Py$^+$-Me-PF the drop occurs earlier (≈3 log reduction of bioluminescence after 15 min) than for the other porphyrins. After 15 min of irradiation, Tri-Py$^+$-Me-CO_2Me caused bioluminescence reduction of 0.46 log (both with 0.5 and 1.0 μM), while Tetra-Py$^+$-Me caused 0.89 log and 2.46 log decrease with 0.5 and 1.0 μM, respectively. For the highest concentration, at short irradiation periods (15 min) the photoinactivation with Tetra-Py$^+$-Me is slightly higher than that of Tri-Py$^+$-Me-PF. However, after 90 min of irradiation, all the three porphyrins are similarly effective, reaching the detection limit.

Control samples

The results of the experiments carried out under artificial light conditions, show that the viability of the recombinant bioluminescent *E. coli* was not affected either by irradiation itself (light control) or by any of the PS tested (dark control) (Figs. 3a, 4a, 5a). The same was observed in the light and dark controls during solar light experiments. This clearly indicates that the cell viability reduction observed after irradiation of the treated samples is due to the photosensitization effect of the porphyrins.

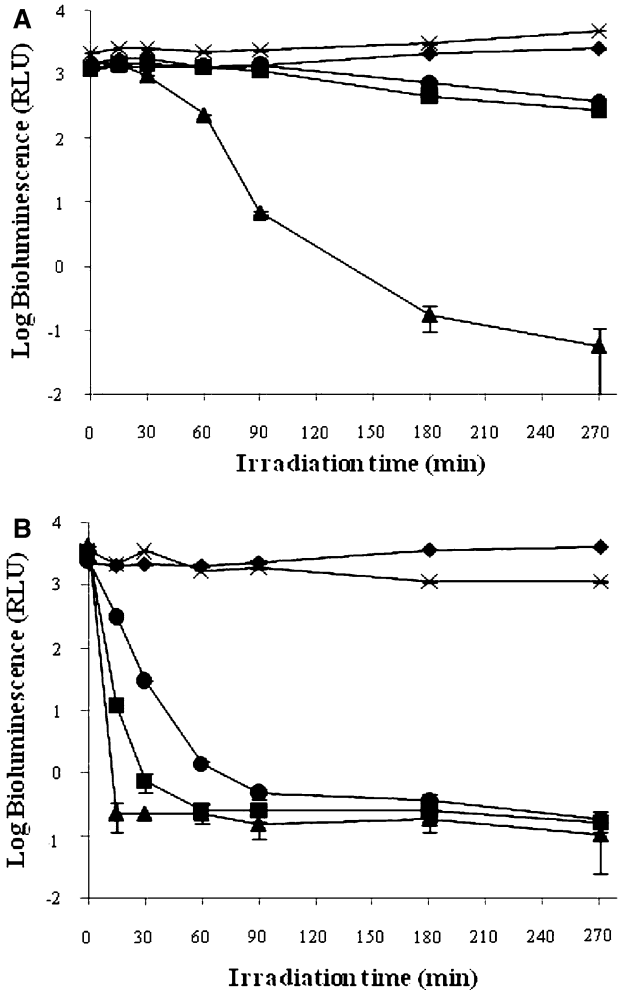

Fig. 5 Bioluminescence monitoring of *E. coli* treated with Tetra-Py$^+$-Me. **a** Irradiation with artificial light (40 W m^{-2}). **b** Irradiation with solar PAR light (620 W m^{-2}). The values are expressed as the means of two independent experiments; *error bars* indicate the standard deviation; *cross* light control, *filled diamond* dark control, *filled circle* 0.5 μM, *filled square* 1.0 μM, *filled triangle* 5.0 μM

Discussion

Photodynamic antimicrobial therapy has been considered a promising alternative to treat skin lesions and dental infections [8, 51, 52]. The possibility to use this approach in the wastewater treatment, in order to avoid or reduce the environmental pollution and to promote the reuse of water for crop irrigation, for example, has led to the study of the efficiency of different PS on the inactivation of different microorganisms under different light fluencies [1–3, 6, 53]. The conventional methods used to test microbial PI in vitro require laborious techniques of plating, overnight incubation and the time-consuming counting of CFU. To this end, simpler, faster, cheaper and sensitive methods, as bioluminescence methods, are desirable for PI studies.

In the present study, we demonstrate that it is possible to rapidly photoinactivate bioluminescent *E. coli* with cationic *meso*-substituted porphyrin derivatives as PS. The bioluminescent indicator strain was obtained by transformation of an *E. coli* strain with the lux operon (*luxCDABE*) that emits light continuously without the addition of exogenous substrates [20]. The use of stable bioluminescent bacteria allows following the progress of the PI process with real time results. The bioluminescence was followed as a function of time to show that the transformed bacteria are stable light producers and the light production correlated with the growth of the bacteria (Fig. 2).

The relationship between cell viability and luminescence intensity in the presence and in the absence of the sensitizer is similar (Fig. 2), showing that the sensitizer is not toxic to the transformed bacterium and, consequently, does not affect the relationship between bacterial growth and luminescence. Moreover, the photoinactivation pattern obtained with the bioluminescence method is similar to that determined by the conventional plating technique for the *E. coli* strain with the Tri-Py$^+$-Me-PF sensitizer used in this assay [44].

The eventual metabolic stress introduced in the transformed bacterium during the plasmid insertion does not seem to affect the photoinactivation kinetics. The viability of bioluminescent strain is not affected by the porphyrin after 4 h of incubation in the dark (dark control in Figs. 3, 4, 5) or by light irradiation (light control in Figs. 3, 4, 5). The bioluminescent strain is only affected by irradiation in the presence of the sensitizer (test samples in Figs. 3,4, 5), indicating that the decrease in bacterial viability is due to the photoinactivation process and not due to any kind of metabolic stress related to the plasmid insertion.

The experiments carried out with artificial light, with a fluence rate of 40 W m^{-2}, show that the three porphyrins used are efficient PS against bioluminescent *E. coli*, causing a reduction on light signal to the limit of detection of the luminometer. The differences between the three porphyrins are more noticeable at lower concentrations of PI, since the PI by tricationic porphyrins is significantly higher than that by tetracationic porphyrin. At 5.0 μM the differences are still observed for short irradiation periods but after 270 min of irradiation, all porphyrins show equal effectiveness.

Under solar irradiation with a fluence rate of ≈620 W m^{-2}, Tri-Py$^+$-Me-PF is more effective than Tetra-Py$^+$-Me and Tri-Py$^+$-Me-CO_2Me for low concentrations. With this porphyrin the PI is much faster (more than a 3 log decrease after only 15 min of irradiation). At 5.0 μM, at lower irradiation periods, the Tetra-Py$^+$-Me is the more effective PS. As expected, when solar light is used the PI occurs faster due to the higher light fluence rate.

This study shows that the PI of bioluminescent *E. coli* is achieved with both light fluences of 40 and ≈620 W m^{-2}. It is worth to refer that the results obtained in this study for artificial light (40 W m^{-2}) are in agreement with the PI patterns previously obtained for these PS where the conventional method of CFU count was carried out and a non-transformed *E. coli* stain was used [44]. The major difference is that, in the present study, the effect of the photosensitizers is observed immediately after the irradiation just by measuring bioluminescence of the test cell suspension. Since it does not require expensive equipment or materials, it can be used as a rapid method for monitoring PI experiments. For this reason, it can be concluded that the bacterial bioluminescence method is a sensitive, simple and cost-effective real time approach,.

The complete inactivation of Gram (−) bacteria (to the limit of detection) with solar radiation shows that, using the adequate PS, the photodynamic antimicrobial therapy can be used for environmental applications, namely for the disinfection of wastewater under natural irradiation conditions. In order to make this method an inexpensive, easily applicable and an environmental-friendly technology to remove sewage bacteria from wastewater, the recovery and reutilization of these porphyrins is required. For that, their immobilization on solid supports is already being tested in our laboratory.

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