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Photosensitization of Wild and Mutant Strains of *Escherichia coli* by *meso*-Tetra (*N*-methyl-4-pyridyl)porphine

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Wild type *Escherichia coli* cells as well as some mutant strains lacking specific DNA repair systems are efficiently killed upon visible light-irradiation after 5 min-incubation with *meso*-tetra(4*N*-methyl-pyridyl)-porphine (T_4MPyP). The presence of oxygen is necessary for cell photoinactivation. The porphyrin appears to exert its phototoxic activity largely by impairing some enzymic and transport functions at the level of both the outer and cytoplasmic membrane. Thus, SDS–PAGE electrophoresis shows a gradual attenuation of some transport protein bands as the irradiation proceeds, while a complete loss of lactate and NADH dehydrogenase activities is caused by 15 min-exposure to light. On the other hand, DNA does not represent a critical target of T_4MPyP photosensitization as suggested by the closely similar photosensitivity of the wild *E. coli* and *E. coli* strains defective for two different DNA repair mechanisms, as well as by the lack of any detectable alteration of the pUC19 plasmids extracted from photosensitized *E. coli* TG1 cells.

The increasing diffusion of resistance of bacteria, especially Gram-negative bacteria, to commonly used antibiotics, stimulates the development of new modalities for the treatment of microbial infections as well as for the sterilization of different media (e.g. water). Photosensitization can represent a useful approach for the killing of microbial cells since it was shown that several porphyrins and related compounds, once activated by visible light, exhibit phototoxicity against many types of microbial agents, including bacteria, mycoplasma and yeasts (1, 2); Moreover, the antibacterial effect is independent of the antibiotic sensitivity of the treated pathogen (3). However, photosensitization of bacteria is peculiar in that, while Gram-positive bacteria are photosensitive and can be efficiently killed under illumination in the presence of various types of neutral or anionic photosensitizers, Gram-negative bacteria cannot be photoinactivated unless the structure of the outer membrane is altered by pretreatment with EDTA or polycations (4, 5). It is likely that the outer membrane of Gram-negative bacteria, located outside the cell wall, acts as a permeability barrier preventing the access of the photosensitizer molecules to the inner cytoplasmic membrane (6), as well as intercepting the cytotoxic reactive species (e.g. singlet oxygen or hydroxyl radicals) which are generated by those photosensitizer molecules bound to the outer cell structures (7). More recently, it was shown that cationic porphyrins (8) and phthalocyanines (9) can efficiently photosensitize the inactivation of different strains of Gram-negative bacteria even in the absence of membrane-permeabilizing agents. Apparently, the ability to photoinactivate Gram-negative bacteria is not related to particular photophysical properties of these photosensitizers (10). It appears that the positive charge orientates the photosensitizer to particular cellular sites whose initial damage facilitates the penetration of photosensitizer molecules to the inner membrane and/or cytoplasm. In order to obtain further information on the mechanism by which cationic photosensitizers induce the photoinactivation of Gram-negative bacteria, we have undertaken a detailed study of *Escherichia coli* photosensitization by meso-tetra(N-methyl-4-pyridyl)porphine (T_4MPyP), whose photocydal activity against bacteria had been previously investigated in our laboratory.

MATERIALS AND METHODS

Chemicals. *meso*-Tetra(N-methyl-4-pyridyl)porphine tetratosylate (T_4MPyP) was supplied by Porphyrin Products (Logan, UT, U.S.A.) and dissolved in water (ϵ = 194000 $M^{-1}cm^{-1}$ at 422 nm). Unless specified otherwise, all chemicals were analytical grade reagents and were used without further purification.

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Bacterial strains. *Escherichia coli* strain O4 (wild type) was grown aerobically at 37°C in brain heart infusion broth (Difco, Detroit, MI, U.S.A.). *E. coli* strain WP2 TM9 was kindly provided by Prof. Baccicchetti (Department of Pharmaceutical Sciences, University of Padova, Italy); this strain is uvrA$^-$, trp$^-$ and contains the plasmid R46 carrying the resistance to ampicillin; it was grown in a minimal broth containing 0.2% glucose, 0.1 mg/ml tryptophan and 25 μg/ml ampicillin (Sigma, St Luis, MO, U.S.A.). *E. coli* strain TG1 was transfected with the plasmid pUC19, after treatment with cold 0.1 M $CaCl_2$ (11), and maintained in Luria-Bertani broth containing 100 μg/ml ampicillin. *E. coli* Bs-1 (ATCC 23224) is an hcr$^-$ strain and was grown in nutrient broth (Difco). In all cases cells in the stationary phase of growth were harvested by centrifugation, washed twice with 10 mM phosphate-buffered saline (PBS) at pH = 7.4, containing 2.7 mM KCl and 0.14 M NaCl and diluted in PBS to an absorbance of 0.7 at 650 nm, corresponding to $10^8 - 10^9$ cells/ml.

Porphyrin binding experiments. The amount of cell-bound T_4MPyP was estimated by spectrophotofluorimetric analysis on the cell pellet obtained by centrifugation at the end of the incubation time (0 washing) or after one or three washings with PBS. The cell pellets were resuspended in 2% aqueous SDS, incubated overnight with the surfactant and diluted in the same solvent; the fluorescence emitted by T_4MPyP (600–750 nm) was measured after excitation at 400 nm by means of a MPF4 spectrophotofluorimeter (Perkin-Elmer) and the concentration of the sensitizer was calculated by measuring the intensities of the emission peaks and interpolating the data on a calibration plot. The protein content of the samples was assayed by the method of Lowry (12) and was used to express the recovery as nmoles of sensitizer/mg of protein.

Irradiation experiments and cell survival assays. In a typical experiment, 5 ml of cell suspension were incubated with 10 μM T_4MPyP for 5 min. in the dark at room temperature and then irradiated at a fluence rate of 150 mW/cm^2 with white light emitted by a Penta PTL halogen lamp (Teclas, Lugano, Switzerland). During irradiation, the suspension was magnetically stirred and kept at 37°C by circulating water. For the experiments under anoxic conditions the cell suspensions were degassed by evaporating half of the buffer with a vacuum pump, incubated with the photosensitizer and irradiated in an Atmos-bag (Sigma, St. Louis, Mo, U.S.A.) filled with N_2.

Cell survival was tested by plating aliquots of the suspensions on brain heart agar after dilution and counting the number of colonies formed after 18–24 h incubation at 37°C.

Enzymic assays. The activity of various enzymes of the cytoplasmic membrane was measured in lysed spheroplasts prepared by resuspending the treated cells in Tris-HCl 0.05 M buffer at pH = 6.8 containing 0.01 M EDTA, 0.3 M saccarose and lysozyme at a concentration of 1 mg/ml. The samples were incubated at 37°C for 1 h and centrifuged at 2000 g for 10 min. The pellet was resuspended in water and the spheroplasts were lysed by sonication in an ice bath.

NADH dehydrogenase activity was measured according to Marriott *et al.* (13) by following the oxidation of NADH spectrophotometrically at 340 nm in the presence of $K_3Fe(CN)_6$ as electron acceptor. Lactate and succinate dehydrogenase activities were measured by the methods described by Santos *et al.* (14) and Osborn *et al.* (15), respectively. ATPase activity was evaluated by assaying the amount of inorganic phosphate produced from ATP hydrolysis (16); the enzyme was rapidly inactivated by the addition of 10% trichloroacetic acid and the phosphate present in the supernatant obtained after sample centrifugation was measured by a kit supplied by Boehringer (Mannheim, Germany). Protein content of the samples was measured by the Lowry procedure; the activities of the enzymes are expressed as percentage of the specific activity of the control samples.

Protein electrophoresis. Outer membrane proteins were isolated as described by Rapp *et al.* (17). After determination of the protein concentration in the samples by the Lowry method, about 50 μg of proteins were loaded in a 12% polyacrylammide gel in the presence of 2% SDS for electrophoretic analysis (18). The gel was stained with Coomassie brilliant blue and destained with methanol:acetic acid: water (4:3:33, v:v:v).

TABLE 1

Recovery of Porphyrin from Different Strains of *E. coli* Cells, Incubated for 5 Min with 10 μM T_4MPyP and Subjected to Different Washing Steps

	T_4MPyP recovery (nanomoles/mg of protein)		
E. coli strains	0 washings	1 washing	3 washings
O4	3.08 ± 0.51	1.29 ± 0.52	0.60 ± 0.19
WP2	2.76 ± 0.71	0.97 ± 0.18	0.67 ± 0.29
Bs-1	3.28 ± 0.69	1.37 ± 0.19	0.50 ± 0.12
TG1	3.47 ± 0.31	1.37 ± 0.08	1.10 ± 0.20
TG1 + pUC19	4.62 ± 0.79	2.09 ± 0.05	1.01 ± 0.26

Plasmid DNA purification and electrophoresis. Plasmid DNA from control and irradiated cells of *E. coli* TG1 was extracted following the procedure described by Maniatis (11). Briefly, the cell pellets obtained from 5 ml of suspension were resuspended for 5 min. in 100 μl of a sterile solution at pH = 8 containing 50 mM glucose, 25 mM Tris and 10 mM EDTA. Cells were then lysed by adding 200 μl of 0.2 M NaOH containing 1% SDS and incubating the samples for 5 min. in an ice bath. After the addition of 150 μl of a cold K^+ acetate/acetic acid solution (3 M K^+ and 5 M acetate final concentrations), the samples were incubated for 5 min. in ice and centrifuged again. The DNA in the supernatant was precipitated with 2 volumes of absolute ethanol, centrifuged, washed with 1 ml of 70% ethanol and resuspended in 20 μl of 10 mM Tris, 1 mM EDTA buffer at pH = 8. The samples were then treated with RNAase, digested, when necessary, with Bam HI and denaturated by boiling for 3 min. Electrophoresis was performed on 1% agarose gel in 0.045 mM Tris buffer containing 0.045 mM boric acid and 1 mM EDTA at pH = 8. The DNA was stained with 0.25 μg/ml ethidium bromide.

RESULTS

T_4MPyP exhibited a similar affinity for the strains O4, WP2, Bs-1 and TG1 of *E. coli* cells. As shown in Table 1, essentially identical amounts of porphyrin were recovered from the four strains after 5 min. incubation. Moreover, similar percentages of the initially bound T_4MPyP were removed after 1 or 3 washing steps and about 20% of the accumulated porphyrin was tightly bound under our conditions. Exposure of the cell suspensions to white light caused a marked inhibition of the bacterial cell growth; a decrease of about 5 log in cell survival was observed after 30 min. irradiation in air-equilibrated phosphate buffer for the *E. coli* wild strain O4 (Fig. 1); the WP2 and Bs-1 strains, defective for two different DNA repair mechanisms, were photoinactivated with very similar efficiency. On the contrary, no decrease in cell survival was observed when irradiation of *E. coli* O4 was performed under identical experimental conditions but in the absence of O_2 (Fig. 1); control experiments pointed out that *E. coli* cells kept in the darkness under anoxic conditions for 30 min. underwent no detectable decrease in survival.

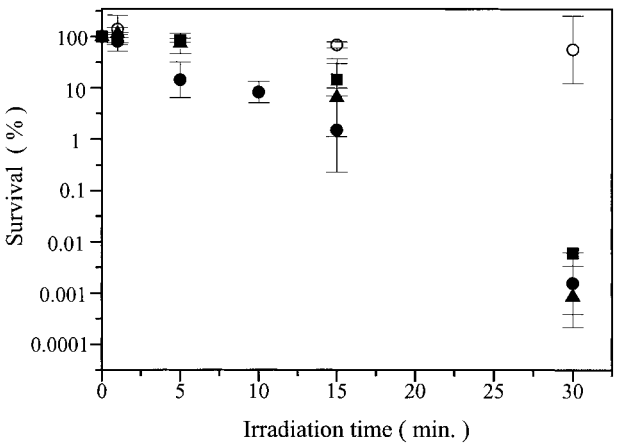

FIG. 1. Survival of *E. coli* O4 (○, ●), Bs-1 (▲), WP2 (■) cells incubated for 5 min. with 10 μM T_4MPyP and irradiated for different times. *E. coli* O4 cells were irradiated in air-equilibrated (●) or deoxygenated (○) 10 mM phosphate buffer, pH = 7.4.

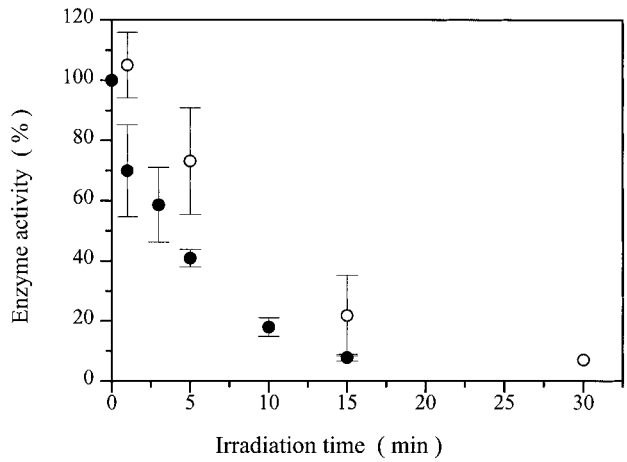

FIG. 3. NADH dehydrogenase (○) and lactate dehydrogenase (●) activities in *E. coli* O4 cells incubated for 5 min. with 10 μM T_4MPyP and irradiated for different times.

SDS–PAGE analysis of the *E. coli* O4 outer membrane proteins showed an alteration of the electrophoretic mobility in the irradiated samples (Fig. 2). In particular, some protein bands in the 14–20 and 25–30 kdalton ranges and bands corresponding to a molecular weight around 50 kdalton and higher than 66 kdalton appeared to be attenuated or undetectable in the photosensitized samples. Moreover, in the 15- and 30 min.-irradiated samples high molecular weight products were observed as aggregated material on the top of the gel.

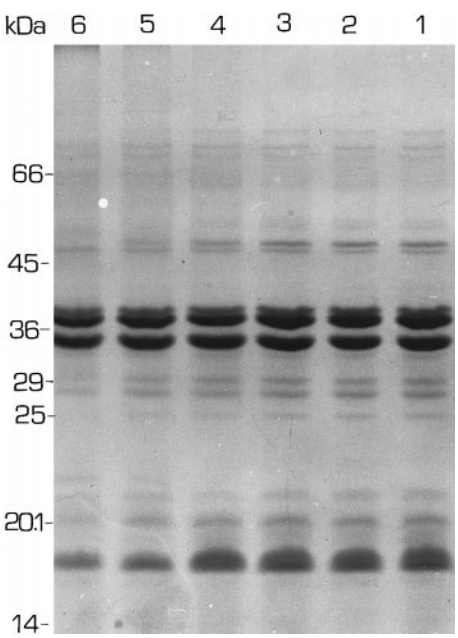

FIG. 2. SDS–PAGE of the outer membrane proteins of *E. coli* O4 cells. Lane 1: proteins from control cells; lanes 2–6: proteins from cells incubated for 5 min. with 10 μM T_4MPyP and irradiated for 0, 1, 5, 15, 30 min., respectively.

The damage of the cytoplasmic membrane proteins in T_4MPyP-photosensitized cells was demonstrated by the impairment of different enzymic activities: lactate and NADH dehydrogenases were readily inactivated by irradiation in the presence of T_4MPyP and a complete loss of enzymic function was observed after 15 and 30 min. irradiation, respectively (Fig. 3), while ATPase and succinate dehydrogenase were also inactivated although to a lesser extent (Fig. 4).

Figure 5 shows the survival of *E. coli* TG1 strain, incubated with the cationic photosensitizer and irradiated under the same conditions used for the other strains. An approximately 3 log decrease in cell survival was observed after 30 min. irradiation of both the TG1 strain transfected with the pUC19 plasmid and the plasmid-free strain. Moreover, no difference in the colony forming ability was observed when the irradi-

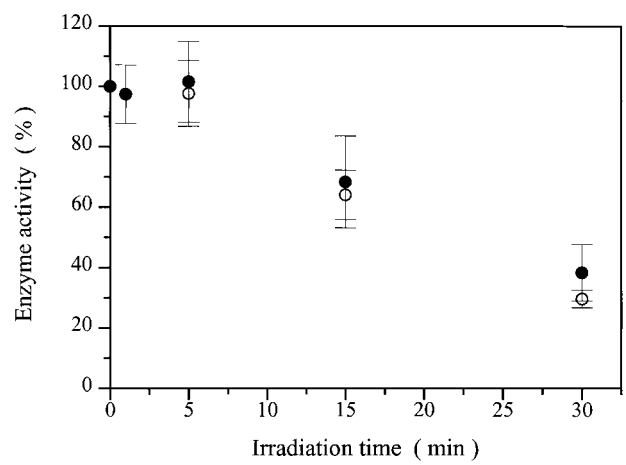

FIG. 4. ATPase (●) and succinate dehydrogenase (○) activities in *E. coli* O4 cells incubated for 5 min. with 10 μM T_4MPyP and irradiated for different times.

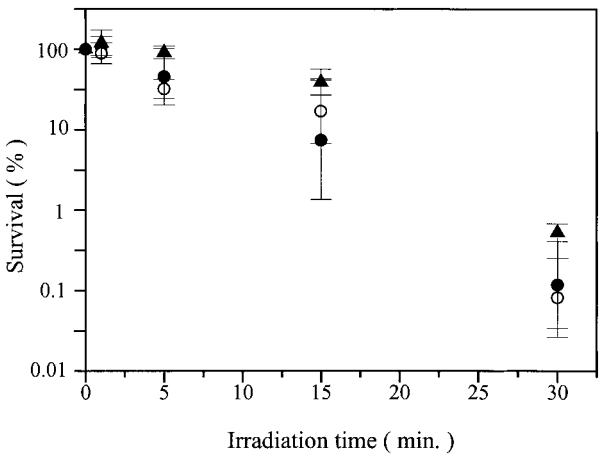

FIG. 5. Survival of *E. coli* TG1 normal cells (▲) and cells transformed with pUC19 plasmid (○, ●) incubated for 5 min. with 10 μM T_4MPyP and irradiated for different times. Transformed cells were plated on LB agar in the presence (○) and in the absence (●) of 100 μg/ml ampicillin.

ated samples were plated in the presence and in the absence of ampicillin (Fig. 5).

The effect of T_4MPyP photosensitization on plasmidial DNA extracted from *E. coli* TG1 cells was studied by mean of gel electrophoresis, as shown in Fig. 6. The different forms of the plasmid are present in every sample and no appearance of new bands could be detected, both under native conditions and after digestion and denaturation. Nevertheless, a marked decrease of plasmid DNA extracted from the *E. coli* cells irradiated for 15 and 30 min. was observed.

DISCUSSION

Previous studies from our laboratory (8) have clearly shown that T_4MPyP represents an efficient photosensitizer for both Gram-positive and Gram-negative bacteria. We have now undertaken a study on various *E. coli* strains characterized by different genotypes, in order to elucidate the mechanism of Gram-negative microorganisms photosensitization by T_4MPyP. This cationic porphyrin appears to act as a typical "photodynamic sensitizer" which requires the presence of oxygen to exert its cytotoxic activity (Fig. 1). The quantum yield of singlet oxygen production by T_4MPyP in aqueous solution is around 0.7 (10, 19), hence it is likely that singlet oxygen is involved in the photoinactivation of *E. coli* cells, even though the participation of other oxygen reactive species cannot be excluded. The photosensitivity of *E. coli* cells to the combined action of T_4MPyP and visible light is mainly influenced by the amount of bound porphyrin. As shown in Fig. 1, both the wild strain O4 and WP2 and Bs-1 strains, which display a closely similar pattern of T_4MPyP binding, undergo a very similar decrease in cell survival as a function of the irradiated time. WP2 and Bs-1 strains are defective for two different DNA repair mechanisms: the *uvr*A gene, mutated in the WP2 strain, is involved in the repair of many kinds of DNA lesions which deform the double helix, while the Bs-1 is an UV and X-ray sensitive strain. It is expected that cells with an impaired DNA repair system should show a greater sensitivity to any DNA damaging agent. The fact that the defective *E. coli* strains show no increased sensitivity toward T_4MPyP photosensitization as compared to the wild strain O4 appears to rule out an important role of DNA damage in determining the survival of the bacteria. Our hypothesis is further supported by the results obtained with the *E. coli* TG1 strain, which has been used as a wild type or after transformation with the pUC19 plasmid. The photosensitivity of this strain is lower than that typical of the other *E. coli* strains and is independent of the presence of the plasmid (Fig. 5). Moreover, the cell photoinactivation does not involve any primary damage to plasmidial DNA since surviving cells retain the resistance to the antibiotic, carried by the plasmid. Accordingly, gel electrophoresis of the plasmid extracted from the irradiated cells shows no significant alteration of the migration pattern.

Several cationic porphyrins, including T_4MPyP, are known to interact with DNA in vitro by both intercalation and binding externally to the double helix (20 and references therein). Such interaction can cause DNA photocleavage, as detected by single strand break formation in plasmidial DNA (19, 21). Our results do not confirm such effect at least in the case of *E. coli* cells: porphyrin concentrations and light doses which induce a drastic decrease in the cell survival do not cause a detectable DNA damage, as revealed by plasmidial DNA analysis and by the studies on the DNA repair deficient strains. It is possible that in the cellular environment the porphyrin interacts with targets other than DNA which are sensitive to photodynamic

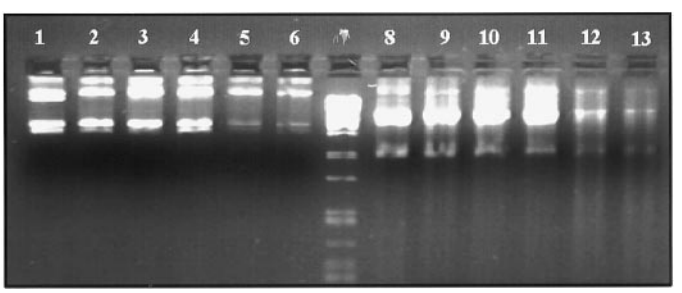

FIG. 6. Agarose gel electrophoresis of native plasmidial DNA (lanes 1–6) and of plasmidial DNA after digestion with Bam HI and denaturation (lanes 8–13) from *E. coli* TG1 cells. Lanes 1 and 8: DNA from control cells. Lanes 2–6 and 9–13: DNA from cells incubated with 10 μM T_4MPyP and irradiated for 0, 1, 5, 15 and 30 min., respectively. Lane 7 represents PstI-digested λ phage.

processes. The lower plasmid DNA content extracted from the cells irradiated for 15 and 30 min. as compared to the untreated cells may be explained with a decreased efficiency of the DNA extraction from the damaged/dead cells.

In fact, protein damage seems to be an important effect of *E. coli* photosensitization under our conditions: the proteins of both the outer and cytoplasmic membrane are clearly modified already after the first few minutes of irradiation. The alterations of the outer membrane proteins (Fig. 2) are rather selective. Thus, the disappearance of the bands corresponding to molecular weights higher than 66 kdalton could indicate damage of various proteins involved in the transport of different ferric ion chelates (22). The band localized in the 50 kdalton position can be ascribed to the lamB protein involved in the passage of maltose and maltodextrins (22) and appears to be strongly attenuated and almost undetectable in the 15 and 30 min.-irradiated samples. On the other hand, porins, which are the most abundant proteins of the outer membrane and are characterized by a molecular weight around 37–38 kdalton, are not significantly affected by T_4MPyP photosensitization. In any case, such modifications of the outer membrane could cause an increased permeability of the cells to the porphyrin during the initial stages of the photoprocess (10). The influx of T_4MPyP from the external medium would potentiate the phototoxic activity of the porphyrin.

The cytoplasmic membrane proteins are also photodamaged with a certain degree of selectivity. Thus, ATPase and succinate dehydrogenase are affected by T_4MPyP photosensitization but retain about 40% of their activity when the cell survival has undergone a 5 log decrease (Fig. 4); on the other hand, lactate and NADH dehydrogenase completely loose their activity under the same conditions (Fig. 3). Such difference in sensitivity is likely to reflect the different localization of the enzymes in the microenvironment of the cytoplasmic membrane: succinate dehydrogenase and ATPase are characterized by transmembrane subunits which can be partially protected from the phototoxic activity of the porphyrin by the hydrophobic bilayer; on the contrary, according to Cronan *et al.* (23), in *E. coli* cells, lactate and NADH dehydrogenase are flavoproteins associated to the cytoplasmic side of the membrane through hydrophobic interactions and represent quite accessible targets for an hydrophilic photosensitizer. The complete loss of these two enzymatic functions causes a serious impairment in the oxidative metabolism of the cells, blocking both the first step of the electron transport chain, and the glycolytic process, hence this can be considered a crucial event in the inactivation of *E. coli* cells by T_4MPyP photosensitization.

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