

# Phenothiazinium Antimicrobial Photosensitizers Are Substrates of Bacterial Multidrug Resistance Pumps

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**Antimicrobial photodynamic therapy (PDT) combines a nontoxic photoactivatable dye, or photosensitizer (PS), with harmless visible light to generate singlet oxygen and free radicals that kill microbial cells. Although the light can be focused on the diseased area, the best selectivity is achieved by choosing a PS that binds and penetrates microbial cells. Cationic phenothiazinium dyes, such as methylene blue and toluidine blue O, have been studied for many years and are the only PSs used clinically for antimicrobial PDT. Multidrug resistance pumps (MDRs) are membrane-localized proteins that pump drugs out of cells and have been identified for a wide range of organisms. We asked whether phenothiazinium salts with structures that are amphipathic cations could potentially be substrates of MDRs. We used MDR-deficient mutants of *Staphylococcus aureus* (NorA), *Escherichia coli* (TolC), and *Pseudomonas aeruginosa* (MexAB) and found 2 to 4 logs more killing than seen with wild-type strains by use of three different phenothiazinium PSs and red light. Mutants that overexpress MDRs were protected from killing compared to the wild type. Effective antimicrobial PSs of different chemical structures showed no difference in light-mediated killing depending on MDR phenotype. Differences in uptake of phenothiazinium PS by the cells depending on level of MDR expression were found. We propose that specific MDR inhibitors could be used in combination with phenothiazinium salts to enhance their photodestructive efficiency.**

Photodynamic therapy (PDT) combines a nontoxic photoactivatable dye, or photosensitizer (PS), with harmless visible light of the correct wavelength to excite the dye to its reactive triplet state, which will then generate reactive oxygen species, such as singlet oxygen and superoxide, that are toxic to cells (4). Although discovered more than 100 years ago by its killing effect on microorganisms (25), PDT has found most success as a treatment for cancer (9) and age-related macular degeneration (3).

A relatively novel application of PDT is to employ its ability to kill pathogenic microbes in the treatment of localized infections (12). Most of the PSs that are under investigation for the treatment of cancer and other tissue diseases are based on the tetrapyrrole nucleus, such as porphyrins, chlorins, bacteriochlorins, and phthalocyanines (4). However, other dyes that are frequently proposed as antimicrobial PSs have different molecular frameworks. These include halogenated xanthenes, such as Rose Bengal (RB) (33); perylenequinones, such as hypericin (17); and phenothiazinium salts, such as toluidine blue O (TBO) (1), methylene blue (MB) (11), and azure dyes (37). It is known that gram-positive bacterial species are much more sensitive to photodynamic inactivation (PDI) than gram-negative species (22) and that the ideal PS for killing bacteria should possess an overall cationic charge and preferably multiple cationic charges (12). Phenothiazinium salts possess one intrinsic quaternary nitrogen atom and have been used as PSs to kill tumor cells in vitro (39) as well as to treat tumors in

animal models (10). There is an increasing body of evidence for their phototoxic efficiency against a broad range of microorganisms (29, 38), such as *Escherichia coli*, *Staphylococcus aureus* (29), streptococci (28), *Listeria monocytogenes* (32), and *Vibrio vulnificus* (41). At present, the only PSs used clinically for antimicrobial treatments are phenothiazinium salts. For instance, MB or TBO and red light are used to disinfect blood products and sterilize dental cavities and are proposed to treat periodontitis (36).

Efflux mechanisms have become broadly recognized as major components of microbial resistance to many classes of antibiotics (30). Some efflux pumps selectively extrude specific antibiotics, while others, referred to as multidrug resistance pumps (MDRs) expel a variety of structurally diverse compounds with differing modes of action. It has been suggested that amphipathic cations represent the existing natural substrates of MDRs (19), and these molecules have frequently been used to study bacterial MDR-mediated efflux. A classical example of an amphipathic cation is ethidium bromide, and its uptake by microbial cells can easily be quantified by measuring intracellular fluorescence generated when the dye binds to nucleic acids (2). The existence of MDRs makes the discovery of antimicrobial compounds that are recognized by them problematic in standard screens that employ cells carrying MDRs. The development of MDR mutants gave an answer to this apparent paradox (13). Disabling of MDRs in gram-negative species led to a striking increase in antimicrobial activity for numerous plant substances (35).

In the present study, we asked whether phenothiazinium salts, which are structurally characterized as amphipathic cations, could potentially be substrates of microbial MDRs. We used MDR-deficient and MDR-overexpressing mutants of the

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TABLE 1. Bacterial strains

Bacterial strain	Genotype <sup>a</sup>	Characteristic
<i>S. aureus</i> 8325-4 1758 QT1	WT $\Delta norA::cat$ $norR::cat$	Wild type Knockout Overexpressing (NorA is overexpressed)
<i>E. coli</i> K-12 KLE701	WT $\Delta tolC::tet$	Wild type Knockout
<i>P. aeruginosa</i> PA767 PAO1 prototroph K1119 PAM1032	WT $\Delta mexAB-oprM$ $nalB$	Wild type Knockout Overexpressing (MexAB-OprM is overexpressed)

<sup>a</sup> WT, wild type; cat, chloramphenicol transferase; tet, tetracycline.

human pathogens *S. aureus*, *E. coli*, and *Pseudomonas aeruginosa*, together with a range of PSs, including both phenothiazinium salts and non-phenothiazinium-based PSs.

## MATERIALS AND METHODS

**Microbial strains and culture conditions.** Bacterial strains used in this study are listed in Table 1. Cells were cultured in brain heart infusion broth with aeration at 37°C. Cells were used for experiments in mid-log growth phase (10<sup>8</sup> per ml).

**Photosensitizers and light sources.** Toluidine blue O, methylene blue, and 1,9-dimethylmethylene blue (DMMB), all as chloride salts (Sigma-Aldrich, St. Louis, MO), were used as phenothiazinium-based PSs. We used a poly-L-lysine-chlorin<sub>e6</sub> conjugate (pL-c<sub>e6</sub>), which had an average of 110 lysine residues with four chlorin e6 molecules attached (8), and Rose Bengal (Sigma-Aldrich) as non-phenothiazinium-based PSs. The chemical structures of all of the PSs are shown in Fig. 1. Stock solutions were prepared in water at a concentration of 2 mM and stored for a maximum of 2 weeks at 4°C in the dark before use. Spectra of stock solutions diluted 140- to 280-fold in methanol were recorded on a UV visible-spectroscopy system (Waldbronn, Germany). A noncoherent light source with interchangeable fiber bundles (LC122; LumaCare, London, United Kingdom) was employed. Thirty-nanometer band-pass filters at ranges of 540 ± 15 nm for RB, 635 ± 15 nm for TBO and DMMB, and 660 ± 15 nm for MB and pL-c<sub>e6</sub> were used. The total power output provided out of the fiber bundle

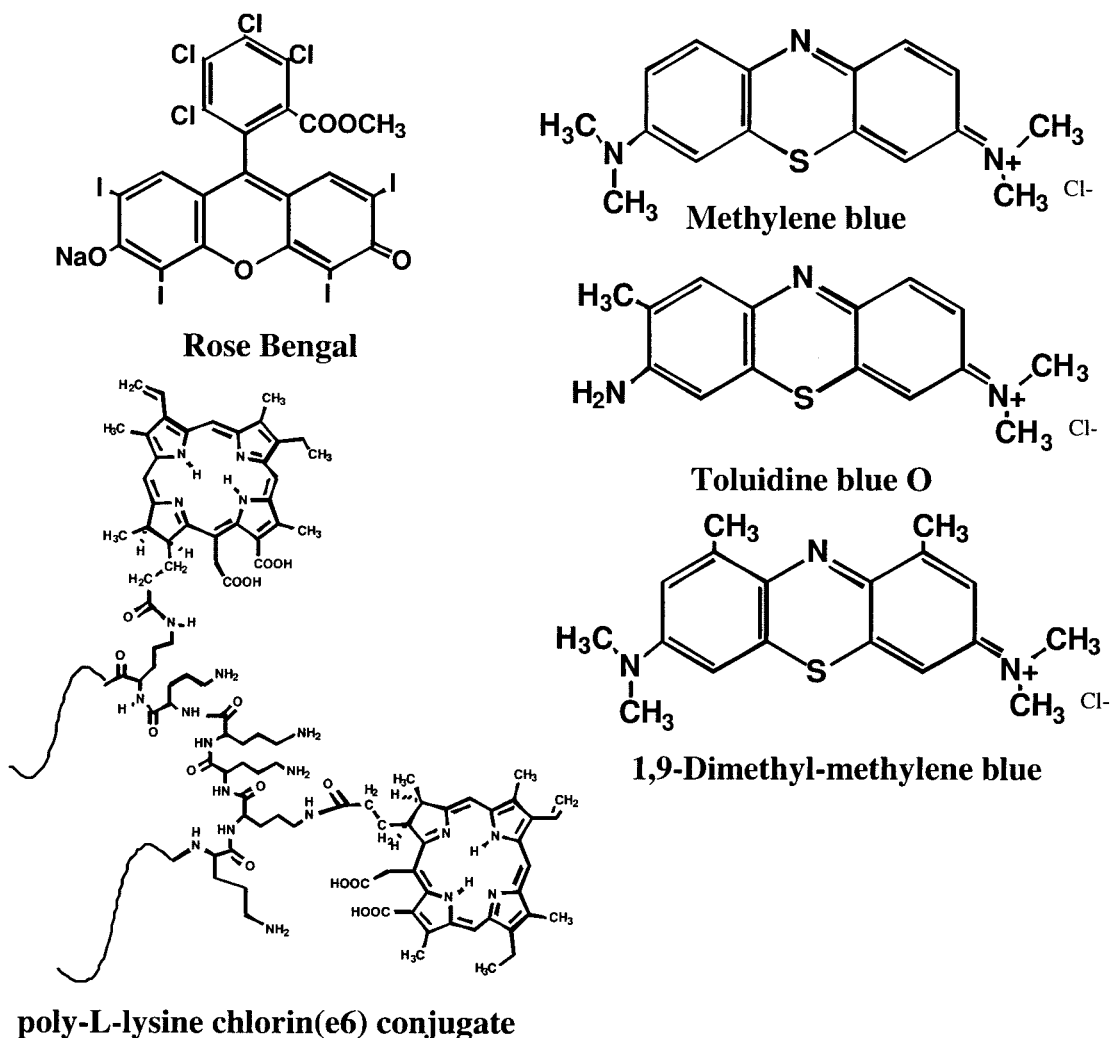


FIG. 1. Chemical structures of the PSs used.

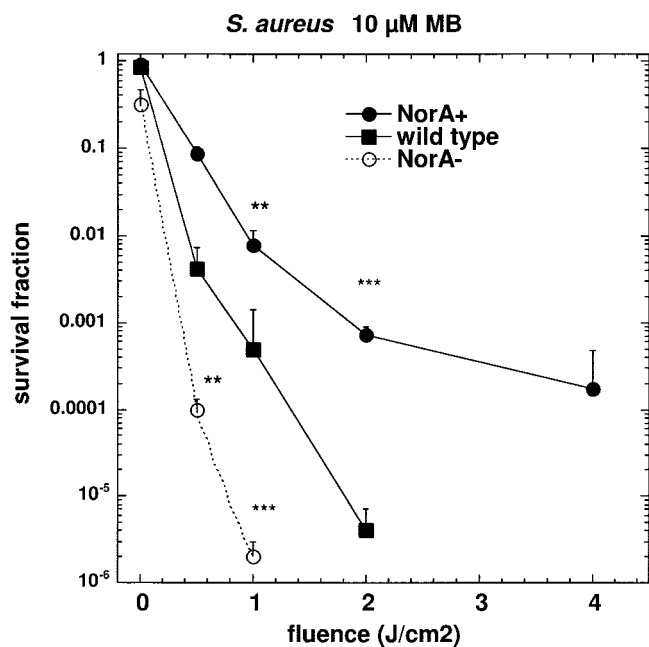


FIG. 2. Phototoxicity of MB after incubation at a concentration of 10  $\mu\text{M}$  by *S. aureus* NorA knockout (NorA<sup>-</sup>), wild-type, and NorA-overexpressing (NorA<sup>+</sup>) strains. Incubation with the PS was for 30 min followed by a wash. Bacteria were then illuminated with 100 mW  $\text{cm}^{-2}$  660-nm light, and the survival fractions were determined as described in Material and Methods. Values are means of three separate experiments, and bars are SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (compared to the wild type).

ranged from 300 to 700 mW. The spot was arranged to give an irradiance of 100  $\text{mW}/\text{cm}^2$ .

**PDI studies.** Bacterial suspensions in phosphate-buffered saline (PBS) (initial concentration,  $10^8$  cells  $\text{ml}^{-1}$ ) were incubated with PSs in the dark at room temperature for 30 min at concentrations from 1  $\mu\text{M}$  to 300  $\mu\text{M}$ . The cell suspensions were centrifuged at 12,000 rpm and then washed twice with sterile PBS. The bacterial suspensions were placed in wells of 96-well microtiter plates (Fisher Scientific) and illuminated with appropriate light at room temperature. Fluences ranged from 0 to 20  $\text{J}/\text{cm}^2$  at a fluence rate of 100  $\text{mW}/\text{cm}^2$ . During illumination, aliquots of 10  $\mu\text{l}$  were taken to determine the CFU. The contents of the wells were mixed before sampling. The aliquots were serially diluted 10-fold in PBS to give dilutions of  $10^{-1}$  to  $10^{-6}$  times the original concentrations and were streaked horizontally on square brain heart infusion agar plates as described by Jett et al. (14). This allowed a maximum of 7 logs of killing to be measured. Plates were incubated at 37°C overnight. Two types of control conditions were used: illumination in the absence of PS and incubation with PS in the dark.

**Uptake studies.** Bacterial suspensions ( $10^8$  cells/ml) were incubated in PBS in the dark at room temperature for 30 min with PS in the same concentrations as were used for the PDI experiments. Incubations were carried out in triplicate. The cell suspensions were centrifuged ( $9,000 \times g$  for 1 min), the PS solution was aspirated, and bacteria were washed twice in 1 ml of sterile PBS and centrifuged as described above. Finally, the cell pellet was dissolved by digestion in 3 ml of 0.1 M NaOH-1% sodium dodecyl sulfate (SDS) for at least 24 h to give the cell extract as a homogenous solution. Fluorescence in the extracts was measured with a spectrofluorometer (model FluoroMax3; SPEX Industries, Edison, N.J.). For TBO and DMMB, the excitation wavelength was 620 nm and the range for emission was 627 to 720 nm. For MB, the excitation wavelength was 650 nm and the range for emission was 655 to 720 nm. For pL-C<sub>60</sub>, the excitation wavelength was 400 nm and the emission spectra of the solution were recorded from 580 to 700 nm. For RB, the excitation wavelength was 552 nm and the emission was recorded in the range from 555 to 620 nm. The fluorescence was calculated from the heights of the peaks recorded. If necessary, the solution was diluted with 0.1 M NaOH-1% SDS to reach a concentration of the PS where the fluorescence response was linear. Calibration curves were made from pure PS dissolved in

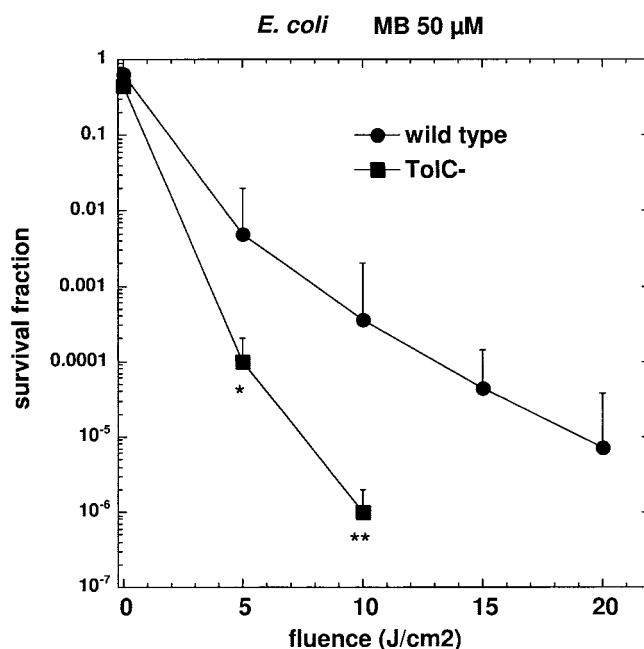


FIG. 3. Phototoxicity of MB after incubation at concentration of 50  $\mu\text{M}$  by *E. coli* wild-type and TolC knockout (TolC<sup>-</sup>) strains. Conditions are as described in the legend for Fig. 2.

NaOH-SDS and used for determination of PS concentration in the suspension. Uptake values were obtained by dividing the number of nanomoles of PS in the dissolved pellet by the number of CFU obtained by serial dilutions, and the number of PS molecules/cell was calculated by using Avogadro's number.

**Statistics.** Values are means of three separate experiments, and bars are standard errors of the means (SEM). Differences between means were tested for significance by an unpaired two-tailed Student  $t$  test assuming equal or unequal variations as appropriate. The significance level was set at a  $P$  value of  $< 0.05$ .

## RESULTS

**NorA expression protects against MB phototoxicity in *S. aureus*.** The three isogenic strains of *S. aureus* were incubated with 10  $\mu\text{M}$  MB for 30 min and then washed free of unbound dye by centrifugation and resuspension in PBS and illuminated with 660-nm light. Figure 2 shows the resulting light-dose-dependent phototoxicity. The wild-type strain showed 3 logs of killing after 1  $\text{J}/\text{cm}^2$ , 5 logs after 2  $\text{J}/\text{cm}^2$ , and 7 logs after 4  $\text{J}/\text{cm}^2$ . The NorA knockout strain showed complete killing after 1  $\text{J}/\text{cm}^2$ , while the NorA-overexpressing strain was significantly protected compared to the wild type (1 log less killing at 1  $\text{J}/\text{cm}^2$ , 2 logs less killing at 2  $\text{J}/\text{cm}^2$ , and 3 logs less killing at 4  $\text{J}/\text{cm}^2$ ).

***E. coli* TolC knockout mutant is more susceptible to MB PDI.** It was necessary to use higher overall PDI doses to kill gram-negative *E. coli* than to kill gram-positive *S. aureus*. We selected a concentration of MB of 50  $\mu\text{M}$  with the same 30-min incubation and wash by centrifugation, together with light doses up to 20  $\text{J}/\text{cm}^2$ . Under these conditions, as shown in Fig. 3, the TolC knockout mutant showed 2 logs more killing than the wild type at 5  $\text{J}/\text{cm}^2$  and three logs more at 10  $\text{J}/\text{cm}^2$ , with the knockout mutant being totally eliminated at higher light doses. When the MB concentration was raised to 250  $\mu\text{M}$ ,

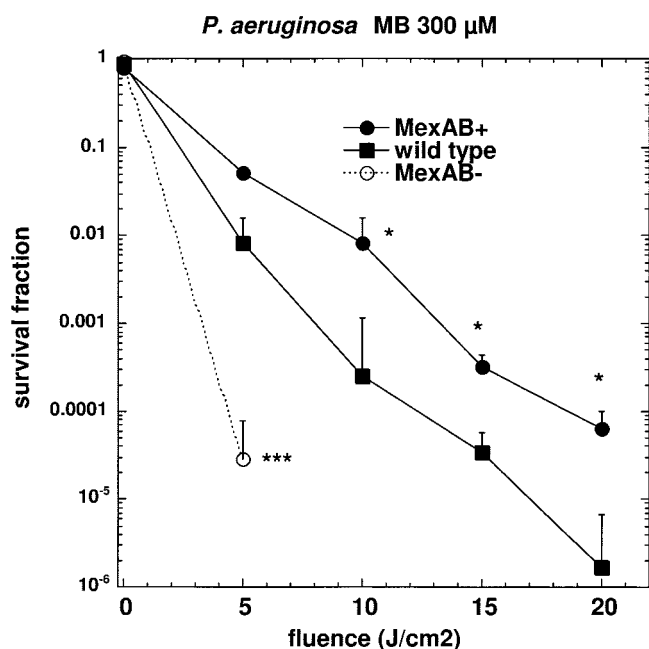


FIG. 4. Phototoxicity of MB after incubation at concentration of 300  $\mu\text{M}$  by *P. aeruginosa* MexAB knockout (MexAB<sup>-</sup>), wild-type, and MexAB-overexpressing (MexAB<sup>+</sup>) strains. Conditions are as described in the legend for Fig. 2.

both the wild-type and TolC knockout strains were totally eliminated after 20 J/cm<sup>2</sup> (data not shown).

***P. aeruginosa* MexAB expression determines phototoxicity of MB PDI.** It was necessary to use concentrations of MB that were even higher than those used for *E. coli* in order to effect light-dependent killing of *P. aeruginosa*. The three isogenic strains were therefore incubated with 300  $\mu\text{M}$  under the same conditions used previously. Figure 4 shows that the wild-type strain showed 5 logs of killing after 20 J/cm<sup>2</sup>. The MexAB knockout strain showed 2 logs more killing at 5 and 10 J/cm<sup>2</sup> and was completely eliminated at 15 J/cm<sup>2</sup>. The MexAB-overexpressing strain was protected at all light doses by about 1 log.

**Multiple phenothiazinium PSs are substrates of *S. aureus* NorA MDR.** To establish the recognition of phenothiazinium dyes as a class by NorA, we repeated the experiments described for Fig. 2 with the phenothiazinium compounds TBO and DMMB. The results shown in Fig. 5a and b show a susceptibility pattern similar to that found for MB. The NorA knockout strain is eliminated by 1 J/cm<sup>2</sup> in the case of TBO and by 0.5 J/cm<sup>2</sup> in the case of DMMB. By contrast, the wild-type strain is comparatively resistant, demonstrating 2 to 4 logs less killing. The NorA-overexpressing strain shows even less killing than the wild type (roughly 2 logs), and these differences are significant. The overall order of efficiency of killing was DMMB > TBO > MB.

**Activity of nonphenothiazinium PS is unaffected by MDR phenotype.** In order to show that the differences in killing we observed with the various MDR phenotypes were dependent on MDR recognition of phenothiazinium dyes rather than some alternative alteration in microbial physiology that could potentially alter susceptibility to PDI, we studied two antimicrobial PSs with non-phenothiazinium-based molecular struc-

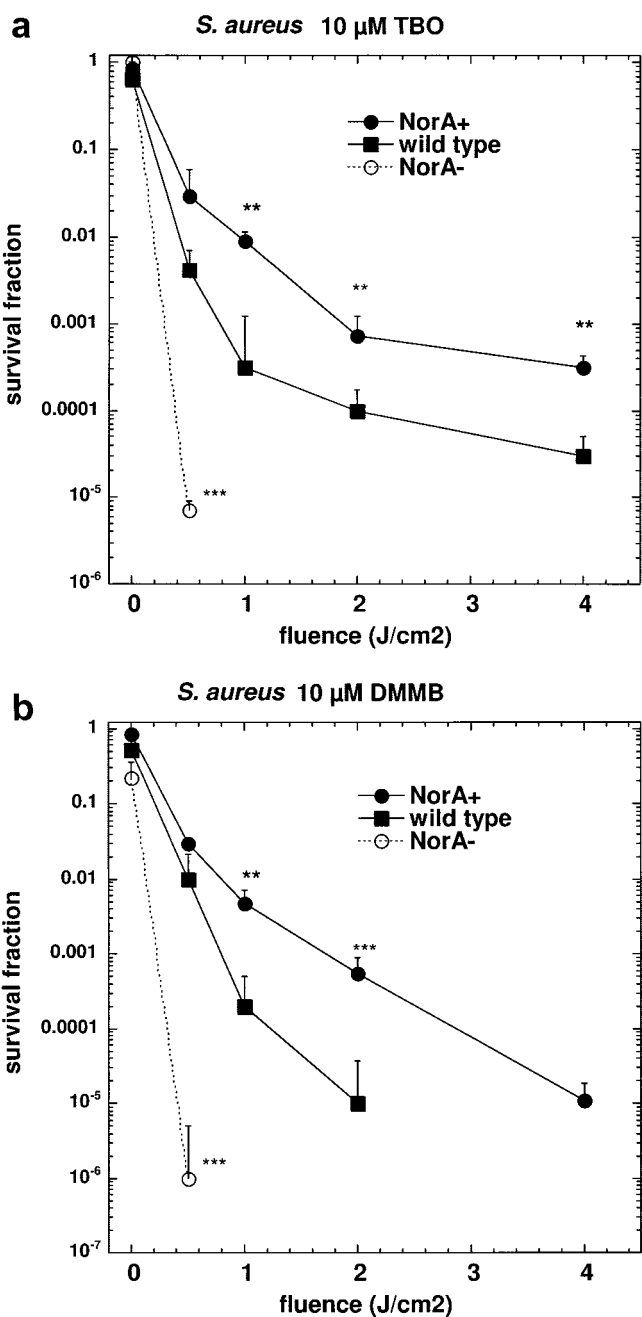


FIG. 5. Phototoxicity of (a) TBO and (b) DMMB after incubation at concentration of 10  $\mu\text{M}$  by *S. aureus* NorA knockout (NorA<sup>-</sup>), wild-type, and NorA-overexpressing (NorA<sup>+</sup>) strains. Incubation with the PS was for 30 min followed by a wash. Bacteria were then illuminated with 100 mW cm<sup>-2</sup> 635-nm light for both TBO and DMMB.

tures. RB is a xanthene dye that has four aromatic rings, but these are positioned differently than phenothiazinium dyes, and in addition RB possesses an overall negative charge. pL-c<sub>6</sub> is a macromolecular conjugate between the tetrapyrrole PS chlorin e6 and a poly-L-lysine chain with an overall polycationic charge that is thought to be taken up by bacteria by disturbing their membrane structure. As seen in Fig. 6a and b, there were no differences in killing between the three *S. aureus* NorA

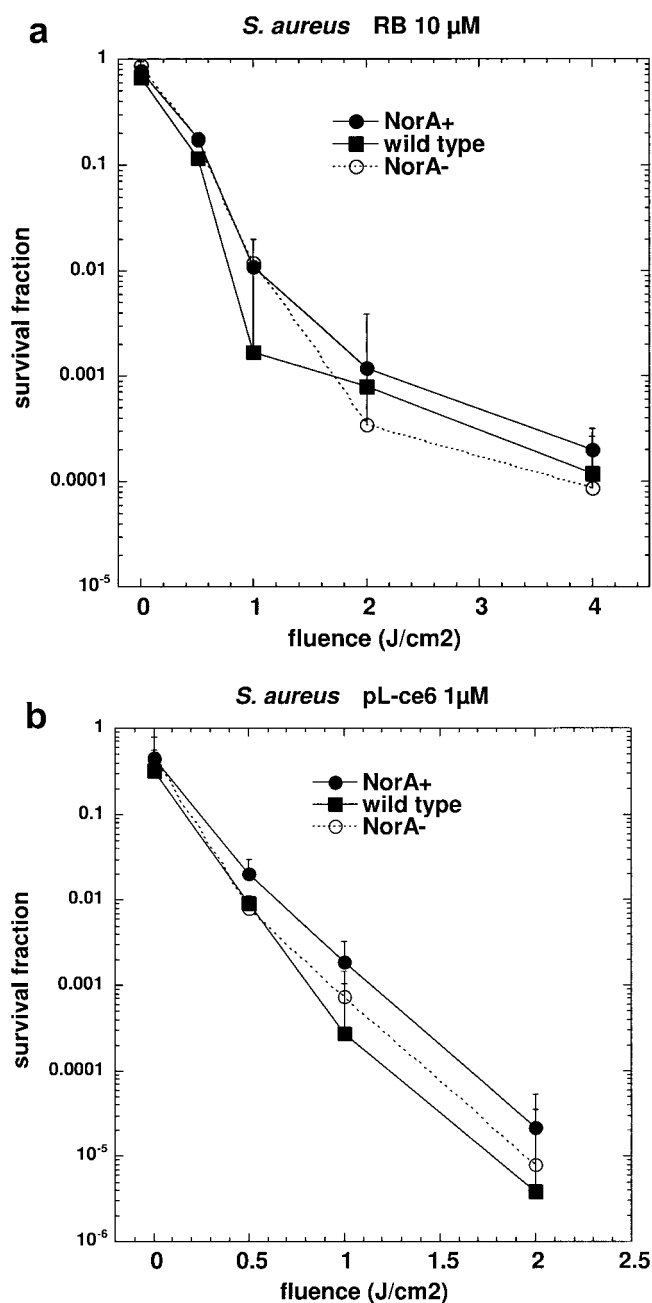


FIG. 6. Phototoxicity of (a) RB at 10  $\mu\text{M}$  and (b) pL-*c<sub>6</sub>* at 1  $\mu\text{M}$ , both with a wash with *S. aureus* NorA knockout (NorA<sup>-</sup>), wild-type, and NorA-overexpressing (NorA<sup>+</sup>) strains, followed by illumination with 100 mW cm<sup>-2</sup> 540-nm light for RB and 660-nm light for pL-*c<sub>6</sub>*.

phenotypes with either PS. pL-*c<sub>6</sub>* was significantly more effective than RB since only 1/10 the concentration produced more killing with the same light fluence. We also carried out pL-*c<sub>6</sub>*- and RB-mediated PDI with both *E. coli* wild-type and TolC knockout strains and with the MexAB-overexpressing, wild-type, and MexAB knockout strains of *P. aeruginosa*. There were no significant differences between the extents of killing with either PS that were dependent on MDR phenotype. This shows that PSs in general are not recognized by gram-negative

MDRs that generally have broader substrate specificity and also are helped dramatically by the outer membrane structure.

**MDR phenotype affects bacterial uptake of phenothiazinium PS but not of other structures.** To confirm our hypothesis that the MDRs reduce intracellular concentrations of phenothiazinium PS by an active efflux mechanism, we measured uptake of the dye by the cells by extraction and fluorescence quantification. The cells were incubated with same concentrations of the dye that were used for the killing experiments. Figure 7a shows that the uptake of the two phenothiazinium dyes tested (MB and TBO, both at 10  $\mu\text{M}$ ) by the three *S. aureus* strains were significantly different according to NorA phenotype. The NorA knockout strain took up  $(1.34 \pm 0.32) \times 10^9$  and  $(1.22 \pm 0.22) \times 10^9$  molecules/cell of TBO and MB, respectively, compared to  $(0.16 \pm 0.02) \times 10^9$  and  $(0.06 \pm 0.01) \times 10^9$  for the wild type and  $(0.114 \pm 0.016) \times 10^9$  and  $(0.021 \pm 0.003) \times 10^9$  for the NorA-overexpressing strain. All of these differences were significant. By contrast, the uptakes of the nonphenothiazinium dyes RB (10  $\mu\text{M}$ ) and pL-*c<sub>6</sub>* (1  $\mu\text{M}$ ) showed no significant differences between NorA phenotypes. Figure 7b depicts the uptakes of two phenothiazinium PSs (MB and TBO) by the *E. coli* wild-type and TolC null cells (concentration used was 50  $\mu\text{M}$ ) and by the three MexAB phenotypes of *P. aeruginosa* (concentration used was 300  $\mu\text{M}$ ). It is interesting to observe that the uptakes of the phenothiazinium dyes by all of the MDR knockout mutants of different bacterial species are fairly similar ( $1.2 \times 10^9$  to  $3.2 \times 10^9$  molecules per cell). In all cases, TBO uptake was higher than MB uptake. This similarity in uptakes between different bacteria is remarkable because of the widely different PS concentrations used (10 to 300  $\mu\text{M}$ ). These values indicate that there is a necessary amount of PS per cell to mediate efficient PDI and show that levels of bacterial uptake of phenothiazinium dyes vary between bacterial classes and species. This variation may be due to intrinsic permeability differences or to the fact that some species (such as *P. aeruginosa*) may have many separate but related MDRs, and knocking out MexAB may still leave other functional MDRs to pump out phenothiazinium dyes.

## DISCUSSION

This study has demonstrated for the first time that phenothiazinium-based PSs are substrates of MDRs in bacteria. The fact that this effect was observed with three separate but related molecular structures (MB, TBO, and DMMB), which have all been frequently used in the literature as antimicrobial PSs, suggests that it is a general phenomenon applicable to all photoactive phenothiazinium dyes. By contrast, the complete absence of any differences in susceptibility by use of the nonphenothiazinium-based antimicrobial PSs (RB and pL-*c<sub>6</sub>*) provides evidence that the MDR phenotype of the bacterial cells does not affect other physiological parameters that could influence susceptibility to PDI. These parameters could have included such variables as membrane structure, DNA and other cellular repair systems, and levels of antioxidant enzymes. RB and light probably kill bacteria by generating extracellular singlet oxygen that destroys the membrane from the outside (7, 8, 33), while polycationic polymeric pL-*c<sub>6</sub>* is probably taken up into bacterial cells by a self-promoted uptake pathway, as described for other polycationic peptides (24). The finding that uptake levels of both MB and TBO by the



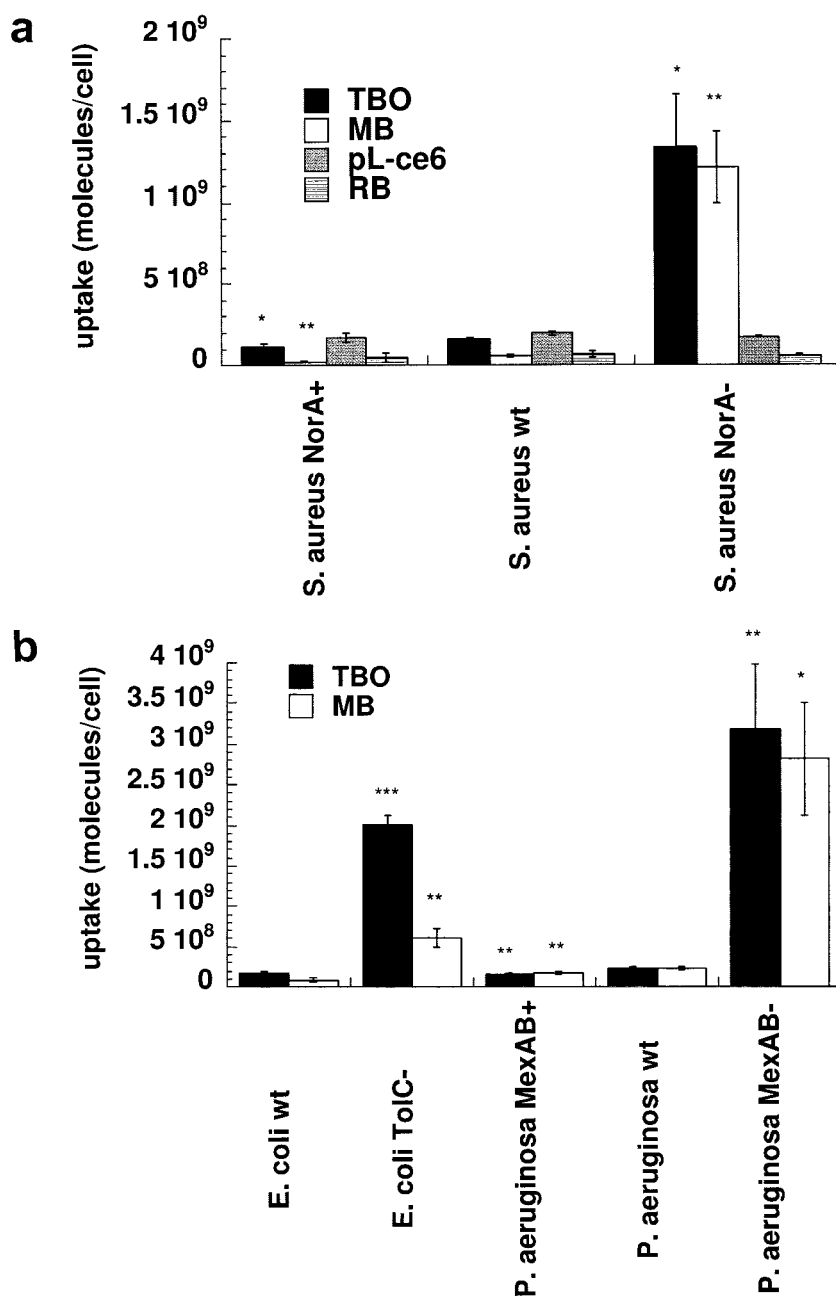


FIG. 7. (a) Uptake of PS in terms of molecules per cell by *S. aureus* NorA knockout (NorA<sup>-</sup>), wild-type (wt), and NorA-overexpressing (NorA<sup>+</sup>) strains. Concentrations were 10  $\mu$ M for MB, TBO, and RB and 1  $\mu$ M for pL-*c<sub>e6</sub>*. PSs were incubated for 10 min, washed, and fluorescence extracted and measured as described in the text. Values are means of three separate determinations, and bars are SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (compared to the wild type). (b) Uptake of MB and TBO in terms of molecules per cell by *E. coli* TolC knockout (TolC<sup>-</sup>) and wild-type strains and *P. aeruginosa* MexAB knockout (MexAB<sup>-</sup>), wild-type, and MexAB-overexpressing (MexAB<sup>+</sup>) strains. Concentrations were 50  $\mu$ M for *E. coli* and 300  $\mu$ M for *P. aeruginosa*. Values are means of three separate determinations, and bars are SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (compared to the wild type).

NorA knockout mutant, wild-type, and overexpressing strains of *S. aureus* were proportional to levels of NorA expression suggests that the role of the MDRs is to pump out the PS from the cells and thereby lessen the phototoxicity observed upon illumination. The similarity of the levels of uptake of the non-phenothiazinium PSs between the various NorA phenotypes suggests that these compounds are not recognized by MDRs.

Alternatively, a less likely explanation is that the nonphenothiazinium PSs were not taken up into the cells but merely bound to the outside layers of the cell coat. This is unlikely because pL-*c<sub>e6</sub>* was the most potent PS active at a 1- $\mu$ M concentration, and this high activity is best explained by the polycationic structure mediating intracellular uptake. The correlation of phenothiazinium dye (MB and TBO) uptake with TolC

levels in *E. coli* and with MexAB levels in *P. aeruginosa* further confirms the role of MDRs to pump out these PSs.

We found that the MDR recognition of phenothiazinium PSs applied equally to two different molecular efflux systems present in three different bacterial species. The NorA MDR of *S. aureus* is a member of the MF family and protects the cells from norfloxacin and a number of amphipathic cations, such as the common disinfectants benzalkonium chloride and cetrimide (13). RND pumps are frequently found in gram-negative bacteria. The TolC protein of *E. coli*, through its interaction with AcrA and AcrB, is thought to form a tripartite continuous protein channel that expels substrates from the cell. AcrAB-TolC assembles into an alpha-helical transperiplasmic tunnel, which is embedded in the outer membrane by a contiguous beta-barrel channel (16). AcrAB-TolC and its homologues thus provide large exit ducts for a wide range of substrates, including organic solvents, fluorescent lipids, bile acids, erythromycin, and cloxacillin (40, 42), recognized by the AcrAB proteins. *P. aeruginosa* carries genes for at least 11 distinct (but related) RND family pumps (30). Mex-Opr substrates include biocides, dyes, detergents, metabolic inhibitors, organic solvents, and molecules involved in bacterial cell-cell communication. The total number of MexAB-OprM units per wild-type cell was calculated to be about 400 assemblies, and the turnover rate of a single pump unit was predicted to be about 500 molecules per second (27).

There have been some reports of compounds with structures somewhat similar to those of phenothiazinium dyes being substrates or inhibitors of bacterial MDRs. The cationic xanthen dye pyronin Y was reported to be a substrate of RND pumps in *P. aeruginosa* (23) and also of NorA in *S. aureus* (15). Similarly, the cationic acridinium antimicrobial acriflavine was reported to be a substrate of NorA (15). Noncationic phenothiazine compounds such as chlorpromazine derivatives have been reported to be inhibitors of MDRs in *E. coli* (26), and prochlorpromazine inhibited NorA in *S. aureus* (15).

It has been said that the present times represent the "end of the antibiotic era" (31), due to increasing development of bacterial resistance to multiple classes of antibiotics. PDT uses otherwise harmless dyes and light, and it has been proposed that microbes would be unlikely to develop resistance to the destructive effects of photochemically generated reactive oxygen species that can cause irreversible oxidative damage to essential cellular constituents such as proteins, lipids, and nucleic acids. There is one report of the failure to produce resistance by repeated cycles of PDI using a nonphenothiazinium polycationic-PS conjugate (18). Our data however raise the possibility of bacteria developing resistance to phenothiazinium-based PDI due to selective survival of strains with increased MDR expression levels.

We generally found bigger differences in susceptibility between MDR knockout and wild-type strains than between wild-type and overexpressing strains. This suggests that the wild-type species we tested had high levels of functioning MDRs and provides a possible reason why the concentrations of phenothiazinium PS necessary to efficiently kill both gram-positive and gram-negative bacteria upon illumination is significantly higher (10 to 300  $\mu$ M [37, 41]) than the concentrations of alternative cationic PS that have been used; for instance, Maisch et al. (21) reported a cationic porphyrin de-

rivative in combination with blue light that mediated killing *S. aureus* at a concentration of only 5 nM.

Phenothiazinium dyes have been long established as non-toxic and clinically useful compounds both for staining living tissues (6) (for instance, in the detection of dysplasias [20]) and for some pharmacological indications (5, 34). This consideration together with their ready availability has probably been important in their selection as antimicrobial PSs for the few clinical indications in which antimicrobial PDT is carried out. The present discovery that these compounds are substrates for bacterial MDRs raises the possibility of combining the phenothiazinium dye with an MDR inhibitor. For instance, the dye solution applied to sterilize a dental cavity when illuminated could contain an MDR inhibitor applicable to the MDRs expressed by the oral pathogens causing dental caries.

Our future work will ask whether phenothiazinium dyes are also substrates of MDRs expressed by fungi (e.g., *Candida albicans*) and to what degree selected MDR inhibitors can potentiate antimicrobial PDI of pathogenic microorganisms.

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#### REFERENCES

- Bhatti, M., A. MacRobert, S. Meghji, B. Henderson, and M. Wilson. 1998. A study of the uptake of toluidine blue O by *Porphyromonas gingivalis* and the mechanism of lethal photosensitization. *Photochem. Photobiol.* **68**:370–376.
- Bohnert, J. A., and W. V. Kern. 2005. Selected arylpiperazines are capable of reversing multidrug resistance in *Escherichia coli* overexpressing RND efflux pumps. *Antimicrob. Agents Chemother.* **49**:849–852.
- Bressler, N. M., and S. B. Bressler. 2000. Photodynamic therapy with verteporfin (Visudyne): impact on ophthalmology and visual sciences. *Investig. Ophthalmol. Vis. Sci.* **41**:624–628.
- Castano, A. P., T. N. Demidova, and M. R. Hamblin. 2004. Mechanisms in photodynamic therapy. I. Photosensitizers, photochemistry and cellular localization. *Photodiagn. Photodyn. Ther.* **1**:279–293.
- Clifton, J., II, and J. B. Leikin. 2003. Methylene blue. *Am. J. Ther.* **10**:289–291.
- Czarnecki, D. J., H. K. Feider, and G. F. Splittgerber. 1989. Toluidine blue dye as a breast localization marker. *Am. J. Roentgenol.* **153**:261–263.
- Dahl, T. A., W. R. Midden, and D. C. Neckers. 1988. Comparison of photodynamic action by Rose Bengal in gram-positive and gram-negative bacteria. *Photochem. Photobiol.* **48**:607–612.
- Demidova, T. N., and M. R. Hamblin. 2005. Effect of cell-photosensitizer binding and cell density on microbial photoinactivation. *Antimicrob. Agents Chemother.* **49**:2329–2335.
- Dolmans, D. E., D. Fukumura, and R. K. Jain. 2003. Photodynamic therapy for cancer. *Nat. Rev. Cancer* **3**:380–387.
- Frimberger, A. E., L. Cincotta, S. M. Cotter, and J. W. Foley. 1998. Photodynamic therapy of naturally occurring tumors in animals using a novel benzophenothiazine photosensitizer. *Clin. Cancer Res.* **4**:2207–2218.
- Gad, F., T. Zahra, T. Hasan, and M. R. Hamblin. 2004. Effects of growth phase and extracellular slime on photodynamic inactivation of gram-positive pathogenic bacteria. *Antimicrob. Agents Chemother.* **48**:2173–2178.
- Hamblin, M. R., and T. Hasan. 2004. Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochem. Photobiol. Sci.* **3**:436–450.
- Hsieh, P. C., S. A. Siegel, B. Rogers, D. Davis, and K. Lewis. 1998. Bacteria lacking a multidrug pump: a sensitive tool for drug discovery. *Proc. Natl. Acad. Sci. USA* **95**:6602–6606.
- Jett, B. D., K. L. Hatter, M. M. Huycke, and M. S. Gilmore. 1997. Simplified agar plate method for quantifying viable bacteria. *BioTechniques* **23**:648–650.
- Kaatz, G. W., V. V. Moudgal, S. M. Seo, and J. E. Kristiansen. 2003. Phenothiazines and thioxanthenes inhibit multidrug efflux pump activity in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **47**:719–726.

16. **Koronakis, V.** 2003. TolC—the bacterial exit duct for proteins and drugs. *FEBS Lett.* **555**:66–71.
17. **Kubin, A., F. Wierrani, R. H. Jindra, H. G. Loew, W. Grunberger, R. Ebermann, and G. Alth.** 1999. Antagonistic effects of combination photosensitization by hypericin, meso-tetrahydroxyphenylchlorin (mTHPC) and photofrin II on *Staphylococcus aureus*. *Drugs Exp. Clin. Res.* **25**:13–21.
18. **Lauro, F. M., P. Pretto, L. Covolo, G. Jori, and G. Bertoloni.** 2002. Photoinactivation of bacterial strains involved in periodontal diseases sensitized by porphycene-polylysine conjugates. *Photochem. Photobiol. Sci.* **1**:468–470.
19. **Lewis, K.** 1999. Versatile drug sensors of bacterial cells. *Curr. Biol.* **9**:403–407.
20. **Ligthelm, A. J., A. Weber, P. J. van Niekerk, and W. F. van Heerden.** 1989. Diagnosis of oral precancer and cancer. *J. Dent. Assoc. S. Afr.* **1989**(Suppl. 1):2–5.
21. **Maisch, T., C. Bosl, R.-M. Szeimies, N. Lehn, and C. Abels.** 2005. Photodynamic effects of novel XF porphyrin derivatives on prokaryotic and eukaryotic cells. *Antimicrob. Agents Chemother.* **49**:1542–1552.
22. **Malik, Z., H. Ladan, and Y. Nitzan.** 1992. Photodynamic inactivation of Gram-negative bacteria: problems and possible solutions. *J. Photochem. Photobiol. B* **14**:262–266.
23. **Mao, W., M. S. Warren, D. S. Black, T. Satou, T. Murata, T. Nishino, N. Gotoh, and O. Lomovskaya.** 2002. On the mechanism of substrate specificity by resistance nodulation division (RND)-type multidrug resistance pumps: the large periplasmic loops of MexD from *Pseudomonas aeruginosa* are involved in substrate recognition. *Mol. Microbiol.* **46**:889–901.
24. **Minnock, A., D. I. Vernon, J. Schofield, J. Griffiths, J. H. Parish, and S. B. Brown.** 2000. Mechanism of uptake of a cationic water-soluble pyridinium zinc phthalocyanine across the outer membrane of *Escherichia coli*. *Antimicrob. Agents Chemother.* **44**:522–527.
25. **Moan, J., and Q. Peng.** 2003. An outline of the hundred-year history of PDT. *Anticancer Res.* **23**:3591–3600.
26. **Molnar, J., A. Hever, I. Fakla, J. Fischer, I. Ocsovski, and A. Aszalos.** 1997. Inhibition of the transport function of membrane proteins by some substituted phenothiazines in *E. coli* and multidrug resistant tumor cells. *Anticancer Res.* **17**:481–486.
27. **Narita, S., S. Eda, E. Yoshihara, and T. Nakae.** 2003. Linkage of the efflux-pump expression level with substrate extrusion rate in the MexAB-OprM efflux pump of *Pseudomonas aeruginosa*. *Biochem. Biophys. Res. Commun.* **308**:922–926.
28. **O'Neill, J., M. Wilson, and M. Wainwright.** 2003. Comparative antistreptococcal activity of photobactericidal agents. *J. Chemother.* **15**:329–334.
29. **Phoenix, D. A., Z. Sayed, S. Hussain, F. Harris, and M. Wainwright.** 2003. The phototoxicity of phenothiazinium derivatives against *Escherichia coli* and *Staphylococcus aureus*. *FEMS Immunol. Med. Microbiol.* **39**:17–22.
30. **Poole, K.** 2005. Efflux-mediated antimicrobial resistance. *J. Antimicrob. Chemother.* **56**:20–51. [Online.] doi:10.1093/jac/dki171.
31. **Poole, M. D.** 1993. Are we facing the end of the antibiotic era? *Ear Nose Throat J.* **72**:433.
32. **Romanova, N. A., L. Y. Brovko, L. Moore, E. Pometun, A. P. Savitsky, N. N. Ugarova, and M. W. Griffiths.** 2003. Assessment of photodynamic destruction of *Escherichia coli* O157:H7 and *Listeria monocytogenes* by using ATP bioluminescence. *Appl. Environ. Microbiol.* **69**:6393–6398.
33. **Schafer, M., C. Schmitz, R. Facius, G. Horneck, B. Milow, K. H. Funken, and J. Ortner.** 2000. Systematic study of parameters influencing the action of Rose Bengal with visible light on bacterial cells: comparison between the biological effect and singlet-oxygen production. *Photochem. Photobiol.* **71**:514–523.
34. **Schirmer, R. H., B. Coulibaly, A. Stich, M. Scheiwein, H. Merkle, J. Eubel, K. Becker, H. Becher, O. Muller, T. Zich, W. Schiek, and B. Kouyate.** 2003. Methylene blue as an antimalarial agent. *Redox Rep.* **8**:272–275.
35. **Tegos, G., F. R. Stermitz, O. Lomovskaya, and K. Lewis.** 2002. Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. *Antimicrob. Agents Chemother.* **46**:3133–3141.
36. **Wainwright, M.** 2003. The use of dyes in modern biomedicine. *Biotech. Histochem.* **78**:147–155.
37. **Wainwright, M., D. A. Phoenix, S. L. Laycock, D. R. Wareing, and P. A. Wright.** 1998. Photobactericidal activity of phenothiazinium dyes against methicillin-resistant strains of *Staphylococcus aureus*. *FEMS Microbiol. Lett.* **160**:177–181.
38. **Wainwright, M., D. A. Phoenix, J. Marland, D. R. Wareing, and F. J. Bolton.** 1997. A study of photobactericidal activity in the phenothiazinium series. *FEMS Immunol. Med. Microbiol.* **19**:75–80.
39. **Walker, I., S. A. Gorman, R. D. Cox, D. I. Vernon, J. Griffiths, and S. B. Brown.** 2004. A comparative analysis of phenothiazinium salts for the photosensitisation of murine fibrosarcoma (RIF-1) cells in vitro. *Photochem. Photobiol. Sci.* **3**:653–659.
40. **Whitney, E. N.** 1971. The *tolC* locus in *Escherichia coli* K12. *Genetics* **67**:39–53.
41. **Wong, T. W., Y. Y. Wang, H. M. Sheu, and Y. C. Chuang.** 2005. Bactericidal effects of toluidine blue-mediated photodynamic action on *Vibrio vulnificus*. *Antimicrob. Agents Chemother.* **49**:895–902.
42. **Yu, E. W., J. R. Aires, and H. Nikaido.** 2003. AcrB multidrug efflux pump of *Escherichia coli*: composite substrate-binding cavity of exceptional flexibility generates its extremely wide substrate specificity. *J. Bacteriol.* **185**:5657–5664.