Efflux-mediated response of *Staphylococcus aureus* exposed to ethidium bromide

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**Objectives:** By adapting an antibiotic-susceptible *Staphylococcus aureus* strain to increasing concentrations of ethidium bromide, a known substrate of efflux pumps (EPs), and by phenotypically and genotypically analysing the resulting progeny, we characterized the molecular mechanisms of *S. aureus* adaptation to ethidium bromide.

**Methods:** *S. aureus* ATCC 25923 was grown in increasing concentrations of ethidium bromide. The MICs of representatives of eight classes of antibiotics, eight biocides and two dyes against ATCC 25923 and its ethidium bromide-resistant progeny ATCC 25923EtBr were determined with or without six efflux pump inhibitors (EPIs). Efflux activity in the presence/absence of EPIs was evaluated by real-time fluorometry. The presence and expression of eight EP genes were assayed by PCR and quantitative RT–PCR (qRT–PCR), respectively. Mutations in *grlA*, *gyrA* and *norA* promoter regions were screened by DNA sequencing.

**Results:** Compared with its parental strain, ATCC 25923EtBr was 32-fold more resistant to ethidium bromide and also more resistant to biocides and hydrophilic fluoroquinolones. Resistance to these could be reduced by the EPIs chlorpromazine, thioridazine and reserpine. Increased efflux of ethidium bromide by ATCC 25923EtBr could be inhibited by the same EPIs. qRT–PCR showed that *norA* was 35-fold over-expressed in ATCC 25923EtBr, whereas the remaining EP genes showed no significant increase in their expression. Sequencing of the *norA* promoter region revealed a 70 bp deletion in ATCC 25923EtBr.

**Conclusions:** Exposure of *S. aureus* to quaternary compounds such as ethidium bromide results in decreased susceptibility of the organism to a wide variety of compounds, including quinolones and biocides through an efflux-mediated response, which for strain ATCC 25923 is mainly NorA-mediated. This altered expression may result from alterations in the *norA* promoter region.

Keywords: staphylococci, efflux pumps, NorA, overexpression

**Introduction**

*Staphylococcus aureus* is one of the most common human pathogens, being responsible for a wide variety of infections, many of which can be life-threatening. Besides its pathogenic potential, this bacterium also shows many different mechanisms of resistance towards antibiotics. Several of these mechanisms are well known, and have been characterized: resistance to β-lactam antibiotics mediated by PBP2a, encoded by the *meC* gene, or resistance to fluoroquinolones resulting from mutations in either topoisomerase IV or gyrase genes.¹ On the other hand, antibiotic resistance based on efflux systems capable of extruding the drug or other noxious agents from the cell is less well characterized for these bacteria.

To date, more than 10 efflux pumps (EPs) have been described for *S. aureus*.² Most of these pumps belong to the major facilitator superfamily, namely the chromosomally encoded NorA, NorB, NorC, MdeA and SdrM as well as the plasmid-encoded QacA/B pumps.³–⁸ Other types of pumps have also been described for *S. aureus* such as MepA, a member of...
Ethidium bromide-induced efflux in S. aureus

the multidrug and toxic compound extrusion family, as well as Smr, which belongs to the small multidrug resistance (SMR) family, and SepA. Although these pumps show different substrate specificity, most of them are capable of extruding compounds of different chemical classes, thus providing the cell with the means to develop a multidrug resistance (MDR) phenotype or to survive in a hostile environment.

In order to find specific inhibitors of these pumps or simply to demonstrate the presence of efflux activity, different compounds have been tested for their ability to hinder the activity of S. aureus EPs, including the proton motive force uncoupler carbonyl cyanide m-chlorophenylhydrazine (CCCP), the alkaloid reserpine, as well as different members of the phenothiazines, among others.

In this work, a fully antibiotic-susceptible S. aureus strain was challenged with increasing concentrations of a well-known substrate of EPs, ethidium bromide, and the mechanisms by which the cells adapted to this compound were evaluated by different methods. This experimental approach allowed us to study for the first time the response of an S. aureus strain that is forced to cope with high concentrations of a noxious pump substrate, in particular, to evaluate the efflux system(s) preferentially used by the cells under such conditions.

Materials and methods

Bacterial strains

The study was conducted with S. aureus strain ATCC 25923, a clinical isolate collected at Seattle in 1945, fully antibiotic-susceptible, β-lactamase-negative, with an ethidium bromide MIC of 6.25 mg/L. This strain was initially grown in 5 mL of ethidium bromide and incubated at 37°C. When visible growth was obtained, the culture was transferred to new media, containing increasing concentrations of ethidium bromide (5, 10, 20, 40, 60, 80 and 100 mg/L). During an 82 day period, 7 cultures were obtained, with MICs of ethidium bromide (5, 10, 20, 40, 60, 80 and 100 mg/L). During this period, the means to develop a multidrug resistance (MDR) phenotype or to survive in a hostile environment.

Growth conditions

Cultures were grown in tryptic soy broth (TSB) or agar (TSA) (Oxoid Ltd, Basingstoke, UK) at 37°C. When necessary, the strain ATCC 25923 was grown in a medium supplemented with 50 mg/L of ethidium bromide. For determination of the MICs of the agents employed in this study, cultures were grown in Mueller–Hinton broth (MHB), and for Kirby–Bauer antibiotic susceptibility assays, in Mueller–Hinton agar, at 37°C.

Antibiotics

Antibiotics in powder form were purchased from different sources, as follows: nalidixic acid, erythromycin, tetracycline, rifampicin and chloramphenicol were purchased from Sigma-Aldrich (St Louis, USA); norfloxacin was purchased from ICN Biomedicals Inc. (OH, USA) and ciprofloxacin from Fluka Chemie GmbH (Buchs, Switzerland). Kirby–Bauer discs containing nalidixic acid (30 μg), norfloxacin (10 μg), ciprofloxacin (5 μg), tetracycline (30 μg), oxacillin (1 μg), gentamicin (10 μg), cefotaxime (30 μg), penicillin (10 U) and fusidic acid (10 μg) were acquired from Oxoid Ltd.

Efflux pump inhibitors (EPIs)

CCCP, thioridazine, chlorpromazine, verapamil and reserpine were purchased from Sigma-Aldrich. Ouabain was purchased from Fluka Chemie GmbH. Solutions of thioridazine, chlorpromazine, verapamil and ouabain were prepared in deionized water; reserpine was prepared in DMSO and CCCP in 50% methanol (v/v). All solutions were prepared on the day of the experiment and kept protected from light.

Drug susceptibility testing

Antibiotics. MICs were determined by the broth microdilution method, as per the guidelines of CLSI and carried out in triplicate. Results were evaluated according to the CLSI breakpoints, except for nalidixic acid, for which there are no defined breakpoints. Kirby–Bauer antibiotic susceptibility assays were conducted as outlined by the CLSI standards and performed in triplicate. The zones of inhibition were measured in millimetres and analysed by the CLSI breakpoints, except for fusidic acid, which was analysed by the BSAC standards and nalidixic acid, for which there are no defined breakpoints.

Biocides and dyes. MICs were determined by the broth microdilution method. Briefly, cultures were grown overnight in TSB, at 37°C, diluted in PBS and the suspension turbidity adjusted to a 0.5 McFarland standard. Twenty microlitres of this suspension were then transferred to each well of 96-well plates containing 2-fold dilutions of each agent to be tested, diluted in MHB. The plates were then incubated at 37°C for 18 h and the MIC values, corresponding to the lowest concentration of compound that inhibited visible growth, recorded. Each MIC determination was carried out in triplicate.

Efflux pump inhibitors. Each EPI employed in this study was evaluated for its ability to reduce or reverse antibiotic resistance to given antibiotics and biocides, both of which are characteristics that define the agent as an inhibitor of efflux pump activity. The evaluation of an agent for EPI activity was conducted at a concentration of the agent that did not inhibit the growth of the organism in a medium containing varying concentrations of an antibiotic or biocide and a bacterial inoculum corresponding to the one used for MIC determination. Parallel cultures were tested in media containing no EPI and varying concentrations of the antibiotic or biocide. The cultures were incubated for 18 h and their contents (growth) evaluated visually; minimum bactericidal concentrations were determined by counting colony-forming units. The final concentrations of the EPIs used, which correspond to half of the MIC determined for each EPI unless otherwise stated, were: thioridazine (12.5 mg/L), chlorpromazine (25 mg/L), verapamil (200 mg/L), reserpine (20 mg/L), ouabain (100 mg/L) and CCCP (0.18 mg/L). The MIC of each EPI was determined by the broth microdilution method, as described above. All assays were performed in triplicate.

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Preparation of chromosomal DNA

Genomic DNA was extracted with the QIAamp DNA minikit (QIAGEN, Hilden, Germany), with an additional step of 30 min digestion with lysostaphin (Sigma) (200 mg/L) prior to extraction.

Macrorestriction analysis

Cultures were typed by pulsed-field gel electrophoresis (PFGE) analysis, using well-established protocols. Briefly, agarose discs containing intact chromosomal DNA were prepared as described previously and restricted with Smal, according to the manufacturer’s recommendations (New England Biolabs, Ipswich, MA, USA). Restriction fragments were then resolved by PFGE, which was carried out in a contour-clamped homogeneous electric field (CHEF) apparatus (CHEF-DRII, Bio-Rad, Hercules, CA, USA), as described previously.

Preparation of plasmid DNA

The QIAPrep Spin Miniprep kit (QIAGEN) was used, with the following modification: prior to extraction, cells were incubated with lysostaphin (35 mg/L) at 37°C for 90 min, as described previously.

RNA extraction

Total RNA was extracted from the parent ATCC 25923 strain unexposed to ethidium bromide and the ATCC 25923EtBr strain grown in TSB supplemented with 50 mg/L of ethidium bromide with the Rneasy Mini Kit (QIAGEN), as per the manufacturer’s instructions. Before extraction of total RNA, the cultures were treated with an RNAProtect bacterial reagent (QIAGEN). The extracted RNA was treated with RNase-free DNase (QIAGEN) for 2 h at room temperature and re-purified using the same kit.

PCR amplification of efflux pump genes

Separate fragments of DNA internal to each of eight EP genes previously described for S. aureus were amplified by PCR, using the primers described in Table 1. The reaction mixture (50 μL) contained 2.5 U of *Taq* Polymerase (Fermentas Inc., Ontario, Canada), 1× *Taq* buffer (Fermentas), 25 pmol of each primer, 200 μM dNTP and 1.75 mM MgCl₂. The PCRs were conducted in a thermocycler Mastercycler personal 5332 (Eppendorf AG, Hamburg, Germany). Amplification conditions were as follows: DNA was denatured at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 45°C (norA) or 53°C (norB, norC, mdeA, mepA, norAp) for 30 s, extension at 72°C for 1 min, with a final extension step at 72°C for 7 min.

Table 1. Primers used in this study

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<tr>
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</table>
and sepA) for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. The PCRs for genes qacA/B and smr were conducted under the following conditions: DNA was denatured at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 40°C (qacA/B) or 48°C (smr) and extension at 72°C for 1 min, followed by a step of final extension at 72°C for 5 min. The amplification products were visualized in 1% agarose gel electrophoresis.

**Screening of mutations in the norA promoter region**

A 449 bp region upstream of norA was amplified using primers NorAp_Fw and NorAp_Rv (Table 1). The amplification conditions were the same as used for norB amplification. Amplification products were purified and sequenced in both strands. Sequences were analysed and aligned using programs BioEdit, version 7.0.8.0, and ClustalW, respectively.

**Screening of mutations in grlA and gyrA genes**

Fragments internal to the grlA and gyrA genes were amplified using the primers GrlA_Fw/GrlA_Rv and GyrA_Fw/GyrA_Rv, described in Table 1. The amplification conditions were as follows: DNA was denatured at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. Amplification products were purified and sequenced in both strands. Sequences were analysed and aligned using the programs BioEdit and ClustalW, respectively.

**qRT–PCR protocol**

Quantitative RT–PCR (qRT–PCR) was performed in triplicate using a Quantitect SYBR Green RT–PCR Kit (QIAGEN). The primers used in these assays are described in Table 1. The relative quantity of mRNA from the eight efflux pumps was determined by the comparative threshold cycle (Ct) method in a Rotor-Gene 3000™ thermocycler using real-time analysis software (Corbett Research, Sydney, Australia). The method was applied to the analysis of three assays conducted with three independent total RNA extractions. Negative controls and a genomic DNA contamination control were included. 16S rRNA was used as an endogenous control.

**Assessment of efflux activity**

**Ethidium bromide agar screening method.** Efflux activity was first assayed by the ethidium bromide agar screening method previously described. Briefly, each culture was swabbed onto TSA plates containing ethidium bromide concentrations ranging from 0.5 to 2.5 mg/L. The plates were incubated at 37°C for 16 h after which the minimum concentration of ethidium bromide that produced fluorescence under UV light associated with the bacterial mass was recorded. The plates were then incubated at 4°C for another 16 h, after which the minimum ethidium bromide concentration that produced fluorescence was recorded and compared with the minimal concentration of ethidium bromide that produced fluorescence at 37°C.

**Semi-automated fluorometric method.** A second method, recently developed in our laboratory, was also used for the assessment of efflux activity. This method is based on the accumulation of a given EP substrate (in this case ethidium bromide) inside the cells, followed by the fluorometric measurement of its efflux in a Rotor-Gene 3000™ thermocycler using real-time analysis software (Corbett Research).

For the accumulation of ethidium bromide, cultures were grown in TSB medium at 37°C and 150 rpm to an OD600 of 0.6, centrifuged and washed twice in PBS. The OD600 of the cellular suspension was then adjusted to 0.3 in PBS. The conditions used to maximize accumulation were: incubation with 3 mg/L ethidium bromide and 25 mg/L chlorpromazine, the most active EPI, at 25°C, and in the absence of glucose, for a 60 min period. The ethidium bromide and chlorpromazine concentrations were used at half MIC for the cultures in order to ensure that no effect was produced on the viability of the cells.

For the efflux assay, the ethidium bromide-loaded cells were collected by centrifugation and re-suspended in PBS to an OD600 of 0.3. Glucose to a final concentration of 0.4% was added to a set of microtubes containing an aliquot of 1 mL of cellular suspension. One microtube was left without glucose, to which 25 mg/L chlorpromazine was added. Afterwards, aliquots of 95 μL were transferred to 0.2 mL microtubes. Aliquots of 5 μL from aqueous stock solutions of the EPIs chlorpromazine, thioridazine and reserpine were added to the PCR microtubes in order to achieve final concentrations of: 5, 10, 15 and 25 mg/L; 5, 10 and 12.5 mg/L; and 20, 40, 60 and 100 mg/L, respectively. Duplicate tubes without glucose and/or without EPI were also used as controls. The efflux assays were then conducted in the Rotor-Gene 3000™ at a temperature of 37°C, and the fluorescence of ethidium bromide was measured at the end of every cycle of 60 s, for a total period of 25 min. Each assay was performed at least in triplicate and the viability of the cells was checked at the end of the assay by plating dilutions of the culture in TSA.

The raw data obtained was then normalized against data from non-effluxing cells (cells from the control tube with 25 mg/L chlorpromazine and no glucose) at each point, which were considered to be the maximum fluorescence values that could be obtained during the assay. The relative fluorescence thus corresponds to the ratio of fluorescence that remains per unit of time relative to the ethidium-bromide-loaded cells.

**Results**

**Adaptation to ethidium bromide**

The parent ATCC 25923 strain, susceptible to 6.25 mg/L ethidium bromide, was serially cultured in stepwise increases of ethidium bromide beginning with 5 mg/L ethidium bromide. After 82 days, the resistance of this strain to ethidium bromide was raised from an MIC of 6.25 to 200 mg/L and this ethidium bromide-adapted culture was designated as ATCC 25923_EtBr.

**Contamination control**

The parent ATCC 25923 and its adapted ethidium-bromide-resistant progeny, ATCC 25923_EtBr, were typed by PFGE analysis. Since the Smal macrorestriction patterns of both the parental and the ethidium bromide-adapted cultures were identical (data not shown), one can rule out any contaminant being introduced during the process by which the parent strain became resistant to ethidium bromide.

**Susceptibility to biocides and dyes**

The MIC values of several biocides and dyes against the parental strain and its ethidium bromide-induced-resistant progeny,
Briefly, the MICs against the ethidium bromide-adapted strain have all increased, especially those of the biocides TPP and dequalinium, for which an 8-fold increase is evident.

The antibiotic susceptibility of the two cultures against different classes of antibiotics was first screened by Kirby–Bauer assays. Whereas the initial parent strain was susceptible to all antibiotics, the adapted strain showed evidence of decreased susceptibility to norfloxacin, ciprofloxacin and nalidixic acid (with zones of inhibition of 27, 28 and 14 mm for ATCC 25923 and 19, 21 and 11 mm for ATCC 25923EtBr, respectively). However, the diameters of the zones of inhibition for ATCC 25923EtBr still fall within the susceptibility range.

The MIC of each antibiotic against the parental and ethidium bromide-adapted strains is shown in Table 2. According to the Kirby–Bauer antibiotic disc assay, the ethidium bromide-adapted strain was 4-fold more resistant than its parental strain to norfloxacin and ciprofloxacin. No alteration was observed in the MIC of nalidixic acid or any of the other antibiotics tested.

For ciprofloxacin, the MIC obtained for ATCC 25923EtBr (1 mg/L) equals the breakpoint for distinguishing susceptibility from intermediate resistance, according to the CLSI breakpoint values. However, measured against EUCAST breakpoint values, the MIC of ciprofloxacin for this strain would correspond to resistance, thus further illustrating the development of an MDR phenotype in this strain.28

The two strains were then tested for the effect of different EPIs at half or less of their MIC on the MIC of several efflux pump substrates. MICs of the different inhibitors were determined in liquid media, and did not differ between the two cultures. MICs were as follows: CCCP, 0.375 mg/L; thioridazine, 25 mg/L; chlorpromazine, 50 mg/L; verapamil, >200 mg/L; reserpine, >100 mg/L; and ouabain, >200 mg/L.

### Table 2. MICs of biocides and antibiotics in the presence and absence of EPIs

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<th>CPZ</th>
<th>VER</th>
<th>CCCP</th>
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<td>100</td>
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<tr>
<td></td>
<td>ATCC 25923EtBr</td>
<td>&gt;800 (4×)</td>
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<tr>
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</table>

Values in bold type correspond to a decrease of 4-fold or higher on the MIC values in comparison to the ones in the absence of inhibitor. Values in parentheses indicate the MIC increment relative to the one of the original culture. The concentration of each EPI used is defined in the Materials and methods section.

Ethidium bromide; TPP, tetraphenylphosphonium bromide; DC, dequalinium chloride; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; PT, pentamidine; BAC, benzalkonium chloride; CHX, chlorhexidine; CTAB, cetyltrimethylammonium bromide; RD, rhodamine; BER, berberine; NOR, norfloxacin; CIP, ciprofloxacin; CCCP, carbonyl cyanide m-chlorophenylhydrazone; TZ, thioridazine; CPZ, chlorpromazine; VER, verapamil; RES, reserpine. Additional antibiotics tested included nalidixic acid, tetracycline, erythromycin, chloramphenicol and rifampin, with no alteration in the MICs between the two cultures. The effect of ouabain in the MICs was also tested for EtBr, TPP, DC, DAPI and PT, with no observable effect.

ATCC 25923EtBr, are summarized in Table 2. Briefly, the MICs against the ethidium bromide-adapted strain have all increased, especially those of the biocides TPP and dequalinium, for which an 8-fold increase is evident.

### Susceptibility to antibiotics

**Kirby–Bauer assays.** The antibiotic susceptibility of the two cultures against different classes of antibiotics was first screened by Kirby–Bauer assays. Whereas the initial parent strain was susceptible to all antibiotics, the adapted strain showed evidence of decreased susceptibility to norfloxacin, ciprofloxacin and nalidixic acid (with zones of inhibition of 27, 28 and 14 mm for ATCC 25923 and 19, 21 and 11 mm for ATCC 25923EtBr, respectively). However, the diameters of the zones of inhibition for ATCC 25923EtBr still fall within the susceptibility range.

**MIC determination.** The MIC of each antibiotic against the parental and ethidium bromide-adapted strains is shown in Table 2. According to the Kirby–Bauer antibiotic disc assay, the ethidium bromide-adapted strain was 4-fold more resistant than its parental strain to norfloxacin and ciprofloxacin. No alteration was observed in the MIC of nalidixic acid or any of the other antibiotics tested.

For ciprofloxacin, the MIC obtained for ATCC 25923EtBr (1 mg/L) equals the breakpoint for distinguishing susceptibility from intermediate resistance, according to the CLSI breakpoint values. However, measured against EUCAST breakpoint values, the MIC of ciprofloxacin for this strain would correspond to resistance, thus further illustrating the development of an MDR phenotype in this strain.28

**Effect of EPIs on MICs of antibiotics, biocides and dyes**

The two strains were then tested for the effect of different EPIs at half or less of their MIC on the MIC of several efflux pump substrates. MICs of the different inhibitors were determined in liquid media, and did not differ between the two cultures. MICs were as follows: CCCP, 0.375 mg/L; thioridazine, 25 mg/L; chlorpromazine, 50 mg/L; verapamil, >200 mg/L; reserpine, >100 mg/L; and ouabain, >200 mg/L.
As evident from Table 2, the two phenothiazines (thioridazine and chlorpromazine), as well as reserpine, reduced the resistance of the ATCC 25923 EtBr strain to many of the agents to which resistance had been induced by exposure to increasing concentrations of ethidium bromide. A reduction of the MICs to at least a quarter of their original values in the presence of the EPI was considered as indicative of the presence of efflux activity. The resistance to the agents affected by EPIs indicates a role of an efflux pump system.

**Assessment of efflux activity**

**Ethidium bromide agar screening method.** Both ATCC 25923 and ATCC 25923 EtBr were evaluated for the presence of EP activity by the ethidium bromide agar screening method. While the initial parental culture began to exhibit fluorescence at a concentration of 0.5 mg/L ethidium bromide at 37°C, the adapted strain began to show fluorescence at an ethidium bromide concentration of 2 mg/L (Figure 1). Following transfer to 4°C, the minimal ethidium bromide concentration-producing fluorescence for the adapted strain decreased to 1 mg/L (Figure 1), whereas the transfer of the parent culture to 4°C did not alter the minimal concentration of ethidium bromide that caused the fluorescence of the bacterial mass. These results suggest that the adapted strain is expressing a system (or systems) capable of extruding ethidium bromide, a process that has been previously shown to be temperature-dependent.19,26

**Semi-automated fluorometric method.** The semi-automated fluorometric method demonstrated that there was no appreciable efflux activity in the ATCC 25923 culture (Figure 2a), whereas the ethidium bromide-adapted culture showed pronounced efflux activity, which was inhibited by the three EPIs tested, in a concentration-dependent manner (Figure 2b).

**Genetic analysis**

The two cultures were evaluated for: (i) the presence of mutations in the chromosome generally associated with fluoroquinolone resistance and (ii) the presence and expression of genes coding for several efflux pumps.

**Mutations in the grlA and gyrA genes.** The two cultures were screened for mutations in the chromosome most commonly associated with fluoroquinolone resistance in *S. aureus*, namely the ones in the quinolone resistance-determining regions (QRDRs) of both the grlA and gyrA genes.29–31 None of the mutations generally described for these two genes were found in any of the cultures that could be associated with the decrease in fluoroquinolone susceptibility observed for ATCC 25923 EtBr.

**Screening of genes coding for S. aureus EPs.** Genes that coded for the most common EPs in *S. aureus* were screened by PCR, using the primers described in Table 1. From the eight genes screened, six (norA, norB, norC, mepA, mdeA and sepA) were detected by PCR amplification. No amplification products were obtained with primers for qacA/B and smr genes. The presence of these plasmid-encoded EPs (QacA/B and Smr) was ruled out by the absence of plasmids in these cultures (data not shown).

**qRT–PCR analysis of the expression level of genes coding for S. aureus EPs.** The expression of the six EP genes previously found for the two cultures was then evaluated by a qRT–PCR analysis, which showed that norA is overexpressed in the adapted strain (34.38 ± 3.36). The remaining genes show much lower variation in their expression: mepA (2.32 ± 1.97), norB (1.91 ± 1.25), sepA (1.71 ± 1.34), mdeA (1.25 ± 1.20) and norC (0.82 ± 0.16).

**norA promoter sequencing.** In order to understand the reasons for the overexpression of norA of ATCC 25923 EtBr, the promoter region of this gene was sequenced in both the parental and the ethidium bromide-adapted cultures. Sequencing of the norA promoter region for both ATCC 25923 and ATCC 25923 EtBr revealed that a 70 bp deletion occurred in ATCC 25923 EtBr, immediately downstream of −10 sequence, that encompasses the majority of the 5′-UTR, the site of the so-called flqB mutation, which has been related to increased resistance to

Figure 1. Evaluation of efflux activity of ATCC 25923 original (left) and ATCC 25923 EtBr (right) cultures by the ethidium bromide agar screening method. Cultures were swabbed in TSA plates containing increasing concentrations of ethidium bromide. Following overnight incubation at 37°C (upper panels) and after transfer to 4°C for 16 h (lower panels) fluorescence was detected under UV light.
fluoroquinolones, and the site of the assigned transcription initiation (Figure 3).32,33

Discussion
The results of this study show that continuous and prolonged exposure of the *S. aureus* strain ATCC 25923 to increasing concentrations of ethidium bromide results in an impressive increase in resistance to this agent. In parallel, resistance to fluoroquinolones as well as to other known EP substrates is also augmented, in particular, to the biocides tetraphenylphosphonium and dequalinium. The larger increase in resistance to ethidium bromide compared with that observed for the other NorA substrates may be explained by a greater capacity of NorA to extrude ethidium bromide, inasmuch as this pump has been described as conferring only low-level resistance to fluoroquinolones.12 However, this difference may also result from increased cell adaptation to ethidium bromide during the 82 day selection with this drug, whereas challenge by the other drugs tested only occurred during the 18 h period of the susceptibility assay. One may further hypothesize that cellular factors other than the pump itself may also play a role, namely the involvement of sensor systems that are specifically activated by each class of

Figure 2. Evaluation of ethidium bromide efflux activity by the semi-automated screening method for ATCC 25923 (a) and ATCC 25923_{Etb} (b). Assays were run at 37°C, in the presence of glucose, with or without the most active EPIs. The data presented were normalized against the conditions of accumulation (no efflux), and the relative fluorescence was determined. CPZ, chlorpromazine; TZ, thioridazine; RES, reserpine.
substrate recognized by MDR EPs, such as NorA. Recently, we have documented such interplay between EP activity, their regulation and altered cell permeability to antibiotics in Escherichia coli.4,6,7,9,10,13 Other and SdrM, whose genes are localized in the chromosome.

QacA/B, Smr, QacG, QacE

Escherichia coli

regulation and altered cell permeability to antibiotics in

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(0.88 μM). Concentrations of CCCP of 100 μM have been recently shown to produce significant killing of bacteria. Ng et al. noted that when working with 1 μM CCCP, reversal of norfloxacin accumulation by S. aureus cells corresponded to only 6%–10% of that observed with 100 μM CCCP.

The fact that thioldazine, chlorpromazine and reserpine also had some effect on the MICs for the original ATCC 25923 culture towards the same drugs suggests basal intrinsic efflux activity, in accordance with that reported by Kaatz and Seo, who described low-level expression of NorA in an S. aureus strain not exposed to any drug. This low-level expression may account for the slight decrease in fluorescence observable in the fluorometric assay for the parental strain, after 18 min incubation in the absence of EPIs (Figure 2a).

While this paper was in review, a report by Garvey and Piddock was published describing the selection of efflux-mediated MDR Streptococcus pneumoniae after exposure to reserpine. Their observations confirm the results in this study that demonstrate the potential for non-antibiotic efflux substrates to select for MDR in S. aureus. Since this MDR phenotype was correlated at the genetic level with overexpression of NorA, we infer that, among the several ethidium bromide chromosomally encoded EPs, this is the one that cells use as a first-line response to extrude this noxious agent. A recent study revealed that among S. aureus clinical isolates showing increased expression of EPs, the majority over-expressed a single EP gene, which for most cases was norA, whereas the remaining strains over-expressed two or more genes, most commonly norB and norC. These findings support the hypothesis made here of the eventual key role played by NorA in multidrug efflux by S. aureus.

S. aureus ATCC 25923 has several efflux systems, of which at least one is expressed at basal levels, and when these cells are challenged with increasing concentrations of a noxious agent that is a substrate of these efflux systems, the cells respond by increasing its efflux and survive, provided that the initial concentration of the agent is sufficiently low. This process may mirror the one that eventually takes place when a patient is infected with a bacterium and is given an antibiotic whose final concentration is below the one necessary to effectively kill the microorganism. By activating and/or over-expressing the cellular systems of drug efflux, the bacteria may be able to survive non-lethal concentrations of the drugs and become refractory to therapy.

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Transparency declarations

None to declare.

References

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