Identification and characterization of a novel efflux-related multidrug resistance phenotype in *Staphylococcus aureus*

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Moxifloxacin is a C8-methoxy (C8-OMe) fluoroquinolone that is highly active against *Staphylococcus aureus*, including many strains resistant to older fluoroquinolones such as ciprofloxacin. Available data indicate that it is a poor substrate for the NorA multidrug efflux pump. We produced a mutant of *S. aureus* in vitro (SA-K2068) with a novel non-NorA-mediated multidrug resistance phenotype characterized by raised MICs of several fluoroquinolones, including the C8-OMe fluoroquinolones, moxifloxacin and gatifloxacin, and the organic cations ethidium and tetraphenylphosphonium. Reserpine reduced MIC increases by two- to eight-fold. SA-K2068 also demonstrated reduced accumulation of moxifloxacin, gatifloxacin and enoxacin, and increased efflux of ethidium, activities that were completely blocked by carbonyl cyanide m-chlorophenyl hydrazone (CCCP); competition experiments indicated that a single pump was responsible for the phenotype. The effect of CCCP and ionophores identified the proton motive force as the source of energy for efflux. These data, combined with previous work from our laboratory and genome sequence data, indicate that *S. aureus* possesses several multidrug efflux pump proteins and it is apparent that C8-OMe fluoroquinolones can be substrates for such pumps.

Keywords: *Staphylococcus aureus*, multidrug efflux

Introduction

Moxifloxacin is a C8-methoxy (C8-OMe) fluoroquinolone with excellent potency against *Staphylococcus aureus* associated with relatively balanced inhibition of DNA gyrase and topoisomerase IV, possibly reducing the likelihood that resistance will emerge during therapy.¹,² Strains possessing mutations in single topoisomerase genes can have raised moxifloxacin MICs, but achievable serum levels are high enough that these strains may remain clinically susceptible.³,⁴

It has been shown that moxifloxacin is a poor substrate for the *S. aureus* NorA multidrug (MDR) efflux pump. Accumulation of the drug by a norA-overexpressing strain and its parent was equivalent,³ as were its MICs for a strain deleted for norA or the same strain transformed with a multicopy vector carrying norA (G. W. Kaatz, unpublished data). These characteristics suggest that moxifloxacin may be useful in the therapy of infections involving *S. aureus*, including strains containing resistance mechanisms that result in high MICs of older fluoroquinolones.

Analysis of *S. aureus* genome data indicates that it is likely to possess several MDR efflux pumps; we have shown that at least one non-NorA MDR efflux pump exists.⁶,⁷ It is conceivable that although moxifloxacin is a poor NorA substrate, other MDR pump gene(s) may exist that can transport this compound. Low- to moderate-level resistance conferred by such a pump may be the first step towards the appearance of higher-level resistance in the same strain associated with target mutations.

In this report we describe the production of an MDR mutant of *S. aureus* capable of effluxing multiple unrelated compounds, including C8-OMe fluoroquinolones. This occurrence establishes that these drugs are not immune to *S. aureus* efflux systems.

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Materials and methods

Bacterial strains

*S. aureus* SA-1199B (*norA* overexpressor) was used in some experiments.9 *S. aureus* NCTC 8325-4 (hereafter referred to as SA 8325-4) was used for the production of mutants. In an effort to produce MDR derivatives the organism was passed serially on gradient plates containing increasing concentrations of moxifloxacin and ethidium bromide (EtBr). This approach was used because no single-step mutants could be recovered on plates containing low concentrations of both compounds (mutation frequency of \(6 \times 10^{-10}\) at two-fold the MIC of each). Once growth occurred across a plate that included the target drug concentrations, a single-colony purified derivative was selected for analysis (SA-K2068).

DNA sequence determination

The quinolone resistance determining regions (QRDRs) of *gyrA*, *grlA*, *gyrB* and *grlB*, and the *norA* gene and its promoter, were amplified from chromosomal DNA using previously described procedures and primers.9 The nucleotide sequence of PCR products was determined using the dideoxynucleotide chain termination method.10

MIC determinations

MICs were determined by microdilution techniques according to the NCCLS guidelines.11 The effect of reserpine (20 mg/L) on MICs was also determined. Results were expressed as the geometric mean of at least four determinations, and four-fold or greater differences were considered significant.

Fluoroquinolone accumulation

Whole-cell accumulation of moxifloxacin and gatifloxacin was determined using a fluorometric method as described previously, modified by the use of Mueller–Hinton II broth (MHB; Becton-Dickinson, Cockeysville, MD, USA) as the growth medium.5 The effect of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, 100 µM) on drug accumulation was also assessed. Protein concentrations were determined using a commercially available kit (Bio-Rad Laboratories, Hercules, CA, USA), and results were expressed as ng drug accumulated/mg cell protein. Enoxacin accumulation was determined using a radiometric method as described previously, employing the same conditions and manipulations as described for the fluorometric assay.12

EtBr efflux

The loss of EtBr from EtBr-loaded cells was determined fluorometrically as described previously.7 Results were expressed as mean total efflux over a 5 min time course, and a four-fold change in efflux compared with the parent strain was considered significant.

In order to establish whether selected compounds could compete with EtBr for efflux, a baseline total EtBr efflux by SA-K2068 and SA-1199B was established. Efflux experiments were then repeated, except that cells were loaded concurrently with EtBr and potential inhibitors (each at 10- to 50-fold molar excess compared with the EtBr concentration). Loaded cells were pelleted and then resuspended in fresh MHB, resulting in a \(>40\)-fold reduction in residual concentrations of putative inhibitors, and fluorescence was followed as described above. Potential inhibitors tested included tetraphenylphosphonium bromide (TPP), moxifloxacin, gatifloxacin, enoxacin, chloramphenicol and linezolid.

The method of Mitchell et al.13 was employed to determine the effect of various concentrations of valinomycin or nigericin on efflux. EtBr efflux was monitored exactly as described previously, except that cells were resuspended in 50 mM KH2PO4 (pH 7.0) containing 1 mM MgSO4 plus 125 mM sodium formate. ATP concentrations following exposure to CCCP or valinomycin were measured using a commercially available kit (ENLITEN ATP Assay System; Promega Corp., Madison, WI, USA).

norA expression

RNA was isolated using the RNeasy kit (Qiagen Inc., Valencia, CA, USA). *norA* and *sarA* (included as a control for normalization purposes) mRNAs were labelled using the Primer Extension System-AMV Reverse Transcriptase kit (Promega). The oligonucleotides used were end-labelled with \(\gamma\)-[32P]ATP (3000 Ci/mmol; NEN Life Science Products, Inc., Boston, MA, USA) using procedures recommended by the manufacturer of the Primer Extension System. Primer extension products were quantified using a phosphorimaging system (Storm 860; Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analysis

Drug accumulation data were analysed using the Student’s *t*-test. The Mann–Whitney rank-sum test was used when the power of a particular comparison was \(< 0.8\); significance was defined as \(P < 0.05\).

Results and discussion

SA-K2068 had an MDR phenotype (Table 1). There were no significant mutations in any QRDR. Non-QRDR mutations associated with fluoroquinolone resistance have been found in *S. aureus*, but only one (resulting in P25H in GrlB) would have been missed by our analysis.14,15 There were no mutations present in the *norA* region, and *norA* expression in SA-K2068 was unchanged from that observed for SA 8325-4.
These data indicate that the SA-K2068 MDR phenotype is unrelated to NorA (data not shown). Significant (≥four-fold) rises in the MICs of ciprofloxacin, norfloxacin, enoxacin, gatifloxacin and moxifloxacin were observed, whereas modest to minimal increases were observed for sparfloxacin, levofloxacin and trovafloxacin. Several organic cations, all of which are NorA substrates, were also evaluated and significant MIC increases were observed for EtBr and TPP (∼eight- and seven-fold, respectively). Minimal MIC increases occurred for acriflavine, benzalkonium chloride (BAC) and rhodamine.

Reserpine significantly influenced the susceptibility of SA-K2068 to ciprofloxacin, norfloxacin, gatifloxacin, EtBr and TPP (reserpine effect –four-fold or greater; see Table 1). A reserpine effect of at least four-fold was also observed for acriflavine and BAC despite little change in the MICs of these compounds; this phenomenon is likely to be related to reserpine-mediated inhibition of other pumps for which they are substrates. In fact, the marked reserpine effect observed for acriflavine, BAC, EtBr and TPP for the parent strain suggests that these compounds are avidly effluxed by wild-type S. aureus.

Despite MIC rises of four-fold or greater for moxifloxacin and enoxacin for SA-K2068, the presence of reserpine resulted in a lower than expected (i.e. <four-fold) MIC reduction. Work done with Bmr, a Bacillus subtilis MDR transporter and NorA homologue, suggests that reserpine binds to the transporter and it is reasonable to expect that it may interact with other MDR pumps in a similar fashion. A reserpine–pump interaction may alter the active site of a transporter such that efflux of moxifloxacin and enoxacin can continue, albeit at a reduced level, whereas the efflux of other substrates is strongly inhibited and ≥four-fold MIC reductions are observed. Alternatively, a reduced reserpine effect could result from low-level activity of one or more efflux pumps resistant to the inhibitor.

With respect to organic cations, it has been shown that NorA mediates the efflux of EtBr and TPP more effectively than that of either acriflavine or BAC (eight- to 14-fold increases in MICs versus four-fold, respectively).8,10 EtBr and TPP also appear to be better substrates for the SA-K2068 efflux process. A similar preference was found for an MDR pump studied in a norA-disrupted strain of S. aureus.7 Perhaps EtBr and TPP have structural characteristics that favour recognition and transport; however, what these characteristics might be is unclear at the present time.

Compared with SA 8325-4, significantly enhanced EtBr efflux was observed for SA-K2068 (Figure 1a). CCCP inhibited EtBr efflux, suggesting the presence of a membrane-based, proton-motive force (pmf)-dependent MDR pump. SA-K2068 also showed reduced accumulation of several fluoroquinolones compared with the parent strain (Figure 1b–d; P < 0.03 for all time points prior to CCCP addition). SA-K2068 accumulated 2.4-, 2.9- and 4.4-fold less enoxacin, moxifloxacin and gatifloxacin, respectively, than did the parent strain. CCCP eliminated differences between strains for each fluoroquinolone.

Results for competition experiments are shown in Figure 2. For SA-K2068, all compounds except chloramphenicol and linezolid competed with the EtBr efflux. TPP was the most

<table>
<thead>
<tr>
<th>Drug</th>
<th>Relative resistance</th>
<th>Reserpine effect</th>
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<tbody>
<tr>
<td></td>
<td>SA 8325-4</td>
<td>SA-K2068</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1 (0.39)</td>
<td>13.2</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>1 (0.87)</td>
<td>7.2</td>
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<td>Enoxacin</td>
<td>1 (0.96)</td>
<td>6.5</td>
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<tr>
<td>Gatifloxacin</td>
<td>1 (0.18)</td>
<td>4.9</td>
</tr>
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<td>Moxifloxacin</td>
<td>1 (0.08)</td>
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</tr>
<tr>
<td>Sparfloxacin</td>
<td>1 (0.15)</td>
<td>3.3</td>
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<tr>
<td>Levofloxacin</td>
<td>1 (0.39)</td>
<td>1.6</td>
</tr>
<tr>
<td>Trovafloxacin</td>
<td>1 (0.07)</td>
<td>1.6</td>
</tr>
<tr>
<td>Acriflavine</td>
<td>1 (7.93)</td>
<td>1.6</td>
</tr>
<tr>
<td>BAC</td>
<td>1 (1.15)</td>
<td>1.1</td>
</tr>
<tr>
<td>EtBr</td>
<td>1 (2.99)</td>
<td>8.4</td>
</tr>
<tr>
<td>Rhodamine</td>
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<td>2.5</td>
</tr>
<tr>
<td>TPP</td>
<td>1 (10.66)</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Table 1. Susceptibility of study strains to various drugs, and the influence of reserpine on MICs

*aRatio of the MIC for the indicated strain to that for SA 8325-4.

*bRatio of the MIC in the absence of reserpine to that in its presence (20 mg/L).

*cThe MIC of each drug for SA 8325-4 is given in parentheses.
efficient competitor, as a 10-fold molar excess resulted in a greater reduction in efflux efficiency than did 50-fold molar excesses of the fluoroquinolones. The TPP effect observed represents true competition and not a secondary effect of dissipation of the transmembrane potential ($\Delta \psi$) by the compound, as we evaluated the effect of a maximal residual external concentration (5 mg/L) in standard EtBr efflux assays and found no effect versus SA-K2068 (data not shown).

The lack of competition by chloramphenicol and linezolid indicated that the reduced EtBr efflux observed following exposure to TPP and the fluoroquinolones was not due to a generalized antimicrobial effect. For SA-1199B, TPP again was an efficient competitor but moxifloxacin and gatifloxacin essentially did not compete with NorA-mediated EtBr efflux. These data indicate that in SA-K2068 a single pump is responsible for the observed phenotype and confirm that its substrate profile is different from that of NorA.

In potassium-buffered solutions, valinomycin specifically collapses $\Delta \psi$, whereas nigericin specifically collapses the transmembrane pH gradient. A concentration-dependent inhibition of EtBr efflux occurred for both ionophores in whole cells of SA-K2068 and SA-1199B (concentrations tested, 0.25–4 µM; data not shown). Only minor differences in inhibitory potency were observed between the ionophores for SA-1199B, but for SA-K2068 valinomycin was 34% (at 4 µM) to 79% (at 0.25 µM) more potent than nigericin.

In whole cells, increased sensitivity to valinomycin versus nigericin could occur in either an ATP- or a pmf-coupled system. By collapsing $\Delta \psi$, valinomycin, like CCCP, can uncouple the $F_0F_1$ proton-translocating ATPase and in its hydrolytic mode the enzyme can deplete intracellular ATP. If efflux were ATP dependent, depletion of the intracellular pool would result in a cessation of the process. With CCCP exposure the ATP content of SA-K2068 was only 14% of the control and the EtBr efflux was reduced by 90%, data consistent with an uncoupling of the ATPase (data not shown). Conversely, the highest concentration of valinomycin employed (4 µM) inhibited EtBr efflux by >80% but the ATP content was 41% of the control. The maintenance of a significant ATP pool, despite strong inhibition of EtBr efflux in the presence of valinomycin, supports the conclusion that efflux in SA-K2068 is coupled to the pmf and not to ATP. The ionophore data also suggest that $\Delta \psi$ is the most important component of the pmf for the SA-K2068 efflux process, but this requires verification using everted vesicles once the genetic determinant has been isolated.
Our data establish that one pmf-dependent pump is responsible for the observed MDR phenotype in SA-K2068. The data also indicate that efflux of C8-OMe fluoroquinolones as a mechanism for reduced susceptibility can occur in S. aureus. We now have evidence favouring the existence of three chromosomally encoded S. aureus MDR efflux pumps that have overlapping, but distinct, substrate profiles. Whether or not overlapping substrate profiles imply functional relatedness requires the identification of the natural function(s) of these proteins.

The fact that S. aureus possesses numerous MDR efflux pumps and can adapt so well to antimicrobial stress through their activity underscores the urgent need to understand these processes. The isolation of the non-norA genes associated with MDR efflux phenotypes, further characterization of their protein products and a detailed study of the regulation of expression of these genes are the next logical steps.

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References


