A multidrug efflux phenotype mutant of *Streptococcus pyogenes*

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We describe a mutant of *Streptococcus pyogenes* NCTC 8198 with a multidrug efflux phenotype. A mutant selected with ethidium bromide showed a four-fold rise in MIC of norfloxacin, a 16-fold rise in MIC of ethidium bromide and an eight-fold rise in MIC of acriflavine when compared with the parent strain. The MICs were unaffected by the efflux pump inhibitors reserpine, rescinnamine and verapamil. The mutant’s ethidium bromide MIC was reduced two-fold by norfloxacin. Ethidium bromide accumulation after 10 min was 58% lower in the mutant compared with the parent. This difference was not affected by carbonyl cyanide m-chlorophenyl-hydrazone.

Introduction

Efflux-mediated fluoroquinolone resistance has been described previously in several Gram-positive bacteria, including *Streptococcus pneumoniae* and viridans group streptococci. Such isolates display multidrug resistance with decreased susceptibilities to a variety of compounds, including ethidium bromide, acriflavine and some fluoroquinolones.

Fluoroquinolone resistance in *S. pyogenes* associated with point mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* has been described. Little is known, however, about the role of efflux-mediated fluoroquinolone resistance in *S. pyogenes*. Although *S. pyogenes* remains universally susceptible to penicillin, the newer fluoroquinolones with improved Gram-positive activity may be useful therapeutically in the management of soft tissue infections. It is therefore important to understand the mechanisms of fluoroquinolone resistance in *S. pyogenes*.

Materials and methods

Selection of efflux mutants

Mutants were selected from *S. pyogenes* NCTC 8198. An inoculum of ~10⁹ cfu was plated onto Columbia agar (Oxoid, Basingstoke, UK) supplemented with 5% horse blood containing norfloxacin, ciprofloxacin, levofloxacin, moxifloxacin or ethidium bromide. After incubation in air at 37°C for 48 h, mutant colonies were recovered, subcultured onto non-selective media and incubated overnight.

Susceptibility testing

The MICs of ethidium bromide, acriflavine and a range of fluoroquinolones were determined by an agar dilution method using Iso-sensitest agar (Oxoid) supplemented with 5% horse blood and an inoculum of 10⁴ cfu/spot. After incubation at 37°C in air for 20 h, the MIC was taken as the lowest concentration of the compound that inhibited growth completely. Norfloxacin susceptibility testing was also carried out in the presence of efflux inhibitors reserpine (10 and 30 mg/L), rescinnamine (20 mg/L) and verapamil (25 mg/L). Ethidium bromide testing was also carried out in the presence of reserpine. An efflux phenotype was considered likely if a mutant showed a four-fold or greater increase in the MICs of ethidium bromide, acriflavine and norfloxacin when compared with the parent strain.

To determine whether norfloxacin could reduce the MIC of ethidium bromide for mutant 1EB1 by competitive inhibition, ‘half chequerboard’ broth MICs were carried out in Iso-sensitest broth (Oxoid) supplemented with lysed blood by the British Society for Antimicrobial Chemotherapy (BSAC) susceptibility testing method; norfloxacin was used at 0.5 and 1 mg/L.
Ethidium bromide accumulation

Ethidium bromide accumulation in *S. pyogenes* was determined using a method previously described for *S. pneumoniae*, and was determined in the presence and absence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 100 µM).

**QRDR sequencing**

The QRDRs of *gyrA*, *gyrB*, *parC* and *parE* in the parent strain and mutant were amplified by PCR using the following primers: *gyrA*, 5′-TATGCACTAGTGCTATTG-3′ and 5′-ATATGAGGCGGAATGTTAG-3′; *gyrB*, 5′-GCAATTCAGAAGTGGTTAAG-3′ and 5′-AGCTTCTAGAACCAGGTCTCA-3′; *parC*, 5′-GGCGGTATTCAGATAATATCAT-3′ and 5′-CAGACGCATCAATCCTT-3′; *parE*, 5′-GCTAGATTATCGAGAAGGA-3′ and 5′-GATTACGCATGAGTGTCATTGT-3′. The conditions used for *gyrA*, *parC* and *parE* QRDR amplification were an initial extended denaturation step of 95 °C for 5 min followed by 30 cycles of 30 s at 48 °C, 1 min at 72 °C and 30 s at 95 °C. For *gyrB* QRDR amplification the initial denaturation step was followed by 30 cycles of 30 s at 46 °C, 1 min at 72 °C and 30 s at 95 °C. The PCR products were sequenced by automated fluorescence sequencing (Lark Technologies, Saffron Waldon, UK).

**Results**

**Susceptibilities of selected mutants**

Agar dilution susceptibility testing was carried out on 35 mutants selected with norfloxacin at concentrations between 4 and 8 µg/mL and a total of 12 mutants selected with ciprofloxacin, levofloxacin and moxifloxacin. All 47 mutants showed two- to eight-fold increases in the MIC of norfloxacin, four- to 16-fold increases in the MIC of ciprofloxacin, and two- to four-fold increases in the MIC of ofloxacin when compared with the parent strain. The increase in the MIC of norfloxacin was not reversed by reserpine. None of the mutants displayed an increase in the MIC of ethidium bromide when compared with the parent strain. These mutants were assumed to be topoisomerase mutants and were not studied further.

Ethidium bromide selection yielded 22 mutants at concentrations between 3 and 8 µg/mL. The greatest increase in MIC (determined by agar dilution susceptibility testing) was shown by 10 mutants with identical phenotypes. A representative of these, designated 1EB1, was chosen for further work. Mutant 1EB1 showed a 16-fold increase in the MIC of ethidium bromide, which was not reversed by reserpine, and an eight-fold or greater increase in the MIC of acriflavine (Table 1). The MIC of norfloxacin increased four-fold compared with the parent; this was not significantly changed in the presence of any one of the three efflux inhibitors. The MIC of ciprofloxacin was increased two-fold. No change was observed in the MIC of other fluoroquinolones tested, or of erythromycin, azithromycin or clindamycin (Table 1).

The half chequerboard MICs determined on 1EB1 by broth dilution susceptibility testing were as follows: norfloxacin alone = 2 µg/mL; ethidium bromide alone = 8 µg/mL; ethidium bromide plus norfloxacin at 0.5 µg/mL = 8 µg/mL; ethidium bromide plus norfloxacin at 1 µg/mL = 4 µg/mL. Thus norfloxacin at 1 µg/mL resulted in a two-fold reduction in the ethidium bromide MIC.

**Ethidium bromide accumulation**

The mutant 1EB1 accumulated significantly less ethidium bromide than the parent strain (Figure 1). After 10 min, the level of accumulation in the mutant was 58% less than that observed in the parent strain. In the presence of CCCP this difference was unchanged.

**QRDR sequencing**

Sequencing of the QRDRs of *gyrA*, *gyrB*, *parC* and *parE* in the parent and 1EB1 revealed no differences between the two strains.

**Discussion**

Selection of *S. pyogenes* mutants with an efflux phenotype was attempted using ethidium bromide and a variety of...
due to an efflux pump. It is also most likely, from parallels to other bacteria with efflux pumps unaffected by reserpine, that the putative pump will require further study.

Interestingly, mutant 1EB1 appears to differ from that recently described by Boos et al.9 They described an S. pyogenes mutant that shows an eight-fold decrease in ciprofloxacin MIC in the presence of reserpine. This mutant was selected from a clinical isolate using gemifloxacin. The method they used involved serial subculture of the organism in broth containing antibiotic. The differences between the mutant selected by Boos et al.9 and that selected by ourselves raises the possibility that S. pyogenes has more than one efflux system for quinolones. This would not be unexpected since other bacteria have multiple efflux pumps,10 and genome sequence data indicate this is likely to be the case for S. pyogenes M1 (http://www.genome.ou.edu/strep.html; last accessed 17th October 2002). Our observation that the erythromycin and azithromycin MICs were unchanged in mutant 1EB1 indicates that the putative efflux phenotype we describe is independent of that conferred by mef.11

The S. pyogenes mutant 1EB1 was not affected by the proton pump inhibitor CCCP, perhaps because the concentration of CCCP was too low or because the putative efflux pump is not a proton motive force-dependent pump. ATP-dependent pumps can also cause multidrug resistance in other pumps such as Bmr and NorA, that in 1EB1 there is a regulatory mutation that up-regulates expression of an S. pyogenes efflux pump.

The putative efflux mechanism found in S. pyogenes mutant 1EB1 is unusual in that it is not inhibited by reserpine, rescinnamine or verapamil. This is in contrast to many other efflux systems that are inhibited by one or more of the inhibitors tested in our study.2,4,7 Neyfakh et al.7 have shown that different efflux pumps are inhibited by different concentrations of reserpine. When Bmr and NorA were cloned into Bacillus subtilis, Bmr was inhibited at a reserpine concentration of 5 mg/L and NorA at a concentration of 20 mg/L. However, even a reserpine concentration of 30 mg/L did not change the MICs of ethidium bromide, acriflavine and fluoroquinolones displayed by 1EB1. Mutants of other Gram-positive bacteria with efflux pumps unaffected by reserpine have been described. For example, mutations in the Bmr pump in B. subtilis can cause the pump to become resistant to reserpine, even though it is still able to efflux antibacterial compounds.3 It is possible that the putative efflux mechanism of 1EB1 is naturally resistant to reserpine. The use of reserpine alone for efflux recognition may therefore be limited and a variety of efflux inhibitors and pump substrates should be used to detect efflux phenotype strains.

We attempted to see whether there was any competition between norfloxacin and ethidium bromide for the putative pump by carrying out half chequerboard MICs. Unfortunately, the ethidium bromide MIC was only reduced two-fold by 1 mg/L norfloxacin; this is a small decrease and may not be significant. The nature of competitors for, and inhibitors of, the putative pump will require further study.

Figure 1. Accumulation of ethidium bromide in S. pyogenes NCTC 8198 (squares) and 1EB1 (triangles). Each point represents the mean of three experiments. Error bars represent one standard deviation.

fluoroquinolones. None of the mutants selected by any of the fluoroquinolones displayed an efflux phenotype. We have found that levofloxacin and moxifloxacin appear to be poor efflux pump substrates for PmrA and do not easily select efflux mutants of S. pneumoniae (N. P. Brenwald & M. J. Gill, unpublished data). Failure to select efflux mutants with these compounds was therefore not unexpected. Norfloxacin and ciprofloxacin are both substrates of several multidrug efflux pumps1–3 and yet failed to select efflux mutants of S. pyogenes, perhaps because topoisomerase mutants of this species are preferentially selected by fluoroquinolones. Ethidium bromide, which is also an efflux pump substrate, was therefore used to select for efflux mutants.

The ethidium bromide-selected mutant 1EB1 showed decreased susceptibility to several different compounds, including ethidium bromide, acriflavine and some fluoroquinolones. This susceptibility pattern is similar to multidrug efflux pump phenotypes in S. pneumoniae and viridans group streptococci.2,4 The ethidium bromide accumulation data are also consistent with an efflux mechanism, with mutant 1EB1 accumulating significantly less ethidium bromide when compared with the parent strain. Analysis of the QRDRs of 1EB1 and the parent strain showed no mutations. Although a general reduction in permeability due to changes in cell wall structures such as peptidoglycan or capsule cannot be entirely ruled out in 1EB1, these changes are likely to introduce only very small changes in MICs. Given the magnitude of the differences in norfloxacin and ethidium bromide MICs between 1EB1 and its parent, such permeability changes are unlikely. The phenotype displayed by 1EB1 is therefore most probably due to an efflux pump. It is also most likely, from parallels
Gram-positive bacteria, for example LmrA in *Lactococcus lactis*. It is possible that the putative efflux mechanism in 1EB1 is ATP dependent. Further work is required to investigate the relationship between the 1EB1 efflux system and those in other Gram-positive bacteria.

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References


