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# DX-619, a Novel Des-Fluoro(6) Quinolone Manifesting Low Frequency of Selection of Resistant *Staphylococcus aureus* Mutants: Quinolone Resistance beyond Modification of Type II Topoisomerases

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**DX-619, a novel des-fluoro(6) quinolone, was 16- to 32-fold, twofold, and four- to eightfold more potent than ciprofloxacin, gemifloxacin, and garenoxacin, respectively, against wild-type *Staphylococcus aureus*. DX-619 manifested equal fourfold increases in MIC against a common *parC* mutant and a common *gyrA* mutant and selected for mutants at up to two- to fourfold its MIC, consistent with dual-targeting properties. Of the four independent single-step mutants selected, two had new single mutations in *parC* (V87F and R17H), and two shared a new *gyrA* mutation (A26V), one with an additional deletion mutation in *parE* (Δ215-7). By allelic exchange, the ParC but not the GyrA or ParE mutation was shown to be fully responsible for the resistance phenotypes, suggesting an as yet undefined mechanism of resistance operating in conjunction with type II topoisomerase mutations contributed to resistance to DX-619. Studies with purified topoisomerase IV and gyrase from *S. aureus* also showed that DX-619 had similar activity against topoisomerase IV and gyrase (50% stimulation of cleavage complexes concentration, 1.25 and 0.62 to 1.25 μg/ml, respectively). Susceptibility studies with DX-619 and an array of efflux pump substrates with and without reserpine, an inhibitor of efflux pumps, suggested that resistance in DX-619-selected mutants is affected by mechanisms other than mutations in topoisomerases or known reserpine-inhibitable pumps in *S. aureus* and thus are likely novel.**

To execute their bactericidal activity, quinolones interact with the type II topoisomerases, DNA gyrase, and topoisomerase IV (topo IV) (7). Quinolone resistance occurs stepwise by mutations in the two topoisomerase target enzymes, with the first mutation generally occurring in the more sensitive enzyme (11). *Staphylococcus aureus* mutants selected stepwise with quinolones usually first manifest a mutation in topo IV followed by a mutation in gyrase (18, 25, 30), or as has been recently reported for topo IV, mutation in the promoter region leading to reduced enzyme expression (16). In addition, several chromosomally encoded efflux pumps in *S. aureus*, including NorA (24, 34), NorB (32), and MepA (20), mediate low-level quinolone resistance when overexpressed.

With the increased use of quinolones and the subsequent emergence of resistance (4, 10), new quinolones should be active against pathogens carrying multiple resistance mechanisms in order to remain clinically effective. An important characteristic of fluoroquinolones to limit the selection of resistance in wild-type bacteria is dual activity, in which the activity against both DNA gyrase and topo IV is the same (6, 37).

DX-619 is a novel des-fluoro(6) quinolone with enhanced activity against resistant gram-positive bacteria that is currently under development (9; H. Ishida, K. Fujikawa, M. Chiba, M. Tanaka, T. Otani, and K. Sato, Abstr. 44th Intersci. Conf. Antimicrob. Agents Chemother., abstr. F-1935, 2004; M. Tanaka, K. Fujikawa, Y. Murakami, T. Akasaka, M. Chiba, T. Otani, and K. Sato, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. F-1060, 2003). We undertook to define the effects of established resistance mechanisms on DX-619 activity, to characterize mechanisms of resistance to DX-619 of DX-619-selected mutants, and to define its potency against purified topoisomerase target enzymes.

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## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Becton Dickinson, Sparks, MD). For mutant selection *S. aureus* strains were grown in brain heart infusion (BHI) (Becton Dickinson, Sparks, MD) broth, and for MIC testing in Mueller-Hinton (MH) broth (Becton Dickinson, Sparks, MD). All strains were grown at 37°C, except for *E. coli* with plasmid pSAGA1, which was grown at 25°C for induction with arabinose. Ampicillin was used at 100 μg/ml. Ampicillin and all other chemicals, unless stated otherwise, were purchased from Sigma Chemical Co. (St. Louis, MO).

**Drug susceptibility determinations.** DX-619 (Daiichi Pharmaceutical Co. Ltd., Tokyo, Japan), garenoxacin (Bristol-Myers Squibb Co., Princeton, NJ), gemifloxacin (GlaxoSmithKline, Research Triangle Park, NC), and ciprofloxacin (Bayer Corporation, West Haven, CT) were kindly provided by the manufacturers. Novobiocin was purchased from Sigma Chemical Co. (St. Louis, MO). MICs for the above-mentioned compounds were determined by the agar dilution method (22). The MIC was the lowest concentration of antibiotic that yielded no visible growth after incubation at 37°C for 24 h. The MICs of quinolones, ethidium bromide, Hoechst 33324, rhodamine, cetrimide, and tetraphenylphosphonium bromide were determined by the broth microdilution method (22) and by agar dilution. MICs were determined in broth with and without reserpine (20 μg/ml) to screen for changes in efflux pump expression (28).

**Frequency of selection of mutants.** Mutants were selected by plating appropriate dilutions of overnight cultures of *S. aureus* ISP794 and its isogenic mutants on BHI agar containing DX-619, ciprofloxacin, gemifloxacin, or garenoxacin at increasing concentrations at or above the MIC of each drug, up to the limit at which no mutants could be selected (5). Plating of dilutions of the same cultures on drug-free BHI agar was used to determine the number of CFU plated on the selection plates. When needed for selection with DX-619, large (150 mm by

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| *S. aureus* strains | | |
| ISP794 | 8325 *pig-131* | 29 |
| SS1 | 8325 *pig-131 gyrB142* (Ile102Ser, Arg144Lys) *gyrA* (Ser84Leu) | 2 |
| MT5224c4 | 8325 *nov (gyrB142) hisG15 pig-131 parC542* (Ser80Phe) | 31 |
| EN1252a | 8325 *nov (gyrB142) hisG15 pig-131 parC542 gyrAΩ 1051* (Erm) $Nov^+$ | 2 |
| QT1 | ISP794 *mgrA*::*cat* | 33 |
| MT23142 | 8325 *pig-131 flqB* | 24 |
| KL820 | RN4220 *norA*::*cat* | 12 |
| DX-619-C | ISP794 *gyrA* (A26V) | This study; spontaneous $DX\text{-}619^r$ |
| DX-619-E | ISP794 *gyrA* (A26V), *parE* (Δ215–217) | This study; spontaneous $DX\text{-}619^r$ |
| DX-619-JJ | ISP794 *parC* (V87F) | This study; spontaneous $DX\text{-}619^r$ |
| DX-619-MM | ISP794 *parC* (R17H) | This study; spontaneous $DX\text{-}619^r$ |
| DX-619-E-ΔE-AE1 | ISP794 *gyrA* (S84L), *parE* (Δ215–217) | This study; spontaneous $DX\text{-}619^r$ |
| DX-619-E-ΔE-AE2 | ISP794 *gyrA* (E88K), *parE* (Δ215–217) | This study; spontaneous $DX\text{-}619^r$ |
| *E. coli* strains | | |
| DH5α | $F^-$ *ϕ80dlacZΔM15Δ(lacZYA-argF)U169 deoR recA1 endA1 phoA hsdR17* ($r_K^-$ $m_K^-$) *supE44* $\lambda^-$ *thi-1 gyrA96 relA1* | Gibco-BRL |
| TOP10 | $F^-$ *mcrAΔ*(*mrr-hsdRMS-mcrBC*) ϕ80*lacZΔM15ΔlacX74 recA1 araΔ139Δ*(*ara-leu*)*7697 galU galK rpsL* ($Str^r$) *endA1 nupG* | Invitrogen |
| Plasmids | | |
| pBAD/Thio-TOPO | 4.5-kb linearized expression vector, $Ap^r$ | Invitrogen |
| pTrcHisB-gyrB | *gyrB* cloned into cloning vector pTrcHisB | Invitrogen |
| pCL52.1 | 8,100-bp plasmid containing the replicon of pGB2, $Sp^r$ (*E. coli*) and temperature-sensitive replicon of pE194, $Tc^r$ | 21 |
| pQT8 | pSK950-*norB* | 32 |

[a] Abbreviations: $1734^r$, WCK-1734 resistant; $Cip^r$, ciprofloxacin resistant; $Ap^r$, ampicillin resistant.

15 mm) petri dishes were used to plate approximately $10^{11}$ CFU. Selection plates were incubated at 37°C. The frequency of selection of resistant mutants was calculated as the ratio of the number of resistant colonies at 48 h to the number of CFU plated, and the mutant prevention concentration was defined as the lowest antibiotic concentration at which no resistant colonies were detected upon plating of $10^{11}$ CFU. Selected colonies of various sizes were purified on plates containing the same concentration of drug. Mutants were then maintained at −80°C in BHI broth containing 10% glycerol.

**Sequence analysis.** Chromosomal DNA from various mutants of *S. aureus* ISP794 was isolated using the Easy-DNA kit (Invitrogen, Carlsbad, CA) after lysing the cells with lysostaphin (Ambi, Lawrence, NY) at 0.1 mg/ml in phosphate-buffered saline and was used as a template for PCRs. The entirety of the *parC*, *parE*, *gyrA*, and *gyrB* structural genes and the promoter regions of *parE*, *gyrB*, and *norA* were individually amplified by PCR, as previously described (24, 30). DNA sequencing of the PCR products was performed using the *Taq* DyeDeoxy Terminator method (Applied Biosystems) with the ABI 3700 PRISM automated sequencer (Massachusetts General Hospital core facility). All selected mutants were first sequenced for at least the first 500 bases of the *parC* and *gyrA* genes. All genetically defined mutants selected with DX-619 were sequenced for the entirety of the *parC*, *parE*, *gyrA*, and *gyrB* genes.

**Allelic exchange.** For the allelic exchange experiments, the following gene fragments were amplified with upstream and downstream primers containing engineered EcoRI and BamHI sites, respectively. For the *gyrA* mutation, the region between nucleotides 1746 and 2784 of the *gyrB-gyrA* tandem genes (GenBank accession number D10489) from mutant DX-619-C was amplified as previously described (30). For the *parC* mutants, the region between nucleotides 2019 and 2919 of the *parE-parC* tandem genes (GenBank accession number D67075) from mutants DX-619-JJ and DX-619-MM were amplified with the upstream (5′TTAGTAGAATTCTAAAGGCAAAACAAAGCGAGTTG) and downstream (5′AATTAAGGATCCGTGGTGGTATATCTGTCGCGC) primers containing engineered EcoRI and BamHI sites, respectively. The annealing temperature was 55°C, and the extension time was 56 s for these PCRs.

For the *parE* mutant we used the forward (5′TTAGTAGAATTCGAAATT GTCGATAACTCCGTCGAT) and reverse (5′AATTAAGGATCCAATAGA ATGGCAATTTGTCTGCAA) primers to amplify the region encompassing nucleotides 511 to 1477 of the *parE* gene; the annealing temperature was 52°C, and the extension time was 59 s. Following gel extraction with the QIAquick gel extraction kit (QIAGEN, Valencia, CA), the PCR products were ligated into the EcoRI and BamHI sites of pCL52.1, a thermosensitive shuttle vector, and the recombinant plasmids were electroporated into *E. coli* DH5α. The plasmid clones were then transformed into *S. aureus* RN4220 and subsequently into *S. aureus* ISP794, as previously described (26). In addition, mutant *parE* and *gyrA* alleles were cloned into pCL52.1, their sequences were confirmed, and the plasmid clones were transformed into the isogenic strains DX-619-C-AE-α and DX-619-E ΔE-AE, respectively. Allelic exchange was performed as previously described (21). The resulting colonies were screened for susceptibility to tetracycline at a concentration of 5 μg/ml and reduced susceptibility to DX-619 at a concentration of 0.008 μg/ml. Allelic exchange was confirmed by DNA sequencing of the mutant alleles in the allelic exchange mutants.

**Cloning and expression of *S. aureus* ISP794 *parC*, *parE*, *gyrA*, and *gyrB* genes.** The *S. aureus* ISP794 *parC*, *parE*, *gyrA*, and *gyrB* genes were cloned into pTrcHisC, pTrcHisA, pBAD/Thio-TOPO, and pTrcHisB vectors, respectively, and overexpression and purification of the corresponding proteins ParC, ParE, GyrA, and GyrB was performed as previously described (18, 30).

**Topoisomerase catalytic and DNA cleavage assays.** Enzyme assays were carried out as described previously (30). DNA products were resolved by electrophoresis in 1% agarose, stained with ethidium bromide, photographed, and visualized under UV light. All enzyme assays were done at least twice, with reproducible results.

## RESULTS

**Activities of DX-619, ciprofloxacin, gemifloxacin, and garenoxacin against genetically defined mutants.** We first determined the MICs of DX-619 against genetically defined mutants of *S. aureus* and compared them to those of ciprofloxacin, gemifloxacin, and garenoxacin (Table 2). The MICs of ciprofloxacin, gemifloxacin, and garenoxacin determined in this study were identical to or within one twofold dilution of those previously reported (18, 19, 30). A *gyrA* mutation produced no change in the MIC of ciprofloxacin, but a mutation in topo IV caused an eightfold increase in the MIC of ciprofloxacin, a pattern that is consistent with its principally targeting topo IV.

TABLE 2. Activity of DX-619, ciprofloxacin, gemifloxacin, and garenoxacin against genetically defined strains of *S. aureus*[a]

| Strain | Strain property | MIC[b] (μg/ml) | | | |
|---|---|---|---|---|---|
| | | DX-619 | CIP | GEM | GAR |
| ISP794 | Wild type (parent) | 0.004–0.008 | 0.125–0.25 | 0.008–0.016 | 0.032 |
| MT5224c4 | *parC* (Ser80Phe) | 0.016–0.032 | 1.0 | 0.032–0.064 | 0.128 |
| SS1 | *gyrA* (Ser84Leu) | 0.016–0.032 | 0.125–0.25 | 0.032 | 0.064–0.128 |
| EN1252a | *parC* (Ser80Phe) *gyrA* (Ser84Leu) | 0.062–0.125 | 16.0 | 1–2 | 4.0 |
| QT1 | *mgrA* knockout | 0.008 | ND | ND | ND |
| MT23142 | *norA* overexpressor | 0.004–0.008 | ND | ND | ND |
| KL820 | *norA* knockout | 0.004–0.008 | ND | ND | ND |
| ISP794(pQT8) | *norB*-encoding plasmid | 0.004–0.008 | ND | ND | ND |

[a] Determined by agar dilution.
[b] CIP, ciprofloxacin; GEM, gemifloxacin; GAR, garenoxacin; ND, not done.

A strain with mutations in both *gyrA* and *parC* was highly resistant to ciprofloxacin, with a 128-fold increase in the MIC in comparison to the wild-type strain. Relative to ciprofloxacin, DX-619 was 16- to 32-fold more potent against the wild-type strain, fourfold more potent against a common *parC* mutant and a common *gyrA* mutant, and 16- to 32-fold more potent against the dual *parC gyrA* mutant. In contrast to ciprofloxacin, both *parC* and *gyrA* single mutants exhibited similar slight increases in MIC of DX-619, suggesting that DX-619 has similar activity against both target enzymes. Relative to gemifloxacin and garenoxacin, DX-619 was twofold and four- to eightfold, respectively, more potent against the wild-type strain, and 16-fold and 32- to 64-fold, respectively, more potent against the dual *parC gyrA* mutant.

**Frequency of selection of mutants.** The range of frequencies of selection of single-step resistant mutants of wild-type strain ISP794 with ciprofloxacin was similar to those previously published (15, 30). DX-619 was more active than ciprofloxacin, gemifloxacin, and garenoxacin and thus had lower frequencies of selection of mutants at comparable drug concentrations of gemifloxacin and garenoxacin. Normalizing for the differences in activity, with ciprofloxacin, mutants could be selected at up to fivefold the MIC (Table 3). With DX-619, gemifloxacin, and garenoxacin, however, mutants could be selected at low frequency at twofold the MIC but were not detected at fourfold the MIC (18, 19). Correspondingly, the mutant prevention concentration of ciprofloxacin was eightfold higher than its MIC, whereas DX-619, gemifloxacin, and garenoxacin selected for mutants at up to two- to fourfold their respective MICs (Table 3).

**Characterization of DX-619-selected single-step mutants.** To ascertain the primary cellular target of DX-619 we selected and characterized four independent mutants selected with DX-619 at two- to fourfold the MIC of ISP794 (0.016 μg/ml) (Table 4). Mutant DX-619-C had increased MICs of eightfold for both DX-619 and ciprofloxacin, and mutant DX-619-E had an increased MIC of fourfold for both DX-619 and ciprofloxacin. Both of these mutants had a novel Ala26Val mutation in GyrA. Such an increase in the MIC of ciprofloxacin was unexpected, because ciprofloxacin is known to have topo IV as a primary target. In addition, as has been previously demonstrated with the isogenic strains SS1 and 1734-J-AE-15, bearing the Ser84Leu and the Gly82Asp mutations in GyrA, respectively, the MIC of ciprofloxacin was similar to or only twofold higher than that of the wild type (30). Therefore, to account for the additional resistance phenotype of these mutants, we sequenced the entirety of the *gyrA*, *gyrB*, *parC*, and *parE* genes as well as the promoter region of *parE* or *gyrB*, the former of which has been recently described as a quinolone resistance mechanism through reduced enzyme expression (16). We found only a novel deletion of amino acids Arg, Gln, and Glu at positions 215 to 217 of ParE in DX-619-E but no additional mutation in DX-619-C.

Two other mutants, DX-619-JJ and DX-619-MM, had two- to fourfold increased MICs of DX-619 and a four- to eightfold increased MIC of ciprofloxacin. Sequencing the entirety of *gyrA*, *gyrB*, *parC*, and *parE* identified two novel mutations, Val87Phe within the QRDR of ParC in DX-619-JJ and Arg17His in ParC in DX-619-MM. Thus, in each of the four characterized mutants selected with DX-619, a novel mutation

TABLE 3. Frequency of selection of resistant mutants of strain ISP794

| Selecting drug concn (μg/ml) | Frequency of selection of mutants (fold MIC) | | | |
|---|---|---|---|---|
| | Ciprofloxacin | DX-619 | Gemifloxacin | Garenoxacin |
| 0.008 | | $5.9 \times 10^{-7}$–$9.0 \times 10^{-6}$ (×1) | | |
| 0.016 | | $7.6 \times 10^{-11}$–$1.8 \times 10^{-9}$ (×2) | $8.2 \times 10^{-6}$–$1.5 \times 10^{-5}$ (×1) | |
| 0.032 | | <1.4–<$4.6 \times 10^{-11}$ (×4) | $6.9 \times 10^{-10}$–$1.1 \times 10^{-9}$ (×2) | $6.8 \times 10^{-6}$–$1.1 \times 10^{-5}$ (×1) |
| 0.062 | | | Not detected (×4) | $3.1 \times 10^{-10}$–$1.9 \times 10^{-9}$ (×2) |
| 0.25 | $8.0 \times 10^{-6}$–$3.0 \times 10^{-5}$ (×1) | | | Not detected (×4) |
| 0.5 | $2.4 \times 10^{-7}$–$7.8 \times 10^{-6}$ (×2) | | | |
| 1.0 | $8.5 \times 10^{-9}$–$7.7 \times 10^{-8}$ (×4) | | | |
| 1.25 | $4.6 \times 10^{-10}$–$5.4 \times 10^{-10}$ (×5) | | | |
| 2.0 | <$2.6 \times 10^{-11}$ (×8) | | | |

TABLE 4. Characteristics of *S. aureus* ISP794 mutants selected with DX-619 and of allelic exchange isogenic strains

| Strain | Selecting drug concn (μg/ml) | MIC[a] (μg/ml) | | | | | Mutation(s) in: | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | DX-619 | CIP | GEM | GAR | NOV | ParC | ParE | GyrA | GyrB |
| ISP794 | | 0.004–0.008[b] | 0.125–0.25 | 0.008[b] | 0.032[b] | 0.16–0.32 | | | | |
| DX-619-C | 0.016 | 0.062[b] | 1–2 | 0.125[b] | 0.5[b] | 0.04 | None | None | A26V | None |
| DX-619-E | 0.016 | 0.032[b] | 0.5–1 | 0.125[b] | 0.5[b] | 0.04 | None | Δ215-7 | A26V | None |
| DX-619-JJ | 0.016 | 0.016[b] | 1 | 0.062 | 0.125 | 0.16 | V87F | None | None | None |
| DX-619-MM | 0.016 | 0.016[b] | 1 | 0.032 | 0.125 | 0.16 | R17H | None | None | None |
| DX-619-C-AE-α | | 0.008–0.016 | 0.25 | 0.016[b] | 0.062[b] | 0.16 | | | A26V | |
| DX-619-JJ-AE | | 0.016 | 1 | 0.032 | 0.062 | 0.16 | V87F | | | |
| DX-619-MM-AE | | 0.008–0.012 | 0.5 | 0.016 | 0.032 | | R17H | | | |
| DX-619-E-ΔE-AE | | 0.016 | 0.5–1 | 0.062 | 0.125 | 0.16 | | Δ215-7 | | |
| DX-619-E-ΔE-AE1 | 0.016 | 0.062–0.125 | 1 | 0.25–0.5 | 0.5–1 | | None | Δ215-7 | S84L | None |
| DX-619-E-ΔE-AE2 | 0.016 | 0.062 | 1 | 0.25–0.5 | 0.5–1 | | None | Δ215-7 | E88K | None |

[a] Determined by agar dilution. CIP, ciprofloxacin; GEM, gemifloxacin; GAR, garenoxacin; NOV, novobiocin.
[b] The MIC for these strains did not change with the addition of reserpine (20 μg/ml).

in either *gyrA* or *parC* was found. The mutants selected with DX-619 also manifested increased resistance to gemifloxacin and garenoxacin, with two- to fourfold increases in MIC relative to the wild type than DX-619. Thus, DX-619 maintained higher potency than gemifloxacin and garenoxacin against mutants selected with DX-619.

**Evaluation of the role of new mutations in resistance by allelic exchange.** To evaluate further the contribution of the novel *gyrA* and *parC* mutations in the resistance phenotype and to determine the gene targets of DX-619, we performed allelic exchange experiments for the new Ala26Val GyrA mutation found in DX-619-C and DX-619-E, for the Val87Phe and Arg17His ParC mutations found in DX-619-JJ and DX-619-MM, respectively, and for the deletion mutation at positions 215 to 217 of ParE. After growing the cells at the permissive temperature (30°C) to allow excision of the integrated plasmid pCL52.1, cells were screened for susceptibility to tetracycline and resistance to DX-619.

For mutants DX-619-C and DX-619-C-AE-α, the allelic exchange mutant, the MICs of DX-619, ciprofloxacin, gemifloxacin, and garenoxacin differed (Table 4). For DX-619-C-AE-α, the MICs of DX-619 (0.008 to 0.016 μg/ml), ciprofloxacin (0.25 μg/ml), gemifloxacin (0.016 μg/ml), and garenoxacin (0.062 μg/ml) were similar to or only twofold higher than those of the wild-type strain, unlike the eightfold increase for the original mutant DX-619-C. DNA sequencing of DX-619-C-AE-α confirmed the presence of the mutation at codon 26 in the *gyrA* gene encoding a change from Ala to Val. Thus, the Ala26Val mutation in GyrA could only partially account for the resistance of the original strain DX-619-C.

For mutant DX-619-E, the MICs of DX-619 (0.032 μg/ml) and gemifloxacin (0.125 μg/ml) were twofold higher than for the allelic exchange mutant DX-619-E-ΔE-AE, for garenoxacin (0.5 μg/ml) the MIC was fourfold higher, and the MICs for ciprofloxacin were the same (0.5 to 1 μg/ml). DNA sequencing also confirmed the presence of *parE* deletion mutation in the alleles exchanged.

For mutant DX-619-JJ, the MICs of DX-619 (0.016 to 0.032 μg/ml) and ciprofloxacin (1 μg/ml) were the same as for the allelic exchange mutants DX-619-JJ-AE-1 and -3, indicating that the Val87Phe ParC mutation was responsible for the resistance phenotype, whereas the MICs of gemifloxacin and garenoxacin were twofold lower for the allelic exchange strain. For mutant DX-619-MM, the MIC of DX-619 (0.016 μg/ml) was 1.5- to 2-fold higher, the MICs of ciprofloxacin (1 to 2 μg/ml), and gemifloxacin (0.032 μg/ml) were twofold higher, and the MIC of garenoxacin (0.125 μg/ml) was fourfold higher than those for the allelic exchange mutants DX-619-MM-AE-1 and -2, indicating that the ParC Arg17His mutation was responsible for almost all of the increases in MICs of the original mutant for DX-619, ciprofloxacin, and gemifloxacin but not for garenoxacin. DNA sequencing confirmed the presence of the *parC* mutations (encoding Val87Phe or Arg17His) in the alleles exchanged.

To study further the role of the combined *gyrA* and *parE* mutations in mutant DX-619-E, we attempted to perform two allelic exchange experiments in which we used an allelic exchange mutant as a recipient for the second allelic exchange experiment in order to reconstruct the double mutant. We were, however, unable to identify an allelic exchange double mutant despite screening over 1,000 colonies for each of these experiments. Thus, the *parC* and, less so, *parE* mutations were sufficient to confer most of the resistance phenotype of the selected mutants, whereas the *gyrA* mutation contributed only partially to the resistance phenotype. Thus, as with the effects of the defined common *parC* (Ser80Phe) and *gyrA* (Ser84Leu) mutations on the MICs of DX-619, the primary target in DX-619-E determined from DX-619-selected mutants remained ambiguous, suggesting that this quinolone has the property of highly similar interactions with both gyrase and topo IV in *S. aureus* in whole cells.

**Screening for contributions of efflux to the mutant resistance phenotype.** The MICs of DX-619 for ISP794 and ISP794 transformed with pQT8 (encoding the NorB efflux pump), MT23142 (*norA* overexpressor), and KL820 (*norA* knockout) were the same, indicating that increased expression of the NorA and NorB pumps has little or no effect on the activity of DX-619. The MIC for mutant QT1 (*mgrA* knockout), however, increased twofold relative to the MICs for ISP794 (Table 2), suggesting that there may be a contribution to resistance to DX-619 by another mechanism that is under the control of a global regulator such as MgrA. Whether this resistance results from expression of another efflux pump under the control of MgrA remains to be determined, but the properties of some mutants selected with DX-619 add support to this possibility.

TABLE 5. Susceptibilities of strains to quinolones and other agents with and without the addition of reserpine[a]

| Strain | RES | MIC (μg/ml) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | NOR | CIP | MOXI | SPAR | EB | H33324 | Rhodamine | TPP | Cetrimide |
| ISP794 | − | 0.5–1 | 0.125 | 0.062 | 0.062 | 2–4 | 1 | 0.5–1 | 4 | 0.5–1 |
| | + | 0.25–0.5 | 0.125 | 0.062 | 0.062 | 0.25–0.5 | 0.5–1 | 0.5 | 4 | 0.125–0.25 |
| DX-619-C | − | 4–8 | 1 | 0.5 | 0.5 | 1 | 1–2 | 0.25–0.5 | 2 | 0.5–1 |
| | + | 2–4 | 0.5 | 0.5 | 0.5 | 0.125 | 0.5 | 0.25–0.5 | 1 | 0.25 |
| DX-619-E | − | 2–4 | 0.5–1 | 0.5 | 0.5 | 1–2 | 1–2 | 0.5–1 | 4 | 0.5–1 |
| | + | 1–2 | 0.5 | 0.5 | 0.5 | 0.25 | 0.5 | 0.25–0.5 | 2 | 0.25 |
| DX-619-C-AE-α | − | 1 | 0.25 | 0.062–0.125 | 0.062–0.125 | 2–4 | 1 | 0.5–1 | 4 | 1 |
| | + | 0.5 | 0.125–0.25 | 0.062–0.125 | 0.062–0.125 | 0.25 | 0.5–1 | 0.25–0.5 | 2–4 | 0.25–0.5 |

[a] RES, reserpine (20 μg/ml); NOR, norfloxacin; CIP, ciprofloxacin; MOXI, moxifloxacin; SPAR, sparfloxacin; EB, ethidium bromide; H33324, Hoechst 33324; TPP, tetraphenylphosphonium bromide.

Because the resistance levels of mutants DX-619-C and DX-619-E could not be completely accounted for by the target enzyme mutations found, we screened for the possibility that increased active efflux could also have contributed to resistance in these mutants, using an array of compounds known to be substrates of efflux pumps in *S. aureus* and reserpine, a known inhibitor of a number of efflux pumps (23, 32, 36). As shown in Table 5, the MICs of norfloxacin and ciprofloxacin, which are substrates of efflux pumps NorA (35), NorB (32), and MepA (20), but not MdeA (13), and the MICs of sparfloxacin and moxifloxacin, which are substrates of NorB (32), for mutant DX-619-C increased eightfold and for DX-619-E increased four- to eightfold relative to that of ISP794, and these increases were partially abolished or, in the case of sparfloxacin and moxifloxacin, unchanged by reserpine.

The MICs of DX-619 for the four first-step-selected mutants, however, did not change with the addition of reserpine (Table 4), which is an inhibitor of NorA, NorB, MepA, and MdeA. Thus, overexpression of these specific pumps is unlikely to contribute to resistance to DX-619. Interestingly, the MICs of ethidium bromide (which is a substrate for many efflux pumps) for mutants DX-619-C and DX-619-E but not DX-619-C-AE-α decreased twofold relative to that of ISP794, suggesting that there is a regulatory mechanism that mediates downregulation of some pumps together with regulation of other genes, the expression of which can affect DX-619 activity. These other genes might encode an as yet unidentified reserpine-resistant efflux pump or might mediate another resistance mechanism in mutants DX-619-C and DX-619-E. It is noteworthy that other regulators, such as MgrA, have been shown to act as both positive and negative regulators of different pumps (32, 33).

**Characterization of DX-619-selected second-step mutants from DX-619-E-ΔE-AE.** Three out of four first-step DX-619-selected mutants had novel mutations outside the QRDR that contributed only partially to their resistance phenotypes. To assess if stepwise mutations in the two topoisomerase target enzymes can be selected with DX-619, we selected at onefold the MIC second-step mutants of the allelic exchange mutant DX-619-E-ΔE-AE containing the ParE 215 to 217 deletion. We analyzed two different mutants, which were selected at a frequency of $3.5 \times 10^{-5}$. The MICs of DX-619 for the DX-619-E-ΔE-AE1 and DX-619-E-ΔE-AE2 mutants were increased four- to eightfold (0.062 to 0.125 μg/ml) and fourfold (0.062 μg/ml), respectively, whereas the MICs of ciprofloxacin for the two mutants were the same or increased twofold (1.0 μg/ml). Sequencing the entirety of *gyrA*, *gyrB*, *parC*, and *parE* revealed, in addition to the deletion in *parE*, two previously characterized mutations in the QRDR of *gyrA*, S84L in DX-619-E-ΔE-AE1 and E88K in DX-619-E-ΔE-AE2. Thus, as expected, second-step mutants selected from first-step mutants harboring a topo IV mutation had gyrase mutations. Unlike the first-step mutants, the second-step mutants selected with DX-619 had mutations within the QRDR of GyrA.

**Comparative activities of ciprofloxacin and DX-619 against purified topo IV and gyrase.** Having found results suggesting similar targeting of DX-619 for gyrase and topo IV in whole cells, we then tested its effects against the purified target enzymes, using the cleavage complex formation assay. Ciprofloxacin, which primarily targets topo IV, was used for comparison. Ciprofloxacin was 20- to 24-fold more potent in stimulating half-maximal intensity of linear plasmid pBR322 DNA cleavage complex formation with topo IV than gyrase. DX-619, on the other hand, was more potent than ciprofloxacin overall and also showed an almost identical potency for gyrase and topo IV, with half-maximal cleavage complex formation values of 0.62 to 1.25 μg/ml and 1.25 μg/ml, respectively (Table 6). Thus, the in vitro data show that *S. aureus* gyrase and topo IV are equally sensitive to DX-619, a finding consistent with the results with established mutants.

TABLE 6. DX-619 and ciprofloxacin stimulation of cleavage complex formation with topoisomerase IV and gyrase

| Drug | $CC_{50}$ (μg/ml)[a] | |
|---|---|---|
| | Topo IV | Gyrase |
| DX-619 | 1.25 | 0.62–1.25 |
| Ciprofloxacin | 1.25–2.5 | 30–50 |

[a] $CC_{50}$, concentration causing half-maximal intensity of linear plasmid pBR322 DNA.

## DISCUSSION

We studied the resistance mechanisms of a novel des(6) fluoroquinolone, DX-619, in *S. aureus*. Against a wild-type strain and defined isogenic mutants, DX-619 manifested exceptionally low MICs, a narrow mutant prevention concentration-MIC ratio, and an exceptionally low frequency of selection of resistant mutants. Based on these findings and the almost equal inhibitory activity against purified topo IV and gyrase, DX-619 appears to have dual targeting activity. Dual-targeting quinolones manifest a similar and slight increase in the MIC with each single target enzyme resistance-conferring mutation (27, 30). We have selected four independent mutants resistant to DX-619 and found new mutations in all, suggesting the possibility that DX-619 interacts with type II topoisomerases distinctly from other quinolones.

The increase in the MICs of DX-619 for each of the mutants bearing five independent mutations, in gyrase or topo IV, as determined from the allelic exchange experiments, and DX-619-E-ΔE-AE1 (*gyrA* mutation identical to the isogenic strain SS1), was two- to fourfold, and consistent with a dual targeting activity of DX-619. In analyzing the resistance mechanisms of DX-619-C, however, we found that type II topoisomerase mutations did not explain the level of resistance to DX-619 and the other quinolones, ciprofloxacin, gemifloxacin, and garenoxacin, in this mutant. Thus, an as yet undefined mechanism other than changes in type II topoisomerases must contribute to the resistance pattern of these mutants.

The increase in the MIC of DX-619 attributable to the novel Arg26Val and the classic Ser84Leu GyrA mutations, as determined by strains DX-619-C-AE-α and SS1, respectively, was similar. Thus, it is unclear why we did not identify the Ser84Leu and Glu88Lys GyrA mutations, which were found in second-step mutants, among the first-step DX-619-selected mutants. It is noteworthy that mutants DX-619-C and DX-619-E manifested a four- to eightfold decrease in the MIC of novobiocin relative to that of wild-type ISP794. The Arg136Leu mutation in *E. coli gyrB* and the Asn470Gln mutation in *S. aureus parE* mutants are known to confer novobiocin hypersusceptibility (1, 8). Although we did not find these mutations in DX-619-C or DX-619-E, a novel 215 to 217 deletion mutation in ParE was found in the latter mutant, but, as determined from the allelic exchange strain DX-619-E-ΔE-AE, this deletion mutation was not responsible for this phenotype. Thus, the genetic basis of novobiocin hypersusceptibility in these mutants remains undefined.

The MIC of ethidium bromide, a substrate of several efflux pumps in *S. aureus* (23, 32, 36), for DX-619-C but not its allelic exchange mutant DX-619-C-AE-α (Arg26Val GyrA mutation) decreased two- to fourfold relative to that of wild-type *S. aureus*, and this effect was abolished by the addition of reserpine (a known inhibitor of several multidrug resistance pumps). For mutant DX-619-C, reserpine did not affect the MIC of DX-619, gemifloxacin, and garenoxacin and reduced the MIC of ciprofloxacin twofold. These findings suggest that DX-619-C also harbors a mutation that reduces the efflux of ethidium bromide and to lesser extent ciprofloxacin without an effect on DX-619. This pattern suggests a regulatory mutation with pleiotropic effects on efflux pump expression and possibly other properties. Further studies will be required to characterize the full spectrum of resistance mechanisms in mutant DX-619-C.

Novel quinolones select for resistant mutants with distinctive mutations located outside the QRDR (14, 15, 17–19), suggesting that the manner of interaction of these quinolones with a target enzyme-DNA complex may vary among the different drugs, resulting in a varying degree of cross-resistance among them. Interestingly, the novel mutations selected with DX-619 differ from those selected with garenoxacin (18), another desfluoroquinolone, suggesting that interactions with the target enzymes differ between different desfluoroquinolones. This property complicates attributing the reduced frequency of selected mutants for such novel drugs solely to their ability to target both gyrase and topo IV similarly. In addition, DX-619 is a prime example of how a potent dual-targeting quinolone selects for an array of resistance mechanisms beyond novel mutations outside of the QRDR.

Determination of whether such novel and potent quinolones as DX-619 have additional actions within the cell to account for their exceptional potency and unusual pattern and low frequency of mutants must await further studies. The increased potency of DX-619 has also recently been shown in clinical isolates of *S. aureus* that have high-level resistance to other quinolones and usually multiple mutations (3, 9). Thus, the potency of DX-619 may allow its use for at least some clinical isolates of *S. aureus* that are already highly resistant to older quinolones, as often is the case with methicillin-resistant strains.

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