ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1993, p. 1086–1094
0066-4804/93/051086-09$02.00/0
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Vol. 37, No. 5

Efflux-Mediated Fluoroquinolone Resistance in *Staphylococcus aureus*

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Received 8 July 1992/Accepted 15 February 1993

Transport processes are used by all organisms to obtain essential nutrients and to expel wastes and other potentially harmful substances from cells. Such processes are important means by which resistance to selected antimicrobial agents in bacteria is achieved. The recently described *Staphylococcus aureus norA* gene encodes a membrane-associated protein that mediates active efflux of fluoroquinolones from cells. SA-1199B is a fluoroquinolone-resistant strain of *S. aureus* from which we cloned an allele of *norA* (*norA1199*). Similar to that of *norA*, the protein product of *norA1199* preferentially mediates efflux of hydrophilic fluoroquinolones in both *S. aureus* and an *Escherichia coli* host, a process driven by the proton motive force. Determination of the nucleotide sequence of *norA1199* revealed an encoded 388-amino-acid hydrophobic polypeptide 95% homologous with the *norA*-encoded protein. Significant homology with other proteins involved in transport processes also exists, but especially with tetracycline efflux proteins and with the *Bacillus subtilis* Bmr protein that mediates active efflux of structurally unrelated compounds, including fluoroquinolones. In *S. aureus*, the *norA1199*-encoded protein also appears to function as a multidrug efflux transporter. Southern hybridization studies indicated that *norA1199* (or an allele of it) is a naturally occurring *S. aureus* gene and that related sequences are present in the *S. epidermidis* genome. The nucleotide sequence of the wild-type allele of *norA1199*, cloned from the fluoroquinolone-susceptible parent strain of SA-1199B, did not differ from that of *norA1199* throughout the coding region. Northern (RNA) and Southern hybridization studies showed that increased transcription, and not gene amplification, of *norA1199* is the basis for fluoroquinolone resistance in SA-1199B.

Staphylococcus aureus is an important community- and nosocomially acquired pathogen. This organism can be resistant to multiple antimicrobial agents, which severely limits therapeutic options in selected instances. Development of the fluoroquinolone class of antimicrobial agents advanced the possibility of an effective option for the therapy of serious infections caused by multiresistant strains of *S. aureus*, even by the oral route of administration. Unfortunately, shortly after introduction of these agents into clinical use the emergence of fluoroquinolone-resistant *S. aureus* was noted, especially among methicillin-resistant strains (6, 23, 27). The mechanisms by which *S. aureus* develops resistance to fluoroquinolones has been the subject of intensive research, and these investigations have defined at least three ways by which resistance to these agents is accomplished. The first mechanism consists of a mutational alteration of DNA gyrase, an essential bacterial enzyme involved in DNA replication and repair (35). DNA gyrase is a tetrameric protein consisting of two A and B subunits, encoded by the *gyrA* and *gyrB* genes, respectively. Fluoroquinolones interact with either the A subunit itself or a complex of DNA gyrase and DNA (through the A subunit) to inhibit enzymatic function (8). Several point mutations in *gyrA* have been described that confer high-level fluoroquinolone resistance upon an organism that possesses them, with most of these mutations lying within a small region of the gene (5, 30). The second means by which resistance to fluoroquinolones is produced is mediated by the recently described *cfx-ofx* locus (32). This locus confers lower-level resistance than that seen with *gyrA* mutations, and the mechanism(s) by which it does so has not been investigated. The third mechanism of resistance consists of efflux of fluoroquinolones from the cell, which also confers relatively lower-level resistance than *gyrA* mutations. We and others have cloned the gene involved in such efflux (*norA*), and its sequence has been reported by one group (10, 34). Fluoroquinolone efflux mediated by *norA* is an active process that is sensitive to protonophores, such as dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), suggesting that the proton motive force is important in the efflux process.

Achievement of a better understanding of this and other related efflux processes may yield information important in the development of ways to circumvent this type of resistance mechanism. With this end in mind, further characterization of *norA1199*, the *norA* allele cloned in our laboratory, forms the basis of this report.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. aureus* SA-1199 and its ciprofloxacin-resistant derivative SA-1199B have been described previously (10). *Escherichia coli* $DH_{10}B$ and AG100 were used as hosts for *norA1199* and its wild-type (wt) allele (see below) (4, 15). *Klebsiella pneumoniae* ATCC 10031 and *E. coli* JM83 were used as donors of chromosomal DNAs for Southern hybridization (33; see below). *S. aureus* 8325-4 was used as a donor of control chromosomal DNA for pulsed-field electrophoresis experiments (22; see below). Plasmid pUC19 was obtained from Bethesda Research Laboratories (BRL), Gaithersburg, Md. *NorA1199* was cloned into the polylinker region of pUC19 as described previously, and the resultant plasmid (pK21 [10]) was transformed into both *E. coli* $DH_{10}B$ and AG100 by standard techniques (25).

Antimicrobial susceptibility testing. MICs were determined

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in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) cation adjusted with calcium and magnesium in accordance with National Committee for Clinical Laboratory Standards guidelines (17). The effect of reserpine (final concentration, 20 μg/ml) on the MICs of selected compounds for *S. aureus* also was evaluated. At least four determinations were made for each agent tested, and the daily variation was no more than twofold. Results were expressed as geometric means.

Preparation of everted membrane vesicles. Everted membrane vesicles of *E. coli* AG100 with and without pK21 were prepared as described by Cohen et al. (3). Vesicles were stored at −70°C until used.

Uptake of [^{3}H]norfloxacin. Unless otherwise noted, all reagents were the highest grade available and were obtained from Sigma Chemical Co., St. Louis, Mo. Uptake studies were performed for whole cells of *E. coli* AG100 as described previously, by using an incubation temperature of 30°C (10). Briefly, a concentrated suspension of late-exponential growth phase cells in cation-adjusted Mueller-Hinton broth was exposed to [^{3}H]norfloxacin (13.4 mCi/mg; Merck, Rahway, N.J.) at a final concentration of 0.25 μM (0.08 μg/ml). Aliquots were removed at frequent intervals, placed on GF/C filters (Whatman International, Maidstone, United Kingdom), and washed once with 6 ml of 0.85% NaCl poured over the filters under vacuum suction. Additional aliquots were removed simultaneously for determination of protein content (protein assay kit; Bio-Rad Laboratories, Richmond, Calif.). In some studies, the effect of addition of CCCP (final concentration, 50 μM) on [^{3}H]norfloxacin uptake was assessed. Radioactivity on filters was quantitated by scintillation counting. Nonspecific binding of [^{3}H]norfloxacin to filters and the contribution of the growth medium to the protein content of samples were subtracted. Results were expressed as nanograms of norfloxacin per milligram of cell protein.

For everted membrane vesicles, a modification of the method of Cohen et al. was used (3). Vesicles were suspended in assay buffer (50 mM potassium phosphate, pH 7.5) at a concentration of 1 mg/ml and an incubation temperature of 30°C. [^{3}H]norfloxacin (specific activity, 2.3 mCi/mg) was added to a final concentration of 0.25 μM (0.08 μg/ml). After a 15-min equilibration period, a 50-μl aliquot was withdrawn and diluted into 10 ml of wash buffer (0.1 M lithium chloride, 0.1 M potassium phosphate, pH 7.5). Vesicles were collected on GF/C filters by vacuum suction and then washed with 4 ml of wash buffer. All subsequent aliquots were collected and treated in this manner, and additional aliquots were withdrawn simultaneously for determination of protein content. After an additional 5 min of incubation, a second sample was obtained and lithium lactate was added as an energy source to a final concentration of 20 mM. Additional aliquots were obtained 1, 5, and 10 min later. CCCP then was added to a final concentration of 100 μM, and aliquots were obtained 1 and 5 min later. Filters were air dried, and the radioactivity contained on them was quantitated by scintillation counting. Nonspecific background binding of [^{3}H]norfloxacin to filters was subtracted. Results were expressed as nanograms of norfloxacin per milligram of vesicle protein.

DNA sequence determination. The nucleotide sequences of *norA1199* and its wt allele (see below) were determined by the dideoxy-chain termination method with [^{35}S]dATP (1,000 Ci/mmol; New England Nuclear, Boston, Mass.) (26).

Restriction endonuclease digestions. Unless otherwise noted, all restriction endonucleases were obtained from BRL and used in accordance with the manufacturer's guidelines.

Preparation of the *norA1199* gene probe. Following digestion of *norA1199* with *Hinc*II and separation of the fragments in an agarose gel, an internal 702-bp fragment of the gene was cut from the gel and purified by repeat electrophoresis in and recovery from low-melting-point agarose (BRL). This fragment was labeled with [α-^{32}P]dATP (800 Ci/mmol; New England Nuclear) and used for both Southern and Northern (RNA) hybridization experiments (see below).

Isolation of chromosomal DNAs. Chromosomal DNAs were isolated from SA-1199 and SA-1199B, eight clinical strains of *S. aureus* (four methicillin susceptible and four methicillin resistant), six clinical isolates of *S. epidermidis* (three methicillin susceptible and three methicillin resistant), four clinical strains of *Enterococcus faecalis* (two gentamicin susceptible and two gentamicin resistant), *K. pneumoniae* ATCC 10031, and *E. coli* $DH_{10}B$ and JM83. All strains except SA-1199B were fluoroquinolone susceptible. For *S. aureus*, lysostaphin (20 μg/ml; Sigma) was used to digest cell walls; this was followed by treatment with sodium dodecyl sulfate (SDS; 0.6%) and proteinase K (120 μg/ml; BRL) for 1 h at 37°C. DNA was phenol and ether extracted, precipitated with ethanol, and then suspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). For *S. epidermidis*, the procedure was the same except that the concentration of lysostaphin was increased to 1.8 mg/ml and it was used in conjunction with lysozyme (0.5 mg/ml; Sigma). For *E. faecalis*, lysozyme (0.65 mg/ml) was substituted for lysostaphin. Gram-negative organisms were lysed directly with SDS and treated with proteinase K; this was followed by extraction and precipitation of DNA as described above.

Southern blotting. Chromosomal DNAs from organisms of interest were digested with *Hin*dIII, *Eco*RI, or *Sma*I (for pulsed-field gels, see below). Restriction fragments were separated in agarose gels, transferred to nylon membranes, and hybridized with the *norA1199* probe (29). Stringency conditions were varied for some experiments (high stringency, 42°C and 50% formamide; medium stringency, 42°C and 25% formamide; low stringency, 42°C and no formamide). Pulsed-field gels were probed only under high-stringency conditions. Positive and negative controls consisted of the probe itself and *Hin*dIII-digested bacteriophage λ DNA (BRL), respectively; for pulsed-field gels, the positive control was *S. aureus* 8325-4 DNA, to a 262-kb fragment of which a *norA* probe has been shown to hybridize (32), and the negative controls were MegaBase I and II standards (BRL).

Northern blotting. Total cellular RNAs were isolated from equal numbers of cells of SA-1199 and SA-1199B by the guanidinium thiocyanate-phenol-chloroform procedure, modified for *S. aureus* by use of lysostaphin (250 μg/ml) to digest the cell wall and SDS (0.7%) to lyse protoplasts (2, 25). RNA was separated in glyoxal-containing agarose gels and then transferred to a nylon membrane (25). Hybridization with the *norA1199* gene probe then was carried out by standard techniques (29).

Application of equal amounts of RNA to the glyoxal gel was ensured by two methods. (i) The RNA content of each sample was estimated by use of the ratio of optical densities at 260 and 280 nm. (ii) Each sample was run in duplicate in glyoxal gels, and the gel was divided after electrophoresis. One half was stained with ethidium bromide, and the other was blotted. Direct visualization of the rRNA bands in the stained portion of the gel allowed a reasonable estimation of the equality of the RNA content of each sample.

Pulsed-field electrophoresis. A modification of the pulsed-field electrophoresis method of Smith et al. was used (28). SA-1199, SA-1199B, and *S. aureus* 8325-4 were grown in 10 ml of L broth to the logarithmic growth phase. Cells were washed and then concentrated in buffer (10 mM Tris, 1 M NaCl, pH 7.6; final volume, 2 ml). One milliliter of cells was mixed with an equal volume of 2% low-melting-point agarose, dispensed into molds, and chilled on ice. The agarose inserts then were suspended in EC buffer (6 mM Tris, 1 M NaCl, 100 mM EDTA, 0.5% Brij 58, 0.2% deoxycholate, 0.5% Sarkosyl, pH 7.6) containing 20 μg of RNase (Sigma) per ml and 100 μg of lysostaphin per ml. After overnight incubation at 37°C, the inserts were suspended in ES buffer (0.5 M EDTA, 1% Sarkosyl, pH 9.5) containing 2 mg of proteinase K per ml and incubated overnight at 50°C. Inserts were incubated at room temperature for 2 h in TE buffer containing 1 mM phenylmethylsulfonyl fluoride and then incubated twice for 2 h each time at room temperature in TE buffer alone. Inserts then were treated overnight with *Sma*I at room temperature, suspended in ES buffer, and incubated for 2 h at 50°C. This was followed by an additional 2 h of incubation at 50°C in ES buffer containing proteinase K (2 mg/ml). The inserts then were placed in the wells of a 0.9% agarose gel. Electrophoresis was done in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) at 14°C with a CHEF-DR II system (Bio-Rad). The electrophoresis parameters were 180 V, 27 h, and a pulse time of 27 s.

Cloning of the wt *norA1199* allele. Purified chromosomal DNA was obtained from SA-1199 by the method of Lindberg et al. (14). The polymerase chain reaction (PCR) was used to amplify the wt *norA1199* allele [*norA1199*(wt)]. On the basis of the nucleotide sequence of *norA1199*, oligonucleotide primers were designed to simplify the cloning of the resultant PCR product into pUC19. The primers were synthesized by the Macromolecular Core Facility at Wayne State University and consisted of 5′-TACATTCAACGGTACCTTCGC CTT-3′ (*norA1199* positions −278 to −255, with an artificial *Kpn*I site at position −264) and 5′-CGTTAATGAATTC GGTATGTGG-3′ (complementary to positions 226 to 247 bp beyond the end of *norA1199*, with an artificial *Eco*RI site at position 238). PCRs were performed with the GeneAmp PCR Reagent Kit (Perkin Elmer Cetus, Norwalk, Conn.) and *Taq* DNA polymerase (12). The PCR parameters used were 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min for 25 cycles with a DNA Thermal Cycler 480 (Perkin Elmer Cetus). PCR products were digested with *Kpn*I and *Eco*RI and then ligated into a similarly digested pUC19 vector. *E. coli* $DH_{10}B$ then was transformed with this DNA by conventional techniques (25). The presence of an insert of the appropriate size (1.7 kb) in plasmids of transformants was verified by restriction endonuclease analysis.

Ohshita et al. have reported that a single nucleotide change (adenine to cytosine) at position 1085 of the wt allele of *norA*, which results in substitution of alanine for aspartic acid at position 362 of the protein product, is responsible for fluoroquinolone resistance in strains bearing *norA* (21). However, these workers did not determine the nucleotide sequence of the entire gene. To determine whether this or another mutation(s) important for fluoroquinolone resistance is present in *norA1199*, the nucleotide sequence of *norA1199*(wt) in four independent PCR-generated clones was determined as described previously and a consensus sequence was generated.

Analysis of nucleotide 1085. To generate an estimate of the incidence of occurrence of either adenine or cytosine at position 1085 of the *norA* locus, we designed oligonucleotide primers for use in PCR that included this site in the amplification product. The primers consisted of 5′-ACTATACA CAGCTGACAAGG-3′ (*norA1199* positions 677 to 696) and 5′-GAATTAGGTATGTGGATTGC-3′ (complementary to positions 162 to 181 bp beyond the end of *norA1199*). We employed the chromosomal DNA from each of the eight clinical strains of *S. aureus* described previously as a PCR template. PCR parameters were identical to those outlined above. The amplification product from each DNA sample then was subjected to digestion with *Sfc*I (New England BioLabs, Beverly, Mass.). This enzyme has a single recognition site in *norA1199* that includes position 1085 (C̲T-purine-pyrimidine-AG). *Sfc*I digestion of an amplification product having cytosine at this position results in restriction fragments of 408 and 323 bp, whereas the presence of adenine eliminates the site and leaves the 731-bp PCR product intact.

Cytosine is present at position 1085 of both *norA1199* and *norA1199*(wt) (see below). The effect of changing this nucleotide to adenine, and thus introducing the putative wild-type aspartic acid residue of Ohshita et al. at position 362 of the protein product (21), on norfloxacin resistance was determined with the Transformer Site-Directed Mutagenesis Kit (Clontech Laboratories, Palo Alto, Calif.). The primer used to introduce this change consisted of 5′-CAATTTATATG GATATAGGTG-3′ (*norA1199* positions 1073 to 1093). The nucleotide sequence of the mutagenized *norA1199* gene was determined to verify the presence of the desired change.

Nucleotide sequence accession numbers. The GenBank accession numbers assigned to *norA1199* and *norA1199*(wt) are M80252 and M97169, respectively.

RESULTS

Antimicrobial susceptibility studies. The MICs of selected fluoroquinolones and nalidixic acid for SA-1199 and SA-1199B are given in Table 1. With the exception ethidium bromide, no pleiotropic resistance to members of nonquinolone drug classes was seen (data not shown). The ethidium bromide MICs for SA-1199 and SA-1199B were 3.13 and 25 μg/ml, respectively.

Hirai et al. have determined the hydrophobicity indexes of several fluoroquinolones, and these values also are shown in Table 1 (7). A higher hydrophobicity index indicates a more hydrophobic compound. For SA-1199B, the MICs of hydrophilic fluoroquinolones tended to rise to a greater degree than those of more hydrophobic compounds, with the notable exception of pefloxacin (a more hydrophobic drug). Table 1 also gives the MICs of selected fluoroquinolones for *E. coli* $DH_{10}B$ and AG100 with and without pK21 (plasmid containing *norA1199*). The trend seen in SA-1199B is more obvious in these data; hydrophilic compounds are affected to a greater degree than hydrophobic ones. This phenomenon has been reported previously by Yoshida et al. for the *norA* gene (34). No pleiotropic resistance to nonquinolones developed in *E. coli* strains carrying pK21 (data not shown).

The MICs of norfloxacin and ethidium bromide were decreased two- and fourfold and eight and eightfold for SA-1199 and SA-1199B, respectively, in the presence of 20 μg of reserpine per ml (data not shown).

[^{3}H]norfloxacin uptake studies. We have shown that uptake of [^{3}H]norfloxacin by SA-1199B is markedly reduced compared with that of SA-1199 (10). Uptake of norfloxacin by this strain is nearly normalized following addition of dinitrophenol or CCCP, agents that disrupt the proton motive

TABLE 1. MICs for study strains

Quinolone	H.I.[a]	MIC (fold increase vs parental strain)			
		S. aureus		*E. coli*	
		SA-1199	SA-1199B	$DH_{10}B$/+pK21[b]	AG100/+pK21[b]
Enoxacin	0.007	1.47	29.7 (20)	0.04/0.95 (24)	0.08/1.25 (20)
Norfloxacin	0.01	1.59	79.4 (50)	0.04/1.25 (31)	0.08/5.00 (50)
Ciprofloxacin	0.02	0.25	5.96 (24)	0.04/0.12 (3)	0.02/0.50 (25)
Fleroxacin	0.08	0.60	6.25 (10)	0.04/0.12 (3)	0.04/0.16 (4)
Ofloxacin	0.33	0.40	3.29 (8)	0.03/0.13 (4)	0.04/0.16 (4)
Pefloxacin	1.32	0.65	12.5 (19)	0.03/0.05	0.08/0.16 (2)
Nalidixic acid	3.92	31.3	313 (10)	2.32/3.13	6.25/6.25

[a] H.I., hydrophobicity index.
[b] MIC in the background strain/MIC in the same strain transformed with pK21.

force. Figure 1 shows the results of drug uptake studies with *E. coli* AG100 with and without pK21. The presence of pK21 markedly reduced uptake of [^{3}H]norfloxacin, a phenomenon fully reversed by addition of CCCP. Figure 1 also shows the results of studies performed with everted membrane vesicles prepared from *E. coli* AG100 with and without pK21. Addition of an energy source (lactate) resulted in a slight increase in vesicle-associated [^{3}H]norfloxacin for the plasmid-free strain and a marked increase in the vesicle-associated drug in the strain carrying pK21. Addition of CCCP to the system resulted in swift loss of the drug from vesicles.

Nucleotide sequence of *norA1199*. The nucleotide sequence of the *S. aureus* chromosomal DNA contained in pK21 is shown in Fig. 2. This 2,627-bp clone contained two putative open reading frames (ORFs), one on each of the complementary strands. The largest of these (1,167 bp) reads from left to right in Fig. 2 and is *norA1199*. This gene encodes a 388-amino-acid polypeptide very similar to that encoded by *norA* (34; see Fig. 3). At the DNA level, *norA1199* and *norA* are 91% homologous, and their deduced protein products are 95% homologous. Clearly, *norA1199* is an allele of *norA*. A search of available data banks revealed that the *norA1199* protein has significant homology with several tetracycline (Tet) efflux proteins, including those encoded by *E. coli* plasmid pBR322 (24%), *E. coli* transposon Tn*10* (23%), and *Bacillus stearothermophilus* plasmid pTHT15 (21%). Other proteins to which some homology exists include the *S. aureus qacA* protein (19%), a membrane-based polypeptide involved in the efflux of intercalating dyes and quaternary ammonium-type antiseptic compounds (24), the rat brain glucose transporter (17%), and the xylose-H^+ transporter (19%), the arabinose-H^+ symport (19%), and the lactose permease (18%) of *E. coli*. The highest degree of homology

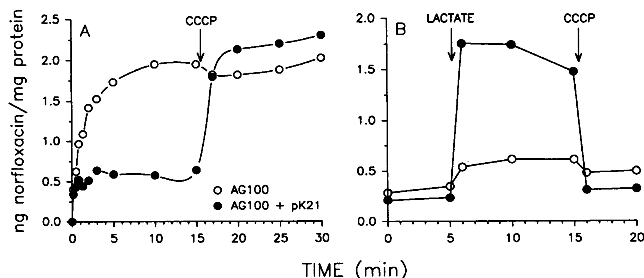

FIG. 1. Uptake of [^{3}H]norfloxacin by whole cells (A) and everted membrane vesicles (B) of *E. coli* AG100 with and without pK21. Lactate and CCCP were added at the times indicated.

was to the product of the *B. subtilis bmr* gene, the Bmr protein (45%), an integral membrane polypeptide capable of mediating the active efflux of several structurally unrelated compounds, including fluoroquinolones (18, 19). In Fig. 3, the homology between the deduced protein products of *norA1199* and *norA*, the *B. subtilis* Bmr protein, and a representative Tet protein, that from pBR322, is apparent.

A second putative ORF (ORF A) is present on the strand complementary to that which encodes the *norA1199* protein. ORF A reads from right to left in Fig. 2, and as for *norA1199*, it is preceded by appropriate promoter-like sequences, the −35 region of which overlaps that of *norA1199*. The entire coding region is not contained within the cloned DNA, but translation of the partial sequence revealed an abbreviated polypeptide (88 residues) with some sequence similarity to ORF 188 (19%; putative *qacA* regulatory protein [24]) and the TetR repressor from Tn*10* (21%; 1).

A Kyte-Doolittle plot of the *norA1199* protein, with that of *norA* superimposed for comparison, is shown in Fig. 4 (11). There are 12 hydrophobic domains, 6 each of which are placed on either side of a central hydrophilic region. The plots for the Tet protein of pBR322 and the *B. subtilis* Bmr protein are very similar in appearance (data not shown). As shown in Fig. 4, the hydropathic profile of *norA1199* is nearly identical to that of *norA*, despite the presence of 19 amino acid differences between the proteins.

Southern blotting. Under high-stringency conditions, the *norA1199* gene probe hybridized only with *S. aureus* DNA (data not shown). Hybridization with *Eco*RI- or *Hin*dIII-digested chromosomal DNA occurred with 18- and 2.7-kb fragments (one strain), 18- and 5.4-kb fragments (five strains), and 8.1- and 5.4-kb fragments (two strains), respectively. Methicillin susceptibility had no relationship to any hybridization pattern. For SA-1199 and SA-1199B, hybridization occurred with 18-kb *Eco*RI and 2.7-kb *Hin*dIII fragments.

Reduction to medium-stringency conditions resulted in hybridization of the probe with *S. epidermidis* DNA. Hybridization occurred with a >23-kb *Eco*RI fragment and a 20-kb *Hin*dIII fragment in one strain and with 2.8-kb *Eco*RI and *Hin*dIII fragments in five strains (data not shown). As for *S. aureus*, methicillin susceptibility had no relationship to any hybridization pattern.

No hybridization of the *norA1199* probe occurred with DNA from the *E. faecalis* strains or the gram-negative organisms tested, even when low-stringency conditions were used.

Northern blotting. The *norA1199* probe hybridized with RNAs from both SA-1199 and SA-1199B; hybridization with

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       GTTTACACGTTTTACTGGTAACAGATCTGCTCTTTATAATGGATTATTCGAGCAGTTAAGGTTACCGAGTCATTATACAAAAAGAAGCATAACAAAGCAA
-472   CAAATGTGCAAAATGACCATTGTCTAGACGAGAAATATTACCTAATAAGCTCGTCAATTCCAATGGCTCAGTAATATGTTTTTCTTCGTATTGTTTCGTT

       CAATTTAACTTTTTAAAATTTTGCTGCAGTAGTGTACGTGGTTACGGCGACTGACTACATTTACAATTCAGAACCAGTAGACGTTTCAAACAATATGTAA
-372   GTTAAATTGAAAAATTTTAAAACGACGTCATCACATGCACCAATGCCGCTGACAGATGTAAATGTTAAGTCTTGGTCATCTGCAAAGTTTGTTATACATT

                                                         <--- Start ORF A          <-- SD                <--
       GTTGCTATAGAAGCGGAAAAAGATTGTGATCATCATATCATACTAATGAAAAAACGTTAAAGTATACTAGTTAGGGGAAATAAAATTATACAGTAATTAA
-272   CAACGATATCTTCGCCTTTTTCTAACACTAGTAGTATAGTATGATTACTTTTTTGCAATTTCATATGATCAATCCCCTTTATTTTAATATGTCATTAATT

       -10                                                 <-- -35
       TATGTTAATTTACCTTTTATCACTATTAATGTTTCTTTTTTATAACAGTTTACATCATTATAACATTATGTTATATCTTTGAATAATGCTTATAAATCGA
-172   ATACAATTAAATGGAAAATAGTGATAATTACAAAGAAAAAATATTGTCAAATGTAGCAATATTGTAATACAATATAGAAACTTATTACGAATATTTAGCA
                                                          -35 -->          -10 -->
                                                                    <-dyad symmetry->

-72    TGAATTGCAATCTGTCGTGGAAAAGAAAAATAACAGCTTGAAGAGTGACAAGTAGAAAAAAGAGGTGAGCAAATGAATAAACAGATTTTGGTATTATATT
                                                                    SD -->        Start norA1199 --->
                                                                                  (nucleotide 1)
29     TTAATATTTTCTTAATTTTTTTAGGTATCGGTCTAGTGATACCAGTCTTACCTGTTTATTTAAAAGATTTGGGGTTAACTGGTAGTGATTTAGGATTATT

129    AGTTGCTGCTTTCGCCTTATCTCAAATGATTATATCGCCATTCGGTGGTACGTTAGCTGATAAATTAGGAAAGAAATTAATTATATGTATAGGATTAATT

229    TTGTTTTCAGTGTCAGAATTTATGTTTGCTATCGGTCAGAATTTTTTAATTTTGATGTTATCAAGGGTTATCGGTGGTATGAGTGCTGGTATGGTTATGC

329    CTGGGGTGACAGGTTTAATAGCTGATATTTCACCAAGCCATCAAAAAGCAAAAAACTTTGGCTACATGTCAGCGATTATCAATTCAGGATTCATTTTAGG

429    ACCAGGGATTGGTGGATTTATGGCAGAAGTTTCACATCGTATGCCATTTTATTTTGCAGGTGCATTAGGTATTCTAGCATTTATAATGTCAATTGTATTG

529    ATTCACGACCCTAAAAAAGTTTCGACAAATGGATTCCAAAAGTTGGAGCCACAATTGCTAACGAAAATTAACTGGAAAGTGTTTATTACACCAGTTATTC

629    TAACACTTGTATTATCGTTTGGTTTATCTGCATTTGAAACATTGTATTCACTATACACAGCTGACAAGGTAAATTATTCACCTAAAGATATTTCGATTGC

729    AATTACAGGTGGCGGTATCTTTGGTGCACTTTTCCAAATTTATTTCTTTGATAAATTTATGAAATACTTCTCTGAGTTAACATTTATTGCATGGTCATTA

829    ATATATTCAGTTATTGTATTAGTGCTATTAGTTATTGCCGATGGTTACTGGACAATTATGGTAATAAGCTTTGTTGTCTTTATCGGGTTCGATATGATAA

929    GACCTGCTATTACAAATTATTTTTCAAATATTGCTGGTGATAGACAGGGATTTGCAGGTGGGTTAAACTCAACATTCACTAGCATGGGGAATTTTATAGG

1029   TCCTTTAATCGCAGGTGCGTTATTTGATGTGCACATTGAAGCCCCAATTTATATGGCTATAGGTGTGTCATTAGCTGGTGTTGTCATTGTTTTAATTGAA

1129   AAGCAACATAGAGCTAAGTTAAAACAACAAGATTTGTAATATCGCACATGGTTGTCATTCGATTAATGTTTTTCGACAACTAAAGCATTTCAAAATAAGC
                                           End norA1199
                                           (nucleotide 1167)
+62    ATCGATTTTCTTACAATTATAGTGAAGAAAAGTCGATGCTTTAAATTTATAAAGATTAAAAACTTTTAAATGTTTTAGTCTTTATATTTAAATGTTATAT
           transcription terminator

+162   GTAACAAAAAATGATTTTGAGTAATAAACGTGTTACAAATATTACATTCTTTTTAAATTGCAATCCACATACCTAATTCATTAACGTTAATGTGTTAAGA

+262   TGATAAAAAATGAGTAAGGAAATGTGGGTAAGGGGATGACAGTAAAAAATTTATTTTTAGGCTTTGTTGCTGTAATATTAACTGTTTGTTTAATTGGTTT

+362   ATTAATTTTAGCAACAAATGAAGATGCGCTTGCTAAGGTACATAAAACGATTAATACGCTTAACGCGATAAATGTATCAACTGAAGATACTTATAAAAAG

+462   AAAATGGATATTCTCAATATTCATACTGCTAAAGCATCTGAAGTGAATGAAAATGTGAAAAAGCAAAATCATTTTAAGCATCGTGTGAATGCAAATAAAT

+562   CAAATTCTTTTAACGAACAAGAGTGCCAAGTTATTGCTGATCGTTATGCAGATAAGCATATCAATGATAATTATGGTTTAGAAAGAATTTCTAAGACAAA

+662   TCATGGATATAATTATGTTTATTCCAATGATAATTCAACGAGTAAGCAACATGTAAGTATTTCAAATCAAGGCATAATAACGAAATAATAGATGGAACAG

+762   TGTATTCTAATTGGATATACTGTTTTTATTTTGCAATATTTTAATTTAAAAAGGTGAAATCAACTTATAAAATGATGTAAATGTTATGTCAAAATCAACC

+862   AATCCGTAATGTATTTTAAAATGTTAATATAGTTCTGAAGAAGTATAAATGAGGTGTTGAAATGGCTAAAAATAAGAAAACGAACGCGATGCGTATGCTT

+962   GATCGTGCAAAAATTAAATACGAAGTT
```

FIG. 2. Nucleotide sequence of the *S. aureus* DNA contained in pK21. A portion of the complementary strand also is shown. Minus and plus signs indicate nucleotides that precede or follow, respectively, the coding region of *norA1199*. For both *norA1199* and ORF A, the −35, −10, and Shine-Dalgarno (SD) sequences are noted, as are the transcription terminator sequences of *norA1199*. The transcription initiation and stop codons are underlined and in boldface. The region of dyad symmetry surrounding the −10 region of *norA1199* is indicated.

```
norA1199   MNKQIL---VLYFNIFLIFLGIGLVIPVLPVYLKDLGLTGS---DLGLLV    44
norA       MNKQIF---VLYFNIFLIFLGIGLVIPVLPVYLKDLGLTGS---DLGLLV    44
BSBmr      MEKKNITLTILLTNLFIAFLGIGLVIPVTPTIMNELHLSGT---AVGYMV    47
TetPBR     M-KSNNALIVILGTVTLDAVGIGLVMPVLPGLLRDIVHSDSIASHYGVLL    49
           * *      ..  .. .  .*****.** *  ....  ...      * ..

norA1199   AAFALSQMIISPFGGTLADKLGKKLIICIGLILFSVSEFMFAIGQNFLIL    94
norA       AAFALSQMIISPFGGTLADKLGKKLIICIGLILFSVSEFMFAVGHNFSVL    94
BSBmr      ACFAITQLIVSPIAGRWVDRFGRKIMIVIGLLFFSVSEFLFGIGKTVEML    97
TetPBR     ALYALMQFLCAPVLGALSDRFGRRPVLLASLLGATIDYAIMATTPVLWIL    99
           * .*. *.. .*  *   *..*.. ..  .*.  ...  .....    .*

norA1199   MLSRVIGGMSAGMVMPGVTGLIADISPSHQKAKNFGYMSAIINSGFILGP   144
norA       MLSRVIGGMSAGMVMPGVTGLIADISPSHQKAKNFGYMSAIINSGFILGP   144
BSBmr      FISRMLGGISAPFIMPGVTAFIADITTIKTRPKALGYMSAAISTGFIIGP   147
TetPBR     YAGRIVAGITGATG-AVAGAYIADITDGEDRARHFGLMSACFGVGMVAGP   148
             .*...*...     . ... ****.  . ... .* *** .. *.. **

norA1199   GIGGFMAEVSHRMPFYFAGALGILAFIMSIVLIHDPKKVSTNGFQKLEPQ   194
norA       GIGGFMAEVSHRMPFYFAGALGILAFIMSIVLIHDPKKSTTSGFQKLEPQ   194
BSBmr      GIGGFLAEVHSRLPFFFAAAFALLAAILSILTLREPERNPENQEIKGQKT   197
TetPBR     VAGGLLGAISLHAPFLAAAVLNGLNLLLGCFLMQESHKGERRPMPLRAFN   198
             **.....  . **  *.... *. ...   ......   .     .

norA1199   LLTKINWK--VFITPVILTLVLSFGLSAF--ETLYSLYTADKVNYSPKDI   240
norA       LLTKINWK--VFITPVILTLVLSFGLSAF--ETLYSLYTADKVNYSPKDI   240
BSBmr      GFKRIFAP--MYFIAFLIILISSFGLASF--ESLFALFVDHKFGFTASDI   243
TetPBR     PVSSFRWARGMTIVAALMTVFFIMQLVGQVPAALWVIFGEDRFRWSATMI   248
             ...    . ... ....   . * .   ..*. .. ...  .... *

norA1199   SIAITGGGIFGALFQIYFFDKFMKYFSELTFIAWSLIYSVIVLVLLVIAD   290
norA       SIAITGGGIFGALFQIYFFDKFMKYFSELTFIAWSLLYSVVVLILLVFAN   290
BSBmr      AIMITGGAIVGAITQVVLFDRFTRWFGEIHLIRYSLILSTSLVFLLTTVH   293
TetPBR     GLSLAVFGILHALAQAFVTGPATKRFGEKQAIIAGMAADALGYVLLAFAT   298
           .. ..  .*  *. *    .   . *.*   *  ..  ..    **. .

norA1199   GYWTIMVISFVVFIGFDMIRPAITNYFSN-IAGDRQGFAGGLNSTFTSMG   339
norA       GYWSIMLISFVVFIGFDMIRPAITNYFSN-IAGERQGFAGGLNSTFTSMG   339
BSBmr      SYVAILLVTVTVFVGFDLMRPAVTTYLSK-IAGNEQGFAGGMNSMFTSIG   342
TetPBR     RGWMAFPI-MILLASGGIGMPALQAMLSRQVDDDHQGQLQGSLAALTSLT   347
                . .  ... . .. .**. . .*. .... **   *  . .**..

norA1199   NFIGPLIAGALFDVHIEAPIYMAIGVSLAGVVIVLIEKQHRAKLKQQDL    388
norA       NFIGPLIAGALFDVHIEAPIYMAIGVSLAGVVIVLIEKQHRAKLKEQNM    388
BSBmr      NVFGPIIGGMLFDIDVNYPFYFAT-VTLAIGIALTIAWKAPAHLKA-ST    389
TetPBR     SITGPLIVTAIYAASASTWNGLAWIVGAALYLVCLPALRRGAWSRATST    396
           .  **.* . ...   .     .* *. *  .    . .  *  .. .
```

FIG. 3. Relatedness of the deduced protein products of *norA1199*, *norA*, and the *B. subtilis bmr* gene (BSBmr) and the Tet protein of pBR322 (TetPBR). Asterisks show identical residues; dots show conservative replacements; dashes indicate gaps introduced into the respective sequences to optimize the alignments.

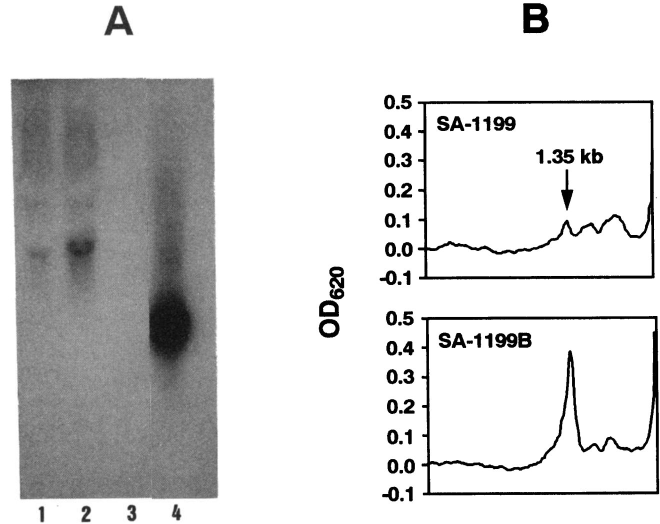

FIG. 5. Northern blot autoradiogram of SA-1199 and SA-1199B following hybridization with the *norA1199* probe (A) and densitometric scans of the autoradiogram (B). Lanes: 1, SA-1199; 2, SA-1199B; 3, *E. coli* $DH_{10}B$ (negative control); 4, the probe itself (positive control). OD_{620}, optical density at 620 nm.

a transcript of approximately 1.35 kb occurred (Fig. 5). The hybridization signal for SA-1199B was significantly stronger than that for SA-1199.

Pulsed-field electrophoresis. The *Sma*I restriction patterns of SA-1199 and SA-1199B were identical but differed somewhat from that of *S. aureus* 8325-4 (Fig. 6). The probe hybridized with a 262-kb fragment of *S. aureus* 8325-4 DNA (Pattee D fragment [22]) and an identical-size fragment in strains SA-1199 and SA-1199B. The intensities of the hybridization signals for both strains were identical (densitometric scanning; data not shown).

Nucleotide sequence of *norA1199*(wt). The consensus nucleotide sequence of *norA1199*(wt) revealed no differences from *norA1199* over the entire coding region (data not shown). All four PCR-generated clones possessed a cytosine at position 1085 and an alanine at position 362 of the deduced protein product. The single difference that was identified was the presence of thymine at position −89 of *norA1199*(wt) instead of the adenine found at this position in *norA1199*.

One of the PCR-generated clones contained no *Taq* DNA-polymerase-induced errors throughout the coding region and was found to confer norfloxacin resistance upon an *E. coli* host organism. The plasmid containing this clone was named pK38, and compared with a plasmid-free strain, its presence resulted in an 8-fold rise in the norfloxacin MIC for *E. coli*

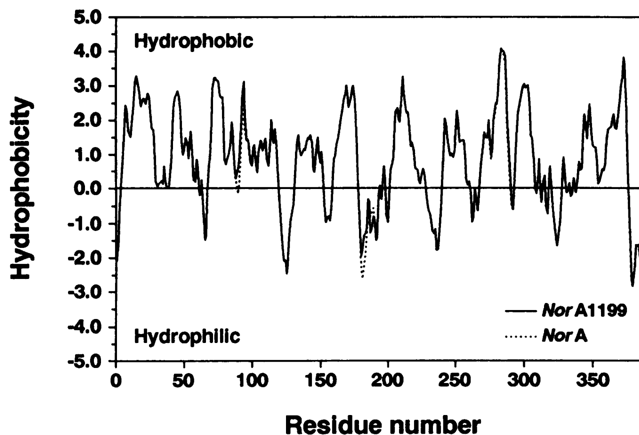

FIG. 4. Hydropathic profile of the *norA1199*-encoded protein. The profile of the *norA*-encoded protein is superimposed for comparison.

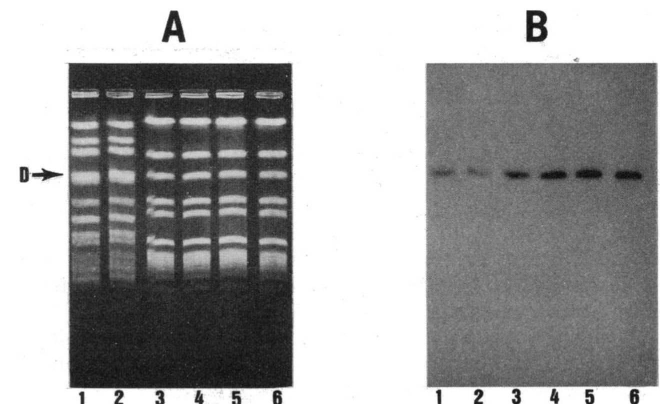

FIG. 6. (A) Pulse-field gel of *Sma*I-digested chromosomal DNAs of *S. aureus* NCTC 8325-4 (lanes 1 and 2), SA-1199 (lanes 3 and 5), and SA-1199B (lanes 4 and 6). The position of the D fragment is indicated. (B) Southern blot autoradiogram following hybridization with the *norA1199* probe.

$DH_{10}B$ (0.04 to 0.31 μg/ml, respectively); a 31-fold rise was conferred by pK21. None of the other PCR-generated clones conferred norfloxacin resistance upon an *E. coli* host organism. Each demonstrated a single *Taq* DNA polymerase-induced error within the coding region of the gene. One clone had a stop codon introduced through a single nucleotide substitution, and the remaining two clones possessed a single nucleotide deletion that resulted in a frame shift. Each of these errors occurred near the 5′ end of the gene.

An insufficient quantity of ORF A was present in the cloned DNA contained in pK38 to allow a meaningful comparison with the ORF A found in pK21.

Analysis of nucleotide 1085. *Sfc*I digestion of the PCR products that included nucleotide 1085 from each of eight clinical strains of *S. aureus* revealed a preservation of the restriction site and thus the presence of cytosine at position 1085 (data not shown). Substitution of adenine for cytosine at this position of *norA1199*, and thus substitution of aspartic acid for alanine at position 362 of the protein product, did affect the ability of the protein to confer norfloxacin resistance upon *E. coli* $DH_{10}B$ (the norfloxacin MIC was reduced from 1.25 to 0.08 μg/ml).

DISCUSSION

Transport mechanisms, including efflux processes, are utilized by prokaryotic and eukaryotic cells in many situations, including acquisition of essential nutrients, establishment of the proper charge and pH gradient across the cytoplasmic membrane, and extrusion of potentially toxic compounds (16, 31). Active efflux of selected antibiotics has been described for several genera of bacteria and is one mechanism by which such organisms resist the inhibitory or lethal effect(s) of these drugs. The most-studied antimicrobial agent efflux system is that for Tet. A family of highly related *tet* genes have been described, and their protein products are integral membrane polypeptides that are Tet-H^+ antiporters the functions of which are dependent upon the proton motive force (13). Tet^r of this type tends to be plasmid or transposon based and is widespread among both gram-positive and -negative bacteria. The *norA1199*-encoded protein has many similarities to Tet proteins. It has significant homology with many of them, and its hydropathic profile is similar to those of Tet proteins (suggesting that it also is an integral membrane protein). Additionally, on the basis of [^{3}H]norfloxacin uptake studies using whole cells and everted membrane vesicles (in which drug uptake is equivalent to drug efflux in whole cells), it mediates the efflux of a drug and its activity is dependent upon the proton motive force. However, the *norA1199* protein differs from Tet proteins in that it originates from a chromosomal gene that occurs naturally in the *S. aureus* genome and it does not confer Tet^r. Southern hybridization data also indicate that related sequences are present in the *S. epidermidis* genome. No similar gene appears to be present in the *E. faecalis* genome or in gram-negative organisms, such as *E. coli* and *K. pneumoniae*. The polymorphism in the restriction fragment sizes of the *S. aureus* and *S. epidermidis* DNAs that hybridized with the *norA1199* probe likely is the result of point mutations in both genomes leading to acquisition or loss of restriction endonuclease recognition sites.

An even higher degree of homology than that seen with Tet proteins exists between the *norA1199*-encoded protein and the *B. subtilis* Bmr protein, which is an integral membrane polypeptide 389 amino acids long. This protein also does not confer Tet^r, but it does mediate the efflux of structurally unrelated compounds, such as chloramphenicol, ethidium bromide, and tetraphenylphosphonium bromide, and also is capable of efflux of fluoroquinolones (18, 19). The function of Bmr is interfered with by reserpine and verapamil, compounds that also inhibit the function of the mammalian multidrug efflux transporter P-glycoprotein. P-glycoprotein confers resistance to structurally unrelated antineoplastic agents in selected tumor cells (9). ATP hydrolysis is a requirement for the function of P-glycoprotein, differentiating it from both the *norA1199* protein and Bmr. In addition, Bmr and the *norA1199*-encoded protein have minimal homology with any P-glycoprotein species. Neyfakh et al. have cloned *norA1199* into a *B. subtilis* vector and have observed expression of the gene in a *B. subtilis* host (20). In this system, the *norA1199*-encoded protein functions as a multidrug efflux transporter and its activity is inhibited by reserpine. With respect to norfloxacin and ethidium bromide, we have found the same to be true in both SA-1199 and SA-1199B, as reserpine reduces the MICs of both compounds for both strains. That the wild-type strain is affected at all suggests that *norA1199*(wt) functions endogenously at a low level. We previously have published [^{3}H]norfloxacin uptake data for SA-1199 and SA-1199B that also support this conclusion (10).

The reason why the *norA1199*-encoded protein (and the *norA*-encoded protein) transports hydrophilic fluoroquinolones much more effectively than hydrophobic ones is not clear. Possibly these proteins form, or are part of, channels through the cytoplasmic membrane through which hydrophilic compounds preferentially pass, possibly unidirectionally (outward). Alternatively, the *norA1199*-encoded protein may function as a carrier protein with a greater affinity for hydrophilic compounds. Hydrophobic compounds may not be able to utilize either of the above-described systems efficiently. Since *norA1199* (and its allele[s]) is a naturally occurring *S. aureus* gene, its protein product must have a function other than fluoroquinolone transport, as these compounds are completely synthetic substances not likely to be encountered in nature. Discovery of its natural function may help to explain the preferential transport of hydrophilic fluoroquinolones.

The existence of ORF A upstream of *norA1199* suggests an intriguing possibility. The regulatory proteins for both the *S. aureus qacA* gene and the *tet* gene of Tn*10* are encoded by genes upstream of and on the strand complementary to that of the genes they regulate (1, 24). The distance between the resistance determinant and the regulatory gene is small, and portions of the promoter sequences overlap in both the *qacA* and Tn*10 tet* situations, similar to the circumstance that exists between *norA1199* and ORF A. An area of dyad symmetry, consisting of an 8-bp inverted repeat which may represent a regulatory protein-binding site, is present upstream of *norA1199* (Fig. 2). It is possible that ORF A encodes a regulatory protein that recognizes this putative regulatory region and controls transcription of *norA1199*. Determination of the complete nucleotide sequence of ORF A would be the first step in a more complete evaluation of the possible functions of the encoded protein, and we do possess earlier clones containing additional nucleotides upstream of *norA1199* that should contain the entire coding region.

The sequence data for *norA1199*(wt) differ from those reported by Ohshita et al. (21). There is no mutational alteration of residue 362 in the *norA1199*-encoded protein. In fact, no nucleotide differences between *norA1199* and its wt allele were identified throughout the coding region. How-

ever, our site-directed mutagenesis data do verify the findings of Ohshita et al. that substitution of aspartic acid for alanine at residue 362 of the *norA1199*-encoded protein reduces its ability to confer norfloxacin resistance upon an *E. coli* host. The activity of the protein is not completely eliminated; its presence confers a stable twofold increase in the norfloxacin MIC for *E. coli* $DH_{10}B$ compared with a plasmid-free strain (0.08 versus 0.04 μg/ml, respectively). This twofold rise also was found by Ohshita et al. by using a different *E. coli* host strain. Despite this apparent agreement, additional data we have generated lead us to a different conclusion regarding the putative adenine-to-cytosine mutation at position 1085 that Ohshita et al. have concluded is responsible for converting a wild-type *norA* allele into one that confers hydrophilic fluoroquinolone resistance. *Sfc*I analysis of PCR amplification products generated from all of the *S. aureus* strains that we have examined verified the presence of cytosine at position 1085, and sequencing data verified the presence of the same nucleotide at this position in both *norA1199* and *norA1199*[wt]. These data indicate that cytosine likely is the naturally occurring nucleotide at this position. Ohshita et al. studied fluoroquinolone-susceptible and -resistant strains of *S. aureus* that were not isogenic; it is possible that their wt strain actually possessed a cytosine-to-adenine transition at position 1085. Why this substitution has such a negative effect will be the subject of future studies.

Our data indicate that rather than a mutational alteration in the *norA1199* gene, increased transcription of *norA1199* is the likely mechanism of fluoroquinolone resistance in SA-1199B. Northern hybridization data showed a much stronger mRNA signal for SA-1199B than for SA-1199 (Fig. 5). On the basis of Southern hybridization studies of pulsed-field gels, gene amplification as a mechanism of resistance did not occur (Fig. 6). Gene amplification is the mechanism behind the multidrug resistance phenotype for cells carrying the *B. subtilis bmr* gene and is one mechanism by which mammalian tumor cells expressing P-glycoprotein resist the effects of selected antineoplastic agents (9, 19). Increased transcription of *norA1199* may be the result of an unidentified alteration in the regulatory mechanism(s) for the gene, possibly involving ORF A. Additionally, the thymine [*norA1199*(wt)]-to-adenine (*norA1199*) transversion at position −89, which lies just downstream of the −10 region of the promoter, may have some effect on transcription of the gene. This transversion also may have something to do with the fact that *norA1199*(wt) confers a slightly lower degree of norfloxacin resistance upon *E. coli* $DH_{10}B$ than does *norA1199* (MICs, 0.31 and 1.25 μg/ml, respectively). Site-directed mutagenesis experiments will allow determination of the importance of this nucleotide in the expression of the resistance phenotype.

Expression of norfloxacin resistance in *E. coli* $DH_{10}B$ carrying *norA1199*(wt) on a multicopy plasmid (pK38) supports the conclusion that an increased quantity of the protein product is responsible for the resistance observed. However, in plasmid-containing *E. coli* strains this increased quantity is the result of increased gene dosage rather than increased transcription of a single copy of the gene. Additional evidence favoring increased transcription of *norA1199* as the basis for fluoroquinolone resistance in SA-1199B would be obtained by placing *norA1199*(wt) under the control of an inducible promoter. Induction to various levels of transcription should correlate with the level of fluoroquinolone resistance in the *E. coli* host strain.

REFERENCES

1. **Bertrand, K. P., K. Postle, L. V. Wray, Jr., and W. S. Renznikoff.** 1983. Overlapping divergent promoters control expression of Tn10 tetracycline resistance. Gene **23:**149–156.
2. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
3. **Cohen, S. P., D. C. Hooper, J. S. Wolfson, K. S. Souza, L. M. McMurry, and S. B. Levy.** 1988. Endogenous active efflux of norfloxacin in susceptible *Escherichia coli*. Antimicrob. Agents Chemother. **32:**1187–1191.
4. **George, A. M., and S. B. Levy.** 1983. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in Escherichia coli: involvement of a non-plasmid-determined efflux of tetracycline. J. Bacteriol. **155:**531–540.
5. **Goswitz, J. J., K. E. Willard, C. E. Fasching, and L. R. Peterson.** 1992. Detection of *gyrA* mutations associated with ciprofloxacin resistance in methicillin-resistant *Staphylococcus aureus*: analysis by polymerase chain reaction and automated direct DNA sequencing. Antimicrob. Agents Chemother. **36:**1166–1169.
6. **Harnett, N., S. Brown, and C. Krishnan.** 1991. Emergence of quinolone resistance among clinical isolates of methicillin-resistant *Staphylococcus aureus* in Ontario, Canada. Antimicrob. Agents Chemother. **35:**1911–1913.
7. **Hirai, K., H. Aoyama, T. Irikura, S. Iyobe, and S. Mitsuhashi.** 1986. Differences in susceptibility to quinolones of outer membrane mutants of *Salmonella typhimurium* and *Escherichia coli*. Antimicrob. Agents Chemother. **29:**535–538.
8. **Hooper, D. C., and J. S. Wolfson.** 1991. Mode of action of the new quinolones: new data. Eur. J. Microbiol. Infect. Dis. **10:**223–231.
9. **Juranka, P. F., R. L. Zastawny, and V. Ling.** 1989. P-glycoprotein: multidrug-resistance and a superfamily of membrane-associated transport proteins. FASEB J. **3:**2583–2592.
10. **Kaatz, G. W., S. M. Seo, and C. A. Ruble.** 1991. Mechanisms of fluoroquinolone resistance in *Staphylococcus aureus*. J. Infect. Dis. **163:**1080–1086.
11. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**1063–1065.
12. **Lawyer, F. C., S. Stoffel, R. K. Saiki, K. B. Myambo, R. Drummond, and D. H. Gelfand.** 1989. Isolation, characterization, and expression in *Escherichia coli* of the DNA polymerase gene from the extreme thermophile, *Thermus aquaticus*. J. Biol. Chem. **264:**6427–6437.
13. **Levy, S. B.** 1992. Active efflux mechanisms for antimicrobial resistance. Antimicrob. Agents Chemother. **36:**695–703.
14. **Lindberg, M., J.-E. Sjöström, and T. Johansson.** 1972. Transformation of chromosomal and plasmid characters in *Staphylococcus aureus*. J. Bacteriol. **109:**844–847.
15. **Lorow, D., and J. Jessee.** 1990. Focus 12:19.
16. **Mueckler, M., C. Caruso, S. A. Baldwin, M. Panico, I. Blench, H. R. Morris, W. J. Allard, G. E. Lienhard, and H. F. Lodish.** 1985. Sequence and structure of a human glucose transporter. Science **229:**941–945.
17. **National Committee for Clinical Laboratory Standards.** 1990. Approved standard M7-A2. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 2nd ed. National Committee for Clinical Laboratory Standards, Villanova, Pa.
18. **Neyfakh, A. A.** 1992. The multidrug efflux transporter of *Bacillus subtilis* is a structural and functional homolog of the *Staphylococcus* NorA protein. Antimicrob. Agents Chemother. **36:** 484–485.
19. **Neyfakh, A. A., V. E. Bidnenko, and L. B. Chen.** 1991. Efflux-mediated multidrug resistance in *Bacillus subtilis*: similarities and dissimilarities with the mammalian system. Proc. Natl. Acad. Sci. USA **88:**4781–4785.
20. **Neyfakh, A. A., C. M. Borsch, and G. W. Kaatz.** 1993. Fluoroquinolone resistance protein NorA of *Staphylococcus aureus* is a multidrug efflux transporter. Antimicrob. Agents Chemother. **37:**128–129.
21. **Ohshita, Y., K. Hiramatsu, and T. Yokota.** 1990. A point

mutation in *nor*A gene is responsible for quinolone resistance in *Staphylococcus aureus*. Biochem. Biophys. Res. Commun. **172**:1028–1034.

22. **Patel, A. H., T. J. Foster, and P. A. Pattee.** 1989. Physical and genetic mapping of the protein A gene in the chromosome of *Staphylococcus aureus* 8325-4. J. Gen. Microbiol. **135**:1799–1807.
23. **Peterson, L. R., J. N. Quick, B. Jensen, S. Homann, S. Johnson, J. Tenquist, C. Shanholtzer, R. A. Petzel, L. Sinn, and D. N. Gerding.** 1990. Emergence of ciprofloxacin resistance in nosocomial methicillin-resistant *Staphylococcus aureus* isolates. Arch. Intern. Med. **150**:2151–2155.
24. **Rouch, D. A., D. S. Cram, D. DiBerardino, T. G. Littlejohn, and R. A. Skurray.** 1990. Efflux-mediated antiseptic resistance gene *qac*A from *Staphylococcus aureus*: common ancestry with tetracycline- and sugar-transport proteins. Mol. Microbiol. **4**:2051–2062.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **82**:5463–5467.
27. **Shalit, I., S. A. Berger, A. Gorea, and H. Frimerman.** 1989. Widespread quinolone resistance among methicillin-resistant *Staphylococcus aureus* isolates in a general hospital. Antimicrob. Agents Chemother. **33**:593–594.
28. **Smith, C. L., P. E. Warburton, A. Gaal, and C. R. Cantor.** 1986. Analysis of genome organization and rearrangements by pulsed field gradient gel electrophoresis, p. 45–70. *In* J. K. Setlow and A. Hollaender (ed.), Genetic engineering, principles and methods. Plenum Press, New York.
29. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
30. **Sreedharan, S., M. Oram, B. Jensen, L. R. Peterson, and L. M. Fisher.** 1990. DNA gyrase *gyrA* mutations in ciprofloxacin-resistant strains of *Staphylococcus aureus*: close similarity with quinolone resistance mutations in *Escherichia coli*. J. Bacteriol. **172**:7260–7262.
31. **Tisa, L. S., and B. P. Rosen.** 1990. Transport systems encoded by bacterial plasmids. J. Bioenerg. Biomembr. **22**:493–507.
32. **Trucksis, M., J. S. Wolfson, and D. C. Hooper.** 1991. A novel locus conferring fluoroquinolone resistance in *Staphylococcus aureus*. J. Bacteriol. **173**:5854–5860.
33. **Yanisch-Perron, C., J. Vieria, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
34. **Yoshida, H., M. Bogaki, S. Nakamura, K. Ubukata, and M. Konno.** 1990. Nucleotide sequence and characterization of the *Staphylococcus aureus norA* gene, which confers resistance to quinolones. J. Bacteriol. **172**:6942–6949.
35. **Wang, J. C.** 1985. DNA topoisomerases. Annu. Rev. Biochem. **54**:665–697.