

## Inducible NorA-Mediated Multidrug Resistance in *Staphylococcus aureus*

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**The NorA protein of *Staphylococcus aureus* mediates the active efflux of hydrophilic fluoroquinolones from the cell, conferring low-level resistance upon the organism. This protein also is capable of transporting additional structurally diverse compounds, indicating that it has a broad substrate specificity. Increased transcription of the *norA* gene, leading to a greater quantity of the NorA protein within the cytoplasmic membrane, is felt to be the mechanism by which strains possessing such changes resist fluoroquinolones. *S. aureus* SA-1199 and its in vivo-selected derivative SA-1199B are fluoroquinolone-susceptible and -resistant isolates, respectively; SA-1199B resists hydrophilic fluoroquinolones via a NorA-mediated mechanism in a constitutive manner. SA-1199-3 is an in vitro-produced derivative of SA-1199 in which NorA-mediated multidrug resistance is expressed inducibly. Compared with organisms exposed to subinhibitory concentrations of a NorA substrate for the first time, preexposure of SA-1199-3 to such a compound followed by growth in the presence of that substrate results in the elimination of a 2- to 6-h period of organism killing that occurs prior to the onset of logarithmic growth. The uptake of radiolabeled fluoroquinolone is markedly reduced by preexposure of SA-1199-3 to NorA substrates; such prior exposure also results in a dramatic increase in RNA transcripts that hybridize with a *norA* probe. Preexposure of SA-1199 and SA-1199B to such substrates results in small increases or no increases in these transcripts. No sequence differences between SA-1199 and SA-1199-3 within the *norA* gene or flanking DNA were found. It appears likely that the regulation of *norA* in SA-1199-3, which may be effected by one or more genetic loci outside the *norA* region of the chromosome, differs from that of SA-1199 and SA-1199B.**

*Staphylococcus aureus* is an important community-acquired and nosocomially acquired pathogen. Therapeutic options for infections caused by this organism can be quite limited, especially when methicillin-resistant strains are involved. The fluoroquinolones held much promise as useful agents for the treatment of infections caused by multidrug-resistant strains of *S. aureus*, but shortly after their introduction into clinical use, high-level resistance became widespread among methicillin-resistant strains (5, 18, 21). This occurrence virtually eliminated the utility of these drugs as useful alternatives for the therapy of presumptive *S. aureus* infections in which methicillin-resistant strains might be involved.

There are at least three mechanisms by which *S. aureus* can develop resistance to fluoroquinolones, two of which have been investigated in some detail. The first consists of a mutational alteration(s) of the *gyrA* gene, which encodes the A subunit of DNA gyrase, a topoisomerase and an established target of fluoroquinolone action (4, 6, 24). Such a mutation(s) can result in the continued function of this essential bacterial enzyme despite the presence of concentrations of fluoroquinolones lethal to wild-type strains. The second mechanism involves the efflux of hydrophilic fluoroquinolones from the cell, which generally results in a lower level of resistance than that seen in *gyrA* mutants (7, 8, 25, 26). This type of resistance is mediated by the NorA protein, which is located in the cytoplasmic membrane and is the product of the *norA* gene. We have found that in *S. aureus* increased quantities of NorA, resulting from either increased transcription of *norA* or an increase in the stability of its mRNA, confer resistance not

only to hydrophilic fluoroquinolones but also to several other structurally diverse substances. These include a number of lipophilic and monocationic compounds such as cetrимide, ethidium bromide, benzalkonium chloride, acriflavine, and others (8) (this study and unpublished data). A similar efflux-mediated mechanism of multidrug resistance in *Bacillus subtilis* has been described by Neyfakh et al. (13). This resistance results from overproduction of the membrane-based Bmr protein by way of amplification of the chromosomal *bmr* gene. Bmr and NorA are related proteins that have 45% sequence homology at the amino acid level (8, 12). The mechanism by which these proteins are capable of recognizing structurally dissimilar substrates is unclear. The third described mechanism of fluoroquinolone resistance in *S. aureus* involves a genetic locus on the *Sma*I A fragment of the chromosome called *flqA* (25). Strains containing this allele show relatively low-level resistance to fluoroquinolones, the mechanism of which has yet to be elucidated but which may involve a mutational alteration of another topoisomerase (i.e., *grlA*) (3).

Fluoroquinolone resistance due to a *gyrA* mutation(s) is constitutive in nature. No work toward answering the question of constitutive versus inducible resistance has been described to date for strains expressing *flqA*. We have done much work with *S. aureus* SA-1199 and its fluoroquinolone-resistant derivative SA-1199B (7, 8). SA-1199B expresses low-level but clinically relevant fluoroquinolone resistance by way of NorA-mediated efflux in what appears to be a constitutive manner (this study and unpublished data). Using in vitro techniques, we attempted to isolate and characterize a mutant of SA-1199 having resistance to fluoroquinolones similar to that observed in SA-1199B. This report describes our work with such a mutant, which appears to express NorA-type resistance in an inducible manner.

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## MATERIALS AND METHODS

**Bacterial strains and plasmids.** SA-1199 and its fluoroquinolone-resistant derivative SA-1199B have been described previously (7, 8). *Escherichia coli* DH<sub>10</sub>B was used as a host for plasmids (10). Plasmid pUC19 was used as a cloning vector (Gibco BRL/Life Technologies, Gaithersburg, Md.).

**Determination of antimicrobial susceptibilities.** Unless otherwise noted, all reagents were the highest grade available and were obtained from Sigma Chemical Co., St. Louis, Mo. Antimicrobial agents were obtained from their respective manufacturers. MICs were determined following overnight growth of test organisms in the presence or absence of cetrимide, ethidium bromide, or norfloxacin at concentrations of 1/4, 1/8, and 1/10 the MIC, respectively, using Mueller-Hinton II (MH-II) broth (BBL Microbiology Systems, Cockeysville, Md.) and a microdilution technique according to the guidelines of the National Committee for Clinical Laboratory Standards (11). The effect of reserpine (final concentration, 20 µg/ml) on selected MICs also was determined. Results were expressed as geometric means of multiple (at least three) determinations.

**Production of mutants.** SA-1199 was grown in MH-II broth until exponential growth phase was achieved (optical density at 620 nm [OD<sub>620</sub>], ≈0.8). Organisms were concentrated by centrifugation, and approximately 10<sup>9</sup> CFU was spread evenly over the surface of Mueller-Hinton agar plates (Difco Laboratories, Detroit, Mich.) containing ciprofloxacin at a concentration of 1.25 µg/ml (approximately five times the MIC for SA-1199). After 48 h of incubation at 35°C, the plates were examined for growth. Mutants resistant to this concentration of ciprofloxacin were recovered at a frequency of approximately 10<sup>-9</sup>. Plates containing visible colonies were replicated onto Mueller-Hinton agar containing 15 µg of ethidium bromide per ml (≈2.5 times the MIC for SA-1199). Four mutants that demonstrated multidrug resistance by having the ability to grow on these concentrations of both ciprofloxacin and ethidium bromide were recovered, and one strain (SA-1199-3) was selected for further analysis.

**Growth characteristics.** SA-1199B and SA-1199-3 were grown overnight in MH-II broth containing or lacking ethidium bromide or norfloxacin at concentrations of 1/4 and 1/10 the MIC, respectively. Both of these compounds are substrates of NorA (8, 14). These organisms then were diluted in fresh MH-II broth with or without an identical concentration of the same NorA substrate present. The effect of pregrowth in the presence of a NorA substrate on subsequent growth in the presence of that substrate was compared with the growth of naive organisms exposed to such a compound for the first time by determining the CFU per milliliter for cultures at frequent intervals. Doubling times were calculated from these data.

**Uptake of [<sup>14</sup>C]enoxacin.** Uptake studies were performed with whole cells of *S. aureus* as described previously, modified by the use of [<sup>14</sup>C]enoxacin (15.9 µCi/mg; generously provided by Parke-Davis Pharmaceutical Research, Ann Arbor, Mich.) at a final concentration of 40 µg/ml (7). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used to dissipate the proton motive force across the cytoplasmic membrane (final concentration, 100 µM). For some studies, SA-1199-3 was grown in the presence of cetrимide, ethidium bromide, norfloxacin, or tetracycline at concentrations of 1/4, 1/8, 1/10, and 1/4 the MIC, respectively, prior to performance of the uptake studies.

**Cloning of *norA* from SA-1199-3.** Primers for use in PCR were synthesized by the Macromolecular Core Facility at Wayne State University. Purified chromosomal DNA was obtained from SA-1199-3 by the method of Lindberg et al. (9), and PCR was used to amplify *norA* from this preparation. The primers utilized have been described previously and result in an amplification product consisting of *norA* plus approximately 250 bp of upstream and downstream DNA (8). These primers introduce artificial *KpnI* and *EcoRI* sites into the amplification product to simplify its cloning into pUC19. PCRs were performed with Vent<sub>r</sub> DNA polymerase (New England Biolabs, Beverly, Mass.). PCR parameters consisted of 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, Norwalk, Conn.). PCR products were digested with *KpnI* and *EcoRI* (Gibco BRL/Life Technologies) and then ligated into a similarly digested pUC19 vector. *E. coli* DH<sub>10</sub>B was transformed with this DNA by conventional techniques (19). The presence of an insert of the appropriate size was verified by restriction endonuclease analysis.

With DNA obtained from SA-1199B, PCR was employed to generate a 790-bp internal fragment of *norA* for use as a probe. The conditions of PCR were the same as those noted previously. The probe was labeled with [<sup>32</sup>P]dATP (800 Ci/mmol) (New England Nuclear, Boston, Mass.) by using a random primer kit (Gibco BRL/Life Technologies).

**DNA sequence determination.** The nucleotide sequence of the *norA* gene from SA-1199-3 was determined by the dideoxy chain-termination method using <sup>35</sup>S-dATP (1,000 Ci/mmol) (New England Nuclear) (20). Three independently generated PCR products were sequenced to verify the fidelity of the polymerase.

**Northern (RNA) blotting.** Protoplasts of SA-1199, SA-1199B, and SA-1199-3 were produced by exposing organisms to lysostaphin (30 µg/ml) in SMM buffer (0.5 M sucrose; pH 6.8) for 45 min on ice (16). Total cellular RNA was isolated from protoplasts by the method of Chomczynski (2). Equivalent amounts of RNA (30 µg) from each strain were applied to and separated in a formaldehyde-containing agarose gel. The RNA was transferred to a nylon membrane, and hybridization with the *norA* gene probe was carried out under high-stringency conditions (42°C, 50% formamide) (23).

The application of equal amounts of RNA to the formaldehyde gel was

TABLE 1. Susceptibilities of study strains to selected NorA and non-NorA substrates

Compound	MIC (µg/ml) <sup>a</sup> for:		
	SA-1199	SA-1199B	SA-1199-3
Ciprofloxacin	0.3	6.0	1.6
Enoxacin	1.5	29.8	6.3
Norfloxacin	1.6	79.4	18.8
Norfloxacin + reserpine	0.4	6.3	1.6
Sparfloxacin	0.1	0.2	0.1
Acriflavine	6.3	25.0	25.0
Benzalkonium chloride	0.8	3.1	3.1
Cetrимide	0.4	6.3	12.5
Ethidium bromide	6.3	25.0	50.0
Ethidium bromide + reserpine	1.6	6.3	6.3
TPP <sup>b</sup>	17.7	125.0	250.0
Chloramphenicol	17.6	13.0	12.5
Tetracycline	1.4	1.9	0.8

<sup>a</sup> Results expressed as geometric means of multiple determinations.

<sup>b</sup> TPP, tetraphenylphosphonium bromide.

ensured by two methods. First, the RNA content of each sample was estimated by use of the OD<sub>260</sub>/OD<sub>280</sub> ratio. Second, each sample was run in duplicate in a formaldehyde gel, which was divided after electrophoresis. One half was stained with ethidium bromide, and the other half 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 contents of the samples.

For some experiments, organisms were grown in the presence of subinhibitory concentrations of cetrимide (1/4 the MIC), ethidium bromide (1/8 the MIC), or norfloxacin (1/10 the MIC) prior to the isolation of RNA.

## RESULTS

**Antimicrobial susceptibilities.** The MICs of various compounds for the strains used in this study are shown in Table 1. Overnight growth of any of the test organisms in the presence of subinhibitory concentrations of cetrимide, ethidium bromide, or norfloxacin did not result in any appreciable changes in MICs (data not shown). The MICs for SA-1199-3 were qualitatively similar to those for SA-1199B. Quantitative differences included approximately fourfold-lower and twofold-higher MICs, respectively, for the hydrophilic fluoroquinolones ciprofloxacin, enoxacin, and norfloxacin and the nonfluoroquinolone NorA substrates cetrимide, ethidium bromide, and tetraphenylphosphonium bromide. No changes in the MICs of the non-NorA substrates chloramphenicol and tetracycline were observed. The MICs of the hydrophobic fluoroquinolone sparfloxacin, which is a poor NorA substrate (26), were minimally elevated for SA-1199B and unchanged for SA-1199-3 compared with the MIC for SA-1199. The addition of reserpine resulted in a 12-fold reduction in the MICs of norfloxacin for both SA-1199B and SA-1199-3; ethidium bromide MICs were reduced four- and eightfold, respectively. The presence of reserpine also resulted in a fourfold reduction in the MICs of norfloxacin and ethidium bromide for SA-1199.

**Growth characteristics.** The growth characteristics of SA-1199B when exposed to subinhibitory concentrations of ethidium bromide or norfloxacin are illustrated in Fig. 1A and B. No untoward effect on doubling time was observed regardless of whether the organism was preexposed to either of these compounds prior to subsequent growth in their presence. The doubling times of organisms exposed to ethidium bromide or norfloxacin for the first time (30 and 34 min, respectively) were not significantly different from those of pregrown organisms (29 and 35 min, respectively) or controls (33 min). There was no delay in the onset of exponential growth for SA-1199B regardless of any prior exposure to a NorA substrate.

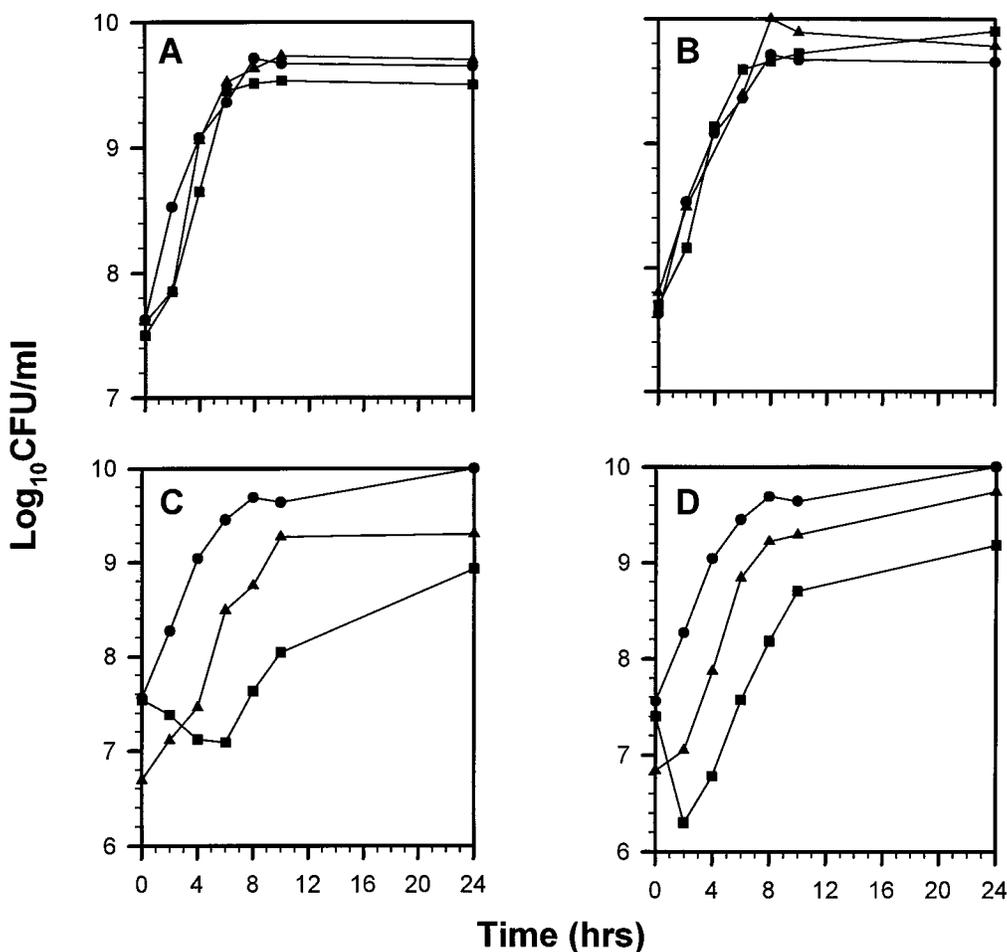


FIG. 1. Growth characteristics of *S. aureus* strains in the presence of NorA substrates following pregrowth in the presence or absence of the same substrates. (A) SA-1199B and ethidium bromide; (B) SA-1199B and norfloxacin; (C) SA-1199-3 and ethidium bromide; (D) SA-1199-3 and norfloxacin. ●, control; ■, naive organisms subsequently exposed to drug; ▲, organisms preexposed to drug and then grown in the presence of the same compound.

When SA-1199-3 was exposed to subinhibitory concentrations of either ethidium bromide or norfloxacin for the first time, a 2- to 6-h period of reduced viability was observed to precede the onset of exponential growth (Fig. 1C and D). This period of bacterial killing was eliminated by preexposure of SA-1199-3 to either of these NorA substrates. With the onset of exponential growth, naive organisms growing in the presence of ethidium bromide or norfloxacin had doubling times of 44 and 34 min, respectively, compared with a doubling time of 32 min for control organisms. Pregrowth in the presence of ethidium bromide or norfloxacin followed by growth in the presence of the same compound shortened these doubling times to 37 and 27 min, respectively.

**[<sup>14</sup>C]enoxacin uptake.** The uptake of radiolabeled enoxacin by study organisms with and without pregrowth in the presence of a subinhibitory concentration of ethidium bromide is shown in Fig. 2. The same results were generated with subinhibitory concentrations of cetrimeid or norfloxacin (data not shown). Without preexposure of SA-1199-3 to a NorA substrate or with preexposure of the organism to the non-NorA substrate tetracycline, its uptake profile was comparable to that of SA-1199B (data not shown for tetracycline preexposure), but with preexposure to a NorA substrate, the uptake profile paralleled that of SA-1199B. The addition of CCCP resulted in a marked increase in cell-associated enoxacin for SA-1199B and preex-

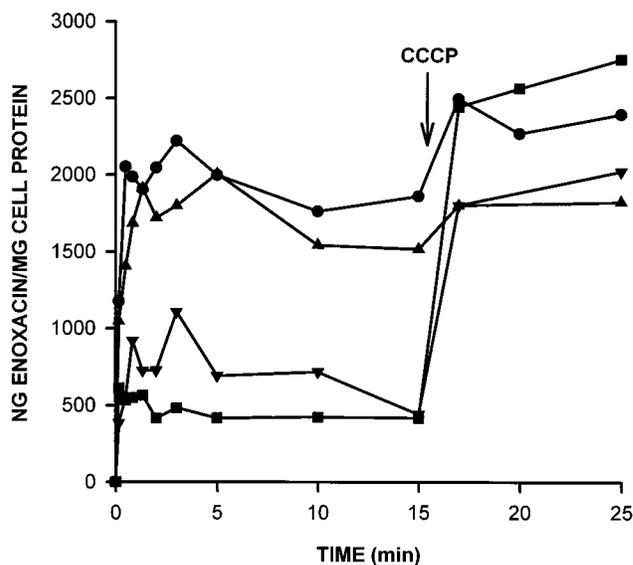


FIG. 2. Uptake of [<sup>14</sup>C]enoxacin by *S. aureus* strains. ▲, SA-1199; ▼, SA-1199B; ●, naive SA-1199-3; ■, SA-1199-3 pregrown in the presence of ethidium bromide. CCCP was added at the indicated time.

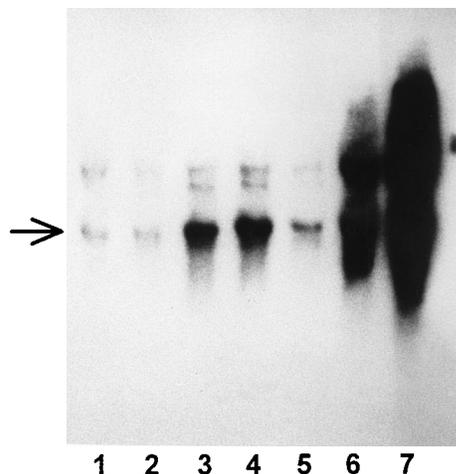


FIG. 3. Northern blot autoradiogram following hybridization with the *norA* probe. Lane 1, naive SA-1199; lane 2, SA-1199 pregrown with cetrimide; lane 3, naive SA-1199B; lane 4, SA-1199B pregrown with cetrimide; lane 5, naive SA-1199-3; lane 6, SA-1199-3 pregrown with cetrimide; lane 7, SA-1199-3 pregrown with norfloxacin. The position of the 1.35-kb *norA* transcript (arrow) is indicated.

posed SA-1199-3; a much smaller increase was observed for SA-1199 and SA-1199-3 not pregrown in the presence of ethidium bromide.

**Nucleotide sequence of *norA* from SA-1199-3.** The nucleotide sequences of the *norA* regions of the chromosomes of SA-1199 and SA-1199B have been published previously (8). The only difference found between these sequences was a T (SA-1199)-to-A (SA-1199B) transversion at a position 89 nucleotides upstream of the *norA* start codon. With respect to SA-1199-3, no changes were observed at any position within the coding region or in the 250 bp of upstream and downstream DNA contained in the PCR product generated from chromosomal DNA (data not shown). The nucleotide at position -89 was the wild-type T that is present in SA-1199.

**RNA contents of study strains.** The autoradiogram of the Northern blot is shown in Fig. 3. As has been shown previously, the quantity of *norA* mRNA (1.35-kb transcript) is increased in SA-1199B compared with that in SA-1199 (8) (Fig. 3, compare lanes 1 and 3). Pregrowth in the presence of cetrimide resulted in a slight increase in the quantity of *norA* mRNA for SA-1199B (Fig. 3, compare lanes 3 and 4). Two larger transcripts also were observed to hybridize with the *norA* probe in both cetrimide-preexposed and naive SA-1199 and SA-1199B (seen more clearly in the original autoradiogram). As for the 1.35-kb transcript, the quantity of these additional transcripts hybridizing with the probe increased slightly with cetrimide exposure in SA-1199B.

The quantity of *norA* transcript for NorA substrate-naive SA-1199-3 was slightly greater than that of SA-1199 (Fig. 3, compare lanes 1 and 5), but when SA-1199-3 was pregrown in the presence of either cetrimide (lane 6) or norfloxacin (lane 7), a dramatic increase in the quantity of RNA species hybridizing with the *norA* probe was seen. Compared with substrate-naive SA-1199-3, the increases observed included the 1.35-kb transcript and at least one of the larger transcripts. These increases again were seen more clearly on the original autoradiogram. The same results were observed with pregrowth in the presence of ethidium bromide or norfloxacin (data not shown).

## DISCUSSION

From the data presented here, it is clear that SA-1199-3 is resistant to fluoroquinolones by way of NorA-mediated efflux. The organism appears to possess a mutation(s) at an as-yet-unidentified position on the chromosome that affects the expression of the *norA* gene at the level of transcription in an inducible manner.

SA-1199B and SA-1199-3 are qualitatively similar with respect to their susceptibility profiles, despite the fact that with induction more mRNA hybridizing with the *norA* probe is present in SA-1199-3 (Fig. 3). This finding might reasonably be expected to result in NorA substrate MICs greater than those for SA-1199B; however, prior exposure of SA-1199-3 to sub-inhibitory concentrations of inducers did not result in increased MICs of these substrates. This apparent discrepancy may be indicative of a saturable process. A maximum number of NorA molecules may be tolerated within the membrane regardless of the quantity of message present. Alternatively, the *norA* message in SA-1199-3 may be shorter-lived than that of SA-1199B, resulting in quantitatively similar amounts of NorA protein being present in the membranes of the two organisms.

Some consistent differences in MICs were observed between SA-1199B and SA-1199-3. SA-1199-3 generally is more susceptible to fluoroquinolones (fourfold) and more resistant to several nonquinolone NorA substrates (twofold; Table 1) than SA-1199B. An alteration in the NorA protein itself, with a resultant change in affinity of the protein for selected substrates, cannot account for these differences, as the DNA sequences of the *norA* gene region for the two organisms were identical. One explanation for these observations is that the non-NorA substrates might be better inducers of *norA* in SA-1199-3; however, this seems unlikely, as quantitatively similar amounts of *norA* message are produced when this organism is induced with either norfloxacin or cetrimide (Fig. 3). A second possibility is that these compounds are not as rapidly bactericidal as the fluoroquinolones; in this situation, the induction process can occur prior to cell death. It also is possible that some as-yet-unidentified gene product(s) may be involved in modulating the resistances observed in these organisms. This gene product(s) may affect *norA* expression in a manner similar to the way the regulatory protein BmrR affects *bmr* expression (1). In that system, substrates of Bmr bind to BmrR and increase its affinity for the *bmr* promoter. Binding of BmrR to the *bmr* promoter enhances transcription of the gene. There is a potential regulatory protein binding site (8-bp inverted repeat) upstream of *norA* that includes the -10 region of the putative promoter (8). The nucleotide sequences of this region in SA-1199B and SA-1199-3 were identical. However, selected substrates may interact with a regulator of *norA* expression encoded elsewhere on the chromosome in variable manners in these organisms (as a result of mutational alteration) and may affect the binding of that regulator to this site differentially. All of these possibilities require further study.

The inhibition of NorA function by reserpine occurs by an undefined mechanism. Reserpine also inhibits the function of the mammalian multidrug efflux transporter P-glycoprotein in what is thought to be a competitive manner (17). Studies done with the NorA homolog Bmr suggest that the same may be true in that system (1). Whether this is the case for NorA has yet to be established, although it is possible on the basis of the functional and structural similarities of NorA and Bmr. The reduction in ethidium bromide and norfloxacin MICs observed in the presence of reserpine for SA-1199 suggests that NorA functions in wild-type strains at a low level. This is not surpris-

ing, as *norA* is known to be a naturally occurring *S. aureus* gene (8).

The growth characteristics of SA-1199-3 in the presence of subinhibitory concentrations of a NorA substrate are dependent upon whether the organism was previously exposed to that substrate. Naive organisms show a 2- to 6-h period of reduced viability followed by recovery and exponential growth (Fig. 1). Preexposed organisms have no such delay in the onset of exponential growth. These findings are suggestive of an inducible phenomenon. The prolonged induction period observed here has been described for other inducible systems involving membrane-based efflux proteins (22). SA-1199B does not demonstrate an inducible response to NorA substrates. Preexposed and naive organisms show identical growth characteristics when grown in the presence of a NorA substrate, a finding consistent with a constitutively expressed resistance trait.

Further evidence in favor of inducible NorA-mediated resistance in SA-1199-3 can be found in the results of the [<sup>14</sup>C]enoxacin uptake studies. NorA substrate-naive SA-1199-3 had an uptake profile comparable to that of SA-1199, whereas organisms preexposed to three different NorA substrates had results comparable to those of SA-1199B. The induction phenomenon appeared to be specifically effected by NorA substrates, as preexposure to tetracycline (not a substrate) did not result in an SA-1199B-like uptake profile.

The strongest evidence in favor of inducible NorA-mediated multidrug resistance in SA-1199-3 can be found in the RNA data. A small increase in *norA* mRNA occurs in response to pregrowth in the presence of a NorA substrate for SA-1199B, but a remarkable increase in the quantity of RNA transcripts having homology to *norA* occurs in response to such preexposure for SA-1199-3. This increase correlates with the aforementioned change in the profile of fluoroquinolone uptake from one similar to that observed with SA-1199 to one similar to that of SA-1199B. The small increase in RNA transcripts with homology to *norA* observed when SA-1199B was preexposed to a NorA substrate does not correlate with any alterations in growth kinetics. Although it appears that in SA-1199B a slight induction of *norA* transcription occurs in response to exposure to NorA substrates, this increase seems to have little biologic significance, and the NorA-mediated multidrug resistance in this organism is a functionally constitutive phenomenon.

The additional bands hybridizing with the *norA* probe observed in all strains, but especially with SA-1199-3 pregrown in the presence of a NorA substrate, that can be seen in Fig. 3 may represent degradation products (lower-molecular-weight bands) or possibly a transcript originating from a promoter outside of *norA* directing the synthesis of a longer transcript that includes *norA* (higher-molecular-weight bands). On the basis of the nearly equal signal intensities of the higher-molecular-weight RNA species and the *norA* transcript seen with induction of SA-1199-3, it is possible that the induction of *norA* transcription is occurring from a putative outside promoter or both such a promoter and the *norA* promoter. These possibilities require further study.

The nucleotide sequence of the *norA* region of SA-1199-3 revealed no differences from that of its parent strain. This includes the -89 position, a mutational alteration of which has been suggested to be responsible for NorA-mediated resistance (15). A change at this position is not responsible for the resistance observed in SA-1199-3, nor is a change anywhere else within the segment of DNA from SA-1199-3 that was sequenced in this study. Clearly, multidrug resistance in this strain is the result of a mutation(s) elsewhere on the chromo-

some involving the regulation of *norA* expression. Significant up-regulation appears to occur in response to exposure to a substrate. It is truly remarkable that a noninducible system has become inducible after a mutation(s). It is possible that a preexisting induction system (for a genetic locus other than *norA*) has been altered mutationally to recognize the *norA* promoter. Another possibility is that there is a naturally occurring *norA* induction system responsive to one set of inducers (possibly its natural substrate[s] but not the compounds studied in this work) that is now able to recognize a different set of inducers (i.e., fluoroquinolones, ethidium bromide, and other NorA substrates). The regulation of *norA* expression is an area for which little information currently exists; identification of the natural substrate(s) of the protein would be a good beginning in understanding this process.

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