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Characterization of NorR Protein, a Multifunctional Regulator of *norA* Expression in *Staphylococcus aureus*

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We characterized a *Staphylococcus aureus norA* gene expression regulator, NorR, initially identified from its binding to the *norA* promoter. The *norR* gene was 444 bp in length, located ~7 kb upstream from the *norA* gene, and encoded a predicted 17.6-kDa protein. Overexpression of *norR* in wild-type *S. aureus* strain ISP794 led to a fourfold decrease in sensitivity to quinolones and ethidium bromide and an increase in the level of *norA* transcripts, suggesting that NorR acts as a positive regulator of *norA* expression. Overexpression of *norR* in *sarA* and *agr* mutants did not alter quinolone sensitivity or levels of *norA* transcription, indicating that the presence of these two global regulatory systems is necessary for NorR to affect the expression of *norA*. Insertion and disruption of *norR* in ISP794 increased resistance to quinolones by 4- to 16-fold but had no effect on *norA* transcription, suggesting that NorR acts as a repressor for another unidentified efflux pump or pumps. These mutants also exhibited an exaggerated clumping phenotype in liquid media, which was complemented fully by a plasmid-encoded *norR* gene. Collectively, these results indicate that NorR is a multifunctional regulator, affecting cell surface properties as well as the expression of NorA and likely other multidrug resistance efflux pumps.

Staphylococcus aureus is a leading cause of human infectious diseases worldwide, ranging from superficial skin lesions to systemic and life-threatening infections, such as osteomyelitis, endocarditis, pneumonia, and septicemia. The virulence of *S. aureus* has been associated with the production of a large number of extracellular toxins, enzymes, and cell-surface-associated proteins, encoded by diverse genes, the expression of which is controlled by the accessory gene regulator locus (*agr*) (35, 50). The *agr* regulatory effector is a 510-base RNA molecule (RNAIII) (42). The *agr* locus regulates most target genes at the level of transcription, but was also shown to affect translation of some genes (35, 42). *sarA* is the second known global regulatory locus that was also involved in the regulation of virulence factors in *S. aureus* (4, 34). SarA activates α-toxin gene transcription but represses transcription of genes for serine protease and protein A (7, 9, 10). SarA acts partly through the *agr* regulatory pathway by binding to *agr* promoters and stimulating the transcription of *agr* (8, 11, 45). Since *agr* and *sarA* loci play such important roles in diverse gene regulation, they may also participate in regulation of efflux pump expression, which causes resistance to multiple antibiotics, including fluoroquinolones in *S. aureus*.

Fluoroquinolones are synthetic antimicrobial agents and have been used for treatment of a broad range of bacterial infections (22). Increases in resistance to diverse antibiotics, including fluoroquinolones, have limited the choice of antimicrobial agents in some clinical settings. The genetics and mechanisms of bacterial resistance to fluoroquinolones have been studied extensively. Fluoroquinolones act on DNA gyrase and topoisomerase IV to inhibit bacterial DNA replication (12). Mutations in *gyrA* and *gyrB* encoding the subunits of DNA gyrase (21, 23, 26) and *grlA* and *grlB* encoding the subunits of DNA topoisomerase IV of *S. aureus* (13, 38) lead to quinolone resistance in gram-positive and gram-negative bacteria. One of the most intriguing mechanisms underlying resistance to fluoroquinolones as well as a range of other antimicrobial agents involves the extrusion of a variety of structurally unrelated compounds due to active efflux by membrane pumps (19, 51). On the basis of bioenergetic and structural criteria, the multidrug transporters have been divided into five major families (5): the ATP-binding cassette family (ABC), the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion transporters (MATE), the drug/metabolite transporters (DMT), and the resistance/nodulation/division transporters (RND) (31, 44). The *S. aureus* NorA protein belongs to the MFS group frequently found in bacteria.

Overexpression of the NorA multidrug resistance (MDR) efflux pump causes resistance to some quinolones (25, 27, 37, 39, 53). It is characterized by the presence of 12 transmembrane segments (37, 39) and is related to Bmr, an efflux pump of *Bacillus subtilis* (1, 36, 55). NorA protects the cell from a number of lipophilic and monocationic compounds, such as ethidium bromide, cetrimide, benzalkonium chloride, tetraphenylphosphonium bromide, and acriflavine, as well as hydrophilic quinolones (25, 27). While the physiological function of NorA as a self-sufficient multidrug transporter was demonstrated with cytoplasmic membrane vesicles and reconstituted proteoliposomes (54), the regulation of the expression of the NorA efflux pump is still not well understood.

Previous studies demonstrated that the expression of *norA* is increased by mutations in the promoter region that increase mRNA stability, reported in the case of the chromosomal quinolone resistance locus, *flqB*, which is linked to *fus* and the transposon insertion Ω*1108* on the *Sma*I D fragment (17), and by mutation in the *arlS* gene that alters the two-component regulatory system *arlRS*, reported in the case of the insertion-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotypes or relevant characteristic(s)	Source or reference
Strains		
S. aureus		
RN4220	8325-4 r^-	30
ISP794	8325 *pig-131*	49
MT23142	8325 *pig-131 flqB*	39
MT1222	8325 *pig-131 grlA flqB gyrA*	39
BF16	8325 *pig-131 arlS*::Tn*917*LTV1	15
QT1	ISP794 *norR*::*cat*	This study
QT2	ISP794 *norR*::*cat*	This study
KL820	RN4220 *norA*::*cat*	25
RN6390	8325-4 Hla^+ Prt^+	41
RN6911	RN6390 *agr*::*tetM*	42
ALC136	RN6390 *sar*::Tn*917*LTV1	10
ALC135	RN6390 *agr*::*tetM sar*::Tn*917*LTV1	6
SH1000	Functional *rsbU* derivative of 8325-4 $rsbU^+$	24
E. coli		
DH5α	F^- φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 deoR recA1 endA1 phoA hsdR17*(r_k^- m_k^-) *supE44* λ^- *thi-1 gyrA96 relA1*	Gibco-BRL
BL21(DE3)	*E. coli* B F^- *dcm ompT hsdS*(r_B^- m_B^-) *gal*λ(DE3)	Stratagene
Plasmids		
pGEM3-zf(+)	2.9-kb *E. coli* cloning vector, Ap^r	Promega
pCL52.2	Temperature-sensitive *E. coli*-*S. aureus* shuttle vector	47
pL150	Shuttle cloning vector (Ap^r Cm^r)	47
pSK950	10.5-kb plasmid carrying the *attP* site of phage L54a, replicon of pE194; Tc^r Em^r (*S. aureus*)	40
pTrcHisA	Cloning and His-tag-expressing vector in *E. coli*	Invitrogen
pQT1	pGEM3-zf(+)-*norR*	This study
pQT2	pGEM3-zf(+)-*norR*::*cat*	This study
pQT3	pCL52.2-*norR*::*cat*	This study
pQT4	pSK950-*norR*	This study
pQT5	pTrcHisA-*norR*	This study

disruption of *arlS* by Tn*917*LTV1 in the *S. aureus* chromosome, but it is not known if the effects of *arlRS* on *norA* expression are direct or indirect (15, 16). In order to identify direct regulatory elements involved in *norA* expression, we have purified the 17.6-kDa protein that binds to the *norA* promoter, identified its gene, and characterized the effects of its disruption and overexpression. Our data show that this putative regulatory protein, which we have named "NorR," functions as an activator of *norA* expression but is also multifunctional, affecting cell surface properties and acting as a negative regulator for expression of other effectors of the MDR phenotype that likely represent other as-yet-uncharacterized MDR efflux pumps.

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MATERIALS AND METHODS

Bacterial strains, plasmids, growth media, and other materials. The bacterial strains and plasmids used in this study are listed in Table 1. Staphylococci were cultivated in brain heart infusion broth (BHI) (Difco, Sparks, Md.) at 37°C unless otherwise stated. *Escherichia coli* cells were grown in Luria-Bertani (LB) medium. Lysostaphin was obtained from AMBI Products, Inc., New York, N.Y. Ciprofloxacin and moxifloxacin were obtained from Bayer Corp., Westhaven, Conn. Sparfloxacin was obtained from Parke-Davis Pharmaceutical Research Division, Ann Arbor, Mich. 2′-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole (Hoechst 33342), nalidixic acid, norfloxacin, ethidium bromide, cetrimide, tetracycline, erythromycin, reserpine, and chloramphenicol were obtained from Sigma Chemical Co., St. Louis, Mo. All primers used in this study were synthesized at the Tufts University Core Facility, Boston, Mass., and are listed in Table 2.

MIC determinations. MICs of quinolones, ethidium bromide, cetrimide, and Hoechst 33342 were determined out by serial agar dilution on Trypticase soy agar (TSA). All plates were incubated at 30 or 37°C for 24 h before being read. Determinations of MICs of quinolones and other chemical compounds for transformants containing pSK950 and pQT4 were done on TSA containing 5 μg of erythromycin per ml to ensure maintenance of the plasmid, with incubation at 30°C. The effect of reserpine on quinolone susceptibility was determined by broth (BHI) dilution in the presence and absence of 10 and 20 μg of reserpine per ml.

DNA isolation. Plasmid DNA isolation was performed with the Qiagen midiprep kit (Qiagen, Inc., Valencia, Calif.) as recommended by the manufacturer. *S. aureus* was transformed with plasmid DNA by electroporation, as previously described (39). Chromosomal DNA from *S. aureus* was prepared with the Easy DNA kit (Invitrogen Life Technologies, Carlsbad, Calif.) as recommended by the manufacturer.

Southern hybridization. Restriction endonuclease-digested staphylococcal chromosomal DNA was resolved by electrophoresis at 100 V in 0.9% agarose for 8 h. The DNA was transferred to Hybond-N+ nylon membrane by alkaline blotting (Amersham, Pharmacia Biotech, Little Chalfont, United Kingdom). Target genes were detected by hybridization with a gel-purified DNA probe that was nonradioactively labeled with the ECL (enhanced chemiluminescence) direct nucleic acid labeling kit (Amersham, Pharmacia Biotech, United Kingdom).

Cloning and overexpression of *norR*. To clone the *norR* gene, primers based on flanking sequences (NCTC8325; Oklahoma University) (B. A. Roe, Y. R. Tian, H. Jia, S. Li, S. Lin, S. Kenton, H. Lai, J. D. White, A. Dorman, F. Z. Najar, S. Clifton, V. Worrell, and J. Iandolo, *Staphylococcus aureus* Genome Sequencing Project, 2002) were synthesized by the Tufts University Core Facility (Boston, Mass.). A 1.3-kb fragment was amplified by PCR from *S. aureus* ISP794 chromosomal DNA with primers *norR*Ba and *norR*Ps, which generated flanking *Bam*HI and *Pst*I sites, respectively (Table 2). The amplified *norR* gene was digested with *Pst*I and *Bam*HI and then ligated into the *Pst*I and *Bam*HI site of the plasmid pGEM3-zf(+) to yield pQT1 and introduced into *E. coli* DH5α.

TABLE 2. Primers used for this study

Primers	Primer sequence[a]	Comments
*NorR*Ba	5′-AAACGGATCCTATGTCTGGAGG-3′	Upstream of *norR, Bam*HI site added
*NorR*Ps	5′-TATG*CTGCAG*CGAACATTTATGA-3′	Downstream of *norR*, *Pst*I site added
*NorR*Ec	5′-AAACTATCAAGAATTCCTCGCTA-3′	Downstream of *norR, Eco*RI site added
*catpvu*1	5′-GATCCAGCTGAAGCACCCATTAGT-3′	Upstream of *cat* gene in plasmid pL150, *Pvu*II site added
*catpvu*2	5′-GATCCAGCTGAGTGACATTAGAAA-3′	Downstream of *cat* gene, *Pvu*II site added
*norA*1	5′-TGCAATTTCATATGATCAATCCC-3′	Upstream of *norA* (−35), amplifies a 150-bp *norA* promoter
*norA*2	5′-AGATTGCAATTCATGCTAAATATT-3′	Downstream of *norA* (−10), amplifies a 150-bp *norA* promoter
*norR*1	5′-AAAATGATAACATATATATATTAA-3′	Upstream of *norR* putative promoter
*norR*2	5′-CGTTTTTTTCTCTTTTCGGATTGGT-3′	Downstream of *norR* putative promoter, before the putative Shine-Dalgarno region

[a] Endonuclease restriction sites added to the primer sequences are underlined.

Plasmids extracted from ampicillin-resistant colonies were screened for the *norR* fragment insertion by restriction endonuclease digest patterns and confirmed by DNA sequencing.

To generate a plasmid for expression of *norR* in *S. aureus*, the *norR* gene was subcloned in *E. coli* into the temperature-sensitive shuttle plasmid pSK950 to yield pQT4. This plasmid was then electroporated into *S. aureus* RN4220 (8325 r^-) to generate transformants, and the structure of pQT4 in *S. aureus* was confirmed by restriction mapping. Electrocompetent strain ISP794 and other strains were transformed with this plasmid isolated from RN4220. Tetracycline- and erythromycin-resistant colonies isolated at 30°C were confirmed to have intact pQT4 by restriction mapping.

Construction of a *norR* mutant by allelic exchange. To generate a *norR* mutant, the 800-bp DNA fragment containing the *cat* gene was amplified from plasmid pLI50 with primers catpvu1 and catpvu2. The PCR product was digested with *Pvu*II and then ligated into an *Acc*I site, previously blunted with Klenow fragment enzyme and deoxynucleoside triphosphates (dNTPs), within the putative *norR* coding region of plasmid pQT1. The resultant plasmid, containing the 2.1-kb *norR*::*cat* fragment, termed "pQT2," was confirmed by restriction mapping and sequencing. The 2.1-kb *norR*::*cat* fragment was subcloned into the temperature-sensitive shuttle plasmid pCL52.2 to yield pQT3. This plasmid was then introduced into RN4220 by electroporation to generate chloramphenicol- and tetracycline-resistant transformants. Putative transformants were confirmed by restriction mapping and DNA sequencing. Electrocompetent ISP794 was subsequently transformed with pQT3 isolated from RN4220. Colonies grown at 30°C that were resistant to chloramphenicol and tetracycline were selected for the allelic exchange after screening. ISP794 harboring pQT3 was grown in BHI broth with tetracycline (3 μg/ml) at 30°C, diluted 1:1,000 in fresh medium, and propagated at 42°C for 24 h. The culture was grown again at 30°C without selection for 48 h. Chloramphenicol-resistant, tetracycline-sensitive colonies, representing possible double-crossover events, were screened for and tested for *cat* insertion into *norR* by Southern hybridization, PCR, and sequencing of the PCR fragment containing the junctional fragment.

DNA mobility shift analysis. To perform the gel shift assay, a pair of primers based on the *norA* DNA sequence were synthesized (*norA*1 and *norA*2) (Table 2) and used to amplify a fragment from the Shine-Dalgarno sequence extending 150-bp upstream and containing the entire *norA* promoter region. One of the primers was biotinylated by Gibco BRL (Rockville, Md.). The gel mobility shift assay was carried out using the LightShift Chemiluminescent EMSA (electrophoretic mobility shift assay) kit (Pierce, Rockford, Ill.), as recommended by the manufacturer. The biotin-labeled DNA was incubated with the indicated amount of purified proteins from *S. aureus* ISP794 in 20 μl of binding buffer (10 mM HEPES [pH 8], 60 mM KCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mg of bovine serum albumin per ml, 0.25 mM dithiothreitol) containing 1 μg of poly(dI-dC), 200 ng of sheared herring sperm DNA, and 10% glycerol. The reaction mixture was incubated for 20 min at room temperature and analyzed by 5% nondenaturing polyacrylamide electrophoresis.

Identification of the NorR protein from cell extracts. Cell extracts collected from 5 liters of *S. aureus* cells at late exponential phase (optical density at 600 nm [OD_{600}] of 0.9) were used to purify the NorR protein as previously described (14). The 150-bp biotinylated DNA fragment described above was immobilized on magnetic beads with covalently coupled streptavidin (Dynabeads M-280; Dynal) according to the manufacturer's protocol. DNA bound to beads was incubated with protein extract in binding buffer (10 mM HEPES [pH 8], 60 mM KCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mg of bovine serum albumin per ml, 0.25 mM dithiothreitol) containing herring sperm DNA (200 ng) for 20 min at room temperature. Beads were washed twice with binding buffer containing herring DNA and twice with binding buffer without DNA. Proteins were then eluted in binding buffer containing 0.5 M NaCl. Eluted proteins were dialyzed against water, concentrated, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The 18-kDa protein was blotted onto polyvinylidene difluoride (PVDF) membrane for N-terminal amino acid sequencing by the Edman degradation method (Dana Farber Research Institute Core Facility, Boston, Mass.).

Purification of NorR protein. The *norR* gene was amplified by PCR from *S. aureus* ISP794 chromosomal DNA with primers *norR*Ba and *norR*Ec, which generated flanking *Bam*HI and *Eco*RI sites, respectively (Table 2). After digestion with *Eco*RI and *Bam*HI, *norR* was ligated into the *Eco*RI and *Bam*HI site of the plasmid pTrcHisA (Invitrogen, Carlsbad, Calif.) to yield pQT5 and then introduced into *E. coli* BL21(DE3). The purification of the histidine-tagged NorR was carried out as recommended by the manufacturer. *E. coli* BL21(DE3) cells harboring pQT5 were grown to mid-log phase in LB medium, at which time, isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) was added to the culture. After 3 h, the cells were harvested by centrifugation and then resuspended in 20 mM sodium phosphate buffer (pH 7.4). The cells were lysed with lysozyme (0.02%) and then centrifuged (100,000 × *g*) for 90 min. The supernatant was applied to the nickel affinity column (iminodiacetic acid [IDA]-Sepharose-Ni; Amersham Pharmacia Biotech, Uppsala, Sweden) and then washed with start buffer supplemented with concentrations of imidazole increasing from 10 to 60 mM. NorR protein was eluted with 300 mM imidazole. The homogeneity of the eluted protein was verified by SDS-PAGE.

RNA analysis. Total *S. aureus* RNA was prepared by extraction from lysostaphin-treated cells grown to the postexponential phase at 37 or 30°C, by using the RNeasy mini kit (Qiagen, Valencia, Calif.). The concentration of RNA was determined spectrophotometrically as A_{260}. For Northern blot analysis, 10 μg of total RNA was electrophoresed through a 0.9% agarose–0.66 M formaldehyde gel in morpholinepropanesulfonic acid (MOPS) and blotted onto Hybond-N+ membranes as previously described (39). DNA probes were amplified from the ISP794 chromosome and labeled with psoralen for the detection of specific transcripts (*norR* and *norA*) by using the Northern Max kit (Ambion, Inc., Austin, Tex.) as recommended by the manufacturer. Blots were hybridized with probes overnight at 42°C, washed, and autoradiographed with Kodak X-Omat film.

RESULTS

Identification of NorR from its binding to the *norA* promoter. In searching for regulatory elements that directly control the expression of the *norA* structural gene, we had previously identified in cell extracts an ~18-kDa protein that binds to the *norA* promoter, producing specific band shifts of a 150-bp DNA fragment containing the entire *norA* promoter region (14).

In order to identify this protein, we first isolated the protein from cell extracts of the wild-type strain ISP794 by using magnetic beads coupled to the 150-bp DNA fragment as an affinity reagent (14). The eluted proteins were separated by SDS-PAGE then transferred to PVDF membrane for N-terminal amino acid sequencing. The first 14 amino acids of the N terminus (XDQHNLXEQLCFSL) were then used to search a data bank of the *S. aureus* genome NCTC 8325 (Roe et al., *Staphylococcus aureus* Genome Sequencing Project, 2002). The database contained a putative protein of 147 amino acids and a predicted molecular mass of 17.6 kDa that contained an identical N-terminal amino acid sequence. Analysis of this amino acid sequence allowed the identification of a 444-bp open reading frame (ORF) on the *S. aureus* genome (Fig. 1).

NorR purification and promoter binding. The putative gene was then expressed by cloning the 444-bp ORF coding region into pTrcHisA, a His-tag expression vector (Invitrogen). After induction with IPTG and purification on a nickel affinity column, we isolated a protein of ~21 kDa (size includes the NorR protein [17.6 kDa] plus the His tag and the anti-Xpress antibody epitope). The SDS-PAGE gel shows one protein band at ~21 kDa in the first eluted fraction and an additional band at ~55 to 60 kDa in the second elution fraction (Fig. 2A). No other protein species were identified by silver staining. We speculate that the larger band is a multimeric form of NorR because it as well as the 21-kDa band is immunoreactive with antibody to the anti-Xpress antibody epitope encoded by pTrcHisA (Invitrogen) (data not shown). The purified 21-kDa NorR from the first eluted fraction (fraction 1) when incubated with the 150-bp *norA* promoter fragment showed a clear shift in the DNA banding pattern (Fig. 2B). With increasing concentrations of proteins, the intensity of the shifted band increased, and band shifts were reduced in the presence of 100-fold excess unlabeled 150-bp *norA* DNA but remained unchanged in the presence of excess herring sperm DNA,

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  1  CCGTTAATTA TGGATATTCT TATTTTTTCA TCTTTATTAT TTTTCATTTT

 51  ATATTCTCTT TTCTATTACA GTTATTTTTA AAAATGATAA CATATATATT

101  AAATTATCTT TTTGCTTGAT TTAATCAAAA ATATCTTCTA AACTTTAAAC
                                putative promoter region 1
151  ATACTTTAAT TTTAGCATGG CATTGAATCA AAGAAAGTTG TGAATTAATA
                                                putative
201  AACAATCAAC TTTTTAATGA TTACCAATCC GAAAAGAGAA AAAAACGGAT
        promoter region 2
251  AGTATGTCTG GAGGAGAACT TTAATGTCTG ATCAACATAA TTTAAAAGAA
                SD region     ATG - norR coding sequence-
301  CAGCTATGCT TTAGTTTGTA CAATGCTCAA AGACAAGTTA ATCGCTACTA

351  CTCTAACAAA GTTTTTAAGA AGTACAATCT AACATACCCA CAATTTCTTG

401  TCTTAACAAT TTTATGGGAT GAATCTCCTG TAAACGTCAA GAAAGTCGTA

451  ACTGAATTAG CACTCGATAC TGGTACAGTA TCACCATTAT TAAAACGAAT

501  GGAACAAGTA GACTTAATTA AGCGTGAACG TTCCGAAGTC GATCAACGTG

551  AAGTATTTAT TCACTTGACT GACAAAAGTG AAACTATTAG ACCAGAATTA

601  AGTAATGCAT CTGACAAAGT CGCTTCAGCT TCTTCTTTAT CGCAAGATGA

651  AGTTAAAGAA CTTAATCGCT TATTAGGTAA AGTCATTCAT GCATTTGATG

701  AAACAAAGGA AAAATAATTA ACTTTTGTCA TGACAATTAA AGTAATGTTT
                - TAA
751  AGAATTTATT AAGAATAGAA AAACAATTAG CACGCGTAAG CTTGTTAGTT

801  AAAAAACTGC TTGAAAGGTT TCTTAGCCTA TCAAGCAGTTTTTTTATGCAT
        putative terminator region
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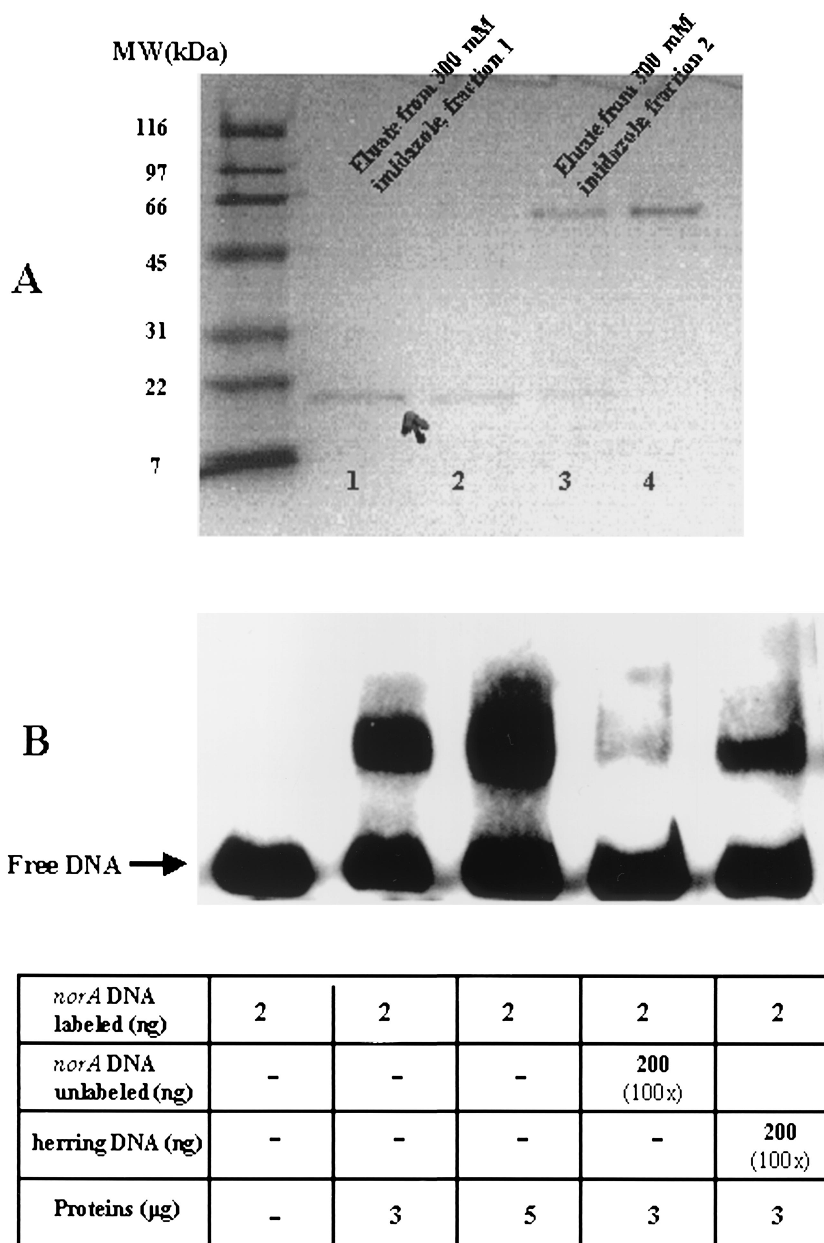
FIG. 1. Nucleotide sequence of 851 bp of *S. aureus* DNA containing the *norR* gene from ISP794 (complete sequence shown). The putative promoter regions, the Shine-Dalgarno sequence, and a putative transcription terminator are underlined. The coding region of *norR* is marked by the ATG start and TAA stop codons.

indicating specific binding to the *norA* promoter fragment. We named this protein "NorR" because its amino acid sequence showed homology with SarR (35% amino acid identity), a protein of the SarA family, and with the MarR (40% identity) protein of the MarR family. The aligned amino acid identity between NorR and QacR and BmrR was less than 10% for both proteins. The NorR protein and its DNA sequence are 100% conserved in the genomes of the sequenced *S. aureus* strains Mu50, N315, COL, and MW2.

To determine whether NorR is also involved in its own regulation via direct binding, we amplified a 150-bp DNA fragment from the region upstream of the *norR* ATG start codon that contains two putative promoters and a putative Shine-Dalgarno region (primers are listed in Table 2). No band shift was found upon incubation of NorR with this DNA fragment (Fig. 3).

***norR* mRNA levels in wild-type and mutant strains.** Northern blots of ISP794 (wild type), RN4220 (8325-4 r^-), MT23142 (*flqB*), MT1222 (pleiotropic), BF16 (*arlS*), and RN6390 (wild type) were probed with a biotin-labeled *norR* fragment to ascertain *norR* expression in these backgrounds. A *norR*-hybridizing transcript was found to be ~500 nucleotides in length by Northern analysis. Because a putative transcriptional termination signal was found 83 bp downstream from the stop codon, this *norR* transcript is probably monocistronic. With the same amount of total RNA, all mutant strains showed a slight decrease of twofold in the levels of *norR* transcription to that of ISP794, RN6390, and RN4220 at the postexponential growth phase (Fig. 4A). The decrease in *norR* transcripts of these mutants suggests an additional regulatory mechanism to regulate the level of MDR efflux pump possibly by NorA-mediated efflux of an effector required for *norR* expression.

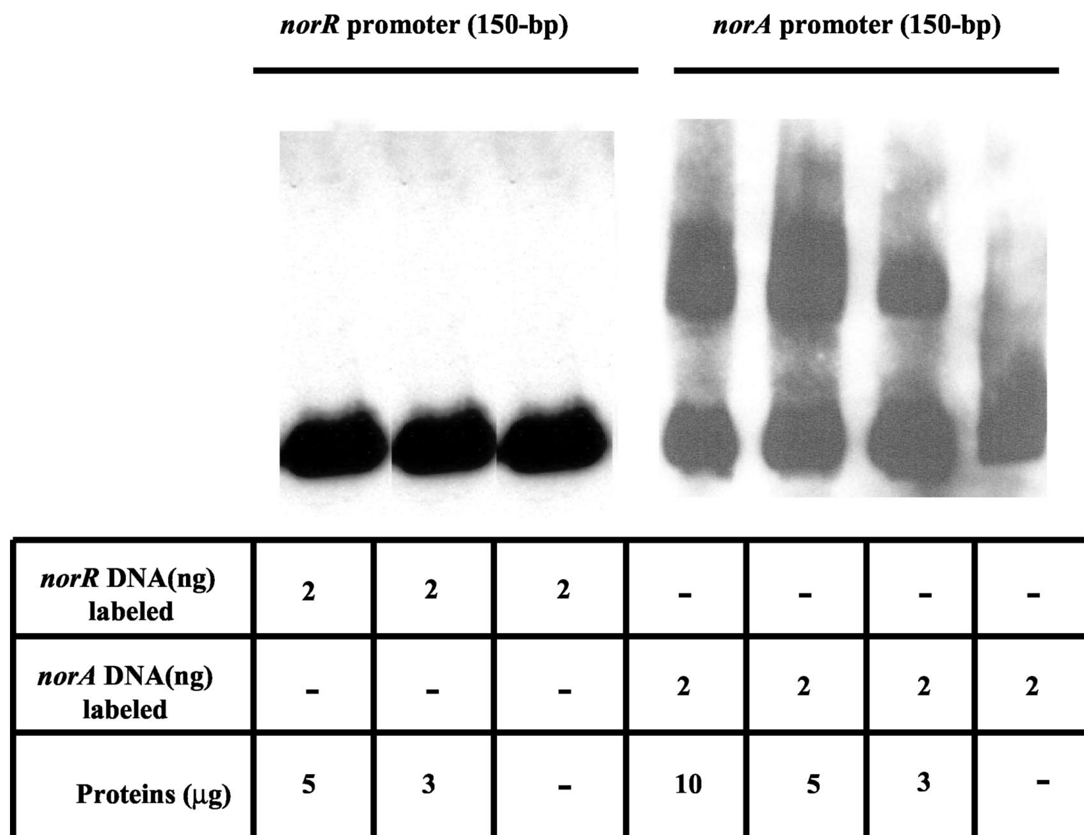
Effects of *norR* overexpression. (i) Resistance to quinolones and other compounds. To address the effect of *norR* overexpression in vivo, we transformed a series of wild-type and mutant strains with plasmid pSK950 into which we had cloned the *norR* gene (pQT4), and then we determined the MICs of quinolones and ethidium bromide for strains with the *norR* plasmid pQT4. The MIC determinations were carried out at 30°C in the presence of erythromycin (5 μg/ml) to ensure the stability of plasmid pQT4. In order to detect any artifact caused by erythromycin in the MIC determinations, strains



norA DNA labeled (ng)	2	2	2	2	2
norA DNA unlabeled (ng)	-	-	-	200 (100x)	
herring DNA (ng)	-	-	-	-	200 (100x)
Proteins (μg)	-	3	5	3	3

FIG. 2. (A) SDS-PAGE analysis of NorR-His purification by Ni affinity chromatography. NorR protein in the crude cell extracts was adsorbed to the Ni column. The column was washed with buffers containing 10 and 60 mM imidazole, and the purified protein was eluted with buffer containing 300 mM imidazole. The gel was stained with Coomassie blue followed by silver staining. MW, molecular mass in kilodaltons. (B) Gel mobility shift analysis of the interaction of purified by NorR-His protein with the biotinylated 150-bp *norA* promoter fragment. Competing unlabeled *norA* promoter DNA and herring sperm DNA were used to determine the specificity of promoter binding. Protein and DNA concentrations and ratios of unlabeled to labeled DNA used in this assay are indicated in the table below the gel.

with the vector plasmid pSK950 were also included in the experiment and tested in the presence of erythromycin. Strains with and without pSK950 showed no change in susceptibility to the drugs tested. Fourfold increases in the MICs of norfloxacin and ethidium bromide, which are known substrates of NorA, were seen with ISP794(pQT4). We also found a twofold increase in the MIC of ciprofloxacin, but no change in MICs of sparfloxacin, moxifloxacin, and nalidixic acid for ISP794 (pQT4). ISP794(pQT4) also showed slight (twofold or less) increases in the MICs of the nonquinolone NorA substrates cetrimide and Hoechst 33342 (Table 3). To evaluate the contribution of *norA* overexpression to the resistance phenotype when *norR* was overexpressed, we used a *norA* knockout strain, KL820, into which we introduced plasmid pQT4. Only a slight increase in the MICs of norfloxacin, ciprofloxacin, and ethidium bromide was found in contrast to the fourfold changes in the ISP794 ($norA^+$) background, indicating that *norA* overexpression accounts for most of the resistance associated with *norR* expression from pQT4 (Table 3). Thus, although *norR* could have additional positive regulatory effects on genes en-



***norR* DNA(ng) labeled**	2	2	2	-	-	-	-
***norA* DNA(ng) labeled**	-	-	-	2	2	2	2
Proteins (μg)	5	3	-	10	5	3	-

FIG. 3. Gel mobility shift analysis of the interaction of purified NorR-His protein with the biotinylated *norA* and *norR* promoter fragments. The protein and DNA concentrations used in this assay are indicated in the table below the gel.

coding other MDR transporters, its major effect on resistance when overexpressed appears to be mediated through *norA*. Mutants that overexpress *norA* by other mechanisms, such as BF16, MT1222, and MT23142, did not demonstrate an additional increase in the MICs of quinolones and dyes when pQT4 was introduced (Table 3).

To assess a possible interaction of *norR* overexpression with the *agr* and *sarA* global regulatory loci, we introduced pQT4 into *sarA* and *agr* single- and double-mutant backgrounds. In contrast to the findings with the agr^{+} $sarA^{+}$ background, *norR* expression from pQT4 in the *agr* and *sarA* mutants caused no increase in the resistance phenotype (Table 3), suggesting that the resistance phenotype of *norR* overexpression is dependent on intact *agr* and *sarA* loci. Increased *norR* expression from pQT4 was maintained in these strains (Fig. 4B), and thus the dependence of the *norR*-overexpression resistance phenotype on intact *agr* and *sarA* cannot be explained by a dependence on these loci for *norR* to be expressed from pQT4.

(ii) Effect on *norA* transcription. Northern blots of RNA from ISP794 with and without pQT4 and from MT23142 were probed with biotin-labeled *norA* and also separately with biotin-labeled *norR* to assess the effect of overexpression of *norR* on *norA* expression. RNA levels of *norR* were documented to be higher in ISP794(pQT4) than in ISP794, as expected (Fig. 5A). Notably, *norA* expression in ISP794(pQT4) was increased substantially to a level as high as that observed in the mutant MT23142, which carries the *flqB* promoter mutation responsible for the overexpression of *norA* in this strain (Fig. 5A). Thus, *norR* behaves as a positive regulator of *norA* transcription. Since ISP794 is a σ^{B} mutant, we also checked the effect of an overexpression of *norR* on *norA* expression by using a σ^{B} wild-type strain (SH1000) (24). The results obtained with strain SH1000 were similar to those with ISP794 (data not shown), indicating that these effects are not modulated by σ^{B}. In addition, in the *agr* and *sarA* mutants, the presence of pQT4 produced increases in *norR* transcripts (Fig. 4B) but no increase in *norA* transcripts (data not shown), further supporting the interactions of *norR* with the *agr* and *sarA* global regulatory systems in its effects on *norA* overexpression.

Properties of a *norR* mutant. In order to assess further the role of NorR as a regulator, we generated a *norR* mutant. We first transformed the wild-type strain ISP794 with a temperature-sensitive plasmid (pCL52.2) that contained a *cat* gene cassette within the *norR* coding region (plasmid pQT3), and then transformants were first selected for resistance to chloramphenicol and tetracycline at permissive temperature (30°C). After successive growth at 42°C with tetracycline and 30°C without tetracycline to enrich for the insertion-excision event of the plasmid pQT3 in the *norR* gene, we screened for and identified tetracycline-sensitive, chloramphenicol-resistant colonies. All candidate *norR*::*cat* mutants were confirmed by PCR, Southern blotting, and DNA sequencing.

The presence of the *cat* gene in *norR* and the correct insertion-excision of the plasmid pQT3 were verified by PCR amplification with primers that flanked the *norR* gene. The size of the PCR fragment as well as direct sequencing of the PCR

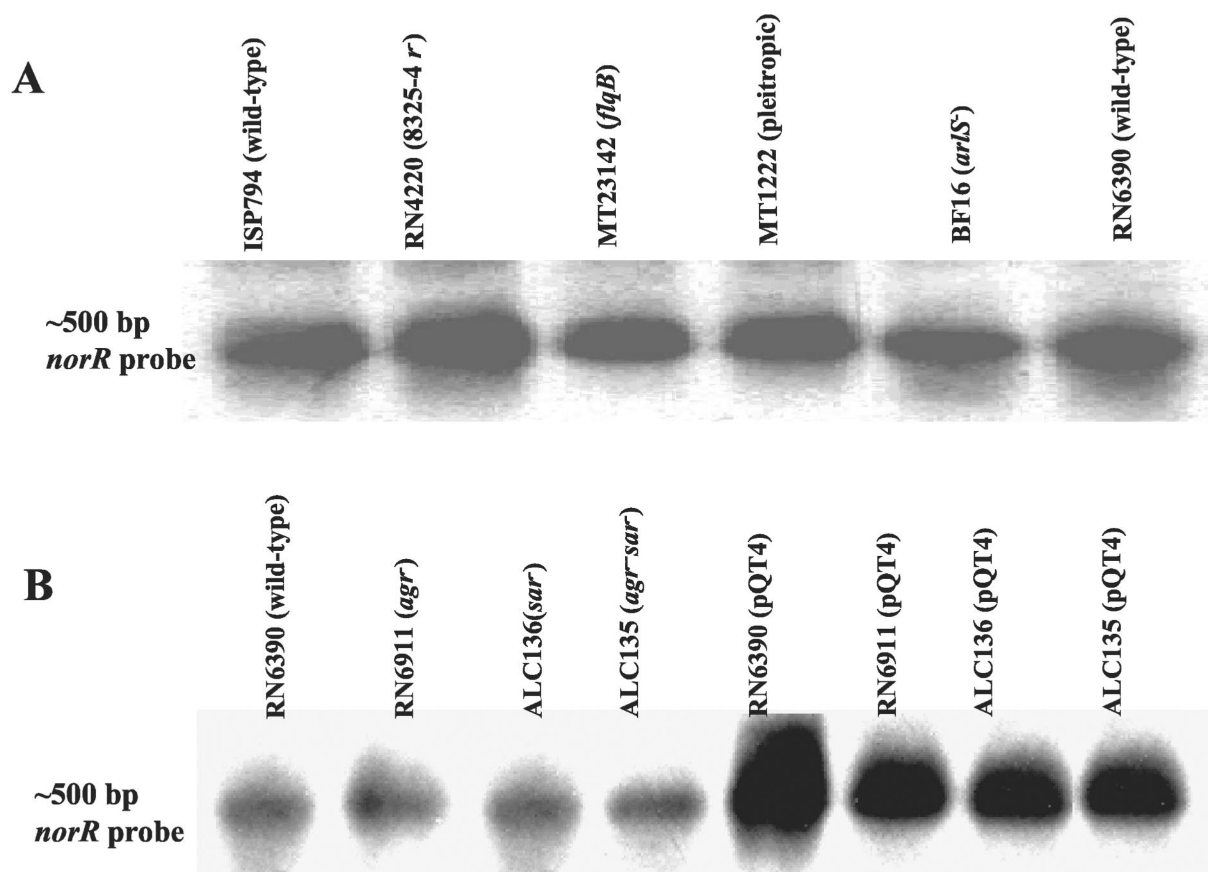


FIG. 4. (A) Northern hybridization of RNA extracted from *S. aureus* strains in the late exponential phase of growth (OD_{600} = 0.9) and probed with the *norR* gene, to observe the transcription level of *norR* in wild-type and mutant strains. (B) *norR* transcripts in *agr sarA* mutants with and without pQT4 (*norR* overexpressed).

fragment confirmed that a double-crossover event had taken place between the plasmid and the chromosome. Southern hybridization of *Dra*I chromosomal digests with fluorescently labeled *norR* revealed that *norR* mutants QT1 and QT2 carried a single *norR*::*cat* insertion in the chromosome, while QT3 resulted from a single crossover that left two copies of the *norR* gene (*norR* and *norR*::*cat*) in its chromosome (Fig. 6).

The *norR* mutants QT1 and QT2 exhibited a resistance phenotype, with 16-fold increases in the MICs of sparfloxacin (2 μg/ml); 8-fold increases in the MICs of norfloxacin (4 μg/ml), ciprofloxacin (2 μg/ml), and moxifloxacin (0.5 μg/ml); 4-fold increases in the MICs of cetrimide (2 μg/ml) and ethidium bromide (4 μg/ml); a 2-fold increase in the MIC of nalidixic acid (400 μg/ml); and no change in MIC of Hoechst 33342 (2 μg/ml) (Table 3). The level of resistance of *norR* mutants to quinolones was similar to those of *flqB* (MT23142) and *arlS* (BF16) mutants that overexpress *norA* (Table 3), except for the MICs of sparfloxacin and moxifloxacin, which showed 16- and eightfold increases in MICs for QT1 and only a 4-fold increase and no increase in the respective MICs for MT23142 (*flqB*) (Table 3). In the presence of reserpine, the *norR* mutants QT1 and QT2 both became less resistant to quinolones and ethidium bromide. We observed a fourfold decrease in the MICs of norfloxacin, ciprofloxacin, moxifloxacin; an eightfold decrease in the MIC of sparfloxacin; and a twofold decrease in the MICs of ethidium bromide when these mutants were tested in the presence of reserpine (Table 3). The level of *norA* expression in Northern blots in QT1 and QT2, however, did not differ from those of wild-type strain ISP794 (data not shown), for which the level of susceptibility to quinolones and ethidium bromide was not affected by reserpine. Thus, these findings further suggest that *norR* also serves as a negative regulator of other effectors of multidrug resistance, likely representing as-yet-undefined MDR efflux pumps. In order to detect any effect of NorR on the transcription of *arlS*, we performed Northern hybridization with strains with and without *norR*. No change in the level of *arlS* transcripts was found among all strains tested (data not shown).

Further supporting the multiple regulatory roles of *norR*, the mutation in *norR* mutants (QT1 and QT2) also strikingly modified the growth of these strains in liquid media without a perceptible change in colony morphology on solid media. Figure 7 illustrates the clumping of the mutant in BHI broth after overnight growth.

In order to assess whether the disruption of *norR* itself or a polar effect of the *cat* insertion was responsible for the *norR*::*cat* mutant phenotype, we transformed the strain with plasmid pQT4. Expression of *norR* from pQT4 in strain QT1 was documented by Northern hybridization (Fig. 5B). Complementation with plasmid-encoded *norR* completely reversed the

TABLE 3. Susceptibilities of strains to quinolones and other agents

Strain (plasmid)[a]	Genotype	Presence/ absence of reserpine[b]	MIC in μg/ml in (factor change in resistance)[c]							
			NOR	CIP	SPAR	MOXI	NAL	CTR	H33342	EB
ISP794	8325 *pig-131*	−	0.5 (1)	0.25 (1)	0.125 (1)	0.06 (1)	200 (1)	0.5 (1)	2 (1)	1 (1)
ISP794		+	0.5 (1)	0.25 (1)	0.125 (1)	0.06 (1)				1 (1)
MT23142	8325 *pig-131 flqB*	−	8 (16)	2 (8)	0.5 (4)	0.06 (1)	800 (4)	8 (16)	16 (8)	8 (8)
MT23142		+	2 (4)	0.5 (2)	0.25 (2)	0.06 (1)				2 (4)
BF16	8325 *pig-131 arlS*::Tn*917*LTV1	−	2	1	0.25	0.06	400	2	2	2
BF16		+	0.5 (1)	0.25 (1)	0.125 (1)	0.06 (1)				1 (1)
QT1	8325 *pig-131 norR*::*cat*	−	4 (8)	2 (8)	2 (16)	0.5 (8)	400 (2)	2 (4)	2 (1)	4 (4)
QT1		+	1 (2)	0.5 (2)	0.25 (2)	0.125 (2)				2 (2)
QT2	8325 *pig-131 norR*::*cat*	−	4 (8)	2 (8)	2 (16)	0.5 (8)	400 (2)	2 (4)	2 (1)	4 (4)
QT2		+	1 (2)	0.5 (2)	0.25 (2)	0.125 (2)				2 (2)
KL820	RN4220 *norA*::*cat*	−	0.33 (.6)	0.16 (.6)	0.06 (.4)	0.06 (1)	400 (2)	2 (4)	2 (1)	0.33 (.3)
RN6390	8325-4 Hla$^+$ Prt$^+$	−	0.5	0.25	0.125	0.06	200	0.5	2	1
RN6911	RN6390 *agr*::*tetM*	−	0.5	0.25	0.125	0.06	200	0.5	2	1
ALC136	RN6390 *sar*::Tn*917*LTV1	−	0.5	0.25	0.125	0.06	200	0.5	2	1
ALC135	RN6390 *agr*::*tetM sar*::Tn*917*LTV1	−	0.5	0.25	0.125	0.06	200	0.5	2	1
MT1222	8325 *pig-131 grlA flqB gyrA*	−	256	64	8	8	1000	32	16	16
MT1222		+	64	32	4	4				4
ISP794(pQT4)		−	2 (4)	0.5 (2)	0.125 (1)	0.06 (1)	200 (1)	2 (4)	3 (1.5)	4 (4)
MT23142(pQT4)		−	8	2	0.5	0.06	800	8	64	8
BF16(pQT4)		−	2	1	0.25	0.06	400	2	4	2
QT1(pQT4)		−	2 (4)	0.25 (1)	0.125 (1)	0.06 (1)	200 (1)	2 (4)	2 (1)	4 (4)
QT2(pQT4)		−	2	0.25	0.125	0.06	200	2	2	4
KL820(pQT4)		−	0.5	0.25	0.06	0.06	400	2	2	0.5
RN6390(pQT4)		−	1	0.5	0.125	0.06	200	0.5	2	2
RN6911(pQT4)		−	0.5	0.25	0.125	0.06	200	0.5	2	1
ALC136(pQT4)		−	0.5	0.25	0.125	0.06	200	0.5	2	1
ALC135(pQT4)		−	0.5	0.25	0.125	0.06	200	0.5	2	1
MT1222(pQT4)		−	256	64	8	8	1,000	32	16	16

[a] Strains harboring plasmid pQT4 were grown in the presence of erythromycin (5 μg/ml).
[b] −, reserpine absent; +, reserpine present.
[c] NOR, norfloxacin; CIP, ciprofloxacin; SPAR, sparfloxacin; MOXI, moxifloxacin; NAL, nalidixic acid; CTR, cetrimide; EB, ethidium bromide; H3342, Hoechst 33342.

clumping phenotype (Fig. 7) and resulted in a change in a resistance phenotype pattern to one similar to that of ISP794 (pQT4) (Table 3). For drugs that are good NorA substrates (e.g., norfloxacin) complementation resulted in little change in resistance, likely due to increased *norA* expression upon restoration of *norR*. In contrast, for sparfloxacin and moxifloxacin, which are poor NorA substrates, *norR* complementation resulted in increases in drug susceptibility. Thus, disruption of *norR* itself is responsible for both the cell-clumping phenotype and for resistance to sparfloxacin, moxifloxacin, and likely other quinolones and other drugs with increased MICs in QT1.

DISCUSSION

NorR is a DNA-binding protein. The regulation of expression of *norA* encoding a major multidrug efflux transporter in *S. aureus* is little understood. In our effort to identify new elements that regulate *norA* expression, we identifed NorR, a putative regulatory protein that has homology with SarR and MarR, two known regulators of gene expression. Purified NorR protein binds specifically to the *norA* promoter region, thus suggesting a direct role for NorR in expression of the *norA* gene. In contrast, gel mobility shift assays showed no evidence that NorR binds to its own promoter, thus suggesting that the *norR* locus is not directly autoregulatory.

NorR functions as an activator of *norA* expression. To study further the effect of *norR* on *norA* expression, we overexpressed the *norR* gene from a plasmid and observed changes in the level of *norA* expression in *S. aureus* strains with different genetic backgrounds. By Northern hybridization, we found an increase in the level of *norA* transcripts in the wild-type strain, ISP794, harboring *norR* cloned on a plasmid. This increase in *norA* transcripts correlated with a fourfold increase in the MICs of norfloxacin and ethidium bromide, suggesting that NorR is a direct transcriptional activator of *norA* expression. Thus, *norR* is the first regulatory locus identified to act directly on the *norA* promoter in *S. aureus*.

Although there was a slight resistance phenotype in a *norA* knockout strain in which *norR* is overexpressed, the major effect of *norR* overexpression appears to be attributable to overexpression of NorA. This finding (i.e., the level of increased susceptibility) also implies that the additional role of NorR as a repressor of other efflux pumps (discussed below) appears to be maximal at basal levels of expression and is not further augmented by NorR overexpression.

***norR* mutants and other possible roles of NorR in the bacterial cell.** *norR* mutants also exhibited a resistance phenotype and a cell-clumping phenotype in liquid media, suggesting that *norR* has a complex regulatory role involving cell surface properties and likely other MDR pumps. The pleiotropic nature of the *norR* resistance phenotype and the reduction in resistance in the presence of reserpine argue that resistance is likely due to MDR pumps. Additional studies are ongoing to identify the specific effectors of MDR in *norR* mutants. The absence of increased levels of *norA* transcripts and some noteworthy dif-

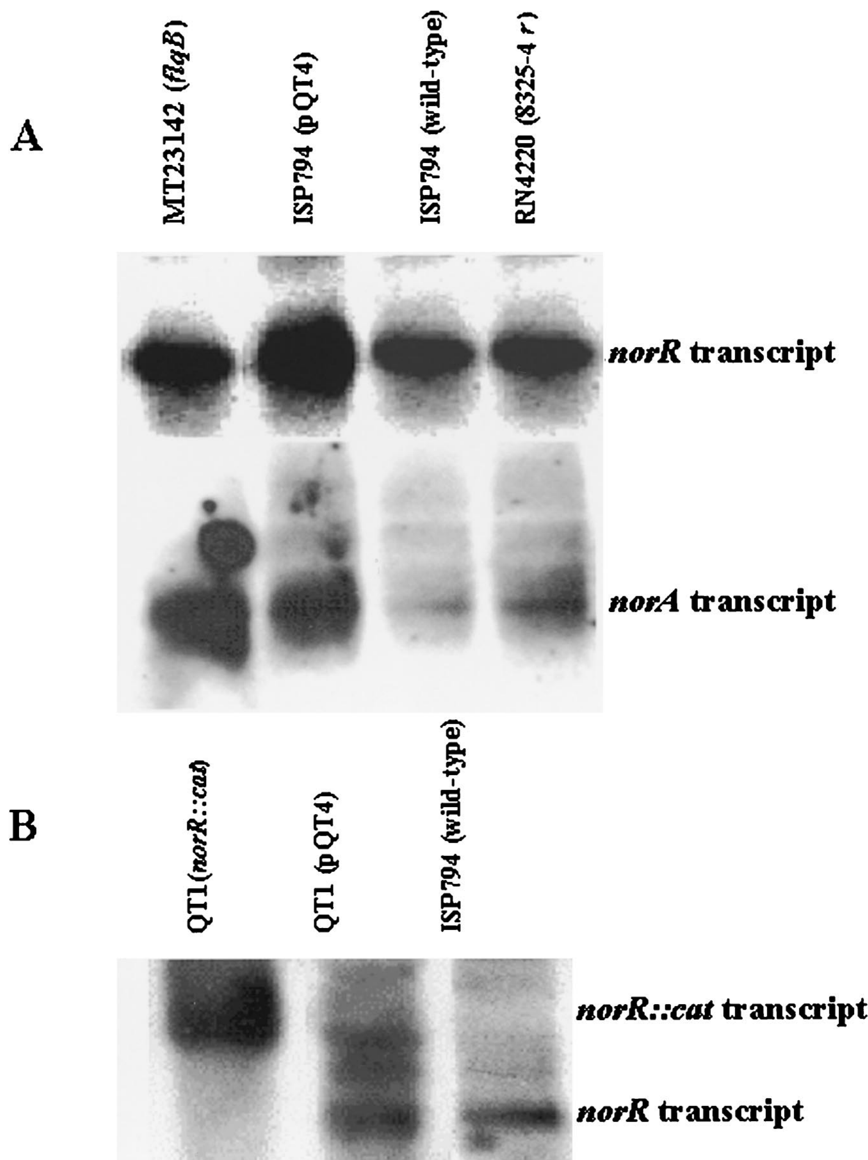


FIG. 5. (A) Effects of *norR* overexpression on *norA* expression. Northern blots of RNA extracted from *S. aureus* strains MT23142, ISP794 (with and without pQT4), and RN4220 in the late exponential phase and probed with either the *norR* or *norA* gene. (B) *norR* transcripts in QT1 with and without pQT4. The *norR*::*cat* transcript is larger than *norR* as expected.

ferences in the resistance profile (resistance to sparfloxacin and moxifloxacin) of the *norR* mutants relative to strains overexpressing *norA* also argue for *norR* as a negative regulator of MDR pumps other than NorA that may have these more hydrophobic quinolones as substrates.

For regulatory genes in which overexpression and reduced expression both have similar phenotypes, reversal of mutant phenotypes with plasmid-encoded complementing genes may be difficult because of the precise titration of the complementing gene product needed to restore the wild-type phenotype. In this study, complementation of *norR* mutant QT1 with pQT4 (*norR* overexpressed) changed the antibiotic resistance pattern to that of ISP794(pQT4), which overexpresses *norA*. We propose that the resistance phenotype of *norR* mutants is in fact due to the disruption of NorR itself for three reasons. First, *norR* complementation of pQT1 reversed the mutant-clumping phenotype completely. Second, *norR* has a transcriptional stop site at its terminus, and its transcripts appear to be monocistronic, suggesting that insertion of the *cat* gene into *norR* does not disrupt an operon. Furthermore, the putative gene adjacent to *norR* is located 200 bp downstream from the *norR* TAA stop codon and appears to have a putative promoter region at 80-bp upstream from its ATG start codon. No change in the levels of transcripts of this ORF was seen in comparing ISP794 and QT1 (data not shown). Third, for two quinolones, sparfloxacin and moxifloxacin, which are poor NorA substrates and thus are little affected by *norA* overexpression, *norR* complementation of QT1 was complete. Work is under way to identify and characterize the efflux pump or pumps that we postulate underlie the

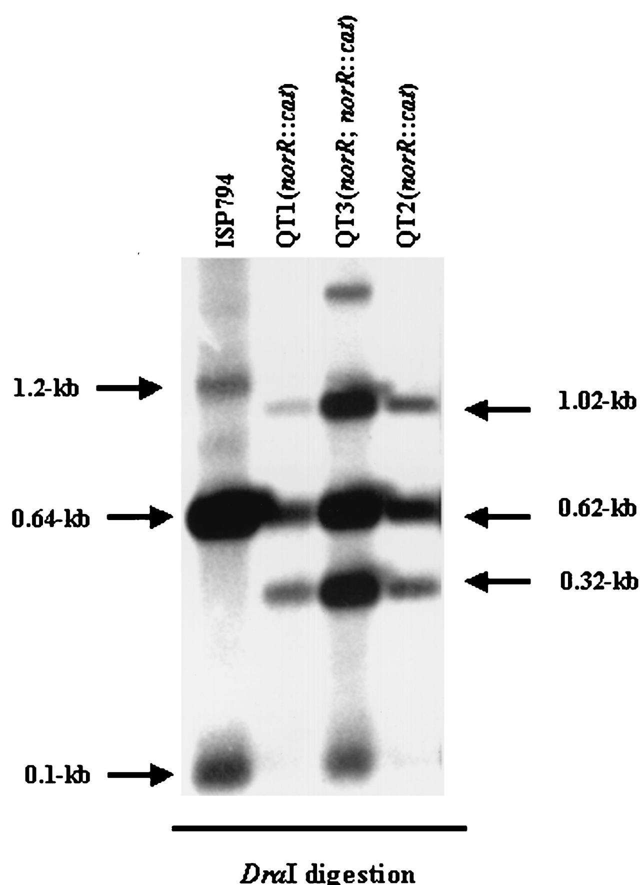


FIG. 6. Southern hybridization of *Dra*I restriction digests of chromosomal DNA from the *S. aureus* wild type and mutants. Changes occurred in the band size, and the numbers of the putative insertion mutation relative to the parental type indicate potential insertion of *cat* into *norR. Dra*I cleaves both *norR* and *cat* genes (once inside each gene). The 0.7-kb *norR* DNA probe hybridizes with 0.64- and 0.1-kb fragments of *norR* gene in ISP794 DNA, while the same 0.7-kb probe hybridizes with 1.02-, 0.62-, and 0.32-kb fragments of *norR*::*cat* in DNA of mutants QT1 and QT2. The 1.2-kb fragment seen in ISP794 DNA came from incomplete enzyme digestion. QT3 resulted from a single crossover between the plasmid and the chromosome, leading to a combined hybridization pattern of wild-type and mutant *norR*.

resistance phenotype of the *norR* mutant and to define directly the role of *norR* in their regulation.

Role of NorR in regulatory networks. NorR differs from SarR and MarR in having direct binding to a target gene promoter rather than acting indirectly on other transcriptional regulators that themselves control target gene expression. SarR and MarR are both autoregulatory, in keeping with their central role in complex regulons (2, 3, 33). Purified NorR, however, appears not to bind upstream of *norR* and thus is unlikely to be directly autoregulatory. It is as yet unclear what factors regulate *norR* expression. Other mutants that have been shown to affect *norA* expression do not appear to affect expression of *norR*. Thus, the effects of the *arlRS* two-component regulatory system on *norA* expression appear not to be mediated through changes in *norR* expression, suggesting that additional factors mediate increased *norA* expression in *arlRS* mutants.

Interestingly, although *norR* overexpression in a wild-type background causes overexpression of *norA* and increased resistance to NorA substrates, *norR* overexpression does not further increase the expression of *norA* or further increase resistance in mutants that exhibit *cis*-acting *norA* overexpression (*flqB norA* promoter mutant) (17, 39) or *trans*-acting overexpression (*arlRS* mutant) of *norA* (15, 16). Thus, these mutations appear to be epistatic to *norR* overexpression. Since sustained high levels of expression of some efflux pumps are likely harmful to the cell, it is possible that as-yet-undefined counterregulatory factors come into play when *norA* expression exceeds certain levels. Additional data obtained from overexpression of *norR* in *agr* and *sarA* mutants further indicate that the effect of *norR* overexpression on *norA* expression requires intact *agr* and *sarA*. The nature of these requirements and interactions is as yet unclear but could involve modification of NorR protein or a requirement for coordinate binding to the *norA* promoter by other factors under the control of the *agr* and *sarA* global regulators.

MarR in *E. coli* exhibits indirect effects on the expression of the AcrB MDR efflux pump (18, 32, 43) as well as affecting the expression of outer membrane proteins. Thus, NorR and MarR both mediate multiple functions within the bacterial cell that include MDR efflux pumps. Other known regulators of MDR efflux pump expression, such as BmrR of *B. subtilis*, QacR of *S. aureus*, EmrR of *E. coli*, and MexR of *Pseudomonas aeruginosa* regulate transcription of *bmr*, *qacA*, *emrB*, and *mexAB-oprM* operons encoding the MDR pumps Bmr, QacA, EmrB, and MexAB-OprM, respectively (20, 46, 52, 55). BmrR, QacR, and EmrR are also known to mediate substrate induction of transcription. EmrR is also known to regulate the expression of the plasmid-encoded *mcb* operon, which directs the synthesis of microcin B17. NorR is less closely related to BmrR, QacR, EmrR, and MexR than it is to MarR, and these four regulators have not been shown to have physiologic roles apart from their direct regulation of expression of their respective MDR pumps. In addition, the *bmrR*, *qacR*, *emrR*, and *mexR* genes, in contrast to *norR* (Fig. 2) and *marR*, are closely linked to the structural genes of the pumps that they regulate, consistent with their presumed targeted role of specific substrate induction of a specific pump (20, 48, 55). NorR is the first identified direct regulator of efflux pump expression in *S. aureus*, and it appears to function as both a positive and negative regulator of different pumps. It is noteworthy that the two best-characterized MDR pumps in *Lactococcus lactis*, LmrA and LmrP, are reciprocally expressed (W. Konings, personal communication), suggesting that regulators with opposing effects on expression of different pumps, like NorR, may also be present in other species. Whether or not NorR protein binds pump substrates is not known, nor is its role in mediating the quinolone induction of *norA* expression reported for one mutant strain (28).

Regulation of expression of *norA* is multifactorial and is known to include effects due to expression of the *arlRS* two-component regulatory system and mutations that affect the stability of *norA* mRNA (15, 17). NorR is now identified as a third component in the regulation of *norA* that acts at the level of transcription. Variations in band-shift patterns observed with the cell extracts obtained from the pleiotropic mutants MT1222 and BF16 (*arlS*) (data not shown) suggest that addi-

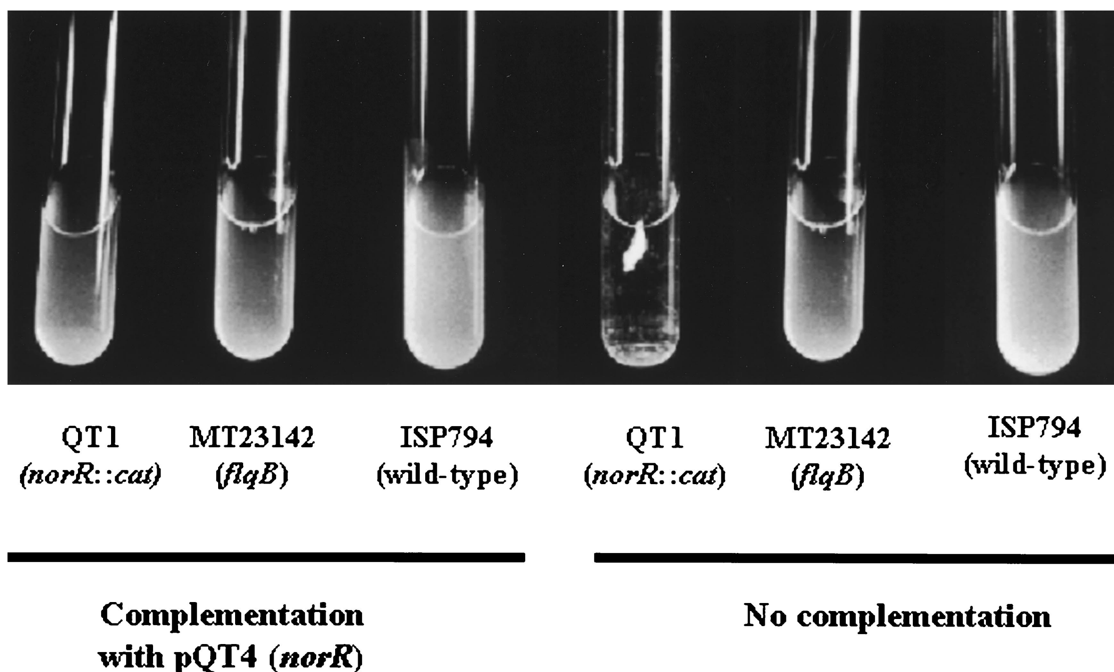


FIG. 7. Overnight cultures of wild-type and mutant *S. aureus* strains, with and without complementation by plasmid pQT4 containing the *norR* gene.

tional regulatory factors also likely bind to the *norA* promoter, and experiments are underway to identify any such additional proteins. Such proteins might also underlie the requirement for intact *agr* and *sarA* for *norR* to increase *norA* expression. As noted above, other mutants not yet genetically characterized have also been described to have a *norA*-mediated resistance phenotype that is inducible with norfloxacin (27–29). Thus, regulation of *norA* and other efflux pumps warrants further study, particularly to define the global networks that mediate regulation of efflux pump expression and to define those environmental conditions under which physiologic overexpression of various pumps reduces antibiotic action and which promote selection of resistant mutants due to efflux pump overexpression. NorR itself, functioning as both a positive and negative regulator of efflux pump expression, appears likely to have a central role in a regulatory network for MDR efflux pumps that likely also interacts in complex ways with established global regulators.

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