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Expression of the Multidrug Resistance Transporter NorA from *Staphylococcus aureus* Is Modified by a Two-Component Regulatory System

BÉNÉDICTE FOURNIER,[1,2] RAHUL ARAS,[1] AND DAVID C. HOOPER[1]*

Infectious Disease Division and Medical Services, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114-2696,[1] and Unité de Biochimie Microbienne, Institut Pasteur, 25, rue du Docteur Roux, 75724 Paris Cedex 15, France[2]

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To dissect genetically the regulation of NorA, a multidrug transporter of *Staphylococcus aureus*, we analyzed the differential expression of the *norA* promoter using a transcriptional fusion with a β-lactamase reporter gene. Expression studies with an *arlS* mutant revealed that the *norA* promoter is ArlS dependent. The *arlR-arlS* locus was shown to code for a two-component regulatory system. The protein ArlR has strong similarity to response regulators, and ArlS has strong similarity to protein histidine kinases. We have also analyzed the 350-bp region upstream of the Shine-Dalgarno sequence of *norA* by gel mobility shift experiments. It was shown that only the 115-bp region upstream of the promoter was necessary for multiple binding of an 18-kDa protein. From transcriptional fusions, we have localized four different putative boxes of 6 bp, which appear to play a role in the binding of the 18-kDa protein and in the up-regulation of *norA* expression in the presence of the *arlS* mutation. Furthermore, the gel mobility shift of the 18-kDa protein was modified in the presence of the *arlS* mutation, and the *arlS* mutation altered the growth-phase regulation of NorA. These results indicate that expression of *norA* is modified by a two-component regulatory system.

For many years, antibiotics have been effective in the treatment of many infectious diseases caused by a range of pathogens, including *Staphylococcus aureus*. The occurrence of antibiotic resistance, however, has transformed some previously treatable diseases into a new threat to public health. One of the mechanisms underlying antibiotic resistance involves the extrusion of the compounds by an efflux pump or carrier (29). The most intriguing mechanisms of drug extrusion are those that include a wide variety of structurally unrelated compounds as substrates for multidrug resistance (MDR) transporters. On the basis of bioenergetic and structural criteria, the known transporters are subdivided into (i) ATP-binding cassette-type transporters and (ii) secondary transporters. The secondary transporters use the electrochemical proton gradient or proton motive force across the cytoplasmic membrane to extrude drugs, whereas the first group utilizes the free energy of ATP hydrolysis (4). The secondary transporters comprise the largest group of known drug extrusion systems in bacteria. They have been subdivided into three different groups: the major facilitator superfamily (MFS), the resistance nodulation and cell division family, and the family of small multidrug resistance (Smr). The MFS family is characterized by the presence of either 12 or 14 putative transmembrane segments. In *S. aureus*, an MDR pump named NorA was previously sequenced and characterized (14, 17, 23, 24, 38). NorA belongs to the MFS family frequently found in bacteria (4). 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 some hydrophilic quinolones (14, 17, 23, 24).

The regulation and the physiological function of NorA, however, is not known. Efflux pumps, such as Bmr of *Bacillus subtilis*, which has similarity to NorA, possess a regulatory gene downstream or upstream of the structural gene. *bmrR*, which is downstream of *bmr*, is responsible for the regulation of expression of Bmr (1). For NorA, the protein encoded by the open reading frame found upstream of *norA* on the chromosome lacks similarity to any known regulator (data not shown).

In order to elucidate the regulation of *norA*, we analyzed the differential expression of the *norA* promoter using a transcriptional fusion with a β-lactamase reporter gene. We found that *norA* expression is affected by ArlS, a member of a newly described two-component regulatory system (B. Fournier and D. C. Hooper, unpublished data). We also performed gel mobility shift experiments on fragments containing the *norA* promoter: it was shown that only the 115-bp region upstream of the promoter was necessary for multiple bindings of an 18-kDa protein and that the binding of this protein was modified in the *arlS* mutant.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. Staphylococci were cultivated in Trypticase soy broth (TSB) at 37°C unless otherwise stated. *Escherichia coli* cells were grown in Luria-Bertani medium.

To construct transcriptional fusions of *norA* with the β-lactamase gene *blaZ* (*norA*::*blaZ*), PCR-generated DNA fragments, L4-R1, L1-R1, and L5-R1, located upstream of the Shine-Dalgarno sequence of *norA* (Fig. 1C) were cloned in pGEM3-zf(+), introduced into *E. coli* DH5α, and sequenced. The DNA fragments were then subcloned into the promoter-probe vector pWN2018 using *Kpn*I and *Pst*I sites to generate plasmids pBF8-30 (L4-R1), pBF4-3 (L1-R1), and pBF15-5 (L5-R1). To construct pBF4-3, an *Eco*RV site present 132 bp upstream of the Shine-Dalgarno sequence and the *Sma*I site of the vector were used to remove 67 bp of pBF8-30.

To construct a plasmid containing the *arlR-arlS* locus, a 2.4-kb product containing *arlR* and *arlS* was amplified by PCR using Vent DNA polymerase (New England Biolabs), chromosomal DNA of ISP794, and two primers containing the *Bam*HI site. The PCR product contained about 300 bp upstream and 100 bp downstream of the *arlR-arlS* locus. PCR products were digested by *Bam*HI and ligated into the *Bam*HI site of pGB2 (9). The resulting plasmid containing the

* Corresponding author. Mailing address: Infectious Disease Division, Massachusetts General Hospital, 55 Fruit St., Boston, MA 02114-2696. Phone: (617) 726-3812. Fax: (617) 726-7416. E-mail: dhooper@partners.org.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristic(s)[a]	Source or reference
Strains		
S. aureus		
ISP794	8325 *pig-131*	34
MT23142	8325 *pig-131 flqB*	24
BF15	8325 *pig-131 flqB arlS*::Tn*917*LTV1	This study
RN4220	8325-4 r^-	18
KLE820	RN4220 *norA*::*cat*	14
RN6390	8325-4 Hla^+ Prt^+	26
RN6911	RN6390 *agr*::*tetM*	27
ALC136	RN6390 *sar*::Tn*917*LTV1	8
ALC135	RN6390 *agr*::*tetM sar*::Tn*917*LTV1	7
E. coli DH5α	F-ϕ80dlacZΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 phoA hsdR17* (r_K^- m_K^-) *supE44O* λ^- *thi-1 gyrA96 relA1*	Gibco BRL
Plasmids		
pGEM3-zf(+)	2.9-kb *E. coli* cloning vector, Ap^r	Promega
pGB2	4.3-kb *E. coli* cloning vector, Sm^r	9
pWN2018	10.5-kb *S. aureus* promoter-probe vector, Cm^r	39
pE194	3.7-kb *S. aureus* cloning vector, Em^r	33
pSK265	3-kb *S. aureus* cloning vector, Cm^r	32
pBF8-30	315 bp containing the entire promoter of *norA* cloned upstream of the *blaZ* gene of pWN2018	This study
pBF3-50	250 bp containing a truncated promoter of *norA* cloned upstream of the *blaZ* gene of pWN2018	This study
pBF4-3	130 bp containing a truncated promoter of *norA* cloned upstream of the *blaZ* gene of pWN2018	This study
pBF15-5	110 bp containing a truncated promoter of *norA* cloned upstream of the *blaZ* gene of pWN2018	This study
pBF16-4	2.4-kb PCR product containing the *arlR-arlS* locus cloned into pGB2 + pE194	This study
pBF17	2.4-kb PCR product containing the *arlR-arlS* locus cloned into pGB2 + pSK265	This study

[a] Resistance determinants: Ap, ampicillin; Sm, streptomycin; Cm, chloramphenicol; Em, erythromycin.

arlR-arlS locus in pGB2 was cut with *Pst*I and introduced into the *Pst*I site of plasmid pSK265, which has the *S. aureus* replicon of pC194 to give pBF17, or into the *Pst*I site of plasmid pE194 to give pBF16-4 (Table 1).

These plasmids were introduced into *S. aureus* RN4220, a restriction-deficient strain, by electroporation before being introduced into the derivatives of strain ISP794.

DNA manipulations. Plasmid DNA isolation was performed using the Qiagen midiprep kit. *S. aureus* was transformed with plasmid DNA by electroporation (11). Chromosomal DNA from *S. aureus* was prepared as described previously (34).

Enzyme assays. In order to measure *norA* promoter activity, cells containing different plasmids in which the *norA* promoter controls β-lactamase expression were grown in TSB at 37°C to an OD_{600} of 0.9. The whole culture was assayed for β-lactamase activity using nitrocefin as a substrate as described by Ji et al. (16), except that incubation was done at room temperature. β-Lactamase activities are expressed in micromoles of nitrocefin hydrolyzed per hour per gram of

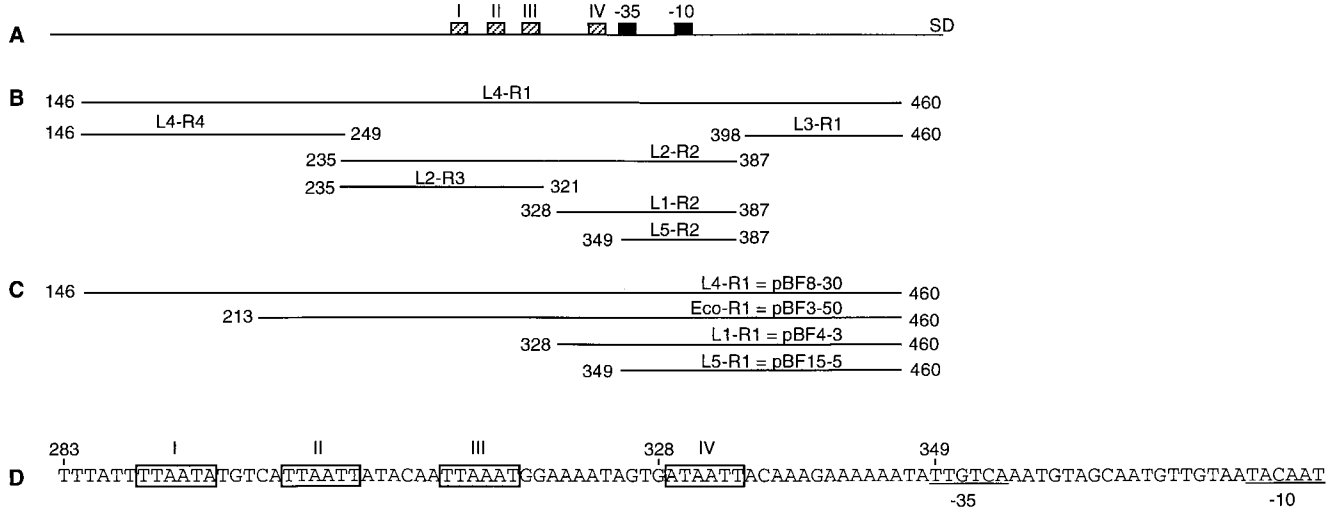

FIG. 1. Maps of the different DNA segments of the *norA* promoter examined in this study. The numbers indicate the nucleotide positions according to Yoshida et al. (38). (A) Schematic map of the *norA* promoter. The −35 and −10 consensus sequences are indicated by black boxes, and the Shine-Dalgarno site is marked SD. The repeated sequences, shown in panel D, are indicated by hatched boxes. (B) PCR fragments used in band shift experiments. (C) Schematic map showing the DNA cloned upstream of the β-lactamase gene in transcriptional fusions. (D) Sequence of the region upstream of the −35 consensus sequence. Repeated sequences are boxed, and the −35 and −10 sequences are underlined.

cell protein. Assays of chloramphenicol acetyltransferase (CAT) activity were used as a control for the copy number of the fusion plasmids. Crude extracts were prepared by lysis with lysostaphin (80 μg/ml) for 30 min at 37°C, and CAT activity was determined as previously described (26). Protein concentrations were determined by the Bradford method (Bio-Rad).

Preparation of cell-free extracts. Cell-free extracts were prepared as previously described with some modifications (22). Cells (OD_{600} = 0.9) were washed once in buffer A (20 mM Tris-HCl, 50 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol) and frozen at −70°C overnight. The pellet was suspended in 10 ml of buffer A, containing 0.1 mg of lysostaphin per ml, and incubated 3.5 h on ice. The suspension was frozen overnight at −70°C. After thawing on ice, 6 ml of buffer A containing 1.3 M KCl was added and incubated on ice for 30 min. The bacterial lysate was left 30 min at room temperature before centrifugation at 40,000 × *g* for 30 min to remove debris. The supernatant was dialyzed 3 h against water and frozen at −70°C.

Gel mobility shift analysis. A gel electrophoresis DNA mobility shift assay was used to identify DNA-binding proteins. DNA fragments were synthesized by PCR (Fig. 1B). One of the primers in each reaction was labeled with [γ-^{32}P]ATP using polynucleotide kinase.

Radiolabeled DNA fragments (20,000 counts/min/reaction) were incubated with the indicated amount of protein extract from *S. aureus* in 10 μl of binding buffer (10 mM HEPES [pH 8.0], 60 mM KCl, 4 mM $MgCl_2$, 0.1 mM EDTA [pH 8.0], 0.1 mg of bovine serum albumin (BSA) per ml, 0.25 mM dithiothreitol) containing 1 μg of poly(dI-dC), 200 ng of sheared herring sperm DNA, and 10% glycerol as previously described (13). In the case of the purified protein, 100 ng of poly(dI-dC) and 5% glycerol were used in addition to the binding buffer. The reaction mixture was incubated 15 min at room temperature and analyzed by 5% nondenaturing polyacrylamide electrophoresis.

Purification of the protein binding to the *norA* promoter. DNA fragment L2-R2 (150-bp) (Fig. 1B) was generated by PCR using a purified biotinylated primer (Gibco BRL), separated by agarose gel electrophoresis, and after cutting out the band, purified by QiaQuick (Qiagen). Nine micrograms of DNA was immobilized on 2 mg of magnetic beads with covalently coupled streptavidin (Dynabeads M-280; Dynal) according to the manufacturer's protocol. As previously described (22), DNA bound to beads was incubated with 200 μg of protein extract in 800 μl of binding buffer containing 600 μg of herring sperm DNA per ml for 15 min at room temperature. Beads were washed once with binding buffer containing 5 mg of herring sperm DNA per ml without BSA and twice with binding buffer without BSA. Proteins were eluted in 100 μl of binding buffer containing 0.5 M NaCl. Two different elutions were pooled, dialyzed against water for 1 h, and concentrated in a Speed-Vac evaporator. The samples were separated on a sodium dodecyl sulfate (SDS)–11% polyacrylamide gel. Proteins were detected by silver staining (Bio-Rad).

RESULTS

Effect of inactivation of *arlS* on *norA* expression. In order to find loci involved in the regulation of *norA*, we used a library of Tn*917*LTV1 insertions in the chromosome of strain MT23142 using selection for higher and lower levels of resistance to tetraphenylphosphonium bromide, a substrate of NorA. MT23142 carries the *flqB* mutation. *flqB*, a *cis*-acting mutation of *norA*, is localized downstream of the initiation start site of *norA* and overexpresses *norA* (24). The mutant BF15 showed a slight increase of resistance to tetraphenylphosphonium bromide and contained a Tn*917*LTV1 insertion in the *arlS* gene from the *arlR-arlS* locus (Fournier and Hooper, unpublished). The *arlR-arlS* locus codes for a two-component regulatory system. The protein ArlR has strong similarity to response regulators from the PhoB-OmpR family, and ArlS has similarity to protein histidine kinases (Fournier and Hooper, unpublished).

To determine the effect of chromosomal *arlS* and *flqB* mutations on *norA* expression, plasmid pBF8-30 carrying the full promoter region of *norA* fused to *blaZ* was introduced into strains ISP794 (wild type), MT23142 (*flqB*), and BF15 (*arlS*). β-Lactamase activity was increased 2.6-fold in BF15 relative to ISP794 (Table 2), but there was no difference in activity between MT23142 and ISP794 (data not shown). The difference of 2.6-fold of *norA* expression between ISP794 and BF15 (Table 2) was obtained with a culture grown until an OD_{600} of 0.9. When the culture was grown until an OD_{600} of 1.5, the β-lactamase activity was 5,900 U of β-lactamase per g of proteins for ISP794 and 36,600 U of β-lactamase per g of proteins for BF15, indicating that for the *arlS* mutant (BF15) *norA* expression was sixfold higher than that of the wild-type strain (ISP794) during early stationary phase. In order to verify that the β-lactamase activity increase was not due to differences in plasmid copy number, CAT activity of the fusion plasmid was determined, and no differences were observed in the three strains (data not shown). Introduction of plasmid pBF16-4 carrying the *arlR-arlS* locus into BF15 (pBF8-30) decreased the expression of *norA* seen in BF15 (Table 2), whereas introduction of the same plasmid into ISP794 (pBF8-30) did not modify *norA* expression (Table 2). Thus, the *flqB* mutation does not affect *norA* expression in *trans*, but disruption of *arlS* itself contributes to increased *norA* expression.

TABLE 2. Effect of the *arlS* deletion and truncated *norA* promoters on *norA* expression

Plasmid	Strain			
	ISP794		BF15	
	β-Lactamase activity[a]	Ratio[b]	β-Lactamase activity[a]	Ratio[b]
pWN2018	≤2,000		≤2,000	
pBF8-30	9,400 ± 330	1.0	24,300 ± 180	1.0
pBF8-30 plus pBF16-4	9,200 ± 146	1.0	16,700 ± 1430	0.7
pBF3-50	11,700 ± 390	1.2	25,500 ± 850	1.0
pBF4-3	9,510 ± 510	1.0	7,770 ± 420	0.3
pBF15-5	9,230 ± 810	1.0	10,700 ± 1540	0.4

[a] Results are expressed in units of β-lactamase per gram of proteins. All determinations were performed in triplicate.

[b] Ratios of β-lactamase activities with respect to that of the wild-type promoter in both strains.

An 18-kDa protein binds to the *norA* promoter. In order to determine how the *arlR-arlS* locus might control *norA* expression, we analyzed the protein(s) that binds to the *norA* promoter by gel mobility shifts using different DNA fragments. As seen in Fig. 2A, the first fragment L4-R1 (Fig. 1B) of 315 bp, containing the entire *norA* promoter from the Shine-Dalgarno sequence extending 200 bp upstream, exhibits several shifts with the protein extract from the wild-type strain ISP794. With increasing concentrations of protein, the intensity of the bands increased. Band shifts were reduced by increasing amounts of the unlabeled L4-R1 DNA but were not affected by nonspecific DNA, indicating that the protein(s) bound was specific to L4-R1 DNA. We then tested three separate DNA fragments, L4-R4, L2-R2, and L3-R1, which constituted separate domains of L4-R1 (Fig. 1B). The cell extract (2 μg of proteins) mixed with fragments L4-R4 and L3-R1 produced no band shifts (data not shown). In contrast, the cell extract mixed with fragment L2-R2 produced a band shift pattern identical to that of L4-R1 (Fig. 2B), indicating that the protein(s) binds to this region. The multiple bands seen in mobility shift assays suggested that L2-R2 DNA bound different numbers of protein molecules. Competition experiments with unlabeled specific and nonspecific DNA also confirmed that binding to L2-R2 was specific (Fig. 2B).

To localize further the site of protein binding, fragment L2-R2 was divided in two smaller fragments, L2-R3 and L1-R2 (Fig. 1B). Each of these fragments showed only two band shifts (Fig. 2C and D), in contrast to L2-R2, which exhibited at least five band shifts. These results indicate that either several different proteins bind to the fragment L2-R2 or the same protein binds in multiples to L2-R2. The specificity of the binding was again demonstrated by competition experiments (Fig. 2C and D). The last fragment tested, L5-R2, a smaller fragment of L1-R2 (Fig. 1B), did not show any shift when mixed with 2 μg

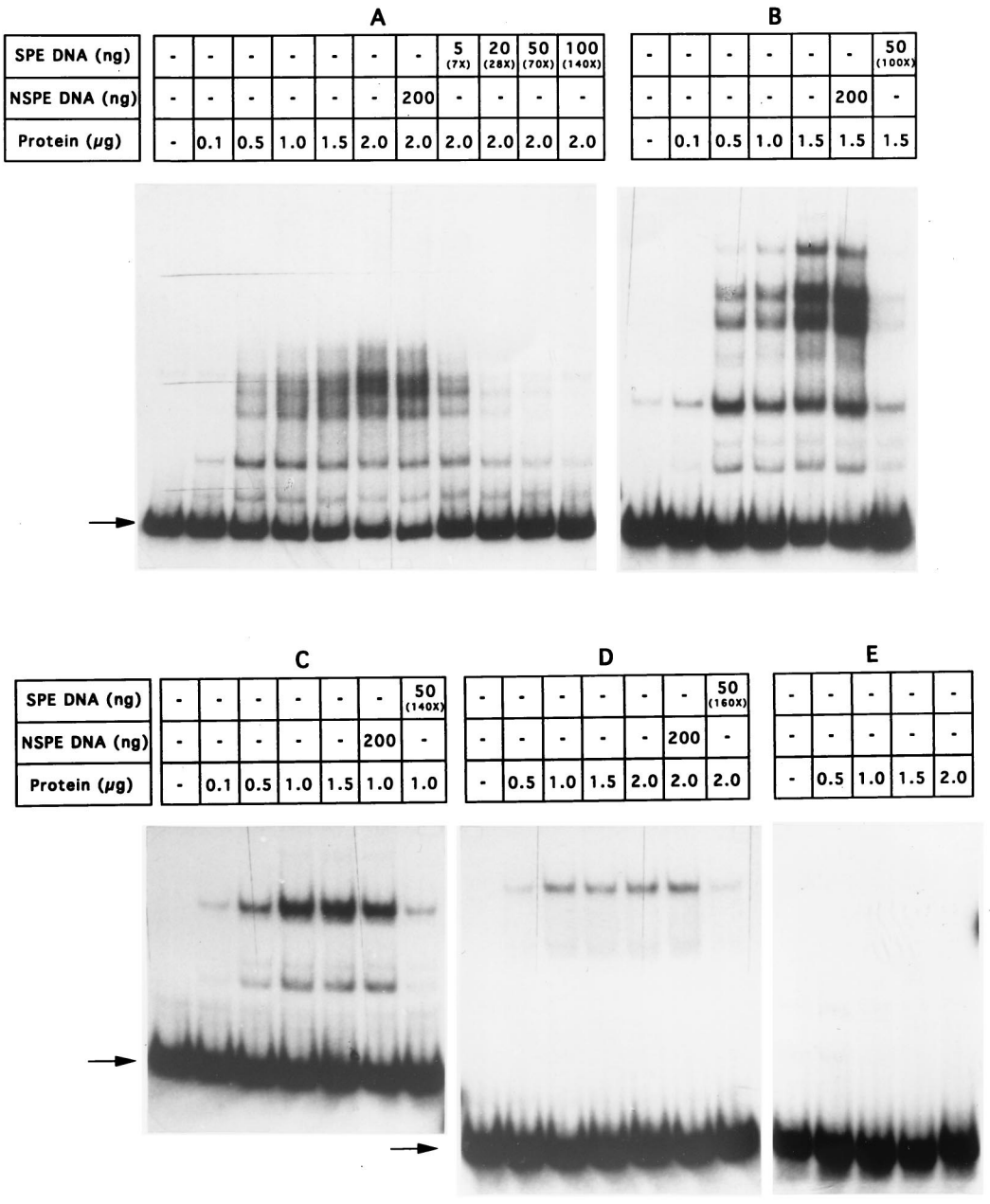

FIG. 2. Gel mobility shift analysis of the interaction of protein extracts from the wild-type strain ISP794 with different fragments of the *norA* promoter and the effect of unlabeled DNA. The radiolabeled fragment (arrow) was incubated with increasing amounts of protein extracts. The labeled fragments used in these experiments are L4-R1 (315 bp) (A), L2-R2 (153 bp) (B), L2-R3 (87 bp) (C), L1-R2 (60 bp) (D), and L5-R2 (39 bp) (E). The protein(s) binds to the tested fragment and retards its mobility (a different gel was used for each fragment). An unlabeled fragment of 350 bp amplified by PCR from a *Klebsiella oxytoca* promoter and an unlabeled fragment of the tested fragment serve as specificity control (NSPE DNA and SPE DNA, respectively). Protein and DNA concentrations and ratios of unlabeled fragments to labeled fragments used in this assay are indicated in the tables above the figures.

of protein extracts (Fig. 2E). Higher concentrations of protein extracts were needed to generate a shift of fragment L1-R2, in comparison to the fragment L2-R3, indicating weaker binding (Fig. 2C and D). Together, these results indicate that the protein binding site on L1-R2 is located between positions 328 and 349 (Fig. 1B).

In order to determine if the mobility shift was due to one or several proteins, we used magnetic beads coupled to the L2-R2 DNA fragment to isolate the bound protein(s). The crude extract of the wild-type strain ISP794 was adsorbed to these beads, and the bound protein(s) were eluted and separated by SDS-polyacrylamide gel electrophoresis (PAGE). A single 18-kDa protein was identified (Fig. 3A, lane 1). To confirm that only one protein species bound to the fragment L2-R2, we then performed the same experiment to capture protein bound by the two smaller fragments L2-R3 and L1-R2, and the same protein was found (Fig. 3A, lanes 2 and 3). To verify that this single protein was responsible for the multiple shifts observed for the fragment L2-R2, a band shift experiment was done using the eluted protein obtained from the fragment L2-R2. The same pattern of multiple band shifts was seen with the eluted protein as with the crude extract (Fig. 3B), indicating

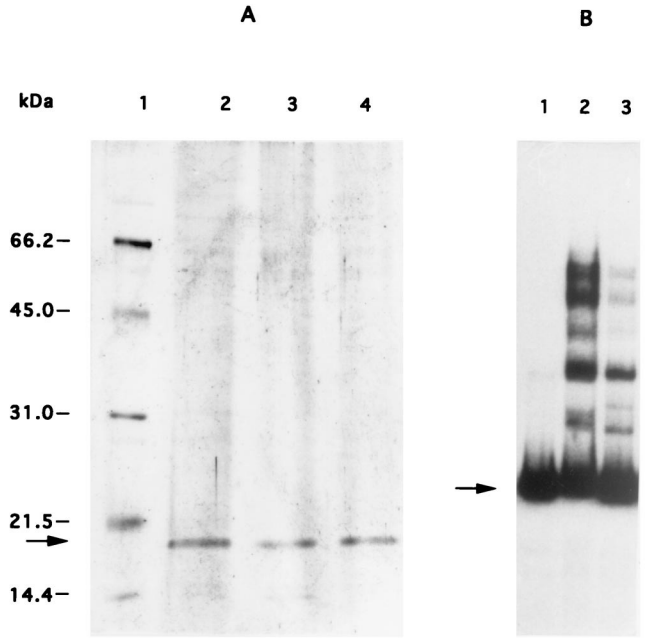

FIG. 3. Isolation of the protein from the wild-type strain ISP794 binding to different fragments of the *norA* promoter. Different fragments of DNA were immobilized on magnetic beads. Proteins binding to these fragments were then used for different analyses. (A) SDS-PAGE analysis of protein released from DNA affinity magnetic beads. Lane 1, standard proteins (in kilodaltons); lane 2, fragment L2-R2; lane 3, fragment L1-R2; lane 4, fragment L2-R3. The 18-kDa protein is indicated by an arrow on the left. (B) Gel mobility shift analysis of fragment L2-R2 with affinity-purified extracts from strain ISP794. Lane 1, control DNA without protein; lane 2, purified protein; lane 3, 0.5 μg of protein from crude extracts of ISP794. Free DNA is indicated by an arrow.

that this single protein was sufficient to generate the multiple band shifts.

The previous experiment suggested that several molecules of the same protein bound to multiple or at least two binding sites. In order to find the repeated sequence to which the 18-kDa protein bound, we analyzed the sequence between nucleotides 235 and 349 and found four repeated sequences (boxes) (Fig. 1D). The consensus sequence of these boxes was TTAATT. The fourth putative box (ATAATT) was present between nucleotides 328 and 349 (Fig. 1D), consistent with our data indicating that a binding site was located between 328 and 349. Fragment L2-R3 contained three putative boxes, a finding that correlates with the stronger binding of the 18-kDa protein to this fragment relative to fragment L1-R2, which contains only one box (Fig. 2C and D). Footprinting experiments will be necessary to confirm that these boxes are the binding sites of the 18-kDa protein.

In order to understand further the role of upstream DNA sequences shown to be involved in binding of the 18-kDa protein in *norA* expression, we constructed transcriptional fusions encompassing varying sequences of the upstream region of the *norA* promoter (Fig. 1C). These plasmids were introduced into ISP794 (wild type) and BF15 (*arlS*). In ISP794, little or no difference in β-lactamase expression was observed between the different truncated promoters (pBF3-50, pBF4-3, and pBF15-5) and the complete promoter (pBF8-30) (Table 2). In contrast, in the *arlS* mutant BF15 expression of β-lactamase from plasmids pBF4-3 and pBF15-5 was reduced by 70 and 60%, respectively (Table 2). The truncated promoter of pBF4-3 corresponds to the fragment L1-R2 used for the band shift experiments (Fig. 2D). Thus, the increase in *norA* expression in the *arlS* mutant is dependent on sequences between nucleotides 213 and 328 (Fig. 1C). Since binding of the 18-kDa protein to L2-R3 sequences contributes to full binding pattern of the larger L2-R2 DNA fragment and removal of the L2-R3 sequence reduces *norA* expression, it is possible that binding of this protein modulates *norA* expression.

Binding of the 18-kDa protein is modified in the *arlS* mutant. To study further the effect of *arlR-arlS* on *norA* expression, gel mobility shift experiments were done with crude extracts of the *arlS* mutant (BF15). As seen in Fig. 4B and 5B, extracts from the *arlS* mutant gave a band shift pattern different from that of the wild-type strain ISP794 (Fig. 4A). The first band was identical to that of the wild type, whereas the three other bands migrated slightly differently. When we complemented the *arlS* mutant with the plasmid pBF17 encoding *arlR-arlS*, the band shift pattern became identical to that of the wild type (Fig. 4C). In addition, the amount of shifted bands was consistently lower with extracts containing identical amounts of total protein from BF15 in comparison to BF15 (pBF17) and ISP794 (Fig. 4). Using magnetic beads to which the L2-R2 fragment (Fig. 1B) was coupled, we isolated the 18-kDa protein from the wild-type strain and from the *arlS* mutant (Fig. 5A). A band shift experiment with the protein eluted from the *arlS* mutant gave a pattern similar to that of the crude extract (Fig. 5B), indicating that the 18-kDa protein is present in the wild-type strain and in the *arlS* mutant. However, the pattern and extent of binding of this protein to the *norA* promoter is modified in the *arlS* mutant.

The *arlR-arlS* locus alters the growth-phase regulation of NorA. In *S. aureus*, regulation of many proteins is affected by growth phase (30). To analyze the effect of growth phase on *norA* expression, an overnight culture was diluted 1/50 in TSB, and every half hour, OD_{600} and β-lactamase activity were determined. The ratio of β-lactamase activity/OD_{600} as an estimate of specific activity was then calculated. For the parental strain, β-lactamase-specific activity decreased throughout the logarithmic phase (Fig. 6). For the *arlS* mutant, the β-lactamase-specific activity was over twofold higher than that of the parental strain and also decreased during logarithmic phase (Fig. 6). In contrast to the parental strain, the *arlS* mutant exhibited a plateau and slight rebound in β-lactamase-specific activity as early stationary phase was entered. Thus, growth-phase regulation of *norA* expression is also altered in the *arlS* mutant.

Because growth-phase regulation of protein expression is mediated by the *agr* and *sar* loci (30), we evaluated the effects of mutations in these loci on *norA* expression. We introduced the plasmid pBF8-30 in wild-type strain RN6930, *agr* (RN6911), *sar* (ALC136), and *agr sar* (ALC135) isogenic mutants. β-Lactamase activity of mutant cells was similar to that of the wild-type strain (data not shown).

DISCUSSION

Here we have shown that the *arlR-arlS* locus encoding an apparent two-component regulatory system is involved in the expression of the multidrug efflux pump NorA and in the binding of an 18-kDa protein to the *norA* promoter region.

The 18-kDa protein binding to the *norA* promoter does not appear to have any effect on *norA* promoter expression under normal growth conditions in the wild-type strain, whereas a modified pattern of its binding is associated with increased promoter expression when *arlS* is disrupted (Table 2). In the wild-type strain, the 18-kDa protein might function as a regulator that is activated in the presence of increased concentrations of a putative inducer. In the *arlS* mutant, several hypotheses could be considered to explain increased expression of

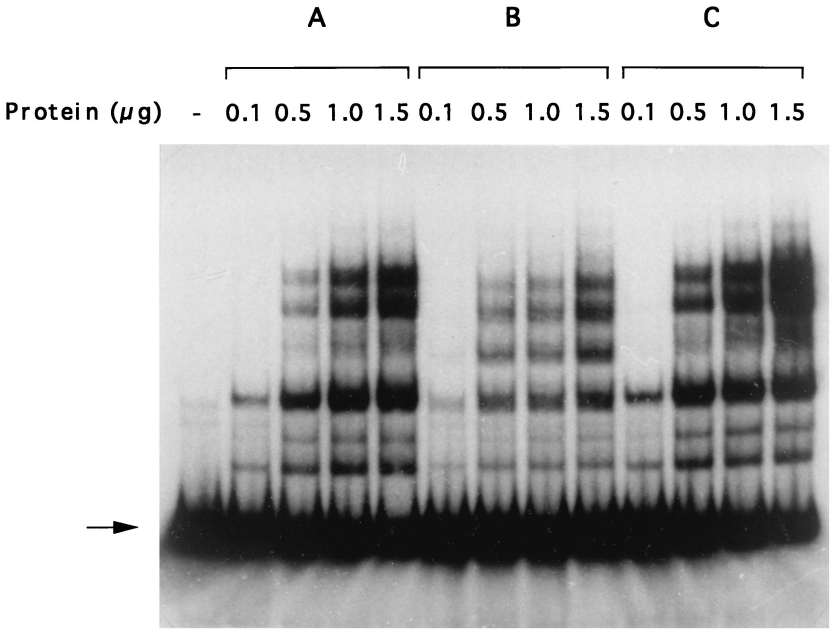

FIG. 4. Gel mobility shift analysis of the interaction of the protein extracts from different strains with the complete *norA* promoter. The radiolabeled fragment L2-R2 (arrow) was incubated with increasing amounts of protein extracts. The protein(s) binds to the tested fragment and retards its mobility. Lanes: A, strain ISP794; B, *arlS* mutant BF15; C, *arlS* mutant BF15 containing pBF17.

norA. First, the *arlR-arlS* locus might directly control NorA. Because the 18-kDa protein modified its binding when *arlS* was disrupted, the protein that binds to the *norA* promoter could be ArlR, the response regulator of the *arlR-arlS* locus. ArlR-ArlS appears to constitute a two-component regulatory system (Fournier and Hooper, unpublished), such as those that mediate adaptative responses of bacteria to their environment. These systems are composed of a transmembrane sensor (histidine protein kinase) and its associated response regulator (35). In general, the transmembrane protein binds a specific ligand, the signal, and autophosphorylates at a conserved histidine residue. The phosphorylated sensor then relays the phosphate to aspartic residues in the response regulator (28). The response regulator can in turn stimulate or repress target genes at the level of transcription. The protein ArlR belongs to the PhoB-OmpR group. These regulators are known to bind a region upstream of the promoter of their target genes and to

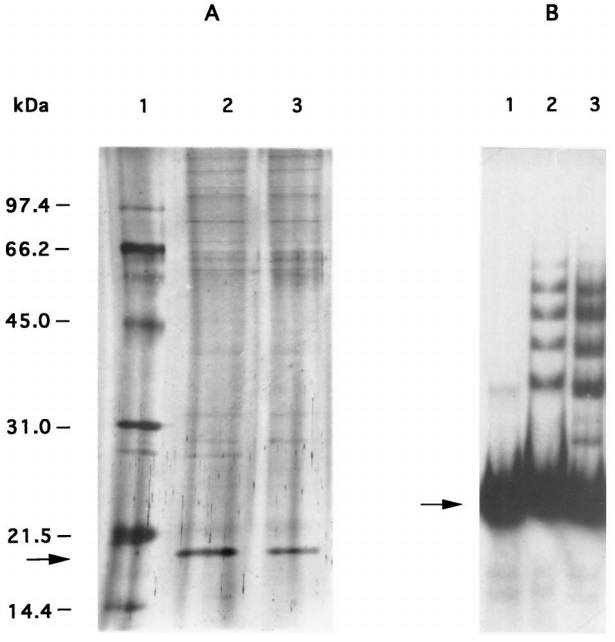

FIG. 5. Isolation of the protein from the mutant BF15 binding to the fragment L2-R2. (A) SDS-PAGE analysis of protein released from affinity-purified extracts from different strains. Lane 1, standard proteins (in kilodaltons); lane 2, purified protein from ISP794; lane 3, purified protein from BF15. The 18-kDa protein is indicated by an arrow on the left. (B) Gel mobility shift analysis of fragment L2-R2 with affinity-purified protein extracts from BF15 and fragment L2-R2. Lane 1, control DNA without protein; lane 2, purified protein; lane 3, 0.5 μg of protein from crude extracts of BF15. Free DNA is indicated by an arrow.

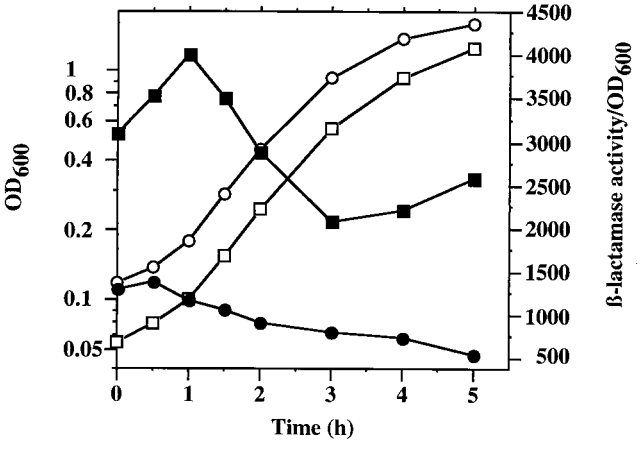

FIG. 6. Effect of the *arlS* mutation on NorA regulation during the growth. The parent strain MT23142 (circles) and the *arlS* mutant BF15 (squares) containing the plasmid pBF8-30 were grown at 37°C. The ratio of β-lactamase/OD_{600} was calculated as an estimate of specific activity. Open symbols indicate OD_{600}, and solid symbols indicate the ratio of β-lactamase activity/OD_{600}.

modify gene expression (e.g., PhoB in the phosphate regulon [21] or OmpR in the porin regulon [19, 37] of *E. coli*). The pattern of band shift from the purified 18-kDa protein from the wild-type strain and the *arlS* mutant was similar to that from the crude extract from each. Although we cannot rule out the purification of a complex protein inducer, this suggests that the difference in the pattern of band shift was due to the protein itself and not to another component present in the crude extract. As a protein histidine kinase, ArlS likely phosphorylates the response regulator ArlR. This phosphorylation might modify the binding of the regulator (2). In the case of OmpR, phosphorylation by EnvZ (31) modifies OmpR binding to the promoter of its target gene *ompF* (37). Moreover, response regulators such as PhoB or OmpR often have one or several consensus sequences that function as regulator-binding sites upstream of the promoter (20, 35, 37), such as the putative TTAATT boxes associated with binding of the 18-kDa protein. We can speculate that, in the absence of ArlS, ArlR is not phosphorylated and that its binding to the *norA* promoter as well as *norA* expression is modified. In such a case, removal of the binding sites of the regulator protein would also modify *norA* expression (Table 2). Together, these studies suggest that the protein binding to the *norA* promoter could be the response regulator ArlR. However, response regulators such as ArlR are dephosphorylated due to an autophosphatase activity. The half-lives of hydrolysis of phosphoaspartate groups in regulator proteins at neutral pH and ambient temperature range from only a few seconds to several hours, with most exhibiting intermediate values of several minutes (36). Therefore, it seems unlikely that ArlR remained phosphorylated throughout the complete DNA affinity purification, which lasted at least 3 h. Furthermore, the size of ArlR predicted from its amino acid sequence is 25.5 kDa (Fournier and Hooper, unpublished), rather than the 18 kDa observed for the protein binding to the *norA* promoter. If ArlR is itself directly involved in modulation of *norA* expression by binding to the promoter region, then additional processing of ArlR must have occurred.

A second possibility is that the 18-kDa protein is phosphorylated by ArlS. Cross talk may result from cross-specificities in which sensors of similar sequence phosphorylate nonpartner regulators (40). For example, the histidine kinase CheA can phosphorylate the Ntr transcription factor NR1 (25). We can speculate that the 18-kDa protein not derived from ArlR is directly phosphorylated by ArlS.

Finally, the *arlR-arlS* locus might affect *norA* expression indirectly by modifying another gene affecting the activity of the 18-kDa protein (for example, the gene producing the physiological inducer of NorA). In such a case, the 18-kDa protein would bind differently to the *norA* promoter in the presence and in the absence of the inducer. For the related efflux pump Bmr, its regulator BmrR binds to the *bmr* promoter and enhances expression in the presence of some inducing substrates. It has been shown that the Bmr substrates that induce Bmr expression interact directly with BmrR (1). The binding of inducers to its C-terminal domain converts BmrR into an activator of transcription from the *bmr* promoter. This activation is likely to occur through untwisting of the spacer region of the promoter, which serves as the BmrR-binding site. This untwisting leads to proper positioning of the promoter motifs binding RNA polymerase and thus initiates transcription (41). Recently, it has been shown in *B. subtilis* that the two MDR pumps, Bmr and Blt, that have high similarity with NorA, are regulated by a global transcriptional activator, Mta, a member of the Mer family of bacterial regulatory proteins. Thus, these pumps are controlled by specific transcriptional activators, BmrR and BltR, and by a global regulator, Mta. The individually expressed N-terminal DNA-binding domain of Mta interacts directly with the promoters of *bmr* and *blt* and induces transcription of these genes (3). Since no regulator gene was found around *norA*, we can speculate that *norA* is controlled only by the 18-kDa protein that could be a global regulator. Moreover, we found another mutant, MT1222, which also modifies *norA* expression and for which no modification was found in the *arlR-arlS* locus, indicating that an additional locus is also involved in the *norA* regulation (Fournier and Hooper, unpublished). Thus, the mutant locus of MT1222 could represent the gene encoding the protein binding to the *norA* promoter. The identity of the 18-kDa protein that binds to the *norA* promoter will be further studied.

The expression of *norA* is affected by the growth phase (Fig. 6). *norA* expression appears to increase during early logarithmic phase followed by a decrease during late logarithmic and early stationary phases. Further decrease in expression occurs in stationary phase since the supernatant culture medium from an overnight culture (mixed 50% with TSB) decreases β-lactamase activity of ISP794 (pBF8-30) twofold compared to that of late logarithmic phase (data not shown). Thus, a component secreted by *S. aureus* in the medium acts directly or indirectly to reduce *norA* expression in different phases of growth.

Because the *arlR-arlS* locus, which affects *norA* expression, is involved in autolysis of *S. aureus* (Fournier and Hooper, unpublished), we can speculate that NorA is perhaps also involved in autolysis and protects the cell by removing autolysins or products from autolysis, which would be toxic if allowed to accumulate. In *S. aureus*, another two-component regulatory system, *lytS-lytR*, is also involved in autolysis (5) and regulates a gene, *lrgA*, encoding a protein showing characteristics in common with the bacteriophage murein hydrolase transporter family of proteins known as holins (6). As some murein hydrolases lack N-terminal signal sequences, it has been speculated that holin-like proteins might be involved in the export of bacterial murein hydrolases (10). However, Triton X-100- or penicillin-induced autolysis does not stimulate *norA* expression in the wild-type strain (data not shown), and the *norA* mutant KLE820 had a rate of Triton X-100-induced autolysis similar to that of its parent strain RN4220 (data not shown). A third two-component regulatory system *agrC-agrA* and another related locus, *sar*, are also involved in autolysis in *S. aureus* (12). The *agr* and *sar* loci regulate other cellular functions: synthesis of extracellular toxins and enzymes (i.e., alpha-toxin, beta-hemolysin, enterotoxins, lipases, proteases, etc.) and synthesis of cell-surface proteins (protein A, fibronectin-binding protein, capsular polysaccharide type 5, and coagulase) (15, 27, 30), indicating the multiplicity of functions of the two-component regulatory systems. *norA* expression in *agr* and/or *sar* mutants was similar to that in the wild-type strain, indicating that neither *agr* and *sar* loci nor cellular functions controlled by these loci modified *norA* expression. Thus, we can speculate that the *arlR-arlS* locus might regulate other physiological functions that affect the native substrate of NorA.

Our findings identify for the first time several components likely involved in the complex regulation of *norA* expression, including a two-component regulatory system, ArlR-ArlS, and specific binding of an 18-kDa protein to the *norA* promoter. Substances accumulating in the medium of stationary-phase cells may act through these and other regulatory elements. Further studies will be required, however, to identify the 18-kDa protein, which binds to the *norA* promoter. Nevertheless, our findings of *norA* regulation by a two-component regulatory system open a new avenue for investigation of the molecular

mechanisms of the multidrug efflux pumps, their regulation, and their physiological role.

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