JOURNAL OF BACTERIOLOGY, July 2003, p. 3703–3710 Vol. 185, No. 13
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.13.3703–3710.2003

mgr, a Novel Global Regulator in *Staphylococcus aureus*

Thanh T. Luong, Steven W. Newell,† and Chia Y. Lee*

Department of Microbiology, Molecular Genetics and Immunology, University of Kansas Medical Center, Kansas City, Kansas 66160

Received 10 February 2003/Accepted 10 April 2003

The virulence determinants of *Staphylococcus aureus* are coordinately controlled by several unlinked chromosomal loci. Here, we report the identification of CYL5614, derived from strain Becker, with a mutation that affects the expression of type 8 capsular polysaccharide (CP8), nuclease, alpha-toxin, coagulase, protease, and protein A. This novel locus, named *mgr*, was linked by transposon Tn*917* and mapped by three-factorial transduction crosses. The region containing the *mgr* locus was cloned and sequenced. Deletion mutagenesis and genetic complementation showed that the locus consisted of one gene, *mgrA*. Interestingly, *mgrA*-null mutants exhibited a phenotype opposite to that of CYL5614. This was due to a T-to-C mutation upstream of *mgrA* that resulted in a four- to eightfold increase in *mgrA* transcription in strain CYL5614. Thus, these results indicate that *mgrA* is an activator of CP8 and nuclease but a repressor of alpha-toxin, coagulase, protease, and protein A. In addition, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses showed that the *mgr* locus profoundly affected extracellular protein production, suggesting that the locus may regulate many other genes as well. The translated MgrA protein has a region of significant homology, which includes the helix-turn-helix DNA-binding motif, with the *Escherichia coli* MarR family of transcriptional regulators. Northern slot blot analyses suggested that *mgr* affected CP8, alpha-toxin, nuclease, and protein A at the transcriptional level.

Staphylococcus aureus is an important human pathogen responsible for a wide range of diseases. The pathogenicity of the organism is largely determined by its ability to coordinately produce a plethora of extracellular toxins, enzymes, and surface antigens under various environmental conditions. Recently, several regulatory loci which globally affect the expression of many of these virulence genes have been identified. Among these global regulators, *agr* and *sarA* have been studied most extensively.

agr is a complex quorum-sensing regulatory system consisting of two divergent transcriptional units, P2 and P3. The P2 operon contains four genes, *agrBDCA*, of which the *agrBD* genes are involved in the production and export of an autoinducing peptide. As the cell density increases to a certain level, the accumulated peptide activates, through the two-component system encoded by *agrCA*, both the P2 and P3 promoters. The P3 operon encodes an RNA effector, RNAIII, which then regulates the target genes (reviewed in reference 21). RNAIII has been shown to control target gene expression largely at the transcriptional level; however, the mechanism is unknown. At the translational level, RNAIII has been shown to regulate alpha-toxin by an antisense mechanism (21).

The *sarA* locus consists of three overlapping transcripts initiating from three different promoters but terminating at a common 3′ end. All three transcripts contain the major open reading frame (ORF), *sarA*, within the overlapping region. The SarA protein has been shown to be required for activating transcription of the P2 and P3 operons of the *agr* locus by binding to the *agr* promoters. Although *sarA* seems to work in concert with the *agr* system, it can also regulate some genes independently. The direct binding of SarA to a consensus motif with a long stretch of AT upstream of the −35 sequence of the promoter is required for regulation of its target genes (5, 21).

Several *sarA* homologs have been identified in the *S. aureus* chromosome. Studies of two of the homologs, *sarH1* (also known as *sarS*) and *sarR* showed that they were involved in *agr*/*sarA* regulation. The *sarH1* gene has been shown to activate *spa* (protein A) in an *agr*-dependent manner and to repress *hla* (alpha-toxin) in a *sarA*-dependent manner (4, 32). The *sarR* gene has recently been shown to repress *sarA* by binding to all three *sarA* promoters (18). Two other *sarA* homologs, *sarU* and *sarT*, have been shown to affect RNAIII production, indicating that they are involved in the *agr*/*sarA* regulatory system as well (19, 27). Another *sarA* homolog, *rot*, was shown to have global regulatory effects on many genes and was thought to regulate some genes downstream of *agr* in the global regulatory network (20, 23).

Besides the *agrCA* two-component system, a number of two-component regulatory systems have also been shown to be involved in global regulation. These include *sae* (8), *arl* (7), and *ssr* (33). The *arl* system has been shown to interact with both the *agr* and *sarA* loci, and the *ssr* system at least partially regulates its target through the *agr* locus. On the other hand, a *sae* mutation was shown not to affect the transcription of *agr* or *sarA*. While the *agr* system senses cell density, *ssr* has been shown to sense the oxygen level in the environment; however, no known environmental stimuli have been implicated in the *arl* and *sae* systems. In addition, the effect of the growth phase on global regulation has been shown to be through the alternative sigma factor σ^B (encoded by *sigB*), which modulates *agr*/*sarA* expression in a growth phase-dependent manner by repressing RNAIII and activating *sarA* (2).

In a study to identify genes that regulate capsule production,

* Corresponding author. Mailing address: Department of Microbiology, Molecular Genetics and Immunology, Room 3025, WHW, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160. Phone: (913) 588-7156. Fax: (913) 588-7295. E-mail: clee@kumc.edu.

† Present address: Naval Research Laboratory, Stennis Space Center, MS 39529.

TABLE 1. *S. aureus* strains and plasmid vectors

Strain or plasmid	Relevant characteristics	Reference
S. aureus		
Becker	CP8 strain	
RN4220	8325r$^-$	13
CYL5614	Becker::*cat mgr5614*; MgrA high producer	This study
CYL175	Becker::Tn*917*; Tn*917* linked to *mgr*	This study
CYL180	Becker::Tn*917*; Tn*917* linked to *mgr*	This study
CYL183	CYL5614::Tn*917*; Tn*917* linked to *mgr5614*	This study
CYL402	Becker::*cat402*	This study
CYL412	Becker::*cat412*	This study
CYL896	Becker Δ(SA639-640)::*cat*	This study
CYL978	Becker Δ*spa*	This study
CYL1040	Becker Δ*mgrA*::*cat*	This study
CYL1065	Becker ΔSA642::*cat*	This study
Plasmid vector		
pTV1	Tn*917* delivery vector	34
pCL52.2	Temperature-sensitive cloning vector	26
pLL28	Temperature-sensitive cloning vector	17
pCL95	Cloning vector	10
pCL83	Single-copy cloning vector	15

we found a mutant that affected not only the production of type 8 capsular polysaccharide (CP8) but also that of protein A, alpha-toxin, nuclease, lipase, protease, and coagulase. The phenotypic changes caused by this mutation were different from those caused by mutations in other global regulatory loci. We named this locus *mgr* for multiple gene regulator. In this report, we describe the mapping, cloning, and inactivation of this new locus. Our results showed that *mgr* is a new global regulatory locus that contains only one regulatory gene, *mgrA*, which shares a helix-turn-helix motif with several bacterial transcriptional regulators.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. *Escherichia coli* strain XL1-Blue was used as the host strain for plasmid constructions. The *S. aureus* strains and plasmid vectors used in this study are listed in Table 1. *S. aureus* RN4220 (13) was used as the recipient for plasmid electroporations. *S. aureus* strains were cultivated in Trypticase soy medium (Difco Laboratories, Detroit, Mich.). *E. coli* strains were routinely cultivated in Luria-Bertani medium (Difco). Electroporation was carried out by the procedure of Kraemer and Iandolo (12). Plasmid transduction using phage 52A was carried out as previously described (28).

Phenotypic characterization. CP8 production was determined as described before (17) with the following modification. Two milliliters of an 18-h culture adjusted to an optical density at 660 nm (OD_{660}) of 5.0 were pelleted and resuspended in 100 μl of phosphate-buffered saline. The suspension was treated consecutively with the following enzymes at 37°C: 100 μg of lysostaphin/ml for 15 min, 300 U of DNase I/ml for 15 min, and 100 μg of proteinase K (Sigma, St. Louis, Mo.)/ml for 1 h. The proteinase K was subsequently inactivated at 75°C for 10 min. The crude CP8 preparations were assayed by the immunoblotting method as described previously (17).

Crude extract of protein A was prepared in the same way as that of CP8 except that proteinase K treatment was omitted. Serial dilutions of the extract were blotted onto nitrocellulose membranes using a dot blot or slot blot apparatus (Bio-Rad). The membranes were incubated with a horseradish peroxidase-conjugated rabbit anti-guinea pig immunoglobulin G for 1 h, washed, and developed by color reagent (Bio-Rad). A semiquantitative assay of protein A was performed by immunoblot assay of colonies on agar plates.

Coagulase production was assayed by measuring the coagulation activities of equal-volume mixtures of whole-cell cultures and twofold serial dilutions of rabbit plasma (Difco). DNase production was assayed by reduction of the OD_{260} upon incubation of herring sperm DNA with culture supernatant as described by Smeltzer et al. (29). Alpha-toxin was assayed by Western blotting of the crude cell extracts using affinity-purified anti-alpha-toxin antibody (Toxin Technology, Sarasota, Fla.) and donkey anti-sheep antibody– horseradish peroxidase conjugate (Sigma). The semiquantitative assay of alpha-toxin was performed by streaking cultures on sheep blood agar plates. The protease assay was performed essentially as described previously (29).

DNA manipulations. Standard DNA manipulations were carried out as described by Sambrook et al. (24). Rapid small-scale plasmid DNA isolation was performed according to the procedure of Holmes and Quigley (9). Qiagen (Chatsworth, Calif.) DNA isolation kits were used for general plasmid purification. Bulk chromosomal DNA from *S. aureus* was isolated using a chromosomal DNA purification kit (Promega, Madison, Wis.). The enzymes used in DNA manipulation were obtained from GIBCO-BRL (Gaithersburg, Md.), New England Biolabs, Inc. (Beverly, Mass.), or Promega Corp. PCR amplification was carried out with the Advantage cDNA PCR kit (Clontech, Palo Alto, Calif.). The transfer of DNA to nitrocellulose membranes was performed according to the method of Southern (30).

RNA extraction and Northern hybridization. A Blue FastRNA kit (Bio 101, Inc., Vista, Calif.) was used in RNA extraction (3). Northern hybridization was carried out as described by Sambrook et al. (24). Total RNAs were resolved in a 1% agarose gel containing formaldehyde and transferred to a nitrocellulose membrane by using a TurboBlotter (Schleicher & Schuell, Keene, N.H.). For Northern slot blotting, denatured total RNAs were blotted to nitrocellulose paper using a slot blotting apparatus. The conditions for hybridization and washing were previously described (29).

Cloning of Tn*917* flanking sequence by inverse PCR. Chromosomal DNA from strain CYL175 was digested with *Hpa*II, religated, and used as a template for inverse PCR amplification as described by Ochman et al. (22). Two inverse PCR primers (5′-CACAATAGAGAGATGTCACC-3′ and 5′-GCTATGCTCG AGTGAGTACG-3′), facing away from each other and located at the right end of the Tn*917* sequence, were used for PCR amplification. The amplified fragment was cloned into the pGEM-T vector (Promega) and sequenced.

Construction of chromosomal *cat* insertion by allele replacement. The basic strategy for allele replacement was described previously (26). In brief, upstream and downstream fragments (>0.5 kb) of the target region to be replaced were amplified by PCR and sequence verified or obtained by restriction digestion. The two fragments and the *cat* gene of pC194 were cloned into pCL52.2 or pLL28 in such a way that the *cat* gene was flanked by the two fragments in the same orientation as in the chromosome. The resultant plasmids were first electroporated into RN4220 and then transferred to strain Becker by phage transduction at 30°C. The strains containing the plasmids were subjected to temperature shift as described previously (26), and the desired mutants were verified by PCR or Southern hybridization.

RESULTS

Identification of a mutant with a pleiotropic phenotype. During the construction of chromosomal insertions in strain Becker using the chloramphenicol resistance gene (*cat*) of the

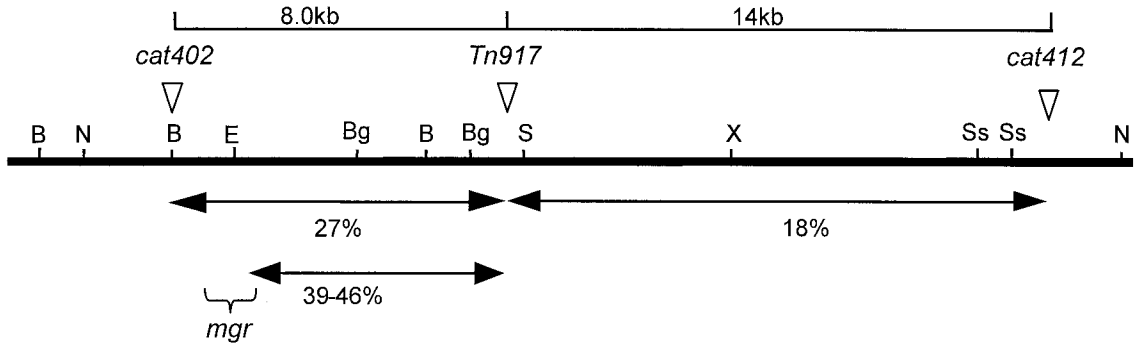

FIG. 1. Genetic mapping of the *mgr* locus. The insertion sites of Tn*917* (in CYL175) and *cat* (in CYL402 and CYL412) are indicated by triangles. The rates of cotransduction are indicated as percentages. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; N, *Nco*I; S, *Sal*I; Ss, *Sst*I; X, *Xba*I.

S. aureus plasmid pC194 by allele replacement to study the regulation of CP8, we unexpectedly isolated a mutant, CYL5614, that produced a larger amount of CP8 and smaller amounts of alpha-toxin and protein A than the wild-type strain (not shown). To determine whether the *cat* insertion in strain CYL5614 was responsible for the mutant phenotype, we transduced the *cat* insertion back to strain Becker. The mutant phenotype did not correlate with the insertion in the backcross experiments, indicating that the *cat* insertion is not responsible for the pleiotropic effect. Thus, these results indicate that another mutation(s) in the chromosome of CYL5614, which probably occurred spontaneously, is responsible for the phenotype. Since the phenotype is different from those of *agr*, *sarA*, *sae*, *ssr*, and *arl* mutants, it is most likely that the mutation(s) represents a new global regulatory locus. We named this locus *mgr* for multiple gene regulator and designated the mutation in CYL5614 *mgr5614*.

Linking the *mgr* locus by transposon Tn*917*. To clone the *mgr* gene(s), we screened a plasmid library of strain Becker for complementation of protein A (Spa) or alpha-toxin (Hla) (i.e., using Spa or Hla as the phenotype for *mgr*, since CYL5614 produced markedly reduced protein A and alpha-toxin). However, we failed to identify any positive clone. Since there is no selection for the *mgr* locus, we sought an alternative approach to clone the gene by linking transposon Tn*917* to *mgr* (11, 16). Random transposition was carried out by temperature shift using strain Becker harboring pTV1 (34), which contains Tn*917* on a thermosensitive replicon. The Tn*917* insertion library thus generated was transduced to strain CYL5614 by phage 52A, and the resulting Tn*917*-encoded erythromycin-resistant (Em^r) transductants were screened for Spa^+ colonies representing cotransduction of Tn*917* and *mgr*. The rationale for this experiment is that if Tn*917* inserts at a location close to the wild-type *mgr* locus in Becker, the two markers could then be cotransduced by phage to strain CYL5614. The closer the two markers are, the higher the percentage of the cotransduction will be. By screening ~25,000 transductants, we found that two, CYL175 and CYL180, produced wild-type levels of protein A, suggesting that these two strains have Tn*917* inserted near *mgr*. The two transductants also produced wild-type levels of CP8, indicating that the Tn*917* insertion indeed links to the *mgr* pleiotropic locus (data not shown). The Tn*917* insertions in CYL175 and CYL180 were transduced back to CYL5614, and the transductants were screened for protein A production. These backcross experiments showed that the cotransduction frequencies for Tn*917* insertion and *mgr5614* using CYL175 and CYL180 as the donors were 11 and 18%, respectively. However, when Hla (alpha-toxin) was used as a marker in later experiments, we found an ~43% link between *mgr5614* and Tn*917*. The probable reason for this discrepancy was that Spa was rather difficult to detect by the colony immunoblotting method, which resulted in underestimating the Spa^+ phenotype. These data indicate that we have isolated two strains with Tn*917* inserted near the *mgr* locus. The chromosomal DNAs from CYL175 and CYL180 were purified; digested with *Eco*RI, *Sau*3A, *Hin*dIII, and *Hpa*II; and subjected to Southern analysis using both halves of Tn*917* as probes. The Southern patterns of the two strains were the same, indicating that Tn*917* inserted at the same location in both strains (not shown).

The DNA sequence flanking one side of the Tn*917* insertion was obtained by inverse PCR amplification as described in Materials and Methods. An ~500-bp fragment (as expected from the Southern analysis) was obtained and cloned into the pGEM-T vector. Sequencing of the cloned insert revealed that the PCR fragment contained the expected Tn*917* sequences and a 444-bp Becker DNA sequence adjacent to the right side of the Tn*917* insertion site.

Fine genetic mapping of *mgr* by transduction. Since Tn*917* was inserted near the *mgr* locus, to map the position of *mgr5614* in reference to the Tn*917* insertion site by three-factor transduction and to subsequently clone the *mgr* gene(s), we need to isolate large DNA fragments encompassing this region of the chromosome. To this end, the 500-bp DNA fragment from the inverse PCR experiment was used as a probe to screen a cosmid library of strain Becker constructed in *E. coli* (25) using a low-copy-number cosmid vector, pLAFR3. Several cosmid clones with overlapping inserts spanning an ~55-kb region were obtained, and their restriction maps were determined. The partial composite map is shown in Fig. 1. In addition, the Tn*917* insertion site was mapped between the *Bgl*II and *Sal*I sites by Southern blotting.

To precisely map the *mgr* locus, we employed fine genetic mapping by three-factor transductional crosses. To perform such experiments, however, an additional marker is needed. To provide this new marker, we inserted the *cat* gene of pC194 into the chromosome of strain Becker ~8.0 kb to the left of the Tn*917* insertion site (of strain CYL175) by allele replacement,

TABLE 2. Transduction frequencies of three-factor crosses with CYL183 (Em^r Hla$^-$ Cm^s) as the donor[a]

Transduction (recipient)	Transductant phenotype	Frequency (%)
1 (CYL402 [Em^s Hla$^+$ Cm^r])	Em^r Hla$^+$ Cm^s	2
	Em^r Hla$^-$ Cm^s	25
	Em^r Hla$^+$ Cm^r	59
	Em^r Hla$^-$ Cm^r	14
2 (CYL412 [Em^s Hla$^+$ Cm^r])	Em^r Hla$^+$ Cm^s	9
	Em^r Hla$^-$ Cm^s	9
	Em^r Hla$^+$ Cm^r	55
	Em^r Hla$^-$ Cm^r	37

[a] Transductants were selected for Em^r, and 100 colonies were scored for Hla and Cm phenotypes.

which resulted in strain CYL402 (i.e., Becker::*cat402*). In addition, another strain, CYL412 (i.e., Becker::*cat412*), with a *cat* insertion ~14 kb to the right of the Tn*917* insertion (of strain CYL175), was also constructed. The *cat* insertion sites of both CYL402 and CYL412 are shown in Fig. 1. These two strains, which are phenotypically Em^s Hla$^+$ Cm^r, were then used as the recipients for the three-factor cross experiments described below. However, we did not have a donor strain with the Em^r Hla$^-$ Cm^s phenotype suitable for the transduction experiments. Therefore, we transduced Tn*917* from CYL175 (containing Tn*917* encoding Em^r) to CYL5614 with the selection of Em^r and then screened for the Hla$^-$ phenotype (i.e., the Mgr5614 phenotype). The resultant strain, CYL183, with the Tn*917* insertion and the *mgr5614* genotype, was used as the donor for the transduction experiments. It should be noted that CYL183 is in fact Cm^r due to a *cat* insertion in the chromosome of CYL5614. However, this *cat* insertion is unlikely to have interfered with our transduction experiments because the insertion is too far away to be cotransduced with Tn*917* (~630 kb apart, according to the published *S. aureus* genome sequence [14]).

After the donor and recipient strains were constructed, genetic crosses were carried out by phage 52A transduction from CYL183 to CYL402 and CYL412 with Em^r selection. The transductants were then scored for Hla and Cm phenotypes. The results in Table 2 show that the frequency of cotransduction for all three markers, *mgr5614*, Tn*917*, and *cat402*, was 25% (Em^r Hla$^-$ Cm^s phenotype [row 2 in transduction 1]). In contrast, the frequency of cotransduction for *mgr5614*, Tn*917*, and *cat412* was 9% (row 2 in transduction 2). These results indicate that *mgr* is closer to *cat402* than to *cat412*, suggesting that *mgr* is located to the left of Tn*917*. To determine the order of *mgr5614*, *cat402*, and Tn*917*, we examined the data in Table 2, transduction 1. The fact that the frequency of Em^r Hla$^+$ Cm^s transductants was the lowest (2%) among the four possible phenotypes indicates that obtaining transductants with this phenotype (i.e., crossover of Tn*917* and *cat* but not *mgr5614* markers) requires the most (at least four) crossovers. These data, therefore, unambiguously positioned *mgr5614* to the right of the *cat402* insertion. Thus, the order of these markers was, from left to right, *cat402-mgr5614*-Tn*917-cat412*, as shown in Fig. 1. Also shown in Fig. 1 are the cotransduction frequencies for these markers: 39 to 46% between *mgr5614* and Tn*917* (25% plus 14% [Table 2, transduction 1] and 9% plus 37% [Table 2, transduction 2]), 27% between *cat402* and Tn*917* (2% plus 25% [Table 2, transduction 1]), and 18% (9% plus 9% [Table 2, transduction 2]) between Tn*917* and *cat412*. The cotransduction frequencies for *cat402* and Tn*917* and those for *cat412* and Tn*917* correspond well to the actual physical distances between the markers, indicating that there is little, if any, marker effect due to differences in the transduction rates of individual markers.

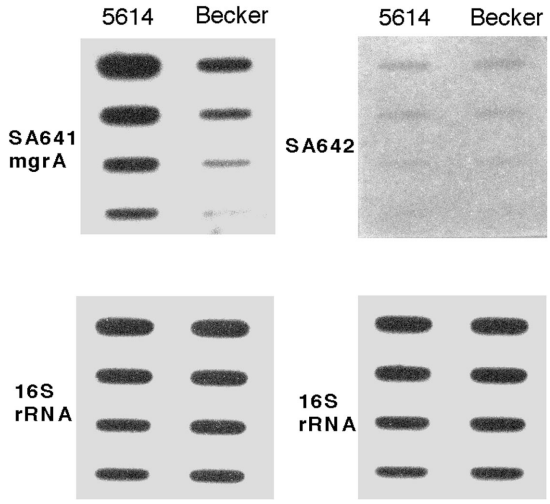

FIG. 2. Northern slot blot of twofold serial dilutions of Becker and CYL5614 total RNAs probed with *mgrA* or SA642. Blots of 16S rRNA were used as controls.

Cloning of the *mgr* gene. The results of the genetic crosses described above indicate that the *mgr* locus is located to the left of Tn*917* near the *cat402* insertions. To clone the *mgr* gene(s), we subcloned the DNA fragments that most likely contained the *mgr* locus from cosmid clones to pCL95, an *E. coli-S. aureus* multicopy shuttle vector (10), or to pCL83, a single-copy vector (15), with tetracycline resistance selection in *S. aureus*. These subclones were used to complement CYL5614 for its ability to produce alpha-toxin. Surprisingly, none of the inserts in either the multicopy or single-copy vector complemented the mutant (not shown). Clones containing inserts further to the left or right also showed no complementation. These results were rather unexpected and were consistent with our failed efforts earlier to clone the gene by screening a plasmid library by complementation. To search further for the *mgr* gene(s), we deleted several regions, including a 9-kb region that encompassed both Tn*917* and *cat402* insertion sites. To our surprise, the 9-kb knockout strain, CYL776, exhibited a phenotype entirely opposite to that of CYL5614 (i.e., slightly decreased CP8, slightly increased protein A, and moderately increased alpha-toxin). The backcrossed strain also showed the same phenotype, indicating that the resulting phenotype is due to the 9-kb deletion (data not shown). We next sequenced an ~5-kb region that most likely contained the *mgr* gene(s), based on genetic mapping. By a sequence comparison between Becker and CYL5614, we found one point mutation with a T-to-C transition in a 227-bp intergenic noncoding region between two divergent ORFs (444- and 927-bp ORFs corresponding to SA0641 and SA0642 of the sequenced N315 strain [14], respectively) (see Fig. 3A). The mutation is located 155 bp upstream

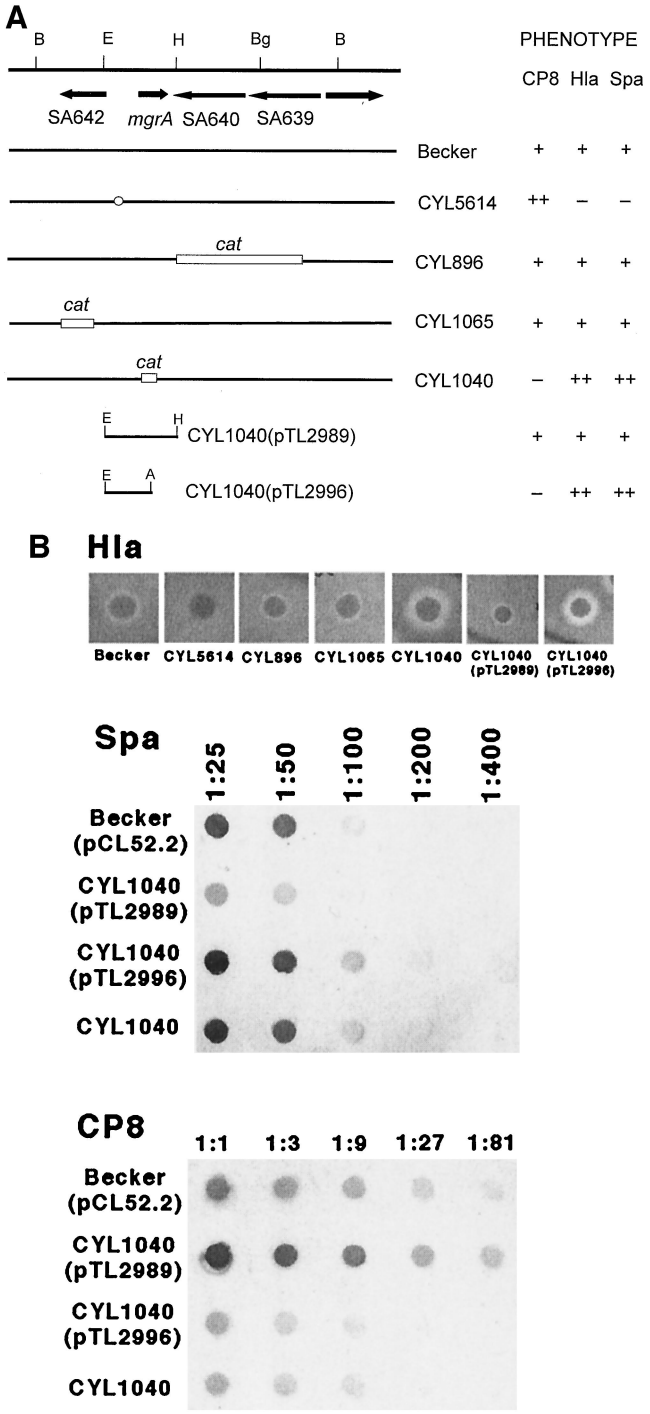

FIG. 3. Localization of the *mgr* locus. (A) Specific segments of the chromosomal region containing the *mgr* locus were deleted by allele replacement with the *cat* gene. The corresponding mutants are indicated. The bottom two lines indicate the DNA fragments used for complementation of strain CYL1040. The phenotypes of each mutant and complemented strain are shown on the right. ++, strong producer; +, moderate producer; −, nonproducer. Note that the phenotypic characteristics of CYL1040(pTL2989) are the same as those of Becker, although pTL2989 excessively complemented CYL1040 due to a multiple-copy effect (see the text). The T-to-C transition in CYL5614 is indicated by a small circle. H, *Hin*dIII; A, *Acc*I. (For the other abbreviations, see the legend to Fig. 1.) (B) Production of alpha-toxin (Hla) from strains shown in panel A was semiquantitatively assayed on sheep blood agar. The production of protein A (Spa) and CP8 by the strains used in the complementation tests shown in panel A was analyzed by Western dot blot analysis of samples serially diluted as indicated above the blots.

of the SA641 ORF and 72 bp upstream of the SA642 ORF. One possible mechanism leading to these results could be that the *mgr5614* mutation in CYL5614 is an up mutation that increases *mgr* expression instead of a down mutation. In this scenario, we would not be able to complement the mutant and a deletion of the *mgr* gene(s) would result in a reverse phenotype of the mutant. To test this hypothesis, Northern slot blots of the total RNAs isolated from Becker and CYL5614 using the internal fragments of both SA0641 and SA0642 as probes were compared. The results showed that the mutant strain produced four- to eightfold more message of SA641 than the wild-type Becker, whereas there was no difference in the amount of SA642 message (Fig. 2). These results strongly indicate that the 444-bp SA641 is the *mgr* gene responsible for the phenotype found in CYL5614. We named this ORF *mgrA*.

To determine whether *mgrA* indeed encodes the *mgr* regulatory factor, we constructed a mutant strain with an *mgrA*-specific deletion by allele replacement. The mutant strain CYL1040, with a *cat* gene replacing a 269-bp internal fragment of the *mgrA* ORF, showed exactly the same phenotype as the 9-kb deletion strain, CYL776 (Fig. 3). To further confirm that the mutant phenotype is due to the deletion of *mgrA*, we attempted to complement CYL1040 with the 0.9-kb *Eco*RI-*Hin*dIII fragment containing the intact *mgrA* ORF and with the 0.6-kb *Eco*RI-*Acc*I fragment containing the truncated *mgrA*. Our results (Fig. 3) showed that, as expected, the plasmid pTL2996 with the 0.6-kb *Eco*RI-*Acc*I fragment containing the truncated *mgrA* did not complement CYL1040. However, pTL2989, with a 0.9-kb *Eco*RI-*Hin*dIII fragment containing the intact ORF *mgrA*, complemented the mutant CYL1040 excessively, particularly with respect to CP8 and protein A production [i.e., CYL1040(pTL2989) produced much less protein A and much more CP8 than the wild type (Fig. 3B)]. The phenotype of CYL1040(pTL2989) could be explained by the fact that *mgrA* was cloned in a multiple-copy plasmid vector, which likely resulted in overexpression of *mgrA*. These results clearly showed that *mgrA* was involved in *mgr* regulation. A BLAST search showed that MgrA had homology with the MarR family of helix-turn-helix transcriptional regulators, of which the multiple-antibiotic resistance regulatory gene *marR* of *E. coli* is the prototype (1, 31). The homologous region contains a potential helix-turn-helix DNA-binding motif from amino acid 56 to 76 of the MgrA protein (Fig. 4). MgrA (SA641) of the recently sequenced *S. aureus* strain N315 was designated a conserved hypothetical protein (14).

Defining the *mgr* locus. From the N315 genome, we found that the third ORF (SA638) to the right of *mgrA* (Fig. 3A) is the *bcaA* gene involved in bacitracin resistance, indicating that this gene is not likely to be involved in *mgr* regulation. The second ORF (SA643) to the left of *mgrA* had been disrupted by the *cat402* insertion without altering the Mgr phenotype, suggesting that it is not part of the *mgr* locus either. Thus, from these results, we believe that the *mgr* regulatory locus consists of at most four ORFs (SA642, *mgrA*, SA640, and SA639). To

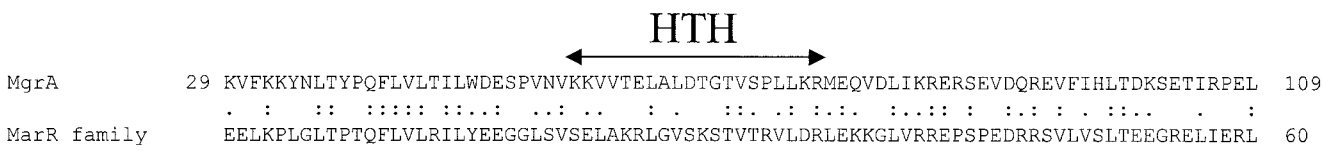

FIG. 4. Comparison of MgrA sequence and MarR family consensus sequence. The helix-turn-helix motif (HTH) is indicated. Colons and periods indicate identical and similar residues, respectively.

define the boundaries and the number of genes in the *mgr* locus, we constructed chromosomal deletion mutants of these three uncharacterized genes in this region by replacing the deletion regions with the pC194 *cat* gene. Strain CYL1065, with a 610-bp deletion within SA0642, and strain CYL896, with a 2.7-kb deletion of both SA640 and SA639, showed no change in Mgr phenotype (Fig. 3). These results indicate that the three genes flanking *mgrA* are not part of the *mgr* regulatory system. Thus, the *mgr* locus appears to consist of only the *mgrA* gene.

Phenotypic characterization of *mgr*. To further study the effect of *mgr* on its target gene products and to test whether *mgr* affects additional virulence factors, we quantitatively measured the production of CP8, alpha-toxin, nuclease, protein A, protease, and coagulase by strains Becker, CYL1040, and CYL5614 as described in Materials and Methods. In the alpha-toxin assay by Western analysis, to prevent protein A interference in our analysis, we constructed a *spa*-null mutant strain, CYL978, that produced undetectable protein A (not shown). As shown in Fig. 5A (top), the inclusion of the *spa*-null strain allowed us to unambiguously identify alpha-toxin in the gel. The results confirmed that MgrA repressed Hla. The results shown in Fig. 5A (middle and bottom) also confirmed that *mgrA* repressed Spa but activated CP8. In addition, our results (Table 3) also showed that *mgr* up-regulated nuclease but down-regulated protease and coagulase (although the coagulase activities in CYL1040 and Becker were the same). We found that the *mgr5614* mutation seemed to have greater effects on the production of the target products than the *mgrA*-null mutation, although the two mutations had opposite effects. However, in general, the extent of regulation was proportional to the quantity of *mgrA* expression.

The results described above showed that *mgr* regulates CP8, protein A, and four other extracellular proteins. To estimate the extent of the effect of *mgr* on gene expression in *S. aureus*, proteins from whole-cell lysates and extracellular supernatants of broth cultures from the wild-type strain Becker, an *mgr* deletion mutant (CYL1040), and an *mgr* overproduction strain (CYL5614) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 5B, no major difference between the wild type and the mutant strains was observed in cell extracts. Increasing or decreasing the amount of cell extract in the gel also did not show a difference between the strains (not shown). On the other hand, major differences were found between these strains in the extracellular supernatants. The intensities of at least four bands increased drastically, and the intensities of a large number of bands with molecular masses ranging from 40 to 80 kDa decreased significantly in the *mgr*-overproducing strain CYL5614. In the *mgr*-null mutant CYL1040, the intensities of many bands increased greatly, especially at ~70 kDa. Although we were unable to show the exact number of proteins affected by the mutations due to the low resolution of the one-directional SDS-PAGE system, these results clearly show that *mgr* profoundly affects the production of the secreted proteins but only slightly affects the production of the cellular proteins. Thus, *mgr* is likely to control the expression of a great number of extracellular proteins.

***mgr* affects the transcription of target genes.** To determine whether *mgr* affects the transcription of its target genes, serial dilutions of the total RNAs from strains Becker, CYL1040, and CYL5614 were slot blotted to nitrocellulose membranes. The membranes were hybridized to gene probes specific for CP8, protein A, nuclease, or alpha-toxin. The results (Fig. 6) showed that the *mgr5614* mutation and the null mutation affected the mRNA production of each of the gene products tested. The patterns were very comparable to those of the phenotypic characterization shown in Fig. 5 and Table 3, suggesting that *mgr* most probably regulates *cap8*, *spa*, *nuc*, and *hla* at the transcriptional level.

DISCUSSION

Several regulatory systems affecting the expression of a number of *S. aureus* genes have been discovered recently. In this study, we identified *mgr* as a novel global regulator that controlled the expression of multiple genes. The *mgr* locus was originally defined from an unknown mutation. The locus was initially linked to a Tn*917* insertion and mapped to a short region on the chromosome by fine genetic crosses. Our subsequent knockout, sequencing, and Northern blotting showed that the original *mgr5614* mutation in CYL5614 was a point mutation resulting in overproduction of *mgrA*. These results led us to perform further knockout and complementation experiments, which definitively showed that *mgrA* was the only gene in this locus that was involved in *mgr* regulation. The finding that *mgr5614* is an up mutation explains why our initial attempts to clone the *mgr* gene by complementation failed, because CYL5614 already had high expression of *mgrA*. Our strategy employed in this study, though complicated and time-consuming, was therefore warranted.

mgr5614 was isolated fortuitously during the construction of a mutant to interrupt a gene with a 14-transmembrane domain by allele replacement using temperature shift protocol (unpublished results). It is therefore interesting to know whether such a mutation represents a common event in *S. aureus* and, if so, whether the condition used in the allele replacement procedure or the mutation in the gene with a 14-transmembrane domain induced the mutation. To test this, we simply screened the CP8 production of strain Becker by immunoblotting. We found CP8-overproducing colonies at a frequency of $\sim 10^{-4}$. Preincubation of the cultures at 42°C for 24 to 48 h did not increase the frequency. However, none of these isolates showed Hla production different from that of the wild type, and sequencing of four isolates from each condition showed no mutation similar to *mgr5614*, suggesting that growth at 37 or

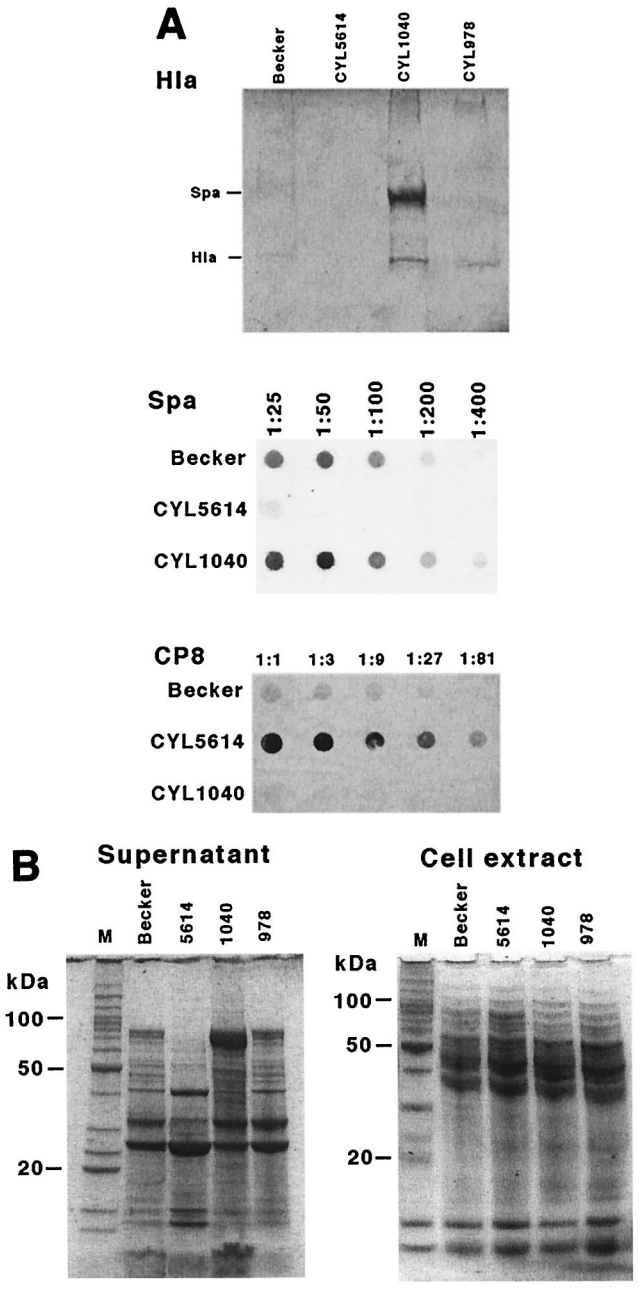

FIG. 5. Phenotypic analysis of *mgr*. (A) The production of alpha-toxin was performed by Western analysis using anti-alpha-toxin antibody after cellular proteins were subjected to SDS–8% PAGE. The production of protein A and CP8 was analyzed by Western dot blot analysis of samples serially diluted as indicated above the blots. (B) SDS-PAGE of whole-cell extracts and supernatants. Samples were run on a 5 to 20% gradient gel.

42°C does not contribute to *mgr5614*-like mutation. Whether the mutation of the 14-transmembrane-domain protein increases the frequency of isolating an *mgr5614* type of mutation is unknown.

It is interesting that the up-regulation of *mgrA* in CYL5614 is the result of a point mutation located 155 bp upstream of the gene. Examination of the mutation showed that the T-to-C point mutation was located within a 12-bp perfect inverted repeat (not shown). It is therefore tempting to speculate that the inverted repeat may serve as a repressor-binding site and that the *mgr5614* mutation affects repressor binding, thereby increasing transcription. However, it is also possible that the mutation results in enhancing the promoter activity or stabilizing the mRNA. These possibilities are under investigation.

TABLE 3. Quantitative analysis of nuclease, protease, and coagulase production[a]

Strain	Production		
	Protease[b]	Nuclease[c]	Coagulase[d]
Becker	2.48 ± 0.37	0.490 ± 0.181	100 ± 0
CYL5614	0.53 ± 0.92	0.632 ± 0.180	14.8 ± 17.0
CYL1040	3.33 ± 0.33	0.013 ± 0.023	100 ± 0

[a] All data are averages (± standard deviations) of three independent experiments.
[b] Protease production was analyzed by measuring depletion of 3 mg of azocasein/ml in 17 h, monitored by OD_{340}.
[c] Nuclease production was analyzed by measuring depletion of 5 mg of salmon sperm DNA/ml monitored by OD_{260}.
[d] Coagulase was measured by a tube coagulation test of threefold serial dilution samples. The numbers represent the highest dilution and are standardized by setting the activity of Becker as 100.

Apparently, the expression of the target genes of *mgr* correlates well with the amount of MgrA produced. For example, the most CP8 is produced by the *mgrA*-overexpressing strain CYL5614, followed by the wild-type strain, while the *mgrA*-null strain CYL1040 produces the least (Fig. 5A). Furthermore, CYL1040 harboring the multiple-copy pTL2989 containing the intact *mgrA* resulted in excess complementation, whereas *mgrA* cloned in a single-copy vector showed no excess complementation in a *mgrA*-null strain derived from strain Newman (results not shown). Thus, the degree of regulation by *mgr* is proportional to the amount of MgrA expressed. The fact that MgrA contains a helix-turn-helix motif within the homologous region shared with the MarR transcriptional regulator family strongly suggests that *mgrA* regulates its target genes by DNA binding at a specific binding site. This hypothesis is consistent with our conclusion that *mgrA* exerts its regulation at the transcriptional level, based on the Northern hybridization and phenotypic characterization of the expression of CP8, protein A, nuclease, and alpha-toxin.

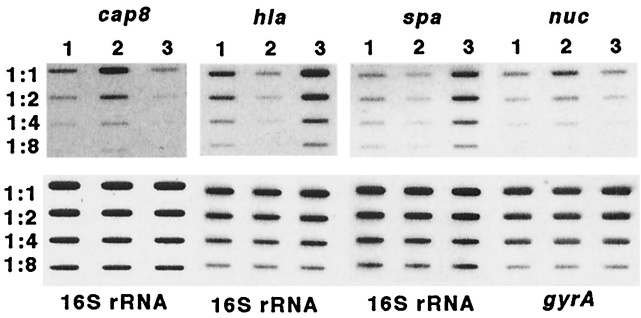

FIG. 6. Effect of *mgr* on expression of *cap8*, *hla*, *spa*, and *nuc* by Northern slot blot analysis. Twofold serial dilutions of the total RNAs from Becker (lanes 1), CYL5614 (lanes 2), and CYL1040 (lanes 3) were transferred to membranes and probed with specific gene probes. The loading controls were probed with either *gyrA* (gyrase A subunit) or 16S rRNA probes.

It has been shown by microarray analyses that *agr* and *sar* regulate the production of a broad range of target genes in *S. aureus*, including many genes not likely to be involved in virulence (6). The number of target genes that *mgr* regulates is not known. However, from the SDS-PAGE analyses shown in Fig. 5B, we found that *mgrA*, though it slightly affected cellular protein production, profoundly affected extracellular protein production in *S. aureus*, suggesting that *mgr* may control a large number of genes.

The discovery to date of several global regulatory systems, including the *mgr* system reported here, indicates that coordinate regulation of virulence genes in *S. aureus* is highly complicated. These systems could interact with one another to control the expression of various virulence genes. Indeed, *sigB*, *sarR*, *ssr*, and *arl* have all been shown to affect RNAIII levels and/or *sarA* expression (2, 7, 18, 33). The individual global regulatory systems could also exert their effects independently of one another. Since the spectrum of target genes regulated by *mgr* overlapped with other global regulatory loci, *mgr* is expected to interact with these loci. However, our Northern slot blotting experiments did not reveal detectable difference in RNAIII levels between the wild type and the *mgrA* mutants (data not shown). Although these results suggest that *mgrA* does not regulate its targets through *agr* directly, we cannot rule out the possibility that regulation is through other regulators, such as *sarA*. Further studies of the interactions of *mgr* with other global regulators will be actively pursued in our laboratory.

ACKNOWLEDGMENTS

We thank Ali Fattom for providing CP8-specific antibodies.

This work was supported by grants AI37027 and AI54607 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Alekshun, M. N., Y. S. Kim, and S. B. Levy.** 2000. Mutational analysis of MarR, the negative regulator of *marRAB* expression in *Escherichia coli*, suggests the presence of two regions required for DNA binding. Mol. Microbiol. **35:**1394–1404.
2. **Bischoff, M., J. M. Entenza, and P. Giachino.** 2001. Influence of a functional *sigB* operon on the global regulators *sar* and *agr* in *Staphylococcus aureus*. J. Bacteriol. **183:**5171–5179.
3. **Cheung, A. L., K. Eberhardt, and V. A. Fischetti.** 1994. A method to isolate RNA from gram-positive bacteria and mycobacteria. Anal. Biochem. **222:** 511–514.
4. **Cheung, A. L., K. Schmidt, B. Bateman, and A. C. Manna.** 2001. SarS, a SarA homolog repressible by *agr*, is an activator of protein A synthesis in *Staphylococcus aureus*. Infect. Immun. **69:**2448–2455.
5. **Chien, Y., A. C. Manna, S. J. Projan, and A. L. Cheung.** 1999. SarA, a global regulator of virulence determinants in *Staphylococcus aureus*, binds to a conserved motif essential for *sar*-dependent gene regulation. J. Biol. Chem. **274:**37169–37176.
6. **Dunman, P. M., E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E. L. Brown, R. J. Zagursky, D. Shlaes, and S. J. Projan.** 2001. Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. J. Bacteriol. **183:**7341–7353.
7. **Fournier, B., A. Klier, and G. Rapoport.** 2001. The two-component system ArlS-ArlR is a regulator of virulence gene expression in *Staphylococcus aureus*. Mol. Microbiol. **41:**247–261.
8. **Giraudo, A. T., A. Calzolari, A. A. Cataldi, C. Bogni, and R. Nagel.** 1999. The *sae* locus of *Staphylococcus aureus* encodes a two-component regulatory system. FEMS Lett. **177:**12–22.
9. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114:**193–197.
10. **Jana, M., T. T. Luong, H. Komatsuzawa, M. Shigeta, and C. Y. Lee.** 2000. A method for demonstrating gene essentiality in *Staphylococcus aureus*. Plasmid **44:**100–104.
11. **Kleckner, N., J. Roth, and D. Botstein.** 1977. Genetic engineering in vivo using translocatable drug-resistance elements. New methods in bacterial genetics. J. Mol. Biol. **116:**125–159.
12. **Kraemer, G. R., and J. J. Iandolo.** 1990. High-frequency transformation of *Staphylococcus aureus* by electroporation. Curr. Microbiol. **21:**373–376.
13. **Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature **305:**709–712.
14. **Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu.** 2001. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. Lancet **357:**1225–1240.
15. **Lee, C. Y., S. L. Buranen, and Z. H. Ye.** 1991. Construction of single-copy integration vectors for *Staphylococcus aureus*. Gene **103:**101–105.
16. **Luchansky, J. B., and P. A. Pattee.** 1984. Isolation of transposon Tn*551* insertions near chromosomal markers of interest in *Staphylococcus aureus*. J. Bacteriol. **159:**894–899.
17. **Luong, T., S. Sau, M. Gomez, J. C. Lee, and C. Y. Lee.** 2002. Regulation of *Staphylococcus aureus* capsular polysaccharide expression by *agr* and *sarA.* Infect. Immun. **70:**444–450.
18. **Manna, A., and A. L. Cheung.** 2001. Characterization of *sarR*, a modulator of *sar* expression in *Staphylococcus aureus*. Infect. Immun. **69:**885–896.
19. **Manna, A., and A. L. Cheung.** 2003. *sarU*, a *sarA* homolog, is repressed by SarT and regulates virulence genes in *Staphylococcus aureus*. Infect. Immun. **71:**343–353.
20. **McNamara, P. J., K. C. Milligan-Monroe, S. Khalili, and R. A. Proctor.** 2000. Identification, cloning, and initial characterization of *rot*, a locus encoding a regulator of virulence factor expression in *Staphylococcus aureus*. J. Bacteriol. **182:**3197–3203.
21. **Novick, R. P.** 2000. Pathogenicity factors and their regulation, p. 392–407. *In* V. A. Fischetti, R. P. Novick, J. J. Ferriti, D. A. Portnoy, and J. I. Rood (ed.), Gram-positive pathogens. ASM Press, Washington, D.C.
22. **Ochman, H., J. W. Ajioka, D. Garza, and D. L. Hartl.** 1989. Inverse polymerase chain reaction, p. 105–111. *In* H. A. Erlich (ed.), PCR technology: principles and applications for DNA amplification. M. Stonon Press, New York, N.Y.
23. **Said-Salim, B., P. M. Dunman, F. M. McAleese, D. Macapagal, E. Murphy, P. J. McNamara, S. Arvidson, T. J. Foster, S. J. Projan, and B. N. Kreiswirth.** 2003. Global regulation of *Staphylococcus aureus* genes by Rot. J. Bacteriol. **185:**610–619.
24. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Sau, S., and C. Y. Lee.** 1996. Cloning of type 8 capsule genes and analysis of gene clusters for the production of different capsular polysaccharides in *Staphylococcus aureus*. J. Bacteriol. **178:**2118–2126.
26. **Sau, S., J. Sun, and C. Y. Lee.** 1997. Molecular characterization and transcriptional analysis of type 8 genes in *Staphylococcus aureus*. J. Bacteriol. **179:**1614–1621.
27. **Schmidt, K. A., A. C. Manna, S. Gill, and A. L. Cheung.** 2001. SarT: a repressor of α-hemolysin synthesis in*Staphylococcus aureus*. Infect. Immun. **69:**4749–4758.
28. **Shafer, M. W., and J. J. Iandolo.** 1979. Genetics of staphylococcal enterotoxin B in methicillin-resistant isolates of *Staphylococcus aureus*. Infect. Immun. **25:**902–911.
29. **Smeltzer, M. S., M. E. Hart, and J. J. Iandolo.** 1993. Phenotypic characterization of *xpr*, a global regulator of extracellular virulence factors in *Staphylococcus aureus*. Infect. Immun. **61:**919–925.
30. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
31. **Sulavik, M. C., L. F. Gambino, and P. F. Miller.** 1995. The MarR repressor of the multiple antibiotic resistance (*mar*) operon in *Escherichia coli*: prototypic member of a family of bacterial regulatory proteins involved in sensing phenolic compounds. Mol. Med. **1:**436–446.
32. **Tegmark, K., A. Karlsson, and S. Arvidson.** 2000. Identification and characterization of SarH1, a new global regulator of virulence gene expression in *Staphylococcus aureus*. Mol. Microbiol. **37:**398–409.
33. **Yarwood, J. M., J. K. McCormick, and P. M. Schlievert.** 2001. Identification of a novel two-component regulatory system that acts in global regulation of virulence factors of *Staphylococcus aureus*. J. Bacteriol. **183:**1113–1123.
34. **Youngman, P. J., J. B. Perkins, and R. Losick.** 1983. Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with *Streptococcus faecalis* transposon Tn*917*. Proc. Natl. Acad. Sci. USA **80:**2305–2309.