Identification of *sarV* (SA2062), a New Transcriptional Regulator, Is Repressed by SarA and MgrA (SA0641) and Involved in the Regulation of Autolysis in *Staphylococcus aureus*

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The expression of genes involved in the pathogenesis of *Staphylococcus aureus* is known to be controlled by global regulatory loci, including *agr*, *sarA*, *sae*, *arlRS*, *lytSR*, and *sarA*-like genes. Here we described a novel transcriptional regulator called *sarV* of the SarA protein family. The transcription of *sarV* is low or undetectable under in vitro conditions but is significantly augmented in *sarA* and *mgrA* (*norR* or *rat*) (SA0641) mutants. The *sarA* and *mgrA* genes act as repressors of *sarV* expression, as confirmed by transcriptional fusion and Northern analysis data. Purified SarA and MgrA proteins bound specifically to separate regions of the *sarV* promoter as determined by gel shift and DNase I footprinting assays. The expression of 19 potential target genes involved in autolysis and virulence, phenotypes affected by *sarA* and *mgrA*, was evaluated in an isogenic *sarV* mutant pair. Our data indicated that the *sarV* gene product played a role regulating some virulence genes and more genes involved in autolysis. The *sarV* mutant was more resistant to Triton X-100 and penicillin-induced lysis compared to the wild type and the *sarA* mutant, whereas hyperexpression of *sarV* in the parental strain or the *sarV* mutant rendered the resultant strain highly susceptible to lysis. Zymographic analysis of murein hydrolase activity revealed that inactivation of the *sarV* gene results in decreased extracellular murein hydrolase activity compared to that of wild-type *S. aureus*. We propose that *sarV* may be part of the common pathway by which *mgrA* and *sarA* gene products control autolysis in *S. aureus*.

Staphylococcus aureus is an important human pathogen that has become a growing public health concern, primarily due to the emergence of antibiotic-resistant strains within the hospital environment (4, 5, 25). The spectrum of infections caused by this organism is extremely broad, ranging from cutaneous infections such as impetigo, folliculitis, and carbuncles to deep-seated infections such as pneumonia, endocarditis, septicemia, osteomyelitis, and other metastatic complications. Besides invasion syndromes, *S. aureus* can also cause toxin-mediated diseases such as food poisoning, toxic shock syndrome, and scalded skin syndrome. The primary site of infection is generally the skin or a wound from which the organism can spread to the bloodstream and subsequently disseminate into various host tissues. Once *S. aureus* establishes its presence in the tissue, it produces a large number of bacterial components and secreted products that include surface-associated protein adhesins, enzymes, exotoxins, and capsular polysaccharides, gene products that facilitate tissue colonization, tissue destruction, and immune evasion. The expression of many of these genes is coordinately controlled by regulatory loci (2, 5, 33, 35). These regulatory loci include *agr*, *sarA*, *saeRS*, *sigB*, *lytSR*, *arlRS*, and *sarA*-like genes.

The *agr* locus consists of two divergent transcripts, RNAII and RNAIII, which encode *agrDBCA* and *hld*, respectively (19, 20, 33). It is also a complex quorum-sensing two-component

regulatory system in *S. aureus*. The RNAII transcriptional unit codes for four genes, of which *agrBD* are involved in the generation and secretion of an autoinducing octapeptide. As the autoinducing peptide reaches a threshold concentration in the growth medium during the transition from exponential to postexponential phase, activation of *agrC* and *agrA*, encoding the two-component regulatory system, would occur, leading to the transcription of RNAII and RNAIII, the *agr* regulatory molecule responsible for the up-regulation of exoproteins and down-regulation of cell wall-associated proteins (20, 33). The *sarA* locus comprises a major open reading frame (ORF), *sarA*, driven by three distinct promoters, resulting in three overlapping transcripts with a common terminating end (8, 29). The *sarA* locus up-modulates the expression of selected cell wall proteins and exoproteins (e.g., α- and β-hemolysins). DNA binding studies have revealed that SarA, the major *sarA* effector molecule, binds to several target gene promoters (e.g., *agr*, *hla*, and *spa*) to modulate gene transcription (10), thus implicating both *agr*-dependent and *agr*-independent pathways for SarA-mediated regulation.

Using affinity chromatography and genomic scan, we and others have identified several SarA homologs that are involved directly or indirectly in gene regulation. SarR, a 115-residue-long polypeptide (30), represses SarA expression during the postexponential phase by binding to the *sarA* promoter region. SarS (also called SarH1) is a 250-residue protein (9, 44) repressed by *agr* to activate *spa* expression and, to a lesser extent, to down-regulate *hla* expression. *rot*, also a *sarA* homolog, is a negative regulator for hemolysins (*hla* and *hlb*), lipase (*geh*), and serine proteases (*sspB* and *splA*) as well as a positive

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regulator for cell surface proteins (*clfB*/clumping factor, *spa*/protein A, *sdrC*/putative cell surface adhesin) (32, 39). SarT is a repressor of *hla* and RNAIII transcription (41). Contiguous to *sarT*, but transcribed in the opposite orientation, is *sarU* (*sar*H2), whose expression is negatively controlled by SarT. Phenotypic and transcriptional analysis revealed that *sarU* is a positive regulator of RNAIII and contributes to the expression of virulence genes controlled by *agr* (31). More recently, *mgrA* (also called *rat* or *norR*), a homolog of *marR* and also *sarA*, has been found to negatively regulate autolysis genes without significantly affecting *sarA* and *agr* expression (17). The *mgrA* gene also impacts upon the expression of type 8 capsular polysaccharide (*cap8*), α-hemolysin (*hla*), protein A (*spa*), *norA*, lipase, protease, and coagulase (27, 45).

Besides regulating virulence genes, the *agr* and *sarA* loci are also involved in the regulation of genes involved in the autolysis process, but the exact mechanism (e.g., direct versus indirect) is not known. Two-component regulatory systems such as ArlRS and LytSR have been shown to participate in the regulation of autolysis genes (6, 12, 13, 16). Downstream of the dicistronic *lytSR* operon is the *lrgAB* locus, the expression of which is dependent on activation by LytSR. Based on homology alone, it has been speculated that LrgA may function as an antiholin to inhibit murein hydrolase activity, presumably by interfering with the holin molecule that has been postulated to form a pore for the transport of murein hydrolases across the cell membrane (4, 7, 14). Although the exact mechanism of autolysis and their mode of regulation are not well defined, it is hypothesized that a diminution in transport of autolysins across the cell membrane would reduce the autolytic process.

We have identified another homolog of SarA, a 116-residue-long polypeptide called SarV (SA2062 and protein GI13702067) (21). The expression of *sarV* is repressed by *sarA* and *mgrA* (SA0641 and protein GI 13700577), known to be involved in the control of virulence and autolysis genes. Inactivation of *sarV* did not affect the expression of regulatory genes such as *sarU*, *sarR*, *sarS*, *sarT*, *sarA*, *rot*, and *sae* but had discernible effects on the expression of *agr* RNAII and RNAIII, *lytSR*, and *arlRS*. As *sarA* and *mgrA* are involved in the regulation of autolysis and virulence genes, we also evaluated the expression of 19 potential target genes involved in these phenotypes (33) (*spa*, *coa*, *clfA*, *fnbA*, *fnbB*, *hla*, *atl*, *lytM*, *lytN*, *lrgAB*, *geh*, *scdA*, *abcA*, *pbp4*, *splA*, *scp*, *aur*, *norA*, and *cap8*) by Northern blotting and transcriptional fusions in *sarV* mutants. Importantly, the *sarV* gene product is involved in the regulation of autolysin and protease genes (*scdA*, *lrgB*, *atl*, *splA*, and *aur*). Lytic assays with Triton X-100 and penicillin indicated that the *sarV* mutant, contrary to the effect of *mgrA* and *sarA* mutants, is more resistant to lysis than the parent, while hyperexpression of *sarV* renders the strain sensitive. Analysis of the *sarV* mutant indicated that this gene regulates extracellular and intracellular murein hydrolase activity, possibly by regulating selective genes involved for autolysis. Because *sarV* likely does not regulate *mgrA* or *sarA*, we propose that *sarV* constitutes an important "hub" for the control of autolysis by *mgrA* and *sarA*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Phage φ11 was used as a generalized transducing phage for *S. aureus* strains. *S. aureus* strain RN4220, a restriction-deficient derivative of strain 8325-4, was used as the initial recipient for the transformation of plasmid constructs. *S. aureus* cells were grown at 37°C with aeration in Trypticase soy broth (TSB) or 03GL agar medium supplemented with antibiotics when necessary. Luria-Bertani medium was used for growing *Escherichia coli*. Antibiotics were used at the following concentrations: for *S. aureus*, erythromycin at 5 μg/ml, kanamycin at 50 μg/ml, tetracycline at 5 μg/ml, and chloramphenicol at 10 μg/ml; for *E. coli*, ampicillin at 50 μg/ml, chloramphenicol at 30 μg/ml, erythromycin at 200 μg/ml, and spectinomycin at 75 μg/ml.

Genetic manipulations in *E. coli* and *S. aureus*. Based on homology with SarA, the *sarV* gene product was initially identified in the *S. aureus* N315 genome database (21; www.tigr.org/ and www.ncbi.nlm.nih.gov/genomics). To construct a *sarV* mutant, the *sarV* gene together with flanking sequences on both sides was amplified by PCR with the primers 5′-AACTGTCGATGGATTTAACGTTA-3′ and 5′-AGTTTAGTATTAGGTACAGCGA-3′, using chromosomal DNA from strain RN6390 as the template. The 2.2-kb PCR fragment was cloned into cloning vector pCR 2.1 (Invitrogen, San Diego, Calif.) in *E. coli*. The HindIII-EcoRV DNA fragment containing the 2.2-kb fragment was then cloned into the HindIII and HincII sites of pUC19. A 565-bp fragment, comprising the *sarV* coding region as well as 137 and 78 bp of the respective upstream and downstream regions, was deleted by restricting with BsaBI and SphI and then replacing the deleted fragment with a ~1.2-kb *ermC* gene at these sites. The fragment containing the *ermC* replacement of the deleted *sarV* gene was cloned into the temperature-sensitive shuttle vector pCL52.2. Construction and selection of the putative *sarV* mutant (tetracycline-sensitive and erythromycin-resistant colonies) were performed as described elsewhere (30, 31). A phage φ11 lysate of the putative *sarV* mutant was then prepared to infect fresh strain RN6390 cells to reconstruct the *sarV* mutant, in an attempt to avoid any putative genomic mutations that might have occurred during the temperature shift to promote homologous recombination. The correct mutation was confirmed by PCR, Northern, and Southern hybridization with *sarV* and *ermC* probes as described previously (28, 30). One clone, designated ALC2319, was selected for further study.

To complement the *sarV* mutation, a 1.5-kb fragment encompassing the *sarV* gene and 295 bp upstream of the *sarV* translation start site were cloned into shuttle plasmid pSK236. The recombinant plasmid was electroporated into RN4220, selecting for chloramphenicol-resistant colonies. The correct transformant was verified by restriction analysis of the recombinant plasmid. The plasmid from RN4220 was then electroporated into parental strain RN6390 and the *sarV* mutant (ALC2319) to construct strains ALC2375 and ALC2376, respectively.

Isolation of RNA and Northern blot hybridization. Total RNA from *S. aureus* was prepared by using a TRIzol isolation kit (Gibco BRL, Gaithersburg, Md.) and a reciprocating shaker as described elsewhere (8, 30, 31). The optical density at 650 nm OD_{650} of various cultures was determined with a spectrophotometer (Spectronic 20). The concentration of total RNA was determined by measuring the absorbance at 260 nm, and 10 μg of each total RNA sample was analyzed by Northern blotting as described previously (30). The genes coding for *agr* RNAII, *agr* RNAIII, *sarA*, *sarR*, *sarT*, *sarS*, *sarU*, *rot*, *mgrA*, *sae*, *lytS*, *lytR*, *arlS*, *arlR*, *spa*, *coa*, *clfA*, *fnbA*, *fnbB*, *hla*, *lytM* and -*N*, *atl*, *lrgB*, *geh*, *scdA*, *abcA*, *pbp4*, *splA*, *scp*, *aur*, *norA*, and *cap8* were either amplified by PCR or excised from the plasmids containing the desired genes with restriction endonucleases. An internal fragment of the 16S rRNA gene (nucleotides [nt]777 to 1500; GenBank accession no. X68417) was used as a loading control. For detection of specific transcripts, gel-purified DNA probes were radiolabeled with [α-^{32}P]dCTP by using the random-primed DNA labeling kit (Roche Diagnostics GmbH) and hybridized under aqueous-phase conditions at 65°C. The blots were subsequently washed and autoradiographed.

Transcriptional fusion studies of *agr* RNAII, *agr* RNAIII, *hla*, *sarA*, *sarV*, *arlR*, and *lytS* promoters linked to the *gfp_{uvr}* reporter gene. Construction of the plasmid pALC1743, -1742, and -1740 containing *agr* RNAIII, *agr* RNAII, and *hla* promoter fragments linked to the *gfp_{uvr}* reporter gene were described earlier (18, 31). A 268-bp *sarV* promoter fragment (position 21 to 288 nt upstream from the start ATG codon) was amplified by PCR using chromosomal DNA of *S. aureus* strain RN6390 and primers with flanking EcoRI or XbaI sites. An EcoRI and XbaI fragment containing the *sarV* promoter was cloned into shuttle plasmid pALC1484, generating plasmid pALC2489 containing the transcriptional fusion of the *sarV* promoter to the *gfp_{uvr}* reporter gene. The construction of plasmid pALC1484 and modification of *gfp_{uv}* (Clontech, Palo Alto, Calif.) to *gfp_{uvr}* with an S65T mutation to yield a red shift (excitation maxima from 395 to 488 nm) were described earlier (18). Similarly, DNA fragments containing *arlS*, *lytR*, and *mgrA* P1 promoters were cloned into shuttle plasmid pALC1484, generating plasmids containing transcriptional fusions to the *gfp_{uvr}* reporter gene (17). Restriction analysis and DNA sequencing confirmed the orientation and authenticity of the promoter reporter gene constructs. The recombinant plasmids,

TABLE 1. Strains and plasmids used

Strain or plasmid	Reference or source	Comment
S. aureus strains		
RN4220	30	Mutant of 8325-4 that accepts foreign DNA
RN6390	30	Laboratory strain that maintains its hemolytic pattern when propagated on sheep erythrocyte agar (parental strain)
RN6911	6	*agr* mutant of RN6390 with an Δ*agr*::*tetM* mutation
JC1413	43	*atl* mutant of *S. aureus*
ALC1342	Laboratory strain	A *sarA* mutant with deletion of ORF3 and the *sarA* ORF and replaced with an *ermC* gene, Δorf3*sarA*::*ermC*
ALC488	29	*sarA* mutant of RN6390 with *sarA*::*ermC*
ALC1629	Laboratory strain	ALC1342 with *sarB* (nt 1 to 1349 plus 180 bp of upstream sequence) integrated into the *geh* locus on the chromosome
ALC1713	30	*sarR* mutant of RN6390 with Δ*sarR*::*ermC*
ALC1905	41	*sarT* mutant of RN6390 with Δ*sarT*::*ermC*
ALC2272	31	*sarU* mutant of RN6390 with Δ*sarU*::*ermC*
ALC1893	9	*sarS* mutant of RN6390 with Δ*sarS*::*ermC*
ALC2530	17	*mgrA* mutant of RN6390 with Δ*mgrA*::*ermC*
ALC2531	17	ALC2530 complemented with *mgrA* gene into the *geh* locus on the chromosome
ALC2319	This study	*sarV* mutant of RN6390 with a deletion of ORF of the *sarV* gene and replaced with an *ermC* gene
ALC2375	This study	RN6390 with pALC2366
ALC2376	This study	ALC2272 with pALC2366
E. coli strains		
XL-1 Blue	28	Host strain for cloning
Topo InvαF′	Invitrogen	Host strain for the TA cloning vector
Primers		
pCL52.2	30	Temperature-sensitive *E. coli*-*S. aureus* shuttle vector
pCR2.1	Invitrogen	*E. coli* cloning vector for direct cloning of PCR products
pSK236	31	Shuttle vector containing pUC19 cloned into the HindIII site of pC194
pUC19	28	*E. coli* cloning vector
pALC1484	18	Modified pSK236 shuttle vector with a promoterless *gfp*$_{uvr}$ reporter gene preceded by an *S. aureus* ribosome binding site
pALC2231	This study	pUC19 containing a 2.2-kb EcoRVIHindIII fragment containing *sarV* gene
pALC2280	This study	pCL52.2 containing a 2.84-kb DNA fragment which has a deletion of a 565-bp BsaBl/SphI fragment that includes complete ORF of the *sarV* gene and an insertion of the 1.2-kb *erm*C gene at the BsaBI/SphI sites
pALC2366	This study	pSK236 with a 1.4-kb DNA fragment containing the *sarV* gene with a 295-bp upstream sequence at the EcoRI site
pALC2489	This study	pALC1484 with a 273-bp promoter fragment of *sarV* fused with the *gfp*$_{uvr}$ reporter gene at the EcoRI/XbaI sites

containing the respective promoter regions, were first introduced into *S. aureus* strain RN4220 by electroporation. Plasmids purified from RN4220 transformants were then electroporated into RN6390, its isogenic *sarV*::*ermC* (ALC2319), *sarA*::*ermC* (ALC1342), *mgrA*::*ermC* (ALC2530), *mgrA*::*ermC*/*sarA*::*kan* (ALC2535) mutants, and single-copy complemented *mgrA* (ALC2531) and *sarA* (ALC1629) strains as required.

After overnight culture, *S. aureus* strains harboring the recombinant plasmids were diluted 1:100 in TSB containing chloramphenicol (10 μg/ml) and grown at 37°C with shaking at 250 rpm. Aliquots (200 μl) were transferred hourly to microtiter plates to assay for OD_{650} and fluorescence for 10 h in an FL600 fluorescence reader (BioTek Instrument, Winooski, Vt.). Promoter activation was plotted as mean fluorescence per OD_{650} over time, using the average values from triplicate readings.

Purification of SarA and MgrA proteins. The cloning and purification of the His$_6$ tagged fusion SarA protein were described earlier (10). The 444-bp DNA fragments containing the full-length *mgrA* gene (SA0641of *S. aureus* N315) were amplified by PCR using chromosomal DNA from *S. aureus* RN6390 as the template and primers containing flanking restriction sites (NdeI and BamHI) to facilitate in-frame cloning into the expression vector pET14b (Novagen, Madison, Wis.). The recombinant plasmids containing the full-length *mgrA* coding region were confirmed by restriction digestion and DNA sequencing. The recombinant plasmids were then transformed into *E. coli* BL21(DE3)pLysS. The recombinant His$_6$-MgrA protein expression and purification were done in a manner similar to that for the SarA protein as described elsewhere (10). The authenticity and purity of the purified His$_6$-tagged MgrA fusion protein were confirmed by sodium dodecyl sulfate (SDS) gels stained with Brilliant Blue R-250 and microsequenced for the first 20 residues from the N terminus. The purified His$_6$-tagged SarA and MgrA proteins were found to be more than 98% pure in an SDS–12% polyacrylamide gel. The concentration of the purified proteins was determined by the Bradford protein assay (Bio-Rad, Hercules, Calif.), using bovine serum albumin as the standard.

Gel shift analysis and DNase I footprinting. To determine if the recombinant SarA or MgrA proteins bind to the *sarV* promoter region, a 191- or 268-bp fragment (positions 21 to 211 or 288), representing the *sarV* promoter region, was end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. Labeled fragment (0.1 ng or 0.5 fmol) was incubated at room temperature (RT) for 20 min with various amounts of purified SarA or MgrA protein in 25 μl of binding buffer (25 mM Tris-Cl [pH 7.5], 0.1 mM EDTA, 75 mM NaCl, 1 mM dithiothreitol, and 10% glycerol) containing 0.5 μg of calf thymus DNA (Amersham Pharmacia Biotech). The reaction mixtures were analyzed in an 8.0% nondenaturing polyacrylamide gel. The band shifts were detected by exposing dried gels to X-ray films.

Footprinting assays with template DNA fragment and DNase I were performed as previously described (3, 10, 30). To label PCR products, only one of the primers was labeled at one end. For the assay, the binding reactions were carried out in a 100-μl reaction volume containing 20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 5 mM MgCl$_2$, 1 mM CaCl$_2$, 2 mM dithiothreitol, 10 μg of bovine serum albumin, 0.4 μg of calf thymus DNA, radiolabeled template DNA (20,000 cpm), and various amounts of the purified SarA or MgrA proteins at RT for 30 min. DNase I (0.02 U; Boehringer, Mannheim, Germany) was added and allowed to incubate for 1 min at RT. The reaction mixtures were extracted with phenol-chloroform, ethanol precipitated, washed with 70% ethanol, dried, and resuspended in loading buffer (98% deionized formamide, 10 mM EDTA [pH 8.0], 0.025% [wt/vol] xylene cyanol FF, 0.025% [wt/vol] bromophenol blue). DNA samples were denatured at 95°C for 5 min and analyzed on a 6% denaturing polyacrylamide sequence gel. The positions of the protected region were derived by comparing the footprint with the A+G sequencing ladder of the same fragment (28).

Primer extension analysis. Mapping of the 5′ end of the *sarV* transcript by primer extension was performed using the primer 5′-CTTCAATTGAAATCTT ATGTGTTG-3′, complementary to the *sarV* coding strand and located from nt positions 94 to 71 downstream from the putative start codon ATG. Reverse

transcription was carried out as described elsewhere by using total RNA isolated from wild-type RN6390 or the *sarA* mutant as described previously (3, 30).

Lytic assays with Triton X-100 and penicillin. These assays were performed as described elsewhere (12, 17, 27). In brief, overnight-grown bacteria were diluted to an OD_{650} of 0.05 in TSB containing 1 M NaCl and allowed to grow at 37°C with shaking till the OD_{650} reached 0.7 to 1.2. Cells were harvested, washed twice with ice-cold water, and then resuspended in 0.05 M Tris-Cl (pH 7.2) in the original volume with or without 0.05% Triton X-100. Cells were incubated at 30°C with shaking and checked for lysis by measuring the progressive decrease in absorbance (OD_{650}) at 30-min intervals. To assess the sensitivity of the *sarV* mutant to penicillin, inoculations were done in TSB from overnight cultures to yield a starting OD_{650} of 0.05. Cultures were grown at 37°C with shaking to reach exponential phase (OD_{650} ~0.5). Penicillin G was added to a concentration of 0.4 μg/ml. Cultures were incubated further, and the OD_{650} was measured every hour for 7 to 8 h.

Zymographic analysis. For the detection of extracellular and intracellular murein hydrolases, SDS-polyacrylamide gel electrophoresis-based zymographic analysis was performed as described by Groicher et al. (16). In brief, various strains were grown in TSB medium for 16 h at 37°C and 250 rpm. Extracellular murein hydrolases were isolated by pelleting 16-h cultures at $6,000 \times g$ for 20 min at 4°C. The supernatant was filter sterilized and concentrated 100-fold by using polyethylene glycol 8000, followed by dialysis in phosphate-buffered saline. To obtain intracellular and cell wall-associated murein hydrolases, the cell pellets were extracted as essentially described by Groicher et al. (16), and final fractions were dialyzed against phosphate-buffered saline buffer.

The concentrations of total proteins in each sample were determined by using the Bradford assay (Bio-Rad) according to the manufacturer's procedures. Various protein fractions were resolved on an SDS–12% polyacrylamide gel containing either *Micrococcus luteus* (Sigma Chemical Co.) or autoclaved and lyophilized *S. aureus* RN6390 cells (1 mg [dry weight] of cells per ml of gel). After electrophoresis, gels were washed with water and incubated overnight in 25 mM Tris-Cl, pH 8.0, containing 1% Triton X-100 at 37°C to allow hydrolysis of the embedded bacterial cells. After incubation, gels were stained with 1% methylene blue (Sigma Chemical Co.) and destained in water. Following destaining the gels were scanned in a scanner, and the white bands in the gels (zones of hydrolysis) indicated regions of murein hydrolase activity. Quantitative cell wall hydrolysis assays were performed as described by Rice et al. (38) as the turbidity of the samples was determined by measuring the absorbance at 580 nm with a spectrophotometer.

RESULTS

Identification of the *sarV* gene. Searching for additional SarA homologs in the recently published *S. aureus* N315 genome, we found at least 12 proteins homologous to SarA (SA0573, protein GI 13700508), using a default setting of 30 as the cutoff for the BLAST search (21; www.ncbi.nlm.nih.gov /genomes/staphylococcus). Some of these homologs included SarT (SA2286 or SarH3, protein GI 13702448; e-value of $9e^{-17}$) (41), SarS (also called SarH1 or SA0108, protein GI 13700028; e-value of $1e^{-16}$) (9, 44), SarU (also called SarH2 or SA2287, protein GI 13702449; e-value of $2e^{-15}$) (31), SarR (SA2089, protein GI 13702095; e-value of $9e^{-12}$) (30), and Rot (SA1583, protein GI 13701558; e-value of 0.003) (32). MgrA (also called Rat, NorR, or SA0641; protein GI 13700577; e-value of $4e^{-04}$), more homologous to MarR than to SarA, has been shown to play a role in the regulation of genes involved in autolysis and virulence (17, 26, 45). The remaining homologs of unknown function were SA0668 (protein GI13700559; e-value of $1e^{-04}$ and 46% homology), SA2174 (protein GI13702335; e-value of 0.002 and 53% homology), and two more proteins (protein GI 1301144 and 13700062; e-values of 0.003 and 0.030 and homologies of 48 and 48% within a small region of proteins, respectively). SarV (SA2062 and protein GI 13702067) is the seventh protein that came up in our homology search, with SarT, -S, -U, and -R, SA0668, and MgrA being the first six hits (21). SarV is a 116-residue

polypeptide (13,985 Da) that shares 52, 48, 44, 52, 51, 45, 49, and 60% homology to SarR, SarA, SarU1 (residues 1 to 124), SarU2 (residues 125 to 247), SarS1 (residues 1 to 125 of SarS), SarS2 (residues 126 to 250 of SarS), SarT, and MgrA, respectively. Based on sequence alignment, it can be observed that specific residues of SarV are conserved within the SarA protein family, including K23, L36, K48, K80, R82, D86, E87, R88, Q98, L101, and I109 of SarV (data not shown). In a BLAST comparison of SarV against other microbial genomes with the same default setting, we found a number of putative transcriptional regulators with unknown functions (e-values of $2e^{-06}$ to 9.0). Apart from *S. aureus*, significant homologs include *Bacillus subtilis* hypothetical protein *ybfA* (Z99105; e-value of $2 e^{-04}$ with 29% identity and 52% similarity) and a probable transcriptional regulator of *Clostridium perfringens* (AP003188; e-value of 0.001 with 23% identity and 50% similarity).

Expression of *sarV* in RN6390 and an assortment of isogenic mutants. To determine the phenotypic effects of *sarV*, we constructed a *sarV* mutant in RN6390 by allelic replacement, essentially replacing the *sarV* gene with an *ermC* cassette (see Materials and Methods). A Northern blot assay with a *sarV* probe (350 bp) encompassing the coding region disclosed that *sarV* was poorly transcribed or undetectable in parental strains RN6390 and SH1000 (*S. aureus* 8325-4 *rsbU*⁺ strain; kindly provided by S. J. Foster). As expected, complementation of the wild type or the *sarV* mutant with a plasmid carrying the *sarV* gene (ALC2375 or ALC2376) increased the level of transcript of the *sarV* gene. Interestingly, the *sarV* transcript, sizing at ~500 nt, was significantly enhanced in two *sarA* mutants (ALC488 *sarA*::*ermC* and ALC1342 Δ*orf3sarA*::*ermC*) and the *rat/mgrA* mutant (Δ*mgrA*::*ermC*), but not in the *sarR*, *sarS*, *sarT*, *sarU*, or *agr* mutants (Fig. 1, top panel). The size of the transcript also hinted at the monocistronic nature of the *sarV* locus. Upon introducing a single copy of the *mgrA* gene or of the entire *sarB* locus into the lipase gene (*geh*) of the respective *mgrA* or *sarA* mutant (ALC2531 and ALC1629, respectively), the expression of *sarV* transcript returned to near the parental level, suggesting that the expression of *sarV* is truly repressed by *sarA* or the *mgrA* gene product. To determine whether the expression of *mgrA* was affected in these assorted mutants, we performed Northern analysis with the *mgrA* probe (450 bp) and found no significant changes in the expression of *mgrA* transcript in *agr*, *sarT*, *sarU*, *sarV*, *sarS*, and *sarA* mutants (Fig. 1, middle panel). The expression of *mgrA* gene transcript was slightly elevated in the *sarR* mutant. Notably, the *mgrA* transcript level was more elevated in strains (ALC2375 and ALC2376) harboring a multicopy plasmid carrying the *sarV* gene. Thus, besides repression by *mgrA*, it would seem that increased expression of *sarV* transcript also enhances *mgrA* expression, suggesting that *sarV* may act as an activator for the expression of *mgrA* transcript. However, the significance of this finding remains unclear, since we do not know the physiologic relevance of increased gene dosage in vivo (Fig. 1, top panel). There are no significant growth differences in the growth curves for the *sarV* mutant compared with those for the wild type and various mutants (data not shown). Growth phase variation of various mutants did not make any difference in the data presented above for the expression of *sarV* transcript when Northern blot hybridization was performed with total
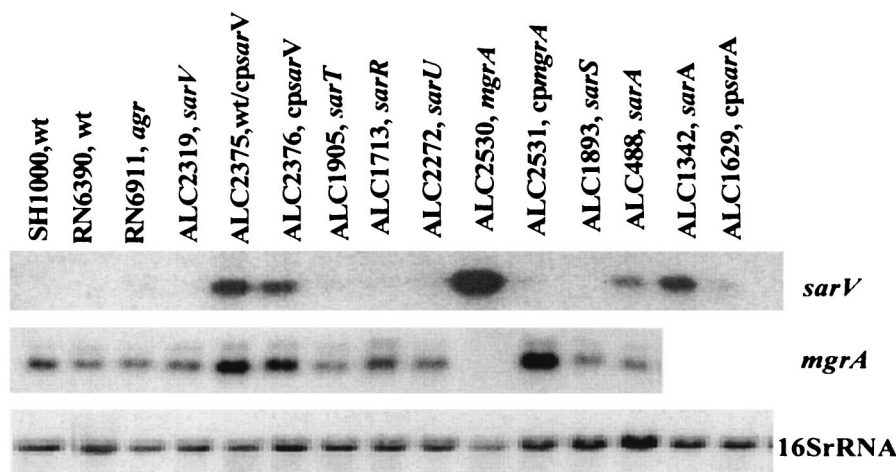
FIG. 1. Northern analysis of the *sarV* and *mgrA* transcripts in the wild type, various isogenic mutants, and complemented strains at postexponential phases (OD_{650}, ~1.7) of growth. A total of 15 µg of cellular RNA was loaded onto each lane. The blots were probed with 360-bp *sarV* (upper panel), 565-bp *mgrA* (middle panel), and 724-bp (lower panel) fragments containing the entire ORF of the *sarV* and *mgrA* genes and internal region of 16S rDNA, respectively. cp indicates complementation with respective genes. The OD_{650} was determined with a spectrophotometer (Spectronic 20).

RNA isolated in the early phase of growth of various mutants (data not shown).

Transcriptional start sites and promoter structure of the *sarV* gene. To determine the transcriptional start site and the promoter sequence, primer extension was performed with total RNA isolated from the wild-type strain RN6390 and its isogenic *sarA* mutant (data not shown). The transcriptional start site was mapped to an A or T which was located 62 or 63 bp upstream of the initiation codon ATG. Based upon the transcriptional start site, the predicted putative promoter boxes are TAGATA(-35)-N_{17}-TATAAT(-10), which has close homology with the -10 and -35 consensus sequences of σ^A-dependent promoters (Fig. 2A; see also Fig. 4D). A strong ribosome binding site, AGGAGG, was located 9 bp upstream of the ATG translational start codon. Analysis of the downstream sequence of the *sarV* gene disclosed the presence of a *rho*-independent transcriptional terminator sequence from the position 1 to 91 bp immediately after the stop codon (TAA). Within this region, a 23-bp inverted repeat sequence from positions 1 to 23 and 68 to 91 after the stop codon of the *sarV* gene (AAAAATAAAAAGCATGCCAATCT-N_{45}-AGATTG GCATGCTTTTTAAGTTTTT) could form a potential hairpin structure with a 24-nt-long heteroduplex.

***sarV* promoter-*gfp_uvr* fusion studies in isogenic *sarA* and *mgrA* strains.** To confirm the repression of *sarV* by *mgrA* and *sarA*, we constructed a transcriptional fusion containing a 268-bp *sarV* promoter fragment upstream of the *gfp*_uvr reporter gene (Fig. 2A). The construct pALC2489 was introduced into the wild-type strain RN6390, *sarA* mutant (ALC1342), *mgrA* mutant (ALC2530), *sarV* mutant (ALC2319), *sarA*/*mgrA* double mutant (ALC2535), complemented *mgrA* mutant (ALC2531), and complemented *sarA* mutant (ALC1629). Promoter activities, assayed as GFP_uvr fluorescence (Fig. 2B), were ~5-, 13- to 17-, and 18- to 28-fold higher at the three time points for *sarA* (lane 3), *mgrA* (lane 5), and *sarA*/*mgrA* (lane 7) mutants, respectively, compared with that of the parental strain RN6390 (lane 1). Complemented *sarA* and *mgrA* mutant strains (Fig.

2B, lanes 4 and 6) had reduced GFP_uvr values compared to the parental strain. These data are consistent with the notion that the expression of *sarV* gene transcript is repressed by *sarA* and *mgrA* gene products. In the double *sarA*/*mgrA* mutant (Fig. 2B, lane 7), the mean GFP_uvr value was higher than that for the single mutant, indicating that SarA and MgrA may repress *sarV* gene expression via divergent mechanisms. Collectively, these data concur with those of Northern analysis, essentially demonstrating that the expression of *sarV* gene transcript was undetectable in the wild type, moderately elevated in the *sarA* mutant, and highly expressed in the *mgrA* mutant. The expression of *sarV* is probably not dependent on its own gene product, as the mean GFP_uvr value (228 to 407 $\pm$ 75 U) in the *sarV* mutant was comparable to that for the parental strain RN6390 (176 to 322 $\pm$ 50 U).

Binding of SarA and MgrA proteins to the *sarV* promoter region. As the level of transcript of *sarV* was increased in *sarA* and *mgrA* mutants (Fig. 1), we speculated that SarA and MgrA may bind to the *sarV* promoter region to modulate *sarV* expression. In previous studies, we reported that members of the SarA protein family (e.g., SarA, -R, -S, and -T) have a conserved helix-turn-helix motif commonly found in DNA binding proteins (10, 30, 31). The MgrA protein, as a MarR homolog and to a lesser extent SarA, likely possesses a helix-turn-helix motif and hence is predicted to be a DNA binding protein. To verify this, we employed a 268-bp or a 191-bp *sarV* promoter fragment (position 21 to 288 or 21 to 211 nt upstream of the initiation codon) upstream of the ribosomal binding site for DNA binding assays. The DNA fragment (0.1 ng or 0.56 fmol per reaction mixture) was end labeled with [γ-^{32}P]ATP and used in gel shift assays with various amounts of purified SarA and MgrA proteins (Fig. 3). Retarded protein-DNA complex could be detected with as little as 0.1 to 0.2 µg of MgrA (2.8 to 5.7 nM, assuming that MgrA is a dimeric protein like SarA or SarR) and ~0.3 µg (10 nM) of SarA. As the concentrations of these proteins increased, the retarded protein-DNA complex became the predominant band, with complete conversion at
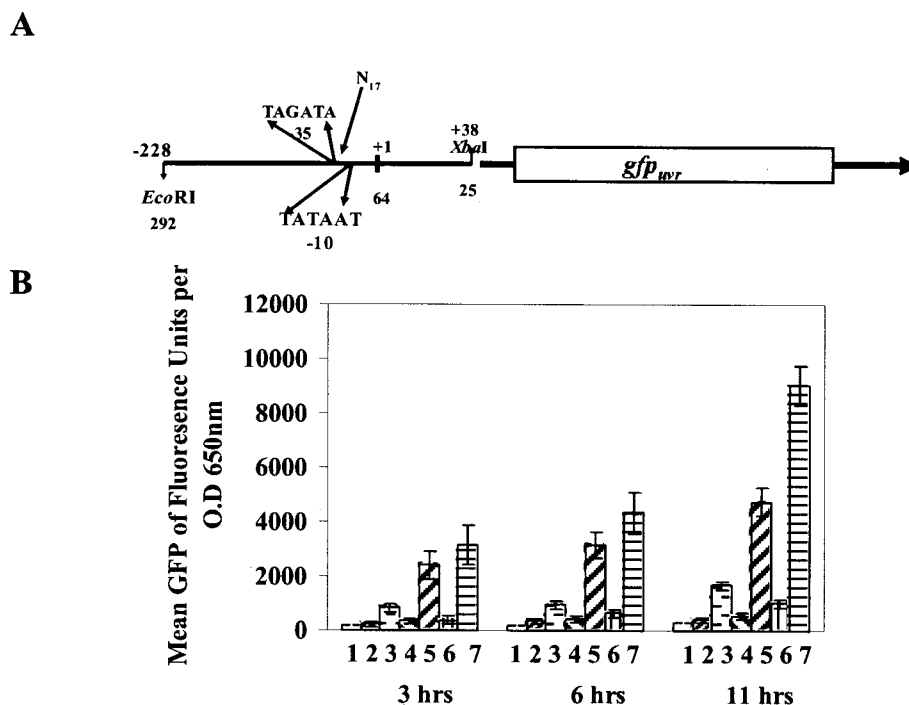
A

B

FIG. 2. Promoter activation of the *sarV* promoter fused to a *gfp*$_{uvr}$ reporter gene. (A) Graphical representation of the 268-bp *sarV* promoter fragment fused to a promoterless *gfp*$_{uvr}$ gene with an *S. aureus* ribosome binding site. The translational start site, labeled as +1, was identified by primer extension, and the putative promoter −10 and −35 boxed sequences are also indicated. The numbers at the line (both top and bottom) are marked according to the transcriptional start site and the translation start codon of the *sarV* gene, respectively. (B) The recombinant shuttle construct pALC2489 containing the 268-bp *sarV* promoter fragment driving the GFP$_{uvr}$ reporter gene was introduced into the wild type, its isogenic mutant strains, and complemented strains. Lane 1, wild-type RN6390; lane 2, *sarV* mutant ALC2319; lane 3, *sarA* mutant ALC1342; lane 4, the *sarB* fragment (28) integrated into the *geh* locus of the *sarA* mutant designated ALC1629; lane 5, *mgrA* mutant ALC2530; lane 6, the 1.5-kb DNA fragment containing the *mgrA* gene integrated into the *geh* locus of the *mgrA* mutant designated ALC2531; lane 7, *mgrA sarA* double mutant ALC2539. To minimize variations in fluorescence attributable to cell density, the data are presented as the average of reported fluorescence unit per OD$_{650}$ in triplicate samples obtained at different growth points.

~1.0 μg for both proteins. Hence, SarA and MgrA proteins can bind to the *sarV* promoter region, presumably acting as a repressor to *sarV* transcription. To verify the DNA binding specificity and to map the binding sites, we performed DNase I protection assays with the same 268- or 191-bp *sarV* promoter fragment labeled with γ-^{32}P at one end in the presence of SarA

or MgrA (Fig. 4). As shown in Fig. 4A, protection from DNase I digestion with SarA was mapped to a region +2 to −42 bp from the +1 transcription start in Fig. 4D. A close analysis of this sequence revealed that the protected regions (box I, −21 to −42 bp from the transcription start; box II, +2 to −19 bp from transcription start in Fig. 4D) closely resemble the SarA
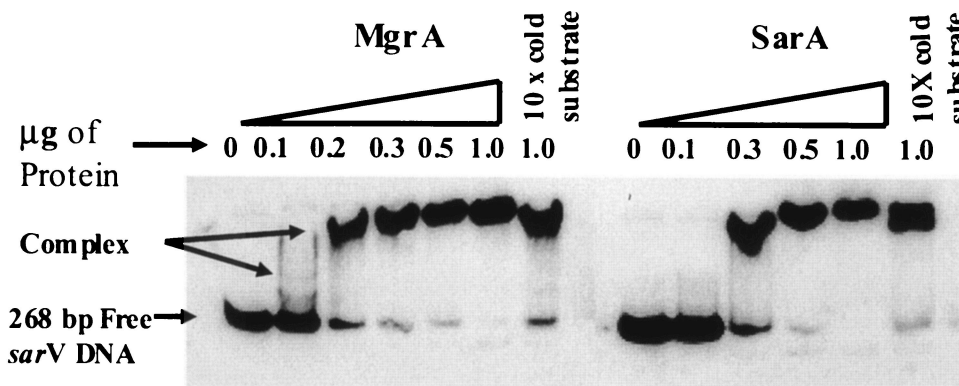

FIG. 3. Autoradiogram of an 8.0% nondenaturing polyacrylamide gel showing gel shifts for purified MgrA and SarA proteins with a 268-bp *sarV* promoter fragment. Mobility of the DNA band in the presence of increasing amounts of either MgrA or SarA protein is indicated on the top. Cold competition lanes with a 10-fold excess (molar ratio) of the unlabeled 268-bp *sarV* fragment are indicated and contained 1.0 μg of the purified MgrA or SarA protein.
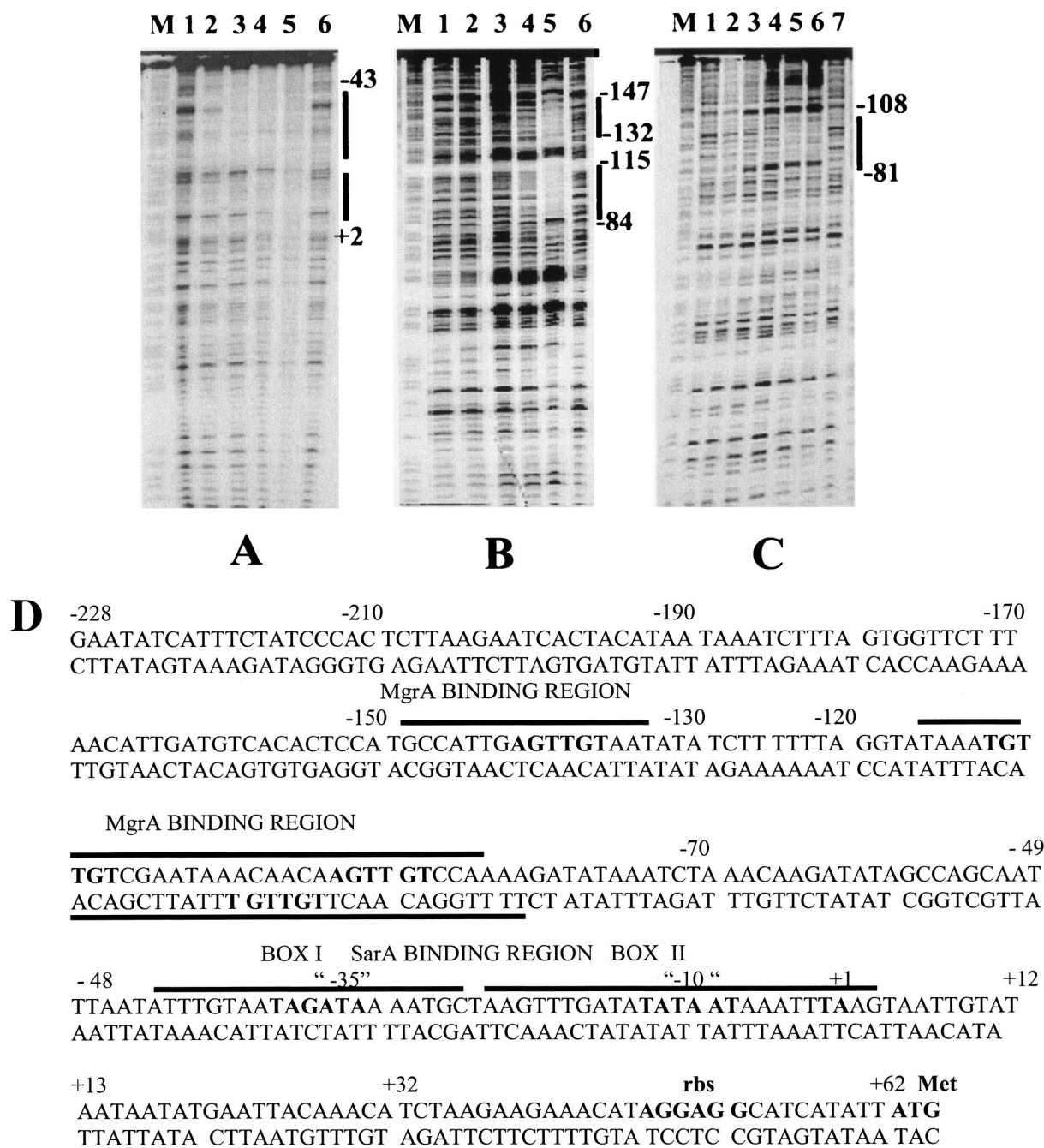
FIG. 4. DNase I protection footprint analysis of SarA and MgrA binding to the *sarV* promoter region of *S. aureus*. (A) Footprint analysis of SarA binding to the *sarV* promoter region. The 191-bp fragment (nt positions −21 to −211 from the start codon ATG) was end labeled (top or coding strand) with [γ-32P]ATP. Lanes 1 and 6, no protein added; lanes 2 to 5, 1, 2, 3, and 4 μg of SarA, respectively. Lane M represents chemical cleavage at purine residues (A/G ladder). (B and C) Footprinting analysis of the MgrA protein binding to the 268-bp *sarV* promoter region (nt positions 21 to 288 from the ATG start codon). The 268-bp fragment was end labeled for top strand (B) and bottom strand (C) and incubated with DNase I in the presence or absence of proteins, as described above. No protein was added for lanes 1 and 6 in panel B or for lanes 1 and 7 in panel C. Lanes 2 to 5 (B) and 4 to 6 (C), 0.2, 0.5, 1.0, and 2.0 μg of protein was added; lane 3 of panel C, 0.3 μg of protein was added. Protected regions are marked by solid lines. (D) Nucleotide sequence of the 293-bp fragment upstream of the *sarV* gene was shown and marked with the putative promoter region and start site as derived from primer extension data. The putative protected regions for SarA and MgrA proteins on the promoter are marked.

consensus DNA binding motif (ATTTGTATTTAATATTTA TATAATTG) previously reported by our investigators (10). Box I and II have 15 of 26 and 19 of 26 matches to the SarA consensus binding sequence, respectively, thus verifying that the DNA binding site of SarA on the *sarV* promoter is likely specific and further confirming our group's earlier studies on the predicted SarA binding consensus sequence. We also mapped the DNA binding site of the MgrA protein on the *sarV* promoter. Using the same end-labeled 191-bp promoter fragment, we initially failed to identify any protected region with

both strands (data not shown). However, when the assays were repeated with the larger 268-bp *sarV* promoter fragment that we have used for the *gfp*_{*uvr*} fusion assay (position −21 to −288 bp from the start codon), we found two closely associated protected regions (Fig. 4B and C). The putative protected regions of MgrA are from positions −84 to −115 bp, −132 to −147 bp (Fig. 4B, top strand of DNA), and −81 to −108 bp upstream of the transcriptional start site (Fig. 4C, bottom strand of DNA, and D). DNA sequence analyses of these regions disclosed a 6-bp conserved sequence (t/aGTTGT) that was repeated four times in the top and bottom strands of the protected sequence (Fig. 4D). The sequence encompassing −84 to −115 bp upstream of the initiation codon was of interest as it contained the sequence t/aGTTGT thrice and was protected in both strands. This led us to speculate that MgrA may bind to this region to facilitate binding to other areas via conformational changes (e.g., by cooperating interaction, bending, or other means of regulation). Whether the conserved 6-bp binding region of MgrA applies to other target loci regulated by MgrA requires additional confirmation. Nevertheless, the results presented in Fig. 3 and 4 clearly demonstrated that SarA and MgrA proteins are likely to bind to different sites on the *sarV* promoter region with a high degree of specificity. The SarA binding sites overlap with the −10 and −35 promoter boxes, whereas the MgrA binding sites are upstream of the *sarV* promoter boxes.

Transcription and transcriptional fusion assays for regulatory genes in a *sarV* mutant and various other isogenic strains. To determine whether inactivation of *sarV* affects other regulatory genes involved in virulence, we performed Northern blot analysis with 11 other regulatory loci (*agr* RNAII, *agr* RNAIII, *sarA*, *sarR*, *sarS*, *sarT*, *sarU*, *mgrA*, *rot*, *sae*, *lytS*, *lytR*, *arlR*, and *arlS*). The functions of many SarA homologs have previously been described, including SarR as a regulator of SarA and *agr* expression, SarS as an activator of protein A expression, SarT as a repressor of α-hemolysin expression, and SarU as an activator of RNAIII that is repressible by SarT (9, 30, 31, 41). The *lytSR* locus is a two-component regulatory system that negatively controls extracellular murein hydrolase activity via positive regulation of the *lrgAB* operon (6, 16). An *arlRS* mutant up-regulates the expression of autolysis genes while augmenting transcription of *agr* and other exoprotein genes, including those of extracellular serine protease activity and the multidrug resistance transporter gene *norA* (12, 13). The *saeRS* locus encodes another two-component regulatory system that positively regulates the expression of α- and β-hemolysins, DNase I, coagulase, and protein A (15). As shown in Fig. 5A, there was a partial reduction in RNAII and, to a lesser extent, RNAIII expression during the postexponential phase in the *sarV* mutant. Likewise, the levels of transcripts of *lytSR* and *arlRS* were also reduced in the *sarV* mutant. In contrast, the transcription levels of *sarR*, *saeRS*, *sarA*, *sarS*, *sarT*, *rot*, and *sarU* were not significantly altered in the *sarV* mutant (data not shown). However, it should be stressed that the expression of *sarS*, *sarT*, and *sarU* was difficult to detect even in the parental strain under routine laboratory culture conditions. Complementation of the parent and *sarV* mutant with a plasmid carrying the *sarV* gene either augmented or restored *agr* RNAII, *agr* RNAIII, *lytSR*, and *arlRS* transcripts to near or above parental levels in the recipient strain. Collectively, these data

suggested that *sarV* might act as an activator either directly or indirectly for the expression of *agr*, *lytSR*, and *arlRS*.

To verify reduction of *agr* RNAII, *agr* RNAIII, *arlS*, and *lytR* transcripts in the *sarV* mutant (ALC2319), we introduced into the isogenic *sarV* strains plasmids containing the *agr* RNAII, *agr* RNAIII, *arlS*, or *lytR* promoter linked to the *gfp*_{*uvr*} gene for promoter fusion analysis. Upon monitoring bacterial growth and serial GFP expression over a 10-h period, we found that the growth rates were comparable between the two strains containing each of the constructs, with early stationary phase appearing after ~6 h of growth. The expression of RNAII and RNAIII during the stationary phase was slightly lower in the *sarV* mutant than in the parental strain (Fig. 5B and C). Thus, both Northern blotting and transcriptional fusion assays were consistent with the slight reduction of levels of transcripts in *agr* RNAII and RNAIII expression in the *sarV* mutant relative to that in the parental strain. Similarly, the expression of *arlS* promoter-*gfp*_{*uv*} and *lytR* promoter-*gfp*_{*uv*} was found to be reduced approximately threefold in the *sarV* mutant compared to the wild-type strain after ~8 h of growth (Fig. 5D). Hence, Northern and transcriptional fusion studies suggested that the *sarV* gene product possibly acts as an activator for the expression of *agr*, *arlRS*, and *lytSR* transcripts. These results were consistent in several repeated experiments. Whether this effect is direct or indirect and also physiologically relevant remains to be determined.

Northern blot analysis for the expression of target genes. The data presented above clearly indicated that the expression of *sarV*, normally undetectable in the parental strain, was elevated moderately in the *sarA* mutant and highly in the *mgrA* mutant. SarA and MgrA, as DNA binding proteins, bind to the *sarV* promoter region, presumably to repress *sarV* transcription. As *sarA* and *mgrA* participate in the control of autolysis as well as virulence genes (4, 8, 10, 12, 17, 26, 45), we determined the expression of target genes known to be involved in autolysis or virulence in the *sarV* mutant. We tested the expression of 19 potential target genes (33), with 11 genes implicated in virulence (e.g., *hla*, *coa*, *geh*, *splA*, *scp*, *norA*, *aur*, *spa*, *clfA*, *fnbA*, and *fnbB*) and 8 involved in autolysis (*lytN*, *lytM*, *atl*, *lrgB*, *scdA*, *abcA*, *pbp2*, and *pbp4*). The transcript levels of genes encoding many cell surface-associated proteins (*fnbA*, *fnbB*, *clfA*, and *spa*) were not markedly altered by the *sarV* mutation during exponential and postexponential phases of growth (data not shown). The expression levels of *geh* (glycerol ester hydrolase) and *coa* (coagulase) were also not significantly affected in the *sarV* mutant (data not shown). The transcript level of the *hla* gene was lower in the *sarV* mutant compared with that in the parental strain but was restored upon complementation (Fig. 6). Transcriptional fusion of *hla*-*gfp*_{*uvr*} also supported the Northern blotting data (1,185 ± 250 versus 2,278 ± 500 mean fluorescence units per OD at 6 h of growth for the *sarV* mutant and parent strain, respectively). A significant reduction in transcripts levels of *splA*, encoding a putative serine protease-like gene product, *aur*, encoding a zinc metalloprotease, and *scp*, encoding staphopain (protease II) (19, 39) was observed in the *sarV* mutant, while transcripts levels of these genes were substantially elevated in the parental strain or returned to a near-normal level in the mutant harboring *trans*-complementation of the *sarV* gene in multicopy (Fig. 6). As predicted from earlier findings, the *agr* mutant also displayed a significant
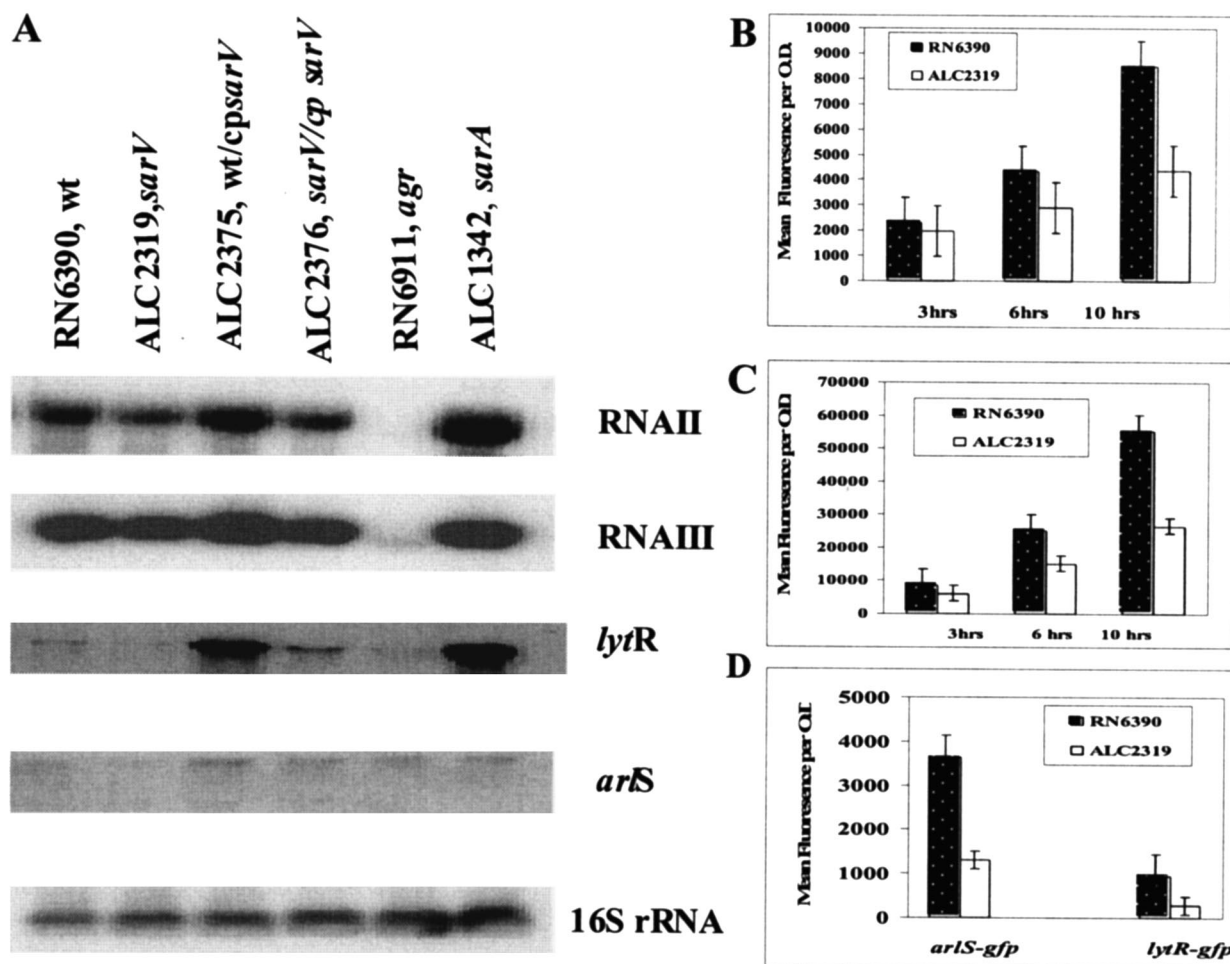
FIG. 5. Analysis of assorted regulatory genes in a *sarV* mutant. (A) Northern blots of *agr* RNAII and RNAIII, *lytR*, *arlS*, and 16S rRNA transcripts in the wild-type RN6390, *sarV* (ALC2319), *trans*-complemented strains (ALC2375, ALC2376), and *sarA* (ALC1342) and *agr* (RN6911) mutants. A total of 10 μg of RNA from the postexponential phase (OD$_{650}$, ~1.7) was applied to each lane, and the blots were probed with the respective gene fragment containing the coding region. (B to D) GFP$_{uvr}$ expression as driven by *agr* RNAII (B), *agr* RNAIII (C), and *arlS-gfp$_{uv}$* and *lytR-gfp$_{uv}$* (D) promoters in the wild-type RN6390 and *sarV* mutant ALC2319. Bar diagrams in panels B and C represent various time points, whereas in panel D the diagram represents the ~8-h time point. The experiments were repeated at least thrice; one representative experiment is shown.

reduction in these proteases, while a *sarA* mutation led to an increase in the expression of these transcripts (19, 39).

The level of transcript of *scdA*, a gene positively associated with decreasing peptidoglycan cross-linking and increased autolysis (7), and the autolysin gene *atl*, which encodes major autolysin (34, 36, 40), was found to be decreased in the *sarV* mutant, thus suggesting positive regulation of *scdA* and *atl* by *sarV* gene; complementation of the *sarV* mutant with a multicopy plasmid (ALC2376) increased the expression of *scdA* or *atl* in the mutant, but the effect was only partial, thus suggesting that regulation of these genes by *sarV* may possibly be indirect or via some unknown factor. Interestingly, a *sarA* mutation increased *scdA* and *atl* transcripts, while *agr* inactivation did not have any significant change (Fig. 6). The *abcA* gene, encoding an ATP binding cassette transporter, is divergently transcribed from *pbp4* (encoding penicillin binding protein 4) and is associated with cell autolysis (42). Interestingly, inactivation of *sarV* augmented the *lrgB* transcript level (Fig. 6), while a *sarA* mutant also increased *lrgB* transcription, consis-

tent with *S. aureus* gene array analysis (DNA chip) data (11). Northern analysis revealed little or no impact effect of *sarV* on *abcA* expression. In contrast, an *agr* mutant, but not a *sarA* mutant, displayed a significant reduction in *abcA* transcript level, in concordance with the data of Schrader-Fischer and Berger-Bächi (42). Under our experimental conditions, the *pbp4* transcript was found to be undetectable in the isogenic *sarV* strains. Additionally, there was no significant difference in *norA*, *pbp2*, *lytN*, and *lytM* transcript levels attributable to the *sarV* mutation (data not shown). Thus, a mutation in *sarV* led to a reduction in transcript level of *atl*, the major autolysin, as well as an alteration in *scdA* and *lrgB* expression profiles consistent with an autolysis-resistant phenotype.

Phenotypic characterization of the *sarV* gene. The alteration in *atl*, *lrgB*, and *scdA* expression levels of transcripts in the *sarV* mutant, coupled with the repressive effect on *sarV* by *mgrA* and *sarA*, suggested that *sarV* may play an important role in controlling autolysis mediated by *mgrA* and *sarA* in *S. aureus*. Accordingly, we tested the *sarV* mutant for susceptibility to lysis in
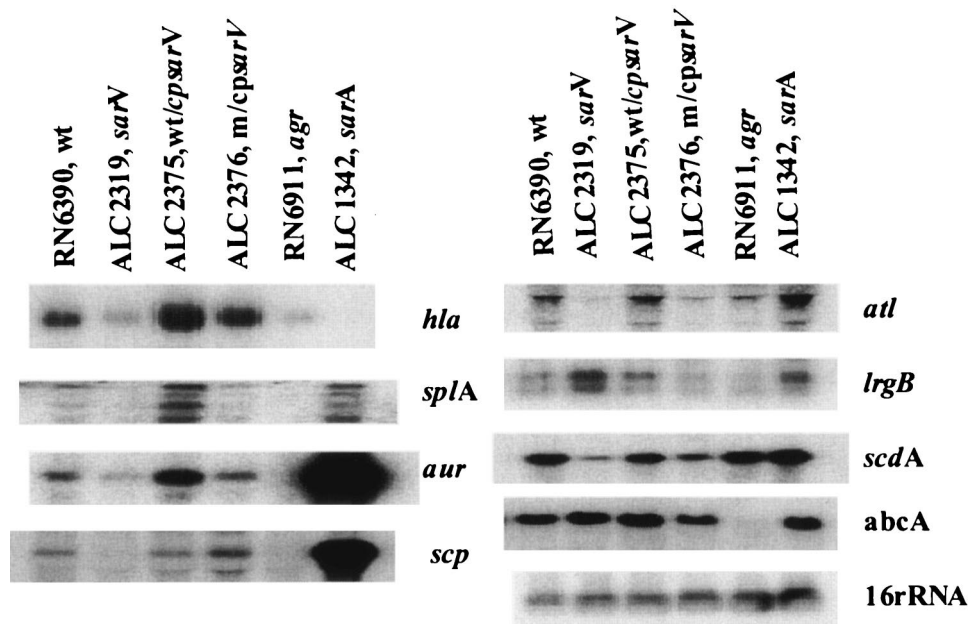
FIG. 6. Northern blot analysis for assorted target genes in the wild type, *sarV* mutant (ALC2319), *trans*-supplemented strains ALC2375 and ALC2376, *sarA* mutant ALC1342, and *agr* mutant RN6911. Northern blots were hybridized with *hla*, *splA*, *aur*, *scdA*, *lrgB*, *atl*, *abcA*, and 16S rRNA DNA fragments containing the coding regions of the respective genes. A total of 10 μg of RNA from the postexponential phase (OD$_{650}$, ~1.7) was applied to each lane, and the OD$_{650}$ was determined with a spectrophotometer (Spectronic 20).

the presence of a detergent and an antibiotic. As shown in Fig. 7A, the *sarV* mutant was more resistant to Triton X-100-induced lysis in 50 mM Tris-Cl buffer, pH 7.2, than the wild-type RN6390 strain. The buffer alone also induced lysis of the wild type, and this effect was amplified by 0.05% Triton X-100. In

contrast, the *sarV* mutant strain remained less susceptible in the buffer. Interestingly, the parental strain carrying the *sarV* gene on multicopy plasmid (ALC2375) became slightly more lytic than the wild-type strain in the presence of Triton X-100. Similarly, the *sarV* mutant carrying the *sarV* gene on multicopy
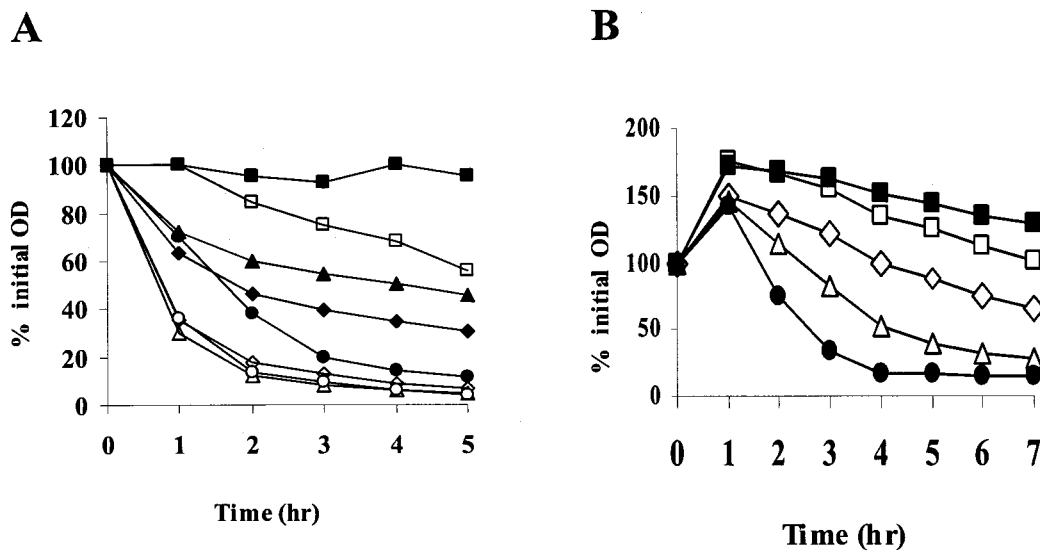

FIG. 7. Phenotypic and *trans*-functional analysis of the *sarV* gene. (A) Effect of the *sarV* mutation on Triton X-100-induced autolysis. ◇ and ◆, wild-type *S. aureus* strain RN6390; □ and ■, *sarV* mutant ALC2319; △ and ▲, wild-type strain containing *trans*-supplemented *sarV* gene in multicopy plasmid pSK236, ALC2375; ○ and ●, *sarA* mutant strain ALC488. Strains were grown as described in Materials and Methods, with 0.05% Triton X-100 (open symbols) and without Triton X-100 (filled symbols), following the decline of A$_{650}$ as an indicator of lysis. (B) Effect of *sarV* on penicillin-induced autolysis. Penicillin G-induced autolysis of wild-type RN6390 (◇), *sarV* mutant ALC2319 (□), RN6390 containing *trans*-supplemented *sarV* gene, ALC2375 (△), *agr* mutant RN6911 (■), and *sarA* mutant ALC1342 (●) was measured as the decline in culture turbidity versus time. Autolysis is expressed as the percent of the initial OD$_{650}$ reading immediately before penicillin was added. The experiments were repeated at least thrice; one representative experiment is shown.
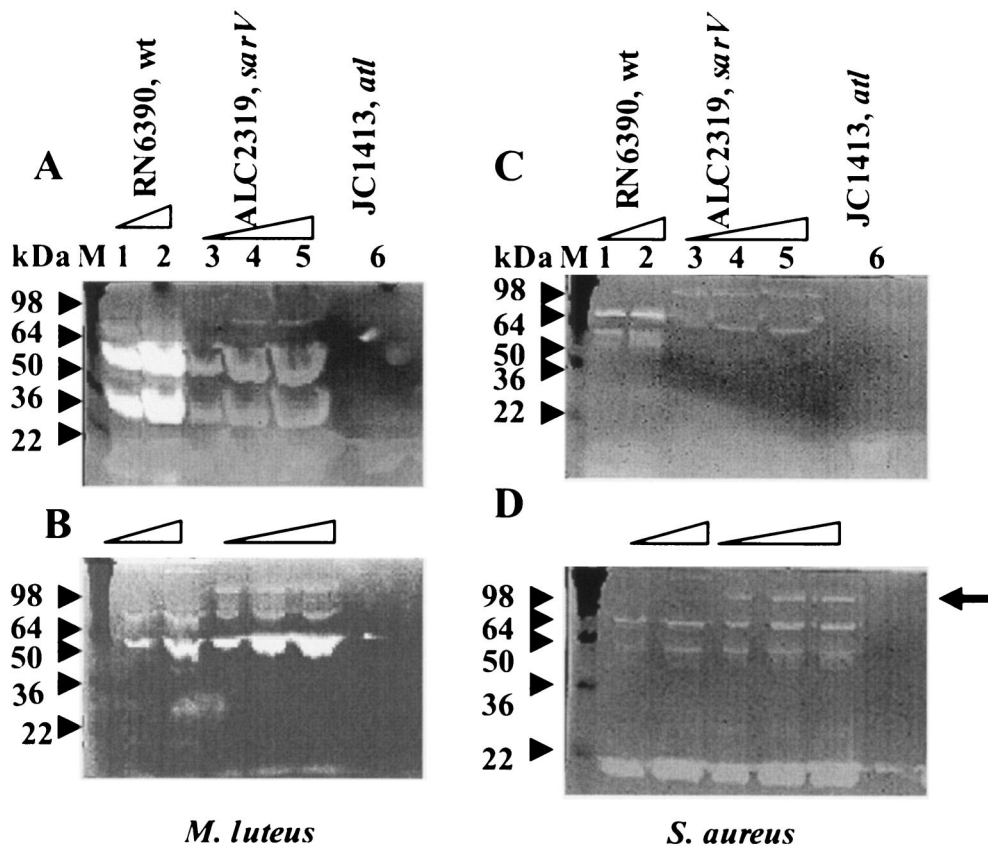
FIG. 8. Zymographic analysis of various fractions of different *S. aureus* strains. Extracellular proteins (A and C) and intracellular proteins (B and D) were isolated, and various amounts were loaded onto an SDS–12% polyacrylamide gel containing 1.0 mg of either *M. luteus* (A and B) or heat-killed RN6390 cells/ml, resolved, and treated with a buffer containing 1% Triton X-100, followed by staining with 1% methylene blue. Lanes 1 and 2, 2 and 4 μg of extra- or intracellular proteins of RN6390 strain; lanes 3 to 5, 2, 4, and 6 μg of extra- or intracellular proteins of the *sarV* mutant (ALC2319); lane 6, 4 μg of extra- or intracellular proteins of the *atl* mutant (JC1413). Molecular size markers in kilodaltons are indicated to the left of each gel.

plasmid (ALC2376) was more susceptible to lysis, but slightly less than its wild-type counterpart (data not shown). We also tested the effect of *sarV* on penicillin-induced lysis. Penicillin G (0.4 μg/ml) was added to a growing culture at early exponential phase ($OD_{650} = 0.5$), and changes in optical density (OD_{650}), as a percentage of initial absorbance (OD), were monitored over a 7-h period. As shown in Fig. 7B, the parental strain RN6390 exhibited sensitivity to penicillin-induced lysis while the *sarV* mutant was resistant, resulting in an OD higher than that of the starting culture even at 6 h after the addition of penicillin. As a positive control, the *sarA* and *agr* mutants displayed high and low sensitivities, respectively, to penicillin-induced lysis, consistent with previously published data (14, 27).

To determine whether the lytic resistance phenotype was attributable to variations in murein hydrolase activity, possibly due to decreased expression of *atl* or *scdA* and/or enhanced expression of *lrgB*, zymographic analyses of culture supernatants and intracellular proteins of various strains were performed using gels containing *M. luteus* or *S. aureus* cells to detect glucosaminidase (GL) (Fig. 8A and B) and amidase (AM) (Fig. 8C and D) activities, respectively. It is known that *atl* (autolysin) is expressed as a 138-kDa precursor protein that is processed by protease to yield GL and AM domains of 54 and 63 kDa, respectively (34). Comparison between various

strains revealed that the culture supernatant of the *sarV* mutant (ALC2319) displayed decreased GL (Fig. 8A) and AM (Fig. 8C) activities compared to the wild-type RN6390 strain. Significantly, no GL and AM activities were detected in supernatant of an *atl* mutant, JC1413 (Fig. 8, lanes 6). The intracellular proteins of the *sarV* mutant showed no significant variation of GL activity in the 54-kDa-size region (Fig. 8B) or AM activity in the 63-kDa-size region (Fig. 9D), but there was an extra zone of hydrolysis for both activities in the >97-kDa-size region compared to results with RN6390. We speculated that the appearance of this band was due to the unprocessed autolysin, which is yet to be confirmed. The cell wall-associated proteins of the *sarV* mutant also showed similar types of variation of the GL and AM activities as were found for intracellular proteins (data not shown). The variation in autolytic activity of autolysin is controlled by multiple factors that include SspA/V8 serine protease, SspB/cysteine protease, *lrgAB* operon/speculated holin and antiholin, another regulatory locus, and or an undefined regulatory mechanism (37). We also speculated that various other proteases, like *aur*, *splA*, and *scp*, which are decreased in a *sarV* mutant, might be involved in the resistance of autolysis.

To quantify the zymographic analysis, cell wall hydrolysis assays were performed as described by Rice et al. (38). Culture
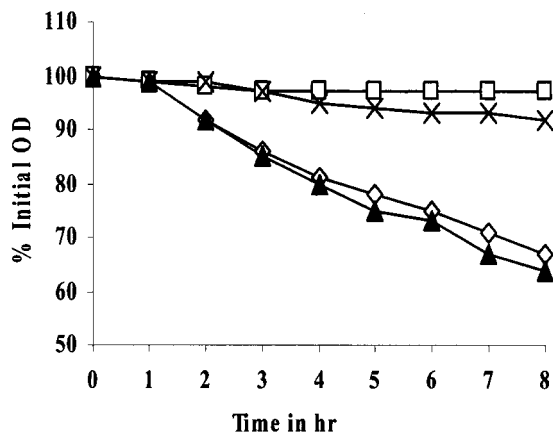
FIG. 9. Quantitative murein hydrolase assays of the *sarV* mutant. Aliquots of 100 μg of the extracellular proteins of various strains were added to a suspension of *M. luteus* cells (1.0 mg per ml), and turbidity was monitored for 8 h. Strains used for quantitative hydrolase analysis were the wild-type RN6390 (◇), *sarV* mutant ALC2319 (□), and the wild-type strain containing *trans*-supplemented *sarV* in multicopy plasmid pSK236, ALC2375 (▲). ×, control in which no extracellular protein was added to the cell suspension. These data are from a single representative experiment and were reproduced several times. OD was determined at 580 nm with a spectrophotometer (Spectronic 20).

supernatants (100 μg) were added to a suspension of *M. luteus* cells (1.0 mg per ml) in 100 mM Tris-Cl (pH 8.0) and incubated at 37°C with shaking, and the turbidity of the samples was monitored at 580 nm. As shown in Fig. 9, the extracellular murein hydrolases of ALC2319 caused no significant decrease in turbidity of *M. luteus* cells after 8 h of incubation, compared to about a 35% decrease in turbidity with the wild-type RN6390 extracellular murein hydrolases. In contrast, the presence of the *sarV* gene in multicopy plasmid in the wild-type RN6390 caused about a 40% decrease in turbidity after 8 h. These results, along with the zymographic analysis, demonstrate that inactivation of the *sarV* gene results in a reduction in the extracellular murein hydrolase activity produced by *S. aureus*.

DISCUSSION

In prior studies, we and others have characterized SarA-like proteins, including SarA (SA0573), SarR (SA2089), SarS (SA0108; SarH1), Rot (SA1583), SarT (SA2286; SarH2), and SarU (SA2287; SarH3), that are involved in the regulation of virulence genes in *S. aureus*. Similar to SarA, SarV has sequence similarity to other SarA homologs, such as SarA, SarR, SarT, and Rot, and also to those with two homologous halves, such as SarS and SarU (data not shown). Unlike MgrA, SarV has a lower degree of sequence similarity to MarR-like proteins (e.g., ~20% identities with MarR-like proteins of *B. subtilis* and *Xanthomonas axonopodis*). Despite this finding, there are specific residues with the protein sequence that are conserved, similar to those found with members of the SarA protein family (see below).

We recently solved the winged-helix structure of SarR, a member of the SarA protein family (24). The dimeric winghelix structure of SarR is a three-domain structure containing a central helical core and two winged-helix motifs. Each of the

winged-helix motifs encompasses a helix-turn-helix motif (residues 52 to 73) and a β-hairpin turn (residues 80 to 95), with both predicted to be DNA binding domains, based upon sequence alignment (23). Interestingly, many of the basic residues are conserved within members of the SarA protein family, consistent with the notion that the DNA binding domains might be highly conserved within this protein family. Indeed, detailed mutational analysis of SarR indicates that many of the conserved residues (e.g., K52, K80, R82, R88, etc.) are involved in DNA binding (A. C. Manna et al., unpublished data). The structures of MarR in *E. coli* and SarS in *S. aureus* were recently solved and were found to be variants of the dimeric winged-helix structure, both containing features that are distinct from SarR (1, 23). Based on structures and sequence alignment, we propose to classify the SarA protein family into three subfamilies: (i) the single-domain proteins (e.g., SarR and SarV); (ii) the two-domain proteins (e.g., SarS and SarU); and (iii) single-domain proteins that are homologous to MarR (e.g., MgrA and its homologs). We predict that members within the same subfamily likely posses similar structures with common DNA binding domains and a divergent activation or repression domain.

The transcription of *sarA*-like genes is highly variable in strain RN6390. For instance, *sarA*, *sarR*, and *mgrA* transcripts are highly expressed in the parental strain, whereas the transcription of *sarU*, *sarT*, and *sarS* is not readily detectable under normal laboratory growth conditions. We also found the *sarV* gene to be poorly transcribed in the parental strain. Northern analyses of *agr*, *sarR*, *sarT*, *sarU*, and *sarS* mutants revealed transcription of the *sarV* gene to be low in these mutants but at a high level in the *sarA* mutant and more so in the *mgrA* mutant. The effect of a *sarA* mutation on *sarV* transcription was also supported by similarly increased mean GFP$_{uvr}$ values in the *sarV* promoter-*gfp*$_{uvr}$ fusion analysis in three different *sarA* mutant strains in our laboratory (data not shown). The finding that the *mgrA* transcript was elevated in the *sarV*-supplemented parent and *sarV* mutant strains (Fig. 1) suggested the plausibility of a feedback loop on *mgrA* expression by the *sarV* gene, possibly to fine-tune *mgrA* expression. This result was also supported by promoter fusion studies (*mgrA* P1 promoter-*gfp*$_{uvr}$) in a pair of isogenic *sarV* strains, documenting a substantial reduction in mean GFP$_{uvr}$ fluorescence in the *sarV* mutant ALC2319 (7,996 ± 750 units per OD$_{650}$) compared the wild-type RN6390 (11,775 ± 1,000 units per OD$_{650}$). However, the applicability of this magnitude of increased *sarV* gene dosage to augment *mgrA* transcription under in vivo conditions was not defined in this study.

In gel shift studies (Fig. 3), we found that purified SarA and MgrA proteins formed complexes with the *sarV* promoter with fairly high affinity. As protein-DNA complexes are shifted substantially, we speculate that multiple SarA or MgrA dimers might interact with each other to bind to the *sarV* promoter region. This is based on our modeling data, showing that three SarA dimers are needed to bind to a single 135-bp *agr* promoter fragment (unpublished data). The specificity of the SarA and MgrA proteins was determined by DNase I footprinting. Two SarA binding regions (box I and II) were found within the 268-bp *sarV* promoter sequence, locating and overlapping with the −10 and −35 region of the *sarV* promoter. Interestingly, each of these two binding regions resembles the SarA consen-

sus binding motif or the SarA box, previously reported by our group (10). In contrast, the MgrA protein has binding regions outside of the promoter boxes that are distinct from the SarA binding site. These findings are consistent with the notion that MgrA and SarA may repress *sarV* via divergent mechanisms.

Using DNA gene chip technology, Dunman et al. (11) have demonstrated that *agr* and *sarA* regulate the expression of over 100 genes, including known virulence factors as well as other genes involved in metabolism and/or autolysis. Many of the known virulence genes are controlled by regulators like *agr*, *sarA*, *sarA* homologs, *sae*, and *arlRS*. Inactivation of the *sarV* gene has a partial effect on the expression of *agr*, *lytSR*, *arlRS*, and *mgrA*, while the effects on *sae*, *sarA*, *sarR*, *sarT*, *sarS*, *rot*, and *sarU* are minimal. However, the physiological relevance and the mechanism by which the *sarV* gene product, normally undetectable or at a very low level in the wild type under laboratory conditions, acts as a positive regulator (e.g., *agr*) are not clear from these studies.

Interestingly, *sarV* does not have any significant effect on cell surface proteins such as protein A (*spa*), FnbA and -B, and clumping factor A. The regulation of secreted proteins by *sarV* entails up-regulation of *hla*, *splA*, and *aur*, concordant with the notion that *sarV* regulates *agr*. The repression of the *sarV* gene by *mgrA* and *sarA* suggests that the *sarV* gene product may play a regulatory role in cell lysis, since both *mgrA* and *sarA* negatively control genes involved in autolysis, presumably by up-regulating murein hydrolase activity in respective mutants. Remarkably, lytic assays revealed that the *sarV* mutant was more resistant to the lytic effect of Triton X-100 and penicillin G. Surprisingly, an examination of regulatory genes (i.e., *lytSR* and *arlRS*) known to repress murein hydrolase activity disclosed down-regulation of these genes in the *sarV* mutant (Fig. 5 and 6). However, other genes (i.e., *scdA* and *atl* [Fig. 6]), positively linked to increase autolysis, were down-regulated in the *sarV* mutant, exactly what one would expect in a strain less prone to autolysis. The expression of *lrgB*, a part of the *lrgAB* operon, which negatively controls the autolysis process, was substantially higher in the *sarV* mutant than in the parent. Whether *lrgB* may be directly involved in the lytic-resistant phenotype of the *sarV* mutant was not clearly defined in our study. Because *atl*, *scdA*, *aur*, *splA*, and *scp* expression levels are altered in the *sarV* mutant, we speculate that *sarV* might, at least in part, render *S. aureus* cells more susceptible to cell lysis via a mechanism distinct from *mgrA* or *sarA*. Recently, a two-component system, *vraSR*, has been shown to positively regulate the cell wall-associated biosynthesis genes. DNA microarray analysis of *S. aureus* N315 and a *vraSR* mutant treated with vancomycin revealed that vancomycin induces transcription of 139 genes, and SA2062 (*sarV*) is one of them, up-regulated about 4.2-fold in both strains (22). These data supported our finding that *sarV* is an important transcriptional regulator and is involved in the regulation of the virulence and autolysis genes. Collectively, these data indicate that *sarV* impacts negatively upon the expression of proteases (*aur*, *splA*, and *scp*), an autolysin (*atl*), and a gene involved in peptidoglycan cross-linking (*scdA*). These phenotypic alterations would account for the autolysis-resistant phenotype of the *sarV* mutant and the sensitivity of the *sarV*-hyperexpressing strains to autolysis. Interestingly, zymogram analysis revealed that the *sarV* mutant has a different profile from that of an *atl* mutant (Fig. 8), thus indicating that reduced *atl* expression alone cannot account for the autolysis-resistant phenotype of the *sarV* mutant. The facts that *sarV* is repressed by negative regulators of autolysis such as MgrA and SarA and that the *sarV* mutant displays a divergent autolytic phenotype from *mgrA* and *sarA* mutants indicate that SarV is likely an important regulator controlled by *mgrA* and *sarA* to modulate cell lysis.

To further define the precise function of the *sarV* gene product, we are in the process of constructing various knockouts of *sarV* with other regulatory genes, particularly with *sarA* and *mgrA*, and investigating the molecular interaction of SarV with the upstream region of its target genes. Analysis of a *sarV* knockout in combination with mutations in *sarA*, *mgrA*, and other regulatory genes, coupled with our studies on the structure-function relationship of these proteins, may provide a clearer understanding of the mechanism of gene regulation by SarV and other SarA homologs. By unveiling the precise knowledge of these mechanisms and how they control virulence gene expression, this might open up new perspectives for antimicrobial chemotherapy using key inhibitors of these systems.

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