RbsR Activates Capsule but Represses the rbsUDK Operon in Staphylococcus aureus

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ABSTRACT
Staphylococcus aureus capsule is an important virulence factor that is regulated by a large number of regulators. Capsule genes are expressed from a major promoter upstream of the cap operon. A 10-bp inverted repeat (IR) located 13 bp upstream of the −35 region of the promoter was previously shown to affect capsule gene transcription. However, little is known about transcriptional activation of the cap promoter. To search for potential proteins which directly interact with the cap promoter region (Pcap), we directly analyzed the proteins interacting with the Pcap DNA fragment from shifted gel bands identified by electrophoretic mobility shift assay. One of these regulators, RbsR, was further characterized and found to positively regulate cap gene expression by specifically binding to the cap promoter region. Footprinting analyses showed that RbsR protected a DNA region encompassing the 10-bp IR. Our results further showed that rbsR was directly controlled by SigB and that RbsR was a repressor of the rbsUDK operon, involved in ribose uptake and phosphorylation. The repression of rbsUDK by RbsR could be derepressed by D-ribose. However, D-ribose did not affect RbsR activation of capsule.

IMPORTANCE
Staphylococcus aureus is an important human pathogen which produces a large number of virulence factors. We have been using capsule as a model virulence factor to study virulence regulation. Although many capsule regulators have been identified, the mechanism of regulation of most of these regulators is unknown. We show here that RbsR activates capsule by direct promoter binding and that SigB is required for the expression of rbsR. These results define a new pathway wherein SigB activates capsule through RbsR. Our results further demonstrate that RbsR inhibits the rbs operon involved in ribose utilization, thereby providing an example of coregulation of metabolism and virulence in S. aureus. Thus, this study further advances our understanding of staphylococcal virulence regulation.

Staphylococcus aureus produces a large number of virulence factors that endow the organism with the ability to cause a wide range of diseases in humans and animals. The expression of virulence genes is controlled by an equally impressive number of regulators forming a complex regulatory network (1–3). Although the regulation of virulence genes has been the subject of extensive studies recently, our knowledge of the virulence regulatory network in S. aureus is still fragmented. To further understand virulence regulation, we have been studying the S. aureus virulence regulatory network by employing capsule as a model virulence factor (4–7). Capsule is an antiphagocytic virulence factor, and the majority of S. aureus strains produce either type 5 or type 8 capsule (8, 9). Sixteen cap genes, which are organized as a long operon, are required for the biosynthesis of either type of capsule (10). The genetic loci for the type 5 and type 8 capsules (cap5 and cap8) are allelic, with the four genes in the middle of the operon being type specific (11). Because of this allelic organization in the chromosome, the expression of cap5 and cap8 genes is subject to similar transcriptional regulation. To date, a large number of regulators affecting cap gene transcription, some of which are non-DNA-binding factors, have been identified and/or characterized, and they include MgrA, AgrADBC, ArlRS, SaeRS, CodY, KdpDE, SigB, SpoVG, ClpC, ClpP, SbcDC, RpiRC, CcpA, Rot, CcpE, and AirR (4–6, 12–20).

Staphylococcal capsules are involved in immune evasion, but they can also mask cell surface components, such as adhesins, that are important for pathogenesis (21, 22). Thus, the production of capsule must be controlled properly depending on the conditions of the environment in which S. aureus resides. The surprisingly large number of regulators involved in capsule regulation further suggests that capsule is highly regulated and that the capsule regulatory network is very complex. Although many DNA-binding regulators affecting cap gene transcription have been identified, interestingly, only one cis element, a 10-bp inverted repeat (IR) located 13 bp upstream of the −35 region of the cap promoter (Pcap), has been identified to be critical for transcription of the cap genes and for capsule production (23). Among all the transcriptional regulators identified, five (CodY, KdpE, SpoVG, CcpE, and AirR) have been shown to bind directly to the Pcap region (15, 16, 19, 20, 24). However, the 10-bp IR has not been implicated in the binding of these regulators. In this study, we aimed to identify new potential Pcap-binding regulators to further understand capsule regulation. We identified 6 additional proteins that could potentially bind to Pcap in vitro to affect capsule production. We chose to focus on RbsR and showed that it is a DNA-binding regulator...
that directly binds to the 10-bp IR and the flanking sequences. We further demonstrated that rbsR expression is under the direct control of the alternative sigma factor SigB. In addition, we confirmed that RbsR is a repressor of the downstream rbsUDK operon.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Nebraska transposon mutants (30) were obtained through the Network of Antimicrobial Resistance in Nebraska used in this study are listed in Table 1. Nebraska transposon mutants (30) were obtained through the Network of Antimicrobial Resistance in Nebraska.

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μg/ml for chloramphenicol, 10 μg/ml for erythromycin, and 100 μg/ml for ampicillin.

**Plasmid and strain construction.** Primers used for plasmid and strain construction are listed in Table 2. To construct a deletion mutant of NWMN_2027 (CYL12847), DNA fragments flanking the gene were amplified using primer pairs NM2027-1/NM2027-2 and NM2027-3/NM2027-4 and cloned in tandem into plasmid pJB38, followed by allele replacement as described previously (26). The mutation was confirmed by PCR. The transposon mutants CYL12833 (recX::bursa), CYL12837 (xdrA::bursa), CYL12834 (rbsR::bursa), CYL12835 (sarZ::bursa), and CYL12838 (NWMN1391::bursa) were constructed by phage transduction of the transposon insertions from the respective Nebraska transposon mutants to CYL11481 and then verified by PCR.

For complementation of rbsR mutations, pML4233 carrying the S. aureus Newman rbsR gene under the control of Pcap/lutO was constructed by cloning a 1,070-bp PCR fragment, amplified using primer pairs rbsR-3/rbsR-2, into the HindIII and EcoRI sites of pML100. To express the recombinant His$_6$-RbsR protein in E. coli, plasmid pML4237 was constructed by cloning the rbsR gene of S. aureus Newman, amplified with primer pair rbsR-4/rbsR-5, into the Nhel and BamHI sites of pET-28a(+) (Novagen, Madison, WI). For two-plasmid sigB-dependent promoter assays, plasmids pML4261 and pML4262 were constructed by cloning a 153-bp fragment (amplified using primer pair rbsR-9/3-rbsR-10) and a 756-bp fragment (amplified using primer pair rbsR-8/3-rbsR-10) of the rbsR promoter region, respectively, into the promoter probe plasmid pSB40N, at the BamHI and Xhol sites. Plasmid pCL3169, which was used for footprinting analyses, was constructed by inserting a 624-bp Pcap fragment, amplified with primers cp8la.f and cp8la.r, into pGEM-T Easy (Promega, Madison, WI) by T/A cloning. All plasmid constructs were verified by restriction mapping and sequencing of the inserts.

**Fractionation of DNA-binding proteins on heparin-agarose.** An overnight culture (200 ml) of S. aureus Newman pLpcP saeS codY (CYL11391) was pelleted and washed with cold saline, suspended in 10 ml TS buffer (10 mM Tris-Cl, pH 7.6, 150 mM NaCl) with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), and sonicated briefly to dislodge cell aggregates. Cells were lysed with 0.1-mm zirconia-silica beads (BioSpec Products, Bartlesville, OK) in a Fast Prep homogenizer (MP Biomedicals, Solon, OH), using six 40-s pulses at speed 6, with 5-min intervals on ice between pulses. Cell lysates were collected, clarified by centrifugation at 18,000 × g for 20 min at 4°C, and applied to a 1-mL heparin-agarose column (Sigma-Aldrich, St. Louis, MO) to enrich the DNA-binding proteins. Heparin-agarose affinity chromatography was analyzed by 12% SDS-PAGE.

**EMSAs and proteomic analysis.** A 156-bp DNA fragment containing the cap promoter region was generated by PCR amplification from S. aureus Newman chromosomal DNA by using oligonucleotide primers cp8gs6 and cp8gs3. The DNA fragment was labeled with digoxigenin (Dig)-dUTP by using a Dig gel shift kit (Roche Applied Science, Indianapolis, IN), and sonicated briefly to dislodge cell aggregates. Cells were lysed with 0.1-mm zirconia-silica beads (BioSpec Products, Bartlesville, OK) in a Fast Prep homogenizer (MP Biomedicals, Solon, OH), using six 40-s pulses at speed 6, with 5-min intervals on ice between pulses. Cell lysates were collected, clarified by centrifugation at 18,000 × g for 20 min at 4°C, and applied to a 1-mL heparin-agarose column (Sigma-Aldrich, St. Louis, MO) to enrich the DNA-binding proteins. Heparin-agarose affinity column chromatography was analyzed as described by Trubetskoy et al. (32). The fractions of DNA-binding proteins that eluted from the heparin-agarose column were analyzed by 12% SDS-PAGE.

**EMSA and proteomic analysis.** A 156-bp DNA fragment containing the cap promoter region was generated by PCR amplification from S. aureus Newman chromosomal DNA by using oligonucleotide primers cp8gs6 and cp8gs3. The DNA fragment was labeled with digoxigenin (Dig)-dUTP by using a Dig gel shift kit (Roche Applied Science, Indianapolis, IN). Electrophoretic mobility shift assay (EMSA) was performed as described previously (27). To prepare a mutant Pcap fragment (Pcap$^{mut}$), primers cp8gs6 and cp8gs3 were used for PCR amplification of chromosomal DNA from S. aureus strain CYL6401 (which has a 4-bp mutation within the 10-bp IR in Pcap$^{mut}$) (23). To prepare truncated Pcap fragments for competition experiments, primer pairs cp8gs5/cp8gs6 and cp8gs5/cp8gs6 were used for PCR amplification of a 92-bp fragment of Pcap upstream of the 10-bp IR (Pcap$^{mut}$) and a 75-bp fragment of Pcap downstream of the 10-bp IR (Pcap$^{mut}$), respectively. For proteomic analysis, a preparative EMSA gel (1.5-mm thick) was used with unlabeled DNA probes and then stained with Coomassie blue G250. The gel bands were excised and submitted for proteomic analysis by in-gel trypsin digestion followed by liquid chromatography and tandem mass spectrometry (GeLC-MS/MS) at the UAMS Proteomic Core Facility.
His6-RbsR recombinant protein expression and purification. To express the His6-RbsR protein, pML4237 was transformed into E. coli Rosetta2(DE3)(pLysS) (Novagen). Overnight cultures were diluted to an optical density at 600 nm (OD600) of 0.05 in LB medium containing 30 μg/ml kanamycin and 34 μg/ml chloramphenicol, grown at 37°C until an OD600 of about 1, and then induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h at 37°C for protein expression. Bacterial cells were harvested by centrifugation and stored at −80°C until use. The cells were thawed on ice and lysed with SoluLyse (Amsbio, Lack Forest, MA) according to the manufacturer's instructions. The eluted protein was then changed to 20 mM HEPES buffer, pH 7.9, containing 300 mM KCl, 1 mM EDTA, 2 mM dithiothreitol (DTT), and 25% glycerol, using a Zeba Spin desalting column (Thermo Scientific, Waltham, MA) according to the manufacturer’s instructions.

Northern blot analysis. Total RNAs were isolated as described previously (27). For Northern blotting, the 517-bp rpi-specific, 521-bp rps-specific, and 530-bpprs-specific DNA probes were synthesized by using PCR Dig probe synthesis kits (Roche Applied Sciences) with primer pairs rbsR-6/rbsR-7, rbsU-1/rbsU-2, rpi-1/rpi-2, and prs-1/prs-2, respectively. Denaturing RNA gel electrophoresis (1% agarose) was carried out as described by Masek et al. (33), except that the buffer was replaced with TBE (90 mM Tris-borate, 2 mM EDTA) buffer. Northern hybridization was carried out as described previously (27).

Nonradioactive DNase I footprinting. Plasmid pCL3169, which contains a 614-bp Pcap fragment, was used as the template to synthesize a 156-bp probe by PCR, using the 6-carboxyfluorescein (FAM)-labeled primer FAM-FP6 and the VIC-labeled primer VIC-FP3, which correspond to positions −135 to +21 of Pcap with respect to the transcriptional start site of the cap operon (23). The PCR DNA fragments were purified using a Nucleospin column (Clontech, Mountain View, CA). The procedure for DNase I footprinting was essentially as described by Ziani et al. (34). Briefly, the reaction mixture (20 μl), which consisted of 1.36 μg purified His6-RbsR, 80 ng of fluorescent dye-labeled DNA probe, 2 μg of bovine serum albumin (BSA), 0.1 μg of poly(rI-rC) in binding buffer [20 mM HEPES, pH 7.6, 10 mM (NH4)2SO4, 1 mM DTT, 0.2% Tween 20, 30 mM g of poly-L-lysine, and 1 μg of poly(dI-dC) in binding buffer [20 mM HEPES, pH 7.6, 10 mM (NH4)2SO4, 1 mM DTT, 0.2% Tween 20, 30 mM KCl], was incubated at 80°C until use. The experiments were repeated two times. Fifteen microliters of each purified DNA fragment, along with primers rbsR-6/rbsR-7, rbsU-1/rbsU-2, rpi-1/rpi-2, and prs-1/prs-2, was added to the reaction mixture, the mixture was incubated at 23°C for 15 min. DNase I (0.08 U; New England BioLabs) was added to the reaction mixture, the mixture was incubated at 23°C for 4 min, and the reaction was stopped by incubation at 78°C for 10 min. The DNA fragments were purified by use of a Mini Elute PCR kit (Qiagen, Valencia, CA) and eluted in 25 μl of H2O. The experiments were repeated two times. Northern blot analysis. Total RNAs were isolated as described previously (27). For Northern blotting, the 517-bp rpi-specific, 495-bp rps-specific, 521-bp rps-specific, and 530-bp rps-specific DNA probes were synthesized by using PCR Dig probe synthesis kits (Roche Applied Sciences) with primer pairs rbsR-6/rbsR-7, rbsU-1/rbsU-2, rpi-1/rpi-2, and prs-1/prs-2, respectively. Denaturing RNA gel electrophoresis (1% agarose) was carried out as described by Masek et al. (33), except that the buffer was replaced with TBE (90 mM Tris-borate, 2 mM EDTA) buffer. Northern hybridization was carried out as described previously (27).

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TSS determination. The transcriptional start site (TSS) of rbsR was determined by using the adaptor- and radioactivity-free (ARF-TSS) method of Wang et al. (35). Briefly, 5 μg of total RNA isolated from S. aureus strain 11481 was used for cDNA synthesis with the 5′-end-phis-
phorylated primer rbsR-T1, using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNAs were then degraded using 0.25 N NaOH and neutralized with 0.25 N HCl. cDNAs were purified with a Mini Elute PCR kit (Qiagen). The 5'-phosphoryl-terminating single-stranded cDNA was then covalently joined to the 3' end (TSS) of the cdNA by using T4 RNA ligase (Epícentre, Madison, WI). Circularized cDNAs were amplified by inverse PCR using the divergent primers rbsR-T2 and rbsR-T3. The PCR products were cloned into pGEM-T Easy (Promega) and sequenced. A QuantiTect reverse transcription kit (Qiagen) was used for reverse transcription-PCR (RT-PCR) to estimate the ends of the rbsR transcript according to the manufacturer’s instructions, using primer pair rbsR-11/rbsR-12 for the 5' end and primer pair rbsR-13/rbsR-14 for the 3' end. PCR DNA products were analyzed using 2% agarose gel electrophoresis with TBE buffer.

Other methods. To create a two-plasmid system for the SigH-dependent promoter assay, plasmid pMLA261 or pMLA262 containing the PrbsR-lacZ fusion was transformed into E. coli XL1-Blue containing pAC7 or pAC7-sigB, and clones were selected on LBACX-ARA plates as described by Homerova et al. (29). For capsule immunoblotting, capsules were prepared as described previously (6), using TSB without glucose (TSB-0G). Serially diluted samples (1.5 μl each) were applied directly to a nitrocellulose membrane by using a pipette. Membranes were treated with a specific antiscapsule antibody and detected as described previously (6). BlaZ (β-lactamase) assays for Pcap::blaZ fusions were performed with cultures grown in TSB-0G according to a previously described procedure (36). Data for the promoter fusion assays were analyzed by GraphPad Prism (San Diego, CA), using the paired Student t test.

RESULTS

Identification of Pcap-binding proteins. Previously, we identified CodY as a repressor of capsule by identifying proteins bound to the Pcap DNA fragment (15). Because CodY is an abundant cytoplasmic protein, we chose a codY-null strain in our experiment to avoid interference. In addition, because ClpC represses capsule through the Saer two-component system in strain Newman, it was possible that ClpC and Saer would also have a negative impact on our effect. Thus, we used a Newman clpC saer codY triple mutant as the source to prepare cell extracts. The cell extracts were further enriched for DNA-binding proteins by using a heparin column (Fig. 1A). The enriched protein fraction was then used in EMSAs with a 156-bp digoxigenin (Dig)-labeled Pcap DNA fragment as a probe (Fig. 1B). A broad shifted band which was absent in the negative control was identified (Fig. 1C), suggesting the presence of putative DNA-binding proteins other than CodY. To identify these putative DNA-binding regulators, EMSAs were repeated using the same 156-bp Pcap DNA fragment, but without Dig labeling. Both the ethidium bromide- and Coomassie blue-stained gels revealed a shifted band just below the loading well (Fig. 1D; at a position similar to that of the band identified by the Dig-labeled probe, suggesting that the shifted bands contained proteins (as identified by the Coomassie blue stain) interacting with the Pcap DNA fragment (as identified by the ethidium bromide stain). We also included experiments using a mutant Pcap DNA fragment, containing a 4-bp substitution within the 10-bp IR (Pcap<sup>mut</sup>) (Fig. 1B), that was amplified with the same primers. A similar shifted band was also found with the Pcap<sup>mut</sup> fragment, indicating the presence of 10-bp IR-independent DNA-binding proteins in the shifted band (Fig. 1D). The regions containing the shifted bands identified by Pcap and Pcap<sup>mut</sup> and the corresponding region in the negative-control lane (with no DNA) from the Coomassie blue-stained gels (Fig. 1D, right panel) were excised and subjected to protein analysis by GeLC-MS/MS. By comparing the spectral counts, we identified 9 proteins that were present in the EMSA using Pcap or Pcap<sup>mut</sup> but were absent or had much reduced spectral counts in the negative control (data not shown). To test the effects of these proteins on capsule, mutants of the corresponding genes were constructed either by phage transduction from NARSA Nebraska transposon mutants into strain CYL11481 or by allelic replacement in strain CYL11481. We found 6 mutants that had an effect on capsule (Fig. 2A). Among these putative capsule regulators, only XdrA was shown previously to affect cap genes, in a gene profiling study (37). In the present study, we chose to focus on NWMN_0205, which has been annotated RbsR based on homology to a repressor controlling the rbs operon involved in ribose utilization in other bacteria (38–40).

RbsR activates capsule by binding to the cap promoter. The results described above (Fig. 2A) suggest that RbsR is a putative activator of capsule production. To confirm this, we cloned the rbsR gene from strain Newman into pML100 under the control of the Pxyt/tetO promoter (pML4233). As shown in Fig. 2B, the cap-
Suc phenotype of the rbsR mutant (CYL12834) was restored to the wild type when the strain was complemented with pML4233 in the presence of anhydrotetracycline (ATc). Because there was no other gene present in the cloned fragment, the results confirmed that RbsR is an activator of capsule.

RbsR is a putative translational regulator containing a helix-turn-helix DNA-binding motif, suggesting that it regulates its target genes by direct DNA binding. To determine whether RbsR binds to the cap promoter region, a His<sub>6</sub>-RbsR fusion protein was expressed in E. coli and purified by use of a His-Bind resin affinity column. The purified protein was used in EMSAs with the Dig-labeled 156-bp Pcap DNA fragment as a probe. As shown in Fig. 3A, the Pcap fragment could readily be upshifted by His<sub>6</sub>-RbsR. The shifted band could be competed away with a cold Pcap DNA fragment, suggesting that the binding was specific. The binding dissociation constant (K<sub>d</sub>) was then determined to be ~4.9 nM (Fig. 3B). To determine whether the 10-bp IR upstream of the ~35 region of the cap promoter is required for binding, we used the 4-bp mutant Pcap<sup>mut</sup> fragment for cold competition in EMSA. The results (Fig. 3A) showed that the majority of the shifted band remained unchanged. In addition, the shifted band was not out-competed by using a 92-bp DNA fragment upstream (Pcap<sup>5′</sup>) of the 10-bp IR or a 75-bp downstream DNA fragment (Pcap<sup>3′</sup>) (Fig. 3A). Thus, these cold competition results suggest that the 10-bp IR sequence is important for RbsR binding. To further localize the RbsR binding site, we performed a fluorescence-based footprinting experiment. The results in Fig. 4 show that, on the sense strand, RbsR protected a 46-nucleotide (nt) region that centers on the 10-bp IR sequence, which also includes the nearby downstream ~35 region of the promoter. On the antisense strand, it protected a 16-nt region that centers on the ~35 region of the promoter. Although the binding site is much larger than the 10-bp IR sequence, the footprinting results are consistent with those of the EMSAs. In addition, the results showing that RbsR also protected the ~35 region suggest that RbsR may interact directly with RNA polymerase to activate cap mRNA transcription.

**Characterization of rbsR transcription.** To characterize the transcription of rbsR in different growth phases, Northern analyses were performed at different time points. As shown in Fig. 5A, two transcripts, of ~1.0 kb and ~1.4 kb, were identified; both could carry the full-length (999 bp) rbsR gene. These results suggest that rbsR is a monocistronic gene. The larger, ~1.4-kb transcript increased gradually but most prominently in the late exponential growth phase, at an OD<sub>600</sub> of about 2.0 (mid-log phase), whereas the ~1.0-kb transcript increased to the highest level at OD<sub>600</sub> of about 3.5 and 4.1 (early stationary growth phase), suggesting that rbsR transcription is growth phase dependent. These results suggest that rbsR is transcribed from two promoters or transcribed from one promoter with two different 3′ ends. To test these possibilities, we employed the ARF-TSS method (35) to map the 5′ end of the rbsR transcript. We sequenced 8 clones, and all had the same TSS, at the A residue 35 nt upstream of the rbsR ATG start site (Fig. 5B), suggesting that there is a single rbsR promoter. To confirm this prediction, we performed RT-PCR. We were able to amplify a fragment extending from just inside the 3′ end of rbsR to about 200 bp downstream from the stop codon with the primer pair rbsR-13/rbs-14 but were unable to amplify a fragment extending from the 5′ end and encompassing about 300 bp upstream of the start codon by using the primer pair rbsR-11/rbs-12 (results not shown). Thus, taking the results of TSS mapping, RT-PCR, and Northern blotting (Fig. 5A and B) together, we suggest that rbsR is transcribed from one promoter, but with two 3′ ends. The two transcription ends could be due to transcription being terminated at two terminators or to processing of the longer transcript. However, a strong intrinsic terminator has been identified 36 bp downstream of the rbsR gene (41). Based on the start site and the predicted terminator, we estimated the rbsR transcript to be ~1.1 kb, which matches the size of the shorter transcript that we identified by Northern blotting. This suggests that the two transcripts likely resulted from two different terminators rather than from processing of the longer transcript.

**RbsR represses transcription of the rbsUDK operon.** The operonic rbsUDK gene cluster is predicted to encode proteins involved in ribose uptake and phosphorylation. RbsR has been annotated as the repressor of the rbsUDK operon, based on its homology with RbsR repressors in other bacteria (38–40). An in silico analysis also predicted that it binds to a site just upstream of the rbsU gene (42). To test whether RbsR affects rbsUDK expression, we employed Northern hybridization using an internal fragment of the rbsU gene as a probe. The results (Fig. 5C) showed that a pronounced increase in the ~2.5-kb band was detected for the rbsR mutant compared to the wild-type strain (Fig. 5C), indicating that RbsR is a repressor of rbsUDK. The mutant phenotype could readily be complemented with a DNA fragment carrying the

![FIG 2 Immunoblotting of capsule. (A) Various mutants derived from the Newman P18L strain (CYL11481) were grown in TSB-0G for 4 h for capsule isolation. (B) Complementation of the rbsR mutant (CYL12834) with pML4233 (pML100-rbsR) for restoration of the capsule phenotype. Capsules were isolated from cultures grown in TSB-0G in the presence of 2.5 μg/ml chloramphenicol for 2 h.

![FIG 3 EMSAs of Pcap with the His-RbsR protein. (A) The shifted band could be competed away effectively by the cold Pcap fragment but not by the Pcap<sup>mut</sup> fragment, containing a 4-bp mutation; the Pcap<sup>5′</sup> fragment, containing the 5′ half of the 10-bp IR with upstream sequence; or the Pcap<sup>3′</sup> fragment, containing the 3′ half of the 10-bp IR with downstream sequence. (B) A K<sub>d</sub> value of ~4.9 nM was determined by using increasing amounts of His-RbsR and a constant amount of labeled Pcap fragment (0.63 nM).
also assessed by complementation of rbsR
4-h cultures (OD600, H11005 lowercase letters denoting poorly conserved nucleotides (w (B) Map of TSS and predicted promoter of rbsR cap 10-bp IR required for P Protected regions are indicated by brackets. Sequences in red indicate the green peaks in panel A and orange peaks in panel B) indicates protection. peaks in panel A and blue peaks in panel B) compared to that in its absence intensity of DNase I-digested fragments in the presence of 1.9-VIC-labeled antisense strand (B) of the P cap and the 5 = hybridized with the Dig-labeled specific probe indicated below each blot. (A) Expression of FIG 5
wild-type rbsR gene. The size of the rbsU-specific band also indicates that rbsUDK is transcribed as an operon. In many bacteria, ribose is rapidly phosphorylated upon uptake. The resulting ribose-5-phosphate could be converted by Prs to 5-phospho-ribose-1-diphosphate, a precursor for purine, pyrimidine, and his-tidine synthesis, or converted by Rpi to ribulose-5-phosphate in one of the steps of the pentose phosphate cycle. Our Northern blotting results, however, showed that RbsR did not affect the expression of either the prs or rpi gene (Fig. 5C).

SigB directly activates RbsR. Although many regulators have been found to affect capsule production, most of them are likely to regulate capsule indirectly. We therefore speculate that RbsR could serve as a downstream regulator of one or more of these upstream regulators. To test this possibility, the expression of rbsR in various mutants, including agr, mga, clpC, codY, saeRS, arlRS, sigB, and sbcDC mutants, was tested by Northern blotting. Among the proteins examined, only SigB had an apparent effect on rbsR expression (Fig. 6A). Indeed, rbsR was previously shown to be upregulated by SigB in a microarray transcriptional profiling study, and an imperfect SigB box upstream of the rbsR gene has been identified (Fig. 5B) (43), suggesting that SigB may bind to the rbsR promoter directly. To confirm that SigB directly affects rbsR transcription, we employed a two-plasmid system as described by Homerova et al. (29). As shown in Fig. 6B, we found that the rbsR promoter was activated in E. coli only when S. aureus SigB was also expressed, confirming that SigB is required for rbsR activation, most likely by direct promoter binding. SigB has been shown to activate capsule through SpoVG or ArlR (14, 44). Neither SpoVG nor ArlR affected rbsR transcription (not shown), indicating that the SigB-RbsR pathway affecting capsule production is independent of the SpoVG or ArlR pathway.

The repression of rbsUDK by RbsR, but not the activation of cap, is affected by ribose. Ribose has been shown to be an inducer controlling RbsR regulatory function in E. coli but not in Bacillus subtilis (38, 45). To determine whether ribose affects RbsR regulatory function in S. aureus, we performed Northern blotting to determine the effect of RbsR on the expression of the rbsU gene in the presence or absence of D-ribose, using TSB-0G (i.e., TSB without glucose) as the basal medium. As shown in Fig. 7A, we found that the rbsU gene was derepressed by RbsR in the presence of ribose, suggesting that ribose is an inducer that relieves the repression of the rbsUDK operon by RbsR. Likewise, because Pcap is activated by RbsR, we also tested whether RbsR activation of capsule is affected by ribose. To this end, we employed a Pcap-blaZ fusion plasmid and compared the BlaZ activities in the wild-type strain and the rbsR mutant in the presence and absence of D-ribose.

FIG 4 DNase I footprinting analysis of the 5'-FAM-labeled sense strand (A) and the 5'-VIC-labeled antisense strand (B) of the Pcap probe. A reduction in intensity of DNase I-digested fragments in the presence of 1.9 M RbsR (black peaks in panel A and blue peaks in panel B) compared to that in its absence (green peaks in panel A and orange peaks in panel B) indicates protection. Protected regions are indicated by brackets. Sequences in red indicate the green peaks in panel A and orange peaks in panel B) indicates protection.

FIG 5 Transcription analyses. Total RNA (5 M) from each sample used for Northern blotting was denatured in formamide, applied to TBE-agarose gels, and hybridized with the Dig-labeled specific probe indicated below each blot. (A) Expression of rbsR in CYL11481 cultures grown to different OD600s, as indicated. (B) Map of TSS and predicted promoter of rbsR. The SigB binding consensus is shown below, with capital letters denoting highly conserved nucleotides and lowercase letters denoting poorly conserved nucleotides (w = A or T). (C) Effects of RbsR on rbsU, rpi, and prs transcription. Total RNAs were prepared from 4-h cultures (OD600 ~ 4.0) of S. aureus CYL11481 or the rbsR mutant (CYL12834) and hybridized with specific probes, as indicated. The expression of rbsU was also assessed by complementation of rbsR by using pML100-rbsR (pML4233) in the presence of ATC.
in the TSB-0G growth medium. As shown in Fig. 7B, we found that the Pcap promoter activity decreased in the rbsR mutants in both the presence and absence of D-ribose but that there was no significant difference between the absence and presence of D-ribose in either the wild-type strain or the rbsR mutant, suggesting that D-ribose in the medium does not affect RbsR activation of the cap operon.

**DISCUSSION**

*S. aureus* can infect almost any human or animal tissues and can survive outside the host for a long time. It is therefore not surprising that the organism needs to have a large number of regulators to properly regulate various factors required for adapting to different environments. Using capsule as a target virulence factor, we and others have identified more than a dozen regulators involved in capsule gene regulation. In the present study, we attempted to identify transcriptional regulators capable of binding to the promoter region of the cap operon. By using an approach that directly analyzes the proteins bound to Pcap DNA, we found six putative DNA-binding regulators. One of these newly identified regulators, RbsR, was further characterized and shown to bind specifically to the cap promoter region. Although the other five putative regulators have not been characterized fully, our finding is rather surprising, as we did not expect to find that many potential regulators capable of binding to the cap promoter. Adding to the previously known capsule regulators, the remarkable number of regulators devoted to controlling single virulence factors further points to the extreme complexity of the virulence regulatory network in *S. aureus.*

RbsR is a LacI family repressor that has not been characterized previously for *S. aureus.* It shares 29% to 39% amino acid identity with the RbsR proteins from *Lactobacillus sakei,* *E. coli,* and *B. subtilis* (38, 40, 45), which have been shown to repress the rbs operon involved in ribose uptake and phosphorylation. Our results presented here demonstrate that *S. aureus* RbsR is also a repressor of the rbsUDK operon, predicted to be involved in ribose transport and phosphorylation. Ribose can serve as a source for energy, via the pentose phosphate pathway (PPP), and for nucleotide synthesis in many bacteria. In solution, ribose exists as α- and β-pyranose forms, with the latter being the predominant form. In *S. aureus,* ribose is likely imported via RbsU and converted by RbsD to the α form, which can then be recognized by RbsK and converted to ribose-5-phosphate, which either becomes part of the PPP or serves as the precursor for nucleotide synthesis. The RbsU transporter is used by *S. aureus* and *L. sakei* for ribose uptake, whereas a tricomponent ATP-binding cassette transporter composed of RbsABC is operated in *E. coli* and *B. subtilis.*

In this study, we used Northern analyses (Fig. 7A) to show that the repression of the rbsUDK operon by RbsR could be derepressed by ribose in *S. aureus.* These results suggest that ribose or one of its derivatives may be an inducer that interacts directly with RbsR to derepress the negative regulation. This regulation is similar to the ribose induction of rbsDA CKB repressor by RbsR in *E. coli* (38). However, in *B. subtilis,* repression of the rbsKDA CB operon by RbsR does not respond to ribose in the growth medium (45).

In this study, we discovered that RbsR was not only a repressor of the rbs operon but also an activator of the cap genes. *S. aureus* had previously been thought to regulate genes only in the rbs operon. However, Shimada et al. (46) recently showed that *E. coli* RbsR also binds to the promoters of a set of genes resulting in repression and activation of the de novo and salvage purine nucleotide synthesis pathways, respectively. In *Corynebacterium glutamicum,* RbsR affects only the rbs genes, but in association with a coregulator, RbsR can also affect genes involved in the utilization of uridine (47). Since ribose is a direct source of ribose-5-phosphate, which is a key intermediate for synthesizing nucleotides (via phosphorylypyrophosphate), it is not surprising that the genes involved in nucleotide metabolism are also regulated by RbsR in these bacteria. However, our finding that the cap operon is also a direct target of RbsR in *S. aureus* is rather unusual, as the capsule biosynthetic pathway and the ribose utilization pathway are not closely linked.

Although RbsR regulates both the rbs and cap operons, we found that ribose had no effect on the RbsR activation of cap gene expression. These results suggest that the mechanism involved in rbsUDK repression by RbsR is different from that involved in the
activation of the \( \text{cap} \) operon. The consensus RbsR binding site in the promoter region of the \( \text{rbs} \) operon in the \textit{Bacillus/Clostridium} group of bacteria, including \textit{S. aureus}, has been defined by a comparative approach \((41)\). The predicted consensus RbsR box upstream of the \textit{rbsUDK} operon in \textit{S. aureus} bears no resemblance to the RbsR binding site in the \textit{cap} promoter region as defined by footprinting in that study. The 10-bp IR sequence is also not found within a 1,000-bp region upstream of the \( \text{rbs} \) operon. These findings further corroborate that RbsR may regulate capsule production \((48)\). Thus, at least four independent pathways add a third circuit of regulation, through RbsR. Recently, SigB was found to independently activate capsule through SpovG and ArIR \((44)\). Similarly, SpovG was shown to bind a 28-bp region that is 41 bp further upstream of the 10-bp IR \((24)\). However, we reported earlier that deletion of a sequence further upstream of the 10-bp IR had no detectable effect on the \( \text{cap} \) gene expression \((23)\). Because there is no direct evidence that the 28-bp region of the SpovG binding site is involved in \( \text{cap} \) gene transcription, based on our previous results \((23)\), we speculate that SpovG may affect capsule indirectly rather than by binding at this region. In addition to SpovG and ArIR, through which SigB can regulate capsule, in this study we add a third circuit of regulation, through RbsR. Recently, SigB was also found to negatively regulate capsule by activating RsaA, a small RNA that inhibits MgrA translation, thereby reducing capsule production \((48)\). Thus, at least four independent pathways are now known to be involved in SigB regulation of capsule \((Fig. 8)\). The multiple pathways by which capsule can be regulated by SigB suggest that this regulation has a high degree of complexity, which will require additional studies to understand the biological significance of the regulation.

There are ample examples of coregulation of metabolism and virulence in \textit{S. aureus} \((49)\). Our finding that RbsR is involved in ribose utilization as well as capsule production suggests that RbsR could also be an important regulator linking metabolism and virulence regulation. Ribose is present at ~0.1 mM in human blood \((50, 51)\) and in various amounts in other tissues \((52)\). It is likely that the availability of ribose in the tissues affects \textit{S. aureus} pathogenesis by promoting bacterial growth. However, our finding that repression of the \( \text{rbs} \) operon by RbsR, but not activation of capsule, is controlled by ribose suggests that ribose is not likely an effecter linking the two cellular processes. On the other hand, because RbsR is highly regulated by SigB, whose activity is affected by certain \textit{in vitro} and \textit{in vivo} stress conditions \((53–55)\), stress signals that modulate SigB activity are likely to be important effectors for controlling the quantity of RbsR, thereby affecting ribose uptake and capsule production. However, determining which signals are involved and how transduction of these signals through SigB affects the expression of RbsR requires further in-depth studies.

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