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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 (P*cap*), we directly analyzed the proteins interacting with the P*cap* 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 AirSR (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 (P*cap*), 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 P*cap* 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 P*cap*-binding regulators to further understand capsule regulation. We identified 6 additional proteins that could potentially bind to P*cap in vitro* to affect capsule production. We chose to focus on RbsR and showed that it is a DNA-binding regulator

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference/ source
Strains		
S. aureus strains		
RN4220	Restriction-negative laboratory strain	J. Iandolo
Newman	Wild-type CP5 strain	T. Foster
CYL6401	Strain Becker with 4-bp mutation in 10-bp IR	23
CYL11391	Newman Δ*clpC*::*cat* Δ*saeR* Δ*codY*::*ermC*	6
CYL11481	Newman $saeS^{P18L}$	6
CYL12847	CYL11481 Δ*NWMN_2027*	This study
CYL12834	CYL11481 *rbsR*::*bursa*	This study
NE324	USA300 FPR3757 *recX*::*bursa*	NARSA
NE425	USA300 FPR3757 *rbsR*::*bursa*	NARSA
NE567	USA300 FPR3757 *sarZ*::*bursa*	NARSA
NE1445	USA300 FPR3757 *xdrA*::*bursa*	NARSA
NE1781	USA300 FPR3757 *NWMN_1391*::*bursa*	NARSA
GP266	RN4220 $rsbU^{+}$ *sigB1*(Am) Tc^{r}	25
CYL13113	CYL11481 $rsbU^{+}$ *sigB1*(Am) Tc^{r}	This study
E. coli strains		
XL1-Blue	Host strain	Stratagene
CYL3967	Rosetta2(DE3)(pLysS)	Novagen
CYL4242	Rosetta2(DE3)(pLysS)(pML4237)	This study
Plasmids		
pGEM-T Easy	Cloning vector	Promega
pJB38	Vector for allelic replacement	26
pML100	Shuttle vector	27
pML4233	pML100 with *rbsR*	This study
pET28a(+)	Expression vector	Novagen
pML4237	pET28a(+) with *rbsR*	This study
pSB40N	Promoter probe plasmid	28
pAC7	Expression vector with P_{BAD} promoter	28
pAC7-sigB	pAC7 with *sigB*	29
pML4261	pSB40N with 155-bp *rbsR* promoter (P*rbsR*1)	This study
pML4262	pSB40N with 760-bp *rbsR* promoter (P*rbsR*2)	This study
pCL3169	pGEM-T Easy with 614-bp P*cap* fragment	This study
pAM3176	pLL35 with P*cap*::*blaZ*	4

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 *S. aureus* (NARSA) program. Competent *S. aureus* RN4220 was used as the recipient for electroporation by the procedure of Kraemer and Iandolo (31). Phage 52A were used for plasmid and chromosomal DNA transduction between *S. aureus* strains. *Escherichia coli* strain XL1-Blue was used for plasmid construction and maintenance. *S. aureus* strains were cultivated with tryptic soy broth (TSB) or tryptic soy agar (TSA) (Difco Laboratory, Detroit, MI) unless indicated otherwise. *E. coli* was grown in Luria-Bertani broth or agar unless specified otherwise. Antibiotics were added to the culture medium when necessary, at final concentrations of 10 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 by 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 P*xyl/tetO* was constructed by cloning a 1,070-bp PCR fragment, amplified using primer pair 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 NheI 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/rbsR-10) and a 756-bp fragment (amplified using primer pair rbsR-8/rbsR-10) of the *rbsR* promoter region, respectively, into the promoter probe plasmid pSB40N, at the BamHI and XhoI sites. Plasmid pCL3169, which was used for footprinting analyses, was constructed by inserting a 624-bp P*cap* fragment, amplified with primers cp8bla.f and cp8bla.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 *clpC saeR 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 column chromatography was carried out 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 P*cap* fragment ($Pcap^{mt}$), 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 P*cap*) (23). To prepare truncated P*cap* fragments for competition experiments, primer pairs cp8gs5/cp8gs6 and cp8gs3/gp8gs4 were used for PCR amplification of a 92-bp fragment of P*cap* upstream of the 10-bp IR (P*cap*5′) and a 75-bp fragment of P*cap* downstream of the 10-bp IR (P*cap*3′), 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.

TABLE 2 Oligonucleotide primers used in this study

Primer	Sequence (5′–3′)
cp8gs3	CCATTATTTACCTCCCTTAAAAATTTTC
cp8gs4	AACGATATGTAATATGTAAATAC
cp8gs5	ACATATCGTTTAAACAATTAATTACTTT
cp8gs6	CTACTTTAGAGTATAATTATTTTTAATTTC
cp8bla.f	CTGCAGAGCTCGCATTTGAAGATCA
cp8bla.r	GGATCCCTTAGTTTGATTCACTAAA
FAM-FP3	VIC-CCATTATTTACCTCCCTTAAAAATTTTC
FAM-FP6	FAM-CTACTTTAGAGTATAATTATTTTTAATTTC
NM2027-1	GATGATATCGTCGACGAGCTCCTGATAGAATTGAAGCAGGCACATA
NM2027-2	ACGTTGATCTGTTAAATCGAGCGGCCGCTTCTTCGAGATACGGACATACTTCCATC
NM2027-3	TATGTCCGTATCTCGAAGAAGCGGCCGCTCGATTTAACAGATCAACGTACTGCTAA
NM2027-4	GATGATATCGTCGACTAATTAATCCAGATACACCGATTGCTTC
rbsR-2	GATGATATCGAATTCATGATGAGTATATTTCGGAAGATACGTAG
rbsR-3	GATGATATCAAGCTTTAACTGTATGATTAATTACACAATAAAGA
rbsR-4	AGCCATATGGCTAGCATGAAAAAAGTGTCAATTAAAGATGTTGCTA
rbsR-5	CTCGAATTCGGATCCTTAGTTTGAAAGATGATAGCCAGTTGTTG
rbsR-6	TCTCAGCACTGATGCAAAGTATTCATGAC
rbsR-7	ATGCCAATAGCGAGTTCATCGTTAATAG
rbsR-8	GATTCTAGAGGATCCGACAAAATGGCCATTTTCAAATATCAC
rbsR-9	GATTCTAGAGGATCCTATTCTCTCGTCTCAACCTTAATCGTATACTTCAG
rbsR-10	GATGGGCCCCTCGAGTCTTTATTGTGTAATTAATCATACAGTTATATAC
rbsR-11	CAATCACATAGTTCAATATACATCATTTC
rbsR-12	ACTGATACACCAGCTTCTCTAGCAACATC
rbsR-13	CTTCAACGATTACAGCACTTAGATAAATC
rbsR-14	CATGATTTGTTTTTAGTAAAACGTTTTACCAGTGCCATC
rbsU-1	CATCAAATTATCGGTGCTACTGTAGGTACGTTAATC
rbsU-2	AATGACTGCAACAATTACCATACCCATTGCTTG
rpi-1	CTCAAATGGCGCAACTAATTAAAGAACGTGGTTAC
rpi-2	CATATCTAAGAAGTATCCTGTCTCAAACACAC
prs-1	CGTGCTTCTGCAGCAACAATCAATATTGTAG
prs-2	GATAAAACAGGGTGTGTACAACAAGCATATAC
rbsR-T1	CTAAACGTTCTGAAACAGCATGTACGTTTTTTATC
rbsR-T2	CATAATGATAGTCGTTTTTCCGCAAC
rbsR-T3	GTAACTGATACACCAGCTTCTCTAGCAAC

His_6-RbsR recombinant protein expression and purification. To express the His_6-RbsR protein, pML4237 was transformed into *E. coli* Rosetta2(DE3)(pLysS) (Novagen). Overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in LB medium containing 30 μg/ml kanamycin and 34 μg/ml chloramphenicol, grown at 37°C until an OD_{600} 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, CA), and the His_6-RbsR protein was purified using a His-Bind kit (Novagen) according to the manufacturer's instructions. The buffer of 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 *rbsR*-specific, 495-bp *rbsU*-specific, 521-bp *rpi*-specific, and 530-bp *prs*-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 P*cap* 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 P*cap* 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 Zianni et al. (34). Briefly, the reaction mixture (20 μl), which consisted of 1.36 μg purified His_6-RbsR, 80 ng of fluorescent dye-labeled DNA probe, 2 μg of bovine serum albumin (BSA), 0.1 μg of poly-L-lysine, and 1 μg of poly(dI-dC) in binding buffer [20 mM HEPES, pH 7.6, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.2% Tween 20, 30 mM KCl], 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 H_2O. The experiments were repeated two times. Fifteen microliters of each purified DNA fragment, along with primers FAM-FP6 and VIC-FP3 and plasmid pCL3169, was submitted to the Ohio State University Plant-Microbe Genomic Facility for fragment analysis and sequencing using an Applied Biosystems 3730 DNA analyzer. The RbsR DNA-binding sites were determined by aligning the sizes of the fragments and sequences of the probe.

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-phos-

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-terminated single-stranded cDNA was then covalently joined to the 3′ end (TSS) of the cDNA by using T4 RNA ligase (Epicentre, 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 SigB-dependent promoter assay, plasmid pML4261 or pML4262 containing the P*rbsR-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 anticapsule antibody and detected as described previously (6). BlaZ (β-lactamase) assays for P*cap*::*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 P*cap*-binding proteins. Previously, we identified CodY as a repressor of capsule by identifying proteins bound to the P*cap* 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 SaeRS two-component system in strain Newman, it was possible that ClpC and SaeRS would also have a negative impact on our effort. 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 P*cap* 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 P*cap* DNA fragment, but without Dig labeling. Both the ethidium bromide- and Coomassie blue-stained gels revealed a shifted band just below the loading wells (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 P*cap* DNA fragment (as identified by the ethidium bromide stain). We also included experiments using a mutant P*cap* DNA fragment, containing a 4-bp substitution within the 10-bp IR (Pcap^{mt}) (Fig. 1B), that was amplified with the same primers. A similar shifted band was also found with the Pcap^{mt} 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 P*cap* and Pcap^{mt} 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

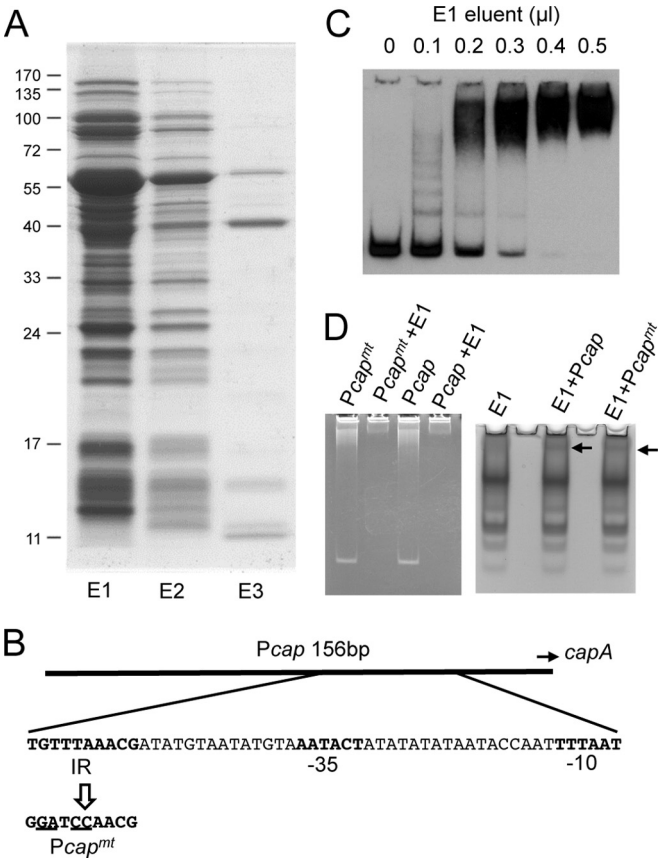

FIG 1 Identification of P*cap* DNA-binding proteins. (A) SDS-PAGE analysis of heparin-agarose column-fractionated proteins. Proteins (10 μl) of each fraction were subjected to 12% SDS-PAGE and stained with Coomassie blue G250. E1, E2, and E3 refer to eluents of three consecutive fractionations. E1 and E2 were eluted with buffer containing 0.5 M KCl. E3 was eluted with buffer containing 1.0 M KCl. (B) *cap* promoter region, with the 10-bp IR and the −35 and −10 sequences shown in bold. The Pcap^{mt} DNA sequence containing the 4-bp substitution (23) is also shown. (C) EMSA using the Dig-labeled 156-bp P*cap* fragment and increasing amounts of the E1 fraction, as indicated at the top. (D) EMSA using P*cap* DNA or Pcap^{mt} DNA (~50 ng) and 4 μl of the E1 fraction. Gels were stained with ethidium bromide (left) or Coomassie blue G250 (right). Arrows indicate shifted bands.

comparing the spectral counts, we identified 9 proteins that were present in the EMSA using P*cap* or Pcap^{mt} 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 P*xyl/tetO* promoter (pML4233). As shown in Fig. 2B, the cap-

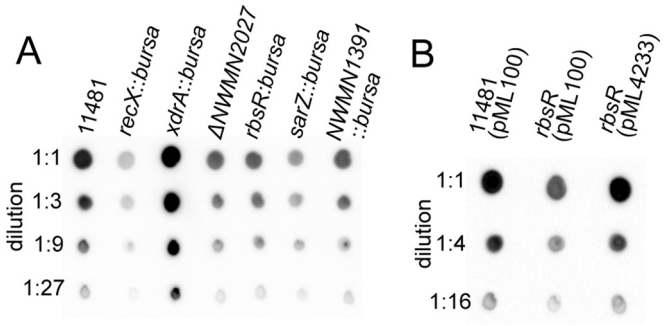

FIG 2 Immunoblotting of capsule. (A) Various mutants derived from the Newman P18L strain (CYL11481) were grown in TSB-0G for 4 h for capsule isolation. (B) Complementation of the *rbsR* mutant (CYL12834) with pML4233 (pML100-*rbsR*) for restoration of the capsule phenotype. Capsules were isolated from cultures grown in TSB-0G in the presence of 2.5 μg/ml chloramphenicol for 2 h and then induced with 200 ng/ml of ATc for 2 h.

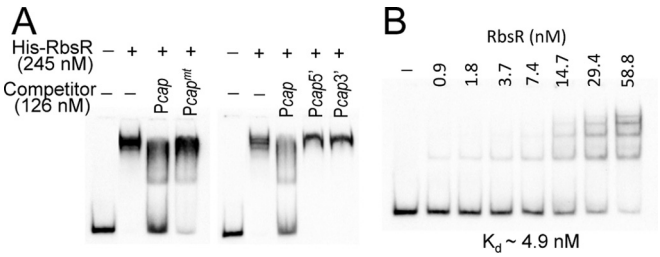

FIG 3 EMSAs of P*cap* with the His-RbsR protein. (A) The shifted band could be competed away effectively by the cold P*cap* fragment but not by the P*cap*^{mt} fragment, containing a 4-bp mutation; the P*cap*5′ fragment, containing the 5′ half of the 10-bp IR with upstream sequence; or the P*cap*3′ fragment, containing the 3′ half of the 10-bp IR with downstream sequence. (B) A K_d value of ~4.9 nM was determined by using increasing amounts of His-RbsR and a constant amount of labeled P*cap* fragment (0.63 nM).

sule 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 transcriptional 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_6-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 P*cap* DNA fragment as a probe. As shown in Fig. 3A, the P*cap* fragment could readily be upshifted by His_6-RbsR. The shifted band could be competed away with a cold P*cap* DNA fragment, suggesting that the binding was specific. The binding dissociation constant (K_d) 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 P*cap*^{mt} 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 outcompeted by using a 92-bp DNA fragment upstream (P*cap*5′) of the 10-bp IR or a 75-bp downstream DNA fragment (P*cap*3′) (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_{600} of about 2.0 (mid-log phase), whereas the ~1.0-kb transcript increased to the highest level at OD_{600}s 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

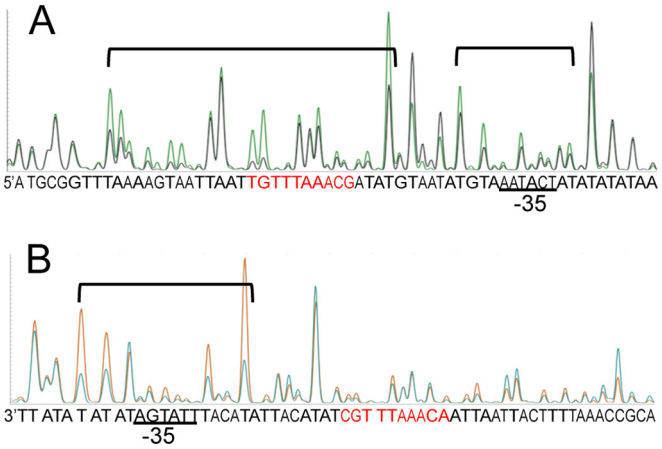

FIG 4 DNase I footprinting analysis of the 5′-FAM-labeled sense strand (A) and the 5′-VIC-labeled antisense strand (B) of the P*cap* probe. A reduction in intensity of DNase I-digested fragments in the presence of 1.9 μM RbsR (black peaks in panel A and blue peaks in panel B) compared to that in its 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 10-bp IR required for P*cap* activation. The results suggest that RbsR binds to the region encompassing the 10-bp IR and the −35 region of the promoter.

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 histidine 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*, *mgrA*, *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 ArlRS 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 P*cap* is activated by RbsR, we also tested whether RbsR activation of capsule is affected by ribose. To this end, we employed a P*cap-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

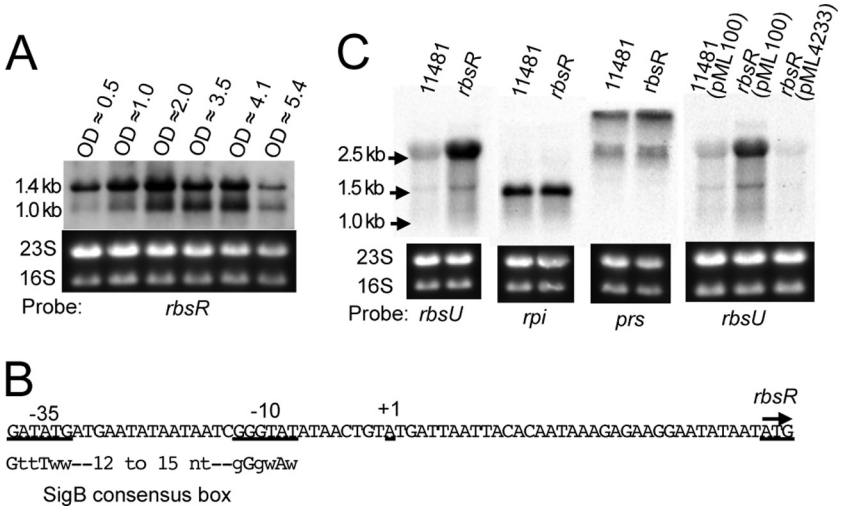

FIG 5 Transcription analyses. Total RNA (5 μg) from each sample used for Northern blotting was denatured in formamide, applied to TBE-agarose gels, and hybridized with the Dig-labeled specific probe indicated below each blot. (A) Expression of *rbsR* in CYL11481 cultures grown to different OD_{600}s, 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 (OD_{600}, ~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.

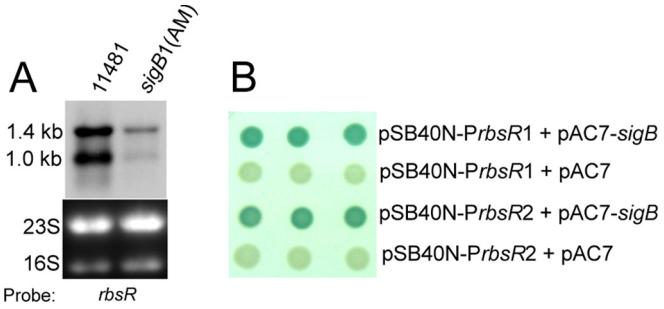

FIG 6 Effect of SigB on *rbsR*. (A) Northern blot analysis. Total RNAs were prepared from 4-h cultures (OD_{600}, ~4.0) of *S. aureus* CYL11481 or the *sigB1*(Am) mutant (CYL13113; contains an amber mutation in *sigB*) and hybridized with an *rbsR*-specific probe. (B) *E. coli* bacterial two-plasmid system. A promoter region of *rbsR*, P*rbsR*1 (153 bp) or P*rbsR*2 (756 bp), was cloned into the promoter probe plasmid pSB40N (giving pML4261 or pML4262, respectively) and transformed into *E. coli* XL1-Blue containing pAC7-*sigB* or pAC7. Clones were selected on LBACX-ARA plates. Positive transformants (containing pAC7-*sigB*) were blue, whereas negative controls (containing the pAC7 vector) were colorless. The longer P*rbsR*2 region was used in the study to include potential unknown upstream promoters.

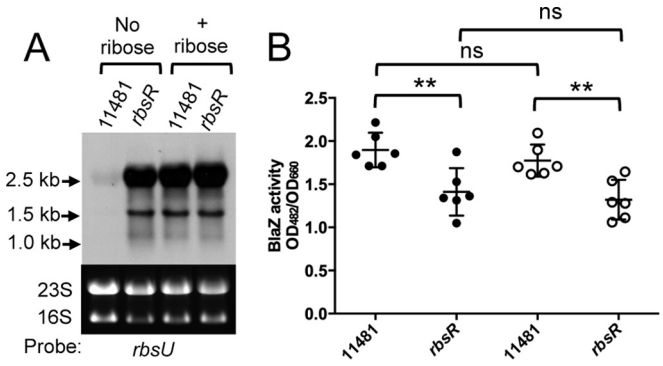

FIG 7 Effect of D-ribose on RbsR regulatory function as assayed in TSB-0G basal medium. (A) Expression of *rbsU* as assayed by Northern analyses. Total RNAs were prepared from 4-h cultures (OD_{600}, ~3.2) of *S. aureus* CYL11481 or the *rbsR* mutant (CYL12834) in the absence or presence of D-ribose and hybridized with an *rbsU*-specific probe. (B) *cap* promoter activities measured by BlaZ reporter assays. Four-hour cultures of CYL11481 or the *rbsR* mutant containing the P*cap-blaZ* fusion plasmid (pAM3176) were assayed for BlaZ activity in the absence (solid circles) or presence (open circles) of D-ribose. ns, not significant; **, $P < 0.01$.

in the TSB-0G growth medium. As shown in Fig. 7B, we found that the P*cap* 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 P*cap* 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 *rbsDACBK* repression by RbsR in *E. coli* (38). However, in *B. subtilis*, repression of the *rbsKDACB* 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. RbsR 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 phosphoribosylpyrophosphate), 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

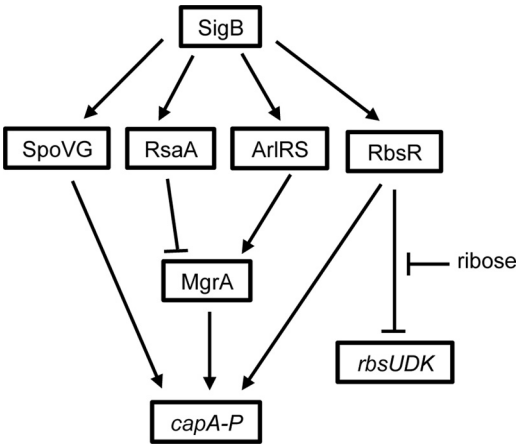

FIG 8 Proposed regulatory circuits for RbsR. RbsR is activated by SigB and in turn represses the *rbs* operon and activates the *cap* operon by directly binding at the promoter. Repression of the *rbs* operon by RbsR is relieved by D-ribose. Arrows indicate activation, and block arrows indicate repression.

activation of the *cap* operon. The consensus RbsR binding site in the promoter region of the *rbs* operon in the *Bacillus/Clostridium* group of bacteria, including *S. aureus*, has been defined by a comparative approach (41). The predicted consensus RbsR box upstream of the *rbsUDK* operon in *S. aureus* bears no resemblance to the RbsR binding site in the *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 *rbsU* open reading frame. These findings further corroborate that RbsR may regulate the *rbs* and *cap* genes by different mechanisms.

SigB is a stress response sigma factor that also controls the expression of a number of virulence factors. SigB has been shown to independently activate capsule through SpoVG and ArlR (44). Recently, 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 *cap* gene expression (23). Because there is no direct evidence that the 28-bp region of the SpoVG binding site is involved in *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 ArlR, 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 *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 *S. aureus* pathogenesis by promoting bacterial growth. However, our finding that repression of the *rbs* operon by RbsR, but not activation of capsule, is controlled by ribose suggests that ribose is not likely an effector linking the two cellular processes. On the other hand, because RbsR is highly regulated by SigB, whose activity is affected by certain *in vitro* and *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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