

σ^B and the σ^B -Dependent *arlRS* and *yabJ-spoVG* Loci Affect Capsule Formation in *Staphylococcus aureus*[∇]

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The alternative transcription factor σ^B of *Staphylococcus aureus* affects the transcription of the *cap* gene cluster, required for the synthesis of capsular polysaccharide (CP), although this operon is lacking an apparent σ^B -dependent promoter. Regulation of *cap* expression and CP production in *S. aureus* strain Newman was shown here to be influenced by σ^B , the two-component signal transduction regulatory system ArlRS, and the *yabJ-spoVG* locus to different extents. Inactivation of *arlR* or deletion of the *sigB* operon strongly suppressed *capA* (CP synthesis enzyme A) transcription. Deletion of *spoVG* had a polar effect on *yabJ-spoVG* transcription and resulted in a two- to threefold decrease in *capA* transcription. Interestingly, immunofluorescence showed that CP production was strongly impaired in all three mutants, signaling that the *yabJ-spoVG* inactivation, despite its only partial effect on *capA* transcription, abolished capsule formation. *trans*-Complementation of the Δ *spoVG* mutant with *yabJ-spoVG* under the control of its native promoter restored CP-5 production and *capA* expression to levels seen in the wild type. Northern analyses revealed a strong impact of σ^B on *arlRS* and *yabJ-spoVG* transcription. We hypothesize that ArlR and products of the *yabJ-spoVG* locus may serve as effectors that modulate σ^B control over σ^B -dependent genes lacking an apparent σ^B promoter.

Staphylococcus aureus is a major nosocomial pathogen with the ability to cause a variety of diseases, including life-threatening infections. Like most microorganisms that are able to cause invasive diseases, *S. aureus* produces extracellular capsular polysaccharides (CPs), which are thought to be of importance in pathogenesis (reviewed in reference 35). Although 11 serologically distinct CPs were identified in *S. aureus*, the majority of clinical isolates produce CPs of serotype 5 (CP-5) or serotype 8 (CP-8). CPs protect *S. aureus* against opsonophagocytic killing by polymorphonuclear leukocytes (16, 17, 25, 53, 56) and enhance virulence in a number of animal models of staphylococcal infection (34, 40, 53, 54, 57). Expression of CPs is known to be influenced by various environmental signals in vitro and in vivo (reviewed in references 35 and 56), and transcription of the *cap* operon was shown to be modulated by regulatory elements, such as *arlRS*, *agr*, *ccpA*, *mgr*, *sae*, and *sarA* (7, 8, 23, 24, 26, 27, 39, 48, 52, 55). Recent microarray analyses added the alternative σ factor σ^B to the regulatory network controlling *cap* operon expression (3, 38) and indicated σ^B to control *capA* transcription in a growth phase-dependent manner (3). However, the lack of an apparent σ^B consensus sequence in the promoter of *capA* suggested that σ^B regulates *cap* transcription indirectly. Candidates for such downstream-acting regulators might be ArlRS and SarA, which are positively controlled by σ^B in *S. aureus* (2, 3), although SarA was previously shown to have only a minor effect on *cap* expression and CP production in *S. aureus* (24). RNAIII of the *agr* locus,

known to positively affect *capA* expression (7, 24, 55), could be excluded as a positive mediator of σ^B activity on *capA* transcription, since RNAIII is known to be repressed by σ^B activity (2, 3, 15). Further candidates for regulators mediating the effect of σ^B might be the putative regulator homologs YabJ and SpoVG (SaCOL0540/1), whose expression is also predominantly controlled by σ^B in *S. aureus* (3). *spoVG* was the first developmentally regulated gene cloned from spore-forming *Bacillus subtilis* (47). Mutations in *spoVG* were shown to impair spore formation of *B. subtilis* stationary-phase cells at stage V (44) and to enhance the stage II defect of a *spoIIB* mutation (29), leading to the assumption that SpoVG is involved in the formation of the spore cortex. More recent findings indicated the primary function of SpoVG to be in the regulation of asymmetric septation in stationary-phase cells (31). Based on the finding that close homologues of SpoVG of *B. subtilis* are present in the genomes of several nonsporulating bacteria as well, we recently suggested that the SpoVG homologues might fulfill different, more general regulatory functions in the latter group of bacteria (3). YabJ is a member of the highly conserved YigF protein family, which is represented in animals, fungi, and bacteria. Many biological processes were shown to be influenced by YigF proteins, but its exact biochemical function remains unknown, although the crystal structure of YabJ of *Bacillus subtilis* has been published (51).

Here, we show that σ^B and the σ^B -modulated *arlRS* and *yabJ-spoVG* loci reduced *capA* transcription and strongly impaired capsule formation in the CP-5-producing strain Newman.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this investigation are listed in Table 1. Bacteria were routinely grown in Luria-Bertani (LB) medium (Difco Laboratories, Detroit, MI) with aeration

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and phenotype ^a	Reference or source
Strains		
<i>S. aureus</i>		
RN4220	NCTC8325-4 r ⁻ m ⁺ <i>rsbU</i>	21
COL	<i>mec</i> ; high-Mc ^r clinical isolate; Mc ^r Tc ^r	20
Newman	Clinical isolate (ATCC 25904); CP-5 producer	9
BF21	RN6390 <i>arlR::cat</i> ; phenotypic <i>arlRS</i> mutant; Cm ^r	10
GP268	NCTC8325 derivative; (<i>rsbU-V-W-sigB</i>) ⁺ - <i>tet</i> (L); Tc ^r	12
IK181	NCTC8325 derivative; Δ <i>rsbUVW-sigB::erm</i> (B); Erm ^r	22
IK183	COL Δ <i>rsbUVW-sigB::erm</i> (B); Erm ^r	22
IK184	Newman Δ <i>rsbUVW-sigB::erm</i> (B); Erm ^r	22
SM1	RN4220 Δ <i>spoVG::erm</i> (B); phenotypic <i>yabJ-spoVG</i> mutant, Erm ^r	This study
SM2	Newman Δ <i>spoVG::erm</i> (B); phenotypic <i>yabJ-spoVG</i> mutant, Erm ^r	This study
SM99	Newman <i>arlR::cat</i> ; Cm ^r	This study
<i>E. coli</i>		
XL1Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI</i> ^q Δ <i>M15 Tn10</i> (Tc ^r)]	Stratagene
Plasmids		
pAC7	Expression plasmid containing the P _{BAD} promoter and the <i>araC</i> gene; Cm ^r	42
pAC7- <i>sigB</i>	pAC7 with a 0.75-kb PCR fragment of the <i>sigB</i> ORF from strain COL; Cm ^r	14
pBT	1.6-kb PCR fragment of the <i>tet</i> (L) gene of pHY300PLK into Alw26I-digested pBC SK(+) (Stratagene); Tc ^r	12
pBus1	<i>E. coli-S. aureus</i> shuttle plasmid with multicloning site from pBluescript II SK (Stratagene) and the <i>rrnT1</i> ₄ terminator sequence from pLL2443; Tc ^r	45
pEC1	pUC derivative containing the 1.45-kb ClaI <i>erm</i> (B) fragment of Tn551; Ap ^r Em ^r	4
pGC2	<i>E. coli-S. aureus</i> shuttle plasmid; Cm ^r	Constructed and obtained by P. Matthews
pSA0455p	pSB40N with a 360-bp PCR fragment covering the σ^B -dependent promoter region preceding <i>yabJ-spoVG</i> fused to the reporter gene <i>lacZα</i> ; Ap ^r	3
pSB40N	Promoter probe plasmid; Ap ^r	19
pSP- <i>luc</i> ⁺	Firefly luciferase cassette vector; Ap ^r	Promega
pSTM01	pEC1 with 0.5- and 1.1-kb PCR fragments covering the <i>spoVG</i> flanking regions; Em ^r Ap ^r	This study
pSTM02	pBT with a 3.1-kb KpnI/HindIII fragment of pSTM01 harboring the <i>spoVG</i> flanking regions and the <i>erm</i> (B) cassette fully replacing the <i>spoVG</i> coding region; Em ^r Tc ^r	This study
pSTM03	pSP- <i>luc</i> ⁺ with a 0.4-kb PCR fragment covering the <i>capA</i> promoter region fused to the reporter gene <i>luc</i> ⁺ ; Ap ^r Em ^r	This study
pSTM04	pBus1 with a 2.1-kb KpnI/EcoRI fragment of pSTM03 harboring the <i>capA</i> promoter- <i>luc</i> ⁺ fusion; Tc ^r	This study
pSTM05	pGC2 with a 1-kb PCR fragment covering the σ^B -dependent <i>yabJ</i> promoter, <i>yabJ</i> , and <i>spoVG</i> ; Cm ^r	This study
pSTM06	pSB40N with a 0.8-kb PCR fragment covering the <i>arlRS</i> promoter fused to the reporter gene <i>lacZα</i> ; Ap ^r	This study
pSTM07	pSB40N with a 0.4-kb PCR fragment covering the <i>capA</i> promoter region fused to the reporter gene <i>lacZα</i> ; Ap ^r	This study
pSTM11	pBus1 with a 0.6-kb PCR fragment covering <i>yabJ</i> and the preceding σ^B -dependent promoter P _{<i>yabJ</i>} ; Tc ^r	This study
pSTM13	pBus1 with a 0.5-kb PCR hybrid including the σ^B -dependent promoter P _{<i>yabJ</i>} fused to the <i>spoVG</i> ORF; Tc ^r	This study

^a Abbreviations are as follows: Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Tc^r, tetracycline resistant.

(200 rpm) at 37°C. Where indicated, mutant strains were grown on antibiotic-supplemented media containing either 100 μ g of ampicillin, 20 μ g of chloramphenicol, 10 μ g of erythromycin, or 10 μ g of tetracycline per ml.

Strain construction. For the construction of an *S. aureus* Newman Δ *spoVG* mutant, DNA fragments covering 0.5 kb of the region upstream of *spoVG* (up fragment) and 1.1-kb of the region downstream of *spoVG* (down fragment) were amplified by PCR, using primer pairs oSTM01/02 and oSTM03/04, respectively, and *S. aureus* Newman DNA as the template (Table 2). The resulting PCR products were KpnI/BamHI and PstI/HindIII digested, respectively, and cloned into plasmid pEC1 (4), with the up fragment preceding the *erm*(B) cassette and the down fragment following the resistance marker. The resulting plasmid, pSTM01, was digested with KpnI and HindIII and the insert cloned into the suicide vector pBT (12), yielding plasmid pSTM02, which was electroporated into RN4220 (Fig. 1). Mutants with the allelic replacement were selected for erythromycin resistance and screened for loss of tetracycline resistance, yielding

strain SM1 [RN4220 *spoVG::erm*(B)], which was subsequently used as a donor for transducing the *spoVG* deletion into the CP-5-producing *S. aureus* strain Newman, yielding strain SM2. The *spoVG* deletion in SM2 was confirmed by PCR and Southern analyses.

The Newman *arlR* mutant SM99 was constructed by transducing the *cat*-tagged *arlR* mutation of BF21 (10) into Newman and selecting for chloramphenicol resistance.

Plasmid construction. For the construction of the *capA* promoter-*luc*⁺ reporter gene fusion plasmid pSTM04, a 0.4-kb DNA fragment covering the *capA* promoter region was amplified by PCR using primer pair capAp-F/capAp-R and *S. aureus* Newman DNA as the template (Table 2). The resulting PCR product was HindIII/NcoI digested and cloned into pSP-*luc*⁺ (Promega) as a 5' fusion to the reporter gene *luc*⁺. A plasmid (pSTM03) harboring the promoter fragment was used to excise the promoter-reporter gene fragment by digestion with HindIII/XhoI, which was subsequently cloned into the multiple cloning site of

TABLE 2. Primers used in this study

Primer	Sequence (5'–3') ^a	Location ^b or reference
arlRprobe+	CAATGTGGACACAGAGTATG	1465970–1465989
arlRprobe–	CAGTTCGTGGCGTTGGG	1465422–1465438
arlSprobe+	CAGCAGTATTAGAAGAATCG	1464595–1464614
arlSprobe–	GAGTCCATTACCGCCTTGAC	1464154–1464173
Asp23probe+	ATGACTGTAGATAACAATAAAGC	11
Asp23probe–	TTGTAAACCTTGTCTTTCTTGG	11
capAp-F	cgcaagcttCAAACATCATATGATTATAAGC	153034–153055
capAp-R	gcgccatgTTTACCTCCCTTAAAAATT	153384–153402
oSTM01	taggtaccTCAAAAAGAAGTTAAACAAG	548665–548684
oSTM02	cggatccATATTAATCGAAAATTATAATTCC	549175–549198
oSTM03	cgctgcagATTATGATGATATGAAAATTATTG	549706–549729
oSTM04	gcgaagcttGACCAATAACAACATCTTCGCC	550760–550781
oSTM20	GAAAATCATTAAACACAACCTT	548806–548826
oSTM21	CTTAATTTTACTTACTAATTC	549155–549175
oSTM28	cgatgatccGTGTTATGAATTTAATGAATGAG	548603–548625
oSTM29	gcgtcgacTTATTGCAAATGTATTACATCGC	549574–549596
oSTM30	CTAAATAAAACAGAGATATATACTATAG	549213–549243
oSTM31	gcgggtaccGTGTTATGAATTTAATGAATGAG	548603–548625
oSTM32	gcgggatccGAAGCTTGATTAACATATTAATCG	549189–549213
oSTM33	gcgggatccTTATTGCAAATGTATTACATCGC	549574–549596
oSTM34	ggattttcatatgACTAAAACCTCCTTTTATGAAAAC	548778–548800
oSTM61	cgcggatccCAAACATCATATGATTATAAGC	153034–153055
oSTM62	gcgctcgagTTTACCTCCCTTAAAAATT	153384–153402
oSTM63	cgcggatccGCAGTAAACCTAAAGTGTCG	1466817–1466836
oSTM64	gcgctcgagTTGTACACCTCATATTACGAC	1466067–1466087
oSTM71	gtgcatatgAAAGTGACAGATGTAAGACTTAG	549259–549281

^a Lowercase letters represent nucleotide additions.

^b Based on the sequence of strain COL (RefSeq accession no. NC_002951).

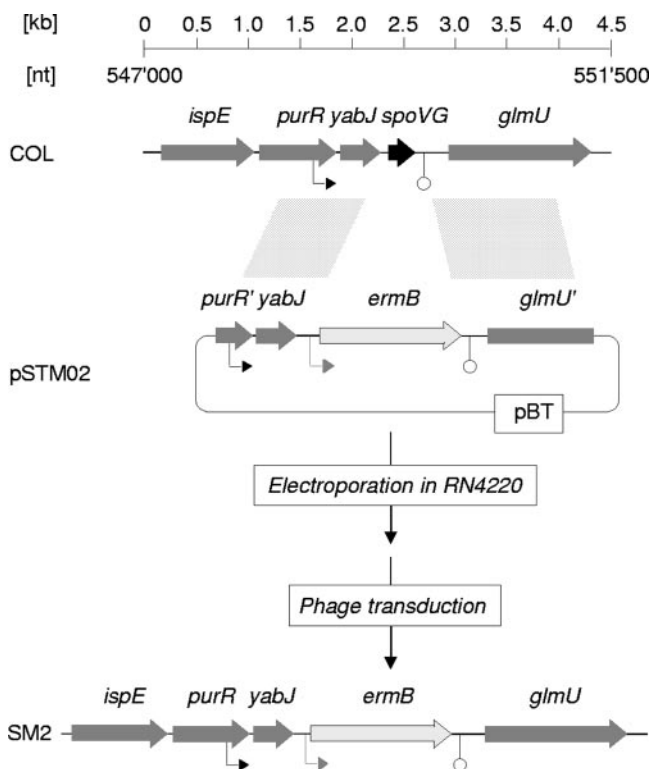


FIG. 1. Genetic organization of the *S. aureus yabJ-spoVG* locus. Schematic representations of the *yabJ-spoVG* region of *S. aureus* and of the strategy used to obtain SM2 are shown. ORFs, promoters, terminators, and regions allowing recombination are indicated. ORF notations and nucleotide (nt) numbers correspond to those of the respective genomic regions of strain COL (RefSeq accession no. NC_002951).

the *Escherichia coli-S. aureus* shuttle vector pBus1 (45) to obtain plasmid pSTM04. pSTM04 was finally used for electroporation of RN4220, from which it was transduced into strains Newman, SM2, SM99, and IK184 by phage transduction.

For the construction of pSTM05, a 995-bp DNA fragment covering the σ^B -dependent *yabJ* promoter, *yabJ*, and *spoVG* was amplified by PCR using primer pair oSTM28/oSTM29 and *S. aureus* Newman DNA as the template. The resulting PCR product was BamHI/SalI digested and cloned into the *E. coli-S. aureus* shuttle vector pGC2 (P. Matthews). pSTM05 was electroporated into RN4220, from which it was transduced into strains Newman and SM2.

For the construction of pSTM06 and pSTM07, DNA fragments representing 771 bp and 370 bp of the *arlR* and *capA* promoter regions of Newman, respectively, were generated by PCR using primer pairs oSTM63/oSTM64 and oSTM61/oSTM62. The PCR products were digested with BamHI and XhoI and cloned into promoter probe plasmid pSB40N (42) upstream of the *lacZ* α reporter gene to obtain pSTM06 (*arlRp*) and pSTM07 (*capAp*).

For the construction of pSTM11, a 612-bp DNA fragment covering the σ^B -dependent *yabJ* promoter (P_{yabJ}) and *yabJ* was amplified by PCR using primer pair oSTM31/oSTM32 and *S. aureus* Newman DNA as the template. The resulting PCR product was BamHI/KpnI digested and cloned into the *E. coli-S. aureus* shuttle vector pBus1. For pSTM13 ($P_{yabJ-spoVG}$), harboring promoter P_{yabJ} fused to *spoVG*, primer pairs oSTM31/oSTM34 and oSTM71/oSTM33 were used together with pSTM05 to amplify 199- and 339-bp DNA fragments covering the σ^B -dependent promoter including the region preceding the *yabJ* open reading frame (ORF) and *spoVG*, respectively. The resulting PCR products were KpnI/NdeI and NdeI/BamHI digested and cloned into pBus1. pSTM11 and pSTM13 were electroporated into RN4220 and subsequently transduced into strains Newman and SM2.

Sequence analyses confirmed the identities of all cloned inserts.

Protease activity assay. The proteolytic activities of Newman, IK184, SM2, and SM99 were determined on casein agar plates as clear zones surrounding colonies.

Northern analyses. Overnight cultures of *S. aureus* were diluted 1:100 into fresh prewarmed LB medium and grown at 37°C and 200 rpm. Samples were removed from the culture at the time points indicated and centrifuged at 14,000 \times g and 4°C for 1 min, the culture supernatants were discarded, and the cell sediments were snap frozen in liquid nitrogen. Total RNAs were isolated according to Cheung et al. (6). Blotting, hybridization, and labeling were performed as previously described (12). Primer pairs arlRprobe+/arlRprobe–, arlSprobe+/arlSprobe–, asp23probe+/asp23probe–, oSTM20/oSTM21, and oSTM29/oSTM30 were used to generate

digoxigenin-labeled *arlR*-, *arlS*-, *asp23*-, *yabJ*-, and *spoVG*-specific probes by PCR labeling.

Luciferase assays. Luciferase activity was measured as described earlier (2), using the luciferase assay substrate and a Turner Designs TD-20/20 luminometer (Promega).

LightCycler RT-PCR. The *capA* and *gyrB* transcripts were quantified by LightCycler reverse transcription (RT)-PCR as described earlier (48), using RNA samples obtained from cultures grown for 8 and 24 h in LB at 37°C and 200 rpm.

CP-5 determination. CP-5 production was determined by indirect immunofluorescence from cultures grown for 8 and 24 h in LB medium as described earlier (48), using mouse immunoglobulin M monoclonal antibodies to CP-5 (13). Quantification of CP-5-positive cells was done by determining the numbers of DAPI (4',6'-diamidino-2-phenylindole)- and CY-3-positive cells using the program CellC 1.11 (Institute of Signal Processing, Tampere University of Technology, Finland).

Two-plasmid analysis. Testing of the interaction of *S. aureus* promoters with RNA polymerase containing *S. aureus* σ^B was done essentially as described earlier (14). The promoter-containing plasmids pSA0455p (*yabJ* promoter), pSTM06 (*arlR* promoter), and pSTM07 (*capA* promoter) were transformed into *E. coli* XL1Blue containing a compatible plasmid, either pAC7-*sigB* or empty pAC7. Clones were selected on LBACX-ARA plates (LB medium containing lactose [5 mg ml⁻¹], ampicillin [100 μ g ml⁻¹], chloramphenicol [40 μ g ml⁻¹], 5-bromo-4-chloro-3-indolyl-D-galactopyranoside [20 μ g ml⁻¹], and arabinose [2 μ g ml⁻¹]) and analyzed for color production (43).

RESULTS

Construction of SM2 and SM99. Inactivation of *spoVG* and *arlR* in *S. aureus* Newman yielded strains SM2 and SM99, respectively. Southern blots probed with *spoVG* or the C-terminal part of *arlR* confirmed the constructs (data not shown).

Transcriptional analysis of the *yabJ-spoVG* locus in Newman and SM2. Our recent microarray analyses suggested that *yabJ* and *spoVG* form a bicistronic operon that is predominantly controlled by σ^B activity (3). To test whether and how the deletion of *spoVG* affected the expression of the *yabJ-spoVG* locus, we analyzed the transcription of *yabJ* and *spoVG* in cells of Newman and SM2 during growth in LB. Our Northern analyses identified *yabJ*-specific signals with sizes of 1.2 and 0.6 kb and *spoVG*-specific signals with sizes of 1.2 and 0.5 kb in Newman. The 1.2-kb signal detected with both the *yabJ*- and the *spoVG*-specific probes appeared to be the major transcript of the *yabJ-spoVG* locus. It was present already after 1 h of growth and increased within time, while the minor 0.6-kb, *yabJ*-specific signal and the 0.5-kb, *spoVG*-specific signal appeared only after 3 h of growth (Fig. 2). In line with our Southern results, no *spoVG*-specific transcripts were detected in SM2. Based on our hypothesis that *yabJ* and *spoVG* are predominantly transcribed as a bicistronic mRNA and taking into account that the deletion of *spoVG* did not alter the genetic organization of *yabJ* and its promoter(s) in SM2, we expected the allelic replacement of *spoVG* with *ermB* to affect the sizes of the *yabJ* transcripts rather than the intensities of *yabJ* transcription. Surprisingly, we detected only a series of faint signals in our *yabJ* Northern analysis of SM2, indicating that the deletion of *spoVG* apparently had a polar effect on the expression of the preceding *yabJ*. We therefore considered SM2 to represent a phenotypic *yabJ-spoVG* mutant.

Phenotypic characterization of SM2 and SM99. The successful allelic replacement of *spoVG* and its impact on *yabJ* indicated that these genes, like *sigB* and *arlR*, were not essential for growth in vitro. Deletions of *sigB* and *arlR* were previously shown to affect the growth rates of the mutants (12, 23). While inactivation of *arlR* reduces growth during the early

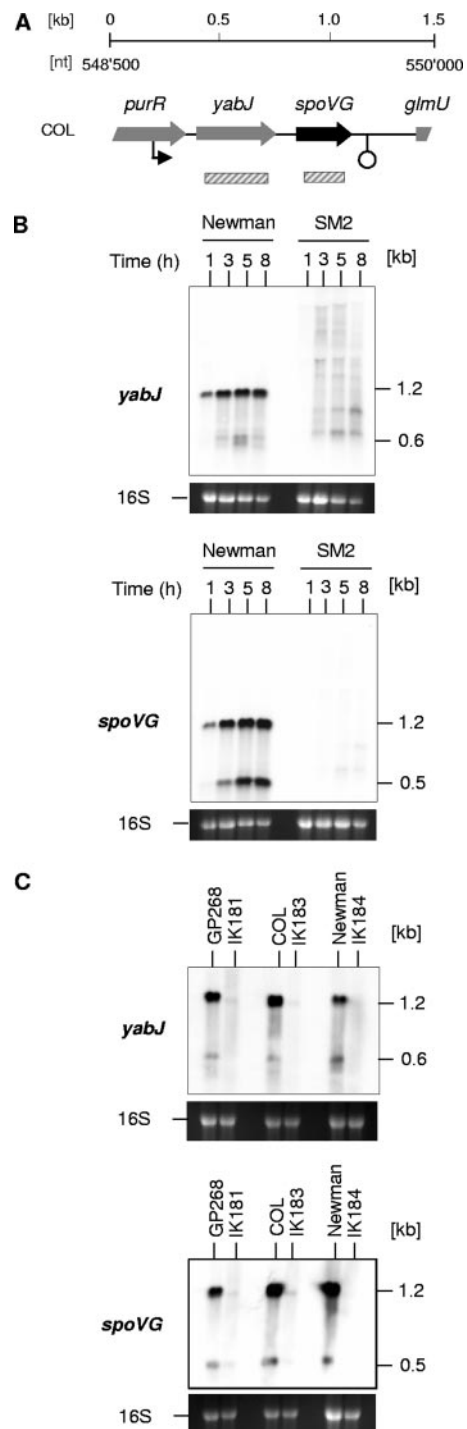


FIG. 2. Expression of *yabJ-spoVG* in *S. aureus*. (A) Schematic representation of the *yabJ-spoVG* region of *S. aureus* COL. ORFs, promoters, terminators, and the probes used are indicated. nt, nucleotide. (B) Northern blot analyses of the *yabJ* and *spoVG* transcriptions in Newman and SM2 (Δ *spoVG*) during growth in LB. (C) Effect of σ^B on *yabJ* and *spoVG* expression. Northern blot analyses of the *yabJ* and *spoVG* transcriptions in strains GP268, COL, and Newman and their isogenic Δ *rsbUVW-sigB* mutants after 8 h of growth. Relevant transcript sizes are indicated. Ethidium bromide-stained 16S rRNA patterns are shown as an indication of RNA loading.

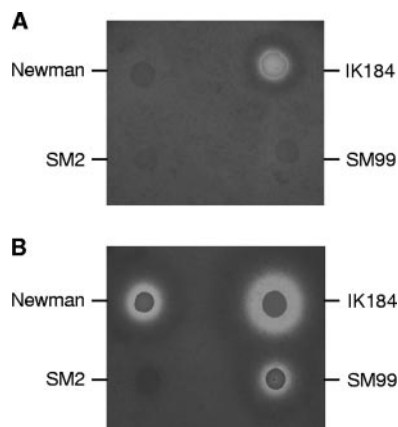


FIG. 3. Protease production of Newman, IK184 (Δ *rsbUVW-sigB*), SM2 (Δ *spoVG*), and SM99 (*arlR*) grown on casein agar plates for 24 h at 37°C (A) and then incubated for 72 h at room temperature (B).

stages (23), inactivation of *sigB* affects only the later stages of growth (12). Monitoring growth of strains Newman, SM2, and SM99 in LB over a period of 8 h confirmed the negative impact of the *arlR* mutation on the growth rate of SM99, while the growth curves of Newman and SM2 were virtually identical, indicating that deletion of *spoVG* was not associated with the growth defect observed for *sigB* mutants (data not shown). Inactivation of *sigB* is further known to prevent formation of staphyloxanthin (12, 22, 33), the orange end product of *S. aureus* carotenoid biosynthesis (30). Neither inactivation of *arlR* nor deletion of *spoVG* reduced pigment formation, indicating that ArlR and YabJ/SpoVG were not involved in the regulation of pigment production of *S. aureus* (data not shown). Mutations in *sigB* and *arlRS* are also known to affect the proteolytic activities of the mutants (10, 18, 23). We therefore tested the proteolytic activities of Newman and its derivatives IK184 (Δ *rsbUVW-sigB*), SM2 (Δ *spoVG*), and SM99 (*arlR*) on casein agar plates. After 24 h of incubation at 37°C, only IK184 produced a clear zone surrounding the colony, while neither Newman, SM2, nor SM99 exhibited such a clear zone (Fig. 3A). However, after storage of the incubated plates for 72 h at room temperature, clear zones surrounding colonies were also observed for Newman and SM99 but not for SM2 (Fig. 3B), suggesting that the inactivation of the *yabJ-spoVG* locus caused a suppression of extracellular protease production or activation in *S. aureus*.

Effect of σ^B on *arlRS* and *yabJ-spoVG* expression. We recently observed that *arlRS* transcription, like *yabJ-spoVG* transcription, is positively controlled by σ^B activity during later growth stages (3). While the positive effect of σ^B on *yabJ-spoVG* transcription was found in different *S. aureus* genetic lineages, including COL, GP268 (*rsbU*-positive NCTC8325 derivative), and Newman (3, 38), the positive impact of σ^B on *arlRS* expression seemed to be strain dependent and was seen only in *S. aureus* Newman so far (3). We therefore determined the *arlRS* and *yabJ-spoVG* expression patterns in cells of COL, GP268, Newman, and their isogenic Δ *rsbUVW-sigB* mutants grown in LB for 8 h. While clear *yabJ*- and *spoVG*-specific signals were visible in COL, GP268, and Newman at this growth stage, these transcripts were missing in all of the

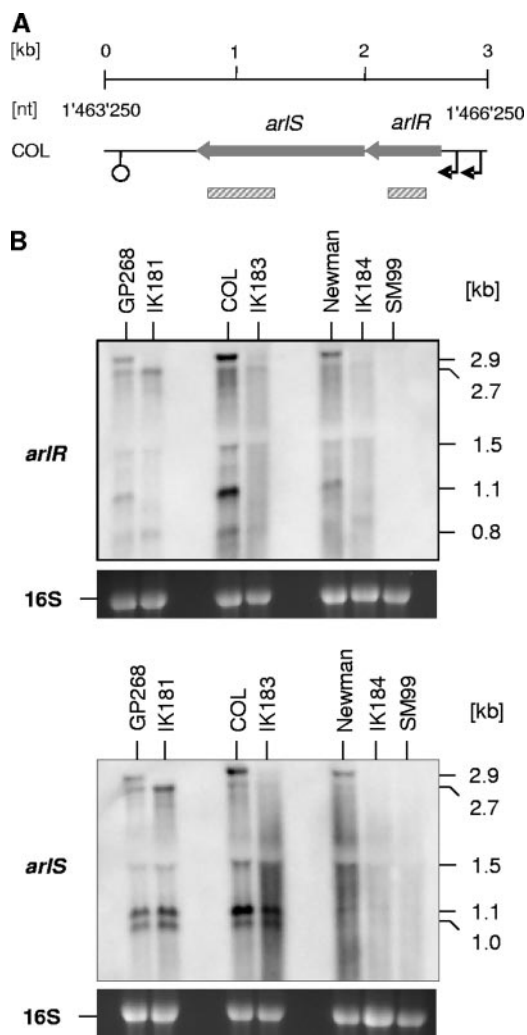


FIG. 4. Effect of σ^B on *arlRS* expression. (A) Schematic representation of the *arlRS* region of *S. aureus* COL. ORFs, promoters, terminators, and the regions used as probes are indicated. ORF notations and nucleotide (nt) numbers correspond to those of the respective genomic regions of strain COL (RefSeq accession no. NC_002951). (B) Northern blot analyses of the *arlR* and *arlS* transcriptions in strains GP268, COL, and Newman and their isogenic Δ *rsbUVW-sigB* mutants after 8 h of growth in LB at 37°C. Relevant transcript sizes and the probes used are indicated. Ethidium bromide-stained 16S rRNA patterns are shown as an indication of RNA loading.

Δ *rsbUVW-sigB* mutants (Fig. 2C), confirming the importance of σ^B activity for the expression of the *yabJ-spoVG* locus. Interestingly, our Northern analyses of *arlRS* transcription revealed a clear σ^B dependence in all genetic lineages analyzed (Fig. 4) and identified several new signals in a strain-dependent manner in addition to the 2.7- and 1.5-kb transcripts that have been reported for *arlRS* before (10). Hybridizing the total RNA samples with a probe specific for *arlR* produced signals with sizes of 2.9, 2.7, 1.5, 1.1, and 0.9 kb in all parental strains, while the 2.9- and 1.1-kb signals were missing in all Δ *rsbUVW-sigB* mutants. All signals were missing in the *arlR* mutant SM99, confirming that the signals were *arlR* specific. Hybridizing the same RNAs with a probe specific for *arlS* resulted in signals with sizes of 2.9, 2.7, and 1.5 kb in all parental strains,

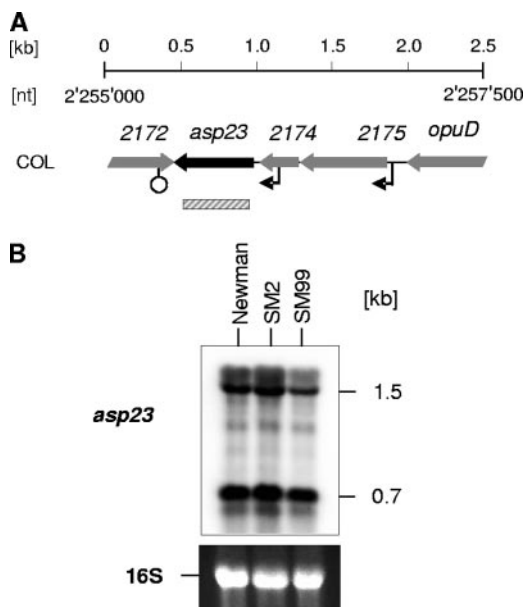


FIG. 5. Effect of *arlRS* and *yabJ-spoVG* on σ^B activity. (A) Schematic representation of the *asp23* region. ORFs, promoters, terminators, and the regions used as probes are indicated. ORF notations and nucleotide (nt) numbers correspond to those of the respective genomic regions of strain COL (RefSeq accession no. NC_002951). (B) Northern blot analysis of σ^B -dependent *asp23* transcription in Newman, SM2 (Δ *spoVG*), and SM99 (*arlR*) grown for 8 h in LB at 37°C. Relevant transcript sizes are indicated. Ethidium bromide-stained 16S rRNA patterns are shown as an indication of RNA loading.

while clear 1.1- and 1.0-kb signals were present only in COL and GP268. As for the *arlR* pattern, the 2.9-kb signal was detectable only in the wild-type strains and missing in the Δ *rsbUVW-sigB* mutants. However, the 1.1- and 1.0-kb signals were present in the COL and GP268 Δ *rsbUVW-sigB* derivatives as well, indicating that these transcripts are produced independently from σ^B activity and suggesting that the 1.1-kb signals observed with *arlR* and *arlS* were not identical. Only weak signals were identified with *arlS* in the Newman *rsbUVW-sigB* mutant IK184, and none of the signals were detected in SM99. The impact of σ^B on *arlRS* expression appeared to be strongest in Newman but was also visible in the COL and GP268 backgrounds.

Effect of *ArlRS* and *YabJ/SpoVG* on σ^B activity. To assess whether *ArlRS* and *YabJ/SpoVG* might have an impact on σ^B activity, we analyzed the transcription of *asp23*, a marker gene for σ^B activity in *S. aureus* (11, 12, 22), during a later growth stage in Newman, SM2, and SM99 (Fig. 5). No difference in *asp23* expression was found between the wild type and its Δ *spoVG* derivative, while the *arlR* mutant showed a slight but reproducible reduction in the expression of the 1.5-kb transcript but not of the 0.7-kb transcript. Interestingly, variations in the expression levels of these two directly σ^B -controlled transcripts were recently observed in a Newman *hemB* mutant, indicating the presence of factors modulating σ^B activity in the recognition of its promoter consensus sequences under certain circumstances (49).

Effect of the *arlRS* locus on *yabJ-spoVG* transcription and vice versa. The inactivation of *arlR* did not affect *yabJ-spoVG*

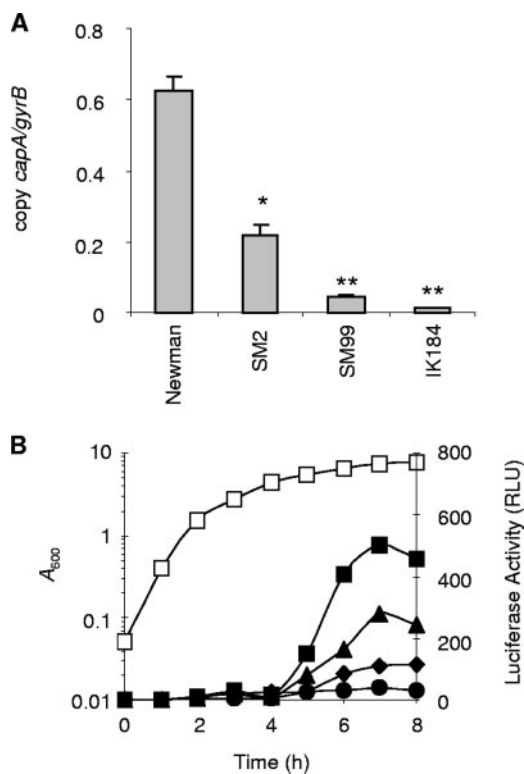


FIG. 6. Expression of *capA* in Newman and its derivatives. (A) Quantitative transcript analysis of *capA* by LightCycler RT-PCR of strains Newman, SM2 (Δ *spoVG*), SM99 (*arlR*), and IK184 (Δ *rsbUVW-sigB*) grown for 8 h at 37°C in LB. Transcripts were quantified in reference to the transcription of gyrase (in numbers of copies per copy of *gyrB*). Values from two separate RNA isolations and two independent RT-PCRs each were used to calculate the mean expression levels (\pm standard errors of the mean). *, $P < 0.05$ for derivative versus Newman; **, $P < 0.01$ for derivative versus Newman. (B) Growth curve of Newman (open squares) and transcriptional activity of the *capA* promoter in plasmid pSTM04-carrying strains Newman (squares), SM2 (triangles), SM99 (diamonds), and IK184 (circles). *capA* promoter activity was determined by measuring the luciferase activity of the *capAp-luc*⁺ fusion. Shown are representative results for at least three independent experiments.

transcription, nor did the deletion of *spoVG* alter *arlRS* expression, suggesting that both loci are independent from each other (data not shown).

Effect of σ^B , *ArlRS*, and the *yabJ-spoVG* locus on *capA* transcription. Previous studies showed that transcription of the *cap* operon is growth phase dependent and affected by various global regulators in *S. aureus*. Expression of the *cap* operon is predominantly driven by the major promoter located at the beginning of the operon, although several internal promoters with weak activities have been identified in some *cap* gene clusters (36, 37, 46). To confirm the previously observed impact of σ^B and *ArlRS* on *cap* expression (3, 27, 28, 38) and to see whether the *yabJ-spoVG* locus is involved in *cap* operon regulation, we determined the *capA* expression levels in strains Newman, IK184, SM2, and SM99 by real-time RT-PCR (Fig. 6A). Monitoring *capA* expression after 8 h of growth showed that all three mutations significantly reduced *capA* transcription in *S. aureus*. While inactivation of *rsbUVW-sigB* and *arlR* was associated with a strong reduction in *capA* expression

(approximately 47-fold for *rsbUVW-sigB* and 15-fold for *arlR*; $P < 0.01$), deletion of *spoVG* resulted in a 2- to 3-fold reduction in *capA* transcription ($P < 0.05$), compared with the wild-type level. After 24 h of growth, *capA* transcript levels were drastically reduced and detectable only in Newman, SM2, and SM99, resulting in very low *capA*-to-*gyrB* transcript ratios (0.09 ± 0.03 copies of *capA* per copy of *gyrB* in Newman, 0.09 ± 0.01 copies in SM2, and 0.004 ± 0.0002 copies in SM99), while the *capA* transcription levels in IK184 were found to be below the detection limit.

The expression of the *capA* promoter during growth was monitored with the *capA* promoter-*luc*⁺ reporter gene fusion plasmid pSTM04 by measuring luciferase activity (Fig. 6B). In line with previous findings (3, 27), luciferase activity values increased in the parental strain Newman in a growth phase-dependent manner, starting at the transition from late-exponential growth phase to stationary phase (i.e., at 4 h), reaching its maximum after 7 h of growth, and declining thereafter. The course of luciferase activity in the $\Delta spoVG$ mutant SM2 followed in principle that in its parental strain, Newman, although the luciferase activity in SM2 was roughly half as strong as that in Newman at most of the time points monitored. In agreement with the real-time RT-PCR results, only a low level of luciferase activity was detectable in the $\Delta rsbUVW-sigB$ derivative IK184 at all time points analyzed. A clear reduction in luciferase activity was also found for SM99, albeit not as strong as expected from the real-time RT-PCR results.

CP-5 production in Newman, IK184, SM2, and SM99. To see whether and how the alterations in *capA* expression observed in IK184, SM2, and SM99 had an impact on capsule production, we investigated the capsule formations of Newman and its derivatives after growth in LB for 8 and 24 h by indirect immunofluorescence (Fig. 7, only 8-h data shown). While more than half of the Newman cells (57%) incubated with the CP-5 antibodies produced clear fluorescence signals at both time points analyzed, indicating the presence of CPs in these wild-type cells after 8 and 24 h of growth, this was not the case with either SM2, SM99, or IK184. Only 1% of the $\Delta spoVG$ cells, 2% of the *arlR*-defective cells, and 1% of the $\Delta rsbUVW-sigB$ cells emitted detectable amounts of fluorescence under these conditions, suggesting that all three mutants were strongly impaired in their abilities to produce CPs.

trans-Complementation of SM2. In order to evaluate whether the decrease in *capA* transcription in SM2 and its impact on CP-5 formation were due to the inactivation of *yabJ-spoVG*, we constructed plasmid pSTM05, carrying the *yabJ-spoVG* operon, and assessed its impact on *capA* transcription and CP-5 production in the *trans*-complemented mutant. Introduction of pSTM05 into SM2 restored the ability of the *trans*-complemented mutant to produce a capsule (Fig. 7B). While 52% of the SM2 cells harboring plasmid pSTM05 were CY-3 positive after 8 h of growth, only 3% of the SM2 cells transformed with the empty control plasmid pGC2 produced detectable amounts of fluorescence. Similarly, introduction of pSTM05 yielded values for *capA* promoter-driven luciferase activity after 8 h of growth that were comparable to those for the wild type (475 ± 31 relative light units [RLU] for Newman and 510 ± 42 RLU for SM2 harboring pSTM05), while introduction of pGC2 into SM2 had no effect on *capA*-dependent luciferase activity (246 ± 30 RLU for SM2 and 208 ± 25 RLU

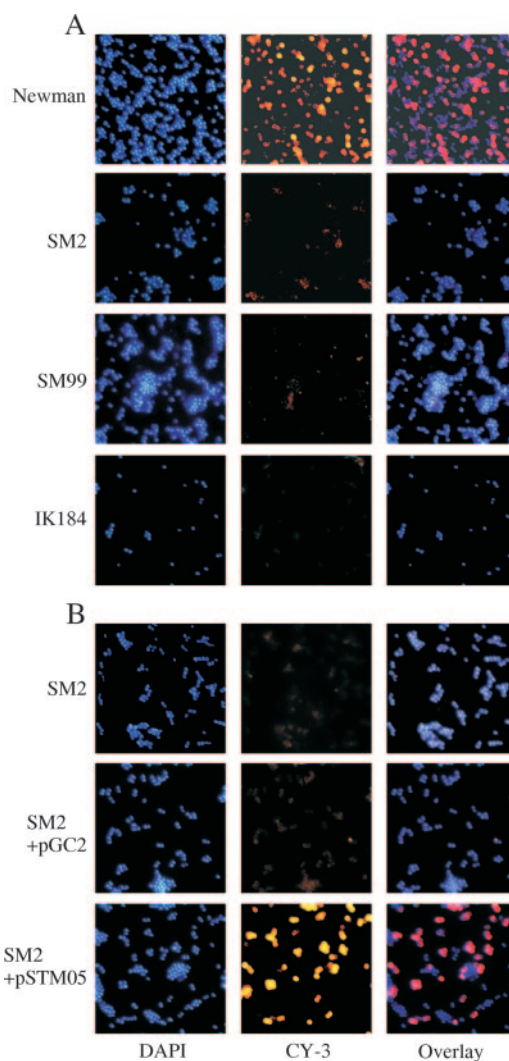


FIG. 7. Capsule production in Newman and its derivatives grown for 8 h in LB at 37°C. (A) CP-5 expression determined by indirect immunofluorescence of strain Newman and its derivatives SM2 ($\Delta spoVG$), SM99 (*arlR*), and IK184 ($\Delta rsbUVW-sigB$). (B) *trans*-Complementation of $\Delta spoVG$ mutant SM2. CP-5 expression was determined by indirect immunofluorescence of SM2, and SM2 transformed with either pSTM05 ($P_{yabJ-spoVG}$) or the empty control plasmid pGC2. Bacteria were stained with DAPI, marked with CP-5-specific monoclonal antibodies, and stained with Cy3-conjugated anti-mouse antibodies (CY-3).

for SM2 harboring pGC2). *trans*-Complementation assays performed with SM2 transformed with plasmids harboring either *yabJ* under the control of its σ^B -dependent promoter (pSTM11) or a $P_{yabJ-spoVG}$ fusion (pSTM13), on the other hand, failed to revert the effect of the *spoVG* deletion on capsule formation, signaling that both *yabJ* and *spoVG* are required to complement SM2 (data not shown).

Two-plasmid testing. The lack of apparent σ^B -specific consensus sequences in the *arlRS* and *capA* promoters suggests the transcription of these operons to be indirectly controlled by σ^B activity. To support this hypothesis, we cloned the promoters of *arlRS* and *capA* into reporter plasmid pSB40N and tested the resulting plasmids pSTM06 (*arlRS*) and pSTM07 (*capA*),

respectively, in a heterologous two-plasmid system that was recently shown to be suitable for the identification of σ^B -dependent *S. aureus* promoters (14). Plasmid pSA0455p, harboring the σ^B -dependent promoter upstream of *yabJ-spoVG* (3), which was used as a positive control, and plasmids pSTM06 and pSTM07, harboring the *arlRS* or *capA* promoter, were each transformed into *E. coli* XL1Blue cells containing either pAC7 or pAC7-*sigB* (14), respectively, and the clones obtained were selected on LBACX-ARA plates (43). Transformants containing pAC7 produced uncolored colonies, indicating that none of the introduced promoters were recognized by any form of *E. coli* RNA polymerase holoenzyme. Transformants containing pAC7-*sigB* and pSA0455p were blue on selective LBACX-ARA plates, demonstrating that the activity of the *yabJ-spoVG* promoter was dependent upon arabinose-induced heterologous expression of the *S. aureus sigB* gene in *E. coli* and indicating that the *S. aureus* σ^B -*E. coli* RNA polymerase holoenzyme hybrid was capable of recognizing the heterologous *S. aureus yabJ-spoVG* promoter. In contrast, transformants containing either pAC7-*sigB* and pSTM06 (*arlRS*) or pAC7-*sigB* and pSTM07 (*capAp*) remained uncolored on selective LBACX-ARA plates, demonstrating that neither the *arlRS* nor the *capA* promoter region was directly recognized by the σ^B -containing RNA polymerase holoenzyme.

Attempts to clone *spoVG* under the control of an inducible promoter. To support our hypothesis that SpoVG might act as regulator downstream of σ^B , we tried to construct a plasmid harboring *spoVG* under the control of an inducible promoter but were not successful with either *E. coli* or *S. aureus*. All our attempts to clone *spoVG* downstream of a σ^B -independent promoter resulted in mutations in the promoter, in the ribosomal binding site, or in the ORF of *spoVG*, signaling that *spoVG* expression needs to be tightly controlled by σ^B or a factor that is dependent on σ^B (data not shown).

DISCUSSION

A recent transcriptional profiling of the σ^B regulon in *S. aureus* indicated the alternative transcription factor to affect the expression levels of 251 genes or operons (3). While most of the genes/operons identified as upregulated by σ^B in that study were also preceded by nucleotide sequences resembling the *S. aureus* σ^B promoter consensus sequence (14), still a significant number of genes/operons found to be upregulated by σ^B lacked such a nucleotide sequence in their promoter regions, including *arlRS* and the *cap* operon (3). Although it is still possible that the latter group of genes/operons might be transcribed by the direct action of a σ^B -containing RNA polymerase holoenzyme, it is more conceivable that σ^B controls the expression of a regulator(s), which would subsequently promote the expressions of these genes/operons. Our Northern analysis performed here confirmed the positive impact of σ^B on *arlRS* and *capA* expression. However, unlike what was suggested by the microarray analyses, the Northern analyses performed here demonstrated *arlRS* expression to be affected by σ^B activity not only in *S. aureus* Newman but in strains COL and GP268 as well. While *arlRS* transcription was found to be highly dependent on σ^B activity in Newman during the later growth stage (8 h of growth), its effect appeared to be less pronounced in COL and the NCTC8325 derivative GP268.

Interestingly, our Northern analysis of the *arlRS* locus identified several further transcripts in addition to the 2.7- and 1.5-kb transcripts that were observed in the NCTC8325 derivative RN6390 (10), suggesting that the *arlRS* locus underlies a complex and strain-dependent regulatory circuit. Regulation of *arlRS* by σ^B is likely to be indirect, since the nucleotide sequence preceding *arlR* was not recognized by a two-plasmid system for the identification of promoters recognized by RNA polymerase containing *S. aureus* σ^B (14), leaving open the question of how σ^B affects *arlRS* expression.

Expression of the *cap* operon and capsule formation in *S. aureus* are known to be under multiple levels of control and affected by various environmental stimuli. In line with previous findings indicating σ^B to take part in the control of *cap* operon expression (3, 38), our quantitative RT-PCR results presented here confirmed σ^B to be important for *cap* operon transcription in *S. aureus*, since *capA* expression was drastically reduced in the Δ *rsbUVW-sigB* mutant IK184. Likewise, the *capA* promoter-driven luciferase activity in IK184 remained at a constantly low level throughout growth, in contrast to that in the wild type, where the luciferase activity increased with time, yielding a 20- to 30-fold difference between Newman and IK184 after 8 h of growth. As could be expected from the strong impact of σ^B on *cap* operon transcription, we could show that σ^B is also essential for capsule formation, since IK184, unlike its parental strain, was drastically impaired in its ability to produce CP-5 after 8 and 24 h of growth. Since the two-plasmid testing of the *capA* promoter sequence failed to identify a direct interaction between *capAp* and the σ^B -containing RNA polymerase, its effect on *cap* operon expression is likely to be indirect and mediated via a downstream-acting regulator(s) that is itself controlled by σ^B activity. One such regulator might be ArIR of the ArIRS system, which we confirmed here to be influenced by σ^B activity in *S. aureus*, and which is known to affect *cap* operon transcription and capsule formation (28). However, since our quantitative RT-PCR results for *capA* in *arlR* mutant SM99 yielded a 15-fold reduction in *capA* transcription in comparison with the wild-type level, and since the *capA* promoter-driven luciferase activity in SM99 was only 4- to 5-fold reduced after 8 h of growth, an additional regulator(s) that mediates the effect of σ^B on *cap* operon expression has to be proposed. To test whether the σ^B -dependent SpoVG homolog of *S. aureus* might fulfill such a function, we constructed strain SM2 lacking *spoVG*. We found that the deletion of *spoVG* in *S. aureus* Newman yielded a strong polar effect on *yabJ* transcription, suggesting the Δ *spoVG* mutant SM2 to represent a phenotypic *yabJ-spoVG* double mutant. We found that deletion of *spoVG* decreased *capA* transcription two- to threefold. Interestingly, deletion of *spoVG* strongly repressed the capacity of the mutant to produce CP-5, although SM2 was found to still produce significant amounts of *capA* transcripts. One possible explanation for the discrepancy between *capA* transcription and capsule production observed in SM2 might be an additional influence of YabJ/SpoVG on the expression of internal promoters of the *cap* operon. Alternatively, the *yabJ-spoVG* locus might affect CP production on transcriptional and posttranslational levels, as has been shown for SarA (24). Interestingly, since inactivation of *sarA* is known to increase extracellular protease activity (5) and capsule stability (24), it might be that the negative effect of the *spoVG*

deletion on the extracellular protease activity contributed to the CP-negative phenotype in SM2.

Our data clearly suggest the effector molecules of the *yabJ-spoVG* locus to contribute to the network of regulatory molecules that control *cap* operon transcription and CP-5 formation in *S. aureus* Newman, albeit on a lower level than ArlRS and σ^B . Moreover, the observation that the deletion of *spoVG* influenced extracellular protease production and *capA* transcription and suppressed CP-5 formation indicates that YabJ and/or SpoVG might indeed function as a regulatory molecule in *S. aureus*. This hypothesis is further supported by our findings that deletion of *spoVG* in methicillin-resistant *S. aureus* and in glycopeptide intermediate-resistant *S. aureus* strains significantly reduced the resistance levels against β -lactams and glycopeptides, respectively (S. Meier and M. Bischoff, unpublished results), two phenomena that have also been associated with σ^B activity (1, 32, 41, 50, 58). Considering the impact of *spoVG* deletion on capsule production and on resistance formation in methicillin-resistant *S. aureus* and glycopeptide intermediate-resistant *S. aureus*, and in the face of our recent findings demonstrating *yabJ-spoVG* expression to be highly dependent on σ^B activity (3), we propose the effector molecules of the *yabJ-spoVG* locus of *S. aureus* to act as regulatory molecules that mediate, together with ArlRS, the effect of σ^B on capsule formation.

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