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RsbU-Dependent Regulation of *Staphylococcus epidermidis* Biofilm Formation Is Mediated via the Alternative Sigma Factor σ^B by Repression of the Negative Regulator Gene *icaR*

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Transposon mutagenesis of *rsbU* leads to a biofilm-negative phenotype in *Staphylococcus epidermidis*. However, the pathway of this regulatory mechanism was unknown. To investigate the role of RsbU in the regulation of the alternative sigma factor σ^B and biofilm formation, we generated different mutants of the σ^B operon in *S. epidermidis* strains 1457 and 8400. The genes *rsbU*, *rsbV*, *rsbW*, and *sigB*, as well as the regulatory cascade *rsbUVW* and the entire σ^B operon, were deleted. Transcriptional analysis of *sarA* and the σ^B-dependent gene *asp23* revealed the functions of RsbU and RsbV as positive regulators and of RsbW as a negative regulator of σ^B activity, indicating regulation of σ^B activity similar to that characterized for *Bacillus subtilis* and *Staphylococcus aureus*. Phenotypic characterization of the mutants revealed that the dramatic decrease of biofilm formation in *rsbU* mutants is mediated via σ^B, indicating a crucial role for σ^B in *S. epidermidis* pathogenesis. However, biofilm formation in mutants defective in σ^B or its function could be restored in the presence of subinhibitory ethanol concentrations. Transcriptional analysis revealed that *icaR* is up-regulated in mutants lacking σ^B function but that *icaA* transcription is down-regulated in these mutants, indicating a σ^B-dependent regulatory intermediate negatively regulating IcaR. Supplementation of growth media with ethanol decreased *icaR* transcription, leading to increased *icaA* transcription and a biofilm-positive phenotype, indicating that the ethanol-dependent induction of biofilm formation is mediated by IcaR. This *icaR*-dependent regulation under ethanol induction is mediated in a σ^B-independent manner, suggesting at least one additional regulatory intermediate in the biofilm formation of *S. epidermidis*.

Staphylococcus epidermidis, a normal inhabitant of human skin and mucous membranes, is the predominant cause of foreign-body-associated infections (67). In addition, *S. epidermidis* is being isolated with increasing frequency as the causative pathogen of nosocomial sepsis and other nosocomial infections and now ranks among the five most frequent nosocomial pathogens (67, 72). The pathogenesis of *S. epidermidis* infections is correlated with the ability to form biofilms on polymeric surfaces (12, 83); cells are more resistant to a variety of antimicrobial substances in such biofilms (39).

Biofilm formation proceeds in two phases (27, 43). Primary attachment of bacterial cells to a polymer surface is a complex process influenced by a variety of factors, including hydrophobic interactions, the presence of host proteins, and specific staphylococcal factors like the capsular polysaccharide adhesin, the autolysin AtlE, and other staphylococcal surface proteins (30, 33, 55, 57, 73, 75). This attachment step is followed by the second phase, leading to the accumulation of bacteria in a multilayered biofilm embedded in an amorphous glycocalyx. Synthesis of the polysaccharide intercellular adhesin (PIA), which mediates cell-to-cell adhesion of the proliferating cells, is essential for *S. epidermidis* cell accumulation (45–47, 50). PIA is synthesized by the gene products of the *icaADBC* gene cluster (23, 31). In addition to its function in intercellular adhesion, PIA is essential for hemagglutination of *S. epidermidis* (19, 48, 66, 69). The significance of PIA as a virulence factor and of biofilm formation as an important process in foreign-body-associated infections was demonstrated in a central venous catheter infection model of the rat and a subcutaneous foreign-body infection model in mice (68, 70, 71). PIA and biofilm expression in *S. epidermidis* are influenced by a variety of environmental stress conditions (17, 20, 36, 37, 64, 65).

Recently, we demonstrated that the inactivation of *rsbU*, the first gene of the σ^B operon in *S. epidermidis*, dramatically decreased *icaADBC* transcription, PIA synthesis, and biofilm formation (36, 49). In contrast, Kies et al. (35) demonstrated that the expression of the *icaADBC* locus in *trans* from plasmid pCN27 (31) in a *sigB* mutant in the *icaADBC*-negative genetic background of *S. epidermidis* Tü3298 was able to form large cell clusters. These findings lead to the conclusion that the σ^B-independent biofilm formation of this strain might therefore be explained by a nonfunctional RsbU-mediated regulatory pathway (35). However, the *icaR* gene, encoding a recently characterized negative regulator of *icaADBC* transcription (14), is lacking in plasmid pCN27, which could also explain the observed biofilm-positive phenotype in the *sigB* mutant.

In contrast to *S. epidermidis* biofilm expression, in which the majority of *icaADBC*-positive strains display biofilm formation in standard media like Trypticase soy broth (TSB) (11, 22, 83), *S. aureus* displays a stronger dependence of biofilm formation

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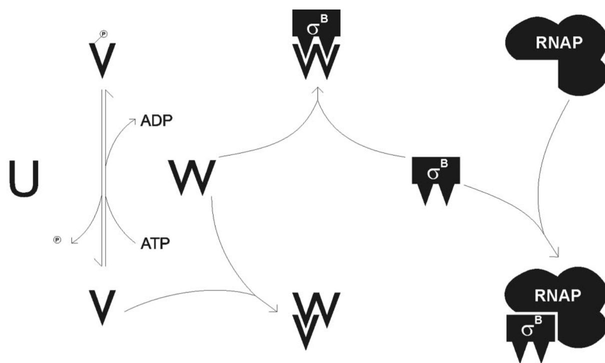

FIG. 1. Model of the regulatory pathway for the activity of the alternative sigma factor σ^B of *S. aureus*, which is homologous to the core regulatory pathway of *B. subtilis*. In these species, σ^B is negatively regulated by the anti-sigma factor RsbW, which additionally acts as a specific kinase for the anti-anti-sigma factor RsbV. RsbV activity depends on phosphorylation status, and inactive phosphorylated RsbV could be activated by dephosphorylation by the specific phosphatase RsbU. Transcriptional analysis of the deletion mutants generated in *S. epidermidis* in this study suggests that regulation of σ^B activity in this species is homologous to that in *S. aureus* and *B. subtilis*.

on distinct growth conditions and requires high sugar concentrations (38). Recently, Valle et al. (74) demonstrated that *icaADBC* transcription in *Staphylococcus aureus* is controlled mainly by SarA. Interestingly, the deletion of σ^B in a *sarA* deletion mutant was able to partially reverse the effect of the *sarA* deletion and restored biofilm formation in this *S. aureus* strain, whereas the deletion of σ^B in the wild-type strain had no effect on biofilm formation (74). For the mucosal isolate *S. aureus* MA12, the influence of σ^B on biofilm formation under high osmolarity was demonstrated (63). However, several other investigated *S. aureus* strains were biofilm-negative despite the expression of the *sigB* gene (63), indicating that the influence of σ^B on the regulation of biofilm formation could be dependent on different genetic *S. aureus* backgrounds. Additionally, the overexpression of σ^B from a tetracycline-dependent promoter could induce biofilm formation in *S. aureus* (2).

The regulation and function of the alternative sigma factor σ^B is well characterized for *Bacillus subtilis* and *S. aureus*. In *B. subtilis*, the alternative sigma factor σ^B is controlled by a complex signal transduction pathway (9, 79). The central module (RsbP, RsbV, and RsbW) is activated by signals of energy stress like carbon, phosphate, or oxygen starvation and directly regulates the activity of σ^B by forming a σ^B and RsbW complex (4, 18, 78). An upstream module of σ^B regulation in *B. subtilis* (RsbX, RsbR, RsbS, RsbT, and RsbU) is activated by environmental stress such as salt, heat, acid, or ethanol shock (1, 3, 5, 7–9, 76). In *S. aureus*, a species closely related to *S. epidermidis*, the σ^B operon consists of only the four genes *rsbU*, *rsbV*, *rsbW*, and *sigB* (40, 80), as was observed for *S. epidermidis* (36). Recently, it was demonstrated that RsbW acts as an anti-sigma factor in *S. aureus* (52) and that RsbU and RsbV act as positive regulators of σ^B activity (56), similar to the case for *B. subtilis* (Fig. 1). In *B. subtilis*, far more than 100 genes which are in part differentially induced by different stress conditions like heat, oxidative, acid, and ethanol stress, as well as starvation, are characterized within the σ^B regulon (28, 29, 32, 58–61, 77). In *S. aureus*, the σ^B regulon also comprises a wide variety of σ^B-regulated genes (25), including several virulence factors like clumping factor, fibronectin binding protein A, and coagulase (53, 54), which are positively regulated, as well as alpha- and beta-hemolysin, thermonuclease, enterotoxin B, serine protease SplA, cysteine protease SplB, metalloprotease Aur, staphopain, and leucotoxin D, which are negatively regulated (41, 82). Regulation of virulence factors can be mediated either directly by σ^B-dependent promoters or indirectly by the influence of σ^B on additional global regulators like SarA and the *agr* system (6).

When these data are taken together, several questions arise with respect to the impact of RsbU on the regulation of the alternative sigma factor σ^B and the regulation of biofilm formation by RsbU or σ^B in *S. epidermidis*. To answer these questions in this study, we deleted each single gene of the σ^B operon as well as the regulatory cascade *rsbUVW* and the entire σ^B operon and investigated the impact of these mutations on biofilm formation and transcription of *icaADBC*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *S. epidermidis* cells were grown in TSB (Becton Dickinson, Cockeysville, Md.) or on Trypticase soy agar (TSB plus 1.5% agar; Becton Dickinson) at 37°C. For phenotypic characterization of the *S. epidermidis* strains, TSB was supplemented with 4% NaCl or 2, 3, or 4% ethanol. *Escherichia coli* cells were grown in Luria-Bertani broth or on Luria-Bertani agar at 37°C. Antibiotics were used at the following concentrations: erythromycin, 100 μg/ml; chloramphenicol, 10 μg/ml; ampicillin, 100 μg/ml; and kanamycin, 50 μg/ml.

Phenotypic characterization. Biofilm formation of *S. epidermidis* was measured by a semiquantitative adherence assay with the indicated media in 96-well tissue culture plates with a NunclonΔ surface (Nunc, Roskilde, Denmark) as described previously (13, 44). For the detection of PIA by immunofluorescence assay, *S. epidermidis* cells were grown in tissue culture dishes (Nunc) for 22 h in the media. Cells were scraped off and diluted in phosphate-buffered saline (PBS) to an optical density at 578 nm of 0.3 to 0.5. The immunofluorescence assay procedure was then performed as described previously with a rabbit antiserum raised against purified PIA (44). For the detection of growth rates, cells were cultured in tissue culture dishes or in 100-ml glass bottles in a horizontal shaker at 200 rpm. Cells were harvested after 2, 4, 6, 8, 10, and 24 h, sonicated for 10 s (3/16-in. tapered Microtip at 50% maximal amplitude) with a Digital Sonifier 250-D (Branson, Danbury, Conn.) to disrupt cell clusters. The optical density of the culture at 600 nm was detected with a DU 530 spectrophotometer (Beckman, Krefeld, Germany). For the assessment of the cell density of stationary-phase cultures, the sonicated cultures were serially diluted and plated on Trypticase soy agar.

Genetic methods. Chromosomal DNA of *S. epidermidis* was prepared as described previously (44). Plasmid DNA of *E. coli* was prepared by using a QIAprep Spin Miniprep kit (QIAGEN, Hilden, Germany). DNA was cleaved with restriction enzymes as suggested by the manufacturer (Pharmacia, Freiburg, Germany), and DNA fragments were separated by electrophoresis in 0.7% agarose gels in Tris-borate buffer. DNA restriction fragments of the expected size were purified from agarose gels with a QIAquick gel extraction kit (QIAGEN). Amplification of DNA fragments was performed by using a DyNazyme DNA polymerase kit (Finnzyme, Espoo, Finland) as described by the manufacturer.

For amplification of fragments used for the allelic gene replacement procedure, the Expand High Fidelity PCR system (Roche, Mannheim, Germany) was used with oligonucleotides shown in Table 2. Amplified fragments were ligated into the pCRII TOPO vector (Invitrogen, Karlsruhe, Germany) and cloned by electroporation into *E. coli* TOP 10. The fragments flanking the erythromycin resistance cassette (*erm*) were ligated at their respective positions in the pCRII background, and the entire fragment was subsequently ligated into the temperature-sensitive, chloramphenicol-resistant *E. coli*/*Staphylococcus* shuttle vector pBT2 (10). The resulting plasmids were cloned into the restriction-deficient staphylococcal genetic background of *S. aureus* RN4220 by electroporation. Plasmids isolated from this staphylococcal host could be transformed by electroporation into mutant M15 and were subsequently transduced into the recipient strain, *S. epidermidis* 1457, by phage transduction with phage 71, kindly provided

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Reference	Comments[a]
S. epidermidis		
1457	50	Isolate from infected central venous catheter
M15	36	*rsbU*-Tn*917* mutant of *S. epidermidis* 1457 used as recipient by electroporation
1457*rsbU*	This study	*rsbU*::*erm* derivate from *S. epidermidis* 1457 derived by allelic gene replacement
1457*rsbV*	This study	*rsbV*::*erm* derivate from *S. epidermidis* 1457 derived by allelic gene replacement
1457*rsbW*	This study	*rsbW*::*erm* derivate from *S. epidermidis* 1457 derived by allelic gene replacement
1457*sigB*	This study	*sigB*::*erm* derivate from *S. epidermidis* 1457 derived by allelic gene replacement
1457*rsbUVW*	This study	*rsbUVW*::*erm* derivate from *S. epidermidis* 1457 derived by allelic gene replacement
1457*rsbUVWsigB*	This study	*rsbUVWsigB*::*erm* derivate from *S. epidermidis* 1457 derived by allelic gene replacement
8400	50	Blood culture isolate
8400-M15	36	*rsbU*-Tn*917* mutant of *S. epidermidis* 8400 derived by phage transduction
8400*rsbU*	This study	*rsbU*::*erm* derivate from *S. epidermidis* 8400 derived by phage transduction
8400*rsbV*	This study	*rsbV*::*erm* derivate from *S. epidermidis* 8400 derived by phage transduction
8400*rsbW*	This study	*rsbW*::*erm* derivate from *S. epidermidis* 8400 derived by phage transduction
8400*sigB*	This study	*sigB*::*erm* derivate from *S. epidermidis* 8400 derived by phage transduction
8400*rsbUVW*	This study	*rsbUVW*::*erm* derivate from *S. epidermidis* 8400 derived by phage transduction
8400*rsbUVWsigB*	This study	*rsbUVWsigB*::*erm* derivate from *S. epidermidis* 8400 derived by phage transduction
E. coli		
TOP 10	Invitrogen	
SJ*rsbU*	This study	TOP 10 containing pSJ*rsbU*
SJ*rsbV*	This study	TOP 10 containing pSJ*rsbV*
SJ*rsbW*	This study	TOP 10 containing pSJ*rsbW*
SJ*sigB*	This study	TOP 10 containing pSJ*sigB*
SJ*rsbUVW*	This study	TOP 10 containing pSJ*rsbUVW*
SJ*rsbUVWsigB*	This study	TOP 10 containing pSJ*rsbUVWsigB*
Plasmids		
pCRII TOPO	Invitrogen	*E. coli* cloning vector for direct cloning of PCR fragments
pBT2	10	ts *E. coli*/*Staphylococcus* shuttle vector
pSJ*rsbU*	This study	ts vector for allelic gene replacement of *rsbU* in *S. epidermidis*
pSJ*rsbV*	This study	ts vector for allelic gene replacement of *rsbV* in *S. epidermidis*
pSJ*rsbW*	This study	ts vector for allelic gene replacement of *rsbW* in *S. epidermidis*
pSJ*sigB*	This study	ts vector for allelic gene replacement of *sigB* in *S. epidermidis*
pSJ*rsbUVW*	This study	ts vector for allelic gene replacement of *rsbUVW* in *S. epidermidis*
pSJ*rsbUVWsigB*	This study	ts vector for allelic gene replacement of *rsbUVWsigB* in *S. epidermidis*

[a] ts, temperature sensitive.

by V. T. Rosdahl, Statens Seruminstitut Copenhagen, Copenhagen, Denmark, as described previously (44). The cells were grown at nonpermissive temperatures, and clones were screened for double crossover with an erythromycin-resistant but chloramphenicol-sensitive phenotype. The correct chromosomal insertion of the cassette into the respective mutants was demonstrated by PCR with *erm*-specific primers paired with primers flanking the genetically manipulated site and subsequent sequencing of the resulting fragments (data not shown). Transduction of the respective mutations into the independent biofilm-producing wild-type strain 8400 was performed essentially as described above with *S. epidermidis* phage 71.

Nucleotide sequence analysis was performed with an ABI Prism 310 sequencer by capillary electrophoresis with an ABI PrismdGTP BigDye Terminator Ready Reaction kit (PE Applied Biosystems, Foster City, Calif.). Nucleotide sequences were subsequently analyzed with HUSAR software (DKFZ, Heidelberg, Germany) or Vector NTI suite II software (InforMax, Frederick, Md.).

RNA preparation. For RNA extraction, cells were cultivated under biofilm conditions in 9.5-cm-diameter cell culture dishes with a NunclonΔ surface (Nunc). RNA was prepared with an RNeasy bacteria kit (QIAGEN) by using a modified protocol. Cells were harvested on ice with a cell scraper and by centrifugation for 5 min at 4°C. The resulting pellets were overlaid with 2 ml of sterile PBS, and cells were resuspended by sonication (10 s with a 3/16-in. tapered Microtip at 70% maximal amplitude; Branson sonifier). A 1.5-ml portion of the cell suspension was mixed with 3 ml of RNA protect solution (QIAGEN) and incubated for 5 min at ambient temperature. Cells were harvested by centrifugation for 10 min at ambient temperature. To remove PIA, cells were resuspended in 10 ml of sterile PBS and treated twice by sonication (3 cycles of 30 s with a 3/16-in. tapered Microtip at 70% maximal amplitude) with a PBS wash step between treatments. After sonication, cells were harvested by centrifugation for 5 min at ambient temperature and the resulting cell pellets were resuspended in 180 μl of Tris-EDTA buffer. Twenty microliters of a lystostaphin solution (1,500 U/ml; Sigma, Deisenhofen, Germany) was added, and the reaction mixture was incubated at 37°C for 10 min. The subsequent extraction of RNA was performed according to the instructions of the manufacturer. Extracted RNA was quantified with a GeneQuant photometer (Pharmacia).

Northern blot analysis. For electrophoresis, 5 μg of total RNA was resuspended in RNA loading buffer and denatured at 70°C for 10 min. The RNA was separated on 1% agarose-formaldehyde gels and blotted on Zeta Probe nylon membranes (Bio-Rad, Munich, Germany) by using capillary transfer with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After 20 h, the RNA was fixed by baking at 80°C for 2 h. After prehybridization at 52°C for 30 min in DIG easy Hyb (Roche), the membranes were incubated at 52°C overnight with specific digoxigenin (DIG)-labeled probes, generated by PCR labeling (PCR DIG probe synthesis kit; Roche) with primers shown in Table 2. Membranes were washed at 52°C twice in 2× SSC–0.1% sodium dodecyl sulfate (SDS), twice in 0.1× SSC–0.1% SDS, and finally in washing buffer (Roche). The membranes were blocked in blocking solution, incubated with anti-digoxigenin-AP Fab fragments, and equilibrated in detection buffer as directed by the manufacturer (Roche). CDP-Star was added as a substrate for the chemiluminescence detection on Lumi Film (Roche). By boiling in 2× SSC–0.1% SDS twice for 6 min, the membranes could be stripped and reprobed up to three times. All transcriptional analyses were performed at least three times with independent RNA preparations.

Real-time RT-PCR analysis. For the reverse transcription (RT)-PCR analysis, 4 μg of RNA was treated with 4 U of RNase-free DNase (Promega, Mannheim, Germany) in a 20-μl total volume for 45 min at 37°C as suggested by the manufacturer. The reaction was diluted 1:10, and 5 μl of DNase-treated RNA solution was used for first-strand cDNA synthesis with the iScript cDNA synthesis kit (Bio-Rad) in a total volume of 20 μl. The cDNA reaction was diluted 1:4,

TABLE 2. PCR primers

Primer	Restriction site	Sequence[a]
Primers used for allelic gene replacement		
*erm*R2	SacI	5′-CTC GAG CTC TGA CGG TGA CAT CTC TCT ATT G-3′
*erm*L1	NheI	5′-CTC GCT AGC GAA AAG TAC CAT AAA CGG TCG-3′
*vrsbU*R1	BamHI	5′-CTC GGA TCC AGC GAA AAT ACC AAC CCA CG-3′
*vrsbU*L1	SacI	5′-CTC GAG CTC GAA ATG CGC CTC CTT ACT TC-3′
h*rsbU*R1	NheI	5′-CTC GCT AGC GAT GGT GTT ACA GAG GCA CG-3′
h*rsbU*L1	KpnI	5′-CTC GGT ACC AGC TGG CAA CCG CAT TTC-3′
h*rsbW*R1	NheI	5′-CTC GCT AGC GTT ATT TCA GAC CAA GGT G-3′
h*rsbW*L1	KpnI	5′-CTC GGT ACC TTA TCA TTC TGT TGT CCC AT-3′
v*sigB*R1	BamHI	5′-CTC GGA TCC CCA ATG AGA CAA GAA GGC AC-3′
v*sigB*L1	SacI	5′-CTC GAG CTC CTT GAG CTT GGC TAT CTT CG-3′
h*sigB*R1	*Nhe*I	5′-CTC GCT AGC CGA AAG AAG CTC AGG TGG AC-3′
h*sigB*L1	KpnI	5′-CTC GGT ACC GAT GCT GAA TAA ACT GAT GCG-3′
Primers used for Northern blot hybridization probes		
erm.for	None	5′-AAT TGG AAC AGG TAA AGG GC-3′
erm.rev	None	5′-AAC ATC TTG GGT ATG GCG G-3′
sigB.for	None	5′-AGA TTT AGT TCA AGT TGG TA-3′
sigB.rev	None	5′-TTA TCA TCT TGT TGT CCC AT-3′
rsbU.for	None	5′-GAA GTG GAA GTA AGG AGG CG-3′
rsbU.rev	None	5′-TCG ATG TGT TAC CAG AAG TCG-3′
sarA.for	None	5′-ATA GGG AGG TTT CAT TAA TGG C-3′
sarA.rev	None	5′-TTT GCT TCT GTG ATA CGG TTG-3′
asp23.for	None	5′-AAA ATC AAA AAG CAC TTG AGC G-3′
asp23.rev	None	5′-AAA AAA TTG CAG GTAT TGC AG-3′
icaA.for	None	5′-GAA TCC AAA ATT AGG CGC AG-3′
icaA.rev	None	5′-AAC ATC CAG CAT AGA GCA CG-3′
icaR.for	None	5′-TCC GAA AAG GGG TAC GAT G-3′
icaR.rev	None	5′-CCT CTT TAT CCA AAG CGA TG-3′
Primers used for quantitative RT-PCR		
asp23.real1	None	5′-TCC AAC TTC TAC AGA TAC GCC-3′
asp23.real2	None	5′-AAA ATT GCA GGT ATT GCA GC-3′
icaA.real1	None	5′-TGT ATC AAG CGA AGT CAA TCT C-3′
icaA.real2	None	5′-GGC ACT AAC ATC CAG CAT AG-3′
icaR.real1	None	5′-TGA AGA TGG TGT TTG ATT TGT G-3′
icaR.real2	None	5′-CCA TTG ACG GAC TTT ACC AG-3′
rsbU.real1	None	5′-AGC GTT TGA GGA AAT TGG TGT G-3′
rsbU.real2	None	5′-CCT CTA CAT CTC GTG CCT CTG-3′
gyrB.real1	None	5′-CTG ACA ATG GCC GTG GTA TTC-3′
gyrB.real2	None	5′-GAA GAT CCA ACA CCG TGA AGA C-3′

[a] Restriction sites are underlined.

and 3 μl was used as a template in real-time PCR analysis with an iQ SYBR Green Supermix (Bio-Rad) with oligonucleotides shown in Table 2 in an iCycler iQ thermal cycler under following conditions: (i) 95°C for 3 min; (ii) 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and (iii) 4°C for 30 min. All RT-PCR analyses were performed in triplicate for at least two independent experiments. Relative transcriptional levels within distinct experiments were determined by using the $2^{-\Delta\Delta C_T}$ method (42).

RESULTS

Construction of deletion mutants. To investigate the relevance of the genes preceding *sigB* in the σ^B operon in *S. epidermidis* to the activity of the alternative sigma factor σ^B, we constructed mutants with deletions of the single genes *rsbU*, *rsbV*, *rsbW*, and *sigB*, as well as the regulatory cascade *rsbUVW* and the entire σ^B operon, by homologous recombination leading to mutants 1457*rsbU*, 1457*rsbV*, 1457*rsbW*, 1457*sigB*, 1457*rsbUVW*, and 1457*rsbUVWsigB* (Fig. 2). For all mutants, the erythromycin resistance cassette (*erm*) was inserted in a positive orientation with respect to the σ^B operon to allow transcription of downstream genes and to avoid gene silencing by induction of antisense RNA transcription. For all mutants, the correct insertion without induction of additional mutations was confirmed by PCR analysis and subsequent sequencing of the chromosomal regions overlapping the manipulated chromosomal regions of the σ^B operon (data not shown). By transcriptional analysis with a *sigB*-specific probe (Fig. 3A), an ~1.7-kb transcript presumably enclosing the internal σ^B-dependent genes *rsbV*, *rsbW*, and *sigB* could be detected for wild-type *S. epidermidis* 1457. The largest amount of *sigB* transcript was observed in the mid-exponential growth phase (7 h under biofilm growth conditions) (data not shown), and all subsequent transcriptional analyses were performed with RNA extracted at this time point. The lack of detection by Northern analysis of the predicted 2.8-kb transcript from the putative σ^A-dependent promoter preceding *rsbU* revealed that this promoter has only low activity. However, low transcriptional activity of *rsbU* was detectable by RT-PCR with RNA prepared from wild-type *S. epidermidis* 1457 and mutants 1457*rsbV*, 1457*rsbW*, and 1457*sigB*, in contrast to mutant 1457*rsbU* (data

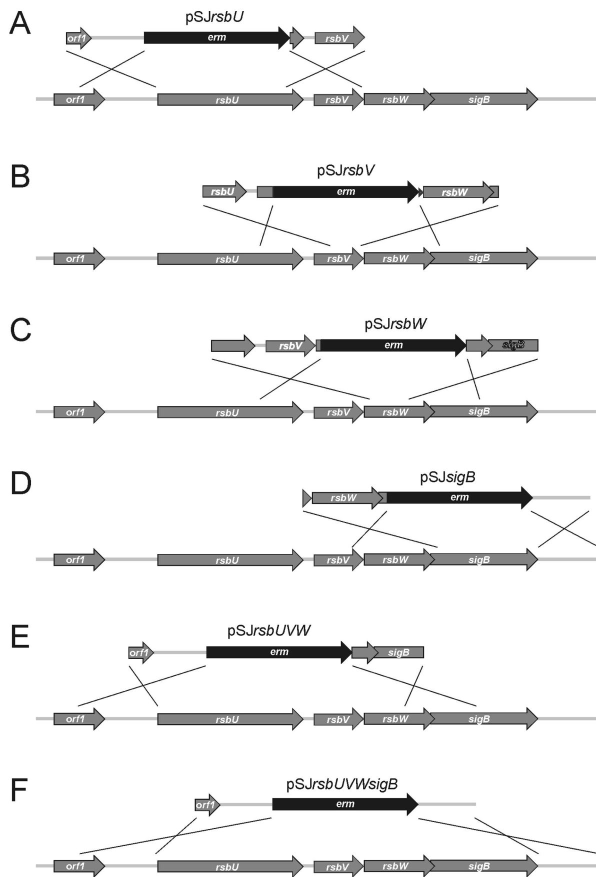

FIG. 2. Physical map of the *sigB* operon of *S. epidermidis* (accession no. AF274004) and construction of deletion mutants. Arrows depict open reading frames and indicate their orientations and sizes. All deleted genes were replaced with the erythromycin resistance gene (*erm*) as indicated. The *erm* gene and chromosomal regions flanking the respective deletions were amplified by PCR and cloned into plasmid pBT2, yielding the integration vectors pSJ*rsbU* (A), pSJ*rsbV* (B), pSJ*rsbW* (C), pSJ*sigB* (D), pSJ*rsbUVW* (E), and pSJ*rsbUVWsigB* (F). The crosses indicate the sites of homologous recombination.

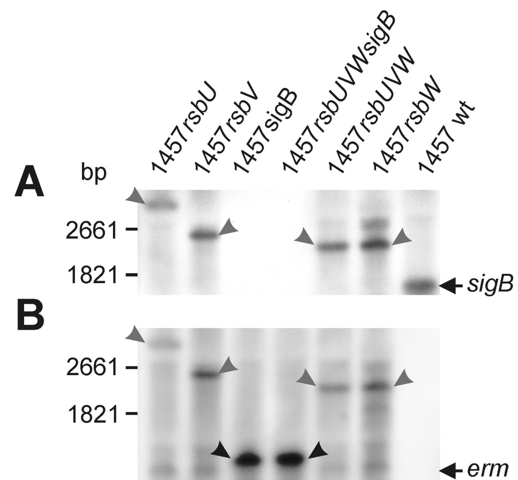

FIG. 3. Influence of deletions on transcription of the *sigB* gene in *S. epidermidis* 1457 and its mutants. (A) Northern blot analysis with a *sigB*-specific probe. *S. epidermidis* 1457 displayed a 1.5-kb transcript. The σ^A-dependent transcript of the entire σ^B operon could not be detected under the conditions used. Mutants with a remaining *sigB* gene displayed transcripts of increased sizes corresponding to the *erm* insertion. In *sigB*-negative mutants, no transcripts were detected. (B) Northern blot analysis with an *erm*-specific probe. For all mutants, a weak transcript comprising only the *erm* gene was detected, indicating a weak terminator following the *erm* gene. In mutants with a remaining *sigB* gene, additional transcripts with sizes identical to those of transcripts for the *sigB*-specific hybridization (A) were observed. In mutants with an inactivated *sigB* gene, the major transcripts detected were only slightly larger than the *erm* transcript, indicating a strong terminator preceding the *sigB* gene closely downstream. The genetic maps for the mutants are shown in Fig. 2. wt, wild type.

not shown). For mutants 1457*sigB* and 1457*rsbUVWsigB*, no *sigB*-containing transcript was observed, whereas for mutants 1457*rsbU*, 1457*rsbV*, 1457*rsbW*, and 1457*rsbUVW*, transcripts of 3.45, 2.60, 2.30, and 2.30 kb, respectively, were detected (Fig. 3A). Transcriptional analysis with an *erm*-specific probe revealed fragments for mutants 1457*rsbU*, 1457*rsbV*, and 1457*rsbUVW* that were identical to the fragments observed with the *sigB* probe. Mutants 1457*sigB* and 1457*rsbUVWsigB* displayed identical fragments of approximately 1.4 kb, whereas for wild-type *S. epidermidis* 1457 no transcript could be detected. For all mutants, an additional weak transcript of approximately 1.3 kb, comprising only the *erm* gene, was observed, indicating a weak terminator downstream of the *erm* gene which is, however, not sufficient to prevent transcription of downstream genes (16). These data revealed the correct insertion of the *erm* cassette with the expected transcriptional behavior for all mutants. These mutants were further analyzed and were used as hosts for the transduction of the mutations into different genetic backgrounds.

Regulation of σ^B in *S. epidermidis*. The relevance of the genes *rsbU*, *rsbV*, and *rsbW* to the regulation of the activity of the alternative sigma factor σ^B was investigated by transcriptional analysis of σ^B-dependent genes in *S. epidermidis* 1457 and their respective mutants. Besides the internal σ^B-dependent promoter of the σ^B operon (36), the only putative σ^B-dependent promoter in *S. epidermidis* published to date is the first of three promoters (P1) of the *sarA* gene locus (21). As an additional control, we decided to use the *asp23* homologue of *S. epidermidis* ATCC 12228 (81). In *S. aureus*, the Asp23 protein was identified as a 23-kDa protein, and this protein was clearly missing in σ^B deletion mutants of *S. aureus* COL and Newman (41). Further studies revealed that *asp23* is transcribed by three independent σ^B-dependent promoters and that this gene is a good marker for σ^B activity in *S. aureus* (24, 26, 41, 52). For the upstream 5,000-bp chromosomal region of the *asp23* homologue of *S. epidermidis*, a consensus sequence search for σ^B-dependent promoters was performed and three putative σ^B-dependent promoters (P1 to P3) could be detected (Fig. 4). The promoters P1 and P2 are conserved between *S. aureus* and *S. epidermidis* with respect to the −35 and −10 sequences, as well as their approximate distance to *asp23*, indicating similar functions in both species. In contrast, the putative promoter P3 in *S. epidermidis* was not conserved compared to that in *S. aureus*. Interestingly, the SA1987 gene, which is preceded by the P3 promoter in *S. aureus*, is missing in this chromosomal region of *S. epidermidis*, whereas the genes

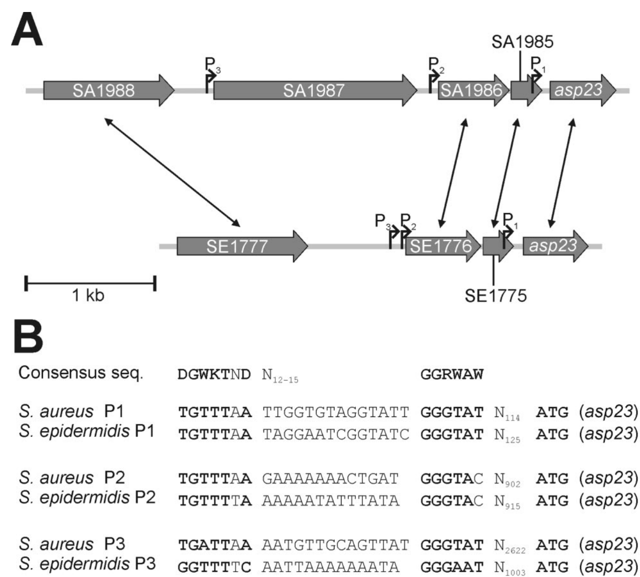

FIG. 4. Homology of the *asp23* promoter regions of *S. aureus* and *S. epidermidis*. (A) Physical maps of the *asp23* promoter regions of *S. aureus* N315 (accession no. AP003136) and *S. epidermidis* ATCC 12228 (accession no. AE016750) are displayed. Homologous genes are indicated by double-headed arrows. In *S. epidermidis*, no open reading frame homologous to the *S. aureus* SA1987 gene could be detected in the *asp23* promoter region. SA1987 is a homologue to the *S. aureus opuD* gene. In *S. epidermidis*, only one *opuD* homologue (SE0259) could be detected in a distinct chromosomal region (accession no. AE016744). (B) Alignment of σ^B promoters within the *asp23* promoter regions of *S. aureus* and *S. epidermidis* with the consensus sequence of σ^B-dependent promoters in *B. subtilis* (59). Bases fitting the consensus sequence are displayed in boldface type. The P1 and P3 promoters of both species represent perfect matches, whereas for the respective P2 promoters one identical mismatch with respect to the consensus sequence was observed.

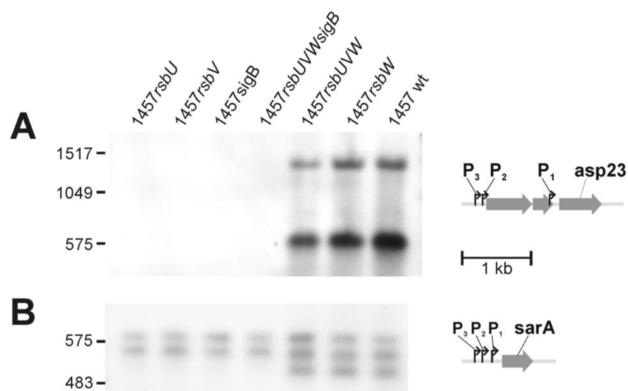

FIG. 5. Influence of deletions on transcription of σ^B-dependent genes *asp23* and *sarA*. Shown are Northern blot analyses with *asp23*-specific (A) and *sarA*-specific (B) probes, as well as maps of the respective genes with published or putative promoter sites. In mutants defective in *sigB* or its function, a lack of σ^B-dependent transcripts of *asp23* and *sarA* transcripts was observed. wt, wild type.

flanking SA1987 in *S. aureus* are conserved in *S. epidermidis* (Fig. 4).

No σ^B-dependent transcript could be observed by Northern blot analysis with *asp23*- and *sarA*-specific probes in mutants 1457*rsbV*, 1457*sigB*, and 1457*rsbUVWsigB* in the mid-exponential growth phase, whereas the σ^A-dependent *sarC* (P2) and *sarB* (P3) designated transcripts of the *sarA* gene (21) were present (Fig. 5). For wild-type *S. epidermidis* 1457 and mutants 1457*rsbW* and 1457*rsbUVW*, the P1-dependent *sarA* transcript (designated *sarA*) and two transcripts of *asp23* could be detected. The transcripts observed with the *asp23*-specific probe were approximately 1.5 and 0.6 kb in size, corresponding to the expected transcript sizes for the putative P1 and P2 promoters. However, the 1.5-kb transcript could represent a double band of transcripts dependent on the closely located putative P2 and P3 promoters in *S. epidermidis* (Fig. 3). Quantitative analysis of transcription by RT-PCR revealed an at-least-1,000-fold down-regulation of *asp23* transcription in σ^B-negative mutants compared to the wild type (data not shown). By overexposure of the film under some conditions, very weak *asp23*- and σ^B-dependent *sarA* transcripts could be detected in 1457*rsbU*, indicating very low σ^B activity remaining in this mutant (data not shown). Transcriptional analysis of mutant 1457*rsbW* compared to wild-type *S. epidermidis* 1457 at various time points (4, 7, 10, 14, 17, 20, and 24 h) revealed that the *asp23* transcripts and the σ^B-dependent *sarA* transcript in 1457*rsbW* were transcribed continuously, whereas in the wild type these genes were up-regulated at the early time points (4 to 14 h) and down-regulated at the late time points (17 to 24 h). The transcriptional levels of these genes in 1457*rsbW* were lower than those in the wild type in the mid-exponential growth phase but higher than those of the wild type in the stationary phase (data not shown), indicating a lack of growth phase-dependent regulation of σ^B activity and functional σ^B overexpression during the post-exponential phase and the stationary phase in mutants with inactivation of *rsbW*.

Biofilm formation in deletion mutants of σ^B operon genes. The impact of the different regulators of σ^B and the influence of σ^B activity on biofilm formation in *S. epidermidis* were investigated under different environmental conditions. In *S. aureus*, the influence of σ^B on biofilm formation in different genetic backgrounds seems to be extremely variable (63, 74). Therefore, we transduced all mutations generated into the independent *icaADBC*-positive genetic background of the clinical *S. epidermidis* isolate 8400. Biofilm assays were performed with TSB and with TSB supplemented with 4% NaCl, 2% ethanol, or 4% ethanol (Fig. 6). Mutants 1457*rsbU* and 8400*rsbU* displayed a phenotype similar to that observed for Tn*917* mutants M15 and 8400-M15 with the Tn*917* insertion site at position 19 of *rsbU* (36). In these mutants, biofilm formation was dramatically decreased or abolished in TSB and in TSB supplemented with NaCl, whereas supplementation of TSB with ethanol was able to restore biofilm formation in these mutants. Phenotypes similar to those detected in *rsbU* mutants were observed in mutants 1457*rsbV*, 1457*sigB*, 1457*rsbUVWsigB*, 8400*rsbV*, 8400*sigB*, and 8400*rsbUVWsigB* (Fig. 6). To exclude the possibility that a delay of growth in dysfunctional σ^B mutants could be responsible for the observed phenotypes, we investigated the growth rates of these strains. Over the time in question, all strains displayed almost identical growth curves in all phases of growth and reached very similar cell densities in the stationary phase (data not shown).

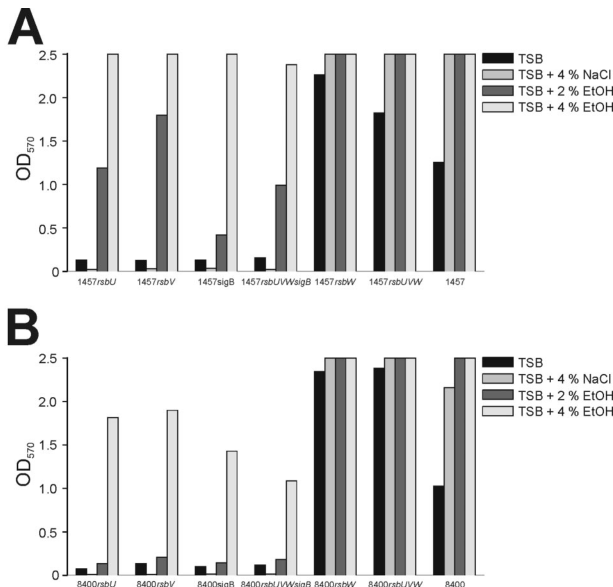

FIG. 6. Biofilm formation of *S. epidermidis* 1457 (A) and *S. epidermidis* 8400 (B) as well as their respective deletion mutants in TSB and in TSB supplemented with 4% NaCl, 2% ethanol (EtOH), or 4% EtOH under different environmental conditions. OD_{570}, optical density at 570 nm.

In contrast to the former mutants, 1457*rsbW*, 1457*rsbUVW*, 8400*rsbW*, and 8400*rsbUVW* displayed increased biofilm formation compared to their respective wild-type strains (Fig. 6). Biofilm formation in these mutants could be induced by NaCl and ethanol, as was observed for the wild-type strains. However, this increase in biofilm formation could not be quantified because the respective optical density values were outside the detection range of the spectrophotometer used (Fig. 6). The lack of PIA synthesis in the biofilm-negative mutants and the presence of PIA under conditions in which biofilm was expressed was demonstrated by an immunofluorescence assay with PIA-specific antibodies (data not shown).

Transcriptional analysis of *icaR* and *icaA* under biofilm conditions. RNA was isolated from *S. epidermidis* 1457 and its respective mutants in the mid-exponential growth phase under biofilm-forming conditions in TSB. Transcriptional analysis revealed that for mutants 1457*rsbV*, 1457*sigB*, and 1457*rsbUVWsigB* no *icaA* transcript could be observed (Fig. 7A) but that for mutant 1457*rsbU* a faint transcript could be observed in some of several experiments (not in the experiment displayed). In mutants 1457*rsbW* and 1457*rsbUVW*, transcription of *icaA* was similar to that observed for wild-type *S. epidermidis* 1457 (Fig. 7A). In contrast to the transcriptional *icaA* profiles of the different mutants, *icaR* encoding a negative regulator of *icaADBC* transcription was up-regulated in mutants 1457*rsbU*, 1457*rsbV*, 1457*sigB*, and 1457*rsbUVWsigB*. In mutants 1457*rsbW* and 1457*rsbUVW*, as well as the wild type, *icaR* transcription was repressed (Fig. 7B). Quantitative analysis of transcription by RT-PCR revealed approximately 25- to 30-fold up- or down-regulation of *icaR* and *icaA* transcription in σ^B-negative mutants compared to that in the wild type under these conditions (data not shown).

Transcriptional analysis with ethanol stimulation for σ^B-inactive mutants. To evaluate the regulatory influence of ethanol induction on biofilm formation in mutants with a deletion of *sigB*, *rsbU*, or the complete σ^B operon, we performed transcriptional analysis of the *icaR*, *icaA*, and *asp23* genes under biofilm formation conditions. RNA was isolated from *S. epidermidis* 1457 and its respective mutants in the mid-exponential growth phase under biofilm-forming conditions in TSB and in TSB supplemented with an optimal stimulating concentration of 3% ethanol. In the mutants defective in *sigB* or its function, 1457*rsbU*, 1457*sigB*, and 1457*rsbUVWsigB*, supplementation of the growth medium with ethanol led to the repression of *icaR* transcription and a subsequent up-regulation of *icaA* transcription (Fig. 8). Despite the increased biofilm formation in wild-type *S. epidermidis* 1457, the *icaR* and *icaA* transcripts were slightly repressed by ethanol supplementation. In all investigated mutants with a lack of *sigB* function, no *asp23* transcript could be detected under these conditions (data not shown).

DISCUSSION

Biofilm formation of *S. epidermidis* is regarded as the major virulence factor in this staphylococcal species and is therefore the subject of intense research. The biphasic mode of biofilm formation with a primary attachment step followed by the

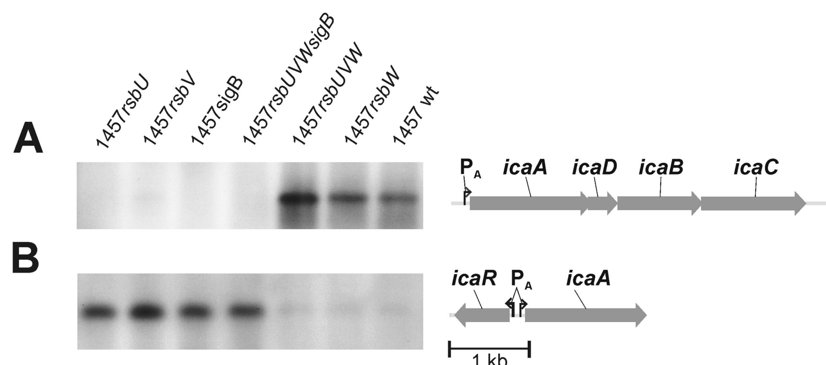

FIG. 7. Influence of deletions on transcription of *icaR* and *icaADBC*. Shown are Northern blot analyses with *icaA*-specific (A) and *icaR*-specific (B) probes, as well as maps of the respective genes with published or putative promoter sites. In mutants defective in *sigB* or its function, *icaADBC* transcription was strongly repressed, whereas *icaR*, encoding a negative regulator (14), was up-regulated. wt, wild type.

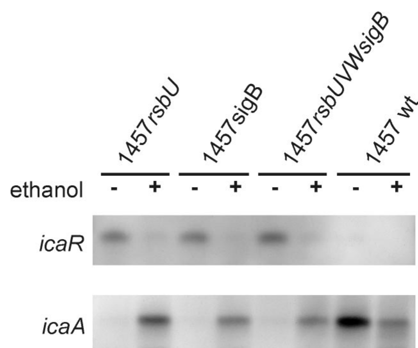

FIG. 8. Influence of ethanol on the transcription of *icaR* and *icaADBC*. Shown are Northern blot analyses with *icaR*- and *icaA*-specific probes. RNA was extracted in the mid-exponential growth phase in TSB supplemented with 3% ethanol. In mutants defective in *sigB* or its function, up-regulated *icaR* mRNA was repressed by the addition of ethanol, leading to increased transcription of *icaADBC*. wt, wild type.

accumulation of cells as a multilayered biofilm is well characterized (27, 43). However, the regulation of the expression of PIA is poorly understood. Some contradictory results regarding the impact of the alternative sigma factor σ^B or its regulator RsbU in the regulation of PIA synthesis and biofilm formation in *S. epidermidis* exist. These data were based on phenotypic characterization of *S. epidermidis* biofilm formation under different environmental conditions (62), the biofilm-negative phenotype of an *rsbU* transposon mutant in an *icaADBC*-positive *S. epidermidis* strain (36), and the biofilm-positive phenotype of a *sigB* mutant in an *icaADBC*-negative genetic background after complementation in *trans* with *icaADBC* (35). In the present study, we evaluated the role of the alternative sigma factor σ^B and its regulators in the biofilm formation of *S. epidermidis* by using allelic gene replacements.

We were able to delete all single genes of the σ^B operon as well as the regulatory cascade *rsbUVW* and the entire operon. The correct insertion of the *erm* cassette used was determined by sequencing the σ^B operons of the generated mutants. Transcriptional analysis with *sigB*- and *erm*-specific probes revealed that genes downstream of the introduced resistance cassette were transcribed from the σ^A-dependent *erm* promoter, which displays activity stronger than that of the putative σ^A-dependent promoter upstream of the σ^B operon. The double bands with fragments of approximately 1.3 and 1.4 kb observed in transcriptional analyses of mutants 1457*sigB* and 1457*rsbUVWsigB* (Fig. 3B) suggest the existence of a strong transcription terminator approximately 150 to 200 bp downstream of the stop codon of the *sigB* gene.

To evaluate the activity of the alternative sigma factor σ^B, we used the σ^B-dependent transcript of the *sarA* locus of *S. epidermidis* (21), which is a good marker when Northern blot analysis after separation of RNA by gel electrophoresis is used as a detection method. However, due to the two additional σ^A-dependent promoters preceding *sarA*, this gene locus is not useful for alternative detection methods like Northern analysis of slot blots or RT-PCR. Therefore, we performed a promoter search with the consensus sequence of σ^B-dependent promoters upstream of the *asp23* gene of *S. epidermidis*. This gene is a homologue to the alkaline shock protein 23 of *S. aureus*, which is solely transcribed from three σ^B-dependent promoters and was used in several studies as a marker gene for σ^B activity in *S. aureus* (24, 26, 41, 52, 81). Three putative σ^B-dependent promoters could be detected upstream of *asp23* in *S. epidermidis*. The P1 and P2 promoters displayed conserved −10 and −35 regions in both species and were located at similar distances upstream of the start codon of the respective *asp23* genes. Even the mismatch of the last base of the −10 region of promoter P2 was found to be conserved in *S. epidermidis* and *S. aureus* when compared to the consensus sequence. These data indicate similar σ^B-dependent transcription of *asp23* from promoters P1 and P2 in both species. Interestingly, the P3 promoters were not conserved in their −10 and −35 region sequences. Additionally, in *S. aureus*, the P3 promoter precedes an open reading frame (SA1987) homologous to the *S. aureus* gene *opuD*, which could not be detected in this chromosomal region of *S. epidermidis*.

The lack of the σ^B-dependent transcript designated *sarA* (21) and the *asp23* transcripts in mutant 1457*sigB* compared to the presence of these transcripts in the wild-type *S. epidermidis* 1457 confirmed the expected σ^B-dependent transcription of these genes, indicating the usefulness of both genes as indicators of σ^B activity. Additionally, the σ^A-dependent transcripts of the *sarA* locus designated *sarB* and *sarC* (21) could act as a control for comparability between different RNA preparations within Northern hybridization experiments. The more-than-1,000-fold decrease in *asp23* transcription in *S. epidermidis* 1457*sigB* compared to the ~30-fold decrease in indirectly σ^B regulated genes detected by quantitative RT-PCR suggests an almost exclusively σ^B-dependent transcription of this gene locus. Therefore, *asp23* seems to be an excellent marker for σ^B activity in *S. epidermidis* when quantitative real time RT-PCR analysis is used.

To evaluate the regulatory roles of the genes *rsbU*, *rsbV*, and *rsbW* on the activity of σ^B in *S. epidermidis*, we performed transcriptional analysis of *asp23* and *sarA* in wild-type *S. epidermidis* 1457 and all of its respective mutants. The dramatic decrease in and lack of σ^B-dependent transcripts in mutants 1457*rsbU* and 1457*rsbV*, respectively, indicate that these genes encode positive regulators of σ^B activity. The phenotypic observation of increased biofilm formation in mutant 1457*rsbW* in contrast to the decreased biofilm formation in mutants with dysfunctional σ^B indicates that this gene encodes a negative regulator of σ^B. However, an increase in σ^B activity compared to that in the wild type could be detected only in the post-exponential growth phase, indicating that for the regulation of σ^B activity during growth, the autoregulation from the internal σ^B-dependent promoter of the σ^B operon is superordinate compared to RsbW regulation. The low σ^B activity in mutant 1457*rsbU* indicates that RsbU is necessary for full σ^B activation in different growth phases and under different environmental conditions. However, the basal σ^B activity in this mutant demonstrates that σ^B could be activated at a low level in an RsbU-independent manner. In view of these data, it can be suggested that the activity of σ^B in *S. epidermidis* is regulated in a manner similar to that described for *B. subtilis* and *S. aureus* (Fig. 1). The high homology between the σ^B operons of gram-positive bacteria (51) also suggests the prediction of similar gene functions in these species (Fig. 1).

Biofilm formation for mutants 1457*rsbU* and 8400*rsbU* dis-

played a phenotype similar to that already observed for mutants M15 and 8400-M15 (36), with dramatic decreases of biofilm formation in TSB and in TSB supplemented with NaCl compared to those for the respective wild types and a strong biofilm-positive phenotype in TSB supplemented with ethanol, indicating that the observed phenotype of mutants M15 and 8400-M15 (36) is mediated by Tn*917* insertion in *rsbU* and not by polar effects of the transposon. Mutants 1457*sigB* and 8400*sigB* displayed phenotypes almost identical to those of mutants 1457*rsbU* and 8400*rsbU*. In these mutants, no differences in transcriptional levels of *rsbU* could be detected, while σ^B activity was abolished, indicating that the RsbU-dependent regulation of biofilm formation is mediated via the alternative sigma factor σ^B. This idea is supported further by the phenotypic properties of mutants 1457*rsbV* and 8400*rsbV*, in which the genes for the second positive regulator of σ^B activity were inactivated. This finding suggests that the observation of Kies et al. (35) of a biofilm-positive phenotype in a *sigB* deletion mutant, *S. epidermidis* TüΔ*sigB*, containing the plasmid pCN27 carrying the *icaADBC* locus seems to be caused by the overexpression of the *icaADBC* genes. This overexpression despite a *sigB*-negative genotype could be explained by the lack of the *icaR* gene (31), encoding the negative regulator of *icaADBC* transcription IcaR (14, 15), in pCN27. However, the possibility that an additional, yet-unknown regulatory intermediate between σ^B and the *icaADBC* operon is missing in an *icaADBC*-negative genetic background cannot be completely excluded. The phenotypic characterization of mutants 1457*rsbW*, 1457*rsbUVW*, 8400*rsbW*, and 8400*rsbUVW* revealed an increase in biofilm formation compared to that of their respective wild-type strains. Apparently, the lack of regulation of σ^B activity during the growth cycle, resulting in constitutive *sigB* transcription with lower σ^B activity during the exponential growth phase and higher activity during the post-exponential phase, leads to overexpression of PIA and biofilm formation.

Transcriptional analysis of the *icaADBC* locus revealed that the positive regulation of biofilm formation in *S. epidermidis* by σ^B is based on transcriptional activation of *icaADBC*, as has already been demonstrated for the *rsbU* mutant M15 (49). However, the lack of a σ^B-dependent promoter upstream of the *icaADBC* transcription start site (31, 36) necessitates a σ^B-dependent regulatory intermediate which controls *icaADBC* transcription. Recently, it was demonstrated that IcaR acts as a negative regulator of *icaADBC* transcription in *S. epidermidis* (14, 15). In *S. aureus*, IcaR was characterized as a DNA binding protein which interacts with the promoter region of *icaADBC* (34). Transcriptional analysis of the *icaR* gene revealed that the positive σ^B regulation of biofilm formation is mediated by negative transcriptional control of IcaR, the negative regulator of *icaADBC* transcription (14), which is upregulated in mutants which are defective in *sigB* or its function. This regulatory pathway could explain the biofilm-positive phenotype of mutants with a lack of σ^B function in media supplemented with ethanol. In these mutants, *icaR* transcription is repressed by ethanol by a yet-unknown mechanism, as was already demonstrated by Conlon et al. for the clinical isolate *S. epidermidis* CSF41498 (15). These data indicate that *icaR* transcription is negatively controlled by two divergent regulatory pathways. Hence, for the σ^B-dependent control of biofilm formation, a negative regulatory intermediate of *icaR* transcription, which is itself under positive control of σ^B, must be predicted. Despite the increased biofilm formation in wild-type *S. epidermidis* 1457, a decrease in *icaADBC* transcription under the influence of ethanol was detected. Recently, a dissociation of *icaADBC* transcription and PIA synthesis was characterized in *S. epidermidis* 1457 with glucose limitation (17). A glucose-dependent posttranscriptional regulator of PIA synthesis was predicted, and this regulator might also be responsible for the observed dissociation between *icaADBC* transcription and biofilm formation under ethanol induction.

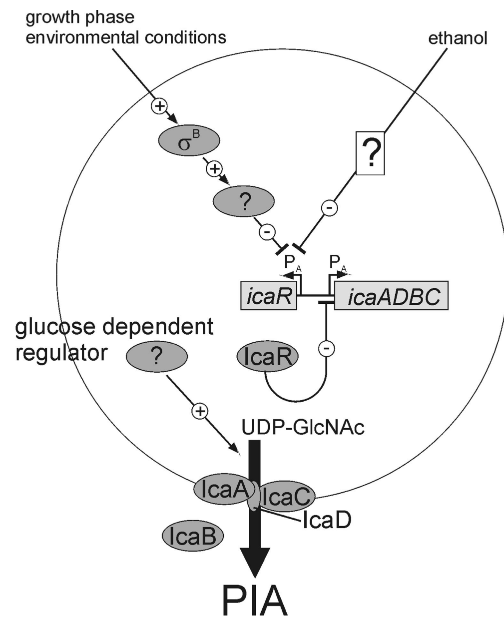

FIG. 9. Model of the regulation of PIA synthesis in *S. epidermidis*. Transcription of *icaADBC* is regulated by the activity of the negative regulator IcaR (14). Transcription of *icaR* is negatively regulated by ethanol (15) and independently by a σ^B-dependent negative regulator, which is yet unknown. Additionally, PIA synthesis is regulated posttranscriptionally by a yet-unknown glucose-dependent regulator (17).

The data obtained in this study together with the current knowledge about the regulation of PIA synthesis in *S. epidermidis* enables us to draw up a model of transcriptional and posttranscriptional regulation (Fig. 9). This model reveals a complex regulation of PIA synthesis, which is regulated by at least three different regulatory pathways. Two of these pathways act through transcriptional regulation of the negative regulator IcaR of *icaADBC* transcription, and the third pathway is a glucose-dependent proteinaceous factor of PIA synthesis (17). However, in this provisional model several gaps of yet-uncharacterized regulatory intermediates remain in this complex regulatory system. The σ^B-dependent negative regulator and the ethanol-mediated negative regulation of *icaR* transcription, as well as the nature of the glucose-dependent posttranscriptional activator of PIA synthesis, are still unknown. The regulatory role of SarA in *S. epidermidis* biofilm formation could not be conclusively assessed. The σ^B-dependent *sarA* transcript was still absent in mutants with dysfunctional σ^B despite a biofilm-positive phenotype under ethanol induction, indicating the lack of influence of SarA on the IcaR-dependent regulation of PIA synthesis in *S. epidermidis*.

In the σ^B-dependent regulatory pathway of PIA synthesis, the possibility of an influence of SarA could not be excluded. However, the *sarB* and *sarC* transcripts were not affected in the mutants with inactivation of σ^B function, indicating a still-present activity of SarA. Interestingly, regulation of PIA synthesis in *S. aureus* seems to be different, as was already predicted by phenotypic characterization (38). In *S. aureus*, regulation of biofilm formation is mainly dependent on SarA, whereas σ^B-dependent regulation plays only a minor role (74). The differential role of the alternative sigma factor σ^B in these species indicates that investigation of the transcriptional profile during biofilm formation in *S. aureus* is not suitable for drawing conclusions about *S. epidermidis* or, presumably, about other staphylococcal species.

PIA synthesis resulting in biofilm formation is the major pathogenetic factor of *S. epidermidis* in foreign-body-associated infections. Therefore, the regulatory system of biofilm formation could be an important target in the prevention and therapy of foreign-body-related infections due to *S. epidermidis*, requiring further studies.

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