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Alternative Transcription Factor σ^B Is Involved in Regulation of Biofilm Expression in a *Staphylococcus aureus* Mucosal Isolate

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Osmotic stress was found to induce biofilm formation in a *Staphylococcus aureus* mucosal isolate. Inactivation of a global regulator of the bacterial stress response, the alternative transcription factor σ^B, resulted in a biofilm-negative phenotype and loss of salt-induced biofilm production. Complementation of the mutant strain with an expression plasmid encoding σ^B completely restored the wild-type phenotype. The combined data suggest a critical role of σ^B in *S. aureus* biofilm regulation under environmental stress conditions.

For numerous pathogenic bacteria, biofilms represent a source of persisting and relapsing infections and thus contribute significantly to pathogenesis (4). Biofilms seem to protect bacteria from unfavorable external conditions, and, in some bacterial ecosystems, the conversion of planktonic cells into a biofilm-producing community is triggered by environmental stress factors (6, 12, 13). In the human pathogen *Staphylococcus aureus*, biofilm formation is mediated by the production of the extracellular polysaccharide adhesin PIA, whose synthesis depends on the expression of the *icaADBC*-encoded enzymes (5, 15). The regulation of biofilm expression in this organism is poorly understood. All *S. aureus* strains analyzed so far contain the entire *ica* gene cluster, but only a few express the operon and produce biofilms in vitro (5). In this study, we investigated whether the alternative transcription factor σ^B is involved in the regulation of *ica* expression. σ^B is known to be a global regulator of the stress response in *S. aureus* and also influences various virulence-associated genes (7, 11, 16, 20). To elucidate the possible role of σ^B in biofilm formation, we used a genetic approach and constructed an *S. aureus sigB*::*ermB* insertion mutant of the biofilm-forming, methicillin-sensitive mucosal isolate *S. aureus* MA12 (18). Biofilm formation and *ica* expression of the mutant were compared with the phenotypes of the corresponding wild-type strain and a complemented strain that carried a *sigB* copy on an expression vector.

Construction of a *sigB* insertion mutant and complementation of the mutation. The inactivation of *sigB* was done by insertion of an erythromycin resistance cassette into the *sigB* gene of *S. aureus* MA12 by double-crossover integration. For this purpose, the temperature-sensitive shuttle vector pSK8, which carries a *sigB*::*ermB* mutation, was constructed. A 937-bp fragment containing the entire *sigB* gene was amplified by PCR from *S. aureus* MA12 by using the primers 5′ CGG GAT CCG GTG TGA CAA TCA GTA TGA C 3′ and 5′ CGG AAT TCG CGA CAT TTA TGT GGA TAC AC 3′. The DNA fragment was inserted into the shuttle vector pBT1 (17), resulting in pSK7. Then the *ermB* cassette of pEC1 (1) was excised by *Xba*I-*Hin*dIII digestion, treated with the Klenow fragment of *Escherichia coli* DNA polymerase, and ligated with *Eco*RV-digested pSK7, resulting in plasmid pSK8. Following passage through the restriction-negative strain *S. aureus* RN4220, pSK8 was reisolated and transformed into *S. aureus* MA12 by electroporation (19). Replacement of the chromosomal *S. aureus* MA12 *sigB* wild-type gene was achieved by double-crossover integration of the *sigB*::*ermB* insert of pSK8 following a temperature shift to the nonpermissive temperature of the shuttle vector (42°C). Erythromycin-resistant and chloramphenicol-sensitive colonies were isolated, and the *sigB*::*ermB* integrations were confirmed by Southern hybridization, PCR, and nucleotide sequencing (data not shown). From these experiments, the *sigB*::*ermB* insertion mutant *S. aureus* MA12.2 was selected for further analysis. To restore the σ^B function, the *S. aureus* MA12.2 *sigB* mutant strain was complemented with plasmid pSK9. Plasmid pSK9 was constructed by inserting the *sigB* PCR fragment into the expression shuttle vector pHPS9 (8). Prior to transformation into *S. aureus* MA12.2, the vector was transformed into the restriction-negative cloning host *S. aureus* RN4220. The plasmid was reisolated and transferred into *S. aureus* MA12.2, resulting in the complemented strain *S. aureus* MA12.2-1(pSK9).

Analysis of the σ^B function. Inactivation of the *sigB* gene in *S. aureus* MA12.2 and restoration of its function in the complemented strain *S. aureus* MA12.2-1(pSK9) was investigated by Northern analysis of the *asp23*-specific gene expression. *asp23* encodes an alkaline shock protein, and the gene was shown to be preceded by a σ^B-dependent promoter (7, 11). Recent studies have shown that *asp23* transcription is absent in *S. aureus sigB* mutants. It has therefore been concluded that *asp23* transcription reliably reflects the activity of the σ^B factor (7, 11, 16).

The Northern hybridization experiments revealed a strong *asp23*-specific signal in both the wild-type and the complemented strains. In contrast, no *asp23*-specific transcription was detected in the mutant strain *S. aureus* MA12.2 (data not shown). Furthermore, as expected for a σ^B-negative strain (2, 11), and in contrast to both the wild-type and complemented strains, the mutant strain had lost its yellow pigmentation and showed enhanced hemolysis on blood agar plates (data not shown). Taken together, these data indicate that *S. aureus* MA12.2 is a *sigB* mutant and that the *sigB* mutation is restored in the complemented strain *S. aureus* MA12.2-1(pSK9).

Effect of the *sigB* mutation on biofilm production and *ica* expression. Quantitative biofilm measurement was done in a microtiter assay as described previously (3, 22). Bacteria were grown overnight in 96-well, flat-bottomed tissue culture plates

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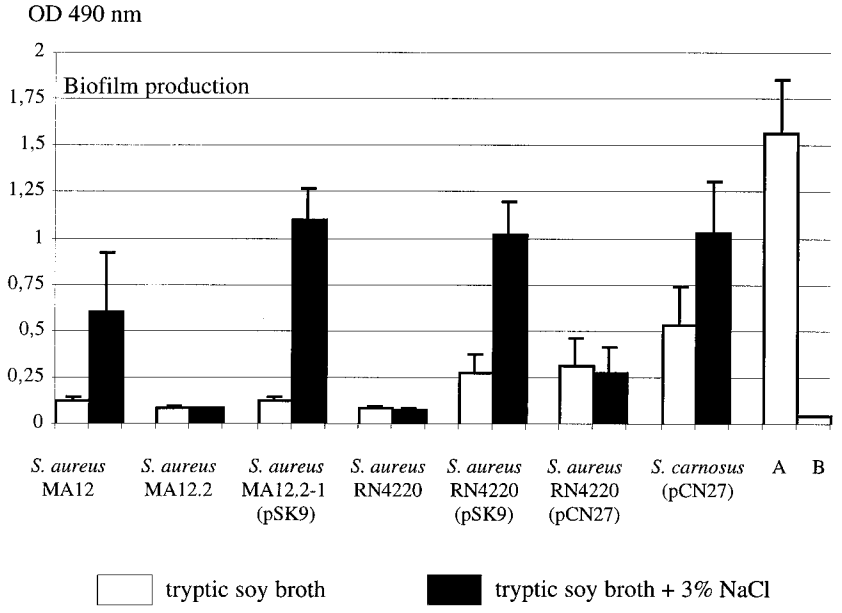

FIG. 1. Biofilm formation on polystyrene tissue culture plates of the wild-type strain (*S. aureus* MA12), the *sigB*::*ermB* insertion mutant (*S. aureus* MA12.2), the complemented strain [*S. aureus* MA12.2-1(pSK9)], the cloning host *S. aureus* RN4220, *S. aureus* RN4220(pSK9), and *S. aureus* RN4220(pCN27) carrying the *icaADBC* operon of *S. epidermidis* on plasmid pCN27 (9), and *S. carnosus* TM300(pCN27) (9) after growth in unsupplemented TSB and TSB supplemented with 3% sodium chloride, respectively. A, *S. epidermidis* RP62A (positive control); and B, *S. carnosus* TM300 (negative control).

(Greiner, Nürtingen, Germany) at 37°C using either tryptic soy broth (TSB) (Difco, BBL, Detroit, Mich.) or TSB supplemented with 3% sodium chloride as growth medium. Based upon the optical densities (OD) of the biofilms, the strains were classified as nonadherent strains (OD $\leq$ 0.120), weak biofilm producers (0.120 $<$ OD $\leq$ 0.240), or strongly adherent strains (OD $>$ 0.240) according to the scheme introduced by Christensen et al. (3). As shown in Fig. 1, the biofilm formation of *S. aureus* MA12 was weak when the strain was grown in TSB, but it could be significantly stimulated under osmotic stress conditions (that is, TSB containing 3% sodium chloride). In contrast, the *S. aureus* MA12.2 *sigB* insertion mutant failed to produce any detectable biofilm, irrespective of whether it was grown in TSB alone or in TSB containing 3% sodium chloride. In the case of *S. aureus* MA12.2(pSK9), in which the inactivated chromosomal copy of *sigB* was complemented by an expression plasmid carrying *sigB*, both basal and the osmotic stress-stimulated biofilm formation were restored.

Next, we examined the effect of the *sigB* inactivation on the transcription of the *ica* gene cluster by Northern blotting experiments. To this end, RNA was isolated from mid-log cultures of the wild-type strain, the *sigB* mutant, and the complemented strain, which were grown in TSB containing 3% sodium chloride. Hybridization with a ^{32}P-radiolabeled *icaA*-specific gene probe revealed a strong signal in the *S. aureus* MA12 wild-type strain (Fig. 2, lane 1). In contrast, *ica* transcription was drastically diminished in the *sigB* mutant (Fig. 2, lane 2) and restored in the complemented strain *S. aureus* MA12.2SK(pSK9) (Fig. 2, lane 3). These data are consistent with the observed differences in biofilm production under high-salt conditions between the wild-type and mutant strains and again support the conclusion that *sigB* is involved in the control of biofilm formation in *S. aureus* MA12.

We then extended the study to another *S. aureus* strain, RN4220. *S. aureus* RN4220 is a restriction-negative strain that is commonly used as a cloning host for plasmids prior to their transformation into the staphylococcal strain of interest (10). *S. aureus* RN4220 had been derived from *S. aureus* 8325, which was described recently as a spontaneous *sigB*-negative mutant with an 11-bp deletion in the *rsbU* regulatory gene (11, 20). Normally, *S. aureus* RN4220 does not produce biofilms, under either low-salt or high-salt conditions (Fig. 1). We found that, when transformed with plasmid pSK9, this strain formed biofilms and, moreover, that the biofilm production was induced under high-salt conditions. These results led us to propose that the biofilm-negative phenotype of *S. aureus* RN4220 is caused by the absence of σ^B. Obviously, this conclusion appears to contradict previous results which demonstrated that *S. aureus* RN4220 exhibits a biofilm-positive phenotype when the *icaADBC* genes are provided on a plasmid (5, 14). Therefore, we have done an additional set of experiments. We have transformed plasmid pCN27 (9), which carries the entire *ica* operon of *Staphylococcus epidermidis*, into *S. aureus* RN4220. Consistent with previous data (14), the strain formed a biofilm when grown in TSB (Fig. 1). However, in obvious contrast to the pSK9 complementation experiments described above, no in-

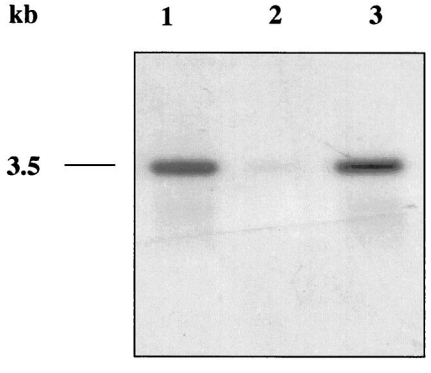

FIG. 2. Northern blot analysis of *ica* transcription in the *S. aureus* MA12 wild-type strain (lane 1), the *sigB*::*ermB* insertion mutant *S. aureus* MA12.2 (lane 2), and the complemented strain *S. aureus* MA12.2-1(pSK9) (lane 3) after growth in TSB supplemented with 3% sodium chloride.

duction of biofilm production was observed under osmotic stress conditions. Finally, we found that propagation of pCN27 in *Staphylococcus carnosus* TM300 (9) increased the biofilm production of this strain under high-salt conditions. The combined data led us to hypothesize that the biofilm-forming phenotype of *S. aureus* RN4220(pCN27) may result from a basal, vector-driven *ica* expression, which is enhanced by the copy effect of the plasmid. The absence of biofilm induction, however, suggests that σ^B is required for the activation of biofilm formation in response to osmotic stress.

Conclusions. In this study, we provide evidence that the biofilm-forming phenotype observed in a mucosal isolate of *S. aureus* can be induced by changing environmental conditions (in this case, osmotic stress). The results are consistent with our recent data obtained for *S. epidermidis* (S. Rachid and W. Ziebuhr, unpublished data), which demonstrated that the expression of the biofilm-mediating *ica* operon is enhanced by high osmolarity, high temperature, and subinhibitory concentrations of certain antibiotics. In contrast to *S. epidermidis*, all *S. aureus* strains analyzed so far carry the *ica* gene cluster, but only a few spontaneously express biofilms in vitro (5). It is thus tempting to speculate that, in these biofilm-negative strains, the *ica* expression may be tightly controlled. The data presented in this study strongly support the idea that this suppression might be overcome by activation of σ^B in response to external stress. Another explanation for the biofilm-negative phenotype in the majority of the *S. aureus* strains would be the presence of mutations in the *sigB* gene itself or in its adjacent *rsbU*, *rsbV*, and *rsbW* regulatory genes (11, 20). It is also conceivable that *S. aureus* biofilm production may be influenced by mechanisms that have been shown to be used by *S. epidermidis* in the control of the *ica* expression (e.g., phase variation and gene rearrangements) (21–23). All these hypotheses still need careful experimental evaluation. It should also be noted that, in this study, we have characterized only the mucosal isolate *S. aureus* MA12 in detail. Even though we have obtained conclusive evidence for the critical involvement of the σ^B factor in biofilm formation in this isolate, the actual role of σ^B in *ica* expression remains to be determined. The nucleotide sequence immediately upstream of the *ica* operon does not resemble any of the known σ^B-dependent promoter structures, suggesting an indirect activation of the *ica* expression by additional unknown regulatory factors. This idea is also supported by the identification of *S. aureus* strains which express the *sigB* gene but, nevertheless, are biofilm negative (Rachid and Ziebuhr, unpublished). To answer the question of whether σ^B is directly or indirectly involved in *S. aureus ica* expression, more experimental work, including primer extension analyses under different growth conditions, is needed. Finally, a broad range of *S. aureus* strains has to be analyzed to identify possible variations in biofilm regulation among different strains.

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