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Identification of the *sigB* Operon in *Staphylococcus epidermidis*: Construction and Characterization of a *sigB* Deletion Mutant

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The role of the alternative sigma factor σ^B in *Staphylococcus epidermidis* was investigated by the construction, complementation, and characterization of a *sigB* deletion mutant. Electrophoretic analyses confirmed a profound influence of σ^B on the expression of exoproteins and cytoplasmic proteins. Detailed investigation revealed reduced lipase and enhanced protease activity in the σ^B mutant. Furthermore, no significant influence of σ^B on heterologous biofilm formation or on the activity of the global regulator *agr* was detected.

In several bacteria, various alternative sigma factors modulate gene expression in response to environmental and metabolic signals (12). Based on the information available for the *Staphylococcus aureus* genome, staphylococci appear to possess only one alternative sigma factor, σ^B. Therefore, σ^B is presumed to play a crucial role in global regulation. Expression of virulence factors in *S. aureus* has been shown to depend on σ^B (17) and on at least two global regulatory systems: *agr* (accessory gene regulator) (20) and *sar* (staphylococcal accessory regulator) (4). Since *sar* is responsible for *agr* activation and σ^B influences SarA expression (5), σ^B represents the superior global regulator in the *S. aureus* regulatory network controlling exoprotein expression.

Staphylococcus epidermidis ranks among the most important nosocomial pathogens, mainly because of its ability to colonize indwelling medical devices by forming a biofilm (9, 10, 29). In addition, many antibiotics lose their effectiveness against *S. epidermidis* in the biofilm environment because of the impenetrable slime capsule (6, 28). A further factor contributing to the severe threat of *S. epidermidis* to public health is the occurrence of multiresistant and vancomycin-resistant strains (24). Virtually nothing is known about the regulation of virulence factors in *S. epidermidis*. An *agr* deletion mutant has recently been constructed and characterized by our group (30), but no other global regulator has been studied so far.

A region of the chromosome of *S. epidermidis* Tü3298 comprising four open reading frames with strong sequence similarities to the *S. aureus sigB* operon was sequenced by direct chromosomal sequencing (GenBank accession number AF359562), starting with primer S1 (CACGAAGATTTAGT TCAAGTTGGTATGGTGG), derived from the *S. aureus* sequence. The striking similarity in both sequence and overall genetic organization suggested that this region is the *sigB* operon of *S. epidermidis* (Fig. 1).

In order to analyze its function in *S. epidermidis, sigB* was replaced by a spectinomycin resistance gene (*spc*) from Tn*554* (19) by homologous recombination (Fig. 1). Since the *rsb* genes are tightly clustered and *rsbW* and *sigB* overlap, we decided not to replace the first bases of *sigB*, leaving the anti-sigma factor *rsbW* intact. Fragments of about 870 bp upstream and downstream of *sigB* were amplified using primer pairs SigEcoRI and SigBamHI (GATTAAAGTGAATTCATGTAGGGTATAGG and CAGGTGATGGATCCCTAGCTGATTTCGAC) for fragment 1 and SigSphI and SigHindIII (GCTGCATGCCAGTA AACGAGTTGTTAAC and GAGGAAAAGCTTAGTCCCT GATTAAAAACATC) for fragment 2. The fragments were cloned into the polylinker region of plasmid pBT2 (2), flanking the spectinomycin resistance gene. The resulting plasmid, pBTΔsigB, was introduced into *S. epidermidis* Tü3298 by electroporation (1). Allelic replacement of the wild-type *sigB* gene by *spc* was carried out as previously described (2). The correct insertion of the antibiotic resistance marker in the resulting strain, *S. epidermidis* TüΔsigB, was confirmed by Southern blot analysis and chromosomal sequencing.

To investigate the influence of σ^B on protein expression, protein samples from stationary phase (16 h) cultures were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein profiles of the σ^B mutant *S. epidermidis* TüΔsigB and the wild-type strain *S. epidermidis* Tü3298 were compared with the complemented mutant *S. epidermidis* TüΔsigB(pTXsigB) and *S. epidermidis* Tü(pTXsigB), which overexpresses *sigB*. To generate plasmid pTXsigB, the *sigB* gene of *S. epidermidis* Tü3298 was amplified by PCR with primers SigUBamHI (GATTAAGGATCCAAAAAAAGAG CAGGTGCG) and SigUMluI (CTCTGTTAACAACGCGTT TACTGTCTTGCAGC) and cloned in plasmid pTX15 (23, 31). *S. epidermidis* TüΔsigB(pTX16) and *S. epidermidis* Tü(pTX16) served as control strains to show that complementation and overexpression effects are not caused by the pTX plasmid. Plasmid pTX16 (23, 31) is used as a negative control in pTX15 expression studies, because cloning in vector pTX15 deletes a lipase gene which had also been deleted in pTX16.

Strains were cultivated in B-medium (1% peptone [Difco], 0.5% yeast extract [Gibco-BRL], 0.5% NaCl, 0.1% K_2HPO_4, 0.1% glucose). Vectors pTX15 and pTX16 allow xylose-inducible and glucose-repressible gene expression (23, 31), and therefore strains containing these plasmids were grown in modified B-medium containing 0.5% xylose and lacking glucose. Cultures were adjusted to the same cell density and

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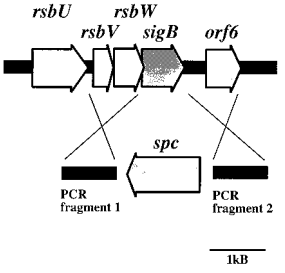

FIG. 1. Physical map of the *sigB* operon of *S. epidermidis* and construction of a *sigB* deletion mutant using plasmid pBTΔsigB. Arrows depict open reading frames and indicate their orientation and size. The *sigB* gene was replaced with the spectinomycin resistance gene (*spc*) as shown in the lower part of the figure. The *spc* gene and two PCR-amplified regions flanking *sigB* were cloned into plasmid pBT2, yielding integration vector pBTΔsigB. The crosses indicate the sites of homologous recombination.

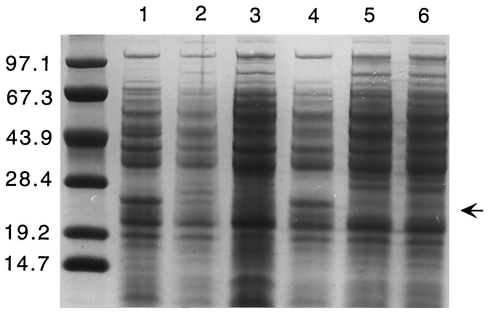

FIG. 3. Influence of *S. epidermidis* σ^B on production of cytoplasmic proteins. Protein profiles of 16-h cultures of the following strains are shown: *S. epidermidis sigB* deletion mutant *S. epidermidis* Tü3298ΔsigB (lane 1), *S. epidermidis* wild-type Tü3298 (lane 2), the complemented mutant *S. epidermidis* TüΔsigB(pTXsigB) (lane 3), the control strain *S. epidermidis* TüΔsigB(pTX16) (lane 4), the σ^B-overexpressing strain *S. epidermidis* Tü(pTXsigB) (lane 5), and its corresponding control strain *S. epidermidis* Tü(pTX16) (lane 6). The proteins were separated on SDS–10% polyacrylamide gels and stained with Coomassie brilliant blue G250. The arrow indicates the position of a 27-kDa protein present or absent in the different strains. The molecular masses (in kilodaltons) of size standard proteins are shown on the left.

grown for 16 h. Exoprotein samples were precipitated with 1/9 volume of trichloracetic acid, and cytoplasmic and membrane fractions were prepared as described previously (22). The growth rates of all the strains tested were the same.

Deletion of *sigB* in *S. epidermidis* resulted in a pleiotropic alteration of the protein pattern of exoproteins (Fig. 2) and cytoplasmic proteins (Fig. 3), while the composition of membrane proteins was not altered significantly (data not shown). The most striking differences in the σ^B -mutant samples containing exoproteins were the absence of an approximately 27-kDa protein and clearly more pronounced 44-kDa and 38-kDa proteins. The profiles of cytoplasmic proteins showed a distinct change in the pattern, especially in the range from about 25 to 30 kDa. Most remarkable in this respect is a protein of about 27 kDa, which is present in significantly higher amounts in the mutant strain. Comparison of protein profiles show that the σ^B -minus phenotype was complemented by plasmid pTXsigB; in contrast, the wild-type strain bearing the same plasmid did not show a protein pattern different from that of the wild type. Our studies suggest a crucial role for σ^B in *S. epidermidis* gene regulation. Since the most striking differences in the protein patterns were seen in the exoprotein fractions, we decided to analyze exoprotein expression in more detail.

Lipases and proteases are known to be virulence factors in *S. aureus* (8, 11) and may also contribute to the pathogenicity of *S. epidermidis*. Therefore, lipase and protease activity in the supernatant of the *S. epidermidis* wild-type and Δ*sigB* mutant strains was analyzed by zymography (Fig. 4). Exoprotein samples were separated on nondenaturing SDS-polyacrylamide

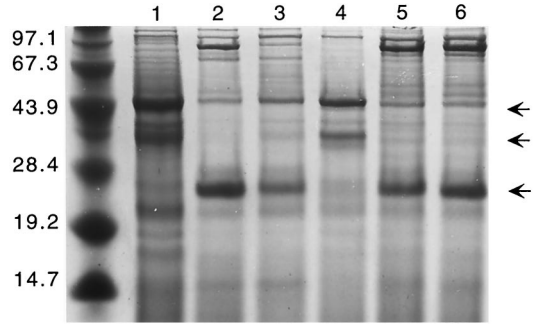

FIG. 2. Influence of *S. epidermidis* σ^B on production of exoproteins. Protein profiles of 16-h cultures of the following strains are shown: *S. epidermidis sigB* deletion mutant *S. epidermidis* Tü3298ΔsigB (lane 1), *S. epidermidis* wild-type Tü3298 (lane 2), the complemented mutant *S. epidermidis* TüΔsigB(pTXsigB) (lane 3), the control strain *S. epidermidis* TüΔsigB(pTX16) (lane 4), the σ^B-overexpressing strain *S. epidermidis* Tü(pTXsigB) (lane 5), and its corresponding control strain *S. epidermidis* Tü(pTX16) (lane 6). The proteins were separated on SDS–10% polyacrylamide gels and stained with Coomassie brilliant blue G250. The arrows mark the positions of 27-kDa, 38-kDa, and 44-kDa proteins present or absent in the different strains. The molecular masses (in kilodaltons) of size standard proteins are shown on the left.

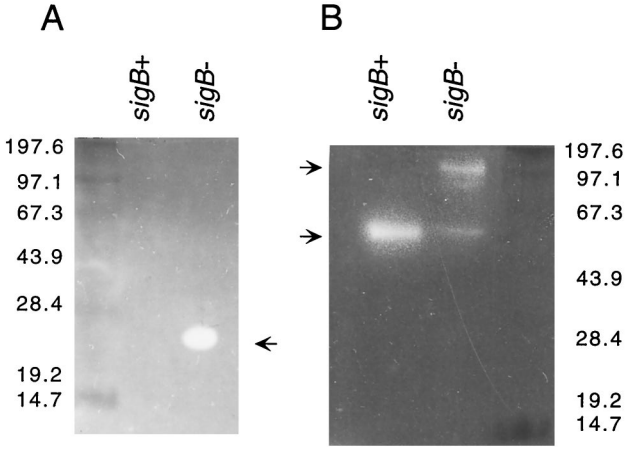

FIG. 4. Zymographic analysis of protease (A) and lipase (B) activity. Exoprotein samples of strains *S. epidermidis* Tü3298 (wild-type; *sigB*$^+$) and *S. epidermidis* TüΔsigB (*sigB* deletion mutant; *sigB*$^-$) were separated on SDS–10% polyacrylamide gels. Zymographic analysis of the gels was carried out on protease or lipase agarose test plates. Arrows indicate the positions of proteolytic or lipolytic bands. The molecular masses (in kilodaltons) of size standard proteins are shown on the left (A) and right (B).

gels and incubated on agarose test plates containing 1% Tween (for lipase detection) or 1% casein according to the method of Hammersten (Amersham Pharmacia Biotech, Freiburg, Germany) for protease detection as described previously (30). Staphylococcal lipases are organized as preproenzymes and are secreted in the prolipase form (11, 27). Lipase test plates revealed a single lipolytic band at about 50 kDa in the exoprotein sample from the *S. epidermidis* wild-type strain, which was less pronounced in samples from the σ^B mutant strain. A second lipolytic band of about 100 kDa was also present in samples from the mutant strain; the size corresponds to that of the proform of staphylococcal lipase (11, 27). Thus, the processing of this lipase shows pronounced σ^B dependence. In contrast, lipase production was enhanced in three different *S. aureus* σ^B mutants compared to their isogenic wild-type strains (17). These data indicate that the involvement of σ^B in lipase gene expression may vary among staphylococcal species. Protease test plates of the σ^B mutant strain showed a distinct proteolytic band at about 25 kDa which was not detectable in the wild-type strain. For strain *S. epidermidis* Tü3298, an extracellular protease with a corresponding molecular mass has not been reported so far. The results indicate that the expression or secretion of this novel protease is repressed by σ^B.

In *S. aureus* and *Bacillus subtilis*, σ^B modulates expression of several stress and starvation proteins. We carried out heat shock (54°C, 10 min) and salt stress (2 M) studies in B-medium. The wild-type strain and the σ^B mutant had similar growth patterns after stress exposure (data not shown). In *B. subtilis*, the regulatory factor RsbU is essential for full σ^B activity under stress conditions but is not required for activation of σ^B during the stationary phase (13). The occurrence of natural *rsbU* mutants of *S. aureus* harboring an 11-bp deletion has been reported (3, 16, 17), but based on sequence comparisons, *rsbU* appears to be intact in *S. epidermidis* Tü3298. Since the signal transduction pathway influencing RsbU expression in bacilli and in staphylococci is not known, we speculate that *S. epidermidis* Tü3298 might contain a mutation in this pathway, leading to the nonresponsiveness of σ^B expression to environmental stress.

In *S. epidermidis*, biofilm formation and cell aggregation are dependent on the production of the polysaccharide intercellular adhesin (PIA) (18), which is synthesized by the gene products of the *ica* gene locus. Strain *S. epidermidis* Tü3298 used in this study lacks the *ica* genes. This was proven by PCR, Southern blot analysis, and direct chromosomal sequencing (data not shown). Plasmid pCN27 containing the *S. epidermidis* RP62A *ica* genes has been shown to lead to the formation of cell clusters and PIA expression in the heterologous host *Staphylococcus carnosus* (14).

To investigate the impact of σ^B in biofilm formation, plasmid pCN27 was introduced into *S. epidermidis* Tü3298 and *S. epidermidis* TüΔsigB. Light microscopy analysis of 16-h cultures of the two strains showed formation of large cell clusters but did not reveal any significant differences in cell aggregation between the two strains. The same result was obtained with cultures that had been exposed to heat or salt stress before growth (data not shown). External stress has been demonstrated to induce biofilm formation in *S. aureus* (25) and *S. epidermidis* (26), and the response was shown to depend on functional RsbU (15). The nonresponsiveness of cell aggregation in strain *S. epidermidis* Tü3298 to the presence or absence of σ^B might therefore be explained by a nonfunctional RsbU-mediated signal transduction pathway. Furthermore, as *S. epidermidis* Tü3298 lacks the *ica* genes, regulatory elements needed for the control of *ica* gene expression might also be missing.

To understand a putative regulatory cascade of global regulators in *S. epidermidis*, we elucidated the role of σ^B in *agr* expression. Staphylococcal delta toxin is encoded within the gene coding for RNAIII, the effector molecule of the *agr* regulation system. Therefore, quantitative analysis of delta toxin allows the measurement of *agr* activity in different strains. The delta toxin concentration was directly detected by analytical high-pressure liquid chromatography is the supernatant of *S. epidermidis* Tü3298 and *S. epidermidis* Tü3298ΔsigB, as described previously (21). Delta toxin production was measured in five independent stationary-phase cultures of each strain. The amount of delta toxin in the two strains was not significantly different (wild-type strain, 7.6 μg/ml; σ^B deletion mutant, 7.0 μg/ml), indicating no direct influence of σ^B on *agr* activity.

Given that σ^B regulates *sar* activity, as has been shown for *S. aureus*, we cannot rule out that at least some of the alterations reported in this study are caused by reduced transcription of the *sar* regulatory locus. To understand the regulation of virulence genes in *S. epidermidis*, it will be important to distinguish the Sar and σ^B target genes and to test the virulence of a σ^B mutant in an animal model.

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