The role of $\sigma^B$ in persistence of Staphylococcus epidermidis foreign body infection

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INTRODUCTION

Coagulate-negative staphylococci (CoNS) and in particular Staphylococcus epidermidis have become important causes of nosocomial infections due to their ability to form a biofilm on foreign bodies such as prosthetic heart valves or urinary tract and central venous catheters (O’Gara & Humphreys, 2001). Treatment of CoNS foreign body infections (FBIs) is difficult. Although antibiotics generally suppress the symptoms, they rarely succeed in eradicating the infection without removal of the foreign device (Costerton et al., 1999). A better understanding of the genes and complex pathways that regulate biofilm formation during FBI may help to identify new treatment targets.

Formation of adherent multilayered biofilms embedded into a glycolcalyx is a multi-step process. The attachment step is affected by multiple factors, including hydrophobic interactions, presence of host proteins, and specific surface proteins such as the autolysin AtlE (Heilmann et al., 1997) and the capsular polysaccharide adhesin PS/A (Muller et al., 1993). In the accumulation phase, polysaccharide intercellular adhesin (PIA) synthesis at the level of the entire $\sigma^B$ operon. Both wild-type strains and the constitutively $\sigma^B$-expressing rsbUVW mutant showed a strong biofilm-positive phenotype. The rsbUVW mutant was, however, thinner and more evenly spread than the wild-type biofilm. Inactivation of SigB in the rsbUVW mutant or mutation of the positive regulator Rsbu reduced both the number of sessile bacteria and polysaccharide intercellular adhesin synthesis. These differences between the wild-types and their respective mutants appeared after 6 h in vitro biofilms but only after 4 days in vivo biofilms. Our results provide additional evidence for a role for $\sigma^B$ in biofilm formation. They also suggest a role for $\sigma^B$ in biofilm maturation and stability that is independent of PIA or accumulation-associated protein (Aap) and point to significant differences in the temporal development between in vitro and in vivo biofilms.

Abbreviations: CLSM, confocal laser-scanning microscopy; CoNS, coagulate-negative staphylococci; FBI, foreign body infection; gDNA, genomic DNA; PIA, polysaccharide intercellular adhesin; SEM, scanning electron microscopy.
completely independent of PIA, consistent with the observation of ica-independent biofilms (O’Gara, 2007; Rohde et al., 2005).

Alternative sigma factors have been shown to be important for survival under extreme conditions and to affect virulence and pathogenicity in several bacterial species (Wise & Price, 1995). In staphylococci there are three known sigma factors: $\sigma^A$, the housekeeping sigma factor, and two alternative sigma factors $\sigma^B$ and $\sigma^H$ (Pane-Farre et al., 2006). In contrast to $\sigma^A$, the alternative sigma factor $\sigma^B$ has been studied intensively (Bischoff et al., 2004) and is presumed to play a crucial role in the global regulation of gene expression (Kies et al., 2001). The $\sigma^B$ operon in staphylococci contains four genes: rsbU, rsbV, rsbW and sigB. The rsbW gene product is a key negative regulator, responsible for an inactive SigB. The rsbU and rsbV gene products stimulate SigB activity. RsbV acts as a positive regulator; in its dephosphorylated form, RsbV can bind competitively to RsbW, resulting in the release of free and active SigB. The binding of RsbW to SigB or to RsbV depends on the phosphorylation status of RsbV, which is modulated by the phosphatase RsbU (Wise & Price, 1995). A SigA-dependent promoter, $P_\lambda$, is found upstream of rsbU, and a SigB-dependent promoter, $P_{\beta_b}$, precedes the last three genes of the operon. SigB increases its own transcription as a consequence of its activation, further inducing the entire $\sigma^B$ regulon (Fouet et al., 2000). $\sigma^B$ may act only indirectly via an additional, unknown factor or RsbU may, by itself, be a regulator of icaADBC transcription. Transcriptional analysis of icaR revealed that the positive $\sigma^B$ regulation of biofilm formation is mediated by negative transcriptional control of IcaR, the negative regulator of icaADBC transcription, which is upregulated in mutants defective in sigB or its function (Knobloch et al., 2004). Activation of PIA expression by different stress stimuli apparently uses different pathways. This implies that a number of environmental conditions and regulatory systems can influence the expression of staphylococcal biofilms, reflecting the magnitude of the complexity associated with biofilm formation.

In the present study we characterized biofilm formation and biofilm stability of S. epidermidis wild-type strains 10b, 8400 and 1457 and different $\sigma^B$ operon mutants.

**METHODS**

**Bacterial strains, genes and growth conditions.** Strains used in this study are listed in Table 1. The staphylococcal strains were cultured in brain heart infusion (BHI, Oxoid) at 37 °C. Growth rates of the different strains were compared by means of growth curves. Starting from an OD$_{600}$ of 0.002, a sample was taken every hour from 0 until 9 h.

Based on the complete genome of the non-biofilm-forming S. epidermidis strain ATCC 12228 (NC_004461, http://www.ncbi.nlm.nih.gov) primers were designed to amplify gmk ($\text{accession no. AE015929}$), aap (AJ249487), rsbU (AF274004) and asp23 (SE1773) in S. epidermidis 10b. Primers to amplify icaA (U43366) in S. epidermidis 10b were based on the S. epidermidis RP62A genome. PCR was performed on a GeneAmp PCR System 9700 (PE Applied Biosystems). The PCR products of all genes were cloned in the pGEM-T Easy vector system (Promega) according to the instructions of the manufacturer. Pure plasmid DNA was obtained and quantified with the GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech).

**Comparison of catheter colonization rates.** Bacterial adhesion on polyurethane catheters (polyurethane triple-lumen intravenous catheter, Arrow International) was compared between different wild-type strains: S. epidermidis 10b, 8400 and 1457. In vitro, an inoculum of 10$^7$ cells ml$^{-1}$ was prepared in BHI and catheter fragments (7 mm) were added at t=0. The number of bacteria on each catheter was quantified after 2, 4 and 6 h and 1 and 2 days of incubation at 37 °C. In vivo, pre-incubated catheter fragments were subcutaneously implanted in rats (see below) and bacterial adhesion was determined at 0, 2 and 4 h and 1, 2 and 4 days after implantation.

After gentle cleaning with 0.9 % NaCl, catheters from in vitro or in vivo experiments were placed in a tube containing 1 ml 0.9 % NaCl. Tubes were vortexed for 10 s, sonicated for 10 min at 40 000 Hz in a water bath (Branson 2200, Branson Ultrasonics) and again vortexed for 10 s. Thereafter, tube contents were diluted and 50 µl aliquots of 10-fold dilutions were plated on TSA (Tryptone Soya Agar, Oxoid) plates using a Spiral Plating system. Colonies on all plates were counted manually and the number of bacteria was defined as the mean of at least eight quantitative cultures.

To determine the infection rate of different wild-type strains, the number of catheters still infected with bacteria after 4 days of implantation in vivo was counted.

**DNA and RNA isolation.** Genomic DNA (gDNA) and RNA extraction from planktonic and sessile bacteria in vitro and in vivo were performed as described earlier (Vandecasteele et al., 2001). Briefly, 1 ml bacterial suspension (planktonic) was pelleted for 5 min at 12 000 g at

<table>
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<th>Table 1. Strains used in this study</th>
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<tr>
<td><strong>Strain</strong></td>
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<tr>
<td>10b</td>
</tr>
<tr>
<td>8400</td>
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<tr>
<td>8400rsbU</td>
</tr>
<tr>
<td>8400rsbUVW</td>
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<td>8400rsbUVWsigB</td>
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<tr>
<td>1457</td>
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<tr>
<td>1457rsbU</td>
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<tr>
<td>1457rsbUVW</td>
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<td>1457rsbUVWsigB</td>
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4 °C. The pellet was resuspended in 500 μl NAES buffer (50 mM NaOAc, pH 5.1; 10 mM EDTA, 1 % SDS) and added to a FastRNA tube-blue (Bio 101) supplemented with 500 μl acidified phenol/ chloroform (5:1, v/v), pH 4.5 (Ambion). For sessile bacteria, catheter fragments, gently cleaned with 0.9 % NaCl to remove planktonic bacteria, were added directly to the FastRNA tubes, filled with NAES and acidified phenol/chloroform. Tubes were shaken for 23 s at 6000 r.p.m. (FastPrep, FP120, Bio 101). After shaking, tubes were centrifuged for 5 min at 12,000 g. Supernatant (450 μl) was precipitated with 520 μl isopropyl alcohol and 35 μl 3 M NaOAc and the pellets were washed with 70 % ethanol and resuspended in 150 μl RNase-free water. Fifty microlitres of this sample was diluted in 450 μl sterile distilled water (1/10) and used for gDNA quantification. The remaining 100 μl sterile distilled water purified with the RNeasy Mini kit (Qiagen) according to the manufacturers’ instructions. Reverse transcriptase reaction was performed as described earlier (Vandecasteele et al., 2001).

**Taqman quantitative PCR.** Taqman primers and probes were constructed by use of Primer Express 1.0 (PE Applied Biosystems) and are shown in Table 2. Gene quantification was done on a 2 μl sample (purified cDNA or gDNA), 12.5 μl 2× Taqman PCR master mix (PE Applied Biosystems), 0.9 μM of each primer and 0.2 μM of the probe in a final volume of 25 μl. The thermal cycling conditions were as previously published (Vandecasteele et al., 2001). To allow gene quantification, a standard dilution of a known quantity of the plasmid was included in each run. Every run included a negative control (distilled water) and an RNA sample without reverse transcriptase step (to determine gDNA contamination). Relative gene control (distilled water) and an RNA sample without reverse transcriptase step (to determine gDNA contamination). Relative gene expression values were obtained by the quotient of the number of transcriptase step (to determine gDNA contamination). Relative gene control (distilled water) and an RNA sample without reverse transcriptase step (to determine gDNA contamination). Relative gene expression values were obtained by the quotient of the number of transcriptase step (to determine gDNA contamination).

**Gene expression in vitro.** Two hundred microlitres of an overnight *S. epidermidis* culture was inoculated in 50 ml fresh BHI and incubated at 37 °C in a shaking incubator at 250 r.p.m.; at time 0 catheter fragments were added. At different time points (10), from 0 until 8 h after inoculation, 16 samples, from three independent experiments, were taken for gDNA and RNA extraction, from both planktonic and sessile bacteria.

**In vivo model.** A Fisher rat strain was inbred and has been maintained under germ-free conditions in the Rega Institute, University of Leuven, since 1965 (Van Wijngaerden et al., 1999; Vandecasteele et al., 2002). The first-generation descendants of these germ-free rats, born and reared under normal non-germ-free conditions were used. These rats, exposed to rat flora from birth, were termed EGF-Fisher rats (for ex-germ-free Fisher rats). Animal experimentation guidelines were followed throughout this study.

Prior to implantation, catheter fragments were incubated for 20 min at 37 °C in a physiological salt solution, containing *S. epidermidis*, and thus contaminated with a low inoculum of *S. epidermidis* (1.09×10⁴ cells per catheter (95% CI 9.78×10³–2.00×10⁴)). After incubation, catheters were placed on ice and implanted in EGF-Fisher rats. Anaesthesia was done by a short inhalation period of enflurane gas (Alyrane, Pharmacia). Thereafter rats were kept asleep during the implantation procedure by a gaseous mix of enflurane (20 %) and oxygen (80 %). The back of each rat was shaved and disinfected with chlorhexidine 0.5 % in 70 % alcohol. After a few minutes, a 10 mm incision was made and the subcutis was carefully dissected to create three subcutaneous tunnels. Nine catheter fragments were implanted in each rat. For catheter explantation, rats were euthanized by CO₂ inhalation. The skin was disinfected and catheter fragments were gently removed from the subcutaneous tissue.

**Gene expression in sessile bacteria during in vivo FBI.** Catheter fragments were incubated in a *S. epidermidis* solution and subsequently implanted in rats. Catheters (*n=281*) were explanted 15 and 30 min, 1, 1.5, 2, 4, 6, 12, 24, 48 and 96 h and 1 and 2 weeks after implantation. Subsequently gDNA and RNA isolation was done.

**Influence of σB operon mutations on foreign body colonization.** In vitro *S. epidermidis* 8400 and 1457 wild-type strains and their respective mutants with deletion of rsbU, rsbUVW or the entire rsbUVWsigB operon, were inoculated in BHI (10⁵ cells ml⁻¹), with catheter fragments. At 2 and 4 h and 1, 2 and 4 days after inoculation the number of bacterial cells in the biofilm was determined. We quantified the number of gDNA copies of the housekeeping gene guanylate monokinase (*gmk*) recovered from each catheter. As previously demonstrated (Vandecasteele et al., 2002), the number of

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**Table 2.** Taqman primers and probes (5’–3’).

Probes are labelled FAM-5’ and TAMRA-3’. F, forward primer; P, probe; R, reverse primer.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer or probe sequence</th>
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<tr>
<td>gmk</td>
<td>F  AAGGTGCTAAGCAGAAAGAAAATAATT</td>
</tr>
<tr>
<td></td>
<td>P  ATGCCTTGGCTATATTTTAAATCCCATC</td>
</tr>
<tr>
<td></td>
<td>R  CAAACACGCTTCCTTCAGCTATCAT</td>
</tr>
<tr>
<td>icaA</td>
<td>F  AAGATGGATGTTATGGGATACGTGA</td>
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<tr>
<td></td>
<td>P  TTGCCTTCTCATGGAAAATCCCATCCTTTTGTATA</td>
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<tr>
<td></td>
<td>R  CATAGGACCGTGGTTCGTAATA</td>
</tr>
<tr>
<td>aap</td>
<td>F  AGAACCTCACAACCTGAGAACCCTGGA</td>
</tr>
<tr>
<td></td>
<td>P  TATAGTTTCCTCGTTTTATGTTCTTATTAAGCATTCGC</td>
</tr>
<tr>
<td></td>
<td>R  ACCCTTTCAACGGATGGCGGTAAT</td>
</tr>
<tr>
<td>rsbU</td>
<td>F  TGGCGGATGACCTGGCTATAT</td>
</tr>
<tr>
<td></td>
<td>P  CGTTTGGGAAATGCGTGGCAGGAA</td>
</tr>
<tr>
<td></td>
<td>R  GTTTTGGGACTGATCTTTAAAGC</td>
</tr>
<tr>
<td>asp23</td>
<td>F  CAGCACGCTTTTTTTTCTC</td>
</tr>
<tr>
<td></td>
<td>P  TGTTACCGTTGGAAAGGCA</td>
</tr>
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<td></td>
<td>R  CATGAAAGGTTGGCTTCAGC</td>
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gmk copies per catheter correlated very well with the number of c.f.u. per catheter.

In vivo differences in biofilm formation were determined by gDNA quantification of gmk, 4 h, 1, 2 and 4 days, and 2 and 3 weeks after subcutaneous implantation.

Confocal laser-scanning microscopy (CLSM). Five hundred microlitres of an overnight culture were inoculated in 4.5 ml fresh BHI and incubated at 37 °C in a shaking incubator at 250 r.p.m. for 1.5 h. Bacteria were pelleted and resuspended in 3 ml BHI. One millilitre of this bacterial suspension was added to a glass disc (coverslips 12 mm diameter, Menzel-Glaser), covered with a thin polyurethane layer (Opsite spray, Smith&Nephew), and incubated at 37 °C. Biofilm formation was visualized by CLSM at different time points. Wheatgerm agglutinin (WGA) conjugated with fluorescent Alexa Fluor 633 (Molecular Probes), which binds to PIA, was used to study matrix production in biofilms. Stock solutions of this lectin (500 μg ml−1) were prepared in phosphate-buffered saline (PBS) and stored frozen at −20 °C. One hundred microlitres of this stock solution was carefully applied directly on top of the biofilms. After incubation for 30 min in the dark at room temperature, excess staining solution was removed by washing twice with PBS. Counter-staining of bacterial cells within the biofilm was performed by use of the fluorescent DNA-binding stain SYTO 9 (component A of the LIVE/DEAD BacLight Bacterial Viability kit, Molecular Probes) for dead bacteria. After lectin staining, the biofilms were treated with 100 μl of a freshly prepared solution containing 1 μl SYTO 9 and 1 μl SYTOX Orange per millilitre of physiological salt solution. Biofilms were incubated for 30 min in the dark at room temperature. Excess staining was removed by washing once with PBS.

Stained biofilms were examined using an LSM 510 confocal laser-scanning microscope (Zeiss). The system consisted of a laser-scanning module that was mounted on an Axiovert 100 M BP inverted microscope (Zeiss), an argon laser (458, 488 and 514 nm) and two helium-neon lasers (543 and 633 nm). Images were recorded at an excitation wavelength of 488 nm and an emission wavelength of 500 nm for SYTO 9, at an excitation wavelength of 543 nm and an emission wavelength of 570 nm for SYTOX Orange and at an excitation wavelength of 633 nm and an emission wavelength of 647 nm for Alexa Fluor 633 labelled lectins. Digital image analysis of the CLSM optical thin sections was performed with the Zeiss LSM software (version 4.1).

Scanning electron microscopy (SEM). In vivo biofilm formation was visualized by SEM. At explantation, catheter fragments were 99% NaCl before fixation. Adherent bacteria were fixed for 12 h at 4 °C in 2.5% glutaraldehyde in 0.1 mol l−1 sodium cacodylate buffer at pH 7.4, rinsed in 0.2 mol l−1 sodium cacodylate buffer at pH 7.4 for 1 h with three changes, rinsed with distilled water for 1 min and then dehydrated in 10 min steps in a series of ascending ethanol baths (25%, 50%, 75%, 95% and 100%). Dehydrated samples were air-dried following a bath of hexamethyldisilazan and sputter coated with platinum (Agar, Auto Sputter Coater). Fixed samples were observed with a JEOL JSM7401F field emission scanning electron microscope in a high-vacuum mode with a conventional Everhart–Thornley detector.

Statistical analysis. All statistical analyses were performed with Prisim (GraphPad Prism 4.0). Data from both in vitro and in vivo experiments were log10-transformed to satisfy the requirements of normality (Shapiro–Wilk test for normality).

One-way analysis of variance, ANOVA, was used to test if there was a significant different evolution in gene expression between the different time points. A significant difference in the evolution over time of the gene expression levels between the planktonic versus sessile group was tested by two-way ANOVA analysis. When ANOVA analysis was significant, multiple t-tests with a correction for multiple comparisons (Bonferroni multiple comparison test) were used to locate the significant differences.

A significant change in bacterial colonization over time within one strain was tested by one-way ANOVA analysis. The Bonferroni multiple comparison test was used to determine which time points differed at α=0.05, with a correction for multiple comparisons. Significant differences in bacterial adhesion over time between the different S. epidermidis strains in vitro and in vivo were examined using two-way ANOVA. The Bonferroni multiple comparison test was used to locate the significant differences.

RESULTS

Bacterial growth and catheter colonization rates

Bacteria from an overnight culture were resuspended in 50 ml BHI at a density equivalent to an OD600 of 0.002 (1 × 10⁶ cells ml⁻¹). No differences in bacterial growth rate and growth yield could be found between the different wild-type and mutant strains; stationary phase was attained after approximately 7 h (data not shown).

Bacterial adhesion of the wild-type strains, 10b, 8400 and 1457, was compared in vitro and in vivo. The number of c.f.u. per catheter was determined at different time intervals from 2 h up to 2 days in vitro and up to 4 days in vivo. The number of c.f.u. was equal for the three strains in vitro (two-way ANOVA; Bonferroni; P=0.2513) and in the in vivo foreign body rat model (two-way ANOVA; Bonferroni; P=0.4630). Also, the number of catheters still colonized after 4 days in vivo did not differ between the wild-type strains (two-way ANOVA; Bonferroni; P=0.9939). After 4 days, out of a total of 63 catheters implanted in vivo, 81% and 83% and 83% were still colonized with adherent bacteria for S. epidermidis 10b, 8400 and 1457, respectively (data not shown).

Comparison of gene expression in biofilm forming and planktonic bacteria in vitro

S. epidermidis was inoculated in BHI. Gene expression patterns of S. epidermidis 10b are shown in Fig. 1. In planktonic bacteria an initial increase in icaA expression was followed by decreased expression in the stationary growth phase (significant evolution over time, one-way ANOVA, P<0.01) (Fig. 1b). In sessile bacteria the initial increase in icaA expression was more pronounced (significant change over time, one-way ANOVA, P<0.0001) and expression remained at a high level until it started to decrease from 7 h onwards. Expression of aap in both planktonic and sessile bacteria initially decreased (significant change over time, one-way ANOVA, P<0.0001, for both groups) (Fig. 1c). In sessile bacteria, however, a temporary increase in aap expression was observed between 4 and 7 h after the start of the experiment.
For all $$\sigma^B$$ operon genes (only $$rsbU$$ expression is shown, Fig. 1e) we found in planktonic bacteria a simultaneous increase in expression levels in the early exponential growth phase leading to maximal expression levels in the mid-exponential growth phase followed by a gradual decrease. In sessile bacteria $$rsbU$$ expression increased significantly more than in planktonic bacteria up to 5 h post-inoculation (two-way ANOVA, $$P<0.0001$$). The expression levels of $$asp23$$ (Fig. 1d) mirrored the expression pattern of $$\sigma^B$$ in planktonic and in sessile bacteria.

**Gene expression analysis in biofilm-forming bacteria in vivo**

*In vivo* gene expression in *S. epidermidis* 10b is summarized in Fig. 2. After implantation of the catheter fragments in
the rats, icaA expression increased temporarily and subsequently slowly decreased up to 2 weeks after implantation (significant change over time, one-way ANOVA; \(P<0.0001\)). Expression of aap declined progressively from 15 min after implantation of the catheter fragments (significant evolution over time, one-way ANOVA; \(P<0.0001\)). For rsbU, however, expression initially increased and peaked after 1 h, followed by a decrease in rsbU expression and from 1 day onwards again an increase leading to high expression levels in the 1- and 2-week-old biofilm. Expression levels of asp23 confirmed the upregulation of \(\sigma^B\) in the older biofilms. The evolution of rsbU and asp23 expression over time was significant (one-way ANOVA; \(P<0.0001\), for both groups).

**Effect of sigB mutations on foreign body colonization in vitro**

Both *S. epidermidis* strains 8400 and 1457, and their rsbU, rsbUVW and rsbUVWsigB mutants, were incubated in BHI. Catheter fragments were added and bacterial colonization was followed over a period of 4 days (Fig. 3). For both strains, there were no significant differences in the number of colonizing cells between the wild-type and the rsbUVW mutants (two-way ANOVA; \(P>0.05\)). However, mutation of rsbU alone and mutation of the entire rsbUVWsigB operon led to a significant reduction of the number of colonizing cells from 6 h after incubation onwards (two-way ANOVA; \(P<0.0001\) from 6 h onwards, for both groups in comparison to wild-types and rsbUVW mutants).

CLSM data confirmed these results. After 24 h incubation in BHI, clusters of cells and PIA production could be visualized for the 1457 wild-type and 1457 rsbUVW::erm strain. By employing CLSM, the biofilm thicknesses of five independent image stacks recorded with the 100 \(\times\) lens were averaged. The wild-type biofilm had a fairly uniform thickness [16.91 \(\mu\)m (95% CI 15.19–18.75)] of up to 18.75 \(\mu\)m and a mean disc covering of 50.44%. The 1457rsbUVW::erm strain presented a more dispersed and opaque biofilm over a larger surface (mean disc covering: 71.91%) with a mean thickness of 4.33 \(\mu\)m. In both strains, extracellular matrix was equally present and a limited number of dead cells were scattered throughout the biofilm. For both 1457rsbU::erm and 1457rsbUVWsigB::erm, fewer bacteria were visible, and only a small number of cell clusters could be found. For both of these mutants almost no PIA could be detected (PIA non-specific dot blot, results not shown). Similar
confocal images were obtained for the 8400 wild-type strain and its SigB mutants: only a small number of cell clusters and almost no PIA could be visualized for mutants with inactive SigB (data not shown).

**Effect of sigB mutations on foreign body colonization in vivo**

All previously mentioned strains were subcutaneously implanted in EGF-Fisher rats. Catheter colonization was evaluated at different time intervals, starting immediately before implantation (0 h), until 3 weeks after implantation (Fig. 4a, b). For all strains, we observed that the number of colonizing bacteria increased initially by a factor of 10.47 (95 %, 8.28–12.66) and decreased again by a factor of 21.38 (95 %, 19.61–23.15) in the 2- and 3-week-old biofilm. As observed for catheters in vitro, there were no significant differences between both wild-types and their respective rsbUVW mutants (two-way ANOVA; P>0.05). For these strains, the number of colonizing bacteria increased for 2–4 days after implantation and then on decreased (significant evolution over time, one-way ANOVA; P<0.01, for both groups). Mutation of rsbU alone had the same effect as mutation of the entire rsbUVWsigB operon (two-way ANOVA, P>0.05). Bacterial colonization also increased initially but started to decrease earlier and to a greater extent than for the wild-type strains.

CLSM data of in vivo biofilms could not confirm these data due to the presence of rat tissue and immune cells that distorted the confocal images. SEM data confirmed the differences in catheter colonization and in PIA production (Fig. 4c–f). These pictures show differences in the nature of the extracellular matrix between the strains. Strains with active SigB formed biofilms covered with an opaque and dense well-formed matrix, in contrast to strains with inactive SigB, which showed adherent cells linked to each other by a filamentous network.
DISCUSSION

We observed identical expression patterns for rsbU, rsbV, rsbW and sigB in S. epidermidis strain 10b. Although rsbU transcription depends on the PA promoter and rsbV, rsbW and sigB depend on the PB promoter, it has been shown in Bacillus subtilis that genes downstream of the PB promoter can be transcribed by polymerase activity originating at PA (Wise & Price, 1995). Knobloch et al. (2004), on the other hand, only found an rsbU transcriptional signal that was at least 10-fold lower compared to the other genes of the $\sigma^B$ operon. A possible explanation is that these differences are strain-dependent (Blevins et al., 2002; Ziebuhr et al., 1997).

A role for sigB in biofilm formation is consistent with the large difference in expression levels between planktonic and sessile bacteria. Foreign body colonization in vitro induced a sharp increase in SigB activity, significantly higher than in planktonic bacteria. In vivo expression data confirmed these in vitro data and showed an initial upregulation of all $\sigma^B$ operon genes, shortly after implantation in the rat, and a second upregulation, in the late phase of FBI. The initial increase in expression of rsbU and of all the other $\sigma^B$ operon genes in vitro as well as in vivo was, however, not immediate but only occurred after a lag period of 2–5 h. Expression levels differed between the different strains, but evolution over time was identical among S. epidermidis strains 10b, 8400 and 1457 (data not shown).

The increase in icaA expression clearly preceded the increase in rsbU expression in vitro as well as in vivo. This is consistent with factors other than SigB inducing icaADBC operon expression (Conlon et al., 2002; Mack et al., 2000, 2007). Increased expression of icaA and aap is suggestive of S. epidermidis cell accumulation and biofilm formation (Hussain et al., 1997; Mack et al., 1996). We have no explanation as to why the transiently increased expression of aap after 4 h in vitro was not found in vivo. There are no data regarding the effect of SigB on aap.

The role of SigB in biofilm formation was confirmed by the effect of $\sigma^B$ operon mutations on the biofilm-forming capacity of different S. epidermidis strains with a strong biofilm-positive phenotype (Knobloch et al., 2004; Mack et al., 1992). Comparison of biofilm formation in vitro by the wild-type strains with their respective mutants showed no significant differences after 2 h. After 6 h, however, differences were clearly apparent, coinciding with the highest expression level of the $\sigma^B$ operon. In the rsbU and

![Graphs showing expression levels over time for strains 8400 and 1457.](image-url)

**Fig. 4.** Biofilm formation by wild-type strains S. epidermidis 8400 (a, white bars) and 1457 (b, white bars), and their respective mutants with inactivation of rsbU (dark grey bars), rsbUVW (light grey bars) or the entire $\sigma^B$ operon (black bars), was quantified in an in vivo foreign body model. Catheters were explanted from 0 to 3 weeks after implantation. The number of adherent bacteria was quantified as the log$_{10}$ of gDNA copies of the housekeeping gene *gmk* per catheter. The error bars represent the 95% CI. Eighteen independent samples were assessed at each time point. Bacterial colonization in rsbU and rsbUVWsigB mutant strains decreased significantly earlier and to a higher extent (two-way ANOVA; **P<0.001) than in the wild-type and rsbUVW mutant strains. Low (c, e 1000×) and high (d, f 5000×) magnification SEM of sessile cells of S. epidermidis strains 1457 (c, d) and 1457rsbU (e, f) on catheter surface, 1 week after implantation in vivo.
rsbUVWsigB mutants (both without SigB activity) there was reduced biofilm formation. Our data show that this was due to both a reduced number of cells in the biofilm and reduced PIA formation. CLSM confirmed these data. For strains 1457rsbU::erm and 1457rsbUVWsigB::erm only a small number of dispersed cell clusters could be found. The wild-type and rsbUVW::erm strain both showed multi-layered adherent cells and PIA production. However, biofilms formed by the 1457 and 8400 wild-type strains versus their respective rsbUVW mutants were different with respect to the thickness of the biofilm and the continuity of the layer. The wild-type biofilm was significantly thicker and less spread out. These differences are probably due to a different sigB expression pattern. Both in vitro and in vivo wild-type strains showed a time-dependent evolution of sigB expression, whereas in the rsbUVW mutant strains sigB was constitutively expressed at a constant level (data not shown). This suggests a role for temporal variations of sigB expression in the biofilm structure.

Bacteria with inactive SigB seemed able to attach to the foreign body but not to produce PIA. This is consistent with earlier data (Knobloch et al., 2001; Mack et al., 2000). Data from in vitro experiments (Jager et al., 2005) suggest that PIA is necessary for biofilm stability. This could explain why even after 4 days, biofilms formed by mutants without SigB activity still consisted of dispersed clusters of a small number of bacteria.

In vivo studies confirmed the in vitro data, although the differences in SigB-deficient biofilms were slower to appear. Throughout the first day after implantation, there were no significant differences between the mutants without SigB activity and the wild-types and rsbUVW mutants in terms of the number of adherent cells. However, after 2 days, catheter colonization by the rsbU and rsbUVWsigB mutants was reduced, and this reduced level of colonization was maintained for the rest of the implantation period. Reduction of the number of colonizing cells was also observed in vivo in the wild-type strains and the rsbUVW mutants but the decrease was less pronounced than in the rsbU and rsbUVWsigB mutants. In addition, SEM pictures of catheters 1 week after implantation in the rat showed clear differences in extracellular matrix production between the different strains. Strains with active SigB produced a dense matrix surrounding and protecting the biofilm-forming cells, in contrast to mutant strains with inactive SigB, which produced a fibrous network connecting the attached cells. Even though the number of adherent cells was decreased in mutants with a dysfunctional σB gene, there remained dispersed clusters of adherent bacteria not covered by measurable amounts of extracellular matrix.

Both in vitro and in vivo, rsbU and σB operon mutations seemed to have no effect on the early phase of biofilm formation, i.e. catheter attachment, in accordance with the expression patterns of the σB operon genes. The effect of these mutations was only seen in the later phases of biofilm formation, suggesting that absence of SigB activity leads to an inherent reduction in the persistence of the biofilm, perhaps because protection by the extracellular matrix becomes more important for the biofilm-forming cells. Morphological differences between wild-type and rsbUVW mutant strains suggest a role for temporal variations in sigB expression in biofilm structure.

Although absence of sigB expression does not completely abolish the biofilm-forming capacity of the strains that were examined, these data confirm an important role for SigB in the ultrastructure and stability of biofilms.

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