The role of σ^B in persistence of *Staphylococcus epidermidis* foreign body infection

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Staphylococcal biofilm formation depends on the transcription factor σ^B . We further investigated the role of σ^B in biofilm formation and persistence *in vitro* and *in vivo* in a subcutaneous rat model. As expected, expression of all σ^B operon genes was transiently higher in the first 6 h of biofilm formation compared to planktonic bacteria, concurrent with a temporary upregulation of *icaA* and *aap* expression. However, we also observed a second upregulation of *sigB* expression in biofilm more than 2 days old without upregulation of *icaA* or *aap*. Biofilm formation by *Staphylococcus epidermidis* strains 8400 and 1457 was compared to that of isogenic mutants with inactivation of *rsbU*, of *rsbUVW* and of the entire σ^B operon. Both wild-type strains and the constitutively *sigB*-expressing *rsbUVW* mutant showed a strong biofilm-positive phenotype. The *rsbUVW* mutant biofilm was, however, thinner and more evenly spread than the wild-type biofilm. Inactivation of SigB in the *rsbUVWsigB* mutant or mutation of the positive regulator RsbU reduced both the number of sessile bacteria and polysaccharide intercellular adhesin (PIA) synthesis. These differences between the wild-types and their respective mutants appeared after 6 h in *in vitro* biofilms but only after 4 days in *in vivo* biofilms. Our results provide additional evidence for a role for σ^B in biofilm formation. They also suggest a role for σ^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.

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INTRODUCTION

Coagulase-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.

Abbreviations: CLSM, confocal laser-scanning microscopy; CoNS, coagulase-negative staphylococci; FBI, foreign body infection; gDNA, genomic DNA; PIA, polysaccharide intercellular adhesin; SEM, scanning electron microscopy.

Formation of adherent multilayered biofilms embedded into a glycocalyx 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), synthesized by products of the *icaADBC* operon, mediates cell-to-cell adhesion and is essential for bacterial cell accumulation (Dunne, 2002; Gerke *et al.*, 1998). Expression of the *icaADBC* operon depends on a variety of environmental conditions, and at least four unlinked genes (*icaR*, σ^B , the *purR* locus and SarA) are known to control PIA synthesis at the level of transcription (Conlon *et al.*, 2002; Mack *et al.*, 2000, 2007). However, cell-wall-linked Aap (accumulation-associated protein) and teichoic acids (Hussain *et al.*, 1992) can also mediate intercellular adhesion and biofilm formation,

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: σ^A , the housekeeping sigma factor, and two alternative sigma factors σ^B and σ^H (Pane-Farre *et al.*, 2006). In contrast to σ^H , the alternative sigma factor σ^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 σ^B operon in staphylococci contains four genes: *rsbU*, *rsbV*, *rsbW* and *sigB*. The *rsbW* gene product is 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_A , is found upstream of *rsbU*, and a SigB-dependent promoter, P_B , precedes the last three genes of the operon. SigB increases its own transcription as a consequence of its activation, further inducing the entire σ^B regulon (Fouet *et al.*, 2000). σ^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 σ^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 σ^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 OD₆₀₀ 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* (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 10⁵ 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 000g at

Table 1. Strains used in this study

Strain	Reference	Comments
10b	Vandecasteele <i>et al.</i> (2001)	Isolate from a patient with a proven catheter-related bloodstream infection
8400	Mack <i>et al.</i> (1992)	Blood culture isolate
8400 <i>rsbU</i>	Knobloch <i>et al.</i> (2004)	<i>rsbU::erm</i> derivative from <i>S. epidermidis</i> 8400 derived by phage transduction
8400 <i>rsbUVW</i>	Knobloch <i>et al.</i> (2004)	<i>rsbUVW::erm</i> derivative from <i>S. epidermidis</i> 8400 derived by phage transduction
8400 <i>rsbUVWsigB</i>	Knobloch <i>et al.</i> (2004)	<i>rsbUVWsigB::erm</i> derivative from <i>S. epidermidis</i> 8400 derived by phage transduction
1457	Mack <i>et al.</i> (1992)	Isolate from infected central venous catheter
1457 <i>rsbU</i>	Knobloch <i>et al.</i> (2004)	<i>rsbU::erm</i> derivative from <i>S. epidermidis</i> 1457 derived by allelic gene replacement
1457 <i>rsbUVW</i>	Knobloch <i>et al.</i> (2004)	<i>rsbUVW::erm</i> derivative from <i>S. epidermidis</i> 1457 derived by allelic gene replacement
1457 <i>rsbUVWsigB</i>	Knobloch <i>et al.</i> (2004)	<i>rsbUVWsigB::erm</i> derivative from <i>S. epidermidis</i> 1457 derived by allelic gene replacement

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 expression values were obtained by the quotient of the number of copies of cDNA per ml (a measure of the amount of RNA) and the number of bacteria per ml (gDNA copies).

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* [1.09×10^4 cells per catheter (95% CI $9.78 \times 10^3 - 2.00 \times 10^4$)]. 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 σ^B 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 (10^5 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 *gmk* recovered from each catheter. As previously demonstrated (Vandecasteele *et al.*, 2002), the number of

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

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

Gene		Primer or probe sequence
<i>gmk</i>	F	AAGGTGCTAAGCAAGTAAGAAAGAAATT
	P	ATGCGTTGTTTCATATTTTTAGCGCCTCCA
	R	CAACAAGACGTTCTTTCAAGTCATCT
<i>icaA</i>	F	AAAGATGTAGGTTATTGGGATACTGACA
	P	TTGCTGTTTCATGGAACTCCATCTTTTGATTA
	R	CATAGAGCACGTGGTTCGTACTTAA
<i>aap</i>	F	AGAACCTACAACCTTCAGAACCTGTGAA
	P	TATAGTTCTCCGTTTATGTCCTTATTAAGCATGCCTGC
	R	ACCTTTAACCGTAGTTGGCGGTAT
<i>rsbU</i>	F	TGGGCATGAACCTGGCTATAT
	P	CGTTTGAGGAAATTGGTGTGCGAGGAA
	R	GTTTTGGGACTCACACTTAGAACT
<i>asp23</i>	F	CAGCAGCTTGTTTTCTCCA
	P	TGTTACCGTTTGAGAAAGCG
	R	CATGAAAGGTGGCTTCACAG

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 living and SYTOX Orange (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 rinsed with 0.9 % 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 hexamethyldisilazane 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 Prism4 (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 testing (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 $\alpha=0.05$, 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 %, 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.

Fig. 1. (a) *In vitro* growth of planktonic (○) and sessile (●) *S. epidermidis* 10b in BHI. The number of bacteria is given as the log₁₀ of the number of gDNA copies of the housekeeping gene *gmk*. (b–e) *icaA*, *aap*, *asp23* and *rsbU* expression (cDNA/gDNA) in planktonic (□) and sessile (■) bacteria. Bars show the 95 % CI. Sixteen samples from three independent cultures were assessed at each time point.

For all σ^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 σ^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

Fig. 2. *icaA*, *aap*, *asp23* and *rsbU* expression in sessile *S. epidermidis* 10b from 0 until 2 weeks (336 h) after implantation *in vivo*. Gene expression is quantified as $\log_{10}(\text{cDNA/gDNA})$. The expression level at time point 0 represents the expression level just prior to implantation. The error bars represent the 95 % CI. Eighteen independent samples were assessed at each time point.

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 σ^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 μm (95 % CI 15.19–18.75)] of up to 18.75 μm and a mean disc covering of 50.44 %. The 1457*rsbUVW::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 μm . In both strains, extracellular matrix was equally present and a limited number of dead cells were scattered throughout the biofilm. For both 1457*rsbU::erm* and 1457*rsbUVWsigB::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

Fig. 3. Biofilm formation *in vitro* in BHI from 0 to 4 days. The 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), and the entire σ^B operon (black bars), were investigated. The number of adherent bacteria is given on the y-axis as log₁₀ of gDNA copies of the housekeeping gene *gmk* per catheter. Bars represent the 95% CI. Fifteen samples from three independent cultures were assessed at each time point. Catheter colonization was significantly reduced in the *rsbU* and *rsbUVWsigB* mutants (two-way ANOVA; $*P < 0.01$, $**P < 0.001$), whereas no significant differences were detected between the wild-type and the *rsbUVW* mutant strains (two-way ANOVA; $P > 0.05$). (c) CLSM of sessile bacteria. Bacteria were grown overnight in BHI and then incubated with polyurethane coated glass discs in BHI. Pictures were taken after 24 h incubation. For each time point images show the *xy*-, *xz*- (green line) and *yz*- (red line) planes. The *xy*- and orthogonal images of *S. epidermidis* biofilms showed living (blue, SYTO 9) and dead (green, SYTOX Orange) cells embedded in extracellular matrix (red, WGA- Alexa Fluor 633). Experiments were performed in triplicate and biofilms were viewed at 100 $\times$ magnification.

confocal images were obtained for the 8400 wild-type strain and its σ^B operon 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 from 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.

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 σ^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 *rsbUVW* σ^B 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 $\times$) and high (d, f 5000 $\times$) magnification SEM of sessile cells of *S. epidermidis* strains 1457 (c, d) and 1457*rsbU* (e, f) on catheter surface, 1 week after implantation *in vivo*.

DISCUSSION

We observed identical expression patterns for *rsbU*, *rsbV*, *rsbW* and *sigB* in *S. epidermidis* strain 10b. Although *rsbU* transcription depends on the P_A promoter and *rsbV*, *rsbW* and *sigB* depend on the P_B promoter, it has been shown in *Bacillus subtilis* that genes downstream of the P_B promoter can be transcribed by polymerase activity originating at P_A (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 σ^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 σ^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 σ^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 σ^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 σ^B operon. In the *rsbU* and

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 1457*rsbU::erm* and 1457*rsbUVWsigB::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 σ^B gene, there remained dispersed clusters of adherent bacteria not covered by measurable amounts of extracellular matrix.

Both *in vitro* and *in vivo*, *rsbU* and σ^B 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 σ^B 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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