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Das *extracellular matrix binding protein* von *Staphylococcus epidermidis* vermittelt Biofilmbildung und Adhärenz an Fibronektin

Dissertation

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„Der Gelehrte und seine Wissenschaft sind in den gesellschaftlichen Apparat eingespannt, ihre Leistung ist ein Moment der Selbsterhaltung, der fortwährenden Reproduktion des Bestehenden, gleichviel, was sie sich selbst für einen Reim darauf machen.“

Max Horkheimer

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The giant extracellular matrix-binding protein of *Staphylococcus epidermidis* mediates biofilm accumulation and attachment to fibronectin

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Summary

Virulence of nosocomial pathogen *Staphylococcus epidermidis* is essentially related to formation of adherent biofilms, assembled by bacterial attachment to an artificial surface and subsequent production of a matrix that mediates interbacterial adhesion. Growing evidence supports the idea that proteins are functionally involved in *S. epidermidis* biofilm accumulation. We found that in *S. epidermidis* 1585v overexpression of a 460 kDa truncated isoform of the extracellular matrix-binding protein (Embp) is necessary for biofilm formation. Embp is a giant fibronectin-binding protein harbouring 59 Found In Various Architectures (FIVAR) and 38 protein G-related albumin-binding (GA) domains. Studies using defined Embp-positive and -negative *S. epidermidis* strains proved that Embp is sufficient and necessary for biofilm formation. Further data showed that the FIVAR domains of Embp mediate binding of *S. epidermidis* to solid-phase attached fibronectin, constituting the first step of biofilm formation on conditioned surfaces. The binding site in fibronectin was assigned to the fibronectin domain type III12. Embp-mediated biofilm formation also protected *S. epidermidis* from phagocytosis by macrophages. Thus, Embp is a multifunctional cell surface protein that mediates attachment to host extracellular matrix, biofilm accumulation and escape from phagocytosis, and therefore is well suited for promoting implant-associated infections.

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Introduction

Staphylococcus epidermidis has emerged as a leading pathogen in nosocomial infections (Wisplinghoff *et al.*, 2003; Karlowsky *et al.*, 2004; Mack *et al.*, 2006). Most frequently, *S. epidermidis* infections are observed after implantation of foreign-materials like prosthetic joints, central venous catheters and artificial heart valves (Rohde *et al.*, 2006). The tremendous success of *S. epidermidis* is based on the species' ability to establish adherent multilayered bacterial communities on natural and artificial surfaces (Mack, 1999; Götz, 2002). These so-called biofilms render *S. epidermidis* less susceptible to phagocytosis (Vuong *et al.*, 2004a; Kristian *et al.*, 2008) and antibacterials (Knobloch *et al.*, 2002; 2008), leading to persistent, difficult-to-treat infections. Biofilm formation is a two-step process during which the bacteria first adhere to the surface to be colonized (primary attachment), and subsequently accumulate into a complex, multilayered biofilm architecture (accumulative phase) (Rohde *et al.*, 2006). *S. epidermidis* factors binding to host extracellular matrix components like fibrinogen-binding protein Fbe (Nilsson *et al.*, 1998), vitronectin-binding autolysins AtlE (Heilmann *et al.*, 1997) and Aae (Heilmann *et al.*, 2003) are thought to critically determine primary attachment. The accumulative phase of biofilm formation depends on the expression of intercellular adhesive properties which are thought to be responsible for the integration of *S. epidermidis* cells in the complex biofilm structure (Mack *et al.*, 2006). The polysaccharide intercellular adhesin (PIA) (Mack *et al.*, 1996; 2009) has been intensively studied as an important component of the *S. epidermidis* biofilm matrix. PIA turned out to be a virulence determining factor in two foreign-body animal infection models (Rupp *et al.*, 1999a,b) and a *Caenorhabditis elegans* infection model (Begun *et al.*, 2007). In

addition, epidemiological studies describing *icaADBC* to be highly prevalent in invasive isolates suggested that PIA is also essential for the *S. epidermidis* pathogenicity in humans (Ziebuhr *et al.*, 1997; Frebourg *et al.*, 2000; Galdbart *et al.*, 2000; Rohde *et al.*, 2004). *IcaADBC*-negative strains were therefore regarded as prototypic commensal, avirulent strains (Zhang *et al.*, 2003; Li *et al.*, 2005). However, there is growing epidemiological evidence that PIA is not by all means necessary for *S. epidermidis* biofilm formation and the establishment of persistent infections. Several studies identified significant numbers of *icaADBC*-negative, clinically relevant *S. epidermidis* strains in strain collections from infected central venous catheters, cerebro-spinal fluid shunts, pacemakers and prosthetic joint infections (Klug *et al.*, 2003; Frank *et al.*, 2004; Bradford *et al.*, 2006; Kogan *et al.*, 2006; Petrelli *et al.*, 2006; Hennig *et al.*, 2007; Rohde *et al.*, 2007). In line with these observations, *icaADBC*-negative strains producing no biofilms *in vitro* displayed the same pathogenicity compared with *icaADBC*- and biofilm-positive strains in a guinea pig tissue cage infection model (Francois *et al.*, 2003; Chokr *et al.*, 2007), questioning the exclusive role of PIA in biofilm-related *S. epidermidis* infections. More recently, we were able to demonstrate that the cell wall-linked accumulation-associated protein Aap can functionally substitute PIA as an intercellular adhesin (Rohde *et al.*, 2005). Thereupon, further studies supported the idea that Aap plays an important role in *S. epidermidis* biofilm formation (Sun *et al.*, 2005; Petrelli *et al.*, 2006; Rohde *et al.*, 2007; Stevens *et al.*, 2008). Hence, at present it is clear that *S. epidermidis* biofilm accumulation is a redundantly organized, multifactorial process, potentially involving a plethora of adhesive factors.

It is well known that *S. epidermidis* biofilm formation is subject to phase variation leading to the spontaneous switch between a biofilm-positive and -negative phenotype. This can in PIA-producing *S. epidermidis* strains genetically be linked to the reversible or irreversible inactivation of *icaADBC* or superimposed regulatory systems (Ziebuhr *et al.*, 1999; Conlon *et al.*, 2004; Nuryastuti *et al.*, 2008). In addition, it must be emphasized that mechanisms involved in *icaADBC*-independent biofilm formation could be silent under *in vitro* culture conditions (Rohde *et al.*, 2005). In turn, a biofilm-negative phenotype displayed under these conditions not necessarily reflects the general inability of a *S. epidermidis* isolate to form biofilms. By enriching for adherent cells we obtained biofilm-positive *S. epidermidis* variant 1585v from clinically significant, *in vitro* biofilm-negative strain 1585. *S. epidermidis* 1585v biofilm formation is mediated by a novel mechanism of biofilm formation based on expression of a 460 kDa isoform of the giant 1 MDa extracellular matrix-binding protein (Embp). Embp is sufficient for biofilm formation in *icaADBC*- and *aap*-negative *S. epidermidis* and combines intercellular adhesive and extracellular matrix binding properties. A comprehensive understanding of the different mechanisms contributing to biofilm formation is of major importance, as from here potential targets for preventive strategies like surface modifications or vaccine development can evolve (Götz, 2004; Rohde *et al.*, 2006).

Results

Identification and characterization of biofilm-positive S. epidermidis *1585v*

By passaging and enrichment of adherent cells a biofilm-positive variant 1585v was selected from clinically significant, *icaADBC*- and *aap*-negative *S. epidermidis* 1585 (Fig. 1A). In contrast to the parent strain *S. epidermidis* 1585, *S. epidermidis* 1585v formed large cell clusters under planctonic growth conditions, showing that the biofilm-positive phenotype most likely resulted from the expression of intercellular adhesive cell surface properties (data not shown). As biofilms of *S. epidermidis* 1585v were readily disrupted by proteinase K, but not by PIA degrading β-1,6-hexosaminidase DspB (Fig. 1B), intercellular adhesion presumably depends on proteinaceous structures.

Transposon mutagenesis of biofilm-positive S. epidermidis *1585v*

Aiming at identifying the mechanisms involved in biofilm formation of *icaADBC*- and *aap*-negative *S. epidermidis* 1585v, Tn*917* transposon mutagenesis was carried out using the plasmid-cured, heat-adapted *S. epidermidis* $1585v_{hc}$ (see *Experimental procedures*). By screening 3515 Tn*917* mutants, two mutants TM135 and TM84 displaying an abolished or significantly reduced biofilm-forming capability were identified (Fig. 1A). The corresponding transposon insertions were back transduced into *S. epidermidis* 1585v by phage transduction, resulting in strains M84 and M135. As these transductants M84 and M135 displayed an impaired biofilm-forming capability comparable to that of the respective transposon mutants in *S. epidermidis* $1585v_{hc}$, genetic linkage between transposon insertions and altered phenotypes was proven.

No significant quantitative difference between *S. epidermidis* 1585v and M135 in primary attachment to NunclonΔ was found (Fig. 1C), providing strong evidence that the biofilm-negative phenotype of the respective mutants resulted from the direct or indirect insertional inactivation of structures involved in intercellular adhesion, thereby impairing the accumulative phase of biofilm formation.

Analysis of transposon mutants M84 and M135

By applying a PCR strategy for amplification and subsequent sequencing of staphylococcal chromosomal

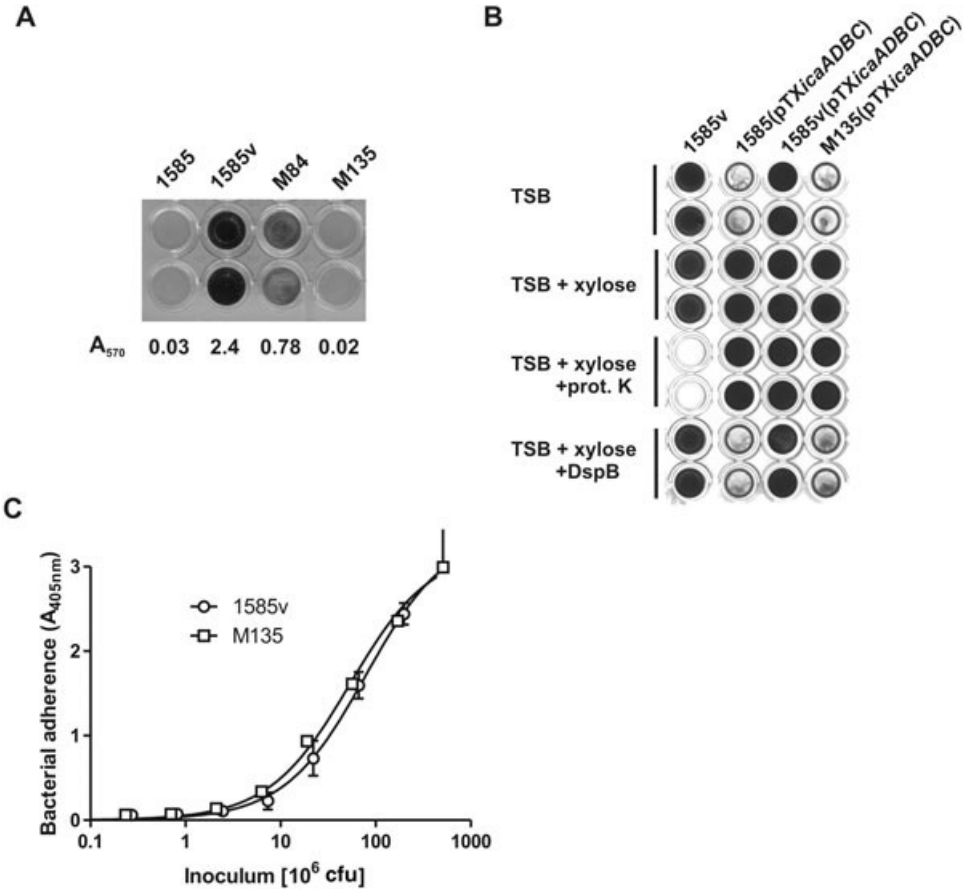

Fig. 1. Analysis of biofilm formation in *S. epidermidis* 1585, 1585v, mutant M135 and corresponding transductants expressing *icaADBC*.
A. Biofilm phenotype of clinically significant *S. epidermidis* 1585 and the corresponding biofilm-positive variant *S. epidermidis* 1585v. By transposon mutagenesis of *S. epidermidis* 1585v, mutants M84 (impaired biofilm formation) and M135 (biofilm-negative phenotype) were isolated. Biofilm formation was tested in NunclonΔ 96-well microtitre plates (Nunc, Roskilde, Denmark). Quantification of biofilm formation is given as A_{570}.
B. Effect of proteinase K and PIA-degrading β-1,6-hexosaminidase DspB on established biofilms of *S. epidermidis* 1585v, 1585(pTX*icaADBC*), 1585v(pTX*icaADBC*) and M135(pTX*icaADBC*). After overnight cultivation in TSB or TSB + 1% (w/v) xylose, medium was aspirated and replaced by TSB or TSB + 1% (w/v) xylose supplemented with proteinase K (1 mg ml^{-1}) or DspB (10 μg ml^{-1}), respectively, where indicated.
C. Primary attachment of biofilm-positive *S. epidermidis* 1585v and the biofilm-negative mutant M135 to unconditioned polystyrene (NunclonΔ, Roskilde, Denmark). Similar attachment characteristics were found with mutant M84 (data not shown).

sequences flanking Tn*917* insertions the exact localization of the respective transposon insertions were identified in M135 and M84 (Fig. 2A). BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) comparison of the obtained sequences against the nucleotide sequence databases at NCBI found perfect matches for the transposon flanking sequences of both mutants within a single 30 612 bp open reading frame (ORF) of *S. epidermidis* RP62A (SERP1011), encoding for the 1 MDa Embp (Williams *et al.*, 2002). The respective transposon insertions were located in the 3′ third of the gene encoding Embp (Fig. 2A).

Organization and expression of embp *in* S. epidermidis *1585, 1585v, M84 and M135*

Using independent primer pairs (Table S1, Fig. 2A) *embp* transcription in late exponential growth phase was analysed by real-time RT-PCR in *S. epidermidis* 1585 and 1585v. While e*mbp* expression in biofilm-negative *S. epidermidis* 1585 was near the detection limit of the RT-PCR, relative transcription analysis using primer pairs *embp*-real2, 3 and 4 (Fig. 2A) showed a significant increase in *embp* transcription in biofilm-positive *S. epidermidis* 1585v compared with *S. epidermidis* 1585 (Table 1). In contrast, using primer pair *embp*real1 (Fig. 2A), no difference in expression levels was found between *S. epidermidis* 1585 and 1585v (Table 1), suggesting that in *S. epidermidis* 1585v, *embp* is comprised of two ORFs transcribed from independent promoters. Indeed, 5′ rapid amplification of cDNA ends (RACE) analysis found an in-frame fusion between *msrR* (nt 1–461; SERP0932) and *embp* at nt 18 213 in *S. epidermidis* 1585v (Fig. 2A and C). As a consequence, a truncated 12 858 bp *embp–msrR* hybrid gene referred to as *embp1* is expressed in *S. epidermidis*

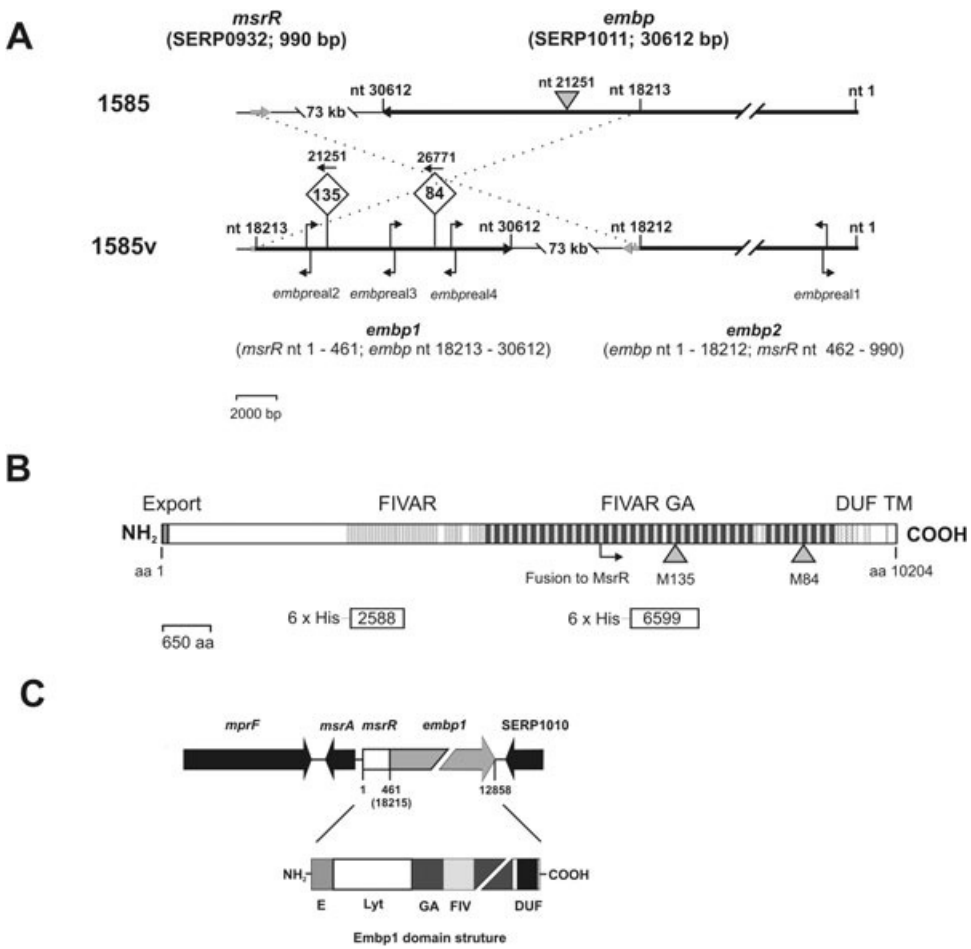

Fig. 2. *embp* in *S. epidermidis* 1585 and 1585v.
A. Schematic representation of *embp* of *S. epidermidis* 1585 wild type and 1585v. Direct sequencing revealed that in *S. epidermidis* 1585 *embp* (SERP1011) comprises 30 612 nucleotides (black arrow). As deduced from chromosomal organization in *S. epidermidis* RP62A, *msrR* (SERP0932; grey arrow) is located, in opposite transcriptional direction, about 73 kb upstream of *embp*. Resulting from an inversion (indicated by dotted lines) of an approximately 90 kb chromosomal fragment, in *S. epidermidis* 1585v nt 18 213–30 612 of *embp* are in frame fused to nt 1–461 of *msrR*. The resulting 12 858 bp ORF is referred to as *embp1*. In addition, an in-frame fusion of nucleotides 1–18 212 of *embp* and nt 462–990 of *msrR* was identified. Transposon insertions in *embp1* leading to mutant M135 (position in wild-type *embp* nt 21 251) and M84 (position in wild-type *embp* nt 26 771) are depicted (arrows above the square indicate the transcriptional direction of erythromycin resistance gene *ermB* located on Tn*917*). A triangle indicates Tn*917* position in *embp* of transductant 1585-M135. The positions of primer pairs *embp*real1–4 used for transcription analysis are given.
B. Schematic view of the modular Embp domain architecture based on bioinformatics analysis (http://smart.embl-heidelberg.de) of the deduced Embp amino acid sequence from *S. epidermidis* 1585. In *S. epidermidis* 1585v Embp is at aa 6072 fused with MsrR (indicated by an arrow). Triangles show relative positions of Tn*917* insertions in the *embp1* or *embp* coding sequence in mutants M84, M135 and 1585-M135 respectively. The positions of His_6-tagged recombinant proteins rEmbp2588 (containing FIVAR domains alone) and rEmbp6599 (containing FIVAR and GA domains) are indicated.
C. Detailed representation of the *msrR–embp* fusion gene (referred to as *embp1*) in *S. epidermidis* 1585v. Nucleotide sequence was determined by sequence analysis of 5′-RACE-PCR amplicons (see *Experimental procedures*) and sequencing of chromosomal DNA from *S. epidermidis* 1585v. *embp1* is a 12 858 bp open reading frame coding for a 4132 aa protein with a deduced molecular weight of 463 kDa. The lower panel shows the domain structure of Embp1 as determined by bioinformatics analysis at SMART (http://www.smart.embl-heidelberg.de). In Embp1, the truncated wild-type Embp is N-terminally fused with 124 amino acids encoded by *msrR*. As a consequence, an export signal (E, aa 1–25) and a LytR_cpsA_psr domain (Lyt, aa 71–171; PFO 3816) are introduced into the molecule. GA, GA module; FIV, FIVAR domain; DUF, domain of unknown function. The C-terminally located, potential *trans*-membrane domain is not depicted. Figure is not to scale.

1585v (Figs 2A and C and 3A and B). Additional sequence analysis showed that in *S. epidermidis* 1585v fusion between *msrR* nucleotides 1–461 and *embp* nucleotides 18 213–30 612 most probably resulted from an inversion of a large 90 kb chromosomal fragment, fusing 3′ parts of *msrR* (nt 462–990) in frame with 5′ parts of *embp* (nt 1–18 212) (Fig. 2A). In contrast, chromosomal organization of *msrR* and *embp* in *S. epidermidis* 1585 was identical compared with the published sequence of *S. epidermidis* RP62A (Fig. 2A).

Table 1. Relative *embp* transcription in *S. epidermidis* 1585v, M84 and M135 compared with *S. epidermidis* 1585 (x-fold regulation).[a]

	Primer pairs			
Strain	*embp*real1[b]	*embp*real2[b]	*embp*real3[b]	*embp*real4[b]
1585v	**0.58** (0.49; 0.63)	**56** (31; 88)	**144** (114; 190)	**123** (77; 181)
M84	**1.18** (0.79; 1.52)	**87** (57; 137)	**156** (106; 185)	**2.34** (1.15; 3.03)
M135	**2.01** (1.32; 2.96)	**117** (51; 194)	**1.40** (1.07; 1.59)	**1.74** (1.07; 2.09)

a. Relative *embp* expression compared with 1585 with *gyrB* as reference gene using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The resulting factor indicates the increase or decrease of transcription compared with reference strain *S. epidermidis* 1585v. The cut-off for significant differences in transcription was defined as 2.5-fold up- or downregulation.
b. See Fig. 2 for localization of primer pairs in *embp*. Factors are means (indicated in bold) obtained in three independent experiments using independent RNA preparations. Numbers in brackets give the range of values estimated (minimum; maximum).

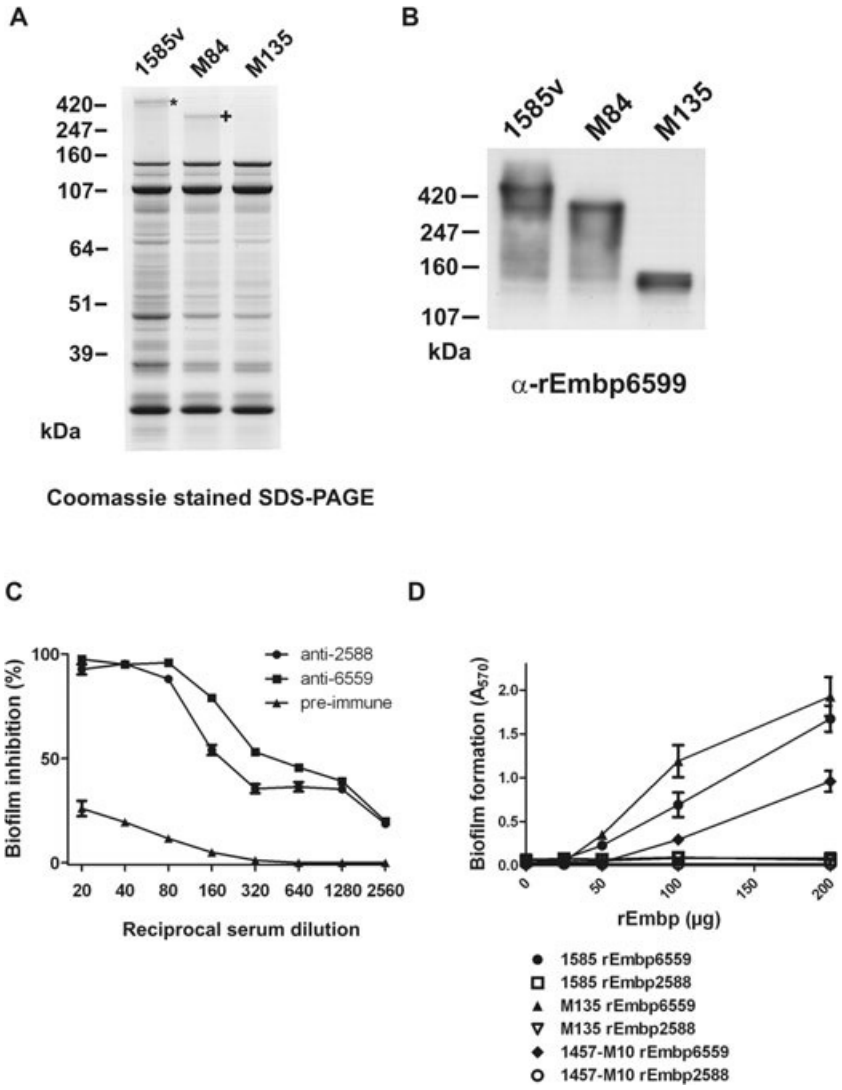

Fig. 3. Analysis of *embp1* in *S. epidermidis* 1585v.
A. Separation of cell surface-associated proteins by SDS-PAGE using a 4–12% gradient Bis-Tris gel (Invitrogen, Karlsruhe, Germany). Bands were made visible by Coomassie staining. The high-molecular-weight 460 kDa protein in 1585v [indicated by asterisk (*)] represents Embp as identified by mass spectrometry. Mutant M84 expresses a truncated, 320 kDa isoform of Embp [indicated by plus symbol (+)].
B. Analysis of cell surface proteins from *S. epidermidis* 1585v, M84 and M135 by immunoblot. After transfer of electrophoretically separated cell surface-associated proteins onto PVDF membrane and blocking with protein-free blocking reagent (Pierce, Rockford, USA) anti-rEmbp$_{6599}$ antiserum diluted 1:10.000 in PBST was used for detection of Embp. Bound antibodies were made visible by chemiluminescence using an anti-rabbit antibody coupled to peroxidase.
C. Inhibition of *S. epidermidis* 1585v biofilm formation by antisera directed against rEmbp2588 (FIVAR domains alone) and rEmbp6599 (alternating FIVAR and GA domains). The corresponding pre-immune sera served as a negative control. Relative inhibition was calculated by using the formula $(1 - A_{570\ \text{with antiserum}}/A570_{\text{without antiserum}}) \times 100$.
D. Influence of rEmbp6599 (comprised of alternating FIVAR and GA domains) and rEmbp2588 (comprising only FIVAR domains) on the biofilm phenotype of *S. epidermidis* 1585, M135 and 1457-M10. Purified Embp fragments rEmbp6599 and rEmbp2588 were added in different concentrations to the growth medium (TSB) and cells were allowed to grow in wells of a 96-well Nunclon∆ microtitre plate (Nunc, Roskilde, Denmark). Addition of PBS served as a negative control. The plot shows mean values from eight data points obtained in two independent experiments. Upper and lower error bars represent the standard deviation.

In transductants M135 and M84 transcription from *embp1* upstream of the respective transposon insertions (Fig. 2A) was still increased compared with *S. epidermidis* 1585 (Table 1). However, by using primer pairs located downstream of the respective transposon insertions (Fig. 2A), no significant increase in *embp1* transcription levels was found (Table 1), indicating that the transposon insertions interfere with *embp1* transcription in the mutants.

The findings of *embp* sequence and transcriptional analysis were corroborated by the exploration of cell surface-associated proteins by SDS-polyacrylamid gel electrophoresis (SDS-PAGE), demonstrating the presence of a 460 kDa Embp isoform in *S. epidermidis* 1585v (Fig. 3A) and, according to the location of Tn*917*, 360 kDa and 115 kDa Embp isoforms in mutant M84 and M135 respectively (Fig. 3A and B).

Embp domain structure

Analysis of the predicted Embp amino acid sequence of *S. epidermidis* RP62A and 1585 using SMART (http://smart.embl-heidelberg.de) and SignalP software (http://www.cbs.dtu.dk/services/SignalP/) revealed a highly organized modular domain structure (Fig. 2B). An N-terminal export signal peptide containing a YSIRK motif (aa 58–84) is followed by a region containing several coiled-coil domains. Amino acids 2579–4497 are characterized by 21 repetitive Found In Various Architectures (FIVAR) domains (PFO7554), amino acids 4504–9407 contain 38 alternating FIVAR and G-related albumin-binding (GA) domains (PFO1468). At the C-terminus four domains of unknown function (DUF1542) are followed by a putative *trans*-membrane domain. In *S. epidermidis* 1585v, as a result of the fusion to *msrR*, Embp1 carries an alternative N-terminal export signal and a LytR_cpsA_psr domain (aa 71–171; PFO 3816), both encoded by *msrR*-derived sequences (Fig. 2C). The LytR domain is followed by the wild-type Embp organization (aa 6072–10 203; Fig. 2A–C) containing alternating 27 FIVAR and 26 GA domains.

Role of Embp in S. epidermidis *biofilm accumulation*

The functional involvement of Embp1 in *S. epidermidis* 1585v biofilm formation was demonstrated in biofilm inhibition experiments using specific polyclonal rabbit antisera raised against fragments containing FIVAR domains alone or FIVAR and GA domains in combination; (Fig. 2B). Both antisera inhibited *S. epidermidis* 1585v biofilm formation in a concentration-dependent manner up to a dilution of 1:2560 (Fig. 3C), whereas the respective pre-immune sera containing no Embp-specific antibodies had only weak inhibitory activity at lower dilutions (Fig. 3C). Furthermore, by addition of a recombinant Embp fragment containing FIVAR domains and GA modules (Fig. 2B) to the growth medium it was possible to induce cell aggregation (data not shown) and biofilm formation in wild-type *S. epidermidis* 1585 and mutant M135 in a dose-dependent manner (Fig. 3D). This effect was also observed with genetically independent, biofilm-negative mutant 1457-M10 (Fig. 3D). In contrast, addition of a recombinant Embp fragment solely containing FIVAR domains had no biofilm-inducing properties at the concentrations tested, together suggesting that the combination of FIVAR and GA domains is necessary for Embp-mediated biofilm formation (Fig. 3D).

To study the role of wild-type, full-length Embp on intercellular adhesion and biofilm formation we constructed mutant 1585$P_{xyl/tet}$ *embp* allowing regulated *embp* expression under the control of a tetracycline-inducible promoter $P_{xyl/tet}$ (Geissendörfer and Hillen, 1990; Bateman *et al.*, 2001) (Fig. 4A). By RT-PCR-based relative transcription analysis, 3 h after addition of tetracycline to an exponentially growing culture of strain 1585$P_{xyl/tet}$ *embp*, a significant 14.6- (primer pair *embp*real1; standard deviation $\pm$ 7.4) and 18.6-fold (primer pair *embp*real3; standard deviation $\pm$ 10.5) increase of *embp* transcription was detected compared with the non-induced control. No significant upregulation of *embp* was found in non-induced 1585$P_{xyl/tet}$ *embp* and *S. epidermidis* 1585 in the presence of tetracycline (data not shown). After addition of tetracycline increasing Embp amounts were detected in preparations of cell surface-associated proteins from 1585$P_{xyl/tet}$ *embp* using a semi-quantitative dot immuno assay (Fig. 4B). In mutant 1585$P_{xyl/tet}$*embp* SDS-PAGE analysis of cell surface protein extracts prepared after growth under *embp*-inducing conditions detected a protein band running above the 420 kDa marker with an estimated molecular weight consistent with the expected 1 MDa size of full-length Embp (Fig. 4C). This high-molecular-weight protein was not found in strain 1585$P_{xyl/tet}$*embp* after growth without tetracycline and was also absent in *S. epidermidis* 1585 wild type (Fig. 4C), indicating that it indeed represents Embp. Thus, results from transcriptional *embp* and surface protein analysis demonstrate that the artificial promoter is functional in *S. epidermidis* 1585$P_{xyl/tet}$*embp* and that in this strain, full-length *embp* is expressed. By immunofluorescence microscopy Embp was detected on surfaces 1585$P_{xyl/tet}$*embp* cells grown in the presence of tetracycline, showing that wild-type Embp is a cell surface-associated protein (Fig. 4D). In addition, under inducing conditions 1585$P_{xyl/tet}$*embp* formed cell clusters not found in the uninduced control, demonstrating that full-length Embp possesses intercellular adhesive properties. Indeed, mutant 1585$P_{xyl/tet}$*embp* formed adherent biofilms when grown under *embp*-inducing conditions (Fig. 4E), providing

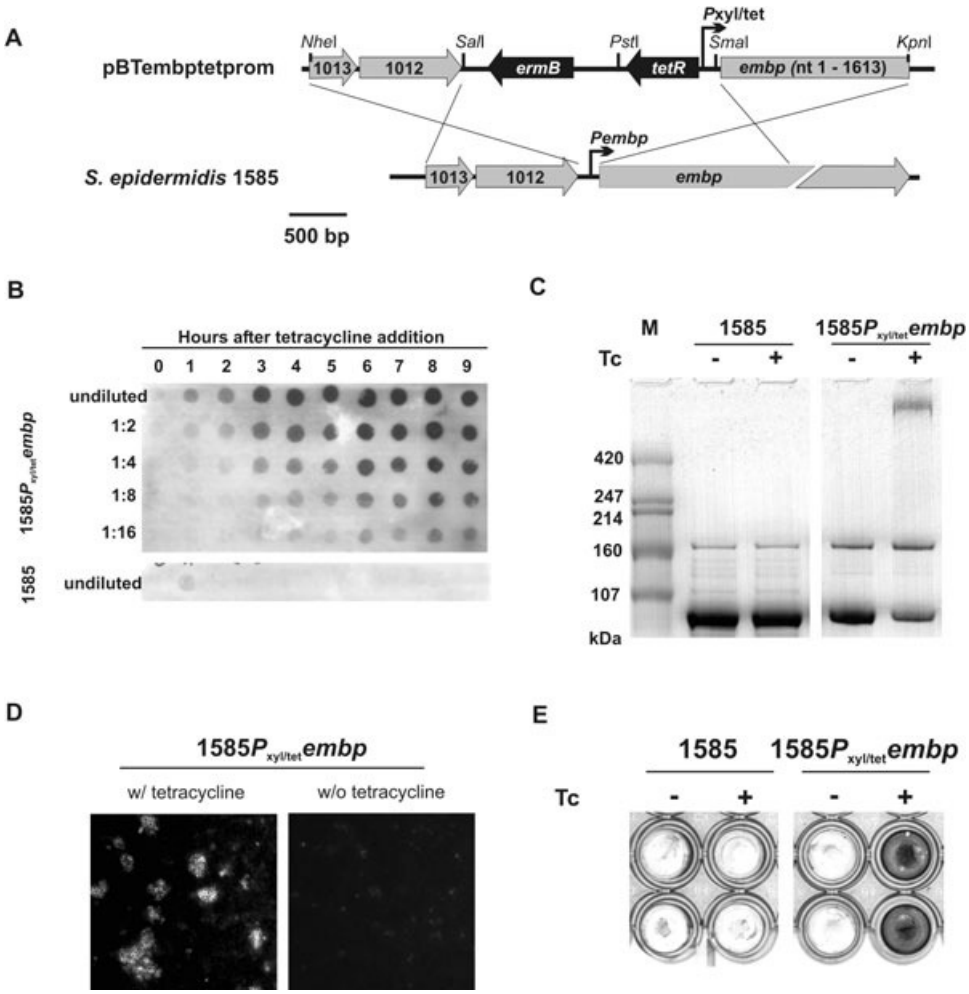

Fig. 4. Construction and analysis of mutant 1585$P_{xyl/tet}$*embp*.
A. Schematic representation of SERP1013, SERP1012 and *embp* in wild-type *S. epidermidis* 1585 (lower panel). Arrows depict open reading frames and their orientation. The natural *embp* promoter is indicated (*Pembp*). In mutant 1585$P_{xyl/tet}$*embp* the natural promoter is deleted and substituted by tetracycline-inducible promoter $P_{xyl/tet}$. The upper panel shows the genetic organization in plasmid pBTembptetprom used for allelic replacement, where erythromycin resistance cassette *ermB*; tetracycline-responsive repressor *tetR* and promoter $P_{xyl/tet}$ are flanked by wild-type sequences SERP1013, SERP1012 and *embp* (nt –36 to 1613). Restriction sites used for construction of pBTembptetprom are indicated.
B. Semi-quantitative analysis of Embp expression in *S. epidermidis* 1585$P_{xyl/tet}$*embp* by dot immunoassay. *S. epidermidis* 1585$P_{xyl/tet}$*embp* and wild-type 1585 were grown overnight in TSB without tetracycline at 37°C with shaking. Cultures were diluted 1:100 in TSB with or without tetracycline (125 ng ml^{-1}) and grown at 37°C. At indicated time points, aliquots were removed, chilled on ice and adjusted to identical optical densities. Cell surface-associated proteins were extracted by ultra sonication and, after clearance by centrifugation at 4°C, serial dilutions were prepared in PBS, from which 10 µl aliquots were spotted onto a PVDF membrane. After overnight blocking, Embp was detected by anti-rEmbp6599 antiserum and chemiluminescence.
C. Detection of Embp in mutant 1585$P_{xyl/tet}$*embp*. Cell surface-associated proteins from *S. epidermidis* 1585, mutant 1585$P_{xyl/tet}$*embp* were extracted after growth in the presence or absence of tetracycline (Tc; 125 ng ml^{-1}) and loaded onto a 4–12% gradient Bis-Tris gel (Invitrogen, Karlsruhe, Germany). After separation bands were made visible by Coomassie staining. In the presence of tetracycline a protein running high above the highest marker band (420 kDa) and the 460 kDa Embp isoform expressed by *S. epidermidis* 5185v is detected in mutant 1585$P_{xyl/tet}$*embp*. Using the ImageJ software the picture's blue RGB channel has been subtracted to remove background staining. M, marker (HiMark, Invitrogen).
D. Localization of Embp expressed by mutant 1585$P_{xyl/tet}$*embp* in the presence of tetracycline (125 ng ml^{-1}) was analysed by immunofluorescence microscopy. Cells grown in the absence of tetracycline were used as a negative control. Full-length Embp is located on the cell surface. In addition, induction of Embp synthesis results in cell cluster formation, indicative for intercellular adhesion.
E. Analysis of biofilm-forming capability of mutant 1585$P_{xyl/tet}$*embp*. Overnight cultures of *S. epidermidis* 1585, 1585v and mutant 1585$P_{xyl/tet}$*embp* were diluted 1:100 in TSB with or without tetracycline (125 ng ml^{-1}) and added into 96-well microtitre plates. After 18 h growth at 37°C the plates were washed with PBS and adherent biofilms were detected by Gentiana violet staining.

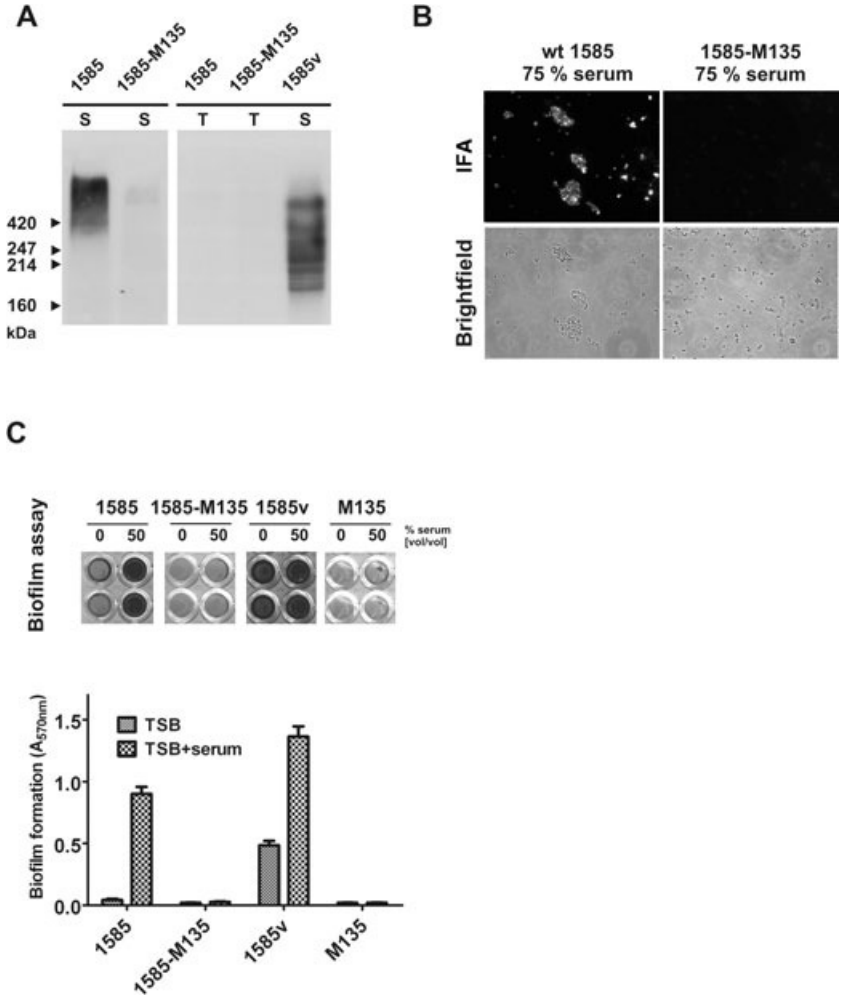

Fig. 5. Embp production and biofilm formation in goat serum.
A. Immunoblot analysis of cell wall-associated proteins from *S. epidermidis* 1585, 1585-M135 and 1585v after growth in TSB (T) or goat serum supplemented with 25% (v/v) TSB (S). Proteins were separated on a 4–12% Bis-Tris gradient gel (Invitrogen, Karlsruhe, Germany) and blotted onto a PVDF membrane. Embp was detected by using anti-rEmbp6599 antiserum. The smear observed with protein preparation from *S. epidermidis* 1585 and 1585v grown in serum most likely results from unspecific degradation.
B. Detection of surface-associated Embp by immunofluorescence assay (IFA). After 48 h growth in goat serum bacterial cells were, after washing with PBS, adjusted to an A_{600} 0.1 and spotted onto immunofluorescence slides. Anti-rEmbp6599 antiserum and FITC-coupled anti-rabbit IgG were used to detect Embp. *S. epidermidis* 1585 grown in 75% (v/v) goat serum formed cell clusters that strongly reacted with anti-rEmbp6599. In mutant 1585-M135 only small cell aggregates were found that displayed almost no immuno-reactivity.
C. Biofilm phenotypes of *S. epidermidis* 1585, transductant 1585-M135, strain 1585v and mutant M135 after 48 h growth in the presence of 50% (v/v) goat serum. The upper panel shows biofilm formation in Nunclon∆ microtitre plates (Nunc, Roskilde) in the presence or absence of goat serum; the lower panel shows the corresponding quantitative read-out. Means were calculated from 16 values obtained in three independent experiments. Error bars represent standard error of mean. Growth in the presence of 75% (v/v) serum also induced biofilm formation in *S. epidermidis* 1585; however, under these conditions biofilms were less strong due to interference of serum components with primary attachment and weak growth.

unambiguous genetic evidence that Embp expression is sufficient for *S. epidermidis* biofilm formation. No differences in primary attachment to the unconditioned Nunclon∆ surface were detected between induced and uninduced 1585$P_{xyl/tet}$*embp* cells (data not shown).

Growth in the presence of serum induces Embp expression, cell aggregation and biofilm formation in wild-type S. epidermidis *1585*

Analysis of 1585$P_{xyl/tet}$*embp* does not completely rule out the possibility that only artificial overexpression of Embp is associated with *S. epidermidis* biofilm formation. *S. epidermidis* 1585 expresses Embp only when grown in the presence of serum (Fig. 5A). Therefore we asked if in wild type in *S. epidermidis* 1585 Embp expression from its natural promoter leads to cell aggregation and biofilm formation. To give answer to this question Tn*917* insertion from mutant M135 was transduced into *S. epidermidis* 1585, resulting in strain 1585-M135 (Fig. 2A). PCR and sequencing proved that phage transduction resulted in transfer of Tn*917* insertion in *embp*, but not of the *msrR–embp* rearrangement into *S. epidermidis* 1585. After 48 h growth in the presence of 75% (v/v) goat serum immunoblot analysis of cell surface-associated proteins demonstrated that 1585 expresses large Embp amounts, migrating above the 420 kDa marker band (Fig. 5A). In contrast, no Embp was detected in mutant 1585-M135, showing that Tn*917* insertion at position of bp 21 251 (Fig. 2A) interferes with Embp production in this strain (Fig. 5A). Using anti-rEmbp6599 antiserum Embp was detected on the surface of wild-type *S. epidermidis* 1585 grown in the presence of serum, but not mutant 1585-M135 (Fig. 5B). Moreover, Embp-producing *S. epidermidis* 1585 formed large cell clusters not found with Embp-negative mutant 1585-M135 (Fig. 5B), showing that Embp possesses intercellular adhesive properties. In line with this, 48 h of growth in the presence

of 50% (v/v) goat serum induced a biofilm-positive phenotype in *S. epidermidis* 1585, quantitatively comparable with *S. epidermidis* 1585v grown under identical conditions (Fig. 5C). In contrast, mutant 1585-M135 did not form a biofilm (Fig. 5C), giving clear genetic evidence that Embp expression is necessary and sufficient for biofilm formation in *S. epidermidis* 1585.

Relation between Embp1- and PIA-mediated biofilm formation

In order to study the relation between Embp1- and PIA-mediated biofilm accumulation, plasmid pTX*icaADBC* (Gerke *et al*., 1998) was transduced into *S. epidermidis* 1585, 1585v, and mutant M135, allowing targeted *icaADBC* expression and analysis of PIA function in the presence or absence of Embp1. Growth under *icaADBC*-inducing conditions led to a biofilm-positive phenotype in *S. epidermidis* 1585(pTX*icaADBC*) and mutant M135(pTX*icaADBC*) (Fig. 1B), indicating that PIA functions as an intercellular adhesin in the absence of Embp. No significant alteration of quantitative biofilm formation was observed in *S. epidermidis* 1585v(pTX*icaADBC*) compared with the uninduced control (Fig. 1B). Exposure of mature biofilms to proteinase K or DspB led to a differential effect. Biofilms of 1585(pTX*icaADBC*) and M135(pTX*icaADBC*) were disintegrated by DspB, but not proteinase K. In contrast, biofilms of 1585v(pTX*icaADBC*) grown under *icaADBC*-inducing conditions were resistant against both enzymes, proteinase K and DspB (Fig. 1B). This demonstrates that Embp1 and PIA can act in parallel and independently of each other as intercellular adhesins.

Interaction of Embp with fibronectin

Binding of recombinant fragments of Embp and its *Staphylococcus aureus* homologue Ebh to fibronectin has been demonstrated (Clarke *et al*., 2002; Williams *et al*., 2002). To investigate the contribution of Embp to bacterial attachment to immobilized fibronectin, we compared adherence of *S. epidermidis* 1585, 1585v, mutant M135 and 1585$P_{xyl/tet}$*embp* grown under inducing and non-inducing conditions to fibronectin-coated polystyrene surfaces (Greiner, Frickenhausen, Germany) (Fig. 6A). Importantly, this polystyrene surface does not support binding of *S. epidermidis* (Mack *et al*., 2001) and therefore served as a negative control. Embp-expressing strains adhered significantly stronger to the fibronectin-coated surface compared with Embp-negative strains (Fig. 6A), indicating that Embp mediates *S. epidermidis* adherence to fibronectin. No adherence to the native uncoated surface was observed (Fig. 6A). Furthermore, a quantitative association between fibronectin amounts used for plate coating and *S. epidermidis* 1585v adherence was found, indicating that here, fibronectin is essential for bacterial binding (Fig. 6B). These findings suggest that Embp plays a role during primary attachment to conditioned surfaces.

Indeed, *S. epidermidis* 1585v formed biofilms on the Greiner polystyrene surface only after coating with fibronectin, whereas no biofilm formation was observed on the untreated polystyrene (Fig. 6C). Thus, under these experimental conditions Embp-mediated adherence to immobilized fibronectin is necessary for primary attachment and subsequent biofilm accumulation.

We next investigated fibronectin binding to recombinant Embp fragments rEmbp6599 and rEmbp2588 in solid-phase ELISA. rEmbp6599 attached to fibronectin-coated polystyrene plates in a dose-dependent manner (Fig. 7A). Interestingly, the same binding characteristics were observed for FIVAR-only rEmbp2558 (Fig. 7A). Interaction of rEmbp2588 and rEmbp6599 with fibronectin was further analysed with surface plasmon resonance (SPR). rEmbp2588 and rEmbp6599 were found to interact with fibronectin at low nanomolar concentrations when injected across a fibronectin surface. The sensorgrams for injection of a 40 nM sample showed slightly differing kinetics of rEmbp2588 and rEmbp6599 (Fig. 7C); however, the response plots did not allow a detailed quantitative kinetic analysis of binding constants using BIAevaluation software (Biacore). Taken together these data demonstrate that FIVAR domains exert fibronectin binding activity.

In order to map the domains used by Embp to interact with fibronectin we recombinantly expressed and affinity purified overlapping fibronectin type III domains (III1–5, III4–7, III7–10, III10–12, III12–14 and III13–15) and used these and the proteolytic, N-terminal 70 kDa fibronectin fragment encompassing fibronectin type I and II domains in Far Western blotting experiments with rEmbp6599 as a ligand. Here we found that recombinant rEmbp6599 bound to fibronectin III10–12 and III12–14 fragments (Fig. 7C). No binding to the proteolytic N-terminal 70 kDa fibronectin fragment containing type I and II domains was detected (Fig. 7C). These results show that Embp binds to fibronectin type III domains, and suggest that fibronectin domain III12 is a major binding site.

Embp-mediated biofilm formation protects S. epidermidis *from phagocytosis*

A hallmark of *S. epidermidis* infections is the inability of the host innate immune system to eradicate infection. Therefore we asked if Embp-mediated biofilm formation contributes to *S. epidermidis* immune escape. To this end surface-grown *S. epidermidis* 1585v and Embp-mutant M135 were exposed to mouse macrophages J774A.1 (Fig. 8). Microscopic analysis of differentially stained intra- and extracellularly located bacteria 4 h after addition of

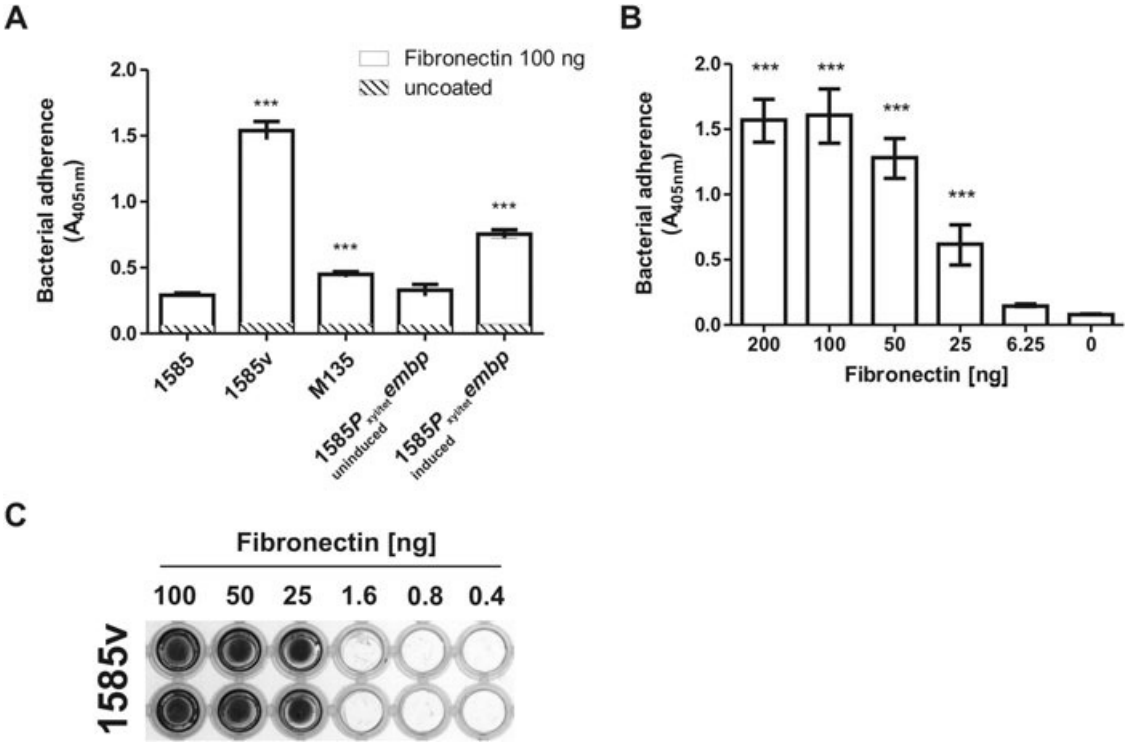

Fig. 6. Interaction of *Staphylococcus epidermidis* with fibronectin-coated surfaces.
A. Adherence of *S. epidermidis* 1585, 1585v, M135, 1585$P_{xyl/tet}$*embp* (uninduced) and 1585$P_{xyl/tet}$*embp* (induced) to fibronectin-coated surfaces. Adherence was tested by adding 10^8 bacteria to 96-well polystyrene plates (Greiner, Frickenhausen, Germany) coated with human fibronectin. After 1 h of incubation at 37°C plates were washed and adherent bacteria were detected using a poly-valent anti-staphylococcal antiserum and alkaline phosphatase-coupled secondary antibodies. Uncoated surfaces were used as a reference negative control. Asterisks indicate significant stronger adherence compared with wild-type *S. epidermidis* 1585 as determined by Bonferroni's test for multiple comparison after one-way analysis of variance.
B. Ninety-six-well microtitre plates (Greiner, Frickenhausen, Germany) coated with various amounts of fibronectin were inoculated with 10^7 colony-forming units (cfu) *S. epidermidis* 1585v. After 1 h at 37°C plates were washed and bacterial adherence was quantified using an anti-*S. epidermidis* antiserum and alkaline phosphatase-conjugated secondary antibody. Column height and error bars represent mean and SD from four replicates. Asterisks denote results significantly different from control (0 ng of fibronectin) as determined by Dunnett's test after one-way ANOVA ($P < 0.05$).
C. *S. epidermidis* 1585v biofilm formation on polystyrene surfaces (Greiner) coated with different fibronectin amounts. Biofilm formation only occurs on surfaces coated with fibronectin amounts sufficient to support bacterial adherence (see also A).

phagocytes showed the direct physical contact of macrophages with biofilm-forming and -negative *S. epidermidis* cells (Fig. 8A). Enumeration of intra- and extracellular bacteria, however, showed that significantly ($P < 0.0001$, unpaired *t*-test with Welch's correction) more Embp- and biofilm-negative M135 cells (mean number 76.9, SD 22.7) were taken up compared with isogenic, Embp- and biofilm-positive *S. epidermidis* 1585v (mean number 20.4, SD 14.0; Fig. 8A and B). Since macrophages are important for early defence against microbial pathogens, these results indicate that Embp-mediated biofilm is of importance for *S. epidermidis* immune evasion.

Discussion

We here describe the identification of a new mechanism of *S. epidermidis* biofilm formation depending on the expression of the 1 MDa giant surface protein Embp. Several lines of evidence suggest that Embp is a proteinaceous intercellular adhesin sufficient for mediating biofilm formation. Initial genetic evidence comes from transposon mutagenesis of biofilm-positive *S. epidermidis* 1585v, leading to the isolation of biofilm-negative mutants M135 and M84 which carry transposon insertions within *embp*. A mutant 1585$P_{xyl/tet}$*embp* allowing the targeted expression of full-length wild-type Embp formed a biofilm when grown under *embp*-inducing conditions. Moreover, also wild-type *S. epidermidis* 1585 formed a biofilm after induction of Embp expression using serum, whereas a corresponding Embp-negative mutant 1585-M135 was biofilm-negative. These results give unambiguous genetic evidence that indeed, Embp is necessary and sufficient for *S. epidermidis* biofilm formation. In addition, specific anti-Embp antisera inhibited biofilm formation in *S. epidermidis* 1585v and recombinantly expressed Embp

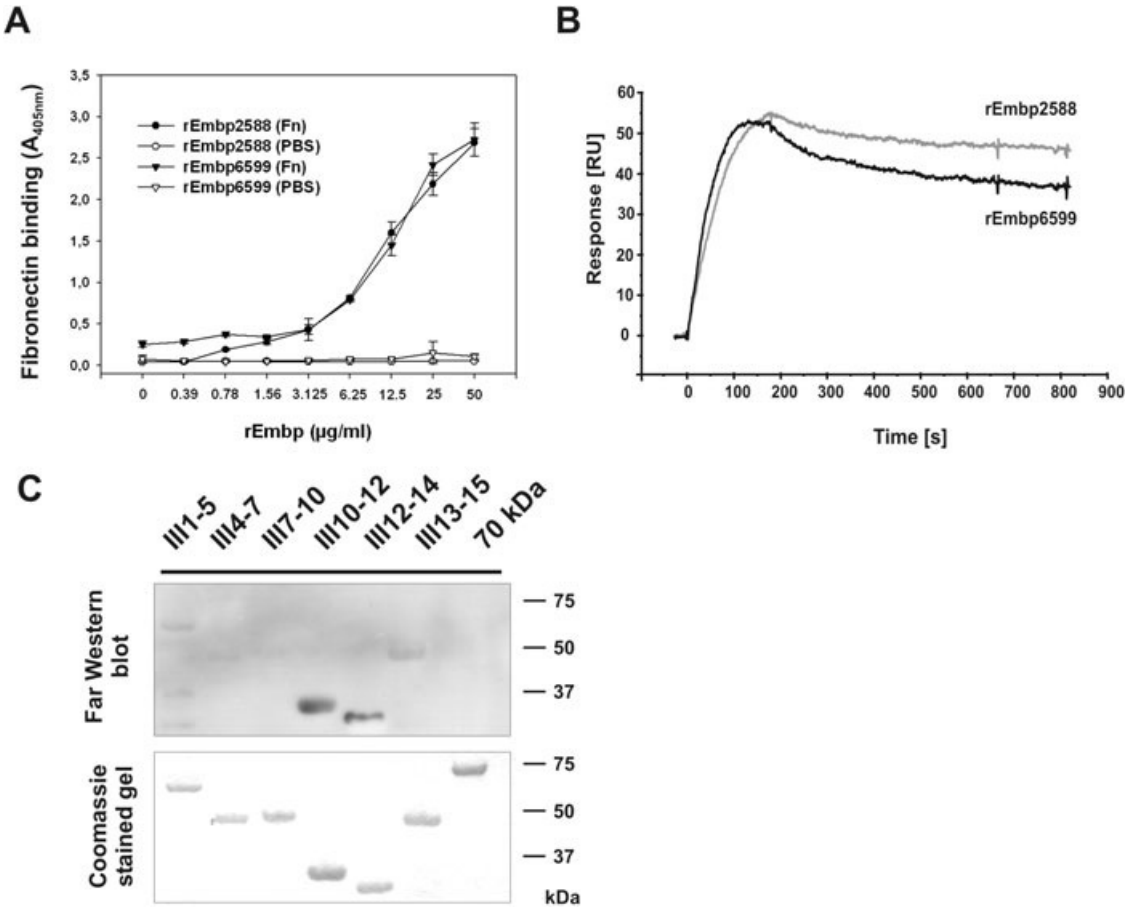

Fig. 7. Interaction of recombinant Embp fragments with fibronectin.
A. ELISA-based analysis of rEmbp2588 and rEmbp6599 binding to immobilized fibronectin. Ninety-six-well flat-bottom polystyrene microtitre plates (Greiner, Frickenhausen, Germany) were coated with 100 µl of fibronectin (10 µg ml^{-1}) overnight at 4°C. After blocking using protein-free blocking agent (Pierce, Rockford, USA) plates were incubated with serial dilutions of the respective recombinant protein. After washing bound Embp fragments were detected by specific antisera (anti-rEmbp2588 and anti-rEmbp6599 respectively) and an alkaline phosphatase-coupled, anti-rabbit antiserum. Uncoated wells served as a negative control.
B. Analysis of rEmbp2588 and rEmbp6599 binding to fibronectin using surface plasmon resonance. The figure shows double-referenced sensorgrams of rEmbp2588 (40 nM) and rEmbp6599 (40 nM) interacting with immobilized fibronectin. Experiments were performed as described in *Experimental procedures*.
C. Analysis of Embp binding to fibronectin subdomains by far Western ligand blotting (upper panel). Recombinantly expressed fibronectin type III domains and the proteolytic N-terminal 70 kDa fibronectin fragment (Sigma, Munich, Germany) were separated by SDS-PAGE and blotted onto a PVDF membrane. Subsequently, membranes were probed with rEmbp6599 (10 µg ml^{-1}). After washing bound proteins were detected using anti-rEmbp6599 rabbit antiserum and peroxidase-coupled anti-rabbit IgG (Dianova, Hamburg, Germany) and chemiluminescence detection (GE Healthcare, Uppsala, Sweden). Coomassie staining (lower panel) proved that similar protein amounts were used in the experiment.

fragment rEmbp6599 had biofilm-inducing properties not only in *S. epidermidis* 1585 and M135, but also in the genetically independent, biofilm-negative *S. epidermidis* 1457-M10 (*icaA*::.Tn*917*).

Proteins have early been recognized as components of the biofilm matrix (Hussain *et al*., 1993) and specific surface proteins with intercellular adhesive properties have been described in *S. epidermidis* (Aap) (Rohde *et al*., 2005; Sun *et al*., 2005; Qin *et al*., 2007), *S. aureus* (Bap) or *Enterococcus faecalis* (Esp) (Toledo-Arana *et al*., 2001; Lasa and Penades, 2006). In contrast to these proteins, Embp does not possess a LPXTG motif, suggesting a non-covalent attachment to the cell surface. In addition, Embp is mainly composed of FIVAR and GA domains, which have not been described in intercellular adhesins so far. FIVAR domains are found in cell wall-associated FmtB protein from *S. aureus* (Komatsuzawa *et al*., 2000) and the gellan lyase from *Bacillus* sp. GL1 (Hashimoto *et al*., 1998), being hypothetically implicated in binding to *N*-acetylglucosamine. GA domains are present in protein PAB of *Finegoldia magna* and protein G of group C and G streptococci (Lejon *et al*., 2004) and are involved in binding to human serum albumin (de Chateau *et al*., 1996). Anticipating that Embp must interact with

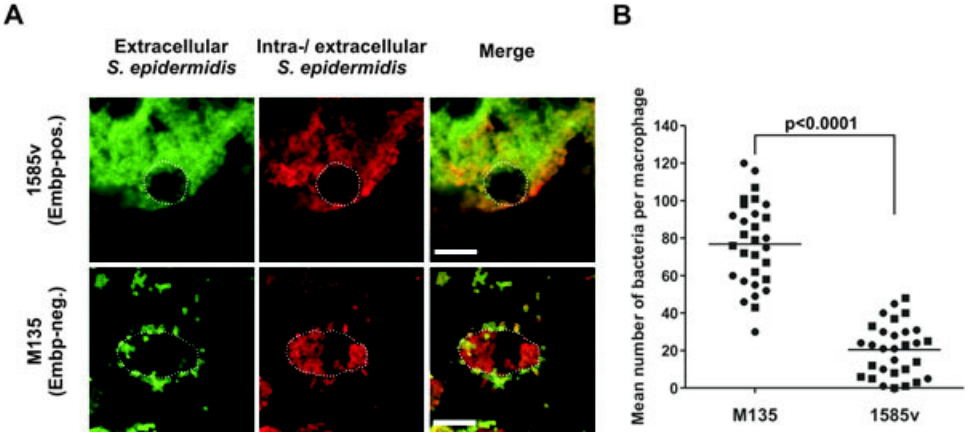

Fig. 8. Phagocytosis of *S. epidermidis* 1585v and M135.
A. Microscopic analysis of bacterial uptake by differential inside–outside staining. Four hours after addition of murine J774A.1 macrophages transfected with pmaxGFP to statically grown *S. epidermidis* 1585v and M135, extracellular bacteria were detected using an anti-*S. epidermidis* antiserum and a Cy5-coupled goat anti-rabbit antibody (green). Intracellular bacteria were, after cell permeabilization, detected by an anti-*S. epidermidis* antiserum and a Alexa568-coupled goat anti-rabbit antibody (red). Macrophage cell boundaries (dotted white line) were determined by detection of intracellular GFP. Colourization was performed with the Adobe Photoshop software package. White bar = 10 μm.
B. Quantitative analysis of *S. epidermidis* 1585v and M135 uptake. The scatter plot illustrates the number of intracellular bacteria for 30 macrophages counted in two independent experiments (data from the respective experiments are indicated by filled circles or filled bars). Mean numbers are indicated by horizontal lines (M135, 78.9, SD 22.7; 1585v, 20.4, SD 14.0). Difference between means (56.43, 95% confidence interval 46.65–66.22) was found statistically significant by unpaired *t*-test with Welch's correction ($P < 0.0001$, significance level 0.05).

bacterial cell surface structures to function as an intercellular adhesin, especially the *N*-acetylglucosamine binding activity of FIVAR domains appeared as a potential functional feature determining the intercellular adhesive properties of Embp. However, recombinantly expressed rEmbp2588 solely containing FIVAR domains exhibited no biofilm-inducing activity. In contrast, rEmbp6599 containing FIVAR and GA domains induced biofilm formation in *S. epidermidis* 1585 and 1457-M10. Although it cannot be excluded that rEmbp2588 binds to the bacterial cell surface, obviously, for the induction of cell aggregation, at least the combination of FIVAR and GA domains is necessary. Here, homo- as well as heterophilic interactions of Embp with the cell surface could be of functional relevance. Interaction analysis between rEmbp2588 and rEmbp6599 using SPR spectroscopy gave no indication for homotypic interactions (data not shown). As in addition, rEmbp6599 also induced biofilm formation in *S. epidermidis* 1585 and 1457-M10 producing no Embp under the conditions employed, the involvement of heterophilic Embp interactions with cell surface structures can be anticipated. Moreover, the finding that mutant M84 expressing 31 FIVAR-GA modules still formed a weak biofilm whereas mutant M135 was biofilm-negative despite the expression of 18 FIVAR GA modules suggests a quantitative association between FIVAR-GA module numbers and promotion of intercellular adhesion. This is also reflected by our observation that rEmbp6599 induced biofilm formation only at high concentrations. Interestingly, the crystal structure of FIVAR-GA repeats from *S. aureus* Embp-homologue Ebh has been resolved recently (Tanaka *et al.*, 2008). It was proposed that each FIVAR-GA repeat consists of two distinct three-helix bundles which are connected along the long axis of the molecule, resulting in a rod-like structure with a length of 320 nm. Anticipating that Embp possesses a structure similar to Ebh, the protein could form a proteinaceous, bacteria-derived matrix, promoting the formation of a complex, multidimensional *S. epidermidis* biofilm architecture. In addition to FIVAR and GA modules, DUF1542 domains as well as the potential *trans*-membrane region might be of additive functional importance for full intercellular adhesive Embp properties. The exact contribution of FIVAR, GA , DUF1542 and *trans*-membrane domains to Embp-mediated *S. epidermidis* biofilm formation is subject of current studies.

The combination of FIVAR and GA domains has been found to be involved in the fibronectin binding activity of Embp (Williams *et al.*, 2002) and the Embp homologue in *S. aureus*, Ebh (Clarke *et al.*, 2002). Indeed, binding of *embp* transposon insertion mutant M135 to fibronectin-coated microtitre plates was severely impaired compared with parent strain *S. epidermidis* 1585v, indicating that Embp acts as a fibronectin-binding protein which is necessary for primary attachment and subsequent biofilm accumulation on fibronectin-conditioned surfaces that otherwise do not support adherence. So far, the fibronectin binding activity of Embp and Ebh has been attributed to

regions consisting of FIVAR and GA domains (Clarke *et al*., 2002; Williams *et al*., 2002). Importantly, by using recombinant protein rEmbp2588 we here demonstrate for the first time that in fact, FIVAR domains alone are sufficient for fibronectin binding.

Fibronectin is composed of 12 type I, 2 type II and 15–17 (dependent on alternative splicing) type III domains (Wierzbicka-Patynowski and Schwarzbauer, 2003). These domains carry specific functions during fibronectin matrix assembly, being fundamental for cell proliferation, differentiation and migration. Many bacterial pathogens specifically bind fibronectin, and this interaction is of key importance for the infective process. Most notable *S. aureus* uses fibronectin-binding protein FnBPA to interact with soluble fibronectin via binding to N-terminal type I domains. This interaction is crucial for adherence to endothelial cells (Schröder *et al*., 2006) and endocarditis pathogenesis (Que *et al*., 2005). In contrast, we here provide first evidence that *S. epidermidis* binds to fibronectin type III domains, most probably domain III12. So far, binding to fibronectin type III domains has been demonstrated for *Salmonella enterica* protein ShdA and *Bartonella henselae* protein Pap31 (Kingsley *et al*., 2004; Dabo *et al*., 2006), both proteins interacting with fibronectin type III domain 13. In contrast, *Borrelia burgdorferi* surface protein BBK32 target type III1–3 (Prabhakaran *et al*., 2009). Importantly, BBK32 has been shown to directly modulate fibronectin structures, manipulating host cell activities. It will therefore be most interesting to analyse potential functional consequences of Embp – Fn III12 binding for host–pathogen interactions during pathogenesis of *S. epidermidis* foreign material-associated infections.

A major consequence of *S. epidermidis* biofilm formation is the failure of professional phagocytes to eradicate infection. Especially PIA has been implicated in biofilm-associated *S. epidermidis* immune evasion by rendering the bacteria less susceptible to phagocytosis (Vuong *et al*., 2004a,b). Our results show that in addition to PIA, Embp-mediated biofilm formation protects *S. epidermidis* from phagocytosis. The demonstration that increased *S. epidermidis* resistance against phagocytosis is not exclusively linked to PIA-mediated biofilm formation but can also be observed with Embp-mediated biofilms puts forward the more general question which molecular events on the phagocyte's part finally result in impaired pathogen uptake, potentially opening ways for reprogramming and optimization of host immune responses to biofilm-associated *S. epidermidis* infections.

Interestingly, under standard *in vitro* culture conditions, in wild-type *S. epidermidis* 1585 *embp* is only expressed at low levels, which is a plausible cause for the biofilm-negative phenotype of that strain. A similar low *embp* expression as detected by immunoblot analysis was found in *S. epidermidis* 1457 and additional 20, genetically independent *embp*-positive *S. epidermidis* strains isolated from prosthetic joint infections (H. Rohde, unpubl. results), indicating a general low *in vitro* expression level which might account for the failure to identify Embp as an intercellular adhesin in independent screening approaches using transposon mutagenesis (Heilmann *et al*., 1996; Mack *et al*., 2000). In line with findings obtained by using *S. epidermidis* O-47 (Sellman *et al*., 2005) we found that in *S. epidermidis* 1585 *embp* is expressed only in the presence of serum, resembling the *in vivo* situation. Consequently, similar to *S. epidermidis* protein Fbe (Sellman *et al*., 2008), Embp appears to be preferentially expressed *in vivo*. Elucidating the regulators involved in *embp* expression control under different conditions, for example the *agr* system shown to be a negative regulator of *embp*-homologue *ebh* in *S. aureus* (Clarke *et al*., 2002), will shed important light on the mechanisms used by *S. epidermidis* to adapt to different environmental conditions.

Our functional studies and the well-documented broad distribution of *embp* in invasive *S. epidermidis* isolates from blood cultures (Rohde *et al*., 2004), prosthetic joint infections (Rohde *et al*., 2007) and intraocular infections (Duggirala *et al*., 2007) suggest that *embp* is of additional relevance for the pathogenesis of foreign material-associated infections similar to *icaADBC*. Potentially, as Embp and PIA can function as intercellular adhesins independently or in parallel, Embp in combination with PIA can be used for the development of a poly-valent *S. epidermidis* vaccine. In the future it will be of major interest to further characterize the role of Embp in the pathogenesis of *S. epidermidis* foreign material-associated infections and the relative importance compared with PIA using specific mutants in independent *S. epidermidis* strains and appropriate animal models.

Experimental procedures

Bacterial strains and cell lines

Clinical *S. epidermidis* isolate 1585 was obtained from a patient of the University Hospital Hamburg-Eppendorf (*S. epidermidis* 1585). Using specific primers (Rohde *et al*., 2004; 2005) neither *aap* nor *icaADBC* was detected in this isolate. Strains were identified as *S. epidermidis* by a negative clumping factor, negative catalase and by ID32Staph gallery (bioMerieux, Marcy l′ Etoile, France). *S. epidermidis* 1457 and the biofilm-negative transposon mutant 1457-M10 have been described elsewhere (Mack *et al*., 1992; 2001). Additional strains used in this study are summarized in Table 2. Murine macrophages J774A.1 (ATCC TIB 67) were grown in RPMI 1640 growth medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), L-glutamine and streptomycin/penicillin at 37°C. Cells were transfected with vector pmaxGFP (Amaxa, Cologne, Germany) using the

Table 2. Bacterial strains and plasmids used in this study.

Strains/plasmids	Properties	Reference
Strains		
S. epidermidis 1457	Biofilm-, *icaADBC*- and PIA-positive strain	Mack *et al.* (2001)
S. epidermidis 1457-M10	1457*icaA*::Tn*917*; PIA- and biofilm-negative	Mack *et al.* (2000)
S. epidermidis 1457-M10(pTX*icaADBC*)	Mutant 1457-M10 containing plasmid pTX*icaADBC* allowing expression of *icaADBC* under the control of a xylose-inducible promoter	Mack *et al.* (2000); Gerke *et al.* (1998)
S. epidermidis 1585	Isolated from port-catheter infection; biofilm-, *aap*-, *icaADBC*- and PIA-negative	Rohde *et al.* (2005)
S. epidermidis 1585v	Biofilm-positive subpopulation of *S. epidermidis* 1585	This study
S. epidermidis 1585v_c	*S. epidermidis* 1585v cured from cryptic 6.3 kb plasmid	This study
S. epidermidis 1585v_{ch}	*S. epidermidis* 1585v_c adopted to grow at 45°C	This study
S. epidermidis 1585$P_{xyl/tet}$*embp*	*S. epidermidis* 1585 carrying *tetR* and promoter $P_{xyl/tet}$ upstream the natural *embp* start codon	This study
Mutant M135	*S. epidermidis* 1585v carrying a Tn*917* insertion within *embp* (nucleotide position 21 251)	This study
Mutant M84	*S. epidermidis* 1585v carrying a Tn*917* insertion within *embp* (nucleotide position 26 771)	This study
S. epidermidis 1585(pTX*icaADBC*)	*S. epidermidis* 1585 containing plasmid pTX*icaADBC*	This study
S. epidermidis 1585v(pTX*icaADBC*)	*S. epidermidis* 1585v containing plasmid pTX*icaADBC*	This study
M135(pTX*icaADBC*)	Mutant M135 containing plasmid pTX*icaADBC*	This study
1585-M135	Transductant of wild-type *S. epidermidis* 1585 carrying Tn*917* in *embp* (nucleotide position 21 251)	This study
E. coli TOP10	Cloning host, genotype F^-*mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74recA1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
E. coli BL21AI	Expression host; genotype F^- *ompT hsdS*$_B$ (r_B-m_B-) *gal dcm araB*::*T7RNAP-tetA*	Invitrogen
Plasmids		
pALC2073	Plasmid carrying *tetR* and the $P_{xyl/tetO}$ promoter	Bateman *et al.* (2001)
pBT2	Temperature-sensitive shuttle vector used for allelic replacement	Brückner (1997)
pBTembptetprom	pBT2 containing *exoS*, *ermB*, *tetR*, $P_{xyl/tet}$, *embp* (nt −36 to 1613) fusion used for allelic replacement of the natural *embp* promoter	This study
pCR4.0	Vector for cloning of PCR amplicons	Invitrogen
pENTR/D-Topo	Entry vector for Gateway® technology	Invitrogen
pDEST17	Expression vector; N-terminal His_6-tag fusion	Invitrogen
pDEST*embp*$_{2588}$	pDEST17 containing *embp* fragment encoding isolated FIVAR repeats (aa 2588–3187)[a]	This study
pDEST*embp*$_{6599}$	pDEST17 containing *embp* fragment encoding alternating GA and FIVAR domains (aa 6599–7340)[a]	This study

a. Amino acid positions refer to the deduced amino acid sequence of *embp* from *S. epidermidis* RP62A (GenBank Accession No. SERP1011).

FuGENE transfection reagent (Roche, Mannheim, Germany) with 2 μg of plasmid DNA per 1×10^6 cells following the instructions of the manufacturer.

Enrichment of biofilm-forming variants

A fresh colony of the *S. epidermidis* 1585 was inoculated into 5 ml of TSB and incubated in uncoated NunclonΔ cell culture bottles (Nunc, Roskilde, Denmark) under static conditions. After 22 h, the bottles were heavily shaken and the medium was changed. After establishment of a visible biofilm on the bottom of the bottle (usually after 3–5 days), the adherent cells were washed twice with 5 ml of phosphate-buffered saline. Then cells were scraped off from the surface and plated onto Columbia blood agar plates. Subsequently, single colonies were picked and tested for biofilm formation in the biofilm assay. One clone was chosen for further analysis and is referred to as *S. epidermidis* 1585v. Subcultivation of 1585v on Columbia blood agar plates revealed the stability of the biofilm-positive phenotype for at least 30 passages.

Adherence assay, in vitro *biofilm formation and Embp-immunofluorescence assay*

Primary attachment to native NunclonΔ (Nunc, Roskilde, Denmark) surfaces was assessed by using a specific ELISA (Mack *et al.*, 2001) except that bacteria from late exponential growth phase were used. The NunclonΔ surface is optimized for cell binding in tissue culture and support adherence of *S. epidermidis* (Mack *et al.*, 2001). Alternatively, in order to investigate *S. epidermidis* binding to fibronectin, 96-well ELISA polystyrene plates (Greiner, Frickenhausen, Germany) were used. In contrast to the NunclonΔ surface the Greiner polystyrene surface does not support binding of *S. epidermidis* (Mack *et al.*, 2001). Wells were coated with 100 μl of human fibronectin (Sigma, Munich, Germany) as described (Rupp *et al.*, 1999a), and uncoated surfaces served as a negative control. Purity of fibronectin preparations was checked by SDS-PAGE. No fibrinogen contaminations were found in immunoblotting experiments using an anti-fibrinogen antibody.

Biofilm formation was tested in TSB (Trypticase soy broth, Becton Dickinson, Cockeysville, USA) using the semi-quantitative microtitre plate test (biofilm assay) as described (Mack *et al.*, 2001). For biofilm inhibition experiments, anti-Embp antisera were added to the culture in concentrations as indicated in *Results*. Inhibition was estimated using the formula $(1 - A_{570\ \text{with inhibitor}}/A570_{\text{without inhibitor}}) \times 100$. Biofilm-positive *S. epidermidis* 1457 (Mack *et al.*, 1992) and its corresponding, biofilm-negative *icaADBC* transposon mutant 1457-M10 (Mack *et al.*, 1994) served as positive and negative controls respectively. Biofilm stability against protease or DspB treatment was tested as published elsewhere (Rohde *et al.*, 2007).

For analysis of biofilm formation in the presence of serum bacteria were grown overnight in TSB. Two hundred microlitres of a 1:100 dilution in TSB was used to fill wells of a Nunclon∆ microtitre plates and bacteria were statically grown at 37°C. After 6 h the medium was carefully removed and replaced by goat serum (Difco, Heidelberg, Germany) supplemented with 50% (v/v) 2× TSB. After additional 48 h of incubation the medium was poured away and wells were carefully washed twice with PBS. After drying at 37°C adherent bacteria were stained with gentiana violet and biofilms were quantified by measuring the absorbance at 570 nm using a spectrophotometer.

Detection of Embp by immunofluorescence was performed as described (Mack *et al.*, 2001). In brief, overnight cultures were diluted 1:100 in TSB and grown in Nunclon∆ (Nunc, Roskilde, Denmark) at 37°C under static conditions. For detection of Embp in mutant *S. epidermidis* 1585*P*xyl/tet*embp* tetracycline (125 μg ml^{-1}) was added. After 6 h bacteria were harvested by centrifugation, washed and re-suspended in PBS to a final A_{600} of 0.1. Aliquots of the respective cell suspensions (20 μl) were spotted onto microscopic slides and air dried. Embp was subsequently detected with epitope saturating concentrations of anti-rEmbp rabbit antiserum and an Alexa Fluor 488-coupled secondary antibody (Invitrogen, Karlsruhe, Germany) diluted 1:200 in phosphate-buffered saline containing 0.1 ml l^{-1} Tween 20 (PBST) with 50 ml l^{-1} protein-free blocking solution (Pierce, Rockford, USA) respectively.

Transposon mutagenesis and phage transduction

Staphylococcus epidermidis 1585v harbours two cryptic plasmids of about 1.5 kb and 9 kb respectively. Tn*917* preferentially inserted into the 9 kb plasmid, making plasmid curing necessary that was carried out as described earlier (Nedelmann *et al.*, 1998). In short, strains with Tn*917* insertion in the plasmid to be eliminated were grown overnight in YETS broth (TSB containing 0.3% Difco yeast extract) with subinhibitory concentrations of SDS (62 μg ml^{-1}, 0.5-fold minimum inhibitory concentration), plated in appropriate dilution onto YETS-Agar and screened for loss of transposon-encoded resistance. As the resulting strain *S. epidermidis* 1585v_c only insufficiently eliminated pTV1ts at 43°C, a more heat-resistant subpopulation was selected by subcultivation on blood agar and incubation at step-wise elevated temperatures, resulting in strain 1585 Ra_{ch} that eliminated pTV1ts after growth at 45°C with an approximately elimination rate 75% (data not shown). Otherwise, 1585v_{ch} displayed no alterations of the phenotype compared with 1585v with respect to antibiotic resistance pattern and biofilm formation (data not shown). For transposon mutagenesis the temperature-sensitive plasmid pTV1ts carrying transposon Tn*917* was introduced into *S. epidermidis* 1585v_{ch} from *S. epidermidis* 1457 × pTV1ts using phage A6C as previously published (Mack *et al.*, 2001). Mutants carrying chromosomal transposon insertions were selected as described (Mack *et al.*, 1994) and tested for a biofilm-negative phenotype in the biofilm assay. Biofilm-negative transposon mutants displaying the identical growth characteristics as the parent strain were further analysed. The respective Tn*917* insertion sites were back transduced into *S. epidermidis* 1585v using phage A6C. Similarly, the Tn*917* insertion in *embp* was transduced from transductant M135 into wild-type 1585, resulting in strain 1585-M135.

For transduction of plasmid pTX*icaADBC* phage A6C was propagated on *S. epidermidis* 1457-M10(pTX*icaADBC*) (Mack *et al.*, 2000). The resulting phage lysate was used to transduce pTX*icaADBC* into *S. epidermidis* 1585, 1585v, and mutant M135, resulting in strains 1585(pTX*icaADBC*), 1585v(pTX*icaADBC*) and M135(pTX*icaADBC*). Successful transduction was proven by plasmid preparations and PCR using *icaA*-specific primers (Rohde *et al.*, 2004).

*DNA preparation, sequencing of Tn*917 *insertion sites and* embp

Staphylococcal DNA was isolated using the Qiagen DNA kit (Qiagen, Hilden, Germany) essentially following the instructions of the manufacturer with the exception that cells were lysed with 15 U of lysostaphin added to buffer P1. The sequence of regions flanking Tn*917* insertion sides was determined by the method of Knobloch *et al.* (2003) and sequences were subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For full-length sequencing of *embp* from *S. epidermidis* 1585 and 1585v fragments of about 1000 bp were amplified with the TripleMaster PCR kit (Eppendorf, Germany). The resulting amplicons were cloned into vector pCR4.0 (Invitrogen, Karlsruhe, Germany) and subsequent sequencing and data analysis was performed as published (Rohde *et al.*, 2001). Alternatively, sequencing was carried out by MWG (Ebersberg, Germany).

Transcription analysis and RACE

For RNA preparation, overnight cultures in TSB were diluted 1:100 in fresh medium and incubated in Nunclon∆ 9 cm tissue culture dishes (Nunc, Roskilde, Denmark) at 37°C under static conditions (Dobinsky *et al.*, 2003). Total RNA was isolated from two dishes (20 ml) as described (Franke *et al.*, 2007). Quality of isolated RNA was verified by an average optical density (OD) OD_{260}/OD_{280} nm absorption ratio of 1.92 (range 1.75–2.01).

For transcription analysis, RNA was digested with 1 U μg^{-1} RNA RNase-free RQ1 DNase (Promega, Madison, USA) for 45 min at 37°C, 500 ng of RNA was reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany) following the instructions of the manufacturer. PCR was performed on an iCycler thermal cycler (Bio-Rad,

Munich, Germany) in 25 μl reaction volumes using iQ SYBR Green Supermix (Bio-Rad, Munich, Germany) and commercially generated (MWG, Munich, Germany) primers listed in Table S1 at a final concentration of 300 nM each. Cycling conditions for all experiments were as following: denaturation (95°C 5 min); 40 cycles of amplification and quantification (30 s at 95°C, 30 s at 55°C, 30 s at 72°C, plate read); melting curve (65–95°C). Gradient PCR confirmed 55°C as appropriate annealing temperature for all primers. All samples were run in triplicates in each of three independent experiments. Relative expression levels were estimated by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) with *gyrB* as internal control (Knobloch *et al.*, 2005) and *S. epidermidis* 1585 as the calibrator. Variance was calculated on the level of Ct-values and propagated according to the rules of error propagation (Livak and Schmittgen, 2001). Identical amplification efficiency (E) for all primer pairs was assured by analysing 10-fold serial dilutions of genomic DNA (Pfaffl, 2001).

For 5′-RACE-PCR, mRNA was enriched from whole cell RNA of *S. epidermidis* 1585v and mutant M84 using the MicrobeExpress Bacterial mRNA enrichment kit (Ambion, Austin, USA) essentially following the instructions of the manufacturer. Using 250 ng of enriched mRNA, 5′-RACE-PCR was performed using the GeneRacer kit (Invitrogen, Karlsruhe, Germany) following the provided standard protocol. In brief, after 5′ linker ligation, cDNA synthesis was performed using primer *embp*revRT18040 or *embp*-revRT19043 and SuperScriptIII reverse transcriptase. The resulting, RNase H-treated cDNA was used as template for amplification with Phusion polymerase (Finnzymes, Espoo, Finland) using primers *embp*rev 18881 and GeneRacer 5′ (Invitrogen, Karlsruhe, Germany). The resulting amplicons were cloned into vector pCR4.0 (Invitrogen, Karlsruhe, Germany) and sequenced.

Primer sequences are given in Table S1.

Construction of a tetracycline-inducible embp *promoter*

For the construction and chromosomal insertion of tetracycline-inducible promoter *P*xyl/tet, a 1332 bp fragment containing parts of SERP1012 and SERP1013 using primers *exoS*_for and *exoS*_rev and a 1652 bp *embp* fragment (nt –36 to 1613) containing the presumable Shine–Delgarno sequence and anticipated *embp* start codon using primers *embp*-36_for and *embp*1613_rev were amplified from chromosomal DNA of *S. epidermidis* 1585 respectively (Fig. 4A). A 1304 bp amplicon encoding *ermB* was amplified using primers *ermB*343_for and *ermB*1648_rev and DNA from mutant M135 DNA as a template. All amplifications were performed using proof-reading Phusion polymerase (Finnzymes, Espoo, Finland) under conditions as recommended by the manufacturer. The *embp* fragment was ligated into shuttle vector pBT2 via KpnI and SalI restriction sites, resulting in plasmid pBTembp. The *ermB* fragment was ligated with linearized pBTembp using BamHI and SalI restriction sites, and the resulting plasmid pBTembperm was, after linearization with SalI and NheI, ligated with the SERP1012/1013 fragment, giving plasmid pBTembpermexo. A 800 bp fragment containing the *tetR* gene and the *xyl/tetO* promoter was cut from pALC2073 (Bateman *et al.*, 2001) using SmaI and PstI, and the resulting fragment was ligated into pBTembpermexo linearized with SmaI and PstI, giving pBTembptetprom. In this constructed, *ermB*, *tetR* and the *xyl*/*tetO* promoter are flanked by the SERP1012/1013 fragment and the *embp* fragment, respectively, replacing the anticipated natural *embp* promoter structure (Fig. 4A). pBTembptetprom was electroporated into *S. aureus* RN4220, and further introduced into *S. epidermidis* mutant 1457-M12 (Mack *et al.*, 2000) by electroporation as described (Knobloch *et al.*, 2004). From mutant 1457-M12 the plasmid was moved into *S. epidermidis* 1585 by phage transduction using phage AGC (Rohde *et al.*, 2005). Allelic replacement was carried out as described (Knobloch *et al.*, 2004), and erythromycin-resistant, but chloramphenicol-susceptible mutants were selected for further analysis. Successful replacement of the natural promoter insertion of the tetracycline-inducible promoter was verified by sequencing. One mutant designated *S. epidermidis* 1585$P_{xyl/tet}$*embp* was chosen for further testing. Embp expression was induced by adding subinhibitory tetracycline concentrations (125 ng ml^{-1}) to the growth medium (TSB). The addition had no influence on growth of wild-type *S. epidermidis* 1585 or mutant 1585$P_{xyl/tet}$*embp*. Primer sequences are given in Table S1.

Expression of recombinant Embp and fibronectin subfragments, and generation of antisera

In order to clone and overexpress defined Embp, subdomains chromosomal fragments of *S. epidermidis* 1585 were amplified using primer pairs *embp*7762_for combined with *embp*9561_rev, and *embp*19675_for combined with *embp*22020_rev using the Platinum *Pfx* DNA polymerase (Invitrogen, Karlsruhe, Germany). The resulting amplicons were cloned into pENTR/D-TOPO and subcloned into pDEST17 (Invitrogen, Karlsruhe, Germany) according to the instructions of the manufacturer, giving plasmids pDEST*embp*$_{2588}$ (Embp amino acids 2588–3187) and pDEST*embp* (Embp amino acids 6599–7340). Expression was performed in *Escherichia coli* BL21AI. The recombinant proteins were affinity purified using HiTrap chelating HP columns (GE Healthcare, Freiburg, Germany) and finally eluted with sodium-phosphate buffer (0.05 M $NaPO_4$, 0.5 M NaCl, pH 7.4) via HiTrap desalting columns (GE Healthcare, Freiburg, Germany) to remove imidazole. SDS-PAGE and subsequent Coomassie staining tested integrity of the respective protein preparations. Expression and purification of fibronectin subfragments was carried out as described (Ohashi and Erickson, 2005).

For generation of specific antisera, rabbits were, after obtaining pre-immune sera, subcutaneously immunized with 100 μg of the respective recombinant proteins emulsified in Freund's complete adjuvant. A booster immunization was performed after 4 weeks using 100 μg of the respective recombinant proteins emulsified in Freund's incomplete adjuvant. After an additional 4 weeks the rabbit was sacrificed and serum was stored at –20°C. Western blotting using the recombinant proteins used for immunization proved the lack of Embp-specific antibodies in the pre-immune sera, whereas the sera obtained after immunization strongly reacted with the respective Embp fragments.

Preparation of cell wall proteins, SDS-PAGE and mass spectrometry

For preparation of cell wall-associated, non-covalently linked proteins bacterial cells were grown overnight in NunclonΔ dishes (9 cm diameter), harvested, re-suspended in LDS buffer (Invitrogen, Karlsruhe, Germany) and boiled for 5 min. After centrifugation, the supernatant was recovered for further analysis. Alternatively, for preparation of cell surface-associated proteins, bacteria were grown overnight in 10 ml of TSB in cell culture dishes (9 cm diameter, Nunc, Roskilde, Denmark). Bacterial cells were scrapped off the surface, washed once with PBS and were finally re-suspended in 5 ml of PBS. Proteins were then removed from the bacterial surface by sonification (30 s, Branson Ultrasonifier). Extracts were cleared by centrifugation and the supernatants were further analysed. For staphylococcal surface protein preparations after growth in serum bacteria were grown in goat serum (Invitrogen, Karlsruhe, Germany) supplemented with 25% (v/v) TSB for 48 h at 37°C with shaking. After centrifugation bacteria were washed using ice-cold PBS. Subsequently, cell suspensions were adjusted to an A_{600} of 1 and boiled in LDS buffer (Invitrogen, Karlsruhe, Germany) for 5 min. After centrifugation supernatants were used for further experiments. Protein concentrations were estimated using the Lowry assay as recommended by the manufacturer (Bio-Rad, Munich, Germany). SDS-PAGE, Western blot analysis of cell wall-associated proteins and mass spectrometry have been described elsewhere (Stürenburg *et al.*, 2002).

SPR analysis of recombinant Embp fragments

Binding interaction between rEmbp2588 and rEmbp6599 and fibronectin was analysed using a Biacore 3000 instrument (Biacore, GE Healthcare, Uppsala, Sweden). SPR experiments were performed with C1 sensor chips (research-grade) at 25°C using HBS-EP running buffer [10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Tween 20]. Purity of protein preparations (> 95%) was checked before use of samples by SDS-PAGE (12.5% polyacrylamide) under non-reducing and reducing conditions, followed by Coomassie blue staining. Fibronectin (10 $\mu g\ ml^{-1}$ in 10 mM sodium acetate, pH 5.0) was immobilized (520 response units, RU) to sensor chip surfaces using the Amine Coupling kit (Biacore, GE Healthcare, Uppsala, Sweden) according to the manufacturer's recommendations (Karlsson and Falt, 1997). Reference surfaces were prepared using activation and immediate deactivation by ethanolamine (1 M, pH 8.5) without protein coupling or by immobilizing BSA (450 RU). rEmbp2588 and rEmbp6599 (0–160 nM diluted in HBS-EP) were injected at 40 $\mu l\ min^{-1}$ (180 s injection + 600 s dissociation). Surfaces were regenerated with two 5 s pulses of 100 mM NaOH. All binding data were double-referenced (Myszka, 1999) before analysis. It was not possible to evaluate the data using standard kinetic analysis in the BIAevaluation 4.1 software (Biacore, GE Healthcare, Uppsala, Sweden). A plot of steady-state binding response (Rmax) versus Embp fragment concentrations indicated that the used analyte concentrations did not saturate available binding sites.

Phagocytosis assay

For evaluating uptake of *S. epidermidis* 1585v and mutant M135 overnight cultures were diluted 1:100 in 2 ml of fresh TSB and bacteria were grown on 12 mm glass coverslips placed in six-well cell culture plates (Nunc, Roskilde, Denmark) overnight at 37°C without shaking. Plating of serial dilutions proved similar bacterial cell numbers for *S. epidermidis* 1585v and mutant M135. After removal of TSB medium and addition of RPMI medium without antibiotics 10^6 J774A.1 cells transfected with pmaxGFP were added to bacteria at a ratio of roughly $1{:}10^3$. Four hours after addition of the macrophages co-cultures were fixed using 2% (v/v) paraformaldehyde for 10 min at room temperature. Subsequently, a previously described protocol was applied for differential staining of intra- and extracellular bacteria (Heesemann and Laufs, 1985). In brief, after washing with PBS and blocking with 10% (v/v) FCS in PBS extracellular bacteria were detected using an anti-*S. epidermidis* rabbit antiserum (Mack *et al.*, 1992) (1:100 in PBS containing 1.5% FCS) and an Cy5-coupled goat anti-rabbit IgG (Molecular Probes, Karlsruhe, Germany; 1:100 in PBS containing 1.5% FCS). After washing with PBS macrophages were permeabilized by addition of PBS containing 0.2% Triton X-100 for 5 min and intracellular bacteria were detected using the anti-*S. epidermidis* rabbit antiserum (1:100 in PBS containing 1.5% FCS) and an Alexa568-coupled goat anti-rabbit IgG (Molecular Probes, Karlsruhe, Germany; 1:100 in PBS containing 1.5% FCS). Microscopic analysis was carried out with a Zeiss Axiovert 200M confocal laser scanning microscope at 60-fold magnification using the Volocity 4.3 Improvision Software package. Quantification of intracellular bacteria was carried out in two independent experiments and analysis of 15 representative macrophage cells for each strain per experiment.

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Supporting information

Additional supporting information may be found in the online version of this article.

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Table S1: Primers used in this study

Primer name	Primer sequence	Primer position (nt)[a]	Function / Feature
embpreal1_for	5`-CAGTAGTTGCAGAAGCATACCATGA-3`	2744	Detection of embp transcripts
embpreal1_rev	5`-TGTATCGCTACTAAACGGAACGCT-3`	2896	Detection of embp transcripts
embpreal2_for	5`-AACTTCAAGATGCAAAGACAGATGC-3`	20339	Detection of embp transcripts
embpreal2_rev	5`-AATGACGCTTTGGCTACTGCA-3`	20534	Detection of embp transcripts
embpreal3_for	5`-GCAACTAACGGATGGTGTAAATC-3`	24438	Detection of embp transcripts
embpreal3_rev	5`-GCCAATAATAGCCGCAACTTC-3`	24604	
embpreal4_for	5`-TGATGAGGCGCTACAACAAG-3`	27384	Detection of embp transcripts
embpreal4_rev	5`-GATCTTTGCTCTTCTCAGTTGC-3`	27517	
gyrBreal_for	5`-CTGACAATGGCCGTGGTATTC-3`	n.a.	Detection of gyrB transcript
gyrBreal_rev	5`-GAAGATCCAACACCGTGAAGAC-3`	n.a.	Detection of gyrB transcript
embprevRT18040	5`-CATGAAGATTAGATTTAGCTTGAGTTAC-3`	18040	cDNA synthesis
embprevRT19043	5`-GCAGAATCTTCATTTGTATAATCAC-3`	19043	cDNA synthesis
embprev 18881	5`-CTGAGTTTTTCACGTTGTGCATCTGTTAAATCTG-3`	18881	Amplification of cDNA

			ends
GeneRacer 5´	5`-CGACTGGAGCACGAGGACACTGA-3`	n.a.	Amplification of cDNA ends; Invitrogen
fmtC1_for	5`-AGCACCTATTGGAGTAATGAGAGA-3`	n.a.	
fmtC2_for	5`-TGCGACTATATATACTTATGCGTCA-3`	n.a.	
exoS_for	5`-**GTCGAC**TCATTGGTTTATGGTTAGAGGTG-3`	n.a.	Amplification of exoS fragment; SalI restriction site (bold)
exoS_rev	5`-**GCTAGC**AGAAGAACAAATCCTACCAGTCA-3`	n.a.	Amplification of exoS fragment; NheI restriction site (bold)
ermB343_for	5`-<u>GGATCC</u>**CTGCAG**agagtgattggtcttgcgt-3`	n.a.	Amplification of ermB; BamHI restriction site (underlined), PstI restriction site (bold)
ermB1648_rev	5`-**GTCGAC**AGTACCATTCAAATTTATCCTTA-3`	n.a.	Amplification of ermB; SalI restriction site (bold)
embp-36_for	5`-**GGATCC**<u>CCCGGG</u>TAGAACAAATAAATTGTTGTTCAA-3`	-36	Amplification of embp; BamHI restriction site (bold), SmaI restriction site (underlined)
embp1613_rev	5`-**GGTACC**atttgatctggtttctcactatttagca-3`	1613	Amplification of embp; KpnI restriction site (bold)
embp7762_for	5`-**CACC**AGTAAAGAGAACAAAACACCATCAAGT-3`	7762	Entry site for directional cloning in pENTR/D-Topo (bold).
embp9561_rev$_{9561}$	5`-**CTA**TGGTTGACTATTAATCGCTTGATC-3`	9561	Contains additional stop

			codon (bold).
embp19675_for	5`-**CACC**TTAATTAATAATGCTGATACTCGA-3`	19675	Entry site for directional cloning in pENTR/D-Topo (bold).
embp22020_rev	5`-**TTA**CAGTGCTTTCATAGCTTCAT-3`	22020	Contains additional stop codon (bold).

[a] Nucleotide positions refer to the 5´ (forward primers) and 3´ (reverse primers) annealing positions according to the embp coding sequence of S. epidermidis RP62A (GeneBank accession number SERP1011).

2. Darstellung der Publikation

Hintergrund

Staphylococcus epidermidis ist der Hauptbestandteil der epidermalen Standortflora des Menschen (Noble 1997). Bereits in den ersten Lebenstagen beginnt die Kolonisation der gesamten Körperoberfläche und aller Schleimhäute. Wie die meisten koagulasenegativen Staphylokokken (KNS) gilt der Keim als harmloser Saprophyt, dessen Präsenz auf der Haut vermutlich sogar die Ansiedlung pathogener Mikroorganismen erschwert (Iwase et al. 2010). Durch seine ausgeprägte Umweltresistenz ist *S. epidermidis* im Rahmen von Alltagskontakten leicht übertragbar. Für eine effektive Verbreitung sind, anders als beim artverwandten *Staphylococcus aureus*, die im Rahmen von Infektionsprozessen zeitweilig auftretenden hohen lokalen Erregerkonzentrationen nicht erforderlich. Dementsprechend findet man bei *S. epidermidis* auch kein vergleichbares Arsenal dedizierter Pathogenitäts- und Virulenzfaktoren(Otto 2009). Die klinische Relevanz von *S. epidermidis* resultiert vor allem aus der hohen Prävalenz bei fremdmaterialassoziierten Infektionen (Tabelle 1). Als Folge der zunehmenden Verbreitung entsprechender Zugänge und Implantate gehört *S. epidermidis* mittlerweile zu den häufigsten Auslösern einer nosokomialen Infektion (Karlowsky et al. 2004). Trotz in der Regel wenig foudroyanter Verläufe mit niedriger Mortalität sorgen Schwierigkeiten bei der zeitnahen Diagnose (wegen der ubiquitären Besiedelung des Menschen werden KNS beim Erstnachweis häufig als Kontamination interpretiert) und ein hoher Anteil primär resistenter Isolate (etwa 70 % aller *S. epidermidis* Blutkulturisolate sind einer Behandlung mit Betalaktamantibiotika nicht zugänglich (Casey et al. 2007)) für eine erhebliche Gesamtmorbidität.

Tabelle 1: Infektionsraten und relative Häufigkeit KNS-bedingter Infektionen bei häufig verwendeten Implantaten in Deutschland (modifiziert nach Mack et al. 2006).

Implantiertes Fremdmaterial	Anwendungen pro Jahr	Infektionsrate [%]	Anteil KNS bedingter Infektionen [%]
Zentralvenöse Katheter	1.750.000	0,5 – 5	30 – 40
Gelenkprothesen	300.000	1 – 5	20 – 40
Herzschrittmacher	70.000	1 – 3	45 – 60
Künstliche Herzklappen	18.000	1 – 3	15 – 40
Liquorableitungen	10.000	2 – 20	50 – 70

Als Ursachen für die besondere Bedeutung von *S. epidermidis* als Erreger fremdkörperassoziierter Infektionen gelten die aus der Besiedlung der menschlichen Haut resultierende regelhafte Kontamination von prothetischem Material im Rahmen der Implantation und die Fähigkeit zur Bildung mehrschichtig organisierter bakterieller Gemeinschaften (Biofilme) auf der Fremdkörperoberfläche (Götz 2002, Mack 1999). Biofilme bieten den Erregern Schutz vor Phagozytose durch Zellen des Immunsystems (Kristian et al. 2008, Vuong et al. 2004b) und vermindern die Empfindlichkeit gegenüber antimikrobiell wirksamen Substanzen (Knobloch et al. 2002, Stewart et al. 2001, Wiederhold et al. 2005). Die genaue Kenntnis der dem Prozess der Biofilmbildung zu Grunde liegenden Mechanismen und der beteiligten Faktoren gilt daher als wichtige Voraussetzung für die Entwicklung neuer Strategien zur Prävention und Therapie fremdkörperassoziierter Infektionen.

Die bakterielle Biofilmbildung lässt sich funktionell in mehrere Phasen unterteilen (Abbildung 1). Der Adhäsion einzelner Bakterien an eine künstliche oder natürliche Oberfläche (primäre Anheftung) folgt die Vermehrung und mehrschichtige Organisation (Akkumulation). Ein zunehmender Anteil der Bakterien verliert dabei den direkten Kontakt zur Oberfläche und wird durch interzelluläre Adhärenz im Biofilm gehalten (Rohde et al. 2006). Zudem sind spezifische Mechanismen beschrieben, die es einzelnen Bakterien ermöglichen, sich aus dem Verband zu lösen und die so die Bildung von Absiedlungen der Gemeinschaft (Streuung der Infektion) befördern könnten (Vuong et al. 2004a, Wang et al. 2011).

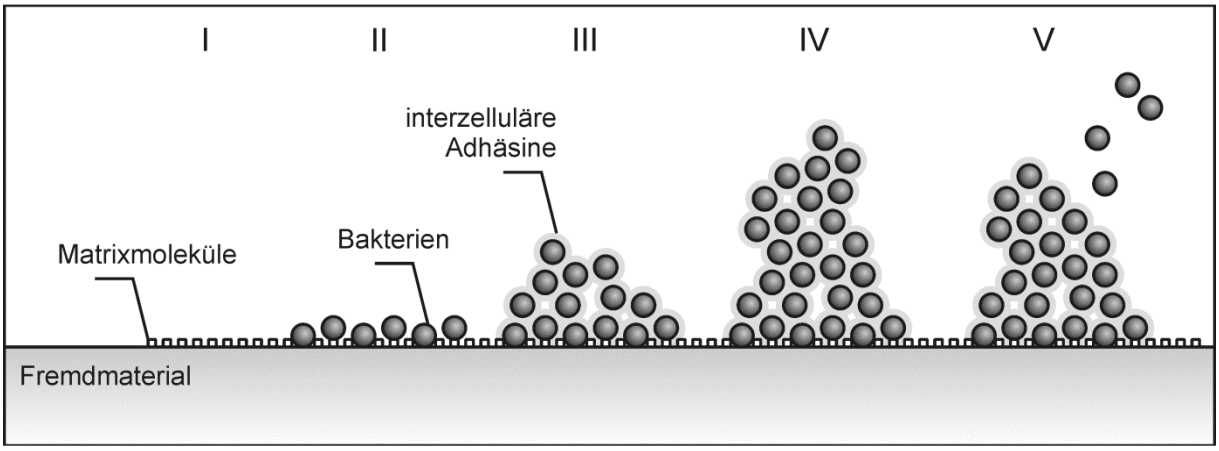

Abbildung 1: Phasen der bakterielle Biofilmbildung (modifiziert nach Otto 2009).
Konditionierung der Fremdmaterialoberfläche (I) durch Ablagerung von Plasma- und/oder Matrix-Komponenten (II), primäre Adhärenz von Bakterien (III), Akkumulation durch Vermehrung der Bakterien und Expression interzellulärer Adhäsine (IV, V), Biofilmreifung und Streuung (IV).

Die primäre Adhärenz an native künstliche Oberflächen wird vor allem über unspezifische hydrophobe Interaktionen vermittelt (Vacheethasanee et al. 1998). Da jedoch Fremdkörperoberflächen in vivo durch die Ablagerung von Plasmaproteinen und Komponenten der extrazellulären Matrix innerhalb kurzer Zeit erheblich modifiziert werden (von Eiff et al. 2002), sind gerade auch bakterielle Faktoren, die die Bindung an die beteiligten Matrixkomponenten und Plasmaproteine vermitteln (*microbial surface components recognizing adhesive matrix molecules*, MSCRAMMs), an der primären Anheftung beteiligt.

Entscheidend für das akkumulative Wachstum von Biofilmen ist die interzelluläre Adhärenz. Diese kann durch die Interaktion mit Oberflächenstrukturen anderer Bakterien oder mit extrazellulär abgelagerten Substanzen vermittelt werden (Mack et al. 2006). Am besten dokumentiert ist dabei die Rolle des beta-1-6-glykosidisch verknüpften Polysacharids PIA (Mack et al. 1996). Die Produktion dieses polymeren Zuckers war in unterschiedlichen Tiermodellen mit der Pathogenität der getesteten Erreger assoziiert und konnte bei verschiedenen Stämmen als hinreichende Bedingung für die *in vitro* Biofilmbildung identifiziert werden (Begun et al. 2007, Rupp et al. 1999a, Rupp et al. 1999b). Darüber hinaus sind mit den Oberflächenproteinen Aap und Bap bereits zwei Mediatoren einer PIA-unabhängigen Biofilmbildung bei *S. epidermidis* beschrieben (Rohde et al. 2005, Tormo et al. 2005). Der wiederholte Nachweis von biofilmpositiven Isolaten, bei denen keiner dieser bekannten Akkumulationfaktoren gefunden wurde, spricht für das Vorhandensein weiterer, bisher nicht charakterisierter Mechanismen der Biofilmbildung bei *S. epidermidis*.

Voraussetzungen und Ziel der Arbeit

Das klinische *S. epidermidis*-Isolat 1585 wurde in 5 unabhängigen Blutkulturen und an der Portkatheterspitze eines Patienten einer pädiatrischen hämatologisch-onkologischen Station nachgewiesen. Der Stamm bildet in Kulturmedien mit hohem Serumanteil Biofilm auf zellkulturtauglichen Polystyrenoberflächen. Durch Passage in serumfreiem Medium mit Anreicherung der adhärenten Subpopulation konnte eine auch in serumfreiem Medium biofilmpositive Variante 1585v isoliert werden. Weder 1585 noch 1585v produzieren einen der bekannten Mediatoren der Biofilmbildung bei *S. epidermidis* (PIA, Aap oder Bap).

Das Ziel der vorliegenden Arbeit war die Aufklärung eines bisher nicht beschriebenen Mechanismus der Biofilmbildung bei *S. epidermidis*. Die beteiligten Faktoren sollten durch Transposonmutagenese von 1585v identifiziert und anschließend funktionell charakterisiert werden.

Wesentliche Ergebnisse

Der Biofilm von 1585v erschien licht- und elektronenmikroskopisch als vielschichtige Ansammlung dicht gepackter Bakterienzellen. Elektronenmikroskopisch konnten keine massiven Ansammlungen extrazellulären Materials dargestellt werden (Abbildung 2). Die Empfindlichkeit wachsender und bereits etablierter 1585v-Biofilme gegenüber der proteolytischen Aktivität von Proteinase K bei gleichzeitiger Unempfindlichkeit gegenüber der PIA-spaltenden Hexosaminidase Dispersin B (Kaplan et al. 2003) wiesen auf bakterielle Proteine als Vermittler der interzellulären Adhäsion hin (Abbildung 2).

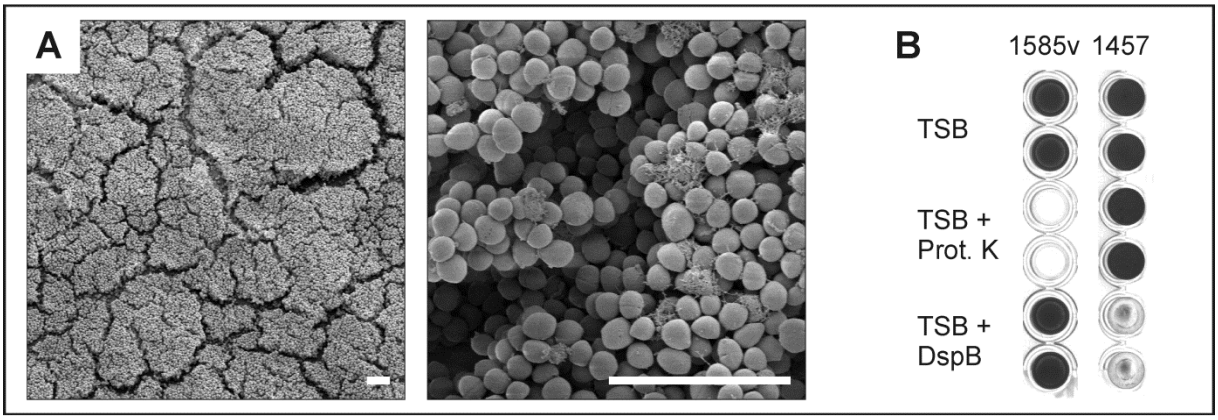

Abbildung 2: Biofilmbildung bei 1585v.
A. Elektronenmikroskopische Aufnahmen des Biofilms von 1585v. Maßstab: 5 µm.
B. Differentielle Wirkung von Proteinase K und Dispersin B (Hexosaminidase) auf etablierte Biofilme von *S. epidermidis* 1585v und 1457 (PIA-vermittelt).

Vor Durchführung der Transposonmutagense wurde der Zielstamm 1585v durch mehrfache Passage hitzeadaptiert (Youngman et al. 1983) und durch Kultur bei subinhibitorischen SDS-Konzentrationen vom größeren seiner beiden kryptischen Plasmide befreit (Sonstein und Baldwin 1972). Mit dem angepassten Stamm 1585vhc konnten, nach Transduktion des transposontragenden hitzelabilen Plasmids pTV1ts, die für eine effiziente Mutagenese erforderlichen hohen temperaturabhängigen Plasmideliminationsraten (> 90 %) erzielt werden.

Insgesamt wurden im Rahmen der Transposonmutagenese 3515 Mutanten erzeugt und im Biofilmtest phänotypisch charakterisiert. Dabei zeigte sich bei sechzehn Stämmen eine im Vergleich zu 1585v signifikant reduzierte Biofilmbildung, die nur bei zwei Stämmen auf eine deutlich reduzierte Wachstumsrate zurückgeführt werden konnte. Durch Transduktion der jeweiligen Transposoninsertion in den Ausgangsstamm 1585v konnte der veränderte

Phänotyp bei allen Mutanten als spezifische Folge der Transposoninsertion verifiziert werden. Position und Orientierung des inserierten Transposons wurden durch Amplifikation und Sequenzierung der an die Transposoninsertion angrenzenden chromosomalen Abschnitte identifiziert (Knobloch et al. 2003). Bei dreizehn Stämmen ergab der Vergleich mit Sequenzen des publizierten Genoms von *S. epidermidis* RP62A Transposoninsertionen im Gen *embp* (SERP1011 bei RP62A) für das fibronektinbindende Oberflächenprotein Embp (*extracellular matrix binding protein*). Die eingehender charakterisierten Transduktanden M135 und M84 repräsentieren dabei die am weitesten proximal bzw. distal im *embp*-Gen gelegenen Insertionen.

Die vollständige Sequenzanalyse von *embp* und der flankierenden Chromosomenabschnitte bei 1585 und 1585v offenbarte deutliche Unterschiede in der Organisation des Genortes zwischen den beiden Stämmen. Die für 1585 ermittelte Embp-kodierende Sequenz stimmte nahezu vollständig mit den entsprechenden Sequenzen im publizierten Genom von RP62A überein. Das Gen *embp* kodiert hier für ein 10204 Amiosäuren umfassendes Riesenprotein mit einem errechneten Molekulargewicht von über 1 MDa und einem ausgeprägt modularem Aufbau (Abbildung 3). Die hinteren drei Viertel der Primärstruktur bestehen nahezu vollständig aus repetitiven Elementen, wobei sich drei Regionen anhand der Art der wiederholten Motive unterscheiden lassen: ein etwa 1900 Aminosäuren umfassender Bereich mit 21 FIVAR-Domänen, ein etwa 4900 Aminosäuren umfassender Bereich, in dem 38 weitere FIVAR Domänen im gleichmäßigen Wechsel mit 38 GA-Domänen angeordnet sind, und ein etwa 400 Aminosäuren umfassender C-terminaler Abschnitt mit vier DUF1542 Domänen. Beim konstitutiv biofilmpositiven Stamm 1585v war der entsprechende offene Leserahmen (*open reading frame*, ORF), vermutlich in Folge einer Inversion eines ca. 90 kb großen Chromosomenabschnitts, zweigeteilt. Der distale Teil von *embp* (Nukleinsäuren 18213-30612) war mit dem proximalen Teil (Nukleinsäuren 1-461) des Gens *msrR* für das membranassoziierte regulatorische Protein MsrR verschmolzen, der proximale Teil von *embp* (Nukleinsäuren 1-18212) mit dem distalen Teil von *msrR* (462-990). Da das Leseraster im Rahmen dieser Genfusionen nicht verschoben worden war, resultierte aus der beschriebenen Umordnung bei 1585v ein *msrR-embp* Hybridgen (*embp1*) unter Kontrolle des *msrR*-Promotors (Abbildung 3). Die Transkription von *embp1* wurde durch Detektion und Analyse der entsprechenden mRNA mittels 5‘-RACE (*rapid amplification of cDNA ends*) belegt. Die Primärstruktur des Hybridproteins Embp1 wird zu 90 % aus den repetitiven Elementen des Embp-Anteils gebildet; das dominierende Strukturelement sind wechselseitig angeordnete FIVAR- (27 Elemente) und GA-Domänen (26 Elemente).

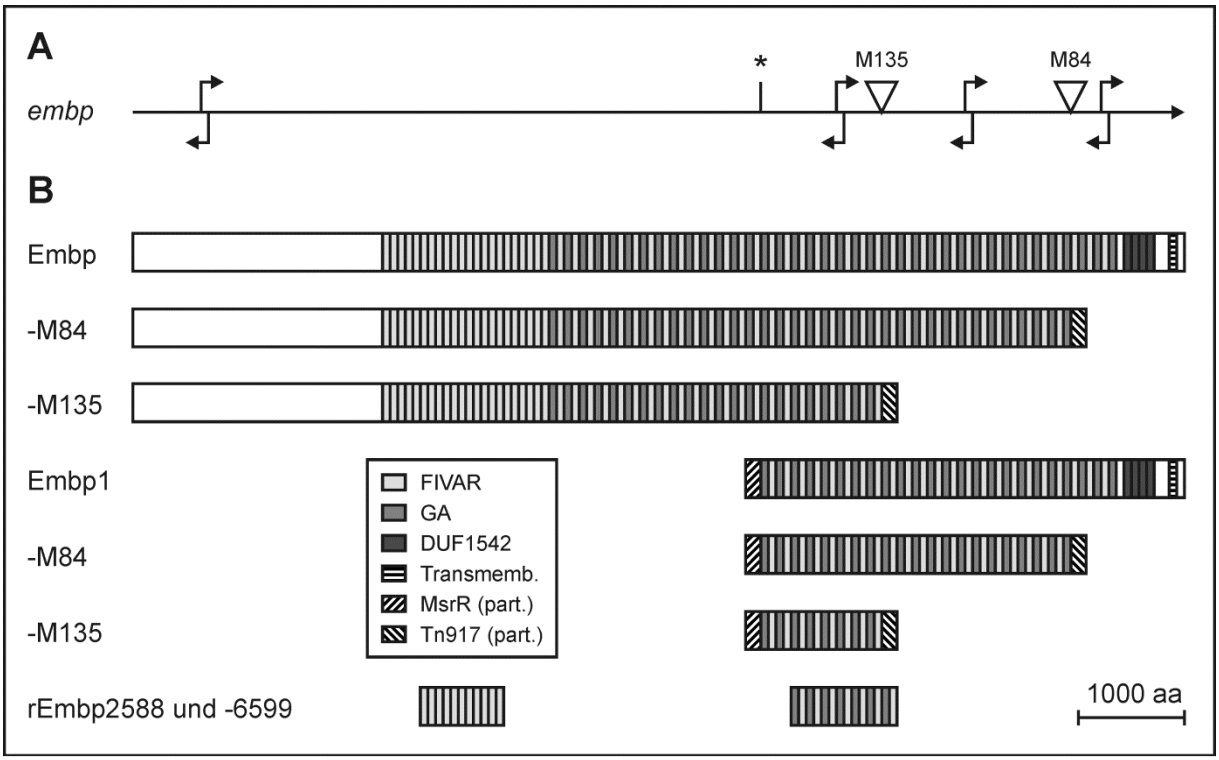

Abbildung 3: Embp.
A. Das *embp*-Gen von *S. epidermidis* 1585. Pfeile markieren die Bindungsstellen der zur Analyse der *embp*-Expression verwendeten *real-time* RT-PCR Primer; Dreiecke markieren die Lokalisation der Transposoninsertionen bei den Mutanten M84 und M135; der Stern markiert die Position der rekombinationsbedingten Genteilung bei 1585v.
B. Vergleichende Darstellung der Domänenarchitektur von Embp (*S. epidermidis* 1585), Embp1 (*S. epidermidis* 1585v), der zugehörigen Transposonmutanten und der beiden rekombinanten Embp-Fragmente rEmbp2588 und rEmbp6599.

Die durch quantitative *real-time* RT-PCR in der spätexponentiellen Wachstumsphase in serumfreiem Medium gemessene relative Transkription des Hybridgens *embp1* bei 1585v betrug etwa das 100-fache der Transkription von *embp* bei 1585. Die Analyse von Oberflächenproteinpräparationen von 1585v mittels SDS-Gelelektrophorese und Western-Blot bestätigte die Expression großer Mengen des 480 kDa-Proteins Embp1. Im Einklang mit den Ergebnissen der Transkriptionsanalyse konnte Embp in serumfreien Kulturen von 1585 mit den genannten Methoden nicht detektiert werden.

Eine differentielle Transkriptionsanalyse mit verschiedenen Primerpaaren zur Detektion von *embp*-Sequzenzen proximal und distal der jeweiligen Transposoninsertionsstellen (Abbildung 3) zeigte die Transkription verkürzter *embp1*-Varianten bei den Transduktanden M84 und M135. Das gemessene Transkriptionsniveau war dabei vergleichbar mit dem von *embp1* bei 1585v. Proteine mit dem erwarteten scheinbaren Molekulargewicht von 340 kDa (Embp1-M84) bzw. 130 kDa (Embp1-M135) konnten in Oberflächenproteinpräparationen beider

Stämme detektiert und mittels Massenspektrometrie und *western-blot* als Embp-Varianten identifiziert werden.

Mit einem polyvalenten Antiserum gegen das rekombinant exprimierte Embp-Fragment rEmbp6599 konnte zudem die Dichte entsprechender Epitope an der Oberfläche von 1585v, M84 und M135 direkt im IFT quantifiziert werden. Die gemessene Fluoreszenzintensität war mit der Stärke der Biofilmbildung des entsprechenden Stammes korreliert (Abbildung 4).

Das gleiche anti-rEmbp Antiserum war geeignet, um die Biofilmbildung von 1585v zu inhibieren. Der direkte Nachweis der aggregativen Eigenschaften von Embp konnte durch den Einsatz rekombinant exprimierter Anteile des Proteins erbracht werden. Die Zugabe des etwa 70 kDa großen, aus alternierend angeordneten FIVAR- und GA-Domänen bestehenden Embp-Fragments rEmbp6599 zum serumfreien Kulturmedium war hinreichend, um Biofilmbildung bei den Stämmen 1585 und M135 auszulösen. Die Stärke der Biofilmbildung korrelierte dabei mit der Menge des zugesetzten Proteins. Dagegen hatte ein allein FIVAR-haltiges Fragment vergleichbarer Größe (rEmbp2588) auch in hoher Konzentration keinen Einfluss auf Zellaggregation und Biofilmbildung.

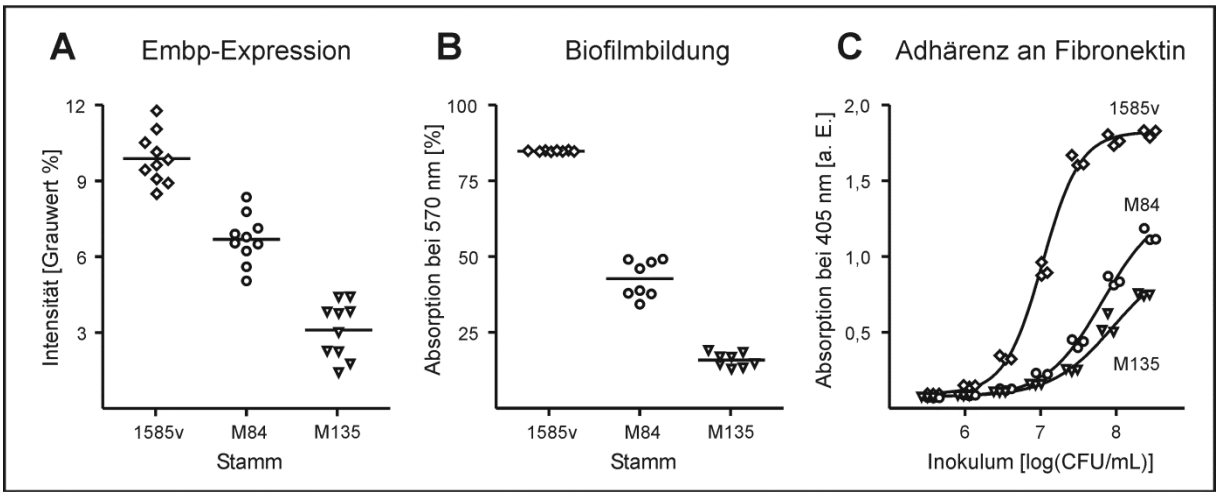

Abbildung 4: Biofilmbildung und Fibronektinadhärenz bei Stämmen mit unterschiedlich starker Embp-Expression.
A. Embp-Expression im quantitativen Immunfluoreszenztest. Die Detektion von zellulärem Embp erfolgte mit anti-rEmbp6599 Antiserum und Alexa-488 markierten Zweitantikörpern. Die Fluoreszenzintensität ist dargestellt als mittlerer Grauwert im Fluoreszenzkanal über die Fläche der in der korrespondierenden Hellfeldaufnahme detektierten Bakterien.
B. Biofilmbildung auf Polystyrol. Nach 20Stunden statischer Kultur in TSB-Medium wurden adhärente Bakterien mit Gentianaviolett gefärbt und über die Absorption bei 570 nm quantifiziert.
C. Bakterienadhärenz an eine fibronektinbeschichtete Polystyrenoberfläche. Unterschiedliche Bakterienkonzentrationen wurden für eine Stunde bei 37°C in beschichteten Mikrotiterplatten inkubiert. Nach mehrmaligem Waschen wurden adhärente Bakterien mit einem anti-Staphylokokken Antiserum und alkalische Phosphatase konjugierten Zweitantikörpern detektiert.

Die aggregativen Eigenschaften von Embp1 in serumfreiem Medium konnten durch Austausch des natürlichen Embp-Promotors bei 1585 gegen einen tetrazyklininduzierbaren Promotor auch für das intakte Embp demonstriert werden. Das *embp*-Transkriptionsniveau konnte beim resultierenden Stamm 1585$P_{xyl/tet}$*embp* durch Induktion um den Faktor 10 gegenüber der nicht induzierten Kontrolle und dem Wildtyp 1585 erhöht werden. In der Folge war oberflächenassoziiertes Embp mittels SDS-Gelelektrophorese und IFT nachweisbar und der Stamm bildete einen Biofilm.

Die Rolle von Embp bei der Biofilmbildung des Wildtyps 1585 in serumhaltigem Kulturmedium wurde durch Transduktion der Transposoninsertion von M135 in 1585 bestätigt. Die Oberflächendichte der mittels Embp-Antiserum im IFT detektierbaren Epitope bei Kultur in 50 % Serum war bei 1585-M135 im Vergleich zu 1585 signifikant reduziert. Wie die 1585v-Transposonmutante M135 hatte auch der Stamm 1585-M135 in Folge der Transposoninsertion die Fähigkeit zur Biofilmbildung verloren.

Zusammenfassung und Diskussion

Im Rahmen der vorliegenden Arbeit wurde das Oberflächenprotein Embp als bisher nicht beschriebener Mediator der Biofilmbildung bei *S. epidermidis* identifiziert. Dem Molekül konnten durch eine Reihe funktioneller Untersuchungen sowohl aggregative als auch adhäsive Eigenschaften attestiert werden.

Entsprechend der Zielsetzung der vorliegenden Arbeit wurde Embp im Rahmen eines offenen Screeningverfahren als essentielle Determinante der Biofilmbildung des Laborstamms 1585v identifiziert. Der anschließende Nachweis der funktionellen Relevanz konnte auf Grund der Größe des *embp*-Gens und der mangelhaften Expression unter den üblichen *in vitro* Bedingungen nicht über den klassischen Ansatz einer knock-out-Mutante und deren Komplementierung erfolgen. Stattdessen wurden die Eigenschaften des Moleküls zunächst aus Untersuchungen an rekombinanten Proteinfragmenten, der Wirkung spezifischer Antiseren und dem Verhalten der Stämme 1585 (keine relevante Embp-Expression in serumfreiem Kulturmedium, kein Biofilm), 1585v (Überexpression der N-terminal verkürzten Embp-Variante Embp1, Biofilm), M84 (C-terminal verkürztes Embp1, schwacher Biofilm) und M135 (C-terminal verkürztes Embp1, kein Biofilm) abgeleitet. Die auf diese Weise für Proteinfragmenten bzw. verkürzte Embp-Varianten gezeigten aggregativen und adhäsiven Eigenschaften konnten schließlich durch die induzierte Expression von Volllängen-Embp beim Wildtypstamm 1585 auch für das native Molekül belegt werden.

Durch das Aggregationsverhalten der Mutanten mit C-terminal verkürztem Embp (M135, M84) und der biofilminduzierenden Wirkung rekombinanter Embp-Fragmente ließen sich die aggregativen Eigenschaften von Embp dem C-terminalen Molekülanteil zuordnen. Die in diesem Bereich lokalisierten repetitiven FIVAR-GA-Module zeigen bereits in Abwesenheit von Serumbestandteilen eine unmittelbar aggregative Wirkung, die die ausschließlich aus FIVAR-Domänen bestehenden Molekülabschnitte offenbar nicht entfalten. Die Rolle der ebenfalls in diesem Molekülabschnitt lokalisierten DUF1542-Domänen sowie der relative quantitative Beitrag einzelner Module muss in folgenden Untersuchungen mit geeigneten rekombinant exprimierten Embp-Konstrukten geklärt werden. Auch die Frage, ob die Embp-abhängige Bakterienaggregation (insbesondere *in vivo*) auf Wechselwirkungen von Embp-Molekülen untereinander oder auf Interaktionen mit anderen bakteriellen Oberflächenmolekülen oder Serumbestandteilen zurückzuführen ist, kann mit den vorliegenden Daten nicht abschließend beantwortet werden. Bei Oberflächenplasmonresonanzanalysen der in dieser Arbeit generierten Embp-Fragmente

ergab sich kein Anhalt für homotype Interaktionen zwischen den entsprechenden Molekülabschnitten. Zudem kann die Induzierbarkeit von Aggregation und Biofilmbildung durch Zugabe eines rekombinanten Embp-Fragments zu *S. epidermidis* Isolaten ohne detektierbare Embp-Eigenexpression als Hinweis auf heterotype Interaktionen gewertet werden. Da jedoch keine Transposonmutanten mit Insertionen in anderen zellwandassoziierten Genen gefunden wurden, fehlen greifbare Hinweise zur Identität heterotyper Interaktionspartner auf der bakteriellen Zelloberfläche. Weitere Aufklärung könnten Interaktionsstudien von Embp mit Zellwand- und Serumkomponenten erbringen.

Bereits in früheren Arbeiten wurde die fibronektinbindende Aktivität von FIVAR-GA-haltigen Embp-Abschnitten beschrieben (Williams et al. 2002). Die Interaktion mit Fibronektion könnte sowohl die bakterielle Aggregation in Serum (im Sinne einer oben beschriebenen heterotypen Wechselwirkung) befördern als auch Embp-exprimierende Stämmen zur Adhärenz an fibronektinbeschichtete Oberflächen befähigen (Abbildung 4). Im Rahmen der vorliegenden Arbeit konnte die funktionelle Relevanz dieser Wechselwirkung durch Bakterienadhärenzteste an fibronektinbeschichteten Polymeroberflächen und an extrazellulärer Matrix von Mausfibroblasten belegt werden. Außerdem wurden die für die Wechselwirkung relevanten Molekülbestandteile beider Interaktionspartner (FIVAR Domänen von Embp und Typ-III Domänen von Fibronektin) mittels Far-Western Blot und Oberflächenplasmonresonanzanalyse identifiziert. Die eingehendere Charakterisierung der Fibronektin-Embp-Interaktion ist bereits Gegenstand weiterer Untersuchungen (Rohde et al. 2011).

Da die in dieser Arbeit erzeugten adhärenz- und aggregationsdefizienten Embp-Mutanten bisher nicht in einem der etablierten Tiermodelle für fremdmaterialassoziierte Infektionen untersucht wurden, stehen eindeutige Belege für die biologische Relevanz der aufgezeigten aggregativen und fibronektinbindenden Eigenschaften von Embp bei der Pathogenese von *S. epidermidis* Infektionen derzeit aus. Allerdings konnte in einer vergleichenden funktionellen Analyse gezeigt werden, dass Embp vermittelte Biofilme die phagozytische und inflammatorische Aktivität von einer makrophagenähnlichen Mauszellline (J774A.1) in gleichem Ausmaß beeinträchtigen, wie die gut charakterisierten Biofilme auf Basis des Polysacharids PIA (Schommer et al. 2011). Einen weiteren indirekten Hinweis auf die Bedeutung PIA-unabhängiger Mechanismen der Biofilmbildung lieferten Arbeiten an einem Meerschweinchenmodell für fremdkörperassoziierte Infektionen, bei denen die Pathogenität

der getesteten KNS nicht auf der Grundlage eines PIA-Nachweises prognostiziert werden konnte (Chokr et al. 2007, Francois et al. 2003).

Unter infektionsassoziierten *S. epidermidis* Isolaten ist das *embp*-Gen weit verbreitet (Rohde et al. 2007, Rohde et al. 2004). Da das Protein jedoch von keinem der bisher von uns untersuchten Isolate unter den Standardbedingungen des *in vitro* Biofilmtests (Kultur in serumfreiem Medium) in relevantem Maße exprimiert wird, muss davon ausgegangen werden, dass die Fähigkeit zur Embp-vermittelten Biofilmbildung bei früheren phänotypischen Screenings übersehen wurde. Der hier geführte Nachweis eines bisher nicht beschriebenen, in der Regel nur unter *in vivo* ähnlichen Bedingungen nachweisbaren Mechanismus‘ der Biofilmbildung bei *S. epidermidis* liefert eine mögliche Erklärung für den regelhaften Nachweis „biofilmnegativer“ Isolate als Auslöser klassischer biofilmassoziierter Infektionen (Klug et al. 2003, Petrelli et al. 2006, Rohde et al. 2007). Jeder neu entdeckte Mechanismus verringert zudem die Erfolgsaussichten spezifischer, allein auf die PIA-assoziierte Biofilmbildung abzielender Präventionsstrategien (Hennig et al. 2007).

Beiträge des Promovenden

Der Promovend war im Rahmen der vorgelegten Publikation für die Transposonmutagenese, die genotypische und phänotypische Charakterisierung der Transposonmutanten, die Untersuchung der Bakterienadhärenz und die statistische Analysen verantwortlich.

Der Promovend war an der Konzeption der Arbeit (mit D. Mack, M. Aepfelbacher und H. Rohde), der quantitativen Analyse der *embp*-Expression (mit G. Franke), der Herstellung rekombinanter Embp-Fragmente (mit G. Kroll), der Analyse der Wechselwirkung zwischen Embp und Fibronektin mittels Oberflächenplasmonresonaz (mit C. Schulze) sowie der Abfassung des Manuskripts (mit H. Rohde, D. Mack und M. Aepfelbacher) und der Erstellung von Abbildungen (mit H. Rohde) maßgeblich beteiligt.

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4. Lebenslauf

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Publikationen als Erst- oder Letztautor

Wolters M, Rohde H, Maier T, Belmar-Campos C, Franke G, Scherpe S, Aepfelbacher M, **Christner M**. 2011. MALDI-TOF MS fingerprinting allows for discrimination of major methicillin-resistant Staphylococcus aureus lineages. *Int J Med Microbiol,* 301**,** 64-8.

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5. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

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