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## ***Staphylococcus aureus* DsbA is a membrane-bound lipoprotein with thiol-disulfide oxidoreductase activity**

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**Abstract** DsbA proteins, the primary catalysts of protein disulfide bond formation, are known to affect virulence and penicillin resistance in Gram-negative bacteria. We identified a putative DsbA homologue in the Gram-positive pathogen *Staphylococcus aureus* that was able to restore the motility phenotype of an *Escherichia coli dsbA* mutant and thus demonstrated a functional thiol oxidoreductase activity. The staphylococcal DsbA (SaDsbA) had a strong oxidative redox potential of  $-131$  mV. The persistence of the protein throughout the growth cycle despite its predominant transcription during exponential growth phase suggested a rather long half-life for the SaDsbA. SaDsbA was found to be a membrane localised lipoprotein, supporting a role in disulfide bond formation. But so far, neither in vitro nor in vivo phenotype could be identified in a staphylococcal *dsbA* mutant, leaving its physiological role unknown. The inability of SaDsbA to interact with the *E. coli* DsbB and the lack of an apparent staphylococcal DsbB homologue suggest an alternative re-oxidation pathway for the SaDsbA.

**Keywords** DsbA · *Staphylococcus aureus* · Disulfide · Lipoprotein · Cysteine

### **Introduction**

Disulfide bonds between cysteine residues support the correct folding of proteins and maintain their structural integrity and activity. Disulfide bond containing proteins are usually localised in extracytoplasmic compartments or secreted into the medium, but are rare in the reducing environment of the cytoplasm. Although disulfide formation can occur spontaneously under oxidising conditions, this reaction is unspecific and slow (Anfinsen 1973). In vivo, enzymatic systems ensure efficient and correct disulfide bond formation. The primary catalyst of this reaction in *Escherichia coli* is the extensively studied periplasmic thiol-disulfide oxidoreductase DsbA. It is a monomeric 21-kDa protein containing a redox active C-X-X-C sequence embedded in a thioredoxin fold, typical for the proteins of this family (reviewed in Kadokura et al. 2003). Upon catalysing disulfide bond formation, the two cysteines of DsbA become reduced and are subsequently re-oxidised by the inner membrane protein DsbB, which passes the electrons to the electron transfer chain via quinone reduction (Guilhot et al. 1995; Kishigami et al. 1995; Kishigami and Ito 1996; Bader et al. 1998, 1999).

*Escherichia coli dsbA* mutants have a pleiotropic phenotype and, amongst others, lack motility due to the improper assembly of the flagellar motor (Jones et al. 1990; Dailey and Berg 1993). Interestingly, *dsbA* mutants of several Gram-negative pathogenic bacteria also exhibit a reduced virulence, due to the absence of critical disulfide bonds in exotoxins, adherence factors or pili (Yu and Kroll 1999; Yu et al. 2001; Stenson and Weiss 2002; Ha et al. 2003; Burall et al. 2004; Miki et al. 2004). So far, an involvement of a DsbA homologue in virulence of a Gram-positive pathogen has not been documented.

In Gram-negative bacteria, disulfide bond formation occurs in the periplasm. Gram-positive bacteria do not possess a classic periplasmic compartment, and very little is known about disulfide bond formation in these organisms. The *Bacillus brevis* Bdb was the first DsbA

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**Table 1** Bacterial strains and plasmids

	Relevant properties	Source or reference
<b>Strains</b>		
<i>Escherichia coli</i>		
Rosetta DE3	BL21 derivative	Novagen
AD13	JCB571, pAD2	This study
AD15	JCB571, pBR322	This study
AD28	JCB571, pAD11	This study
AD29	JCB571, pISC2	This study
AD44	Rosetta DE3, pmSaDsbA, Ap <sup>r</sup>	
AD59	JCB818, pAD2, Km <sup>r</sup> , Ap <sup>r</sup>	This study
AD60	JCB818, pAD11, Km <sup>r</sup> , Ap <sup>r</sup>	This study
AD189	JCB818, pBR322, Km <sup>r</sup> , Ap <sup>r</sup>	This study
AD190	JCB818, pEC52, Km <sup>r</sup> , Ap <sup>r</sup>	This study
AD191	JCB570, pBR322, Km <sup>r</sup> , Ap <sup>r</sup>	This study
JCB570	MC1000 <i>phoR zih12::Tn 10</i>	Bardwell et al. 1991
JCB571	JCB570 <i>dsbA::kan1</i>	Bardwell et al. 1991
JCB818	MC1000 <i>dsbA::kan1, dsbB::kan, Km<sup>r</sup></i>	Jonda et al. 1999
<i>Staphylococcus aureus</i>		
8325-4	NCTC8325 derivative, cured of all prophages	Peng et al. 1988
AD19	RN4220 <i>dsbA::tet; Tc<sup>r</sup></i>	This study
AD21	BB270 <i>dsbA::tetL; Tc<sup>r</sup></i> , methicillin resistant (Mc <sup>r</sup> )	This study
AD22	Newman <i>dsbA::tetL; Tc<sup>r</sup></i>	This study
AD23	MSSA1112 <i>dsbA::tetL; Tc<sup>r</sup></i>	This study
AD188	8325-4 <i>dsbA::tetL; Tc<sup>r</sup></i>	This study
BB270	NCTC8325 <i>mec; Mc<sup>r</sup></i>	Berger-Bächli and Kohler 1983
MB261	BB270 ( <i>dsbAp::luc+</i> )-pBT	This study
Newman	ATCC 25904, clinical isolate	Duthie and Lorenz 1952
RN4220	8325-4; restriction negative	Rhoads Kraemer and Iandolo 1990
<b>Plasmids</b>		
pAD2	pBR322 derivative; containing a 1.4 kb fragment of <i>S. aureus</i> BB270 encoding SA2198 and <i>dsbA; Tc<sup>r</sup>, Ap<sup>r</sup></i>	This study
pAD7	pEC4 derivative ; containing the double crossover construct for the inactivation of <i>dsbA</i> in <i>S. aureus; Tc<sup>r</sup> ; Em<sup>r</sup> ; Ap<sup>r</sup></i>	
pAD11	pISC2 derivative; containing the sequence for a translational fusion of the OmpA signal peptide and the truncated (aa 19-199) DsbA of <i>S. aureus</i> under the control of an arabinose inducible promoter; Ap <sup>r</sup>	This study
pBR322	<i>E. coli</i> cloning vector; Tc <sup>r</sup> , Ap <sup>r</sup>	Bolivar et al. 1977
pBT	<i>S. aureus</i> suicide vector; containing the <i>tetL</i> gene; Tc <sup>r</sup>	Giachino et al. 2001
pBT <i>dsbA::luc+</i>	pBT with 1 kb PCR fragment of the <i>dsbA</i> promoter from strain BB270 fused to the luciferase gene <i>luc+</i> ; Tc <sup>r</sup>	This study
pEC4	Suicide vector ; containing the <i>tetL</i> gene; Tc <sup>r</sup>	Brückner 1997
pEC52	pISC2 derivative; containing the sequence for a translational fusion of the OmpA signal peptide and the truncated CcmG of <i>E. coli</i> under the control of an arabinose inducible promoter; Ap <sup>r</sup>	Fabianek et al. 1998
pET11a	<i>E. coli</i> cloning vector; Ap <sup>r</sup>	Novagen
pISC2	<i>E. coli</i> expression vector; Ap <sup>r</sup>	Thöny-Meyer et al. 1998
pmSaDsbA	pET11a derivative; encoding a truncated DsbA without signal sequence; Ap <sup>r</sup>	This study
pSP- <i>luc+</i>	Firefly luciferase cassette vector; Ap <sup>r</sup>	Promega
pSP- <i>dsbA::luc+</i>	1 kb PCR fragment of <i>dsbA</i> promoter from strain BB270 in pSP- <i>luc+</i> ; Ap <sup>r</sup>	This study

*aa* amino acids

homologue of a Gram-positive bacterium shown to have thiol oxidase activity by complementing the motility of an *E. coli dsbA* mutant (Ishihara et al. 1995). Subsequently, the *Bacillus subtilis* BdbC and BdbD were demonstrated to be functional homologues of the *E. coli* DsbA and DsbB, respectively, and to be involved in the

development of competence (Erlendsson and Hederstedt 2002; Meima et al. 2002). Although some of the secreted virulence factors produced by the human pathogen *Staphylococcus aureus*, such as all staphylococcal enterotoxins and the beta-haemolysin, are known to contain disulfide bonds in their active forms (Hovde et al.

**Table 2** Primers

Primers	Sequences 5' - 3' <sup>1</sup>	Position of the 3' end <sup>2</sup>	Restriction site
Seq1	catgatcgcgattatcatg	-38	
Seq2	ctgcatgactcgcacgcaacc	253	
Seq3	ctcgtacaattaaaattgtc	33	
Seq4	gctataatagtgtgtact	-71	
Pr1f	<u>gggaattcatgtttgacgggtatag</u>	-431	<i>EcoRI</i>
Pr1r	<u>ggaaagctfactttgtcataaagtgc</u>	914	<i>HindIII</i>
PEX1	taatgttagtaatttttagtc	3	
PEX2	atcacctgtgcctttttgg	-256	
PEX3	cacaaatcgctcaatgctc	-385	
DsbApf	<u>gaggtaccaatttcacgtctgcattgcg</u>	-977	<i>Asp718</i>
DsbApr	<u>tagccaaggtaactcctgttcatactagttcg</u>	-26	<i>KpnI</i>
SaDsbAf	<u>cctagctagcatgcatcaccatcaccacatggtaaaaaagaatcagcaacgacatcc</u>	84	<i>NheI</i>
SaDsbAr	<u>ccgcggatccctattttatcttttaataacttctcataacttc</u>	525	<i>BamHI</i>
Pr3f	<u>cagaggcctcaaaaaagaatcagcaacg</u>	78	<i>StuI</i>
Pr3r	<u>cgggaattcgaagaatacacctatcctg</u>	755	<i>EcoRI</i>
Pr4f	cgaaaagtgccacctgacg		
Pr4r	aactaatggtttccggttttc	88	
TetL +	cctattgtgtataaagtg		
TetL -	cctgtataaaaaaggatc		

<sup>1</sup>The recognition sites of restriction enzymes are underlined  
<sup>2</sup>The indicated position is relative to the first nucleotide of *dsbA*

1994; Dziewanowska et al. 1996), the mechanism by which these disulfide bonds are formed has never been examined. In this study we describe the identification and functional characterisation of a putative DsbA homologue (SA2197) in *S. aureus* and analyse its importance for virulence and antibiotic resistance.

## Materials and Methods

### Bacterial strains, plasmids, and media

The strains and plasmids used in this study are listed in Table 1. The cells were grown at 37°C in Luria-Bertani (LB) medium. When required, tetracycline 10 µg/ml, or ampicillin 100 µg/ml were added.

### DNA manipulations

Preparation of chromosomal and plasmid DNA, PCR, and Southern blot analysis were performed by standard procedures (Sambrook et al. 1989). Probes were labelled with the PCR DIG Probe Synthesis Kit (Roche, Rotkreuz, CH, USA), following the manufacturer's instructions. Transductions were done with phage 80α as described previously (Berger-Bächi 1983). Primers used for PCR, sequencing and primer extension reactions are listed in Table 2.

### Sequence analysis

Direct chromosomal sequencing of the SA2198-*dsbA* operon of strain BB270 was done according to the protocol of A. Wada (Wada 2001) on a ABI Prism 310 Sequencer (Perkin Elmer) with the primers Seq1, Seq2,

Seq3, Seq4 and PEX2. The sequences were compared to genome sequences of *S. aureus* NCTC8325 (NC\_002954), Mu50 (NC\_002758), N315 (NC\_002745), MW2 (NC\_003923) (Kuroda et al. 2001), COL (TIGR), MSSA476 (NC\_002953) and MRSA252 (NC\_002952). Homology searches were performed using the program BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) (Altschul et al. 1990). The sequence was deposited in GenBank under the accession number AF321274.

### Isolation and analysis of total RNA

Total RNA was isolated according to the protocol of Cheung (Cheung et al. 1994), using the FastRNA kit (BIO 101, Vista, CA, USA). Northern blot analysis was performed as described by Sambrook (Sambrook et al. 1989). The transcriptional start was determined by primer extension using the <sup>32</sup>P-labeled primers PEX1, PEX2 and PEX3 as described by Rossi (2003).

### *dsbA* promoter-reporter gene construct

A 1 kb DNA fragment covering the SA2198-*dsbA* promoter region (*dsbAp*) of *S. aureus* was amplified using the primers DsbApf and DsbApr, digested with *KpnI* and *NcoI* and cloned in front of the luciferase gene of plasmid pSP-*luc+*. A 2.6 kb *KpnI-EcoRI* fragment, including the *dsbA* promoter region fused to the luciferase coding region was subsequently subcloned into suicide plasmid pBT (Giachino et al. 2001) yielding plasmid pBT *dsbAp::luc+*, which was electroporated into RN4220. A co-integrate was selected and used as a donor to transduce strain BB270, resulting in strain MB261. The luciferase activity was measured as described earlier (Bischoff et al. 2001).

## SaDsbA-isolation and antibody preparation

The primers SaDsbAf and SaDsbAr were used to amplify a fragment encoding the presumed mature part of SaDsbA lacking its signal sequence (mSaDsbA). The fragment was cloned into pET11a using *Nhe*I and *Bam*HI restriction sites. The cloning procedure did add four supplementary amino acids M-A-S-M to the N-terminus of mSaDsbA, thereby introducing a new start methionine to the mSaDsbA. The resulting plasmid pmSaDsbA was transformed into Rosetta DE3 cells (Novagen) for overexpression. Cells were grown in LB medium to an OD<sub>600</sub> of 0.8 and induced with 1 mM IPTG. After 3 h, cells were collected by centrifugation and resuspended in 20 mM Tris/HCl pH 8.0. The cells were disrupted (Cell Cracker, EMBL, Heidelberg Germany), and the debris separated by centrifugation for 30 min at 15,000 *g*. The supernatant was applied onto a QA-52 anion-exchange column (Whatman, Brentfort, UK) pre-equilibrated with 20 mM Tris/HCl pH 8.0. Proteins were eluted with a 0–1 M gradient of NaCl in 20 mM Tris/HCl pH 8.0. Fractions containing mSaDsbA were pooled, dialysed against 20 mM HEPES/NaOH pH 8.0 and applied onto a SE52 cation-exchange column (Whatman). mSaDsbA was eluted with a gradient of 0–1 M NaCl in 20 mM HEPES/NaOH pH 8.0. One milligram of the eluted, homogenous mSaDsbA was used to raise chicken antibodies (DAVIDS Biotechnologie, Regensburg, Germany).

## Fluorescence spectroscopy and determination of the redox potential

The redox potential of mSaDsbA was determined essentially as previously described (Grauschopf et al. 2003) by measuring the redox equilibrium of mSaDsbA with glutathione using a specific fluorescence intensity change at 327 nm between the oxidised and reduced state of the protein.

## Localisation of SaDsbA

Exponentially growing Newman cells harvested at OD<sub>600</sub> 2 were disrupted and fractionated into supernatant, cell wall, cell membrane and cytoplasm, according to Schneewind (1993). Protein fractions corresponding to a 1-ml culture were subjected to Western analysis using antigen-purified anti-DsbA antibodies at a dilution of 1:5,000. To demonstrate the lipoprotein nature of SaDsbA, growing cells were challenged with 50 µg/ml globomycin (Sankyo Corp., Tokyo, Japan) at OD<sub>600</sub> 0.4 and harvested at OD<sub>600</sub> 2. Aliquots containing 15 µg whole protein were subjected to Western blot analysis using the conditions given above.

## Complementation of an *E. coli dsbA* mutant by the *dsbA* gene of *S. aureus*

A 1.4-kb fragment covering the SA2198- *dsbA* operon as well as the SA2198- *dsbA* promoter was amplified using

the primers Pr1f and Pr1r. The obtained insert was cloned into the EcoRV site of pBR322, yielding pAD2. Plasmid pAD11, carrying a translational in-frame fusion of the *E. coli* *OmpA* signal peptide, MKKTAIAIAVA-LAGFATVAQA, with the putative mature part of SaDsbA (aa 19–199) was constructed by amplifying a 735-bp fragment encoding an SaDsbA lacking its 18 N-terminal amino acids with the primers Pr3f and Pr3r, and cloning it into the StuI-EcoRI restricted pEC52 (Fabianek et al. 1998). In this construct the codons for cysteine 19 and glycine 20 were changed to codons for alanine and serine, respectively, thereby eliminating the signal peptidase II recognition site of SaDsbA.

To determine the complementation of the non-motile *E. coli dsbA* mutant strain JBC571 (Bardwell et al. 1991), plasmids pAD2, pAD11 as well as the empty vectors pBR322 and pISC2 were electroporated into *E. coli* JCB571. The motility of the resulting strains AD13, AD28, AD15 and AD29, respectively, was tested by placing filter discs of 6 mm diameter (bioMérieux) soaked in the respective overnight cultures, onto an LB plate containing 0.3% agar and 0.2% arabinose and incubating for 12.5 h at 37°C. To test the dependence of the complementation on the presence of DsbB, plasmids pAD2, pAD11, pBR322 and pISC2 were electroporated into the *E. coli dsbA dsbB* double mutant JCB818, yielding strains AD59, AD60, AD189 and AD190, respectively. As a control, pBR322 was electroporated into the wild-type strain JCB570, yielding AD191. The dependence of the complementation of the *dsbA* and *dsbA dsbB* mutants on the presence of cystine was demonstrated by growing the strains overnight in liquid M9 medium containing 4 µg/ml of each amino acids except cysteine, 0.4% glycerol and 100 µg/ml ampicillin. Thirty microlitre of the overnight culture, resuspended to an OD<sub>600</sub> of 0.2, were placed on 6 mm filter discs which were transferred to an M9 agar plate containing 0.3% agar, 0.2% arabinose and 4 µg/ml of each amino acid except cysteine. When required, 25 µg/ml cystine was added to the medium. The distance reached by motile swarming cells was determined after overnight incubation.

## Insertional inactivation of *dsbA*

The *dsbA* gene from *S. aureus* was inactivated by replacing a 249 bp internal fragment with a 1.6-kb *tetL* cassette. An 845-bp fragment upstream of *dsbA* was amplified using pAD2 as a template and the primers Pr4f and Pr4r, digested with EcoRI, and inserted into the 4.7-kb fragment *EcoRI-HpaI* fragment of pAD2. The three bases GTT at the 3'-end of the insert restored an *HpaI* site after ligation with the vector. The 1.6-kb *tetL* fragment was amplified by PCR from plasmid pBT using the primers TetL+ and TetL- and inserted into the restored *HpaI* site. From this product the 2.7-kb *EcoRI-BamHI* fragment was excised and inserted into the corresponding sites of pEC4, yielding plasmid pAD7. Tetracycline

resistant clones obtained after electroporation of pAD7 into the *S. aureus* RN4220 were screened for loss of erythromycin resistance. About 5% of the tetracycline resistant clones were sensitive to erythromycin, indicating loss of the plasmid backbone due to a double crossover. The *dsbA::tetL* allele of the resulting strain AD19 was transferred to strains BB270, Newman, MSSA112, and 8325-4 by phage transduction, resulting in strains AD21, AD22, AD23, and AD188 respectively. The deletion of the *dsbA* fragment on the chromosome was confirmed by Southern blot analysis.

#### Characterisation of the *S. aureus dsbA* mutant

Susceptibility to dithiothreitol (DTT) was tested by placing sterile filter discs of 6 mm diameter impregnated with 20 µl of a 1 M DTT on a lawn of cells diluted to McFarland 0.5 in 0.8% NaCl and spread on Müller-Hinton plates. The inhibition zone was measured after overnight incubation at 37 °C. Susceptibility to antibiotics was assessed by E-test (AB Biodisk, Solna, Sweden) following the recommendations of the manufacturer. The relative resistance to oxacillin of the strain pair BB270/AD21 was compared on plates containing a gradient of oxacillin from 0 to 200 µg/ml.

#### Measurement of the haemolytic activity

The haemolytic activities of strain 8325-4 and its *dsbA* defective derivative AD188 were essentially determined following the protocol of Nilsson and co-workers (Nilsson et al. 1997). After incubation of the washed erythrocytes with diluted culture supernatant, the samples were centrifuged for 1 min at 7000 g. The optical density at 415 nm due to the released haemoglobin was measured and plotted against the dilution factor of the culture supernatant.

#### Animal models

Strain Newman and its *dsbA* mutant AD22 were used in two pathogenicity assays. The *Caenorhabditis elegans* killing assay was performed according to the protocol by Sifri and co-workers (Sifri et al. 2003). In the mouse model for septic arthritis, the protocol described by Bremell and co-workers (Bremell et al. 1991) was followed, using 15 mice and a bacterial load of  $6 \times 10^6$  cells.

## Results

#### Identification of a putative DsbA homologue

*B. subtilis* BdbD was previously identified as a close *E. coli* DsbA homologue in a Gram-positive organism

(Fabianek et al. 2000). A BLAST search for Bdb homologues in the *S. aureus* NCTC8325 genome database resulted in the identification of a 199-amino acid ORF with 41% similarity to BdbD that was tentatively termed SaDsbA. No further Dsb or Bdb homologues were found in any of the published genome sequences of *S. aureus*. Direct chromosomal sequencing of the NCTC8325 derivative BB270 over the region covering *dsbA* showed an exact match with the corresponding published genome sequences of NCTC8325 and COL. In *S. aureus* MW2, Mu50, N315, MRSA252 and MSSA476, a G to A transition causes the valine residue at position 38 to be changed to a isoleucine. In addition, a G to T transversion causes the change of leucine at position 52 to a phenylalanine in strain MRSA252.

The deduced sequence of the 20 N-terminal amino acids of the *S. aureus* DsbA revealed the characteristics of a lipoprotein signal sequence with an ILTACG predicted signal peptidase II cleavage site (PROSITE motive PS00013) (Tjalsma et al. 2000; Sutcliffe and Harrington 2002), suggesting SaDsbA to be an extracytoplasmic lipoprotein with a lipid attachment site at cysteine 19.

The predicted size of 181 aa of the deduced mature SaDsbA was similar to that of DsbA from other bacteria, and conserved residues were found over the entire length of its primary structure (Fig. 1). SaDsbA harbours, with its C-P-Y-C motif, the C-X-X-C active site characteristic for thiol-disulfide oxidoreductases, and possesses a proline residue at position 154, which aligns well with the *cis*-proline at position 151 of *E. coli* DsbA (Fig. 1). The *cis*-proline, strongly conserved in all members of the thioredoxin family, and positioned close to the C-X-X-C motif in their native form (Martin 1995), has a critical role in the resolution of the mixed disulfide intermediate (Kadokura et al. 2004) and affects the stability of DsbA proteins (Charbonnier et al. 1999).

Upstream of the *S. aureus dsbA*, and separated by 21 nucleotides, a 360-bp open reading frame (ORF), SA2198, was identified. This ORF encodes a putative protein of 120 amino acids that shares no apparent similarity to any other protein with known function. SA2198 appears to have a lipoprotein signal sequence with a FISGCG predicted signal peptidase II cleavage site. The sequence of this ORF was found to be identical in all the published *S. aureus* genome sequences, suggesting that its gene product is functional in *S. aureus*. Additionally, a similar genetic organisation was found in the closely related *Staphylococcus epidermidis*, with the two predicted lipoproteins, SE1987 sharing 68% sequence similarity with SaDsbA, and SE1988 sharing 65% similarity with SA2198.

#### Analysis of the *dsbA* transcript

Northern blot analyses with probes covering either *dsbA* or SA2198-*dsbA*, yielded a single 1.3-kb transcript

<i>S. aureus</i>	18 <sup>1</sup>	VVYGDYKCPYCKELD	149	HIKTPTAFINGEK	AAG41993	181 aa <sup>2</sup>
<i>S. epidermidis</i>	18	VVIYGDYKCPYCKKTE	149	HIKQTPAFVNDNK	NP_765542	180 aa
<i>B. subtilis</i>	24	VVEFGDYKCPCKVFN	151	NIQATPTIYVNDKV	C70041	196 aa
<i>B. anthracis</i>	30	VVEFGDFKCPAORTWD	158	KVQGAPSVVNGNL	NP_654476	189 aa
<i>N. meningitidis 1</i>	50	VLEFFGYFCPHOAHLE	171	QISGTPTVIVGGKY	NP_284883	213 aa
<i>N. meningitidis 2</i>	49	VLEFFGYFCPHCAHLE	172	QIDGTPTVIVGGKY	NP_284900	214 aa
<i>N. meningitidis 3</i>	26	VLEFFGYFCVHCHHFD	147	RIDSTPTVVVGGKY	NP_284776	195 aa
<i>E. coli</i>	22	VLEFFSFFCPHCYQFE	146	QLRGVPAMFVNGKY	P24991	189 aa
<i>S. typhimurium</i>	22	VLEFFSFFCPHCYQFE	145	QLQGVAMFVNGKY	O30848	188 aa
<i>Y. pestis</i>	22	VLEFFSFFCPHCYQFE	145	QLRGVPAMFVNGKY	NP_671106	188 aa
<i>H. influenzae</i>	22	VLEFFSFFCPHCYAFE	144	KVRGVPDFVNGKF	P31810	183 aa
<i>V. cholerae</i>	22	VNEFFSFFCPHCNTFE	144	GLTGVAVVNNRY	P32557	181 aa
<i>P. aeruginosa</i>	26	VVELFWYGCPHCYAFE	146	QVTGVPTMVVNGKY	NP_254176	189 aa

**Fig. 1** Alignment of the conserved active site and of the *cis*-proline region of some members of the DsbA protein family. The alignment was performed with the program ClustalW (Thompson et al. 1994) using the default parameters. Identical residues are underlined in black. Dark grey and grey shaded residues correspond to conserved and semi-conserved substitutions, respectively. <sup>1</sup>The position indicated refers to the first aa in the alignment of the mature protein. The position of the cleavage site was predicted by the program SignalP using the standard parameters (Bendtsen et al. 2004). <sup>2</sup>The indicated length refers to the mature protein. Sequences shown are from *S. aureus*, *S. epidermidis*, *B. subtilis*, *Bacillus anthracis*, *Neisseria meningitidis*, *E. coli*, *Salmonella typhimurium*, *Yersinia pestis*, *Haemophilus influenzae*, *Vibrio cholerae* and *Pseudomonas aeruginosa*

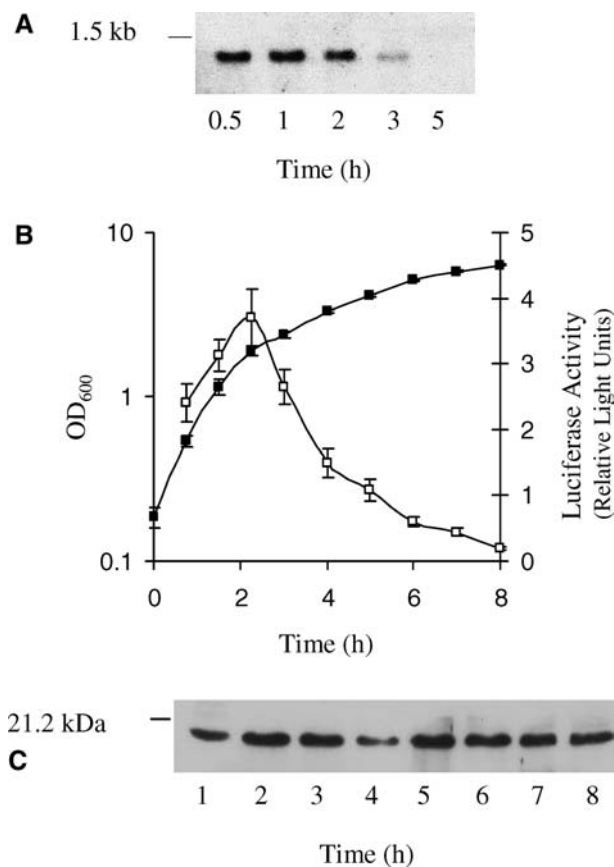
(Fig. 2a), indicating that SA2198 and *dsbA* are co-transcribed, thereby forming a bi-cistronic operon. The level of the 1.3-kb transcript was high during early exponential growth (30 min to 2 h) and strongly decreased thereafter (3 and 5 h). A *dsbA* promoter-luciferase reporter gene fusion, co-integrated into the *S. aureus* chromosome of strain MB261, confirmed the growth phase-dependent expression pattern of SA2198-*dsbA* (Fig. 2b). Western blot analysis, however, demonstrated SaDsbA to be present at all time points analysed, irrespective of the transcription pattern of its gene (Fig. 2c).

Primer extension experiments, using primers binding either within *dsbA* or SA2198 coding sequences, yielded one transcriptional start point 42 bp upstream of SA2198 (Fig. 3), confirming the co-expression of these two genes. The identified transcriptional start point is preceded by a sequence (TTGTAG<sub>-20</sub>-TATAGT), which is fairly well resembling the consensus promoter sequence of the house-keeping  $\sigma^A$  factor (TTGACA<sub>-16-18</sub>-TATAAT) (Helmann 1995; Rao et al. 1995), with the predicted -35 and -10 regions being separated, however, by an unusually long spacer region of 20 nucleotides.

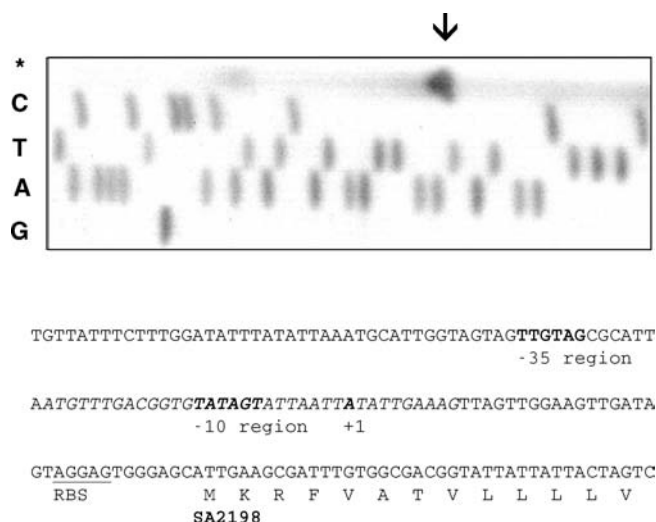
#### Production of SaDsbA in *E. coli* and determination of its redox potential

We produced the predicted mature part of SaDsbA in the cytoplasm of *E. coli* strain AD44 containing plasmid pmSaDsbA. After the two-step purification using an anion-exchange and a cation-exchange column, 80 mg of pure mSaDsbA were obtained from an original 6 l

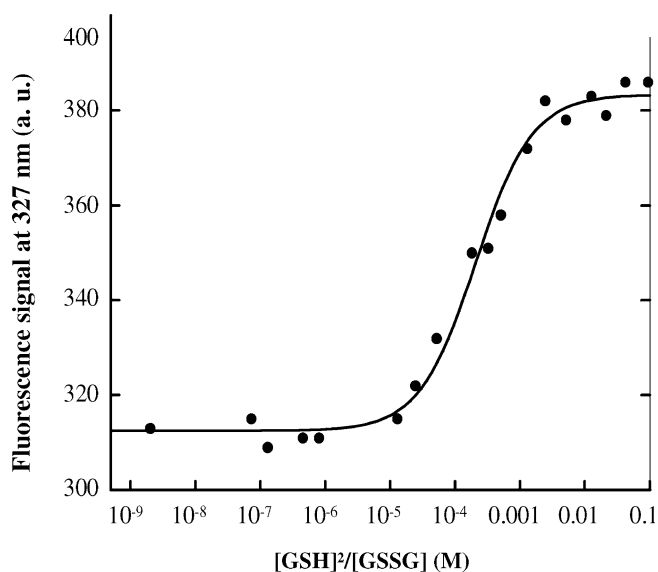
culture. We found that oxidised mSaDsbA, similarly to its *E. coli* homologue exhibited an increase in tryptophane fluorescence at 327 nm upon reduction of the disulfide bond by dithiothreitol. We made use of this redox-state-dependent fluorescence to determine the intrinsic redox potential of mSaDsbA. By equilibrating the protein with different glutathione redox buffers, a



**Fig. 2** Expression pattern of *dsbA* transcript during growth. **A** Northern blot analyses of *dsbA* expression in strain BB270 using a DIG-labelled DNA-probe specific for SA2198 and *dsbA*. **B** The transcriptional activity of the *dsbA* promoter was determined by measuring the luciferase activity of a *dsbA::luc* + fusion (open symbols). Bacterial growth was measured as the OD<sub>600</sub> (solid symbols). **C** Detection of SaDsbA by Western blot analysis. Fifteen micrograms obtained from whole-cell extracts were loaded in each lane

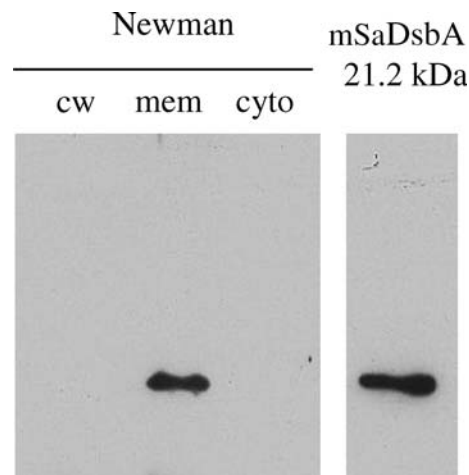


**Fig. 3** Determination of the transcription start site of the SA2198-*dsbA* transcript by primer extension. The signal detected using the  $\gamma$ - $^{32}$ P labelled primer PEX3 is designated by an *arrow*. The same start site was obtained with the primers PEX1 and PEX2 (data not shown). The portion of the sequence represented in the picture is written in *italics*. The -10 region, the mapped transcriptional start, the ribosome binding site and the first amino acids of SA2198 are indicated



**Fig. 4** Redox equilibrium of SaDsbA with glutathione. SaDsbA was incubated in the presence of 1 mM GSSG and varying concentrations of GSH in 100 mM sodium phosphate, pH 7.0 at 25 °C. The tryptophane fluorescence at 327 nm was recorded, and data were fitted as described (Grauschopf et al. 2003)

single transition with an equilibrium constant ( $K_{eq}$ ) of  $2.09 \pm 0.27 \times 10^{-4}$  M was detected, corresponding to a redox potential ( $E'_{o}$ ) of -131 mV (Fig. 4). Thiol-disulfide oxidoreductases of the thioredoxin family have redox potentials ranging from -270 mV for the *E. coli* thioredoxin to -122 mV for the most oxidative of all, the *E. coli* DsbA.



**Fig. 5** Subcellular localisation of SaDsbA. Cells of strain Newman were fractionated in cell wall (*cw*), cell membrane (*mem*) and cytoplasmic (*cyto*) fractions. As control, 30 ng of purified mature staphylococcal DsbA (*mSaDsbA*) was loaded. The protein fractions were separated on a 12% SDS-PA gel and analysed by Western blotting using the anti-DsbA antibody. The amount of protein of each fraction corresponded to a 1-ml culture

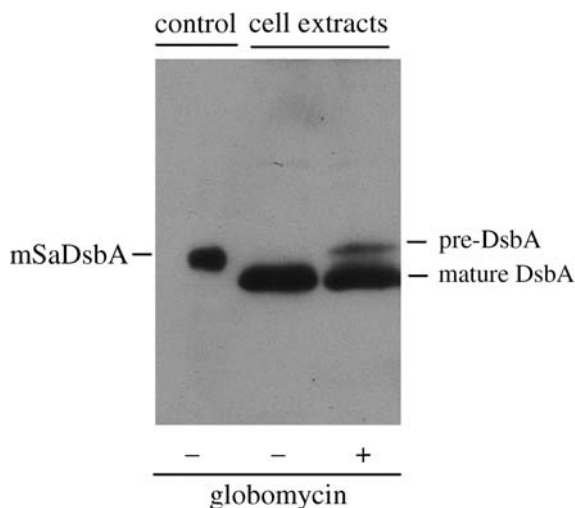
#### Localisation of SaDsbA

To confirm that SaDsbA is a lipoprotein, cells of strain Newman were fractionated into cell wall, cell membrane and cytoplasm. The fractions were subjected to Western blot analysis using polyclonal antibodies raised against the purified SaDsbA. An antigen corresponding to the expected size of 21.0 kDa was detected exclusively in the membrane fraction (Fig. 5). Additionally, no SaDsbA was detected among TCA precipitated proteins obtained from the culture supernatant. In order to confirm the lipoprotein character of SaDsbA, we challenged exponentially growing cells of strain Newman with globomycin, an antibiotic which inhibits the cleavage of the lipoprotein signal sequence by blocking signal peptidase II, resulting in the accumulation of an unprocessed form of the protein that typically runs 2–3 kDa higher than the mature lipoprotein (Inukai et al. 1984). This specific effect of globomycin has been previously used for analysis of lipoprotein maturation in *S. aureus* (Navarre et al. 1996). Protein extracts from cells treated with globomycin contained a second band running at a slightly higher molecular weight, as expected for the unprocessed SaDsbA (Fig. 6).

#### Complementation of an *E. coli dsbA* mutant

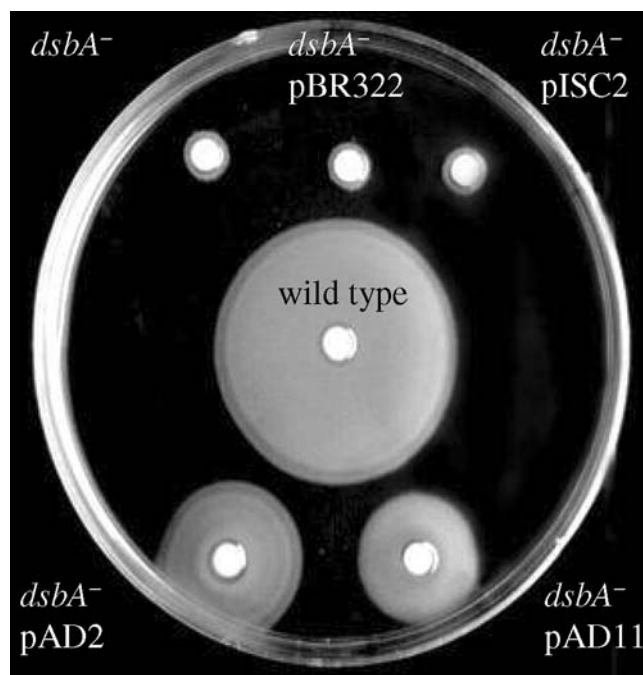
*E. coli dsbA* mutants are impaired in motility due to their inability to form the disulfide-bond of the flagellar protein FlgI (Jones et al. 1990; Dailey and Berg 1993). This phenotype has been used to identify genes





**Fig. 6** Influence of globomycin on the maturation of DsbA. Bacterial cultures were treated with 0 or 50 µg/ml globomycin, respectively. Fifteen micrograms of proteins from whole-cell extracts were analysed by Western blotting using the anti-DsbA antibody. As control, 30 ng of the purified (mSaDsbA) was loaded

that complement *dsbA* mutants (Ishihara et al. 1995; Ng et al. 1997). To demonstrate the functionality of SaDsbA, we complemented the *E. coli dsbA* mutant strain JCB571 with plasmid pAD2, containing SA2198-*dsbA*. This construct clearly restored the motility of the *dsbA* mutant, although not to wild-type levels, whereas complementation with the empty plasmid pBR322, had no effect on motility of the mutant (Fig. 7). To exclude that the efficiency of complementation was impaired because of incompatibilities of the staphylococcal gene at the transcriptional or export level in *E. coli*, we complemented JCB571 with plasmid pAD11, harbouring a sequence encoding a chimeric protein where the signal sequence of the *E. coli* outer membrane protein OmpA was fused to the predicted mature part of SaDsbA. Complementation of the *dsbA* mutant with pAD11 restored the motility of JCB571 in a way that was almost identical to that obtained with pAD2 (Fig. 7). In order to assess whether the activity of SaDsbA in the *E. coli* mutant was dependent on the presence of *E. coli* DsbB, the motility of an *E. coli dsbA dsbB* double mutant was tested in a similar way. Since it is known that cystine, a di-amino acid resulting from the oxidation of cysteine, can functionally complement a *dsbB* mutant (Dailey and Berg 1993), the motility tests were performed on an M9-based swarm agar devoid of cysteine. On this medium, none of the plasmids encoding the *S. aureus* DsbA were able to complement either the *dsbA dsbB* double mutant (Fig. 8), or the *dsbA* single mutant (not shown). When the swarm agar was supplemented with 25 µM cystine, the strains containing pAD2 or pAD11 partially restored motility of both the single and the double mutant (Fig. 8).

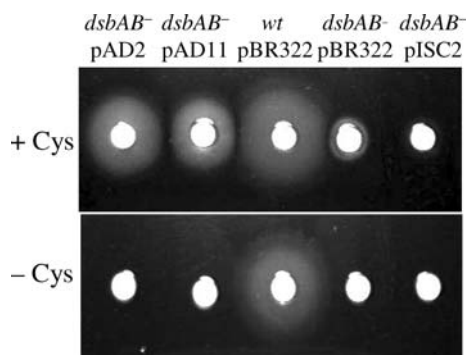


**Fig. 7** Complementation of the motility of an *E. coli dsbA* mutant. Partial restoration of the *E. coli dsbA* deficient strain JCB571 by the staphylococcal DsbA, alone (pAD11) and in combination with SA2198 (pAD2)

#### Inactivation of *dsbA* in *S. aureus*

In order to analyse the function of *dsbA* in *S. aureus*, a *dsbA* mutant was constructed by replacing an internal 249-bp *dsbA* fragment with a *tetL* resistance cassette. The *dsbA* deficient strains were not impaired in aerobic growth. Since *dsbA* mutants of *E. coli* have been shown to have an increased sensitivity to reduced dithiothreitol (DTT) (Missiakas et al. 1993), we tested the *S. aureus* mutant strains for growth inhibition by this substance. The sensitivities of strains AD21, AD22 and AD23 to this substance were the same as those of the respective wild types (data not shown). Another reported phenotype of *E. coli dsbA* is a reduced resistance to the β-lactam antibiotic benzylpenicillin (Missiakas et al. 1993). Since many proteins of *S. aureus* involved in resistance to antibiotics contain cysteine residues that might form disulfide bonds, we tested the resistance of the *S. aureus* mutants to various antibiotics using *E* test or antibiotic gradient plates. The tested antibiotics belonged to various classes, targeting cell wall biosynthesis (beta-lactams and glycopeptides), protein synthesis (aminoglycosides, lincosamides, macrolides), DNA gyrase activity (fluoroquinolones) or RNA synthesis (rifampicin). However, no significant difference could be seen between wild-type and *dsbA* mutant strains with respect to the resistance levels of all antibiotics analysed (data not shown).

The *S. aureus* beta-haemolysin, also termed sphingomyelinase C, requires a disulfide bond for activity



**Fig. 8** Complementation of the motility of an *E. coli dsbA dsbB* double mutant. Where indicated (+ Cys), cystine was added at a concentration of 25  $\mu$ g/ml

(Dziewanowska et al. 1996). We therefore compared the appearances and diameters of the beta-haemolysis-zones of the *dsbA* defective strains AD19, AD21, AD22 and AD23 and their respective parental strains RN4220, BB270, Newman and MSSA1112 on sheep blood agar plates, but were unable to observe notable differences. Similarly, no significant differences were found in the release of haemoglobin from sheep blood cells challenged with supernatants from stationary phase cultures of the beta-haemolysin producing strain 8325-4 or its corresponding *dsbA* mutant AD188 (data not shown).

To assess the impact of DsbA on virulence, strain Newman and its *dsbA* defective derivative AD22 were tested in a *Caenorhabditis elegans* killing assay (Sifri et al. 2003). There was no significant difference between wild type and mutant strain, neither with respect to killing rate, nor killing kinetics (C.D. Sifri, unpublished results). The same strains were also tested in a mouse model for septic arthritis (Bremell et al. 1991). The severity of arthritis, weight decrease and mortality were monitored over a period of 13 days, but no notable difference was observed between Newman and AD22 (I.M. Jonsson, unpublished results).

## Discussion

The family of DsbA proteins is known to constitute the primary catalyst of protein disulfide bond formation in Gram-negative bacteria (reviewed in Kadokura et al. 2003). Members of the DsbA family have been associated in Gram-negatives with pathogenicity and penicillin resistance (Missiakas et al. 1993; Yu and Kroll 1999). With the exception of the Bdb proteins of the genus *Bacillus* (Ishihara et al. 1995; Bolhuis et al. 1999; Erlendsson and Hederstedt 2002), nothing is known about how disulfide bond formation is accomplished by Gram-positives. We have identified a potential DsbA homologue in the human pathogen *S. aureus*. This is of particular interest, since the active forms of various staphylococcal virulence factors, such as enterotoxins and beta-haemolysin, require disulfide bond formation

(Hovde et al. 1994; Dziewanowska et al. 1996). The localisation of SaDsbA as a membrane-anchored lipoprotein supports a putative role in disulfide bond formation of excreted virulence factors.

Heterologous complementation of an *E. coli dsbA* mutant demonstrated that SaDsbA had in fact a functional thiol-disulfide oxidoreductase activity. Moreover, the measured redox potential of SaDsbA was very similar to that of *E. coli* DsbA, indicating that the purified staphylococcal protein is strongly oxidative. However, the apparent lack of a phenotype, either in vitro or in animal infection models, leaves the role of SaDsbA in *S. aureus* open.

Despite its predominant transcription during exponential growth, Western blot analysis demonstrated the presence of SaDsbA throughout the growth. This indicated that SaDsbA showed a rather long half-life and that it might interact with surface associated, as well as excreted proteins, which are predominantly produced by *S. aureus* during the exponential and stationary growth phases, respectively. The anchoring of SaDsbA to the membrane is unusual for the oxidative members of the DsbA family, which are generally secreted through the Sec pathway and cleaved by signal peptidase I, resulting in soluble periplasmic proteins (Schierle et al. 2003). However, recently Tinsley and co-workers reported that two of the three DsbA homologues produced by the Gram-negative pathogen *Neisseria meningitidis* are associated with the inner membrane via a lipoprotein anchor (Tinsley et al. 2004). The particularity of neisseria to secrete toxin-filled outer-membrane vesicles (Pettit and Judd 1992) was postulated to lead to the loss of periplasmic enzymes, in particular the non-membrane-associated DsbA homologue (Tinsley et al. 2004). Gram-positive bacteria, missing an outer-membrane, have to prevent the diffusion of critical enzymes. A thick, almost impermeable cell wall defining a pseudo-periplasm, as in *B. subtilis*, could compensate for the lack of it (Merchante et al. 1995). *B. brevis*, whose DsbA homologue Bdb is predicted to have a cleavable signal sequence (Ishihara et al. 1995), probably makes use of this diffusion barrier. An alternative strategy to prevent diffusion of secreted proteins is anchoring them to the membrane via a lipoprotein anchor or a transmembrane anchor, as postulated for BdbD of *B. subtilis* (Erlendsson and Hederstedt 2002). Lipoproteins of Gram-positive bacteria have been proposed to constitute the functional equivalent of Gram-negative periplasmic proteins (Nielsen and Lampen 1982), which may be the case for SaDsbA.

In *E. coli*, re-oxidation of DsbA is achieved by the action of DsbB (Guilhot et al. 1995; Kishigami et al. 1995; Kishigami and Ito 1996), and in *B. subtilis*, BdbD is re-oxidised by BdbC (Erlendsson and Hederstedt 2002).

The experimental results of the present study strongly suggest that the *E. coli* DsbB was not able to re-oxidise SaDsbA. However, further biochemical experiments would be required to definitely confirm the lack of

interaction between those two proteins. The absence of any DsbB or DsbC homologue, however, indicates that the re-oxidation of the staphylococcus DsbA may be carried out differently. A BLAST search through all sequenced bacterial genomes revealed that a large majority of species encoding a DsbA/DsbD homologue also harbour a DsbB/DsbC homologue. Notable exceptions besides *S. aureus* and *S. epidermidis* were the insect symbionts *Buchnera aphidicola* and *Wigglesworthia glossinidia*. Both organisms feature very small genomes (616 respectively 697 kb) that resulted from reductive genome evolution as a consequence of their endosymbiotic life forms (Akman et al. 2002; van Ham et al. 2003). Neither function nor activity of DsbA in these organisms are established, but the fact that the gene has been conserved while hundreds of others were discarded by the evolution speaks for its importance. Additionally, it suggests that some bacteria may use a different way to re-oxidise DsbA. The di-amino acid cystine for instance was shown to relieve the phenotype of an *E. coli* mutant lacking *dsbB* at concentrations as low as 20.8  $\mu$ M (Dailey and Berg 1993). Interestingly, cystine is present at a concentration of 40  $\mu$ M in human plasma (Chawla et al. 1984; Jones et al. 2000). Since *S. aureus* is a human pathogen and is therefore often in contact with blood plasma, cystine might contribute to the re-oxidation of SaDsbA under such conditions. Another potential redox partner of SaDsbA could be SA2198. Since no homologue of SA2198 has been found outside the *Staphylococcus* genus, it is currently not feasible to estimate its function. The findings that *dsbA* and SA2198 are co-transcribed, the similar genetic organisation in *S. epidermidis*, and that SA2198 is predicted to be localised in the same cellular compartment as SaDsbA, support the possibility that both proteins are functionally linked. However, the lack of cysteine residues in SA2198, which are essential for DsbB activity (Guilhot et al. 1995), and the observation that plasmid pAD2, containing SA2198-*dsbA*, was not more efficient at restoring the motility of the *E. coli* mutants than plasmid pAD11, encoding only SaDsbA, seemed to exclude a DsbB-like action of SA2198 on SaDsbA.

In conclusion, despite its low sequence similarity to *E. coli* DsbA, and being a membrane anchored protein, the staphylococcal DsbA can functionally replace *E. coli* DsbA. SaDsbA is thus the first *S. aureus* protein shown to have the potential for extracellular disulfide bond formation. The finding that *S. aureus*, as well as some other bacteria, encode a DsbA but lack a DsbB homologue, leads us to postulate the existence of an alternative disulfide formation pathway distinct from the classical DsbA-DsbB electron cascade. The exact function of SaDsbA in staphylococci, and its importance for virulence of the human pathogens *S. aureus* and *S. epidermidis* remains to be elucidated.

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