Characterisation of a cell wall-anchored protein of \textit{Staphylococcus saprophyticus} associated with linoleic acid resistance

Nathan P King, Türkan Sakinç, Nouri L Ben Zakour, Makrina Totsika, Begoña Heras, Pavla Simerska, Mark Shepherd, Sören G Gatermann, Scott A Beatson and Mark A Schembri

Abstract

**Background:** The Gram-positive bacterium \textit{Staphylococcus saprophyticus} is the second most frequent causative agent of community-acquired urinary tract infections (UTI), accounting for up to 20% of cases. A common feature of staphylococci is colonisation of the human skin. This involves survival against innate immune defenses including antibacterial unsaturated free fatty acids such as linoleic acid which act by disrupting bacterial cell membranes. Indeed, \textit{S. saprophyticus} UTI is usually preceded by perineal skin colonisation.

**Results:** In this study we identified a previously undescribed 73.5 kDa cell wall-anchored protein of \textit{S. saprophyticus}, encoded on plasmid pSSAP2 of strain MS1146, which we termed \textit{S. saprophyticus} surface protein F (SssF). The \textit{sssF} gene is highly prevalent in \textit{S. saprophyticus} clinical isolates and we demonstrate that the SssF protein is expressed at the cell surface. However, unlike all other characterised cell wall-anchored proteins of \textit{S. saprophyticus}, we were unable to demonstrate a role for SssF in adhesion. SssF shares moderate sequence identity to a surface protein of \textit{Staphylococcus aureus} (SasF) recently shown to be an important mediator of linoleic acid resistance. Using a heterologous complementation approach in a \textit{S. aureus} sasF null genetic background, we demonstrate that SssF is associated with resistance to linoleic acid. We also show that \textit{S. saprophyticus} strains lacking sssF are more sensitive to linoleic acid than those that possess it. Every staphylococcal genome sequenced to date encodes SssF and SasF homologues. Proteins in this family share similar predicted secondary structures consisting almost exclusively of \alpha-helices in a probable coiled-coil formation.

**Conclusions:** Our data indicate that SssF is a newly described and highly prevalent surface-localised protein of \textit{S. saprophyticus} that contributes to resistance against the antibacterial effects of linoleic acid. SssF is a member of a protein family widely disseminated throughout the staphylococci.

Background

Urinary tract infections (UTIs) are a universal source of human morbidity, with millions of cystitis and pyelonephritis episodes reported annually [1]. An estimated 40-50% of all women will experience at least one UTI in their lifetime, and one in three women will have had at least one clinically diagnosed UTI by the age of 24 [2]. Direct health care costs due to UTI exceed $1 billion each year in the USA alone [2]. \textit{Staphylococcus saprophyticus}, a coagulase-negative staphylococcus, is the second most common causative agent of community-acquired urinary tract infection after \textit{Escherichia coli} [3], and is responsible for up to 20% of cases. \textit{S. saprophyticus} is of particular significance to sexually active young women, accounting for over 40% of UTI in this demographic [4]. \textit{S. saprophyticus} UTI symptoms mirror those of \textit{E. coli} [5] and recurrence is common, affecting 10-15% of infected women [6].

Three cell wall-anchored proteins, featuring a conserved characteristic C-terminal LPXTG motif, have previously been identified in \textit{S. saprophyticus}. These proteins (i.e. Sdr1, UafA and UafB) are all involved in adhesion [7-9], a crucial first step in the colonisation process. \textit{S. saprophyticus} also possesses non-covalently surface-associated Aas [10,11].

© 2012 King et al; licensee BioMed Central Ltd This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
and Ssp [12] surface proteins that are implicated in virulence. Other than surface proteins, S. saprophyticus produces abundant urease which contributes to its ability to grow in urine [13]. Other putative virulence factors include cell surface hydrophobicity [14], slime [15] and D-serine deaminase [16].

Apart from rare complications, S. saprophyticus is only known to infect the urinary system [17-19]. The primary niches of this organism are in the human gastrointestinal and genitourinary tracts [4,20]. S. saprophyticus UTI is often preceded by colonization of the perineal area; thus it can survive despite the innate immune defences of the skin. In this study, we have identified a previously undescribed LPXTG motif-containing cell wall-anchored protein of S. saprophyticus, termed SssF. The sssF gene is plasmid-encoded in S. saprophyticus strains ATCC 15305 and MS1146 and is highly prevalent in clinical isolates. We show that SssF belongs to a family of proteins conserved among staphylococcal species and contributes to survival against the staphylococcal free fatty acid linoleic acid - a component of the human innate immune defence system.

Results
Analysis of plasmid pSSAP2
S. saprophyticus strain MS1146, a clinical UTI isolate, has been described previously [7]. Its genome contains three plasmids - pSSAP1, pSSAP2 and pSSAP3. Sequence analysis of the 36 907 bp pSSAP2 plasmid revealed the presence of 35 predicted protein-coding genes, six pseudogenes and a mean G + C content of 29.9% (Figure 1 and Additional file 1: Table S1). Like other staphylococcal plasmids previously described, pSSAP2 has a mosaic structure with evidence of multiple insertions and deletions of discrete sequence blocks.

Plasmid pSSAP2 contains the repA gene and an approximately 17 kb region (from position 4 124 to 21 247) which share 96% and 97-99% nucleotide identity, respectively, with the chromosome of S. saprophyticus ATCC 15305 (Figure 1). A large proportion of the proteins encoded in this region are of unknown function or hypothetical, with the exception of a putative permease and several analogues of enzymes of the ribulose monophosphate pathway (Additional file 1: Table S1). Of note, the corresponding region in S. saprophyticus ATCC 15305 is longer (26 kb) and contains an arsenic resistance operon ArsRBC and a putative lipase, both absent from pSSAP2. This region is also framed by two copies of the IS element IS431, which is frequently involved in the recombinase-mediated integration of transposons and plasmids in methicillin-resistant S. aureus (MRSA) chromosomes [21,22]. Therefore, this region is likely to be an integrative plasmid of strain ATCC 15305; positioned upstream is a truncated integrase (SSP1642), for which an intact copy can be found in the S. saprophyticus MS1146 chromosome (Figure 1).

Another region of pSSAP2, ranging from position 21 529 to 33 235, shares ~99% nucleotide identity with plasmid pSSP1, which was originally described from S. saprophyticus ATCC 15305 [8]. The most notable feature of this region is the presence of a gene encoding for a LPXTG domain containing protein that we have designated sssF (see below).

Sequence analysis of SssF staphylococcal homologues
The S. saprophyticus MS1146 sssF gene is 1962 bp in length and the full-length translated SssF (S. saprophyticus surface protein F) protein contains 654 residues with a predicted molecular mass of 73.5 kDa (Figure 2A). SssF contains a predicted signal peptide of 45 residues (SignalP) [23] and an LPDTG anchor motif at the C terminus (Figure 2A), involved with covalent attachment of the mature protein to the cell wall. No conserved functional protein domains were detected, except for a possible albumin-binding GA module (Pfam PF01468, residues 58-109, E-value = 0.00039).

Sequence searches using the SssF amino acid sequence revealed similar proteins in other staphylococci. As expected, the SssF homologue encoded by pSSP1 in S. saprophyticus ATCC 15305 is near-identical at the protein level with only seven amino acid substitutions. Of note, every other sequenced staphylococcal genome contains an sssF-like gene, all chromosomally located except in S. saprophyticus (Additional file 2: Figure S1). Multiple alignment of the C-terminal regions (corresponding to the C-terminal 402 residues of SssF sequence) of one representative SssF-like protein from each sequenced staphylococcal species demonstrates there is variation from blocks of conserved and similar residues to regions of less similar sequence. This showed an overall protein identity ranging from 30.3-47.6%, versus Staphylococcus pseudintemmedius HKU10-03 and Staphylococcus carnosus TM300, respectively, and an average amino acid identity of approximately 37% with the remaining SssF-like proteins. In terms of protein sequence similarity, these values range from 41.7% (S. pseudintemmedius HKU10-03) to 84.4% (S. carnosus TM300). The N-terminal sequences are considerably more divergent.

All SssF-like proteins have a predicted signal peptide of between 35 and 45 residues, according to SignalP predictions. It is noted that the annotated Staphylococcus hae- molyticus JCSC1435 SssF-like protein has an incorrectly called start codon, artifically truncating the signal peptide sequence. All of the SssF-like proteins have a C-terminal sortase motif, implying cell surface localisation. Of the ten illustrated in Additional file 2: Figure S1, four have the canonical LPXTG motif, five have an alanine
residue in the fourth position, and the *Staphylococcus luedunensis* protein has a serine in this position.

**Structural prediction of SssF**

Secondary structure predictions using PSI-PRED [24] indicate that SssF contains long, almost uninterrupted segments of α-helices (Figure 2B), which are likely to wrap around each other forming a rope-like coiled-coil structure. In order to predict its three-dimensional fold we carried out a fold-recognition analysis of SssF sequence using Phyre [25] (Protein Homology/AnalogY Recognition Engine). This server allows a pairwise alignment of the SssF sequence to a library of known protein structures available from the Structural Classification of Proteins (SCOP) [26] and the Protein Data Bank (PDB) [27] databases and generates preliminary models of the protein by mapping the sequence onto the atomic coordinates of different templates. Although SssF shares very low sequence identity with proteins in the PDB (range from 5-9%), this analysis identified several structural homologues of SssF with a confidence level of 100%. All the structures identified as likely analogues of SssF correspond to proteins that have a coiled-coil fold, including various types of the filamentous proteins such as tropomyosin [28] (PDB code: 1C1G) or alpha-actinin [29] (PDB code 1HCI) (Figure 2C), strongly suggesting that this protein shares a similar three-dimensional structure. Each of the SssF-like proteins (complete mature forms) of the other ten staphylococcal species indicated in Additional file 2: Figure S1 is also predicted to almost exclusively consist of α-helical coiled-coils with the same Phyre-predicted structural analogue as SssF (data not shown).

**The sssF gene is highly prevalent in *S. saprophyticus***

To assess the prevalence of sssF in *S. saprophyticus* we used PCR to screen our collections of clinical isolates originating from Australia, Germany and the USA. The sssF gene was detected in 84.6% (55/65) of Australian isolates, 90.9% (10/11) of American isolates and 88.3% (53/60) of German isolates.

**SssF is expressed at the *S. saprophyticus* cell surface**

In order to study the cellular localisation and function of the SssF protein, we generated an isogenic *S. saprophyticus* MS1146 sssF mutant (MS1146sssF) by insertional inactivation with a group II intron using the TargeTron system. We then complemented the sssF mutation by the introduction of a pPS44 staphylococcal vector containing the cloned sssF gene, to create MS1146sssF(pSssF). Western blot analysis of whole-cell lysates from *S. saprophyticus* MS1146, MS1146sssF and MS1146sssF(pSssF) using rabbit polyclonal anti-SssF serum raised against a recombinant truncated SssF protein, demonstrated expression of SssF in MS1146 but not MS1146sssF. Complementation of sssF restored SssF expression in MS1146sssF(pSssF) (Figure 3A).
anti-SssF serum was used in conjunction with immunogold labeling and electron microscopy to demonstrate localisation of the SssF protein at the cell surface. MS1146 and MS1146

SSSF (pSSSF) exhibited abundant gold labeling whereas MS1146

SSSF was devoid of labeling (Figure 3B).

SssF does not mediate adhesion to uroepithelial cells or colonisation of the mouse bladder

Initial investigations into the function of SssF found no evidence of adhesion (to T24 and 5637 human bladder carcinoma cells [American Type Culture Collection; ATCC], exfoliated human urothelial cells or a wide range of ECM and other molecules, including human serum albumin), invasion of 5637 bladder cells, cell surface hydrophobicity modulation, biofilm formation or serum resistance that could be attributable to SssF (data not shown). Strain MS1146 and derivatives colonised the mouse bladder in similar numbers in a mouse model of UTI (4.8–5.8 \times 10^6 c.f.u. per 0.1 g bladder tissue), indicating that SssF does not contribute to colonisation in this infection model.

S. saprophyticus strains containing the ssf gene are more resistant to linoleic acid than those lacking sssf

The above results prompted us to analyse the sequences of the family of SssF-like proteins to predict a function
for SssF. The staphylococcal SssF-like proteins are all hypothetical proteins of unknown function except for SssF, which contributes to resistance of *S. aureus* to linoleic acid [30]. The mechanism of this phenotype remains unexplored. To determine whether SssF had a similar phenotype to the *S. aureus* SasF protein, linoleic acid survival assays were performed with *S. saprophyticus* MS1146 wild-type, MS1146 sssF and MS1146 sssF (pSssF) strains. No differences in survival among the strains were observed (data not shown). Following the lack of an observable phenotype for SssF in *S. saprophyticus* MS1146, we modified the linoleic acid emulsion assay to examine the survival of *S. saprophyticus* isolates that contain and do not contain the *sssF* gene in the presence of 0.85 M NaCl. Under these conditions, we observed a 30-fold difference in survival between the *sssF* and *sssf* strains (*P* = 0.008; Figure 4). Using this modified protocol, we still observed no difference between the *S. saprophyticus* MS1146 wild-type and *sssf* mutant at linoleic acid concentrations of up to 25 mM (data not shown).

**SssF is associated with resistance to linoleic acid**

Survival assays were carried out with a *S. aureus* SH1000 genetic background, with the aim of determining if SssF could restore linoleic acid resistance of a *S. aureus* SH1000sasF knockout mutant (Figure 5). In agreement with a previous study [30], mutation of *sasF* in *S. aureus* SH1000 resulted in enhanced sensitivity to linoleic acid and this effect could be complemented by the introduction of a *sasF*-containing plasmid [SH1000sasF(pSKSasF)]. When the *sssF* gene from *S. saprophyticus* MS1146 was introduced into *S. aureus* SH1000sasF, resistance to linoleic acid was also restored, demonstrating that SssF contributes to the survival of *S. aureus* in the presence of linoleic acid.

**Discussion and conclusion**

*S. saprophyticus* is a major cause of community-acquired UTI in young women. Knowledge of the virulence mechanisms of *S. saprophyticus* has advanced in recent years, particularly with the acquisition and analysis of whole genome sequence data. The majority of acknowledged virulence
factors of *S. saprophyticus* are proteins tethered to the cell surface, which with the exception of the Ssp lipase [12], are all involved in adhesion: Aas is an autolysin that also binds to fibronectin [10]; UafA adheres to uroepithelial cells via an unidentified ligand [8]; SdrI binds to collagen I and fibronectin [9,31] and UafB binds to fibronectin, fibrinogen and urothelial cells [7]. Here we have identified another cell wall-anchored protein produced by *S. saprophyticus* that we have termed SssF - the sixth surface protein described for this species.

The *sssF* gene was identified in the sequence of the pSSAP2 plasmid of *S. saprophyticus* MS1146 due to the presence of the canonical LPXTG sortase motif in the translated protein sequence. A copy of the *sssF* gene is also located on the pSSP1 plasmid of *S. saprophyticus* ATCC 15305 (99% nucleotide identity; Figure 1), but it was not acknowledged as encoding an LPXTG motif-containing protein [8]. We recently characterised another plasmid-coded LPXTG motif-containing protein of *S. saprophyticus* MS1146, UafB, as an adhesin [7]. We first sought to investigate whether SssF was another adhesin, since a considerable proportion of characterised Gram-positive covalently surface anchored proteins have adhesive functions [32], including every other known *S. saprophyticus* LPXTG motif-containing protein. No evidence of an adhesion phenotype for SssF was detected.

SssF protein sequence searches with the BLAST database provided an output of uncharacterised staphylococcal proteins with a maximum 39% amino acid identity to SssF across the entire protein sequence, mostly annotated as
hypothesised cell wall-anchored proteins. In contrast to \textit{S. saprophyticus}, the genes encoding these SssF-like proteins are located on the chromosome, rather than on a plasmid, in every other sequenced staphylococcal species. Some of these staphylococcal SssF-like proteins contain atypical sortase motifs. At this stage it is not known whether all of these proteins are sorted to the cell surface efficiently, but SasF has been shown to be associated with the cell wall of \textit{S. aureus} 8325-4 even with the non-classical LPKAG sortase motif [33]. There was a distinct lack of phenotypic data for these SssF-like proteins until a role for SasF was recently uncovered. Kenny et al. [30] observed that \textit{SasF} was the most upregulated gene in \textit{S. aureus} MRSA252 microarray and qRT-PCR experiments upon challenge with linoleic acid. The protective function of SasF was apparent when examined in a linoleic acid emulsion agar plate-based bacterial survival assay. Our hypothesis focused on the possibility that SssF possessed a similar function to SasF, but no linoleic acid resistance phenotype for SssF was observed in the \textit{S. saprophyticus} MS1146 genetic background. Using the linoleic acid emulsion agar plate bacterial survival assay in the presence 0.85 M NaCl, we observed a higher survival amongst \textit{S. saprophyticus} strains that harbour the \textit{sssF} gene than those that lack \textit{sssF}. We then successfully expressed SssF heterologously in a \textit{S. aureus} SH1000\textit{sasF} host and demonstrated restored resistance to linoleic acid. We found \textit{S. saprophyticus} MS1146 to be intrinsically more resistant to linoleic acid than \textit{S. aureus} SH1000. This remains to be explored but could be due to a number of species/strain specific factors including the action of redundant \textit{S. saprophyticus} MS1146 resistance mechanisms or variations in surface components such as capsule or teichoic acids.

We found that the survival of \textit{S. aureus} SH1000 and its derivatives was markedly increased in the presence of linoleic acid at pH 6.0 compared to pH 7.4. This result is consistent with previous studies of the staphylococcal fatty acid modifying enzyme (FAME), an unidentified but partially characterised protein secreted by most staphylococci which detoxifies free fatty acids by esterifying them to an alcohol [34,35]. The FAME of \textit{S. aureus} and \textit{S. epidermidis} demonstrate optimal activity at pH 6.0, and have little activity at pH 7.4 [35,36]. This is congruent with human skin having a slightly acidic pH of 5.5–6 [37]. RP-HPLC experiments using linoleic acid and crude protein extracts demonstrated that SssF activity is distinct from FAME activity (data not shown). Other antimicrobial fatty acids such as sapienic acid have yet to be examined as substrates for SssF or SasF. We hypothesise that some or all of the other uncharacterised SssF-like proteins exhibit fatty acid resistance activity, but this remains to be demonstrated experimentally.

There are precedents for bacterial surface structures that provide protection against bactericidal free fatty acids. Gram-positive bacterial cell wall teichoic acids provide protection against free fatty acid mediated killing of \textit{S. aureus} [38]. The IsdA protein of \textit{S. aureus} reduces bacterial hydrophobicity when expressed at the cell surface under the cue of iron starvation to resist fatty acid membrane attack and also promotes fatty acid resistance of \textit{S. aureus} in a volunteer human skin survival model [39]. Our studies however found that expression of SssF does not influence cell surface hydrophobicity of \textit{S. saprophyticus}, and this corresponds with matching data for SasF and \textit{S. aureus} [30].

No conserved motifs that might predict the functional residues of SssF-like proteins were identified. The observation that the SssF-like proteins are structurally related to myosin is noteworthy, especially in light of the recent characterisation of myosin cross-reactive antigens of \textit{Streptococcus pyogenes} and \textit{Bifidobacterium breve} as fatty acid hydratases [40,41]. These enzymes act to detoxify unsaturated free fatty acids, including linoleic acid. Homologous proteins with modest primary sequence identity but similar tertiary structures are acknowledged in both bacterial [42] and mammalian [43] lipid-binding protein families. It is possible that conserved tertiary protein structure between SssF-like proteins contributes to their function.

\textit{S. saprophyticus} is a uropathogen, but SssF is unlikely to have evolved to facilitate survival in the urinary tract. A common trait of staphylococci is skin colonisation. Staphylococcal free fatty acids (especially unsaturated) are present on human skin [44] and are also active in staphylococcal abscesses [45]. Furthermore, linoleic acid is one of the most abundant polyunsaturated fatty acids on human skin [46], and is also present in vaginal secretions [47]. SssF may be an important determinant for survival of \textit{S. saprophyticus} in the events preceding urethral entry in community-acquired UTI - colonisation of perineal and periurethral tissue. This would account for the absence of SssF involvement in the mouse model of UTI, in which the inocula are delivered directly into the bladder.

The location of \textit{sssF} on a plasmid in both sequenced \textit{S. saprophyticus} strains is intriguing, particularly as every other staphylococcal SssF-like protein is chromosomally encoded. It has been observed that many genes that are located on plasmids encode for traits which have extracellular functions [48], and \textit{sssF} falls into this category. Furthermore, plasmid genes have often been noted to confer selective advantage to the bacteria in some environmental niches but not others [49]. Every pathogenic staphylococcal species known to carry a chromosomal \textit{sssF}-like gene is known to commensally inhabit the skin, and this can be considered their main niche. \textit{S. saprophyticus}, on the other hand, primarily resides in the genitourinary and gastrointestinal tracts [4,20]. It is feasible that since human skin is not the major habitat of \textit{S. saprophyticus}, \textit{sssF} has been...
retained as an accessory gene required for survival on the skin during non-UTI periods. Nonetheless, it may still be the case that \textit{sssF} is found on the chromosome of some \textit{S. saprophyticus} strains.

\textit{SssF} represents the fourth LPXTG motif-containing protein described in \textit{S. saprophyticus}. We present here evidence that the \textit{S. saprophyticus} \textit{SssF} protein has a role in the protection against free fatty acid mediated killing, and that it is a member of a newly identified protein family broadly distributed throughout the \textit{Staphylococcus} genus.

\section*{Materials and methods}

\subsection*{Bacterial strains and plasmids}

The bacterial strains and plasmids used in this study are listed in Table 1. The clinical \textit{S. saprophyticus} isolate collection used in this study is as previously described [7]. In addition, 60 clinical isolates from Germany were also tested. \textit{S. saprophyticus} ATCC 15305 was described previously [8]. Staphylococcal strains were cultured in/on Brain Heart Infusion (BHI) broth/agar (Oxoid) supplemented with erythromycin or chloramphenicol (10 μg ml\(^{-1}\)) as required. \textit{E. coli} strains were cultivated in/on Luria-Bertani (LB) broth/agar supplemented with ampicillin (100 μg ml\(^{-1}\)) as required.

\subsection*{DNA manipulations and genetic techniques}

Genomic and plasmid DNA were isolated as previously described [7]. PCR assays to determine the presence of \textit{sssF} (primers 1127 and 1128) were performed using Taq DNA polymerase (NEB) under the following conditions: 2 min at 94°C, 25 cycles of 15 s at 94°C, 30 s at 55°C, 20 s at 72°C, 1 cycle of 3 min at 72°C, 4°C hold. Primers were synthesised by Sigma and are listed in Table 2. PCR amplification of the \textit{sssF} gene was performed using Phusion Hot Start DNA Polymerase (Finnzymes).

\subsection*{Bioinformatic analysis and identification of \textit{sssF}}

The \textit{sssF} gene was identified in plasmid pSSAP2 of \textit{S. saprophyticus} MS1146. The final pSSAP2 sequence was finished to Q40 standard with an average Sanger read depth of ~23 × coverage, which corresponds to an estimated number of four pSSAP2 plasmid copies per cell, based on the observed chromosomal read coverage (data not shown). Annotation of plasmid pSSAP2 was carried out manually using Artemis [55] and BLAST [56] similarity searches of publicly available sequence databases. The complete nucleotide sequence of \textit{S. saprophyticus} plasmid pSSAP2 is available from the GenBank/EMBL/DDJB database under accession number HE616681. The multiple alignment (Additional file 2: Figure S1) was created with CLUSTAL W2 [57] and edited with Jalview [58]. Figure 1 was produced using Easyfig [59].

\section*{Construction and complementation of staphylococcal mutants}

Plasmid construct pNK24 (Table 1), specifically retargeted to the \textit{sssF} gene of \textit{S. saprophyticus} MS1146, was prepared using the Sigma TargeTron Gene Knockout System, as per the manufacturer’s instructions. Retargeting PCR primer sequences (1001-1003, Table 2) were determined by the TargeTron online design site, followed by a retargeting PCR and cloning of the PCR product into the provided shuttle vector, pNL9164 (Table 1). The construct was sequenced to verify correct inserts using primer 1011 (Table 2). The retargeted plasmid was then purified with a Qiagen Maxiprep kit and introduced into \textit{S. saprophyticus} MS1146 by protoplast transformation as previously described [10], followed by CdCl\(_2\) induction and colony PCR screening to identify the \textit{sssF} mutant (MS1146\textit{sssF}). The \textit{S. aureus} SH1000 \textit{sasF} gene was also interrupted with the TargeTron system as above, using primers 2065-2067 (Table 2). The retargeted plasmid (pNK41, Table 1) was passaged through a restriction-deficient \textit{S. aureus} strain (RN4220), then electroporated into \textit{S. aureus} SH1000 and induced to create the \textit{sasF} mutant (SH1000\textit{sasF}). For complementation of the \textit{S. saprophyticus} MS1146 \textit{sssF} mutation, the \textit{sssF} gene was initially amplified from \textit{S. saprophyticus} MS1146 (primers 839 and 840, Table 2) and cloned into the \textit{S. aureus} SH1000 plasmid pPS44 with BamHI cloning sites (data not shown). The final pSSA2 construct contained the base pair \textit{sssF} gene, as required.

\section*{Purification of truncated \textit{SssF}, antibody production and immunoblotting}

For antiserum production, a 1330 bp segment from \textit{sssF} from \textit{S. saprophyticus} MS1146 (Figure 2A) was amplified with primers 873 and 874 (Table 2), digested with Xhol/EcoRI and ligated into Xhol/EcoRI-digested pBAD/HisB. The resultant plasmid (pSssFHis) contained the base pairs 181-1510 of \textit{sssF} fused to a 6 × His-encoding sequence. This \textit{sssF} sequence corresponds to amino residues 39-481 of the \textit{SssF} sequence. Protein induction and
purification, inoculation of rabbits, staphylococcal cell lysate preparation and immunoblotting were performed as described previously [7], except NuPAGE Novex 4-12% Bis-Tris precast gels with NuPAGE MES SDS running buffer (Invitrogen) were used for the SDS-PAGE and S. saprophyticus MS1146 sssF-adsorbed rabbit anti-SssF serum was used as the primary serum for the Western blot.

Microscopy and image analysis
Immunogold labeling and transmission electron microscopy (TEM) were performed as described previously.

Table 1 Strains and plasmids used in this study

| Strain or plasmid | Description | Reference or source |
|-------------------|-------------|---------------------|
| **E. coli strains** | | |
| DH5α | F− thi-1 gyrA96 relA1 supE44 l− thi-1 gyrA96 relA1 | Grant et al. [50] |
| BL21 | F− ompT hsdSd(r− m−) gal dcm | Stratagene |
| MS2066 | DH5α containing pSssFHis | This study |
| MS2067 | BL21 containing pSssFHis | This study |
| **S. saprophyticus strains** | | |
| ATCC 15305 | Type strain (genome sequenced) | Kuroda et al. [8] |
| MS1146 | Clinical isolate | AstraZeneca |
| MS1146 sssF | MS1146 isogenic sssF mutant | This study |
| MS1146 sssF (pSssF) | Complemented MS1146 sssF mutant | This study |
| **S. aureus strains** | | |
| SH1000 | Functional rsbU-repaired derivative of S. aureus 8325-4 | Horsburgh et al. [51] |
| SH1000 sssF | SH1000 isogenic sasF mutant | This study |
| SH1000 sssF (pSKsasF) | SH1000 sasF mutant complemented with sasF | This study |
| SH1000 sssF (pSKsasF) | SH1000 sssF mutant complemented with sasF | This study |
| SH1000 sssF (pSKsasF) | SH1000 sssF mutant complemented with empty pSK5632 vector | This study |
| **S. carnosus strains** | | |
| TM300 | Wild-type SK311 | Schleifer & Fischer [52] |
| TM300 (pSssF) | TM300 containing pSSsF | This study |
| **Plasmids** | | |
| pBAD/HisB | Cloning and protein expression vector, containing N-terminal 6 × His tag; Ap<sup>R</sup> | Invitrogen |
| pNL9164 | E. coli/S. aureus TargeTron shuttle vector (temperature sensitive); Ap<sup>R</sup> Em<sup>R</sup> | Sigma |
| pSK5632 | Cloning and expression E. coli/S. aureus shuttle vector; Ap<sup>R</sup> Cm<sup>R</sup> | Grkovic et al. [53] |
| pPS44 | Staphylococcal vector, contains replicon and cat gene of pC194; Cm<sup>R</sup> | Wieland [54] |
| pSssFHis | 1330 bp MS1146 sssF fragment, amplified with primers 873 and 874, digested with EcoRI/XhoI and cloned into EcoRI/XhoI-digested pBAD/HisB, with in-frame N-terminal 6 × His tag; Ap<sup>R</sup> | This study |
| pNK24 | pNL9164 shuttle vector retargeted with primers 1001-1003, EBSU to knock out MS1146 sssF (TargeTron system); Ap<sup>R</sup> Em<sup>R</sup> | This study |
| pNK41 | pNL9164 shuttle vector retargeted with primers 2065-2067, EBSU to knock out SH1000 sasF (TargeTron system); Ap<sup>R</sup> Em<sup>R</sup> | This study |
| pSKsasF | 2394 bp fragment, including entire sasF gene from MS1146, amplified with primers 839 and 840 and cloned into the BamHI site of pSK5632; Ap<sup>R</sup> Cm<sup>R</sup> | This study |
| pSssF | 2400 bp BamHI/XbaI fragment, containing sssF gene, subcloned from pSKsasF into BamHI/XbaI-digested pPS44; Cm<sup>R</sup> | This study |
| pSKsasF | 2175 bp fragment, including sasF gene from S. aureus NCTC 8325, amplified with primers 2084 and 2085 and cloned into the HindIII site of pSK5632; Ap<sup>R</sup> Cm<sup>R</sup> | This study |
Table 2 PCR primers used in this study

| Primer    | Sequence (5’-3’) | Description                                                                 |
|-----------|------------------|------------------------------------------------------------------------------|
| 1127      | GGTGAAGCAATTTGGAAGAAC | ssfF screen forward                                                         |
| 1128      | TTCTTCAATTAGTACCAACTCAAC | ssfF screen reverse                                                        |
| 839       | GCTAGGACCTCTCATACCTAATTAAGAAATGACAGG | ssfF cloning forward. Contains BamH1 site (underlined) |
| 840       | ACTAGGATCCCTCATCAAGGTGCCTCCATTA | ssfF cloning reverse. Contains BamH1 site (underlined) |
| 873       | GCTCACGAGTTCGAACACCATTACGAGAACG | ssfF fragment PCR for cloning into pBAD/HisB, for antibody production, forward. Contains XhoI site (underlined) |
| 874       | GCTCGAATCAAAGGCCTTTAGCTTTAGCATC | ssfF fragment PCR for cloning into pBAD/HisB, for antibody production, reverse. Contains EcoRI site (underlined) |
| 1001      | AAAAAAGCCTTATAATTATCTTAAATGTCAAGATCTGATAGCTGATAGCTGCTT | ssfF TargetTron IBS |
| 1002      | CAGATTGTTCAAATTGGTGAATACAGATAATGTAATTATCTTAACTTACTTCACCCATATCAAC | ssfF TargetTron EBS1d |
| 1003      | TGAACGCAATTTCTAATTTCTCGATTTCTGATAGATAAGGAAAATGCTT | ssfF TargetTron EBS2 |
| 2065      | AAAAAAGCCTTATAATTATCTTAAATGTCAAGATCTGATAGCTGATAGCTGCTT | ssfF TargetTron IBS |
| 2066      | CAGATTGTTCAAATTGGTGAATACAGATAATGTAATTATCTTAACTTACTTCACCCATATCAAC | ssfF TargetTron EBS1d |
| 2067      | TGAACGCAATTTCTAATTTCTCGATTTCTGATAGATAAGGAAAATGCTT | ssfF TargetTron EBS2 |
| 2068      | CAGATTGTTCAAATTGGTGAATACAGATAATGTAATTATCTTAACTTACTTCACCCATATCAAC | ssfF cloning forward. Contains HindIII site (underlined) |
| 2069      | CAGATTGTTCAAATTGGTGAATACAGATAATGTAATTATCTTAACTTACTTCACCCATATCAAC | ssfF cloning reverse. Contains HindIII site (underlined) |
| 1011      | TCTTTAGGTGTAACATATACGG | Sequencing primer to check for correct 350 bp retargeted intron fragments for TargetTron |

Additional file 1: Table S1 Predicted protein-coding genes of pSSAP2.
Additional file 2: Figure S1 ClustalW alignment of the C-terminal 402 amino acid residues of S. propionicus MS1146 SssF protein (61% of entire sequence) with corresponding sequence from other staphylococcal SssF-like proteins, showing clusters of amino acid conservation. Only one representative protein from each species is shown. Sequences are sorted (in descending order) by similarity to S. propionicus MS1146 SssF sequence, which ranges from 31.1% (S. pseudintermedius HKU010-03) to 48.5% (S. camus TM300). Jalview was used to colour-code the alignment by percentage identity. The C-terminal sortase anchor motifs are indicated by a red box. GenBank accessions for the SssF-like proteins are as follows: S. camus TM300, CAL29334; S. capies (S. aureus) SK14, EEE48467; S. capitis C87, EF516450; S. epidermidis 86/3A, AAW53125; S. warneri SJ14, EEE48467; S. pseudintermedius XSC1435, BA503665; S. hominis SK119, EEK11979; S. aureus NCTC 8325, ABD31969; S. lugdunensis HKU09-01, ADC86449; S. pseudintermedius HKU010-03, ADV06726.

Acknowledgements

This work was supported by grants from the Australian National Health and Medical Research Council to M.A.S. (569676) and S.A.B. (S11224), and a University of Queensland Early Career Researcher grant to S.A.B. M.A.S. is supported by an Australian Research Council (ARC) Future Fellowship (FT100100662) and S.A.B. is supported by an ARC, Australian Research Fellowship (DP0881247).

Author details

1Australian Infectious Diseases Research Centre, School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD 4072, Australia. 2Department of Medical Microbiology, Institute for Hygiene and Microbiology, Ruhr-Universitat Bochum, Universitaetsstr. 150, 44780 Bochum, Germany. 3Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD 4072, Australia. 4Center for Infectious Diseases and Travel Medicine, University Medical Center Freiburg, Freiburg, Germany. 5School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, UK.

Additional material

Linoleic acid survival assay

S. aureus linoleic acid survival assays were performed essentially as described by Kenny et al. [30]. Briefly, serial dilutions of overnight cultures (2.5 μl spots) were plated in duplicate onto BHI agar, pH 6.0, containing 0 mM or 1 mM linoleic acid. All agar media contained a final concentration of 1% ethanol. Colonies were counted after overnight incubation at 37°C. Mean values were compared using Student’s t test. S. propionicus survival assays were performed similarly, but with agar plates containing 5 mM linoleic acid, supplemented with 0.85 M NaCl.

Structural predictions of SssF

Secondary structure and three-dimensional fold predictions were performed using PSI-PRED [24] and Phyre [25], respectively.

Additional material

[7], using 1:10 anti-SssF serum as the primary antibody. No negative staining was performed.

Acute phase responses

Linoleic acid survival assay

S. aureus linoleic acid survival assays were performed essentially as described by Kenny et al. [30]. Briefly, serial dilutions of overnight cultures (2.5 μl spots) were plated in duplicate onto BHI agar, pH 6.0, containing 0 mM or 1 mM linoleic acid. All agar media contained a final concentration of 1% ethanol. Colonies were counted after overnight incubation at 37°C. Mean values were compared using Student’s t test. S. propionicus survival assays were performed similarly, but with agar plates containing 5 mM linoleic acid, supplemented with 0.85 M NaCl.

Structural predictions of SssF

Secondary structure and three-dimensional fold predictions were performed using PSI-PRED [24] and Phyre [25], respectively.

Additional material

[7], using 1:10 anti-SssF serum as the primary antibody. No negative staining was performed.

Linoleic acid survival assay

S. aureus linoleic acid survival assays were performed essentially as described by Kenny et al. [30]. Briefly, serial dilutions of overnight cultures (2.5 μl spots) were plated in duplicate onto BHI agar, pH 6.0, containing 0 mM or 1 mM linoleic acid. All agar media contained a final concentration of 1% ethanol. Colonies were counted after overnight incubation at 37°C. Mean values were compared using Student’s t test. S. propionicus survival assays were performed similarly, but with agar plates containing 5 mM linoleic acid, supplemented with 0.85 M NaCl.

Structural predictions of SssF

Secondary structure and three-dimensional fold predictions were performed using PSI-PRED [24] and Phyre [25], respectively.

Additional material

[7], using 1:10 anti-SssF serum as the primary antibody. No negative staining was performed.
References

1. Schappert SM. Ambulatory care visits to physicians, hospital outpatient departments, and emergency departments: United States, 1997. Vital Health Stat 1999; 13(43):1-36.

2. Foxman B, Barlow R, D’ArCY H, Gillespie B, Sobel JD. Urinary tract infection: self reported incidence and associated costs. Ann Epidemiol 2000, 10(8):509-515.

3. Hooton TM, Stamm WE. Diagnosis and treatment of uncomplicated urinary tract infection. Infect Dis Clin North Am 1997, 11(3):551-581.

4. Rupp ME, Soper DE, Archer GL. Coagulase-negative staphylococci - pathogens associated with medical progress. Clin Infect Dis 1994, 19(2):231-243.

5. Faro S, Fenner DE. Urinary tract infections. Clin Obstet Gynecol 1998, 41(3):744-754.

6. King NP, Beaton SA, Totsika M, Ulett GC, Alm RA, Manning PA, Schernb M. UafB is a serine-rich repeat adhesin of Staphylococcus saprophyticus that mediates binding to fibronectin, fibrinogen and human uroepithelial cells. Microbiology 2011, 157:1161-1175.

7. Kuroda M, Yamashita A, Hirakawa H, Kumano M, Morikawa K, Higashide M. Staphylococcus saprophyticus: a common cause of urinary tract infections. Rev Infect Dis 1984, 6(3):328-337.

8. Rup ME, Soper DE, Archer GL. Staphylococcus saprophyticus: a multifunctional protein: localization of the fibronectin-binding site. FEBS Lett 2000, 491(1-2):174-178.

9. Roche FM, Massey R, Peacock SJ, Day NPJ, Visai L, Speziale P, Lam A. Improved prediction of SssF and prepared Figure 2B and 2C. PS participated in the research and assisted in writing and editing the manuscript. MAS directed research and assisted in writing and editing the manuscript. All authors read and approved the final manuscript.

Received: 3 October 2011 Accepted: 15 January 2012

References

1. Schappert SM. Ambulatory care visits to physicians, hospital outpatient departments, and emergency departments: United States, 1997. Vital Health Stat 1999, 13(43):1-36.

2. Foxman B, Barlow R, D’ArCY H, Gillespie B, Sobel JD. Urinary tract infection: self reported incidence and associated costs. Ann Epidemiol 2000, 10(8):509-515.

3. Hooton TM, Stamm WE. Diagnosis and treatment of uncomplicated urinary tract infection. Infect Dis Clin North Am 1997, 11(3):551-581.

4. Rupp ME, Soper DE, Archer GL. Coagulase-negative staphylococci - pathogens associated with medical progress. Clin Infect Dis 1994, 19(2):231-243.

5. Faro S, Fenner DE. Urinary tract infections. Clin Obstet Gynecol 1998, 41(3):744-754.

6. King NP, Beaton SA, Totsika M, Ulett GC, Alm RA, Manning PA, Schernb M. UafB is a serine-rich repeat adhesin of Staphylococcus saprophyticus that mediates binding to fibronectin, fibrinogen and human uroepithelial cells. Microbiology 2011, 157:1161-1175.

7. Kuroda M, Yamashita A, Hirakawa H, Kumano M, Morikawa K, Higashide M. Staphylococcus saprophyticus: a common cause of urinary tract infections. Rev Infect Dis 1984, 6(3):328-337.

8. Rup ME, Soper DE, Archer GL. Staphylococcus saprophyticus: a multifunctional protein: localization of the fibronectin-binding site. FEBS Lett 2000, 491(1-2):174-178.

9. Roche FM, Massey R, Peacock SJ, Day NPJ, Visai L, Speziale P, Lam A. Improved prediction of SssF and prepared Figure 2B and 2C. PS participated in the research and assisted in writing and editing the manuscript. MAS directed research and assisted in writing and editing the manuscript. All authors read and approved the final manuscript.

References

1. Schappert SM. Ambulatory care visits to physicians, hospital outpatient departments, and emergency departments: United States, 1997. Vital Health Stat 1999, 13(43):1-36.

2. Foxman B, Barlow R, D’ArCY H, Gillespie B, Sobel JD. Urinary tract infection: self reported incidence and associated costs. Ann Epidemiol 2000, 10(8):509-515.

3. Hooton TM, Stamm WE. Diagnosis and treatment of uncomplicated urinary tract infection. Infect Dis Clin North Am 1997, 11(3):551-581.

4. Rupp ME, Soper DE, Archer GL. Coagulase-negative staphylococci - pathogens associated with medical progress. Clin Infect Dis 1994, 19(2):231-243.

5. Faro S, Fenner DE. Urinary tract infections. Clin Obstet Gynecol 1998, 41(3):744-754.

6. King NP, Beaton SA, Totsika M, Ulett GC, Alm RA, Manning PA, Schernb M. UafB is a serine-rich repeat adhesin of Staphylococcus saprophyticus that mediates binding to fibronectin, fibrinogen and human uroepithelial cells. Microbiology 2011, 157:1161-1175.

7. Kuroda M, Yamashita A, Hirakawa H, Kumano M, Morikawa K, Higashide M. Staphylococcus saprophyticus: a common cause of urinary tract infections. Rev Infect Dis 1984, 6(3):328-337.
42. Arpigny JL, Jaeger KE: Bacterial lipolytic enzymes: classification and properties. Biochem J 1999, 343:177-183.
43. Stoich J, McDermott L: Structural and functional analysis of fatty acid-binding proteins. J Lipid Res 2009, 50:S126-S131.
44. Ricketts CR, Squire JR, Topley E, Lilly HA: Human skin lipids with particular reference to the self-sterilising power of the skin. Clin Sci 1951, 10(1):89-111.
45. Dye ES, Kapral FA: Survival of Staphylococcus aureus in intraperitoneal abscesses. J Med Microbiol 1981, 14(2):185-194.
46. Capkin RS, Ziboh VA, Marcelo CL, Voorhees JJ: Metabolism of essential fatty acids by human epidemal enzyme preparations - evidence of chain elongation. J Lipid Res 1986, 27(9):945-954.
47. Huggins GR, Preti G: Volatile constituents of human vaginal secretions. Am J Obstet Gynecol 1976, 126(1):129-136.
48. Rankin DJ, Rocha EPC, Brown SP: What traits are carried on mobile genetic elements, and why? Heredity 2011, 106(1):1-10.
49. Eberhard WG: Why do bacterial plasmids carry some genes and not others? Plasmid 1989, 21(3):167-174.
50. Grant SGN, Jessee J, Bloom FR, Hanahan D: Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants. Proc Natl Acad Sci USA 1990, 87(12):4645-4649.
51. Horsburgh MJ, Aish JL, White IJ, Shaw L, Lithgow JK, Foster SJ: Sigma(B) modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from Staphylococcus aureus 8325-4. J Bacteriol 2002, 184(19):5457-5467.
52. Schleifer KH, Fischer U: Description of a new species of the genus Staphylococcus - Staphylococcus carnosus. Int J Syst Bacteriol 1982, 32(2):153-156.
53. Girkovic S, Brown MH, Hardie KM, Firth N, Skurray RA: Stable low-copy-number Staphylococcus aureus shuttle vectors. Microbiology 2003, 149:785-794.
54. Wieland B: Der Xyl-Promotor aus Staphylococcus xylosus als Grundlage der transriptionale Regulation von Genen in Staphylococcus carnosus, PhD thesis. PhD thesis Tübingen, Germany: Universität Tübingen; 1993.
55. Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B: Artemis: sequence visualization and annotation. Bioinformatics 2000, 16(10):944-945.
56. Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997, 25(17):3389-3402.
57. Chenina R, Sugawara H, Koke T, Lopez R, Gibson TJ, Higgins DG, Thompson JD: Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 2003, 31(13):3497-3500.
58. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ: Jalview Version 2 - a multiple sequence alignment editor and analysis workbench. Bioinformatics 2009, 25(9):1189-1191.
59. Sullivan MJ, Petty NK, Beaton SA: Easyfig: a genome comparison visualiser. Bioinformatics (Oxf) 2011, doi: 10.1093/bioinformatics/btr039.

doi:10.1186/1471-2180-12-8
Cite this article as: King et al.: Characterisation of a cell wall-anchored protein of Staphylococcus saprophyticus associated with linoleic acid resistance. BMC Microbiology 2012 12:8.