UafB is a serine-rich repeat adhesin of *Staphylococcus saprophyticus* that mediates binding to fibronectin, fibrinogen and human uroepithelial cells

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Staphylococcus saprophyticus is an important cause of urinary tract infection (UTI), particularly among young women, and is second only to uropathogenic *Escherichia coli* as the most frequent cause of UTI. The molecular mechanisms of urinary tract colonization by *S. saprophyticus* remain poorly understood. We have identified a novel 6.84 kb plasmid-located adhesin-encoding gene in *S. saprophyticus* strain MS1146 which we have termed uro-adherence factor B (*uafB*). UafB is a glycosylated serine-rich repeat protein that is expressed on the surface of *S. saprophyticus* MS1146. UafB also functions as a major cell surface hydrophobicity factor. To characterize the role of UafB we generated an isogenic *uafB* mutant in *S. saprophyticus* MS1146 by interruption with a group II intron. The *uafB* mutant had a significantly reduced ability to bind to fibronectin and fibrinogen. Furthermore, we show that a recombinant protein containing the putative binding domain of UafB binds specifically to fibronectin and fibrinogen. UafB was not involved in adhesion in a mouse model of UTI; however, we observed a striking UafB-mediated adhesion phenotype to human uroepithelial cells. We have also identified genes homologous to *uafB* in other staphylococci which, like *uafB*, appear to be located on transposable elements. Thus, our data indicate that UafB is a novel adhesin of *S. saprophyticus* that contributes to cell surface hydrophobicity, mediates adhesion to fibronectin and fibrinogen, and exhibits tropism for human uroepithelial cells.

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INTRODUCTION

Urinary tract infections (UTIs) are among the most common infectious diseases of humans and are a major cause of morbidity. In the USA alone, community-acquired UTIs are estimated to be the basis of seven million doctor visits each year (Schappert, 1999), with associated costs totalling \$1.6 billion annually (Foxman *et al.*, 2000).

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Abbreviations: ECM, extracellular matrix; TEM, transmission electron microscopy; UPEC, uropathogenic *E. coli*; UTI, urinary tract infection.

The GenBank/EMBL/DDBJ accession number for the complete nucleotide sequence of *S. saprophyticus* plasmid pSSAP1 is FR687301.

A supplementary table, showing predicted protein-coding genes and other features of pSSAP1, is available with the online version of this paper.

Staphylococcus saprophyticus, a coagulase-negative staphylococcus, causes 5–20 % of community-acquired UTIs (Hooton & Stamm, 1997). *S. saprophyticus* is responsible for over 40 % of UTIs in sexually active young women (Rupp *et al.*, 1992) and is second only to *Escherichia coli* as the most common cause of UTIs in this subpopulation (Hovelius & Mårdh, 1984). Symptoms of *S. saprophyticus* UTI are indistinguishable from those caused by *E. coli* (Rupp & Archer, 1994), and recurrence is common, affecting 10–15 % of infected women (Faro & Fenner, 1998). Infection with *S. saprophyticus* is rarely associated with nosocomial UTI (Rupp & Archer, 1994).

To establish infection of the urinary tract, bacteria must adhere to the epithelial cell lining (Mårdh *et al.*, 1979). Most pathogens, including *S. saprophyticus*, express cell surface proteins which adhere to host cell molecules, including those of the extracellular matrix (ECM). A subset

of Gram-positive surface molecules – cell wall-anchored proteins – share a common set of features which comprises an N-terminal signal peptide, functional domains that may contain several serine-rich tandem repeats, and a hydrophobic cell wall-spanning sorting region at the C terminus that contains a conserved LPXTG motif and a charged tail (Navarre & Schneewind, 1999). The extensive tandem repeats can be of varying sequence, size and frequency, and have been implicated in providing sites for O-linked glycosylation via the serine residues (Stephenson *et al.*, 2002) and exposing the adhesive domain of the protein beyond the bacterial capsule (Shivshankar *et al.*, 2009). The LPXTG motif is required for anchoring of the protein at the cell surface, and is cleaved by a sortase enzyme between the threonine and glycine residues during assembly of the peptidoglycan cell wall (Navarre & Schneewind, 1999).

Currently, at least 11 serine-aspartate repeat (Sdr) proteins have been identified in staphylococci, including ClfA, ClfB and SdrC–SdrI (McCrea *et al.*, 2000; Sakinç *et al.*, 2006). Proteins in this family are adhesins and are characterized by the presence of a typical signal peptide, a non-repeat region (A region), often up to five long non-serine-rich tandem repeats (B-repeat regions), a C-terminal span of serine-aspartate tandem repeats, and an LPXTG anchor region.

Distinct from the Sdr proteins, serine-rich repeat proteins represent another family of Gram-positive cell wall-anchored proteins, and have been best described in staphylococci and streptococci (Zhou & Wu, 2009). The genes encoding these high-molecular-mass proteins are generally located together with additional genes that encode their own dedicated secretory and glycosylation systems. All of the characterized members of this group are adhesive glycoproteins (Zhou & Wu, 2009). The Fap1 and GspB proteins are the best-characterized serine-rich repeat proteins and can be considered the prototypes of this family of adhesins. Fap1 of *Streptococcus parasanguis* binds to saliva-coated hydroxylapatite (Wu *et al.*, 1998) and GspB of *Streptococcus gordonii* mediates adhesion to human platelets, a precursor step in infective endocarditis (Bensing *et al.*, 2004b). The GspB protein contains an atypical signal sequence that directs its export via the accessory Sec system (Bensing *et al.*, 2007), while the non-repeat region flanked by the serine-rich repeats encodes the GspB binding domain (Takamatsu *et al.*, 2005). Other serine-rich repeat proteins have also been shown to be virulence factors: SraP of *Staphylococcus aureus* (Siboo *et al.*, 2005) and HsaP of *Streptococcus gordonii* (Bensing *et al.*, 2004b) also bind to human platelets, while Srr-1 of *Streptococcus agalactiae* promotes blood–brain barrier penetration in a mouse meningitis model (van Sorge *et al.*, 2009) and PsrP of *Streptococcus pneumoniae* binds to keratin 10 on lung cells and contributes to invasive pneumococcal disease (Shivshankar *et al.*, 2009).

A core set of genes is conserved in every genome that contains serine-rich repeat protein-encoding genes (Zhou

& Wu, 2009), consisting of *secA2*, *secY2*, three accessory secretory protein genes (*asp1–3*) and two glycosyltransferase genes (*gtfA*, *gtfB*). The *fimS* accessory locus of *Streptococcus salivarius* contains two extra accessory secretory proteins (Zhou & Wu, 2009). The *secA2* gene is present in many pathogenic bacteria. The SecA2 protein of *Streptococcus gordonii* is an ATPase, like SecA (Bensing & Sullam, 2009), but these proteins are distinct. In *Streptococcus parasanguis* it has been shown that polyclonal antibodies to SecA and SecA2 do not cross-react (Chen *et al.*, 2006). The two glycosyltransferases are thought to form part of a glycosylation complex (Zhou & Wu, 2009), which performs the protein glycosylation in the cytosol prior to export (Bensing *et al.*, 2004a). The GspB protein consists of 10% (w/w) carbohydrate (Bensing *et al.*, 2004a), and this glycan modification is required for the stability and solubility of the protein (Takamatsu *et al.*, 2004b). Protein glycosylation may also protect against extracellular proteolytic digestion (Upreti *et al.*, 2003). Glycosylation of Fap1 contributes to a number of phenotypes, including adhesion (Peng *et al.*, 2008). All of the streptococcal serine-rich repeat protein accessory loci also contain additional glycosyltransferase (*gly*) and nucleotide sugar synthetase (*nss*) genes (Zhou & Wu, 2009). These genes affect the glycoprotein carbohydrate composition but are not essential for glycosylation (Takamatsu *et al.*, 2004a, b).

Only a small number of surface-associated proteins of *S. saprophyticus* have been described. The *S. saprophyticus* surface-associated protein (Ssp) has been identified as a lipase (Sakinç *et al.*, 2005), and the autolysin/adhesin of *S. saprophyticus* (Aas) protein is a multifunctional autolysin, sheep erythrocyte haemagglutinin and fibronectin-binding adhesin (Gatermann & Meyer, 1994; Hell *et al.*, 1998). The *S. saprophyticus* ATCC 15305 genome sequence reveals the presence of the uro-adherence factor A (UafA) protein, which also agglutinates sheep erythrocytes and mediates binding to human uroepithelia (Kuroda *et al.*, 2005). The serine-aspartate repeat protein I (SdrI) collagen- and fibronectin-binding adhesin is the most recently characterized *S. saprophyticus* surface protein (Sakinç *et al.*, 2009, 2006). Among these surface-associated proteins, UafA and Aas are conserved in 100%, Ssp in over 90%, and SdrI in 5–10% of all *S. saprophyticus* strains so far tested (Kleine *et al.*, 2010).

In this study, we have identified and characterized a novel serine-rich repeat glycoprotein (termed UafB) from *S. saprophyticus*. The *uafB* gene was shown to be located on a large plasmid and flanked by putative transposon sequences, suggesting mobility. We demonstrate that UafB is an adhesin that promotes cell surface hydrophobicity and mediates binding to fibronectin, fibrinogen and human bladder epithelial cells.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Clinical *S. saprophyticus*

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<i>E. coli</i> strains		
DH5 α	F [−] ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K [−] m _K ⁺) <i>phoA supE44 λ[−] thi-1 gyrA96 relA1</i>	Grant <i>et al.</i> (1990)
BL21	F [−] <i>ompT hsdS_B</i> (r _B [−] m _B [−]) <i>gal dcm</i>	Stratagene
MS1616	DH5 α containing pUafB	This study
MS2017	DH5 α containing pUafBHis	This study
MS2050	BL21 containing pUafBHis	This study
MS2777	DH5 α containing pNK27	This study
<i>S. saprophyticus</i> strains		
ATCC 15305	Type strain (genome sequenced)	Kuroda <i>et al.</i> (2005)
MS1146	Clinical isolate	AstraZeneca
MS2827	MS1146, cured of endogenous pSSAP1 plasmid	This study
MS1146 <u>uafB</u>	MS1146 isogenic <i>uafB</i> mutant	This study
Plasmids		
pGEM-T Easy	T/A cloning vector; Ap ^r	Promega
pBAD/HisB	Cloning and protein expression vector, containing N-terminal 6 $\times$ His tag; Ap ^r	Invitrogen
pUafB	7653 bp fragment, including <i>uafB</i> gene from MS1146, amplified with primers 373 and 434 and cloned into pGEM-T Easy; Ap ^r	This study
pUafBHis	1314 bp <i>uafB</i> fragment, amplified with primers 761 and 762, digested with <i>EcoRI/XhoI</i> and cloned into <i>EcoRI/XhoI</i> -digested pBAD/HisB, with in-frame N-terminal 6 $\times$ His tag; Ap ^r	This study
pNL9164	<i>E. coli/S. aureus</i> TargeTron shuttle vector (temperature-sensitive); Ap ^r Em ^r	Sigma
pNK27	pNL9164 shuttle vector retargeted with primers 1412–1414, EBSU to knock out <i>uafB</i> (TargeTron system); Ap ^r Em ^r	This study
pNK37	640 bp <i>uafB</i> non-repeat region, amplified with primers 2056 and 2057 and cloned into the SspI site of LIC E vector, with in-frame N-terminal 6 $\times$ His tag and thioredoxin fusion; Ap ^r	This study

isolates were collected from the UCLA Medical Center, Los Angeles, CA, USA (five isolates including MS1146), the Christiana Hospital, Newark, DE, USA (five isolates), and Sullivan Nicolaides Pathology, Brisbane, Australia (65 isolates). *S. saprophyticus* ATCC 15305 has been described before (Kuroda *et al.*, 2005). *S. saprophyticus* strains were cultured in/on brain heart infusion (BHI) broth/agar (Oxoid) supplemented with erythromycin (10 μ g ml^{−1}) as required. *E. coli* strains were cultivated in/on Luria–Bertani (LB) broth/agar supplemented with ampicillin (100 μ g ml^{−1}) or kanamycin (50 μ g ml^{−1}) as required.

DNA manipulations and genetic techniques. Plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen). For staphylococcal strains, 100 μ g ml^{−1} recombinant lysostaphin (Ambi Products) was added to the resuspension buffer prior to incubation at 30 °C. Restriction endonucleases were used according to the manufacturer's specifications (New England Biolabs; NEB). *S. saprophyticus* genomic DNA was extracted as described by Wilson (2001), with the exception that overnight cultures were treated with lysostaphin and incubated for 1–2 h at 30 °C prior to the lysis step. PCR amplification of the *uafB* gene was performed using the Expand Long Template PCR system according to the manufacturer's instructions (Roche). PCR assays to determine the presence of *uafB* (primers 256 and 257), *uafA* (primers 258 and 259) and *sdri* (primers 260 and 261) 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, 1 min at 72 °C, one cycle of 7 min at 72 °C, 4 °C hold. Primers were synthesized by Sigma and are listed in Table 2.

Sequencing and annotation of plasmid pSSAP1. A draft genome sequence of *S. saprophyticus* MS1146 was determined using a

combination of Sanger sequencing and 454 GS-FLX (Roche) pyrosequencing. This revealed the presence of three plasmids, including the *uafB*-containing plasmid pSSAP1. Assemblies were carried out using the Consed/Phrap/Phred software package (Gordon *et al.*, 1998). In order to sequence across the extensive repeats in the *uafB* gene sequence, an Ez-Tn5 <Kan-2> insertion kit (Epicentre) was employed. The *uafB* gene was PCR-amplified using primers 373 and 434, digested with *HindIII/EcoRI* and ligated into the *HindIII/EcoRI* sites of pGEM-T Easy to generate plasmid pUafB. Plasmid pUafB was incubated with the transposon mixture as per the manufacturer's protocol. A portion of the transposition mixture was introduced into competent *E. coli* DH5 α cells, and the transformation mixture was plated onto LB agar containing kanamycin. Plasmid purification was performed from a random selection of the resultant transformant clones. The purified plasmids were digested with *NotI* and *SphI* to define the location of the transposon and to assist the sequence assembly process. The final pSSAP1 sequence was finished to Q40 standard with an average Sanger read depth of $\sim 16 \times$ coverage. Annotation of plasmid pSSAP1 was carried out using Artemis (Rutherford *et al.*, 2000) and BLAST (Altschul *et al.*, 1997) similarity searches of publicly available sequence databases.

Construction of *S. saprophyticus* MS1146 mutants. Strain MS2827 was isolated as a derivative of *S. saprophyticus* MS1146 that had reduced cell surface hydrophobicity due to loss of the *uafB*-containing plasmid pSSAP1. Plasmid construct pNK27 (Table 1), specifically retargeted to the *uafB* gene of *S. saprophyticus* MS1146, was prepared using the Sigma TargeTron Gene Knockout system, as per the manufacturer's instructions. Briefly, optimal intron insertion sites and primer sequences (1412–1414, Table 2) were determined by the TargeTron online design site, followed by a retargeting PCR and cloning of the PCR product into the shuttle vector provided,

Table 2. PCR primers used in this study

Primer	Sequence (5'–3')	Description
256	GAGGATTTAGGTTTCAGCAATCG	<i>uafB</i> screen forward
257	AAACCGTATCCCTGTTTAGCC	<i>uafB</i> screen reverse
258	GTGCAACGAATACTGCGAAA	<i>uafA</i> screen forward
259	CGTGTGCTCGTGTGTCTCTT	<i>uafA</i> screen reverse
260	ACGCAGTAAGGAAGGCTCAA	<i>sdrI</i> screen forward
261	CTGACGGTTTAACTGCTTGG	<i>sdrI</i> screen reverse
373	ATTAAGCTTCAACATTATGCTTAGCCTCTTTG	<i>uafB</i> cloning reverse
434	GCATAGAATTCCAACAGAACCCGGCATAAAT	<i>uafB</i> cloning forward
761	GCTCACTCGAGGAAAGCATCGGCAGTTATTG	<i>uafB</i> fragment PCR for cloning into pBAD/HisB, for antibody production, forward. Contains <i>XhoI</i> site (underlined)
762	GCTCGGAATTCGCTGTCCATGTAGTTGTTAAACC	<i>uafB</i> fragment PCR for cloning into pBAD/HisB, for antibody production, reverse. Contains <i>EcoRI</i> site (underlined)
1011	TTCTTTAGGTGATGAACATATCAGG	Sequencing primer to check for correct 350 bp retargeted intron fragments for TargeTron
1412	AAAAAAGCTTATAATTATCCTTAAATC-TCCTAAAAGTGCGCCAGATAGGGTG	<i>uafB</i> TargeTron IBS
1413	CAGATTGTACAAATGTGGTGATAACAGA-TAAGTCCTAAAAGTTAACTTACCTTTCTTTGT	<i>uafB</i> TargeTron EBS1d
1414	TGAACGCAAGTTTCTAATTTTCGGTTAGAT-TCCGATAGAGGAAAAGTGTCT	<i>uafB</i> TargeTron EBS2
2056	TACTTCCAATCCAATGCGGATTTAGGTTTCAGCAATCG	<i>uafB</i> fragment PCR for cloning the non-repeat region into LIC E vector, forward. Contains LIC extension (underlined)
2057	TTATCCACTTCCAATGTTATTCGCTTGTAGGATCCGTAAC	<i>uafB</i> fragment PCR for cloning the non-repeat region into LIC E vector, reverse. Contains LIC extension (underlined)
EBSU	CGAAATTAGAACTTGCGTTCAGTAAAC	TargeTron EBS universal
FUP	CGTTGTAAAACGACGGCCAG	M13 Universal forward
RUP	AGGAAACAGCTATGACCATG	M13 Universal reverse

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 *S. saprophyticus* MS1146 by protoplast transformation, as described by Hell *et al.* (1998), followed by CdCl₂ induction and colony PCR screening to identify the *uafB* knockout (MS1146*uafB*).

Purification of truncated UafB, rUafB_{nrr}, antibody production and immunoblotting. For antiserum production, a 1308 bp segment from *uafB* was amplified with primers 761 and 762 (Table 2), digested with *XhoI/EcoRI* and ligated into *XhoI/EcoRI*-digested pBAD/HisB. The resultant plasmid (pUafBHis) contained base pairs 213–1520 of *uafB* fused to a 6×His encoding sequence. *E. coli* BL21 was transformed with plasmid pUafBHis and induced with 0.2% arabinose, and the resultant 6×His-tagged protein was purified using the Qiagen Ni-NTA Spin kit according to the manufacturer's instructions. Protein purity was assessed by SDS-PAGE analysis. Polyclonal anti-UafB serum was raised in rabbits by the Institute of Medical and Veterinary Sciences (South Australia) following a standard protocol. To construct the recombinant rUafB_{nrr} fusion protein, a 640 bp segment of *uafB* encoding the non-repeat region (nrr) was amplified with primers 2056 and 2057 (Table 2). The PCR product was then inserted into a pET21a-based LIC vector. The construct (pNK37) contained base pairs 999–1638 of *uafB* fused to upstream 6×His and thioredoxin-encoding sequences. *E. coli* BL21 harbouring pNK37 was induced with 1 mM IPTG, and the fusion

protein was purified under native conditions using a Qiagen Ni-NTA Superflow column as per the manufacturer's instructions. The negative control protein (6×His–thioredoxin, no *uafB* fusion) was induced and purified in the same way. Buffer-exchanged recombinant proteins were analysed by SDS-PAGE and quantified using a bicinchoninic acid kit (Sigma). Staphylococcal cell lysates were prepared as follows: 10 ml of overnight culture was pelleted by centrifugation, resuspended in 300 µl lysostaphin buffer (0.15 M NaCl, 0.05 M Tris/HCl, pH 8.0) containing recombinant lysostaphin (100 µg ml⁻¹) and Benzonase endonuclease (Sigma, 50 U), and incubated for 1 h at 30 °C. The cells were then sonicated with a Branson sonicator (four times 10 s) on ice. The sonicated cells were centrifuged, and the supernatant was removed, added to SDS-PAGE loading buffer and boiled for 5 min. For immunoblotting, whole-cell lysates were subjected to SDS-PAGE using NuPAGE Novex 3–8% Tris-acetate precast gels with NuPAGE Tris-acetate SDS running buffer, and subsequently transferred to PVDF microporous membrane filters using the iBlot dry blotting system as described by the manufacturer (Invitrogen). For the Western blot analysis, serum raised against UafB was used as primary serum and the secondary antibody was alkaline phosphatase-conjugated anti-rabbit IgG (A3687, Sigma). Sigma Fast BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) was used as the substrate in the detection process. Glycoprotein detection was performed using a DIG Glycan Detection kit (Roche) as per the manufacturer's instructions.

Microscopy and image analysis. Immunogold labelling and transmission electron microscopy (TEM) were performed essentially as described previously (Ulett *et al.*, 2007a) but with some modifications. Cells were fixed with 4 % paraformaldehyde in PBS (5 min) prior to quenching. Anti-UafB serum was used undiluted as the primary antibody. Air-dried grids were negatively stained with 1 % uranyl acetate before TEM analysis.

Cell surface hydrophobicity assay. The microbial adhesion to hydrocarbons (MATH) assay was performed essentially as described by Martin *et al.* (1989). Briefly, overnight cultures (10 ml) were pelleted by centrifugation, washed twice with saline, resuspended in 10 ml saline and adjusted to OD₆₀₀ 0.3. In triplicate, 5 ml bacterial suspensions were overlaid with 0.4 ml n-hexadecane and mixed on a platform shaker at 37 °C (400 r.p.m., 15 min). After 10 min equilibration at room temperature, the lower aqueous phase was aspirated and the OD₆₀₀ measured. The hydrophobicity index (HPBI) was calculated as follows: [OD (initial) – OD (final)]/OD (initial). The results were analysed by ANOVA.

Human uroepithelial cell adhesion assays. T24 bladder epithelial cells (ATCC no. HTB-4) were maintained in McCoy's 5a modified medium (Gibco), with fetal bovine serum (FBS) added to a final concentration of 10 %. The interaction of *S. saprophyticus* MS1146, MS1146uafB and MS2827 with T24 epithelial cell monolayers was studied essentially as previously described (Valle *et al.*, 2008). Briefly, wells were seeded with 1.1×10^5 cells in 24-well tissue culture plates (eight replicates for each bacterial strain). Once cells were confluent (1.2×10^6 cells per well), the culture medium was removed and cells were washed three times with PBS. Overnight bacterial cultures were diluted and grown to OD₆₀₀ 0.55, pelleted by centrifugation, washed three times with PBS and resuspended in 1:10 diluted McCoy's medium (without FBS). Bacteria (6.6×10^6 cells per well) were added to the T24 cell monolayers at an m.o.i. of 60 and incubated for 80 min at 37 °C in 5 % CO₂. The inoculating dose of bacteria was confirmed by serial dilution and plating. Monolayers were washed four times with PBS to remove non-adherent bacteria. The remaining bacteria were released by eukaryotic cell lysis with 0.1 % Triton X-100, and the number of adherent bacteria was determined by serial dilution and plating. The results were analysed by ANOVA. To test adhesion of the *S. saprophyticus* strains to fresh exfoliated uroepithelial cells, mid-stream urine was collected, pooled and centrifuged (500 g, 15 min) to harvest the uroepithelial cells. Overnight cultures of bacteria were pelleted by centrifugation and resuspended in a one-tenth volume of PBS. The epithelial cells and bacteria were mixed in equal parts and incubated at 37 °C, 175 r.p.m. for 1 h. The epithelial cells were washed four times with PBS (500 g, 3 min) and resuspended in PBS. Cells were examined using phase-contrast microscopy ($\times 63$ magnification).

Immobilized fibronectin and fibrinogen adhesion assay. Microtitre plate (Nunc U96 Maxisorp) wells were coated with 2 µg fibronectin, fibrinogen or BSA (Sigma) in 100 µl PBS per well overnight at 4 °C. Wells were then washed three times with 200 µl PBS, quenched with 170 µl 2 % BSA in PBS for 1 h, and then washed twice with 200 µl PBS before the addition of bacteria. Overnight bacterial cultures were washed and resuspended in PBS to a concentration of 1×10^9 c.f.u. ml⁻¹. The microtitre plate was inoculated with the bacterial suspensions (100 µl cells per well) and incubated for 2 h at 37 °C. For negative control wells, 100 µl PBS was added instead of bacteria. The wells were washed three times with 200 µl PBS and the adherent cells were fixed with 150 µl 25 % aqueous formaldehyde for 30 min at room temperature. Wells were washed twice with 200 µl PBS, stained with 150 µl 0.1 % crystal violet for 30 min, washed again with water and incubated with 150 µl ethanol/acetone (80:20) mixture for 30 min at room temperature

with gentle agitation. A₅₇₀ measurements were obtained using a microtitre plate reader and results were analysed by ANOVA.

rUafB_{nrr} ELISA. Microtitre plate (Nunc U96 Maxisorp) wells were coated with 5 µg fibronectin, fibrinogen and BSA (Sigma) in 100 µl PBS overnight at 4 °C. Wells were then washed twice with 200 µl Tris-buffered saline, 0.1 % Tween 20 (TBST), quenched with 150 µl Tris-buffered saline (TBS), 1.5 % BSA for 1 h and then washed three times with 200 µl TBST. Recombinant 6 × His–Trx–UafB_{nrr} and 6 × His–Trx proteins were added at a concentration of 5 µM (total volume of 100 µl per well) and incubated for 1 h at room temperature. The wells were washed four times with TBST before the addition of 100 µl 1:2500 mouse anti-polyhistidine antibodies (H1029, Sigma) diluted in TBS, 1.5 % BSA. Plates were incubated for 1 h at room temperature, washed six times with TBST and then incubated with 100 µl 1:7500 rabbit anti-mouse alkaline phosphatase-conjugated antibodies (A4312, Sigma) in TBS, 1.5 % BSA for 1 h at room temperature. Wells were washed six times with TBST. A 1 mg ml⁻¹ *p*-nitrophenyl phosphate (pNPP) (N2765, Sigma) substrate solution dissolved in 0.1 M glycine, 1 mM MgCl₂, 1 mM ZnCl₂ buffer, pH 10.4, was added and incubated at 37 °C for 30 min. The reaction was stopped with 50 µl 3 M NaOH and the A₄₀₅ was read with a microtitre plate reader. Mean values were compared using Student's *t* test.

Mouse model of UTI. The mouse model of UTI was performed as previously described (Ulett *et al.*, 2007b). Female C57BL/6 mice (9 weeks) were purchased from the University of Queensland Animal Facility. For colony counts, bladder and kidney homogenates, as well as the urine, were serially diluted in PBS and plated onto BHI agar. Means were compared by ANOVA.

RESULTS

The surface hydrophobicity of *S. saprophyticus* MS1146 is mediated by a plasmid-encoded factor

S. saprophyticus MS1146 possesses a high cell surface hydrophobicity index, a feature which is significantly different from the genome-sequenced *S. saprophyticus* ATCC 15305 (Fig. 1a). *S. saprophyticus* MS1146 contains three endogenous plasmids, and we hypothesized that a factor(s) encoded on one of these plasmids might be associated with this difference in hydrophobicity. We therefore screened a large number of *S. saprophyticus* MS1146 colonies obtained following repeated passaging in BHI medium for their cell surface hydrophobicity index. Using this approach, we identified one mutant (MS2827) that had significantly reduced hydrophobicity (Fig. 1a). Further analysis of MS2827 using PCR primers designed to target each individual plasmid revealed that it lacked one plasmid, which we have termed plasmid pSSAP1. Thus, our focus was targeted at the analysis of plasmid pSSAP1, and the identification and characterization of factors associated with cell surface hydrophobicity in *S. saprophyticus* MS1146.

Bioinformatic analysis of plasmid pSSAP1

Sequence analysis of the 66 104 bp plasmid pSSAP1 revealed the presence of 46 predicted protein-coding genes, eight pseudogenes or degenerate insertion sequence (IS) elements, and several repeat regions (Fig. 1b, Supplementary

Fig. 1. (a) Cell surface hydrophobicity of *S. saprophyticus* ATCC 15305, MS1146, MS1146uafB and MS2827. Measurements were made using a two-phase aqueous–hydrocarbon system. The results are presented as the mean of three replicates + SEM from three independent experiments. A significant difference in cell surface hydrophobicity was observed between MS1146 and ATCC 15305 ($P < 0.001$; *t* test) as well as between MS1146 and MS1146uafB/MS2827 ($P = 0.001$; ANOVA). (b) Genetic map of the *S. saprophyticus* MS1146 pSSAP1 plasmid. Arrows either side of the outer circle denote the protein-coding regions in the plasmid. The regions encoding uro-adherence factor B (*uafB*) and accessory genes, *IS431*, the *rep* replication initiation gene, the cadmium resistance loci (*cadCA*, *cadD*) and arsenic resistance loci (*arsA*, *arsRBC*) are labelled. Coding sequences are coloured according to predicted function: *uafB* or accessory gene, red; transposase or IS-related function, purple; plasmid-related function, pink; other predicted function or conserved hypothetical protein, blue; hypothetical protein, grey. Inverted repeats formed by degenerate IS4 elements either side of the *uafB* locus are shown as black bars. In the inner circles, arcs indicate regions longer than 1 kb of high nucleotide identity to other staphylococcal sequences: *S. epidermidis* SK30 plasmid SAP107A, 85–92% identity (gold); *S. saprophyticus* ATCC 15303 pSSP1 (olive) and pSSP2, both >98% identity (cyan); and the *S. haemolyticus* JCSC1435 genome sequence, 96–98% identity (aqua) (accession nos GQ900456, AP008935, AP008936 and AP006716). The two innermost circles represent deviation from the mean G+C content (32.4%) and GC-skew, respectively. Sizes are given in kilobase pairs (kb).

Table S1). Like other staphylococcal plasmids, pSSAP1 has a mosaic structure with evidence of multiple insertions and deletions of discrete sequence blocks, many carrying genes for heavy metal resistance. For example, there is a high level of nucleotide sequence identity to the *cadCA* cadmium resistance and *arsRBC* arsenic resistance operons of *S. aureus* pI258 (Endo & Silver, 1995; Nucifora *et al.*, 1989). The most notable feature of pSSAP1 is the presence of a 17 kb cluster containing a novel gene encoding a serine-rich repeat adhesin (which we have termed uro-adherence factor B or *uafB*) and seven genes encoding a putative accessory expression system. The accessory expression genes are located immediately downstream of the *uafB* gene and putatively encode SecA2, SecY2, three accessory secretory proteins and two glycosyltransferases (Fig. 2a). This locus is located within a pair of ~210 bp inverted repeats formed from degenerate IS4 sequences (Fig. 1b). Immediately downstream of the left-hand inverted repeat is an intact IS431 transposase, which is often found encoded within SCCmec elements. Other genes encoded by pSSAP1 are

hypothetical or have general functional predictions only (Fig. 1b, Supplementary Table S1). The remainder of this study aimed to characterize the function of UafB.

Sequence analysis of UafB

The *uafB* gene is highly repetitive in nature and required the use of an *in vitro* transposon mutagenesis strategy to complete its sequence. The *uafB* gene was PCR-amplified and cloned in pGEM-T Easy to generate plasmid pUafB. *In vitro* transposon mutagenesis was employed to generate a library of overlapping transposon mutants, which enabled sequencing across the repetitive region of the gene. This revealed that the *uafB* gene is an intact coding region of 6840 bp in length. The size of the complete *uafB* gene sequence corresponded with that of the *uafB* PCR product (data not shown).

The unmodified UafB protein consists of 2279 amino acid residues (Fig. 2b). The N terminus contains an atypical

Fig. 2. (a) Conserved gene arrangement in a selection of *uafB*-like loci. Serine-rich repeat genes are coloured red. Genes encoding homologous secretion machinery and glycosylation accessory proteins in different loci are shown in the same colour. The *uafB* cluster has the same components as the *S. aureus* *sraP* cluster, but lacks two genes present in the *Streptococcus gordonii* *gspB* cluster. (b) Features of the *S. saprophyticus* MS1146 UafB protein and three staphylococcal UafB homologues. Corresponding features of the different proteins are shown in the same colour. The N terminus (containing the putative signal peptide), C terminus (containing the LPXTG sortase motif), non-repeat region and serine-rich repeat regions are indicated. (c) Amino acid identities of the UafB protein with UafB homologues. The repeat sequences represent consensus sequences of the major tandem repeat units. ND, Not determined, because assembled repeat sequences were not available.

signal peptide, putatively 93 residues in length based on previous analyses of other serine-rich repeat proteins (Bensing *et al.*, 2007). A hydrophobic cell wall-spanning segment at the C terminus contains an LPETG sortase motif followed by a charged tail. UafB contains three distinct serine-rich tandem repeat regions. The longest of these comprises 68.5 % of the total protein sequence; the three repeat regions together encompass 81.5 % of UafB. Based on studies of other serine-rich repeat proteins, we predicted that the putative binding domain of UafB would reside within the 213-residue non-repeat region between the serine-rich repeat regions. No known conserved protein domains were detected in the non-repeat region sequence, and this region also lacks the corresponding sequence that mediates bacterial cell aggregation recently identified in the *Streptococcus pneumoniae* PsrP serine-rich repeat protein (Sanchez *et al.*, 2010). The predicted molecular masses of the complete UafB protein and the mature N- and C-terminally processed UafB protein are 218.1 and 203.1 kDa, respectively.

By searching GenBank for matches to the non-repeat region of UafB, we identified genes encoding UafB homologues, complete with accessory expression system loci, in both *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*. The 42.6 kb *S. epidermidis* SK30 plasmid SAP107a (GenBank accession no. GQ900456.1) contains a gene encoding a 2681-residue serine-rich repeat protein which is homologous to UafB, but has been misannotated as two individual genes, namely *sdrI* and *fliK*. Additionally, we detected sequences representing the N terminus, non-repeat region, C terminus and accessory expression genes of a UafB homologue in the draft genome of *S. epidermidis* W23144 (GenBank accession no. ACJC000000000; ZP_04797573.1, ZP_04797572.1, NZ_ACJC01000147). Each of these sequence fragments is located at the very beginning of a separate contig and it is apparent that these contigs have not yet undergone final assembly. Based on the draft assembly, it is not clear whether this putative *uafB* homologue is chromosomally or plasmid located. Gene SH0326 (GenBank accession no. 3484004) of the sequenced *S. haemolyticus* JCSC1435 strain encodes a 3607-residue UafB homologue protein which is followed by accessory expression system genes.

The closest matches to the *S. saprophyticus* UafB non-repeat region are from the above-mentioned coagulase-negative staphylococci (Fig. 3a). The amino acid identity of each section of the *S. saprophyticus* MS1146 UafB protein with the *S. epidermidis* and *S. haemolyticus* homologues is shown in Fig. 2(c). The *S. epidermidis* UafB homologues have good matches to each of the UafB subsequences, including the serine-rich repeat units (note that no repeat sequence data are available for *S. epidermidis* W23144). All three UafB homologues share high amino acid similarity (≥ 80 %) with the non-repeat region of UafB (Fig. 2c and Fig. 3b), which contains the predicted ligand-binding residues. While having an 81 % identity with the UafB non-repeat region, the *S. haemolyticus* homologue protein

termini and serine-rich repeats are noticeably more divergent. The accessory protein sequences (concatenated) of the *S. epidermidis* homologues also share high similarity with the corresponding UafB sequence (SAP107a, 91 %; W23144, 95 %), while the concatenated *S. haemolyticus* accessory proteins share 58 % similarity (data not shown). A number of transposase sequences are present in close proximity to the *uafB*-like loci in *S. haemolyticus* JCSC1435 and in the *S. epidermidis* SK30 SAP107a plasmid. A comparison of *S. saprophyticus* pSSAP1 and *S. epidermidis* SAP107a plasmids revealed that apart from genes encoding the serine-rich repeat and accessory expression proteins, only transposase genes and a malate:quinone oxidoreductase gene were shared. Of note, pSSAP1 contains one copy of IS431 located downstream of the *uafB* locus compared with three copies of IS431 downstream of the *uafB* homologue locus in SAP107a.

UafB contributes to cell surface hydrophobicity

To evaluate the putative role of UafB on cell surface hydrophobicity, we constructed a *uafB* knockout mutant employing the TargeTron system (Sigma). The correct isogenic *uafB* knockout (referred to as MS1146*uafB*) was verified by colony PCR and sequencing, as well as PCR screening for the *S. saprophyticus* MS1146 endogenous plasmids to ensure that none had been lost in the process. The hydrophobicity indices of *S. saprophyticus* MS1146, MS1146*uafB* and MS2827 were compared by examining adhesion to n-hexadecane. In comparison with *S. saprophyticus* MS1146, MS1146*uafB* and MS2827 displayed a 44 % decrease in cell surface hydrophobicity in this assay ($P=0.001$; Fig. 1a). Thus, UafB is a major contributor to the cell surface hydrophobicity of *S. saprophyticus* MS1146, and the generation of a specific *uafB* deletion strain confirms the contribution of UafB in the pSSAP1 plasmid-cured strain MS2827.

Prevalence of *uafB* in *S. saprophyticus*

To assess the prevalence of *uafB* in *S. saprophyticus* we screened a collection of 76 clinical isolates from three different locations. The isolates were screened for the presence of the *uafB* gene, as well as the two previously described LPXTG motif-containing adhesin genes of *S. saprophyticus* (i.e. *uafA* and *sdrI*). PCR screening detected *uafB* in 5.3 % (4/76) of the *S. saprophyticus* strains, *uafA* in 100 % (76/76) and *sdrI* in 6.6 % (5/76). Southern blotting confirmed the *uafB* prevalence result (data not shown). None of the strains harboured both *uafB* and *sdrI*.

UafB is a glycoprotein expressed by *S. saprophyticus* MS1146

Examination of whole-cell lysates prepared from *S. saprophyticus* MS1146, MS1146*uafB* and MS2827 by SDS-PAGE and Western blotting employing a UafB-specific antiserum (directed at amino acid residues 72–506)

Fig. 3. Comparison of non-repeat regions of UafB and UafB homologues. (a) Unrooted phylogram produced from a CLUSTAL W alignment of the non-repeat regions of staphylococcal and streptococcal serine-rich repeat proteins, using the neighbour-joining algorithm implemented under CLUSTAL X. UafB clusters with homologues from *S. epidermidis* and *S. haemolyticus*. Bootstrap values out of 100 are indicated. Distance is measured in substitutions per site. *S. saprophyticus* UafB is shown in bold type. (b) CLUSTAL W alignment of the non-repeat regions of UafB and its staphylococcal homologues, showing high amino acid sequence conservation. Residues identical to the *S. saprophyticus* UafB sequence are denoted with a dot. Residue positions relative to the start of the serine-rich repeat protein sequences are as follows: *S. saprophyticus* MS1146 UafB, 334–544; *S. epidermidis* plasmid SAP107a, 359–570; *S. haemolyticus* JCSC1435, 284–497. Proportions of hydrophobic and polar/charged residues of the non-repeat regions are consistent amongst the homologues and are as follows (hydrophobic, polar/charged): *S. saprophyticus* MS1146 UafB, 31 %, 55 %; *S. epidermidis* plasmid SAP107a, 32 %, 55 %; *S. haemolyticus* JCSC1435, 33 %, 55 %; *S. epidermidis* W23144, 33 %, 55 %.

demonstrated expression of UafB in *S. saprophyticus* MS1146 but not in MS1146uafB or MS2827 (Fig. 4a). The apparent molecular mass of UafB was >460 kDa,

significantly larger than its expected molecular mass of 203.1 kDa. DIG glycan analysis detected a high-molecular-mass band for *S. saprophyticus* MS1146 but not for the

Fig. 4. Expression of UafB. (a) Western blot analysis of whole-cell lysates prepared from *S. saprophyticus* MS1146, MS1146uafB and MS2827 using a polyclonal antiserum directed against UafB. Lanes: M, HiMark prestained protein marker (Invitrogen); 1, MS1146; 2, MS1146uafB; 3, MS2827. Expression of UafB was detected in wild-type *S. saprophyticus* strain MS1146 but not in its isogenic uafB mutant or the pSSAP1 plasmid-cured mutant. The lower-molecular-mass bands common to all strains are not related to UafB. (b) Carbohydrate analysis of total cell lysates prepared from *S. saprophyticus* MS1146, MS1146uafB and MS2827 using a DIG Glycan Detection kit (Roche). Lanes: 1, MS1146; 2, MS1146uafB; 3, MS2827. A high-molecular-mass band was detected in wild-type *S. saprophyticus* strain MS1146 but not in its isogenic uafB mutant or the pSSAP1 plasmid-cured mutant. A positive control of human salivary glycoproteins was used and reacted strongly in this assay (data not shown). (c) Immunogold TEM of *S. saprophyticus* MS1146 and MS1146uafB. Expression of UafB at the cell surface of *S. saprophyticus* MS1146 was demonstrated by abundant labelling with UafB–gold particles. In contrast, *S. saprophyticus* MS1146uafB was virtually devoid of gold labelling. Bars, 200 nm.

MS1146uafB and MS2827 mutants (Fig. 4b). This band corresponds to the UafB band detected by Western blotting and shows that UafB is glycosylated.

UafB is located at the cell surface

The salient features of UafB suggest that it is covalently anchored to the cell wall and exposed at the cell surface. To confirm this, we used the UafB-specific antiserum for immunogold labelling and electron microscopy of *S. saprophyticus* MS1146. Wild-type *S. saprophyticus* MS1146 cells displayed abundant surface labelling with UafB–gold particles. In contrast, *S. saprophyticus* MS1146uafB and MS2827 (data not shown) were devoid of labelling with UafB-specific antibodies (Fig. 4c). These data show that the uafB gene is functional and results in the expression of a large protein at the cell surface in *S. saprophyticus* MS1146.

UafB mediates binding to bladder epithelial cells, fibronectin and fibrinogen

The contribution of UafB to adhesion to human T24 bladder epithelial cells was examined. We observed an eightfold reduction in binding to T24 bladder epithelial cells by the *S. saprophyticus* MS1146uafB and MS2827 mutants compared with *S. saprophyticus* MS1146 ($P<0.001$; Fig. 5a). Recently, we have shown that T24 bladder cells contain a high amount

of fibronectin (Valle *et al.*, 2008), and thus we also examined the ability of UafB to mediate binding to this ECM protein. In contrast to wild-type *S. saprophyticus* MS1146 cells, MS1146uafB and MS2827 adhered significantly less to fibronectin ($P<0.001$; Fig. 5b), demonstrating the involvement of UafB in this adhesion phenotype. We also found that UafB bound to fibrinogen ($P<0.001$; Fig. 5b). To examine the specificity of binding to fibronectin and fibrinogen, we also tested *S. saprophyticus* MS1146 for its ability to bind to other ECM proteins. *S. saprophyticus* MS1146 did not adhere to laminin, collagen I, collagen II, collagen IV or vitronectin (data not shown).

The non-repeat region of UafB mediates binding to both fibronectin and fibrinogen

To further characterize the binding of UafB to its receptors we sought to determine whether the non-repeat region of the protein contained the binding domain. To facilitate these experiments, the non-repeat region of UafB was PCR-amplified, cloned, expressed and purified as a fusion to the thioredoxin scaffold sequence. Using an ELISA approach, we found that the non-repeat region of UafB binds to both fibronectin and fibrinogen. The control recombinant protein lacking the UafB sequence (i.e. thioredoxin alone) bound significantly less in both cases ($P<0.001$; Fig. 5c). Neither recombinant protein bound to BSA.

The *uafB* gene does not contribute to colonization of the mouse bladder

To study the role of UafB in virulence we examined the ability of *S. saprophyticus* MS1146, MS1146uafB and MS2827 to colonize the mouse urinary tract. The mouse UTI model is well established for uropathogenic *E. coli* (UPEC), and has been used successfully to define the role of several virulence factors (Connell *et al.*, 1996; Ulett *et al.*, 2007b). *S. saprophyticus* MS1146 efficiently colonized the mouse bladder [8.8×10^5 c.f.u. ml^{-1} (0.1 g bladder tissue) $^{-1}$]; this level of colonization is similar to that which we routinely observe for the UPEC strain CFT073 (Ulett *et al.*, 2007b; Valle *et al.*, 2008). However, *S. saprophyticus* MS1146uafB and MS2827 also colonized the mouse bladder with equal efficiency [8.3×10^5 and 8.0×10^5 c.f.u. ml^{-1} (0.1 g bladder tissue) $^{-1}$], suggesting that UafB does not affect adhesion in this infection model. A similar level of colonization was also

Fig. 5. (a) Adhesion of *S. saprophyticus* MS1146, MS1146uafB and MS2827 to human T24 bladder epithelial cells. T24 bladder epithelial cell monolayers were inoculated with bacteria and incubated for 80 min at 37 °C, and adherent bacteria were enumerated by direct plating and colony counts. Results are the mean $\pm$ SEM of eight replicates from three independent experiments. A significant difference in adhesion was observed between MS1146 and MS1146uafB/MS2827 ($P < 0.001$; ANOVA). (b) Adhesion of *S. saprophyticus* MS1146, MS1146uafB and MS2827 to immobilized fibronectin and fibrinogen. Cell suspensions of each strain were added to microtitre plate wells coated with fibronectin, fibrinogen and BSA (negative control) and incubated for 2 h at 37 °C. Adherent bacteria were stained with 0.1% crystal violet and quantified by A_{570} . Data represent the mean $\pm$ SEM of 12 replicates from three independent experiments. Significant differences in adhesion to fibronectin and fibrinogen were observed between MS1146 and MS1146uafB/MS2827 ($P < 0.001$; ANOVA). (c) Direct binding of the recombinant UafB non-repeat region to fibronectin and fibrinogen. Microtitre plate wells coated with fibronectin, fibrinogen or BSA were incubated with recombinant 6xHis-UafB non-repeat region thioredoxin fusion or 6xHis-thioredoxin proteins at 37 °C for 1 h. A schematic representation of the recombinant 6xHis-Trx-UafB_{nrr} protein is shown in the inset. Detection was performed using anti-polyhistidine antibodies. Following 30 min development, A_{405} was read with a microtitre plate reader. Data represent the mean $\pm$ SEM of three replicates from three independent experiments. The recombinant protein containing the UafB non-repeat region adhered to both fibronectin ($P < 0.001$) and fibrinogen ($P < 0.001$) significantly more than the thioredoxin-only control protein (*t* test).

observed in the urine of infected mice, while no significant colonization of the kidneys was observed (data not shown).

UafB promotes specific adhesion to human uroepithelial cells

The above results suggest that UafB may mediate differential binding to human and mouse bladder cells. To investigate this further, we examined the ability of *S. saprophyticus* MS1146, MS1146uafB and MS2827 to bind to exfoliated uroepithelial cells freshly harvested from human urine. *S. saprophyticus* MS1146 mediated very strong binding to these cells, while *S. saprophyticus* MS1146uafB and MS2827 did not adhere (Fig. 6). Thus, UafB appears to be an adhesin specifically associated with adherence to human bladder epithelial cells.

DISCUSSION

S. saprophyticus is a common cause of community-acquired UTI, especially among the sexually active young female demographic. However, surprisingly little is known about the molecular mechanisms that underpin the ability of *S. saprophyticus* to cause infection, with only a handful of molecular genetic studies undertaken to date. Acknowledged

virulence factors include urease (Gatermann & Marre, 1989), a cell surface lipase (Sakinç *et al.*, 2005), slime production (Atmaca *et al.*, 2000), cell surface hydrophobicity (Schneider & Riley, 1991) and three adhesins (Aas, UafA and SdrI) (Hell *et al.*, 1998; Kuroda *et al.*, 2005; Sakinç *et al.*, 2006). *S. saprophyticus* strains are known to bind collagen (Sakinç *et al.*, 2006), laminin (Paulsson *et al.*, 1992) and fibronectin (Gatermann & Meyer, 1994). Here we have identified a novel plasmid-located gene in *S. saprophyticus* MS1146 which we have termed uro-adherence factor B (*uafB*). The *uafB* gene is the third LPXTG motif-encoding *S. saprophyticus* gene described to date.

UafB is a cell wall-anchored adhesin that belongs to a group of serine-rich repeat surface proteins that are dependent on a proprietary export system for glycosylation and transport across the cell membrane; it is the first such protein identified in *S. saprophyticus*. Serine-rich repeat proteins have been identified from other staphylococci, streptococci and a lactobacillus. A locus of core genes encoding SecA2, SecY2, three accessory secretory proteins and two glycosyltransferases is located immediately downstream of the *uafB* gene. The *uafB* cluster lacks the *gly* and *nss* genes present in the *Streptococcus gordonii* *gspB* cluster [consistent with all other known staphylococcal serine-rich repeat protein accessory loci (Zhou & Wu, 2009)], but still produces a functional adhesive glycoprotein. The UafB atypical signal sequence contains the key glycine residues and conserved KSGKXW motif (Bensing *et al.*, 2007) that are present in the signal sequences of other members of the serine-rich repeat protein family.

Searches for other proteins with non-repeat regions similar to those of UafB resulted in the identification of a chromosomal homologue in *S. haemolyticus*, a previously misannotated homologue on an *S. epidermidis* plasmid and a putative homologue in an *S. epidermidis* draft genome. *S. epidermidis* and *S. haemolyticus* are commensal coagulase-negative staphylococci that tend to cause opportunistic

infections, especially in the immunocompromised. Interestingly, both *S. epidermidis* and *S. haemolyticus* can also cause UTIs. The finding that *uafB* cluster homologues are found in other staphylococci suggests that *uafB* is not just a rare *S. saprophyticus* adhesion factor, and the presence of the nearby transposase sequences is strong evidence that the *uafB* and *uafB*-like loci were once part of mobile elements. Indeed, our sequence data suggest that the *uafB* cluster was mobilized *en bloc* onto pSSAP1 via transposition.

Cell surface hydrophobicity represents an important virulence determinant in bacterial urinary pathogens, including coagulase-negative staphylococci (Martin *et al.*, 1989). Hydrophobic forces contribute to the initial events that lead to irreversible adhesion of bacteria to a surface (Arp, 1988), and hydrophobic interactions promote adhesion between bacteria and mammalian cells (Tylewska *et al.*, 1979). Some bacterial cell surface proteins are known to modulate cell surface hydrophobicity. For example, expression of the fibronectin-binding CshA adhesin increases the cell surface hydrophobicity of *Streptococcus gordonii* (McNab *et al.*, 1999). Here we have demonstrated that UafB is a major cell surface hydrophobicity determinant of *S. saprophyticus* MS1146.

In order to functionally characterize the UafB protein, we generated two independent mutants. First, we cured the pSSAP1 plasmid from *S. saprophyticus* MS1146 (whilst maintaining the other two endogenous plasmids), and second an isogenic *uafB* knockout was constructed by insertional inactivation with a group II intron. To our knowledge, this is the first instance in which the Sigma TargeTron system has been used to generate a gene-specific knockout in *S. saprophyticus*. For every assay employed in this work, the phenotypes for both of these independent mutants were consistent. We were unable to complement MS1146*uafB* or MS2827 despite repeated attempts to transform these strains with an *E. coli*-*S. aureus* shuttle plasmid (pCU1) containing *uafB*. The transformation of *S.*

Fig. 6. Adhesion of *S. saprophyticus* MS1146, MS1146*uafB* and MS2827 to exfoliated human uroepithelial cells. Uroepithelial cells were harvested from fresh pooled human urine, mixed with bacteria and incubated for 1 h at 37 °C. The cells were visualized by phase-contrast microscopy (×63 magnification). *S. saprophyticus* MS1146 adhered strongly to uroepithelial cells, while no adhesion was observed for MS1146*uafB* or MS2827. Bars, 10 µm.

saprophyticus requires the generation of protoplasts, and in our hands *S. saprophyticus* MS1146 is not amenable to transformation with plasmids larger than 10 kb.

S. saprophyticus MS1146, MS1146uafB and MS2827 were used in conjunction with a UafB-specific antiserum to demonstrate that UafB is expressed by *S. saprophyticus* MS1146 and located on the cell surface. The size of the UafB protein identified by Western blot analysis was considerably larger than its expected apparent molecular mass. Since two genes encoding putative glycosyltransferases were identified in the *uafB* accessory secretory locus, we hypothesized that this discrepancy in mass may be due to post-translational carbohydrate modification of UafB. We confirmed the glycosylation of UafB using a DIG Glycan Detection kit (Roche). The identity of the modifying glycan residues was not investigated.

S. saprophyticus MS1146 adhered in significantly greater numbers to T24 bladder epithelial cells than the *uafB*-negative strains MS1146uafB and MS2827. *S. saprophyticus* strains are known to bind to the ECM proteins collagen (Sakinç *et al.*, 2006), laminin (Paulsson *et al.*, 1992) and fibronectin (Gatermann & Meyer, 1994), and thus we also examined the adherence of these strains to a range of ECM molecules. Our data show that UafB plays a role in the binding of *S. saprophyticus* MS1146 to immobilized fibronectin. The background fibronectin binding by MS1146uafB and MS2827 is likely due to the Aas protein. Fibronectin is a large dimeric glycoprotein that is present in both its soluble and cellular forms in the ECM of epithelial cells. Numerous fibronectin-binding adhesins from Gram-positive bacteria have been characterized, including Fba proteins, Sfb proteins and M1 protein from *Streptococcus pyogenes*; FnBA and FnBB from *Streptococcus dysgalactiae*; PavA from *Streptococcus pneumoniae*; FbpA and CshA from *Streptococcus gordonii*; FnBPA and FnBPB from *S. aureus* and Aas and SdrI from *S. saprophyticus* (Henderson *et al.*, 2011; Sakinç *et al.*, 2009). Binding of epithelial cell fibronectin can also lead to invasion by pathogenic staphylococci and streptococci (Joh *et al.*, 1999). Future work will investigate whether UafB is involved in internalization of *S. saprophyticus* MS1146 in uroepithelial cells. A number of bacterial fibronectin-binding adhesins, such as FnBPA of *S. aureus* and SfbI of *Streptococcus pyogenes*, can also bind fibrinogen, a soluble plasma glycoprotein. The fibronectin- and fibrinogen-binding domains of these adhesins are typically located at separate termini of the protein, unlike UafB, in which the N-terminal non-repeat region mediates adhesion to both receptors. Most, but not all, serine-rich repeat proteins are lectins; this has not yet been established for UafB. Due to the strong similarity between the non-repeat regions of UafB and those of its homologues, we speculate that these homologues may share binding specificities with UafB. Adhesins recognizing fibronectin and fibrinogen have been identified and characterized in *S. epidermidis*, but functional redundancy is also well established for bacterial adhesins. *S. haemolyticus* is known to bind fibronectin, among other host proteins (Paulsson *et al.*,

1992), but very little is known about the mechanisms of adhesion of this species.

The UafB phenotypic data obtained from the *in vitro* human cell adhesion experiments were not corroborated in the *in vivo* mouse UTI model. *S. saprophyticus* MS1146 efficiently colonized the bladders and urine of the mice, but no role for UafB was apparent. It is possible that this is due to the UafA adhesin, which has been shown to mediate adhesion to human T24 bladder cells in the genome-sequenced *S. saprophyticus* strain ATCC 15305 (Kuroda *et al.*, 2005). However, further work is required to confirm this and also to evaluate the contribution of the Aas adhesin to *S. saprophyticus* MS1146 colonization of the mouse bladder. It is possible that the expression of redundant adhesins disguises the phenotype of UafB in the mouse UTI model. The lack of mouse kidney colonization is in line with previous experiments employing UPEC strains and C57BL/6 mice in our laboratory (Ulett *et al.*, 2007b; Valle *et al.*, 2008).

Other studies have demonstrated that *S. saprophyticus* exhibits a higher level of adhesion to uroepithelial cells than other types of epithelia, including buccal and skin epithelial cells (Colleen *et al.*, 1979; Mårdh *et al.*, 1979), and almost exclusively causes infections of the urinary tract. Although *S. saprophyticus* can colonize the murine urinary tract, the lack of UafB-mediated adhesion in the mouse model prompted us to examine its ability to mediate adherence to freshly harvested human uroepithelial cells. We observed a striking difference in adherence between *S. saprophyticus* MS1146 and the *uafB*-negative strains MS1146uafB and MS2827, suggesting the possibility that UafB surface expression mediates tropism for human uroepithelial cells. The precise mechanism by which UafB binds to uroepithelial cells remains to be determined.

The *S. saprophyticus uafB* prevalence (5.3 %) in this study is comparable with that of the *sdrI* gene (6.6 %) for the same collection of strains. Importantly, *uafB*-positive *S. saprophyticus* strains were identified amongst clinical isolates collected from both Australia and the USA. Further work needs to be done to assess the prevalence of the *uafB* homologues in *S. epidermidis* and *S. haemolyticus* clinical isolates, and to study their functions. It also remains to be seen whether *uafB* is exclusively plasmid-encoded in *uafB*-positive *S. saprophyticus* strains. However, the plasmid location of *uafB*, together with the fact that homologous *uafB* loci are located on apparently transposable elements in other staphylococci, suggests the possibility that this adherence-encoding factor is mobile and can be spread between different species of staphylococci.

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