The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation

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Staphylococcus aureus colonizes the moist squamous epithelium of the anterior nares. One of the adhesins likely to be responsible is the <u>S</u>. <u>aureus s</u>urface protein <u>G</u> (SasG), which has sequence similarity with the proteins Pls (<u>pl</u>asmin <u>s</u>ensitive) of *S. aureus* and Aap (<u>a</u>ccumulation <u>a</u>ssociated <u>p</u>rotein) of *Staphylococcus epidermidis.* Expression of SasG by a laboratory strain of *S. aureus* could not be detected by Western immunoblotting. To enable investigation of SasG, the gene was cloned into two expression vectors, the IPTG-inducible pMUTIN4 and the tetracycline-inducible pALC2073, and introduced into *S. aureus.* Expression of SasG masked the ability of exponentially grown *S. aureus* cells expressing protein A (Spa), clumping factor B (ClfB) and the fibronectin binding proteins A and B (FnBPA and FnBPB) to bind to IgG, cytokeratin 10 and fibronectin, respectively. SasG also masked binding to fibrinogen mediated by both ClfB and the FnBPs. Western immunoblotting showed no reduction in expression of the blocked adhesins following induction of SasG. SasG size variants with eight, six or five B repeats masked binding to the ligands, whereas variants with four, two or one repeats had no effect. SasG-expressing strains formed peritrichous fibrils (53.47 ± 2.51 nm long) of varying density on the cell wall, which were labelled by immunogold negative staining with anti-SasG antibodies. SasG-expressing strains of *S. aureus* also formed biofilm independently of the polysaccharide intercellular adhesin (PIA). SasG variants with eight, six and five repeats formed biofilm, whereas variants with four, two or one repeats did not. It was concluded that the fibrillar nature of SasG explains its ability to mask binding of *S. aureus* microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) to their ligands and to promote formation of biofilm. In addition, the strong adhesion of SasG to desquamated nasal epithelial cells likely compensates for its blocking of the binding of *S. aureus* ClfB to cytokeratin 10, which is important in adhesion to squames by cells lacking SasG. Several clinical isolates expressed SasG at levels similar to those of SH1000 *sasG* :: pMUTIN4, indicating that the properties described in the laboratory strain SH1000 may be relevant *in vivo*.

INTRODUCTION

Staphylococcus aureus causes community-acquired and nosocomial infections including infections associated with indwelling medical devices. The natural habitat of *S. aureus* is the moist squamous epithelium in the anterior nares. About 20 % of the human population carry *S. aureus* permanently in their noses and another 60 % of individuals are intermittent carriers (Kluytmans *et al.*, 1997). Infection rates are higher in carriers than in non-carriers and invasive disease is often caused by the patient's own isolate (Peacock *et al.*, 2001).

The ability of *S. aureus* to cause infection is due, in part, to cell surface-associated proteins that mediate attachment to the host extracellular matrix termed MSCRAMMs (<u>micro</u>bial <u>s</u>urface <u>c</u>omponents <u>r</u>ecognizing <u>a</u>dhesive <u>m</u>atrix <u>m</u>olecules) (Foster & Hook, 1998). Clumping factors A and B (ClfA and ClfB), protein A and the fibronectin binding proteins A and B (FnBPA and FnBPB) are characterized by an N-terminal signal sequence for Sec-dependent secretion and a C-terminal cell wall-anchoring domain (Mazmanian *et al.*, 2001; Navarre & Schneewind, 1999) for covalent linkage to the cell wall by sortase (Navarre & Schneewind, 1999).

A novel surface protein identified by analysis of genome sequences called SasG has sequence similarity to proteins Pls (plasmin sensitive) of *S. aureus* and Aap (accumulation associated protein) of *Staphylococcus epidermidis* (Roche *et al.*, 2003a). The N-terminal A region of SasG consists of a unique 157 residue subdomain not present in Pls or Aap and a conserved 224 residue subdomain which is 52 and 59 % identical to that of Pls and Aap, respectively (Fig. 1). SasG of strain 8325-4 has seven 128 residue B repeats and one partial B repeat of 65 amino acids, which are 60–67 % identical to those of Aap and 65 % identical to those of Pls (Fig. 1) (Roche *et al.*, 2003a).

The ability of *S. aureus* to colonize the nasal epithelium is in part due to the MSCRAMMs ClfB (O'Brien *et al.*, 2002) and IsdA (Clarke *et al.*, 2006), which promote adhesion to desquamated epithelial cells. SasG can also promote adhesion to squames, and might contribute to colonization (Roche *et al.*, 2003b). Although strain 8325-4 has a copy of the *sasG* gene, expression of SasG protein is not detected by immunoblotting (Roche *et al.*, 2003b). Paradoxically, weak constitutive expression of SasG from plasmid pKS80 in *S. aureus* 8325-4 partially blocks the ability of MSCRAMMs to interact with ligands in adhesion assays (Roche *et al.*, 2003b), a phenomenon that was originally reported for Pls (Savolainen *et al.*, 2001). Previous studies of SasG are incomplete due to the weak, uncontrollable expression from the vector pKS80 (Roche *et al.*, 2003b). In this paper we achieve high-level controlled expression of SasG, which enables more thorough investigation of the masking effect and the role of SasG in adherence to squamous epithelial cells.

Gram-positive cocci can express fibrillar appendages with adhesive functions that project outwards from the cell wall. Fibrils occur on *Streptococcus gordonii* (McNab *et al.*, 1999), *Streptococcus sanguis* (Handley *et al.*, 1985), *Streptococcus mitis* (Elliott *et al.*, 2003) and *Streptococcus salivarius* (Weerkamp *et al.*, 1986). Fibrils have adhesive functions equivalent to those of fimbriae which have recently been identified on some Gram-positive bacteria (Telford *et al.*, 2006). Very recently, the SasG homologue Aap from *Strep. epidermidis* has been shown to be present as fibrils on the cell surface (Banner *et al.*, 2007). Thus, we investigated if SasG formed fibrils on the surface of *S. aureus*.

Biofilms are bacterial communities that are resistant to host immune responses and antimicrobial agents (Chuard *et al.*, 1991; Rohde *et al.*, 2005). Biofilm formation occurs in several stages. Initially, cells adhere to a surface, and in *S. epidermidis* this is mediated by the autolysin AtlE (Heilmann *et al.*, 1997). Bacteria then accumulate in multiple layers, leading to the formation of a glycocalyx (Mack, 1999; Mack *et al.*, 2004). The accumulative phase of both *S. aureus* and *S. epidermidis* biofilms is usually supported by the polysaccharide intercellular adhesin (PIA) (Mack, 1999), encoded by the *icaADBC* locus (Heilmann *et al.*, 1996). However, *S. epidermidis* strains that lack *ica* can form biofilms, for example mediated by the Aap protein (Rohde *et al.*, 2005). Biofilm formation also occurs with strains of *S. aureus* that lack *ica* (Fitzpatrick *et al.*, 2005; Toledo-Arana *et al.*, 2005). It was of interest, therefore, to determine whether SasG could promote biofilm formation.

METHODS

Bacterial strains, plasmids and culture conditions. Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown on Luria (L) agar or in L broth. *S. aureus* was grown in tryptic soy broth (TSB) at 37 °C with aeration (200 r.p.m.) or statically in brain heart infusion (BHI) broth. Media were supplemented with glucose (0.5 %, w/v), ampicillin (100 μg ml^{-1}), erythromycin (10 μg ml^{-1}), chloramphenicol (10 μg ml^{-1}) or tetracycline (2 μg ml^{-1}), where appropriate. SasG expression by *sasG*::pMUTIN4 and pALC2073*sasG*$^+$ in the exponential phase was induced by adding 1 mM IPTG or 60 ng tetracycline ml^{-1}, respectively, with the inoculum (stationary-phase culture diluted 1:100). Expression in stationary phase was induced with 1 mM IPTG or 160 ng tetracycline ml^{-1}, respectively, added to cultures at OD$_{600}$ 0.6 and incubated for 3 h. IPTG (1 mM) or 60 ng tetracycline ml^{-1} was added to wild-type and pALC2073-containing strains, respectively, as an inducer control.

Molecular techniques. Standard procedures were used (Sambrook & Russell, 2001). Restriction enzymes and ligase (New England Biolabs or Roche) were used according to the manufacturer's protocol, as was Pfu DNA polymerase (Roche). Oligonucleotides were purchased from Sigma-Genosys.

Plasmid and strain construction. To construct pMUTIN4*sasG'*, a 1 kb fragment of the *sasG* gene incorporating the ribosome-binding site but not the promoter sequence was amplified by PCR from *S. aureus* strain 8325-4. The forward primer (5′-GGGGAATTCGTAAG-TAAAGTGGAAAATATGG-3′) and reverse primer (5′-GCGGGATC-CTCCAACCATTTGTGAATTACC-3′) incorporated a 5′ *Eco*RI and a

Fig. 1. Schematic organization of SasG of *S. aureus* 8325-4. The A domain lies between the signal sequence (S) and the first B repeat (B1). The positions of the wall/membrane-spanning regions (W/M) and the LPKTG motif, required for anchoring of the protein to the cell wall by sortase, are indicated. The conserved domain also present in Aap and Pls is indicated by the dotted box.

Table 1. Bacterial strains and plasmids used in this study

Abbreviations: Em, erythromycin; Tc, tetracycline; Ap, ampicillin; Cm, chloramphenicol.

Strain or plasmid	Relevant characteristics	Reference
Strains		
E. coli		
XL1-Blue	Host for cloning	Stratagene
S. aureus		
8325-4	NCTC 8325 cured of prophages	Novick (1967)
8325-4 *sasG*::pMUTIN4	8325-4 containing pMUTIN4*sasG'* chromosomal insertion (Em^r)	This study
RN4220	Restriction-deficient mutant of 8325-4 capable of stably maintaining shuttle plasmids	Kreiswirth *et al.* (1983)
SH1000	Strain 8325-4 with repaired defect in *rsbU*	Horsburgh *et al.* (2002)
SH1000 *sasG*::pMUTIN4	SH1000 containing pMUTIN4*sasG'* chromosomal insert (Em^r)	This study
SH1000 Δ*ica*	SH1000 containing a deletion in the *ica* gene (Tc^r)	Fitzpatrick *et al.* (2005)
38, 148, 3151	Clinical isolate st1	Day *et al.* (2001)
56, 157, 3096	Clinical isolate st5	Day *et al.* (2001)
125, 315, 3076	Clinical isolate st8	Day *et al.* (2001)
207, 410, 3093	Clinical isolate st15	Day *et al.* (2001)
40, 49, 3178	Clinical isolate st22	Day *et al.* (2001)
Plasmids		
pMUTIN4	Suicide vector which allows insertion mutagenesis (Ap^r, Em^r)	Vagner *et al.* (1998)
pMUTIN4*sasG'*	pMUTIN4 containing internal 1 kb *sasG* fragment (Ap^r, Em^r)	This study
pALC2073	*S. aureus*/*E. coli* shuttle vector containing the *tetR* gene and *xyl/tetO* promoter (Ap^r, Cm^r)	Bateman *et al.* (2001)
pALC2073*sasG*$^+$	pALC2073 containing full-length *sasG* (Ap^r, Cm^r)	This study
pALC2073*	pALC2073 with mutated *Xmn*1 sites (Ap^r, Cm^r)	This study
pALC2073**sasG*$^+$	pALC2073* containing full-length *sasG* (Ap^r, Cm^r)	This study
pALC2073*sasG*$^+$ 6R, 5R	pALC2073*sasG*$^+$ containing six or five B repeats (Ap^r, Cm^r)	This study
pALC2073**sasG*$^+$ 4R, 2R, 1R	pALC2073**sasG*$^+$ containing four, two or one B repeats (Ap^r, Cm^r)	This study

3′ *Bam*HI site, respectively (restriction sites underlined). The PCR fragment was cloned into the *Eco*RI and *Bam*HI sites of pMUTIN4 and transformed into *E. coli*. pMUTIN4*sasG'* was introduced into *S. aureus* RN4220 by electroporation, in which it integrated into the chromosomal copy of *sasG*. It was then transduced into *S. aureus* strains SH1000 and 8325-4 (Foster, 1998).

pALC2073*sasG*$^+$ was constructed by amplifying the entire *sasG* gene using the forward primer 5′-CGG<u>GGTACC</u>GTAAGTAAAGTGGA-AAATATGG-3′ and the reverse primer 5′-GGC<u>GAGCTC</u>GTCT-TTCAATCCAACTTTTGG-3′, which incorporate a 5′ *Kpn*I and a 3′ *Sac*I site, respectively (restriction sites underlined). The PCR product was cloned into pALC2073 between the *Kpn*I and *Sac*I sites. These primers were also used to amplify *sasG* from clinical isolates in order to estimate the length of the gene and the number of B repeats.

Mutagenesis of two *Xmn*1 sites present in the pALC2073 to create pALC2073* was carried out using overlap primer PCR. Complementary primers (5′-GACTCCTGTAAAAAATGACTTCAA-AG-3′ and 5′-CTTTGAAGTCATT<u>T</u>TTTACAGGAGTC-3′; mutagenic nucleotides underlined) were used to amplify the circular template. The PCR product was treated with *Dpn*I to digest parental DNA and transformed into *E. coli* XL1-blue. The second *Xmn*1 site was eliminated using complementary primers (5′-CATTGGAA-AACG<u>C</u>TCTTCGGGGGCG-3′; mutagenic nucleotide underlined) and (5′-CGCCCCGAAGA<u>G</u>CGTTTTCCAATG-3′; mutagenic nucleotide underlined), using the same procedure.

Variants of *sasG* expressing five and six B repeats were isolated during transduction of pALC2073*sasG*$^+$ into *S. aureus* SH1000 and 8325-4. Strains expressing SasG with one, two and four repeats were obtained

by partial restriction digestion of the *sasG* gene in pALC2073* with *Xmn*1. Partial digests were purified, ligated and transformed into XL1-blue.

Coupling of biotin to human fibronectin. Biotin was prepared as previously described (Fitzgerald *et al.*, 2006). Human fibronectin (0.5 mg ml^{-1} in PBS) was incubated with 2 mg biotin ml^{-1} for 20 min at room temperature. The reaction was stopped by addition of 10 mM NH$_4$Cl. Excess biotin was removed by dialysis against PBS overnight at 4 °C.

Ligand and Western immunoblot analysis. Cell wall-associated proteins of *S. aureus* were prepared as previously described (Roche *et al.*, 2003a). Exponential-phase cultures were grown to OD$_{600}$ 0.6 with the appropriate inducer. Cells were harvested, washed in PBS and resuspended to OD$_{600}$ 1 in lysis buffer (50 mM Tris/HCl, 20 mM MgCl$_2$, pH 7.5) supplemented with 30 % (w/v) raffinose and 40 μl ml^{-1} protease inhibitors (Roche). Cell wall proteins were solubilized by incubation with lysostaphin (200 μg ml^{-1}) for 10 min at 37 °C. Protoplasts were recovered by centrifugation and resuspended in lysis buffer with 40 μl ml^{-1} protease inhibitors. Supernatant and protoplast fractions were separated on 7.5 % (w/v) polyacrylamide gels, electrophoretically transferred onto PVDF membranes (Roche), blocked in 10 % (w/v) skimmed milk (Marvel) and probed with rabbit anti-SasG A domain (1 : 20 000; a gift from Dr M. Meehan, Department of Microbiology, Trinity College, Dublin) or anti-ClfB (1 : 5000; a gift from Dr E. Walsh, Department of Microbiology, Trinity College, Dublin) antibodies. Bound antibodies were detected using protein A–peroxidase (POD) (1 : 500; Sigma). Protein A was detected using horseradish peroxidase (HRP)-conjugated chicken

anti-protein A antibodies (1:5000; Gallus). FnBPs were detected using biotinylated fibronectin and POD-conjugated streptavidin (1:5000; Roche). Proteins were visualized using the LumiGLO reagent and peroxide detection system (Cell Signaling Technology).

Whole-cell immunoblotting. *S. aureus* cells in exponential phase were resuspended to OD_{600} 1 in PBS. Doubling dilutions (5 µl) were spotted on a nitrocellulose membrane (Protran). The membrane was blocked and developed as described above.

Adherence assays. Flat-bottomed 96-well microtitre plates (Sarstedt) were coated with doubling dilutions of fibrinogen (Calbiochem) or fibronectin (Calbiochem) in PBS ranging in concentration from 10 to 0.16 µg ml^{-1}. Plates were coated overnight at 4 °C and blocked for 2 h at 37 °C with 5 % (w/v) BSA. *S. aureus* cells (100 µl of washed exponential-phase cells, resuspended at OD_{600} 1 in PBS) were added and incubated for 2 h at 37 °C. After washing with PBS, adhering cells were fixed with formaldehyde, stained with crystal violet and the A_{570} measured. Binding assays with recombinant cytokeratin 10 (a gift from H. Miajlovic, Department of Microbiology, Trinity College, Dublin) or human IgG (Baxter) were performed with Nunc plates using sodium carbonate buffer instead of PBS.

Transmission electron microscopy. Negative staining was performed by a modification of the method of Handley *et al.* (1985). Strains were grown to mid-exponential phase and SasG expression was induced with tetracycline at 60 ng ml^{-1} for 3 h. For negative staining, cells were harvested by centrifugation (10 000 g, 2 min), washed three times in distilled water, resuspended in 1:100 volume distilled H_2O and a drop applied to a Formvar-coated copper grid (600 mesh; Agar Scientific) that had been carbon-coated beforehand (Bio-Rad E6200 carbon coater) and plasma-glowed (Plasma Barrel Etcher model PT 7150, Fisons). Bacteria were negatively stained with 2 % (w/v) methylamine tungstate (pH 6.5) (Agar Scientific). Fibril length was measured from the cell wall to the end of the longest fibril at four typical points per cell using Adobe Photoshop CS2 software.

Immunogold negative staining was carried out using a modification of the method of McNab *et al.* (1999). Bacterial cells and grids were prepared as above. Grids were placed for 5 min onto 25 µl drops of cell suspension. The grids were then inverted onto a 25 µl drop of IG buffer [0.05 M Tris, pH 8.0, containing 1 % (w/v) ovalbumin, 0.1 % (w/v) gelatin and 0.05 % (v/v) Tween 20] and then onto a 25 µl drop of anti-SasG antibody diluted in IG buffer. They were subsequently incubated at ambient temperature for 30 min, washed five times in drops of distilled H_2O, and incubated for 30 min with 10 nm diameter gold–anti-rabbit IgG. Grids were washed five times on top of drops of distilled H_2O and stained with 2 % (w/v) methylamine tungstate (pH 6.5) (Agar Scientific). Control grids were prepared using either pre-immune serum or no primary antibody to test for non-specific binding of the gold particles. Cells were photographed using an FEI Tecnai 12 electron microscope at 100 kV.

Bacterial adherence to desquamated epithelial cells. Adherence assays were performed as described previously (O'Brien *et al.*, 2002). Briefly, desquamated nasal epithelial cells were harvested from healthy donors by vigorous swabbing of the epithelium of the nasal septum. After washing in PBS, cells were adjusted to 1×10^5 ml^{-1}. Bacterial cells were washed with PBS and adjusted to 1×10^9 cells ml^{-1}. Volumes (100 µl) of bacterial and epithelial cells were mixed and incubated at 37 °C for 1 h with occasional shaking before being captured on 12 µm isopore polycarbonate filters, washed with PBS, fixed and stained with 5 % (w/v) crystal violet. The filters were mounted onto glass slides. The number of bacteria per 100 squames was counted using light microscopy.

Biofilm formation. *S. aureus* cells were grown overnight in TSB and diluted 1:200 in BHI with glucose (0.5 %, w/v). Volumes of 200 µl were added to sterile 96-well round-bottomed polystyrene plates (Sarstedt) and incubated at 37 °C for 24 h. Wells were washed three times with PBS and dried by inversion for 1 h. Adherent cells were stained with 5 % (w/v) crystal violet and the A_{570} measured. To test the role of proteases, α_2-macroglobulin (0.5 U, Roche) was added to inoculated wells and the assay continued as described above.

RESULTS

Expression of SasG by *S. aureus*

pMUTIN4 is a suicide vector that allows a gene in the *S. aureus* chromosome to be connected to the IPTG-inducible pSPAC promoter (Vagner *et al.*, 1998). A 5′ fragment of the *sasG* gene containing the ribosome-binding site and approximately 1000 bp of the coding sequence for the signal sequence and part of the A domain (*sasG′*), was amplified by PCR and cloned into the multiple cloning site of pMUTIN4 in *E. coli* forming pMUTIN4*sasG′*. The plasmid was transformed into *S. aureus* RN4220 selecting for erythromycin resistance. The plasmid underwent a single crossover with the chromosomal *sasG* gene, creating a pSPAC–*sasG* fusion and simultaneously fusing the 5′ *sasG* truncated gene fragment to *lacZ* (Vagner *et al.*, 1998). The *sasG*::pMUTIN4 element was transduced into SH1000 and 8325-4, selecting for erythromycin resistance.

pALC2073 is a multicopy shuttle plasmid that allows expression of genes from the TetR-controlled *xyl/tetO* promoter (Bateman *et al.*, 2001). The entire *sasG* gene, including the ribosome-binding site but lacking the promoter, was cloned into the multiple cloning site to generate pALC2073*sasG*$^+$. The plasmid was transformed into *S. aureus* RN4220 and transduced into SH1000 and 8325-4, selecting for chloramphenicol resistance.

Expression of SasG after induction of SH1000 *sasG*::pMUTIN4 by IPTG and SH1000 (pALC2073*sasG*$^+$) by tetracycline was measured by whole-cell immunoblotting (Fig. 2). SasG expression directed by pSPAC was only detected in the IPTG-induced sample. No expression was detectable in the uninduced samples, consistent with previous reports that repression in *S. aureus* is tight (Zhang *et al.*, 2000). In contrast, uninduced SH1000 (pALC2073*sasG*$^+$) expressed levels of SasG similar to those of fully induced SH1000 *sasG*::pMUTIN4, whereas a 16-fold higher level of expression occurred when the cells were induced with tetracycline. Addition of IPTG or tetracycline to wild-type SH1000 and SH1000 (pALC2073), respectively, had no effect on expression of SasG, and while IPTG had no effect on growth rate, the addition of tetracycline had a slight inhibitory effect (data not shown). Altogether, a 64-fold higher level of expression of SasG compared to the plasmid-free control was achieved. This is consistent with earlier reports that repression by the TetR repressor over the *xyl/tetO* promoter is incomplete (Zhang *et al.*, 2000).
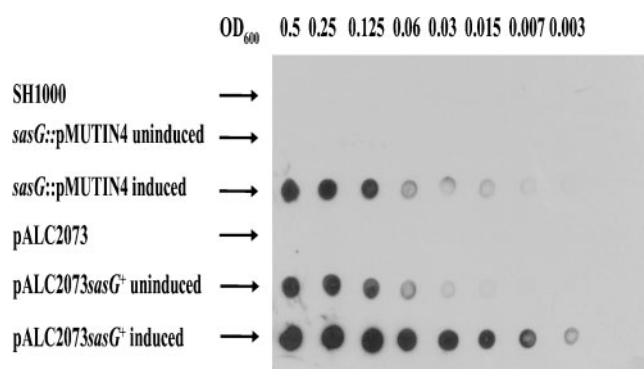
OD$_{600}$ 0.5 0.25 0.125 0.06 0.03 0.015 0.007 0.003

SH1000   →

sasG::pMUTIN4 uninduced   →

sasG::pMUTIN4 induced   →

pALC2073   →

pALC2073*sasG*⁺ uninduced   →

pALC2073*sasG*⁺ induced   →

Fig. 2. Whole-cell dot immunoblot of *S. aureus* SH1000, SH1000 *sasG*::pMUTIN4 uninduced and induced, SH1000 (pALC2073) empty vector, and SH1000 (pALC2073*sasG*⁺) uninduced and induced. Serial dilutions of cells were applied to a nitrocellulose membrane and probed with rabbit anti-SasG A domain antibodies and protein A–POD. Similar results were seen for 8325-4 vector-containing strains.

Expression of SasG inhibits adherence of *S. aureus* to immobilized ligands

High-level constitutive expression of Pls in *S. aureus* completely inhibited adherence to fibronectin and fibrinogen, despite normal expression of MSCRAMMs FnBPA and ClfA (Roche *et al.*, 2003b). Partial inhibition of adhesion by SasG was also observed (Roche *et al.*, 2003b). The difference was attributed to the modest level of expression of SasG compared to that of Pls (Roche *et al.*, 2003b). To determine the extent of interference with adhesion by SasG, *S. aureus* SH1000, SH1000 *sasG*::pMUTIN4 and SH1000 (pALC2073*sasG*⁺) were tested for adhesion to immobilized fibrinogen, fibronectin, cytokeratin 10 and IgG (Fig. 3 and data not shown).

Wild-type SH1000, SH1000 *sasG*::pMUTIN4 uninduced and SH1000 (pALC2073) adhered strongly to cytokeratin 10 (Fig. 3a) and fibrinogen (Fig. 3b) in a dose-dependent and saturable manner. A marked reduction in adherence was observed with IPTG-induced SH1000 *sasG*::pMUTIN4 and uninduced SH1000 (pALC2073*sasG*⁺), whereas induced SH1000 (pALC2073*sasG*⁺) failed to adhere to either ligand. Similar results were obtained with immobilized IgG and fibronectin (data not shown). This is consistent with the level of SasG expression in Fig. 2, and strongly suggests that high-level expression of SasG can block the adhesins responsible.

Expression of ClfB, the FnBPs and protein A after induction of SasG

The ability of SH1000 cells from the exponential phase of growth to bind to cytokeratin 10, fibronectin and IgG is due to ClfB, FnBPA and B, and protein A, respectively, each of which is expressed optimally in this phase (McAleese, 2002; Ni Eidhin *et al.*, 1998; Saravia-Otten

et al., 1997). In contrast, interactions with fibrinogen are more complex, with ClfB and the FnBPs contributing in the exponential phase. ClfA is the dominant MSCRAMM that binds fibrinogen in cells from the stationary phase (Zhang *et al.*, 2000).

To determine if induction of SasG affected the level of expression of the surface proteins, Western immunoblotting or ligand blotting was performed with proteins that were solubilized by lysostaphin during the formation of stable protoplasts. No significant reduction in the level of ClfA, ClfB, FnBPs or protein A was detected in any of the samples.

To ensure that ClfB, protein A and the FnBPs were being sorted and covalently attached to the peptidoglycan as normal, the protoplast fractions of the SasG-expressing strains were analysed in the same way, and levels of ClfB, protein A and the FnBPs compared to wild-type and uninduced strains (data not shown). No reactive protein was detected in the protoplast fractions, but intense bands were seen in the solubilized cell wall fraction (Fig. 3c and data not shown). Thus, sorting of MSCRAMMs appeared to be unimpeded in strains expressing SasG.

SasG forms peritrichous surface fibrils

Negative staining showed that *S. aureus* SH1000 (pALC2073) and 8325-4 (pALC2073) had few cell-surface fibrils. Cells had a very smooth outer surface (Fig. 4a). A very small number of fibrils were detected on a few cells (data not shown). However, short peritrichous fibrils were expressed on most SH1000 (pALC2073*sasG*⁺) cells, both with and without induction. The density of the fibrils varied considerably. Most cells had fibrils of intermediate density (Fig. 4b), but about 10 % had very dense fibrils (Fig. 4c). SH1000 (pALC2073*sasG*⁺) expressed more fibrils than 8325-4 (pALC2073*sasG*⁺). Fibrils of *S. aureus* SH1000 (pALC2073*sasG*⁺) were 53.47±2.51 nm in length. The fibrils are extremely thin, and usually individual fibrils cannot be distinguished due to the very close packing, which results in a confluent edge to the fibrillar fringe (Fig. 4c).

To show that fibrils were composed of SasG, SH1000 (pALC2073*sasG*⁺) cells were immunogold-labelled using anti-SasG A domain antibodies. Gold particles labelled the fibrillar fringe (Fig. 4d–f) and covered the whole cell surface. When fibrils were densely packed, the gold was also more densely packed. Both induced (Fig. 4d, e) and uninduced cells (Fig. 4f) of SH1000 (pALC2073*sasG*⁺) showed densely packed fibrillar fringes labelled with the anti-SasG A antibodies. Labelled fibrils covered the surface of dividing cells (Fig. 4f). After division, the newly exposed surface at the septum showed a patch without fibrils (Fig. 4e). This is presumed to be a temporary bare patch, because other non-dividing spherical cells were completely covered in fibrils (data not shown). Individual fibrils were usually very difficult to detect after staining due to masking by the antibodies and gold.
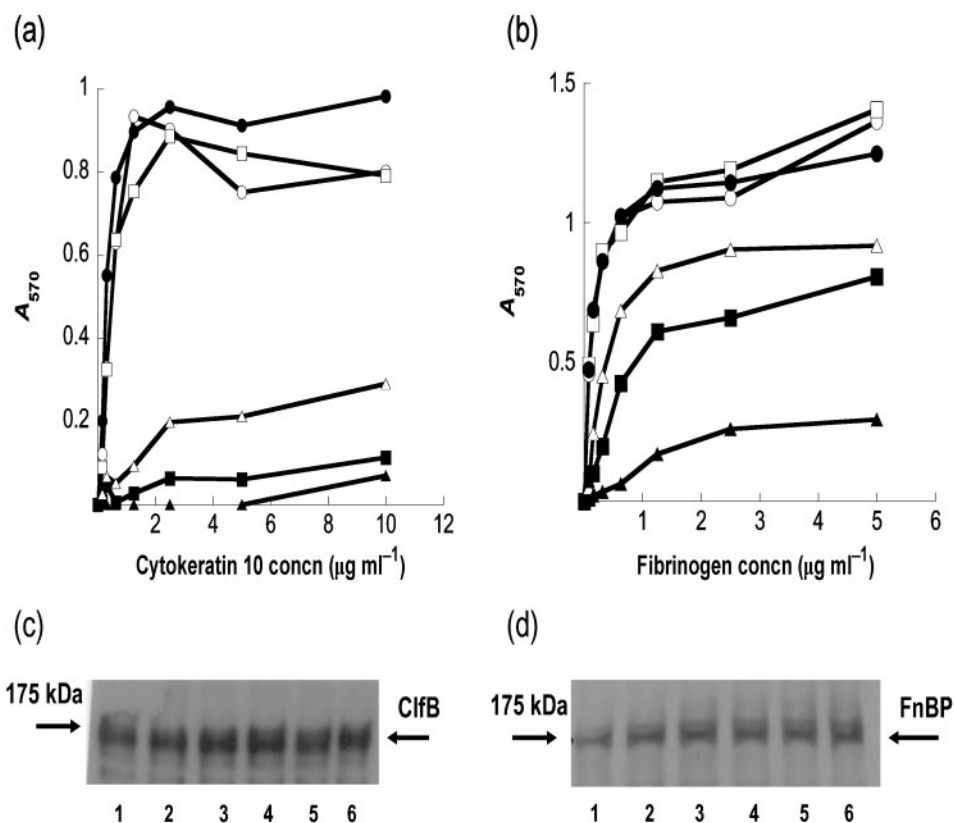
Fig. 3. Adherence of *S. aureus* to cytokeratin 10 and fibrinogen, and analysis of cell wall protein expression. (a, b) Adherence of *S. aureus* SH1000 with IPTG (○), SH1000 (pALC2073) with tetracycline (●), SH1000 *sasG*::pMUTIN4 uninduced (□) and induced (■), and SH1000 (pALC2073*sasG*$^+$) uninduced (△) and induced (▲), to immobilized cytokeratin 10 and fibrinogen. All bacteria were grown to exponential phase. Experiments were done in triplicate. Similar results were seen for IgG and fibronectin binding, and using 8325-4 vector-containing strains. (c) Western immunoblot and (d) ligand affinity blotting showing expression of ClfB and the FnBPs in *S. aureus* SH1000 strains. Lanes: 1, SH1000 wild-type; 2, SH1000 *sasG*::pMUTIN4 uninduced; 3, SH1000 *sasG*::pMUTIN4 induced; 4, SH1000 (pALC2073); 5, SH1000 (pALC2073*sasG*$^+$) uninduced; 6, SH1000 (pALC2073*sasG*$^+$) induced. Cell wall proteins were released by lysostaphin digestion of SH1000, run on 7.5 % SDS-PAGE gels and probed with anti-ClfB antibodies or biotinylated fibronectin. The positions of standard molecular mass proteins (in kDa) are marked.

The control SH1000 (pALC2073) did not label with the gold-labelled antiserum. Since fibrils are always present when SasG is expressed, and they label with anti-SasG A domain antibodies, it can be concluded that they are composed of SasG.

Mechanism of inhibition of bacterial attachment by SasG

SasG inhibits adhesion of *S. aureus* to the ligands fibrinogen, fibronectin, cytokeratin 10 and IgG. One explanation for this inhibition is steric hindrance, whereby the B repeats of SasG project the A domain from the cell surface and sterically hinder adhesin–ligand interactions. To investigate this, variants of SasG with reduced numbers of B repeats were isolated either during transduction of

pALC2073*sasG*$^+$ or by partial digestion at *Xmn*I sites within each repeat encoding region. Size variants were identified by cleavage with *Kpn*I and *Sac*I (Fig. 5a), which separates the insert from the vector.

Six out of eight possible *sasG* size variants were transduced into SH1000. Western immunoblotting demonstrated that the size of the proteins corresponded to the number of B repeats (Fig. 5b). Adherence assays were performed to measure the ability of shorter SasG variants to prevent adhesion to cytokeratin 10 (Fig. 6a), fibrinogen (Fig. 6b) and fibronectin (data not shown). Strains expressing variants of SasG with eight, six or five repeats masked binding to all three ligands. Variants expressing four, two or one repeats did not block binding. This indicates that the length of SasG is crucial for masking the binding of *S. aureus* to ligands.
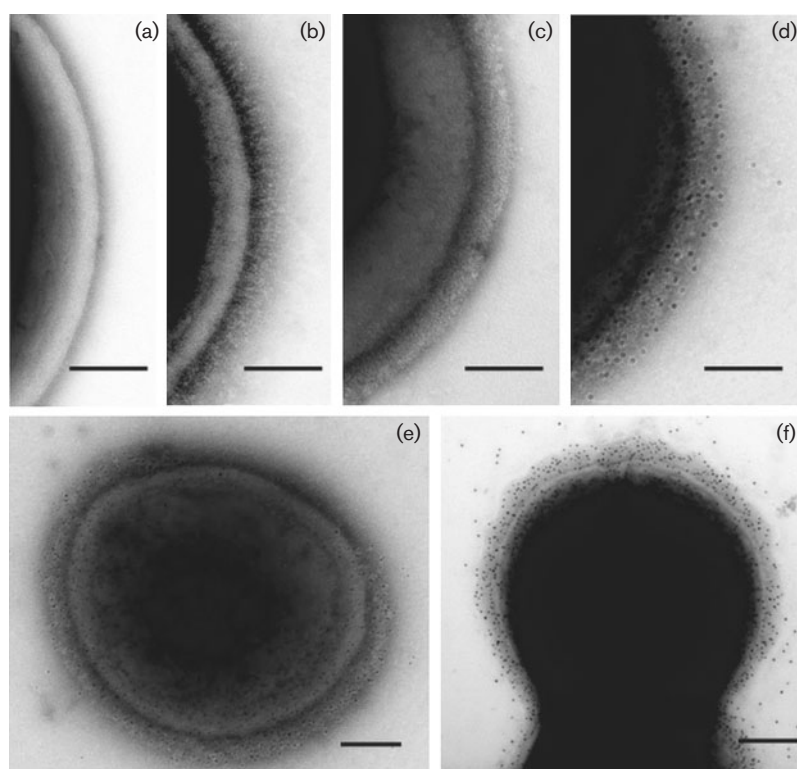
Fig. 4. (a) 8325-4 (pALC2073); (b–f) SH1000 (pALC2073*sasG*$^+$); (a–c) *S. aureus* cells negatively stained with 2 % methylamine tungstate; (d–f) *S. aureus* cells negatively stained and immunogold-labelled with anti-SasG A domain antibodies and 10 nm gold-conjugated secondary antibody. (a) Early stationary-phase cell showing a typically smooth cell surface. (b) After 3 h induction, showing peritrichous fibrils (53.47 ± 2.51 nm long) with an intermediate density. (c) After 3 h induction, 10 % of isolates show a dense layer of closely packed fibrils. Individual fibrils cannot be detected at this fibril density. (d) After 3 h induction, showing gold particles labelling the SasG A domains at the distal end of the fibrils. This cell has densely packed fibrils and the gold labelling is also dense. (e) After 3 h induction, showing gold particles labelling the fibrils. This cell, however, displays a bald patch with no fibrils and no gold labelling to one side of the cell. (f) Early stationary-phase cell with no induction, showing gold particles evenly labelling the peritrichous fibrillar fringe of this dividing cell. Fibrils are not visible due to the pale negative staining. Bars, 100 nm.

SasG promotes adherence to desquamated nasal epithelial cells

Surface proteins ClfB and IsdA promote adhesion to squames (Clarke *et al.*, 2006; O'Brien *et al.*, 2002). To confirm that SasG can promote adhesion (Roche *et al.*, 2003b), the protein was expressed by inducing SH1000 *sasG*::pMUTIN4 and pALC2073*sasG*$^+$. Both adhered about three times more strongly to squamous cells than wild-type SH1000 and uninduced SH1000 *sasG*::pMUTIN4 (Fig. 7). Uninduced SH1000 (pALC2073*sasG*$^+$) adhered to squames at the same level as induced SH1000 (pALC2073*sasG*$^+$). These experiments were performed using bacteria from the exponential phase of growth, at which ClfB is optimally expressed but its adhesive properties would be masked by SasG (Fig. 3). However, adhesion to squames promoted by SasG (~5500 adherent bacteria per 100 squames) more than compensated for that determined by ClfB (1800 bacteria per 100 squames) seen in the controls in which SasG was not expressed.

Expression of SasG in *S. aureus* causes biofilm formation

The SasG homologue Aap induces biofilm formation independently of *ica* (Rohde *et al.*, 2005). Here, the possible role of SasG in *S. aureus* biofilms was investigated. SH1000 expressing SasG from either *sasG*::pMUTIN4 or pALC2073*sasG*$^+$ grew as biofilms, whereas wild-type

SH1000 and SH1000 (pALC2073) did not (Fig. 8a). Uninduced cultures of SH1000 (pALC2073*sasG*$^+$) expressed sufficient levels of protein for biofilm formation. Biofilm formation by SasG occurred independently of *ica* (Fig. 8a).

Biofilm formation by SasG is dependent on the level of protein induction

To investigate whether biofilm formation was dependent on the level of SasG, IPTG at concentrations ranging from 0 to 0.7 mM was added to SH1000 *sasG*::pMUTIN4 cultures and biofilm formation was measured (Fig. 8b). IPTG did not stimulate biofilm formation by SH1000 wild-type. This showed that biofilm formation is dependent on the level of SasG expression.

Formation of a biofilm is dependent on the length of SasG

SH1000 variants carrying pALC2073 expressing SasG with eight, six and five B repeats resulted in the formation of a biofilm. Strains expressing four, two or one B repeats did not form biofilms (Fig. 8c). This showed that in SasG, five B repeats is the minimum number required for a biofilm to form. The level of SasG expression by B-repeat variants was shown to be the same by whole-cell immunoblotting with anti-A domain antibodies (data not shown).
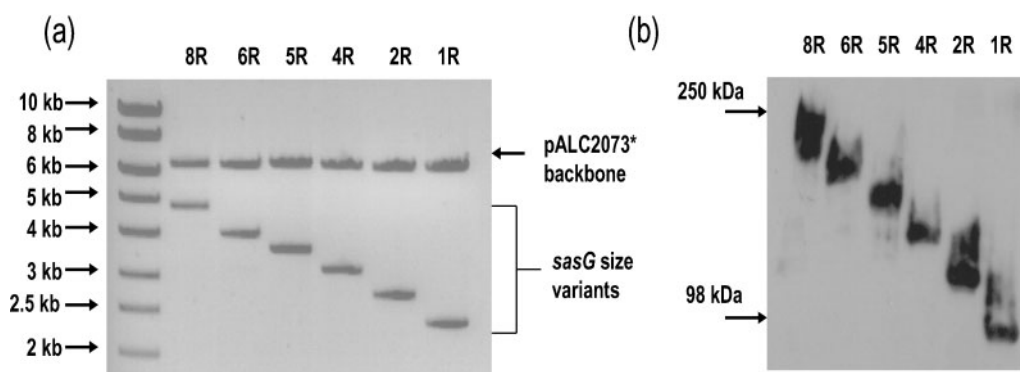
Fig. 5. (a) Gel of a restriction digest showing six different pALC2073*sasG*+ or pALC2073**sasG*+ size variants. The different sizes of *sasG* can be visualized by cutting the entire gene out of pALC2073*sasG*+/pALC2073**sasG*+ with *Kpn*I and *Sac*I. The different numbers of B repeats (R) present in each gene are indicated. The positions of the molecular mass markers are indicated (in kb). (b) Western immunoblot of the six different pALC2073*sasG*+/pALC2073**sasG*+ size variants. The different numbers of B repeats (R) present in each gene are indicated. The positions of standard molecular mass proteins (in kDa) are marked.

Proteolysis of SasG is essential for biofilm formation

To determine whether, as for Aap, proteolytic processing of SasG is required for biofilm formation, the protease inhibitor α_2-macroglobulin was added to culture-containing wells and biofilm formation was measured (Fig. 8d). This experiment showed that α_2-macroglobulin has the ability to block biofilm formation in SasG-expressing strains. This ability was lost by inactivating the protease inhibitor by heating at 65 °C for 20 min. It can be concluded from this that SasG must undergo proteolytic processing for a biofilm to form.

SasG expression by clinical isolates of *S. aureus*

Comparative genomic DNA microarray analysis of *S. aureus* isolates from community-acquired invasive disease or carriage by healthy donors has identified ten dominant clonal complexes (CCs) based on multilocus sequence typing (MLST) (Lindsay *et al.*, 2006). The *sasG* gene is present in strains from 50 % of the CCs (CC1, CC5, CC8, CC15 and CC22; Lindsay *et al.*, 2006). Fifteen clinical isolates representing strains from these CCs were examined by Southern blotting using a probe designed to recognize the A region of *sasG*. Of the 15 strains tested, all but one, strain 3151, contained the gene (data not shown).

Western immunoblotting was used to determine whether the *sasG*+ clinical strains expressed SasG protein. Eight of the 14 strains expressed SasG, some at levels higher than that of the control 8325-4 *sasG*::pMUTIN4 induced with 0.4 mM IPTG (Fig. 9). As the level of SasG expressed from the control is sufficient to form biofilm (Fig. 8b), it is likely that these clinical isolates can do likewise. The proteins ran at different sizes due to variation in the number of B repeats (Fig. 9). PCR was used to amplify the genes from the eight clinical isolates, and the number of B repeats was determined by comparison with the pALC2073**sasG*+ size variants (data not shown). Strains 40 and 125 contained seven B repeats, strains 207, 3093 and 3178 contained six repeats, and strain 410 contained 10 repeats, implying that these proteins are of sufficient length to contribute to masking, biofilm formation and nasal colonization. Strains 56 and 148 contained three and two B repeats, respectively (data not shown).

DISCUSSION

S. aureus colonizes the moist squamous epithelium of the anterior nares. The ability to adhere to squamous cells is believed to be instrumental in colonization. Surface proteins ClfB and IsdA have been shown to promote adhesion to squames (Clarke *et al.*, 2006; O'Brien *et al.*, 2002) and to be required for colonization of the nares of rodents (Clarke *et al.*, 2006; Schaffer *et al.*, 2006). When expressed in *Lactococcus lactis* and *S. aureus* from the weak constitutive promoter in pKS80, it is evident that SasG can also promote adhesion to squames (Roche *et al.*, 2003b), and this was confirmed here by the strong adhesion seen when SasG was expressed at high levels. The chromosomal *sasG* gene is not expressed at a detectable level by laboratory strains; however, expression has been noted in clinical isolates. Paradoxically, when SasG was expressed at high levels it masked binding of MSCRAMMs to their ligands, including ClfB, which binds cytokeratin 10 and which is important in promoting adhesion to squames (O'Brien *et al.*, 2002). However, the ability to adhere to squames is preserved by the adhesive properties of SasG (Roche *et al.*, 2003b).

The masking effect by SasG can be explained by the fact that it has a fibrillar structure, and individual molecules are
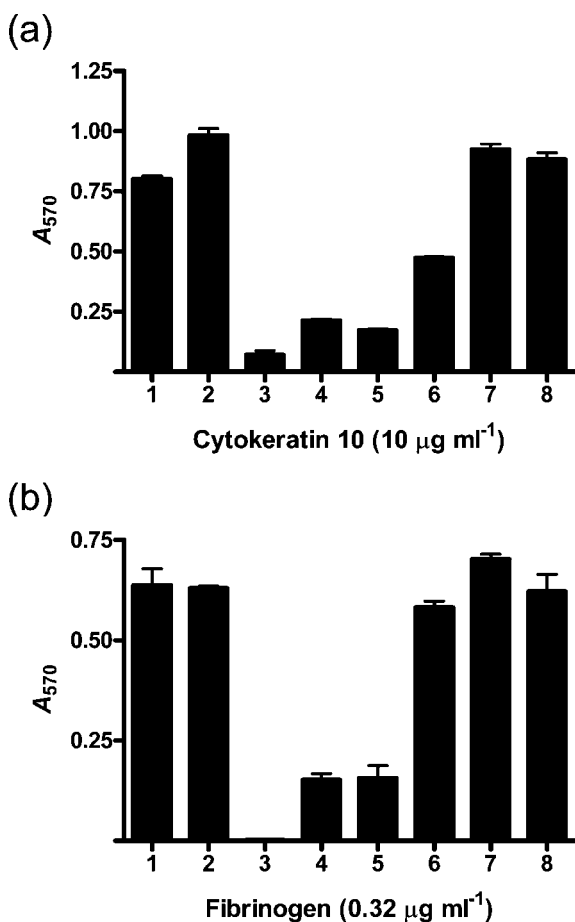
(a)

A570 vs strain (1–8), Cytokeratin 10 (10 µg ml⁻¹)

(b)

A570 vs strain (1–8), Fibrinogen (0.32 µg ml⁻¹)

Fig. 6. Adherence assay showing the masking effect of SasG. (a, b) Binding of 1, SH1000; 2, SH1000 (pALC2073); 3, SH1000 (pALC2073*sasG*⁺); 4, SH1000 (pALC2073*sasG*⁺ 6R); 5, SH-1000 (pALC2073*sasG*⁺ 5R); 6, SH1000 (pALC2073**sasG*⁺ 4R); 7, SH1000 (pALC2073**sasG*⁺ 2R) and 8, SH1000 (pALC-2073**sasG*⁺ 1R) to cytokeratin 10 and fibrinogen, respectively. All strains containing pALC2073 or pALC2073*sasG*⁺ size variants were grown in the presence of 60 ng tetracycline ml⁻¹. Adherence was measured using crystal violet staining and read at A_{570}. Experiments were done in triplicate; error bars show SDs.

Bacterial count per 100 squamous cells vs Strain (1–7)

Fig. 7. Bacterial adherence to desquamated nasal epithelial cells. 1, PBS alone; 2, SH1000 with IPTG; 3, SH1000 (pALC2073) with tetracycline; 4, SH1000 (pALC2073*sasG*⁺); 5, SH1000 (pALC2073*sasG*⁺) with tetracycline; 6, SH1000 *sasG*::pMUTIN4; and 7, SH1000 *sasG*::pMUTIN4 with IPTG. These were tested for their ability to bind to human desquamated epithelial cells. Counts represent the number of bacterial cells adhering to 100 squamous cells. Results are expressed as the mean ± SD of triplicate experiments.

This prediction is confirmed by this study, as the antiserum directed against the A domain appeared to bind to the ends of the fibrils.

The property of masking the adherence of *S. aureus* to the immobilized ligands fibronectin, fibrinogen, cytokeratin 10 and IgG was shown to be dependent on the level of expression of SasG and the number of B repeats, which presumably determines the length of the fibrillar molecule. When the repeats were fewer than five in number, the masking phenotype was lost. Analysis of the sequenced strains of *S. aureus* shows that the number of B repeats can vary from three to eight in different strains, with the *sasG* gene of Mu50 and N315 containing three B repeats, Col containing five, MSSA476 containing six and 8325-4 containing eight (Roche *et al.*, 2003a). When the size of the SasG protein from clinical isolates was examined, the number of B repeats was seen to vary from two to ten. This would imply that SasG has a functional role in masking or biofilm formation in six out of the eight SasG-expressing clinical strains. Expression of proteins containing two and three repeats was noted. SasG would most likely not have a functional role in masking and biofilm formation in these strains. However, it is possible that a protein of this size may have another as yet unknown function.

It might seem contradictory that an *S. aureus* surface protein would prevent adhesion to extracellular matrix components. However, it is possible that at certain stages during infection it is advantageous for bacteria to detach from a ligand to which they are attached, thus allowing

packed quite densely on the cell surface as peritrichous fibrils with a mean length of 53.47 ± 2.51 nm. A similar molecule, Aap, also has a fibrillar morphology, but is longer (122 ± 10.8 nm) and is localized on the cell surface in fibrillar tufts (Banner *et al.*, 2007). Although these two LPXTG-anchored proteins have significant sequence similarity (Roche *et al.*, 2003a) they are organized very differently on the cell surface. The fact that SasG fibrils completely cover the cell surface probably explains why SasG is able to physically mask the functioning of the smaller adhesins closer into the surface, as shown in this paper. It has previously been suggested that the B repeats act as a stalk to project the ligand-binding A region of the protein away from the cell surface (Roche *et al.*, 2003b).
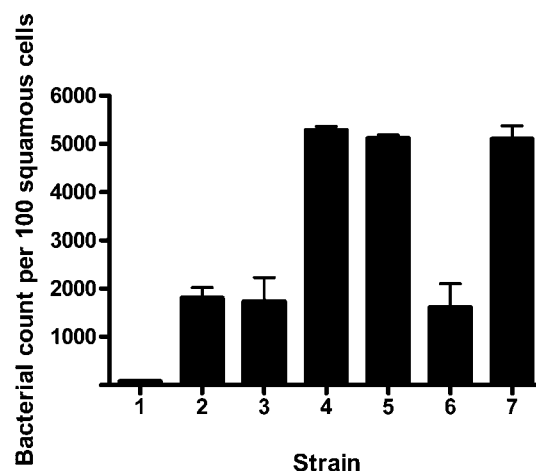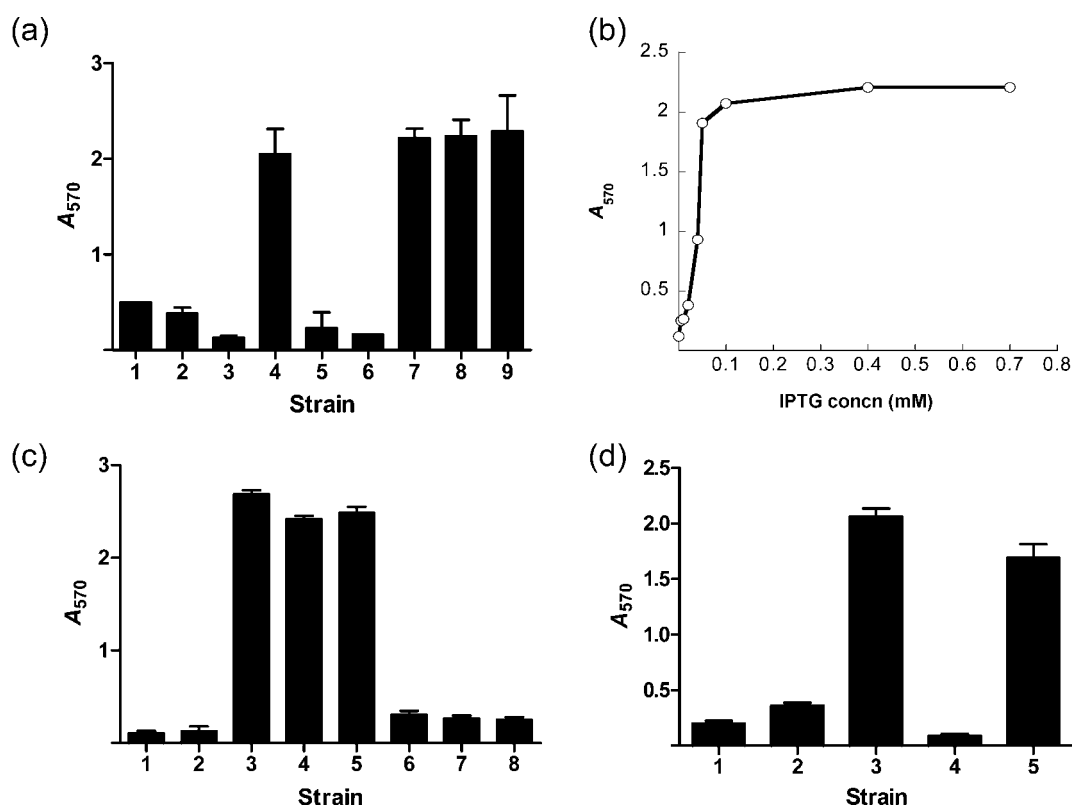
Fig. 8. Biofilm formation by SasG-expressing strains. (a) Biofilm formation by 1, *S. aureus* SH1000; 2, SH1000 with IPTG; 3, SH1000 *sasG*::pMUTIN4; 4, SH1000 *sasG*::pMUTIN4 with IPTG; 5, SH1000 (pALC2073); 6, SH1000 (pALC2073) with tetracycline; 7, SH1000 (pALC2073*sasG*⁺); 8, SH1000 (pALC2073*sasG*⁺) with tetracycline; 9, SH1000 *ica*⁻ (pALC2073*sasG*⁺). (b) Biofilm formation by strain SH1000 *sasG*::pMUTIN4. The biofilm formation was measured with inducer concentrations ranging from 0 to 0.7 mM. (c) Effect of differing B repeat length variants on biofilm formation: 1, SH1000; 2, SH1000 (pALC2073); 3, SH1000 (pALC2073*sasG*⁺); 4, SH1000 (pALC2073*sasG*⁺ 6R); 5, SH1000 (pALC2073*sasG*⁺ 5R); 6, SH1000 (pALC2073**sasG*⁺ 4R); 7, SH1000 (pALC2073**sasG*⁺ 2R); 8, SH1000 (pALC2073**sasG*⁺ 1R). (d) Effect of the protease inhibitor α₂-macroglobulin on SasG-mediated biofilm formation: 1, SH1000; 2, SH1000 (pALC2073); 3, SH1000 (pALC2073*sasG*⁺); 4, SH1000 (pALC2073*sasG*⁺) with α₂-macroglobulin; 5, SH1000 (pALC2073*sasG*⁺) with heat-inactivated α₂-macroglobulin. Biofilm was measured using crystal violet staining and read at A_{570}. All experiments were done in triplicate; error bars show SDs.

dissemination throughout the body via the bloodstream. Furthermore, if adherence to squames is biologically important, then masking of the ClfB adhesin is not an issue, because SasG itself promotes strong adhesion to these cells. Also, formation of biofilm might be involved in colonization of the nasal epithelium by promoting stronger adhesion to the squamous epithelium and allowing bacterial cells to adhere to each other.

As with masking, biofilm formation was clearly shown to be dependent on the level of expression of the protein and the number of B repeats. It was also shown to be independent of the *ica*-encoded polysaccharide PIA. The related protein Aap from *S. epidermidis* can also promote biofilm formation independently of *ica* (Rohde *et al.*, 2005). In this case, proteolytic cleavage of the A domain is a necessary precursor for cell–cell interactions by the exposed B domains (Rohde *et al.*, 2005). Biofilm formation
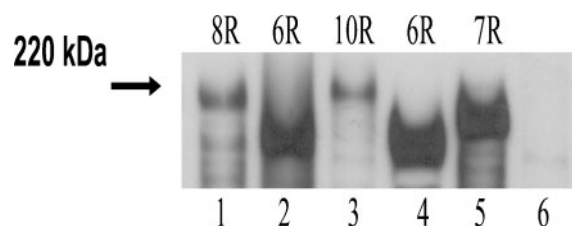

Fig. 9. Western immunoblot showing expression of SasG from clinical isolates of *S. aureus*. Lanes: 1, 8325-4 *sasG*::pMUTIN4 induced with 0.4 mM IPTG; 2, strain 207; 3, strain 410; 4, strain 3093; 5, strain 125; 6, strain 315. Cell wall proteins were released by lysostaphin digestion, run on 7.5 % SDS-PAGE gels and probed with anti-SasG A domain antibodies. The different numbers of B repeats (R) present in each gene are indicated. The positions of standard molecular mass proteins (in kDa) are marked.

by SasG is also likely to be protease dependent, because the broad-spectrum protease inhibitor α_2-macroglobulin inhibited the process. One could then hypothesize that the A domain of SasG must be proteolytically cleaved, enabling B–B interactions between proteins on neighbouring cells. This would explain why the repeat length of SasG is important, as the protein would need to be long enough to reach another cell. It is also feasible that a mixed biofilm is promoted by interaction between SasG and Aap if the proteins can interact heterologously. An alternative mechanism of biofilm formation mentioned by Rohde *et al.* (2005) involves the interaction of the B domain with a ligand on the staphylococcal cell surface, in which case the repeats would also need to be of sufficient length to adhere to adjacent cells. The mechanism of biofilm formation promoted by SasG is currently under investigation.

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