The Staphylococcus aureus surface protein SasG and its homologues promote bacterial adherence to human desquamated nasal epithelial cells

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INTRODUCTION

Staphylococcus aureus colonizes the moist squamous epithelium in the anterior nares of about 20% of the human population and is a transient resident of another 60%. The bacterium is an important aetiological agent of human infections such as endocarditis, osteomyelitis and septic arthritis. Nasal colonization is an important risk factor for the pathogenesis of infection (Cole et al., 2001; Kluytmans et al., 1997; Peacock et al., 2001). The ability to adhere to host surfaces is a prerequisite for colonization and initiation of the infection process. S. aureus can bind a multitude of host extracellular matrix proteins including fibrinogen, fibronectin, collagen, elastin and von Willebrand factor. Adherence to these host proteins is mediated by a group of staphylococcal surface proteins termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (Foster & Höök, 1998).

S. aureus binds to human desquamated nasal epithelial cells, a phenomenon likely to be important in nasal colonization. ClfB was identified previously as one staphylococcal adhesin that promoted binding to nasal epithelia. In this study, it is shown that the S. aureus surface protein SasG, identified previously by in silico analysis of genome sequences, and two homologous proteins, Pls of S. aureus and AAP of Staphylococcus epidermidis, also promote bacterial adherence to nasal epithelial cells. Conditions for in vitro expression of SasG by S. aureus were not found. Adherence assays were therefore performed with S. aureus and Lactococcus lactis expressing SasG from an expression plasmid. These studies showed that SasG did not bind several ligands typically bound by S. aureus. Significantly, SasG and Pls did promote bacterial adherence to nasal epithelial cells. Furthermore, pre-incubation of epithelial cells with purified recombinant proteins revealed that the N-terminal A regions of SasG, Pls and AAP, but not the B repeats of SasG, inhibited adherence of L. lactis expressing SasG in a dose-dependent fashion. These results suggest that SasG, Pls and AAP bind to the same as-yet-unidentified receptor on the surface of nasal epithelial cells. In addition, expression of SasG, like Pls, reduced adherence of S. aureus to fibronectin and fibrinogen.

The majority of MSCRAMMs are covalently linked to peptidoglycan and are characterized by common features including an N-terminal signal sequence and a C-terminal region comprising an LPXTG motif involved in covalent linkage to peptidoglycan, a stretch of hydrophobic residues and a short charged tail (Navarre & Schneewind, 1999). S. aureus possesses 21 genes with potential to encode proteins possessing a C-terminal cell-wall-sorting signal (Mazmanian et al., 2001; Roche et al., 2003). Eleven encode known proteins, some of which have been characterized in detail at the structural and functional level. These include the fibronectin-binding proteins FnbpA and FnbpB, which additionally bind to fibrinogen, the collagen-binding protein Cna, protein A, which binds IgG and von Willebrand factor, and the fibrinogen-binding clumping factors A and B (ClfA and ClfB) (reviewed by Foster & Höök, 1998; Wann et al., 2000).

We recently investigated the molecular basis of adherence of S. aureus to human desquamated nasal epithelial cells, a phenomenon which is likely to be important in nasal colonization. ClfB was shown to be an important adhesin mediating attachment of S. aureus to these cells. Furthermore, the ligand for ClfB-mediated adhesion was identified as type I cytokeratin 10, which was shown to be exposed on the surface of epithelial cells (O’Brien et al., 2002).
Ten genes encoding previously uncharacterized \textit{S. aureus} surface (Sas) proteins were identified from genome sequences (Roche et al., 2003). One of these (SasG) has significant sequence identity to two previously documented staphylococcal proteins, namely, Pls of \textit{S. aureus} and AAP of \textit{Staphylococcus epidermidis}. SasG possesses an N-terminal region A which comprises a unique 157-residue subdomain and a conserved 212-residue subdomain which is 52 and 59\% identical to similar domains in protein Pls of \textit{S. aureus} and AAP of \textit{S. epidermidis}, respectively (Fig. 1; Hussain et al., 1997; Savolainen et al., 2001). The A domain of SasG is followed by 128-residue B repeats which are also related to repeats of Pls (65\% identity) and AAP (60–67\% identity) (Fig. 1).

In this study, we have investigated the ability of SasG to promote adhesion of bacterial cells to desquamated nasal epithelial cells by expressing SasG on the surface of the surrogate host \textit{Lactococcus lactis}. We analysed the \textit{in vitro} expression of SasG by \textit{S. aureus} but could only detect protein when the \textit{sasG} gene was present on a multicopy plasmid. We also investigated the ability of Pls and AAP to interfere with SasG-promoted adherence. We propose that SasG, Pls and AAP represent a family of proteins which bind to a common receptor on the surface of nasal epithelial cells and that these proteins are adhesins which, in addition to ClfB, promote binding of \textit{S. aureus} to nasal epithelial cells.

**METHODS**

**Bacterial strains and growth conditions.** \textit{Lactococcus lactis} MG1363 was used for heterologous expression of \textit{sasG} (Gasson, 1983). The plasmid expression vector used was pKS80, a derivative of pTREX1 (Hartford et al., 2001; Wells & Scholfield, 1996). \textit{L. lactis} was grown at 30\°C for 16 h on M17 agar or broth (Difco) containing 0.5\% (w/v) glucose. \textit{S. aureus} strains were grown on brain–heart infusion (BHI; Oxoid) or tryptic soya broth (TSB; Difco). Stationary-phase cultures were grown for 16 h at 37\°C. Exponential-phase cultures were grown for 2 h from an initial inoculum of 1:100 diluted washed stationary-phase cells. For sessile growth, \textit{sasG} was grown on BHI agar. \textit{S. aureus} strains used in this study were 8325-4 (Novick, 1967), COL [sequenced at TIGR (http://www.tigr.org/); Shafer & Iandolo, 1979] and MSA [sequenced at the Sanger Institute (http://www.sanger.ac.uk/projects/S.aureus); Enright et al., 2000]. \textit{Escherichia coli} strains TOPP 3 (Stratagene) and M15 (Qiagen) used for expression of recombinant hexahistidine (His\_6)-tagged proteins were grown in Luria broth (LB) or on L-agar. Where appropriate, media were supplemented with ampicillin (100 \mu g ml\(^{-1}\)), chloramphenicol (10 \mu g ml\(^{-1}\)), erythromycin (5 \mu g ml\(^{-1}\)) and IPTG (1 mM).

**Expression of Sas proteins by \textit{L. lactis} MG1363 and \textit{S. aureus} 8325-4.** The \textit{sasG} gene was amplified by PCR using \textit{Pfu} polymerase. \textit{S. aureus} 8325-4 genomic DNA was used as template. The forward and reverse primers used are outlined in Table 1. The single PCR product was purified, cut with \textit{BclI} and cloned into the \textit{BclI} site of the \textit{lactis} expression vector pKS80. The recombinant plasmid was transformed into competent \textit{L. lactis} MG1363 cells by electroporation as described previously (Hartford et al., 2001; Wells et al., 1993). Transformants were selected on M17 agar containing glucose and erythromycin. Transformants were screened for protein expression by whole-cell Western immuno-dot blotting using specific antibodies to the SasG protein.

To introduce pKS80::\textit{sasG} into \textit{S. aureus} 8325-4, purified pKS80::\textit{sasG} plasmid DNA was firstly transformed into \textit{S. aureus} RN4220 by electroporation and erythromycin-resistant recombinants were selected. pKS80::\textit{sasG} was subsequently transduced into \textit{S. aureus} 8325-4 using bacteriophage 85 (Foster, 1998).

**Expression of Pls by \textit{S. aureus} 8325-4.** The recombinant pCU1 plasmid expressing \textit{pls} (pPLS4) has been described previously (Savolainen et al., 2001). pPLS4 was transformed into \textit{S. aureus} RN4220 by electroporation and chloramphenicol-resistant recombinants were selected. pPLS4 was subsequently transduced into \textit{S. aureus} 8325-4 using bacteriophage 85.

**Construction and purification of recombinant His\_6-tagged fusion proteins.** The recombinant pQE30 plasmid expressing His\_6-tagged rSasG207–428 was constructed as described previously (Roche et al., 2003). \textit{E. coli} TOPP 3 expressing rClfB44–542 was a kind gift from Dr E. Walsh, Department of Microbiology, Trinity College, Dublin, Ireland (Perkins et al., 2001). Gene fragments encoding other recombinant subdomains of SasG, Pls and AAP were generated by PCR using \textit{Pfu} polymerase. The forward and reverse primers are detailed in Table 1. The templates used to amplify \textit{sasG}, \textit{plg} and \textit{AAP} gene fragments were \textit{S. aureus} 8325-4 genomic DNA, pPLS4 (Savolainen et al., 2001) and \textit{S. epidermidis} RP62A genomic DNA, respectively. PCR products were cleaved at appropriate restriction sites for directional cloning, cloned into pQE30 and transformed into \textit{E. coli}. \textit{E. coli} TOPP 3 was used for expression of all recombinant proteins, with the exception of rSasG207–428 where \textit{E. coli} M15 (Qiagen) was used.

For expression of recombinant B repeats (SasG\_B), the primers were designed to anneal to DNA outside that specifying the B repeats. PCR amplification resulted in the generation of a series of products corresponding to a different number of B repeats. This phenomenon is due to repeat-length variation caused by recombination occurring between the highly homologous B repeat DNA (Roche et al., 2003). The mixed PCR products of the B repeat region were cloned into pQE30, and one resultant \textit{E. coli} transformant was used in the present studies.

**Table 1.** PCR primers used to construct recombinant proteins and recombinant pKS80 plasmids

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)*</th>
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<tr>
<td>rSasG207-207</td>
<td>F: CGCCGATCCGCAGCCTGAAAAACATATT R: CCCAAGCTTCAATTCATTCTTTCC</td>
</tr>
<tr>
<td>rSasG207-428</td>
<td>F: CGCCGATCCGTTTATAGAGCTTTTAC R: CCCAAGCCTTTATTCGTAGTTATTTGG</td>
</tr>
<tr>
<td>rSasG_Brep</td>
<td>F: CGCCGATCCGCCGTTTATAGAGCTTTTAC R: CCCAAGCCTTTATTCGTAGTTATTTGG</td>
</tr>
<tr>
<td>rPls48-694</td>
<td>F: CGCGGATCCGCTGAGAATACACAATCTC R: CGGCCGGTTAATCTCCTACCTTTTGTACAAAT</td>
</tr>
<tr>
<td>rAAP53-608</td>
<td>F: CGCGGATCCGAGAAGAACAAGGTTGAC R: CGGAACGTTTATGCTTAGGTTATGTACCATATG</td>
</tr>
</tbody>
</table>

*F, Forward; R, reverse. Restrictions sites used for cloning are underlined.
Examination of the DNA sequence of pQE30::sasG<sub>Gene</sub> revealed that it comprises two complete B-repeat-encoding regions, a hybrid between B1 and B6, B7 and the truncated B8.

His<sub>6</sub>-tagged recombinant proteins were purified from the soluble fractions of <i>E. coli</i> lysates by Ni<sup>2+</sup>-affinity and ion-exchange chromatography in the presence of protease inhibitors (Roche incomplete protease inhibitor cocktail) as described by Perkins et al. (2001). The purified proteins were finally dialysed against PBS (10 mM phosphate buffered salts, 138 mM NaCl, 2.7 mM KCl), concentrated by ultrafiltration and stored at −75 °C. Protein concentration was estimated by the Bio-Rad protein assay using BSA as standard. Mass spectrometry was performed by Dr Richard Turner, Department of Biochemistry, University of Cambridge, UK.

**Bacterial adherence to epithelial cells.** Adherence assays were performed as described previously (O’Brien et al., 2002). Briefly, this involved harvesting epithelial cells from healthy donors by vigorous swabbing of either the epithelium of the nasal septum (nasal epithelial cells) or the buccal epithelium (buccal epithelial cells) followed by washing of the cells with PBS. Immortalized HPV-G keratinocytes from human foreskin were cultured in DMEM-F<sub>12</sub> medium as described previously (O’Brien et al., 2002; Pirisi et al., 1988). For adherence assays, keratinocytes were removed from flasks by scraping into incomplete medium, washing in incomplete medium and disreggregation by passage through a 26-GA needle. Washed epithelial and keratinocyte cells were then quantified and adjusted to 5 × 10<sup>6</sup> cells ml<sup>−1</sup>. Bacterial cells were washed with PBS and adjusted to 1 × 10<sup>9</sup> c.f.u. ml<sup>−1</sup>. Fifty-microlitre volumes of bacterial and epithelial cells were mixed and incubated at 37 °C for 1 h with occasional shaking. Mammalian cells with adherent bacteria were then harvested onto 12 μM isopro propylidene filters, washed with PBS, fixed and stained with 5 % crystal violet. The polycarbonate filters were then mounted onto glass slides and the number of bacteria adherent to 100 epithelial or keratinocyte cells was determined by visual counting using light microscopy.

For blocking experiments, 50 μl volumes of epithelial cells (adjusted to 5 × 10<sup>10</sup> cells ml<sup>−1</sup>) were incubated for 20 min at 37 °C with 10 μl volumes of protein solutions of varying concentrations. Final protein concentrations were in the range 0-0.8–1.0 μM. Bacterial cells were then incubated with these treated epithelial cells for 1 h at 37 °C, and the adherence assay was continued as described above. Blocking experiments performed using 1-0 μM proteins were performed four times in duplicate. Inhibition curves were performed twice in duplicate.

**Ligand-binding experiments.** Experiments to measure adherence of <i>L. lactis</i> and <i>S. aureus</i> cells to immobilized ligands were performed as described previously (Hartford et al., 1997). Briefly, 96-well flat-bottomed plates were coated with increasing concentrations of matrix proteins in PBS overnight. After blocking with 2 % (w/v) BSA, the wells were incubated at 37 °C for 2 h with 100 μl volumes of bacterial cells (OD<sub>595</sub> of 1–0.0). Adherent cells were then fixed with formaldehyde, stained with crystal violet and the level of adherence quantified by measuring absorbance at 570 nm.

**Preparation of anti-SasG antibodies.** Antibodies to rSasG<sub>52–428</sub> were raised in New Zealand White rabbits and partially purified by a number of dialysis steps as described previously (Owen, 1985). The antibodies were then purified using a number of affinity steps. The anti-SasG antibodies were firstly purified on a SasG-affinity column. This involved covalent linkage of purified His<sub>6</sub>-tagged rSasG<sub>52–428</sub> to a HiTrap NHS-activated HP column as described by the manufacturer (Amersham Biosciences). Crude antiserum was passed through the affinity column and bound anti-SasG antibodies were eluted with 0-1 M glycine, pH 2-5. Following dialysis against PBS, anti-SasG antibodies were then passed through an <i>E. coli</i> lysate column (Pierce) to remove any cross-reacting <i>E. coli</i> antibodies. In order to remove antibodies to the His<sub>6</sub> tag, the SasG antibodies were finally passed through an affinity column prepared using an unrelated His<sub>6</sub>-tagged protein (SdrD). The purified antibody was concentrated 10-fold relative to the initial volume of serum and finally dialysed against PBS containing 15 mM sodium azide.

**Preparation of and Western blot analysis of enzymic extracts of <i>S. aureus</i> and <i>L. lactis</i>.** Cell-wall-associated proteins of <i>S. aureus</i> and <i>L. lactis</i> cells were prepared as described previously (Hartford et al., 2001; Roche et al., 2003). Briefly, washed bacterial cells were resuspended (OD<sub>600</sub> of 40) in lysis solution [20 mM Tris/HCl pH 7-5 containing 20 mM MgCl<sub>2</sub>, 30 % (w/v) raffinose and Roche incomplete protease inhibitor cocktail]. <i>S. aureus</i> cells were extracted by incubation for 30 min at 37 °C in the presence of lysozyme (200 μg ml<sup>−1</sup>). <i>L. lactis</i> cells were extracted for 10 min at 37 °C in mutanolysin (500 U ml<sup>−1</sup>) and lysozyme (400 μg ml<sup>−1</sup>). <i>S. aureus</i> and <i>L. lactis</i> protoplasts were finally recovered by centrifugation at 7000 g for 15 min. Ten-microlitre volumes of the supernatant fractions were boiled for 5 min in Laemmli sample buffer prior to SDS-PAGE on either 10 % (w/v) or 12 % (w/v) polyacrylamide separating gels (Laemmli, 1970). Molecular mass markers (Gibco-BRL, Benchmark protein ladder or New England Biolabs pre-stained protein markers) were also included on the SDS-PAGE gels. SDS-PAGE gels were stained with Coomassie brilliant blue or electrotransferred onto PVDF membranes (Roche Molecular Biochemicals). The blots were blocked overnight at 4 °C in 5 % (w/v) dried skimmed milk (Marvel). The rabbit antibodies used to probe the blots were anti-SasG (1 : 20 000), anti-ClfA A domain (1 : 100; a gift from J. Higgins, Department of Microbiology, Trinity College, Dublin, Ireland) or anti-FnbpA A domain (1 : 200; a gift from F. Keane, Department of Microbiology, Trinity College, Dublin, Ireland). Bound antibodies were detected with protein A–peroxidase (1 : 2000; Sigma). The Western blots were finally developed using chemiGLO (New England Biolabs) and visualized by exposure to X-ray film.

**RESULTS**

**Expression of SasG by <i>S. aureus</i> and <i>L. lactis</i>.** The sasG gene of <i>S. aureus</i> 8325-4 was amplified by PCR, cloned into the expression vector pKS80 and transformed into the surrogate Gram-positive host <i>L. lactis</i>. Western immunoblotting with affinity-purified anti-SasG<sub>52–428</sub> antibodies was used to analyse expression of SasG in <i>L. lactis</i> (Fig. 2). <i>S. aureus</i> strain 8325-4 was also tested, along with <i>S. aureus</i> 8325-4(pKS80::sasG). These experiments revealed one major immunoreactive band migrating at an apparent molecular mass of 220 kDa for <i>L. lactis</i> MG1363(pKS80::sasG) (Fig. 2, lane 1). This immunoreactive band was absent in <i>L. lactis</i> MG1363(pKS80). No immunoreactive SasG protein was detected in <i>S. aureus</i> 8325-4 (Fig. 2, lane 4). However, expression of SasG was observed when <i>S. aureus</i> was transformed with pKS80::sasG (Fig. 2, lane 3).

In an attempt to identify conditions under which SasG is expressed in vitro, strains 8325-4, COL and MSSA were grown to exponential phase and stationary phase in TSB and BHI broth and on the surface of BHI agar plates before preparing cell-wall extracts for Western immunoblotting. However, no immunoreactive band was ever detected.
The anti-SasG antibodies can detect as little as 0.75 ng of protein in Western immuno-dot blot experiments (data not shown). The failure to detect SasG in wild-type S. aureus grown under in vitro conditions indicates very low levels of expression. Therefore, in order to conduct meaningful functional analysis of SasG, we studied L. lactis and S. aureus 8325-4 expressing SasG from pKS80, and purified recombinant SasG proteins.

**Ligand binding**

To identify a possible ligand for SasG, adherence assays were conducted. L. lactis MG1363(pKS80::sasG) was tested for its ability to adhere to immobilized fibrinogen, fibronectin, human epidermal keratin, collagen, von Willebrand factor, laminin, heparan sulfate and submaxillary mucin. No adherence to any of these ligands was observed (data not shown).

**SasG promotes adherence to desquamated nasal epithelial cells**

ClfB has recently been shown to promote adherence of S. aureus and L. lactis to desquamated nasal epithelial cells mediated by its interaction with cytokeratin 10. A clfB-negative mutant of S. aureus 8325-4 exhibited a slight but significant reduction in adherence to cytokeratin 10 and squamous cells (O’Brien et al., 2002) suggesting that other surface factors are involved. To test whether SasG may play a role in adherence, L. lactis MG1363(pKS80::sasG) cells were tested for their ability to adhere to squamous epithelial cells isolated from the anterior nares of healthy individuals. Whereas L. lactis MG1363(pKS80) adhered poorly, L. lactis expressing SasG exhibited significant adherence to squamous cells (P = 0.001; Fig. 3). These data suggest that SasG promotes adherence to squamous nasal cells through a specific receptor–ligand interaction. Further support for this comes from analogous adherence assays conducted using S. aureus 8325-4 expressing either SasG or Pls. The results are the mean of triplicate experiments ± SD.

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**Fig. 1.** Schematic representation of the primary structure of SasG of S. aureus 8325, Pls of S. aureus 1061 and AAP of S. epidermidis RP62A. The positions of the signal sequences (S), the B repeats (B), the serine–aspartate (SD) repeats and the wall/membrane spanning regions (W/M) are shown. The A domain of each protein lies between the signal sequence and the B repeats. The conserved domain in the A regions of the three proteins is indicated by a dotted box. Vertical bars indicate stretches of short repeats in Pls and AAP. In the case of Pls, the C-terminal short repeats are SD dipeptide repeats. The regions of the A domains and B repeats corresponding to recombinant His6-tagged proteins are indicated below each of the primary structures.

**Fig. 2.** Western immunoblot analysis of SasG expression in S. aureus and L. lactis. Cell-wall-associated proteins were released following enzymic digestion of stabilized protoplasts of stationary-phase-grown L. lactis MG1363(pKS80::sasG) (lane 1), L. lactis MG1363(pKS80) (lane 2), S. aureus 8325-4(pKS80::sasG) (lane 3) and S. aureus 8325-4 (lane 4). The Western blot was probed with anti-SasG2-428 antibodies followed by protein A–peroxidase. Similar results to that shown in lane 4 were obtained when S. aureus strains COL and MSSA were tested and when the staphylococcal cells were grown to exponential phase and on solid BHI medium. The positions of SasG and faint cross-reacting protein A (PA) are indicated.

**Fig. 3.** Bacterial adherence to desquamated nasal epithelial cells. L. lactis and S. aureus strains were tested for their ability to bind to nasal epithelial cells. The L. lactis strains tested were those carrying the empty vector pKS80 and those expressing SasG. Also tested was S. aureus 8325-4 and strain 8325-4 expressing either SasG or Pls. The results are the mean of triplicate experiments ± SD.
S. aureus, where it was shown that S. aureus 8325-4
(pKS80::sasG) exhibited a 1.7-fold increase in adherence to
squamous cells compared to S. aureus 8325-4 \((P=0.0001)\)
(Fig. 3). In these experiments, stationary-phase-grown
S. aureus cells were used which express very low levels of
ClfB (McAleese et al., 2001; Ní Eidhin et al., 1998). This
eliminates any bacterial adherence promoted by ClfB.

The ability of SasG to promote binding to buccal epithelial
cells isolated from healthy donors and to an immortalized
keratinocyte cell line was also tested. The results of these
experiments showed that there was negligible adherence of
L. lactis MG1363(pKS80::sasG) to keratinocytes and buccal
cells. The counts of L. lactis MG1363(pKS80::sasG) per 100
keratinocytes and buccal cells were 31 ± 25 and 40 ± 19,
respectively. This was similar to the low background binding
of L. lactis MG1363(pKS80) to keratinocytes (11 ± 10
bacteria per 100) and buccal cells (36 ± 23 bacteria per 100).

Recombinant A region of SasG inhibits binding
of L. lactis expressing SasG to nasal epithelial
cells

To confirm that the interaction of SasG with nasal epithelial
cells was specific and to identify the region of SasG involved
in binding, experiments were carried out to test whether
pre-incubation of epithelial cells with recombinant truncated
variants of SasG could block adherence of L. lactis
MG1363(pKS80::sasG). DNA encoding the A region
(residues 52–428), the unique A region subdomain (residues
52–207), the conserved A region subdomain (residues
207–428) and B repeats (Fig. 1) were amplified by PCR
and cloned into the expression vector pQE30 and the recombinant
proteins purified by metal chelate chromatography. The purified
sequences encoding the A regions were therefore cloned into
expression vector pQE30 which a histidyl affinity tag at the N terminus. The resultant recombinant
proteins were purified from E. coli by metal chelate
chromatography. All three proteins gave a single dominant
band on SDS-PAGE gels (data not shown). Mass spectrometry showed the recombinant proteins were of the
anticipated molecular masses (viz. rSasG52–428, 42 987 Da;
rSasG207–428, 71 219 Da; rAAP52–608, 60 504 Da) when analysed by mass
spectrometry.

Pre-incubation of nasal epithelial cells with the purified A
regions of Pls and AAP inhibited L. lactis MG1363
(pKS80::sasG) binding to desquamated nasal epithelial cells by recombinant truncated
SasG proteins. Nasal epithelial cells were pre-incubated with increasing concentrations of rSasG52–207 (○), rSasG207–428 (●), rSasG52–428 (△) and rSasGbrep (□) followed by incubation with L. lactis MG1363(pKS80::sasG). The number of adherent bacteria to 100 squamous nasal cells was counted.

Percentage of binding was determined by taking the number of adherent bacteria to control untreated squamous cells as 100 % binding.

FIG. 4. Inhibition of L. lactis MG1363(pKS80::sasG) binding to
desquamated nasal epithelial cells by recombinant truncated
SasG proteins. Nasal epithelial cells were pre-incubated with increasing concentrations of rSasG52–207 (○), rSasG207–428 (●), rSasG52–428 (△) and rSasGbrep (□) followed by incubation with L. lactis MG1363(pKS80::sasG). The number of adherent bacteria to 100 squamous nasal cells was counted.

Percentage of binding was determined by taking the number of adherent bacteria to control untreated squamous cells as 100 % binding.

Recombinant A regions of Pls and AAP inhibit
adherence of L. lactis expressing SasG to nasal epithelial
cells

SasG protein possesses significant sequence similarity and
has similar domain organization to Pls and AAP (Fig. 1). In
particular a 212/217 residue domain is conserved within the
A regions of the three proteins. It was of interest, therefore,
to determine whether, like SasG, the A regions of Pls and
AAP could also block SasG-promoted bacterial attachment
to squamous epithelial cells. PCR-amplified pls and aap
sequences encoding the A regions were therefore cloned into the
expression vector pQE30 and the recombinant proteins
purified by metal chelate chromatography. The purified proteins gave a single dominant band on SDS-PAGE gels and the anticipated molecular masses (viz. rPls48–694, 71 219 Da; rAAP53–608, 60 504 Da) when analysed by mass
spectrometry.

Pre-incubation of nasal epithelial cells with the purified A
regions of Pls and AAP inhibited L. lactis MG1363
(pKS80::sasG) binding to desquamated nasal epithelial cells (Fig. 5). In contrast, control experiments where epithelial
cells were pre-incubated with recombinant ClfB_N1–3, a
protein known to bind cytokeratin 10 on the surface of these
cells (O’Brien et al., 2002), did not affect L. lactis
MG1363(pKS80::sasG) binding. L. lactis MG1363
(pKS80::sasG) showed 95 % binding to epithelial cells following pre-incubation with 1 μM ClfB. These experiments suggest that Pls and AAP bind to squamous nasal epithelial cells and that they probably recognize the same epithelial cell receptor as SasG.

Pls promotes bacterial adherence to
desquamated nasal epithelial cells

The blocking experiments described above using purified recombinant protein provide good evidence that Pls binds
to nasal epithelial cells. To confirm that the protein can promote bacterial attachment to epithelial cells, the
adherence of S. aureus 8325-4, which does not possess pls,
was compared to strain 8325-4(pPLS4), which expresses a
high level of Pls (Savolainen et al., 2001). These experiments showed that *S. aureus* 8325-4(pPLS4) exhibited a 1.6-fold increase \((P=0.0001)\) in adherence as compared to strain 8325-4 (Fig. 3). This suggests that Pls can promote bacterial attachment to nasal epithelial cells.

Like SasG, Pls did not promote bacterial binding to either buccal epithelial cells or to cultured keratinocytes. The counts of *S. aureus* 8325-4(pPLS4) per 100 keratinocytes and buccal epithelial cells were 43±9 and 208±57, respectively. This was not significantly different to that of *S. aureus* 8325-4 adherence to keratinocytes and buccal cells where the bacterial counts per 100 cells were 44±15 and 204±19, respectively.

**SasG expression prevents adhesion of *S. aureus* to fibrinogen and fibronectin**

It was previously shown that expression of Pls prevented *S. aureus* from binding to fibronectin, fibrinogen, IgG and BSA (Hilden et al., 1996; Savolainen et al., 2001). To determine whether SasG possesses masking ability, adherence of *S. aureus* 8325-4 to fibrinogen and fibronectin was measured and compared to *S. aureus* 8325-4(pKS80::sasG) and to *S. aureus* 8325-4(pPLS4). These tests were performed for *S. aureus* cells grown to both the stationary phase and exponential phase of growth. *S. aureus* shows a growth-phase-dependent expression of surface proteins. Therefore, in the stationary phase of growth, the fibrinogen-binding protein ClfA and not ClfB or the fibronectin-binding proteins (FnbpA and FnbpB) would be expressed. In contrast, all four proteins would be expressed in the exponential phase of growth (McAleese et al., 2001; Ní Eidhin et al., 1998; Saravia-Otten et al., 1997). The results of the adherence assays showed that *S. aureus* 8325-4 adhered to fibrinogen and fibronectin in a dose-dependent fashion.

In contrast, *S. aureus* 8325-4(pPLS4) did not adhere to either ligand. Importantly, *S. aureus* 8325-4(pKS80::sasG) showed a significant reduction in binding to both ligands (Fig. 6a, b). The profile of binding of exponential-phase cells to fibrinogen was similar to that observed for fibronectin (data not shown). Masking of ligand binding was greater in cells from stationary phase than in cells from exponential phase. SDS-PAGE analysis of solubilized cell-wall-associated proteins showed that the level of Pls was much higher than SasG (Fig. 6c, lanes 2 and 3). The apparent breakdown of Pls protein was observed previously (Hilden et al., 1996; Savolainen et al., 2001). One reason for the incomplete masking by the SasG + strain could be the lower level of expression of SasG compared to Pls. Certainly, reduced adherence was not due to a reduction in expression of adhesins since Western immunoblotting showed that the fibrinogen-binding ClfA and fibronectin-binding FnbpA proteins were expressed at similar levels in each strain (Fig. 6d, e).

**DISCUSSION**

SasG was originally identified as a potential MSCRAMM and adhesin by *in silico* analysis of staphylococcal genome sequences (Mazmanian et al., 2001; Roche et al., 2003). The likely importance of SasG in disease was suggested by the observation that *sasG* was significantly associated with invasive disease-causing strains compared to nasal carriage strains. Furthermore, levels of antibodies to SasG protein were higher in sera from many patients who had recovered from *S. aureus* infections (Roche et al., 2003). Here, we show that SasG could promote bacterial binding to squamous nasal epithelial cells. We also provide evidence that Pls and AAP, which exhibit strong homology and similar domain organization to SasG, may also bind to the same epithelial cell receptor.

Our failure to detect SasG expression by *S. aureus* cells would appear not to be due to degradation of the protein since a high molecular mass immunoreactive protein could be detected when the *sasG* gene was expressed by pKS80. The fact that sera from patients recovered from *S. aureus* infections reacted with SasG strongly suggests that the protein is expressed *in vivo* (Roche et al., 2003). Using the expression plasmid pKS80, SasG was successfully surface-expressed in a full-length and undegraded form on both *S. aureus* and the surrogate host *L. lactis*. For both *S. aureus* and *L. lactis*, the lysostaphin- and mutanolysin-solubilized SasG protein ran at an apparent molecular mass of 220 kDa, higher than its molecular mass (179 kDa) predicted from sequence, a feature characteristic of many cell-wall-associated proteins.

Nasal carriage of *S. aureus* is considered to be a key risk factor for development of staphylococcal infection in the community and hospital (Kluytmans et al., 1997; Peacock et al., 2001). The main region of the nasal vestibule where colonization occurs is the moist squamous epithelium which is devoid of hair, cilia and microvilli, a region...
immediately distal to the anterior hairy epidermis (Cole et al., 2001). In vitro studies have shown that S. aureus cells adhere to desquamated nasal epithelial cells isolated from this region of the nasal vestibule (Aly et al., 1977; Bibel et al., 1982; O’Brien et al., 2002).

O’Brien et al. (2002) showed that ClfB promoted staphylococcal binding to nasal epithelial cells. However, significant binding occurred in clfB mutants indicating that other bacterial factors are involved. In this study, we have shown that S. aureus and L. lactis expressing SasG adhered significantly to nasal epithelial cells in contrast to control bacteria.

Previous studies showed that sasG is more significantly associated with invasive isolates than carriage isolates (Roche et al., 2003). As individuals are usually infected with their own carriage isolate (Luzar et al., 1990; Yu et al., 1986) it could be speculated that SasG facilitates transmission from the nose to other body sites. Alternatively, SasG may bind to receptors on other mammalian cell types, facilitating colonization elsewhere. Also, SasG could impede opsonophagocytosis.

The ligand-binding domain of SasG was identified in adherence-inhibition experiments where the recombinant A region but not B repeats inhibited binding of L. lactis expressing SasG to nasal epithelial cells. These experiments strongly suggest the A region contains the ligand-binding domain. This finding is reminiscent of other MSCRAMMs such as ClfA, ClfB and Cna, where the ligand-binding domains are located in the N-terminal A regions (Foster &

**Fig. 6.** Adherence of S. aureus to fibrinogen and fibronectin and analysis of cell-wall-protein expression. (a, b) Adherence of S. aureus 8325-4 (○), S. aureus 8325-4(pKS80::sasG) (●) and S. aureus 8325-4(pPLS4) (□) to immobilized fibrinogen and fibronectin, respectively. (a) Bacterial cells were grown to stationary phase. (b) Bacterial cells were grown to exponential phase. Experiments were repeated three times in triplicate. One representative experiment is shown. (c, d, e) SDS-PAGE and Western blot analysis of cell-wall proteins of S. aureus. Cell-wall-associated proteins were released following lysozyme digestion of stabilized protoplasts of S. aureus 8325-4 (lane 1), S. aureus 8325-4(pKS80::sasG) (lane 2) and S. aureus 8325-4(pPLS4) (lane 3). (c) Was stained with Coomassie brilliant blue. The positions of standard molecular mass proteins (sizes shown in kDa) are marked. PIs is highlighted in lane 2 and SasG is indicated with a dot to the left of lane 2. (d, e) The membrane filters were probed with anti-ClaA and anti-FnbpA region A antibodies, respectively.
Hoök, 1998). The fact that the A regions of Pls and AAP also bound squamous cells strongly implicates the conserved subdomain in ligand binding. However, this region of SasG (residues 207–428) when expressed alone did not inhibit SasG-promoted bacterial adherence to squamous nasal cells. This suggests that additional residues from within the A region are required for function. One likely role for the B repeats could be to act as a stall to project the ligand-binding A region of the protein away from the cell surface. Such a function has been proposed for the serine-aspartate (SD) dipeptide repeats of the Sdr family and the B repeats of SdrD (Hartford et al., 1997; Josefsson et al., 1998).

SasG has significant sequence similarity and structural organization to Pls and AAP. Pls is specifically associated with the Type I staphylococcal cassette chromosome mec (SCCmec) mobile genetic element (Huesca et al., 2002; Savolainen et al., 2001). AAP is associated with the accumulation phase of S. epidermidis biofilm formation (Hussain et al., 1997). Here, we provide strong evidence that the proteins are functionally related. The fact that Pls and AAP inhibited L. lactis expressing SasG binding to nasal epithelial cells indicates that they are likely to bind to the same mammalian cell receptor. Significantly, S. epidermidis is one of the most common inhabitants of the nares (Lina et al., 2003). The possibility that AAP and SasG bind to the same receptor on the surface of squamous nasal epithelial cells suggests that competition for binding may occur. Certainly, bacterial interference between different strains of S. aureus and different bacterial species has been shown to affect S. aureus carriage (reviewed by Peacock et al., 2001). However, several surface components of S. aureus are involved in binding to the squamous epithelium and competition for binding to one receptor is unlikely to be the single determining factor for colonization.

AAP and Pls have both been implicated in promoting bacterial cell–cell interactions (Huesca et al., 2002; Hussain et al., 1997; Savolainen et al., 2001). SasG also causes cellular aggregation since L. lactis cells expressing SasG form aggregates whereas wild-type L. lactis cells do not (F. M. Roche, M. Meehan & T. J. Foster, unpublished data). The ability to form aggregates could be advantageous in promoting colonization of the surface of epithelial cells.

Another property attributed to Pls is masking the adhesive functions of other MSCRAMMs. Savolainen et al. (2001) showed that adherence to fibronectin and IgG was greatly increased in a S. aureus Pls– mutant compared to the isogenic wild-type strain. If such a phenomenon occurs in vivo for Pls, and possibly SasG and AAP, it could be speculated that, at certain points in infection, expression of these proteins may alter staphylococcal adherence characteristics allowing tropism for certain tissue types. In this study, we have shown that SasG also reduced adherence of S. aureus to fibroinogen and fibroinectin compared to wild-type. In contrast, Pls-expressing S. aureus failed to adhere to either ligand. The difference in masking could be attributed to the lower level of expression of SasG compared to Pls. The identity of the mammalian cell receptor for SasG is unknown. Certainly, bacteria have exploited a multitude of host-cell surface components as receptors for adhesins and invasins, including (glyco)proteins, (glyco)lipids and proteoglycans. The fact that SasG does not bind to either buccal cells or non-differentiated keratinocytes would suggest that the receptor for SasG is restricted to desquamated epithelial cells. Our ligand-binding experiments and inhibition experiments suggest that the ligand is not keratin, mucin or heparan sulfate. One candidate receptor for SasG could be ceramides which are present on the cornified envelope of desquamated epithelial cells (Kalinin et al., 2001). Huesca et al. (2002) provided evidence that Pls binds to lipids and glycosphingolipids including those purified from a keratinocyte cell line. In our hands, Pls did not promote bacterial binding to undifferentiated keratinocytes. However, lipid receptors may be masked in these cells. Experiments to elucidate the identity of the receptor for SasG are currently in progress. Elucidating the factors involved in determining nasal colonization could aid in the future development of novel therapeutics to control nasal and skin colonization and hence staphylococcal infection.

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REFERENCES


Staphylococcus aureus


