Consequences of a sortase A mutation in *Streptococcus gordonii*

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Sortase A (SrtA) is required for cell-wall anchoring of LPXTG-containing Gram-positive surface proteins. It was hypothesized, therefore, that disruption of the *srtA* gene would alter surface anchoring and functions of target LPXTG motif-bearing SspA and SspB proteins of *Streptococcus gordonii*. Mutant strains in *srtA* (V288*srtA*[−], DL1*srtA*[−]) were constructed in *S. gordonii* V288 (wtV288) and DL1 (wtDL1). When compared to wtV288, the V288*srtA*[−] mutant showed decreased biofilm formation on polystyrene, and reduced binding to immobilized purified salivary agglutinin (BIAcore analysis). The wtV288 and V288*srtA*[−] strains were similar in ultrastructure, but immunogold-labelled SspA/SspB surface expression was reduced on the V288*srtA*[−] mutant. DL1*srtA*[−] was also complemented to obtain DL1*srtA*⁺. From the wild-type strains (wtV288, wtDL1), *srtA*[−] mutants (V288*srtA*[−], DL1*srtA*[−]), and the complemented mutant (DL1*srtA*⁺), cytoplasmic, cell-wall and released extracellular protein fractions were isolated. Each fraction was analysed by SDS-PAGE and immunoblotting with anti-P1. Spent medium from *srtA*[−] mutant cells contained over-represented proteins, including SspA/SspB (P1 antigen). Mutants showed less P1 on the cell surface than wild-types, as estimated using whole-cell ELISA, and no P1 appeared in the cytoplasmic fractions. Expression of several adhesin genes (*sspA/B*, *cshA/B*, *fbpA*) was generally upregulated in the mutants (V288*srtA*[−], DL1*srtA*[−]), but restored to wild-type levels in DL1*srtA*⁺. These data therefore imply that in addition to its role in processing LPXTG-containing adhesins, sortase A has the novel function of contributing to transcriptional regulation of adhesin gene expression.

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INTRODUCTION

Streptococcus gordonii is a non-cariogenic pioneer colonizer of the tooth surface in the oral cavity (Kuboniwa *et al.*, 2006; Schachtele *et al.*, 2007). Following entry into the

blood, *S. gordonii* can be isolated from prosthetic heart valves and is a frequent cause of infective endocarditis (Douglas *et al.*, 1993; Sommer *et al.*, 1992). The ability of this species to adhere is essential for survival in all known environments. Among the LPXTG-motif-containing surface proteins, SspA/B, CshA/B and Hsa are expressed by *S. gordonii* and are essential for adhesion and colonization (Demuth *et al.*, 1996; Holmes *et al.*, 1998; Jakubovics *et al.*, 2005; McNab & Jenkinson, 1998). *S. gordonii* also utilizes non-LPXTG adhesins, including ScaA, AbpA, AbpB and FbpA (Schachtele *et al.*, 2007). Expressed in *S. gordonii* and virtually all Gram-positive bacteria (Mazmanian *et al.*, 2002; Scott *et al.*, 2002), sortase A (SrtA) is a membrane-localized transpeptidase that specifically cleaves the LPXTG

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Abbreviations: DL1*srtA*[−], sortase A negative mutant in *Streptococcus gordonii* DL1 wild-type; DL1*srtA*⁺, complemented DL1*srtA*[−]; P1, antigen class I/II surface protein of *Streptococcus mutans*; RU, resonance units; V288*srtA*[−], sortase A negative mutant in *S. gordonii* V288 wild-type; wtDL1, *S. gordonii* DL1 wild-type; wtV288, *S. gordonii* V288 wild-type.

sequence, and transfers the processed exported N-terminal polypeptide to peptidoglycan, where it covalently cross-links to the cell wall (Paterson & Mitchell, 2004).

Sortase A is responsible for the anchoring of LPXTG-containing surface proteins to the cell wall (Paterson & Mitchell, 2004). In *Staphylococcus aureus*, a mutation in the *srtA* gene results in defective anchoring of several proteins, including a number of surface-associated adherence factors (Mazmanian *et al.*, 1999). In *Streptococcus mutans* biofilms *in vitro*, genes encoding three putative sortase A-dependent proteins are upregulated when compared to planktonic cells, and mutations in *srtA* reduce biofilm biomass (Levesque *et al.*, 2005). Consistent with sortase A anchoring of specific adhesive proteins, a recombinant M6 protein of *Streptococcus pyogenes* containing the LPXTG motif was expressed in *S. gordonii* GP1223 (Bolken *et al.*, 2001). An isogenic *srtA* knockout expressed markedly less surface M6 protein and showed reduced binding to immobilized human fibronectin and oral colonization of mice.

The LPXTG surface proteins of *S. gordonii* can be modelled by SspA and SspB (El-Sabaeny *et al.*, 2000; Holmes *et al.*, 1998; Williams *et al.*, 1994). SspA and SspB are members of the antigen I/II family of proteins (70 % sequence identity), which are expressed by virtually all species of oral streptococci (Demuth *et al.*, 1997). SspA and SspB contain a specific 35-residue C-terminal sorting signal, consisting of an LPXTG sequence motif and hydrophobic and positively charged domains (Fischetti *et al.*, 1990; Schneewind *et al.*, 1992, 1993).

To show how sortase A affects the distribution and function of an endogenous adhesin, we tested the hypothesis that disruption of the *srtA* gene in *S. gordonii*

changes the localization of SspA and SspB adhesins, biofilm formation, and binding to a specific salivary agglutinin receptor *in vitro*. Since *S. gordonii* must adhere to saliva-coated teeth to survive and avoid extinction, we noted that while the *srtA*[−] mutants showed loss of function, the organism appeared to adapt to the abnormalities in expression of the LPXTG adhesins. Since we have reported (Zhang *et al.*, 2005) that mutations in *S. gordonii* SspA and SspB caused upregulation of other adhesin genes to complement loss of adhesion function, we also explored if sortase A might regulate compensating expression of adhesin genes.

METHODS

Bacterial strains and culture conditions. Streptococci (listed in Table 1) were routinely grown in chemically defined synthetic media (FMC) (Terleckyj *et al.*, 1975) for 16–20 h at 37 °C in 5 % CO₂. *Escherichia coli* DH5α cells were grown aerobically at 37 °C in Luria–Bertani (LB) medium. When required, antibiotics were added to the medium at the following concentrations: erythromycin (Em), 5 µg ml^{−1} (DL1*srtA*[−]/V288*srtA*[−]); kanamycin (Km), 50 µg ml^{−1} (*E. coli*) or 250 µg ml^{−1} (DL1*srtA*⁺).

Genetic manipulations in *S. gordonii* V288 and DL1. Standard recombinant DNA techniques were employed as described (Sambrook *et al.*, 1989). Plasmids (listed in Table 1) were purified from *E. coli* cells using the QIAquick Spin Miniprep Purification Kit (Qiagen). Oligonucleotides were synthesized by Integrated DNA Technologies (listed in Table 2).

Chromosomal DNA was prepared from mutanolysin-treated streptococcal cells using the Qiagen 100/G Genomic Tip System. PCR products were purified using the High Pure PCR Product Purification Kit (Roche). DNA restriction and modification enzymes were used under the conditions specified by the manufacturer (Promega).

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source/reference
<i>E. coli</i>		
DH5α	F [−] ϕ80dlacZΔM15 Δ(<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (r _k [−] m _k [−]) <i>phoA supE44 λ[−] thi-1 gyrA96 relA1</i>	Invitrogen
<i>S. gordonii</i>		
DL1 (Challis)	Wild-type	Pakula & Walczak (1963)
DL1 <i>srtA</i> [−]	Δ(<i>srtA</i>):: <i>ermAM</i>	This study
DL1 <i>srtA</i> ⁺	Δ(<i>srtA</i>):: <i>ermAM</i> complemented with pDL276- <i>srtA</i>	This study
V288	Wild-type	G. Dunny, Univ. Minnesota
V288 <i>srtA</i> [−]	<i>srtA</i> :: <i>ermAM</i>	This study
Plasmids		
pGEM-T Easy	3.0 kb; Ap ^R ; ColE1 <i>ori</i>	Promega
pGEM- <i>srtA.ermAM</i>	pGEM-T Easy derived containing <i>ermAM</i> cassette within flanking sequences of <i>srtA</i> gene	This study
pVA891	5.4 kb; Em ^R , Cm ^R ; pACYC <i>ori</i> ; <i>E. coli</i> –streptococcal shuttle vector	Macrina <i>et al.</i> (1983)
pDL276	6.9 kb; Km ^R ; ColE1 <i>ori</i> ; <i>E. coli</i> –streptococcal shuttle vector	Dunny <i>et al.</i> (1991)
pDL276- <i>srtA</i>	pDL276 derived containing entire <i>srtA</i> gene	This study

Table 2. Primers used in this study

Primer*	Sequence†
srtA.F1	CGGGGTACCCATGGCCTGTAGCTCAATC
srtA.F2	CCATCGATCCTTCTCGTCTTGCAACTC
srtA.R1	GGATCGATGGAAGGAAGCATAAGTTTAATGC
srtA.R2	CGCGAGCTCACCTAAGAGACGGTGACCAG
ermAM.F	CCATCGATGGCCATATCATAAAAAATCGAAACAGC
ermAM.R	CCATCGATGGTAGGGACCTCTTTAGTCTCC
RealsrtA.F	ATGGAAGACACAGCAACTTC
RealsrtA.R	CTGTAATCCCGAAAACATGG
fbpA.F	GCTCAAAGAAGCCGTCAAAC
fbpA.R	GTTGCGGCCAACTAGGATAA

*All primers were designed as part of this study.

†Underlined letters indicate restriction enzyme site.

The V288srtA⁻ mutant was constructed in wtV288 by insertion duplication using the pCR2.1:B210 plasmid (a gift from Sigma) (Bolken *et al.*, 2001). The mutant was selected for resistance to 5 µg erythromycin ml⁻¹ and confirmed by PCR as described previously (Bolken *et al.*, 2001). Unfortunately, attempts to complement the srtA⁻ mutation in strain V288 were unsuccessful. Thus, the closely related strain, DL1 (Challis), was used for this purpose.

The srtA gene of *S. gordonii* DL1 was inactivated by allelic exchange with the erythromycin resistance determinant, ermAM. PCR amplification with primers (Table 2) srtA.F1/srtA.R1 and srtA.F2/srtA.R2 of *S. gordonii* DL1 chromosomal DNA template generated two fragments comprising the flanking sequences of the srtA gene (324 bp, 381 bp), which were ligated via a unique *Clal* site and cloned into pGEM-T Easy. A DNA fragment (1023 bp) containing the ermAM gene was PCR-amplified from plasmid pVA891 using primers ermAM.F/ermAM.R that incorporated *Clal* restriction sites. The PCR product was digested with *Clal* and ligated into the unique site within the combined srtA fragments, generating plasmid pGEM-srtA.ermAM. The insert DNA from this plasmid was PCR-amplified using primers srtA.F1/srtA.R2, purified and transformed into *S. gordonii* DL1, thus generating the srtA deletion mutant, DL1srtA⁻. Confirmation of predicted insertions was obtained by PCR amplification and sequencing.

To complement the DL1srtA⁻ mutant, a DNA fragment (1484 bp) incorporating the entire srtA gene was PCR-amplified from *S. gordonii* DL1 chromosomal DNA using primers srtA.F1/srtA.R2. The PCR product was cloned into *E. coli*-streptococcal shuttle vector pDL276, generating plasmid pDL276-srtA. This construct was purified and used to transform the DL1srtA⁻ mutant. Confirmation of predicted insertions was obtained by PCR amplification and sequencing. Complementation was confirmed by detection of srtA RNA transcript using primers RealsrtA.F/RealsrtA.R (data not shown). RNA extraction and cDNA synthesis were performed as described below.

No significant difference was found in the growth of any of the mutants compared to their respective wild-type strains (data not shown).

Biofilm formation assay. As reported previously (Loo *et al.*, 2000), bacteria were grown in 96-well plastic plates at 37 °C for 16–18 h. The biomass was then stained with 25 µl of 1% (w/v) crystal violet for 15 min and washed to remove planktonic cells and unincorporated stain. To estimate sessile biomass, 250 µl of 70% ethanol was added to each well. From each well, 125 µl of the extracted crystal violet was

transferred to a new well in a polystyrene microtitre plate and the absorbance at 568 nm was determined with an ELISA reader.

Preparation of salivary agglutinin. Salivary agglutinin was prepared by a modification of a technique reported previously (Rundegren & Arnold, 1987). Unstimulated saliva was collected on ice from healthy volunteers and clarified by centrifugation at 8000 g for 20 min at 4 °C. Agglutinin was prepared by adsorption of the clarified saliva with *S. mutans* as described previously (Brady *et al.*, 1992). Equal volumes of clarified saliva, phosphate-buffered saline (PBS; 2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 6.5 mM Na₂HPO₄, pH 7.2) and PBS-washed *S. mutans* NG8 cells (Klett reading of 600) were mixed and incubated on a rotor at 37 °C for 30 min. Cells with adherent agglutinin were removed from the saliva by centrifugation at 2000 g for 15 min and washed once with PBS. Adsorbed agglutinin was eluted from the cells with one volume of PBS containing 1 mM EDTA. Cells were removed by centrifugation (4000 g, 20 min) and the agglutinin preparation was filter-sterilized with a 0.2 µm acrodisc filter (Pall), dialysed against PBS to remove EDTA, and stored in aliquots at -20 °C. Total protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce) and adjusted to 100 ng ml⁻¹.

Binding to salivary agglutinin. The interaction of *S. gordonii* with immobilized agglutinin was analysed using the BIAcore 3000 machine with a Pioneer F1 sensor chip, as described by Oli *et al.* (2006). Salivary agglutinin, isolated as described above, was covalently bound to the activated carboxyl groups of the dextran molecules in the flow cell (FC) 2. The dextran matrix was activated with 35 µl of an equal mixture of *N*-hydroxysuccinimide (115 mg ml⁻¹) and *N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide (750 mg ml⁻¹), as suggested by the manufacturer. Agglutinin (100 ng ml⁻¹) was diluted 1:5 (v/v) in 10 mM sodium acetate buffer (pH 5) and 2–3 × 20 µl was injected manually until the change in resonance units (RU) was ~1500, indicating the immobilization of 1.5 ng protein. The remaining activated dextran was inactivated by injection of 2 × 35 µl of 1 M ethanolamine. All experiments were conducted at 25 °C in modified adhesion buffer (0.78 mM KH₂PO₄, 1.22 mM K₂HPO₄, 50 mM KCl, 1 mM CaCl₂·6H₂O, pH 7.2) (Clark *et al.*, 1978) and the flow rate was maintained at 10 µl min⁻¹ throughout. To serve as a reference surface, FC 1 was treated in the same way, but agglutinin was omitted.

To determine the binding of cells of *S. gordonii* to purified salivary agglutinin, wtV288 and V288srtA⁻ mutant were grown in 10 ml cultures for 16 h at 37 °C in Todd-Hewitt broth (BBL) supplemented with 0.3% (w/v) yeast extract, and with 5 µg erythromycin ml⁻¹ for V288srtA⁻. Cells were harvested by centrifugation and washed once in modified adhesion buffer. After resuspending cells in modified adhesion buffer to ~1 × 10¹⁰ c.f.u. ml⁻¹ (5 ml), the bacterial suspension was sonicated for 30 s to disrupt chains and form a single-cell suspension (Sonic Dismembrator, model 100, Fisher Scientific, setting 5) and washed again. Cells were then resuspended in 5 ml modified adhesion buffer and diluted 1:10 before use.

S. gordonii cells (~1 × 10⁹ c.f.u. ml⁻¹) in modified adhesion buffer were injected at a flow rate of 10 µl min⁻¹ for 60 s, totalling approx. 1 × 10⁷ c.f.u. per injection. Adherent bacterial cells were removed by injection of 10 µl regeneration solution [PBS-0.3% (v/v) Tween-20 with 10 mM Na₂EDTA, 100 mM NaCl and 100 mM NaOH], which allowed repeated reuse of the sensor chip surface. *S. mutans* NG8 cells were analysed for binding as a positive control. Binding of cells to the sensor chip surface was monitored by the change in refractive index over time with resonance signal (RU) plotted on the y-axis and time on the x-axis.

Fractionation of *S. gordonii* cells. Cells were fractionated by a modification of our previously reported method (Herzberg *et al.*,

1990). Overnight bacterial cultures (100 ml) in FMC were harvested (3000 g, 20 min) and the supernatant and cell pellet collected separately. Supernatants were dialysed against 0.2% (w/v) Na₂EDTA, followed by distilled water, concentrated by lyophilization, and designated the extracellular fraction. Cell pellets were resuspended in 10 ml spheroplasting buffer (20 mM Tris/HCl pH 6.8, 10 mM MgCl₂, 26% (w/v) raffinose), and mutanolysin (Sigma) was added at a final concentration of 80 U ml⁻¹. After incubation at 37 °C for 1 h, suspensions were chilled on ice, and the protoplasts and supernatants then separated by centrifugation (12 000 g, 20 min, 4 °C). Supernatants were collected, dialysed, concentrated by lyophilization and designated the cell-wall fraction. Protoplasts were resuspended in 10 ml of 50 mM triethanolamine/250 mM sucrose, lysed by sonication (50 W, 8 min), and insoluble materials were removed by centrifugation (12 000 g, 20 min, 4 °C). Supernatants were collected, dialysed, concentrated by lyophilization and designated the cytoplasmic fraction. Protein concentrations for each fraction were determined using BCA Protein Assay (Pierce). To compare protein profiles, each fraction (20 µg protein) was resolved by 8% (w/v) SDS-PAGE and stained with Coomassie blue.

Western immunoblotting. Proteins were extracted from *S. gordonii* strains and resolved on 8% (w/v) SDS-PAGE gels, as described above. Proteins were transferred onto Trans-Blot nitrocellulose membrane (Bio-Rad) at 15 V for 30 min using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). Membranes were then probed with rabbit anti-*S. mutans* P1 serum (1:1000 dilution) (gift of Neil Hunter, Millenium Institute of Dental Research, Westmead and Sydney, Australia), and the results visualized with goat anti-rabbit alkaline phosphatase-conjugated IgG (Bio-Rad) and 5-bromo-4-chloro-3-indolylphosphate (BCIP)-Nitro Blue Tetrazolium (Sigma) (Brady *et al.*, 1998; Lee & Boran, 2003). As reported previously, the rabbit anti-*S. mutans* P1 antibodies (Forester *et al.*, 1983) specifically recognize SspA and SspB of *S. gordonii* (Hedde *et al.*, 2003; Holmes *et al.*, 1998; Jakubovics *et al.*, 2005).

Immunogold labelling. Bacteria were grown overnight in FMC medium at 37 °C and diluted to OD₆₂₀ 0.3. Bacterial cultures (1 ml) were pelleted by centrifugation, washed with PBS, resuspended in PBS containing 5% (v/v) goat serum, and incubated with a 20 µg ml⁻¹ solution of rabbit anti-P1 antibody for 2 h. At 2 h, bacteria were washed three times with PBS-5% (v/v) goat serum, incubated for 1 h with goat anti-rabbit immunoglobulin conjugated to 10 nm diameter colloidal gold particles (Sigma) diluted 1:50, and then washed three times with PBS. The bacteria were examined by backscatter electron imaging using a Hitachi S-900 field emission scanning microscope (5 keV), as described previously (Olmsted *et al.*, 1993). For quantification, gold particles were counted on 30 randomly selected wtV288 and V288srtA⁻ bacterial cells, the identities of which were blinded from the observer.

Whole-cell ELISA. Overnight cultures (3 ml) of *S. gordonii* strains were harvested, washed and immobilized in the wells (4 × 10⁸ cells per well) of 96-well Maxisorp microtitre plates (Nunc), as described previously (Holmes *et al.*, 1995). The wells were then incubated with 0.1 ml blocking buffer [10 mM Tris/HCl pH 8.0, 0.15 M NaCl, 0.1% (v/v) Tween-20, 2.5% (w/v) BSA] for 16 h at 4 °C. Next, 50 µl of a 1:25 dilution of rabbit anti-P1 antibody in blocking buffer was added to each well and plates were incubated for 2.5 h at room temperature. After three washes with TBST buffer [10 mM Tris/HCl pH 8.0, 0.15 M NaCl, 0.1% (v/v) Tween-20], 50 µl of a 1:125 dilution of goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) in blocking buffer was added to each well. After 2.5 h incubation at room temperature, bound antibody was detected using the Stable Peroxide Substrate Buffer (Pierce), following the manufacturer's instructions.

RNA extraction and cDNA synthesis. RNA extraction from bacterial cultures, confirmation of RNA integrity and purity on agarose gels, and cDNA synthesis were performed as described previously (Zhang *et al.*, 2004, 2005). For each RNA sample, a control cDNA reaction in the absence of reverse transcriptase was performed to check for DNA contamination.

Real-time PCR. Real-time quantitative PCR was modified from the method of Zhang *et al.* (2005) using the Mx3000 Real-Time PCR System (Stratagene). PCR reactions (25 µl) comprised 12.5 µl 2 × Brilliant SYBR Green QPCR Master Mix (Stratagene), 50 pmol each forward and reverse primer and 3 µl template. The 16S rRNA gene was used as the endogenous reference control, and real-time PCR results were quantified based on standard curves, as described previously (Zhang *et al.*, 2005). The primer set used to amplify the *fbpA* gene is given in Table 2, whilst the primer sets used for *sspA*, *sspB*, *scaA*, *abpA*, *abpB*, *hsa*, *cshA*, *cshB* and 16S rRNA were as described previously (Zhang *et al.*, 2005).

Statistics. *S. gordonii* strains were compared for differences in gene expression levels and cell-surface P1 antigen using the Student's *t*-test.

RESULTS

Functional effects

Biofilm assay. Biofilm formation was assessed by release of crystal violet from the biomass that had formed on plastic. The V288srtA⁻ mutant showed a significant decrease (35%, *P*=0.002, *n*=8) in biofilm formation ability when compared to the wild-type (Fig. 1a).

Binding to salivary agglutinin. To investigate the role of sortase A in controlling adhesin surface expression, we determined binding of wtV288 and V288srtA⁻ mutant cells to salivary agglutinin in a BIAcore flow cell assay (Fig. 1b). Salivary agglutinin is the specific receptor on saliva-coated hydroxyapatite for the antigen I/II family of adhesins (Demuth & Irvine, 2002) and for Hsa (Jakubovics *et al.*, 2005; Nobbs *et al.*, 2007). The wtV288 cells bound rapidly to the sensor chip coated with salivary agglutinin. The wtV288 binding curve (sensogram) reflected a change in refractive index (ΔRU) of 206 during the first 60 s, significantly faster than the V288srtA⁻ mutant (ΔRU=3). Overall binding of the V288srtA⁻ mutant was reduced by 97%. Similarly, wtDL1 showed a 60 s ΔRU=184. These data were then compared against those of *S. mutans*, for which the antigen I/II adhesin, SpaP, has been shown to mediate binding to salivary agglutinin (Oli *et al.*, 2006). *S. mutans* NG8 (wild-type) was used as a positive control and the salivary agglutinin-binding negative *S. mutans* PC3370 *spaP*⁻ (isogenic) mutant (Oli *et al.*, 2006) was used as a negative control and these were analysed under identical conditions. Like the V288srtA⁻ mutant, the mutation in the adhesin *spaP* gene resulted in loss of *S. mutans* binding to salivary agglutinin. When the sensor was prepared without agglutinin, overall binding of bacterial cells was 90% less (data not shown). Hence, *srtA* is required for functional expression of an *S. gordonii* adhesin(s) for salivary agglutinin with a binding profile similar to SpaP of *S. mutans*.

Fig. 1. Effects of *srtA*⁻ mutation on *S. gordonii* V288 functions. (a) Biofilm-forming ability. Bacteria were grown in 96-well plates at 37 °C for 16–18 h, and biofilm formation was assessed by crystal violet staining, as described in Methods. Data shown are means $\pm$ SEM. (b) Binding of wtV288 and V288srtA⁻ mutant to salivary agglutinin. Salivary agglutinin was immobilized on an F1 sensor chip and the binding of bacterial cells was followed over time using BIAcore analysis. Cells (1×10^7 c.f.u. in 10 μ l) were injected at a flow rate of 10 μ l min⁻¹ over a 1 min period. Background Δ RU (resonance signal) in a control flow cell lacking agglutinin was subtracted from the experimental values. Binding of wtV288 (solid line) and V288srtA⁻ mutant (dashed line) cells is shown (in arbitrary resonance units, Ru) in this representative surface plasmon resonance sensogram in comparison to the other strains tested.

Structural effects

Electron microscopy. To learn whether the *srtA*⁻ mutation influences cell-wall morphology, the wtV288 and V288srtA⁻ mutant strains were studied with bright-field microscopy, field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM). The wild-type and the mutant strains showed similar ultrastructure (data not shown), but the V288srtA⁻ mutant showed fewer immunogold-labelled particles conjugated to anti-P1 reacting with SspA and SspB proteins than wtV288 (Fig. 2). The wtV288 strain

bound 8.9 ± 2.5 (mean $\pm$ SD) gold particles per cell; the V288srtA⁻ mutant bound 2.8 ± 1.7 particles per cell. This difference was statistically significant ($P < 0.05$).

Protein profiling

The protein profiles of wtV288, wtDL1, V288srtA⁻, DL1srtA⁻ and DL1srtA⁺ were analysed by SDS-PAGE (8%). Fractions were prepared from FMC culture supernatants (extracellular), mutanolysin digests containing cell-wall materials, and residual cytoplasm. The wild-type and

Fig. 2. Immunogold labelling and backscatter electron microscopy of the SspA and SspB antigens of wtV288 (a) and V288srtA⁻ mutant (b). Bacteria were grown overnight in FMC medium at 37 °C, and subsequently stained with immunogold-labelled rabbit anti-P1 antibodies for surface-expressed SspA/B, as described in Methods. Representative images are shown.

Fig. 3. Protein profiles of wild-type *S. gordonii* and *srtA*⁻ mutants. Cytoplasmic, cell-wall and extracellular protein fractions were isolated by mutanolysin digest from wtV288 and V288*srtA*⁻ (a, c) or wtDL1, DL1*srtA*⁻ and DL1*srtA*⁺ (b, d). Proteins (20 µg per lane) were separated by SDS-PAGE (8%) and visualized by staining with Coomassie blue (a, b). Alternatively, proteins were transferred to nitrocellulose membrane and the level of antigen I/II polypeptide detected by immunoblot analysis with polyclonal anti-P1 antibody (c, d). Molecular mass markers are indicated.

mutant strains generally showed similar but non-identical patterns of proteins (Fig. 3a, b). In all strains, lower molecular mass proteins were over-represented in the cytoplasmic and cell-wall fractions when compared to the supernatant fractions. Higher molecular mass proteins were over-represented in the supernatant fractions of V288*srtA*⁻ and DL1*srtA*⁻ when compared to the wild-type strains. Complementation of *srtA* (DL1*srtA*⁺) restored the wild-type phenotype protein profile (Fig. 3b).

To learn if the *srtA* mutation altered the routing of SspA and SspB to the cell wall, the protein fractions were transferred to a nitrocellulose membrane, which was incubated with rabbit anti-P1 antibody (Fig. 3c, d). Anti-P1 antibody reacted with a cell-wall antigen in the wild-type strains, the *srtA*⁻ mutants and the DL1*srtA*⁺ strain. The antigen was not detected in the cytoplasm of any of the strains. P1 antigen was prominent in the supernatant of the deletion mutants (V288*srtA*⁻, DL1*srtA*⁻) when compared to the wild-types or DL1*srtA*⁺ strains, suggesting abundant release of SspA and SspB into the supernatant during growth. The wtDL1 and complemented DL1*srtA*⁺ showed a similar distribution of P1 antigen.

To clarify the seeming inconsistency in expression of cell-surface P1 antigen by the *srtA*⁻ mutant and wild-type, as

observed by electron microscopy and Western immunoblotting, a whole-cell ELISA experiment was carried out. When analysed by whole-cell ELISA, the *srtA*⁻ mutants expressed less cell-surface P1 antigen than the wild-types. Complemented mutant DL1*srtA*⁺ expressed the same level of surface P1 antigen as the wild-type DL1 (Fig. 4).

Fig. 4. Expression of cell-surface SspA/B adhesins of *S. gordonii* strains. Surface expression of antigen I/II polypeptides was estimated using a whole-cell ELISA as described in Methods. *, $P < 0.001$; mean $\pm$ SD, $n = 6$.

Gene expression

Since the *srtA*⁻ deletion mutants showed an abundance of antigen I/II proteins in the supernatant compared to wild-type, bacteria might be upregulating expression of these adhesins in response to the *srtA* mutation.

We therefore studied the possibility that sortase A might be a general regulator of adhesin gene expression. Using real-time PCR, expression of specific mRNA for the LPXTG-bearing adhesin proteins SspA/B, CshA/B and Hsa, and the non-LPXTG adhesins AbpA/B, FbpA and ScaA, were compared in wtV288 and V288*srtA*⁻ (Table 3a) and wtDL1, DL1*srtA*⁻ and DL1*srtA*⁺ (Table 3b). Expression of *sspA/B*, *cshA/B*, *abpA/B* and *fbpA* were significantly upregulated in V288*srtA*⁻ relative to wtV288 (Table 3a). Expression of *scaA* and *hsa* was similar in wtV288 and V288*srtA*⁻. The expression profile of adhesin genes in wtDL1 and DL1*srtA*⁻ (Table 3b) was similar but not identical to wtV288 and V288*srtA*⁻. When compared to wtDL1, DL1*srtA*⁻ genes *sspA*, *cshA/B*, *abpA/B*, *hsa* and *fbpA* were all upregulated, although the typical fold-change was less than in the V288 background. Interestingly, *scaA* was also upregulated 3.2-fold in DL1*srtA*⁻. Confirming that *srtA* regulated expression of the adhesin genes, DL1*srtA*⁺ and wtDL1 showed similar levels of expression of adhesin genes (Table 3b).

DISCUSSION

The LPXTG family of surface proteins is essential for *S. gordonii* adhesion and colonization of the oral cavity, and can be modelled by SspA/B (El-Sabaeny *et al.*, 2000; Holmes *et al.*, 1998; Williams *et al.*, 1994). The tandem *sspA* and *sspB* genes (Demuth *et al.*, 1996) possess individual promoters that are differentially regulated in response to environmental conditions (El-Sabaeny *et al.*, 2000). We hypothesized that functional presentation of SspA/B, the protein products of *sspA* and *sspB* genes, requires accessory genes such as *srtA*. Disruption of the *srtA* gene has now been shown to change the destination and functions of the SspA/B adhesins, consistent with our

hypothesis. Furthermore, sortase A functions more globally in the regulation of adhesin gene expression, apparently optimizing adhesion function in challenging environments and conditions.

Consistent with loss of function of surface adhesins, the sortase A-deficient mutant showed decreased adhesion to immobilized salivary agglutinin, and reduced biofilm-forming ability *in vitro*. Among these adhesins, SspA and SspB from the *srtA*⁻ mutants appeared to be over-represented in the extracellular milieu, while still expressed on the cell surface in reduced amounts, as shown by immunoelectron microscopy (Fig. 2) and whole-cell ELISA (Fig. 4). The reduction in cell-surface P1 antigen is consistent with the loss of sortase function. On the other hand, P1 antigen appeared to be overexpressed in the *srtA*⁻ mutants (Table 3), perhaps accumulating in the interior layers of the cell wall. This speculation is consistent with the observation that total wall-associated SspA and SspB were similar in the wild-type and *srtA*⁻ mutants when cell-wall fractions were analysed by Western immunoblotting (Fig. 3c, d). These data are consistent with the observations reported previously (Mazmanian *et al.*, 2000), indicating that *srtA*⁻ mutants of *Staphylococcus aureus* accumulate non-functional surface protein precursors in their cell wall. Loss of sortase function in the *srtA*⁻ mutants, therefore, causes improper routing of the adhesins through the wall.

In the *srtA*⁻ mutants, LPXTG-containing proteins are synthesized and exported across the plasma membrane into the medium since they cannot bind peptidoglycan in the cell wall. In the absence of sortase A, cell-surface SspA/B is detectable, but less available to bind specific anti-P1 antibodies than on the wild-type strain. Furthermore, the SspA/B expressed on the *srtA*⁻ mutant cell surface appears non-functional, since V288*srtA*⁻, like the prototype *S. mutans* PC3370 *spaP*⁻ mutant (Oli *et al.*, 2006), was unable to bind its preferred (but not exclusive) salivary receptor, high molecular mass agglutinin. Hence, sortase A is required for functional presentation of SspA/B and likely other LPXTG adhesins on the cell surface of *S. gordonii*. In most cases, loss of function could be attributed specifically

Table 3. Relative levels of adhesin-specific RNA in *S. gordonii* *srtA* deletion and complementation mutants compared to parent strains V288 (a) or DL1 (b)

Values given represent mean $\pm$ SD of three independent experiments performed in triplicate.

	Fold change in gene expression								
	<i>sspA</i>	<i>sspB</i>	<i>cshA</i>	<i>cshB</i>	<i>abpA</i>	<i>abpB</i>	<i>hsa</i>	<i>scaA</i>	<i>fbpA</i>
(a) Change relative to wtV288									
V288 <i>srtA</i> ⁻	3.8 $\pm$ 0.27*	2.4 $\pm$ 0.13*	6.3 $\pm$ 0.70*	1.9 $\pm$ 0.08*	2.7 $\pm$ 0.71*	13.0 $\pm$ 1.41*	1.3 $\pm$ 0.28	1.0 $\pm$ 0.14	5.7 $\pm$ 0.47*
(b) Change relative to wtDL1									
DL1 <i>srtA</i> ⁻	2.4 $\pm$ 0.64*	2.2 $\pm$ 0.92	1.6 $\pm$ 0.07*	3.9 $\pm$ 0.49*	2.1 $\pm$ 0.28*	2.7 $\pm$ 0.64*	2.3 $\pm$ 0.35*	3.2 $\pm$ 0.01*	3.7 $\pm$ 1.06*
DL1 <i>srtA</i> ⁺	1.0 $\pm$ 0.07	1.0 $\pm$ 0.28	0.8 $\pm$ 0.14	0.8 $\pm$ 0.07	1.3 $\pm$ 0.07	0.9 $\pm$ 0.01	0.6 $\pm$ 0.07	0.9 $\pm$ 0.01	0.8 $\pm$ 0.07

**P* < 0.05.

to *srtA* since *DL1srtA*⁺, which complemented the *srtA*[−] mutation, showed complete rescue of adhesin gene expression at the transcriptional level, and was indistinguishable from the wild-type strain. Furthermore, a restoration of adhesion function has been specifically demonstrated for *DL1srtA*⁺ (Nobbs *et al.*, 2007).

While the *srtA*[−] mutation affected adhesin-related functions and functional expression of SspA/B, cell morphology was not remarkably altered. The *V288srtA*[−] and *wtV288* cells showed similar bright-field and ultrastructural morphology (data not shown). Furthermore, free-growing *V288srtA*[−] and *wtV288* cells (and *wtDL1*, *DL1srtA*[−] and *DL1srtA*⁺) grew similarly in suspension culture (data not shown). Loss of sortase A activity was, however, associated with a reduction in biofilm formation. During growth of *srtA*[−] mutants, our data indicated that SspA and SspB and other LPXTG adhesins may be inappropriately presented on the cell wall and also overexpressed and exported. Soluble adhesins are likely to competitively inhibit cell binding by inappropriately expressed adhesins that are present on the cell wall. Consequently, cells prepared for electron microscopy and salivary agglutinin adhesion assays were washed in advance to remove extracellular proteins, including released SspA and SspB in the culture media. Yet, the presence of poorly functional LPXTG adhesins and soluble exported LPXTG adhesins released by growing cells remained throughout the *in vitro* biofilm formation assays. Given that adhesion and biofilm formation are adhesin-dependent and that LPXTG adhesins predominate, a sortase A deficiency might, therefore, have been expected to result in greater loss of function than the 36% reduction observed for *V288srtA*[−] relative to *wtV288*. These observations implied the existence of a compensatory mechanism(s) to maintain at least some level of adhesion.

The data presented here clearly demonstrate that the *srtA* mutation was accompanied by compensatory changes in adhesin gene expression. Deletion of *srtA* caused a strain-specific upregulation in adhesin gene mRNAs (*abpA/B*; LPXTG adhesins *sspA/B*, *cshA/B*, *hsa*). Furthermore, these changes were restored upon complementation of *srtA* mutation. In *V288srtA*[−], the magnitude of increase in expression of LPXTG-adhesin genes was greater than in *DL1srtA*[−], but in *DL1srtA*[−], *scaA*, a non-LPXTG-adhesive protein, was substantially upregulated. Hence, *S. gordonii* cells recognized the loss of *srtA*, or inappropriate processing, presentation or routing of LPXTG adhesins, and responded. Several *S. gordonii* adhesins are known to contribute to transcriptional regulation of alternative adhesins. For example, deletion of *sspA/B* increases *scaA* expression at the gene and protein levels (Zhang *et al.*, 2005), HppA and FbpA appear to regulate expression of CshA (McNab & Jenkinson, 1998), while SspB is regulated by SspA (El-Sabaeny *et al.*, 2001). Thus, one might speculate that those adhesin proteins affected by the sortase A mutation subsequently functioned in transcriptional regulation of alternative compensatory adhesins.

While this finding is consistent with a role for sortase A in a regulated network of proteins and genes involved in adhesin-related functions, it remains to be determined how adhesin expression is transcriptionally regulated, although such studies are currently under way. Extensive changes in gene expression are required for *S. gordonii* to achieve a biofilm state (Gilmore *et al.*, 2003; Zhang *et al.*, 2005), including genes encoding adhesins and transcriptional regulators. Its role as a gene regulator may, therefore, provide a further explanation as to the importance of sortase A in enabling biofilm formation.

Collectively, these data strongly suggest that the adhesins of *S. gordonii* contribute to the transcriptional control of an array of adhesin genes that form an adhesin maintenance system (AMS). Furthermore, these adhesin protein domains function independently, with no one adhesin solely responsible for adhesion of a streptococcal cell to a specific biological substrate (Gong & Herzberg, 1997). By modulating the expression of these genes, sortase A serves as an additional component of this AMS. Thus, upon improper presentation of adhesins, as occurs upon loss of *srtA*, the AMS appears to enable the cell wall to remodel its specificity. The cell-wall array of adhesins can be viewed as 'plastic' and accommodating to abnormal adhesion. We posit, therefore, that the AMS provides compensatory fail-safe mechanisms that this commensal species needs to persist within the host in the face of constantly changing environmental conditions, and that sortase A forms an essential part of this mechanism.

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