

Role of the Cell Wall Microenvironment in Expression of a Heterologous SpaP-S1 Fusion Protein by *Streptococcus gordonii*[∇]

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The charge density in the cell wall microenvironment of Gram-positive bacteria is believed to influence the expression of heterologous proteins. To test this, the expression of a SpaP-S1 fusion protein, consisting of the surface protein SpaP of *Streptococcus mutans* and a pertussis toxin S1 fragment, was studied in the live vaccine candidate bacterium *Streptococcus gordonii*. Results showed that the parent strain PM14 expressed very low levels of SpaP-S1. By comparison, the *dlt* mutant strain, which has a mutation in the *dlt* operon preventing D-alanylation of the cell wall lipoteichoic acids, and another mutant strain, OB219(pPM14), which lacks the LPXTG major surface proteins SspA and SspB, expressed more SpaP-S1 than the parent. Both the *dlt* mutant and the OB219(pPM14) strain had a more negatively charged cell surface than PM14, suggesting that the negative charged cell wall played a role in the increase in SpaP-S1 production. Accordingly, the addition of Ca²⁺, Mg²⁺, and K⁺, presumably increasing the positive charge of the cell wall, led to a reduction in SpaP-S1 production, while the addition of bicarbonate resulted in an increase in SpaP-S1 production. The level of SpaP-S1 production could be correlated with the level of PrsA, a peptidyl-prolyl *cis/trans* isomerase, in the cells. PrsA expression appears to be regulated by the cell envelope stress two-component regulatory system LiaSR. The results collectively indicate that the charge density of the cell wall microenvironment can modulate heterologous SpaP-S1 protein expression in *S. gordonii* and that this modulation is mediated by the level of PrsA, whose expression is regulated by the LiaSR two-component regulatory system.

Streptococcus gordonii is a commensal bacterium of the human oral cavity. The bacterium has gained interest as a live oral vaccine vehicle (21). The expression of a number of vaccine antigens has been described previously; however, the levels of protein produced have been very low. The successful use of this bacterium as a vaccine vehicle depends on the ability to produce adequate amounts of heterologous proteins. Little is known about factors governing the yield of secreted proteins following transcription and translation. In our recent studies, we reported that the expression of a heterologous SpaP-S1 protein in *S. gordonii* could be increased using an inducible promoter (27). However, even under the optimal induction condition, the level of SpaP-S1 produced was very low. Similar problems have been reported in *Bacillus subtilis*, where yields of native proteins can be as much as grams per liter (11, 30) but the production of heterologous proteins is significantly lower (33).

It is believed that a number of posttranslational events that limit heterologous protein production exist, including the inefficient translocation and folding of proteins and degradation by proteases (38). Proper folding of secreted proteins is critical to protein production, because improperly folded proteins are highly susceptible to proteolytic degradation. In *B. subtilis*, the folding of secreted proteins is catalyzed by a membrane-asso-

ciated lipoprotein named PrsA (17, 43). PrsA displays homology to peptidyl-prolyl *cis/trans* isomerases. PrsA homologues are found ubiquitously in all Gram-positive bacteria. In *B. subtilis*, the protein secretion rate was linearly proportional to levels of PrsA (42). In addition, overproduction of PrsA resulted in increased expression of the heterologous amylase Q from *Bacillus amyloliquefaciens* and the protective antigen from *Bacillus anthracis* in *B. subtilis* (17, 44). Similarly, increasing levels of PrtM, a PrsA-like protein in *Lactococcus lactis*, led to increased heterologous protein expression in *L. lactis* (28).

Misfolded proteins in the cell wall lead to cellular stress, and *B. subtilis* uses the CssRS two-component regulatory system to sense this stress and activate the CssRS regulon, which includes the *htrA* and *htrB* genes, coding for cell wall-associated serine proteases (8, 13). In *Staphylococcus aureus*, stress caused by cationic antimicrobial peptides led to PrsA upregulation mediated by the VraSR (LiaSR homologue) cell envelope stress two-component regulatory system (32). In *Streptococcus mutans*, LiaSR responds to stress from acidic pH (25).

In this study, we examined the role of the cell wall in modulating heterologous SpaP-S1 production in *S. gordonii*. The results suggest that a negatively charged cell wall can increase SpaP-S1 production. The increase in SpaP-S1 appears to be due to an increase in PrsA production, which is regulated by LiaSR.

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MATERIALS AND METHODS

Bacteria and growth conditions. The bacterial strains used in this study are listed in Table 1. *S. gordonii* DL1 is the parent strain, and all mutants were

TABLE 1. Bacterial strains used in the study

Bacterial strain	Description	Source/reference
<i>S. gordonii</i>		
DL1	Parent strain, Challis	D. LeBlanc
PM14	DL1 carrying pPM14 (Kan ^r)	27
OB219	SspA- and SspB-negative mutant of DL1, <i>sspAB::ermAM</i>	26
<i>dltA</i> mutant	DltA-negative mutant of DL1, <i>dltA::ermAM</i>	5
RJM4	DL1 carrying a single copy of the SpaP-S1 gene on the chromosome, Kan ^r	22
<i>prsA</i> mutant	PrsA-negative mutant of RJM4, <i>prsA::ermAM</i>	This study
SgPL2	RJM4 carrying pSgPL2 (Spc ^r)	This study
<i>liaR</i> mutant	LiaR-negative mutant of RJM4, <i>liaR::ermAM</i>	This study
<i>E. coli</i>		
XL1-Blue	Cloning strain	Stratagene
SgP1H	XL1-Blue carrying pSgP1H (Kan ^r)	This study
SgPL1	XL1-Blue carrying pSgPL1 (Kan ^r)	This study
SgPL2	XL1-Blue carrying pSgPL2 (Spc ^r)	This study

derived from this strain. The *dlt* mutant lacks D-alanine residues on its lipoteichoic acids due to the inactivation of the *dlt* operon (5). OB219 lacks two LPXTG major surface proteins, SspA and SspB (26). Both the *dlt* mutant and OB219 were transformed with pPM14, which carries the SpaP-S1 fusion gene under the control of the tetracycline-inducible *xyl-tetO* promoter (27). *spaP* encodes the LPXTG major surface protein SpaP (also named P1, or antigen I/II) from *S. mutans* (24). The *s1* gene encodes the 179-amino-acid N-terminal fragment of pertussis toxin subunit S1 and is fused to the middle part of *spaP* (23). *S. gordonii* RJM4 carries the SpaP-S1 gene on the chromosome (22) and was used to avoid the potential incompatibility of having two plasmids in the same cell.

S. gordonii strains were cultured on brain heart infusion (BHI) agar or in tryptone-vitamin glucose (TVG) broth (35 mg/ml tryptone, 1 µl/ml vitamin solution [0.04 µg/ml *p*-amino benzoic acid, 0.2 mg/ml thiamine, 1.0 µg/ml nicotinamide, and 0.2 µg/ml riboflavin], 5 mg/ml glucose, pH 7.8) buffered with 100 mM HEPES. When needed, 250 µg/ml of kanamycin, 250 µg/ml spectinomycin, or 10 µg/ml erythromycin was added to the media. The induction of SpaP-S1 expression was achieved by the addition of 10 ng/ml tetracycline to the growth medium (27). *Escherichia coli* was cultured in Luria-Bertani broth (1% tryptone, 0.5% yeast extract, and 1% NaCl, wt/vol) containing 50 µg/ml kanamycin, 50 µg/ml spectinomycin, or 10 µg/ml tetracycline.

For experiments to examine the effects of cations or bicarbonate on SpaP-S1 expression, filter-sterilized stock solutions of KCl, CaCl₂ · 2H₂O, MgCl₂, or NaHCO₃ were added to the growth media at the indicated concentrations (see Fig. 3) prior to inoculation. All cultures grew to the same cell density, indicating that the added ions did not impair growth.

SDS-PAGE and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (19). Bacterial cultures were standardized to the same optical density at 600 nm (OD₆₀₀) following growth. Cells were pelleted by centrifugation (10,000 × *g*, 5 min), resuspended in SDS-PAGE sample buffer, and boiled for 5 min. Alternatively, the entire bacterial cultures were precipitated with 5% trichloroacetic acid and then extracted with SDS-PAGE sample buffer as total cell extracts prior to electrophoresis. Following electrophoresis, the SDS-polyacrylamide gels were stained with Coomassie blue R-250 to ensure equal loading of proteins in the samples. For Western blotting, proteins were transferred to nitrocellulose membranes using methods described by Towbin et al. (39). The SpaP-S1 fusion protein was detected using human anti-pertussis toxin IgG antibody (1/10,000) (3) followed by goat anti-human IgG alkaline phosphatase-conjugated antibody (1/4,000; Sigma-Aldrich Chemical Co., Oakville, ON, Canada). The PrsA protein was detected using mouse anti-PrsA antibody (1/2,000) (see below) followed by goat anti-mouse IgG alkaline phosphatase conjugates (1/8,000; Sigma-Aldrich). The intensities of the full-length 187-kDa SpaP-S1 and 31-kDa PrsA immunoreactive bands were analyzed by digital imaging using the ImageJ program (NIH, <http://rsb.info.nih.gov/ij/>).

Evaluation of bacterial surface charge. Bacterial surface charge was evaluated by two methods. The first method measured the zeta potential values using a zeta potential analyzer (PanKem system 3000; PenKem Inc., Bedford Hills, NY) according to the method described by Speers et al. (35). Mid-exponential-phase (OD₆₀₀ of approximately 0.5) cells were washed once in 20 mM morpholinepropanesulfonic acid (MOPS) buffer, pH 7, and resuspended in the same buffer to 3.5 × 10⁷ CFU/ml. A minimum of 6 measurements was obtained for each triplicate culture of the three strains. The second method used the cationic dye alcian blue 8GX binding assay (4, 5). Mid-exponential-phase cells were washed once with 20 mM MOPS buffer and resuspended in the same buffer to a final OD₆₀₀ of 0.5 (ca. 2.5 × 10⁸ CFU/ml). Alcian blue was added to a final concentration of 65 µg/ml. Samples were rotated at room temperature for 10 min at 3 rpm. As a negative control, MOPS buffer with 65 µg/ml alcian blue was incubated under the same conditions without bacteria. After incubation, bacteria were removed by centrifugation (10,000 × *g*, 5 min). The unbound alcian blue in the supernatant fluids was measured at 530 nm using a spectrophotometer. The amount of alcian blue bound to the bacteria was calculated as (*A*₅₃₀ of supernatant without bacteria – *A*₅₃₀ of supernatant with bacteria)/*A*₅₃₀ of supernatant without bacteria × 100.

Isolation of cellular fractions from *S. gordonii*. Five hundred ml of late-exponential-phase bacterial cultures was centrifuged (10,000 × *g*, 15 min, 4°C). The supernatant was collected and stored at –80°C. The bacterial cells were washed once with phosphate-buffered saline (PBS), resuspended in 2 ml of PBS, and disrupted in a cell disintegrator (Mickle Laboratory Engineering Co. Ltd., Gonshall, Surrey, United Kingdom) for 1.5 h at 4°C to achieve 95% breakage. The supernatant was removed, and the glass beads were washed 4 times with 1 ml of PBS. The supernatant and wash fractions were combined and centrifuged (2,000 × *g*, 2 min) to pellet any remaining glass beads and whole cells. The supernatant was recovered and centrifuged (27,000 × *g*, 30 min, 4°C) to separate the cell wall from the cytoplasm and membranes. The cell wall pellet was resuspended in 5 ml of distilled H₂O (dH₂O) and centrifuged (27,000 × *g*, 30 min, 4°C). The resulting cell wall pellet was resuspended in 500 µl of PBS and stored at –80°C. The cell membrane/cytoplasm supernatant was centrifuged for 1 h at 50,000 × *g* to separate the cell membrane from the cytoplasm. The cell membrane pellet was resuspended in 500 µl of PBS and stored at –80°C. The supernatant, containing the cytoplasm, was also stored at –80°C. All cellular-fraction samples were thawed and standardized by volume prior to electrophoresis and Western immunoblotting.

Analysis of cell wall proteins. Four hundred µl of cell wall was centrifuged (10,000 × *g*, 15 min), resuspended in 50 µl of dH₂O, and freeze-dried. One mg of the dried cell wall was resuspended in 3× sample buffer (20 mM Tris [pH 6.8], 0.3% glucose [wt/vol], 6% SDS [wt/vol], and 0.15% β-mercaptoethanol) and boiled for 20 min to extract non-covalently-bound proteins. The samples were centrifuged (10,000 × *g*, 15 min). The cell wall pellet was washed 8 times with warmed (37°C) dH₂O. The residual cell walls were incubated overnight at 37°C with 100 U of mutanolysin (Sigma-Aldrich) in 100 µl of 50 mM Tris buffer (pH 7.0) containing 10 mM MgCl₂ and 2 mM phenylmethylsulfonyl fluoride. In parallel, 1 mg of unextracted cell wall was digested overnight with mutanolysin. Following digestion, the samples were boiled in sample buffer, centrifuged (10,000 × *g*, 5 min), and analyzed by SDS-PAGE and Western immunoblotting.

Reverse transcription-PCR. Total RNA was extracted from cells using the hot acid phenol method of Peterson et al. (31) as described previously (40). Contaminating DNA was removed by digestion with amplification-grade DNase I (Invitrogen Life Technologies, Burlington, ON, Canada), and the resulting RNA was verified to be free of DNA by PCR for the 16S rRNA gene using primers SL525/SL697 (Table 2). cDNA was synthesized using random primers and SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's instructions. The levels of 16S rRNA and SpaP-S1 gene transcripts were estimated by PCR with *Taq* DNA polymerase and primer pairs SL525/SL697 and SL642/SL643, respectively. The PCR products were incubated at 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min and a final 5-min extension at 72°C. The PCR products were analyzed by gel electrophoresis, and the intensity of the 600-bp *s1* gene fragment was normalized against that of the 16S rRNA gene fragment (500 bp) by using the ImageJ program. The 16S rRNA gene was chosen as the endogenous reference gene, as its expression is relatively stable irrespective of growth conditions (36).

Recombinant PrsA and generation of anti-PrsA antibodies. To produce the recombinant PrsA, the 896-bp DNA fragment encoding the mature PrsA without the LXXC lipoprotein motif was amplified by PCR from the chromosome of DL1 using the primers SL651 and SL669 (Table 2). The PCR product was restricted with *Sfi*I and cloned into the same sites on pSecPrn(–HA). pSecPrn(–HA) is a derivative of pSecCR1 (15); it carries a 0.6-kb DNA insert (Prn) instead of the anti-CR1 gene and the hexahistidine tag but lacks the

TABLE 2. Primers used in the study

Primer name	Primer sequence (restriction enzyme) ^a	Primer function
SL525	GAATTAACACATGCTCCACCGC	Reverse primer for 16S rRNA gene
SL550	CTGGGCCAGCGCGCCGGCCAAAATTTGTTTAT (SfiI)	Forward primer for <i>ermAM</i>
SL551	CCTGGCCGGCCTGGCCGGCAGCGACTCATAGAAT (SfiI)	Reverse primer for <i>ermAM</i>
SL642	ATACATATGGATCCTCCCGCCACCG	Forward primer for <i>s1</i> gene
SL643	ATAGAATTCGGATGTGTAGGGGTTGGG	Reverse primer for <i>s1</i> gene
SL651	CTTGGCCAGGCGGCCGACAAGGACATTATTACTA (SfiI)	Forward primer for <i>prsA</i>
SL667	ATAGGTACCACAAAGGAGTTTTCATTAA (KpnI)	Forward primer for entire <i>prsA</i> gene, including ribosomal binding site and start codon
SL668	ATAGAATTCCTATTTTGTATGAAGAAGATG (EcoRI)	Reverse primer for <i>prsA</i> including stop codon
SL669	CTTGGCCGGCCTGGCCTTTTGTATGAAGAAGATGAAG (SfiI)	Reverse primer for <i>prsA</i> without stop codon
SL692	ACTACATCTTCGTCTACAG	Forward primer for <i>liaR</i> upstream fragment
SL693	CCTGGCCGCCTGGGCCGAACCATTTTCGTGATCATC (SfiI)	Reverse primer for <i>liaR</i> upstream fragment
SL694	CCTGGCCGGCCTGGCCTTCCTCAGGAAGACTTCTGA (SfiI)	Forward primer for <i>liaR</i> downstream fragment
SL695	ACAGGTAGCTTCTGGTAAAC	Reverse primer for <i>liaR</i> downstream fragment
SL697	ATTTATTGGGCGTAAAGCGAGCGC	Forward primer for 16S rRNA gene
SL700	CCTGGCCGCCTGGGCCGCTTCATATGCAGCTTTATA (SfiI)	Reverse primer for <i>prsA</i> upstream fragment
SL701	CCTGGCCGGCCTGGCCACCAGAGGTAAGTGC (SfiI)	Forward primer for <i>prsA</i> downstream fragment

^a Restriction enzyme sites are underlined.

hemagglutinin (HA) tag. The cloning put the *prsA* gene behind the tetracycline-inducible *P_{xyI-tetO}* promoter, the *S. mutans spaP* ribosomal binding site and signal sequence, and an in-frame His tag at the C terminus. The resulting plasmid was named pSgPIH and maintained in *E. coli* XL1-Blue.

Cells from 100-ml cultures of *E. coli* (pSgPIH) induced with tetracycline were harvested by centrifugation (10,000 × g, 10 min) and resuspended in 5 ml of NiCAM wash buffer (50 mM Na₂HPO₄, 300 mM NaCl, and 20 mM imidazole, pH 7). Cells were broken by sonication (Vibra Cell; Sonics & Materials Inc., Danbury, CT) and centrifuged (20,000 × g, 30 min). The clear supernatant was applied to a 4-ml NiCAM column (Sigma-Aldrich), which had been equilibrated with the NiCAM wash buffer. The column was washed with 20 ml of NiCAM wash buffer, and the bound PrsA was eluted with 10 ml of NiCAM wash buffer containing 250 mM imidazole. The eluted PrsA was >95% pure as judged by SDS-PAGE analysis (data not shown).

To generate a polyclonal antibody against PrsA, BALB/c mice (female, 5 weeks old, *n* = 5) were injected subcutaneously, on days 1, 14, and 21, with 10 μg PrsA adsorbed to 2% (wt/vol) aluminum hydroxide gel (Sigma-Aldrich) (16). The animals were euthanized on day 33 and sera saved as the anti-PrsA antibody.

Construction of the *prsA* mutant and the *liaR* mutant. The 5' (446-bp) and 3' (447-bp) regions of *prsA* were amplified from DL1 chromosomal DNA by PCR using the primer pairs SL667/SL700 and SL701/SL668, respectively (Table 2). An erythromycin resistance cassette (*ermAM*) was PCR amplified from a synthetic construct template (6) with the primer pair SL550/SL551. The *prsA* fragments were restricted with SfiI and ligated to the *ermAM* DNA separately. The ligated DNAs were PCR amplified to produce the 1.40-kb 5' *prsA-ermAM* and 1.34-kb 3' *ermAM-prsA* DNA using the SL667/SL551 and SL550/SL668 primer pairs, respectively. The PCR products were mixed in a 1:1 ratio and further amplified by overlapping PCR using the primer pair SL667/SL668 to obtain the final 1.88-kb *prsA::ermAM* construct, which was introduced into *S. gordonii* RJM4 via natural transformation (22). Transformants were selected on erythromycin-BHI agar and verified to carry an insertional inactivation of *prsA* by PCR and Western immunoblotting.

The *liaR* mutant was constructed similarly to the *prsA* mutant. The regions upstream (466 bp) and downstream (532 bp) of *liaR* were amplified from DL1 chromosomal DNA by PCR using the primer pairs SL692/SL693 and SL694/SL695, respectively (Table 2). The PCR products were ligated to *ermAM*, and the 1.6-kb ligated product was PCR amplified using SL692 and SL695. The resulting *liaR::ermAM* construct was transformed into RJM4 to obtain the *liaR* mutant. The mutant was verified by PCR using primer pair SL692/SL695 to carry the 1.6-kb *liaR::ermAM*, while the parent RJM4 had the intact *liaR* (data not shown).

Construction of the PrsA overexpression strain. The full-length *prsA* gene, including its ribosomal binding site, signal sequence, and LXXC lipoprotein motif, was PCR amplified using primers SL667 and SL668. The PCR product was restricted with KpnI and EcoRI and ligated to the same sites on pSecPrn(-HA). The cloning created pSgPL1, with the expression of *prsA* under the control of the tetracycline-inducible *P_{xyI-tetO}* promoter. Because pSgPL1 confers kanamycin resistance, it is not suitable for use in RJM4. Therefore, the 1.95-kb SphI-EcoRI fragment carrying *P_{xyI-tetO}prsA* and the *tetR* repressor gene was subcloned into the

E. coli-Streptococcus shuttle vector pDL278 (Sp^c) (20) to obtain pSgPL2. pSgPL2 was transformed into RJM4 to obtain the PrsA overexpression strain.

Statistical analysis. Statistical significance for the results was evaluated by repeated-measures analysis of variance (ANOVA). This was followed by a Tukey test. A *P* value that was equal to or less than 0.05 was considered to be significant.

RESULTS

***S. gordonii dlt* mutant and OB219(pPM14) express higher levels of SpaP-S1 than the parent strain.** To examine the effects of the cell wall microenvironment on heterologous protein expression, the levels of SpaP-S1 expression were compared between the parent PM14 and two mutant strains of *S. gordonii*. The mutant strains are a *dlt* mutant, which cannot D-alanyl-lipoteichoic acids (5), and OB219, which lacks the major surface proteins SspA and SspB (26). All three strains carry plasmid pPM14. Results from zeta potential measurements and the cationic dye alcian blue 8GX binding assay showed that the mutants have an increase in negative surface charges. The zeta potential values for the *dltA* mutant and OB219(pPM14) were -28.01 ± 0.83 and -27.81 ± 0.29 mV, respectively, and were significantly more negative than that (-21.23 ± 0.49 mV) of PM14 ($P < 0.001$). The results of the alcian blue binding assay showed that the *dlt* mutant and OB219(pPM14) bound $72\% \pm 2\%$ and $73\% \pm 1\%$ of alcian blue, respectively, in comparison to $62\% \pm 2\%$ binding by PM14 ($P < 0.001$). When the three strains were analyzed for SpaP-S1 expression, the *dlt* mutant and OB219(pPM14) produced 2.5- and 2.9-fold more SpaP-S1 than PM14, respectively (Fig. 1). Reverse transcription-PCR analysis showed that the three strains expressed the same level of SpaP-S1 gene transcript (data not shown).

***S. gordonii dlt* mutant and OB219(pPM14) have equivalent amounts of SpaP-S1 anchored to the cell walls.** Since OB219 (pPM14) lacks the LPXTG major surface proteins SspA and SspB, it is conceivable that more anchoring space is available in the cell wall, resulting in the increase in SpaP-S1 expression. To investigate this possibility, cellular fractions were isolated from the mutants and PM14. Western immunoblotting of the isolated fractions was performed to determine the

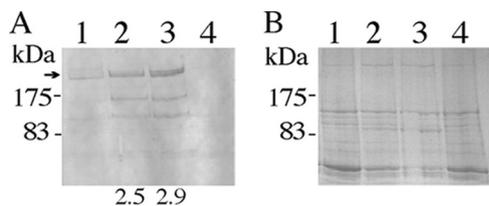


FIG. 1. SpaP-S1 produced by *S. gordonii* PM14, the *dlt* mutant, and OB219(pPM14). (A) Western immunoblot of total cell extracts showing the full-length SpaP-S1 (arrow) and smaller-sized immunoreactive bands, presumably degradation products. (B) SDS-polyacrylamide gel (6.5%) showing equal loading of proteins in the samples. Lanes: 1, PM14; 2, *dltA* mutant; 3, OB219(pPM14); 4, DL1. DL1 does not carry pPM14 and thus does not produce the SpaP-S1 protein. Numbers below the blot indicate the fold increases in SpaP-S1 produced by the mutants compared to the level produced by PM14.

location of SpaP-S1 within the bacterium. Results showed that >95% of the SpaP-S1 protein localized in the cell wall fraction, with the remaining located in the supernatant, the membrane and cytoplasm (data not shown). Consistent with earlier results, the cell walls of both the *dlt* mutant and OB219(pPM14) contained significantly more SpaP-S1 than that of PM14 (Fig. 2). Interestingly, the cell walls from the *dlt* mutant and OB219(pPM14) had similar amounts of covalently bound SpaP-S1. In addition, the cell walls of the *dltA* mutant and OB219(pPM14) contained more proteins than the PM14 cell wall.

Ca²⁺, Mg²⁺, and K⁺ decrease SpaP-S1 expression. The finding that the *dlt* mutant and OB219(pPM14) have a more negatively charged cell surface and produced more SpaP-S1 than the parent suggests that the cell wall charge plays a role in SpaP-S1 expression. Thus, it is of interest to investigate whether SpaP-S1 expression can be modulated by the addition of cations to the cultures. As shown in Fig. 3A, SpaP-S1 production by OB219(pPM14) decreased with increasing concentrations of Ca²⁺. Mg²⁺ has a similar effect of lowering SpaP-S1 production (Fig. 3A). The addition of K⁺ also decreased SpaP-S1 expression, but the decrease was not as drastic as that achieved by Ca²⁺ or Mg²⁺ addition (Fig. 3B). Similar results exerted by

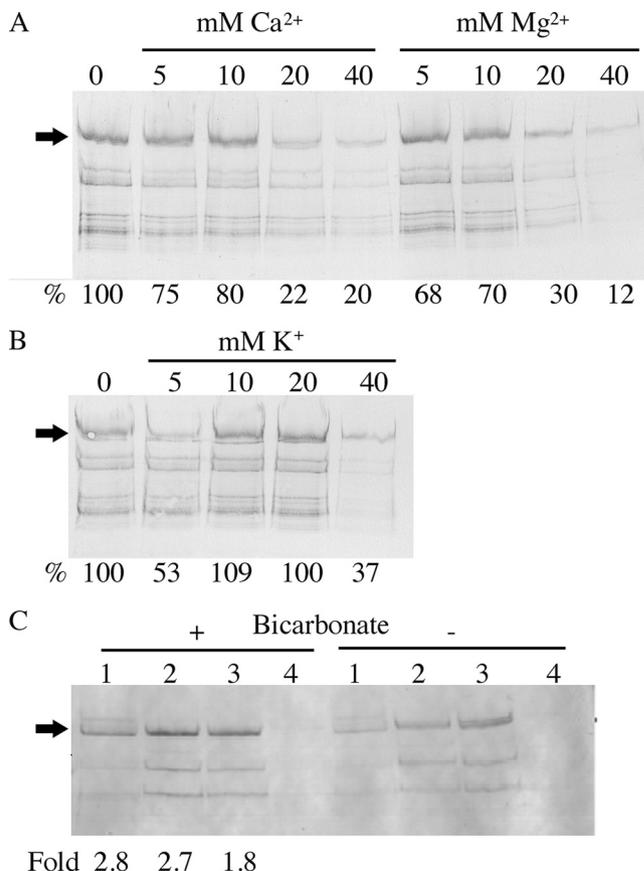


FIG. 3. Effects of cations and bicarbonate on SpaP-S1 expression. (A) Immunoblot of SpaP-S1 produced by OB219(pPM14) following growth in the presence of Ca²⁺ or Mg²⁺ at the indicated concentrations. 0, no added Ca²⁺ or Mg²⁺. (B) Immunoblot of SpaP-S1 produced by OB219(pPM14) following growth in the presence of K⁺ at the indicated concentrations. 0, no addition. (C) Immunoblot of SpaP-S1 produced by *S. gordonii* in the presence (+) or absence (-) of 50 mM bicarbonate. Lanes: 1, PM14; 2, *dlt* mutant; 3, OB219(pPM14); 4, DL1. Numbers below the blots are percentages or fold changes in SpaP-S1 compared to the level for the no-addition control. Arrows indicate the full-length SpaP-S1 protein. The samples were also analyzed on 6.5% SDS-polyacrylamide gels to ensure equal loading of proteins (data not shown).

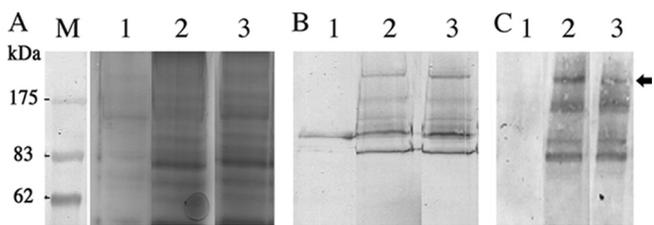


FIG. 2. SpaP-S1 anchored to the cell wall of PM14, the *dlt* mutant, and OB219(pPM14). (A) SDS-polyacrylamide gel (7.5%) of proteins from cell walls before hot-SDS extraction. Each sample is derived from the same quantity of cell wall treated with mutanolysin, as described in Materials and Methods. (B) Western blot of SpaP-S1 in cell wall samples shown in panel A. (C) Western blot of SpaP-S1 on residual cell wall after hot-SDS extraction. Lanes: M, prestained protein markers (New England BioLabs, Mississauga, ON, Canada); 1, PM14; 2, *dlt* mutant; 3, OB219(pPM14). The arrow indicates the full-length SpaP-S1 protein. The lower-mass immunoreactive bands are presumably degradation products.

Ca²⁺, Mg²⁺, and K⁺ were observed for the *dlt* mutant and PM14 (data not shown).

Bicarbonate ion increases SpaP-S1 expression. Dorschner et al. (9) reported that the thickness of the bacterial cell wall is markedly decreased when cells are grown in the presence of a physiological concentration of bicarbonate. To determine whether bicarbonate has an effect on SpaP-S1 expression, *S. gordonii* strains were cultured in the presence of 50 mM NaHCO₃. The results showed that an increase in SpaP-S1 expression was observed in all three strains (Fig. 3C). The parent strain produced 2.8-fold more SpaP-S1 when cultured in the presence of bicarbonate than when cultured without bicarbonate addition. Similarly, both the *dltA* mutant and OB219(pPM14) expressed more SpaP-S1 when grown in the presence of bicarbonate.

Role of PrsA in SpaP-S1 production. Given that heterologous protein production could be increased by PrsA over-

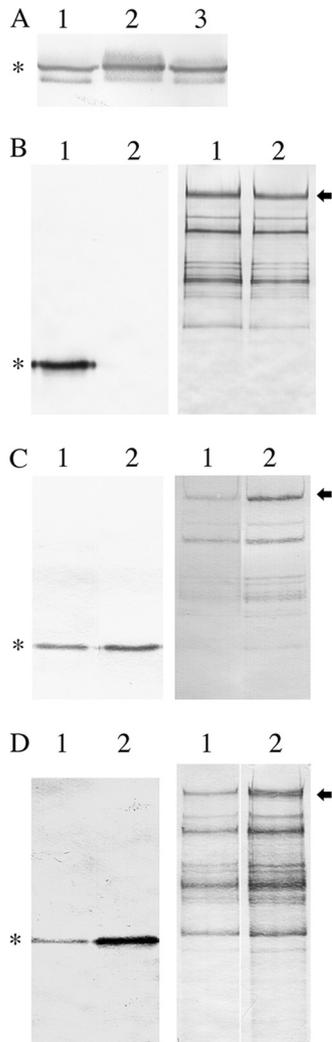


FIG. 4. PrsA and SpaP-S1 expression by *S. gordonii*. (A) Immunoblot of PrsA produced by PM14 (lane 1), OB219(pPM14) (lane 2), and the *dltA* mutant (lane 3). (B) Immunoblots of PrsA (left) and SpaP-S1 (right) produced by RJM4 (lane 1) and the *prsA* mutant (lane 2). (C) Immunoblots of PrsA (left) and SpaP-S1 (right) produced by RJM4 (lane 1) and RJM4 carrying pSgPL2 (lane 2). (D) Immunoblots of PrsA (left) and SpaP-S1 (right) produced by RJM4 (lane 1) and the *liaR* mutant (lane 2). Arrows indicate the full-length SpaP-S1 protein, and asterisks indicate PrsA. The samples were also analyzed on 6.5% or 12.5% SDS-polyacrylamide gels to ensure equal loading of proteins (data not shown).

production in *B. subtilis* (17, 44), it is conceivable that the observed increase in SpaP-S1 production in the *dltA* mutant and OB219(pPM14) of *S. gordonii* was due to an increase in PrsA expression. To examine this possibility, the levels of PrsA in PM14 and the two mutants were analyzed. The results showed that the *dltA* mutant and OB219(pPM14) produced 1.5- and 2-fold more PrsA, respectively, than PM14, suggesting that PrsA plays a role in SpaP-S1 production (Fig. 4A). To help confirm this, the *prsA* gene was insertionally inactivated in *S. gordonii* RJM4. The mutation was confirmed genetically by PCR, which showed the amplification of a 1.9-kb *prsA::ermAM* DNA from the chromosome by use of primer pair SL667/SL668 (data not shown). In addition, the mutant lacked the

31-kDa PrsA protein, as shown by Western blotting using anti-PrsA as the probe (Fig. 4B). The *prsA* mutant produced 35% less SpaP-S1 than the parent strain (Fig. 4B). To further provide support for the role of PrsA in SpaP-S1 production, the *prsA* gene was cloned into pDL278 and transformed into RJM4 to create a PrsA overproduction strain. The resulting strain produced 2.6-fold more PrsA and 3.5-fold more SpaP-S1 than the parent RJM4 (Fig. 4C). The above-described results collectively indicate that the observed increased SpaP-S1 production in the *dltA* mutant and OB219(pPM14) was due to PrsA overexpression.

To investigate whether the upregulation of PrsA was controlled by the cell envelope stress two-component regulatory system LiaSR, the response regulator LiaR in RJM4 was inactivated by the *ermAM* cassette. PrsA was upregulated 9.8-fold in the *liaR* mutant (Fig. 4D). In keeping with earlier results, SpaP-S1 production was increased 4.3-fold in the *liaR* mutant.

DISCUSSION

In this study, the role of the cell wall microenvironment in the expression of heterologous protein SpaP-S1 in *S. gordonii* was investigated. The results indicate that the overall charge density of the cell wall is important to SpaP-S1 production; when the cell wall becomes electronegative, more SpaP-S1 is produced. This statement is supported by results obtained using the *dlt* mutant and OB219(pPM14). Both strains have a more negatively charged cell wall than the parent PM14 and produced higher levels of SpaP-S1 than PM14. On the other hand, cation addition, which presumably makes the cell wall more electropositive, resulted in a decrease in SpaP-S1 production.

The observation that the *dlt* mutant has a more negative cell surface can easily be explained by the fact that lipoteichoic acids are not D-alanylated, due to the inactivation of the *dlt* operon. An increase in cell surface negative charge displayed by *dlt* mutants of other Gram-positive bacteria has been reported previously; thus, our results are consistent with the literature (5, 14, 18, 41). The observation that OB219(pPM14) also has a more negative cell surface is an unexpected result. However, given that SspA and SspB are calcium-binding proteins (10), it is conceivable that in the absence of these two proteins, calcium is not readily recruited to the cell wall, leading to a more negative cell surface. It has been proposed that the recruitment of calcium and other positively charged ions to the cell wall may promote bacterial aggregation by partially reducing the negative surface charge (34, 45).

Increased heterologous protein expression in several *dlt* mutants has been documented. The *B. subtilis* *dlt* mutant had increased expression of heterologous amylase Q from *B. amyloliquefaciens* and the protective antigen from *B. anthracis* (14, 37). Similar results for expression of other heterologous proteins were obtained in *dlt* mutants of *Bacillus licheniformis* and *L. lactis* (7, 29). Thus, our results obtained with the *dlt* mutant are consistent with the literature. However, the results obtained with OB219(pPM14) are novel. To the best of our knowledge, this is the first report that a mutant lacking two surface proteins can have an effect on heterologous protein expression. The results suggest that the increased negative

charge in the cell wall is the reason for the increase in SpaP-S1 production and that the presumably increased availability of LPXTG anchoring sites vacated by SspA and SspB played no role. Our results further showed that the effect induced by *dlt* inactivation and *sspA* and *sspB* inactivation is not limited to SpaP-S1. The cell walls isolated from the two mutants contained more proteins than the parent PM14 cell wall, suggesting that the production of other cell wall-associated proteins is also increased.

The argument that the increase in cell wall negative charge is responsible for the increase in SpaP-S1 production is reinforced by the results of the cation experiments. Addition of Ca^{2+} , Mg^{2+} , and K^{+} resulted in a decrease in SpaP-S1 production. Although we are not able to provide results to show that the cell wall microenvironment became more positive from cation addition, it is reasonable to assume that this is the case. The cell wall is known to function as a cation reservoir (2, 12). With more cations present in the medium, more cations should be in the cell wall. The results of the cation addition experiment are in agreement with reports for *Bacillus brevis* showing that the yield of α -amylase was reduced in the presence of Ca^{2+} , Mg^{2+} , and K^{+} ions (1). Our results further showed that Ca^{2+} and Mg^{2+} ions have a greater effect on SpaP-S1 expression than K^{+} , possibly due to the greater positive charge of the divalent cations than the monovalent ions.

The finding that SpaP-S1 production is increased by bicarbonate is interesting. The simple explanation for the effect of bicarbonate is that bicarbonate makes the cell wall more electronegative, thus leading to an increase in SpaP-S1 production. However, the effect of bicarbonate may be more complex. Bicarbonate can reduce the cell wall thickness via the modulation of cell wall biosynthesis gene expression (9). Thus, the observed increased SpaP-S1 production may be a combined effect of increased cell wall electronegativity and cell wall thickness.

The reason behind the increase in SpaP-S1 expression in the *dltA* mutant and OB219(pPM14) appears to be an increase in PrsA production. This statement is supported by the results that OB219(pPM14) and the *dltA* mutant produced more PrsA than the parent strain and that SpaP-S1 production was increased in the PrsA-overproducing strain and decreased in the PrsA-negative mutant. The role of PrsA in SpaP-S1 production is in keeping with observations made for *B. subtilis* and *L. lactis* that overproduction of PrsA led to larger amounts of foreign protein expression (17, 28, 44). Our results further showed that PrsA expression is upregulated in the *liaR* mutant, and coincident with the increase in PrsA expression, SpaP-S1 production was also increased. This result further supports the idea that PrsA plays a role in SpaP-S1 production. The result also indicates that LiaSR regulates PrsA expression, which is in agreement with findings in *S. aureus* that *prsA* expression is upregulated by LiaSR when cells are stressed with cationic antimicrobial peptides (32). We have attempted to obtain a viable *liaSR* clone in *E. coli* but failed; hence, complementation experiments cannot be performed. In *S. mutans*, LiaSR is needed for cell survival of acidic pH (25), indicating that LiaSR can sense and respond to cell envelope ion concentrations. Thus, it is reasonable to suggest that LiaSR senses and responds to cell wall charges and regulates the expression of

PrsA in *S. gordonii*. PrsA modulates the level of SpaP-S1 production presumably by allowing more efficient protein folding.

In conclusion, heterologous SpaP-S1 protein production in the *dltA* mutant and OB219(pPM14) was higher than that in the parent strain. The increase can be correlated with an increase in the overall cell envelope negative charge and the PrsA level.

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