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A cell wall anchored glycoprotein confers resistance to cation stress in *Actinomyces oris* biofilms

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Abstract

Actinomyces oris plays an important role in oral biofilm development. Like many Gram-positive bacteria, *A. oris* produces a sizable number of surface proteins that are anchored to bacterial peptidoglycan by a conserved transpeptidase named the housekeeping sortase SrtA; however, the biological role of many *A. oris* surface proteins in biofilm formation is largely unknown. Here, we report that the glycoprotein GspA – a genetic suppressor of *srtA* deletion lethality – not only promotes biofilm formation but also maintains cell membrane integrity under cation stress. In comparison to wild-type cells, under elevated concentrations of mono- and divalent cations formation of mono- and multi-species biofilms by mutant cells devoid of *gspA* was significantly diminished, although planktonic growth of both cell types in the presence of cations was indistinguishable. Because *gspA* overexpression is lethal to cells lacking *gspA* and *srtA*, we performed a genetic screen to identify GspA determinants involving cell viability. DNA sequencing and biochemical characterizations of viable clones revealed that mutations of two critical cysteine residues and a serine residue severely affected GspA glycosylation and biofilm formation. Furthermore, mutant cells lacking *gspA* were markedly sensitive to sodium dodecyl sulfate, a detergent that solubilizes the cytoplasmic membranes, suggesting the cell envelope of the *gspA* mutant was altered. Consistent with this observation, the *gspA* mutant exhibited increased membrane permeability, independent of GspA glycosylation, as compared to the wild-type strain.

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AUTHOR CONTRIBUTIONS

A.A.M.A.M. and H.T.-T. conceived and designed all experiments. A.A.M.A.M., C.W., C.C., and B.C.S. performed all experiments. A.A.M.A.M., C.W., C.C., B.C.S., A.D., and H.T.-T. analyzed data. A.A.M.A.M., A.D., and H.T.-T. wrote the manuscript with contribution and approval from all authors.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

Altogether, the results support the notion that the cell wall-anchored glycoprotein GspA provides a defense mechanism against cation stress in biofilm development promoted by *A. oris*.

Keywords

Gram-positive bacteria; *Actinomyces oris* ; biofilm formation; sortase; cation stress; glycosylation; cell wall anchoring

INTRODUCTION

In Gram-positive bacteria, such as *Actinomyces oris*, *Corynebacterium diphtheriae*, *Enterococcus faecalis*, and *Staphylococcus aureus*, cell wall-anchored proteins are important virulence determinants, although they make up a fraction of the bacterial exported proteomes (1-3). To attach surface proteins to peptidoglycan covalently, Gram-positive bacteria utilize a conserved cysteine transpeptidase, called sortase, which recognizes a conserved LPXTG motif preceding a hydrophobic domain, and a tail of positively charged residues (4, 5). These tripartite domains constitute the cell wall sorting signal (CWS) located at the C-terminus of all cell-wall anchored protein substrates (6, 7). Prior to cell wall anchoring, surface protein precursors are translocated across the cytoplasmic membrane by the Sec translocon via their signal peptides, which are removed by a signal peptidase after translocation (8). In the actinobacteria *A. oris* and *C. diphtheriae*, post-translocational folding of the surface proteins with multiple cysteine residues is catalyzed by the thiol-disulfide oxidoreductase MdbA (9, 10). Properly folded proteins are embedded into the cytoplasmic membrane via the hydrophobic domain of the CWS, and the LPXTG motif is subsequently cleaved between the threonine and glycine residues by sortase for succeeding cell wall attachment via the cleaved threonine residue (6).

Like many Gram-positive bacteria, *A. oris* – an oral colonizer that plays a critical role in the development of oral biofilms or dental plaque (11, 12) – encodes a considerable number of cell wall-anchored proteins. The *A. oris* MG1 strain harbors 18 LPXTG-containing surface proteins (13), including 4 fimbrillins (FimA, FimB, FimP and FimQ) that constitute two distinct fimbrial types (14-16). Type 1 fimbriae, composed of the pilus shaft FimP and the tip pilin FimQ, mediate bacterial adherence to proline-rich proteins that coat tooth enamel (11, 14, 16). Type 2 fimbriae, made of the pilus shaft FimA and the tip pilin FimB or CafA, promote polymicrobial interactions or bacterial coaggregation, biofilm formation, and adhesion to host cells.(11, 13, 15, 17). While biofilm formation and bacterial coaggregation are phenotypical attributes of the type 2 fimbrial shaft FimA and pilus tip-localized CafA, (15) respectively, little is known about the biological functions of the remaining surface proteins in *A. oris*.

Among 14 surface proteins of *A. oris*, GspA is of particular interest because of its biochemical connection to cell lethality when the housekeeping sortase *srtA* gene is genetically disrupted; the culprit of this lethality is the accumulation of glycosylated GspA in the cytoplasmic membrane in the absence of SrtA, resulting in toxic glycol-stress and cell arrest (18). In addition, loss of *lcpA* was found to rescue the lethality associated with a sortase mutant (18). LcpA has been proposed to encode the enzyme that catalyzes the

transfer of glycans modifying GspA (19). Of note, no glycosyltransferases were identified as genetic suppressors (18), although a large number of glycosyltransferase-encoding genes are found in the genome of *A. oris* MG1 (http://www.brop.org/), suggestive of redundancy of this class of enzymes. It is worth mentioning that overexpression of GspA in a mutant devoid of *gspA* and *srtA* is lethal, in contrast to overexpression of a GspA mutant missing the CWS (18), confirming that a membrane-stalled glycosylated GspA form is toxic to cells. However, the biochemical nature of the GspA glycans and the biological functions of the glycoprotein GspA still remain to be elucidated.

Here, we demonstrated that the cell-wall anchored protein GspA confers cation-stress resistance to *A. oris* mono- and multiple-species biofilms. Through a genetic screen that find variants suppressing the lethality caused by GspA overexpression in the *srtA* mutant, we revealed key residues affecting GspA glycosylation and resistance to cation stress in biofilms. We also showed that GspA maintains membrane integrity. Altogether, we discussed a model of GspA-mediated cation-stress resistance in *A. oris* biofilms.

MATERIALS AND METHODS

Bacterial strains, primers, plasmids, and media.

A. oris were grown in Heart Infusion (HI) broth (HIB) medium. *E. coli* strains were grown in Luria broth (LB). When needed streptomycin was added to a concentration of 25 and 40 μg/ml, in liquid and agar medium, respectively. *F. nucleatum* were grown in tryptic soy broth (TSB) supplemented with 1% Bacto™ peptone and 0.25% cysteine (TSPC) or on TSPC agar plates with 1% vitamin K1-hemin solution in a Coy anaerobic chamber (5% CO_2, 2% H_2 and 93% N_2). Streptococci were grown in Brain Heart Infusion (BHI) supplemented with 0.5% glucose. Bacterial strains and plasmids used in this study are listed in Table S1 of Supplemental Materials.

Plasmid construction

pCWU9—To generate the *A. oris*/*E. coli* shuttle vector pCWU9, the ampicillin resistance gene of pCVD047, a cyanobacterial broad-host-range vector (20), was replaced with the $Strp^R$ marker from pJRD215 (21). An amplicon containing the pCVD047 without the ampicillin resistance gene was generated using the primers CW1_F and CW1_R (Table S2), and pCVD047 DNA as a template. The fragment containing the $Strp^R$ gene and a multiple cloning site from pJRD215 was then amplified with primers CW2_F and CW2_R (Table S2). Both amplified products were digested with *Bam*HI and *Sal*I and ligated with pCVD047 amplicon without the ampicillin resistance gene to generate pCWU9.

pGspA—The plasmid pGspA was constructed by cloning a *gspA* fragment including its promoter sequence into the *Actinomyces oris*/*E.coli* shuttle vector pCWU9. The *gspA* fragment was PCR-amplified from the *A. oris* chromosomal DNA template using primers AA 64_F and AA64_R (Table S2), with the *Ndh*I and *Eco*RI sites introduced into the amplified fragment. The PCR product was digested with *Ndh*I and *Eco*RI and ligated with pCWU9 precut with the same enzymes. The generated plasmid was introduced into *E. coli* DH5α and plasmid DNA was isolated for DNA sequencing to verify the cloned *gspA*

fragment. The GspA (WP_065362444) sequences can be accessed at https://img.jgi.doe.gov/cgi-bin/m/main.cgi with the gene ID of ANA_13540.

pGspA$_{\Delta CWS}$.—The primers AA118_F and AA119_R (Table S2) were labeled with phosphate by T4 polynucleotide kinase according to the manufacturer's instructions (New England Biolabs, Inc.). These primers were used in an inverse PCR procedure as previously described (22), with pGspA as a template, to generate a plasmid expressing GspA lacking the CWS (residues 286-320). The generated plasmid was introduced into *E. coli* DH5α, and plasmid DNA was isolated for DNA sequencing to verify the truncated *gspA* sequence.

Isolation of spontaneous suppressor mutations for GspA toxicity in the Δ*srtA* mutant.

0.1 μg of pGspA DNA was electroporated into the Δ*gspA*-Δ*srtA* mutant cells (~ 5 x 10^8 CFU) according to a published protocol (23). Electroporated cells were spread on HI agar plates containing 25 μg/ml streptomycin. Plasmids from 38 viable colonies were isolated and subjected to DNA sequence to identify mutations of the *gspA* gene. Viable colonies appeared at a frequency of $8X10^{-4}$, determined by dividing the number of transformants with pGspA by the number of transformants with pCWU9 (empty vector).

Site-directed mutagenesis.

Site-directed mutagenesis of GspA was performed according to a previously published PCR-based assay (18). Briefly, phosphate labeled primer pairs with mutated nucleotides incorporated (Table S2) were used in the PCR reactions with pGspA as template. The PCR products were purified with gel extraction kits (Qiagen), ligated the ends with T4 DNA ligase, and introduced into *E. coli* DH5α by electroporation. Generated plasmids were isolated for DNA sequencing to verify intended mutations.

Cell growth assays.

A. oris strains MG1/pCWU9, Δ*gspA*/pCWU9, Δ*gspA*/pGspA, and Δ*fimA*/pCWU9 were grown overnight in HI broth containing 25 μg/ml streptomycin. Overnight cultures were used to inoculate fresh HI culture supplemented with 25 μg/ml streptomycin and 50 mM KCl and grow to OD_{600} of 0.1. Aliquots of 0.15 ml cell cultures were transferred to 96-well plates, and cell growth was monitored by optical density using a microplate reader (Synergy HT, BioTek) for 24 h with OD_{600} taken every 30 min. Data presented are the means of 3 independent experiments performed in triplicate.

Cell fractionation and immunoblotting.

Overnight cultures of *A. oris* strains were used to inoculate fresh cultures in HI broth (20-fold dilution) and grow at 37°C to OD_{600} between 0.35 and 0.5. Normalized cultures were subjected to cell fractionation as previously reported (24). Protein samples collected from supernatant (S), cell wall (W), and protoplast fractions were solubilized in sodium dodecyl sulfate (SDS) containing sample buffer, separated by SDS-PAGE using 4-20% gradient gels, immunoblotted with specific antibodies against GspA (α-GspA; 1:1000 dilution), and detected by chemiluminescence.

Biofilm assays.

To cultivate monospecies biofilms, overnight cultures were diluted to OD_{600} of 0.05 in HI broth containing 0.5% sucrose and with or without 50 or 100 mM KCl. Aliquots of 1.5 ml cell cultures were transferred to 24-well plates (Greiner Bio-One), which were incubated at 37°C with 5% CO_2 for 48 h (static growth). Biofilm production was quantified according to a published protocol using crystal violet (25). Briefly, cultivated biofilms were washed, air-dried overnight, stained with 0.5% (w/v) crystal violet for 10 min, rinsed with sterile water, air-dried for 3 h, and destained with 1.5 ml of 95% ethanol for 1 h at room temperature. Biofilm production was quantified, with 150 μl aliquots, by measuring absorbance at 580 nm using a Magellan plate reader. The results were expressed as average with standard deviations from three independent experiments performed in triplicate.

For multispecies biofilms, *A. oris, F. nucleatum* and *S. oralis* were used according to a previously reported protocol with some modifications (26). Briefly, *A. oris* cells (~10^8 CFU) were grown in 3 ml HI broth supplemented with 50 mM KCl for 6 h at 37°C in in glass-bottom microwell dishes (35-mm diameter, 14-mm microwell; MatTek Corporation, MA). Unattached cells in wells were aspirated, and 3 ml HI medium with *S. oralis* and *F. nucleatum* (10^8 CFU each) containing 50 mM KCl was added to the wells. Bacterial cells were maintained in an anaerobic chamber for 42 h at 37°C. For *A. oris* Δ*gspA* cells with plasmids expressing $GspA_{\Delta CWS}$ or GspA mutants, the initial 6 h growth was carried out in HI medium with 50 mM KCl and 25 μg/ml streptomycin; the medium was aspirated; and other strains were then added and grown for 42 h in HI supplemented with 50 mM KCl without streptomycin. Biofilms were then washed with PBS (phosphate buffered saline). Biofilm cells were hybridized with FITC, Cy5, or Sp blue-labeled DNA probes specific for *A. oris* (gct acc gtc aac cca ccc) (green), *F. nucleatum* (ccc taa ctg tga ggc aag) (red) and *S. oralis* (cc aca gcc ttt aac ttc aga) (blue), respectively (27), in hybridization buffer (900 mM NaCl, 20 mM Tris-HCl pH8, 0.01% SDS, 20% formamide and 5 mM ethylene diamine tetraacetic acid), and washed with wash buffer (278 mM NaCl, 20 mM Tris-HCl pH7.5 and 0.01% SDS) before conformal microscopic analysis using 40x magnification. The results are representative of three independent experiments performed in triplicate.

SDS sensitivity.

Overnight cultures of various strains were normalized to OD_{600} of 1, and the resulting cultures were serial-diluted 1:10; 5 μl of the cell cultures was spotted onto HI agar plates with or without 0.002% SDS. Cell growth was imaged after incubation at 37°C for 48 h.

Cell membrane permeability.

Cell membrane permeability of *A. oris* strains was determined by the release of intracellular dye calcein according to a published protocol (28). Accordingly, biofilm cells grown for 48h as aforementioned were collected, washed once with 10 mM Na-phosphate buffer, pH 7, and suspended in the same buffer to OD_{600} of 0.1. One ml of cell suspension was treated with 10 μM calcein-AM for 4 h at room temperature, followed by washing twice with an equal volume of 10 mM Na-phosphate. Calcein-AM-loaded bacteria were resuspended in 10 mM Na-phosphate buffer, and 100 μl of the cell suspension was dispensed 96-well microplates (Greiner), with each well containing 100 ul of 10 mM Na-phosphate with or without KCl

(100 or 200 mM). Calcein released from cells was monitored every 10 min for 120 min using a microplate reader, with the fluorescence signal collected at the excitation wavelength of 485 nm and the emission wavelength of 530 nm; cells without calcein were used as a negative control. The results were presented as an average of at least three experiments performed in triplicate, with the presented values as sum of all 10-min readings.

Electron Microscopy.

Surface localization of GspA was determined by immunoelectron microscopy as previously described (13). Briefly, *A. oris* cells of various strains were immobilized onto nickel-coated carbon grids, washed, and stained with α-GspA (1:100), followed by staining with IgG conjugated with 18-nm gold particles. Finally, samples were washed five times with water, stained with 1% uranyl acetate, and viewed with an electron microscope.

Statistical analysis

P values were determined by two-tailed Student's *t*-test for strains compared in parallel in the same experiments. *, ** and *** indicate p values ≤ 0.05, 0.005 and 0.0005, respectively.

RESULTS

GspA is involved in biofilm formation under cation stress.

It was revealed that the cell wall-anchored glycoprotein GspA is genetically linked to the essentiality of the housekeeping sortase SrtA (18); however, the role of GspA in the physiology of *A. oris* is unknown. To identify the potential biological function of GspA, we performed BLAST search, revealing GspA homologs in many *Actinomyces* species, mostly oral bacteria, as well as other actinobacteria commonly isolated from soil, deep-sea sediment, and contaminated ground water (Fig. S1 and Tables S3 & S4). That bacteria from soil, deep-sea sediments encounter high salt conditions (29-31) raises the possibility that *A. oris* GspA might be linked to cation stress. To test this, we cultivated *A. oris* biofilms in the presence of different concentrations of NaCl or KCl, and biofilm production was quantified by a crystal violet method (see Methods). In the absence of cations, both wild-type strain and its mutant lacking *gspA* produced biofilms at a similar level (Fig. 1a). As the concentrations of NaCl and KCl increased, the *gspA* mutant exhibited a significant defect in biofilm formation, as compared to the wild-type strain, and the biofilm defect was more profound with 50 and 100 mM KCl than with those of NaCl (Fig. 1a). This defect was not due to excess chloride as biofilm production in the *gspA* mutant was drastically reduced when biofilms were cultivated in the presence of 20 mM K_2HPO_4 (Fig. 1b). The larger effect of K_2HPO_4, as compared to KCl, in biofilm formation is likely due to potassium ions, although a change in pH or addition of PO_4 ions might also contribute to this process. Significantly, the biofilm defect caused by the high concentrations of potassium was rescued by ectopic expression of GspA in the *gspA* mutant (Fig. 1c). For comparison, a mutant devoid of FimA, a well-characterized fimbrial protein essential for biofilm formation in *A. oris* (15), was included in this assay and exhibited a severe biofilm defect (Fig. 1B, Δ*fimA*), suggesting that GspA may play a different role in biofilm formation when cells are under cation stress. Of note, the growth of the Δ*gspA* mutant was not inhibited by the presence of

50 mM KCl, as compared to that of the WT strain (Fig. 1d), indicating that the defect in biofilm formation is not due to growth deficiency.

Since Na^+ and K^+ are monovalent, we next examined whether divalent cations have any effects on biofilm formation. With a protocol similar to the experiment described in Fig. 1a, biofilms of wild-type and Δ*gspA* mutant strains were cultivated in the presence of increasing concentrations of $MgCl_2$, $FeCl_2$ and $ZnCl_2$. As shown in Fig. 2, the Δ*gspA* mutant displayed at least 2-fold reduction in biofilm formation, as compared to the WT, in the presence of 10 mM $MgCl_2$ (Fig. 2a), 3 mM $FeCl_2$ (Fig. 2b), or 0.5 mM $ZnCl_2$ (Fig. 2c). At higher concentration of $MgCl_2$ (25 mM), $FeCl_2$ (4 mM), or $ZnCl_2$ (1 mM), both wild-type and Δ*gspA* mutant strains produced the same reduced levels of biofilms (Fig. 2); this suggest excessive levels of these cations might be toxic to cells. Altogether, the results support that GspA is required for biofilm formation under cation stress.

GspA maintains multispecies-biofilm formation under cation stress.

Considering that *A. oris* is a key colonizer in the development of oral biofilms (32), we next examined if GspA is required for formation of multispecies biofilms under cation stress. Adapting a previously published protocol (33), we cultivated a three-species biofilm, whereby *A. oris* cells were seeded onto multi-well plates in the absence or presence of 50 mM KCl, followed by seeding the early colonizer *Streptococcus oralis* and then *Fusobacterium nucleatum* cells. The biofilms were maintained in an anaerobic chamber for 42 h, and the presence of each bacterial species in the biofilms was detected by hybridization using fluorescent DNA probes specific for each species (see Methods). As shown in Fig. 3a, with the *A. oris* wild-type strain, the three-species were abundantly detected, in contrast to the biofilms cultivated with the Δ*gspA* mutant that the fluorescent signal for each species was drastically reduced. This defect was rescued by ectopic expression of GspA.

As mentioned above, GspA harbors a CWSS (13), which is essential for attachment to the bacterial cell wall because a protein mutant lacking the CWSS, i.e. $GspA_{\Delta CWS}$, is released into the extracellular milieu and substantially reduced in glycosylation (18, 34). To examine if the released GspA protein contributes to biofilm formation under cation stress, we used the $GspA_{\Delta CWS}$ mutant in the above multi-species biofilm assay. As shown in Fig. 3d, expression of $GspA_{\Delta CWS}$ in the Δ*gspA* mutant failed to rescue the biofilm defect of this mutant, although patches of biofilm cells were observed, as compared to strain expressing the wild-type GspA. It is noteworthy that when the same set of strains used in Fig. 3 was subjected to the three-species biofilm assay without 50 mM KCl, no significant defects in biofilm formation were observed among the 4 aforementioned strains (Fig. S2). Altogether, the results indicate that the cell wall-anchored form of GspA is required for formation of multispecies biofilms under cation stress.

A genetic screen identified *gspA* mutants defective in biofilm formation under cation stress.

Given that GspA is highly glycosylated (18) and GspA is required for biofilm formation under cation stress as reported above, we next examined if GspA glycosylation is needed for this process. Because the biochemical nature of GspA glycans and glycosylation sites

were unknown, we initially sought to address this by a genetic approach based on our previous observation that overexpression of glycosylated GspA in an *A. oris* mutant lacking both *gspA* and *srtA* (Δ*gspA*-Δ*srtA*) is lethal (18). Therefore, we surmised that mutations that affect GspA secretion, glycosylation sites, and surface display would give rise to viable clones (suppressors). To test this, we transformed the Δ*gspA*-Δ*srtA* strain with the plasmid pGspA that overexpressed GspA and found 38 viable clones. Plasmid DNA was isolated and subjected to DNA sequencing to identify mutations in the GspA open reading frame (ORF), revealing missense and in-frame deletion mutations within 8 suppressors and insertion/deletion (frameshift) mutations within the remaining suppressors (Fig. 4a and Table S5). Since the frame-shift mutations disrupt the GspA ORF, we focused on the former consisted of 4 base-substitution mutants (G55R, C58A, G74C and S91P) and 4 in-frame deletion mutants (D1, deletion of residues 10-23; D2, Δ98-139; D3, Δ130-171; and D4, Δ235-318) (Fig. 4a). We then analyzed the sequences within the D1-4 deletion regions and generated alanine-substitution of residues that might potentially be involved in glycosylation. Using pGspA as a template for site-directed mutagenesis, we reconstructed 26 mutants including the four base-substitution mutants. The resulting plasmids were individually introduced into the Δ*gspA* strain by electroporation, and GspA glycosylation was analyzed by western blotting, whereby cells of indicated strains were subjected to fractionation and protein samples in culture medium (S), cell wall (W) and protoplast (P) fractions were immunoblotted with antibodies against GspA (α-GspA) as previously reported (18).

Out of 26 reconstructed mutants, 7 mutants, including the double mutant C58A/C122A, were defective in GspA glycosylation (Table 1). It is worth mentioning that the remaining 19 mutants exhibited a wild-type level of glycosylation (Table 1), although the original mutants were viable in the background of *gspA* and *srtA* deletion. It is likely that in these original mutants additional spontaneous mutations targeting chromosomal genes that suppress lethality of GspA overexpression in the absence of *gspA* and *srtA*. Intriguingly, compared to the wild-type strain, which produced glycosylated GspA polymers ($GspA_P$) found in the cell wall fraction, the C58A, C122A, or C58A/C122A mutant exhibited a severe defect in formation of high molecular mass polymers in the cell wall fractions, leading to accumulation of the low molecular mass species in both cell wall and protoplast fractions (Fig. 4b). Similarly, the G55R and G74C also displayed significant defects in GspA glycosylation (Fig. S3). Considering the essential role of disulfide bond formation in *A. oris* (9, 35), we speculated that C58 and C122 form a disulfide bond that is critical for post-translocational protein folding. Significantly, unlike the wild-type GspA, the S91P mutant was unable to produce wild-type GspA polymers with accumulation of the low molecular mass species in the cell wall and protoplast fractions; it is noteworthy that alanine-substitution of the tryptophan residue preceding S91 also caused a similar glycosylation defect (Fig. 4c). Since serine is involved in bacterial O-linked glycosylation (36, 37), we surmised that S91 may serve as a major glycosylation site in GspA, and W90 may play an important role in GspA glycosylation at this site.

To determine if these mutations affect GspA localization on the cell envelope, we subjected the aforementioned strains to immuno-electron microscopy (IEM) following a previously published protocol (22). Briefly, *A. oris* cells were immobilized on a nickel grid, stained

with α-GspA, followed by staining with IgG-conjugated gold particles, and analyzed by an electron microscope. In the parental strain, GspA signal was observed on the cell surface, esp. more abundant at the cell poles than the septa; the polar localization of GspA was absent in the Δ*gspA* mutant; and the defect was rescued in the Δ*gspA* mutant ectopically expressing GspA (Fig. S4; panels a-c). Similar localization of GspA was also detected in the mutant strains with C58A/C122A, W90A, and S91A mutations (Fig. S4, panels d-f). The results indicate that glycosylation does not affect cell surface localization of GspA.

We next examined if the mutants above have any defects in formation of mono- and multispecies biofilms under cation stress as described in Figures 1 and 3. As shown in Fig. 4D, the three mutants W90A, S91P, and C58A/C122A were significantly defective in formation of monospecies biofilms as compared to the wild-type GspA strain in the presence of 50 mM KCl and biofilm conditions without KCl. Consistent with this result, alanine-substitution of W90 or S91 also caused significant defects in formation of three-species biofilms (Fig. 5).

gspA mutants exhibit increased membrane permeability.

Since GspA confers cation tolerance to *A. oris* biofilms as presented above, we hypothesized that GspA might provide a protective shield to cation assaults. To test this, we first subjected the wild-type and Δ*gspA* mutant strains to a sensitivity assay with sodium-lauryl sarcosinate (SDS), a detergent that is known to solubilize the bacterial cytoplasmic membrane (38). In this assay, overnight cultures of *A. oris* cells were serially diluted and spotted on HI agar plates containing 0.002% SDS, and cell growth at 37°C was recorded after 48 h. As shown in Fig. 6A, the Δ*gspA* mutant was more sensitive to SDS treatment than the WT strain, suggesting that the cell envelope of the Δ*gspA* mutant is compromised.

To investigate further, we determine membrane permeability of *A. oris* strains by measuring the release of intracellular calcein-AM (calcein acetoxymethyl ester) as previously reported (28), whereby after taken up by bacterial cells, calcein-AM is cleaved by intracellular esterases to produce the fluorescent indicator calcein, leakage of which is detected by fluorescence measurement. In this experiment, biofilms of WT and Δ*gspA* strains were cultivated and loaded with Calcein-AM. The calcein release was monitored every 10 min for 2 h in the absence or presence of 50 or 100 mM KCl. Compared to the wild-type strain, the release of calcein was significantly higher in Δ*gspA* mutant (Fig. 6b). To determine whether GspA glycosylation plays roles in the maintenance of membrane integrity, Δ*gspA* biofilm cells bearing the plasmids expressing glycosylation-defective *gspA* mutants were evaluated for the release of the dye. As shown in Fig. 6c, the calcein release from the tested mutants was comparable to that from the wild-type GspA, suggesting that GspA glycosylation is not critical for membrane integrity.

DISCUSSION

In the biogeographical landscape of the oral biofilms, *Actinomyces* spp. are found at the interface between the tooth surface and microbiota (39), interacting with host receptors and a plethora of microbial species including oral streptococci and many early colonizers, promoting formation of a tight-knit, spatially structured community, or oral biofilms (40-43).

As such, *Actinomyces* are expected to encounter a variety of stresses, e.g. oxidation and cations, inflicted by the host cells and the surrounding environment. How *Actinomyces* cope with high concentrations of cations, such as potassium and sodium in the range of 20-50 mM in the oral cavity (44), has not been known. Here we show for the first time that a cell-wall anchored glycoprotein, GspA, promotes cation-stress resistance in *Actinomyces* biofilms.

Informed by homology and BLAST analyses, which revealed GspA homologs are from actinobacteria found in soil and deep-sea sediment, which contain high salt concentrations (Fig. S1 and Tables S1 & S2), a possible role of *A. oris* GspA in cation stress was investigated. Indeed, we showed that GspA is required for biofilm formation under high concentrations of either monovalent or divalent cations (Figures 1 & 2). This cation-stress resistance mediated by GspA is not limited to mono-species biofilms as we demonstrated that GspA is critical for formation of multispecies biofilms cultivated in high concentrations of KCl (Fig. 3). The multispecies-biofilm defect is not due to the defect in cell growth of the tested mutants since they formed biofilms with the same level as the parent strain in the conditions lacking high concentrations of cations (Fig. S2). Because GspA is a cell wall-anchored protein, which requires its CWS, the lack of the CWS caused a significant defect in formation of multispecies biofilms (Fig. 3d); it is interesting to note, however, that some patches of biofilms were observed in this mutant (Fig. 3d; Δ*gspA*/pGspA$_{\Delta CWS}$), unlike the *gspA*-null mutant with a severe defect in biofilm formation (Fig. 3b). It is unclear what contributes to this spotty phenotype; nonetheless, considering that GspA lacking the CWSS is released into the culture medium (18), we speculate that the secreted form of native GspA might offer some protection to biofilms from cation stress.

Given that GspA is highly glycosylated (18), it is logical to examine if glycosylation is required for cation-stress resistance by *A. oris* biofilms. Since the biochemical nature of GspA glycans had not been revealed, we turned to a genetic approach that identified residues severely affecting GspA glycosylation; among those are C58 and S91 (Figures 4 & 5). Considering that disulfide bond formation is important in post-translocational folding of exported proteins (9, 35, 45), we surmised that a disulfide bond formed between C58 and C122 is critical for oxidative folding of GspA, hence its functionality; indeed, alanine-substitution of these residues abrogated GspA glycosylation (Fig. 4B). As serine is a site of bacterial O-glycosylation (36, 37), it is likely that S91 is a major glycosylation site with the preceding residue W90 that might assist in GspA glycosylation. However, it is unclear how G55 and G74 are involved in GspA glycosylation. Future biochemical experiments will examine these intriguing issues.

How does the cell wall-anchored glycoprotein GspA confer cation-stress resistance to biofilms? It is noteworthy that at neutral pH, a condition found in clinically healthy gingiva (46) and laboratory growth medium, e.g. heart infusion broth, matured GspA is predicted to be negatively charged as its isoelectric point (pI) is approximately 4.64. In addition, GspA glycans may possess active groups with negative charges, such as phosphate and carboxylic. Thus, the net negative charge of glycosylated GspA might contribute to ionic interactions that neutralize elevated concentration of cations. How does GspA then contribute to membrane permeability as shown in Fig. 6? Given that glycosylation mutants

display the same level of released fluorescent calcein as the wild-type GspA (Fig. 6c), we think that the loss of the negative charge property of GspA might not account for the loss of membrane permeability. It is possible that as GspA is an abundant protein that traverses through the membrane (18), the lack of GspA, via genetic disruption, might perturb the membrane integrity. As such, our findings show that the cell wall anchored glycoprotein GspA confers cation stress resistance not only to the bacterium but also its community in biofilm conditions, raising the possibility of targeting cell wall anchored proteins could prevent *A. oris*-associated biofilm formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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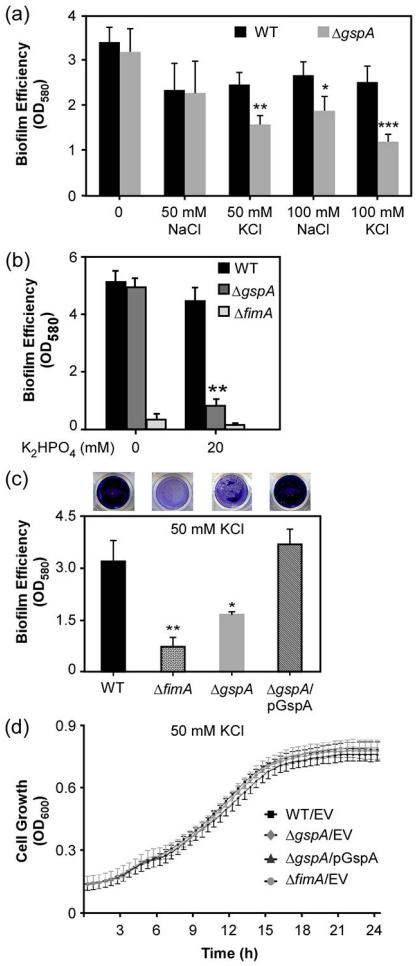

Fig. 1. Requirement of GspA in formation of *A. oris* biofilms in elevated concentrations of monovalent cations.

(a-c) The wild-type MG1 and its variant strains were used to cultivate biofilms at 37°C for 48 h in the absence or presence of indicated cations with various concentrations. Biofilm production was quantified by a crystal violet assay measured at OD_{580}. Representative crystal violet stained wells for biofilm formation in 50 mM KCl were shown in panel C, with the quantification graph below. (d) Cell growth of indicated strains in the presence of 50 mM KCl was monitored by OD_{600} for 24 h. Data presented are the means of 3 independent experiments performed in triplicate; EV, empty vector pCWU9. Statistical analysis was performed with two-tailed Student's *t*-test for strains compared in parallel in the same experiments, using GrapPad Prism; *, ** and *** indicate *p* values ≤ 0.05, 0.005 and 0.0005, respectively.

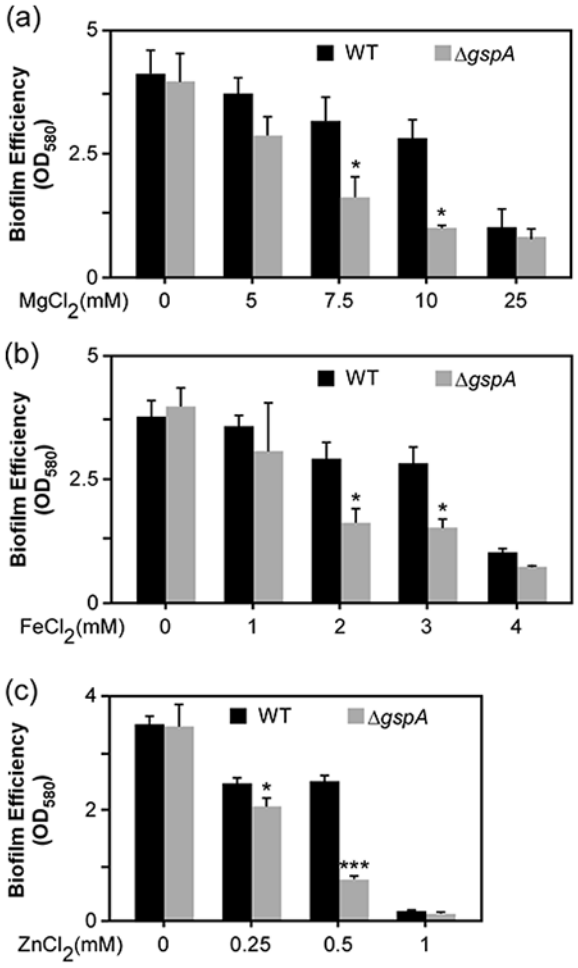

Fig. 2. Requirement of GspA in formation of *A. oris* biofilms in elevated concentrations of divalent cations.

Biofilm experiments and quantification were performed as described in Fig. 1 in the absence or present of various concentrations of $MgCl_2$ (a), $FeCl_2$ (b), and $ZnCl_2$ (c). The results presented are average of at least three independent experiments performed in triplicate; *, ** and *** indicate p values ≤ 0.05, 0.005 and 0.0005, respectively.

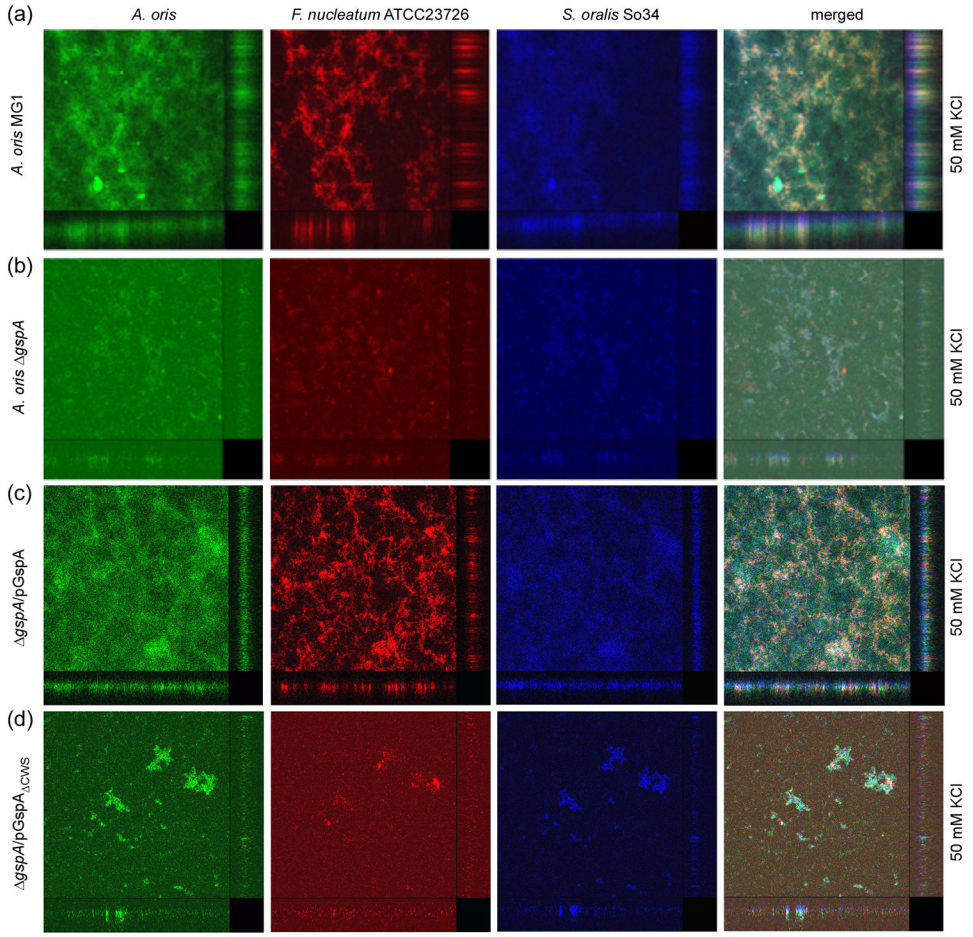

Fig. 3. GspA-mediated tolerance to cations in three-species biofilms.

(a-d) *A. oris* MG1 and its variants were used to cultivate three-species biofilms with *F. nucleatum* ATCC23726 and *S. oralis* So34 in the presence of 50 mM KCl at 37°C for 42 h. Each bacterial species was detected by hybridization with fluorescence tagged DNA labels (FITC, *A. oris*; Cy5, *F. nucleatum*; or Sp blue, *S. oralis*), and biofilms were analyzed by confocal microcopy using 40x magnification. The results presented are representative of three independent experiments performed in triplicate.

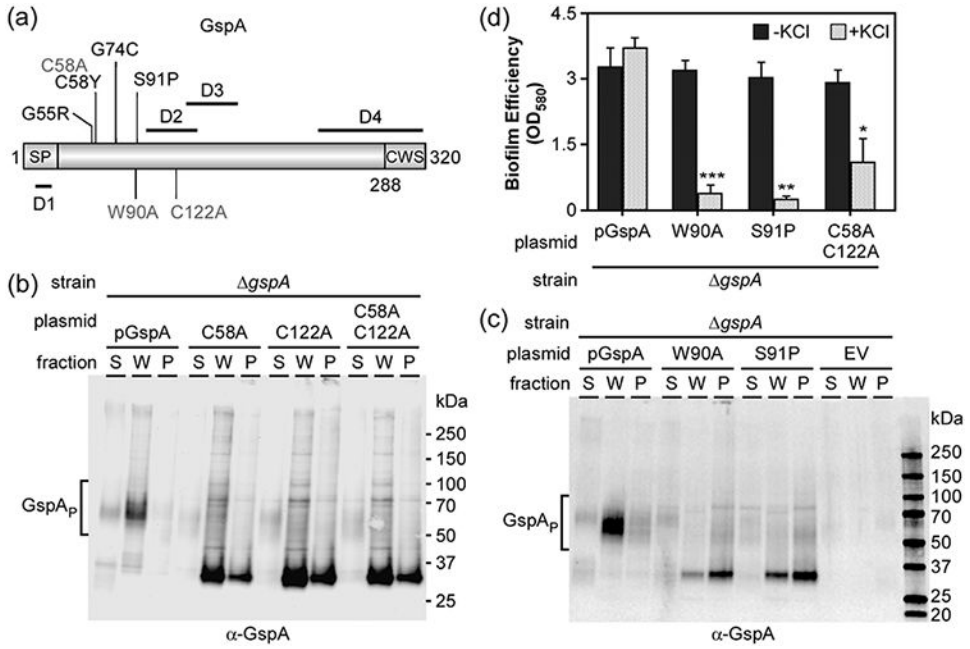

Fig. 4. Involvement of GspA glycosylation in cation tolerance in biofilm formation.

Panels are shown in a counterclockwise direction from (a), which presents a schematic of GspA showing the N-terminal signal peptide (SP) (residues 1-18) and the C-terminal cell wall sorting signal (CWS) (residues 288-320). Point mutations, i.e. G55R, C58Y, etc., and deletion (D) mutations identified by the GspA suppressor screen are marked along the GspA sequence. D1, D2, D3 and D4 are deletion mutations from residues 10-23, 98-139, 130-171 and 235-318, respectively. (b-c) Mid-log phase cells of the Δ*gspA* mutant expressing wild-type GspA (pGspA) or its variants were normalized and subjected to cell fractionation. Protein samples obtained from culture supernatant (S), cell wall (W) and protoplast (P) fractions were analyzed by immunoblotting with a polyclonal antibody against GspA (α-GspA), using 4-20% Tris-glycine gradient gels. GspA polymers (P) and molecular weight markers in kDa are shown. (d) Biofilm formation of the Δ*gspA* mutant expressing wild-type GspA or its variants in the presence or absence of 50 mM KCl was determined by the crystal violet assay as mentioned in Fig. 1c. The data presented is average of at least three experiments performed in triplicate.

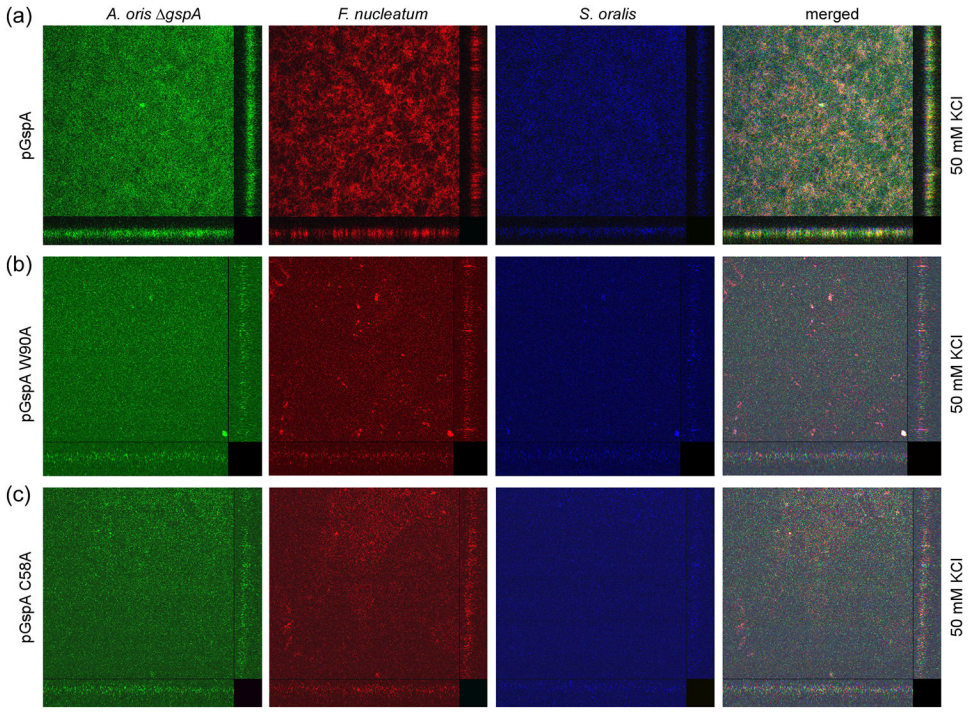

Fig. 5. Requirement of residues affecting GspA glycosylation in formation of multispecies biofilms under cation stress.

(a-c) Multispecies-biofilm formation of indicated *A. oris* strains with *F. nucleatum* and *S. oralis* in the presence of 50 mM KCl was determined by confocal microscopy as described in Fig. 2. The results are representative of three independent experiments performed in triplicate.

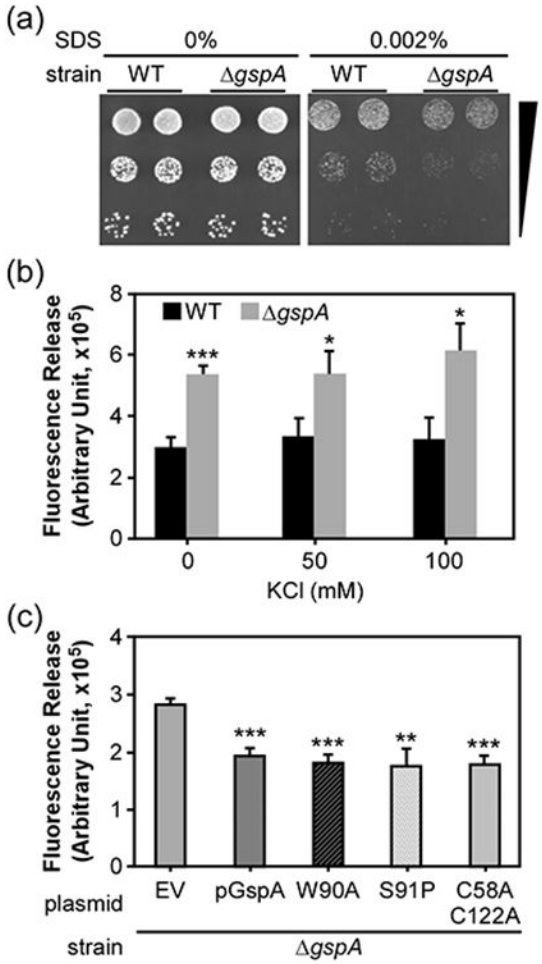

Fig. 6. Involvement of GspA in cell membrane integrity.

(a) Overnight cultures of the wild-type strain and its variant (Δ*gspA*) were normalized to OD_{600} of 1.0. 5-μl aliquots of 1/10 serial diluted cells were spotted onto HI medium without or with 0.002% SDS and grown for 48 h. (b) The cell membrane permeability of *A. oris* WT and Δ*gspA* biofilm cells was determined by a calcein-release assay in the absence and presence of 50 and 100 mM KCl. Release of the fluorescein is expressed as arbitrary unit. (c) The membrane permeability of the Δ*gspA* mutant strains carrying an empty vector (EV), or a plasmid expressing wild-type GspA (pGspA) or its variants was determined similarly as described in (b). All results are presented as an average of at least two independent experiments performed in triplicate.

Table 1:

GspA mutants tested for glycosylation[a].

Mutant	Glycosylation	Mutant	Glycosylation
P52A	ND	N95A	ND
S54A	ND	P97A	ND
G55R	defective	T109A	ND
S56A	ND	S112A	ND
C58A	defective	C122A	defective
T59A	ND	T141A	ND
K61A	ND	Q157A	ND
G74C	defective	P168A	ND
S77A	ND	T173A	ND
N89A	ND	C58A C122A	defective
W90A	defective	QDQQ131-134 to ADAA	ND
S91P	defective	QQ133-34 to AA	ND
TT92-93AA	ND	TY146-147AA	ND

[a]Glycosylation of *A. oris* MG1 and its variants harboring individual GspA mutants (see Table S3) was analyzed by immunoblotting with α-GspA. The wild-type strain MG1 was used as reference for defective or not defective in glycosylation (ND) phenotypes.