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Host Defense Proteins Derived from Human Saliva Bind to *Staphylococcus aureus*

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Proteins in human saliva are thought to modulate bacterial colonization of the oral cavity. Yet, information is sparse on how salivary proteins interact with systemic pathogens that transiently or permanently colonize the oral environment. *Staphylococcus aureus* is a pathogen that frequently colonizes the oral cavity and can cause respiratory disease in hospitalized patients at risk. Here, we investigated salivary protein binding to this organism upon exposure to saliva as a first step toward understanding the mechanism by which the organism can colonize the oral cavity of vulnerable patients. By using fluorescently labeled saliva and proteomic techniques, we demonstrated selective binding of major salivary components by *S. aureus* to include DMBT1$^{gp\text{-}340}$, mucin-7, secretory component, immunoglobulin A, immunoglobulin G, S100-A9, and lysozyme C. Biofilm-grown *S. aureus* strains bound fewer salivary components than in the planctonic state, particularly less salivary immunoglobulins. A corresponding adhesive component on the *S. aureus* surface responsible for binding salivary immunoglobulins was identified as staphylococcal protein A (SpA). However, SpA did not mediate binding of nonimmunoglobulin components, including mucin-7, indicating the involvement of additional bacterial surface adhesive components. These findings demonstrate that a limited number of salivary proteins, many of which are associated with various aspects of host defense, selectively bind to *S. aureus* and lead us to propose a possible role of saliva in colonization of the human mouth by this pathogen.

Saliva plays a key role in host defense against invading pathogens (1–4). Among the more than 2,000 proteins and peptides found in saliva (5), many exhibit direct antimicrobial activity (6). Others can bind to bacteria to facilitate either their colonization on oral surfaces or their clearance from the oral cavity through agglutination (7, 8). It has been suggested that systemic pathogens can be killed, inactivated, or agglutinated by salivary components and, thus, become cleared from the oral cavity through swallowing, thereby preventing them from colonizing the oral cavity of healthy individuals (2, 9). Thus, binding of salivary proteins to pathogens is thought to play an important role in preventing systemic infections. In hospitalized patients, the protective and antimicrobial functions of saliva, which play a crucial role in host defense against invading pathogens (1–3), are frequently impaired by reduction of salivary flow or lack of salivary secretion (9–11). Under such conditions of dry mouth and poor oral hygiene, the normal commensal oral microflora shifts to a community that harbors a higher number of pathogens (12, 13).

Among the various systemic pathogens in the oral cavity, attention has been given to *Staphylococcus aureus* (14, 15), since both endocarditis and pneumonia have been related to oral colonization by this organism (16, 17). Studies have shown the occurrence of *S. aureus* in oral biofilm and saliva of healthy individuals (18), but its frequency was found higher in elderly and institutionalized individuals, including hospitalized and nursing home patients (9, 19). Yet, in spite of the well-described associations between salivary dysfunction, biofilm formation, and bacterial colonization, only a few studies have investigated the adhesive interactions of salivary components with medical pathogens, in particular *S. aureus* (20–24). Here, as a first step toward understanding the mechanism by which pathogens can colonize the oral cavity of vulnerable patients, *S. aureus* was chosen as a model organism to identify specific salivary components that bind to the bacterium and to elucidate the role of biofilm formation for the bacterium's ability to bind salivary proteins.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. aureus* NCTC 8325 and *Staphylococcus epidermidis* RP62a (ATCC 35984) were kindly provided by Steven Gill (25), *S. aureus* strains NCTC 8325-4 (cured of three resident prophages present in NCTC 8325) (26) and DU 5875 (a Δ*spa*::Tcr protein A deletion mutant of NCTC 8325-4) were kindly provided by Mathias Herrmann (27). A Δ*spa*-complemented *S. aureus* strain (DU83/253) was kindly provided by Timothy Foster (27). Five different *S. aureus* isolates from ventilated patients previously characterized by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were also studied (17). All *S. aureus* strains as well as *Streptococcus gordonii* CH1 (Challis) were cultured in tryptic soy broth (TSB; BD Bacto, Franklin Lakes, NJ) supplemented with 5% yeast extract (BD Bacto, Franklin Lakes, NJ) under static conditions aerobically at 37°C overnight as previously described (24, 28). The optical density (OD) of bacterial suspensions was measured at 600 nm using a spectrophotometer (DU 800 UV/visible spectrophotometer; Beckman Coulter, Fullerton, CA) and adjusted to an OD of 1.0, corresponding to about 10^9 organisms per ml, before use in binding assays.

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For testing different growth media which have been described to induce *S. aureus* biofilm formation (29), inocula of overnight cultures (25 μl) were transferred into 5 ml of fresh TSB, TSB supplemented with yeast (TSBY), or TSB supplemented with 0.5% glucose and 3.0% sodium chloride (TSBGN) in 6-well tissue-culture microtiter plates (tissue culture-treated polystyrene, flat-bottom, number 353046; BD Falcon, Franklin Lakes, NJ). The plates were incubated statically at 37°C for 21 h aerobically. Culture supernatants were decanted and nonadherent bacteria removed by rinsing with 5 ml of phosphate-buffered saline (PBS; 20 mM sodium phosphate, 150 mM NaCl, pH 7.2) containing 0.04% NaN_3. For visualization, adherent biofilms were fixed with 100% ethanol and air dried prior to staining for 2 min with 5 ml of 0.4% (wt/vol) crystal violet (C-0775; Sigma, St. Louis, MO) in 12% ethanol. Dye was decanted, and wells were washed with deionized distilled H_2O until negative-control wells became transparent. After the plates were dried, the extent of biofilm formation was documented by photography.

To obtain a large quantity of biofilm-grown cells for saliva-bacterium binding assays, aliquots from overnight cultures (150 μl) were transferred into 30 ml of TSBGN in polystyrene flasks (ventilated plug, tissue culture treated, 250 ml; BD Falcon, Bedford, MA). The flasks were incubated statically at 37°C for 21 h aerobically. Culture supernatants were decanted, and the flasks were gently rinsed twice with 30 ml of PBS to remove nonadherent bacteria. Biofilm-grown cells were harvested from the bottom of the flasks by scraping off the bacteria with a sterile cell scraper (25-cm handle; BD Falcon, Bedford, MA). All bacterial samples were centrifuged at 7,000 × *g* for 5 min, washed three times in PBS, and suspended to an OD_{600} of 1.0 in PBS before use in the saliva-bacterium binding assay.

Collection of saliva and fluorescence labeling. Collection of saliva from a single systemically and dentally healthy individual was performed as approved by the Health Science Institutional Review Board of the University at Buffalo. All saliva samples were collected in 50-ml polypropylene tubes (conical bottom; Corning) on ice. Whole saliva (WS) stimulated by chewing paraffin wax was clarified by centrifugation at 11,000 × *g* for 10 min at 4°C. Parotid saliva and submandibular-sublingual saliva were collected following stimulation of salivary flow by application of 2% citric acid to the dorsum of the tongue. Parotid saliva was collected from the orifice of Stensons's duct using a modified Carlsen-Crittenden device, and submandibular-sublingual saliva was collected from the floor of the mouth with disposable plastic transfer pipettes (VWR International, Radnor, PA) as previously described (21). Protein concentrations of clarified WS were determined using the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockland, IL), with bovine serum albumin (BSA; fraction V; Sigma, St. Louis, MO) as the standard.

For fluorescence labeling of salivary proteins, clarified WS was reacted with Cy3 mono ester (NHS-Cy-dye; GE Healthcare, Piscataway, NJ). For each 6 ml of saliva, 20 μg of NHS-Cy3-dye was added to obtain a final concentration of ~3 μg per ml. The mixture was incubated in the dark at 4°C for 30 min. A total of 120 μg of lysine (Sigma, St. Louis, MO) was then added and incubated for an additional 10 min to quench free NHS-Cy3-dye. Protein concentrations of WS were determined prior to labeling, using the BCA protein assay.

Saliva-bacterium binding assay. Bacterial cells were harvested from 6 ml of bacterial suspension (10^9 organisms/ml) by centrifugation at 7,000 × *g* for 10 min. Growth medium was removed by washing the bacterial pellet three times with 6 ml of PBS containing 0.04% NaN_3. The washed pellet was then resuspended in 6 ml of clarified unlabeled or Cy3-labeled saliva or PBS, as a control, vortexed briefly, and incubated for 30 min at 37°C, occasionally inverting to mix. The suspension was then centrifuged at 7,000 × *g* for 10 min at 4°C, and the bacterial pellet was washed three times with 6 ml of PBS (NaN_3 0.04%). The resulting pellet was resuspended in 1 ml of PBS, transferred into a 1.7-ml microtube, and centrifuged at 7,000 × *g* for 5 min, and the supernatant was removed. To release salivary proteins bound to *S. aureus*, 100 μl of elution buffer (2% SDS in PBS containing 0.1% NaN_3) was added to the pellet, and the suspension was vortexed and then incubated for 30 min at room temperature using a rotator (Roto-Torque; Cole-Parmer, Chicago, IL), followed by centrifugation at 7,000 × *g* for 5 min. For elution of salivary proteins bound to *S. gordonii*, the bacterial suspension was heated for 5 min at 85°C in elution buffer and centrifuged at 7,000 × *g* for 5 min. The supernatants (~ 90 μl), here termed salivary eluate, were recovered and used for analysis of bacterium-bound salivary proteins. Protein concentrations in eluates were determined using the BCA protein assay.

Saliva binding to protein A-coated beads. Sepharose beads coated with staphylococcal protein A (protein A Sepharose CL-4B; GE Healthcare, Piscataway, NJ) and, as controls, uncoated Sepharose beads were hydrated in sterile distilled H_2O by following the instructions of the manufacturer to obtain a final volume of about 0.1 ml. The beads were washed twice in a microtube with 1 ml of sterile distilled H_2O and three times with 1 ml of PBS containing 0.04% NaN_3. The beads were harvested by centrifugation at 100 × *g* for 1 min. The washed beads were resuspended in 1 ml of Cy3-labeled WS or, as a control, PBS and then vortexed briefly and incubated for 30 min at 37°C, with occasional inversion to mix. The mixture was then centrifuged at 100 × *g* for 1 min at 4°C and the beads were washed three times with 1 ml of PBS containing 0.04% NaN_3. The resulting beads were centrifuged at 100 × *g* for 1 min, and the supernatant was removed. To elute bound salivary proteins, the beads were resuspended in 100 μl of SDS elution buffer, vortexed briefly, and then incubated for 30 min at room temperature using a rotator (Roto-Torque; Cole-Parmer, Chicago, IL) and centrifuged at 100 × *g* for 1 min. Protein concentrations in SDS eluates were determined using the BCA protein assay.

Gel electrophoresis and staining of proteins and glycoproteins. Proteins were separated by SDS-PAGE (8 to 16% gradient gel; Novex, Carlsbad, CA) under reducing conditions. Salivary samples were denatured in an equal volume of 2× sample buffer (Novex, Carlsbad, CA). For detection of $DMBT1^{gp\text{-}340}$ by Western blotting, 2-mercaptoethanol was omitted from the sample buffer. Salivary eluates derived from bacteria contained already 2% SDS as part of the elution buffer. They were complemented with glycerol (Fisher Scientific, Fair Lawn, NJ) and bromophenol blue (Bio-Rad Laboratories, Richmond, CA) to final concentrations of 5% and 0.0125%, respectively. Saliva and salivary eluates were standardized to 20 μg of protein in 30 μl, reduced by 2-mercaptoethanol (3%), heated for 5 min (95°C), and loaded per lane. For comparative experiments, equal volumes (25 μl) of eluates were loaded, corresponding to an equal number of bacteria (1.5×10^9). For Western blotting, 10 μg of WS, 25 μl of eluate, and 25 μl of bacterial surface extract were loaded per lane. For detection of immunoglobulin components by Western blotting, 2 μg of WS, 5 μl of eluate, and 5 μl of bacterial surface extract were loaded. As controls, 1.25 μg of purified human IgG (Athens, GA) and human S-IgA (Cappel Laboratories, West Chester, PA) were loaded per lane (data not shown). Gels were run at room temperature for 1.5 h at a constant voltage of 150 V. Gels were stained using Coomassie blue (R-250; Bio-Rad, Hercules, CA), followed by periodic acid-Schiff reagent to locate proteins and glycoproteins, respectively, as previously described (30). Protein bands of interest were excised from gels for identification of proteins by liquid chromatography-tandem mass spectrometry (LC-MS/MS). For detection of Cy3-labeled salivary proteins, gels were scanned using a fluorescence scanner (Typhoon 9410; GE Healthcare, Piscataway, NJ). Cy3-labeled protein bands were revealed by scanning at a laser wavelength of 532 nm. Following fluorescence scanning, the identical gels were counterstained by Coomassie blue (R-250; Bio-Rad, Hercules, CA).

On-bacteria limited trypsin digestion. For limited trypsin digestion of bacterium-bound proteins, a modification of the on-bead digestion method was employed (31). Bacterial pellets (10^9 bacteria) carrying bound salivary proteins were washed three times with 50 mM ammonium bicarbonate buffer, pH 8 (1 ml). Excess buffer was removed by thorough aspiration. Trypsin was reconstituted in 40 μl of 50 mM acetic acid to a final concentration of 0.5 μg/μl. The pellets were resuspended in ammonium bicarbonate buffer (50 μl). The trypsin was added to achieve a ratio of trypsin to protein of about 1:20 (wt/wt). The suspension was incubated at 37°C for 2.5 h with occasional vortexing. After centrifugation, the su-

pernatant was collected with utmost caution not to touch the pellet. Proteins were reduced with dithiothreitol to a final concentration of 10 mM at 37°C for 45 min and subsequently alkylated with iodoacetamide to a final concentration of 50 mM in the dark for 30 min. A full trypsin digest was then performed using a ratio of trypsin to protein of 1:10 (wt/wt), and the mixture was incubated at 37°C overnight. The digests were acidified using formic acid to a final concentration of 2%, dried completely, and reconstituted by adding 20 μl of 2% formic acid before LC-MS/MS analysis.

Identification of protein by mass spectrometry (LC-MS/MS). Mass spectrometric identifications of proteins were performed as previously described (32). In brief, slices of chosen protein bands or samples derived from on-bacteria limited trypsin digests were subjected to trypsin digestion and peptides were analyzed by liquid chromatography-nanoelectrospray-tandem mass spectrometry (LC-ESI-MS/MS) using a nanoACQUITY UPLC (Waters Corporation) coupled through a nebulization-assisted nanospray ionization source to a Q-ToF Premier mass spectrometer (Waters/Micromass). The LC consisted of a trap column (Symmetry C_{18}, 5 μm, 180 μm by 20 mm; Waters), followed by separation on an analytical column (Atlantis C_{18}, 3 μm, 100 μm by 10 cm; Waters). The tryptic digests (4 μl of each sample) were loaded, trapped, and washed at a flow rate of 4 μl/min with 99% solvent A (0.1% formic acid)-1% solvent B (acetonitrile containing 0.1% formic acid) for 5 min. Peptides were eluted with a gradient of 99% solvent A-1% solvent B to 65% solvent A-35% solvent B for 50 min at 0.4 μl/min, followed by 10% solvent A-90% solvent B for 7 min at 1.2 μl/min. Throughout the gradient, the Q-ToF Premier mass spectrometer was programmed (data-dependent acquisition experiment [DDA]) to monitor ions and select those with mass/charge (*m/z*) in the range of 300 to 1,500 and ions with +2 to +5 charges for MS/MS analysis using the preset DDA collision energy parameters.

Database search and protein identification. The generated MS/MS spectra were transformed to the PKL file format using Proteinlynx Global server version 2.3 (Waters/Micromass) and default parameters of MaxEnt3 (Waters/Micromass). All PKL files were imported into the Swiss-Prot database (released in 2010, containing 517,802 sequences) using a locally installed version of MASCOT (Matrix Science; version 2.2.2). PKL files were searched against the *Homo sapiens* or firmicutes (Gram-positive bacteria) subset database. The database search parameters were as follows: the proteolytic enzyme used was trypsin with two possible missed cleavages, carboxyamidomethylation of cysteine was set as a fixed modification, while oxidation of methionine was set as a variable modification. The allowable mass error was 100 ppm for peptides and 100 mD for fragment ions, the peptide charges were set to 2+ and 3+, and the instrument option was set to ESI-QUAD-TOF. Proteins were identified by finding at least two peptides from the parent protein with statistically significant ion scores, and in certain instances a decoyed database was used in MASCOT to minimize the false-positive error rate. For certain proteins identified from the on-bacteria limited trypsin digestion, only one peptide could be identified, and these proteins are listed in Table 2 only if they were also identified in Table 1 and if their ion scores were above the threshold for significance.

Western blotting. Salivary proteins were separated by SDS-PAGE and then transferred to nitrocellulose membranes (0.45 μm, BA 85; Whatman Protran) using an electroblotting transfer unit (TE 77, ECL Semi-Dry; Amersham Biosciences) under constant current of 45 mA/gel for 2.5 h in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Blots were then washed in TBS (20 mM Tris-Cl, 150 mM NaCl, 0.02% NaN_3) for at least 10 min to remove excess transfer buffer. Blots were blocked for 1 h in 2% nonfat dry milk (Carnation) in TBS containing 0.05% Tween 20 (TBST-milk). For detection of immunoglobulin components, 3% BSA was substituted for milk. In experiments involving rabbit antisera, dog gamma globulin (Jackson ImmunoResearch Inc., West Grove, PA) at a dilution of 1:1,000 was included in the blocking buffer to avoid unwanted binding of these antibodies by staphylococcal protein A (SpA) in the transferred eluates or extracts (33). Primary antibodies used for immunoblotting included mouse anti-human gp-340 (1G4, HYB 213-06-02; AntibodyShop, Rockford, IL), rabbit anti-human MG2 (34) and rabbit anti-human cystatin (35) (both by courtesy of Michael J. Levine), rabbit anti-human amylase (Calbiochem, Darmstadt, Germany), and rabbit anti-human IgA α chain (IGHA) (DakoCytomation, Glostrup, Denmark) antibodies. Mouse anti-human secretory component (SC) and mouse anti-protein A (SpA) antibodies were purchased from Sigma (St. Louis, MO). Goat anti-human IgG Fc and goat anti-human IgG $F(ab')_2$/F(ab) were obtained from Jackson ImmunoResearch Inc. (West Grove, PA). Goat anti-human carbonic anhydrase (CA) VI, goat anti-human SPLUNC2, goat anti-human lysozyme C, and goat anti-human calgranulin-B (S100-A9) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). For detection of $DMBT1^{gp\text{-}340}$, the gels were run under nonreducing conditions.

Blots were incubated for 1 h with the indicated primary antibody in the appropriate blocking buffer. Blots were washed three times with TBS containing 0.05% Tween 20 (TBS-Tween) for 5 min each and then incubated for 1 h with the appropriate secondary antibody, including goat anti-mouse IgG (H+L)-AlexaFluor488, chicken anti-goat IgG (H+L)-AlexaFluor488, or goat anti-rabbit IgG (H+L)-AlexaFluor488 (all from Invitrogen, Eugene, OR). Each indicated secondary antibody was diluted 1:1,000 in the appropriate blocking buffer. Blots were washed three times with TBS-Tween for 5 min each, dried, and then scanned by a fluorescence scanner (Typhoon 9410; GE Healthcare, Piscataway, NJ) at a wavelength of 488 nm (blue laser).

RESULTS

A limited number of salivary proteins bind to *S. aureus*. Whole saliva (WS), glandular parotid (PAR), and submandibular-sublingual (SMSL) secretions were separated by SDS-PAGE to obtain a baseline reference for the protein and glycoprotein components present in these fluids (Fig. 1A). The pattern of salivary protein bands detectable in the eluate from *S. aureus* 8325 exposed to WS (Fig. 1B) showed some distinct differences in the banding pattern to that of unabsorbed WS (Fig. 1A). Salivary protein binding increased proportionally with the concentration of bacteria in saliva, and optimum binding was achieved with a ratio of $\sim 10^9$ bacteria per ml of whole saliva (data not shown). To discern if the visible protein bands in eluates from *S. aureus* were indeed derived from saliva, the bacteria were incubated under analogous conditions in buffer alone (Fig. 1B). Under these conditions, no discrete protein bands were distinguishable in the eluate, even when the buffer eluate was derived from a 10-fold-increased number of bacteria. When exposed to WS, the same 10-fold increase in bacteria resulted in stronger protein bands in the eluate. For all further experiments, eluates were derived from 6×10^9 bacterial organisms incubated in 6 ml of WS, a condition that consistently provided strong salivary protein bands but minimized the risk of contamination by bacterial components.

All major protein bands in the eluates varied depending on the type of salivary secretion to which the bacteria had been exposed (Fig. 1C). Thus, when *S. aureus* was exposed to PAR secretion instead of WS, a diffuse band above 250 kDa became more prominent, whereas a diffuse band at ~150 kDa and a strong band at 50 kDa disappeared. When *S. aureus* was exposed to SMSL secretion, bands at the top of the gel as well as those above 250 kDa and at ~150, 25, and ~15 kDa became stronger. After exposure to either WS or SMSL secretion, the protein band at 50 kDa was enhanced in eluates but was absent when bacteria were exposed to PAR saliva. Compared to unabsorbed saliva, the band at 25 kDa became stronger in eluates derived from all glandular secretions. Other protein bands, clearly detectable in the original salivary secretions, appeared weaker or disappeared in eluates (e.g., band at

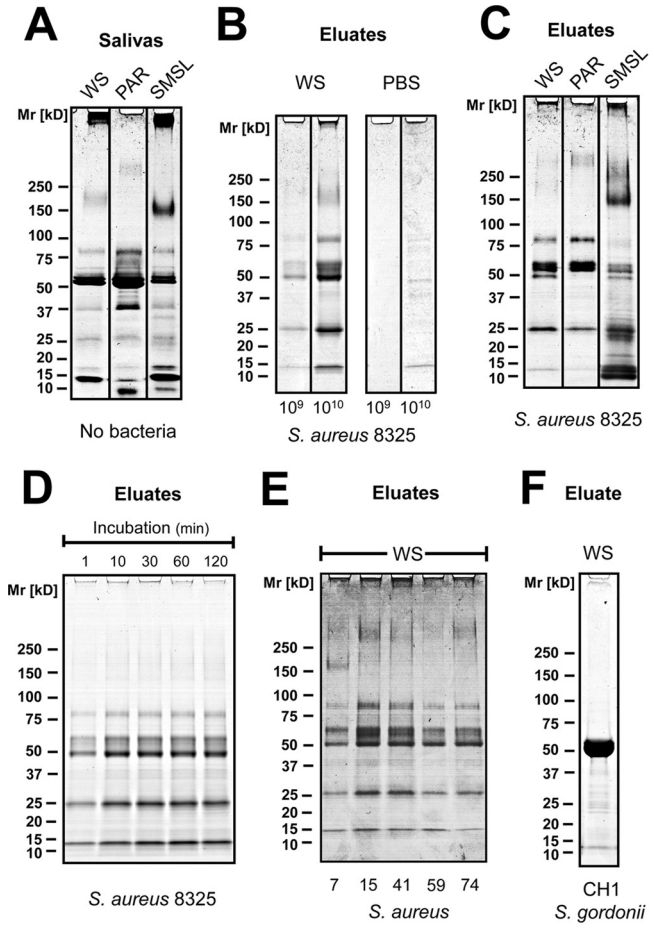

FIG 1 Profiles of salivary proteins bound to *S. aureus*. Bacteria were exposed to salivary secretions and washed to remove unbound proteins. Bound proteins were eluted from the bacterial organisms and separated by SDS-PAGE. Protein and glycoprotein bands were revealed by Coomassie blue and periodic acid-Schiff staining, respectively. (A) Saliva from different glandular sources: whole saliva (WS), parotid saliva (PAR), and submandibular-sublingual saliva (SMSL). (B) Eluates from 10^9 and 10^{10} organisms following exposure to WS or PBS. The ratio of bacteria to WS or PBS was kept constant (i.e., 10^9 organisms/ml). (C) Eluates from *S. aureus* after exposure to WS, PAR, or SMSL. (D) Eluates from *S. aureus* 8325 after exposure to WS for different periods of time. (E) Eluates from several clinical isolates of *S. aureus* after exposure to WS. (F) Eluate from *S. gordonii* CH1 after exposure to WS.

37 kDa). Since the bands at the origin of the gel, above 250 and at ~150 kDa, were intensely stained by periodic acid-Schiff stain (color version of image not shown), they were predicted to represent salivary mucin 5B (>1,000 kDa), $DMBT1^{gp\text{-}340}$ (300 to 400 kDa), and salivary mucin-7 (~150 kDa). Together, these observations lend further support to selectivity in the binding of salivary proteins by *S. aureus* and confirmed that the protein bands observed in the eluates were of salivary and not of bacterial origin.

Kinetics of binding showed that virtually all protein bands in the eluate were detectable following 1 min of exposure of the bacteria to saliva (Fig. 1D). Saturation levels were reached within 10 min, and banding patterns remained stable for up to 2 h.

Because nonspecific binding mechanisms have been suggested to guide very early events of bacterial binding to salivary substrates (36), the extent to which electrostatic and hydrophobic interactions control salivary protein binding to the surface of *S. aureus* was examined. Neither NaCl up to 2 M (37) nor urea up to 4 M had any influence on protein binding (see Fig. S1 in the supplemental material). Protein bands in the eluates were diminished only after exposure to 6 M urea (see Fig. S1B), likely due to the chaotropic effects of high-concentration urea (38). From these findings, it was inferred that nonspecific forces did not play a dominant role in the binding of salivary components to *S. aureus*.

Next, the salivary protein binding to different *S. aureus* strains was examined (Fig. 1E). The overall qualitative pattern of bands in salivary eluates obtained from different clinical isolates of *S. aureus* (17) matched generally well with the pattern seen with the type strain 8325 (Fig. 1C, WS). However, variations mostly in the intensities of certain bands were recognized among the eluates from different strains. For instance, the band at ~150 kDa was clearly visible in eluates from strain 7 and strain 8325 (compare Fig. 1B and C) but faintly or not at all in eluates derived from the other clinical isolates. The type strain 8325 was chosen for further experiments because it is widely studied and its genome is fully sequenced and annotated (25). Also, all further experiments were performed using WS since it is the physiologically relevant biological fluid present in the oral cavity.

To examine possible species-associated differences in salivary protein binding, the oral commensal bacterium, *S. gordonii*, which has been well characterized in terms of its saliva-binding properties (28), was used as a comparison. The pattern of bands detected in the salivary eluate obtained from *S. gordonii* was different than that from *S. aureus*. Only one predominant band slightly above 50 kDa was detected in the eluate from *S. gordonii* CH1 (Fig. 1F), which represents salivary α-amylase (28). The finding that salivary proteins bound by *S. aureus* were not bound by *S. gordonii* and vice versa suggested that binding of salivary components could depend on the species of bacteria.

Proteomic identification of salivary proteins bound to *S. aureus*. Two approaches were used to identify salivary proteins bound to *S. aureus*. The first approach consisted of excising the major visible protein bands from SDS-PAGE, subjecting them to in-gel trypsin digestion, and analyzing the peptide profiles by LC-MS/MS (Table 1). The second approach used a limited trypsin digest to cleave bound salivary proteins directly from the surface of *S. aureus*, followed by a complete trypsin digestion and subsequent analysis by LC-MS/MS as described above (Table 2). The first approach identified a band slightly above 250 kDa as $DMBT1^{gp\text{-}340}$. In addition, secretory component (80 kDa), IgA and IgG heavy chains (55 kDa and 50 kDa, respectively), immunoglobulin light chains (25 kDa), and S100-A9 (below the15-kDa marker) were identified. A faintly visible band at 37 kDa was also cut out and identified as carbonic anhydrase VI (CA VI). Mucin-5B (MG1) could not be identified as a component of the band retained at the origin of the gel in the eluate, although it was easily identifiable in the same region in the saliva control (data not shown). Likewise, mucin-7 (MG2) was not identifiable in the 150-kDa band in eluates. In WS, mucin-7 could be identified, but only one peptide matched, with relatively low ion score, presumably because the protein backbone of this mucin is densely decorated with glycans (39).

By on-bacteria limited trypsin digestion, proteins that were eluted matched well with the proteins identified by the SDS-PAGE approach (Table 2). Using this alternative approach, only zymogen granule protein 16 homolog B (ZG16B) and SPLUNC2 were additionally detected with significant scores (Table 2). There were

TABLE 1 Identification of human whole-saliva proteins bound to *S. aureus* 8325 in eluates by in-gel trypsin digestion (LC-MS/MS)

UniProtKB/Swiss-Prot accession no.	Protein name	Gene name	Nominal mass (Da)	Individual ion score	No. of queries matched	emPAI[a]	Sequence coverage (%)
Q9UGM3	Deleted in malignant brain tumor 1 protein (glycoprotein 340, salivary agglutinin)	DMBT1 (GP340)	268,039	229	9	0.02	4
P01833	Polymeric immunoglobulin receptor (PIgR; secretory component as cleaved PIgR)	PIGR	84,429	440	6	0.16	12
P01860	Ig gamma-3 chain C region	IGHG3	42,287	231	12	0.46	18
P01876	Ig alpha-1 chain C region	IGHA1	38,486	386	11	0.78	36
P01877	Ig alpha-2 chain C region	IGHA2	37,301	315	10	0.81	38
P01857	Ig gamma-1 chain C region	IGHG1	36,596	521	22	1.38	50
P01859	Ig gamma-2 chain C region	IGHG2	36,505	209	11	0.84	26
P01861	Ig gamma-4 chain C region	IGHG4	36,431	138	13	0.42	22
P23280	Carbonic anhydrase 6 (carbonic anhydrase VI, CA-VI)	CA6	35,459	95	4	0.31	11
P01591	Immunoglobulin J chain	IGJ	18,543	33	2	0.18	15
P61626	Lysozyme C	LYZ	16,982	227	3	0.44	27
P12274	Protein S100-A9 (calgranulin-B, calprotectin L1H subunit, MRP-14)	S100A9	13,291	64	2	0.58	24
P01834	Ig kappa chain C region	IGKC	11,773	467	15	3.66	80
P01842	Ig lambda-1 chain C regions	IGLC1	11,401	115	3	0.30	23

[a] emPAI, exponentially modified protein abundance index.

generally fewer peptides found for the salivary proteins identified from the on-bacteria limited trypsin digestion (Table 2) than from the in-gel digestion procedure (Table 1), which was attributed mainly to a possible ion suppression effect due to competition for ionization during mass spectrometry from the abundance of digested bacterial (nonsalivary) proteins in these samples. Data obtained by both approaches confirmed selectivity of binding by showing that only a limited number of salivary proteins were found to bind the surface of *S. aureus*.

Saliva-bacterium binding assessed using Cy3-labeled saliva and identification of bound salivary components. To optimize the conditions for more sensitive and specific detection of salivary components, saliva was labeled with different concentrations of Cy3 before separation of proteins by SDS-PAGE. A concentration of 3 μg Cy3 per ml was found optimal for revealing a wide range of salivary proteins for all subsequent experiments (see Fig. S2 in the supplemental material). Saliva labeled with Cy3 was employed in saliva-*S. aureus* binding assays (Fig. 2). The salivary eluates were resolved by SDS-PAGE, and the gel was scanned at different PMT sensitivities. At lower sensitivity (550V), the protein profile revealed by fluorescence scanning was similar to that revealed by Coomassie-periodic acid-Schiff (COOM/PAS) counterstaining. High-molecular-range (>75-kDa) salivary glycoprotein bands, only faintly detected by COOM/PAS, became more apparent at higher PMT sensitivities (600 to 650 V). For example, the protein band above 150 kDa, representing salivary mucin-7, was well de-

TABLE 2 Identification of human whole-saliva proteins bound to *S. aureus* 8325 in eluates by on-bacteria limited trypsin digestion (LC-MS/MS)

UniProtKB/Swiss-Prot accession no.	Protein name	Gene name	Nominal mass (Da)	Individual ion score	No. of queries matched	emPAI[a]	Sequence coverage (%)
Q9UGM3	Deleted in malignant brain tumor 1 protein (glycoprotein 340, salivary agglutinin)	DMBT1 (GP340)	268,039	105	1	0.01	0
P01833	Polymeric immunoglobulin receptor (PIgR; secretory component as cleaved PIgR)	PIGR	84,429	97	1	0.04	2
P01876	Ig alpha-1 chain C region	IGHA1	38,486	178	12	0.18	15
P01859	Ig gamma-2 chain C region	IGHG2	36,505	55	3	0.09	8
P01861	Ig gamma-4 chain C region	IGHG4	36,431	63	3	0.19	8
P23280	Carbonic anhydrase 6 (carbonic anhydrase VI [CA-VI])	CA6	35,459	188	3	0.20	7
Q96DR5	Short palate, lung, and nasal epithelium carcinoma-associated protein 2 (BPI fold-containing family A member 2, parotid secretory protein)	SPLUNC2 (BPIFA2)	27,166	52	3	0.12	17
Q96DA0	Zymogen granule protein 16 homolog B	ZG16B	22,725	256	3	0.51	17
P61626	Lysozyme C	LYZ	16,982	300	7	1.07	35
P01834	Ig kappa chain C region	IGKC	11,773	248	6	0.67	35
P0CG04	Ig lambda-1 chain C regions	IGLC1	11,401	64	1	0.30	14

[a] emPAI, exponentially modified protein abundance index.

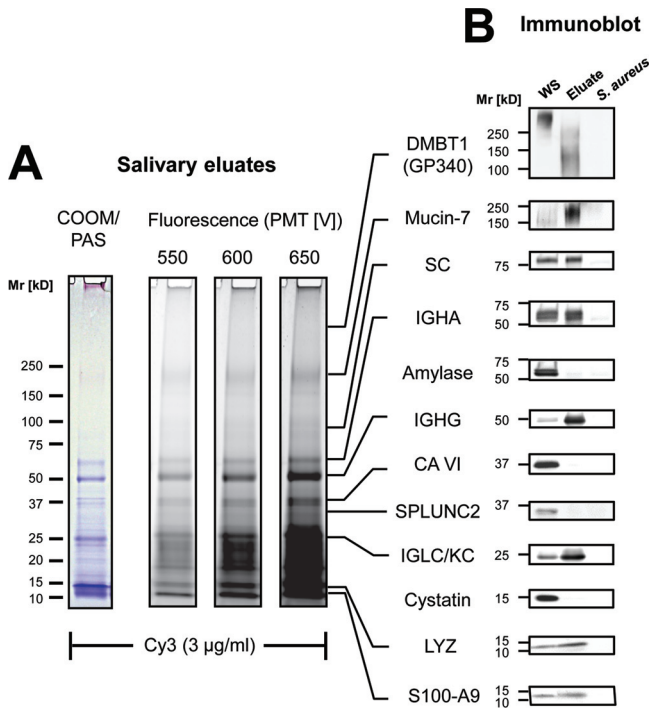

FIG 2 Validation of salivary origin of proteins in eluates by use of fluorescence-labeled saliva and identification by immunoblotting. (A) Saliva-bacterium binding assessed using Cy3-labeled saliva. *Staphylococcus aureus* 8325 was exposed to Cy3-labeled saliva and washed to remove unbound proteins. Bound proteins were eluted from the bacterial surface (salivary eluates) and separated by SDS-PAGE. Gels were scanned using a fluorescence scanner by applying different photomultiplier tube (PMT) voltages (V) to reveal Cy3-labeled protein bands. After being scanned, the identical gels were counterstained by Coomassie blue combined with periodic acid-Schiff staining (COOM/PAS). The locations of proteins suggested by proteomic approaches (Tables 1 and 2) and identified by immunoblot are indicated. (B) Immunoidentification of salivary proteins bound to *S. aureus* 8325. Whole saliva (WS), *S. aureus*-bound salivary proteins (eluate), and extracts of *S. aureus* 8325 surface proteins (*S. aureus*) were separated by SDS-PAGE, electrotransferred, and detected by Western blotting using antibodies directed against the salivary protein components indicated. Abbreviations: SC, secretory component; IGHA, IgA α-chain; IGHG, IgG γ-chain; CA VI, carbonic anhydrase VI; IGLC/KC, Ig κ- and λ-chains; LYZ, lysozyme C.

tected at PMT sensitivity settings of 600 to 650 V. In summary, these observations further confirmed that the salivary eluates were indeed of salivary origin and were not protein components derived from the bacteria. Thus, using fluorescence-labeled saliva allowed us to distinguish proteins and glycoproteins of salivary origin from possible bacterial contaminants. In addition, fluorescence labeling offered greatly enhanced sensitivity for salivary protein detection as well as the flexibility to selectively focus on low- or high-abundant salivary proteins by varying the PMT voltage.

Based on the results derived from the proteomic analyses (Tables 1 and 2), immunoblotting was performed to validate the presence of these putatively bacterium-bound salivary proteins in the eluates and to compare them to their original counterparts in whole saliva (Fig. 2). Immunoglobulin G (IgG) and secretory IgA (S-IgA) components, which were scored in the proteomic analyses with highest significance among the *S. aureus*-bound salivary proteins (Tables 1 and 2), were also readily detected by immunoblotting (Fig. 2B). In the eluates from *S. aureus*, IgG (Fc and Fab fragments) was enriched much more than S-IgA (secretory component and IgA heavy chain) relative to their original amounts present in WS. The fact that no evidence for the IgG heavy chain was found in eluates when *S. aureus* was exposed to PAR saliva (see Fig. 1C, missing band at 50 kDa) might be attributable to the fact that IgG is normally present in much smaller amounts in PAR (0.4 μg/ml) than in SMSL secretion or WS (14 μg/ml) (40). From the additional nonimmunoglobulin salivary proteins that were detected by the proteomic approach (Tables 1 and 2), the presence of salivary DMBT1$^{gp\text{-}340}$, mucin-7, lysozyme C, and S100-A9 could be confirmed by immunoblotting (Fig. 2B). When the last three were compared to their original amounts in WS, only mucin-7 appeared to be enriched in *S. aureus* eluates.

DMBT1$^{gp\text{-}340}$ was detected as a diffuse band at lower molecular range in the eluate than in unabsorbed WS, possibly due to its degradation following binding to *S. aureus* (Fig. 2B). Such a putative degradation, however, cannot simply consist of reductive cleavage of DMBT1$^{gp\text{-}340}$ into its subunits, because the antibody used for detection recognizes only unreduced DMBT1$^{gp\text{-}340}$. Amylase, which is abundant in saliva and abundantly bound to *S. gordonii* (see Fig. 1F), could not be detected at all in salivary eluates derived from *S. aureus*. Also, no cystatins could be detected in eluates from *S. aureus* even though a strong signal for cystatins was obtained by immunoblotting in WS at the expected molecular range of 15 kDa (Fig. 2B). These findings fit well with the proteomic analyses, which also failed to detect amylase or cystatins among the components bound to *S. aureus* (Tables 1 and 2). Carbonic anhydrase VI and SPLUNC2, both detected by proteomic analyses as *S. aureus*-bound salivary proteins (Tables 1 and 2), could not be confirmed in eluates by immunoblotting, although bands at the predicted molecular ranges were visible in WS (Fig. 2B).

Biofilm growth diminishes the number of salivary components bound to *S. aureus*. To examine whether its growth state affects the ability of *S. aureus* to bind salivary proteins, the saliva-bacterium binding assay was performed comparing planktonic-grown and biofilm-grown bacteria (Fig. 3). When cultured in glucose-enriched medium (TSBGN) to enhance biofilm formation (Fig. 3A), *S. aureus* bound fewer salivary components than in its planktonic state, most strikingly missing a prominent 50-kDa band in eluates (Fig. 3B). The same band was also missing in salivary eluates of *S. epidermidis*, which is a strain known to spontaneously form biofilms (41) (Fig. 3A and B). Similarly, a 25-kDa band was weaker in eluates from *S. epidermidis*. Analogous results were obtained when *S. aureus* was grown on solid agar (TSBY agar) as a surrogate for biofilm-like growth (data not shown).

Western blots and proteomic analyses have shown that the 50-kDa and 25-kDa bands in salivary eluates from planktonic *S. aureus* contain the heavy and light chains of IgG (Fig. 2). Because it is known that *S. epidermidis* does not express staphylococcal protein A (SpA), an immunoglobulin-binding member of the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family (25, 42, 43), we suspected that SpA might be responsible for binding a subset of the proteins observed in the salivary eluates. To detect the presence of SpA in the salivary eluate from each bacterium, immunoblotting was performed employing anti-SpA antibody (Fig. 3C). SpA was detected slightly above 50 kDa in the eluates from planktonic *S. aureus* 8325 but was absent in biofilm-grown *S. aureus* 8325 as well as *S. epidermidis*. Moreover, SpA was also identified with a high score by pro-

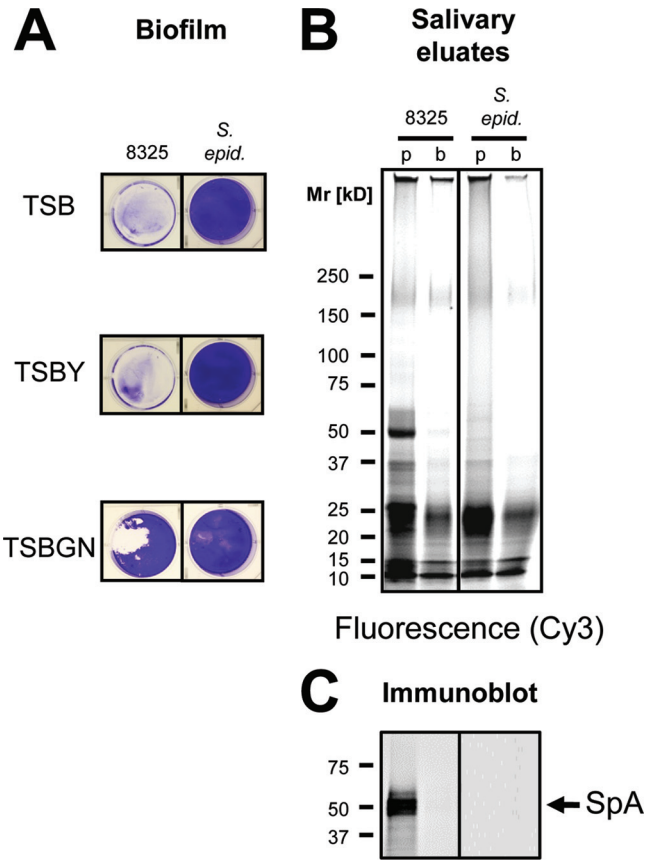

FIG 3 Effect of bacterial growth state on binding of salivary proteins. Bacteria were exposed to Cy3-labeled saliva and washed to remove unbound proteins. Bound proteins were eluted from the bacterial surface (salivary eluates). (A) *S. aureus* strain 8325 and biofilm-forming strain *S. epidermidis* RP62a (*S. epid.*) were grown in tryptic soy broth (TSB), TSB supplemented with yeast extract (TSBY), or TSB supplemented with glucose and sodium chloride (TSBGN) in multiwell tissue culture plates. After being rinsed with buffer, biofilms of bacteria adherent to plates were stained by crystal violet. (B) Salivary proteins were eluted from the surface of *S. aureus* 8325 and *S. epidermidis* RP62a, grown either planktonic (p) or as biofilms (b). Proteins from saliva and salivary eluates were separated by SDS-PAGE, and gels were scanned using a fluorescence scanner to reveal Cy3-labeled bands. (C) The same eluates as in panel B were transferred to a nitrocellulose membrane and probed with anti-SpA antibody.

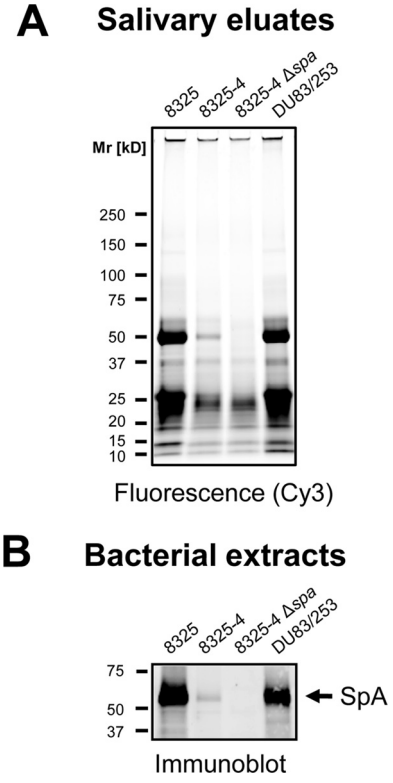

FIG 4 Effects of staphylococcal protein A (SpA) expression on the binding of salivary proteins to *S. aureus*. Wild-type and mutant *S. aureus* strains were exposed to Cy3-labeled saliva and washed to remove unbound proteins. Bound proteins were eluted from the bacterial surface by elution buffer (salivary eluates). (A) Salivary proteins eluted from wild-type strain *S. aureus* 8325, its derivative strain *S. aureus* 8325-4, SpA-deficient mutant (*S. aureus* 8325-4 Δ*spa* mutant), and SpA-complemented strain DU83/253 were separated by SDS-PAGE, and Cy3-labeled protein bands in salivary eluates were revealed by using a fluorescence scanner. (B) Immunoblot probed with anti-SpA antibody of surface extracts from the same bacteria as shown in panel A but not exposed to saliva.

teomic analysis of salivary eluates from *S. aureus* when peptide data were searched against the firmicutes subset bacterial database (data not shown).

Expression of SpA affects the binding of salivary proteins. To determine the role of SpA expression on *S. aureus* for binding of salivary proteins, *S. aureus* 8325, its derivative *S. aureus* 8325-4, SpA-deficient mutant (*S. aureus* 8325-4 Δ*spa* mutant), and SpA-complemented strain DU83/253 were employed in the saliva-bacterium binding assay (Fig. 4). Cy3-labeled saliva was incubated with these planktonic-grown bacteria, and the salivary eluates released from the bacterial surface were analyzed for presence or absence of salivary proteins. Prominent bands of salivary proteins at 25, 50, and 55 kDa were visible in salivary eluates released from the surface of *S. aureus* 8325 and *S. aureus* 8325-4. The latter strain showed weaker binding of the same salivary protein bands, presumably due to lower levels of SpA expression on this strain (44). The salivary proteins at 50 and 55 kDa were missing in salivary eluates released from the surface of the *S. aureus* 8325-4 Δ*spa* mutant (Fig. 4A, lane 3). Binding of these proteins was restored with the SpA-complemented strain DU83/253 (Fig. 4A, lane 4). To validate the expression of SpA on these strains, immunodetection was performed using anti-SpA antibody (Fig. 4B). SpA was detected at ~55 kDa in bacterial extracts from wild-type strain *S. aureus* 8325, its derivative *S. aureus* 8325-4, and SpA-complemented strain DU83/253 but was absent in the SpA-deficient mutant (*S. aureus* 8325-4 Δ*spa* mutant). Overall, the amount of salivary proteins bound to the surface of the bacteria paralleled the expression levels of SpA.

Identification of salivary proteins bound to purified SpA. Purified SpA immobilized onto Sepharose beads was used to determine which of the salivary proteins found in eluates are bound by SpA alone (Fig. 5). The prominent salivary components at 25, 50, and 55 kDa that were seen in the eluate from planktonic-grown *S. aureus* 8325 were also detected in the eluate from SpA-Sepharose but not from uncoated Sepharose beads (Fig. 5A). These bands were identified by immunodetection as IgA α-chain, IgG γ-chain, and Ig light chains. Mucin-7, however, was detected only in the eluate from *S. aureus*

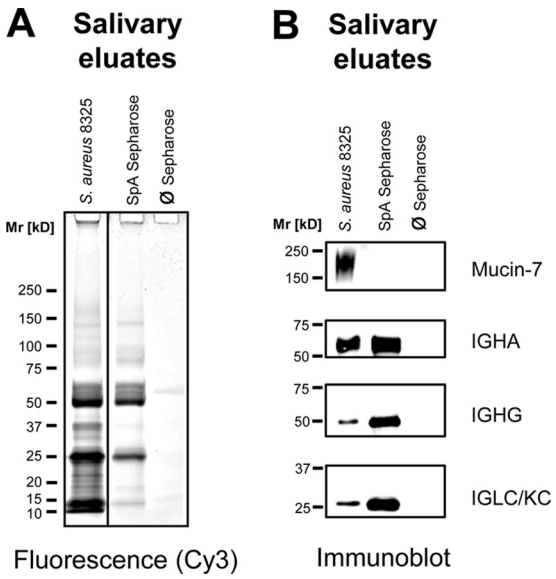

FIG 5 Identification of the salivary proteins bound by purified staphylococcal protein A (SpA) immobilized to Sepharose. *Staphylococcus aureus* organisms or Sepharose beads were exposed to Cy3-labeled saliva and washed to remove unbound proteins. Bound proteins were eluted from the surfaces of bacterial organisms and Sepharose beads (salivary eluates). (A) Salivary proteins eluted from *S. aureus* 8325 as well as from SpA-coated (SpA Sepharose) and uncoated Sepharose beads (Ø Sepharose) were separated by SDS-PAGE, and Cy3-labeled salivary protein bands were revealed by a fluorescence scanner. (B) The same eluates as in panel A were transferred to a nitrocellulose membrane and probed with anti-mucin-7, anti-IgA α-chain (IGHA), anti-IgG Fc (IGHG), and anti-IgG F(ab′)$_2$/F(ab) (IGLC/KC) antibodies.

(Fig. 5B, lane 1). No components could be detected in eluates from uncoated Sepharose beads (Fig. 5B, lane 3).

DISCUSSION

Saliva is believed to act as a gatekeeper in the human oral cavity at the entrance to the gastrointestinal and respiratory tracts (1–3). Yet, our knowledge on whether and how medical pathogens are affected by the salivary proteome during their passage through or residence within the mouth is limited (20–24). In this study, we have demonstrated that *S. aureus* rapidly and selectively binds a limited number of salivary proteins to its surface following exposure to saliva. Most of the bound salivary proteins are associated with various aspects of host defense (1–3, 5). Among them, salivary immunoglobulins and mucin-7 were most avidly bound to the *S. aureus* surface. Biofilm-grown *S. aureus* did not bind salivary immunoglobulin components, due to the downregulation of SpA. Since SpA did not mediate binding of salivary mucin-7 to the staphylococcal surface, further staphylococcal surface components must be involved in binding of nonimmunoglobulin salivary proteins.

Proteomic analyses and immunoblotting showed that IgG and IgA were abundantly bound to the staphylococcal surface upon exposure to saliva. Even in the presence of high salt and urea, binding of immunoglobulin components persisted (see Fig. S1 in the supplemental material), suggesting that binding of these components involves particularly strong interactions. The present findings show that biofilm-grown *S. aureus* binds less salivary immunoglobulins to its surface, as presumably the result of SpA downregulation or shedding under this condition (26, 29, 44, 45). While the lack of salivary immunoglobulin binding, as observed here, applies to a monospecies *S. aureus* biofilm, it would be intriguing to determine if a similar downregulation happens in mixed-species biofilms, as they are typically found in the oral cavity (8, 46). It could further be speculated that, when thriving in oral biofilms under conditions of reduced salivary flow (i.e., less salivary immunoglobulin) and reduced oral hygiene (i.e., enhanced biofilm formation), as is often the case in hospitalized patients (11, 47), *S. aureus* would be more resistant to immunological clearance mechanisms.

It is not surprising that SpA was found responsible for binding salivary immunoglobulins to the staphylococcal surface, because it has been shown to preferentially bind not only the Fc fragments of IgG (42, 43) but also the F(ab) fragments of other immunoglobulin classes, including IgA (48, 49). The latter also explains our observation that not only salivary IgG but also S-IgA was bound to the surface of *S. aureus* and to SpA-coated beads. Nevertheless, IgG, although present in saliva in a much lower concentration (14 μg/ml in WS) than S-IgA (194 μg/ml in WS) (40), was still bound by *S. aureus* with highest preference. Although SpA has also been reported to bind proteins other than immunoglobulins (27, 50, 51), it did not account for binding DMBT1$^{gp\text{-}340}$, mucin-7, lysozyme, and S100-A9 to the staphylococcal surface. Yet, all have been reported to interact with *S. aureus* and other microorganisms under various conditions even though they are not commonly regarded as MSCRAMM ligands. For instance, DMBT1$^{gp\text{-}340}$, a member of the scavenger receptor cysteine-rich (SRCR) superfamily of proteins, binds to *S. aureus*, *Streptococcus mutans*, *Helicobacter pylori*, and *S. gordonii* (7, 20, 52). Salivary mucin-7 (MG2) binds to *S. aureus* (24), *Pseudomonas aeruginosa* (30), and *Escherichia coli* (22). Lysozyme can bind to and agglutinate *S. aureus* (53), but *S. aureus* is not killed by lysozyme (54). S100-A9 is also known to inhibit growth of *S. aureus* by chelating of divalent ions, including Zn^{+2} and Mn^{+2}, as nutrient sources (55). Whether mucin-7 and other nonimmunoglobulin salivary proteins become indirectly bound to *S. aureus* as components of larger molecular aggregates with S-IgA (24) remains an interesting question to be resolved. So far, our data do not support this possibility because SpA, when immobilized to beads, did not bind mucin-7 (see Fig. 5B). Thus, additional staphylococcal surface components must be responsible for binding of the nonimmunoglobulin salivary proteins to the *S. aureus* surface. Nevertheless, the question whether protein complexes play a role for binding to the bacterial surface warrants further scrutiny, particularly in view of multiprotein superstructures organized in the form of the recently discovered exosomes in saliva (56, 57). In this context, it has also to be taken into consideration that the whole saliva used in the present experiments was clarified by centrifugation before exposure to the bacteria, which could have potentially altered the composition of salivary proteins by selectively depleting saliva of macromolecular aggregates.

Collectively, our findings suggest that *S. aureus* is able to selectively bind salivary proteins which may influence the oral colonization of this species and, thus, strengthen the notion that salivary proteins ought to be considered important contributors in the host-pathogen interplay, particularly for diseases where medical, nonoral pathogens enter the human body via the oral cavity. To gain more relevant functional insight, it would be helpful to study the salivary status of hospitalized patients at risk for infection by respiratory and gastrointestinal pathogens. Identification of such

interactions may suggest future strategies to inhibit oral colonization by pathogens.

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