

Fluid- or Surface-Phase Human Salivary Scavenger Protein gp340 Exposes Different Bacterial Recognition Properties

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Salivary scavenger receptor cysteine-rich protein gp340 aggregates streptococci and other bacteria as part of the host innate defense system at mucosal surfaces. In this article, we have investigated the properties of fluid-phase gp340 and hydroxylapatite surface-adsorbed gp340 in aggregation and adherence, respectively, of viridans group streptococci (e.g., *Streptococcus gordonii* and *Streptococcus mutans*), non-viridans group streptococci (e.g., *Streptococcus pyogenes* and *Streptococcus suis*), and oral *Actinomyces*. Fluid-phase gp340 and surface-phase gp340 bioforms were differentially recognized by streptococci, which formed three phenotypic groupings according to their modes of interaction with gp340. Group I streptococci were aggregated by and adhered to gp340, and group II streptococci preferentially adhered to surface-bound gp340, while group III streptococci were preferentially aggregated by gp340. Each species of *Streptococcus* tested was found to contain strains representative of at least two of these gp340 interaction groupings. The gp340 interaction modes I to III and sugar specificities of gp340 binding strains coincided for several species. Many gp340 interactions were sialidase sensitive, and each of the interaction modes (I to III) for *S. gordonii* was correlated with a variant of sialic acid specificity. Adherence of *S. gordonii* DL1 (Challis) to surface-bound gp340 was dependent upon expression of the sialic acid binding adhesin Hsa. However, aggregation of cells by fluid-phase gp340 was independent of Hsa and involved SspA and SspB (antigen I/II family) polypeptides. Conversely, both gp340-mediated aggregation and adherence of *S. mutans* NG8 involved antigen I/II polypeptide. Deletion of the *mga* virulence regulator gene in *S. pyogenes* resulted in increased cell aggregation by gp340. These results suggest that salivary gp340 recognizes different bacterial receptors according to whether gp340 is present in the fluid phase or surface bound. This phase-associated differential recognition by gp340 of streptococcal species of different levels of virulence and diverse origins may mediate alternative host responses to commensal or pathogenic bacterial phenotypes.

Tissue fluids, like saliva and tears, contain soluble glycoproteins that promote aggregation and clearance of bacteria in the fluid phase (38). In contrast, other secreted proteins, like acidic proline-rich proteins (PRPs) in saliva, promote microbial adherence and colonization when adsorbed to surfaces, although they lack microbial cell binding activity in the fluid phase (11). Moreover, fluid and surface forms of fibronectin and laminin differ in their bacterial recognition properties (31, 46).

Salivary agglutinin, which is secreted by cells associated with the parotid gland, mediates aggregation of commensal (e.g., *Streptococcus gordonii*) and cariogenic (e.g., *Streptococcus mutans*) oral viridans group streptococci (9, 36), non-viridans group streptococci (e.g., *Streptococcus pyogenes* and *Streptococcus agalactiae*), and other pathogenic bacteria (e.g., *Helicobacter pylori*) (38). Salivary agglutinin also provides a substrate, when bound to hydroxylapatite surfaces, for adherence of oral streptococci (6, 22). The adherence levels of *S. mutans* Ingbritt to hydroxyapatite coated with saliva from caries-prone subjects are higher than those to hydroxylapatite coated with saliva from caries-resistant subjects (41). This adherence, which

largely involves binding to salivary agglutinin, is also modulated by allelic acidic PRP variants (41). Salivary agglutinin may therefore facilitate bacterial clearance on the one hand and favor biofilm formation in vivo on the other (8). However, the relative adhesive capacities of fluid-phase and surface-adsorbed agglutinin toward oral commensal and cariogenic streptococci and other bacterial ligands remain largely unknown.

Salivary agglutinin is a 5×10^6 -Da oligomeric protein complex of the scavenger receptor cysteine-rich (SRCR) glycoprotein gp340, secretory immunoglobulin A, and an 80-kDa protein (36, 38). The gp340 monomer is composed of SRCR ($n = 14$), CUB ($n = 2$), and ZP ($n = 1$) domains (16, 17). Gp340 stimulates random migration of macrophages (48) and binds collectins SP-A and SP-D (16, 48). Furthermore, gp340 is a spliced form of DMBT1, a tumor suppressor protein (33), and gp340/DMBT1 affects epithelial cell differentiation and proliferation (34). Recently, an SRCR domain-derived peptide was found to induce aggregation of streptococcal ligands (2). However, little is known about the ability of gp340 to distinguish between different bacterial phenotypes in a given species.

Viridans group streptococci commonly express conserved, though polymorphic, cell surface antigen I/II (Ag I/II) adhesins or polypeptides: e.g., SspA and SspB in *S. gordonii* and SpaP in *S. mutans* (20). *S. mutans* binds salivary agglutinin (gp340)

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through SpaP (Ag I/II), which is thought to contain separate domains for saliva-mediated aggregation and adherence (3). The interaction with agglutinin (gp340) of *S. gordonii*, which expresses two sialic acid-sensitive adhesins, SspB (7) and Hsa, a polypeptide with highly repetitive serine-rich domains (44), is partly inhibited by sialyl oligosaccharides (7). The relative roles of Ag I/II family polypeptides and of Hsa-like polypeptides in gp340-mediated aggregation and adherence of viridans group streptococci are not fully understood.

The aim of this study was to characterize (i) the adhesive behavior of fluid- and surface-phase gp340 toward streptococci and other bacterial ligands and (ii) the molecular determinants for defined behaviors. We therefore tested a panel of viridans and non-viridans group streptococci, as well as specific adhesin mutants, for aggregation by fluid-phase gp340 and for adherence to gp340 adsorbed onto hydroxyapatite surfaces. We suggest that fluid-phase gp340 and surface-immobilized gp340 expose different binding properties and, consequently, differentially recognize adhesive phenotypes of diverse bacterial species.

MATERIALS AND METHODS

Bacterial strains. Strains or clinical isolates of *S. gordonii*, *Streptococcus sanguinis* (*sanguis*), and *Streptococcus oralis* (SK strains) were from M. Kilian (Århus University, Denmark); *S. gordonii* Blackburn and strains of *Actinomyces* were obtained from the Culture Collection of the University of Göteborg (CCUG); *S. mutans* Ingbritt was provided by J. Carlsson (Umeå University, Umeå, Sweden); *S. pyogenes* A8173 was from K. Kunas (National Public Health Institute, Kuopio, Finland); *S. pyogenes* 71676 was from A. Podbielski (37); and other clinical *S. pyogenes* isolates (KTL strains) were from P. Huovinen (National Public Health Institute of Finland, Turku, Finland). The *S. gordonii* isogenic mutant strain UB1360 Δ (*sspA sspB*) (14) was generated by allelic replacement of a 9774-bp fragment containing the *sspA* and *sspB* genes (bp 389 to 10162; GenBank accession no. U40027) with a 900-bp fragment containing the *aad9* gene (37) encoding spectinomycin resistance (500 μ g/ml). *S. gordonii* UB1545 Δ *hsa* was generated from strain DL1 by allelic replacement of a 6,024-bp fragment comprising most of the *hsa* gene (bp 711 to 6734; GenBank accession no. AB029393) with a 1,300-bp fragment containing the *aphA3* kanamycin (250 μ g/ml) resistance determinant (19). *S. gordonii* UB1552 Δ (*sspA sspB*) Δ *hsa* was generated from strain UB1360 by transformation with DNA extracted from strain UB1545 and selection for kanamycin resistance. Allelic replacements were confirmed by appropriate PCR amplifications from the mutant chromosomes. *S. mutans* 834 *spaP*, a derivative of wild-type strain NG8, and *S. pyogenes* A8173-1 Δ *nga*, a derivative of wild-type strain A8173, were generated by allelic replacement (28) or transposon mutagenesis (18), respectively. The *sspA* gene was cloned into the vector pTREX1-*usp45L*S and expressed on the surface of *Lactococcus lactis* MG1363 as previously described (15).

Cultivation of bacteria. All streptococci, except *S. pyogenes*, were grown at 37°C in Jordans broth, containing (per liter) 5 g of Trypticase, 5 g of yeast extract (Merck, Darmstadt, Germany), 5 g of K₂HPO₄, 4 g of glucose, 0.5 ml of salts solution (0.8 g of MgSO₄·7H₂O, 0.04 g of FeSO₄·7H₂O, 0.019 g of MnCl₂·4H₂O in 100 ml of distilled water) and 5 ml of Tween 80. Strains of *S. pyogenes* were grown in Todd-Hewitt broth (Difco) supplemented with 2 g of yeast extract per liter (Difco). *Actinomyces* strains were grown on Columbia II-agar base plates (Becton Dickinson Microbiology Systems, Cockeysville, Md.) supplemented with a human erythrocyte suspension (30 ml/liter), in candle jars. Isogenic mutants were cultured as described above in media containing the appropriate antibiotics (erythromycin at 5 μ g/ml, tetracycline at 5 μ g/ml, spectinomycin at 100 or 500 μ g/ml, or kanamycin at 250 μ g/ml). *Lactococcus lactis* MG1363 and MG1363 expressing SspA polypeptide were cultured in M17 broth without or with erythromycin (5 μ g/ml), respectively (15).

Purification of salivary gp340. gp340 was purified from human saliva by adsorption to *S. mutans* Ingbritt cells as described previously (38). Briefly, fresh stimulated parotid saliva samples from 6 to 10 healthy donors were pooled and mixed with bacterial cells at 37°C for 60 min. After pelleting of the bacterium-gp340 aggregates by centrifugation, gp340 was released from the bacterial cells by adding 20 mM EDTA and further purified by gel filtration. The protein concentration was determined with the Bio-Rad DC protein assay (Bio-Rad

Laboratories, Hercules, Calif.) with bovine serum albumin (BSA) as a standard. The gp340 preparations showed the same gp340 band, although no other protein bands, upon gel electrophoresis and Coomassie brilliant blue staining and showed virtually identical adhesion and aggregation patterns with selected reference strains.

Aggregation and adherence assays. The ability of gp340 to aggregate bacterial cells was assessed at 37°C as described elsewhere (38). Briefly, washed bacterial cells were suspended in phosphate-buffered saline (PBS; 10 mM K-phosphate buffer, 150 mM NaCl, pH 6.8) supplemented with 1 mM CaCl₂ at an optical density at 700 nm [OD₇₀₀] of 1.0. To this suspension, untreated or glycosidase-treated gp340 was added at a final concentration of 1 μ g/ml. Aggregation was recorded by measuring the OD₇₀₀ at 1-min intervals over 1 h with a Beckman DU-50 series spectrophotometer. The extent of aggregation was expressed as a percentage after 30 or 60 min and calculated with the formula [(*t*₀ at A₇₀₀ - *t*₆₀ at A₇₀₀)/*t*₀ at A₇₀₀] × 100.

Adherence of bacteria to gp340-coated hydroxyapatite (Bio-Rad Laboratories) beads was measured in microtiter plates (5 mg of hydroxyapatite/well) at room temperature essentially as described previously (10). Briefly, the beads were hydrated overnight with buffered KCl (1 mM KH₂PO₄-K₂HPO₄ buffer, pH 6.5, containing 50 mM KCl, 1 mM CaCl₂, and 0.1 mM MgCl₂) at 4°C and coated with 125 μ l of purified gp340 (6 μ g/ml in buffered KCl) for 60 min. After incubation of the wells with 5% (wt/vol) BSA, 125 μ l of [³⁵S]methionine-labeled bacteria (5 × 10⁸ CFU/ml in buffered KCl supplemented with 0.5% BSA) was added for 60 min. After repeated washes, bound bacteria were measured by scintillation counting. Adherence (percentage of input) was calculated as follows: [(cpm of bacteria bound to gp340-coated beads - cpm of bacteria bound to BSA-coated beads)/cpm of input bacteria] × 100.

In control experiments, the aggregation and adherence assays produced the same results regardless of whether they were performed at 37°C or at room temperature.

Sialidase and glycosidase treatment of gp340. gp340 was depleted of sialic acid residues by treatment with sialidase from *Clostridium perfringens* (type X; Sigma, St Louis, Mo.). For aggregation tests, 0.5 U of sialidase and 25 μ g of gp340 were incubated in a total volume of 50 μ l of Na-acetate buffer, pH 5.4, at 37°C for 24 h. For adherence tests, sialidase treatment was performed by incubating the gp340-coated hydroxyapatite beads for 30 min with 0.1 U of sialidase in 20 mM phosphate buffer, pH 6.0. As a control, gp340 was incubated in buffer without sialidase, and as another control, reference strains were treated with sialidase without affecting their gp340 interaction patterns compared to those of the nontreated strains. For aggregation tests, gp340 (25 μ g) was treated at 37°C with each of the following enzymes and appropriate buffers (within parentheses) in a final volume of 50 μ l: endo- β -galactosidase (5 mU, 10 mM Na-acetate buffer, pH 5.6) (Seikagaku, Falmouth, Mass.), α -fucosidase (125 mU, K-phosphate buffer, pH 6.0) (Sigma), α -galactosidase (675 mU, buffer supplied by the manufacturer Glyco, Novato, Calif.) or β -galactosidase (125 mU, buffer supplied by the manufacturer, Glyco).

Inhibition by SL. Inhibition of adherence or aggregation by sialyllactose (SL; a mixture of 2-3 and 2-6 sialyllactose) (Sigma, St. Louis, Mo.) was tested by addition of SL to the bacterial suspension (final concentration, 1 mg/ml) before assaying for adherence or aggregation.

Detection of Ag I/II polypeptides. Production of Ag I/II polypeptides was determined by Western immunoblot analysis as previously described (15). Bacteria were incubated with mutanolysin to release cell wall-associated proteins. These were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto nitrocellulose membrane, and incubated with polyclonal antibodies to *S. mutans* Ag I/II. These antibodies cross-react with all streptococcal Ag I/II family polypeptides tested (20). Antibody reactive bands were detected with horseradish peroxidase-conjugated secondary antibody.

DNA hybridization and detection of Hsa-like polypeptides. The presence of the *hsa* gene in streptococci was measured by slot blot DNA hybridization with a digoxigenin (DIG)-labeled *hsa* gene probe. The probe (1,480 bp) was generated by PCR amplification of chromosomal DNA from *S. gordonii* DL1 with the forward primer ACGAAGTTGAACGTGTTACGC and the reverse primer TGCTGCTGCAACTGCTTCTC, and the PCR DIG probe synthesis kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The probe comprised the coding sequence for the N-terminal nonrepeated region and the first part of the serine-rich region of the deduced Hsa amino acid sequence (44). Chromosomal DNA (3 μ g), purified from streptococci (40), was blotted onto nylon membrane (Hybond-N+; Amersham, Little Chalfont, United Kingdom), denatured, fixed by heating (80°C, 2 h) and then incubated with denatured DIG-labeled *hsa* gene probe at 46°C. The hybridizations were detected with the DIG luminescent detection kit (Roche) after washings with 0.5% SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS at 65°C. The degree of

TABLE 1. Differential recognition of streptococcal ligands by salivary gp340 in the fluid phase (aggregation) or surface-bound phase (adherence)

Group and species	Strain	% gp340-mediated ^a :		Strain origin (reference)
		Aggregation	Adherence	
Mode I (aggregation and adherence)				
Viridans group				
<i>S. gordonii</i>	DL1 Challis	51*	37*	Not recorded (possibly human blood)
<i>S. mutans</i>	Ingbritt	61*	39*	Human dental plaque (25)
<i>S. sanguinis</i>	SK 112	40*	14*	Human dental plaque (21)
Non- <i>viridans</i> group				
<i>S. agalactiae</i>	SK 870	58	25*	
<i>S. pyogenes</i>	KTL 7	45*	16*	Human skin infection (P. Huovinen)
<i>S. pyogenes</i>	A 8173	47	24*	
<i>S. pyogenes</i>	71676	18	24*	
<i>S. suis</i>	KU5	65*	19*	Pig tonsillae (25)
Mode II (aggregation << adherence)				
Viridans group				
<i>S. gordonii</i>	SK 12	10	26*	Human oral cavity (21)
<i>S. gordonii</i>	SK 184	1	18*	Human dental plaque (21)
<i>S. gordonii</i>	SK 120	4	21*	Human oral cavity (21)
<i>S. oralis</i>	SK 105	1	14*	Human dental plaque (21)
Non- <i>viridans</i> group				
<i>S. pyogenes</i>	KTL 31	0	29*	Human blood (P. Huovinen)
<i>S. pyogenes</i>	KTL 26	6	26*	Human throat (P. Huovinen)
Mode III (aggregation >> adherence)				
Viridans group				
<i>S. gordonii</i>	M5	43*	1	Human dental plaque (B. Rosan)
<i>S. gordonii</i>	Blackburn	46*	3	Not recorded (possibly human blood)
<i>S. mutans</i>	LT11	54	1	Human oral cavity
<i>S. mutans</i>	NG8	21*	5	Human dental plaque (K. Knox)
<i>S. oralis</i>	SK 113	38*	4	Human sputum (21)
Non- <i>viridans</i> group				
<i>S. suis</i>	836	53	0	Pig lung (12)
<i>S. suis</i>	628	71	0	Human brain (12)

^a Shown is the percentage of aggregating cells, measured by decrease in OD₇₀₀, of the total number of added bacteria. Also shown is the percentage of adhering cells of the total number of added bacteria. Asterisks denote interactions that are either inhibited or reduced by treatment with sialidase.

^b An origin is given with the reference or source when well defined.

hybridization was scored from 0 to 4+, based on densitometric measurements (MCID-M5 Plus image analyzer; Imaging Research Corp., St. Catharines, Ontario, Canada) in which 0 = <0.05, 1+ = 0.05 to <0.1, 2+ = 0.1 to <0.15, 3+ = 0.15 to <0.20, and 4+ = ≥0.20.

For detection of Hsa-like polypeptides, mutanolysin-released cell wall-associated proteins were separated by SDS-PAGE and blotted onto nitrocellulose membrane as described above. Blots were then incubated with 2 µg of biotinylated succinylated wheat germ agglutinin (sWGA) per ml, followed by 0.2 µg of peroxidase-conjugated streptavidin per ml (1). sWGA binds *N*-acetylglucosamine residues that are covalently linked to streptococcal Hsa-like proteins. Protein expression was scored on the basis of densitometric measurements of 0 = <0.05, 1+ = 0.05 to <0.1, and 2+ = >0.10.

HA and saccharide inhibition. Hemagglutination (HA) was determined by mixing equal volumes (10 µl) of bacteria (5 × 10⁹ cells/ml) and erythrocytes (4%) in PBS for 5 min. The extent of HA was scored visually as 0, 1+, 2+, 3+, or 4+. The effect of sialidase treatment of the erythrocytes on HA was evaluated by treating 1 ml of a 4% (vol/vol) erythrocyte suspension in PBS with 0.1 U of sialidase at 37°C for 30 min. The minimum sugar concentration (millimolar) needed for 50% inhibition of HA (e.g., 2+ to 1+ and 4+ to 2+) was established by adding reciprocal dilutions of the following oligosaccharides to the bacterial suspension prior to the HA assay: SL (NeuNAcα2-3/6Galβ1-4Glc), 3'SL (NeuNAcα2-3Galβ1-4Glc), 6'SL (NeuNAcα2-6Galβ1-4Glc), sTn (NeuNAcα2-6GalNAcα1-3 Ser), sLex (NeuNAcα2-3Galβ1-4(Fucα1-3)GlcNAc), LSTb (Galβ1-3(NeuNAc 2-6)GlcNAcβ1-3Galβ1-4Glc), Gal-sulfate (D-Gal-6-O-SO₃), and 3'-

and 6'SL-human albumin conjugates. SL, 3'SL, 6'SL, and sLex were from Sigma; sTn was from Calbiochem (La Jolla, Calif.), and Gal-sulfate and LSTb were from Dextra Laboratories (Berkshire, United Kingdom).

RESULTS

Differential recognition by streptococci of fluid-phase or surface-bound gp340. We screened a panel of viridans and non-*viridans* group streptococci for aggregation by fluid-phase gp340 and for adherence to hydroxyapatite beads coated with gp340 (Table 1). The strains tested could be divided into three groups based upon their modes of interaction with gp340. Group I (mode I interaction) strains were aggregated by and adhered to gp340, and group II strains preferentially adhered to surface-immobilized gp340, while group III strains were preferentially aggregated by gp340 (mode III). Each species of *Streptococcus* contained strains that exhibited either two or all three of phenotypes I to III. This observation held true regardless of whether the streptococci were viridans or non-*viridans* group (Table 1).

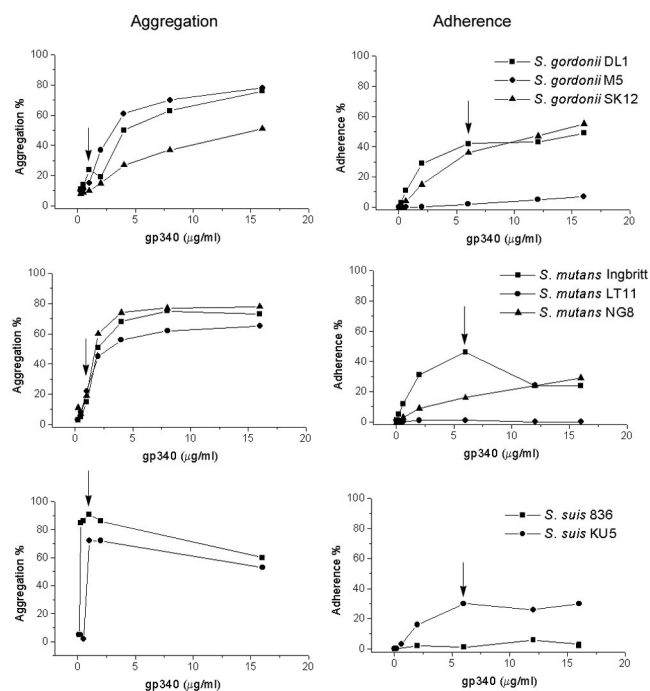


FIG. 1. Aggregation by fluid-phase gp340 and adherence to surface-adsorbed gp340 of *S. gordonii*, *S. mutans*, and *S. suis* strains as a function of gp340 concentration. The arrows marks the fixed amounts of gp340 used for aggregation and adherence of all streptococcal and *Actinomyces* strains by salivary gp340.

Dependence of aggregation or adhesive phenotypes on gp340 concentration. We then tested selected strains representative of the different gp340 interaction modes for aggregation and adherence over a range of fluid-phase or surface-immobilized gp340 concentrations (Fig. 1). Although *S. gordonii* M5, *S. mutans* LT11 and *S. suis* 836 group III cells were rapidly aggregated by gp340, they were deficient in adherence to surface bound gp340, even at the highest gp340 concentrations tested (Fig. 1). Thus, surface-immobilized gp340 receptors are entirely cryptic for some aggregating phenotypes. In contrast, while *S. gordonii* SK12 and DL1, *S. mutans* Ingbritt and LT11, and *S. suis* KU5 group I and II cells all adhered more avidly to surface-bound gp340, their aggregation reactions were similar to, if not somewhat weaker than, those of the group III cells. These results confirm that fluid-phase gp340 and surface-bound gp340 have truly different adhesive behaviors.

Relationship between gp340 interaction mode and virulence. No direct correlations between gp340 interaction phenotypes and commensal or pathogenic potential of streptococci were observed. Thus, commensal *S. gordonii* (an early colonizer on teeth) and pathogenic *S. pyogenes* (implicated in tonsillitis) both showed adhering (mode II) and aggregating (modes I or III) phenotypes (Table 1). Similarly, no general conclusions can be drawn, based on the low number of strains tested, about a potential relationship between gp340 interaction phenotype and oral commensal versus cariogenic (*S. mutans*) viridans group streptococci. However, oral isolates of *S. mutans* ($n = 3$) all showed high gp340 aggregation activity, with gp340 adherence ranging from zero to moderate (modes I and III). In contrast, most oral isolates of *S. gordonii* (three out of four

strains) were preferentially adherent (mode II), while *S. gordonii* isolates of unknown origin ($n = 2$) were preferentially aggregative (modes I and III). The affinities of the tested strains for fluid-phase gp340 (aggregation) were in the order *S. suis* > *S. gordonii* (Fig. 1).

Relationship between gp340 interaction mode and sugar specificity. Incubation of gp340 with sialidase inhibited the aggregation or adherence reactions of members of all three *S. gordonii* gp340 interaction phenotypes (I to III), *S. suis* KU5, and of some of the *S. pyogenes* phenotypes (Tables 1 and 2). The gp340-mediated aggregation or adherence reactions for the other bacteria tested were either simply reduced or not affected at all by sialidase treatment of gp340. Some of the sialidase-sensitive gp340 interactions, such as those exhibited by *S. gordonii* SK12 and *S. suis* KU5, were partially inhibitable by SL and by other sialyl oligosaccharides (Table 2). In contrast, none of the sialidase-insensitive gp340 interactions was inhibitable by these oligosaccharides.

We next investigated differences in sugar binding specificity as they relate to the gp340 interaction phenotypes. Each of the three sialidase-sensitive phenotypes of *S. gordonii* coincided with a variant sialic acid binding specificity, being associated with unique sialidase-sensitive HA and saccharide inhibition patterns (Table 2). These *S. gordonii* phenotypes (I to III) recognized sialic acid receptors on gp340 with (modes I and II) or without (mode III) mimicry on erythrocytes. SL inhibition of their gp340 interactions followed in the order I > II > III (data not shown). Moreover, *S. suis* KU5 (with specificity for sialyl polylactosamine structures) and *S. suis* 628 and 836 (both with specificity for Gal α 1-4Gal structures) showed different gp340 interaction modes (Table 2).

Actinomyces adhesive phenotypes also differ in gp340 interaction mode. Viridans group streptococci and *Actinomyces* species predominate in early biofilms formed on teeth and oral mucosal surfaces (35). The *Actinomyces* species have been classified into six groupings on the basis of their adherence interactions with host and bacterial partners (23). *Actinomyces odontolyticus* PK984 and *Actinomyces naeslundii* ATCC 12104, which are representative strains of two of these six *Actinomyces* groupings, showed different gp340 interaction modes and sugar specificities (Table 2). *A. odontolyticus* PK984 with Sia α 2-6GalNAc specificity showed sialidase-sensitive gp340-mediated aggregation and adherence (mode I). In contrast, *A. naeslundii* ATCC 12104 with Gal β specificity showed preferential gp340-mediated adherence (mode II), which was increased to desialylated gp340 via exposure of underlying Gal β receptors.

Functions of Hsa and Ag I/II in gp340 interactions. *S. gordonii* DL1 (Challis) produces a cell surface-anchored polypeptide designated Hsa, a sialic acid-specific adhesin (44), and two Ag I/II family cell surface-anchored polypeptides designated SspA and SspB that interact with gp340 (15).

We screened all the streptococcal strains in Table 1 for the presence of *hsa*-like genes and expression of Hsa-like proteins and for production of Ag I/II proteins. Accordingly, the streptococci were designated Hsa⁺ Ag I/II⁺ (e.g., *S. gordonii*), Hsa⁻ Ag I/II⁺ (e.g., *S. mutans*) or Hsa⁻ Ag I/II⁻ (e.g., *S. pyogenes*). These designations appear to be independent of gp340 interaction modes I to III. To investigate the functions of Hsa and Ag I/II (SspA and SspB) polypeptides in the interactions of *S. gordonii* DL1 with gp340, we tested isogenic mutants with de-

TABLE 2. Coinciding gp340 interaction mode and sugar binding specificity within several *Streptococcus* and other bacterial species

Species and strain	Mode	% gp340 binding ^a		Sialidase sensitive ^b	Sugar specificity (reference) ^c	Hemagglutination		Adhesins		
		Aggregation	Adherence			Specificity ^d	Sialidase sensitive ^e	<i>hsa</i> ^f	Hsa ^g	AgI/II ^h
<i>S. gordonii</i>										
DL1	I	51 ⁱ (1)	37 ⁱ (1)	Yes	sLe ^x /3'SL	Ho, Go, Ra, Ch, Hu	Yes	++	++	++
SK 12	II	10	26 ⁱ (0)	Yes	Sialic acid	Go	Yes	++	+	++
SK 184	II	1	18 ⁱ (0)	Yes	Sialic acid	Go, Ch	Yes	+	++	++
SK 120	II	1	21 (0)	Yes	Sialic acid	Ch, Hu	Yes	+	++	++
M5	III	43 (3)	2	Yes	Sialic acid	0	NT ^j	+++	++	++
Blackburn	III	46 (8)	3	Yes	Sialic acid	0	NT	+	++	0 ^k
<i>S. mutans</i>										
Ingbritt	I	61 (46)	39 (20)	Yes	Sialic acid	0	NT	0	0	++
NG 8	III	21 (12)	5	Yes	Sialic acid	0	NT	0	0	++
LT11	III	54 (55)	1	No	Unknown	0	NT	0	0	++
<i>S. suis</i>										
KU5	I	59 (0)	19 ⁱ (0)	Yes	Sia2-3galβ1-4GlcNAc (12, 27, 30)	Hu	Yes	0	0	0
836	III	53 (50)	0	No	Galα1-4Gal (12, 27, 30)	Hu	No	0	0	0
628	III	71 (67)	0	No	Galα1-4Gal (12, 27, 30)	Hu	No	0	0	+/-
<i>Actinomyces</i>										
PK984	I	45 ⁱ (37)	26 ⁱ (2)	Yes	3'SL/sTn	Ho, Go, Ra, Ch, Hu	Yes	NT	0	NT
ATCC 12104	II	1 (3)	34 (50)	No	GalNAcβ (13, 29, 43)	Ho, Go, Ra, Ch, Hu	No	NT	0	NT

^a Shown is the percentage of aggregating cells, measured by decrease in OD₇₀₀, of the total number of added bacteria. Also shown is the percentage of adhering cells of the total number of added bacteria. Values in parentheses represent the percentage of aggregation or adherence after sialidase treatment of gp340.

^b Sialidase treatment of gp340 reduced adherence or aggregation, while other glycosidases did not (see Material and Methods).

^c Saccharide specificity either through inhibition of HA with a panel of saccharides (sLe^x/3'SL or 3'SL/sTn) or via inhibition of gp340-mediated binding by SL or sialidase (sialic acid) or from the literature (12, 13, 27, 29, 30, 43). The minimum sugar concentrations for 50% inhibition of HA were as follows for the most active substances: 0.25 mM 3'SL and sLe^x for *S. gordonii* DL1 and 0.5 mM sTn and 1.0 mM 3'SL for *A. odontolyticus* PK984. The HA of *S. gordonii* strains SK12, SK120, and SK184 was not inhibited by any of the tested saccharides at the final concentrations of 4 mM (SL), 2 mM (3'SL, 6'SL, sTn, Gal-sulfate LSTb) or 1 mM (sLex).

^d Positive HA with horse (Ho), goat (Go), rabbit (Ra), chicken (Ch), or human (Hu) erythrocytes. 0 denotes negative HA.

^e Inhibition of HA by sialidase.

^f Presence of *hsa* genes measured by DNA hybridization with an *hsa*-specific probe from *S. gordonii* DL1. Signal strength: 0, no signal; +, weak; ++, moderate; and +++, strong.

^g Surface expression of Hsa as measured by staining with sWGA. Signal strength: 0, no signal; +, weak; ++, strong.

^h Surface expression of Ag I/II as measured by binding of anti-Ag I/II sera to bacterial cell extract. Reaction strength: 0, no reaction; +, weak; ++, strong.

ⁱ SL-inhibitible binding.

^j NT, not tested.

^k Blackburn secreted Ag I/II into growth medium.

letions of *hsa* (UB1545) or both *sspA* and *sspB* (UB1360) for gp340 aggregation and adherence. Deletion of *hsa* in *S. gordonii* UB1545 resulted in inhibited adherence of cells to surface-bound gp340, while gp340-mediated aggregation was essentially unaffected (Table 3). On the other hand, deletion of the *sspA* and *sspB* genes in *S. gordonii* UB1360 did not affect adherence to surface bound gp340, but resulted in reduced rate and extent of gp340-mediated aggregation (Table 3). A mutant with the *hsa*, *sspA*, and *sspB* genes deleted (strain UB1552) was inhibited in adherence and reduced in aggregation. Cells of *L. lactis* MG1363 expressing SspA polypeptide, but not vector control cells of *L. lactis* MG1363, were aggregated by gp340, demonstrating that SspA interacts directly with fluid-phase gp340 (Table 3). *S. mutans* 834, an isogenic derivative of strain NG8 in which the *spaP* (Ag I/II) gene is disrupted (28), showed inhibited or reduced gp340-mediated aggregating activity at large or small amounts of gp340, respectively, compared with parental strain NG8 (Table 3). Moreover, *S. mutans* 834 showed inhibited adherence to surface-bound gp340 compared with parental strain NG8. These results indicate that Ag I/II polypeptides play different physi-

ological roles in *S. gordonii* and *S. mutans* interactions with gp340.

gp340-mediated adherence and aggregation of *S. pyogenes* are influenced by Mga. Strains of *S. pyogenes* tested displayed two of the three different interaction modes with gp340 (Table 1). Mga is a well-characterized regulator of virulence factor gene expression in *S. pyogenes* and modulates the expression of several important surface proteins (39). To investigate a possible role for *S. pyogenes* Mga-regulated gene products in interactions with gp340, we determined the gp340 aggregation and adherence phenotypes of *S. pyogenes* A8173 and isogenic derivative strain A8173-1 deficient in Mga expression (Table 3). Compared with the parent strain, aggregation by gp340 of mutant A8173-1 cells was enhanced, while adherence of the Mga⁻ mutant to surface-bound gp340 was reduced (Table 3).

DISCUSSION

This study demonstrates that salivary gp340 recognizes different bacterial ligands according to whether it is present in the fluid phase or immobilized to the surface of hydroxyapatite.

TABLE 3. Delineation of adhesins on *S. gordonii*, *S. mutans*, and *S. pyogenes* interacting with salivary gp340

Species and strain	Phenotype	% gp340-mediated ^a :	
		Aggregation	Adherence
<i>S. gordonii</i>			
DL1 Challis	Hsa ⁺ Ag I/II ⁺	60	37
UB 1545	Hsa ⁻ Ag I/II ⁺	71	1
UB 1360	Hsa ⁺ Ag I/II ⁻	38	39
UB 1552	Hsa ⁻ Ag I/II ⁻	30	<1
<i>L. lactis</i>			
MG1363	Hsa ⁻ Ag I/II ⁻	<1	<1
MG1363 (pTREX1- usp45 sspA)	Hsa ⁻ Ag I/II ⁺	45	8
<i>S. mutans</i>			
NG8	Hsa ⁻ Ag I/II ⁺	36 (74)	5 (24)
834	Hsa ⁻ Ag I/II ⁻	3 (25)	<1 (1)
<i>S. pyogenes</i>			
A8173	Hsa ⁻ Ag I/II ⁻	29	31
A8173-1	Hsa ⁻ Ag I/II ⁻ Mga ⁻	61	18

^a Shown is the percentage of aggregating cells, measured by decrease in OD₇₀₀, of the total number of added bacteria. Also shown is the percentage of adhering cells of the total number of added bacteria. Values in parentheses represent the adherence (percentage) of bacteria to hydroxyapatite beads coated with a larger amount (12 µg/ml) of gp340.

Accordingly, the abilities of streptococci to differentially interact with the two bioforms of gp340 varied markedly, even for strains of the same species. Thus, while some strains within a streptococcal species were preferentially aggregated by gp340, other strains within the same species showed proficient adherence but little or no aggregation. Clearly, fluid-phase as opposed to surface-phase conditions could result in exposure, or in masking, of different bacterium binding epitopes. How precisely this might occur for gp340 is at present unknown, but fluid or surface phases could induce different molecular organization or folding, like the membrane-guided conformation of glycolipid isoreceptors for *Escherichia coli* (42) and the unfolding of the RGD loop of fibronectin under certain surface conditions (24). This interpretation of different epitope exposures in fluid versus surface gp340 had further support in (i) the selective roles of the Hsa and Ag I/II adhesins in gp340-mediated adherence and aggregation of *S. gordonii* DL1, respectively, and (ii) the fact that the receptor binding specificity and interaction mode of strains coincided in several species.

While adherence of *S. gordonii* DL1 to gp340 was fully dependent upon Hsa, aggregation of *S. gordonii* DL1 by gp340 was multimodal, involving both Ag I/II and yet unknown adhesins. The multimodal nature of aggregation may involve both low- and high-affinity binding reactions, since bacterial cell aggregation is subject to lower shear forces than adherence (47). The conclusion that yet unknown surface molecules, in addition to Ag I/II, are normally involved in gp340-mediated aggregation came from the demonstration of residual gp340 aggregation activity in both *S. gordonii* UB1552 (with deletion of the *hsa*, *sspA*, and *sspB* genes) and *S. mutans* 834 (with deletion of *spaP*). These unknown molecules could either be another lectin-like adhesin or a surface component interacting with the gp340 SRCR polypeptide backbone, which is known to aggregate oral streptococci (2).

The different bacterium-gp340 interaction modes (I to III) suggest the involvement of different complements of adhesins or of allelic adhesin variants. The gp340 interactive phenotypes of *S. gordonii* and *S. mutans* are likely to involve allelic Ag I/II and Hsa variants with different receptor binding specificities. First, both Ag I/II and Hsa mediated sialic acid-dependent gp340 interactions of *S. gordonii* DL1. Second, each *S. gordonii* phenotype (I to III) expressed Hsa and Ag I/II together with a unique sialic acid binding specificity. Third, each gp340 interactive phenotype (I and III) of *S. mutans* expressed only Ag I/II, mediating the interactions of *S. mutans* NG8 with gp340. Moreover, the gp340 interaction phenotypes of non-*viridans* group streptococci, which lack Hsa and Ag I/II, are attributable to yet unknown adhesins, and those of the *Actinomyces* phenotypes are attributed to type 1 and 2 fimbrial adhesins (13, 29). Notably, the *Actinomyces* gp340 interaction phenotypes I and II are members of the six *Actinomyces* groupings that each express a unique mosaic of type 1 and type 2 fimbrial subtypes to fit their ecological niches (13, 29). It is possible that, in a similar way, the *S. gordonii* gp340 interaction modes I, II, and III correspond with the three biovars, or taxonomic subpopulations, of *S. gordonii* (4, 21). Generally, gp340 interaction mode did not correlate directly with bacterial virulence potential, since commensal and pathogenic species were represented in each of the gp340 interaction modes I to III. However, although the numbers of strains and species tested were low, the gp340 adherence (mode II) phenotype occurred among commensal *S. gordonii* strains but not among cariogenic *S. mutans* strains, which had aggregating (mode I and III) phenotypes only. Hypothetically, therefore, gp340 may preferentially promote adherence of commensal streptococci while aggregating potentially cariogenic streptococcal phenotypes.

The specificity of strains of *S. gordonii*, *S. suis*, and *A. naeshlundii* for sialic acid or other sugars coincided with their gp340 interaction modes (modes I to III). The gp340 receptors either mimicked common carbohydrate sequences present also on erythrocytes, as for *S. gordonii* modes I and II, or represented carbohydrate structures unique to particular host tissues or bacterial partners (23), as for *S. mutans* (modes I and III) and *S. gordonii* mode III. However, since *S. gordonii* DL1 (mode I) recognized sLe^x, and since other gp340 binding bacteria such as *S. suis* and *H. pylori* recognize sialylα2-3polylactosamine and Le^b/sialylLe^a (32) receptors, respectively, some of the gp340 binding sites may reside on lactosamine oligosaccharides. Moreover, by virtue of the specificity of *A. odontolyticus* PK984 for SAα2-6GalNAcα1-3Ser and binding of *A. naeshlundii* ATCC 12104 to exposed Galβ1-3GalNAc, it is probable that similar binding sites may reside on gp340 as sialylated mucin-type O-linked oligosaccharides. Regardless of the detailed saccharide epitopes being recognized, the array of receptor specificities is likely to determine the biological properties of the phenotype. In this respect, it is noteworthy that the sialic acid specificity of *S. gordonii* DL1 (mode I) mediates adherence to both neutrophils and platelets, an interaction implicated in infective endocarditis (45). Finally, a high level of salivary pellicle adherence of *S. mutans* Ingbritt (mode I) coincides with caries-prone subjects (41). Since this adherence decreases or increases in the presence of the allelic PRP variants, PRP-1 or Db, in saliva, respectively, oral colonization by

S. mutans appears to depend on both the saliva and bacterial phenotypes.

The oligomeric complex of gp340 in saliva may, in a similar way to the extracellular traps formed by neutrophils (5), generate extracellular networks that trap both bacteria and innate defense molecules in close proximity. It remains to be determined, though, if gp340 secreted at host sites outside the oral cavity is organized and behaves similarly to salivary gp340. Nevertheless, since gp340 interacts with collectins, neutrophils, macrophages, and epithelial cells, the gp340 monomer has the ability to target distinct host responses toward bacteria. The high bacterial cell binding capacity of salivary gp340 and its ability to distinguish between bacterial phenotypes may suggest pattern recognition properties of salivary gp340 to direct selective host responses depending upon the bacterial ligand. In this respect, it is noteworthy that deletion of Mga, a protein that regulates expression of several virulence factors (including M-protein, C5a peptidase, and SpeB) in *S. pyogenes*, results in increased gp340-mediated aggregation of group A streptococcal cells. It is possible, therefore, that the attenuated virulence of Mga-deficient *S. pyogenes* (26) may in part be associated with increased gp340-mediated aggregation of bacteria and thus more rapid clearance. It follows then that gp340 could have the ability to distinguish *S. gordonii* and other commensal organisms from potentially pathogenic microorganisms, through a complex recognition pattern that hampers the commensal aggregation reaction. It may be speculated that this, in turn, could introduce an increased affinity of the gp340-bacterium aggregates for tooth and mucosal surfaces. If such a gp340-bacterium communication system operates, then it may act, both directly and indirectly, to suppress pathogenic organisms within the complex biofilm communities present in the mouth and nasopharynx.

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