Salivary Mucin 19 Glycoproteins

INNATE IMMUNE FUNCTIONS IN STREPTOCOCCUS MUTANS-INDUCED CARIES IN MICE AND EVIDENCE FOR EXPRESSION IN HUMAN SALIVA*

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Saliva functions in innate immunity of the oral cavity, protecting against demineralization of teeth (i.e., dental caries), a highly prevalent infectious disease associated with Streptococcus mutans, a pathogen also linked to endocarditis and atheromatous plaques. Gel-forming mucins are a major constituent of saliva. Because Muc19 is the dominant salivary gel-forming mucin in mice, we studied Muc19−/− mice for changes in innate immune functions of saliva in interactions with S. mutans. When challenged with S. mutans and a cariogenic diet, total smooth and sulcal surface lesions are more than 2- and 1.6-fold higher in Muc19−/− mice compared with wild type, whereas the severity of lesions are up to 6- and 10-fold higher, respectively. Furthermore, the oral microbiota of Muc19−/− mice display higher levels of indigenous streptococci. Results emphasize the importance of a single salivary constituent in the innate immune functions of saliva. In vitro studies of S. mutans and Muc19 interactions (i.e., adherence, aggregation, and biofilm formation) demonstrate Muc19 poorly aggregates S. mutans. Nonetheless, aggregation is enhanced upon adding Muc19 to saliva from Muc19−/− mice, indicating Muc19 assists in bacterial clearance through formation of heterotypic complexes with salivary constituents that bind S. mutans, thus representing a novel innate immune function for salivary gel-forming mucins. In humans, expression of salivary MUC19 is unclear. We find MUC19 transcripts in salivary glands of seven subjects and demonstrate MUC19 glycoproteins in glandular mucous cells and saliva. Similarities and differences between mice and humans in the expression and functions of salivary gel-forming mucins are discussed.

Demineralization of teeth (i.e., dental caries) resulting from the production of organic acids by bacteria within overlying biofilms (i.e., dental plaque) continues to be among the most prevalent infectious diseases in developed as well as developing nations (1, 2), particularly among under-privileged groups (3). Streptococcus mutans is highly associated with dental caries worldwide, but it is also linked to bacterial endocarditis and to atheromatous plaques (4, 5), indicating an interconnection between oral health and cardiovascular diseases. Hence, efforts to understand and treat oral diseases have potential ramifications to the development and progression of serious systemic disease.

Through a combination of innate and adaptive immune mechanisms, the oral cavity establishes a homeostasis with the commensal microbiota that is subject to disruption by changes in diet or in immune status, thus allowing opportunistic pathogens to flourish. In particular, recurrent ingestion of fermentable carbohydrates promotes the selection of bacteria proficient at metabolizing carbohydrates to organic acids (i.e., acidogenic organisms) and that then thrive in the resulting low pH environment (i.e., aciduric).

Saliva plays a major role in innate immunity of the oral cavity, protecting hard oral surfaces through its flushing action, buffering of acids, promotion of re-mineralization, bacteriostatic and bactericidal activities, and functioning in selective adherence of commensals and in clearance of acidogenic microorganisms (6–8). Glantz (9) proposed saliva is organized into a continuous phase composed of electrolytes in water, less water-soluble proteins, salivary micelles, other heterotypic complexes, lipid material, and cells (i.e., bacterial and released epithelial cells) all intertwined within a scaffold-like network structure. The network structure is due largely to intermolecular interactions of gel-forming mucins that impart saliva with its weak gel characteristics to function in lubrication and hydration (7, 10, 11). Intermolecular interactions between gel-forming mucins include calcium-mediated cross-links and disulfide-mediated oligomers (12). Mucin monomers as large as 2.9 × 10⁶ Da are also present in whole saliva (13).
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Gel-forming mucins are a major component of whole saliva (14), and in humans are the exocrine products of salivary mucous cells of the major submandibular and sublingual glands as well as numerous minor mucous glands lining the oral cavity (7, 8). Besides lubrication and hydration, these mucins function to control the initial selective adherence of bacteria to tooth surfaces and further provide bacteria with carbohydrate and amino acid nutrients (7, 8, 15).

In humans, MUC5B is well documented as a gel-forming mucin in saliva (16). However, MUC19 transcripts were detected in human submandibular and labial glands (17–19) and glycoproteins localized to salivary mucous cells (19). Conversely, MUC19 was undetected in human saliva in a recent proteomic study (20). It thus remains unclear whether MUC19 contributes to gel-forming mucins in human saliva.

In adult mice, Muc19 is the dominant gel-forming mucin and is synthesized and secreted exclusively by the major sublingual glands and minor mucous glands (21). Unlike humans, rodent submandibular glands produce a small secreted mucin, Muc10 (i.e. submandibular gland mucin), rather than gel-forming mucins (22). Muc5B transcripts are undetectable in the major salivary glands of mice, although the numerous minor mucous glands possess Muc5b, Muc5ac, and Muc2 transcripts in addition to Muc19 (21).

Despite the abundance of in vitro evidence supporting protective functions of individually isolated salivary components (6), the net effects of the loss of a specific salivary constituent on the innate immune functions of saliva in vivo have not been largely explored. Because of the dominant salivary expression of Muc19 compared with other gel-forming mucins in mice, Muc19 represents an attractive candidate to test the impact of deletion of a major gel-forming mucin on the innate immune functions of saliva. We therefore assessed whether the targeted deletion of Muc19 in mice significantly impacts the development of caries upon challenge with S. mutans combined with a highly cariogenic diet. Furthermore, we evaluated changes, in vitro, in innate protective mechanisms of saliva from Muc19 knock-out mice in interactions with S. mutans, and we explored changes in the oral microbiota. We further provide extensive evidence confirming expression of MUC19 transcripts in human salivary glands and further demonstrate MUC19 glycoproteins in stimulated human saliva. Evidence for MUC5AC in saliva is also given.

EXPERIMENTAL PROCEDURES

Materials—Unless indicated, all materials were from Invitrogen. All kits were used according to manufacturer’s instructions.

Animals and Collection of Tissues—The University of FloridaIACUC committee approved all animal procedures. Construction and genotyping of the Muc19−/− mouse model that targets the third through fourth exons encoding Muc19 have been described previously (23). For this study, heterozygous females were backcrossed five times to NFS/NCr males from our own breeding colony at the University of Florida, and heterozygous N5 progeny intercrossed to generate a colony of homozygous Muc19−/− mice.

Five mice of each sex and genotype were examined at 7 weeks of age for outward body characteristics (body shape and size, the number of digits and claws on each limb; fur color and texture on the back and belly; skin appearance; size and shape of the head; the position, size, and shape of ears; the size, position, clarity, and color of eyes; the number, position, size, and color of teeth; whiskers; the size and shape of limbs, genitals, anus, mammary glands; the color and hardness of feces; and the coloration of urine stains of the litter) and for any obvious differences in internal organs (palate, tongue, buccal mucosa, major salivary glands, esophagus, stomach, duodenum, pancreas, spleen, ileum, jejunum, cecum, colon, liver, gallbladder, kidney, adrenal glands, urinary bladder, trachealarynx, thymus, lungs, heart, lymph nodes, mesentry, scrotum, testes, epididymis, seminal vesicle, bulbourethral gland, penis, ovaries, uterus, vagina, Harderian glands, lacrimal glands, and brain).

All animals were maintained under BSL2 conditions. Animals were euthanized by CO2 asphyxiation with confirmation of death via thoracotomy. Mice were genotyped as described previously (23). Unless indicated, tissues were excised, blotted on filter paper, flash-frozen in liquid nitrogen, and stored at −80 °C. Rat sublingual glands were from frozen stocks obtained previously as part of another study (24).

Caries Experimental Protocol and Scoring—The caries protocol was similar to that reported previously (25). Briefly, timed pregnancies were established using 20 breeding pairs each of homozygous NFS/NCr and Muc19−/− mice, and pups were weaned and caged in pairs with non-littermates of the same sex. Mice were weighed weekly and sacrificed 7 weeks after the initial inoculation. The left and right mandibles were removed aseptically and assayed for bacterial colonization of the molars. The buccal, lingual, and proximal surfaces of all mandibular and maxillary molars were scored for smooth surface and sulcal caries using Larson’s modification of the Keyes’ scoring system (26, 27). Scores were compared by analysis of variance with the Tukey-Kramer post hoc test at 99.9, 99.0, and 95% confidence.

Recoveries of S. mutans and Total Bacteria from Molars—Procedures were as described previously (25). Briefly, excised mandibles underwent dissection to isolate the molars. Sonicates of the molars from two non-cagemates were pooled, and serial dilutions were cultured on blood agar to estimate CFU of total bacteria. Recovery of S. mutans was determined from cultures on MSB agar (28).

Comparison of Indigenous Oral Bacteria—Procedures were as described previously (25). Oral swabs from mice (8–10 weeks of age) were streaked on sheep blood agar plates and incubated under aerobic and anaerobic conditions, and total recovered CFUs for each colony morphotype were determined. Colony isolates from each of three mice of each genotype were subjected to colony PCR using degenerate primers (29) complementary to the 5′- and 3′-ends of the 16 S rRNA gene. Consensus sequences were compared with reference sequences (version 13.2) in the Human Oral Microbiome Database (30) and the Ribosome Database Project, Release 10 (31). Alignments were performed via the BLAST server at the Human Oral Microbiome Database.

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Collection of Stimulated Whole Saliva from Mice—Saliva collection was as described previously (25). Mice were anesthetized (ketamine, 75 mg/kg, plus xylazine, 8 mg/kg, i.p.); saliva flow was stimulated (subcutaneous 5 mg/kg pilocarpine, 0.5 mg/kg isoproterenol), and saliva was collected from the cheek pouch at 5-min intervals over a 20-min period.

Biofilms—For all in vitro assays with whole stimulated saliva, we used low-passage S. mutans UA159 containing the ΩKm element integrated into the gtfA gene with the vector pBGK (32), to confer kanamycin resistance (kindly provided by Dr. R. A. Burne). We thus avoided centrifugation of whole stimulated saliva, which promotes precipitation of a mucous gel in addition to removing small amounts of inherent microorganisms. The gtfA gene does not alter carbohydrate metabolism of S. mutans, except for melibiose and raffinose (33). Caries development by this strain is unaffected (34), and collected saliva produced no colonies under anaerobic conditions on blood agar containing 1 mg/ml kanamycin (data not shown).

Assays were carried out in 96-well plates containing hydroxyapatite discs (Clarkson Chromatography Products Inc.). Samples of mouse saliva were resuspended to a concentration of 63% (v/v) in saliva buffer (SB: 50 mM KCl, 1.0 mM KPO4, 1.0 mM CaCl2, and 0.1 mM MgCl2, pH 6.5) plus of 1 mg/ml kanamycin (SBK). Wells received 100 µl of saliva or SBK, and plates were incubated for 30 min at 37 °C. Kanamycin-resistant S. mutans UA159 cultured overnight in brain-heart infusion medium plus kanamycin (1 mg/ml) was diluted 1:10 in the same medium and cultured further at 37 °C in air, 5% CO2 to mid-log phase and then diluted 1:100 in pre-warmed semi-defined biofilm medium (BM: basic salts, vitamins, key amino acids, casamino acids) (35) containing 1% glucose plus 1 mg/ml kanamycin and incubated 4 h at 37 °C in air, 5% CO2. Approximately 106 cells in 150 µl were added to wells to give a final saliva concentration of 25%. After 24 h at 37 °C in air, 5% CO2, the medium was decanted, and biofilms were washed twice gently with 200 µl of sterile 120 mM NaCl to remove planktonic and loosely bound cells. Biofilms were stained with 200 µl of 5 µM Syto-13 in the dark for 25 min at room temperature, and fluorescence was determined. All experimental conditions were performed in triplicate.

Aggregation Assay—Kanamycin-resistant S. mutans UA159 were prepared as described above for biofilm assays. Cell suspensions were mixed with either samples of saliva diluted in SBK to give final concentrations as indicated (v/v) or increasing concentrations of rat Muc19, or a combination of saliva and Muc19. Initial cell suspensions (A600 ~0.65) were transferred to a spectrophotometer with a temperature-controlled multicultu-rette positioner set at 37 °C. After 5 min the A600 of each sample was recorded at 10-min intervals over a 2-h period. Percent aggregation (percent decrease in A600) was calculated as (A600 at 0 min − A600 at 120 min)/A600 at 0 min) × 100.

Adherence of S. mutans—Adherence assays were as described previously (25) using hydroxyapatite discs in 96-well plates and kanamycin-resistant S. mutans UA159. Briefly, wells received 100 µl of diluted saliva, Muc19, or buffer (background controls), and plates were incubated for 1 h at 37 °C with rotary shaking and washed twice with SBK. S. mutans in SBK (100 µl) was added to wells, incubated 1 h at 37 °C, and then washed with SB. Adherent cells were quantified using SYTO-13 and a standard curve. Values were normalized to the mean for cells bound to discs without saliva (100%). All experimental conditions were performed in triplicate.

Isolation of Rat Muc19 Glycoproteins—High density and high molecular weight glycoproteins were isolated from rat sublingual glands as part of a previous study (24). Of all gel-forming mucins (Muc2, Muc5ac, Muc5b, Muc6, and Muc19) only Muc19 was expressed in this gland by RT-PCR (data not shown), and the amino acid composition of high density and high molecular weight mucin glycoproteins isolated from rat sublingual glands are as predicted for rat Muc19 (36). Briefly, gland homogenates were pooled, and the cytosolic fraction was subjected to two rounds of cesium chloride density gradient fractionation under dissipative and reducing conditions. The resultant high density fractions were pooled and further fractionated by gel filtration under dissipative conditions. The high molecular weight material in the void volume and initial included fractions was pooled, dialyzed extensively, and lyophilized. The resultant material was free of lower molecular weight glycoproteins or proteins as assessed by SDS-PAGE (24).

Collection of Stimulated Human Whole Saliva and Human Salivary Glands—Stimulated whole saliva was collected from three healthy Caucasian female subjects, 41–57 years of age, with good oral health. Subjects refrained from oral hygiene procedures, smoking, eating, and drinking for 2 h prior to saliva collection. To collect stimulated whole saliva, subjects chewed a piece of paraffin (about 1.5 g) for 15 min, while refraining from swallowing. The first 30-s sample was discarded. At 30-s intervals, subjects spit into a 50-ml sterile plastic centrifuge tube kept on ice that contained 0.2 ml of protease inhibitor mixture (100-fold concentrated; Sigma). Collected saliva was assessed for volume (assuming a density of 1 g/ml) and resuspended by repeated pipetting with a 10-ml serological pipette, and 1-ml aliquots were frozen at −80 °C. Informed consent was obtained from all subjects. Human sublingual and palatal salivary gland tissues were obtained from human subjects within 36 h after death and immediately frozen in liquid nitrogen. An oral pathologist excised all tissues, and there were no signs of oral disease. Submandibular gland tissues were from pathologically normal tissue during surgery and obtained as frozen samples from the Cooperative Human Tissue Network, part of the Cancer Diagnosis Program of the NCI, National Institutes of Health. The seven samples were from four male and three female Caucasians, aged 40–76, mean age of 57. For immunofluorescence experiments, submandibular gland sections were from a 44-year-old Caucasian female, sublingual gland sections from a 60-year-old Caucasian female, and hard palate sections from a 52-year-old African-American female. Samples were obtained under protocols approved by the University of Florida Institutional Review Board.

RT-PCR—Procedures were as described previously (37). Briefly, RNA was isolated from frozen tissues or LS174T cells, and DNase I-treated RNA (5 µg) was reverse-transcribed with random primers. PCR conditions and specific primers used are shown in Table 1.
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TABLE 1
RT-PCR primers used to detect transcripts for gel-forming mucins in human submandibular glands

The positions of primers are based on reference sequences (GenBank™ accession numbers). PCRs incorporated 1 unit of Accuprime™ Taq high fidelity DNA polymerase, 0.25 mM primers, and 20 ng of cDNA. Cycling conditions were as follows: 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 68 °C followed by 7 min at 68 °C.

| Transcript | Reference sequence | Primer sequence | Exons | Amp | bp |
|------------|--------------------|-----------------|-------|-----|----|
| MUC2       | NM_002457          | 5′-CAAGCCACACGGCCGGGAGTGCAGTGTAGGAGTGTTT3′ | 22    | 441 |    |
|            |                    | 5′-GGTCCGGCTGGCCGACATCGTCC-3′ | 5     | 350 |    |
|            |                    | 5′-GTCCAGACCTTCGGAAGTTCAACAG-3′ | 7     | 294 |    |
| MUC5B      | NM_002458          | 5′-CAAGAGTCCATCGACCACGACG-3′ | 42    | 303 |    |
|            |                    | 5′-CCAGGTTACACGGCCTGAG-3′ | 45/46 | 303 |    |
| MUC6       | U97698             | 5′-AAGCCGGAATGCTGAGACG-3′ | 32    | 820 |    |
|            |                    | 5′-GTCTCATCTGAGCTGCTCC-3′ | 33    |     |    |
| MUC19      | AY236870           | 5′-GGTGGCCAAAAGAACCAACAGAG-3′ | 26    |     |    |
|            |                    | 5′-GGCTTGTAAAACCTGGAAGG-3′ | 25    |     |    |

SDS-PAGE—Procedures were as described previously (21) with minor modifications. For MUC19 and MUC5B Western blots, stimulated whole human saliva was dialyzed (3,500 Mw cutoff) 48 h against water containing a mixture of protease inhibitors (Sigma), lyophilized, and resuspended in SDS-PAGE sample buffer. Samples were briefly sonicated to promote complete solubilization (two 10-s pulses at 30% amplitude with a 60-s interval). Gels were stained for proteins with Coomassie Blue or stained for highly glycosylated glycoproteins with Alcian blue, with or without subsequent silver enhancement (38).

Western Blots—Western blots were conducted as described previously (21) with the following modifications. To detect MUC5B, blots were probed overnight at 4 °C with rabbit anti-MUC5B (1:5000; Santa Cruz Biotechnology). Blots were sub-sequently incubated for 30 min at room temperature with the appropriate secondary antibody and then processed using our published protocol.

Immunofluorescence—Immunofluorescence microscopy was carried out as described previously (23) except frozen tissue sections were fixed in acetone (−20 °C) and then rehydrated in graded ethanol solutions. Sections were probed with chicken anti-MUC19 (1.5 μg/ml) or with preimmune antibody (1.5 μg/ml). Immunodetection was with Alexa Fluor 568 goat anti-chicken IgY (1:500) and DAPI counterstaining.

Statistics—Comparisons between wild type and Muc19−/− mice were conducted with the paired or unpaired two-tailed t test or by analysis of variance with the Tukey-Kramer post hoc test using Prism 5.0 software (GraphPad Software, Inc., San Diego). Curve fitting was also with Prism 5.0.

RESULTS

Characterization of the Muc19−/− Phenotype—We recently described the targeting strategy to produce the Muc19−/− mouse model (23). For this study, we backcrossed Muc19−/− mice five times to NFS/NCr mice because this genetic background produces slightly higher smooth surface caries in control mice, thus permitting better delineation of an experimentally induced decrease in caries development (25, 40). We examined five mice of each sex and genotype at 7 weeks of age for outward body characteristics and for any obvious differences the size, shape, and color of internal organs. In all cases, no differences were noted between Muc19−/− and wild type mice. The number, position, size, and color of teeth were also very similar as were body weight and length (data not shown). Previously, we examined histologically multiple mucin-producing tissues in mice in which we deleted Muc19 expression but inserted a reporter GFP, and we found no abnormalities (21). No differences between the two genotypes were noted in protein profiles of parotid, submandibular, and sublingual glands (Fig. 1A). In rodents, these glands are distinct in secretory acinar cell types and in their major secretion products. Parotid glands contain serous cells with amylase as the major secretion product, and submandibular and sublingual glands contain seromucous cells that produce the low molecular weight and non-gel-forming mucin, Muc10, and sublingual glands contain mucous cells that secrete Muc19. Sublingual glands of Muc19−/− mice do not express detectable Muc19, whereas expression of Muc10 in submandibular glands is unaffected (Fig. 1B). Previously, we demonstrated that sublingual glands of Muc19−/− mice appear normal histologically and ultrastructurally, except for the absence of large and electron translucent (i.e. mucous-like) secretion granules in acinar cells (23).

To evaluate the secretory function of the salivary system of Muc19−/− mice, we studied stimulated saliva flow. Stimulated flow was equivalent between wild type and Muc19−/− mice (Fig. 1C). As shown in Fig. 1D, no differences in protein profiles were noted in whole stimulated saliva from mice of both genotypes. Staining for highly glycosylated glycoproteins indicate a 175-kDa glycoprotein in both samples (Fig. 1E), representing Muc10, as observed in Western blot (Fig. 1F). Salivary agglutinin (i.e. gp340) is well expressed in whole stimulated saliva of wild type and Muc19−/− mice (Fig. 1G). From Fig. 1, E and G, it is apparent that high molecular weight glycoproteins are greatly attenuated in saliva from Muc19−/− mice. Residual glycoproteins in Muc19−/− saliva are due likely to contributions of
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Development of caries and their severities on molars of wild type and Muc19<sup>−/−</sup> mice

Values are means (S.E.) of Larson’s modified Keyes’ scores. 20 mice were in each group. Total smooth surface caries is the sum of buccal, lingual, and proximal caries. E is enamel affected; Ds is dentin exposed; Dm is 3/4 of the dentin affected, and Dx is whole dentin affected.

|                      | Wild type | Muc19<sup>−/−</sup> | p       |
|----------------------|-----------|---------------------|---------|
| **Smooth surfaces**   |           |                     |         |
| Total E              | 8.90 (1.07)| 19.20 (1.74)<sup>a</sup> | 0.001   |
| Total Ds             | 5.35 (1.02)| 15.05 (1.59)<sup>a</sup> | 0.001   |
| Total Dm             | 1.40 (0.48)| 8.25 (1.27)<sup>a</sup>  | 0.001   |
| Total Dx             | 1.25 (0.46)| 7.55 (1.27)<sup>a</sup>  | 0.001   |
| Buccal E             | 2.20 (0.56)| 6.55 (0.83)<sup>a</sup>  | 0.001   |
| Buccal Ds            | 1.15 (0.36)| 5.50 (0.73)<sup>a</sup>  | 0.001   |
| Buccal Dm            | 0.25 (0.14)| 3.50 (0.59)<sup>a</sup>  | 0.001   |
| Buccal Dx            | 0.15 (0.11)| 3.40 (0.57)<sup>a</sup>  | 0.001   |
| Lingual E            | 5.10 (0.51)| 9.80 (0.87)<sup>a</sup>  | 0.001   |
| Lingual Ds           | 2.85 (0.55)| 7.40 (0.79)<sup>a</sup>  | 0.001   |
| Lingual Dm           | 0.75 (0.27)| 3.90 (0.58)<sup>a</sup>  | 0.001   |
| Lingual Dx           | 0.70 (0.27)| 3.45 (0.56)<sup>a</sup>  | 0.001   |
| Proximal E           | 1.60 (0.36)| 2.85 (0.53)         |         |
| Proximal Ds          | 1.35 (0.34)| 2.15 (0.42)         |         |
| Proximal Dm          | 0.40 (0.20)| 0.85 (0.23)         |         |
| Proximal Dx          | 0.40 (0.20)| 0.70 (0.22)         |         |

| **Sulcal surfaces**   |           |                     |         |
| Total E              | 8.50 (0.89)| 13.70 (0.89)<sup>a</sup> | 0.001   |
| Total Ds             | 4.95 (0.79)| 10.55 (0.89)<sup>a</sup> | 0.001   |
| Total Dm             | 1.50 (0.39)| 6.65 (0.73)<sup>a</sup>  | 0.001   |
| Total Dx             | 0.45 (0.15)| 4.65 (0.56)<sup>a</sup>  | 0.001   |

<sup>a</sup> p < 0.001 versus wild type.  
<sup>b</sup> p < 0.01 versus wild type.

Muc5b from minor mucous glands (see under “Discussion”). The absence of Muc19 in Muc19<sup>−/−</sup> mice combined with no other significant differences in phenotypic characteristics validates the use of this model system to assess the impact of salivary Muc19 on caries development.

**Caries Development and Recovery of Bacteria—Muc19<sup>−/−</sup>** and wild type mice were challenged with *S. mutans* strain UA159 and a highly cariogenic diet. Weight gains during this period between the two genotypes were very similar for each gender, although at weeks 5–7 Muc19<sup>−/−</sup> females were ~4% lower in weight (data not shown). Both smooth and sulcal caries were assessed. Smooth surface caries is an evaluation of the proficiency of bacteria to adhere and colonize the smooth vertical tooth surfaces, whereas sulcal caries appraises colonization within the long angular depressions of horizontal surfaces where molars of the upper (maxilla) and lower (mandible) jaws meet, and in which food particles tend to become entrapped (41). As shown in Table 2, the mean of the incidence of total carious lesions to molar smooth surfaces (E score) is more than 2-fold higher in Muc19<sup>−/−</sup> mice compared with wild type. Furthermore, the proportional differences in caries scores further increases with the severity of lesions (i.e. Ds, Dm, and Dx scores), with up to 6-fold higher levels for the most severe type of lesion (Dx; progression into the entire dentin). The increase in smooth surface lesions occurred on surfaces juxtaposed to the tongue (lingual) and cheeks (buccal) but not to areas with silver (Ag). The prominent band at ~175 kDa is Muc10, F, whose stimulated saliva samples (6 μl) from wild type and Muc19<sup>−/−</sup> mice run on a 3–8% gradient gel and probed for mouse Muc10. G, whole stimulated saliva samples (17 μl) from wild type and Muc19<sup>−/−</sup> mice run on a 3–8% gradient gel under nonreducing conditions and either stained for glycoproteins with Alcian blue alone or blotted and probed for mouse gp340. Note that gp340 (~350 kDa) stains weakly with Alcian blue and that high molecular weight mucins barely enter the gel in the absence of prior brief sonication as used in E.
between teeth (proximal). In comparing these two sites, there is a trend for greater proportional increases in lesions on buccal versus lingual surfaces, although these differences are not significant \((p > 0.05, \text{analysis of variance})\).

In comparing lesions on sulcal surfaces, total lesions in \(Muc19^{-/-}\) mice are 1.6-fold higher than in wild type mice. As with smooth surface scores, the proportional differences in sulcal scores increases with lesion severity, to more than 10-fold for Dx scores. The higher levels of caries in \(Muc19^{-/-}\) mice are consistent with recoveries from molars of \(S.\ mutans\) and total microbiota (Fig. 2A). Recovery of \(S.\ mutans\) is nearly 1.7-fold higher from \(Muc19^{-/-}\) mice compared with wild type. For total cultivable bacteria, this difference is 2.4-fold.

**Comparison of Indigenous Oral Bacteria**—We determined whether deletion of Muc19 expression affects the indigenous oral microbiota. From oral swabs of uninfected mice of both genotypes, we consistently obtained seven distinct colony morphologies on blood agar under either aerobic or anaerobic culture conditions, whereas no colonies formed on MSB agar.

**TABLE 3**

| Colony appearance, cell appearance | Gram stain | Catalase activity | Reference sequences | Genus                  | Species                 | GenBank accession no. | Percent identities |
|----------------------------------|------------|------------------|---------------------|------------------------|------------------------|----------------------|-------------------|
| Large gray, short thin rods      | –          | +                | RDP                 | Actinobacillus         | muris                  | AF029526             | 98.5              |
| Large yellow, large cocci        | +          | –                | RDP                 | Haemophilus            | haemolyticus           | HM596277             | 93.9              |
| Medium gray (**-hemolytic**), cocci, chains | +        | –                | RDP                 | Staphylococcus        | xylosus                | AB626129             | 99.9              |
| Large irregular gray, cocci, chains | +        | –                | RDP                 | Staphylococcus        | warneri                | L37603               | 98.3              |
| Medium white, cocci, chains      | +          | –                | HOMD                | Streptococcus         | danielleae             | GQ456229             | 98.7              |
| Medium white, medium to long rods | +          | +                | HOMD                | Streptococcus         | sanguinis              | AF003928             | 97.2              |
| Small gray, short rods           | +          | +                | RDP                 | Streptococcus         | salivarius             | M58839               | 94.7              |
|                                  |            |                  | RDP                 | Streptococcus         | unassigned             | Y09007               | 99.2              |
|                                  |            |                  | RDP                 | Streptococcus         | cristatus              | A538685              | 100               |
|                                  |            |                  | RDP                 | Lactobacillus         | murinus                | AF157049             | 99.9              |
|                                  |            |                  | RDP                 | Corynebacterium       | mastiditis             | A34747               | 99.5              |
|                                  |            |                  | RDP                 | Corynebacterium       | mucicafecines          | X82059               | 95.4              |

We further determined total recoveries of each colony morphotype from \(Muc19^{-/-}\) and wild type mice (see Fig. 2B). There were no differences in total recovered CFUs under aerobic and anaerobic culture conditions (about \(1.4 \times 10^5\) CFUs total). The absence of Muc19 induced a shift in the relative proportions of \(S.\ mutans\), \(S.\ acidominus\), and \(S.\ unassigned\). Most noticeable was about a 100-fold decrease in \(S.\ mutans\) and a 120-fold increase in \(S.\ unassigned\), whereas \(S.\ xylosus\) decreased 5.8-fold. \(S.\ thalattensis\) displayed a trend toward a 10-fold higher proportion in \(Muc19^{-/-}\) mice \((p = 0.10, \text{aerobic}, \text{and } p = 0.09, \text{anaerobic})\). Unchanged in proportion were the species \(Corynebacterium\ mastitiidis\), \(Lactobacillus\ murinus\), and \(Streptococcus\ danielleiae\), the latter representing about 75% of the total cultivable bacteria. Collectively, deletion of Muc19 expression increased the dominance of streptococcal species in the oral cavity from ~77 to 89%.

**In Vitro Assessments of Muc19 Protective Mechanisms**—To interrogate possible mechanisms through which Muc19 may interact with \(S.\ mutans\) to limit its colonization under highly cariogenic conditions, we conducted a series of in vitro assays using either whole saliva from wild type and \(Muc19^{-/-}\) mice or with \(Muc19\) purified from rat sublingual glands (24).

**Adherence of \(S.\ mutans\) to Muc19 and Saliva Pellicles on Hydroxyapatite Discs**—In humans, selective constituents of saliva interact rapidly with enamel surfaces to form an acquired...

**FIGURE 2. Cultivable microflora from mice.** A, recoveries by CFU of total bacteria on blood agar and of \(S.\ mutans\) on MSB agar from sonicates of mandibular molars. Values are means ± S.E. from molar of 20 mandibles prepared from each group. *p < 0.05 for \(Muc19^{-/-}\) mice (KO) compared with the wild type (WT). B, total recoveries for each of seven distinct colony morphotypes from the oral cavities of \(Muc19^{-/-}\) and wild type mice compared with the wild type.
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enamel pellicle (48–51), which promotes initial adherence of Actinomyces species followed by acquisition and predominance of commensal streptococci such as Streptococcus sanguinis, Streptococcus gordonii, Streptococcus mitis, and Streptococcus oralis (48). In contrast, S. mutans adheres poorly to salivary pellicles formed in vitro (52). Because salivary gel-forming mucins display high affinity to naked hydroxyapatite (53–55) and are a component of the acquired enamel pellicle (50, 56–58), we determined whether Muc19 pellicles on hydroxyapatite influence S. mutans adherence. As shown in Fig. 3A, S. mutans adherence is reduced dramatically at low concentrations of Muc19, with near maximal inhibition of ~70% at 2.0 mg/ml, and with 50% of maximal inhibition at 0.03 mg/ml. We next compared adherence after pretreatment of hydroxyapatite with increasing concentrations of saliva from wild type and Muc19−/− mice. At low concentrations, saliva from wild type mice. Adherence is normalized to the percent of cells bound in the absence of Muc19. Adherence is normalized to the percent of cells bound in the absence of saliva. Values are means ± S.E. from five separate experiments. *p < 0.05 versus all higher concentrations of saliva. D, aggregation of S. mutans with increasing concentrations of whole stimulated saliva from Muc19−/− (KO) and wild type (WT) mice. Values are means ± S.E. from 10 separate experiments. *p < 0.05 for saliva from Muc19−/− versus wild type mice. E, percent aggregation of S. mutans with increasing concentrations of whole stimulated saliva from wild type mice. Values are means ± S.E. from 12 separate experiments. *p < 0.05 versus all higher concentrations of saliva. F, detection of transcripts of gel-forming mucins in human submandibular glands—In light of the important role Muc19 plays in protection against caries in mice, we compared expression of MUC19 transcripts in submandibular glands from seven human subjects. Because we previously found expression of MUC19 transcripts in submandibular glands from three human subjects by SDS-PAGE, we localized MUC19 glycoproteins to mucous cells in submandibular (Fig. 4B), major sublingual (Fig. 4C), and minor glands of the hard palate (Fig. 4D) using an antibody raised against gene-specific residues in the N terminus. In examination of stimulated saliva from three human subjects by SDS-PAGE, we observed highly glycosylated glycoproteins at about 110 kDa or higher (Fig. 5A), whereas proteins were localized near the bottom of the gel (< 100 kDa). Interestingly, saliva from subject 3 displays a prominent glycoprotein near the 171-kDa marker, possibly due to high levels of secretion of the lower mass and non-gel-forming mucin, MUC7. We probed blots of all three saliva samples for MUC19 (Fig. 5B), and for comparison we probed for MUC5B (Fig. 5C). There are marked differences between subjects in reactivities of each antibody. For example, saliva from subject 1 displayed much less reactivity for MUC19, but more for MUC5B compared with the other two subjects. Furthermore, MUC19 mucins were more prominent in regions of lower mobilities than were MUC5B mucins.

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Increasing concentrations of whole stimulated saliva from wild type mice produces markedly reduced aggregation compared with the wild type and a small but significant decrease at 10% saliva (Fig. 3D). Although these results suggest Muc19 contributes to the aggregation properties of saliva, it is unclear whether Muc19 interacts with S. mutans directly or possibly indirectly through heterotypic complexation with other salivary constituents. We therefore assessed aggregation with increasing concentrations of Muc19 alone or when added to saliva from Muc19−/− mice. As shown in Fig. 3E, Muc19 alone at concentrations up to 1 mg dry weight/ml has no effect on aggregation. Conversely, aggregation is greatly enhanced with addition of Muc19 to 3% saliva from Muc19−/− mice, with aggregation approaching maximal levels observed with wild type saliva seen in Fig. 3C.

Development of Biofilms—We compared biofilm formation by S. mutans on hydroxyapatite discs in a semi-defined medium containing 1% glucose, without and with saliva from wild type and Muc19−/− mice. As shown in Fig. 3F, biofilm formation was not significantly different among all three conditions.

Detection of Transcripts of Gel-forming Mucins in Human Submandibular Glands—In light of the important role Muc19 plays in protection against caries in mice, we compared expression of MUC19 transcripts in submandibular glands from seven human subjects. Because we previously found expression of MUC19 transcripts in submandibular glands from three human subjects by SDS-PAGE, we localized MUC19 glycoproteins to mucous cells in submandibular (Fig. 4B), major sublingual (Fig. 4C), and minor glands of the hard palate (Fig. 4D) using an antibody raised against gene-specific residues in the N terminus. In examination of stimulated saliva from three human subjects by SDS-PAGE, we observed highly glycosylated glycoproteins at about 110 kDa or higher (Fig. 5A), whereas proteins were localized near the bottom of the gel (< 100 kDa). Interestingly, saliva from subject 3 displays a prominent glycoprotein near the 171-kDa marker, possibly due to high levels of secretion of the lower mass and non-gel-forming mucin, MUC7. We probed blots of all three saliva samples for MUC19 (Fig. 5B), and for comparison we probed for MUC5B (Fig. 5C). There are marked differences between subjects in reactivities of each antibody. For example, saliva from subject 1 displayed much less reactivity for MUC19, but more for MUC5B compared with the other two subjects. Furthermore, MUC19 mucins were more prominent in regions of lower mobilities than were MUC5B mucins.

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thus providing further evidence for MUC19 in stimulated submandibular and sublingual glands, we find three peptides aligning to gel-forming mucins. In stimulated secretions from Project (59) and annotated the domain localization of peptides glands.

FIGURE 4. Expression of transcripts for gel-forming mucins in human submandibular glands and MUC19 immunolocalization in human salivary glands. A, expression of transcripts for gel-forming mucins MUC2, MUC5AC, MUC5B, MUC6, and MUC19 in submandibular gland tissues from seven healthy adults. Shown are RT-PCR products from 20 ng of cDNA. PC, positive controls for all transcripts but MUC19 are from the LS174T colon adenocarcinoma cell line. B, immunolocalization of MUC19 in a human submandibular gland. Chicken anti-MUC19 is detected with Alexa 568-labeled secondary antibody (red). Nuclei are stained blue (DAPI). Both fluorescence (upper image) and differential interference contrast images (lower image) are shown. Mucous cells within acini at the edge of a submandibular gland lobule stain for MUC19 (arrows). Under differential interference contrast optics, the mucous glands have a characteristic crater-like appearance, whereas individual serous acini are poorly distinguished in frozen sections (arrowhead). C, mucous cells within acini (arrows) of a sublingual gland stain with anti-MUC19. D, mucous glands of the hard palate also display anti-MUC19 staining. E, submandibular mucous glands (arrows) and other cellular elements are unstained by preimmune antibody. Bars, 50 μm.

We also surveyed results of the Human Salivary Proteome Project (59) and annotated the domain localization of peptides aligning to gel-forming mucins. In stimulated secretions from submandibular and sublingual glands, we find three peptides aligned to the very N terminus of human MUC19 (see Table 4), thus providing further evidence for MUC19 in stimulated saliva. We also found two peptides unique to MUC5AC, 30 peptides unique to MUC5B, and no peptides aligning to MUC6. One peptide is common to MUC2, MUC5AC, and MUC5B, whereas 18 peptides are common to MUC5AC and MUC5B (Table 4). The presence of multiple peptides shared between MUC5AC and MUC5B reflects the large degree of identity between the two apoproteins (37% by ClustalW alignment, not shown). All peptides are localized outside predicted glycosylated tandem repeat domains of each mucin apoprotein. For reference, a figure of the domain architecture for each mucin apoprotein is given in Fig. 6.

FIGURE 5. Probing human saliva for MUC19 and MUC5B. Stimulated saliva collected from three subjects was dialyzed and lyophilized and then run on 3–8% gradient SDS-polyacrylamide gels. Each lane contains resuspended dry weight material from 100 μl of saliva. A, gel stained first for highly glycosylated glycoproteins with Alcian blue with subsequent silver enhancement (AB-Ag), followed by destaining of the silver enhancement and Coomassie Blue staining for proteins (AB-CB). Proteins stained with Coomassie Blue are in the bottom third of the gel, and Alcian blue-stained glycoproteins are in the upper two-thirds. B, Western blots of identical aliquots of the samples in A probed with either chicken anti-MUC19 (MUC19) or with preimmune antibody (Pre-im). C, samples identical to those in B probed with rabbit anti-MUC5B (MUC5B) or with nonimmune rabbit IgG.

DISCUSSION

Muc19 Protects against S. mutans Colonization and Development of Hard Surface Lesions—In a previous caries study with aquaporin 5−/− mice in which salivary flow is reduced 60%, smooth surface and sulcal caries were only marginally increased (51 and 26%, respectively) (40). In this study, absence of the predominant gel-forming mucin, Muc19, resulted in markedly greater increases in the incidence of total smooth surface and sulcal caries (116 and 61%, respectively). Correlated with increased caries were higher recoveries of S. mutans from molars of Muc19−/− mice. Under highly cariogenic conditions, Muc19 therefore contributes significantly to innate protection of hard oral surfaces against S. mutans by attenuating colonization and subsequent acid-induced lesions in mice. Inference for a negative correlation between caries and salivary gel-forming mucins in humans, as observed in our mouse model, was reported by Banderas-Tarabay et al. (60), in which the index scores in decayed, missing, and filled teeth in healthy human subjects were inversely related to the presence of high molecular mass glycoproteins in resting saliva.

Muc19 Influences the Commensal Oral Microbiota—Nonmutans streptococci represent the predominant commensals of the oral microbiota of wild type and Muc19−/− mice, con-
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Table 4

| MUC2  | MUC5AC | MUC5B | MUC6 | MUC19 |
|-------|-------|-------|------|-------|
| NP_002448.3 | NP_006718462.1 | NP_002449.2 | NP_005952.2 | NP_775871.2 |

Alignment of amino acids to each mucin apoprotein

Unique to MUC19
- R.SGGSFGYSNGSDGGD.D.R.K
- R.KKPSLSVEGSPGETED.D.S.K
- K.KLPSLSVEGSPGETED.D.S.K

Unique to MUC5AC
- R.PGPLCNVYTSHE.C.G
- R.SYVGSDLNENVSH.W.K

Unique to MUC5B
- R.AAYEDENYQ.L.R
- R.GYQCVPLA.DR.C.A
- R.AAQLPDM.EELQGQ.VD.C.M.R
- R.LPLQGSGY.S.S.
- I.PC.TGTT.C.T.C.A.S.
- R.GPGGDK.P.Y.K
- R.KTSF.VR.L
- R.GYQ.VCPVLADIECR.A
- R.KPLFS.LLEFGSPGETED.D.S.K

Common to one or more mucins
- K.TTEDGDRF.R
- R.PGPLCNVYTSHE.C.G
- R.SEDC.LAASSLYYHAC.A
- R.SEDC.LAASSLSYHACA.A
- S.TSYHAC.A.G
- R.WKSPCIDALPKPCTANPR.K
- R.KLSPCIDALPKPCTANPR.K
- R.SPSPCIDALPKPCTANPR.K
- R.PSPCIDALPKPCTANPR.K
- R.KDPTANPR.K
- R.KDPTANPR.K
- R.KDPTANPR.K
- R.KDPTANPR.K

Consistent with previous observations in humans (61) as well as laboratory mice (62). Interestingly, expression of Muc19 promotes oral colonization of A. muris and S. xylosus, while attenuating colonization of S. unassigned. These results highlight the importance of individual salivary constituents to impact the oral microbiota (25) and the necessity for further investigations.
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of homeostatic mechanisms between salivary components and the oral microbiome. Moreover, we acknowledge that differences in oral ecology may have impacted colonization by *S. mutans* in our caries study. For example, increased colonization by *S. unassigned* in *Muc19<sup>−/−</sup>* mice may present a more competitive environment for *S. mutans* (63, 64), but if *S. unassigned* is acidogenic, it may have facilitated *S. mutans* colonization (65). Additional investigations beyond the scope of this study are required to delineate the influence of ecological shifts on subsequent caries development.

**Protective Mechanisms of Muc19 in Interactions with *S. mutans***—Pellicles formed with low concentrations of purified Muc19 markedly reduce adherence of *S. mutans* to hydroxyapatite, consistent with the results of Kishimoto et al. (66) in which pellicles of high mass glycoproteins from human submandibular-sublingual saliva diminish adherence of *S. mutans*. Interestingly, Nieuw Amerongen et al. (67) found that a pellicle of human salivary gel-forming mucins shields the underlying enamel surface from acid demineralization, suggesting a complete protective coating. Hence, the markedly reduced adherence of *S. mutans* to pellicles formed from saturating concentrations of Muc19 or from human high mass glycoproteins (66) indicates salivary gel-forming mucins are functionally analogous in humans and mice, either providing a relatively low density of binding sites for *S. mutans* or entrapping cells within the extended and branched mucin oligosaccharides (68).

Pellicles formed from whole mouse saliva hinder adherence of *S. mutans* onto hydroxyapatite up to 60%, comparable with inhibition observed previously with rat (69) and human saliva (52, 70). The absence of Muc19 increases adherence at very low saliva concentrations, indicating other salivary constituents function in large part to shield hydroxyapatite-binding sites. In our assay, Muc19 may predominate in decreasing adherence at low saliva concentrations due to its selective absorbance to hydroxyapatite, a result of the abundance of gel-forming mucins in saliva (7, 8) and their high affinity for hydroxyapatite (53–55).

We find whole saliva (with or without Muc19) does not effect biofilm formation of *S. mutans* on hydroxyapatite when cultured in semi-defined medium containing vitamins, amino acids, and 1% glucose. To produce energy for growth and biofilm formation, *S. mutans* depends upon fermentation of dietary carbohydrates, and through carbohydrate catabolite repression it can elect to catabolize a preferred carbohydrate when other carbohydrates are available (71). Our results suggest *S. mutans* is unable to degrade salivary constituents, including gel-forming mucins, to provide dietary substrates more preferable to glucose in promoting biofilm formation. Similarly, human gel-forming mucins are unable to support growth of *S. mutans* (72) unless in a defined medium without amino acids (73) or in co-culture with other species that provide enzymes to sufficiently degrade mucins for catabolism (74, 75). Upon the consumption of sucrose by the host, *S. mutans* rapidly metabolizes this disaccharide to produce organic acid and also for production of a matrix of exopolysaccharides (primarily glucans) that serves as a supplemental source of carbohydrates during periods of fasting by the host, and it further promotes adherence of planktonic *S. mutans* expressing glucan-binding proteins (76). Because sucrose is highly preferred by *S. mutans* and readily available in the drinking water and diet of mice in our caries model, the absence of Muc19 likely had no significant direct effect on its survival and growth in supragingival plaque.

Saliva functions to aggregate and clear bacteria from the oral cavity via swallowing. Indeed, the level of aggregation of *S. mutans* by whole saliva from human subjects is inversely correlated with *S. mutans* numbers in plaque (77). Also, a greater capacity of saliva to aggregate *S. mutans* is associated with caries-resistant versus caries-susceptible individuals (78). We find maximal aggregation of *S. mutans* at about 3% saliva, whereas in the absence of Muc19, more than 3-fold higher saliva concentration is required for maximal aggregation. Early studies of aggregation of oral streptococci by crude high molecular weight fractions of saliva demonstrated association with constituents expressing blood group antigens (79), such as high molecular weight mucins (80). However, subsequent studies with highly enriched preparations of human gel-forming mucins found little bacterial binding or aggregating activities toward oral streptococci (81–83), similar to our results with murine Muc19.

Our collective results of murine saliva and purified Muc19 are analogous to previous results with human saliva and salivary gel-forming mucins in their interactions with *S. mutans* in aggregation, adherence to pellicles formed on hydroxyapatite, and support of growth and biofilm formation in monospecies culture. These similarities reinforce the mouse caries model as a tool to explore mechanisms of interactions between the host and *S. mutans*.

**MUC5B and MUC19 in Human Salivary Glands and Saliva**—In prior studies using antibodies to probe saliva or glandular secretions for gel-forming mucins, only MUC5B was identified, but not MUC2, MUC5AC, or MUC6 (84–87). Similarly, mass spectrometry of tryptic digests of isolated salivary gel-forming mucins identified peptides for MUC5B but not for MUC2, MUC5AC, or MUC6 (12, 84, 87). In another study, tryptic peptides from MUC5B were detected, whereas peptides predicted for MUC19 were absent in human resting saliva (20). These collective results argue for MUC5B as the sole gel-forming mucin in human saliva.

In contrast, there is evidence to suggest at least one additional gel-forming mucin in saliva. Troxler et al. (88) detected abundant MUC5B transcripts in sublingual glands by Northern analysis, whereas MUC5B transcripts were barely detected in submandibular glands, and transcripts for MUC2, MUC5AC, or MUC6 were absent in both glands. A subpopulation of unreactive sublingual mucous cells exhibited no immunoreactivity to MUC5B antibody (86). In sublingual gland secretions, one of two populations of mucins with different charge densities was unrecognized by MUC5B antibody (87). Labial gland secretions from 90 subjects were nearly all reactive to anti-MUC5B antibody, whereas secretions from buccal mucous glands (89) were reactive in only 11 subjects (16). MUC19 transcripts were detected in human submandibular and labial glands (17–19), and MUC19 glycoproteins immunolocalized to salivary gland mucous cells (19). We now confirm expression of MUC19 transcripts in submandibular glands from multiple human subjects as well as immunolocalization of MUC19 to mucous elements...
of major and minor salivary glands. More importantly, we
detect MUC19 immunoreactivity to high molecular weight gly-
coproteins in saliva and show that mucins from the same sub-
ject display distinct patterns of MUC19 and MUC5B immuno-
reactivities. We also highlight peptides from MUC19 and
MUC5AC in the salivary proteome database and identify
MUC5AC peptides in acquired enamel pellicle (51, 57).

How Can These Two Conflicting Bodies of Results Be Reconciled?—
Extensive glycosylation can hinder apomucin identification by
blocking anti-peptide epitopes or the access of proteases to
polypeptide domains (84, 85). For example, glycosylated tan-
dem repeat sequences of mucins protect adjacent nonglycosy-
lated regions from denaturation by acid and to proteolysis by
pepsin (90) or cysteine protease (91). Also, a MUC5B antibody
raised against a polypeptide sequence shared in four of the non-
glycosylated WXXW domains displays markedly enhanced
reactivity after reduction of mucins, suggesting the tertiary
structure of these domains in nonreduced mucins shields anti-
body epitopes (86).

In the prior studies described above in which saliva or sali-
vary glands were probed for gel-forming mucins, only one study
probed for MUC19, as the gene was not yet identified, nor were
antibodies available (10, 84, 86, 88, 92). In the one exception,
Rousseau et al. (20), reported the absence of MUC19 in human
saliva, as assessed by mass spectrometry, whereas Muc19 was
identified in saliva of other mammalian species. Because the
complete 5'-end of MUC19 had not been delineated, their
model of the predicted N terminal encompassed the region
containing three VWD domains but excluded the LAT
domain (see Fig. 6), which appears to be unique to humans.
MUC19 peptides identified in the human salivary proteome are
near the 5'-end of the LAT domain, suggesting the more down-
stream region containing three VWD domains is protected
from attack by trypsin. Within the LAT domain are predicted
serine sites of O-glycosylation. Glycosylation within this
domain and/or extensive glycosylation of the tandem repeat
domain bordering the VWD region may therefore hinder
access to trypsin. Moreover, in their assay of tryptic peptides
from the VWD regions of Muc19 from other mammalian spe-
cies, horse, rat, pig, and cow, Rousseau et al. (20) detected 25,
20, 8, and 3 peptides, respectively. Because this region shares
66% protein sequence identity between the four species (20),
their results suggest species differences in steric hindrance to
trypsin, possibly due to differences in the extent of glycosyla-
tion and/or oligosaccharide structures of the tandem repeat
domain (93).

Differences and Similarities in Expression of Salivary Gel-
forming Mucins between Mice and Humans—Although Muc19
and MUC5B appear to represent the predominant gel-forming
mucin in murine and human saliva, respectively, we provide
evidence for MUC19 expression in humans and previously
detected Muc5b transcripts in murine minor mucous glands
(21). Nevertheless, the relative expression of these mucins in
saliva of each species remains unclear. In mice, Muc5b may
account for the minor population of high molecular weight
mucins observed in saliva from Muc19−/− mice. Decreased
expression of human MUC19 may be related to decreased splic-
ing efficiency of exons within the LAT domain, as splicing
within this region is highly variable with some variants produc-
ing nonsense transcripts (19). Interestingly, this domain likely
represents evolutionary remnants of a splice variant of the
Muc19 gene encoding a small glycoprotein, Smgc, using exon 1
and Smgc-specific exons 2–18. Smgc is expressed in mucous
cell precursors during rodent sublingual gland development
prior to differentiation and concomitant switching to the
Muc19 splice variant, utilizing exons 1 and 19–60 (19, 36, 37).

An exponential expansion of the S. mutans population
occurred with the development of agriculture and the accom-
panying increase in dietary starch, with consequential positive
selection in S. mutans for genes involved in sugar metabolism
and acid tolerance (94). Assuming overlapping functions
between MUC5B and MUC19 in controlling S. mutans coloni-
ization, the increase in dietary starch and associated caries
pathogenesis of S. mutans may have favored increased MUC5B
expression in the face of reduced MUC19 expression. Alterna-
atively, other dietary, environmental, or microbial evolutionary
pressures may have been better accommodated by MUC5B.

Previously, MUC5AC and MUC2 were immunolocalized to
sporadic cells within glandular excretory ducts of human major
and minor glands, whereas immunoreactivities were absent in
acinar cells (95, 96). Immunoreactive ductal cells are likely gob-
let cells interspersed within glandular excretory ducts (97, 98).
Our inability to detect MUC2 transcripts within intralobular
tissue fragments of submandibular glands may be due to distrib-
ution of MUC2-positive cells to more distal extralobular
excretory ducts (95, 96). In mice, we reported Muc2 transcripts
are barely detectable in all minor mucous glands and the major
salivary glands, including the serous parotid gland (21). We
thus speculate Muc2 is restricted to goblet cells of extralobular
excretory ducts, similar to humans. Our detection of MUC5AC
transcripts in human submandibular glands, as well as Muc5ac
transcripts in minor mucous glands of mice (21), may also rep-
resent expression in ductal goblet cells. However, low levels of
expression in subpopulations of acinar mucous cells cannot be
ruled out. MUC5AC and MUC2 in glandular ducts may func-
tion in localized innate and adaptive immune responses in
response to bacterial invasion (99).

With respect to Muc6, we previously reported its absence in
murine salivary glands (21), whereas evidence of MUC6 expres-
sion in human glands is conflicting. MUC6 immunoreactivity
was reportedly absent in major or minor glands (95), although
in another report a few submandibular mucous cells were
immunoreactive in 10% of glands sampled (100). Our detection
of MUC6 transcripts in human submandibular glands is more
consistent with the latter study, suggesting that, unlike mice,
MUC6 is a component of saliva, although likely a minor
constituent.

Because of hindrance by glycosylation in identifying gel-
forming mucins, additional studies are warranted that incorpo-
rate deglycosylation strategies to more clearly delineate the
contribution of all gel-forming mucin species to human saliva,
their distributions among the many different salivary mucous
glands, variations in expression among individuals, interactions

2 The abbreviation used is: VWD, von Willebrand factor D.
with *S. mutans*, and correlations with caries prevalence. Information from such studies may provide a framework for development of diagnostic markers or treatments for patients at a high risk for caries, such as those with hypoalimentary disease due either to autoimmune disease or irradiation of the head and neck region (101, 102).

**Indirect Role for Muc19 in *S. mutans* Aggregation**—Interestingly, we find that the addition of Muc19 to saliva from Muc19\(^{-/-}\) mice restores aggregating activity to that of wild type saliva. Because Muc19 alone has very little aggregating activity, Muc19 must participate indirectly to aggregate *S. mutans*, likely through formation of heterotypic complexes with other salivary constituents that bind *S. mutans*. At least 38 human salivary proteins (those with a predicted signal peptide) have orthologs or paralogs in saliva of mice and/or rats (103). Unique to human saliva are additional proteins, including statherin-like proline-rich proteins, histatins, lysozyme, zinc α-glycoprotein, and the Ig saliva secretory complex (103). The 22 proteins unique to mice are thought to be involved mostly in wound healing, identification, or grooming (103).

In human saliva, Wickstrom et al. (13) found oligomeric gel-forming mucins in noncovalent association with salivary sIgA, lactoferrin, lysozyme, MUC7, and GP340. Similarly, Raynal et al. (12) identified noncovalent association of high density mucins with cystatin, amylase, and GP340. MUC7 and GP340 are large glycoproteins that express blood group antigens (6–8, 104) and can bind and aggregate *S. mutans* (81–83). Therefore, MUC7 and GP340 likely contributed to the aggregating activity of crude high molecular weight fractions of saliva in early studies, as the fractions were prepared under nonreductive and nondissociative conditions.

In mice, gp340 binds *S. mutans* as well as other Gram-positive and Gram-negative bacteria (105). Additionally, Liu et al. (106) described mouse Muc10 and human MUC7 as homologues, as both are abundant in saliva, have protein cores of similar lengths, have N-terminal regions with similar compositions of basic amino acids, and contain two cysteines, which in MUC7 are within the binding domain of *S. mutans* (106). Both mucins also have a central region with serine- and threonine-rich tandem repeats, potential sites of O-glycosylation (22, 107). The two mucin genes further share equivalent positions within syntenic genomic regions (108). Importantly, saliva from Muc10\(^{-/-}\) mice display decreased aggregation of *S. mutans* compared with the wild type, suggesting it also binds *S. mutans*.\(^3\)

As described above, salivary gel-forming mucins of humans and mice exhibit similar characteristics in assays with *S. mutans*. Because Muc19 and MUC5B represent predominant gel-forming mucins in murine and human saliva, these two mucins apparently share many of the same functions in suppressing *S. mutans* colonization, suggesting at least some functional redundancies between different gel-forming mucins. We therefore hypothesize that gel-forming mucins participate in the aggregation of *S. mutans* by providing a scaffold for the association of salivary bacteria binding molecules (e.g. MUC7, GP340, and other constituents) as well as bacteriostatic or bactericidal proteins. These complexes thus help sequester, neutralize, and clear bacteria from the oral cavity, working in concert with constituents such as MUC7 and GP340 that may also act alone or within non-mucin-containing complexes to aggregate *S. mutans*. A role for gel-forming mucins in bacterial aggregation and clearance adds yet another protective role for these glycoproteins in the innate immune functions of saliva.

In light of species differences, mucin-protein interactions in mice may not necessarily be predictive of functional interactions in humans. It is therefore important to expand on our current limited information to fully delineate interactions between human gel-forming mucins and other salivary constituents and their role in bacterial aggregation and clearance.

**Consideration of Mucin Glycoforms in Elucidation of Functions**—Salivary gel-forming mucins are polydisperse in mass and charge densities, a result of multiple glycoforms that vary in either their extent of glycosylation and/or in their composition of several hundred unique oligosaccharides with different degrees of sialylation and sulfation (86, 109–111). Interestingly, human major and minor mucous glands display variations in mucin glycoforms (16, 87, 109), suggesting localized functional differences in anti-microbial actions and possibly formation of glycoform-specific heterotypic complexes. Moreover, chronic inflammatory conditions (i.e. ocular rosacea and rheumatoid arthritis) are associated with increased sulfation of salivary oligosaccharides (112, 113), likely due to expression of sulfotransferases in response to systemic inflammatory mediators (114). Mucin sulfation has been shown to promote colonic barrier function (115), adhesion of leukocytes and *Helicobacter pylori* (116), and resistance to bacterial mucin-degrading enzymes (117). Changes in mucin glycoforms in response to inflammation may therefore play a role in associated oral manifestations of systemic diseases (118). One may further postulate that inflammation accompanying severe caries affecting the tooth dentin and pulp may modify the profile of salivary mucin glycoforms and associated functions.

**MUC19 and Systemic Diseases**—In light of the association of *S. mutans* with blood-borne infections (4, 5), elucidation of innate mechanisms to control its oral colonization is relevant to systemic health. Recent genome-wide association studies identified MUC19 as a susceptibility gene of Crohn disease (119–121). Muc19 expression is absent in the gastrointestinal tract of mice (21, 122), as well as in cattle except for inconsistent and sparse expression in jejunum (123). MUC19 expression in the human gastrointestinal tract has not been studied extensively. We speculate that altered salivary expression of MUC19 affecting the oral microbiota may in turn influence the intestinal microbiota and immune homeostasis, through constant swallowing of bacteria aggregated within a complex containing acid-resistant gel-forming mucins. Interestingly, patients with Crohn disease reportedly have higher numbers of decayed tooth surfaces compared with controls (124). Studies of correlations between Crohn disease and salivary MUC19 may further add to the growing list of inter-relationships between the oral microbiota and both oral and systemic health (125, 126).

\(^3\) D. J. Culp, unpublished observations.
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REFERENCES

1. Marcenes, W., Kassebaum, N. J., Bernabé, E., Flaxman, A., Naghavi, M., Lopez, A., and Murray, C. J. (2013) Global burden of oral conditions in 1990–2010: a systematic analysis. J. Dent. Res. 92, 592–597

2. Satcher, D. (2000) Oral Health in America: A Report of the Surgeon General. United States Department of Health and Human Services, NIDCR, National Institutes of Health, Rockville, MD

3. Bowen, W. H. (2002) Do we need to be concerned about dental caries in the coming millennium? Crit. Rev. Oral Biol. Med. 13, 126–131

4. Avilés-Reyes, A., Miller, J. H., Simpson-Haidaris, P. J., Lemos, J. A., and Offner, G. D. (2000) Heterogeneity of high molecular-weight human salivary glycoproteins. J. Biol. Chem. 275, 141–156

5. Kouznetsova, I., Gerlach, K. L., Zahl, C., and Hoffmann, W. (2010) Expression analysis of human salivary glands by laser microdissection: differences between submandibular and labial glands. Cell. Physiol. Biochem. 26, 375–382

19. Zhu, L., Lee, P., Yu, D., Tso, S., and Chen, Y. (2011) Cloning and characterization of human MUC19 gene. Am. J. Respir. Cell Mol. Biol. 45, 348–358

20. Rousseau, K., Kirkham, S., Johnson, L., Fitzpatrick, B., Howard, M., Adams, E. J., Rogers, D. F., Knight, D., Clegg, P., and Thornton, D. J. (2008) Proteomic analysis of polymeric salivary mucins: no evidence for MUC19 in human saliva. Biochem. J. 413, 545–552

21. Das, B., Cash, M. N., Hand, A. R., Shivasad, A., Grieshaber, S. S., Robinson, B., and Culp, D. J. (2010) Tissue distribution of murine Muc19/Smgc gene products. J. Histochem. Cytochem. 58, 141–156

22. Denny, P. C., Mires, L., and Denny, P. A. (1996) Mouse submandibular gland salivary aponglin contains repeated N-glycosylation sites. Glycobiology 6, 43–50

23. Dass, B., Cash, M. N., Robinson, B., Kuhrs, C. S., Latchney, L. R., Fallon, M. A., Elliott, R. W., Hand, A. R., and Culp, D. J. (2013) The old genetic defect: two intronic CA repeats promote insertion of the subsequent intron and mRNA decay. J. Biol. Chem. 288, 14742–14755

24. Watson, G. E., Latchney, L. R., Luo, W., Hand, A. R., and Culp, D. J. (1997) Biochemical and immunological studies and assay of rat sublingual muncins. Arch. Oral Biol. 42, 161–172

25. Culp, D. J., Robinson, B., Parkkila, S., Pan, P. W., Cash, M. N., Truong, H. N., Hussey, T. W., and Gullett, S. L. (2011) Oral colonization by Streptococcus mutans and caries development is reduced upon deletion of carbonic anhydrase VI expression in saliva. Biochim. Biophys. Acta 1812, 1567–1576

26. Keyes, P. H. (1958) Dental caries in the molar teeth of rats. II. A method for diagnosing and scoring several types of lesions simultaneously. J. Dent. Res. 37, 1088–1099

27. Larson, R. (1981) in Animal Models in Cariology (Tanzer, J. M., ed) pp. 195–203, IRL Press, Oxford, UK

28. Emison, C. G., and Bratthell, D. (1976) Growth of Streptococcus mutans on various selective media. J. Clin. Microbiol. 4, 95–98

29. Paster, B. J., Boches, S. K., Galvan, L. J., Ericson, R. E., Lau, C. N., Levanos, V. A., Sahasrabudhe, A., and Dewhirst, F. E. (2001) Bacterial diversity in human subgingival plaque. J. Bacteriol. 183, 3770–3783

30. Chen, T., Yu, W. H., Izard, J., Bananov, O. V., Lakshmanan, A., and Dewhirst, F. E. (2010) The human oral microbiome database: a web accessible resource for investigating oral microbe taxonomic and genomic information. Database 2010, baq013

31. Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., Marsh, T., Garrity, G. M., and Tiedje, J. M. (2009) The ribosomal database project: improved alignment and new tools for rRNA analysis. Nucleic Acids Res. 37, D141–D145

32. Ten, Z. T., and Burne, R. A. (2001) Construction of a new integration vector for use in Streptococcus mutans. Plasmid 45, 31–36

33. Barletta, R. G., and Curtiss, R. 3rd (1989) Impairment of melibiose utilization in Streptococcus mutans serotype c gIaA mutants. Infect. Immun. 57, 992–995

34. Tanzer, J. M., Thompson, A., Wen, Z. T., and Burne, R. A. (2006) Streptococcus mutans: fructose transport, xylitol resistance, and virulence. J. Dent. Res 85, 369–373

35. Lemos, J. A., Abranches, J., Koo, H., Marquis, R. E., and Burne, R. A. (2010) Protocols to study the physiology of oral biofilms. Methods Mol. Biol. 666, 87–102

36. Culp, D. J., Latchney, L. R., Fallon, M. A., Denny, P. A., Denny, P. C., Couwenhoven, R. I., and Chang, S. (2004) The gene encoding mouse Muc19: cDNA, genomic organization and relationship to Smgc. Physiol Genomics 19, 303–318

37. Das, B., Cash, M. N., Hand, A. R., Shivasad, A., and Culp, D. J. (2009) Expression of Muc19/Smgc gene products during murine sublingual gland development: cytodifferentiation and maturation of salivary mucus cells. J. Histochem. Cytochem. 57, 383–396

38. Jay, G. D., Culp, D. J., and Jahnke, M. R. (1990) Silver staining of extensively glycosylated proteins on sodium dodecyl sulfate-polyacrylamide gels: enhancement by carbohydrate-binding dyes. Anal. Biochem. 185, 324–330

39. Denny, P. A., and Denny, P. C. (1982) Localization of a mouse subman-
Expression and Innate Immunity of Salivary Mucin 19

dibular sialomucin by indirect immunofluorescence. Histochem. J. 14, 403–408

40. Culp, D. J., Quivey, R. Q., Bowen, W. H., Fallon, M. A., Pearson, S. K., and Faustoferri, R. (2005) A mouse caries model and evaluation of apq5−/− knockout mice. Caries Res. 39, 448–454

41. Barletta, R. G., Michalek, S. M., and Curtiss, R., 3rd (1988) Analysis of the virulence of Streptococcus mutans serotype c gtA mutants in the rat model system. Infect. Immun. 56, 322–330

42. Bisgaard, M. (1986) Actinobacillus muris sp. nov. isolated from mice. Acta Pathol. Microbiol. Immunol. Scand. B Microbiol. 94, 1–8

43. Johnsson, M., Levine, M. J., and Nancollas, G. H. (1993) Hydroxyapatite binding domains in salivary proteins. J. Dent. Res. 72, 213–221

44. Almiron, M., Traglia, G., Rubio, A., and Sanjuan, N. (2013) Colonization of the mouse upper gastrointestinal tract by Lactobacillus murinus: a histological, immunocytochemical, and ultrastructural study. Curr. Microbiol. 67, 395–398

45. Bore, S., McCartney, C. A., Smelling, T., J. P., Worgan, H. J., and McEwan, N. R. (2010) Isolation of Streptococcus thailandensis from rabbit faeces. Curr. Microbiol. 61, 357–360

46. Li, J., Helmerhorst, E. J., Levine, M. C., Troxler, R. F., Yaskell, T., Haffajee, A. D., Socransky, S. S., and Oppenheim, F. G. (2004) Identification of early microbial colonizers in human dental biofilm. J. Appl. Microbiol. 97, 1311–1318

47. Song, C., Wang, W., and McDonald, E. E. (2013) New insights into the composition and functions of the acquired enamel pellicle. J. Dent. Res. 91, 1100–1118

48. Vaccaro, M. A., and Bowen, W. H. (2000) In situ studies of pellicle formation on hydroxyapatite discs. Arch. Oral Biol. 45, 277–291

49. Zimmerman, J. N., Custodio, W., and Hatibovic-Kofman, S., Lee, Y. H., Xiao, Y., and Siqueira, W. L. (2013) Proteome and peptidome of human acquired enamel pellicle on deciduous teeth. Int. J. Mol. Sci. 14, 920–934

50. Clark, W. B., Bammann, L. L., and Gibbons, R. J. (1978) Comparative estimates of bacterial affinities and adsorption sites on hydroxyapatite surfaces. Infect. Immun. 19, 846–853

51. Johnson, M., Levine, M. J., and Nancollas, G. H. (1993) Hydroxyapatite binding domains in salivary proteins. Crit. Rev. Oral Biol. Med. 4, 371–378

52. Nieuw Amerongen, A. V., Oderkerk, C. H., and Veerman, E. C. (1989) Characterization of the immunologic responses to human in vivo acquired enamel pellicle as a novel means to investigate its composition. Oral Microbiol. Immunol. 18, 183–191

53. Denny, P., Hagen, F. K., Hardt, M., Liao, L., Yan, W., Arelanno, M., Basiliain, S., Bedi, G. S., Boontheung, P., Cociorva, D., Delahunty, C. M., Denny, T., Dunsmore, J. F., Faull, K. F., Gilligan, J., Gonzalez-Begne, M., Halgand, F., Hall, S. C., Han, X., Henson, B., Hewel, J., Hu, S., Jeffrey, S., Jiang, J., Loo, J. A., Ogorzalez Loo, R. R., Malamud, D., Melvin, J. E., Miroshnychenko, O., Navazesh, M., Niles, R., Park, S. K., Prakobphol, A., Ramachandran, P., Richert, M., Robinson, S., Sondej, M., Souda, P., Sullivan, M. A., Takahima, J., Than, S., Wang, J., Whitelegg, J. P., Witkoska, H. E., Wolsinsky, L., Xie, Y., Xu, T., Yu, W., Ytterberg, J., Wong, D. T., Yates, J. R., 3rd, and Fisher, S. J. (2008) The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. J. Proteome Res. 7, 949–2006

54. Bandera-Tarabay, J. A., Zacarias-D’Oleire, I. G., Garduno-Estrada, R., Aceves-Luna, E., and Gonzalez-Begne, M. (2002) Electrophoretic analysis of whole saliva and prevalence of dental caries. A study in Mexican dental students. Arch. Med. Res. 33, 499–505

55. Lazarevic, V., Whiteson, K., Huse, S., Hernandez, D., Farinelli, L., Os teras, M., Schrenzel, J., and Francois, P. (2009) Metagenomic study of the oral microbiota by Illumina high throughput sequencing. J. Microbiol. Methods 79, 266–271

56. Chun, J., Kim, K. Y., Lee, J. H., and Choi, Y. (2010) The analysis of oral microbial communities of wild-type and toll-like receptor 2-deficient mice using a 454 GS FLX Titanium pyrosequencer. BMC Microbiol. 10, 1

57. Takehashi, N., and Nyvad, B. (2008) Caries ecology revisited: microbial dynamics and the caries process. Caries Res. 42, 409–418

58. Kishimoto, E., Hay, D. I., and Gibbons, R. J. (1989) A human salivary protein which promotes adhesion of Streptococcus mutans serotype c strains to hydroxyapatite. Infect. Immun. 57, 3702–3707

59. Nieuw Amerongen, A. V., Oderkerk, C. H., and Driessen, A. A. (1987) Influence of the blood group reactive substances in saliva on the virulence of Actinobacillus actinomycetemcomitans. Infect. Immun. 57, 85–92

60. Bradshaw, D. J., Homer, K. A., Marsh, P. D., and Beighton, D. (1994) Metabolic cooperation in oral microbial communities during growth on salivary pellicles. Arch. Oral Biol. 39, 701–706

61. Mothey, D., Buttaro, B. A., and Piggot, P. J. (2014) Mucin can enhance growth, biofilm formation, and survival of Streptococcus mutans. FEMS Microbiol. Lett. 350, 161–167

62. McHenry, M., Butler, B. P., and Begg, D. (1994) Bacterial and host interactions of Streptococcus mutans spp, in young and elderly human dental students. Arch. Med. Res. 25, 179–184

63. Banas, J. A. (2004) Virulence properties of Streptococcus mutans. Front. Biosci. 9, 1267–1277

64. Cailling, A., Olsson, J., and Ramberg, P. (1996) Saliva mediated adhesion, aggregation and prevalence in dental plaque of Streptococcus mutans, Streptococcus sanguis and Actinomyces spp, in young and elderly humans. Arch. Oral Biol. 41, 1133–1140

65. Rosan, B., Appelbaum, B., Golub, E., Malamud, D., and Mandel, I. D. (1982) Enhanced saliva-mediated bacterial aggregation and decreased bacterial adhesion in caries-resistant versus caries-susceptible individuals. Infect. Immun. 38, 1056–1059

66. Ligtenberg, A. J., Veerman, E. C., de Graaff, J., and Nieuw Amerongen, A. V. (1990) Influence of the blood group reactive substances in saliva on the aggregation of Streptococcus rattus. Antonie Van Leeuwenhoek 57, 97–107
80. Prakobphol, A., Leffler, H., and Fisher, S. J. (1993) The high molecular-weight human mucin is the primary salivary carrier of ABH, Le(a), and Le(b) blood group antigens. Crit. Rev. Oral Biol. Med. 4, 325–333.

81. Litgembre, A. J., Walgreen-Weterings, E., Veerman, E. C., van Soest, J., de Graaff, J., and Amerongen, A. V. (1992) Influence of saliva on aggregation and adherence of Streptococcus gordonii HGG 222. Infect. Immun. 60, 3878–3884.

82. Murray, P. A., Prakobphol, A., Lee, T., Hoover, C. I., and Fisher, S. J. (1992) Adherence of oral streptococci to salivary glycoproteins. Infect. Immun. 60, 31–38.

83. Slomiany, B. L., Piotrowski, J., Czajkowski, A., Shovlin, F. E., and Slomiany, A. (1993) Differential expression of salivary mucin bacterial aggregating activity with caries status. Int. J. Biochem. 25, 935–940.

84. Issa, S. M., Schulz, B. L., Packer, N. H., and Sheehan, J. K. (1999) Structural and functional characteristics of the minor salivary glands. Int. Rev. Cytol. 190, 1–14.

85. Thornton, D. J., Howland, M., Devine, P. L., and Sheehan, J. K. (1995) Methods for separation and deglycosylation of mucin subunits. Anal. Biochem. 227, 162–167.

86. Wickström, C., Davies, J. R., Eriksen, G. V., Veerman, E. C., and Carlstedt, I. (1998) MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage. Biochem. J. 334, 685–693.

87. Thornton, D. J., Khan, N., Mehrotra, R., Howard, M., Veerman, E., Packer, N. H., and Sheehan, J. K. (1999) Salivary mucin MG1 is comprised almost entirely of different glycosylated forms of the MUC5B gene product. Glycobiology 9, 293–302.

88. Troxler, R. F., Jontcheva, I., Oppenheim, F. G., Nunes, D. P., and Offner, G. D. (1997) Molecular characterization of a major high molecular weight mucin from human sublingual gland. Glycobiology 7, 965–973.

89. Hand, A. R., Pathmanathan, D., and Field, R. B. (1999) Morphological features of the minor salivary glands. Arch. Oral Biol. 44, S3–S10.

90. Loomes, K. M., Senior, H. E., West, P. M., and Roberton, A. M. (1999) Functional protective role for mucin glycosylated repetitive domains. Eur. J. Biochem. 266, 105–111.

91. van der Post, S., Subramani, D. B., Bäckström, M., Johansson, M. E., Vester-Christensen, M. B., Mandel, U., Bennett, E. P., Clausen, H., Dahlén, G., Sorka, A., Potempa, J., and Hansson, G. C. (2013) Site-specific O-glycosylation on the MUC2 mucin protein inhibits cleavage by the Porphyromonas gingivalis secreted cysteine protease (RgpB). J. Biol. Chem. 288, 14636–14646.

92. Nielsen, P. A., Bennett, E. P., Wandall, H. H., Therkildsen, M. H., Hansson, J. L., and Clausen, H. (1997) Identification of a major human high molecular weight salivary mucin (MG1) as tracheobronchial mucin MUC5B. Glycobiology 7, 413–419.

93. Gerken, T. A., Gupta, R., and Jentoft, N. (1992) A novel approach for chemically deglycosylating O-linked glycoproteins. The deglycosylation of submaxillary and respiratory mucins. Biochemistry 31, 639–648.

94. Cornejo, O. E., Lefebvre, T., Bitar, P. D., Lang, P., Richards, V. P., Ellerton, K., Do, T., Beighton, D., Zeng, L., Ahs, S. J., Burne, R. A., Siepel, A., Bustamante, C. D., and Stanhope, M. J. (2013) Evolutionary and population genomics of the cavity causing bacteria Streptococcus mutans. Mol. Biol. Evol. 30, 881–893.

95. Alos, L., Lujan, B., Castillo, M., Nadal, A., Carreras, M., Caballero, M., de Bolos, C., and Cardesa, A. (2005) Expression of membrane-bound mucins (MUC1 and MUC4) and secreted mucins (MUC2, MUC5AC, MUC5B, MUC6 and MUC7) in mucoepidermoid carcinomas of salivary glands. Am. J. Surg. Pathol. 29, 806–813.

96. Handra-Luca, A., Lamas, G., Bertrand, J. C., and Fouret, P. (2005) MUC1, MUC2, MUC4, and MUC5AC expression in salivary gland mucoepidermoid carcinoma: diagnostic and prognostic implications. Am. J. Surg. Pathol. 29, 881–889.

97. Pinkstaff, C. A. (1980) The cytology of salivary glands. Int. Rev. Cytol. 63, 141–261.

98. Rother, P. (1963) The secretory function of the parotid duct and the submandibular duct. Acta Histochem. 15, 325–346.

99. Brandtzaeg, P. (2013) Secretory immunity with special reference to the oral cavity. J. Oral Microbiol. 10.3402/jom.v6i26102.

100. Nakayama, K., Ota, H., Zhang, M. X., Sano, K., Honda, T., Ishii, K., and Nakayama, J. (2003) Expression of gastric gland mucous cell-type mucin in normal and neoplastic human tissues. J. Histochem. Cytochem. 51, 1689–1698.

101. Castro, I., Sepúlveda, D., Cortés, J., Quez, A. F., Barrera, M. J., Bahamondes, V., Aguilera, S., Urzúa, U., Alliende, C., Molina, C., González, S., Hermoso, M. A., Leyton, C., and González, M. J. (2013) Oral dryness in Sjögren’s syndrome patients. Not just a question of water. Autoimmun. Rev. 12, 567–574.

102. Dijkema, T., Terhaard, C. H., Roesink, J. M., Veerman, E. C. (2012) MUC5B levels in submandibular gland saliva of patients treated with radiotherapy for head-and-neck cancer: a pilot study. Radiat. Oncol. 7, 91.

103. Dijkema, T., Terhaard, C. H., Roesink, J. M., Veerman, E. C. (2012) MUC5B levels in submandibular gland saliva of patients treated with radiotherapy for head-and-neck cancer: a pilot study. Radiat. Oncol. 7, 91.

104. Dijkema, T., Terhaard, C. H., Roesink, J. M., Veerman, E. C. (2012) MUC5B levels in submandibular gland saliva of patients treated with radiotherapy for head-and-neck cancer: a pilot study. Radiat. Oncol. 7, 91.

105. Madsen, J., Tornøe, I., Terhaard, C. H., Roesink, J. M., Raaijmakers, C. P., van den Keijbus, P. A., Brand, H. S., and Veerman, E. C. (2012) MUC5B levels in submandibular gland saliva of patients treated with radiotherapy for head-and-neck cancer: a pilot study. Radiat. Oncol. 7, 91.

106. Liu, B., Rayment, S. A., Gyurko, C., Oppenheim, F. G., Offner, G. D., and Troxler, R. F. (2000) The recombinant N-terminal region of human salivary mucin MG2 (MUC7) contains a binding domain for oral streptococci and exhibits candicidial activity. Biochem. J. 345, 557–564.

107. Bobek, L. A., Tsai, H., Biesbrock, A. R., and Levine, M. J. (1993) Molecular cloning, sequence, and specificity of expression of the gene encoding the low molecular weight human salivary mucin (MUC7). J. Biol. Chem. 268, 20563–20569.

108. Williams, O. W., Sharaffkhanen, A., Kim, V., Dickey, B. F., and Evans, C. M. (2006) Airway mucus: from production to secretion. Am. J. Respir. Cell Mol. Biol. 34, 527–536.

109. Böcker, A., Tsai, H., Biesbrock, A. R., and Levine, M. J. (1993) Molecular cloning, sequence, and specificity of expression of the gene encoding the low molecular weight human salivary mucin (MUC7). J. Biol. Chem. 268, 20563–20569.

110. Lichtenberg, A. J., Veerman, E. C., and Nieuw Amerongen, A. V. (2000) A role for Lewis a antigens on salivary agglutinin in binding to Streptococcus mutans. Antonie Van Leeuwenhoek 77, 21–30.

111. Prakobphol, A., Borén, T., Ma, W., Zhixiang, P., and Fisher, S. J. (2005) Expression and Innate Immunity of Salivary Mucin 19
118. Chi, A. C., Neville, B. W., Krayer, J. W., and Gonsalves, W. C. (2010) Oral manifestations of systemic disease. Am. Fam. Physician 82, 1381–1388
119. Kumar, V., Mack, D. R., Marcil, V., Israel, D., Krvupoves, A., Costea, I., Lambrette, P., Grimard, G., Dong, J., Seidman, E. G., Amre, D. K., and Levy, E. (2013) Genome-wide association study signal at the 12q12 locus for Crohn’s disease may represent associations with the MUC19 gene. Inflamm. Bowel Dis. 19, 1254–1259
120. Rivas, M. A., Beaudoin, M., Gardet, A., Stevens, C., Sharma, Y., Zhang, C. K., Boucher, G., Ripke, S., Ellinghaus, D., Burtt, N., Fennell, T., Kirby, A., Latiaro, A., Goyette, P., Green, T., Halfvarson, J., Haritunians, T., Korn, J. M., Kuruvilla, F., Lagacé, C., Neale, B., Lo, K. S., Schumm, P., Torkvist, L., National Institute of Diabetes and Digestive Kidney Diseases Inflammatory Bowel Disease Genetics Consortium (NIDDK IBDGC), United Kingdom Inflammatory Bowel Disease Genetics Consortium, International Inflammatory Bowel Disease Genetics Consortium, Dubinsky, M. C., Brant, S. R., Silverberg, M. S., Duerr, R. H., Altbucher, D., Gabriel, S., Lettre, G., Franke, A., D’Amato, M., McGovern, D. P., Cho, J. H., Rioux, J. D., Xavier, R. J., and Daly, M. J. (2011) Deep resequencing of GWAS loci identifies independent rare variants associated with inflammatory bowel disease. Nat. Genet. 43, 1066–1073
121. Umeno, J., Asano, K., Matsushita, T., Matsumoto, T., Kiyohara, Y., Iida, M., Nakamura, Y., Kamatani, N., and Kubo, M. (2011) Meta-analysis of published studies identified eight additional common susceptibility loci for Crohn’s disease and ulcerative colitis. Inflamm. Bowel Dis. 17, 2407–2415
122. Kerschner, J. E., Hong, W., Taylor, S. R., Kerschner, J. A., Khamiang, P., Wrege, K. C., and North, P. E. (2013) A novel model of spontaneous otitis media with effusion (OME) in the Oxgr1 knock-out mouse. Int. J. Pediatr. Otorhinolaryngol 77, 79–84
123. Hoorens, P. R., Rinaldi, M., Li, R. W., Goddeeris, B., Claerebout, E., Verbruggen, J., and Geldhof, P. (2011) Genome wide analysis of the bovine mucin genes and their gastrointestinal transcription profile. BMC Genomics 12, 140
124. Sundh, B., Johansson, I., Emilson, C. G., Nordgren, S., and Birkhed, D. (1993) Salivary antimicrobial proteins in patients with Crohn’s disease. Oral Surg. Oral Med. Oral Pathol. 76, 564–569
125. Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., and Versalovic, J. (2014) The placenta harbors a unique microbiome. Sci. Transl. Med. 10.1126/scitranslmed.3008599
126. Han, Y. W., and Wang, X. (2013) Mobile microbiome: oral bacteria in extra-oral infections and inflammation. J. Dent. Res. 92, 485–491