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Mucin O-glycans suppress quorum sensing pathways and genetic transformation in Streptococcus mutans

Caroline A Werlang[1], **Wesley G Chen**[1,a], **Kazuhiro Aoki**[2], **Kelsey M Wheeler**[1], **Carly Tymm**[1,b], **Cassidy Mileti**[1,c], **Ana Burgos**[1,d], **Kris Kim**[1,e], **Michael Tiemeyer**[2], **Katharina Ribbeck**[1,*]

[1.]Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA

[2.]Complex Carbohydrate Research Center, University of Georgia, Athens, GA

[a.]Johnson & Johnson, Cambridge, MA 02142

[b.]Dana-Farber Cancer Institute, Boston, MA 02215

[c.]UC Davis Department of Biomedical Engineering, Davis, CA 95616

[d.]Loyola University Medical Center, Maywood, IL 60153

[e.]John Harvard Distinguished Science Fellows Program and the Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138

INTRODUCTORY PARAGRAPH

Mucus barriers accommodate trillions of microbes throughout the human body while preventing pathogenic colonization.[1] In the oral cavity, saliva containing the mucins MUC5B and MUC7 forms a pellicle that coats the soft tissue and teeth to prevent infection by oral pathogens, such as *Streptococcus mutans*.[2] Salivary mucin can interact directly with microbes through selective agglutinin activity and bacterial binding,[2–4] but the extent and basis of saliva's protective functions are not well understood. Using an *ex vivo* saliva model, we identify MUC5B as an inhibitor of microbial virulence. Specifically, we find natively purified MUC5B downregulates the expression of quorum sensing pathways activated by the competence stimulating peptide (CSP) and the *sigX* inducing peptide (XIP).[5] Further, MUC5B prevents the acquisition of antimicrobial resistance through natural genetic transformation, a process activated through quorum sensing. Our data

*Corresponding author: ribbeck@mit.edu.

AUTHOR CONTRIBUTIONS

C.W., W.C., and K.W. generated mucin-related biochemical reagents and protocols. K.A. performed mass spectrometric analysis. C.W., W.C., C.T., C.M., A.B., and K.K. performed transformation and biofilm formation experiments and developed related protocols. C.W. isolated RNA and analyzed gene expression data. M.T. and K.R. supervised the study. All authors contributed to writing and editing the manuscript.

CONFLICTS OF INTEREST

The authors have no conflicts to declare.

DATA AVAILABILITY

High throughput sequencing data presented in Figure 1 and Figure 2 are deposited in the Gene Expression Omnibus (GEO) under accession number GSE163258. All other data are available from the corresponding author upon reasonable request.

CODE AVAILABILITY

Code used for transcriptional analysis is available at https://github.com/cwerlang/Smutans-MUC5B-RNASeq

reveal the effect of MUC5B is mediated by its associated glycans, which are potent suppressors of quorum sensing and genetic transformation, even when removed from the mucin backbone. Together, these results present mucin glycans as a host strategy for domesticating potentially pathogenic microbes without killing.

S. mutans is a causative agent in dental caries and is also highly associated with potentially fatal antibiotic resistant infections of heart valve implants.[6] *S. mutans* is naturally competent, enabling it to uptake foreign DNA and act as a reservoir for antibiotic resistance genes[7–10] that it can share across species through horizontal gene transfer[11,12]. Healthy individuals often carry *S. mutans* asymptomatically,[13,14] but reduced saliva production, such as in people with Sjogren's syndrome, can lead to the outgrowth of *S. mutans* and susceptibility to cavities.[15,16] This suggests that the presence of saliva may be critical to preventing the virulence of *S. mutans.*[17]

To test how healthy saliva impacts the disease-causing ability of *S. mutans,* we focus on biofilm formation, a phenotypic state that enables *S. mutans* to form tenacious plaques that resist environmental stressors[18–20] and is associated with *S. mutans* virulence, outgrowth, and persistent colonization.[21] Whole saliva from healthy volunteers was supplemented with 0.5wt% glucose to sustain bacterial growth, and biofilm formation was measured by counting surface-attached colony forming units after 4 hours. The relative number of viable biofilm cells was lowered by 93% when *S. mutans* was cultured with whole saliva compared to culture medium (Figure 1b). Previous work has revealed that the mucin MUC5B can prevent biofilm formation by *S. mutans* when presented in isolation.[22,23] MUC5B is a complexly glycosylated gel-forming protein with an average molecular weight of 2 MDa.[3,24,25] To test if MUC5B is a predominant biofilm-suppressing molecule in native saliva, we removed the high molecular weight components from saliva using centrifugal filters. We found that when large molecules (>100kDa) were removed, the saliva filtrate was no longer able to prevent biofilm formation of multiple strains of *S. mutans.* This indicates that large molecular weight components - such as mucin - are necessary for saliva to prevent biofilm formation. Addition of purified MUC5B (0.1wt%) to high molecular weight (>10kDa)-depleted saliva restored biofilm inhibition, indicating that this mucin is necessary and sufficient to suppress *S. mutans* biofilms in native saliva. Neither depletion nor supplementation of MUC5B reduced the growth of *S. mutans* (Figure 1c), suggesting that MUC5B is able to reduce biofilm formation by altering its ability to adhere to surfaces rather than killing them. This raises the possibility that MUC5B may be inducing phenotypic shifts in the bacteria through other means.

To determine the extent to which MUC5B regulates *S. mutans* physiology, we performed RNA-sequencing (RNA-seq) on *S. mutans* UA159 cells grown to exponential phase in medium with or without 0.1wt% MUC5B (Figure 1d). MUC5B altered the transcriptional response of *S. mutans,* increasing the expression of 6 genes and decreasing the expression of 61 genes. Among these significantly downregulated genes (Supplementary Table 1), we detected enrichment in pathways regulated by quorum sensing (Figure 1e, Supplementary Table 2). Beyond these major pathways, MUC5B reduced the expression of the *sloABC* operon, which encodes proteins associated with virulence development in endocarditis,[26]

and upregulated the transcription of an ABC transporter operon (SMU_1550c-1554c) that is hypothesized to encode immunity proteins.[27] MUC5B also altered the expression of over a dozen other genes, many of which are uncharacterized.

Quorum sensing is a process by which microbial communities coordinate group behaviors through the secretion and sensing of extracellular signals.[28] In *S. mutans,* quorum sensing pathways control bacteriocin production and competence development, which allows for uptake of foreign genetic elements (Figure 1f).[5] In general, XIP or ComS binds to ComR to induce transcription *sigX*, a sigma factor that controls the expression of late competence genes responsible for DNA uptake and integration, including *comEA*, *comFA*, and *comYA* (also called *comGA*).[29,30] . In parallel, CSP activates the ComDE two-component system to control the transcription of immunity proteins and bacteriocins, including *cipB* which can affect expression of *sigX* and is necessary for CSP-induced increases in competence.[31–33] The XIP-ComR and CSP-ComDE sensory systems have bidirectional feedback, as SigX can promote transcription of *comD* and *comE*.[34,35] It is suggested that *S. mutans* uses these systems to coordinate the killing of competing microbes and incorporation of the subsequently released extracellular DNA.[5,36,37] Our RNA-Seq revealed that MUC5B reduced the expression of competence genes in the ComR and SigX regulons. Additionally, MUC5B lessened the expression of ComE regulated bacteriocins, an effect that was consistent across the regulon although no individual changes in gene expression were significant.

Quorum sensing suppression by MUC5B was validated by RT-qPCR. We find that in medium without mucin, the expression of *cipB* and *comS* spikes during mid-exponential phase, when these genes are typically maximally expressed (Figure 1g, Figure 1h).[38–41] In contrast, exposure to MUC5B suppresses the expression of both genes, underscoring that MUC5B induces a sustained change in gene expression. Integrating the differential area under the curves, we find that cumulative expression of key quorum sensing genes *comC, cipB, comS,* and *comE* is reduced, while the housekeeping genes *tkt* and *gyrA* were not affected (Figure 1i). These results confirm that salivary mucin modulates the expression of the CSP-ComDE and XIP-ComR quorum sensing pathways.

Spatial structuring due to the presence of a large mucin polymer could prevent efficient intercellular communication by separating microbes.[42] To check if the presence of a large polymer is sufficient to alter quorum sensing, we tested the effects of carboxymethyl cellulose (CMC), a polymer commonly used in artificial saliva preparations to mimic the charge and viscoelastic properties of saliva.[43] CMC did not change the expression of representative genes in the quorum sensing cascade (Figure 3i). This suggests that specific biochemistry present on mucin, but not CMC, is required to achieve MUC5B's suppression of quorum sensing.

Beyond its gel-forming properties, MUC5B is heavily glycosylated, with branched O-linked sugars that contribute up to 40–80% of its molecular weight.[44,45] The diverse and varied glycan structures on mucin offer a wealth of biochemical information in minimal space, which may be sensed by *S. mutans*.[46,47] To determine if these mucin glycans drive the suppression of quorum sensing in *S. mutans*, we chemically isolated glycans from human

salivary MUC5B using beta-elimination,[48] allowing us to measure the extent to which these molecules could recapitulate the effects of intact mucin using RNA-seq. Mass spectrometry (MS) analysis of MUC5B glycans shows a diverse array of structures dominated by O-GalNAc Core 1 and Core 2 initiated features (Figure 2a, Supplementary Figure 1).

Like whole mucin, a pooled library of MUC5B glycans triggered significant changes in gene expression in *S. mutans* (Figure 2b). The transcriptional response of MUC5B mucin and glycans are highly similar with a Pearson correlation coefficient of 0.69 (Figure 2c). Specifically, pathway enrichment analysis revealed that MUC5B glycans downregulate quorum sensing and competence machinery (Supplementary Table 2), reducing the expression of almost all genes previously identified to be downstream of ComE (20 of 23), ComR (8 of 8), and SigX (65 of 78) (Figure 2d).[49] In contrast to intact mucin, we find soluble glycans also upregulate galactose metabolism while downregulating the multiple sugar metabolism operon, suggesting a potential role in bacterial metabolism and nutrient sensing. Notably, MUC5B glycans did not alter the growth patterns of *S. mutans* at tested concentrations (Figure 2e).

To determine if the effects of glycans are conserved across mucus types, we also isolated glycans from the mucin MUC5AC, which is present in the mucus barriers in the respiratory and gastric niches, and measured their effects on quorum sensing. MS characterization revealed that MUC5AC glycans were highly similar to MUC5B glycans in their structures (Extended Data 1). Like MUC5B glycans, we found that MUC5AC glycans suppressed quorum sensing gene expression in *S. mutans*, reducing the expression of *cipB* in a dose-dependent manner (Figure 2f). Fitting to a sigmoidal four-parameter inhibitor dose-response curve indicates an inflection point at 0.05wt%, close to the estimated concentration of MUC5B in saliva 0.023wt%.[50] Overall, these results show that glycans from both MUC5B and MUC5AC can elicit highly similar changes in *S. mutans* gene expression (Extended Data 1), suggesting that conserved motifs present in both glycan libraries are potent regulators of quorum sensing pathways in *S. mutans.*

We next sought to test if the mucin-induced transcriptional changes induce phenotypic changes. Quorum sensing regulates several clinically important phenotypes in *S. mutans* beyond biofilm formation, in particular the natural acquisition of competence (Figure 3a). MUC5B reduced transformation frequency relative to the media-only sample in a concentration dependent manner even in media supplemented with 1 μM CSP or 10 μM XIP, where competence is maximally induced (Figures 3b and 3c). Further, this was not a result of spatial structuring, as MUC5B mucin and glycans reduced transformation in both the planktonic and biofilm fractions of culture (Figure 3d).

Mucin may inhibit transformation by preventing the physical import of the quorum sensing peptide XIP, thereby reducing transcription of XIP-ComR regulated genes. In chemically defined media (DMedia), the addition of exogenous synthetic XIP peptides has been shown to increase transformation rates in *S. mutans*[5]; however in complex media (CMedia), the addition of exogenous XIP no longer increases transformation rates (Figure 3e)[30,55]. Instead, in this regime, competence is activated without extracellular XIP signaling; rather it is hypothesized that intracellular ComS binds to and activates ComR.[53] Therefore, if

mucin interferes with competence by blocking XIP import, they should not have an effect in complex media. However, we find that MUC5B mucin and glycans are still able to efficiently reduce transformation in complex CMedia as well as other rich media (Figure 3d, Extended Data 2), indicating that mucin does not reduce transformation rates by blocking import of XIP.

Alternatively, mucin could prevent competence activation by preventing ComDE from responding to CSP. If mucin prevents ComD from sensing CSP, they should not reduce transformation in a deletion mutant of ComE, which no longer responds to external CSP (Extended Data 3). This experiment is possible because *ΔcomE* strains have transformation rates similar to wildtype. However, our data show MUC5B mucin and glycans reduce transformation rates in the *ΔcomE* strain (Figure 3f), indicating that interference in the CSP-ComDE pathway is not the mechanism by which mucin inhibits competence. Further, this is observed in complex media where XIP import is blocked, indicating that mucin glycans are capable of preventing transformation when extracellular signaling in both the CSP-ComDE and XIP-ComR systems is incapacitated (Figure 3g).

To assess whether the reduction of transformation by mucin glycans is unique to their structures or general to nutrient supplementation, we tested mucin *O*-glycans' component monosaccharides to see if they similarly decrease natural genetic transformation (Figure 3h) or alter the transcription of key quorum sensing genes (Figure 3i). RT-qPCR confirms that MUC5B mucin and glycans suppress the expression of bacteriocin and competence genes activated by ComE, ComR, and SigX. By comparison, an equal parts mixture of the component mucin monosaccharides (Monosacch. Mix) did not change the expression of any genes, although it did reduce transformation rates in CMedia. To clarify the effects of monosaccharides, we independently tested the mucin sugars galactose, N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), fucose, and N-acetylneuraminic acid (Neu5Ac, or sialic acid), as well as glucose as a non-mucin sugar control. GalNAc and Neu5Ac both moderately suppress the expression of a subset of competence genes, corresponding with in a reduction in transformation rates, while galactose, glucose, GlcNAc, and fucose had no effect. These findings indicate that the potent effects of mucin glycans are dependent on the complex arrangement, particular linkages, or specific anomeric form of the monomer sugars. Since neither intact MUC5B, MUC5B glycans, nor any of the component monosaccharides in combination or in isolation affect the exponential growth of *S. mutans* (Figure 2f, Extended Data 1), we further conclude that mucin glycan-mediated changes in quorum sensing are not simply the consequence of altered cell density.

To better understand the physiological relevance of mucin glycan-mediated suppression of quorum sensing in *S. mutans*, we monitored transformation in native saliva supplemented with 0.5wt% glucose. Compared to CMedia, there are very low rates of transformation in whole saliva, even when the cultures were supplemented with 1 μM CSP to maximize bacterial competence (Figure 4a). These rates are consistent with observed *in vivo* transformation frequencies of 1 in 10^7,[54] indicating that our *ex vivo* whole saliva model accurately represents transformation activity in the oral cavity. Depletion of high molecular weight components (>100 and >10 kDa) from saliva led to increased rates of transformation,

suggesting that large molecules like mucin are partially responsible for the low rates of genetic transfer observed in saliva. Indeed, re-supplementation of MUC5B to saliva components below 10 kDa restored the transformation-suppressing effects of saliva in a concentration-dependent manner (Figure 4b), affecting cells in both the biofilm and unadhered fractions of the population (Supplementary Figure 2).

We next assayed MUC5B's effect on other *Streptococci* to probe the generality of the transformation suppression phenotype. We found that mucin supplementation can reduce transformation and biofilm formation of other *S. mutans* strains (Extended Data 4). Further, MUC5B reduced the transformation frequency of *Streptococcus sobrinus,* another oral pathogen that is highly associated with cavity formation[55,56], by 98% relative to control in our saliva model (Figure 4c). These results demonstrate that mucin's effect is not isolated to *S. mutans* UA159 and may be applicable to other Streptococci.

Supplementing mucin-depleted saliva with free mucin glycans suppressed genetic transformation and biofilm formation of *S. mutans,* indicating that mucin glycans are sufficient to inhibit these quorum-sensing regulated phenotypes even in the complex saliva environment (Figure 4d). We find that a pool of monosaccharides does not reduce transformation or biofilm formation of *S. mutans* in mucin-depleted saliva, and of the individual monosaccharides tested, only GalNAc is able to suppress genetic transformation, though it did not affect biofilm formation. By contrast, Neu5Ac no longer alters transformation or biofilm formation in the mucin-depleted saliva, suggesting that factors present in saliva may block its activity. Additionally, supplementation with sucrose increased biofilm formation, as previously reported.[22]

How mucin glycans regulate quorum sensing pathways is still an open question. Our initial hypothesis was that mucin gels would reduce the diffusivity of quorum sensing peptides, an effect that has been previously observed to prevent clusters of microbes from sharing quorum sensing signals (Figure 3g).[57] However, mucin glycans freed from the gel-forming protein backbone also reduce quorum sensing (Figure 2), indicating that mucin can act independently of any alterations it makes to the physical environment. Alternatively, these freed mucin glycans could act by competitively blocking the sensing of CSP or import of XIP. However, mucin glycans reduce competence without relying on a CSP-induced response in a *ΔcomE* strain (Figure 3) and in media conditions where XIP import is impeded by other factors, indicating that its main point of action was not at these steps (Figure 3). Together, these results suggest that mucin and mucin glycans do not reduce quorum sensing by any extracellular interaction with quorum sensing peptides or their receptors.

Rather, it appears that mucin glycans may interact with parallel regulators of quorum sensing pathways. There are several signaling systems that can affect competence development upstream or in parallel to the CSP-ComDE and XIP-ComR pathways. The CiaXRH, HdrRM, and VicRK signal transduction systems affect bacteriocin production and competence development.[58–64,64,65] VicR suppresses expression of PepO, an endopeptidase that reduces SigX expression, potentially by degrading XIP or ComS.[66] We do not observe any changes in expression of the CiaR, HdrR or VicR response regulators, nor any alteration in PepO expression, indicating that further analysis of mutants or transcriptome profiles at

earlier timepoints is needed to ascertain whether mucin glycans act through these pathways (Supplementary Table 1). Still, there are other as-of-yet uncharacterized pathways through which mucin could act. Recently, it was hypothesized that a physical interaction between oral streptococci could sequester ComR and prevent it from transcriptionally activating XIP-ComR regulated genes.[67] We find that the transcriptional profiles observed in this report correlate with the response induced by MUC5B glycans (Pearson correlation coefficient of 0.62). Mucin glycans could similarly prevent ComR from acting as a transcriptional regulator as a mechanism of reducing quorum sensing induced competence.

Competence development is highly dependent on the specific nutrients present in the environment, through mechanisms that are not yet entirely understood.[59,66,68–71] Acting through nutrient responsive pathways would be an attractive mechanism for the host to control bacterial virulence, as it would be difficult for bacteria to evolve around these pathways. Mucin contains a large diversity of glycan structures, so we anticipate they may affect multiple different regulatory pathways independently. Indeed, it appears that the biofilm formation and competence phenotypes can be separately regulated; for example, GalNAc reduced transformation rates without impacting biofilm density or bacteriocin gene expression (Figures 3i and 4d). Identifying which structures in the mucin pool are responsible for each effect will enable future studies into mechanistic detail, facilitating screens to identify which *S. mutans* components are necessary for mucin's effect. This will provide insight into how the host environment tunes the behavior of the microbiota and how mucin glycans could be leveraged for therapeutic purposes.

METHODS

Strains and Reagents:

The bacterial strains *Streptococcus mutans* UA159, SJ, and 28BE3 as well as *Streptococcus sobrinus* 6715 were kindly given as a gift by Dan Smith (Forsyth Institute, Cambridge, MA). *S. mutans* UA159 (ATCC 700610) was originally isolated from a child with active caries. *S. mutans* SJ and 28BE3 were isolated from children with active caries. For general propagation and storage, bacteria were cultured and maintained in Bacto Todd-Hewitt broth (TH, BD 249240) or Brain Heart Infusion (BHI, BD 237500) 1.5% agar plates at 37°C with 5% CO_2. All TH media was used within seven days of preparation. Most experiments are conducted in 25% Todd Hewitt broth, called CMedia. Selected experiments used a chemically defined medium previously described[39], called DMedia. *S. mutans* UA159 Δ*comC*::Tc^r, Δ*comDE::*Km^r, Δ*comE::*NPKm^r from Ahn *et. al.*[18] were generously provided by San-Joon Ahn (University of Florida). Stephen Hagen (University of Florida) generously shared *S. mutans* UA159 Δ*comS*::Em^r from Underhill *et. al.*[30] and *S. mutans* UA159 P_{cipB}-GFP-Spec^r from Son *et. al.*[73] Experiments in saliva were performed with this *S. mutans* UA159 P_{cipB}-GFP-Spec^r strain to selectively track *S. mutans* growth and transformation.

Human Saliva Collection:

Submandibular saliva was collected from informed consenting volunteers using a custom vacuum pump setup as described previously.[22] To prevent suction of parotid gland secretions, cotton pads were placed between the molars and cheeks. To collect the

submandibular secretions, the vacuum line was placed underneath the tongue. The collection vessel was placed on ice during this process to prevent degradation of proteins. For whole saliva testing, submandibular saliva was aliquoted into 1 mL volumes, kept on ice, and used within 1 hour of collection. To deplete high molecular weight components, saliva was spun at 10,000x*g* at 4°C in Amicon Ultra Centrifugal Filter Units (UFC5010 and UFC5100). Protocols involving samples from human subjects were approved by the Massachusetts Institute of Technology's Committee on the Use of Humans as Experimental Subjects.

Mucin Purification from Pooled Saliva:

After collection, saliva was diluted with 5.5 M NaCl, such that the final salt concentration was 0.16 M. Antibacterial agents and protease inhibitors were then added at the appropriate final concentrations: sodium azide (0.04wt%), benzamidine HCl (5 mM), dibromoacetophenone (1 mM), phenylmethylsulfonyl fluoride (1 mM), and EDTA (5 mM, pH 7). For solubilization, saliva solutions were gently stirred overnight at 4°C. Solubilized saliva was flash cooled in liquid nitrogen in 40 mL volumes before storage at −80°C. Before chromatography, 200 mL of saliva was thawed at 4°C and centrifuged at 10,000x*g* for 10 minutes in a fixed rotor centrifuge to pellet cellular debris. MUC5B was then purified from the pooled supernatant by fast protein liquid chromatography on a Bio-Rad NGC equipped with an XK 50 column packed with 2 L of Sepharose CL-2B resin (GE HealthCare Biosciences). After dialysis, MUC5B was aliquoted, flash cooled in liquid nitrogen, lyophilized, and stored at −80°C. Our preparation is predominantly MUC5B and contains negligible amounts of another salivary mucin, MUC7.[23] The day before use, lyophilized MUC5B was rehydrated at 2x concentration in Milli-Q-purified water and agitated at 4°C overnight for experiments in CMedia and DMedia. In experiments in saliva, an appropriate volume of high molecular weight depleted saliva (<10kDa saliva) was added to lyophilized MUC5B to reach 0.1wt%; the solution was then vortexed for 5 minutes at 4°C to solubilize the mucin.

Glycan Isolation and Quantification:

Glycans with intact reducing ends were isolated from purified human salivary mucin (MUC5B) or commercially available pig gastric mucin (MUC5AC) using ammonia-based β-elimination using a previously described method.[46,48] Briefly, purified mucin was solubilized in ammonium hydroxide saturated with ammonium carbonate and incubated at 60°C for 40 hours, creating oligosaccharide glycosylamines and leftover partially deglycosylated mucin. Repeated centrifugal evaporation with washes in Milli-Q-purified water removed volatile salts. The deglycosylated mucin backbone was isolated from the soluble glycosylamines using 10kDa molecular weight cut-off centrifugal filters (Amicon). The oligosaccharide glycosylamines were then incubated with 0.5 M boric acid at 37°C for 1 hour, converting them to hemiacetals. Samples were washed with methanol and water, and solvents were removed with centrifugal evaporation. Glycans were quantified using a phenol-sulfuric acid assay with a glucose standard curve.[74] Freeze-dried glycans were stored at −20°C.

Glycan Structural Analysis

Glycan samples were permethylated and analyzed by nanoelectrospray ionization mass spectrometry (NSI-MS). For the detection and characterization of neutral and sialylated glycans lacking sulfate, the permethylated glycans were reconstituted in 50% methanol containing 1 mM NaOH for infusion in positive ion mode and injected into a linear ion trap mass spectrometer with an orbital trap mass analyzer (LTQ Orbitrap; Thermo Fisher). For the detection and characterization of sulfated glycans, the permethylated glycans were reconstituted in 50% methanol containing 1 mM NH_4OAc for infusion in negative ion mode. The nanoelectrospray source was run at a syringe flow rate of 0.60 μL/min and capillary temperature set to 210°C.[75–77]

The LTQ Orbitrap instrument was tuned with a permethylated oligosaccharide standard for positive ion mode, and 3'-sulfated Lewis-X trisaccharide for negative ion mode. For fragmentation by CID (in MS2 and MSn), normalized collision energy of 30~40% was applied. Most permethylated O-glycan components were identified as singly or doubly charged, sodiated species [M + Na] in positive mode. Peaks for all charge states were deconvoluted by the charge state and summed for quantification. All spectra were manually interpreted and annotated. The MS-based glycomics data generated in these analyses and the associated annotations are presented in accordance with the MIRAGE standards and the Athens Guidelines.[78]

RNA Preparation for Gene Expression Analysis

Briefly, overnight cultures grown in 50%TH were diluted 1:20 (to approximately 10^7-10^8 CFU/mL) into 100 μL fresh 25%TH (CMedia) supplemented with 0.1wt% MUC5B, 0.1wt% MUC5B glycans, or other compounds. After 2 hours (unless otherwise noted) of growth with rocking at 25rpm at 37°C with 5% CO_2, samples were collected (at OD_{600} of 0.4–0.6) and pelleted at 3000x*g* at 4°C, supernatant was removed, and the pellets were flash cooled in liquid nitrogen and stored at −80°C for less than one week before extraction. Pellets were thawed on ice and resuspended in 400 μL RNAase free water, 50 μL of 0.1 mm silica spheres (MP Lysing Matrix B) were added, and samples were bead-beaten for 90 seconds in three intervals at 4°C. Total RNA was then extracted using the MasterPure Complete RNA Purification kit (Lucigen). Genomic DNA was removed using the Turbo DNA-free kit (Ambion). Total RNA was measured with an Agilent 2100 Bioanalyzer (Agilent Technologies). Samples were stored at −20°C.

RNA-Seq

RNA-Seq was performed using 2 biological replicates for each condition. The libraries were prepared using the Ribo-Zero Magnetic Kit (Bacteria; Epicentre) and the KAPA RNA HyperPrep kit (Kapa Biosystems). Library sequencing was performed using the Illumina HiSeq platform with a single-end protocol and read lengths of 40 nucleotides.

Reads were mapped to the *S. mutans* UA159 genome NC_004350.2 with BWA (Burrows-Wheeler Aligner) through the Galaxy platform.[79] Reads were counted using the R Bioconductor package.[80] Differential expression and significance was calculated using the

method described by Anders and Huber in 2010[81]. Full results are in Supplementary Table 1.

Pathway Enrichment Analysis was performed using gene to pathway mappings from the KEGG Orthology database.[82] Significance of enrichment was calculated using Fisher's Exact test with adjusted FDRs to correct for multiple hypothesis testing using the Benjamini-Hochberg procedure.[83] Pathways with less than 5 genes were excluded from the analysis. Full results are in Supplementary Table 2.

RT-qPCR

Primers used in this study are available in Supplementary Table 3. First-strand cDNA was synthesized with Protoscript II First-Strand cDNA Synthesis kit (New England Biolabs). The elimination of genomic DNA was confirmed by qPCR amplification of 16SrRNA on negative control samples that did not have reverse transcriptase during cDNA synthesis. 3–5 ng of cDNA was used as a template for quantitative PCR using SYBR Green Master Mix (Thermo Fisher Scientific) performed on a Light Cycler 480 II Real-time PR Machine (Roche). Forward and reverse primers were added at 3 μM each. Melting-curve analyses verified amplification of a single product. Gene expression changes were calculated on the basis of mean change in qPCR cycle threshold compared to 16SrRNA (ΔC_t) and are reported as $\log_2$(fold change) = ΔC_t_CMedia - ΔC_t_sample. Each sample was analyzed with at least 3 technical replicates. Significance values were calculated using a one-sample t test and adjusted to correct for multiple hypothesis testing using the Benjamini-Hochberg procedure.

Biofilm, Transformation, and Growth Assays

Overnight cultures were inoculated from glycerol stocks and grown for 12–18 hours in 50%TH at 37°C with 5%CO_2. Plates are Bacto Brain Heart Infusion with 1.5 wt% agar. Overnight cultures were diluted 1:20 into 60–100μL of CMedia (TH25%), DMedia (CDM), or prepared human saliva in a 96-well polystyrene plate for a starting inoculation of 10^7-10^8 CFU/mL. Saliva was supplemented with 0.5wt% glucose to promote bacterial growth.

To assay bacterial biofilm formation, we incubated the samples for 4 hours at 37°C with 5%CO_2. We then removed the supernatant (which includes the planktonic or unadhered fraction) and washed the adhered cells with 100μL phosphate buffered saline (PBS) twice. We then detached and resuspended the biofilm in 100μL PBS by vigorous scraping with a pipette tip for 30 seconds; the planktonic fraction was also agitated to disperse aggregates. Each culture fraction was serially diluted and plated on BHI agar. Colonies are counted after growth at 37°C with 5%CO_2 for 24–48 hours. Percentage biofilm was calculated as (biofilm CFU/mL) / (biofilm CFU/mL + planktonic CFU/mL). All conditions have at least 2 biological and 2 technical replicates (individual data points shown on graphs).

The transformation assay was performed in parallel with the biofilm assay above. Plasmid DNA pVA838 (ATCC 37160) was purchased from ATCC and maintained in *E. coli* DH5α (BD LB medium, 10 μg/ml chloramphenicol). Plasmid pBSU101 for transformation assays was given to the lab by Barbara Spellerberg (University of Ulm, Ulm, Germany) and maintained in *S. mutans* UA159.[84] We added 1.2 μg/mL of the appropriate plasmid at the beginning of the experiment (t=0 hours) for highest overall transformation efficiency

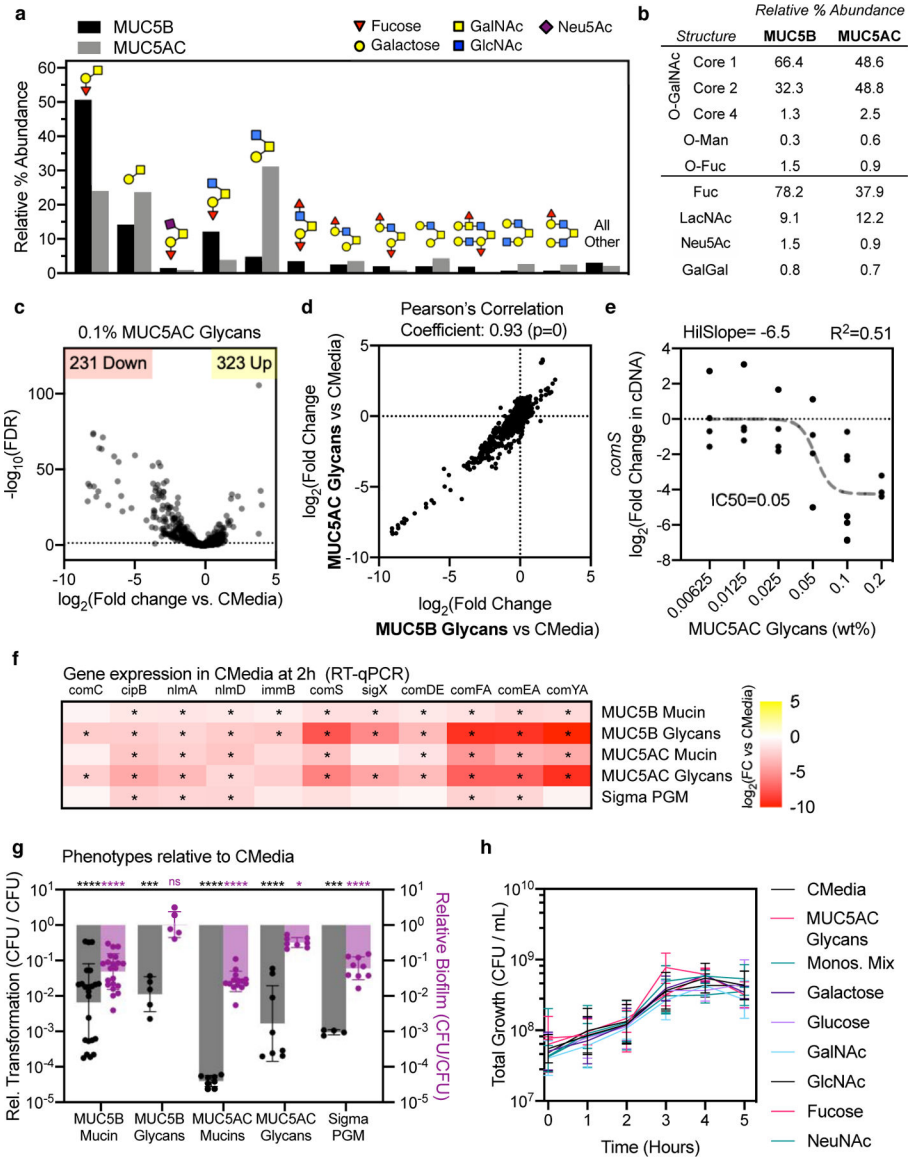
(Supplementary Figure 2). After 4 hours of growth, suspended fractions are collected and the biofilm is resuspended in PBS after two washes. Each culture fraction is serially diluted and plated on BHI and BHI supplemented with erythromycin (10 μg/mL) or spectinomycin (1 mg/mL). After 24–48 hours, colonies are counted and transformation frequency is calculated as (biofilm CFU/mL on antibiotics + planktonic CFU/mL on antibiotics + 50 CFU/mL) / (biofilm CFU/mL + planktonic CFU/mL). The constant 50 CFU/mL is the limit of detection of our assay. Relative transformation is transformation frequency normalized to the media-only control. All conditions have at least 3 biological replicates (individual data points in Source Data). Relative transformation was calculated by normalizing to the mean transformation frequency in media for each biological replicate.

Synthetic peptides XIP (NH_3-GLDWWSL-COOH) and CSP (NH_3-SGSLSTFFRLFNRSFTQALGK-COOH) were synthesized with >95% purity at the Koch Institute Biopolymers and Proteomics Core and purified by HPLC. Peptides were dissolved in water at 1 mM, flash cooled with liquid nitrogen, and stored as aliquots at −80C. Peptides were added (1:100 to 1:1000 dilution) to the culture at t=0.

Statistics and Reproducibility

RNA-seq data was collected from 2 biological replicates. Biofilm and transformation experiments represent at least 3 biological replicates; the exact number of replicates is indicated by dots on graphs, and raw data for Figures 1–4 is available in source data. Reproducibility of biofilm and transformation phenotypes under different experimental conditions was confirmed by measuring several strains in multiple conditions. MUC5B was tested from several purification batches.

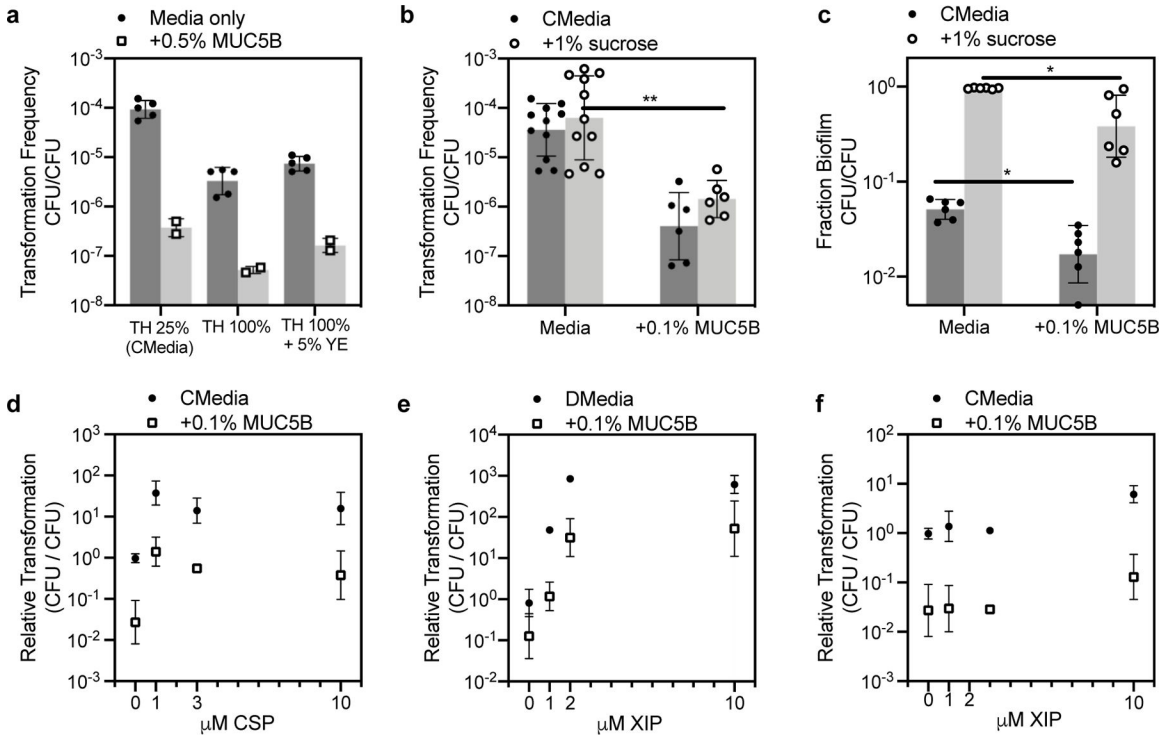
Extended Data



Extended Data Figure 1. MUC5AC glycan pools are highly similar to MUC5B glycan pools in composition and effects on *S. mutans* gene expression and phenotypes.

MUC5AC glycans were isolated from comercially available pig gastric mucin (Millipore Sigma) using beta-elimination.[48] MUC5AC was purified from pig stomachs as detailed previously.[46] Sigma PGM is pig gastric mucin purchased from Millipore Sigma that has been dialyzed (100kDa cutoff) and lyophilized. (a) A comparison on the most abundant glycan structures (at least 1% of the pool) in MUC5B and MUC5AC (n=1). (b) MUC5AC glycans are relatively enriched for Core 2 structures and display less fucose than MUC5B. (c) RNA-Seq was used to profile the effects of 0.1% MUC5AC glycans on gene expression in *S. mutans* in parallel with studies on MUC5B mucin and MUC5B glycans (n=2). (d) The expression profiles of MUC5B glycans and MUC5AC glycans have a Pearson's correlation coefficient of 0.93 (p=0), indicating that they induce

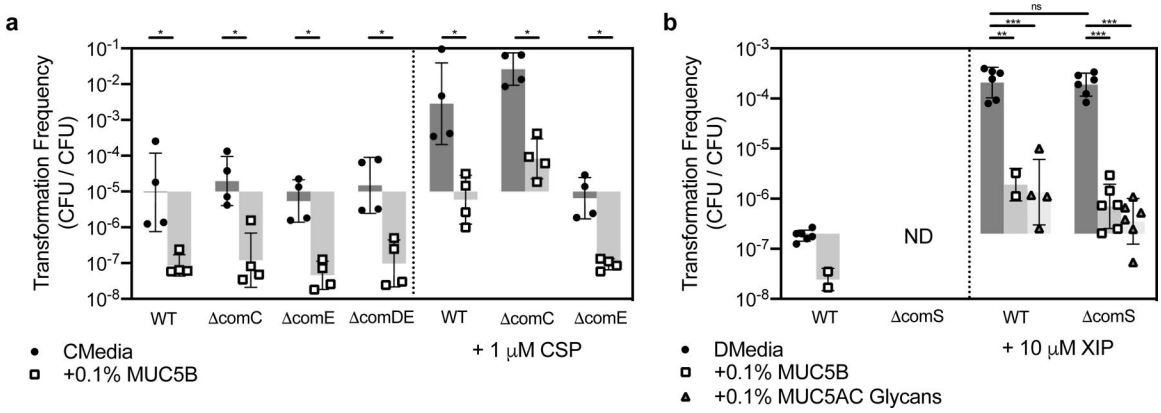
highly similar changes in *S. mutans* global gene expression. (e) MUC5AC glycans have a concentration-dependent influence on *comS* expression. Nonlinear antagonist binding best-fit curves shown (IC50 = 0.05, HillSlope = −6.5, R^2=0.51). (f) RT-qPCR evaluation of *S. mutans* gene expression after 2 hours incubation in CMedia with 0.1wt% substrate. We see that all mucins and mucin glycans downregulate the expression of key quorum sensing genes (n > 4, for full data see Source Data). Interestingly, released mucin glycans (both MUC5B and MUC5AC) have stronger effects than intact mucin proteins (MUC5B and MUC5AC polymers). (g) Mucins (MUC5B and MUC5AC) and mucin glycans (MUC5B and MUC5AC) reduce transformation frequency of *S. mutans* at 0.1wt% in CMedia. While MUC5AC glycans reduce biofilm formation at 0.1wt% in CMedia, MUC5B glycans did not. (h) Growth profiles in CMedia are not altered when supplemented with 0.1wt% of various monosaccharides. KEY: pig gastric mucin (PGM), N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), and N-acetylneuraminic acid (Neu5Ac). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. For **f**, data are means, and significance was assessed using unpaired t tests with the Benjamini-Hochberg correction. For **g**, data are geometric means +/− geometric standard deviations, and significance was assessed using the Kruskal-Wallis test followed by an uncorrected Dunn's test, which does not assume a Gaussian distribution.



Extended Data Figure 2. MUC5B reduces transformation and biofilm formation of *S. mutans* across various media conditions.

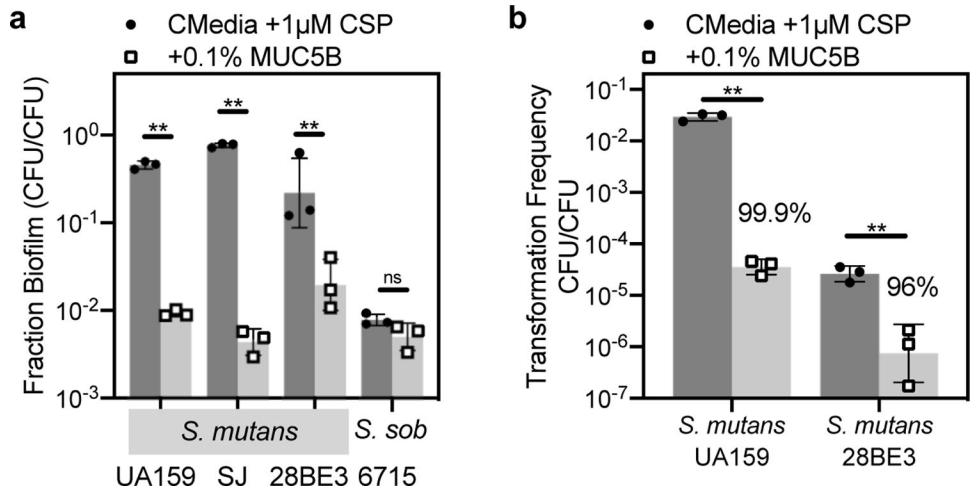
(a) MUC5B reduces transformation frequency in multiple media conditions: CMedia (25%Todd-Hewitt), 100%Todd-Hewitt, 100%Todd-Hewitt + 5% Bacto Yeast Extract. Overall, lower transformation frequencies were observed in richer media. (b) MUC5B reduces transformation rates in CMedia with or without 1% sucrose supplementation to promote biofilm formation. (c) MUC5B prevents biofilm formation in CMedia with

and without 1% sucrose supplementation, as observed previously.[22] (d) MUC5B reduces transformation efficiency in CMedia supplemented with 1–10 μM CSP. (e) MUC5B reduces transformation in DMedia supplemented with XIP and (f) CMedia supplemented with XIP. XIP is less effective at inducing competence in CMedia compared to DMedia. *P<0.05, **P<0.01. Data are geometric mean +/− geometric standard deviation, and significance was assessed using nonparametric Mann-Whitney tests.



Extended Data Figure 3. MUC5B reduces transformation rates in Δ*comC*, Δ*comE, and* Δ*comS* strains.

(a) *S. mutans* UA159 *ΔcomC*, *ΔcomDE,* and *ΔcomE*[18] have natural transformation rates similar to wildtype, and supplementation with 1 μM CSP does not alter transformation rates in *ΔcomE*. MUC5B is able to induce the same reduction of transformation rates in *ΔcomC* and *ΔcomE* strains as in the wildtype, with and without supplemental CSP. (b) We confirm that the *ΔcomS*[30] knockout was not transformable. However, when supplemented with 10 μM XIP, the *ΔcomS* knockout shows transformation rates similar to wildtype. Here, we see that MUC5B mucin and MUC5AC glycans reduce transformation rates in the *ΔcomS* knockout supplemented with 10 μM XIP. *P<0.05, **P<0.01, ***P<0.001, ns = not significant. Data are geometric means +/− geometric standard deviations, and significance was assessed using nonparametric Mann-Whitney tests.



Extended Data Figure 4. MUC5B prevents biofilm formation and transformation in multiple *Streptococcus mutans* strains.

(a) MUC5B reduces biofilm formation of multiple strains of *S. mutans* (UA159, SJ, and 28BE3) in CMedia. MUC5B has no significant effect on biofilm formation of *S. sobrinus 6715.* (b) MUC5B reduces transformation of *S. mutans* strain 28BE3 in CMedia. KEY: *Streptococcus sobrinus* (*S. sob*). **P<0.01, ns = not significant. Data are geometric means +/− geometric standard deviations, and significance was assessed using nonparametric Mann-Whitney tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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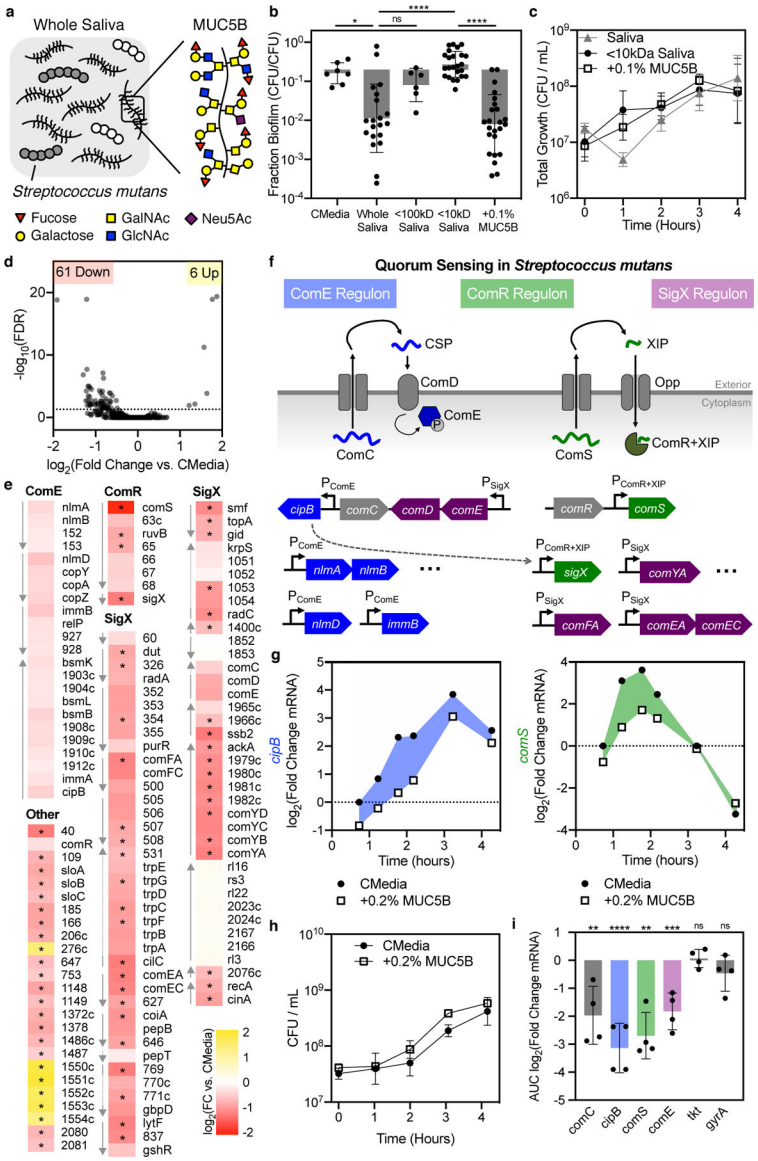

Figure 1: Salivary mucin MUC5B reduces expression of genes in quorum-sensing associated pathways.

(a) Mucus protects the wet epithelial surfaces of the body, hosting much of the microbiota. One mucosal niche is in the mouth, where saliva's viscoelasticity is generated by the highly glycosylated secreted mucin MUC5B. (b) Healthy donor saliva reduces *S. mutans* biofilm formation compared to CMedia. Centrifugal filtration was used to deplete saliva of high molecular weight components, including 2000kDa MUC5B and 150kDa MUC7,[72] removing saliva's ability to reduce biofilm formation. Supplementation of the 10kDa filtered saliva with 0.1wt% MUC5B prevented biofilm formation. (c) Supplementation with MUC5B does not alter the overall growth patterns of *S. mutans* during the experiment. (d) RNA was isolated from *S. mutans* UA159 incubated with and without 0.1wt% MUC5B for two hours in exponential growth in CMedia. RNA-Seq revealed a significant change in expression of 67 genes (n=2, Supplementary Table 1). (e) The majority of significantly downregulated genes (43 of 61) fall within quorum sensing pathways regulated ComE,

ComR, and SigX (*FDR<0.05 with Benjamini-Hochberg correction).[49] Arrows illustrate transcriptionally active regions; dashed arrows represent transcriptional activation in the anti-sense direction.[49] Pathway analysis (Supplementary Table 2) found an enrichment in downregulation of competence-related DNA transport (FDR=3E-7), DNA replication and repair (FDR=0.003), and homologous recombination (FDR=0.05). (f) The ComE, ComR, and SigX regulators are activated by CSP and XIP, which promote transcription of bacteriocin and competence genes. (g) MUC5B suppresses the expression of genes activated by CSP and XIP sensing, *cipB* and *comS* respectively, over time (n=1 of 4 shown, normalized to 16SrRNA). Colored area indicates differential area under the curve. (h) Expression was assayed during exponential phase (n≥2). (i) While quorum sensing associated genes show decreased expression over 4 hours, housekeeping genes *tkt* and *gyrA* are not affected (n=4). KEY: competence stimulating peptide (CSP), *sigX* inducing peptide (XIP), N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), and N-acetylneuraminic acid (Neu5Ac). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. For **b**, data are geometric mean +/− geometric standard deviation. For **g** and **i,** data are area under the curve (AUC) of 6 timepoints over 4 hours; mean and standard deviation are shown. Significance in **b** and **j** was assessed using the Kruskal-Wallis test followed by an uncorrected Dunn's test, which does not assume a Gaussian distribution.

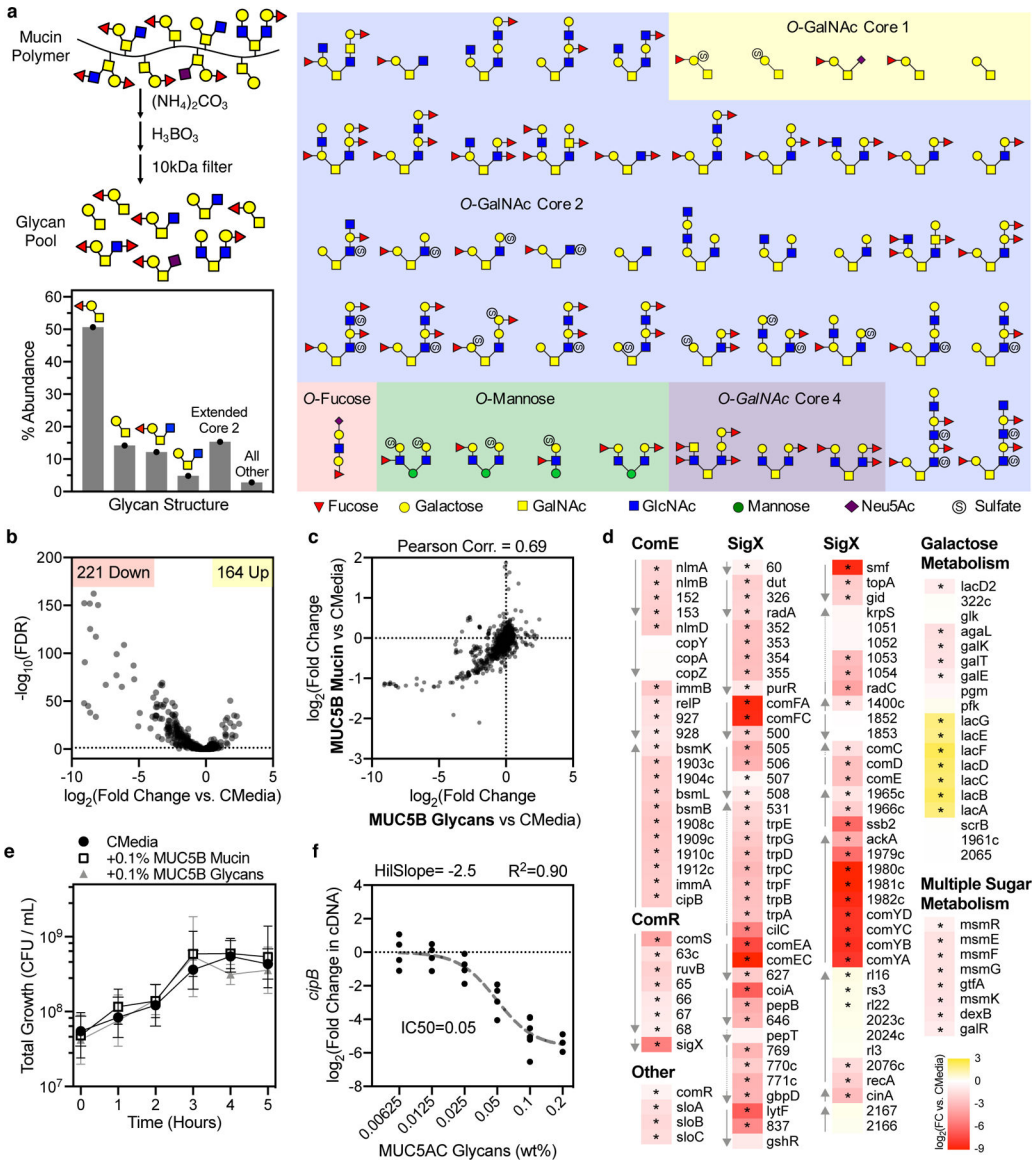

Figure 2: Mucin glycans suppress expression of quorum sensing genes regulating competence, virulence, and bacteriocin production.

(a) Complex mucin glycans were isolated from mucin polymer gels using beta-elimination, leaving their reducing ends intact. Mass spectrometry identified dozens of unique glycan structures (Supplementary Figure 1). MUC5B glycans are dominated by Core 1 and Core 2 glycan structures with high levels of fucose (n=1 pooled sample from 5 donors). (b) RNA was harvested from *S. mutans* UA159 incubated with and without 0.1wt% MUC5B glycans for two hours in exponential growth in CMedia. RNA Seq revealed a significant change in expression of 385 genes (n=2, Supplementary Table 1). (c) MUC5B glycans induce a highly similar change in gene expression as MUC5B mucin; expression profiles have a Pearson's correlation coefficient of 0.69. (d) MUC5B alters expression of almost all genes regulated by ComE, ComR, and SigX (*FDR<0.05 with Benjamini-Hochberg correction). Pathway enrichment analysis (Supplementary Table 2) indicates that MUC5B glycans downregulate quorum sensing (FDR=0.004), downregulate competence-related

DNA transport (FDR=0.0004), and upregulate galactose metabolism (FDR=0.006). Arrows illustrate transcriptionally active regions in the ComE, ComR, and SigX regulons; dashed arrows represent transcriptional activation in the anti-sense direction.[49] (e) Supplementation of CMedia with MUC5B mucin or glycans does not alter the overall growth patterns of *S. mutans* during the experiment (n=4). (f) Mucin glycans have a concentration-dependent influence on *cipB* expression. Nonlinear antagonist binding best-fit curves shown (IC50 = 0.05, HillSlope = −2.5, R^2=0.90). KEY: N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), and N-acetylneuraminic acid (Neu5Ac).

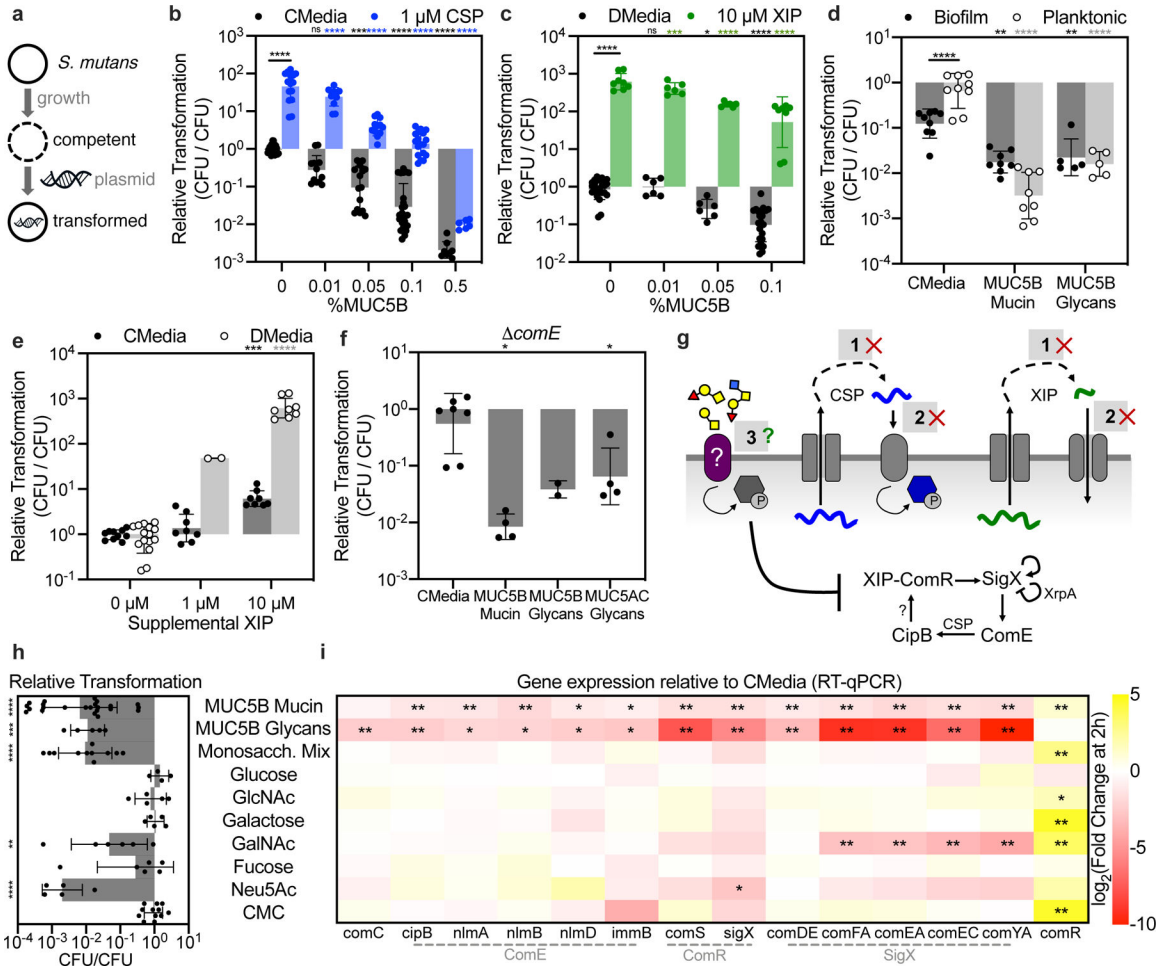

Figure 3: Mucin glycans reduce genetic transformation by suppressing competence in a manner that is dependent on sugar identity and independent of signal peptide transport.

(a) A fraction of the *S. mutans* population is naturally competent in exponential phase growth. Competence development is assayed by measuring transformation frequency in the presence of plasmid DNA with an antibiotic marker. (b) MUC5B reduces transformation in a concentration dependent manner in CMedia with and without 1 μM CSP. (c) MUC5B reduces transformation in a concentration dependent manner in chemically defined media (DMedia[39]) with and without 10 μM XIP. (d) At 0.1wt% MUC5B mucin and glycans reduce transformation in both the biofilm and planktonic fractions of culture in CMedia. (e) Unlike in defined media, external stimulation of transformation by XIP is suppressed in complex media.[51] (f) At 0.1wt% MUC5B mucin, MUC5B glycans, and MUC5AC glycans reduce transformation rates in a *S. mutans* UA159 Δ*comE* strain in CMedia. (g) Hypotheses for how mucin may reduce quorum sensing associated competence development. 1. Mucin does not appear to act by reducing the diffusivity of quorum sensing peptides, since glycans freed from the backbone reduce transformation. 2. Mucin does not appear to act by blocking sensing of CSP or import of XIP. 3. Mucin could prevent competence development through known parallel regulators of quorum sensing or another still uncharacterized mechanism. (h) At 0.1wt% MUC5B mucin, MUC5B glycans, GalNAc, Neu5Ac, and a pool of mucin monosaccharides (Monosacch. Mix; equal parts

galactose, GalNAc, GlcNAc, fucose, Neu5Ac) reduce transformation relative to CMedia alone. (i) Relative expression of quorum-sensing activated genes in CMedia after 2 hours of exposure to treatments at 0.1wt% (RT-qPCR). MUC5B mucin (n=12), MUC5B glycans (n=5), and GalNAc (n=4) reduce expression. Other monosaccharides (n=4) and CMC (n=3) do not. KEY: N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), N-acetylneuraminic acid (Neu5Ac), and carboxymethyl cellulose (CMC). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. For **b, c, d, e, f**, and **h** data show geometric mean +/− geometric standard deviation, and significance was assessed using the Kruskal-Wallis test followed by an uncorrected Dunn's test, which does not assume a Gaussian distribution. For **i**, data show mean, and significance was assessed using unpaired t tests with the Benjamini-Hochberg correction. Significance values not otherwise marked represent comparison to media-only control.

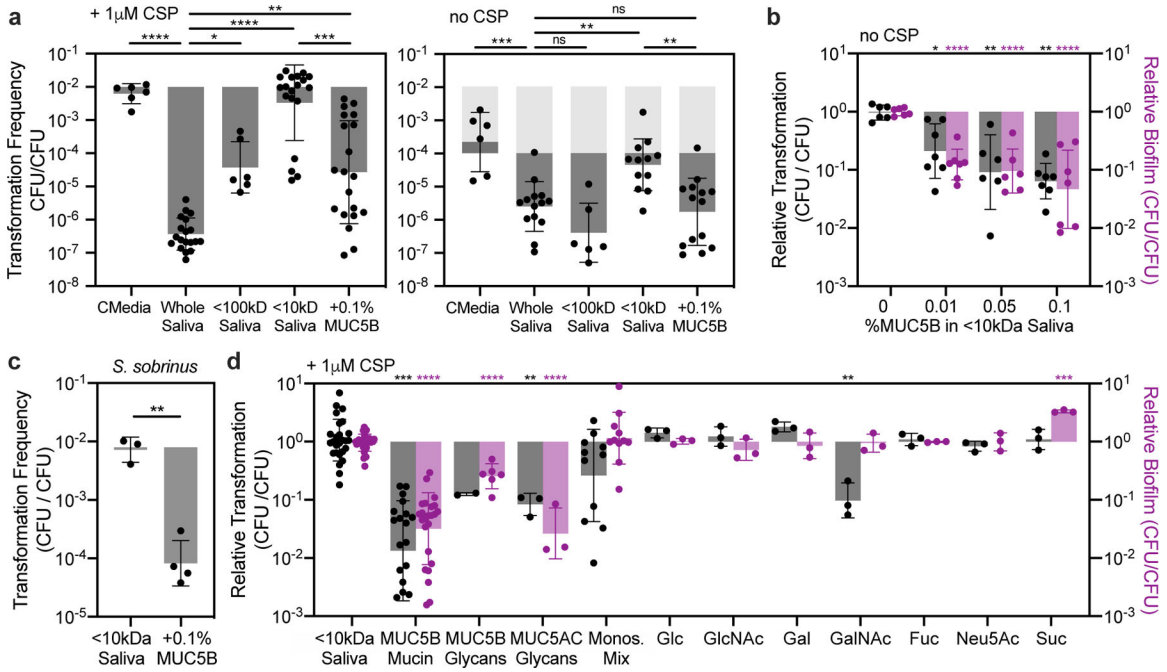

Figure 4: Mucin and mucin glycans reduce natural genetic transformation of *S. mutans* in *ex vivo* human saliva.

Saliva was collected from healthy donors, kept on ice, and used for experiments within one hour. All saliva samples were supplemented with 0.5wt% glucose to promote microbial growth. (a) Centrifugal filtration was used to deplete saliva of high molecular weight components (>100 and >10kDa), removing its ability to reduce transformation. Adding back MUC5B to >10kDa depleted saliva partially restored saliva's ability to suppress transformation. (b) MUC5B reduces transformation in saliva in a concentration-dependent manner. (c) Transformation of *Streptococcus sobrinus* was suppressed by addition of MUC5B. (d) 0.1wt% MUC5B mucin or 0.1wt% MUC5AC glycans added into depleted saliva suppressed biofilm formation and transformation while a monosaccharide pool of equal parts galactose, GalNAc, GlcNAc, fucose, and Neu5Ac (Monos. Mix) did not alter phenotypes. Supplementation with 0.1wt% GalNAc reduced transformation frequency but not biofilm formation. KEY: Fucose (Fuc), Galactose (Gal), N-acetylgalactosamine (GalNAc), Glucose (Glc), N-acetylglucosamine (GlcNAc), N-acetylneuraminic acid (Neu5Ac), and Sucrose (Suc). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Data are geometric mean +/− geometric standard deviation. For **a**, significance was assessed using the Kruskal-Wallis test followed by an uncorrected Dunn's test, which does not assume a Gaussian distribution. For **b, c**, and **d**, significance was assessed using Welch's t-test, which does not assume equal variance across samples. Significance values not otherwise marked represent comparison to media-only control.