The collagen binding protein Cnm contributes to oral colonization and cariogenicity of *Streptococcus mutans* OMZ175.

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Streptococcus mutans is the etiological agent of dental caries and one of the many bacterial species implicated in infective endocarditis. The expression of the Cnm collagen-binding protein by S. mutans has been associated with extra-oral infections, but its relevance in dental caries has only yet been theorized. Due to the collagenous composition of dentinal and root tissues, we hypothesized that Cnm may facilitate colonization of these surfaces, thereby enhancing the pathogenic potential of S. mutans in advancing carious lesions. As shown for extra-oral endothelial cell lines, Cnm mediates invasion of oral keratinocytes and fibroblasts by S. mutans.

In this study, we show that in the Cnm* native strain, OMZ175, Cnm mediates stringent adhesion to dentinal and root tissues as well as collagen-coated surfaces, and promotes both cariogenicity and carriage in vivo. In vitro, ex vivo and in vivo experiments revealed that, while not universally required for S. mutans cariogenicity, Cnm contributes to: (i) invasion of the oral epithelium, (ii) enhanced binding on collagenous surfaces, (iii) implantation of the oral biofilm, and (IV) caries severity by a native Cnm* isolate. Taken altogether, our findings reveal that Cnm is a colonization factor that contributes to the pathogenicity of certain S. mutans strains in its native habitat, the oral cavity.
The oral cavity is colonized by hundreds, if not thousands, of bacterial species that occupy specialized niches within the mouth. One such species, *Streptococcus mutans* is a hard tissue colonizer and considered the major microbial etiological agent of dental caries. In addition, oral streptococci, including *S. mutans*, are implicated as causative agents of extra-oral infections such as infective endocarditis (1). The enhanced capacity to form biofilms in the presence of sucrose, metabolize a wide range of carbohydrates, and high tolerances to fluctuations in pH and nutritional availability, are all considered crucial traits for the organism to survive, persist, and cause dental caries (2-4). Current paradigms of *S. mutans* attachment focus on its ability to bind oral surfaces, particularly to the enamel and proteins within the salivary pellicle via sucrose-dependent and independent mechanisms. Under the extensively studied sucrose-dependent mechanism, secreted glucosyltransferases (Gtfs) serve the dual roles of priming the tooth enamel surface with glucans for adhesion by surface glucan binding proteins (GbPs), and developing an extracellular polysaccharide (EPS) superstructure that anchors the biofilm and supports matrix-delineated pH microenvironments *in situ* (4-9). The sucrose-independent mechanism involves direct substrate recognition by bacterial surface adhesins that bind to either constituents of the salivary pellicle on the tooth surface, such as SpaP (also known as P1, PA or
AntigenI/II) (10, 11), or to components of underlying tissues that become exposed due to demineralization of the enamel; e.g. collagen from dentin and roots (11-13).

*S. mutans* is among several other lactic acid bacteria in the oral cavity that produce organic acids as fermentative end products from dietary carbohydrates which in turn acidifies the surrounding environment. Biofilm accumulation slows the diffusion of organic acids and restricts the access of pH-buffering saliva, subjecting the enamel to repeated and prolonged exposure to acidic pH (pH < 5.5), thereby initiating the dental caries process (3, 14). Left untreated, further demineralization of the enamel and extension of the lesion exposes the underlying dentin, revealing collagen and other additional substrates for bacterial colonization (15, 16). Other oral tissues, such as the cementum, root, and periodontal ligament fibers, are also rich in collagen (17), and if exposed to the oral environment, may be vulnerable to attachment and colonization by microbes equipped with collagen-binding adhesins (13, 18-20).

In *S. mutans*, SpaP, WapA, Cnm, and Cbm have been identified as collagen-binding adhesins, but only SpaP has been thus far examined for its role in attachment to dentin (10). However, the collagen-binding affinity of SpaP has been found to be comparatively marginal to the more recently discovered Cnm and Cbm adhesins (12, 21, 22).

The genes coding for the collagen and laminin-binding surface protein, Cnm, was found in approximately 10-20% of the *S. mutans* clinical isolates (23), and the closely-related cbm was...
prevalent in approximately 2% (24). Although serotype c strains predominate in dental plaque (~ 70-80%), cnm and cbm are mostly found in the less prevalent serotypes e, f and k (23-26).

Previously, we showed that Cnm mediates intracellular invasion of human coronary artery endothelial cells (HCAEC) in vitro, and that Cnm+ strains exhibited increased virulence in the Galleria mellonella insect model (26). Moreover, recent interest in S. mutans collagen binding proteins (CBPs), such as Cnm and Cbm, has linked these adhesins with extra-oral infections, including infective endocarditis, hemorrhagic stroke and atherosclerotic plaque development (1, 27-29). To date, studies have focused on the role of Cnm in S. mutans extra-oral virulence, but have yet to elucidate whether Cnm confers any advantage to S. mutans in colonization of oral tissues and in the development of dental caries. Here, we utilized in vitro, ex vivo and in vivo approaches to evaluate the role of Cnm in colonization, persistence, and virulence in the oral cavity - the natural habitat of S. mutans.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains used in this study (Table 1) were routinely grown in brain heart infusion (BHI) at 37°C under a 5% CO2 atmosphere. The integrative vectors pBGK (30) and pBGE (31) were used to incorporate the non-polar kanamycin (pBGK) and erythromycin (pBGE) resistance cassettes into the gtfA locus of the S. mutans chromosome for
the purpose of strain differentiation during competitive binding assays. The nisin-inducible Cnm-expressing construct, pcnm (26), was introduced into OMZ175 to produce the Cnm-overproducing strain, OMZ175pcnm. Where applicable, kanamycin sulfate (1 mg mL⁻¹), erythromycin (10 μg mL⁻¹), or nisin (500 ng mL⁻¹) was added to the growth medium.

**Attachment and invasion assays of oral cell lines.** The capacity for attachment and invasion of oral cell lines by *S. mutans* was assessed by methods described elsewhere with minor modifications (32). Immortalized human gingival fibroblasts HGF-1 CRL-2014 (ATCC, Manassas VA) were maintained in a 37°C incubator under 5% CO₂ in DMEM with 4.5 g L⁻¹ glucose, sodium pyruvate and L-glutamine (Cellgro, Corning NY) supplemented with 10% fetal bovine serum (Hyclone, Logan UT), and passaged up to 8 times using Accutase (Innovative Cell Technologies, San Diego CA) per manufacturer’s instructions and seeded in 24-well plates for assay purposes. Briefly, stationary phase cultures were harvested, washed, and resuspended in tissue culture medium and kept on ice. Meanwhile, HGF-1 cells were pre-incubated on ice blocks for 30 min followed by co-culturing with *S. mutans* at a multiplicity of infecton (MOI) of 100. Co-cultures were centrifuged for 10 min at 800 rpm, 4°C to synchronize attachment and invasion. For attachment assays, centrifuged co-cultures were maintained on ice for 20 min (total of 30 min incubation for attachment), washed three times with ice-cold
Hank's buffered saline solution (HBSS), and lysed by incubation at 4°C with ice-cold sterile water. Inoculum and lysates were serially diluted and plated on BHI agar for colony forming unit (CFU) enumeration.

Bacterial invasion was assessed using an antibiotic protection assay with the same preparations described above. Centrifuged co-cultures were rapidly warmed for 3 min in a 37°C water bath followed by incubation at 37°C in a 5% CO₂ atmosphere for 2 h followed by washing twice with HBSS. Co-cultures were then incubated for 3 h with fresh culture medium containing penicillin G (10 μg mL⁻¹) and gentamycin (0.3 mg mL⁻¹) to kill extracellular bacteria, followed by washing twice with warm phosphate-buffered saline (PBS). Lysates were recovered by aforementioned procedures.

Human oral keratinocytes (HOK) (ScienCell, Carlsbad CA) were cultured in OKM complete medium with penicillin/streptomycin solution (Sciencell). For assay purposes, cells were seeded in 2x10⁵ cells/well in 24-well plates in medium without antibiotics. Invasion and attachment assays were performed using the same procedures as those described for the HGF-1 assays. For the sake of comparing results to the control strain (OMZ175), attachment and invasion efficiencies for each independent experiment were normalized to the experimental average observed for OMZ175 infections and compared statistically by Kruskal-Wallis tests with
Dunn's comparisons post-hoc. Statistical analyses were performed using Graphpad Prism v. 6.0e for Macintosh (Graphpad Software, San Diego, CA).

Transmission Electron Microscopy of oral cell line invasion by OMZ175. Transmission electron microscopy (TEM) was performed on HGF-1 and HOK cells co-incubated with OMZ175 to observe host invasion and localization of invading cells as described elsewhere (33).

Sections were examined using a Hitachi H-7000 TEM (Hitachi High Technologies America Inc., Schaumburg IL) and images acquired with a Veleta 2k x 2k camera with iTEM software (Olympus Soft-Imaging Solutions Corp, Lakewood CO). Sample fixation, embedding and imaging were performed by the Interdisciplinary Center for Biotechnology Research Electron Microscopy and Bioimaging Core facility at the University of Florida.

Adherence to saliva and collagen-coated hydroxyapatite beads. The binding potential of OMZ175, OMZ175Δcnm and UA159 to hydroxyapatite (HA) beads, a tooth enamel surrogate, coated with either collagen type I, clarified whole saliva or both, was assessed using methods described elsewhere with minor modifications (20, 34, 35). Whole stimulated saliva was obtained from healthy adult volunteers under a protocol approved by the Research Subjects Review Board of the University of Rochester (RSRB#00030432). Briefly, cultures were grown to
mid-exponential phase in low molecular weight broth (LMWG – 0.25% w/v Bacto tryptone, 0.15% w/v Bacto yeast extract, 25 mM KH$_2$PO$_4$, 4 mM MgSO$_4$, pH 7.35, filtered to a 10 kDa cut off and supplemented with 1% glucose) and tritiated thymidine (10 µCi mL$^{-1}$). The cultures were washed with absorption buffer (AB – 1 mM pH 6.5 potassium phosphate buffer plus 50 mM KCl, 1 mM CaCl$_2$ and 0.1 mM MgCl$_2$) and concentrated to 2x volume followed by disruption of cell chains by four brief sonication cycles (15 s at 7 W over 30 s intervals). Culture densities were adjusted to OD$_{600} = 1.0$ with AB and the emitted beta radiation determined by scintillation counts. Ceramic hydroxyapatite type I beads (80 µm, Bio-Rad, Hercules CA) were prepared in one of three ways: [saliva treatment] immersion in clarified saliva on a rocker for 1 h at 37°C followed by 3 washes with buffer AB2 (AB with 0.1 M phenylmethylsulfonyl fluoride + 0.02% sodium azide) and incubated in buffer AB for 1 h at 4°C; [collagen treatment] immersion for 1 h at 4°C in acid solubilized rat tail collagen type I (Sigma, St. Louis MO) diluted in buffer AB (0.5 mg mL$^{-1}$), followed by washes with buffer AB2 and incubated again in buffer AB for 1 h at 4°C; [collagen/saliva treatment] same as collagen treatment protocol followed by incubation in saliva for 1 h at 4°C after washes with AB2. Non-specific binding was minimized by blocking all bead preparations with 0.5 mg mL$^{-1}$ bovine serum albumin (BSA) in AB for 1 h at 37°C. Prepared beads were incubated with culture aliquots on a rocker at 37°C for 1 h followed by washes in AB and emission measurement by liquid scintillation. Bacterial attachment efficiencies were
determined by average emission per CFUs and normalized to the rates of attachment observed for strain OMZ175 and ANOVA was performed on the log_{10} transform Bonferroni’s comparisons post-hoc.

**In vitro collagen binding assay.** The binding potential of study strains to immobilized collagen type I on microtiter plates was assessed using methods described elsewhere (21) with minor modifications. Cells were grown and treated as described for Cnm visualization, followed by washing and resuspension in PBS to achieve 4-fold concentrations. Viable cell densities of cell preparations were checked by serial dilution and plating on BHI agar to ensure similar loading. The resulting Abs_{575} from cells binding to no collagen control wells (background) were subtracted from readings of collagen-bound cells from the same inoculum, and the resulting readings normalized to OMZ175. Statistical analyses were performed on the log_{10} transformations using ANOVA with Bonferroni post-hoc.

**Ex vivo binding competition assays on tooth substrates.** Comparative analysis of *S. mutans* binding avidity and specificity to tooth substrates was evaluated by competition assays using strain pairs mixed 1:1 and co-incubated with sectioned teeth. Surgically extracted non-impacted third molars were cleaned of all soft tissue detritus and cut horizontally using a circular
saw blade at the cementum-enamel junction to separate the crown from the roots (Fig. 3A).

Tooth sections were sonicated in sterile water to remove any bound microorganisms, and screened for microbial contaminants by plating sonicants on tryptic soy agar (TSA). Crown sections intended for binding experiments involving only the enamel surface (identified as "enamel" in this study) were masked with a melted wax cap on the cut face to block binding to the exposed dentin. This cap was removed using a sterile scalpel before the bacterial release step. Tooth particles labeled "dentin", had the apical portion of crown removed and no wax applied. All tooth sections were bathed in clarified sterile saliva for 30 min at 37°C prior to co-infection. Briefly, cultures grown in LMWG medium to mid-exponential phase were mixed 1:1 and incubated with the tooth substrates for 5 min at 37°C in a 5% CO₂ atmosphere. The tooth sections then underwent three washes (1, 2 and 7 min) in 14 mL sterile PBS pH 7.2 on a rotator (60 rpm) to remove loosely-bound cells. Stringently bound cells were released by three sonication cycles of 10 s each with 30 s rests on ice between each interval. Sonicants were then serially diluted in 0.1 M glycine pH 7.2 followed by plating on TSA supplemented with erythromycin or kanamycin. CFUs were enumerated after 48 h incubation at 37°C in a 5% CO₂ atmosphere. For competition assays in the presence of sucrose, cells were grown as described above in LMWG, pelleted, and resuspended in LMW+1% sucrose followed by incubation for 15 min at 37°C in a 5% CO₂ atmosphere prior to 5 min co-incubation with tooth sections. Statistical
analyses were performed on the log_{10} transform of the competitive index using ANOVA with Bonferroni’s comparisons post-hoc to compare substrate preference amongst strain pairs.

**Rat caries model.** The cariogenic potential of OMZ175, OMZ175Δcnm, UA159, UA159-cnms, and UA159-pBGE was assessed in Sprague-Dawley rats using methods described elsewhere (36, 37). Approval for this study was obtained from the University of Rochester Committee on Animal Resources (UCAR#100583) and test subject sample sizes were chosen based upon previously performed studies using similar model parameters to ensure sufficient power to detect distribution differences (38, 39). Female Sprague-Dawley pups aged 19 days were purchased with their dams (Harlan Laboratories, Indianapolis IN). Animals received Diet #2000 (Harlan Laboratories, Indianapolis IN) and sterile 5% sucrose water *ad libitum* for the duration of the study. *S. mutans* cultures were grown to late exponential phase in LMWG and swabbed onto all interior surfaces of the mouth anterior to the oropharynx for 4 consecutive days starting at 19 days of age. Dams were similarly infected on the same days with litter-corresponding cultures and housed with their pups for one week to facilitate strain transmission and implantation. At 26 days old, the pups were paired and housed in wire-bottom cages with those similarly infected from a different litter to minimize litter-based biases (40). Pups were screened for strain implantation at 25, 27 and 29 days of age by oral swabs plated on MSB (*mutans*
salivarius agar + 200 U L\(^{-1}\))

no cross-contamination. The primer pair 1876F/1877R (23) produced a 1.4 Kbp product from UA159 and 7.0 and 7.8 Kbp products for OMZ175 and OMZ175\(\Delta cnm\), respectively.

After 30 days of infection, the pups were killed by CO\(_2\) asphyxiation and the lower left jaws were aseptically removed and placed in sterile saline solution. The jaws were sonicated using the same parameters as described in the \textit{ex vivo} experiments, serially diluted, and plated for total bacterial counts on TSA II (BD Bioscience) and \textit{S. mutans} on MSB. Statistical differences were ascertained by either Kruskal-Wallis tests with Dunn’s comparisons post-hoc, or Kolmogorov-Smirnov tests. Carious lesions were evaluated by a single calibrated examiner blinded to the study groups. The extension and severity of lesions on smooth and sulcal surfaces of the teeth were analyzed using Larson’s modification of Keyes’ method (41, 42) and statistical significances determined by either ANOVA with Bonferroni’s comparisons or Student’s 2-tailed t-test, using the arcsine normalization transform of enumerated lesions (40). Exception to analysis techniques were made for comparison of extensive sulcal lesions in Fig. 4E due to lesion absence in all OMZ175\(\Delta cnm\) infected rats (Kruskal-Wallis with Dunn’s post-hoc).

\textbf{pH drop and acid killing.} The glycolytic capacity of UA159 and OMZ175 and its derivative strains were compared by pH drop experiments using standard protocols (34, 43). For acid
killing experiments, cultures grown to mid-exponential phase were washed once with 0.1 M glycine (pH 7.0) and resuspended in 0.1 M glycine (pH 2.8). Samples were stirred continuously at room temperature, aliquots of cells were removed at pre-determined intervals and viability determined by CFU enumeration on BHI agar. Resulting distributions were analyzed by ANOVA to evaluate survivability distributions for each time point.

Biofilm analyses. Biofilm composition (intracellular, water soluble and insoluble extracellular polysaccharide) and dry weight of S. mutans strains grown in the presence of sucrose were assessed using methods previously described (34, 44). Results within each assessment were compared using Kruskal-Wallis test with Dunn’s comparisons post-hoc.

Cnm detection. Production and localization of Cnm by study strains was confirmed by Western blotting and immunofluorescence techniques previously described (45). Briefly, cells were grown in LMWG to OD$_{600}$ = 0.2, at which time cultures were treated with nisin, where applicable, and grown for an additional 2 h. Whole cell lysates were obtained and resolved by 10% SDS-PAGE, followed by transfer to a polyvinylidene fluoride (PVDF) membrane. The blots were probed for Cnm using rabbit anti-rCnmA (21) with a horseradish peroxidase-coupled secondary antibody. Immunodetection was performed using the ECL chemiluminescent system (GE Life.)
Sciences, Piscataway NJ) with exposures performed on X-OMAT LS film (Carestream, Rochester NY). Validation of Cnm export to the cell surface was performed using immunofluorescent labeling of surface-bound Cnm as detailed elsewhere (45).

RESULTS

Cnm mediates invasion of oral epithelial cell lines. Previously, we and others have shown that Cnm facilitates enhanced invasion and attachment of two non-oral cell lines, HCAEC and human umbilical vein endothelial cells (HUVEC) (22, 26). Here, we determined the contribution of Cnm towards S. mutans attachment to and invasion of two distinct cell lines of the oral epithelium: human gingival fibroblasts (HGF-1) and human oral keratinocytes (HOK). The median intracellular UA159 viable counts recovered from both HGF-1 and HOK was zero. Co-incubation of HGF-1 cells with OMZ175 (Cnm⁺), OMZ175Δcnm (Cnm⁻) or UA159 (Cnm⁻) showed similar levels of attachment to the host cell surface (Fig. 1A, p=0.686). A previous study assessing attachment to HCAECs revealed that Cnm contributed to higher attachment rates by Cnm⁺ S. mutans strains. This could be attributed to both differences in host cell lines and methodology that included centrifugation to the co-culture to facilitate cell-cell contact. Nevertheless, recovery of intracellular S. mutans from HGF-1 revealed significant differences between the Cnm⁺ OMZ175 and the Cnm⁻ strains (p≤0.0089). In addition, co-incubation of these
strains with HOK revealed significant differences between the Cnm+ and Cnm- strains in both attachment (p≤0.0042) and invasion (p≤0.0017) (Fig. 1B). TEM analysis of HGF-1 infected with OMZ175 for 2 h validated the antibiotic protection assay results as it reveals cell membrane extrusions surrounding *S. mutans* cells and intracellular bacteria contained within membrane-bound vacuoles (Fig. 1C and 1D). Thus, our findings suggest that Cnm may confer an advantage to *S. mutans* in colonizing oral epithelial tissues, thereby expanding its possible niches for persistence in the oral cavity.

*Cnm enhances binding to collagen-coated hydroxyapatite surfaces.* Whereas the enamel is a non-collagenous mineralized tissue, the underlying dentin and adjacent roots are much less mineralized and have an organic matrix composed mainly of type I collagen (46). In order to determine whether *S. mutans* binds collagen type I in a context similar to that found in the oral cavity, hydroxyapatite (HA) beads were coated with either collagen type I and/or saliva. The coated beads were incubated with OMZ175, OMZ175Δcnm, and UA159 cells and the number of bound cells quantified (Fig. 2). On HA beads coated only with saliva, UA159 cells bound in higher numbers than OMZ175 or OMZ175Δcnm (p<0.0006). However, on beads coated with either only collagen type I, or a combination of collagen and saliva, the Cnm+ OMZ175 bound significantly better than both Cnm- strains (p<0.0001).
Cnm facilitates enhanced binding to dentinal and root substrates. Based on the observation that OMZ175 displayed an enhanced capacity to bind to collagen-coated HA beads over Cnm⁻ strains, we subsequently evaluated the contribution of Cnm towards binding to biologically pertinent substrates through competitive binding assays. To this end, we developed an ex vivo approach to evaluate the binding avidity of our study strains to: (i) enamel, (ii) exposed dentin and (iii) root surfaces (Fig. 3A). Competitive binding indices (CI) were obtained for the following groups: OMZ175 versus OMZ175Δcnm, OMZ175 versus UA159, and OMZ175Δcnm versus UA159 (Fig. 3B). In all cases, similar binding to enamel surfaces was observed between the three comparative groups. However, the Cnm⁺ strain OMZ175 outcompeted the Cnm⁻ strains on both dentin and root sections by approximately 1-log (p≤0.0153). Inactivation of cnm in OMZ175 impaired its ability to bind dentin and root sections, resulting in similar affinities to these substrates as the Cnm⁻ native UA159. The binding advantage to collagenous tooth substrates conferred by Cnm to OMZ175 was reduced to modest levels upon exposure to sucrose (Fig. S6). While OMZ175 was still able to outcompete the Cnm⁻ strains on dentin and root sections in sucrose, the differences between the indices obtained for collagenous versus non-collagenous substrates were not statistically significant in most cases.
Cnm complementation, overexpression in OMZ175 and expression in UA159. To further confirm that collagen-binding dominance ex vivo was conferred by Cnm in OMZ175, Cnm was expressed in trans (pcnm) using the nisin-inducible pMSP3535 plasmid in the Δcnm strain (OMZ175Δcnm/pcnm) (26). In addition, the effects of Cnm overproduction by pcnm in the native Cnm+ background (OMZ175pcnm), and expression of Cnm in the Cnm− strain (UA159-cnmg), were evaluated in vitro and ex vivo. In vitro binding to collagen type I showed nearly complete restoration of the collagen-binding defect observed in OMZ175Δcnm in the OMZ175Δcnm/pcnm complemented strain (Fig. S1A). This partial restoration may be attributed to Cnm not being expressed in its native fully glycosylated form (Fig. S1B) (45), as the plasmid-encoded Cnm migrate at a slightly smaller size when compared to OMZ175. In the ex vivo competition binding assays, OMZ175Δcnm/pcnm outcompeted OMZ175Δcnm (Fig. S2), although when co-incubated with wild-type OMZ175, it was outcompeted on all substrates. Overexpression of Cnm in OMZ175 (OMZ175pcnm) did not enhance its capacity to bind collagen type I in vitro (Fig. S1 panels a and g). Unexpectedly, the overexpressing strain outcompetes OMZ175 in the ex vivo studies on all three surfaces regardless of apparent collagen content in the substrate. Both the complemented and overexpressing strains displayed inconsistent phenotypes with regards to selective binding to collagenous hard surfaces, and...
were thus not considered useful for study \textit{in vivo}. Next, we evaluated the contribution of Cnm to oral colonization by expressing it in the Cnm\textsuperscript{−} strain UA159. As expected, we observed a gain of function to the strain UA159-cnm as it bound to collagen at levels similar to that of OMZ175 \textit{in vitro} (Fig. S1A). Cnm expression and proper localization to the cell surface by UA159-cnm was confirmed by Western blot and immunofluorescence, respectively (Fig. S1B and S3). In addition, glycosylation by the native machinery in UA159 appears to properly decorate Cnm as shown by a similar migration pattern as OMZ175 by Western blot (Fig. S1B), and conferring stability phenotype upon exposure to proteinase K (45). \textit{Ex vivo} binding assays between UA159 and UA159-cnm showed nearly equal binding to enamel surfaces (Fig. S2), but advantageous adhesion to dentin and root substrates (p=0.063 and 0.005, respectively) by UA159-cnm. Thus, heterologous expression of Cnm confers a clear advantage in adherence to dentin and root surfaces.

\textbf{Inactivation of \textit{cnm} decreases colonization and cariogenic potential of OMZ175.} Our \textit{in vitro} and \textit{ex vivo} findings suggested that Cnm might facilitate the colonization of dentin and roots, leading us to assess the cariogenic potential of Cnm\textsuperscript{−} strains in the rat caries model. Since OMZ175 has never been tested for its ability to cause caries in rats, we included in the study the widely characterized UA159 strain as a reference. Rat pups were infected with either
UA159 (Cnm⁻), OMZ175 (Cnm⁺), or the ∆cnm mutant (OMZ175∆cnm). The average total flora counts recovered from rats infected with OMZ175∆cnm were significantly lower than that found for either UA159 or OMZ175 infections (p<0.001) (Fig. 4A). Similarly, S. mutans carriage was significantly lower in OMZ175∆cnm infections compared to either UA159 or OMZ175 infections (p<0.001) (Fig. 4A). Interestingly, proportions of S. mutans to total flora tended to be higher in OMZ175 infections (60.0 ± 19.8%) compared to those observed for infections by either UA159 or OMZ175∆cnm (43.6 ± 9.6% and 44.4 ± 8.9%, respectively) albeit these differences were not statistically significant (p=0.074).

Scoring of carious lesions on smooth and sulcal surfaces was divided into parameters evaluating either lesion extension (enamel - E), or severity (slight - Ds; moderate - Dm; and extensive - Dx). All three strains produced similar levels of average lesion extension (E) on both smooth and sulcal surfaces (Fig. 4B). However, comparisons of lesion severity (Fig. 4C-E) revealed significant differences resulting from the infecting strains. In most cases, rats infected with OMZ175∆cnm had significantly lower (p<0.05) severity scores compared to both OMZ175 and UA159 on sulcal and smooth surfaces. These results reveal that Cnm plays an important role in the oral colonization and cariogenicity of OMZ175.

Despite an obvious role for Cnm in caries severity by OMZ175 infections, animals infected with UA159 had lesions that tended to be even more severe than those caused by OMZ175. In
an attempt to determine the basis for these differences, UA159, OMZ175 and OMZ175Δcnm were assayed for their ability to withstand (aciduricity) and produce acids (acidogenicity), as well as synthesize extracellular (EPS) and intracellular polysaccharides (IPS) in the presence of sucrose. Collectively, these traits encompass some of the key aspects that denote *S. mutans* cariogenic potential (3). Inactivation of *cnm* in OMZ175 did not significantly alter any of these characteristics compared to the parental strain (Fig. S4). Neither acidogenicity nor aciduricity, revealed significant differences between UA159 and OMZ175 (Fig. S4A and B). However, analysis of biofilms produced by strains grown in the presence of sucrose (Fig. S4C) revealed moderately higher biofilm dry masses produced by UA159 compared to OMZ175 (p<0.08) and significantly higher IPS content (p<0.003). No significant differences in the amounts of soluble and insoluble EPS were observed between UA159 and OMZ175 background strains.

Expression of Cnm in UA159 does not increase cariogenicity. Due to the enhanced binding to collagenous substrates attributed to Cnm expression by UA159 in the *ex vivo* assays, the cariogenic potential of UA159-cnm was evaluated in the rat caries model (Fig. S5). Infections were performed using UA159-cnm and a UA159 variant transformed with the empty pBGE vector, the same vector used to integrate *cnm* into the chromosome of UA159-cnm (21). Although not significant, the total flora and *S. mutans* carriages were slightly lower in the
UA159-\textit{cnm} infected rats compared to those infected with UA159-pBGE (p=0.67). The colonization profiles resulting from infection by either UA159-\textit{cnm} or UA159-pBGE were nearly identical (52.4 ± 13.4\% and 54.9 ± 16.5\%, respectively). In addition, the average lesion extension (E) and severity (Ds, Dm and Dx) was similar between the two strains. Thus, it appears that UA159, a naturally occurring Cnm isolate, employs strategies for tooth adherence, colonization and persistence that outweigh any enhanced function conferred by Cnm expression \textit{in vivo}.

**DISCUSSION**

Bacterial surface proteins that mediate cellular binding to extracellular matrix (ECM) components, known as Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM), are a class of virulence factors that facilitate bacterial infections through adhesion and colonization of host tissues (47, 48). The ability to avidly bind collagenous tissues is an important mechanism utilized by a subset of \textit{S. mutans} expressing CBPs to successfully colonize oral- and extra-oral tissues (1, 22, 24, 26, 27, 49). This study highlights two important factors that can be attributed to Cnm: (i) enhanced invasion to epithelial cell lines relevant to soft tissues of the oral cavity, and (ii) stringent adherence to collagenous oral hard tissues, thereby enhancing the colonization and cariogenic potentials of certain \textit{S. mutans} strains.
Earlier studies have demonstrated that streptococci dominate the intracellular flora of buccal cells in vivo (50) and have also linked expression of Cnm in S. mutans with invasion of HCAEC (26), HUVEC (27) and adherence to hepatocytes (49). These observations prompted our investigation into Cnm-mediated invasion and attachment to oral epithelial cell lines. As observed with HCAEC and HUVEC, we found that expression of Cnm is directly linked to enhanced invasion of HGF-1 and HOK, suggesting that intracellular invasion may serve as a protective reservoir for Cnm\textsuperscript{+} S. mutans. This would facilitate persistence in the oral cavity through mitigating environmental challenges such as host defenses, administered antibiotics, competition with bacterial commensals, and mechanical/chemical removal (51). Moreover, attachment and invasion of epithelial cells, particularly those directly involved in the healing process (e.g. gingival fibroblasts) may further facilitate access of S. mutans into the bloodstream through injury sites.

The presence of collagen on HA surfaces, regardless of coating with saliva, provided the substrate needed for stringent binding by Cnm-expressing strains (OMZ175). Similarly, ex vivo tooth-binding assays demonstrated that Cnm\textsuperscript{+} strains outcompeted their Cnm\textsuperscript{-} variants on adherence to root and dentin sections, but not to the enamel, emphasizing the specificity of Cnm to collagen-rich substrates. The dentin organic matrix is composed of approximately 90% collagen type I, and other surrounding tissues such as the cementum, periodontal ligament.
fibers, and roots are also rich in collagen (17). These tissues are ordinarily shielded from the oral flora by either protective hard surfaces (enamel), or soft tissues (gingiva). However, physical trauma, dentinal-level carious lesions, or gingival recession can expose these various collagenous tissues to the oral flora. Thus, adhesion to and persistence on collagenous tissues, as mediated by Cnm, may further enhance the ability of *S. mutans* to colonize tooth surfaces especially when sucrose availability is low.

*In vivo* caries experiments using rats infected with OMZ175 or its ∆*cnm* counterpart (OMZ175∆*cnm*) strongly supports the hypothesis that Cnm contributes to colonization and persistence of the Cnm*+* native *S. mutans* strain in the oral cavity. As there were no differences between OMZ175∆*cnm* and the parent strain in terms of acid tolerance, acidogenicity, biofilm biomass, and IPS and EPS production, it can be inferred that the decreased caries severity index observed in rats infected with OMZ175∆*cnm* is directly associated with its reduced ability to bind to collagen. Furthermore, Cnm appears to modulate carriage of both *S. mutans* and total biofilm since rats infected with OMZ175∆*cnm* had lower total *S. mutans* and total flora counts compared to the rats infected with the parental strain. Interestingly, animals from the same experiment infected with the Cnm*−* UA159 strain had similar colonization profiles as those infected by OMZ175, but overall, displayed a greater number of severe lesions. Comparisons of biofilm characteristics revealed that UA159 had a significantly higher average IPS accumulation
compared to OMZ175. Heightened production of IPS is considered important to the organism’s long-term persistence and has been linked to a lower bacterial fasting pH, caries active status in human patients and caries development in animal models (52, 53). Additionally, contrary to UA159, OMZ175 lacks the glucan-binding adhesin, GbpA, and expresses low levels of SpaP (54). GbpA has been previously implicated in sucrose-dependent biofilm formation (5) and SpaP is a multi-functional adhesin primarily implicated in mediating adherence to the salivary pellicle (55, 56). This observation is bolstered by the more robust binding by UA159 to HA beads coated with only saliva compared to OMZ175, suggesting that UA159 is a more effective colonizer of intact enamel surfaces. In a study by Nomura et al (22), it was found that collagen binding and HUVEC attachment/invasion were highest in strains expressing only CBPs, followed by strains expressing both CBPs and SpaP, and lowest in strains expressing only SpaP. Taken altogether, it appears that there is a tradeoff between stringent binding to the salivary pellicle or to collagenous surfaces, whereby Cnm\(^{-}\) isolates are able to colonize through optimized adherence via glucan and salivary pellicle-binding, and Cnm\(^{+}\) isolates colonize using collagen as a preferential binding substrate.

Expression of Cnm by UA159 has been previously shown to increase the binding affinity of this strain to collagen and laminin substrates (21). Here, we show that Cnm confers advantageous binding to UA159 on dentin and root surfaces ex vivo. However, these traits
neither increased colonization nor cariogenicity of UA159 in the rat caries model. One reason for this might be that the in vivo model employed here utilizes a high-sucrose diet to induce and accelerate caries development. While effective at evaluating the contribution of glucan-mediated colonization to *S. mutans* pathogenicity, the high sucrose diet may mask the relevance of sucrose-independent adherence mechanisms, such as collagen binding by CBPs. Despite this possibility, it is important to note that the production of glucans from sucrose is a major virulence factor of *S. mutans*, and it may help explain why Cnm⁺ strains do not predominate in the oral cavity. On the other hand, Cnm⁺ strains may take advantage of previously damaged enamel or exposed roots to further the disease process.

The success of *S. mutans* as an etiological agent of dental caries relies upon a variety of mechanisms the organism employs to adhere to hard surfaces in the oral cavity. More recently, mounting evidence suggests that CBPs expressed by a subset of *S. mutans* strains enhances the potential of this pathogen as a provocateur of infective endocarditis. We propose a possible mechanism by which CBPs enable *S. mutans* to exploit situations of pre-damaged tissues to facilitate effective colonization and subsequent pathogenesis, both within and outside the oral cavity (Fig. 5). The expression of CBPs by *S. mutans* confers targeted adhesion to collagenous tissues and may facilitate invasion of the oral epithelium, a niche in which there is likely less competition with commensals and other *S. mutans* strains. Whereas sucrose-mediated
adherence capitalizes on the development of biofilm on non-shedding hard surfaces, stringent adhesion to collagen by CBPs anchor microbes to exposed dentin and root surfaces. Dental procedures, trauma to the mouth, or poor oral hygiene may facilitate the introduction of CBP-expressing strains into the bloodstream where they disseminate throughout the body, creating states of transient bacteremia. Furthermore, pre-existing damage to the vascular endothelium and other host risk predispositions, combined with sufficient bacterial loads, increases the likelihood for extra-oral infections and disease.

In summary, this study reveals that Cnm is an requisite to the virulence of OMZ175 with regard to dental caries, whereas UA159 employs different mechanisms for colonization and pathogenesis in the oral cavity, further bolstering that *S. mutans* strains have enough genetic variability that allows for different colonization, survival and persistence strategies (57).

Therefore, our study reinforces the importance of collagen binding as an important apparatus for oral establishment utilized by a subset of *S. mutans* strains (10). Contemporary studies on the identification, characterization, and clinical surveillance of *S. mutans* expressing collagen-binding adhesins, have focused on the role of these proteins in *S. mutans* colonization and virulence outside the oral cavity. However, our study reveals that Cnm may also contribute to the success of certain strains of *S. mutans* in colonizing, and thus pathogenesis, within its conventional habitat, the oral cavity.
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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
Table 1 - *Streptococcus mutans* strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Experiment§</th>
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<td>(26)</td>
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<td>Cnm⁻</td>
<td>University of Alabama</td>
</tr>
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§ (a) antibiotic protection assays, (b) *in vitro* binding to coated hydroxyapatite beads, (c) *ex vivo* tooth substrate binding competition assay, (d) *in vivo* colonization and caries formation in Sprague-Dawley rats, (e) Western blotting for Cnm, (f) *in vitro* binding to collagen type I, (g) immunofluorescent detection of Cnm.
**FIGURE LEGENDS**

**Figure 1.** Attachment and invasion of human gingival fibroblast (HGF-1) by OMZ175(Cnm+), OMZ175Δcnm (cnm knockout) and UA159 (Cnm-). Antibiotic protection assay to assess *S. mutans* attachment to and invasion of human gingival fibroblasts (A) and human oral keratinocytes (B); median and interquartile range of eight replicates normalized to the average results of OMZ175 infections, asterisks indicate p < 0.05. (C) Transmission electron microscopy of HGF-1 cells co-incubated with OMZ175 for 2 h revealed *S. mutans* attached to HGF-1, enveloped by lamellipodia-like extensions (black arrows), and intracellular within membrane-bound vacuoles (white arrows). (D) Detail of transmission electron micrograph; projections from the surface of OMZ175 were visible (black arrows) and appeared to be in contact with the HGF-1 vesicle wall (white arrows).

**Figure 2.** Binding of *S. mutans* strains to hydroxyapatite beads coated with either saliva, collagen, or saliva over collagen. Averages ± standard deviations (s.d.) of three replicates normalized to OMZ175, asterisks indicate p < 0.05.

**Figure 3.** Ex vivo tooth substrate binding competition assay model. (A) Schematic of tooth sectioning to simulate three distinct substrates for competitive binding assays. (B) Tooth
sections were coated in saliva and co-incubated for 5 min with equal amounts of two strains containing different antibiotic resistance markers. Competition indices were then derived from the ratio of viable cells recovered from the sections compared to those in the inocula. Dotted line indicates equal binding between competing strains. Averages + s.d. presented from at least three independent experiments, asterisks indicate p<0.05.

Figure 4. *In vivo* caries study in Sprague-Dawley rats infected with *S. mutans* strains was carried out for 30 days, twelve animals per infecting strain. (A) Median CFU and interquartile range of total flora and *S. mutans* recovered from the left mandible 30 days post-infection, asterisks indicate p<0.05. (B) Average + s.d. lesion extension and (C-E) severity of lesions on smooth and sulcal tooth surfaces, asterisks indicate p < 0.05.

Figure 5. Proposed mechanism for *S. mutans* infection enhancement by collagen-binding adhesins (CBPs): (A) Dentinal-level lesions or (B) gingival recession expose collagen-rich tissues for stringent adhesion by *S. mutans* expressing CBPs (gray). The capacity to invade the oral epithelium provides an additional colonization reservoir. (C) *In situ* colonization and acidification deepens existing lesions and/or forms subgingival lesions (root caries), exacerbating existing tooth damage and causing new injury sites. (D) Dental procedures,
trauma, and poor oral hygiene enables bloodstream access for *S. mutans* where CBPs act as potential virulence factors for extra-oral infections and pathogenesis.
REFERENCES


