The Collagen-Binding Protein Cnm Is Required for *Streptococcus mutans* Adherence to and Intracellular Invasion of Human Coronary Artery Endothelial Cells

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*Streptococcus mutans* is considered the primary etiologic agent of dental caries, a global health problem that affects 60 to 90% of the population, and a leading causative agent of infective endocarditis. It can be divided into four different serotypes (c, e, f, and k), with serotype c strains being the most common in the oral cavity. In this study, we demonstrate that in addition to OMZ175 and B14, three other strains (NCTC11060, LM7, and OM50E) of the less prevalent serotypes e and f are able to invade primary human coronary artery endothelial cells (HCAEC). Invasive strains were also significantly more virulent than noninvasive strains in the *Galleria mellonella* (greater wax worm) model of systemic disease. Interestingly, the invasive strains carried an additional gene, _cnm_, which was previously shown to bind to collagen and laminin in vitro. Inactivation of _cnm_ rendered the organisms unable to invade HCAEC and attenuated their virulence in _G. mellonella_. Notably, the _cnm_ knockout strains did not adhere to HCAEC as efficiently as the parental strains did, indicating that the loss of the invasion phenotype observed for the mutants was linked to an adhesion defect. Comparisons of the invasive strains and their respective _cnm_ mutants did not support a correlation between biofilm formation and invasion. Thus, _Cnm_ is required for _S. mutans_ invasion of endothelial cells and possibly represents an important virulence factor of _S. mutans_ that may contribute to cardiovascular infections and pathologies.

The oral cavity is colonized by a large number of viridans group streptococci, including primarily soft tissue colonizers, such as *Streptococcus salivarius* and *S. mitis*, and predominantly hard tissue (tooth) colonizers, such as *S. mutans* and *S. gordoni* i. Among tooth colonizers, *S. mutans* is considered the primary etiologic agent of dental caries, an infectious disease that affects 60 to 90% of the population worldwide (12). Strains of *S. mutans* can be grouped into four serotypes (c, e, f, and k) based on the composition and structure of the rhamnose glucose polysaccharide (RGP) associated with the cell wall. Epidemiological studies revealed that serotype c is the most common serotype isolated from dental plaque, being found in nearly 80% of *S. mutans*-positive samples. Serotypes e and f are found in about 20% and 2% of patients, respectively (22, 41, 54). Strains belonging to serotype k are the most infrequent, having been isolated thus far only from subjects from Japan, Thailand, and Finland (29, 42, 44).

In addition to colonizing the teeth in significant numbers, it is not unusual for *S. mutans* to gain access to the bloodstream during dental procedures (16, 21, 26). If a sufficient number of cells enter the circulation, transient bacteremia followed by adhesion to endothelial cells leads to infective endocarditis (IE) (26, 37), particularly in persons with predisposing cardiac conditions. In addition to IE, a significant association between dental infections and the occurrence of coronary atherosclerosis has been demonstrated (36). More specifically, oral streptococci and the periodontal pathogen *Porphyromonas gingivalis* have been associated with atherosclerotic/atheromatous plaques (15, 20, 34, 36, 60). Studies by Nakano and coworkers (38, 44, 45) reported that among bacterial species, *S. mutans* was the most frequently detected in diseased heart valve tissues and atheromatous plaque, suggesting that *S. mutans* may play an important and underestimated role in the onset of cardiovascular disease (CVD) (38). However, detection of bacteria in atheromas has been based on PCR amplification of *S. mutans* DNA, not on isolation of live bacteria. Recently, we demonstrated that two *S. mutans* strains, B14 and OMZ175, belonging to serotypes e and f, respectively, invade and persist in the cytoplasm of human coronary artery endothelial cells (HCAEC), revealing a possible new facet of the pathogenic potential of *S. mutans* and a mechanistic linkage of *S. mutans* to CVD (38).

In some cases, binding to the extracellular matrix (ECM) is the first step in the invasion of host cells (17, 58). The ECM is a macromolecular structure that becomes exposed when tissue integrity is damaged by lesions or traumas (63). Fibronectin, collagen, laminin, and elastin are considered the most common components of ECM (63) and can serve as receptors for bacteria during infection. Some *S. mutans* surface structures, such as the P1 protein (also known as antigen I/II or SpaP), the...
wall-anchored protein A (WapA), the biofilm regulatory protein A (BraA), the autolysin AtlA, the glucosyltransferases (GtfB, GtfC, and GtfD), and the serotype-specific RGP, have been implicated in the pathogenesis of IE by promoting adherence to endothelial tissues and triggering inflammatory responses (17, 55, 61). More recently, a new surface protein with collagen- and laminin-binding activity, Cnm, which has an unique domain structure, has been identified (47, 51, 52). Interestingly, Cnm is found frequently in strains belonging to the uncommon serotype \( \text{cmn} \) (44, 54). Cnm+ strains have been shown to be a major factor that could contribute to systemic infections by the organism. To express the \( \text{cmn} \) gene in trans, the full-length \( \text{cmn} \) gene, including the ribosomal binding site, was amplified by PCR with primers containing BamHI restriction sites and ligated into pMSP5355 (31) which had been digested with BamHI and XbaI. A ligation mixture containing pMSP5355 expressing \( \text{cmn} \) was used to directly transform the \( S. \text{mutans} \) \( \text{cmn} \) knockout strain OMZ175-cnm to generate a complementation strain carrying the gene. Expression of \( \text{cmn} \) from pMSP5355 was induced with 15 ng of nisin ml\(^{-1} \) as described elsewhere (32).

RNA isolation and real-time qRT-PCR. To measure expression levels of \( \text{cmn} \) in strain OMZ175, OMZ175/\( nctc11005 \), and OMZ175/\( nctc11005 \), RNAs were extracted from cells grown to mid-exponential phase (optical density at 600 nm \( [\text{OD}_{600}] = 0.5 \)) in BHI broth as described elsewhere (1). A high-capacity cDNA reverse transcription kit containing random primers (Applied Biosystems, Foster, CA) was used to obtain cDNA from 1 \( \mu \)g each of three independent RNA samples. Quantitative reverse transcriptase PCR (qRT-PCR) was carried out using the \( \text{cmn} \)-specific primers \( \text{cmn} \)-\( f \) and \( \text{cmn} \)-\( r \) (51, 54) and the cDNA, synthesized with \( \text{cmn} \)-\( f \) and \( \text{cmn} \)-\( r \), as template. RNA integrity was assessed by amplification of the 16S rRNA gene by using primers \( \text{cmn} \)-\( f \) and \( \text{cmn} \)-\( r \) (43, 54). The PCR reactions were performed in triplicate for each sample, and the data were expressed as mean \( \pm \) standard deviation. There was no significant difference in the cycle threshold (\( C_{\text{t}} \)) obtained for the \( \text{cmn} \) gene in the different strains (all \( P > 0.05 \)).

Adherence to HCAEC. To test the ability of \( S. \text{mutans} \) strains to adhere to the surfaces of HCAEC, adherent cells were collected by washing the wells three times with PBS, fixed with 2% paraformaldehyde, stained with 0.004% crystal violet, and quantified by a colorimetric assay using a microtiter plate reader. The percentage of adherence was calculated by dividing the number of adherent cells by the total number of cells in the supernatant. The results were expressed as mean \( \pm \) standard deviation.

Data analysis. The results are expressed as mean \( \pm \) standard deviation. The statistical significance of differences between groups was determined by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. A value of \( P < 0.05 \) was considered statistically significant.

TABLE 1. \( S. \text{mutans} \) strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Serotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>UA159</td>
<td>Dental plaque</td>
<td>c</td>
<td>University of Alabama</td>
</tr>
<tr>
<td>OMZ175</td>
<td>Dental plaque</td>
<td>f</td>
<td>B. Guggenheim</td>
</tr>
<tr>
<td>BI4</td>
<td>Dental plaque</td>
<td>e</td>
<td>A. Bleiweis</td>
</tr>
<tr>
<td>MT4653</td>
<td>Dental plaque</td>
<td>e</td>
<td>N. Jakubovics</td>
</tr>
<tr>
<td>13.1</td>
<td>Dental plaque</td>
<td>e</td>
<td>N. Jakubovics</td>
</tr>
<tr>
<td>LML4</td>
<td>Dental plaque</td>
<td>f</td>
<td>N. Jakubovics</td>
</tr>
<tr>
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<td>Dental plaque</td>
<td>p</td>
<td>C. Kaufeld</td>
</tr>
<tr>
<td>OM50E</td>
<td>Dental plaque</td>
<td>p</td>
<td>C. Kaufeld</td>
</tr>
<tr>
<td>OM90E</td>
<td>Dental plaque</td>
<td>f</td>
<td>N. Jakubovics</td>
</tr>
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<td>19</td>
<td>Dental plaque</td>
<td>f</td>
<td>N. Jakubovics</td>
</tr>
<tr>
<td>6139-99</td>
<td>Blood</td>
<td>c</td>
<td>CDC</td>
</tr>
<tr>
<td>1237-00</td>
<td>Blood</td>
<td>c</td>
<td>CDC</td>
</tr>
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<td>2955-00</td>
<td>Blood</td>
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<td>CDC</td>
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<td>190-01</td>
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<td>CDC</td>
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<td>2323-02</td>
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<td>Blood</td>
<td>c</td>
<td>CDC</td>
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<td>OMZ175-cnm</td>
<td>( \text{cmn} ) knockout</td>
<td>f</td>
<td>This study</td>
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<tr>
<td>BI4-cnm</td>
<td>( \text{cmn} ) knockout</td>
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<td>OM50E-cnm</td>
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<td>f</td>
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<tr>
<td>11060-cnm</td>
<td>( \text{cmn} ) knockout</td>
<td>f</td>
<td>This study</td>
</tr>
<tr>
<td>OMZ175-cnm/pMSP5355</td>
<td>Complementation studies</td>
<td>f</td>
<td>This study</td>
</tr>
</tbody>
</table>

- CDC, Centers for Disease Control and Prevention.
assessed in the presence of cytochalasin D (Sigma) as described elsewhere (13), with minor modifications. Briefly, the HCAEC were cultured, seeded, and maintained in the same way as described above. Prior to infection, HCAEC-containing wells were washed three times with Hanks' balanced salt solution (Lonza) and then exposed to EBMD-2 containing 5 μg ml⁻¹ cytochalasin D without antibiotics for 30 min at 37°C in a 5% CO₂ atmosphere. Overnight bacterial cultures were washed twice with phosphate-buffered saline (pH 7.2) and diluted in EBMD-2 containing 5 μg ml⁻¹ cytochalasin D without antibiotics to obtain suspensions containing 1 × 10⁷ CFU ml⁻¹. One milliliter of bacterial suspension was used to infect HCAEC cultures, followed by 30 min of incubation at 37°C in a 5% CO₂ atmosphere. The HCAEC wells were then washed three times with Hanks' balanced salt solution to remove unbound bacteria, followed by HCEMysis with 1 ml of ice-cold sterile water for 20 min. Lysates containing dead HCAEC and intact S. mutans were serially diluted and plated onto BHI agar. All agar plates were incubated for 48 h at 37°C in a 5% CO₂ atmosphere.

Biofilm assay. The capacity of the invasive strains and their respective cnm knockouts to form biofilms in the presence of sucrose or glucose in saliva-coated 96-well microtiter plates was assessed. Briefly, the wells were coated for 1 h at 37°C with 100 μl of human saliva (49). Strains grown in BHI medium to an OD600 of 0.5 were diluted 1:100 in low-molecular-weight agar plates were used to confirm initial inocula. Groups injected with saline solution or with heat-inactivated S. mutans OMZ175 (30 min at 75°C) were used as controls for high and low invasive rates in our experiments. Of the strains isolated from dental plaque, two serotype e strains, LM7 and OM50E, were found to be invasive (Fig. 1A), with 4.8 × 10⁷ and 6.1 × 10⁸ CFU, respectively, recovered from the cytoplasm of HCAEC. Among the blood isolates, serotype f strain NCTC11060 was found to be highly invasive, with 1.6 × 10⁹ CFU recovered from the cytoplasm of HCAEC (Fig. 1B). Notably, while most of the tested strains belonged to the more prevalent serotype c, no serotype c strains were capable of invading HCAEC. On the other hand, 3 of the 5 serotype e and 2 of the 3 serotype f strains tested were capable of invading HCAEC. Note that strains belonging to serotype f were consistently more invasive than serotype e strains, with approximately 5-fold more cells able to reach the cytoplasm of HCAEC.

Invasive strains are more virulent than noninvasive strains in the greater wax worm model. We asked whether G. mellonella could be used to identify differences in the virulence potential of invasive and noninvasive strains. In fact, with the exception of the serotype e strain B14, we found significantly higher mortality rates (P < 0.01) for the groups of worms infected with invasive strains (70 to 100% mortality) within the first 48 h. In contrast, only 10% of the larvae infected with noninvasive serotype c strain UA159 died over the same period (Fig. 2). Six additional noninvasive strains, belonging to sero-
types c (52-03, 2955-00, 190-01, and GS-5), e (2323-02), and f (strain 19), were tested in this model, and all behaved similarly to UA159 (data not shown).

Invasive strains carry the cnm gene. Recently, it was demonstrated that 10 to 20% of S. mutans strains isolated in Asia and Europe carry the cnm gene, which encodes a collagen- and laminin-binding protein (43, 47). Notably, this gene is associated predominantly with serotypes f and k and is rarely found in serotype c and serotype e strains (43, 47, 51). Because there was a strong correlation between non-serotype c strains and HCAEC invasion, we hypothesized that cnm plays a role in cellular invasion by S. mutans. Indeed, we found that of the 33 S. mutans strains tested in the present study and a previous study (2), only the 5 invasive strains harbored a copy of the cnm gene (Fig. 3A).

All invasive strains express cnm. To verify cnm expression levels, the mRNA levels of cnm in exponential-phase cultures of all five invasive strains were measured by qRT-PCR. Strain OMZ175 displayed the highest expression level of cnm \((1.2 \times 10^7\) copies) among all strains. Strain NCTC11060 showed the lowest expression level of cnm \((1.7 \times 10^6\) copies), followed by B14 \((3.3 \times 10^6\) copies), OM50E \((4.6 \times 10^6\) copies), and LM7 \((5.3 \times 10^6\) copies). These results confirmed the expression of cnm in all invasive strains but failed to correlate levels of invasion with higher expression levels of cnm mRNA.

cnm knockout strains have a diminished capacity to adhere to and invade HCAEC. Based on the published sequence of cnm (51), we used a PCR-ligation mutagenesis approach (30) to inactivate the cnm gene in strain OMZ175, using a nonpolar marker. In comparison to the parental strain OMZ175, the cnm mutant strain, designated OMZ175-cnm, did not display any growth defect under standard laboratory growth conditions (data not shown). First, HCAEC were infected separately with UA159 (noninvasive), OMZ175 (invasive), and OMZ175-cnm. The results clearly revealed that inactivation of cnm completely abolished the ability of OMZ175 to invade HCAEC. Based on this finding, we inactivated cnm in the 4 additional invasive strains and observed that, in all cases, a functional cnm gene was required for invasion of HCAEC (Fig. 3B). Adherence assays revealed that both invasive and noninvasive strains could adhere to the surfaces of HCAEC (Fig. 4). However, all invasive strains were recovered in significantly \((P < 0.005)\) larger numbers than the noninvasive strains UA159, 19, and 1237-00. In addition, inactivation of cnm in all of the invasive strains rendered a significant \((P < 0.005)\) 10-fold decrease in the adherence rate (Fig. 4). These results strongly support the hypothesis that Cnm plays an essential role in the invasion process by enhancing bacterial adherence to HCAEC.

cnm knockout strains are less virulent than the parental strains. We tested the cnm mutant strains for the ability to kill...
lateral of \textit{G. mellonella}. The virulence of the \textit{cnm} knockout strain in the OMZ175 background was dramatically attenuated, with killing rates that were identical to those found for noninvasive strains (Fig. 5). An identical pattern was observed for the other four \textit{cnm} knockout strains (data not shown). This result adds further support for an association between intracellular invasion and systemic virulence in \textit{S. mutans} and corroborates the usefulness of the \textit{G. mellonella} infection model to assess bacterial pathogenesis.

The role of Cnm in biofilm formation is strain dependent. The capacity of the invasive strains and their respective \textit{cnm} knockouts (designated B14-cnm, LM7-cnm, OM50E-cnm, 11060-cnm, and OMZ175-cnm) to form biofilms in saliva-coated microtiter plates was assessed. Differences among strains were clear, with LM7 displaying an increased capacity to form biofilms compared to the other strains, regardless of the sugar source (Fig. 6). On the other hand, strains B14 and NCTC11060 showed a diminished capacity to form biofilms, particularly in the presence of glucose (Fig. 6). In sucrose, there was a statistically significant reduction in the amount of biofilm formed by OMZ175-cnm compared to the amount formed by the parent strain OMZ175 ($P = 0.008$) (Fig. 6A). Conversely, biofilm formation in the presence of glucose was significantly enhanced in the OMZ175-cnm and LM7-cnm mutants ($P \leq 0.0035$) compared to the parent strains (Fig. 6B). Overall, the inactivation of \textit{cnm} in the five invasive strains rendered variable biofilm phenotypes among the different strains. Therefore, the role of \textit{cnm} in biofilm formation appears to be strain specific.

Expression of \textit{cnm} in trans in OMZ175-cnm partially restores the capacity to invade HCAEC and the capacity for virulence. The capacity of OMZ175, OMZ175-cnm, and OMZ175-cnm harboring \textit{pcnm} (OMZ175-cnm/\textit{pcnm}; complemented strain) to invade HCAEC was assessed. Although the levels of invasion were not completely restored to wild-type levels, the complemented \textit{cnm} mutant strain displayed an invasive phenotype in the presence of nisin (Fig. 7A). Notably, uninduced OMZ175-cnm/\textit{pcnm} displayed much lower invasion levels than those under nisin-induced conditions (Fig. 7A), suggesting that optimal levels of \textit{cnm} mRNA (or Cnm) could not be achieved in trans.

The virulence of the complemented strain in the \textit{G. mellonella} wax worm model was also assessed. As observed in the invasion assay, the attenuated virulence of the OMZ-cnm strain was partially restored in the complemented strain grown in the presence of nisin (Fig. 7B). The survival rates at 86 h were 10%, 50%, and 90% for OMZ175, OMZ175-cnm/\textit{pcnm}, and OMZ175-cnm, respectively (Fig. 7B).

Cnm does not contribute to growth and survival in human blood. To assess whether Cnm can contribute to survival in blood, the ability of the \textit{cnm} knockout strain in OMZ175 (OMZ175-cnm) and of the invasive (OMZ175, NCTC11060, LM7, B14, and OM50E) and noninvasive (UA159, 19, OM96E, 2955-00, and 2323-02) strains to grow and survive in blood was assessed. Com-

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**FIG. 5.** (A) Killing of \textit{G. mellonella} larvae infected with the noninvasive strain UA159 ( ), the invasive strain OMZ175 ( ), and the \textit{cnm} knockout strain OMZ175-cnm ( ). The experiments were repeated three times, and the results are representative of a typical experiment. (B) Wax worms infected with UA159, OMZ175, and OMZ175-cnm in a typical experiment at 24 h postinjection.

**FIG. 6.** Biofilm assays of cells grown in LMW supplemented with 1% sucrose (A) and 1% glucose (B) on the surfaces of 96-well microtiter plates. Results shown are averages for three separate experiments plus SD. Statistically significant differences between strains were assessed by Student’s $t$ test and are indicated by asterisks.
pared to the parent strain OMZ175, growth and survival of the cnm knockout strain were not affected when cells were cultivated in whole blood, with both strains displaying similar patterns over a 6-day period (data not shown). In addition, comparing invasive strains to noninvasive strains, we did not find a correlation between the invasive phenotype and the ability to grow and survive in blood (data not shown). Finally, there were no obvious differences in survival in blood between strains isolated from dental plaque and those from blood of patients with bacteremia/IE (data not shown).

FIG. 7. Expression of cnm in trans partially restored the invasive phenotype (A) and virulence (B) of OMZ175-cnm.

**DISCUSSION**

Non-serotype c strains of *S. mutans*, comprising serotypes e, f, and k, have been detected at high frequency in specimens from patients who underwent surgery for removal of atheromatous plaque and heart valve replacement (38, 40, 44). It has been speculated that non-serotype c strains were isolated at higher frequency because they were highly persistent in blood (44). However, the results presented herein show no correlation between the different serotypes and survival in blood. Therefore, it is likely that the high recovery of non-serotype c strains from cardiovascular specimens is due to other virulence factors specific to non-serotype c strains. Our observation that, to date, only non-serotype c strains could invade heart endothelial cells suggests that these strains have the potential to persist in the heart tissue by hiding in the intracellular niche, leading to antibiotic treatment failure, chronic inflammation, and increased morbidity. In this study, we identified three new invasive *S. mutans* strains, two belonging to serotype e (LM7 and OM50E) and one belonging to serotype f (NCTC11060), that, in addition to our previously identified serotype e (B14) and f (OMZ175) invasive strains (2), strongly associate invasive behavior with non-serotype c strains. Collectively, our previous and present studies have evaluated the capacity of 33 strains to invade HCAEC and identified a total of 5 invasive strains.

Recently, a number of laboratories (4–6, 8, 9) have demonstrated that the larvae of the greater wax worm *G. mellonella* can be used to model systemic bacterial infections, showing a strong correlation with results obtained for mammals (18, 35, 50). Insects possess a complex, multicomponent innate immune system that kills pathogens by using mechanisms similar to those used by mammals, including the production of enzymes (lysozymes), reactive oxygen species, and antimicrobial peptides (25). In particular, there are significant similarities between the oxidative burst pathways of insect hemocytes and mammalian neutrophils (10). Recently, we demonstrated the usefulness of systemic infection of *G. mellonella* as an adjunct model to study the virulence of *S. mutans* (24). Notably, the invasive strains were more virulent in the *G. mellonella* model than noninvasive strains, establishing for the first time a correlation between specific serotypes and cellular invasion and virulence. In addition, we showed that *cnm*, a gene encoding a collagen- and laminin-binding protein, was present only in invasive strains and that inactivation of *cnm* abolished the capacity of these strains to invade HCAEC and attenuated virulence in *G. mellonella*. It has been suggested that Cnm contributes to caries development and to CVD due to its ability to avidly bind to collagen, a major component of dentin and heart valves (43, 47). Studies are under way to disclose the role of Cnm in *S. mutans* virulence in an animal model of IE.

The distribution of the *cnm* gene in *S. mutans* is on par with the frequency of invasive strains identified in our present study and a previous study (2). While *cnm* is detected in approximately 20% of *S. mutans* populations (40, 47, 51), this gene is overrepresented in the minor serotype f (approximately 80% of strains) (39, 47), suggesting that Cnm-dependent cellular invasion constitutes an important virulence factor of non-serotype c strains. Subsequent adherence experiments confirmed that Cnm acts as an adhesin, as noninvasive and *cnm* knockout strains adhered less efficiently to HCAEC than *cnm*+ strains did. Streptococcal adherence to host cells is mediated by surface proteins and is considered an essential step in the intracellular invasion process (46). Once bacterial adhesins recognize a host receptor and attach to the host surface, host cell signaling cascades can be triggered, leading to various outcomes, such as bacterial internalization, proinflammatory responses, and host cell apoptosis (46). While it is clear that Cnm plays an essential role in the invasion process by enhancing the ability of *S. mutans* cells to adhere to the surfaces of HCAEC, it remains to be determined whether Cnm also participates in the subsequent steps associated with the invasion process.

The ability to bind to surfaces and to form biofilms is considered an important virulence attribute of *S. mutans* (7, 31, 33). In addition, the capacity to adhere to ECM proteins has been suggested to be an important factor in the colonization of the heart valves by oral bacteria (48, 53, 56). Similar to the case for *S. galolyticus* (62), our data revealed that the capacity to
invasion does not seem to be associated with the ability to form biofilms and that Cnm plays a strain-specific role in biofilm formation. In *S. gordonii*, inactivation of glucosyltransferase (gtf), which is responsible for biosynthesis of the extracellular polysaccharide glucan that contributes to the adhesion of streptococci to cultured human umbilical vein endothelial cells (59), led to a significant reduction in the ability of the strain to invade these cells. In *S. mutans*, three glucosyltransferases, GtFB, GtFC, and GtFD, are responsible for the production of the water-insoluble and water-soluble glucans and play a major role in sucrose-dependent biofilm formation (7, 59). The *S. mutans* glucosyltransferases, in particular GtFB and GtFC, contribute to virulence in animal models of caries and endocarditis (55, 64). In addition to the Gtfs, the wall-associated protein A (WapA), which has collagen-binding activity, is thought to participate in the pathogenesis of IE (19). However, inactivation of gtfB, gtfC, and wapA in OMZ175 did not affect the capacity of the mutant strains to invade HCAEC (J. Abranches et al., unpublished data), suggesting that the *S. mutans* invasion process is strongly dependent upon the presence of Cnm.

Among our invasive strains, we observed different invasion efficiency rates, with serotype *f* strains displaying higher invasion rates than serotype *e* strains. Differences in invasion rates have been shown for other oral bacteria, such as *P. gingivalis* and *S. gordonii* (13, 23, 57), as well as for an inhabitant of the gastrointestinal flora, *Streptococcus galactosidicus* subsp. *galactosidicus* (62). Furthermore, certain clinical strains of *S. mutans* display low expression levels of *cmm* mRNA (47). Although some variability in the level of *cmm* mRNA was observed among strains, it was not possible to establish a correlation between invasion rates and the expression levels of Cnm.

In conclusion, we showed that *S. mutans* adhesion to and invasion of HCAEC are intimately linked with the presence of a matrix adhesion-dependent virulence factor, revealing a previously unrecognized mechanism of *S. mutans* pathogenesis. Our current working hypothesis is that the ability to invade HCAEC helps *S. mutans* to evade immune surveillance and antibiotic treatment, thereby increasing the morbidity of IE as well as stimulating chronic inflammatory responses that could contribute to CVD. Furthermore, we propose that the Cnm molecule could serve as a biomarker for screening patients who need to receive preventive treatment prior to dental procedures, as well as being a target for the development of novel therapeutic approaches to treat streptococcal infections.

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