

RESEARCH PAPER

Heterologous expression of *Streptococcus mutans* Cnm in *Lactococcus lactis* promotes intracellular invasion, adhesion to human cardiac tissues and virulence

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ABSTRACT

In *S. mutans*, the expression of the surface glycoprotein Cnm mediates binding to extracellular matrix proteins, endothelial cell invasion and virulence in the *Galleria mellonella* invertebrate model. To further characterize Cnm as a virulence factor, the *cnm* gene from *S. mutans* strain OMZ175 was expressed in the non-pathogenic *Lactococcus lactis* NZ9800 using a nisin-inducible system. Despite the absence of the machinery necessary for Cnm glycosylation, Western blot and immunofluorescence microscopy analyses demonstrated that Cnm was effectively expressed and translocated to the cell wall of *L. lactis*. Similar to *S. mutans*, expression of Cnm in *L. lactis* enabled robust binding to collagen and laminin, invasion of human coronary artery endothelial cells and increased virulence in *G. mellonella*. Using an *ex vivo* human heart tissue colonization model, we showed that Cnm-positive strains of either *S. mutans* or *L. lactis* outcompete their Cnm-negative counterparts for tissue colonization. Finally, Cnm expression facilitated *L. lactis* adhesion and colonization in a rabbit model of infective endocarditis. Collectively, our results provide unequivocal evidence that binding to extracellular matrices mediated by Cnm is an important virulence attribute of *S. mutans* and confirm the usefulness of the *L. lactis* heterologous system for further characterization of bacterial virulence factors.

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Introduction

The association of *Streptococcus mutans* with dental caries, the most prevalent and costly infectious disease worldwide, is well established.^{1,2} The capacity of *S. mutans* to form biofilms in the presence of sucrose, to produce organic acids upon fermentation of dietary carbohydrates, and to tolerate large fluctuations in pH are considered the major cariogenic traits of this bacterium.^{3,4} However, the medical implications of *S. mutans* are not limited to the oral cavity as this organism can cause extra-oral infections and may play an important and underestimated role in the onset of cardiovascular diseases.^{5–9} This facet of *S. mutans* pathogenesis is likely associated with its ability to interact with extracellular matrix (ECM) components such as collagen, which enables colonization of different host tissues.^{10,11}

Infective endocarditis (IE) is a life-threatening bacterial infection of the endocardium with approximately 45,000 occurrences per year in the US¹² and mortality

rates around 15–20%.^{9,13,14} Along with staphylococci and enterococci, viridans streptococci (VS) are a major bacterial group associated with IE,¹² and *S. mutans* is estimated to account for ~20% of all VS-associated IE cases.^{9,15} In addition, *S. mutans* DNA has been detected at a high frequency in cardiovascular tissues from patients that underwent valve replacement surgery and atherosclerotic plaque removal.^{16,17} Yet, the mechanisms employed by *S. mutans* to colonize and persist in human heart tissues are still poorly understood.

The *S. mutans* surface protein Cnm is a collagen- and laminin-binding glycoprotein shown to mediate adhesion to collagen and laminin, invasion of endothelial cells, and virulence in an invertebrate model.^{18–20} Epidemiological studies have shown that *cnm* is present in approximately 15% of *S. mutans* isolates, being found at higher frequency among serotype *e*, *f* and *k* strains but rarely present in serotype *c* strains.^{18,21–23} Interestingly, isolates of *S. mutans* that belong to serotypes *e*, *f* and *k*

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are rare in the oral cavity but commonly detected in patients with IE, atherosclerosis and other systemic infections.^{1,22} Moreover, *in vivo* and cross-sectional studies suggest an association between *S. mutans* serotypes *e*, *f* and *k* expressing Cnm and extra-oral pathologies such as hemorrhagic stroke, cerebral microbleeds and non-alcoholic steatohepatitis,^{5,6,24} indicating that Cnm may contribute to the pathogenic potential of *S. mutans* during systemic infections.

The Gram-positive bacterium *Lactococcus lactis* is a non-pathogenic organism used in the dairy industry for the production of fermented milk products.²⁵ In addition, *L. lactis* is used for the production of heterologous proteins,²⁶ and in live mucosal vaccines.²⁷ The presence of secretory and protein anchoring enzymes (e.g. SecA and SrtA) combined with the low number of genes encoding secreted and surface-anchored proteins makes *L. lactis* a desirable host for the characterization of surface virulence factors of pathogenic Gram-positive bacteria.^{26,28-30} For example, systemic or mucosal delivery of a recombinant *L. lactis* strain expressing the *Streptococcus agalactiae* pilus 1 genes was shown to protect mice from subsequent challenges with *S. agalactiae*.³¹ Other bacterial proteins successfully expressed in *L. lactis* include *Staphylococcus aureus* clumping factor A (ClfA) and fibronectin-binding protein A (FnBPA),³⁰ *Staphylococcus epidermidis* SdrF,³² and *Listeria monocytogenes* LnlA.³³ In each of these studies, heterologous expression of these foreign factors was sufficient to increase the pathogenic potential of this otherwise harmless microorganism. Here, the contribution of Cnm to bacterial virulence was further investigated using the *L. lactis* heterologous expression system. Our results provide unequivocal evidence that Cnm is a major virulence factor of *S. mutans* that contributes to systemic infections.

Results

Optimization of Cnm expression in *L. lactis*

After successful electroporation of pMSP3535::*cnm* into *L. lactis* NZ9800, the concentration of nisin that conferred optimal expression of Cnm without causing growth inhibitory effects was determined. The growth of *L. lactis* was only noticeably affected at 25 ng ml⁻¹ nisin or higher concentrations (data not shown). Western blot analysis showed a concentration-dependent induction of Cnm from pMSP3535::*cnm* by nisin with strong induction observed at 10 ng ml⁻¹ and above (Fig. 1A). Non-induced cultures of *L. lactis* pMSP3535::*cnm* showed basal expression of Cnm, which is in line with previous reports demonstrating that the pMSP3535 system is leaky.^{34,39} Immunofluorescence labeling of intact cells confirmed

expression and surface localization of Cnm (Fig. 1B). Based on these results, we chose 10 ng ml⁻¹ nisin for all subsequent experiments as it yielded robust protein production without interfering with cell growth.

As previously determined in *S. mutans*, *L. lactis* Cnm migrates as a higher molecular weight protein on SDS-PAGE (~90 kDa) compared to its predicted size of 54 kDa (Fig. 2). This version of Cnm corresponds to the unglycosylated variant, which in *S. mutans* displays increased susceptibility to proteinase K degradation.¹⁹ Similar to unglycosylated Cnm from *S. mutans*, Cnm expressed by *L. lactis* was rapidly and completely degraded upon proteinase K treatment (Fig. 2). Nonetheless, unglycosylated Cnm in *S. mutans* retains its functional capacities,¹⁹ thus we proceeded with the characterization of Cnm-mediated phenotypes in *L. lactis*.

Cnm⁺ *L. lactis* binds to ECM and invades HCAEC

The ability of the recombinant *L. lactis* Cnm⁺ strain to bind to collagen and laminin *in vitro* was tested. When compared to the parent strain NZ9800 hosting the empty vector, expression of Cnm from pMSP3535::*cnm* led to robust binding to both collagen and laminin (Fig. 3A and B, respectively). In accordance with our expression analysis (Fig. 1), uninduced pMSP3535::*cnm* produced low levels of Cnm and therefore enabled the recombinant *L. lactis* strain to bind to both ECM proteins, albeit at lower levels than nisin-induced cells ($P < 0.05$). To further demonstrate that the ability to bind to collagen is mediated by Cnm, we performed the collagen-binding assay in the presence of anti-rCnmA antibody. As expected, co-incubation of *L. lactis* cells expressing Cnm with anti-rCnmA antibodies led to significant inhibition of collagen binding activity compared to *L. lactis* incubated with pre-immune serum (Fig. 3C). In agreement with increased collagen and laminin binding activities observed for nisin-induced cells, expression of Cnm enabled *L. lactis* to invade HCAEC (Fig. 3D). While ECM binding was observed in uninduced cultures, HCAEC invasion levels were negligible in the absence of nisin ($P > 0.05$).

Expression of Cnm mediates adherence to aortic valve tissues *ex vivo*

To determine if Cnm bestows an enhanced heart tissue binding capacity to either *S. mutans* or *L. lactis* *ex vivo*, competition binding assays were performed using freshly extirpated human aortic valve tissues. In agreement with the ECM-binding *in vitro* data, the *S. mutans* OMZ175 and nisin-induced Cnm⁺ *L. lactis* strains outcompeted their Cnm⁻ counterparts by approximately 10 and 100-fold, respectively (Fig. 4). In these experiments, the

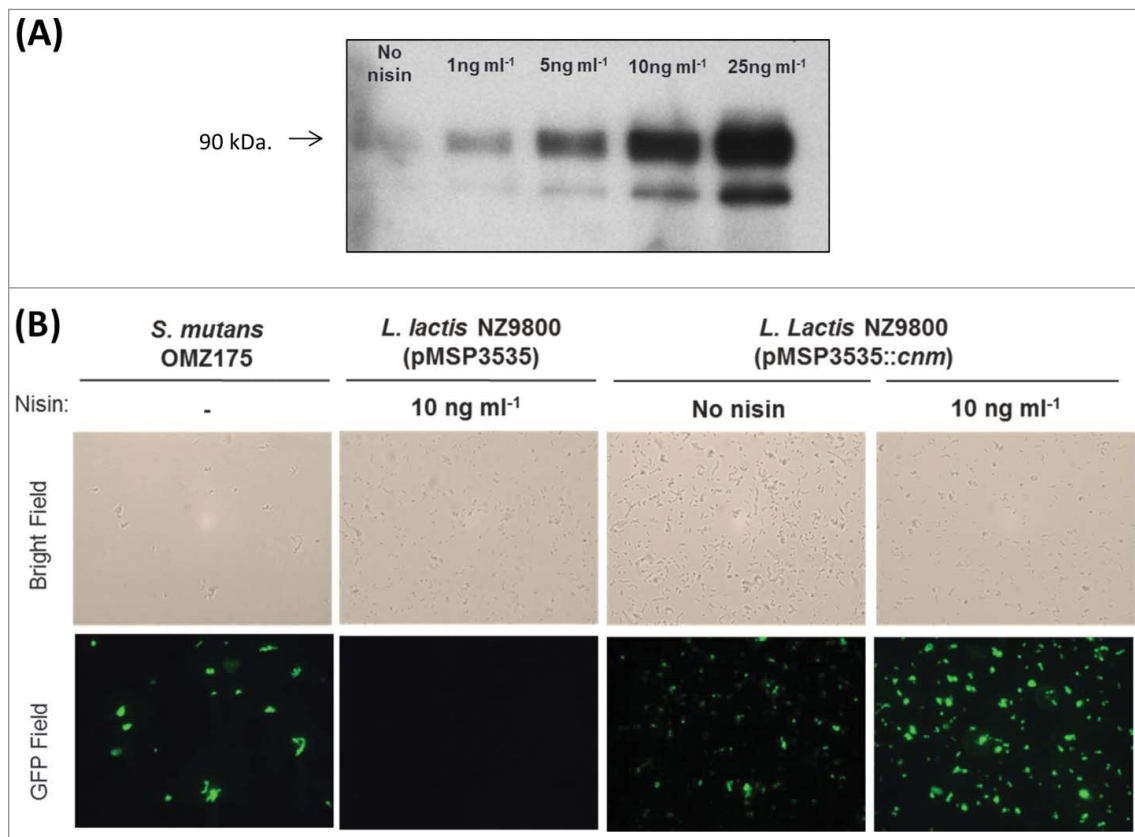


Figure 1. Expression of Cnm from pMSP3535::cnm in recombinant *L. lactis*. (A) Western blot analysis of protein lysates was performed with a polyclonal antibody specific to Cnm. A nisin concentration-dependent induction of Cnm was observed from pMSP3535::cnm, and the uninduced culture showed basal protein expression. (B) Immunofluorescence labeling analysis revealing Cnm expression on the surface of *L. lactis*.

recovered CFUs were on average an order of magnitude higher for *S. mutans* and *L. lactis* Cnm⁺ strains (1.7×10^5 and 3.9×10^5 CFU ml⁻¹, respectively) compared to Cnm⁻ strains (1.7×10^4 and 2.7×10^4 CFU ml⁻¹, respectively). Scanning electron microscopy (SEM) of tissues infected for 90 min with monocultures of *L. lactis* (Fig. 5) or *S. mutans* (data not shown) confirmed that only Cnm⁺ strains could effectively bind to the collagenous fibrils of damaged valve tissues (Fig. 5A). Moreover, while we cannot exclude the possibility that ECM

components from heart valves became exposed during sample manipulation, SEM analysis revealed that Cnm⁺ strains could also adhere to tissue areas where no apparent damage can be observed (Fig. 5B).

Cnm contributes to virulence in galleria mellonella

The ability of the *L. lactis* strains to kill the larvae of *G. mellonella*, a model of systemic infection, was assessed. As shown in Figure 6, the mortality rates of *G. mellonella* were

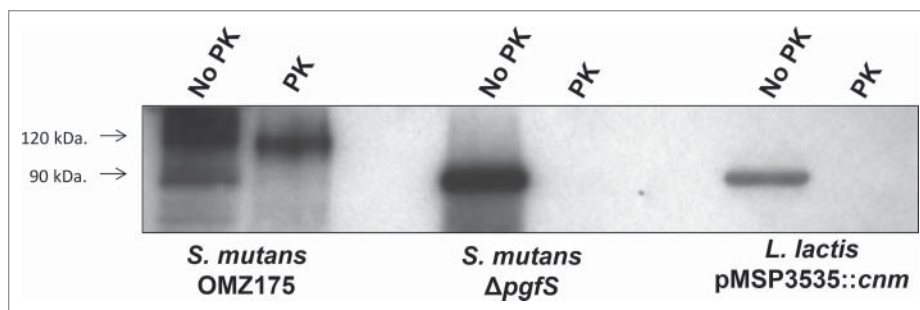


Figure 2. *L. lactis* expresses unglycosylated, proteinase K susceptible Cnm. Whole cells from *S. mutans* OMZ175, ΔpgfS and nisin-induced *L. lactis* pMSP3535::cnm were treated with 30 μg ml⁻¹ of proteinase K for 30 min. After treatment, whole-cell lysates were prepared and Cnm degradation was monitored by protein gel blot using anti-rCnmA.

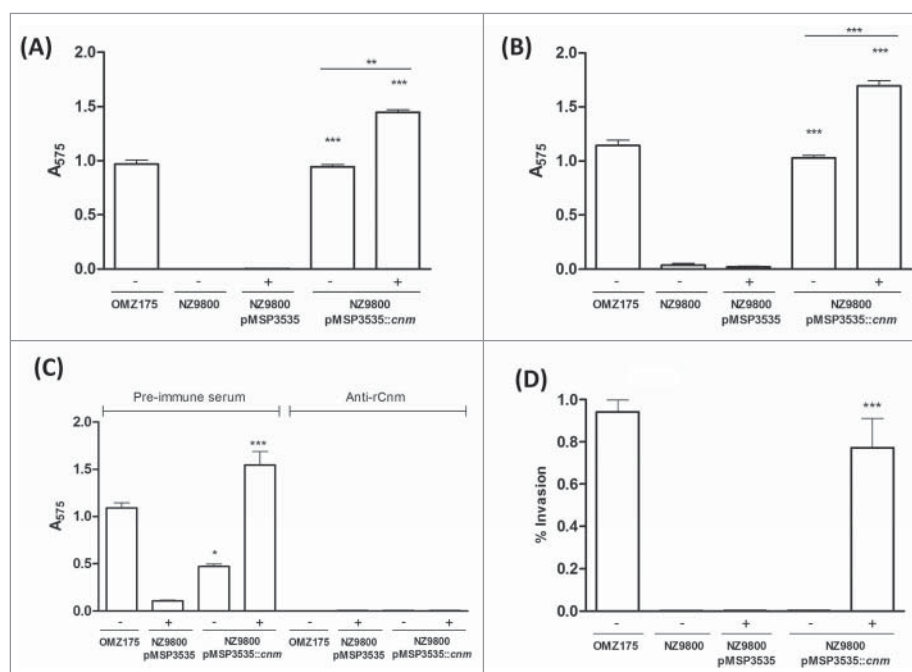


Figure 3. Cnm promotes binding to ECM proteins and invasion of endothelial cells. (A and B) Binding of bacterial cells to extracellular matrix proteins: (A) collagen type I and (B) laminin. (C) Inhibition of binding to collagen type I by anti-rCnmA antibody (1:20). (D) HCAEC invasion assay showing the normalized percentage of invasion for each strain after 3 h of co-incubation (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$, ANOVA followed by Tukey's post-test). The *S. mutans* OMZ175 (Cnm⁺) strain was used as a positive control in all assays. The symbols “+” and “-” in the graphs denote induction or no induction with nisin, respectively.

significantly higher in larvae infected with the Cnm⁺ strain compared to those infected with the wild-type strain harboring the empty vector control ($P < 0.05$). Remarkably when considering the extremely low virulence potential of *L. lactis*, the recombinant Cnm⁺ strain showed a similar

pattern of killing when compared to the Cnm⁺ *S. mutans* OMZ175 strain.

Cnm contributes to endocardium adherence in vivo

A rabbit model of IE³⁸ was employed to examine the effect of Cnm expression on *L. lactis* adherence to endocardium and vegetations. In one experiment, a mixed inoculum of NZ9800 (pCIE) and NZ9800 (pMSP3535::cnm) was injected into rabbits intravenously 1 h after surgery. Two hours later, the rabbits were euthanized and the hearts removed. This time was chosen so as to allow enough time for clearance of bacteria from the bloodstream,⁴⁰ but not so long as to have excessive loss of Cnm due to growth without nisin. Catheters were clamped into place during necropsy to preserve their positions. At this early stage, vegetations were not visible, and the entire endocardial surface in apparent contact with the catheter was excised and homogenized. Following sonication, both the mixed inoculum and the homogenates were plated on selective media to determine strain ratios. In the second experiment, animals were inoculated 2 d after surgery. Vegetations, which were visible in all animals, were removed and homogenized. Because vegetations are composed primarily of platelets and fibrin rather than collagen,⁴¹ the endocardium beneath each vegetation was also collected, homogenized, and plated separately. As shown

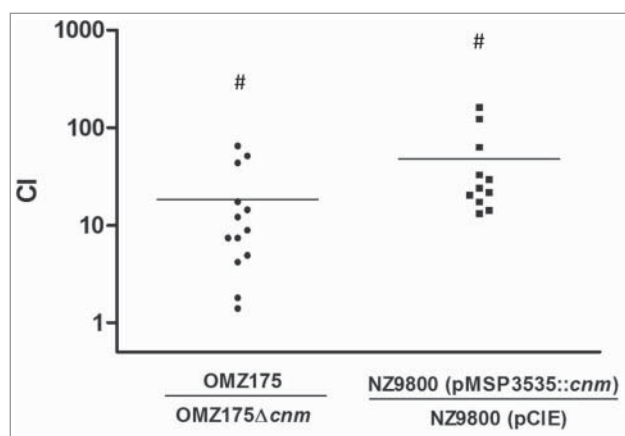


Figure 4. Expression of Cnm mediates bacterial adherence to aortic valve sections. A competition assay was developed to test the contribution of Cnm toward bacterial adherence to human aortic valves *ex vivo*. A 1:1 mixed infection of the valve sections was allowed for 90 min using Cnm⁺ and Cnm⁻ strains. The competitive index (CI) was calculated based on the initial inocula, where a value of “=1” represents no difference and “>1” represents preferred binding by the numerator strain. # $P < 0.05$

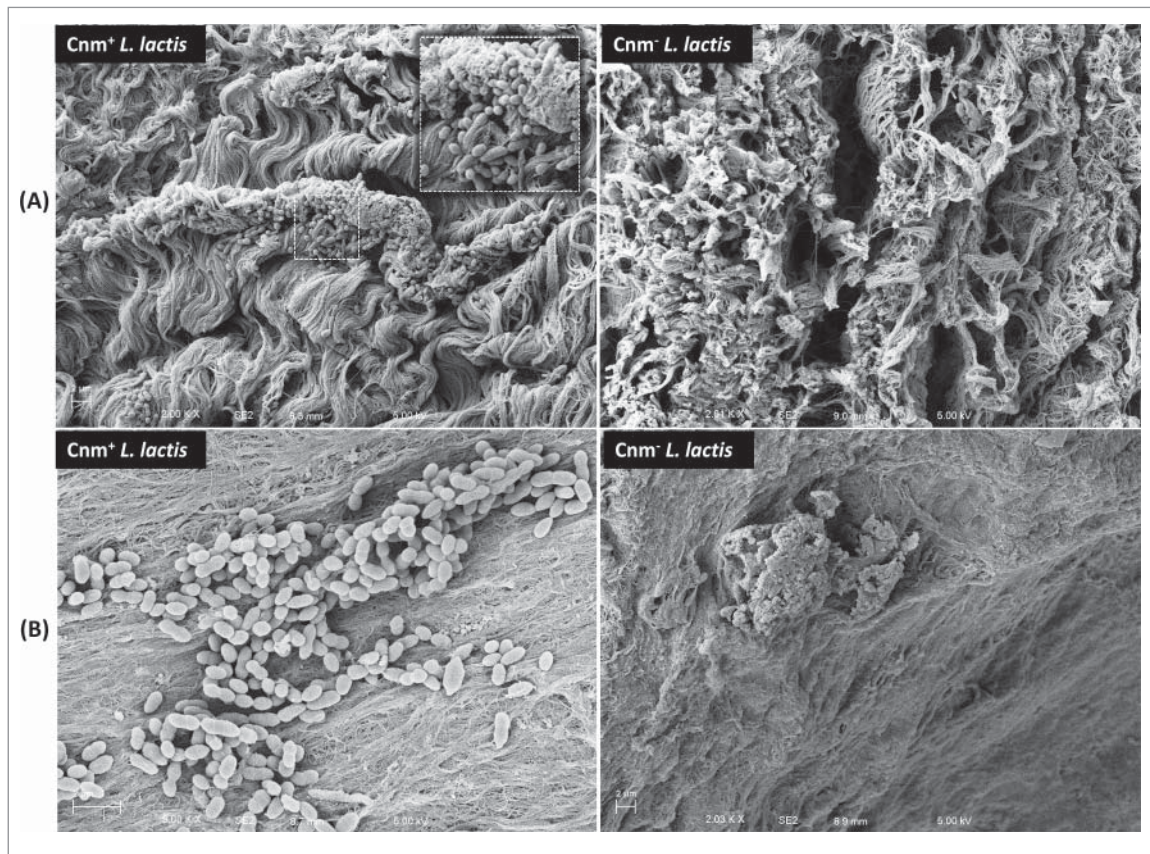


Figure 5. Cnm mediates binding to the collagenous fibrils of damaged and apparently undamaged infected human valve sections. SEM analysis of valve sections incubated with bacterial monocultures showed Cnm⁺ *L. lactis* binding to bundles of collagenous fibrils on the rough edges of the valves whereas no Cnm⁻ *L. lactis* were found attached to this area (A). Cnm⁺ *L. lactis* could also adhere to tissue areas where no apparent damage was observed in contrast with their Cnm⁻ counterparts (B).

in Figure 7, the CI value of the cells harvested from the endocardium were similar in animals inoculated 1 hour or 2 d after catheterization (geometric mean = 1.68 and 1.66, respectively), indicating a ~67% increase in infectivity of the Cnm-expressing strain relative to the control

strain. These values were significantly greater from 1.0 — the value indicating no change in infectivity — whether the 2 experiments were analyzed separately or together. In contrast, there was no significant difference in the CI value for bacteria recovered from vegetations.

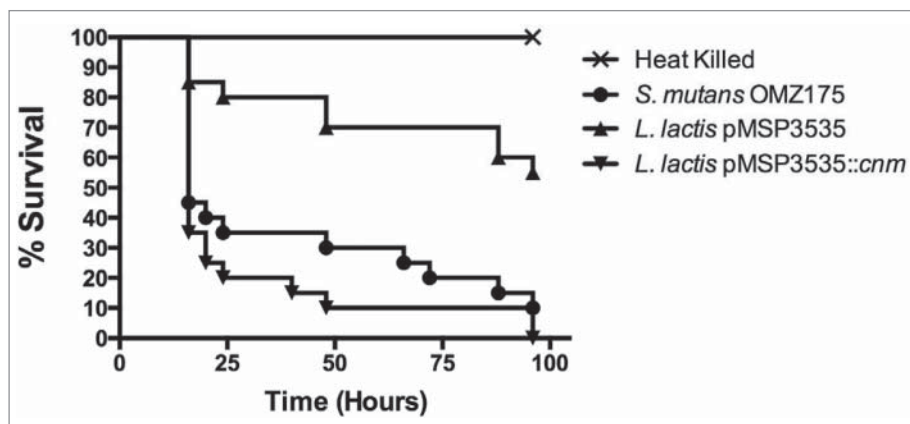


Figure 6. Cnm contributes to virulence in the *G. mellonella* systemic infection model. The Kaplan-Meier curves indicate the percent survival at selected intervals of larvae infected with *S. mutans* OMZ175 and *L. lactis* strains for 96 h. The mortality rates of *G. mellonella* were significantly higher in larvae infected with Cnm⁺ *L. lactis* compared to those infected with the wild-type NZ9800 harboring the empty vector control (pMSP3535) ($P < 0.05$).

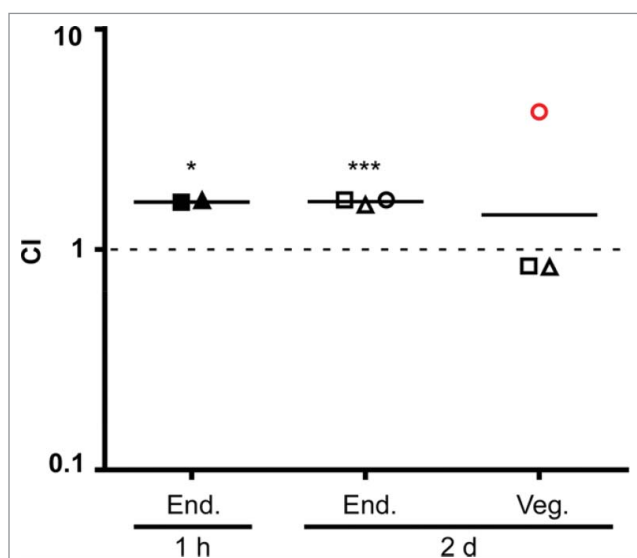


Figure 7. Cnm contributes to bacterial endocardium adherence *in vivo*. Rabbits (n=3) were co-inoculated with Cnm-expressing and control *L. lactis* strains 1 hour or 2 days after insertion of an intracardiac catheter as indicated and then sacrificed 2 hours later. Bacteria were recovered from endocardial surfaces (End.), and in the 2-day samples, from adjacent vegetations (Veg.). Cl values (for *L. lactis* Cnm⁺ over *L. lactis* Cnm⁻) are indicated for each sample, with geometrical mean Cls indicated by a horizontal line. Means significantly different from 1.0 (dashed line) are indicated: **P* < 0.05; ****P* < 0.001. Like symbols in the 2-day experiment indicate samples recovered from the same animal. Red circle, sample from which one of the 2 strains was not recovered (*L. lactis* Cnm⁻); the limit of detection was used for the calculation.

Discussion

The presence of Cnm has been correlated with an increased ability of *S. mutans* to invade and persist intracellularly, and to cause disease.^{6,18,20,21,42,43} In the present study, by expressing Cnm in the non-pathogenic *L. lactis* host we provided unequivocal evidence that Cnm contributes to bacterial virulence.

Various properties of *L. lactis* have justified its attractiveness for heterologous expression of surface proteins. For instance, the fermentative metabolism of *L. lactis* is relatively simple and well known, the genomes of various strains are available and known to encode a very low number of genes encoding secreted and surface-anchored proteins.^{25,44,45} Here, we showed that Cnm expression increased the ability of *L. lactis* to bind to type I collagen and laminin *in vitro*, adhere to aortic valve tissues *ex vivo*, and colonize endocardial surfaces *in vivo*. Of note, type I collagen is the most abundant ECM component in human^{46,47} and rabbit⁴⁸ aortic valves. Although *ex vivo* experiments cannot reproduce the immunologic responses and constant

shear force of blood flow, our findings highlighted the contribution of Cnm in binding of organisms to collagen-rich tissues in both humans and rabbits. Also, while we observed higher levels of ECM binding and virulence *G. mellonella* by Cnm⁺*L. lactis* compared to *S. mutans* OMZ175, during this study we restrained ourselves from making direct comparisons as these species possess fundamental differences that may affect Cnm-dependent phenotypes (e.g., Cnm expression levels, additional surface proteins, growth conditions).

Recently, our group reported that Cnm is a glycoprotein and identified the glycosylation machinery (Pgf) responsible for its post-translational modification. In SDS-PAGE, the native Cnm migrates at approximately 120 kDa whereas the variant from the deletion of the *pgfS* gene was found to migrate at ~90 kDa.¹⁹ When analyzed by Western blot, the Cnm variant produced by *L. lactis* was found to migrate at ~90 kDa, the same size as the unglycosylated version of Cnm produced in *S. mutans*.¹⁹ Moreover, like the unglycosylated Cnm produced by the Δ *pgfS* strain in *S. mutans*, the Cnm expressed by *L. lactis* was highly susceptible to proteinase K degradation.¹⁹ These findings are supported by *in silico* analysis that indicated the absence of Pgf homologs in the available genomes of *L. lactis*. Most importantly, the lack of Cnm glycosylation may explain the difference in adhesion between endocardial tissue *in vivo* and ECM-binding *in vitro* or *ex vivo*. Whether this difference is due to the considerable shear forces and host proteases present in the *in vivo* model, or to some other factor(s) remains to be determined. However, we hypothesize that *L. lactis* expressing glycosylated Cnm will show further increased levels of binding *in vitro*, *ex vivo* and, possibly, *in vivo*. Expression of Cnm had no effect on colonization of preexisting vegetations, which is not surprising considering these vegetations are composed primarily of platelets and fibrin.⁴¹ Nevertheless, adhesins for collagen and other matrix proteins have been identified previously as virulence factors for IE.^{29,49,50} As with these other adhesins, Cnm may allow adherence to damaged endocardium prior to vegetation formation, thereby providing another route for infection in IE.⁵¹ The increased adherence to endocardium observed is also consistent with previous reports of association of Cbm-expressing strains, with both IE and non-IE cardiovascular diseases.^{7,52}

Collectively, our results clearly show that expression of Cnm alone confers virulence to an otherwise non-pathogenic bacterium, and promotes bacterial adherence to cardiac tissue *in vivo* and *ex vivo*. Thus, Cnm could be considered a target for the development of antimicrobial strategies to mitigate virulence of invasive *S. mutans* strains.

Material and methods

Bacterial strains and growth conditions

All strains used in this study are listed in Table 1. The *L. lactis* strains were routinely cultured aerobically at 30°C in M17 broth (Difco) supplemented with 0.5% (w/v) glucose (GM17). When necessary, 10 µg ml⁻¹ erythromycin or 15 µg ml⁻¹ chloramphenicol (Sigma-Aldrich) was added to the growth media (GM17-Erm or GM17-Chlor, respectively). The *S. mutans* strains were routinely cultured in brain heart infusion (BHI) medium at 37°C in a 5% CO₂ atmosphere.

Expression of Cnm in *L. lactis*

The nisin-inducible plasmid pMSP3535³⁴ harboring the *cnm* gene (pMSP3535::*cnm*) from *S. mutans* OMZ175,²¹ was used to express Cnm in *L. lactis*. The pMSP3535::*cnm* plasmid as well as the empty pMSP3535 vector³⁴ and the pCIE plasmids (Dunny *et al.*, unpublished) were electroporated into *L. lactis* NZ9800 as detailed elsewhere³⁵ with some modifications. The pCIE plasmid, conferring resistance to chloramphenicol, was generated from shuttle plasmid pCI13340³⁶ and introduced into *L. lactis* for selection purposes in competition assays. Briefly, cells were grown overnight at 30°C in M17 broth supplemented with 0.5% glucose (GM-17) and sub-cultured (1:100) in fresh GM-17 containing 1% glycine and 0.5 M sucrose (GM17S). The culture was grown to OD₆₀₀ of 0.2, centrifuged at 5,000 rpm for 10 min at 4°C and washed 3 times with cold electroporation buffer (EB) (0.5 M sucrose, 1 mM MgCl₂, 7 mM K₂HPO₄-KH₂PO₄ [pH 7.4]). Electrocompetent cells were resuspended in EB, quickly frozen on dry ice/ethanol bath and stored at -80°C until use. A micro-pulser electroporation apparatus (Bio-Rad Laboratories) (0.1cm cuvette, 1.4 kV, 600 Ω, 10 µF) was used to electroporate the desired plasmids into *L. lactis*. After electroporation, cells were incubated in GM17S for 3 h at 30°C and spread on GM17 plates containing the desired antibiotic. Resulting

colonies were screened for the presence of the desired plasmid by PCR. For induction of Cnm, cells were grown overnight in GM17-Erm in the presence of increasing concentrations of nisin (Sigma-Aldrich). The production and cell surface localization of Cnm was confirmed by Western blotting and by immunofluorescence labeling as detailed below.

Western blot (WB) analysis

Overnight cultures of *S. mutans* OMZ175 and recombinant *L. lactis* strains were used to prepare protein lysates by homogenization with 0.1 mm glass beads using a bead beater (Biospec). Protein lysates were separated on a 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) using standard protocols. The presence of Cnm was detected using rabbit anti-rCnmA polyclonal antibody¹⁹ diluted 1:2000 in 1 x phosphate buffered saline (PBS) with 0.1% Tween 20 pH 7.2 and anti-IgG (goat anti-rabbit) horseradish peroxidase-coupled antibody diluted 1:10000 (Sigma-Aldrich). Membranes were developed using an enhanced chemiluminescent detection kit (GE Life Sciences).

Proteinase K susceptibility assay

The susceptibility of Cnm to protease degradation was assessed as described previously.¹⁹ Briefly, overnight cultures of *S. mutans* OMZ175, Δ*pgfS* and nisin-induced *L. lactis* pMSP3535::*cnm* were pelleted and resuspended in 1x PBS pH 7.4 containing 30 µg ml⁻¹ of proteinase K (Sigma-Aldrich). After 30 min incubation on ice, protease activity was neutralized with the addition of 1x protease inhibitor cocktail (Thermo Scientific). Bacterial cells were then washed once with PBS, and Cnm stability was analyzed by western blotting.

Immunofluorescence labeling of Cnm

Cultures of recombinant *L. lactis* and *S. mutans* OMZ175 were used for cellular localization of Cnm. Cells grown to mid-exponential phase were washed in PBS and incubated in the presence of anti-rCnmA antibody (1:100) followed by anti-rabbit Alexa-488 conjugate antibody (1:100) with 3 washes between each antibody exposure. Cell-antibody complexes were visualized using an Olympus BX41 fluorescent microscope and images were captured with the QCapture Software.

Binding to extracellular matrix (ECM) proteins

Collagen and laminin binding assays were performed as previously described.^{20,21} Briefly, 96-well plates were

Table 1. Strains used in this study.

Strain	Relevant phenotype	Reference
Streptococcus mutans		
OMZ175	Cnm ⁺	Lab collection
OMZ175 (pBGE)	Cnm ⁺ Erm ^r	(21)
Δ <i>cnm</i> Δ <i>pgfS</i>	Cnm ⁻ Kan ^r Cnm ⁺ Kan ^r	(21) (19)
Lactococcus lactis subsp. lactis		
NZ9800	WT, Cnm ⁻	Lab collection
NZ9800 (pMSP3535)	Cnm ⁻ Erm ^r	This study
NZ9800 (pMSP3535:: <i>cnm</i>)	Cnm ⁺ Erm ^r	This study
NZ9800 (pCIE)	Cnm ⁻ , Chlor ^r	This study

Note. WT (wild type); Kan (kanamycin); Erm (erythromycin); Chlor (chloramphenicol).

coated with 40 $\mu\text{g ml}^{-1}$ type I collagen from rat tail (Sigma-Aldrich), or 50 $\mu\text{g ml}^{-1}$ mouse laminin (Becton-Dickinson) for 18 h at 4°C. Individual wells were washed and blocked with 5% bovine serum albumin for 1 h at 37°C. Overnight bacterial cultures were washed twice in PBS and 100 μl aliquots of each bacterial suspension added to the wells, followed by incubation for 3 h at 37°C. Loosely bound bacteria were removed by washing with PBS and adhered cells were stained with 0.05% crystal violet solution and then quantified at 575 nm. Experiments were also performed with bacteria pre-incubated with anti-rCnmA antibody (1:20 dilution in PBS) for 30 min before incubation with collagen. To confirm the requirement of Cnm in collagen binding, experiments were performed as described above but bacteria were pre-incubated with anti-rCnmA or pre-immune serum (1:100) for 30 min before exposure to collagen.¹⁹

Invasion of human coronary artery endothelial cells (HCAEC)

Cultivation of primary HCAECs and invasion assays were performed as previously described.^{18,21} The HCAEC monolayer was incubated with 1 ml of 2% fetal bovine serum (FBS)-endothelial basal medium (EBM-2) containing $1-2 \times 10^7$ CFU ml^{-1} of *S. mutans* OMZ175 or Δcnm , or recombinant *L. lactis* strains for 2 h at a multiplicity of infection of 100:1, followed by 3 h of incubation in 2% FBS-EBM-2 medium containing 300 $\mu\text{g ml}^{-1}$ gentamicin and 50 $\mu\text{g ml}^{-1}$ penicillin G to kill extracellular bacteria. After incubation with antibiotics, HCAEC were lysed with 1 ml of sterile water and the mixture of lysed HCAEC and bacteria serially diluted and plated onto tryptic soy agar (TSA) plates to determine the number of intracellular bacteria. The percentage of invasion for each strain was calculated based on the total colony forming units (CFU) number present in the initial inoculum and the number of intracellular bacteria recovered from HCAEC lysates.

Adherence to human cardiac valve sections ex vivo

The ability of *S. mutans* and *L. lactis* strains to bind to human aortic valve tissues *ex vivo* was assessed by adapting a protocol originally described for porcine heart tissues.³⁷ Human aortic valve tissues were obtained from patients undergoing valve replacement surgery due to calcific stenosis (Institutional Review Board protocol no. RSRB00053016, University of Rochester). Valvular tissues with no signs of calcification, bleeding or inflammation were selected and 3-mm diameter valve sections were obtained using a biopsy punch tool (Acuderm). Valve sections were incubated overnight in 12-well plates

with endothelial cell medium (ECM) supplemented with 5% FBS, 1000 mg L^{-1} hydrocortisone, 12 mg L^{-1} bovine brain extract, 0.1% human recombinant epidermal growth factor (Lonza) plus 300 $\mu\text{g ml}^{-1}$ gentamicin. On the following day, sections were washed twice with Hank's Balanced Salt Solution (HBSS, Corning Inc.) to remove residual antibiotic and then transferred to a new plate. A competition assay was then performed to determine the capacity of Cnm⁺ strains to compete with Cnm⁻ strains. For this, valve sections were infected with approximately 5×10^7 CFU ml^{-1} of a 1:1 suspension of Cnm⁺ and Cnm⁻ strains bearing distinct antibiotic markers for CFU determination of each strain. Plates were incubated for 90 min at 37°C (for *S. mutans*) or 30°C (for *L. lactis*) with gentle rocking. Valve sections were transferred to sterile centrifuge tubes containing 1 ml of HBSS and vigorously agitated 3 times for 5 seconds using a vortex to remove loosely bound bacteria from the tissues. Washed valve sections were homogenized in 500 μl PBS for 2 min using a motorized pestle (Fisher Scientific), serially diluted and plated onto TSA plates with the corresponding antibiotics. The competitive index (CI) was calculated based on the CFUs present in the initial inoculum and the final enumeration of bacteria recovered from the tissue.

Scanning electron microscopy (SEM) analysis of valve sections

To visualize bacterial attachment to heart valve sections, tissue samples were prepared and incubated in the presence of 5×10^7 CFU ml^{-1} *S. mutans* or *L. lactis* monocultures as described in the previous section. Valve samples were washed and fixed with 4% paraformaldehyde, 2.5% glutaraldehyde and 0.1 M sodium cacodylate for 2 d at 4°C, with slow rocking. The samples were analyzed using a Zeiss-Auriga focused ion beam field emission scanning electron microscope (FIB-FE-SEM) and the images captured using the attached Gatan Erlangshen digital camera at the University of Rochester Medical Center Electron Microscopy Core Facility.

Galleria mellonella infection

The systemic infection of *G. mellonella* has been previously detailed elsewhere.^{19,21} Briefly, 5 μl aliquots containing $1-1.5 \times 10^6$ CFU of *S. mutans* OMZ175 (positive control), or recombinant *L. lactis* were injected in the larvae hemocoel via the last left proleg. Larvae injected with heat-killed bacteria (45 min, 80°C) were used as negative controls. After injection, larvae were kept in the dark at 37°C, and survival was periodically monitored for up to 4 d.

In vivo model of infective endocarditis in rabbits

The rabbit model of IE employed for this study was modified from a previously used protocol.³⁸ Specific-pathogen-free, male New Zealand White Rabbits 2 to 3 kg in size were purchased from Robinson Services Inc. After acclimation, rabbits were injected with acepromazine as a sedative, a cocktail of ketamine, xylazine, and buprenorphine for general anesthesia, glycopyrrolate for control of respiratory secretions, and bupivacaine at the incision site. An incision was then made in the neck region, and a 19-gauge catheter (Intracath, BD Bioscience) was inserted through the right internal carotid artery, contacting or passing through the aortic valve to cause minor damage. The catheter was tied off, trimmed, and sutured into place, and the incision closed. Buprenorphine SR Lab was used for post-surgical analgesia. The day prior to inoculation, *L. lactis* strains were cultured overnight in GM17 and antibiotics as described above with 10 ng ml⁻¹ nisin. Twenty hours later, the cultures were diluted 10-fold into pre-warmed media of the same composition and cultured another 3 h. Cells were harvested by centrifugation, washed twice with cold PBS, diluted in PBS, and combined to achieve a concentration of 3 – 5 × 10⁸ CFU ml⁻¹ for each strain. One hour or 2 d after the surgeries, rabbits were inoculated with 0.5 ml of bacterial cells via a peripheral ear vein. Remaining cells were sonicated for 1.5 min at 50% power in a Biologics Model 150 sonicator (Biologic Inc.) to break up clumps and chains and plated with an Eddy Jet spiral plater (Neutec Group Inc.) on GM17 plates containing erythromycin or chloramphenicol to establish the ratio of the 2 strains in the inoculum. Two hours after inoculation, each rabbit was euthanized with intravenous Euthasol (Virbac) and was then checked for proper placement of the catheter tip in the valve region or beneath it in the left ventricle. Any rabbit in which the catheter was not correctly situated was excluded from the study. For rabbits inoculated on the day of surgery, the aortic valves and surrounding endocardium were removed and homogenized in 5 ml PBS using an electric homogenizer (Omni International). For rabbits inoculated 2 d post-surgery, visible vegetations and the endocardium underlying each vegetation were harvested separately and homogenized in 2 ml PBS each using a disposable 15-ml tissue grinder. In all cases, homogenates were sonicated and plated as above for enumeration.

Statistical analysis

All results represent a minimum of 3 independent experiments. Descriptive and inferential statistics were used to analyze the data on Graphpad Prism version 5.0. One-way ANOVA followed by Tukey's post-test was used to analyze the significance of binding and

invasion, with type I error set as 0.05. Analysis of log₁₀-transformed competitive indices was performed using one-way ANOVA with Bonferroni *post hoc* comparisons to determine substrate preferences among strain pairs. For the *G. mellonella* studies, Kaplan-Meier killing curves were plotted and estimation of differences in survival was compared using the log-rank test. Log₁₀-transformed competitive indices were compared to 0 using a one-sample *T* test for rabbit studies.

Abbreviations

BHI	brain heart infusion
CFU	colony forming unit
CI	competition/competitive index
EB	electroporation buffer
EBM	endothelial basal medium
ECM	endothelial cell medium
ECM	extracellular matrix
FBS	fetal bovine serum
FIB-FE-SEM	focused ion beam field emission scanning electron microscope
GM17	M17 broth supplemented with 0.5% glucose
GM17-Erm or GM17-Chlor	M17 broth supplemented with 0.5% glucose plus erythromycin or chloramphenicol (Chlor)
GM17S	GM-17 containing 1% glycine and 0.5 M sucrose
HBSS	Hank's balanced salt solution
HCAEC	human coronary artery endothelial cells
IE	infective endocarditis
PVDF	polyvinylidene fluoride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
TSA	tryptic soy agar
VS	viridans streptococci

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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