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*J Dent Res* 90(4):501-505, 2011

## ABSTRACT

*Streptococcus mutans* is the primary cariogen that produces several virulence factors that are modulated by a competence-stimulating peptide (CSP) signaling system. In this study, we sought to determine if proteases produced by early dental plaque colonizers such as *Streptococcus gordonii* interfere with the subsequent colonization of *S. mutans* BM71 on the existing streptococcal biofilms. We demonstrated that *S. mutans* BM71 colonized much less efficiently *in vitro* on streptococcal biofilms than on *Actinomyces naeslundii* biofilms. Several oral streptococci, relative to *A. naeslundii*, produced proteases that inactivated the *S. mutans* CSP. We further demonstrated that cell protein extracts from *S. gordonii*, but not from *A. naeslundii*, interfered with *S. mutans* BM71 colonization. In addition, *S. mutans* BM71 colonized more efficiently on the *sgc* protease knockout mutant of *S. gordonii* than on the parent biofilms. In conclusion, proteases of early colonizers can interfere with subsequent colonization by *S. mutans in vitro*.

**KEY WORDS:** *Streptococcus mutans*, *Streptococcus gordonii*, *Actinomyces naeslundii*, biofilm, protease, quorum sensing.

DOI: 10.1177/0022034510388808

Received June 17, 2010; Last revision October 5, 2010;  
Accepted October 6, 2010

A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

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# Proteases of an Early Colonizer Can Hinder *Streptococcus mutans* Colonization *in vitro*

## INTRODUCTION

Dental caries is among the most common chronic infectious diseases occurring in humans (NIDCR, CDC, 2002). *Streptococcus mutans* is the primary cariogen that produces several virulence factors. It is also implicated in some cases of infective endocarditis (Blanco-Carrion, 2004). *S. mutans* CSP (encoded by the *comC* gene) and a signal transduction system (a quorum-sensing system encoded by the *comD* and *comE* genes) mediate a variety of virulence characteristics, such as biofilm formation (Li *et al.*, 2002b; Yoshida and Kuramitsu, 2002), bacteriocin production (Yonezawa and Kuramitsu, 2005), acid tolerance (Li *et al.*, 2002a), antimicrobial sensitivity (Matsumoto-Nakano and Kuramitsu, 2006), and natural genetic transformation (Cvitkovitch, 2001).

Although *S. mutans* has been studied extensively relative to cariogenesis, there has been limited information relative to its interactions with other oral bacteria. Experiments carried out on laboratory animals have shown that selective suppression of *S. mutans* inhibits dental caries formation. For example, inoculation of low-virulence bacteria such as *Streptococcus salivarius* reduced the number of *S. mutans* in dental plaque and the subsequent levels of caries (Tanzer *et al.*, 1985). An understanding of the interactions between *S. mutans* and other oral plaque bacteria as demonstrated in this study may provide insight into novel strategies for preventing oral streptococcal infections.

Mono-strain biofilm formation of *S. mutans* has been demonstrated to be a CSP-dependent quorum-sensing phenomenon (Li *et al.*, 2002b; Yoshida and Kuramitsu, 2002). Elimination of CSP by knocking out the *comC* gene results in the formation of abnormal biofilms, either with altered architecture or with reduced biomass. However, dental plaque is composed of multiple bacteria that colonize in a sequential manner. We have demonstrated that one of these proteases, *S. gordonii* chollisin (a serine protease encoded by the *sgc* gene), is able to inactivate *S. mutans* CSP and thereby to antagonize CSP-dependent bacteriocin production (Wang and Kuramitsu, 2005). Since biofilm formation is another property modulated by *S. mutans* CSP, we hypothesize that attenuation of *S. mutans* CSP will also affect *S. mutans*' sequential establishment in multi-species biofilms.

## MATERIALS & METHODS

### Bacterial Strains and Media

*S. mutans* BM71 and its *comC* mutant (erythromycin-resistant), *S. mutans* BM71pTet [*S. mutans* BM71 containing a tetracycline-resistant plasmid (Xie *et al.*, 2007)], *S. mutans* BM71 or GS5, *S. gordonii* Challis and its *sgc* knockout mutant (erythromycin-resistant) (Wang and Kuramitsu, 2005), *S. gordonii* 10558, *Streptococcus mitis* 10712, *Streptococcus oralis* KS32AR, *Streptococcus sanguinis* 10556, as well as *A. naeslundii* 12104 were maintained on Todd-Hewitt

broth (THB) agar plates supplemented with erythromycin or tetracycline (10 µg/mL) where indicated. Bacteria were routinely cultured in THB. A group C streptococcal strain RP66 was used as an indicator for assays of *S. mutans* BM71 bacteriocin activity (Paul and Slade, 1975).

### Sequential Biofilm Formation

To form initial biofilms, we inoculated overnight pure cultures of early colonizers—including *S. gordonii* Challis or 10558, the *S. gordonii* Challis *sgc* mutant, *S. mitis*, *S. oralis*, *S. sanguinis*, *A. naeslundii* or *S. mutans* BM71 or GS5 (as controls) in THB—into autoclaved ¼-strength THB supplemented with 0.01% mucin (Sigma Chemical Co., St. Louis, MO, USA) (BM, biofilm media) (Li *et al.*, 2001) in 48-well polystyrene microtiter plates (10<sup>7</sup> cfu/mL). After a 24-hour incubation at 37°C in the presence of CO<sub>2</sub> under static conditions, the planktonic cells were discarded, and biofilms were washed twice with PBS for further removal of the planktonic cells. For subsequent sequential biofilm formation, BM71pTet in the stationary phase (in THB with tetracycline) was diluted in fresh THB without tetracycline (1:2) and cultured at 37°C for 1 hr to bring the BM71pTet cells to the same growth conditions. The cultured BM71pTet were further diluted in fresh BM (1:1000), and 0.5 mL of BM71pTet was added to each well containing initial biofilms as described above and incubated at 37°C without CO<sub>2</sub> under static conditions for another 4 hrs. The sequentially formed biofilms were washed twice with PBS for removal of the non-adherent bacteria. THB (0.5 mL/well) was added to the washed biofilms, and sonication was applied to each well 5 times by means of a Branson sonifier (output 3, cycle 30). The disrupted biofilm bacteria were further diluted in PBS (1:100), and 10 or 100 µL were plated on THB agar plates supplemented with tetracycline (10 µg/mL). For initial biofilm assessment, the subsequent addition of BM71 was omitted, and the disrupted initial biofilm bacteria were plated on THB agar plates. The plates were cultured at 37°C for 48 hrs under anaerobic conditions for subsequent colony counting.

### Effects of Early Colonizers on the CSP of *S. mutans*

Supernatants from *S. gordonii* or *A. naeslundii* grown to the stationary phase in THB were filtered through 0.22-µm filters to eliminate cellular components. The supernatants were then neutralized with NaOH to pH 7.0-7.5. Exogenous synthetic *S. mutans* CSP [amino acid sequence: SGSLSTFFRLFNRSFTQALGK (Li *et al.*, 2001), synthesized by Sigma-Genosys, The Woodlands, TX, USA; 2.5 µg/mL, final concentration] was incubated with the supernatants at 37°C for 2 hrs. CSP thus treated was added to *S. mutans* BM71 *comC* mutant cells (10<sup>7</sup> cfu) in 0.9 mL of THBY (THB supplemented with 3% yeast extract) to a final concentration of 0.25 µg/mL. After a 24-hour incubation at 37°C, the supernatants from the cultures were passed through 0.22-µm filters, and the bacteriocin level produced by the *comC* mutant was determined by the agar well assays as described below.

### Agar Well Assays

The supernatant fluids containing bacteriocin from the *S. mutans* BM71 *comC* mutant cultures were obtained as described above. The supernatants were added into pre-cut wells in THB agar plates and incubated at 37°C for 24 hrs to facilitate the absorption of the supernatants into the agar surrounding the wells. The wells were then filled using THB with 1% low-melting-temperature agarose, and the plates were overlaid with the indicator strain RP66 (10<sup>6</sup> cfu) in 3 mL THB with 1% low-melting-temperature agarose. After 24 hrs of further incubation at 37°C under anaerobic conditions, the diameters of the inhibition zones surrounding the wells were measured.

### Azocasein Assays

Proteolytic activities against azocasein from several early colonizers were measured according to a modified method (Sojar *et al.*, 1993). Briefly, 50 mL overnight cultures of *S. gordonii* or *A. naeslundii* were centrifuged at 4000 × *g* for 10 min. After being washed once with PBS, the cell pellets were utilized in the azocasein assays. OD<sub>420nm</sub> measured in these assays indicated the levels of protease activity present.

### Extraction of Cell Proteins from Early Colonizers

Cell proteins from *S. gordonii* or *A. naeslundii* were extracted according to a modified method (Wang *et al.*, 2009). Briefly, 50 mL overnight cultures of *S. gordonii* or *A. naeslundii* were centrifuged at 4000 × *g* for 10 min. After being washed once with PBS, the cells were treated with Lysing Matrix B and FastPrep, followed by sonication. The cell extracts were then centrifuged at 12,000 × *g* for 10 min. The supernatants from the cell extracts were concentrated with PEG 8000, and final protein concentrations were adjusted to 100 mg/mL.

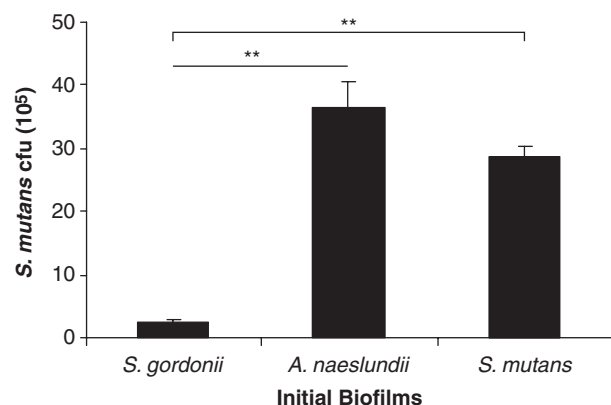
### Statistical Analysis

We performed Student's *t* test to determine significance. A difference was considered significant when a *p* value < 0.05 was obtained.

## RESULTS

### *S. mutans* *in vitro* Colonization is More Efficient on Pre-existing *A. naeslundii* Compared with Biofilms of Other Early Colonizers

*S. mutans* BM71 *in vitro* colonization efficiencies on several pre-formed early-colonizer biofilms, including *S. gordonii* Challis or 10558, *S. mitis*, *S. oralis*, *S. sanguinis*, and *A. naeslundii*, were studied. Pre-formed *S. mutans* BM71 or GS5 biofilms, as well as blank 48-well polystyrene surfaces, served as internal controls. *S. mutans* BM71 colonization was significantly more efficient on the *A. naeslundii* biofilms and the control *S. mutans* biofilms than on those of *S. gordonii* Challis (Fig. 1). *S. mutans* BM71 colonization efficiencies on other early colonizers, such as *S. gordonii* 10558, *S. mitis*, *S. oralis*, and *S. sanguinis*,



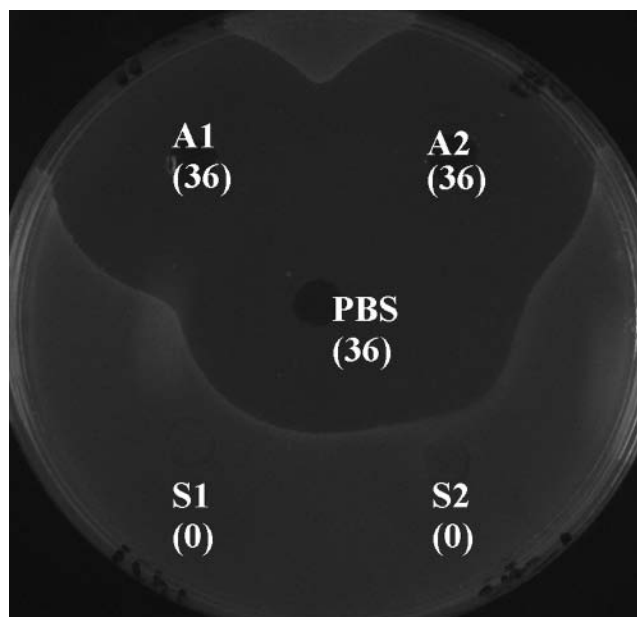
**Figure 1.** Colonization of *S. mutans* BM71 on pre-existing early-colonizer biofilms *in vitro*. Initial biofilms of *S. gordonii* Challis, *A. naeslundii* 12104, and *S. mutans* BM71 (as control) were formed in 48-well polystyrene microtiter plates. *S. mutans* BM71pTet was added on the washed initial biofilms and incubated for another 4 hrs. The sequentially formulated biofilms were disrupted by sonication after the planktonic cells were washed away. The Tet<sup>R</sup> colonies of *S. mutans* BM71pTet were counted on THB agar plates supplemented with tetracycline and adjusted to cfu (10<sup>5</sup>)/well. Data are the mean  $\pm$  standard deviation of triplicate platings from 1 of 3 reproducible experiments. \*\* $p < 0.01$ .

were comparable with that of *S. gordonii* Challis and significantly less relative to those on *A. naeslundii* and *S. mutans* (Appendix Fig.). *S. mutans* BM71 colonization on the blank polystyrene surfaces for 4 hrs was minimal ( $0.56 \pm 0.03 \times 10^5$ /well). The initial biofilms had comparable amounts of bacterial attachment ( $\times 10^5$ ): *S. gordonii* Challis,  $13.5 \pm 0.6$ ; the *S. gordonii* Challis *sgc* knockout mutant,  $12.9 \pm 1.4$ ; *S. gordonii* 10558,  $13.1 \pm 0.5$ ; *A. naeslundii*,  $6.9 \pm 0.4$ ; *S. mitis*,  $6.5 \pm 0.9$ ; *S. oralis*,  $9.4 \pm 1.2$ ; *S. sanguinis*,  $12.6 \pm 0.7$ ; *S. mutans* BM71,  $14.2 \pm 1.3$ ; and *S. mutans* GS5,  $13.6 \pm 1.1$ .

### ***S. gordonii*, but not *A. naeslundii*, Inactivates *S. mutans* CSP**

We have previously demonstrated that oral streptococci such as *S. gordonii*, *S. mitis*, *S. oralis*, and *S. sanguis* inactivated *S. mutans* CSP, which in turn interfered with bacteriocin production, a Com quorum-sensing-dependent phenomenon (Wang and Kuramitsu, 2005). Since *S. mutans* CSP also mediates *S. mutans* mono-biofilm formation, we carried out experiments to compare the ability of *S. gordonii* and *A. naeslundii* to inactivate *S. mutans* CSP. The supernatant fluids from *A. naeslundii* did not inactivate *S. mutans* CSP as did those from *S. gordonii* (Fig. 2). This difference may be one of the mechanisms that might allow *S. mutans* to colonize much more efficiently on *A. naeslundii* than on *S. gordonii* and other CSP-inactivating early colonizers.

We have previously demonstrated that *S. gordonii* did not degrade *S. mutans* bacteriocin in the well assays (Wang and Kuramitsu, 2005), which confirmed that the absence of *S. mutans* bacteriocin in our well assays was due to inactivation of *S. mutans* CSP by *S. gordonii*.



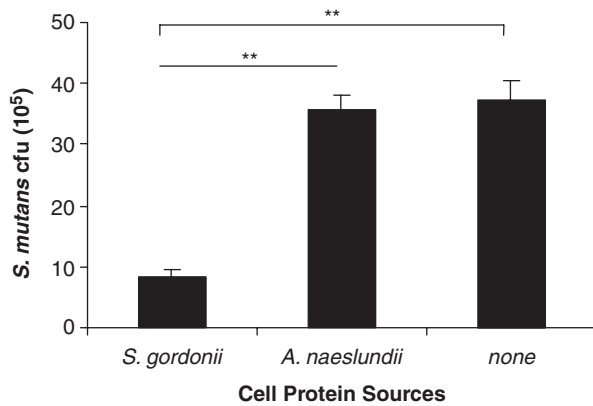
**Figure 2.** Determination of the effects of early colonizers on *S. mutans* CSP in agar well assays. Exogenous synthetic *S. mutans* CSP was incubated with early-colonizer culture supernatants for 2 hrs. CSP thus treated was used to stimulate bacteriocin production in *S. mutans* BM71 *comC* mutant cells. The bacteriocin level produced by the mutant cells was determined by agar well assays. A1 and A2, duplicates of *A. naeslundii* supernatants; PBS, a CSP positive control treated with PBS instead of culture supernatants; S1 and S2, duplicates of *S. gordonii* supernatants. The diameters of the inhibition zones surrounding the wells (mm) are shown in brackets.

### ***S. gordonii* Challis Exhibits Higher Protease Activity than *A. naeslundii***

To further investigate the mechanisms enhancing colonization of *S. mutans* on the *A. naeslundii* biofilms relative to those on *S. gordonii*, we compared the protease activities of *S. gordonii* and *A. naeslundii*. Utilizing azocasein assays to detect non-specific protease activities, we demonstrated that *S. gordonii* has significantly higher protease activity ( $0.39 \pm 0.06$  at OD<sub>420</sub>) than *A. naeslundii* ( $0.09 \pm 0.06$  at OD<sub>420</sub>) ( $p = 0.01$ ). We also carried out zymography using ready-made casein gel and confirmed the results of the azocasein assays (data not shown). These results, along with the evidence that *A. naeslundii* did not inactivate *S. mutans* CSP, suggested that proteases from early plaque colonizers may play a role in subsequent *S. mutans* colonization.

### **Cell Extracts from *S. gordonii*, but not from *A. naeslundii*, Interfered with *S. mutans* Colonization**

We next examined the formation of secondary *S. mutans* biofilms on *A. naeslundii* biofilms, with or without the cell extracts from *S. gordonii* or *A. naeslundii*. The cell extracts from *S. gordonii*, but not those from *A. naeslundii*, significantly interfered with the colonization of *S. mutans* BM71 on *A. naeslundii* (Fig. 3).



**Figure 3.** Effects of cell extracts from early plaque colonizers on the subsequent *S. mutans* BM71 colonization on *A. naeslundii* biofilms *in vitro*. Initial *A. naeslundii* biofilms were formed in 48-well plates. *S. mutans* BM71 pTet, with or without cell extracts (at final concentrations of 1 mg/ml) from *S. gordonii* or *A. naeslundii*, was utilized to form the sequential biofilms on the *A. naeslundii* biofilms as described in MATERIALS & METHODS. *S. mutans* cfu represents the Tet<sup>R</sup> colonies of *S. mutans* BM71 pTet counted on THB agar plates supplemented with tetracycline, adjusted to cfu ( $10^5$ )/well. Data are the mean  $\pm$  standard deviation of triplicate platings from 1 of 2 reproducible experiments. \*\* $p < 0.01$ .

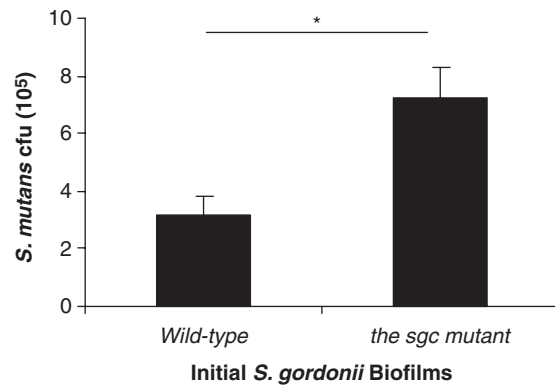
### Deletion of the *sgc* Gene Enhanced the Sequential *S. mutans* Colonization on *S. gordonii* Biofilms

To further confirm the role of proteases, we examined secondary *S. mutans* colonization on pre-existing biofilms of WT *S. gordonii* and its *sgc* knockout mutant *in vitro*. We have previously demonstrated that the *sgc* mutant produces reduced amounts of proteases and does not inactivate CSP (Wang and Kuramitsu, 2005). There was enhanced colonization of *S. mutans* BM71 on the *sgc* mutant biofilms, relative to that on the parental strain (Fig. 4).

## DISCUSSION

We have previously demonstrated that a *S. gordonii* protease, chollisin, is able to inactivate *S. mutans* CSP and thereby to antagonize CSP-dependent bacteriocin production (Wang and Kuramitsu, 2005). In this study, we established a sequential duo-species biofilm model to test our hypothesis that proteases from early dental plaque colonizers also interfere with subsequent *S. mutans* colonization. *S. mutans* BM71 colonization on *A. naeslundii* biofilms was significantly more efficient than that on the biofilms of other early colonizers. One of the mechanisms causing this difference in *S. mutans* colonization might be due to lack of, or a reduced amount of, proteases from *A. naeslundii* biofilms, relative to streptococcal biofilms. Indeed, we demonstrated in this study that *A. naeslundii* cells exhibited much less protease activity relative to *S. gordonii*. This is the first evidence that proteases produced by an early colonizer can interfere with the subsequent colonization of *S. mutans* BM71.

*S. mutans* BM71 exhibited enhanced colonization on the *sgc* mutant biofilms relative to that on the WT biofilms



**Figure 4.** Deletion of the *sgc* gene in *S. gordonii* enhanced the subsequent *in vitro* colonization of *S. mutans* BM71. Initial biofilms of WT *S. gordonii* or its *sgc* knockout mutant were formed in 48-well plates. Sequential biofilms of *S. mutans* BM71 pTet were formed as described in MATERIALS & METHODS. The Tet<sup>R</sup> colonies of *S. mutans* BM71 pTet were counted on THB agar plates supplemented with tetracycline and adjusted to cfu ( $10^5$ )/well. Data are the mean  $\pm$  standard deviation of triplicate platings from 1 of 3 reproducible experiments. \* $p < 0.05$ .

(Fig. 4). However, *S. mutans* colonization was enhanced only two-fold on the *sgc* knockout mutant, whereas there was 10-fold enhancement of *S. mutans* colonization on *A. naeslundii* biofilms, relative to that on those of *S. gordonii*. Therefore, although proteases of *S. gordonii* can play a role in interfering with subsequent *S. mutans* colonization, some other factor(s) may also be involved in the difference of *S. mutans* colonization on the biofilms of *A. naeslundii* and *S. gordonii*, which merits further investigation. In addition, other *S. gordonii* proteases besides chollisin may interfere with subsequent *S. mutans* colonization.

Ultrasonic dispersion has been utilized in other studies (Olsen and Socransky, 1981; Li *et al.*, 2001) to remove attached biofilms of *S. mutans* or disassociate chains formed by *S. mutans* in planktonic cultures before being plated on agar plates. In these studies, the integrity of the oral streptococcal cells after sonication was confirmed. We have verified the viability of planktonic *S. mutans* cells after sonication under our conditions. Higher colony counts were detected after sonication relative to counts before sonication ( $1.70 \times 10^9$ /mL vs.  $1.89 \times 10^9$ /mL, respectively), probably due to dissociation of the chains formed by *S. mutans*. Oral Gram-positive bacteria exhibit thick cell walls that may protect them against the impact of sonication.

Bacteria interact with each other and with their hosts in natural environments. Since quorum sensing in bacteria modulates their virulence, it is reasonable to assume that other bacteria and the host may produce factors that could interfere with the quorum-sensing system of a bacterium for the purpose of either competition for their establishment in the environment or as a defense mechanism. We demonstrated here that the proteases from oral streptococci such as *S. gordonii* could interfere with secondary *S. mutans* colonization in duo-species biofilms. An understanding of interactions among different species of oral

bacteria may provide valuable insight into the prevention of oral infections.

## ACKNOWLEDGMENTS

We thank M.M. Vickerman for helpful discussions. These studies were supported by grant DE017708 from the National Institute of Dental and Craniofacial Research.

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