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ORAL MICROBIOLOGY AND IMMUNOLOGY

# Inhibiting effects of *Streptococcus salivarius* on competence-stimulating peptide-dependent biofilm formation by *Streptococcus mutans*

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**Introduction:** The effects of *Streptococcus salivarius* on the competence-stimulating peptide (CSP)-dependent biofilm formation by *Streptococcus mutans* were investigated.
**Methods:** Biofilms were grown on 96-well microtiter plates coated with salivary components in tryptic soy broth without dextrose supplemented with 0.25% sucrose. Biofilm formations were stained using safranin and quantification of stained biofilms was performed by measuring absorbance at 492 nm.
**Results:** *S. mutans* formed substantial biofilms, whereas biofilms of *S. salivarius* were formed poorly in the medium conditions used. Furthermore, in combination cultures, *S. salivarius* strongly inhibited biofilm formation when cultured with *S. mutans.* This inhibition occurred in the early phase of biofilm formation and was dependent on inactivation of the CSP of *S. mutans*, which is associated with competence, biofilm formation, and antimicrobial activity of the bacterium, and is induced by expression of the *comC* gene. Comparisons between the *S. mutans* clinical strains FSC-3 and FSC-3Δ*glrA* in separate dual-species cultures with *S. salivarius* indicated that the presence of the bacitracin transport ATP-binding protein gene *glrA* caused susceptibility to inhibition of *S. mutans* biofilm formation by *S. salivarius*, and was also associated with the regulation of CSP production by *com* gene-dependent quorum sensing systems.
**Conclusion:** It is considered that regulation of CSP by *glrA* in *S. mutans* and CSP inactivation by *S. salivarius* are important functions for cell-to-cell communication between biofilm bacteria and oral streptococci such as *S. salivarius*. Our results provide useful information for understanding the ecosystem of oral streptococcal biofilms, as well as the competition between and coexistence of multiple species in the oral cavity.

Key words: cell-to-cell communication; competence-stimulating peptide; GlrA; oral biofilm; *Streptococcus mutans*

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Mutans streptococci, including *Streptococcus mutans* and *Streptococcus sobrinus*, are biofilm-forming bacteria considered to be the primary etiological agents of human dental caries (13, 23). They possess a variety of abilities to colonize tooth surfaces and, under certain conditions, are numerically significant in cariogenic biofilms and also form biofilms with other organisms, including other streptococci and bacteria, such as *Actinomyces*, *Neisseria*, and *Veillonella*, in the oral cavity (5, 23, 30). Dental biofilms are produced by bacterial communities, and have been reported to have a vast biodiversity (>700 bacterial species) (2, 20, 33) and high density [$\sim 10^{11}$ cells/g (wet weight)] (14). Oral streptococci have been shown to

compose from 60% to 90% of supragingival plaque biomass structures within the first 24 h of colonization (26, 30). Although numerous studies have elucidated the mechanisms of initial streptococcal adhesion (9, 17, 31, 40) and coaggregation (12, 18) among oral bacteria, the subsequent process of bacterial interaction, accumulation, and proliferation leading to a functional heterogeneous organized sessile community such as dental biofilm has not been well elucidated. It is likely that cooperative interactions between mutans streptococci and other oral streptococci play important roles in the development of dental biofilm and caries in the oral cavity (19).

Oral streptococci are normal inhabitants of the human oral cavity and play a role in resistance to colonization by invading pathogens (43, 45). However, in spite of increased research into the functions of cariogenic bacteria with those of other oral streptococci, little information is available on the competition between and coexistence of surface-associated communities. *Streptococcus salivarius* is one of several oral commensal bacteria and a major constituent of biofilms that colonize the buccal epithelium, tongue, and dorsal epithelium (4). This organism can comprise the majority of the total cultivable flora on soft tissues of the mouth, and possesses a number of important biological activities for lactose uptake and urease enzymes that are thought to contribute to the stability of oral communities (7, 42). Furthermore, products from *S. salivarius* may control oral biofilm formation over a considerable distance to integrate the whole oral cavity into a single, interacting ecosystem (10, 42). In the present study, we studied cell-to-cell communication of *S. mutans* and *S. salivarius*, and attempted to quantify the effects of *S. salivarius* on the biofilm formation by *S. mutans*, by investigating interspecies interactions in 96-well microtiter plates coated with salivary components.

*S. mutans* is known to be resistant to bacitracin and a number of mechanisms of that resistance have been reported for a variety of bacteria (6, 32, 35). In the bacitracin-producing organism *Bacillus licheniformis*, resistance is attributed to the *bcrABC* genes, which encode a putative heterodimeric ATP-binding cassette (ABC) transporter that has been proposed to mediate the active efflux of bacitracin and bacitracin resistance (29, 35). Furthermore, homologs of that transporter have been identified in *S. mutans* (8, 44) and the homolog encoding a protein with 60% identity to the BcrA protein was designated GlrA. In our previous study, the *S. mutans glrA*-deficient mutant FSC-3 (FSC-3Δ*glrA*) showed morphological changes in a flow cell system, including a lower level of biofilm formation in the bottom area and a higher level in the top area when attached to a glass surface, when compared with FSC-3 (28). Therefore, *glrA* is a relating gene for biofilm morphology of *S. mutans*. A recent report has proposed competence-stimulating peptide (CSP), which induces competence and antimicrobial activity and is encoded by *comC*, as one of the key factors for streptococcal biofilm formation (34). Our study showed that expression of *glrA* was regulated by CSP in *S. mutans*.

CSP is recognized by the sensor kinase receptor ComD which is autophosphorylated and transfers a phosphoryl group to the ComE response regulator (16). ComE activates a number of early competence genes. ComX, which acts as an alternative sigma factor, activates late competence genes in *S. mutans* and *Streptococcus pneumoniae* (3, 21). Inactivation of the regulators involved in competence, including *comDE* or *comX*, is likely to interfere with the expression of genes which are regulated by CSP.

In the present study, to investigate the influences of *S. salivarius* to CSP-dependent biofilm formation and *glrA*-dependent morphological changes of biofilm by *S. mutans*, FSC-3Δ*glrA*, and *comC*, *comD*, *comE*, and *comX* mutants, and wild-type strains were used in the present experiments. Our results provide useful information for understanding the ecosystem of oral streptococcal biofilm, as well as competition between and coexistence of multiple species in the oral cavity.

## Materials and methods

### Bacterial strains and culture conditions

*S. mutans* GS5 and UA159, and *S. salivarius* HT9R were used in this study. The *S. mutans* clinical strain FSC-3 and its isogenic mutant FSC-3Δ*glrA* (28), as well as UA159 *com* mutants, were also used to compare dual-species biofilm formation in combination cultures with *S. salivarius.* GS5Δ*comC* was also used for comparison of bacteriocin production in combination culture with FSC-3 and FSC-3Δ*glrA*. The group C streptococcal strain RP66 was used in some assays as an indicator of the bacteriocin activity (47). The tested mutants and wild-type strains are listed in Table 1. All bacteria were grown in brain–heart infusion broth (BHI, Difco Laboratories, Detroit, MI) in an aerobic atmosphere of 5% $CO_2$, 75% $N_2$, and 20% $O_2$ (GasPack $CO_2$, Becton/Deckinson, Sparks, MD) at 37°C before incubation in 96-well microtiter plates.

### Human saliva collection

Whole saliva samples were collected from five healthy human subjects (28–30 years old) after stimulation by chewing paraffin gum and were pooled into ice-chilled sterile bottles over a period of 5 min. The samples were clarified by centrifugation at 10,000 ***g*** for 20 min at 4°C, filter sterilized, and used immediately for biofilm assays in 96-well microtiter plates.

### Biofilm formation assays

Biofilm formation by each strain was assayed using a method described previously (28). To evaluate biofilm formation by mixed cultures of *S. mutans* GS5, FSC-3, FSC-3Δ*glrA*, UA159, and UA159 *com* mutants with *S. salivarius*, 20 μl of each cell suspension [$4.0 \times 10^4$ colony-forming units (CFU)] was mixed with 160 μl tryptic soy broth without dextrose (TSB, Difco Laboratories) supplemented with 0.25% sucrose (TSBS) in the wells of a 96-well (flat bottom) microtiter plate

*Table 1.* Bacterial strains

| Strain | Genotype or phenotype | Source or reference |
|---|---|---|
| *Streptococcus mutans* | | |
| GS5 | Erms, serotype c human isolate | SUNYaB[a] |
| GS5 *comC* mutant | Ermr, GS5 derived, comC deficient | Ref. 46 |
| UA159 | Erms, serotype c human isolate | ATCC[b] |
| UA159 *comC* mutant | Ermr, UA159 derived, comC deficient | This study |
| UA159 *comD* mutant | Ermr, UA159 derived, comD deficient | This study |
| UA159 *comE* mutant | Ermr, UA159 derived, comE deficient | This study |
| UA159 *comX* mutant | Ermr, UA159 derived, comX deficient | This study |
| FSC-3 | Erms, human isolate | Ref. 28 |
| FSC-3 *glrA* mutant | Ermr, FSC-3 derived, *glrA* deficient | Ref. 28 |

[a]SUNYaB: the culture collection in Department of Oral Biology, State of New York, Buffalo, NY.
[b]ATCC: American Type Culture Collection, Manassas, VA.

(Sumitomo Bakelite, Tokyo, Japan). Single culture assays of the tested strains were also performed as controls by mixing 20 $\mu$l of each cell suspension ($4.0 \times 10^4$ CFU) with 180 $\mu$l TSBS. To more closely approximate human oral conditions, salivary components, including antimicrobial agents and receptors of streptococcal adhesions (9), were used to coat the polystyrene surfaces before the addition of the bacterial cell suspension into the wells. The coating of whole saliva was performed for 60 min at 4°C. In some of the experiments with single and dual-species cultures, after the plates were incubated at 37°C for 4, 8, 12, 16, and 20 h under 5% $CO_2$ aerobic conditions, liquid medium was removed and the wells were rinsed a second time with sterile distilled water ($dH_2O$). The plates were air-dried and stained with 0.25% safranin and 0.5% ethanol in $H_2O$ for 15 min, then rinsed with $dH_2O$ to remove excess dye and air-dried. The biofilm mass was measured using a microplate reader (Thermo Bioanalysis Japan, Tokyo, Japan) without dissolving with solvent because the biofilm was formed uniformly on the bottoms of the wells in the 96-well plates (28). Quantification of stained biofilm on the bottom was performed by measuring absorbance at 492 nm.

CSP (amino acid sequence, SGSLSTFFRLFNRSFTQALGK) (22) was synthesized by Asahi Techno Glass (Tokyo, Japan). To analyse the effects of CSP on the cell growth of *S. mutans* UA159 and UA159Δ*comC*, 10 $\mu$l of each cell suspension ($2.0 \times 10^4$ CFU) was mixed with 90 $\mu$l TSBS involving 0, 0.2, 0.5, 1.0, 2.0, 5.0, 7.0, or 10.0 $\mu$M CSP in the 96-well microtiter plate and incubated for 0, 2, 5, 8, 10, 12, 14, and 16 h at 37°C under 5% $CO_2$ aerobic conditions. The cell growth level at each culture time was determined by measuring absorbance at 540 nm. Furthermore, to clarify the effects of CSP on biofilm produced by *S. salivarius* HT9R, 20 $\mu$l of culture supernatant (0, 6.25, 12.5, 25.0, 50.0, and 100 $\mu$g/ml) from HT9R filtered through 0.22-$\mu$m pore-size filters was mixed and incubated with 20 $\mu$l exogenous CSP (70 $\mu$M) at 37°C for 1 h. Then, 40 $\mu$l CSP solution treated in this way was added to *S. mutans* UA159Δ*comC* ($4.0 \times 10^4$ CFU) in 160 $\mu$l fresh TSBS to a final concentration of 7 $\mu$M and incubated at 37°C for 16 h. In other experiments performed for clarification of the inhibition effects of CSP on *S. salivariu*s, exogenous CSP was added to experiments of mixed cultures of UA159 or UA159Δ*comC* and *S. salivarius* HT9R, and of FSC-3 or FSC-3 *glrA* and *S. salivarius* HT9R, with a final concentration of CSP at 1 or 10 $\mu$M.

To confirm which species formed biofilms in dual-species cultures with *S. mutans* GS5 and *S. salivarius* HT9R, 200 $\mu$l of each cell suspension ($4.0 \times 10^5$ CFU) and 1600 $\mu$l TSBS were mixed in the wells of a 24-well (flat bottom) microtiter plate (Corning Incorporated, Corning, NY) with a coating of saliva. After culturing for 4, 8, 12, 16, and 20 h, pH in the supernatant was determined. After washing three times with sterilized phosphate-buffered saline (PBS), the biofilm cells were scraped with a sterilized scraper into 1 ml sterilized PBS. The scraped biofilm was collected and that remaining was collected using a pipette with sterilized PBS, after which 0.25% safranin was added to the well after scraping. No or only slightly stained biofilm remaining was used to confirm the efficacy of the scraping. The biofilm suspension was sonicated using ultrasonic dispersion (60 W power output) for 10 s, then shaken, diluted 1/1000 in PBS, and poured into a Mitis-Salivarius agar plate (Nippon Becton Dickinson Co. Ltd., Tokyo, Japan) using an EDDY JET spiral system (Gunze Sangyo, Inc., Tokyo, Japan) (25). Following aerobic incubation with 5% $CO_2$ for 48 h, *S. mutans* and *S. salivarius* colonies were identified by their characteristic appearance.

### Preparation of inhibiting substance

*S. salivarius* HT9R was incubated aerobically in extra BHI medium after dialysis on a cellulose membrane (passage molecular weight, 14,000, Viskase Companies, Ind., Darien, IL) at 37°C overnight, and then supernatant samples were precipitated by salting out utilizing ammonium sulfate. Next, the precipitates were collected by centrifugation at 10,000 ***g***, and suspended and dialysed in 20 mM Tris–HCl buffer (pH 7.4) at 4°C. Samples greater than 10 kDa were condensed by ultrafiltration using a centrifugal filter device in the sample solution (Amicon Ultra, Millipore, Billerica, MA). Each supernatant sample was then applied to a Sepharose 4B column (26 × 100 cm, Amersham Pharmacia Biotech, Buckinghamshire, UK) pre-equilibrated with the same buffer. After washing the column extensively, 7-ml fractions were collected, then monitored for ultraviolet absorbance and suppression effects toward the formation of biofilm by *S. mutans* GS5. Active fractions were pooled and the proteins were again precipitated by salting out using ammonium sulfate. Then the precipitates were collected by centrifugation at 10,000 ***g***, and suspended and dialysed in PBS (pH 7.4). Protein concentrations in each sample solution were determined using a protein assay kit (BioRad, Richmond, CA) and the samples were also used as supernatant samples in biofilm experiments.

### Construction of *com* mutants

The *comCDEX* genes were identified in the *S. mutans* UA159 database (http://www.genome.ou.edu/smutans.html) and mutants were constructed by double-crossover homologous recombination via insertion of an erythromycin resistance determinant into each gene. The plasmids used for disruption of the *comCDEX* genes were prepared as follows. The polymerase chain reaction (PCR) fragments of the upstream

*Table 2.* Primer list

| Primer | Nucleotides sequence | Amplicon |
|---|---|---|
| ComCAF (Kpn) | 5′-CGGGTACCAAATCTGAACAAGCAGGGG-3′ | comCA |
| ComCAR (Bam) | 5′-CGGGATCCGATAGTGTTTTTTTCATTTTATATCTCC-3′ | |
| ComCBF (Bam) | 5′-CGGGATCCTGGGAAAAT-3′ | comCB |
| ComCBR (Xba) | 5′-CGTCTAGACAGGACATCAATTGCAGGA-3′ | |
| ComXAF (Kpn) | 5′-CGGGTACCGGGCTAATGGTTTCTCCTT-3′ | comXA |
| ComXAR (Bam) | 5′-CCGGATCCAATCTTCACGAGTCCACA-3′ | |
| ComXBF (Bam) | 5′-CCGGATCCGGGAACATCCAGAATTAGAA-3′ | comXB |
| ComXBR (Xba) | 5′-CGTCTAGACATTTAAATCAACACTGGCC-3′ | |
| ComDAF (Kpn) | 5′-CGGGTACCCCATTCATCTGAAACTCAGT-3′ | comDA |
| ComDAR (Bam) | 5′-CCGGATCCAACAGGCAGCAGACCATAA-3′ | |
| ComDBF (Bam) | 5′-CCGGATCCGGCGGGCAATCATATTCTT-3′ | comDB |
| ComDBR (Xba) | 5′-CGTCTAGATCCTGCAATTGATGTCCTG-3′ | |
| ComEAF (Kpn) | 5′-CGGGTACCGCTGCTTTATCTTGGACAG-3′ | comEA |
| ComEAR (Bam) | 5′-CCGGATCCGGTTTCAAGACGTCCTTGT-3′ | |
| ComEBF (Bam) | 5′-CCGGATCCTTATTGACGCTATCCCTG-3′ | comEB |
| ComEBR (Xba) | 5′-CGTCTAGAGCTCTCCTTTGATCAGCAA-3′ | |

Endonuclease recognition sequences are underlined.
A, upstream; B, downstream; F, forward; R, reverse.

and downstream regions of the *com* genes were amplified with pairs of primers (Table 2), using chromosomal DNA from UA159 as a template. Initially, PCR products of the upstream region of *comC* (*comCA*) were cloned into plasmid pUC19. Next, PCR products of the downstream regions of *comC* (*comCB*) were ligated to *comCA*. The resultant plasmid was digested with *Bam*HI, after which *Bam*HI-digested pResEmMCS10 (41) was inserted. The chimeric plasmid was then linearized with *Eco*RI and the linear plasmid was used to transform *S. mutans* UA159. The plasmids for inactivating the *comD*, *comE*, and *comX* mutants were constructed in a similar manner as for the *comC* mutant. Confirmation of plasmid insertions causing gene disruption was performed by either Southern blotting or PCR (data not shown). ComCAF and ComCBR, ComXAF and ComXBR, ComDAF and ComDBR, and ComEAF and ComEBR in Table 2 were used for PCR as *comC*, *comX*, *comD*, and *comE* mutant plasmids.

#### Extraction of RNA

Total RNA was isolated from 1.0 ml log-phase cell cultures for 4 h with or without CSP treatment and a slight modification of the RNeasy Mini Kit protocol (Qiagen GmbH, Hilden, Germany) using on-column DNase digestion with RNase-free DNase I (Qiagen). Briefly, the harvested samples were resuspended in 100 μl TE (10 mM Tris-HCl 1 mM EDTA pH 8.0) buffer containing 6 μl lysozyme (50 mg/ml) and 40 U of mutanolysin, followed by incubation at 37°C for 60 min. The soluble samples were treated using a RNeasy Mini Kit according to the directions of the supplier.

#### Transcription analysis

To confirm whether the expression of *glrA* is dependent on the quorum sensing systems of *com* genes in *S. mutans*, reverse transcription-PCR (RT-PCR) analysis was performed. An overnight culture of the *S. mutans* UA159Δ*comC* was diluted 100-fold in fresh medium and grown at 37°C. After 180, 210, 225, or 235 min of growth, CSP was added to a final concentration of 0.2 μM for each culture. After a total of 4 h incubation, all cultures were used for RNA isolation. For RT-PCR analysis, 1 μg RNA from each sample was used and RT-PCR was performed using the SuperScript III first-strand synthesis system (Invitrogen Corp., Carlsbad, CA) with a random primer (Invitrogen) according to the recommended procedures. First, to check for DNA contamination in the samples, purified total RNA without reverse transcription was used as a negative control for PCR with a pair of universal and *glrA* primers (data not shown). PCR was then performed using the synthesized complementary DNA indicated as templates and primers. The *glrA* primers of *S. mutans* open reading frames (forward: GCGATCAAAGAATTTCGGC, reverse: CAAACGCCAAGATTTAGAA) were used to detect *glrA* expression. The RT-PCR results were normalized using the 16S ribosomal RNA sequences of gram-positive bacteria primers as internal controls (24).

#### Bacteriocin assay

To clarify the effects of sub-purified culture supernatant samples from *S. salivarius* HT9R on CSP activity, 18 μl culture supernatant (0, 3.125, 6.25, 12.5, 25.0, 50.0, and 100.0 μg/ml) filtered through 0.22-μm pore-size filters was mixed and incubated with 2 μl exogenous CSP (1 mM) at 37°C for 1 h. Then, 4 μl CSP solution treated in this way was added to *S. mutans* UA159Δ*comC* ($0.9 \times 10^4$ CFU) in 36 μl fresh TSBS to a final concentration of 10 μM CSP and incubated on the BHI 1% agar (Wako, Tokyo, Japan) plate at 37°C for 24 h. The indicator strain RP66 was grown to an optical density of 0.3 at 550 nm. Each culture was then diluted to 1 : 100 and 0.2 ml of the dilution was transferred by pipette into a tube containing 4 ml molten BHI with 1% agar. The solution was mixed and poured evenly onto the surfaces of the plates, then at 37°C for an additional 24 h, after which the diameters of the zones of inhibition were measured.

To measure CSP production levels by *S. mutans* FSC-3 and FSC-3Δ*glrA*, *S. mutans* GS5 and GS5Δ*comC* were used. Recently, it was reported that the addition of exogenous CSP to *S. mutans* GS-5Δ*comC* in a plate assay using indicator strain RP66 restored the ability to produce bacteriocin (46). The production of CSP from *S. mutans* FSC-3 and FSC-3Δ*glrA* was indicated by the inhibition zones based on bacteriocin produced from *S. mutans* GS-5Δ*comC* in plate assays of *S. mutans* GS5Δ*comC* mixed separately with *S. mutans* FSC-3 and FSC-3Δ*glrA*. Loopfuls of stationary-phase cultures of the *Streptococcus* strains were stabbed into BHI agar on a plate, and then incubated under anaerobic conditions at 37°C for 6 or 24 h. The indicator strain RP66 was also grown and used to measure the diameters of the zones of inhibition revealed by the above-described method.

#### Transformation assays

Transformation efficiency was measured as previously described with minor modifications (1). Briefly, overnight cultures of *S. mutans* UA159 were diluted 1 : 20 in BHI medium containing horse serum (10%, volume/volume). A 0.2-ml aliquot of the cultures was incubated at 37°C for 2 h in a microcentrifuge tube (BIO-BIK, Osaka, Japan) and then added with or without 2 μl synthetic CSP solution (1 μmol/ml), which was treated with 0, 6.25, 12.5, 25.0, 50.0, or 100.0 μg/ml sub-purified culture supernatant sample from *S. salivarius* HT9R for 1 h at 37°C, and overnight culture supernatants of *S. mutans* UA159, FSC-3 or FSC-3Δ*glrA* (1/40 or 1/80 dilution in final concentration). After incubation for 30 min to allow the induction of competence, the cultures were exposed to 200 ng plasmid pDL 276. After 3 h at 37°C, cultures were chilled on ice and transformants and total CFU were enumerated by plating cells in BHI agar plates with or without 500 ng/ml kanamycin. Transformation efficacy was determined after 48 h of incubation and was expressed as the percentage of transformants among the total viable recipient cells.

#### Statistical analysis

Comparisons of biofilm formation levels among the various cultures of *S. mutans* UA159 and UA159Δ*comC* and transformations of plasmid into *S. mutans* UA159 treated with various stimulators were performed by Fisher's protected least significant difference and analysis of variance. A *P*-value of 0.01 or less was considered to indicate statistical significance.

## Results

#### Biofilm formation with dual-species cultures in 96-well microtiter plates

To evaluate the abilities of *S. salivarius* to form biofilms with *S. mutans*, combination cultures of *S. mutans* and *S. salivarius* were grown in 96-well microtiter plates coated with salivary components. The inhibiting effects of *S. salivarius* in dual-species cultures with *S. mutans* GS5 were observed at various time-points (Fig. 1A). Inhibition was recognized at 4 and 8 h after the start of culturing. Each of the culture supernatants, except for the single culture with HT9R, had

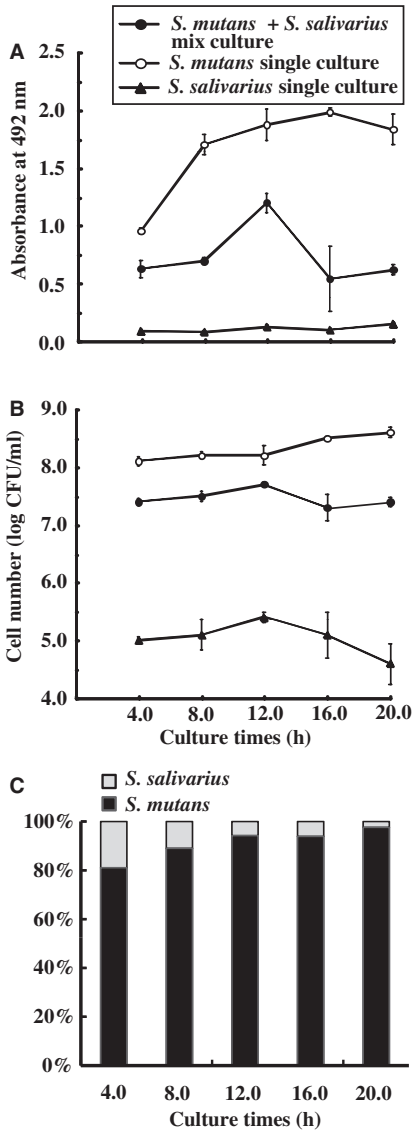


*Fig. 1.* Formation of biofilms by dual-species cultures at various time-points. Graphs show quantification of biofilms formed after 4, 8, 12, 16, and 20 h in cultures of *Streptococcus mutans* GS5 and *Streptococcus salivarius* HT9R in a 96-well microtiter plate (A), colony-forming units (CFU)/biofilm in a 24-well microtiter plate (B) and proportion of *S. mutans* GS-5 or *S. salivarius* HT9R in biofilm (C). The culture conditions were divided into the following three groups: (i) simultaneous culture of GS5 and *S. salivarius*, (ii) single culture of GS5, and (iii) single culture of *S. salivarius*. The results are expressed as the mean ± SD of absorbance at 492 nm obtained in triplicate assays. Representative data from three independent experiments are presented, with similar results obtained in each.

a pH level of around 6.0 after 4 h, while pH levels in the range of 4.6–4.9 were found in the other experiments after 4 h. At all time-points, the total number of cells in the biofilm was lower in the dual-species cultures compared with the single species culture with *S. mutans*, while the number was higher than that in the single species culture of *S. salivarius* (Fig. 1B). Furthermore, the proportion of *S. mutans* cells in the biofilms was greater than 80% after 4 h and then continued to slightly increase until 20 h (Fig. 1C). These results indicate that *S. salivarius* had inhibiting effects on subsequent biofilm growth and cell numbers of *S. mutans* after 4 h of dual-species culture. To confirm whether *S. salivarius* produced a substance that inhibited the biofilm formation of *S. mutans*, the culture supernatant from *S. salivarius* was sub-purified by gel filtration. The sub-purified sample significantly inhibited biofilm formation in a dose-dependent manner (Fig. 3).

### Relationship between biofilm inhibition by *S. salivarius* and CSP

Wang and Kuramitsu reported that some oral streptococci, including *S. gordonii*, *S. sanguinis*, *S. mitis*, and *S. oralis*, have an ability to inactivate *S. mutans* CSP (46). It was also speculated that CSP is inactivated by *S. salivarius* HT9R, while destruction of the CSP-dependent quorum sensing system has been associated with the inhibition of biofilm formation by *S. salivarius* in dual cultures. To clarify the situation, *S. mutans* UA159Δ*comC* was constructed and used for the biofilm formation assay using a pretreatment sample of CSP with the sub-purified sample. Before the biofilm formation assay, the growth of *S. mutans* UA159 and UA159Δ*comC* was studied in the presence of CSP. Addition of exogenous CSP inhibited continuously the cell growth of *S. mutans* UA159 after 8 h of culture (Fig. 2A). In contrast, the cell growth of *S. mutans* UA159Δ*comC* was inhibited by the addition of CSP at 8 h but was restored to the growth level found in conditions without CSP after 10 h of culture (Fig. 2B). It is proposed that the difference in cell growth between *S. mutans* wild-type and the *comC* mutant in the presence of CSP is because the originating CSP from wild-type may combine with the continuous inhibition of cell growth of *S. mutans* UA159 by CSP during 16 h of culture, but the cell growth of *S. mutans* UA159Δ*comC* is only temporarily inhibited by the limiting amount of added CSP under the condition with no originating CSP. As a result, the cell growth of *S. mutans* UA159Δ*comC* in the presence of CSP was similar to that in conditions without CSP by 16 h after incubation.

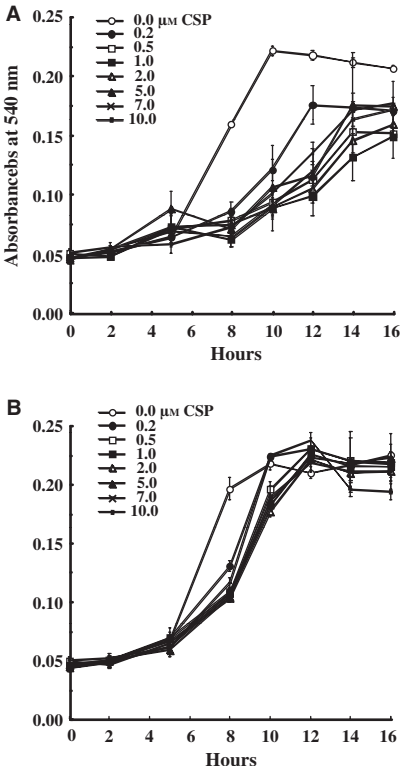


*Fig. 2.* Cell growth of *Streptococcus mutans* UA159 and UA159Δ*comC* in culture with competence-stimulating peptide (CSP). Graphs show quantification of cell growth after 0, 2, 5, 8, 10, 12, 14, and 16 h in culture of *S. mutans* UA159 (A) or UA159Δ*comC* (B) with 0, 0.2, 0.5, 1.0, 2.0, 5.0, 7.0, and 10.0 μM CSP. The results are expressed as the mean ± SD of absorbance at 540 nm obtained in triplicate assays. Representative data from three independent experiments are presented, with similar results obtained in each.

To clarify the inhibition mechanism of *S. salivarius*, CSP was pretreated with a sub-purified culture supernatant sample from *S. salivarius*, and then added to an *S. mutans* UA159Δ*comC* culture and incubated in plates for 16 h. Biofilm formation by the *comC* mutant was significantly inhibited by pretreatment with the CSP-added supernatant sample and the sub-purified sample could not inhibit biofilm formation by *S. mutans* UA159Δ*comC* without CSP (Fig. 3). To obtain clear details of the inhibitory effect of the sub-purified sample, bacteriocin production of *S. mutans* GS-5Δ*comC* and transformation of pDL276 ($Ka^r$) into *S. mutans* UA159 in the presence of CSP, which is another CSP-dependent biological system (1, 47), was also utilized. Bacteriocin production and transformation were clearly and significantly inhibited by pretreatment with the CSP-added supernatant sample in a dose-dependent manner

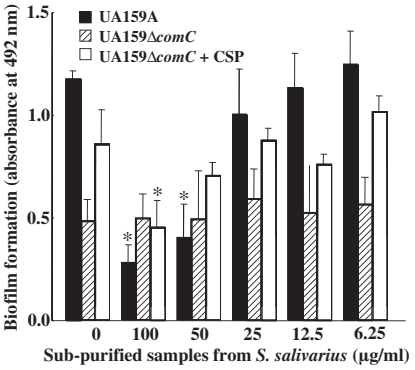


*Fig. 3.* Biofilm formation by *Streptococcus mutans* with culture supernatant from *Streptococcus salivarius*. Graphs show quantification of biofilms formed after 16 h in separate cultures of *S. mutans* UA159 and UA159Δ*comC* with various concentrations (0, 6.25, 12.5, 25.0, and 50.0 μg/ml) of culture supernatant samples from *S. salivarius* in a 96-well microtiter plate. Competence-stimulating peptide (CSP) pretreatment of supernatants was also used to investigate biofilm formation by UA159Δ*comC*. The results are expressed as the mean ± SD of absorbance at 492 nm obtained in triplicate assays. Representative data from three independent experiments are presented, with similar results obtained in each. Asterisks denote significantly different relative levels of biofilm formation ($P < 0.01$, vs. biofilm of UA159, UA159Δ*comC*, or UA159Δ*comC* + CSP without culture supernatant sample).

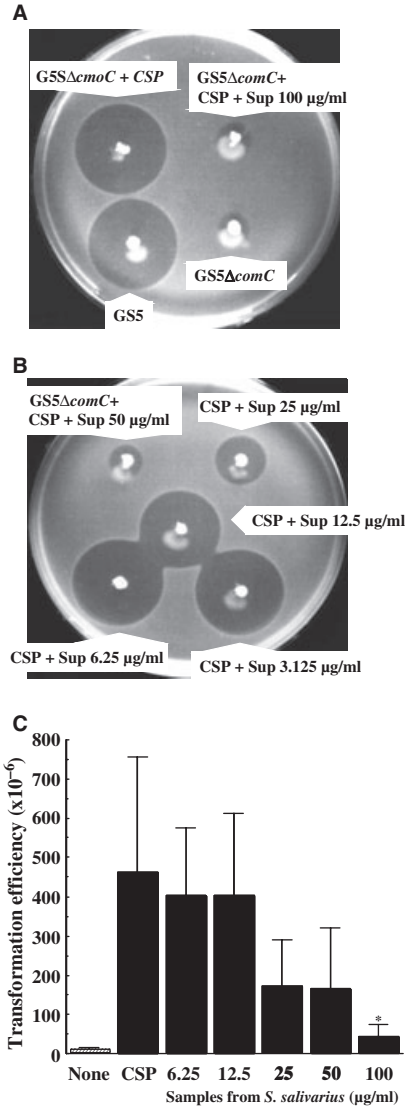


*Fig. 4.* Bacteriocin production and competence activity by *Streptococcus mutans* with competence-stimulating peptide (CSP) and culture supernatant from *Streptococcus salivarius*. The production of bacteriocin was observed in 24-h cultures of GS-5, GS-5Δ*comC*, and GS-5Δ*comC* added with CSP solutions after preincubation with CSP and culture supernatant sample (3.125, 6.25, 12.5, 25.0, 50.0, 100.0 μg/ml) from *S. salivarius* HT9R (A and B). The streptococcal strain RP66 was used as an indicator strain. Representative data from three independent experiments were presented. pDL276 ($Ka^r$) was transformed into *S. mutans* UA159 stimulated with or without CSP which was treated with 0, 6.25, 12.5, 25.0, 50.0, and 100.0 μg/ml of sub-purified culture supernatant sample from *S. salivarius*. Transformation frequency was determined from the ratio of the number of transformants vs. that of the total viable recipients, multiplied by 100 (C). The results were obtained from three independent assays and are expressed as the mean ± SD. Asterisks denote significantly different relative levels of transformation [$P < 0.05$, vs. transformation efficacy in *S. mutans*-treated CSP (1 μmol)].

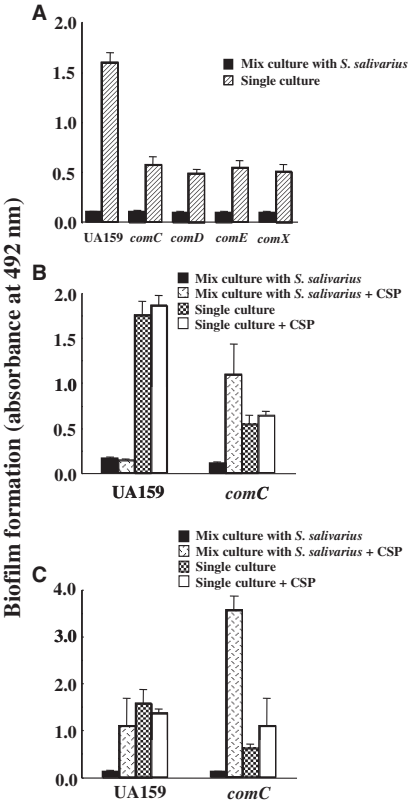


*Fig. 5.* Effects of competence-stimulating peptide (CSP) on biofilm formation by *Streptococcus salivarius* and *Streptococcus mutans*. *S. mutans* UA159, UA159Δ*comC* (comC), UA159Δ*comD* (comD), UA159Δ*comE* (comE), and UA159Δ*comX* (comX) were each incubated with or without *S. salivarius* HT9R (A). UA159 and UA159Δ*comC* were mixed separately with HT9R, after which 1 μM (B) or 10 μM (C) of CSP was added. All mixtures were incubated aerobically for 16 h at 37°C in 96-well microtiter plates. The results are expressed as the mean ± SD of absorbance at 492 nm obtained in triplicate assays. Representative data from three independent experiments are presented, with similar results obtained in each.

(Fig. 4A–C). We therefore considered that biofilm formation was inhibited by CSP inactivation of *S. salivarius*. To analyse still other mechanisms related to the inactivation of CSP in biofilm inhibition, *com* mutants were mixed and cultured with *S. salivarius* cells. The inhibition was greater in the mixed cultures than in the single culture biofilms (Fig. 5A). However, addition of CSP (1 μM) restored biofilm formation in mixed cultures of the *comC* mutant and *S. salivarius* (Fig. 5B). Furthermore, addition of an increased amount (10 μM) of CSP also restored biofilm formation in the mixed cultures of UA159 and the *comC* mutant with *S. salivarius*, and resulted in a more than twofold increase in biofilm formation compared with the wild-type (Fig. 5C).

### Influence of *glrA* on biofilm inhibition in dual-species cultures

Based on our results, we also speculated that morphological differences among the *S. mutans* biofilm formations were related to the inhibition of biofilm development in dual-species cultures with *S. salivarius*. Therefore, we compared FSC-3 with FSC-3Δ*glrA* using dual-species cultures to determine the influence of morphological change on that inhibition. *S. salivarius* inhibited biofilm formation when cultured with FSC-3, but not when cultured with FSC-3Δ*glrA* (Fig. 6). To investigate the relationship between *glrA* and the CSP-dependent quorum sensing system, *glrA* was subjected to RT-PCR with various *com* mutants and the *comC* mutant stimulated by CSP. The results showed that *glrA* was expressed in the *comX* mutant, but not in the *comC*, *comD*, and *comE* mutants (Fig. 7A), though expression was observed in the *comC* mutant 15 min after the addition of CSP (Fig. 7B). In contrast, CSP addition did not affect biofilm formations in mixed cultures of FSC-3 and FSC-3Δ*glrA* with *S. salivarius* (Fig. 7C).

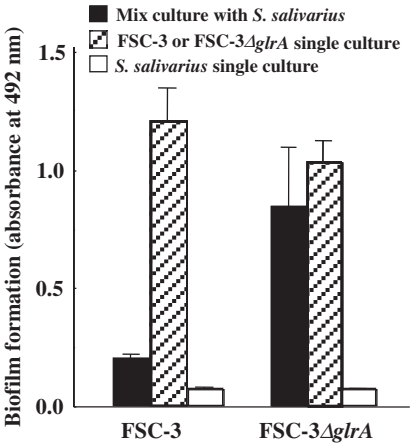


*Fig. 6.* Biofilm formations by FSC-3 and FSC-3Δ*glrA* with *Streptococcus salivarius*. Graphs show quantification of biofilms formed after 16 h of dual-species cultures of *Streptococcus mutans* FSC-3 (A) and FSC-3Δ*glrA* (B) with *S. salivarius* HT9R in 96-well microtiter plates. The culture conditions were divided into the following three groups: (i) simultaneous culture of FSC-3 or FSC-3Δ*glrA* and other streptococci, (ii) single culture of FSC-3 or FSC-3Δ*glrA*, (iii) single culture of *S. salivarius*. The results are expressed as the mean ± SD of absorbance at 492 nm obtained in triplicate assays. Representative data from three independent experiments are presented, with similar results obtained in each.

These findings demonstrated that *glrA* expression in the CSP-dependent quorum sensing system is associated with susceptibility to biofilm inhibition involved in the inactivation of CSP by *S. salivarius.* However, morphological changes, such as a reduction and accretion of volume in the biofilm bottom and top areas of FSC-3Δ*glrA* on a hard surface in comparison with the wild-type FSC-3 (28), were probably associated with the resistance to inhibition by *S. salivarius.* Moreover, we speculated that there was a greater production of CSP by FSC-3Δ*glrA* than by FSC-3, so *S. salivarius* did not inhibit biofilm formation in the dual-species culture with FSC-3Δ*glrA*.

### Influence of *glrA* on CSP-dependent bacteriocin production and genetic transformation assay

To investigate our theory, CSP-dependent bacteriocin production and transformation assays were performed to measure CSP production (1, 47). *S. mutans* GS-5Δ*comC*, which did not show CSP-dependent production of bacteriocin with indicator strain group c streptococcus RP66 cells, was mixed separately with FSC-3 and FSC-3Δ*glrA*, then bacteriocin production was compared between mixed cultures with *S. mutans* GS-5Δ*comC* and FSC-3, or FSC-3Δ*glrA* using plate assays. Greater bacteriocin production was observed in the culture of *S. mutans* GS-5Δ*comC* with FSC-3Δ*glrA* than in that with FSC-3, as indicated by the diameter of the inhibition zone of the indicator strain RP66 (Fig. 7D). However, FSC-3, FSC-3Δ*glrA*, and GS-5Δ*comC* produced minimal amounts of bacteriocin. Furthermore, greater transformation of pDL276 into *S. mutans* UA159 was observed in the culture of *S. mutans* UA159 with 1/40 dilution of culture supernatant from FSC-3Δ*glrA* than in that with FSC-3 (Fig. 7E). These results indicate that FSC-3Δ*glrA* produces greater amounts of CSP than FSC-3.

## Discussion

In the present study, we investigated the effects of *S. salivarius* on CSP-dependent biofilm formation by *S. mutans* in 96-well microtiter plates coated with salivary components. Few biofilms were observed in mono-species cultures of *S. salivarius* HT9R, whereas biofilm formation was inhibited in dual-species cultures with *S. mutans*. *S. salivarius* produces substances that inhibit biofilm formation by *S. mutans*. A prepared culture supernatant sample from *S. salivarius* played a role in the inactivation of CSP, although it was not able to inhibit biofilm formation by UA159Δ*comC* without CSP, it did inhibit biofilm formation by *S. mutans* UA159Δ*comC* and bacteriocin production and transformation of pDL276 into *S. mutans* UA159 after preculturing with CSP (Figs 3 and 4). Furthermore, the addition of CSP restored biofilm formation in dual-species cultures of UA159 and UA159Δ*comC* with *S. salivarius* (Fig. 5B,C).

The inhibition by *S. salivarius* observed in our experiments was not dependent on acid produced by the oral streptococci because the pH levels in the supernatants after culturing were similar in all cultures and the supernatant sample from *S. salivarius* inhibited biofilm formation, although it did not have an influence on culture pH (Fig. 3). We found at least four proteins in the supernatant sample, estimated to range in size from 97.4 to 200 kDa, in a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) assay that utilized Coomassie blue staining (data not shown). The sample did not include the molecular mass (62 kDa) of the large subunit of urease from *S. salivarius* (7). In addition, the *S. salivarius* strain did not produce bacteriocin against *S. mutans* (data not shown). Taken together, our results indicate that the inhibiting substance of *S. salivarius* reacts with CSP to inhibit the development of biofilm formation. Furthermore, it is not an antimicrobial agent, such as acid or bacteriocin that causes direct injury to *S. mutans* cells or a biological agent such as urease.

It is generally considered that the mechanisms employed by healthy microflora to interfere with the adhesion of invading pathogens include competitive exclusion (38), displacement (27), production of antibacterial compounds (15), and the release of biosurfactants (11). Recently, some investigators have proposed quorum sensing systems and virulence response as new targets for strategies to reduce the cariogenicity of organisms such as *S. mutans* (46, 48). Factors such as acid tolerance (47), genetic competence (22), and bacteriocin production (47) in those quorum sensing systems may be modulated by interspecies communication between cariogenic and other types of bacteria in oral biofilms. Another study found that biofilm formation by a *luxS* mutant of *S. mutans* GS5 was complemented by auto-inducer-2 (AI-2) produced by *S. gordonii*, but not that produced by *S. sanguinis* 10556 or *S. salivarius* HT9R (48). However, *S. salivarius* had a two-fold greater level of AI-2 activity than *S. mutans* GS5 in the luminescence assay using *Vibrio harveyi* BB170 and they also noted that additional factors were expressed by some streptococci, which also modulated AI-2 activity. AI-2 plays a role in interspecies signaling, and its concentration was found to be critical for mutualism between *S. oralis* and *Actinomyces naeslundii* grown under conditions that are representative of the human oral cavity (39). In another report, *S. sanguinis* 10556, *S. gordonii* 10558, and *S. mitis* 10712 inhibited bacteriocin production from *S. mutans* GS5, whereas bacteriocin production by *S. mutans* GS5Δ*comC* was restored with the addition of exogenous CSP (46). In the present study, CSP-dependent biofilm formation was also inhibited by *S. salivarius* HT9R. Therefore, auto-inducers including CSP may be target factors for the regulation of biofilm formation by multiple oral streptococci.

To determine whether the ComDE two-component signal transduction system or the alternative sigma factor ComX in addition to ComC, are required for biofilm formation and biofilm inhibition by *S. salivarius*, we performed the single biofilm formation with *comC*, *comD*, *ComE*, and *conX* mutants, and dual-

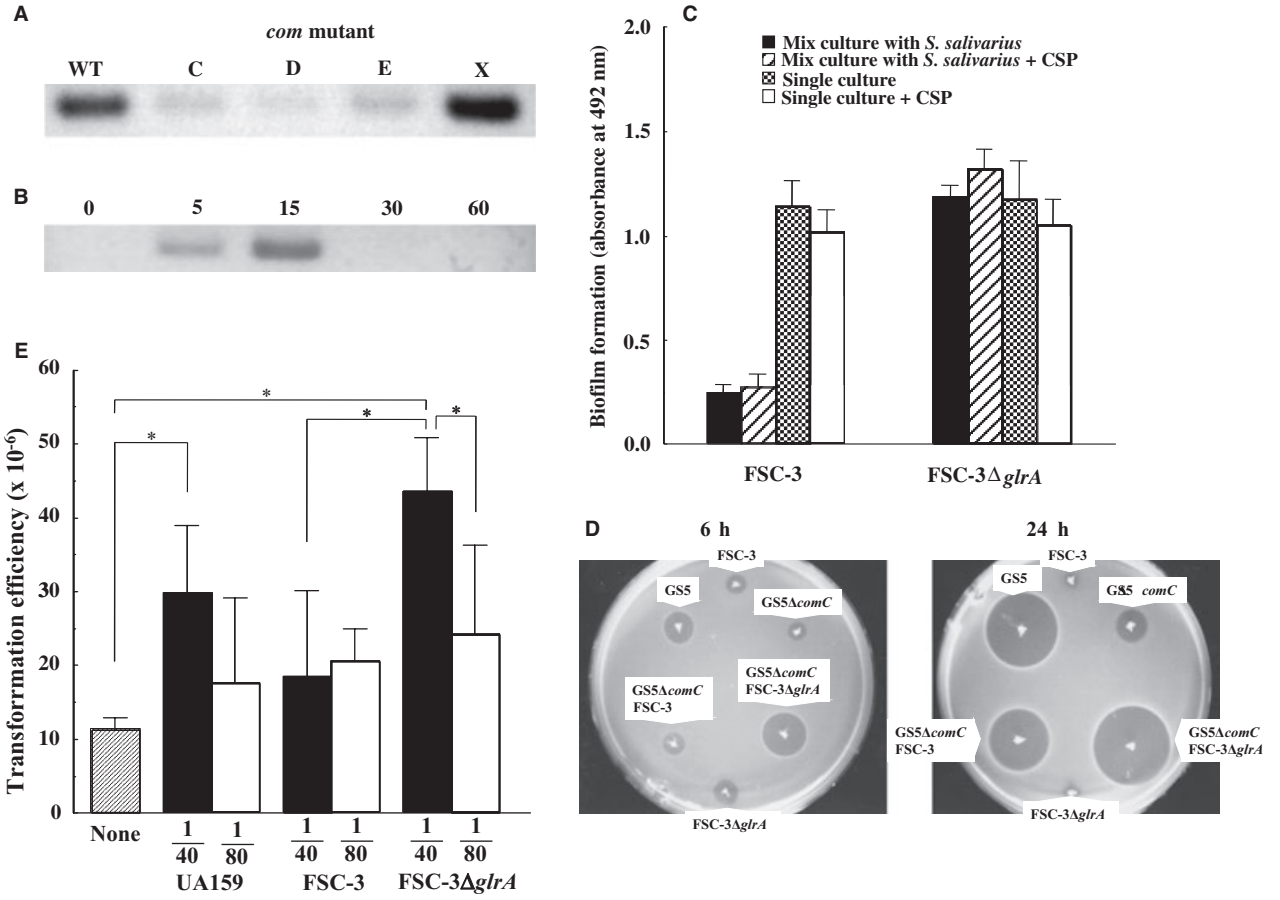


*Fig. 7.* Contribution of *glrA* to competence-stimulating peptide (CSP)-dependent biofilm formation. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *glrA* transcription was performed in log-phase culture without CSP of UA159 (wild-type; WT), UA159Δ*comC* (comC), UA159Δ*comD* (comD), UA159Δ*comE* (comE), and UA159Δ*comX* (comX) (A). An RNA sample isolated from UA159Δ*comC* at 0, 5, 15, 30, and 60 min after the addition of 0.2 μM CSP was subjected to RT-PCR with *glrA* (B). FSC-3 and FSC-3Δ*glrA* were each mixed with or without HT9R, with 0.2 μM CSP added. All mixtures were incubated for 16 h at 37°C under 5% $CO_2$ aerobic conditions in 96-well microtiter plates (C). The results are expressed as the mean ± SD of absorbance at 492 nm obtained in triplicate assays. The production of bacteriocin was observed after 6 and 24 h of single species cultures of FSC-3, FSC-3Δ*glrA*, GS5, and GS5Δ*comC*, and after dual-species cultures of GS-5Δ*comC* and FSC-3, and GS-5Δ*comC* and FSC-3Δ*glrA* (D). The streptococcal strain RP66 was used as an indicator strain. Representative data from three independent experiments are presented, with similar results obtained in each. pDL276 ($Ka^r$) was transformed into *Streptococcus mutans* UA159 stimulated with or without culture supernatant samples (1/40 and 1/80 dilution in final concentration) from *S. mutans* UA159, FSC-3 and FSC-3Δ*glrA* (E). Transformation frequency was determined from the ratio of the number of transformants vs. that of the total viable recipients, multiplied by 100. The results were obtained from four independent assays and are expressed as the mean ± SD. Asterisks denote significantly different relative levels of transformation ($P < 0.01$).

species biofilm formation with the mutants and *S. salivarius*. These results indicated that the CSP-dependent regulatory systems were involved with the biofilm inhibition of *S. mutans*. *S. salivarius* produces an inhibiting factor for the biofilm formation of *S. mutans*, but dual-species biofilm formation was poor and lower than that for single biofilm of these mutants. Therefore, *S. salivarius* may have other inhibiting mechanisms in addition to CSP inactivation.

Dual-species cultures of *S. salivarius* and the *S. mutans* clinical strain FSC-3 had poor biofilm development, whereas those produced in cultures with the *glrA*-deficient mutant, which is regulated by CSP in the quorum sensing system, were substantial. These findings indicate that the interactions between *glrA* in *S. mutans* and CSP-inhibiting substances from *S. salivarius* might be significantly associated with susceptibility to biofilm inhibition by *S. salivarius*. Our results also suggest that *glrA* is expressed by CSP, inhibits CSP production, and regulates biofilm formation in mixed cultures of *S. mutans* and *S. salivarius*. Therefore, *glrA* may respond to excess CSP or to inactivation of CSP by *S. salivarius*, and regulate CSP production and biofilm formation during autoregulation of the *com*-dependent quorum sensing system. In contrast, that resistance to inhibition by *S. salivarius* may not be dependent on morphological changes, because an increase in CSP may relate to the change in biofilm mass in the bottom and top areas of biofilms formed on a hard surface by FSC-3Δ*glrA* and the inhibition occurred in the early phase of biofilm formation. Therefore, the interaction between the regulation system by *glrA* of CSP and CSP inactivation by the inhibiting substance is an important function for cell-to-cell communication between biofilm-forming bacteria such as *S. mutans* and commensal streptococci such as *S. salivarius*.

Several genes involved in biofilm formation have been identified in a variety of organisms (22, 28, 36, 37, 39, 49), though little is known regarding the responsible substances in biofilm formations with mixed species cultures (39, 45, 46). In the present dual-species experiments, we found that the *glrA* gene was associated with susceptibility to biofilm inhibition by

commensal bacteria and was involved in regulating the production of CSP in cariogenic bacteria. However, scant information is available regarding the mechanisms that are involved with transducing CSP signals between *glrA* and other genes or proteins that control biofilm development. Therefore, definitive conclusions regarding the molecular mechanisms used to control the development of biofilms formed by a mixture of species require further investigation to determine how these signals are sensed and how they interact with *glrA* in biofilm-forming bacteria, as well as to elucidate other possible inhibitors of biofilm streptococci.

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