J Infect Chemother 23 (2017) 634–641

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Original Article

Inhibiting effects of fructanase on competence-stimulating peptide-dependent quorum sensing system in *Streptococcus mutans*

Yusuke Suzuki [a, b], Ryo Nagasawa [b], Hidenobu Senpuku [b, *]

[a] Department of Maxillofacial Surgery, Nihon University Graduate School of Dentistry at Matsudo, Chiba, Japan
[b] Department of Bacteriology I, National Institute of Infectious Diseases, Tokyo, Japan

ARTICLE INFO

Article history:
Received 12 October 2016
Received in revised form
22 May 2017
Accepted 16 June 2017
Available online 17 July 2017

Keywords:
Streptococcus mutans
Biofilm formation
Quorum sensing system
Competence-stimulating peptide
Fructanase

ABSTRACT

Streptococcus mutans produces glucosyltransferases encoded by the *gtfB* and *gtfC* genes, which synthesize insoluble glucan, and both insoluble and soluble glucans by conversion of sucrose, and are known as principal agents to provide strong biofilm formation and demineralization on tooth surfaces. *S. mutans* possess a Com-dependent quorum sensing (QS) system, which is important for survival in severe conditions. The QS system is stimulated by the interaction between ComD {Receptor to competence-stimulating peptide (CSP)} encoded by the *comD* and CSP encoded by the *comC*, and importantly associated with bacteriocin production and genetic competence. Previously, we found enzyme fructanase (FruA) as a new inhibitor for the glucan-dependent biofilm formation. In the present study, inhibiting effects by FruA on glucan-independent biofilm formation of *S. mutans* UA159, UA159.*gtfB*$^-$, UA159.*gtfC*$^-$, and UA159.*gtfBC*$^-$ were observed in sucrose and no sucrose sugars-supplemented conditions using the plate assay. The reduction of UA159.*comC*$^-$ and UA159.*comD*$^-$ biofilm formation were also observed as compared with UA159 in same conditions. These results suggested that inhibitions of glucan-independent and Com-dependent biofilm formation were involved in the inhibiting mechanism by FruA. To more thoroughly investigate effects by FruA on the QS system, we examined on CSP-stimulated and Com-dependent bacteriocin production and genetic transformation. FruA inhibited bacteriocin production in collaboration with CSP and genetic transformation in bacterial cell conditions treated with FruA. Our findings show that FruA has multiple effects that inhibit survival functions of *S. mutans*, including biofilm formation and CSP-dependent QS responses, indicating its potential use as an agent for prevention of dental caries.

1. Introduction

Streptococcus mutans is a member of the group of etiological bacteria that cause human dental caries [1–3]. Oral biofilm formation is associated with bacterial attachment to the acquired enamel pellicle, which includes salivary components, as well as ingestion and fermentation of foods containing sugar [2,3]. Colonization and maturing of bacteria are dependent on extracellular polysaccharide (EPS) [4] for bacterial survival in low pH conditions, and when anti-bacterial agents are introduced into the oral cavity [5,6]. *S. mutans* produces glucosyltransferases (GTFs) that form insoluble (α(1,3)-linked) and soluble (α(1,6)-linked) glucans resulting in the formation of sticky biofilm on tooth surfaces [7]. GTF I and GTF SI, which encode the *gtfB* and *gtfC* genes, synthesize both insoluble and soluble glucans by conversion of sucrose, and are known as principal agents to provide strong biofilm formation on tooth surfaces [7–9].

For survival, several different bacteria employ a quorum sensing (QS) system, which interacts with various environmental conditions in the oral cavity and functions as a bacterial intercellular signal mechanism for controlling gene expression in response to population density [10–12]. The Com-dependent QS system of *S. mutans* is known to be stimulated by the competence-stimulating peptide (CSP; amino acid sequence, SGSLSTFFRLFNRSFTQALGK) in conditions of high cell density [10,13]. Com-dependent QS systems mainly consist of various pathways that appear following bacteriocin production, genetic transformation, and acid tolerance, thus

* Corresponding author. Department of Bacteriology I, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo, 162-8640, Japan.
E-mail address: hsenpuku@nih.go.jp (H. Senpuku).

http://dx.doi.org/10.1016/j.jiac.2017.06.006
1341-321X/

QS pathways are controlled by various *com* genes, such as *comA*, *comB*, *comC*, *comD*, and *comE*, as well as others [10,14–18]. The ComCDE system in streptococci has been reported to be a very important model for genetic competence [11,14,15]. In this system, the CSP precursor is translated by ComC and released to outside of the cell. Then, CSP is sensed by a two-component system composed of the sensor-histidine kinase to ComD and response regulator to ComE [13,14,17,18]. Thereafter, it is activated by autophosphorylation and transfers the phosphoryl group to the ComE response regulator, after which response signals are continued for expression of cell activities [18].

Streptococcus salivarius is a numerically predominant oral bacteria and presented as a healthy commensal bacteria in human oral cavity. *S. salivarius* has been noted as an important source of safe and efficacious probiotics, capable of fostering more balanced, and health-associated oral microbiota [19,20]. Clinical trials using *S. salivarius* as a probiotic showed to reduce plaque formation and to lower colonization of *S. mutans* in primary school-aged children [19]. Our recently reported findings indicate that supernatant proteins from *S. salivarius* induce inactivation of CSP-dependent genetic transformation, and also inhibit glucan-dependent biofilm formation and bacteriocin production by *S. mutans* [21,22]. The protein that inhibited biofilm formation in our experiments was identified as fructanase (FruA), an exo-β-D-fructosidase enzyme encoded by the *fruA* gene [22–24]. FruA digests sucrose which is utilized as a substrate to form insoluble (α(1,3)-linked) and soluble (α(1,6)-linked) glucans, and fructan which is β(2,1)- and β(2,6)-linked extracellular fructose polymers, in *S. mutans* [22,25]. However, the function of FruA in the QS system has not reported. In the present study, to clarify the multiple effects of FruA in detail in regard to biofilm formation under various sugar conditions, CSP-dependent bacteriocin production and genetic transformation were examined in conditions with and without FruA. Our results show that FruA has multiple effects related to survival functions of *S. mutans*, including biofilm formation, bacteriocin production, and genetic transformation.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this study are described in Table 1. All strains 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/Dickinson, Sparks, MD) at 37 °C before use in the experiments.

2.2. Construction of mutants

The *gtf* and *com* genes were identified in the *S. mutans* UA159 database (http://www.genome.jp/kegg/), and mutants were constructed using double-crossover homologous recombination via insertion of the resistance determinants kanamycin (Kan; Sigma-Aldrich, St. Louis, MO) and erythromycin (Erm; Sigma-Aldrich) into each gene, as previously described [26]. For polymerase chain reaction (PCR) assays, fragments of the upstream and downstream regions of the *gtf* and *com* genes were amplified with primer pairs (Table 2).

Table 1
Bacterial strains.

Strain	Genotype or phenotype	Reference
Streptococcus mutans		
UA159	Erm, serotype c human isolate	
gtfB mutant	Erm, UA159 derived, *gtfB* deficient	Ref. 26
gtfC mutant	Kan, UA159 derived, *gtfC* deficient	Ref. 26
gtfBC mutant	Erm, Kan, UA159 derived, *gtfBC* deficient	Ref. 26
comC mutant	Erm, UA159 derived, *comC* deficient	Ref. 26
comD mutant	Erm, UA159 derived, *comD* deficient	Ref. 26
GS5	Erm, serotype c human isolate	
comC mutant	Erm, GS5 derived, *comC* deficient	Ref. 26

2.3. Human saliva collection

Whole saliva samples were collected from 3 healthy human volunteers (27–32 years old) after stimulation by chewing paraffin gum and pooled into ice-chilled sterile bottles over a period of 5 min. The samples were clarified by centrifugation at 10,000×*g* for 10 min at 4 °C, sterilized using a 0.22-μm Millex-GP Filter Unit (Merck Millipore, Darmstadt, Germany), and coated onto wells in plates (Sumitomo Bakelite, Tokyo, Japan) for biofilm formation assays.

2.4. Fructanase

For the present study, we used a FruA mixture (Megazyme, Wicklow, Ireland) that included exo-inulinase (FruA)-to-endo-inulinase unit ratios of 10:1 or less of FruA activity. FruA is the sole enzyme involved in utilization of β(2,1)- and β(2,6)-linked extracellular fructose polymers by *S. mutans* [27]. Furthermore, this enzyme has an ability to digest sucrose to fructose and glucose, as well as convert fructan to fructose by hydrolyzation, and is also an important component for the virulence of *S. mutans*.

2.5. Biofilm formation assays

Biofilm formation of by each of the examined strains was assayed using a method previously described, with slight modifications [22]. Briefly, 96-well flat-bottom microtiter plates were coated with whole human saliva and incubated at 4 °C for 60 min. Saliva was removed and the wells were rinsed twice with sterile phosphate-buffered saline (PBS). To evaluate the effects of FruA on biofilm formation by each bacterial cell suspension, the cell suspension was adjusted to OD_{600} = 0.4 using tryptic soy broth without dextrose (TSB; Difco Laboratories), supplemented with 0.25% sucrose (TSBs), 0.25% glucose (TSBg), or 0.25% fructose (TSBf). Thereafter, 20 μl of each cell suspension was mixed with 20 μl of FruA (final concentration, 57 unit/ml) in 160 μl of TSB, TSBs, TSBg, or TSBf in human saliva-coated 96-well flat-bottom microtiter plates. Each biofilm formation assay was performed at 37 °C for 16 h under an aerobic condition with 5% CO_2. After the plates were incubated,

Table 2
Primers for the construction of mutans.

Primer	Nucleotides sequence (5'→3')	Amplicon
GtfBuF	ccccgaattcACAGTTGACAAAACTTCTGAAGC	*gtfB*
GtfBuR	ccccggtaccGCTCTGTGCAGAGCGATCATAAAC	*gtfB*
GtfBdF	cccctctagaTGATGATACAAGTAATCAATTGC	*gtfB*
GtfBdR	ccccaagcttATAGTGTTATCAGCTGTATATC	*gtfB*
GtfCuF	cccccgaattcTGAGTGGTGTATGGCGTCAC	*gtfC*
GtfCuR	cccccggtaccGACCGTTAATGGTTCTGGC	*gtfC*
GtfCdF	cccccctctagaAAACTCTGACTGCTACTGATAC	*gtfC*
GtfCdR	cccccaagcttGAGCAAAGCTGTTAGTGTTATCA	*gtfC*
ComCuF	cccccgaattcAAATCTGAACAAGCAGGGG	*comC*
ComCuR	cccccggtaccGATAGTGTTTTTTTCATTTTATATCTCC	*comC*
ComCdF	cccccctctagaGCCTATCAACATTTTTCCGGC	*comC*
ComCdR	cccccaagcttCCACTAAAGGCTCCAATCGC	*comC*
ComDuF	cccccgaattcCCATTCATCTGAAACTCAGT	*comD*
ComDuR	cccccggtaccAACAGGCAGCAGACCATAA	*comD*
ComDdF	cccccctctagaGGCGGGCAATCATATTCTT	*comD*
ComDdR	cccccaagcttTCCTGCAATTGATGTCCTG	*comD*

liquid medium was removed and the wells were rinsed twice with sterile PBS. The plates were then air dried and stained with 0.25% safranin (MUTO Pure Chemicals, Tokyo, Japan) for 15 min, then rinsed twice with sterile distilled water (DW) to remove excess dye and air dried. The biofilm mass was dissolved with 70% ethanol and measured using a microplate reader (Thermo Bioanalysis Japan, Tokyo, Japan). Quantification of stained biofilms was performed by measuring absorbance at 492 nm.

2.6. Measurement of insoluble and soluble polysaccharides

Insoluble and soluble polysaccharides extraction from *S. mutans* biofilms were assayed using a method previously described, with slight modifications [9]. Briefly, 350 μl of cell confluent suspension ($OD_{600} = 0.4$) was mixed with 2650 μl of TSB, TSBs, TSBg, or TSBf and added to the wells of a 6-well flat-bottom titer plate. After inoculation, the plates were incubated at 37 °C for 16 h under an aerobic condition with 5% CO_2. The biofilms on bottom of the wells in culture supernatant were removed with a sterilized scraper. Biofilm and culture supernatant samples were transferred into centrifugation tube and separated by centrifugation at 10,000×*g* for 10 min. To extract insoluble polysaccharide, the pellets were treated with 3 ml, 1 N NaOH at 37 °C for 2 h, and the biofilm cells were then removed by centrifugation at 10,000×*g* for 10 min. The supernatants containing insoluble polysaccharide were precipitated with 6 ml, 99.5% (v/v) ethanol at −20 °C for 2 h. The precipitate was washed three times with 70% ethanol by centrifugation at 10,000 g for 30 min at 4 °C, then dissolved in DW and used as an insoluble glucan sample. The culture supernatants were filtered through a 0.22-μm Millex-GP Filter Unit and precipitated with 6 ml, 99.5% (v/v) ethanol at −20 °C for 2 h. The precipitate was washed three times with 70% ethanol by centrifugation at 10,000 g for 30 min at 4 °C, then dissolved in DW and used as a soluble glucan sample. Carbohydrate contents in both soluble and insoluble glucan were determined by the phenol-sulfuric acid method.

2.7. Bacteriocin production assays

Bacteriocin production was assayed using a method previously described, with slight modifications [21]. Briefly, 2 μl CSP (1 mM) was pre-treated with 18 μl FruA (15, 29, 57, 114, and 228 unit/ml) in sterile DW at 37 °C for 1 h. Next, 4 μl of each CSP solution was pre-treated with FruA (final concentration, 10 μM CSP) and mixed with 36 μl of a cell suspension (*S. mutans* GS5 or *S. mutans* GS5.*comC*$^-$, $OD_{600} = 0.5$) in TSBs, then spread onto BHI 2.5% agar plates and incubated at 37 °C for 24 h. The plates were overlaid with 4 ml of BHI 1% agar broth including the indicator strain Streptococcus RP66 ($OD_{600} = 0.3$) diluted to 1:100 and incubated at 37 °C for 24 h. Finally, the inhibition zone diameter of each sample was measured. As a control, we used bovine serum albumin (BSA; IWAI Chemicals, Tokyo, Japan) at the same concentrations as FruA.

2.8. Genetic transformation assays

Transformation efficiency was assayed using a method previously described, with slight modifications [21]. Briefly, 2 μl CSP (final concentration, 100 μM) was pre-treated with 18 μl FruA (final concentration, 285 unit/ml) in sterile DW at 37 °C for 1 h and used for the genetic transformation assays. Overnight cultures of *S. mutans* UA159 were diluted 1:20 in BHI medium containing horse serum (10%, vol/vol; Invitrogen, Waltham, MA), then 200 μl diluted cell suspension aliquots were incubated at 37 °C for 4 h under an aerobic condition with 5% CO_2, and then added with or without 1 μM CSP pre-treated with FruA. After incubation at 37 °C for 30 min to assess genetic transformation, cell suspensions were exposed to 700 ng of plasmid pDL276 with a Kan resistance gene. After 2 h of incubation at 37 °C under an aerobic condition with 5% CO_2, the cultures were poured onto BHI agar plates with or without 500 μg/ml Kan. Transformation efficiency was determined after 48 h of incubation at 37 °C under an aerobic condition with 5% CO_2 and is expressed as the percentage of transformants among total viable recipient cells. As a control, BSA was used at the same concentration as FruA.

To clarify the effects of FruA, as an additional effect induced by another method, FruA (final concentration, 15, 29, 57, 114, and 228 unit/ml) was added to the final condition including the diluted cell suspensions and plasmid. Moreover, to characterize its thermal properties, heat-treatment (100 °C for 30 min) of concentrated FruA was also performed.

2.9. Statistical analysis

The statistical significance of differences between groups was determined using Student's *t*-test. For comparisons among multiple groups, one-way analysis of variance (ANOVA) and Bonferroni correction were used. *P*-values of 0.001, 0.01, and 0.05 were considered to indicate a significant difference.

3. Results

3.1. Effects of FruA on biofilm formation by S. mutans in various medium conditions

To confirm production of insoluble glucan and soluble glucan in *S. mutans* UA159 wild type, UA159.*gtfB*$^-$, UA159.*gtfC*$^-$, and UA159.*gtfBC*$^-$ in TSB supplemented with carbohydrates, measurement of polysaccharide was performed in biofilm formation (Fig. 1). In TSBs, significant production of insoluble glucan was recognized in wild type, UA159.*gtfB*$^-$ and UA159.*gtfC*$^-$ but not in UA159.*gtfBC*$^-$ as compared with those in TSB. Amounts of insoluble glucans in UA159.*gtfB*$^-$ and UA159.*gtfC*$^-$ were lower than amounts in wild type. In contrast, significant production of soluble glucans was recognized in all strains, and amounts of soluble glucans in UA159.*gtfC*$^-$ and UA159.*gtfBC*$^-$ were lower than amounts in wild type and UA159.*gtfB*$^-$. In TSBg and TSBf, production of insoluble and soluble glucans were not significantly recognized in all strains, as compared with those in TSB.

As a next, the biofilm formation was measured in same media and strains as previous assay. The level of biofilm formed by *S. mutans* UA159 wild type was slightly up-regulated when incubated with TSBs, TSBg, and TSBf, as compared to with TSB (Fig. 2A). Furthermore, biofilm formation was significantly inhibited by FruA in cultures with TSBs and TSBg, but not with TSBf (Fig. 2A). Interestingly, biofilm formation was also significantly inhibited by FruA when cultured with TSB.

In our next experiment, the effects of FruA on biofilm formation by *S. mutans* UA159.*gtfB*$^-$, in which the contribution of insoluble glucan to biofilm formation is absent, was investigated (Fig. 2B). In cultures with TSB and TSBs, biofilm formation was significantly inhibited by FruA. However, significant inhibition by FruA was not observed in cultures with TSBg and TSBf. Findings obtained with *S. mutans* UA159.*gtfC*$^-$ show reductions of both insoluble and soluble glucans in biofilm formation. Biofilm formation was significantly inhibited by FruA in cultures with TSB, TSBs, and TSBg, but not with TSBf (Fig. 2C). In contrast, FruA did not show significant inhibition of biofilm formation by *S. mutans* UA159.*gtfBC*$^-$, which is completely absent of production of insoluble and soluble glucans (Fig. 2D), whereas slight inhibition by FruA was observed in cultures with TSB, TSBs, and TSBg.

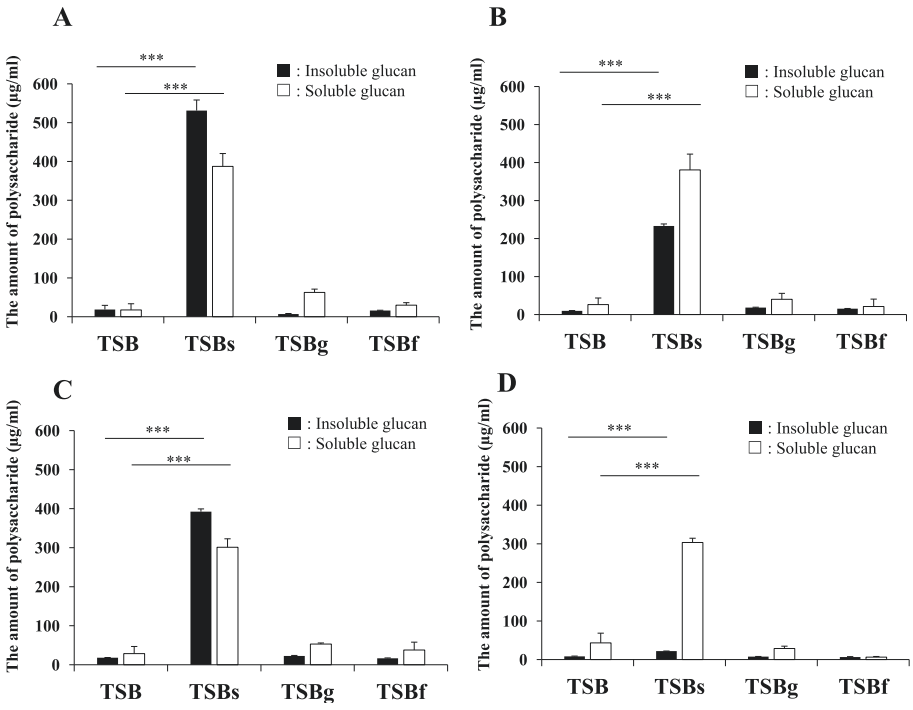

Fig. 1. Quantitative measurement of insoluble and soluble glucan. Insoluble and soluble glucans from *S. mutans* UA159 wild type (A), UA159.*gtfB*$^-$ (B), UA159.*gtfC*$^-$ (C), and UA159.*gtfBC*$^-$ (D) biofilm in cultures with TSB, TSBs, TSBg, and TSBf were extracted and quantitatively measured. Results were obtained from 3 independent assays and are expressed as the mean ± standard deviation (SD). Asterisks indicate significantly different relative levels of polysaccharides production (***$P < 0.005$).

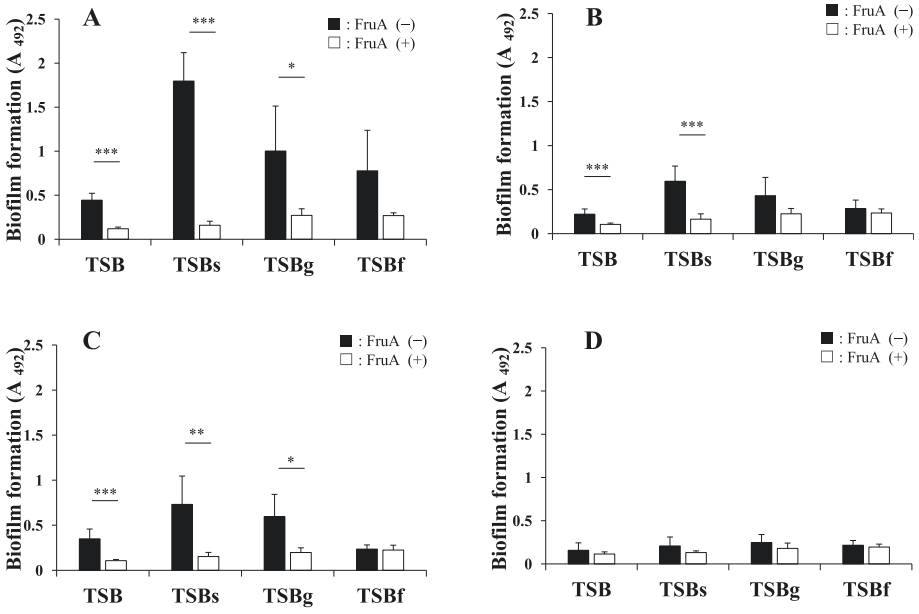

Fig. 2. Effects of FruA on biofilm formation by *S. mutans gtf* mutants in various medium conditions. Effects of FruA (final concentration, 57 unit/ml) on biofilm formation by *S. mutans* UA159 (A), UA159.*gtfB*$^-$ (B), UA159.*gtfC*$^-$ (C), and UA159.*gtfBC*$^-$ (D) in TSB, TSBs, TSBg, and TSBf were observed in human saliva-coated 96-well flat-bottom microtiter plates. Results were obtained from 3 independent assays and are expressed as the mean ± SD. Asterisks indicate significantly different relative levels of biofilm formation (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$).

The QS system is considered to be important for biofilm formation and bacterial cell survival in severe conditions. To confirm the contribution of the QS system to biofilm formation, *S. mutans* UA159.*comC*$^-$ and UA159.*comD*$^-$ were constructed, and used to examine the effects of FruA on biofilm formation. Biofilm levels were significantly reduced by the *comC* and *comD* mutations in *S. mutans* UA159 in cultures with TSBs (Fig. 3A and B). Biofilm levels were reduced by mutations, whereas no significant differences were seen in cultures with TSB, TSBg, and TSBf (Fig. 3A and B). Therefore, we concluded that a Com-dependent QS system contributes to biofilm formation in sugars-supplemented condition. In addition, biofilm formation by *S. mutans* UA159.*comC*$^-$ and UA159.*comD*$^-$ were significantly inhibited by FruA in all of the present medium conditions (Fig. 3A and B).

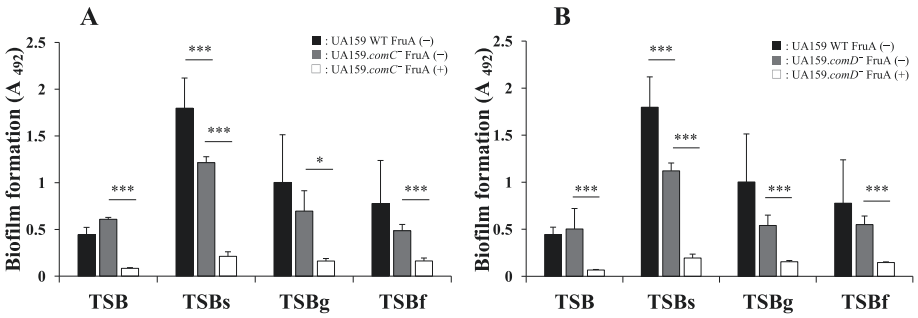

Fig. 3. Effects of FruA on biofilm formation by *S. mutans com* mutants in various medium conditions. Effects of FruA (final concentration, 57 unit/ml) on biofilm formation by *S. mutans* UA159.*comC*⁻ (A) and UA159.*comD*⁻ (B) in TSB, TSBs, TSBg, and TSBf were observed in human saliva-coated 96-well flat-bottom microtiter plates. Results were obtained from 3 independent assays and are expressed as the mean ± SD. Asterisks indicate significantly different relative levels of biofilm formation (*$P < 0.05$ and ***$P < 0.005$).

3.2. Effects of FruA on bacteriocin production by S. mutans

To confirm the effects of FruA on the Com-dependent QS system, CSP-dependent bacteriocin production assays were performed using cultures with CSP treated with or without FruA. *S. mutans* GS5 showed significant inhibition of growth of an indicator strain, while *S. mutans* GS5.*comC*⁻ did not (Fig. 4A). However, bacteriocin production by the latter strain was restored by addition of CSP. FruA treatment to CSP inhibited bacteriocin production in *S. mutans* GS5.*comC*⁻ in a dose-dependent manner (Fig. 4C and D). Furthermore, BSA caused no significant inhibition of bacteriocin production in all of the tested conditions (Fig. 4B and C).

3.3. Effects of FruA on genetic transformation of S. mutans

The genetic transformation of *S. mutans* is mainly controlled by the CSP-dependent pathway in the QS system. To investigate the effects of FruA on CSP-dependent genetic transformation in *S. mutans*, we performed genetic transformation assays using cultures with CSP treated with or without FruA. Direct treatment with FruA to CSP at a high concentration of 285 unit/ml did not show significant effects on bacterial activities (Fig. 5A). Furthermore, BSA at the same concentration, used as a control, also showed no significant influence (Fig. 5B). In culture using CSP treated with FruA, we observed clear effects of FruA on bacteriocin production by *S. mutans* but not on genetic transformation of the bacterium. As another test of the effects of FruA, we examined receptors on bacterial cells considered to be targeted by FruA. For these assays, FruA was added to the final conditions including the diluted cell suspensions and plasmid. Our results showed that FruA at 15 and 29 units/ml inhibited genetic transformation as compared with control without FruA, with no significant difference between treatments with and without FruA (Fig. 5C). In addition, when added at concentrations greater than 57 units/ml, FruA showed a significantly greater inhibition of genetic transformation of the bacterium (Fig. 5C). Genetic transformation inhibition by FruA was restored by heat-treatment of FruA at various concentrations (Fig. 5C). In the present assay, equal protein concentrations were

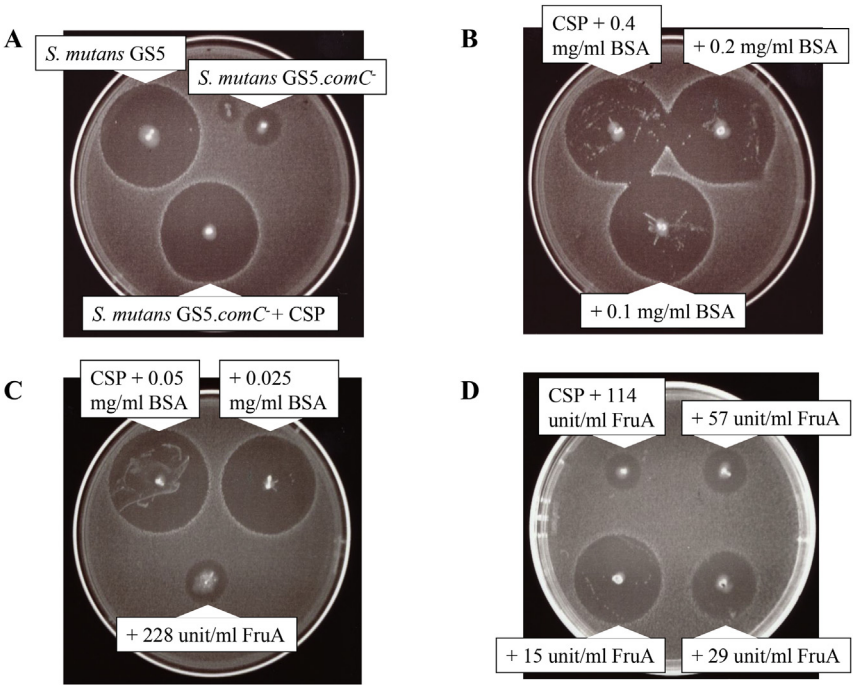

Fig. 4. Effects of FruA on bacteriocin production by *S. mutans*. Production of bacteriocins were observed in *S. mutans* GS5 or *S. mutans* GS5.*comC*⁻, and *S. mutans* GS5.*comC*⁻ with CSP treated with and without FruA (15, 29, 57, 114, and 228 unit/ml). As a control, BSA was used at the same concentrations as FruA. Results shown are representative of 3 independent assays.

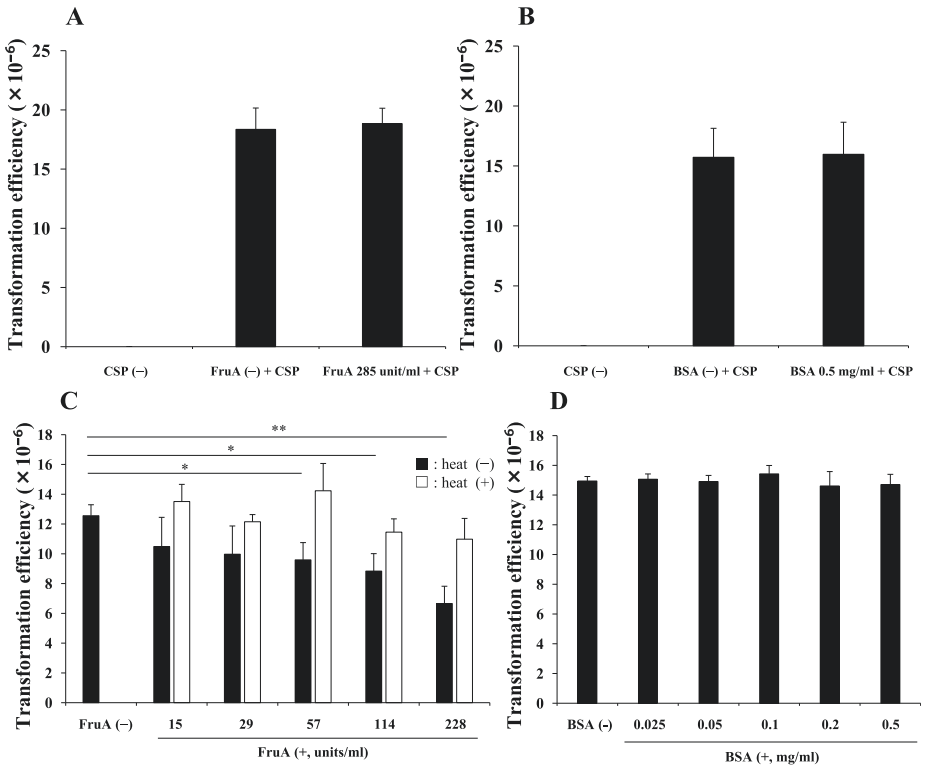

Fig. 5. Effects of FruA on genetic transformation of *S. mutans*. To examine the direct effect of FruA on CSP activity, CSP was pre-treated with FruA (final concentration, 285 unit/ml) in sterile DW at 37 °C for 1 h. Next, cell suspensions were incubated with or without CSP pre-treated with FruA (A). As control, BSA was used at the same concentration as FruA (B). To clarify its effects, FruA (final concentration 15, 29, 57, 114, and 228 unit/ml) was added to the final condition, including the diluted cell suspension and plasmid. Effects of FruA were compared with control without FruA (FruA (−)). Moreover, to characterize its thermal properties, heat-treatment (100 °C, 30 min) of various concentrations of FruA was performed (C). As a control, BSA was used at the same concentrations as FruA (D). Genetic transformation efficiency was examined by determining the ratio of number of transformants as compared to that of the total viable recipients. Results shown were obtained from 3 independent assays and are expressed as the mean ± SD. Asterisks indicate significantly different relative levels of transformation ($^{*}P < 0.05$ and $^{**}P < 0.01$).

determined in BSA and used as a control. However, no significant effects were observed (Fig. 5D).

4. Discussion

Biofilm formation by *S. mutans* occurs by a process of various steps including initial bacterial attachment, colonization, aggregation, and mature formation with polysaccharides such as glucans utilized. Among these steps, glucan-dependent biofilm formation is very important for long-term colonization and survival, and acquisition of resistance to anti-bacterial agents in the oral cavity. In the present study, FruA inhibited biofilm formation in not only cultures with TSBs, and but also those with TSBg and TSBf, which do not produce glucan. These findings show that FruA has additional functions to inhibit the glucan-independent biofilm formation, though it has effect on glucan-dependent biofilm formation.

In a previous report, a biofilm defective mutant of *Streptococcus gordonii* was formed by insertion of the *comD* gene with a transposon [28], and their findings demonstrated that ComD is an important molecule in biofilm formation. Moreover, other studies investigated biofilm formation by *comC, comD*, and *comE S. mutans* mutants in comparison to that by the wild type [12,14], and found that the mutant strains formed biofilms with abnormal morphology and biomass. In the present study, as compared to the wild type, the *comC* and *comD* mutants showed reduced biofilm volume in media supplemented with both sucrose and non-sucrose sugars. Therefore, the Com-dependent QS system is one of functions that are important for biofilm formation, except for glucan-dependent biofilm. FruA inhibited the biofilm formation of wild type until similar level to the biofilm formation inhibited by FruA in UA159.*comC* and UA159.*comD* mutants. These suggested that FruA had inhibiting abilities to both Com-dependent and -independent biofilm formations. Taken together, we suggested that FruA had multiple functions to inhibit both glucan-dependent biofilm formation in the presence of sucrose, and glucan-independent biofilm formation related to the Com-dependent and -independent QS systems in the presence of sugars.

The inhibiting effects of FruA on the QS system could not be completely confirmed from only findings related to biofilm formation because various related physical factors such as attachment, aggregation, extracellular DNA (eDNA) and EPS production in biofilm formation were not directly associated with the QS system. Com-dependent QS system is caused by signal responses following bacteriocin production and genetic transformation. Therefore, we examined the direct effects of FruA on the QS system of *S. mutans* in regard to bacteriocin production and genetic transformation. Bacteriocin is an antibiotic peptide, with that produced by *S. mutans* termed mutacin, and is presented as various types of mutacins, which show activities against closely related species as well as other gram-positive bacteria [11,18]. First, we examined the direct effects of FruA on CSP-dependent bacteriocin production and genetic transformation, and found that it inhibited CSP-dependent bacteriocin production when CSP was directly treated with FruA. CSP undergoes processing by SepM and activates the two-component ComDE system, after which the activated ComE response regulator stimulates the expression of mutacin-encoding genes [12,18]. It is considered that CSP activities are inhibited by FruA before CSP induces the signal activities of the two-component

ComDE system, and bacteriocin production is inhibited by inactivation of CSP. Thus, CSP may be a target for inhibition of bacteriocin production by FruA in the QS system. Bacteriocin and autolysin induce the production of eDNA, which helps with initial attachment and early biofilm formation [29,30]. During the development of mature biofilm, cell death and lysis occur inside of the microcolony, releasing eDNA, which controls the stability of the biofilm structure [31–34]. The present study is the first to show that FruA inhibits production of bacteriocin in the QS system, and may also depress eDNA-dependent initial attachment, early biofilm formation, and biofilm maturity formation in a glucan-independent system.

Our genetic transformation assays revealed that FruA did not inhibit CSP-dependent genetic transformation when directly treated with CSP. Therefore, we speculated that CSP was not a target for inhibition by FruA in the present genetic transformation assay that used pre-treatment of CSP. To observe the effects of FruA in another manner, FruA was additionally applied to bacterial cells under the same final condition as used in that assay, which showed that FruA significantly inhibited genetic transformation of *S. mutans* in a condition that included a mixture of CSP, plasmid pDL276, and bacterial cells. These findings suggest that FruA inhibits the activities of the two-component system on the cell surface or other activities related to transformation of plasmid pDL276. Thus, CSP may not be a target factor for inhibition of genetic competency by FruA in the response of bacterial cell surface components, though we concluded that FruA is an inhibitor of the Com-dependent QS system in *S. mutans*.

FruA showed a higher level of inhibition of bacteriocin production as compared to that of genetic transformation. Bacteriocin production is mainly associated with the CSP-dependent Com pathway [18,35]. *S. mutans* possesses both ComCDE and ComRS systems, which have been shown to regulate genetic competence [11,36]. ComR directly activates *comX* and ComX activities, which are also induced by 2 kinds of signal peptides, CSP-dependent and sigX-inducing peptide (XIP)-dependent competence regulator [11,36]. A previous report showed that CSP does not directly regulate the QS signaling cascades between Com- and XIP-dependent pathways [37]. However, genetic competence is induced via the XIP signaling cascade, which is not regulated by the CSP signaling cascade. Therefore, FruA is not directly associated with CSP in regard to genetic competency, and may inhibit the QS signaling cascades between the Com- and XIP-dependent pathways in the cells.

There is another QS system of LuxS-mediated autoinducer 2 (AI-2) in Gram-positive and -negative bacteria [38,39]. AI-2 is produced to function as a universal QS signal for interaction between bacterial species. *LuxS* mutant was shown to impair biofilm growth, reduced tolerance to acid killing, genetic competence and the production of lantibiotic mutacin I in *S. mutans* [40]. Down-regulation of the *comC* gene 2, 3-fold caused by the *luxS* mutation may be responsible for an attenuated bacteriocin production in the *luxS* mutant strain [41]. Recently, Trappetti et al. reported that FruA was a candidate receptor for AI-2 in *Streptococcus pneumoniae* [42]. Therefore, it is hypothesized that exogenous FruA may bind to autoinducers including CSP, such as AI-2 and inhibit CSP-based signaling which induce genetic competency and bacteriocin production (Fig. 5).

In summary, when FruA is applied through the biofilm formation of *S. mutans* in condition with sucrose, FruA digests sucrose which is utilized as a substrate to form glucan-dependent biofilm, and fructan synthesized from sucrose, which is utilized as a nutritious source in biofilm [22] (Fig. 6). In addition to these effects on biofilm formation in condition with sucrose, FruA loses glucan-independent and QS-dependent abilities in condition with various sugars including sucrose (Fig. 6). These new aspects are notable for development of new agent in oral hygiene to prevent biofilm-associated infectious diseases including dental caries

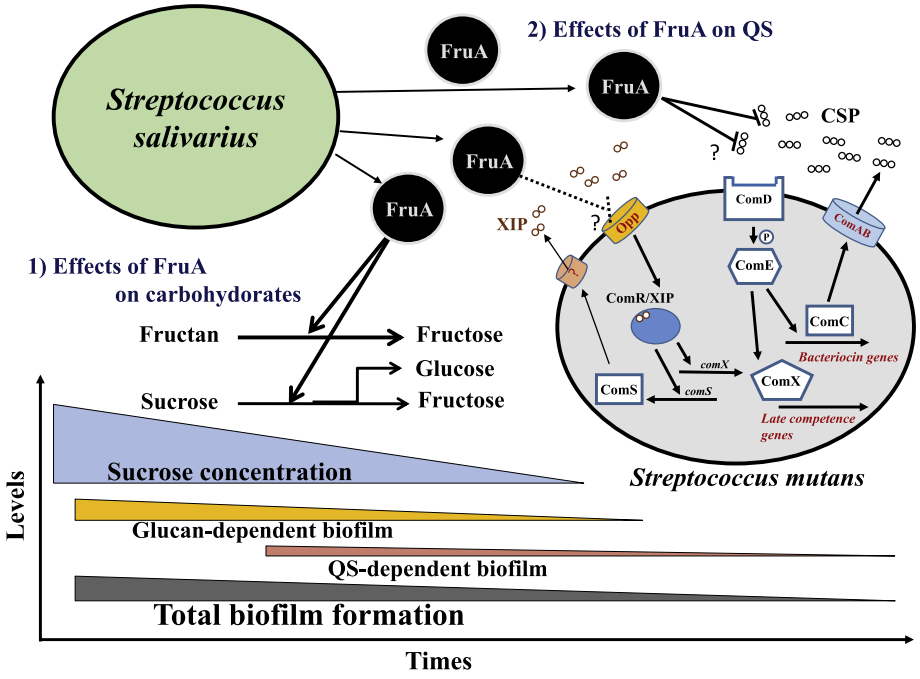

Fig. 6. Schematic image of FruA inhibiting effects on biofilm formation and CSP-responses in *S. mutans*. In previous reports, FruA secreted from *S. salivarius* digested fructan and sucrose to fructose, and glucose and fructose (1). In this study, the inhibition effects by FruA on CSP responses were proposed as one of mechanisms for inhibition of glucan-independent biofilm formation (2). The decrease of sucrose concentration by FruA leaded to the decrease of glucan-dependent biofilm formation and the decrease of QS signals by FruA leaded to the decrease of glucan-independent biofilm formation. Taken together, total biofilm volume was decreased by the inhibition of both glucan-dependent and -independent biofilm formations.

because oral biofilm is formed in condition with not only sucrose, and but also in condition with other sugars. Chemical substances such as triclosan, cetylpyridinium chloride, dextranase, chlorhexidine, etc. were conventionally used as anti-microbial and biofilm agents for oral rinse and paste [43–45]. They do not have additive effects on QS system, which was observed by the treatment of FruA in this study. FruA may be useful for the development of new oral hygiene system. However, principal inhibiting activities of FruA in the interaction between CSP and ComD, and QS signaling cascades among the Com- and XIP- dependent pathways have yet to be clearly identified in the molecular system. It will be important as a future work to study the molecular interaction and chemical responses of FruA with these QS signaling cascades to clarify its multiple functions.

Conflicts of interest

The authors have no conflicts of interest to declare in regard to this study.

Acknowledgments

The authors thank Dr. Toshirou Kondoh and Dr. Naomi Ogura for their advice and valuable discussion. This research was supported in-part by a Grant-in-Aid for Development of Scientific Research (21390506, 24659821 and 16K11537) from the Ministry of Education, Science and Culture of Japan.

References

[1] Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev 1986;50:353–80.
[2] Hamada S, Slade HD. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol Rev 1980;44:331–84.
[3] Gibbons RJ, Cohen L, Hay DI. Strains of *Streptococcus mutans* and *Streptococcus sobrinus* attach to different pellicle receptors. Infect Immun 1986;52:555–61.
[4] Kuramitsu HK, He X, Lux R, Anderson MH, Shi W. Interspecies interactions within oral microbial communities. Microbiol Mol Biol Rev 2007;71:653–70.
[5] Bowden GH, Hamilton IR. Survival of oral bacteria. Crit Rev Oral Biol Med 1998;9:54–85.
[6] Sato Y, Senpuku H, Okamoto K, Hanada N, Kizaki H. *Streptococcus mutans* binding to solid phase dextran mediated by the glucan-binding protein C. Oral Microbiol Immunol 2002;17:252–6.
[7] Bowen WH, Koo H. Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. Caries Res 2011;45:69–86.
[8] Kuramitsu HK, Ingersoll L. Interaction of glucosyltransferases with the cell surface of *Streptococcus mutans*. Infect Immun 1978;20:652–9.
[9] Kawarai T, Narisawa N, Suzuki Y, Nagasawa R, Senpuku H. *Streptococcus mutans* biofilm formation is dependent on extracellular DNA in primary low pH conditions. J Oral Biosci 2016;58:55–61.
[10] Suntharalingam P, Cvitkovitch DG. Quorum sensing in streptococcal biofilm formation. Trends Microbiol 2005;13:3–6.
[11] Conrads G, de Soet JJ, Song L, Henne K, Sztajer H, Wagner-Döbler I, et al. Comparing the cariogenic species *Streptococcus sobrinus* and *S. mutans* on whole genome level. J Oral Microbiol 2014;6. 26189.
[12] Cvitkovitch DG, Li YH, Ellen RP. Quorum sensing and biofilm formation in Streptococcal infections. J Clin Investig 2003;112:1626–32.
[13] Li YH, Tang N, Aspiras MB, Lau PC, Lee JH, Ellen RP, et al. A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. J Bacteriol 2002;184:2699–708.
[14] Ahn SJ, Wen ZT, Burne RA. Multilevel control of competence development and stress tolerance in *Streptococcus mutans* UA159. Infect Immun 2006;74:1631–42.
[15] Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG. Natural genetic transformation of *Streptococcus mutans* growing in biofilms. J Bacteriol 2001;183:897–908.
[16] Hung DC, Downey JS, Ayala EA, Kreth J, Mair R, Senadheera DB, et al. Characterization of DNA binding sites of the ComE response regulator from *Streptococcus mutans*. J Bacteriol 2011;193:3642–52.
[17] Kim JN, Stanhope MJ, Burne RA. Core-gene-encoded peptide regulating virulence-associated traits in *Streptococcus mutans*. J Bacteriol 2013;195:2912–20.
[18] Hossain MS, Biswas I. An extracelluar protease, SepM, generates functional competence-stimulating peptide in *Streptococcus mutans* UA159. J Bacteriol 2012;194:5886–96.
[19] Burton JP, Drummond BK, Chilcott CN, Tagg JR, Thomson WM, Hale JD, et al. Influence of the probiotic *Streptococcus salivarius* strain M18 on indices of dental health in children: a randomized double-blind, placebo-controlled trial. J Med Microbiol 2013;62:875–84.
[20] Wescombe PA, Hale JD, Heng NC, Tagg JR. Developing oral probiotics from *Streptococcus salivarius*. Future Microbiol 2012;7:1355–71.
[21] Tamura S, Yonezawa H, Motegi M, Nakao R, Yoneda S, Watanabe H, et al. Inhibiting effects of *Streptococcus salivarius* on competence-stimulating peptide-dependent biofilm formation by *Streptococcus mutans*. Oral Microbiol Immunol 2009;24:152–61.
[22] Ogawa A, Furukawa S, Fujita S, Mitobe J, Kawarai T, Narisawa N, et al. Inhibition of *Streptococcus mutans* biofilm formation by *Streptococcus salivarius* FruA. Appl Environ Microbiol 2011;77:1572–80.
[23] Zeng L, Burne RA. Comprehensive mutational analysis of sucrose-metabolizing pathways in *Streptococcus mutans* reveals novel roles for the sucrose phosphotransferase system permease. J Bacteriol 2013;195:833–43.
[24] Zeng L, Das S, Burne RA. Genetic analysis of the functions and interactions of components of the LevQRST signal transduction complex of *Streptococcus mutans*. PLoS ONE 2011;6. e17335.
[25] Wen ZT, Burne RA. Analysis of *cis*- and *trans*-acting factors involved in regulation of the *Streptococcus mutans* fructanase gene (*fruA*). J Bacteriol 2002;184:126–33.
[26] Narisawa N, Kawarai T, Suzuki N, Sato Y, Ochiai K, Ohnishi M, et al. Competence-dependent endogenous DNA rearrangement and uptake of extracellular DNA give a natural variant of *Streptococcus mutans* without biofilm formation. J Bacteriol 2011;193:5147–54.
[27] Burne RA, Schilling K, Bowen WH, Yasbin RE. Expression, purification, and characterization of an exo-β-D-fructosidase of *Streptococcus mutans*. J Bacteriol 1987;169:4507–17.
[28] Loo CY, Corliss DA, Ganeshkumar N. *Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes. J Bacteriol 2000;182:1374–82.
[29] Moscoso M, Claverys JP. Release of DNA into the medium by competent *Streptococcus pneumoniae*: kinetics, mechanism and stability of the liberated DNA. Mol Microbiol 2004;54:783–94.
[30] Petersen FC, Tao L, Scheie AA. DNA binding-uptake system: a link between cell-to-cell communication and biofilm formation. J Bacteriol 2005;187:4392–400.
[31] Rice KC, Mann EE, Endres JL, Weiss EC, Cassat JE, Smeltzer MS, et al. The *cidA* murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*. Proc Natl Acad Sci U. S. A 2007;104:8113–8.
[32] Ma L, Conover M, Lu H, Parsek MR, Bayles K, Wozniak DJ. Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. PLoS Pathog 2009;5. e1000354.
[33] Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. Extracellular DNA required for bacterial biofilm formation. Science 2002;295. 1487.
[34] Jennings LK, Storek KM, Ledvina HE, Coulon C, Marmont LS, Sadovskaya I, et al. Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the *Pseudomonas aeruginosa* biofilm matrix. Proc Natl Acad Sci U. S. A 2015;112:11353–8.
[35] Yonezawa H, Kuramitsu HK. Genetic analysis of a unique bacteriocin, Smb, produced by *Streptococcus mutans* GS5. Antimicrob Agents Chemother 2005;49:541–8.
[36] Ahn SJ, Kaspar J, Kim JN, Seaton K, Burne RA. Discovery of novel peptides regulating competence development in *Streptococcus mutans*. J Bacteriol 2014;196:3735–45.
[37] Reck M, Tomasch J, Wagner-Döbler I. The alternative sigma factor sigX controls bacteriocin synthesis and competence, the two quorum sensing regulated traits in *Streptococcus mutans*. PLoS Genet 2015;11. e1005353.
[38] Miller MB, Bassler BL. Quorum sensing in bacteria. Annu Rev Microbiol 2001;55:165–99.
[39] Federle MJ, Bassler BL. Interspecies communication in bacteria. J Clin Investig 2003;112:1291–9.
[40] Merritt J, Kreth J, Shi W, Qi F. LuxS controls bacteriocin production in *Streptococcus mutans* through a novel regulatory components. Mol Microbiol 2005;57:960–9.
[41] Sztajer H, Lemme A, Vilchez R, Schulz S, Geffers R, Yip CYY, et al. Autoinducer-2-regulated genes in *Streptococcus mutans* UA159 and global metabolic effects of the luxS mutation. J Bacteriol 2008;190:401–15.
[42] Trappetti C, McAllister L, Chen A, Wang H, Paton W, Oggioni MR, et al. Autoinducer 2 signaling via the phosphotransferase FruA drives galactose utilization by *Streptococcus pneumoniae*, resulting in Hypervirulence. mBio 2017;8. e02269–16.
[43] van der Hoeven JS, Cummins D, Schaeken MJ, van der Ouderaa FJ. The effect of chlorhexidine and zinc/triclosan mouthrinses on the production of acids in dental plaque. Caries Res 1993;27:298–302.
[44] Ashley FP, Skinner A, Jackson PY, Wilson RF. Effect of a 0.1% cetylpyridinium chloride mouthrinse on the accumulation and biochemical composition of dental plaque in young adults. Caries Res 1984;18:465–71.
[45] Ciardi JE, Rosenthal AB, Bowen WH. Rapid quantitative determination of the effect of antiplaque agents and antisera on the growth, acid production, and adherence of *Streptococcus mutans*. J Dent Res 1981;60:756–62.