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Synergistic inhibition of Streptococcal biofilm by ribose and xylitol

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ABSTRACT

Streptococcus mutans and *Streptococcus sobrinus* are the major causative agents of human dental caries. Therefore, the removal or inhibition of these streptococcal biofilms is essential for dental caries prevention. In the present study, we evaluated the effects of ribose treatment alone or in combination with xylitol on streptococcal biofilm formation for both species. Furthermore, we examined the expression of genes responsible for dextran-dependent aggregation (DDAG). In addition, we investigated whether ribose affects the biofilm formation of xylitol-insensitive streptococci, which results from long-term exposure to xylitol. The viability of streptococci biofilms formed in a 24-well polystyrene plate was quantified by fluorescent staining with the LIVE/DEAD bacterial viability and counting kit, which was followed by fluorescence activated cell sorting analysis. The effects of ribose and/or xylitol on the mRNA expression of DDAG-responsible genes, *gbpC* and *dblB*, was evaluated by RT-qPCR. Our data showed that ribose and other pentose molecules significantly inhibited streptococcal biofilm formation and the expression of DDAG-responsible genes. In addition, co-treatment with ribose and xylitol decreased streptococcal biofilm formation to a further extent than ribose or xylitol treatment alone in both streptococcal species. Furthermore, ribose attenuated the increase of xylitol-insensitive streptococcal biofilm, which results in the reduced difference of biofilm formation between *S. mutans* that are sensitive and insensitive to xylitol. These data suggest that pentose may be used as an additive for teeth-protective materials or in sweets. Furthermore, ribose co-treatment with xylitol might help to increase the anti-cariogenic efficacy of xylitol.

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Abbreviations: DDAG, dextran-dependent aggregation; FACS, fluorescence-activated cell sorting.
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1. Introduction

Streptococcus mutans and *Streptococcus sobrinus* are the major causative agents of human dental caries and are considered to be the principle cariogenic bacteria of all of the oral streptococci.[1] Ecologically driven change in oral biofilms, primarily caused by *S. mutans* and *S. sobrinus*, is responsible for the disease. Thus, the removal of oral biofilms has been an essential target for the prevention of dental caries.[2]

In order to adapt to changes in environments, bacteria exchange signals with one another by synthesizing and releasing substances referred as autoinducers. Such autoinducers mediate quorum sensing through cell-to-cell signalling between bacteria, which enables population-based multicellularity.[3] One of the substances that plays an important role in this networking of bacteria is autoinducer 2 (AI-2).[4,5] Shao et al. showed that AI-2 is required for biofilm growth of *Aggregatibacter actinomycetemcomitans*.[6] Mutation of the gene responsible for AI-2 synthesis caused a remarkable decrease in biofilm formation of *Haemophilus influenzae*.[7] It is generally known that AI-2 flows into the inner cellular matrix in *S. mutans* and *S. sobrinus*.[8–10] This promotes glucan-binding protein C (*gbpC*) and *dblB* gene expression, thus inducing an increase in dextran-dependent aggregation (DDAG).[10] Furthermore, quorum sensing by AI-2 was also reported to play an important role in mutans streptococci,[11,12] suggesting an important role for AI-2 in the cariogenicity of these bacteria.

It is known that *Salmonella*, *Escherichia coli*, *A. actinomycetemcomitans*, and *Streptococcus gordonii* take in AI-2 using Lsr (LuxS-regulated) ABC (ATP binding cassette) transporter, which are the intracellular receptors of AI-2.[13–15] It is known that Lsr ABC transporter has homology to the ribose ABC transporter in *E. coli* and *Salmonella typhimurium*.[16] AI-2 shares structural similarity with pentose, as it bears a furanosyl borate diether form.[17] These resemblances are thought to cause AI-2 and ribose to compete with each other for binding to the Lsr ABC transporter domain. Furthermore, a biofilm growth deficiency was observed when RbsB (ribose binding subunit B, one of the subunits of the ribose ABC transporter) was inactivated.[6] In addition, biofilm growth of *A. actinomycetemcomitans* was reduced in the presence of ribose, which competes with AI-2 for binding to ribose binding subunit of ABC transporter.[6,18] However, there has been little data reporting on the mechanisms of AI-2 uptake by *S. mutans* and *S. sobrinus*.

Xylitol is a sugar alcohol and one of the most effective compounds in preventing dental caries. Trahan et al. showed that xylitol prevents most oral bacteria from producing cariogenic acids.[19] It does so because it cannot be metabolized to acids, rather, it is taken up by cariogenic bacteria and accumulates in cells as a toxic sugar-phosphate, which results in growth inhibition. Xylitol did not affect bacterial attachment, however, bacterial growth inhibition by xylitol may be responsible for the decrease of clinically relevant young biofilms of *S. mutans*.[20] Sato et al. demonstrated that repetitive xylitol treatment induces the emergence of xylitol-insensitive populations, which shows elevated *gbpC* gene expression and DDAG.[21] Banas et al. defined that DDAG of *S. mutans* represents a property of the organism that is beneficial for sucrose-dependent biofilm development, and *gbpC* is responsible for DDAG in *S. mutans* when cultured under defined stressful conditions.[22] Interestingly, contrary to expectations, these xylitol-insensitive streptococci exhibited decreased adhesion to glass surfaces when grown in the presence of sucrose.[21] It has been generally known that binding with insoluble glucan is crucial for attachment of *S. mutans* to solid surfaces for biofilm formation. Therefore, the glucan-binding protein gene family has a critical role in this process. In *S. mutans*, *gbpC* alone is thought to be required for DDAG, because bacterial strains lacking *gbpC* formed biofilms that were structurally different and defective compared to those of wild-type.[23,24] On the other hand, the *S. sobrinus dblB* gene, which is one of the four *gbpC* protein gene homologs, is the primary gene responsible for DDAG in this species.[25,26] Therefore, it is important to reveal the association between *gbpC* or *dblB* gene expression, DDAG, and biofilm formation in mutans streptococci.

In the current study, we first examined the effects of various pentose compounds and reserpine, an ABC-transporter blocker, on oral streptococci biofilm formation. We also analyzed the expression of the *gbpC* and *dblB* genes in *S. mutans* and *S. sobrinus*, respectively. Furthermore, we investigated how ribose and xylitol co-treatment affects streptococcal biofilm formation and the expression of DDAG-responsible genes, in addition to the effect of ribose on biofilm formation of xylitol-insensitive streptococci.

2. Materials and methods

2.1. Bacterial culture

The bacteria tested in this study were *S. mutans* (ATCC 25175) and *S. sobrinus* (ATCC 27607). Streptococci were maintained on brain heart infusion (BHI) medium and were grown under aerobic conditions.

2.2. Effect of pentose, reserpine, and xylitol on Streptococcal biofilm formation

To evaluate the concentration-dependent effect of D-ribose (ribose) and other pentose on streptococcal biofilm formation, *S. mutans* and *S. sobrinus* were cultured for 24 h in 24-well polystyrene plates containing BHI broth and ribose (0–50 mM), in addition to 0.3% and 1% sucrose. Following incubation, bacterial biofilm formation was compared with those formed in medium lacking sucrose. Further experiments were conducted with other pentose such as D-arabinose, L-arabinose, L-ribose, and D-xylose in BHI–0.3% sucrose medium.

To evaluate the combination effect of ribose and xylitol on streptococcal biofilm formation, *S. mutans* and *S. sobrinus* were cultured for 24 h in 24-well polystyrene plates containing BHI broth supplemented with 10 mM ribose and/or 1% (65.7 mM) xylitol. Sucrose was added to the culture broth at a final concentration of 0.3% or 1% and biofilm formation was compared with medium lacking sucrose.

2.3. Counting live, adherent streptococci in biofilm using the LIVE/DEAD counting kit

To mimic the oral cavity, a 24-well polystyrene plate was coated with human whole saliva for 1 h before incubation with