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学位論文の内容の要旨

Summary of the Substance of Dissertation

専攻 Major Field	分子情報制御学	部門 Department	分子腫瘍学
学籍番号 Student No.	13D734	氏名 Name	Khaleque Hasibul
論文題目 Thesis Subject	D-tagatose inhibits the growth and biofilm formation of *Streptococcus mutans*		

(論文要旨)

Summary

Dental caries are an important global health concern, and *Streptococcus mutans* has been established as a major cariogenic bacterial species. Reports indicate that a rare sugar, D-tagatose, is not easily catabolized by pathogenic bacteria. In this study, we examined the inhibitory effects of D-tagatose on the growth and biofilm formation of *S. mutans* GS-5. Monitoring the *S. mutans* growth over a 24-h period showed that D-tagatose prolonged the lag phase without interfering with the final cell yield. This growth retardation was also observed in the presence of 1% sucrose, although it was abolished by the addition of D-fructose. *S. mutans* biofilm formation was significantly inhibited by D-tagatose (1.0 to 4.0%) compared with that in the culture containing sucrose alone, and *S. mutans* formed granular biofilms in the presence of this rare sugar. The inhibitory effect of D-tagatose on *S. mutans* biofilm formation was more evident than that of xylitol. The addition of 1% D-tagatose significantly decreased the expression of *gtfB, fruA*, and D-fructose-specific phosphotransferase genes but not the expression of *ftf* compared with the culture containing 1% sucrose. The activity of cell-associated glucosyltransferase in *S. mutans* was inhibited by 4% D-tagatose. These results indicate that D-tagatose reduces water-insoluble glucan production from sucrose by inhibiting glucosyltransferase activities, which limits access to the free D-fructose released during this process and retards the growth of *S. mutans*.

Materials and Methods

S.mutans GS-5 was incubated in presence of D-tagatose for checking growth and pH. Biofilm was formed and stained with crystal violet for assay. Moreover, biofilm was formed over a plastic disc to study under scanning electron microscope (SEM). Real time PCR analysis was performed to observe sugar metabolism gene expression in *S.mutans* GS-5 during incubation with D-tagatose. Cell associated GTF B enzyme was extracted and incubated with D-tagatose to check the glucan production.

Results; Effects of D-tagatose on *S. mutans* GS-5 growth

First, the effects of D-tagatose on *S. mutans* GS-5 growth in BHI containing 1% sucrose were examined. Sucrose enhanced *S. mutans* GS-5 growth compared with BHI alone, and the pH of the culture decreased to less than 5.0 after 9-h incubation. Interestingly, D-tagatose delayed the transition of *S. mutans* growth to the logarithmic phase despite the presence of sucrose. Correspondingly, the pH decline of the culture was also delayed by

D-tagatose compared with that in sucrose alone.

Effects of D-tagatose on *in vitro S. mutans* GS-5 biofilm formation

We evaluated the effects of D-glucose, xylitol, and D-tagatose on *in vitro S. mutans* GS-5 biofilm formation. The addition of 1% sucrose markedly promoted biofilm formation by *S. mutans* GS-5, which is consistent with many previous reports. Xylitol, D-tagatose, and their combination significantly reduced *S. mutans* GS-5 biofilm formation compared with 1% sucrose alone or when supplemented with 1% glucose.

Scanning electron microscopy examination of *S. mutans* GS-5 biofilms

S. mutans GS-5 was cultured in 1 ml of BHI containing 1% sucrose with or without 1.0% or 4.0% each of xylitol or D-tagatose in 24-well plates with plastic disc inserts. The plates were incubated anaerobically at 37°C for 72 h, and the biofilms formed on the plastic discs were compared. Interestingly, many *S. mutans* GS-5 cell aggregates were observed in the culture containing D-tagatose (especially 4.0%), whereas homogeneous biofilms formed on the discs in the other 1% sucrose-containing cultures tested.

Effects of D-tagatose on the expression of sucrose metabolism genes in *S. mutans* GS-5

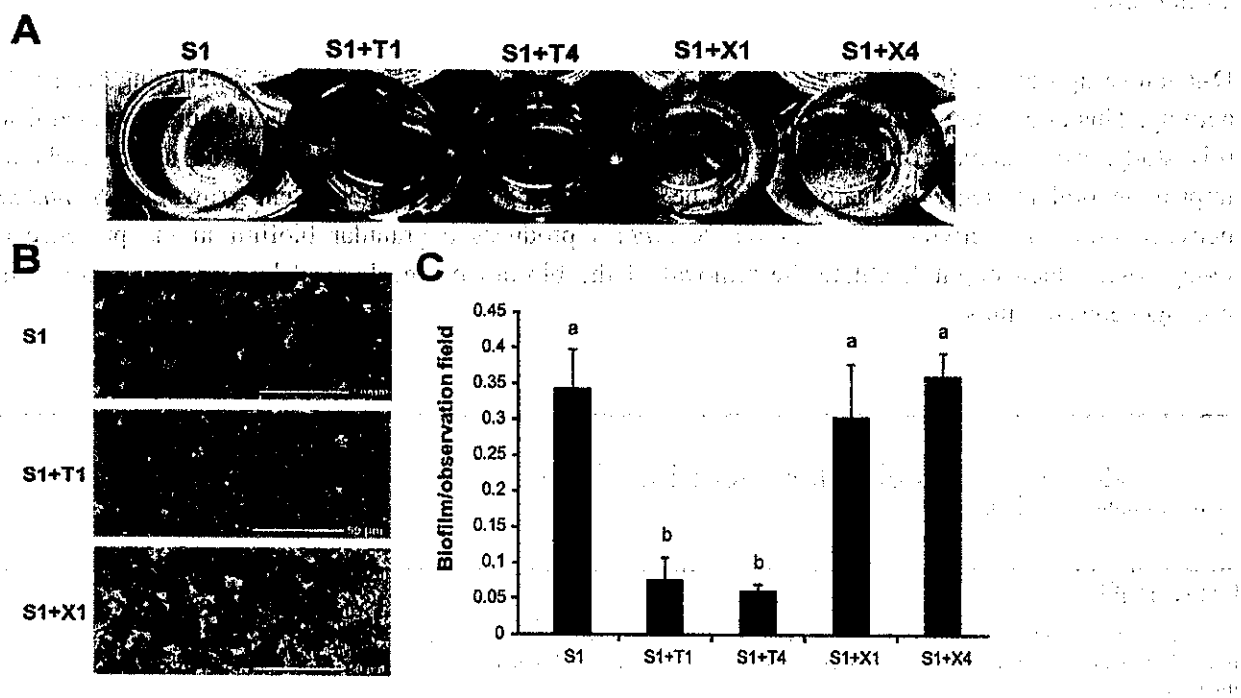
We also confirmed that 1% sucrose increased the expression of *gtfB* but did not increase the expression of the fructosyltransferase gene (*ftf*) compared with bacteria cultured in BHI alone. By contrast, the induction of *gtfB* expression by sucrose was strongly inhibited by D-tagatose (1%). The expression of the exo-β-fructosidase gene (*fruA*), which releases free D-fructose from FTF-producing water-soluble fructan, was also reduced in the presence of D-tagatose. Among the phosphotransferase system (PTS) genes involved in metabolizing sucrose or sucrose-derived monosaccharide (D-glucose and D-fructose), the expression levels of pts^{fru}, $pts^{fru/man}$ and $pts^{glu/man}$ were increased by 1% sucrose, although this increase was inhibited by D-tagatose. GtfB releases D-fructose during glucan synthesis, and these insoluble glucans are degraded by dextranase and utilized as a glucose source. The *fruA* gene product releases free D-fructose from the fructan synthesized by FTF. The repression of these genes as well as the fructose or glucose *pts* genes indicates that D-tagatose limits the ability of *S. mutans* GS-5 to access sucrose-derived monosaccharides, especially D-fructose. Shows that D-fructose is a powerful inducer of *gtfB* expression, and sucrose strongly increases the expression of fructose PTS, thus indicating that D-fructose is a key sugar required for *S. mutans* growth and biofilm formation.

Effects of D-tagatose on *S. mutans* GS-5 GTF activity

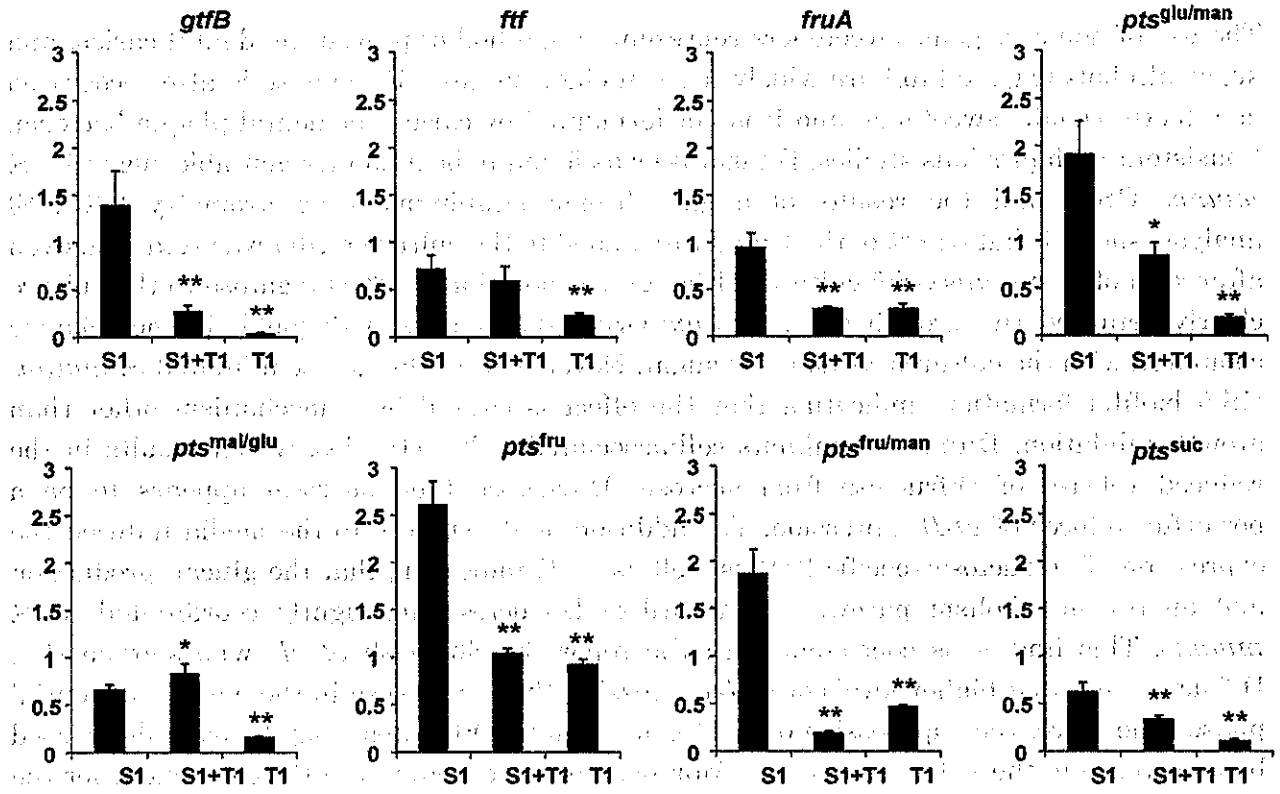
S. mutans GTFs B and C, which produce water-insoluble glucan, are known to be cell-associated. To examine whether D-tagatose directly inhibits the activity of GTF in *S. mutans* GS-5, the cell-associated proteins were extracted with urea from *S. mutans* GS-5 cells cultured in BHI. The water-insoluble glucan production was compared among the renatured enzymes in media with 0.1 M sucrose in the presence or absence of 4% D-tagatose. The addition of 4.0% D-tagatose significantly decreased the water-insoluble glucan production, indicating that the sugar directly inhibits the activity of *S. mutans* GS-5 cell-associated GTF.

Disscussion

The use of non-cariogenic sweeteners represents a method of preventing dental caries, and sugar alcohols (e.g., xylitol) are widely used in chewing gum. D-tagatose is also recognized as a tooth-friendly sweetener, and it is not fermented by cariogenic dental plaque bacteria. Consistent with previous studies, D-tagatose was found to be a non-fermentable sugar for *S. mutans* GS-5, and the results of a gas chromatography-mass spectrometry (GC-MS) analysis showed that 81.6% of the D-tagatose added to the culture media was retained, even after 48 h of *S. mutans* GS-5 culture. Although the addition of 1% D-tagatose to the culture clearly retarded the growth of *S. mutans* GS-5, the final growth yield did not change compared with the cultures without the sugar. Nevertheless, D-tagatose inhibited *S. mutans* GS-5 biofilm formation, indicating that the effect is caused by a mechanism other than growth inhibition. D-tagatose inhibits cell-associated GTF activities, which results in the reduced release of D-fructose from sucrose. D-fructose (and sucrose) appears to be a powerful inducer of *gtfB* expression. The addition of 1% sucrose to the media induced the expression of D-fructose-specific PTS as well as *gtfB*, indicating that the glucan production and energy metabolism pathways that utilize D-fructose are tightly coordinated in *S. mutans*. This finding is consistent with the report by Shemesh *et al.*, who showed that D-fructose induced higher levels of *gtfB* expression than D-glucose in the early exponential phase. Therefore, the suppression of *gtfB* expression by D-tagatose may be partially caused by a decrease in the D-fructose supply. Moreover, genes encoding the EII component for the D-fructose-specific PTS genes (pts^{fru} and $pts^{fru/man}$) were also downregulated in the presence of sucrose. The growth retardation of *S. mutans* GS-5 by D-tagatose is also likely because of the limited D-fructose supply resulting from GTF inhibition because the D-tagatose-induced growth retardation was reversed under D-fructose supplementation. We predict that changes in the availability of this monosaccharide are responsible for the prolongation of the lag-phase of *S. mutans* GS-5 growth by D-tagatose.



Figure; *S.mutans* biofilm inhibition by D-tagatose. Here, S1- 1% Sucrose, S1+T1- 1% Sucrose+ 1% D-tagatose, S1+T4- 1% Sucrose+ 4% D-tagatose, S1+X1- 1% Sucrose+ 1% Xylitol, S1+X4- 1%Sucrose+ 4% Xylitol. Clear biofilm inhibition has seen by D-tagatose



Figure; Effect of D-tagatose on *S.mutans* sugar metabolism gene expression. Here, S1- 1% Sucrose, S1+T1- 1% Sucrose+ 1% D-tagatose, T1- 1% D-tagatose. The expression was measured in BHI media.

Conclusion

D-tagatose appears to inhibit *S. mutans* GS-5 growth and biofilm formation by interfering with GTF activity. This effect may be useful in the prevention of dental caries. Based on the findings obtained from this study, we conclude that foods or preparations containing D-tagatose could be useful tools for improving oral hygiene. D-tagatose might be able to suppress the intermittent growth of *S. mutans* between oral care activities. In addition, *S. mutans* produces a granular biofilm in the presence of D-tagatose, which might facilitate the removal of the biofilm by mechanical brushing compared with homogeneous biofilms.

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