学位論文

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Khaleque Hasibul

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D-tagatose inhibits the growth and biofilm formation of *Streptococcus mutans*

KHALEQUE HASIBUL[1], HARUYUKI NAKAYAMA-IMAOHJI[2], MASAHITO HASHIMOTO[3], HISASHI YAMASAKI[4], TAKAAKI OGAWA[1], JUNPEI WAKI[3], AYANO TADA[2], SOARI YONEDA[2], MASAAKI TOKUDA[5], MINORU MIYAKE[1] and TOMOMI KUWAHARA[2*]

[1]Department of Oral and Maxillofacial Surgery, Faculty of Medicine, Kagawa University, 1750-1 Miki, Kagawa 761-0793; [2]Department of Microbiology, Faculty of Medicine, Kagawa University, 1750-1 Miki, Kagawa 761-0793; [3]Department of Chemistry, Biotechnology, and Chemical Engineering, Graduate School of Science and Engineering, Kagoshima University, 1-21-40 Korimoto, Kagoshima 890-0065; [4]Department of Cellular and Molecular Medicine, Wakayama Medical University Graduate School of Medicine; [5]Department of Cell Physiology, Faculty of Medicine, Kagawa University, 1750-1 Miki, Kagawa 761-0793, Japan.

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Abstract

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*. Thus, foods and oral care products containing D-tagatose are anticipated to reduce the risk of caries by inhibiting *S. mutans* biofilm formation.

Correspondence to: Professor Tomomi Kuwahara, Department of Microbiology, Faculty of Medicine, Kagawa University, 1750-1 Miki, Kagawa 761-0793, Japan. Tel/Fax: +81-87-891-2129; E-mail: tomomi@med.kagawa-u.ac.jp

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Introduction

Oral hygiene care is recognized as an important measure for the prevention of oral diseases (dental caries and periodontitis) and a prophylactic treatment for aspiration pneumonia, type 2 diabetes mellitus (1), and cardiovascular diseases (2). Although dental caries are a worldwide health concern (3), they are preventable through appropriate interventions, especially at the early stage of life (4). However, certain groups cannot easily perform regular oral hygiene care (tooth brushing or flossing) by themselves, including critically ill patients, elderly people, and evacuees after disasters. In addition, many individuals lack the knowledge and motivation for oral hygiene care (5). An interruption of oral hygiene care results in the formation of microbial biofilm called dental plaque on the tooth surface. Biofilm formation on tooth surfaces by cariogenic bacterial communities is the initial step in the development of dental caries.

Streptococcus mutans is a primary aetiologic agent for dental caries (6). The major virulence traits associating with *S. mutans* cariogenicity are acid production from fermentable dietary carbohydrates, acid tolerance, and exopolysaccharide (EPS) formation (7). Acid production promotes demineralization from tooth enamel, and acid tolerance confers survival under the low pH environment within dental plaques. EPS encourages the formation of acidogenic biofilms on the tooth surface, which are bioaggregates resistant to mechanical tooth brushing. Although fluoride-based preparations protect tooth surfaces from acid attacks, their effects are limited unless they are combined with dental plaque control (8). The use of bactericidal compounds for the eradication of cariogenic bacteria is controversial because these compounds disturb the healthy oral microflora and may lead to the development of multidrug-resistant bacteria. The development of specific regulatory measures for cariogenic bacteria is expected to reduce dental plaque formation (9). A number of trials have been performed to determine whether *S. mutans* growth and adhesion are inhibited by various natural products, such as green tea catechins (10), cranberry constituents (11), citrus lemon oil (12), and mushroom extract (13). However, these studies did not evaluate biofilm formation.

Sugar alcohols (polyols) are alternative candidates used for the prevention of dental caries (14). Of these, xylitol is the most evaluated polyol that reduces the risk of caries. Xylitol disturbs the metabolic processes in bacteria and shows a bacteriostatic effect on *S. mutans* by forcing the uptake and efflux of this non-cariogenic sugar alcohol (15). However, the effect of xylitol on *S. mutans* is reduced in the presence of other fermentable sugars (16).

Rare sugars are monosaccharides and their derivatives are infrequently found in nature (17). Recently, rare sugars were the focus of attention as health-supporting sugar substitutes because of their equivalent sweetness but much lower caloric content than sucrose (18). These sugars are expected to reduce calorie intake, thereby decreasing the risk of type 2 diabetes mellitus and obesity (19). The ketohexose D-tagatose has 92% of the sweetness but 38% of the calories of sucrose. D-tagatose is not a preferential substrate for bacterial fermentation, and it has been reported that D-tagatose is not easily catabolized by many lactic acid bacteria or by pathogens such as *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Staphylococcus aureus*, *Bacillus cereus*, and *Yersinia enterocolitica* (20). D-tagatose has been shown to suppress the growth of aerobic and lactic acid bacteria in chopped and formed ham, thereby extending the shelf life of these products by 7-10 days. These findings indicate that D-tagatose-containing foods might suppress cariogenic oral bacteria. In fact, D-tagatose has recently been reported to inhibit the acid production, growth, and water-insoluble glucan production of *S. mutans* GS-5 in the presence of sucrose (21).

In the current study, we evaluated the effect of D-tagatose on *S. mutans* growth and biofilm formation. The results showed that D-tagatose retards *S. mutans* growth and reduces its biofilm formation by interfering with its sucrose utilization.

Materials and Methods

Bacterial strains and growth condition. S. mutans GS-5 (22) isolated from dental caries was used in this study. This strain belongs to serogroup C, which is the most prevalent serogroup in the human oral cavity. A glycerol stock of *S. mutans* GS-5 stored at -80°C was streaked onto Brain-Heart Infusion (BHI; Difco) agar plates and cultured anaerobically at 37°C. Anaerobic cultivation was performed in an anaerobic chamber conditioned with mixed gas (N_2, 80%; H_2, 10%; CO_2, 10%) or in an Anaeropack system using anaerobic jars (Mitsubishi Gas Chemical Co. Ltd.).

Monitoring the growth and pH of S. mutans GS-5 cultures. A fresh *S. mutans* GS-5 colony from a BHI agar plate was inoculated into liquid BHI and anaerobically incubated at 37°C for 24-48 hours (h). BHI broth containing the test sugars (1% or 4% [w/v] sucrose, D-glucose, D-fructose, xylitol, D-tagatose, or their combination) were sterilized by filtration through a 0.22-μm pore filter. D-tagatose was supplied by the Rare Sugar Center of Kagawa University (Kagawa, Japan). The 48-h *S. mutans* GS-5 culture was

then added at 4% (v/v, 0.4 ml) to 10 ml of sugar-supplemented BHI broth. Triplicate cultures were prepared for each test group, and the tubes were incubated at 37°C in an anaerobic chamber. The optical densities at 590 nm (OD_{590}) and the pH values of the cultures were checked every 3 h for 24 h. Vigorous vortexing was performed prior to the OD_{590} measurement to produce a homogenous suspension. Because *S. mutans* GS-5 forms hard and granular aggregates of extracellular polysaccharide (EPS) during growth in BHI supplemented with sucrose, these cultures were incubated with a rotating motion in the anaerobic chamber to prevent firm glucan adherence onto the glass tubes. This modification did not hamper EPS production from *S. mutans* GS-5. A portion of each culture (0.5 ml) was collected to check the pH using a micro-volume pH meter (LAQUA-twin Compact pH Meter-HORIBA, Kyoto, Japan). The initial pH and OD_{590} immediately after incubation were also measured.

Biofilm assay. S. mutans GS-5 pre-culture was prepared by inoculating a fresh colony into 2 ml of liquid BHI and incubating it at 37°C for 48 h. The test sugars (D-glucose, xylitol, or D-tagatose) were dissolved in liquid BHI containing 1% sucrose at 0.5-4.0% (w/v). As controls, liquid BHI containing 1% sucrose alone and liquid BHI without any added sugars were also prepared. The 48-h *S. mutans* GS-5 culture (40 μ l) was mixed with either of the sugar solutions (2 ml), and 0.2 ml of each of the mixtures was dispensed into 96-well plates. Eight wells were used for each sample. The microwell plate was anaerobically incubated at 37°C for 72 h. After a 72-h incubation, the culture media were discarded, and the layers of biofilm adhered to the bottoms of the wells were washed three times with 0.2 ml of phosphate-buffered saline (PBS, pH 7.4). The remaining biofilm was stained with 0.1 ml of 0.01% crystal violet for 20 min at room temperature and then washed four times with 0.2 ml of PBS. The remaining crystal violet was eluted with 0.2 ml of 33% acetic acid with gentle agitation for 20 min at room temperature. The absorbance of the eluents was measured at 550 nm.

Electron microscopy examination. The effects of D-tagatose on *S. mutans* GS-5 biofilm formation were examined by scanning electron microscopy. Sterile plastic discs (Cell Desk, 13.5 mm in diameter, Sumitomo Bakelite Co. Ltd.) were set in 24-well microtitre plates. The 48-h *S. mutans* GS-5 culture (30 μ l) was mixed with 1 ml of BHI containing 1% sucrose with or without xylitol (1% or 4%) or D-tagatose (1% or 4%). The mixture (1 ml) was transferred into each well. After a 72-h anaerobic incubation, the biofilms formed on the discs were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After fixation, they were dehydrated in a graded ethanol series and dried in a Hitachi

PCP-2 critical point drying apparatus. The discs were coated with platinum/palladium in a Hitachi E-102 sputter coater and examined with a JEOL JCM-6000 scanning electron microscope. Biofilm areas were measured as described by Somayaji *et al.* using the auto-selection tool based on colour in Photoshop CS6, and the ratios to the total observation field were calculated (23). At least five randomly selected fields were examined.

RNA isolation and quantitative real-time PCR. Total RNA was extracted from mid-logarithmic phase cultures (OD_{590}=0.4-0.6) of *S. mutans* GS-5 using the hot phenol method. RNA was further purified using an RNeasy CleanUp Kit (Qiagen) and treated with TURBO DNA-*free* (Ambion) to remove contaminating DNA. Total RNA was reverse transcribed using a PrimeScript RT reagent kit (Takara Shuzo Co., Ltd.) with random hexamers at 37°C for 15 min. Reverse transcription was terminated by heating the mixtures at 85°C for 5 s. The cDNA products were subsequently amplified using SYBR Premix Ex-Taq II (Takara) under the following conditions in a StepOne plus apparatus (Applied Biosystems): preheating at 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. All of the samples were run in triplicate. Threshold cycle values were normalized to the levels of 16S ribosomal RNA gene transcripts, and changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method (24). The oligonucleotide sequences of the primers used for each target gene are listed in Table 1.

Preparation of cell-associated glucosyltransferase (GTF). Cell-associated glucosyltransferases (GTFs) of *S. mutans* GS-5 were purified as previously described (25). In brief, *S. mutans* GS-5 was cultured in 800 ml of BHI broth. After a 48-h incubation, the cells were collected by centrifugation at 12,000 *g* for 20 minutes. The bacterial pellet was washed with 10 mM potassium phosphate buffer (KPB, pH 6.0). The cell-bound GTF was extracted with 5 ml of 8 M urea. The cell suspension in urea was incubated at 25°C for 1 h with periodic shaking. The supernatant was collected after centrifugation at 12,000 *g* for 20 min and then dialysed for 24 h at 4°C against 10 mM KPB (pH 6.0). After dialysis, the enzyme preparations were stored at -20°C until use.

Effects of D-tagatose on cell-associated GTF. The effects of D-tagatose on *S. mutans* GS-5 GTF activity were assessed *in vitro* by quantifying the water-insoluble glucan produced in the mixture of sucrose and the cell-associated crude enzyme prepared as described above. A total of 2 ml of 0.1 M KPB (pH 6.0) containing 0.1 M sucrose and 0.01% sodium azide with or without 4% D-tagatose was mixed with 1 ml

of enzyme preparation and incubated anaerobically at 37°C for 48 h. The water-insoluble glucan produced in the mixture was quantified by the phenol-sulfuric acid method (26). In brief, after vigorous vortexing, the mixtures were centrifuged (7,500 $\times g$ for 10 min at 4°C), and then the pellets were washed to remove the water-soluble glucan with 1 ml PBS and subjected to another five washes with distilled water (dH_2O). Subsequently, the pellets were dissolved in 1 ml of 1 N NaOH and centrifuged again. The supernatant (0.5 ml) was mixed with an equal volume of 5% phenol followed by 2.5 ml of sulfuric acid. After allowing to stand at room temperature for 20 min, the OD_{490} of the mixture was measured with a UV-VIS spectrophotometer (Shimadzu Corporation). The quantity of water-insoluble glucan was calculated from the standard curve of known concentrations of D-mannose.

Statistical analysis. Statistical analysis of the data was performed using StatFlex ver. 6.0 software (Artech Co., Ltd., Tokyo). Analysis of variance (ANOVA) was used to compare the means of all groups, and Tukey's post-hoc test was used to compare the means of each of the two groups. Dunnett's test was applied to compare the test groups with the control group. Data were considered to be significantly different if the p value was less than 0.05.

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. As shown in Fig. 1A, sucrose enhanced *S. mutans* GS-5 growth compared with BHI alone, and the pH of the culture decreased to less than 5.0 after a 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 (Fig. 1B). This growth retardation became more evident, and entry into the stationary phase was delayed by 6 h when the D-tagatose concentration was increased to 4%; however, significant differences were not observed in the final OD_{590} after 24 h of cultivation (data not shown). As shown in Figs. 1C and D, the *S. mutans* GS-5 growth delay induced by D-tagatose was abolished when 1% D-fructose (but not D-glucose) was added to BHI containing 1% sucrose. These results indicate that D-tagatose inhibits sucrose catabolism in *S. mutans* GS-5.

Effects of D-tagatose on in vitro S. mutans GS-5 biofilm formation. As described above, the rare sugar D-tagatose is likely to interfere with sucrose metabolism in *S. mutans* GS-5. Because sucrose metabolism plays an important role in water-insoluble glucan production, which is required for biofilm formation, D-tagatose is expected to inhibit *S. mutans* biofilm formation.

We evaluated the effects of D-glucose, xylitol, and D-tagatose on *in vitro S. mutans* GS-5 biofilm formation. As shown in Fig. 2, the addition of 1% sucrose markedly promoted biofilm formation by *S. mutans* GS-5, which is consistent with many previous reports. D-glucose at a final concentration of 1% slightly reduced *S. mutans* biofilm formation. 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.

The effects of xylitol and D-tagatose at varying concentrations (0.5%, 1.0%, 2.0%, and 4.0%) on *S. mutans* GS-5 biofilm formation were examined (Fig. 3). Xylitol inhibited *S. mutans* biofilm formation in a dose-dependent manner, although an additional effect was not observed for concentrations over 2.0%. By contrast, D-tagatose showed clear dose-dependent inhibition of *S. mutans* GS-5 biofilm formation. To determine whether the effects on biofilm formation were caused by high osmolality, *S. mutans* GS-5 biofilm formation was compared in the presence of 1% and 5% sucrose. The biofilm masses in the cultures with 1% and 5% sucrose were similar (OD_{550}; 1.27±0.05 vs 1.09±0.07), indicating that the effects of high osmolality were limited under the conditions used in this study.

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. *S. mutans* GS-5 grew equally in all of the media tested. As shown in Fig. 4A, less biofilm formed on the discs in the cultures containing 1.0% and 4.0% D-tagatose than on those in other media (1% sucrose alone or 1% sucrose plus 1.0% or 4.0% xylitol). 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.

Scanning electron microscopy examination of the discs also showed that there was less biofilm on the discs in the culture with D-tagatose (Fig. 4B). Quantification of the *S. mutans* GS-5 biofilms on the discs showed a significant reduction in the presence of D-tagatose (Fig. 4C).

Effects of D-tagatose on the expression of sucrose metabolism genes in S. mutans GS-5. Sucrose is known to increase the expression of the gene that encodes insoluble-glucan-forming glucosyltransferase B (*gtfB*), which is a major virulence factor of *S. mutans*. 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 (Fig. 5). 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. Fig. 6 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. As shown in Fig. 7, 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.

Discussion

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 (data not shown). 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.

As shown in Fig. 7, 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 (Fig. 6). 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 (27). 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 (Fig. 1C and D). 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.

By contrast, the *ftf* expression levels were not changed by D-tagatose in the presence of 1% sucrose. FTF produces water-soluble inulin-type fructan in *S. mutans*. Because fructan is digested by exo-β-D-fructosidase (FruA) into D-fructose, this fructose polymer is considered to serve as energy storage for *S. mutans*. The downregulation of *fruA* by D-tagatose is expected to limit the D-fructose supply for *S. mutans*. This change in monosaccharide availability might affect *fruA* expression, which is known to be sensitive to the control of carbon catabolite repression via the central regulatory protein CcpA (28).

As mentioned above, D-tagatose appears to inhibit *S. mutans* GS-5 GTFs, and the inhibition of cell-associated GTFs B or C, which produce water-insoluble glucan from sucrose, is considered to be a primary mechanism underlying the biofilm inhibition as well as the growth retardation of *S. mutans* GS-

5 by D-tagatose. Because D-tagatose is a D-fructose epimer at the C-4 position, its structural similarity to D-fructose might interfere with the binding or catalysis of sucrose by the GTFs. In addition, the *S. mutans* GS-5 biofilm that formed in the presence of D-tagatose was granular, whereas the biofilm formed in the culture with 1% sucrose alone or 1% sucrose plus xylitol was uniform (Fig. 4A). This difference may be related to the glucan/fructan imbalance caused by D-tagatose.

Bautista *et al.* reported that many human pathogens are unable to utilize D-tagatose and showed that the sugar is metabolized by a limited member of lactobacilli (20). Recently, probiotic lactobacilli have been reported to suppress the growth of cariogenic bacteria and prevent dental caries (29). Based on our finding that *S. mutans* GS-5 did not preferentially ferment D-tagatose, this sugar is anticipated to prevent *S. mutans* colonization on tooth surfaces by promoting the ability of probiotic oral lactobacilli to resist colonization.

Xylitol is widely used for the prevention of dental caries, although its effects in clinical trials are still controversial (30). *S. mutans* transports xylitol via a fructose-specific PTS, and xylitol-resistant *S. mutans* strains lacking this PTS activity have emerged (31). In addition, the presence of fermentable sugars, such as sucrose, attenuates the effects of xylitol. Therefore, alternative prophylactic treatments for dental caries are required. Xylitol is a non-fermentable sugar for *S. mutans* and exhibits a toxic effect by causing the expenditure of energy for the uptake and export of this non-cariogenic sugar alcohol (32). The mechanism by which xylitol suppresses *S. mutans* appears to be different from that of D-tagatose as described here; therefore, a synergistic effect might be expected for their combination. However, a synergistic effect was not evident in the inhibition of *S. mutans* GS-5 biofilm formation (Fig. 2), which might have been related to interference with xylitol uptake because D-tagatose downregulates the D-fructose-specific PTS genes (pts^{fru} and $pts^{fru/man}$) (Fig. 5).

In 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. Ongoing clinical examinations to evaluate the effectiveness of chewing gum containing D-tagatose as a prophylactic for dental caries in a clinical trial are being performed by our research consortium.

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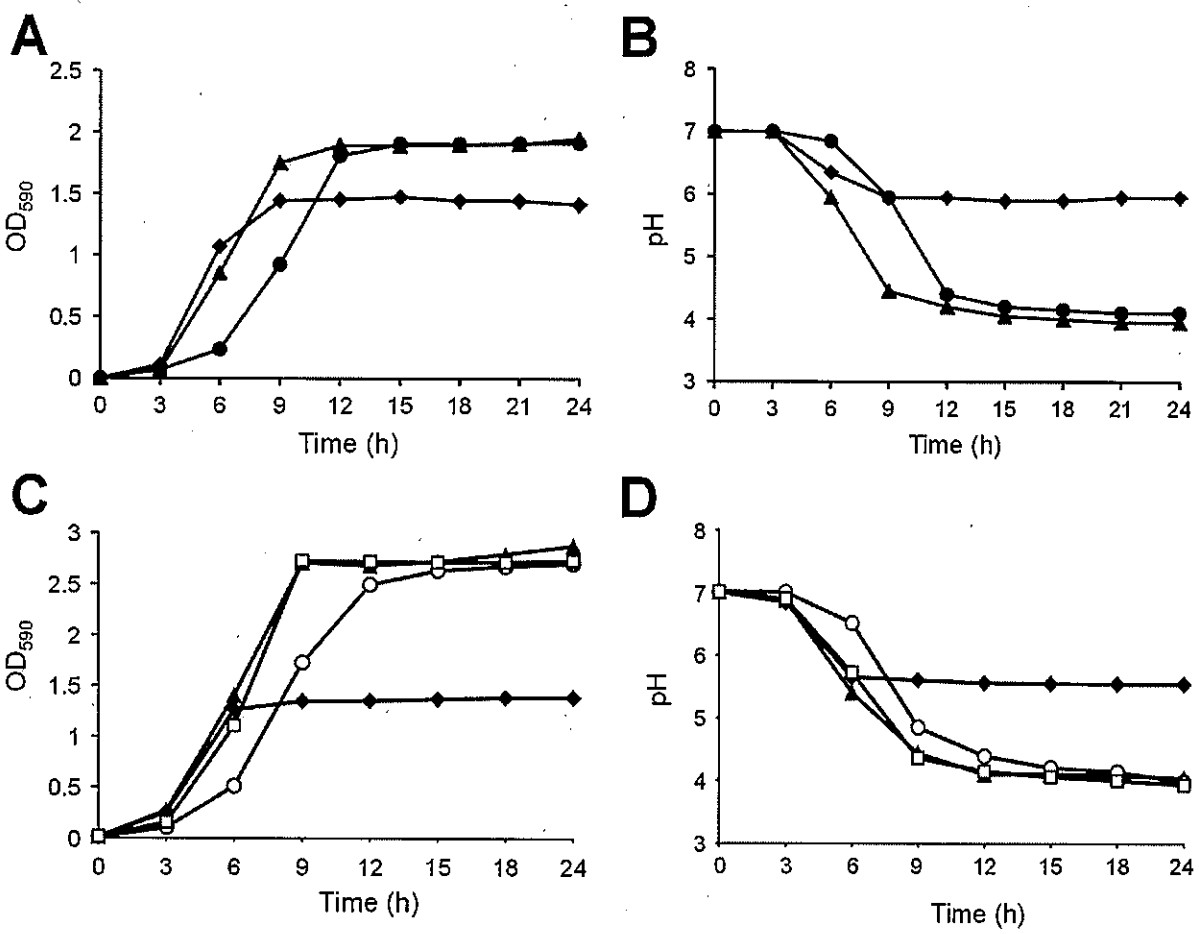

Figure 1. Effects of D-tagatose on *S. mutans* GS-5 growth in the presence of sucrose. The OD_{590} (A) and pH (B) values of *S. mutans* GS-5 cultures grown in BHI (closed diamonds), BHI containing 1% sucrose (closed triangles) or 1% sucrose plus 1% D-tagatose (closed circles). The *S. mutans* GS-5 growth was also checked in BHI containing 1% sucrose plus 1% each of D-tagatose and D-glucose (open circles) or D-fructose (open squares) (panels C and D). The OD_{590} and pH of the cultures were checked every 3 h for 24 h of incubation.

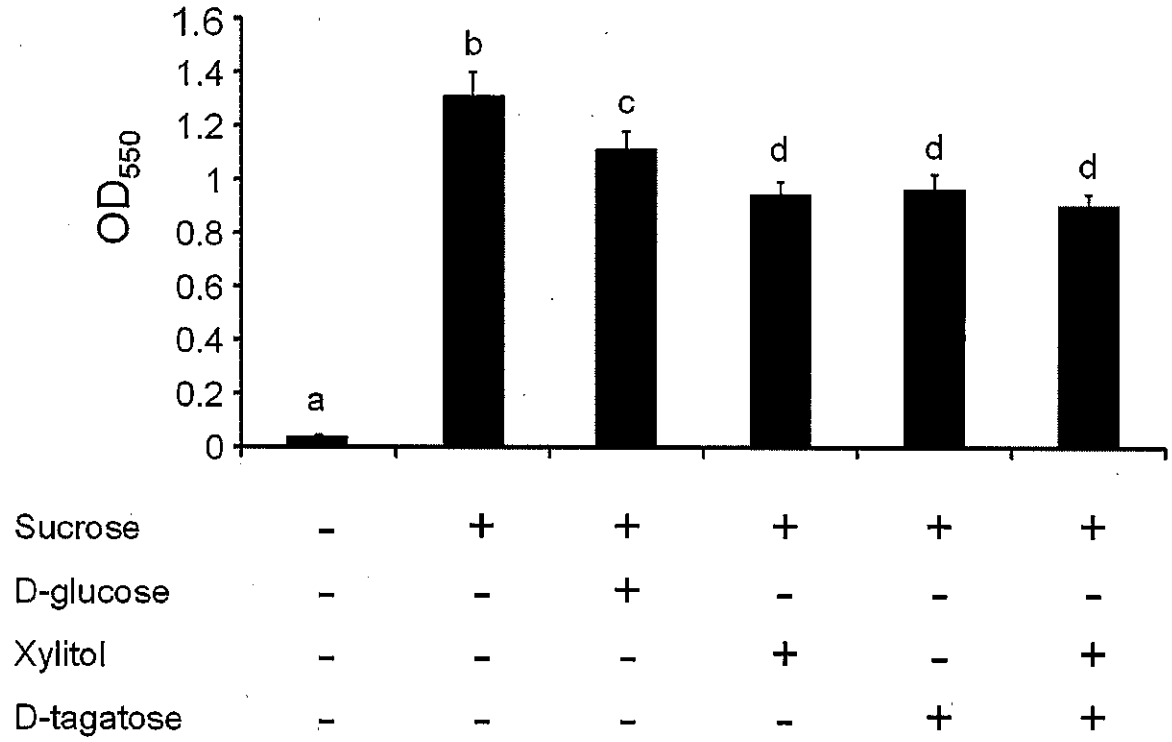

Figure 2. Effects of D-glucose, xylitol, and D-tagatose on the biofilm formation of *S. mutans* GS-5. Biofilm formation in BHI media containing the indicated combinations of the test sugars with 1% sucrose was quantified by crystal violet staining. The data are expressed as the mean ± standard deviation and statistically analysed by ANOVA followed by Tukey's test. Differences were considered to be significant when the *p*-values were less than 0.05. The groups marked by the same letters were not significantly different from each other.

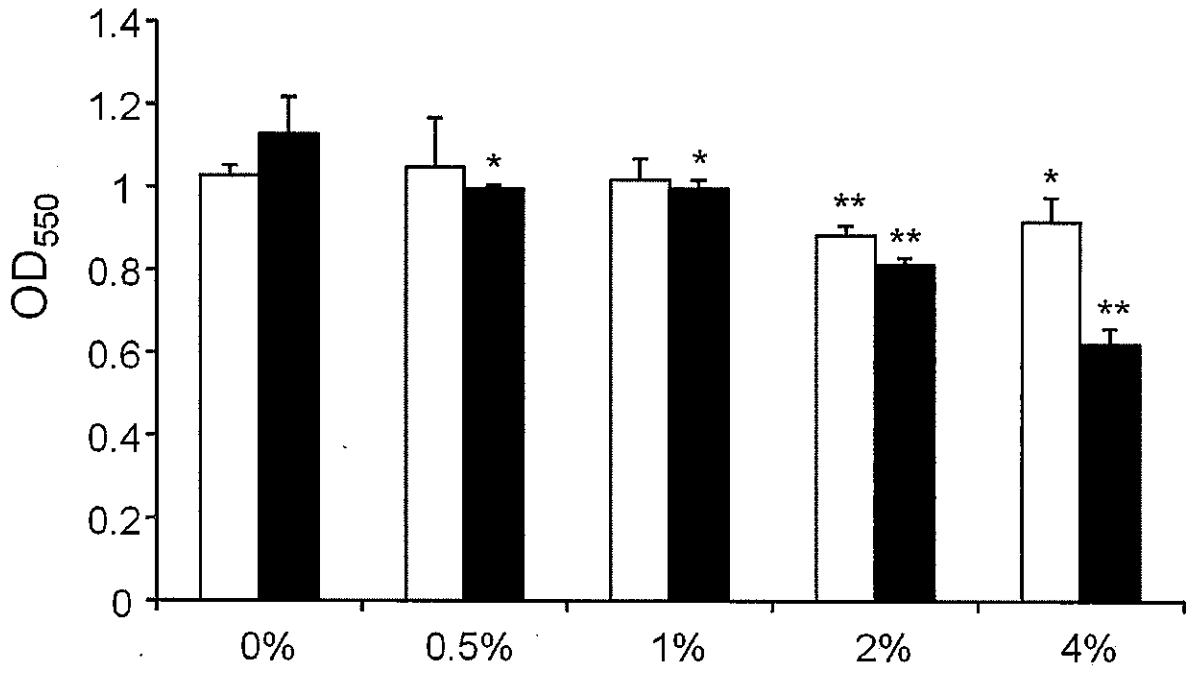

Figure 3. D-tagatose inhibits *S. mutans* GS-5 biofilm formation in a dose-dependent manner. *S. mutans* GS-5 was cultured anaerobically in BHI media containing 1% sucrose and the indicated concentrations of xylitol (white bars) or D-tagatose (black bars). After a 72-h incubation, the biofilms formed on the bottoms of microwells were quantified by crystal violet staining. The data are expressed as the mean ± standard deviation and analysed statistically by ANOVA followed by Dunnett's test. The differences were considered to be significant when the *p*-values were less than 0.05. * Significantly different from 1% sucrose alone ($p<0.05$). ** Significantly different from 1% sucrose alone ($p<0.01$)

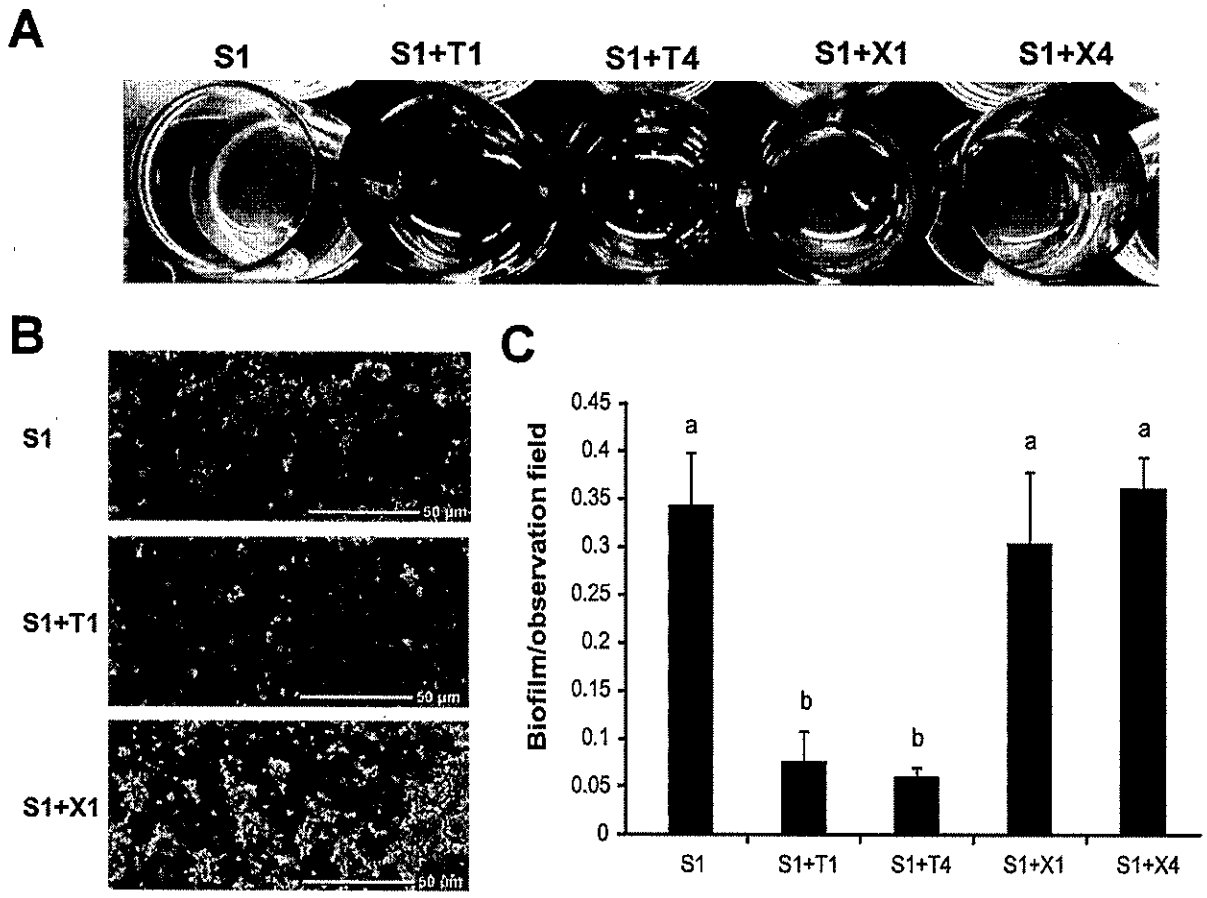

Figure 4. Morphological analysis results for *S. mutans* biofilms. **(A)** Photographs and **(B)** SEM images of *S. mutans* GS-5 biofilms formed on the plastic discs after a 72-h cultivation in BHI supplemented with the indicated sugars. **(C)** Biofilm areas were measured using the auto-selection tool based on colour in Photoshop CS6, and the ratios to the total observation field were calculated. At least three randomly selected fields were examined for each sample. The groups marked by the same letters were not significantly different from each other. S1, 1% sucrose alone; S1+T1, 1% sucrose and 1% D-tagatose; S1+T4, 1% sucrose and 4% D-tagatose; S1+X1, 1% sucrose and 1% xylitol; S1+X4, 1% sucrose and 4% xylitol.

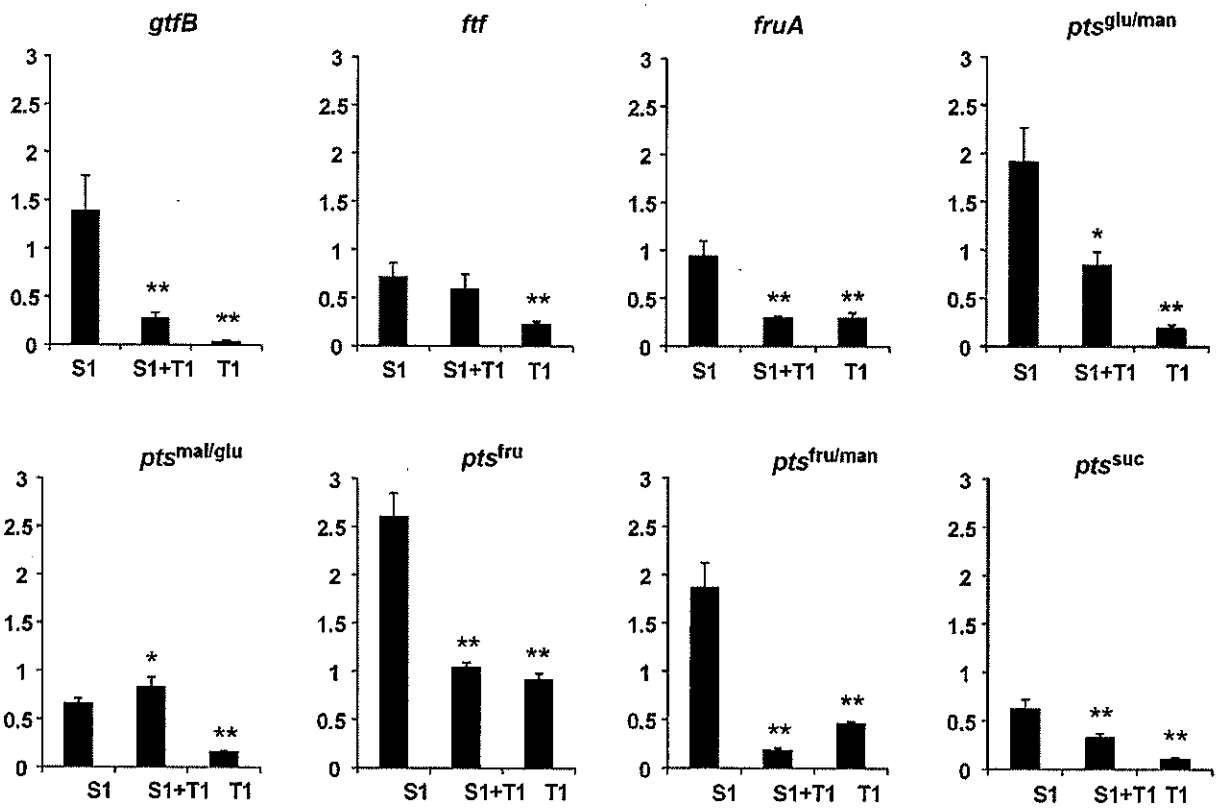

Figure 5. Effects of D-tagatose on the expression of sucrose metabolism genes in *S. mutans* GS-5. The relative expression levels of the genes encoding glucosyltransferase (*gtfB*), fructosyltransferase (*ftf*), and the EII components of the phosphotransferase system specific for sucrose (pts^{suc}), D-fructose (pts^{fru}), D-fructose/D-mannose ($pts^{fru/man}$), D-maltose/D-glucose ($pts^{mal/glu}$), and D-glucose/D-mannose ($pts^{glu/man}$) were evaluated by qPCR. The gene expression levels were normalized to the 16S rDNA expression. The fold change in expression levels relative to that in BHI alone are shown. * Significantly different from 1% sucrose alone ($p<0.05$). ** Significantly different from 1% sucrose alone ($p<0.01$). S1, 1% sucrose alone; S1+ T1, 1% sucrose plus 1% D-tagatose; T1, 1% D-tagatose alone.

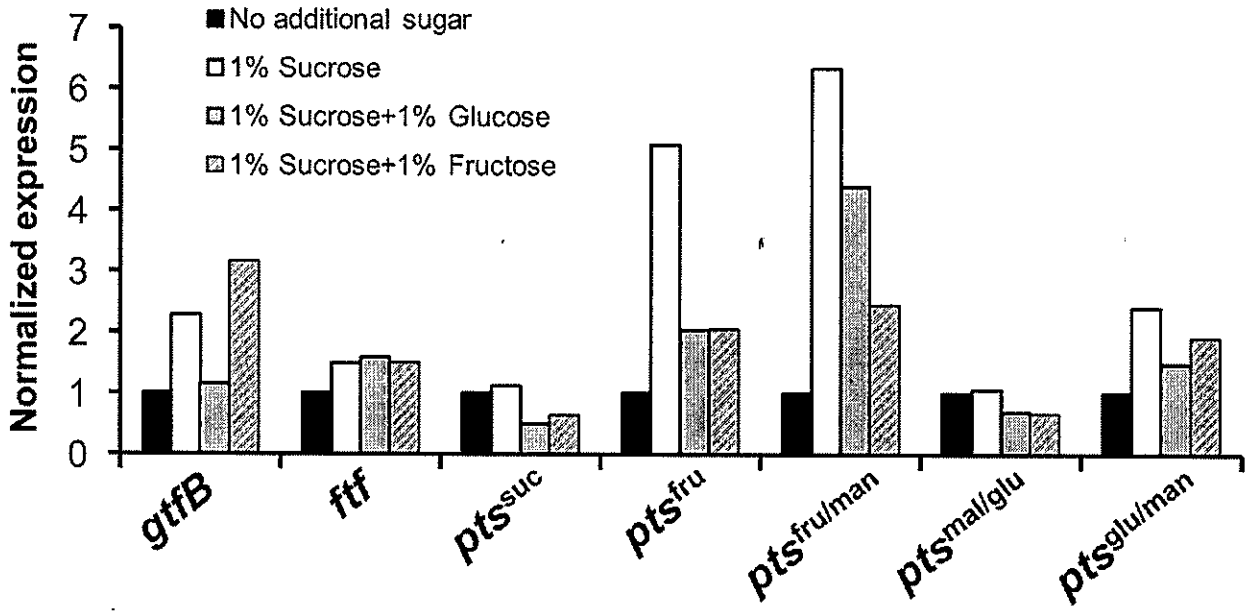

Figure 6. Effects of sucrose and D-fructose on *S. mutans* GS-5 *gtfB* and fructose-specific PTS genes. The relative expression levels of the genes encoding glucosyltransferase (*gtfB*), fructosyltransferase (*ftf*), and the EII components of the PTS specific for sucrose (pts^{suc}), D-fructose (pts^{fru}), D-fructose/D-mannose ($pts^{fru/man}$), D-maltose/D-glucose ($pts^{mal/glu}$), and D-glucose/D-mannose ($pts^{glu/man}$). The gene expression levels were normalized to the 16S rDNA expression. The fold changes in expression relative to that in BHI alone are shown. Sucrose induced *gtfB* expression, and its induction was further enhanced by the presence of D-fructose. Sucrose enhanced the expression of fructose-specific PTS genes more extensively than glucose-specific PTS genes.

Table 1. Oligonucleotide primers used for quantitative real-time PCR performed in this study.

Primers	Target genes	Coding functions	Sequences (5' to 3')	Amplicon (bp)
GtfB-F	SMUGS5_04450	Glucosyltransferase B	agcaatgcagccaatctacaaat	96
GtfB-R			acgaactttgccgttattgtca	
Ftf-F	SMUGS5_09145	Fructosyltransferase	aaatatgaaggcggctacaacg	101
Ftf-R			cttcaccagtcttagcatcctgaa	
EII-fru/man-F	SMUGS5_08805	Fructose/Mannose-specific PTS subunit IIC	aagactctctttacggtgggttc	111
EII-fru/man-R			agtgaaaccaacaaagaatggaa	
EII-glu/man-F	SMUGS5_08440	Glucose/Mannose-specific PTS subunit IIAB	ctaaagctgaccgtatcattgttg	111
EII-glu/man-R			attggtacaacattggccttgaca	
EII-mal/glu-F	SMUGS5_09220	Maltose/Glucose-specific PTS subunit IIABC	acacaatatctttggcggtaaga	105
EII-mal/glu-R			cgaaaccagagataataccaacg	
EII-fru-F	SMUGS5_03875	Fructose-specific PTS subunit IIABC	tgatacttttaaggctgggatca	116
EII-fru-R			aaaagaaccgttgcttcctttac	
EII-sucF	SMUGS5_08275	Sucrose-specific PTS subunit IIABC	ggtgtctttggtgctctgtattc	113
EII-suc-R			gtcaccatgaccagtaccatttt	
16SF	SMUGS5_R09892	16S ribosomal RNA	cctacgggaggcagcagtag	101
16SR			caacagagctttacgatccgaaa	

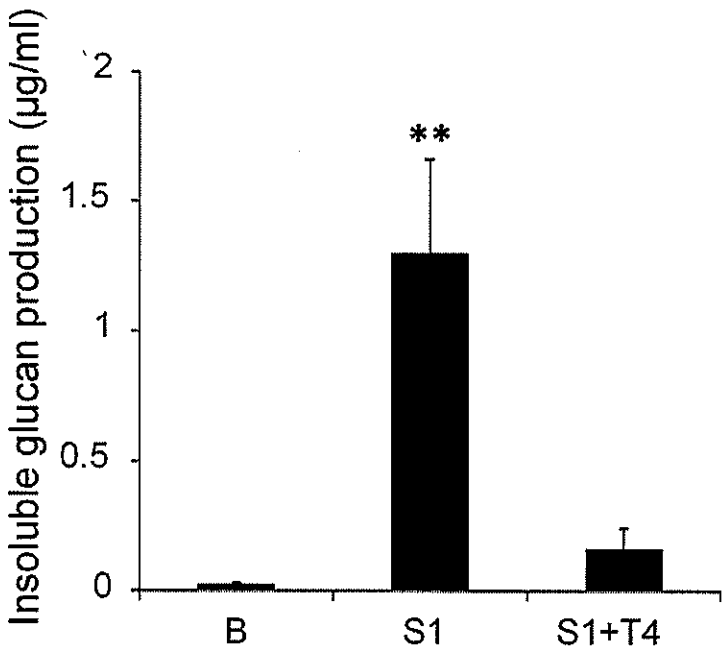

Figure 7. D-tagatose inhibits water-insoluble glucan formation by *S. mutans* GS-5. The effects of D-tagatose on *S. mutans* GS-5 cell-associated glucosyltransferase were assessed by monitoring the production of water-insoluble glucan. The extracted cell-associated proteins were incubated in a phosphate buffer alone (B) or in the same buffers containing 0.1 M sucrose (S) or 0.1 M sucrose with 4% D-tagatose (S+T4). After a 48-h incubation, the mixture was centrifuged and the pellet was washed with and then suspended in buffer. The water-insoluble glucan produced was measured by the phenol-sulfuric acid method, and the amount present was calculated from a standard curve using mannose as a standard. The data are expressed as the mean ± standard deviation and analysed statistically by ANOVA followed by Tukey's test. **Significantly different ($p<0.01$).