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Expression and Secretion of an *Arthrobacter* Dextranase in the Oral Bacterium *Streptococcus gordonii*

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We have constructed a plasmid to express and secrete dextranase in the oral bacterium *Streptococcus gordonii*. The dextranase gene from *Arthrobacter* sp. strain CB-8 was linked to a promoter and a DNA sequence encoding the signal peptide of *Streptococcus downei* glucosyltransferase I (*gtfI*) followed by the *Escherichia coli* $rrnBt_1t_2$ terminator and inserted in the shuttle vector pVA838. *S. gordonii* transformed with this plasmid (pMNK-4) expressed and secreted mature *Arthrobacter* dextranase. The transformant was found to repress the firm adherence of water-insoluble glucan in a coculture experiment with cariogenic bacteria, *Streptococcus sobrinus*, in the presence of sucrose. Such genetically engineered oral bacteria could provide a therapy to prevent dental caries.

Dental caries is produced by plaque-forming oral mutans streptococci. They are divided serologically into eight types (a to h), and most of them colonize the tooth surface even in the absence of sucrose. In the presence of sucrose, however, they adhere firmly to the tooth surface, mediated mainly by a water-insoluble glucan synthesized by glucosyltransferase (9, 13). The water-insoluble glucan is a 1,6-α-linked polymer with a high proportion of 1,3-α-linked glucosyl residues (5, 10, 19). Dextranase is an enzyme which hydrolyzes the 1,6-α-glucosidic linkages of glucan and is known to repress the adherence of mutans streptococci to the tooth surface in vitro (8, 11, 15, 23, 25). This enzyme also retards plaque deposition on the tooth surface and thus inhibits dental caries in experimental animals (2, 7, 12).

In clinical studies, plaque formation is reduced by using toothpaste including dextranase or mouthwashes with highly concentrated dextranase solution (4, 17, 24). However, it would be more effective to maintain high dextranase enzymatic activity levels in the oral cavity. We previously cloned a dextranase gene (*dex*) from *Arthrobacter* sp. strain CB-8. As the pH optimum of this enzyme is 5.5 to 7.5 and it is stable at 37°C, the enzyme is suitable for expression in the oral cavity (26).

In this study, we constructed a plasmid to express and secrete the dextranase in the oral bacterium *Streptococcus gordonii* (18) and tested its effect on the formation of water-insoluble glucan by a cariogenic bacterium, *Streptococcus sobrinus*.

MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* XL-1 Blue and *S. gordonii* Challis were used for cloning and transformation. *S. sobrinus* 6715 (serotype g) was used for the test of the adherence of insoluble materials to a glass surface by coculture with the *S. gordonii* transformant. Plasmid pVA838 (21) was used as a shuttle vector between *E. coli* and *S. gordonii*. Plasmid pMLG5 contained the *gtfI* gene from *Streptococcus downei* MFe28 on pBR322 (6). Plasmid pKK223-3 contained the *E. coli* $rrnBt_1t_2$ terminators (3).

Media. *E. coli* was grown in L broth (1% Bacto tryptone [Difco], 0.5% yeast extract [Difco], 0.5% NaCl), and *S. downei*, *S. gordonii*, and *S. sobrinus* were grown in Todd-Hewitt (Difco) and brain heart infusion (BHI; Difco) broth. Bacterial colonies producing dextranase activity were detected on 0.2% (wt/vol) blue dextran (Pharmacia)–agar plates (22). Antibiotics were used at the following concentrations: ampicillin (Meiji), 50 μg/ml; chloramphenicol (Sigma), 25 μg/ml; and erythromycin (Sigma), 20 μg/ml.

Synthesis of oligonucleotides. The oligonucleotides synthesized are listed in Fig. 1.

***S. gordonii* transformation.** *S. gordonii* was transformed by the method of LeBlanc and Hassell with minor modifications (20). *S. gordonii* was cultured overnight at 37°C in Todd-Hewitt broth supplemented with defibrinated blood (Japan Biotest Laboratories) to 4% (vol/vol). This culture was inoculated into transformation medium, which consisted of 1% Bacto-tryptone, 0.2% yeast extract, 0.2% glucose, and 10% heat-inactivated fetal calf serum (pH 7.8) (Flow Laboratories), and cultured to the early logarithmic phase at 37°C. Plasmid DNA (1 μg/ml of culture) was added, and incubation was continued for 20 min. DNase I was then added to 5 μg/ml, and incubation was continued for an additional 2 h. The cells were plated onto BHI agar plates containing 20 mM glucose and erythromycin and cultured at 37°C for 48 h.

Dextranase assay. *S. gordonii* transformants and *Arthrobacter* bacteria were cultured for 18 h at 37°C in Todd-Hewitt broth containing 200 mM glucose. The dextranase activity of the culture medium was assayed by the size of the transparent halo on the 0.2% blue dextran plate arising from hydrolysis of blue dextran around a 6-mm paper disk. It was standardized to purified *Arthrobacter* dextranase solution by using the corresponding reducing power of liberated sugar from dextran (molecular weight, 100,000 to 200,000) assayed by the method of Somogyi as modified by Nelson (25).

Western blot (immunoblot). The bacterial cells and concentrated culture medium were solubilized in sample buffer, loaded on a 10% polyacrylamide gel, and electrophoresed. Proteins were transferred onto filters (Immobilon-P; Milli-

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Linker 1 : 5' - TCGACCCCGGGTCGCGAG - 3'

3' - GGGGCCCAGCGCTCTTAA - 5'

(*Sal* I - *Sma* I - *Nru* I - *Eco*R I)

Linker 2 : 5' - CGGATCCGTCGACG - 3'

3' - GTACGCCTAGGCAGCTGCTTAA -5'

(*Sph* I - *Bam*H I - *Sal* I - *Eco*R I)

Linker 3 : 5' - CTAGCGCAGAACGCGCCATCACAACCG - 3'

3' - GCGTCTTGCGCGGTAGTGTTGGCAGCT -5'

(*Nhe* I~*Sal* I)

FIG. 1. Oligonucleotides. Nucleotide sequences encoding a part of the *S. downei gtfI* signal peptide and an initial sequence of the mature *Arthrobacter* dextranase are put between *Nhe*I and *Sal*I sites in linker 3.

pore), and dextranase was detected by using anti-*Arthrobacter* dextranase rabbit antibody (26), horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Bio-Rad), and 2,4-diaminobenzidine.

Adherence assay. The *S. gordonii* transformant and *S. sobrinus* 6715 were cocultured in BHI broth containing 1% sucrose and 50 mM sodium phosphate (pH 6.5) in glass tubes (13 by 100 mm; Corning Glass Works) at 37°C for 18 h at a 30° angle. The adherence of insoluble materials to the glass surface was assayed by the procedure reported previously (14).

RESULTS

Construction of a dextranase expression plasmid. To express and secrete the dextranase of *Arthrobacter* sp. strain CB-8 in *S. gordonii* Challis, we fused the structural gene for the *Arthrobacter* dextranase to the promoter and the DNA sequence encoding the signal peptide of glucosyltransferase (*gtfI*) from *S. downei* (6), which is a close relative of *S. gordonii*. The fused gene was followed by the *E. coli* $rrnBt_1t_2$ (3) terminator and inserted into a shuttle vector, pVA838, which can transfer between *E. coli* and *S. gordonii* (Fig. 2 and 3).

DNA containing the promoter and signal peptide sequence of the *S. downei gtfI* gene was released from pMLG5 by *Sau*3AI digestion and cloned into the *Bam*HI site of a pUC19 derivative in which the *Bam*HI site was flanked immediately on the 5′ and 3′ sides by *Sph*I and *Sal*I sites (linker 2 in Fig. 1), respectively. The resulting plasmid was then digested with *Nhe*I and *Sal*I, and the released fragment was replaced with a synthetic oligonucleotide (linker 3 in Fig. 1) in which the *gtfI* signal peptide sequence is fused with a sequence encoding the amino terminus of the mature *Arthrobacter* dextranase gene up to the first *Sal*I site. This fragment (*gtfI* promoter, *gtfI* signal peptide sequence, and 5′ dextranase sequence) with flanking 5′ *Sph*I and 3′ *Sal*I restriction sites (linker 2 in Fig. 1) was then released from the vector by *Sph*I and *Sal*I double digestion and cloned into the *Sph*I and *Sal*I sites of a pVA838 derivative which contained the *E. coli* $rrnBt_1t_2$ terminator previously cloned into the *Sal*I and *Nru*I sites of pVA838 by using linker 1 (Fig. 1). The resulting plasmid was then linearized by *Sal*I digestion, and the *Sal*I fragment encoding the remainder of the *Arthrobacter* dextranase gene from pDEX011 (26) was inserted in the correct orientation between the fusion fragment and the terminator sequence to produce the dextranase expression vector pMNK-4. *E. coli* and *S. gordonii* cells carrying this plasmid were selected by their resistance to chloramphenicol and erythromycin, respectively. The sequence of the upstream region of the reconstructed dextranase gene is shown in Fig. 3.

Expression and secretion of *Arthrobacter* dextranase in *S. gordonii*. To test whether the fused dextranase gene could be expressed in *S. gordonii*, the transformants carrying pMNK-4 were spotted on an agar plate containing 0.2% blue dextran (22). After 12 h of incubation at 37°C, we observed transparent halos which resulted from hydrolysis of blue dextran by the secreted dextranase around colonies of *S. gordonii*(pMNK-4), whereas no halo was detected around cells transformed with only the shuttle vector, pVA838 (Fig. 4).

The dextranase activity in the culture medium of *S. gordonii*(pMNK-4) was assayed after 18 h of incubation at 37°C and quantitated by the size of the transparent halo compared with that of purified dextranase derived from *Arthrobacter* sp. strain CB-8. *S. gordonii*(pMNK-4) secreted 0.2×10^{-2} U of dextranase activity per ml into the medium, and *Arthrobacter* sp. strain CB-8 produced 11.2×10^{-2} U of dextranase per ml in the same medium. One unit of dextranase activity was defined as the amount that increased the reducing power equivalent to 1 μmol of glucose in 1 min. This result indicated that the promoter of the *S. downei* glucosyltransferase gene was active in *S. gordonii* cells. Moreover, the *Arthrobacter* dextranase was secreted in an active form in a heterologous system.

The stability of the plasmid was then examined. The segregation of pMNK-4 in *S. gordonii* with respect to erythromycin resistance was less than 1% after 18 h of culture without the antibiotic. Furthermore, in the erythromycin-resistant colonies, dextranase-nonproducing bacteria were not segregated after 18 h of culture with the antibiotic. This result suggests that plasmid pMNK-4 is maintained stably without antibiotics and that the dextranase gene also does not segregate, even under the stress to produce dextranase and its precursor in the cells, as shown below.

Secretion of dextranase. An *Arthrobacter* dextranase (69 kDa) was detected by Western blot with anti-*Arthrobacter* dextranase antibody. A protein band similar in size to the mature *Arthrobacter* dextranase was observed both from the culture medium and bacterial cellular fraction of *S. gordonii*(pMNK-4) (Fig. 5). From this result, we concluded that the 69-kDa dextranase was expressed and secreted from the *S. gordonii* transformant as mature dextranase after cleavage of the signal peptide. We also detected another larger band (74 kDa) in the cellular fraction but not in the culture medium. This 74-kDa protein was probably an immature dextranase which contained the signal peptide of *gtfI* from *S. downei* at the N-terminal end of the *Arthrobacter* mature dextranase. Thus, the signal peptidase of *S. gordonii* could recognize and cleave the signal peptide of *S. downei gtfI* so as to secrete mature dextranase into the culture medium in an active form. However, the processing of a heterologous signal peptide from *S. downei* in *S. gordonii* would be less effective than in a homologous system, and thus the precursor accumulated in the bacterial cells.

Adherence of *S. sobrinus* 6715 inhibited by *S. gordonii* (pMNK-4). *S. sobrinus* 6715, a mutans streptococcal strain, produces water-insoluble glucan from sucrose and forms dental plaque. To test the effect of the transformant secreting dextranase on the formation of glucans by *S. sobrinus*, we

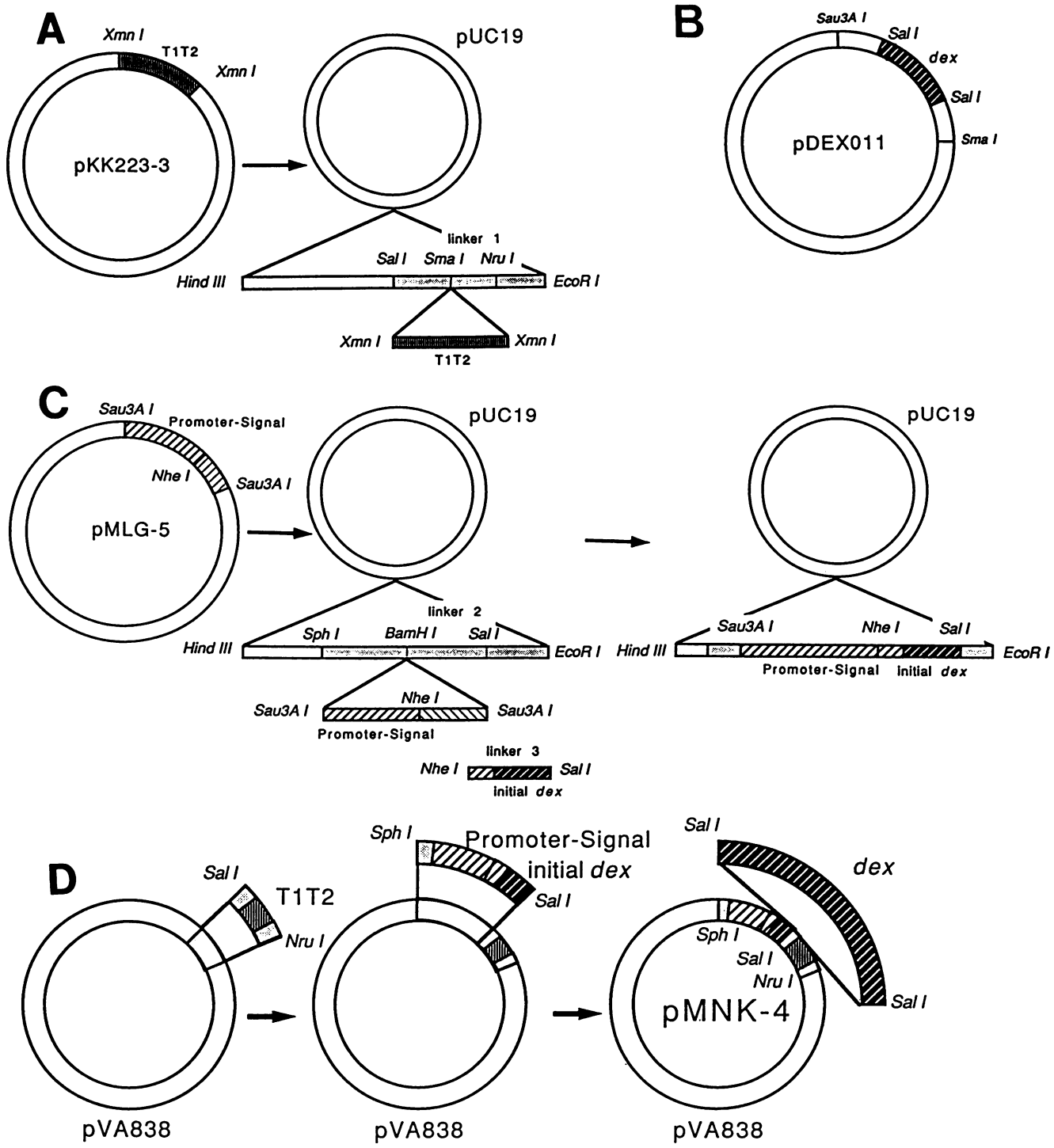
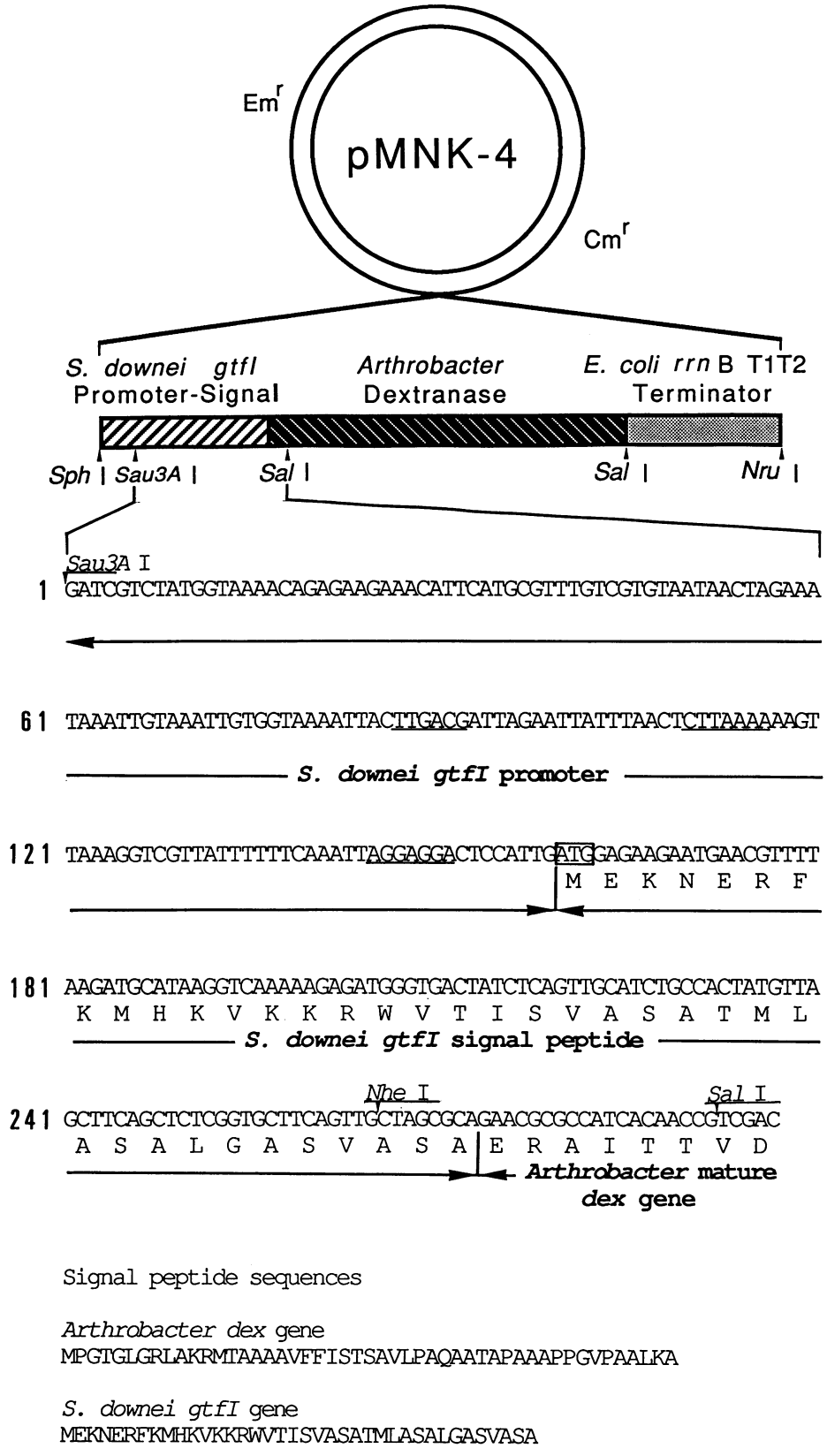


FIG. 2. Schematic of expression vector pMNK-4. See Materials and Methods. (A) Insertion of *E. coli* $rrnBt_1t_2$ terminator into pUC19. (B) *Arthrobacter* dextranase gene (*dex*) in pDEX011. (C) Construction of promoter and N-terminal part of *dex*. (D) Construction of pMNK4.

cocultured *S. gordonii*(pMNK-4) with *S. sobrinus* 6715 in BHI broth containing 1% sucrose in glass tubes. The adherence of the water-insoluble glucans formed was assayed (Fig. 6A). The culture medium in the glass tubes was rotated gently and discarded (fraction A). The residual material was rinsed with water by rotating three times, and the wash was collected as fraction B. Material still adhering to the glass after these treatments was designated adherent cells. The adherent cells were further separated by vortexing, and the supernatant was collected (fraction C). The remaining residue was designated the firm-adherence fraction (fraction D). Water-insoluble material in each fraction was suspended by



Signal peptide sequences

Arthrobacter dex gene
MPGTGLGRLAKRMTAAAAVFFISTSAVLPAQAATAPAAAPPGVPAALKA

S. downei gtfI gene
MEKNERFKMHKVKKRWVTISVASATMLASALGASVASA

FIG. 3. Structure of plasmid pMNK-4. pMNK-4 includes the *Arthrobacter* dextranase gene under the control of the *S. downei gtfI* promoter and its signal sequence. The signal sequence was joined to the sequence coding for mature *Arthrobacter* dextranase. The two genes were joined by using synthetic oligonucleotides. The putative promoter and ribosome-binding site sequences of *S. downei gtfI* are underlined. The signal sequences of the *Arthrobacter dex* gene and *S. gordonii gtfI* gene are shown.

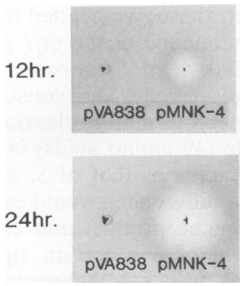


FIG. 4. Secreted dextranase halo assay. *S. gordonii* cells transformed with the dextranase expression vector pMNK-4 or the shuttle vector pVA838 were cultured on an agar plate containing 0.2% blue dextran in BHI medium for 12 and 24 h at 37°C. Halos were observed around colonies of *S. gordonii*(pMNK-4).

an ultrasonic oscillator, and its optical density at 550 nm was measured.

When we compared the coculture of *S. sobrinus* 6715 and *S. gordonii*(pMNK-4) with that of *S. sobrinus* 6715 and *S. gordonii*(pVA838), the control transformants, the total insoluble material produced (fractions A, B, C, and D) decreased to 79.4% of the control value after introduction of the dextranase-producing plasmid (Table 1). The cell adherence index ([C + D/A + B + C + D] × 100) was 28.7% for the coculture of *S. sobrinus* 6715 and *S. gordonii*(pMNK-4) but 42.4% for the coculture of *S. sobrinus* 6715 and *S. gordonii* (pVA838). Furthermore, the firm-adherence index ([D/A + B + C + D] × 100) was 4.0% for the coculture of *S. sobrinus* 6715 and *S. gordonii*(pMNK-4) but 12.5% for the control coculture. Thus, firm adherence was greatly reduced (25.6%), as shown in Fig. 6B.

The growth of the bacteria was examined in a coculture experiment. The growth of both *S. sobrinus* 6715 and *S. gordonii* carrying either pMNK-4 or pVA838 was repressed when they were mixed, but the number of *S. sobrinus* 6715 cells was the same after 18 h of coculture with either *S. gordonii*(pMNK-4) or *S. gordonii*(pVA838). The growth of

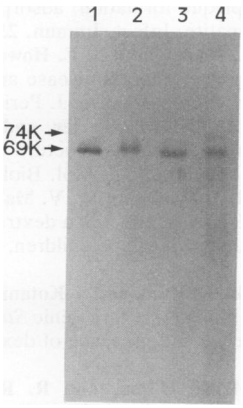


FIG. 5. Detection of *Arthrobacter* dextranase. *Arthrobacter* sp. strain CB-8 and *S. gordonii* Challis(pMNK-4) were cultured. The medium and bacterial cells were separated, lysed in sample buffer, loaded on a 10% polyacrylamide gel, and electrophoresed. The gel was blotted to Immobilon-P, and dextranase was detected with anti-*Arthrobacter* dextranase rabbit antibody. Lanes 1 and 2, *Arthrobacter* sp. strain CB-8; lanes 3 and 4, *S. gordonii* Challis (pMNK-4); lanes 1 and 3, culture medium; lanes 2 and 4, bacterial cells. Sizes are shown in kilodaltons.

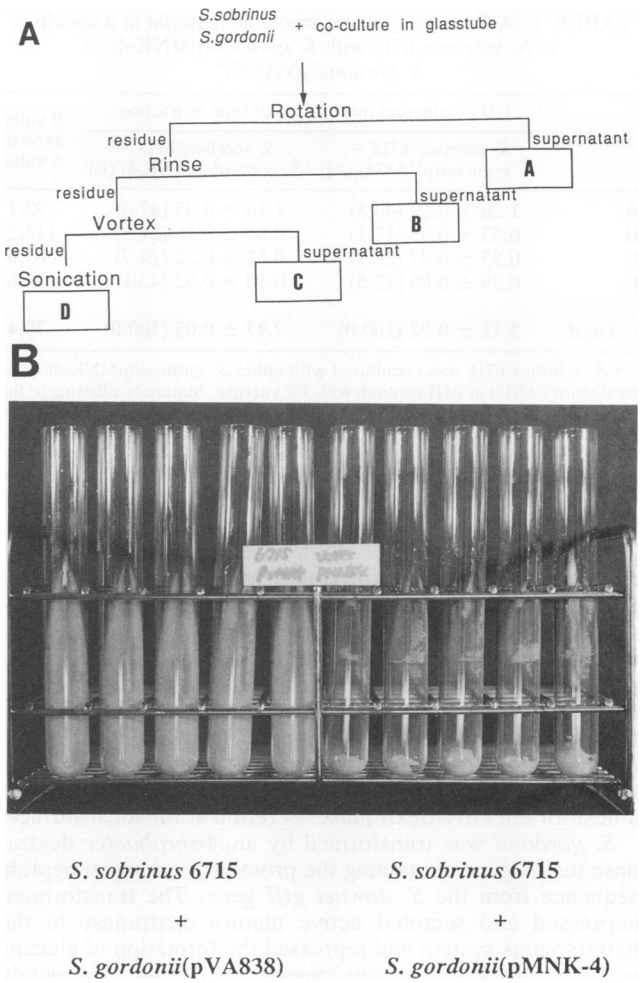


FIG. 6. Adherence assay. (A) Schematic representation of the adherence assay for water-insoluble glucans. (B) Firmly adherent (fraction D) water-insoluble glucan after cocultivation of glucan-producing *S. sobrinus* 6715 with *S. gordonii*(pMNK-4) or *S. gordonii*(pVA838). Each experiment was carried out with five tubes and repeated five times.

S. gordonii also showed no differences after transformation with either pMNK-4 or pVA838. Therefore, the production of dextranase had no effect on the growth of cocultured bacteria, and the reduction in firm adherence caused by pMNK-4 was thought to result from the recombinant dextranase, not from growth inhibition. After the coculture experiment, all *S. gordonii*(pMNK-4) cells maintained the ability to produce dextranase, which indicates plasmid stability. These results indicate that the adherence of the water-insoluble glucan produced by *S. sobrinus* 6715 to a glass wall was suppressed by coculture with *S. gordonii* transformed with the dextranase-secreting plasmid pMNK-4.

DISCUSSION

In the prevention of dental caries, dextranase is one of the effective agents which digest the 1,6-α-glucoside bond of water-insoluble glucan on the tooth surface, and dextranase significantly inhibits the production of water-insoluble glucan and the adherence of mutans streptococci cells in vitro (8, 11, 15, 23, 28). However, rigorous use of mouth rinses

TABLE 1. Adherence of water-insoluble material in a coculture of *S. sobrinus* 6715 with *S. gordonii*(pMNK-4) or *S. gordonii*(pVA838)[a]

Fraction	OD of adherent material (% of total in fraction)		B value as % of A value
	S. sobrinus 6715 + *S. gordonii*(pVA838) (A)	*S. sobrinus* 6715 + *S. gordonii*(pMNK-4) (B)	
A	1.26 ± 0.09 (40.5)	1.16 ± 0.05 (47.0)	92.1
B	0.53 ± 0.08 (17.1)	0.60 ± 0.16 (24.3)	113.2
C	0.93 ± 0.07 (29.9)	0.61 ± 0.22 (24.7)	65.6
D	0.39 ± 0.08 (12.5)	0.10 ± 0.02 (4.0)	25.6
Total	3.11 ± 0.02 (100.0)	2.47 ± 0.05 (100.0)	79.4

[a] *S. sobrinus* 6715 was cocultured with either *S. gordonii*(pMNK-4) or *S. gordonii*(pVA838) in BHI medium with 1% sucrose. Materials adhering to the glass tubes were separated into fractions A through D, and their optical densities (OD) were measured. The percentages of each fraction are shown. Experiments were done five times, and standard deviations are shown. The comparison for each fraction between *S. gordonii*(pMNK-4) and *S. gordonii* (pVA838) is shown as a percentage.

that contain highly concentrated dextranase is necessary for the dispersion and prevention of bacterial plaques in vivo (2, 4, 7, 12, 17, 24). The development of more effective delivery methods would aid the prevention and control of bacterial plaque infections on teeth. Continuous production of dextranase becomes possible by transformation of an oral bacterium. *S. gordonii* is the first organism to colonize dental plaque by interacting with salivary glycoproteins on the tooth surface (1, 29). Consequently, *S. gordonii* is suitable as a host for effective dextranase secretion at the local surface.

S. gordonii was transformed by an *Arthrobacter* dextranase fusion gene containing the promoter and signal peptide sequence from the *S. downei gtfI* gene. The transformant expressed and secreted active mature dextranase in the heterologous system and repressed the formation of glucans in vitro. The cocultivation experiment did not completely protect against the formation of water-insoluble glucan, but firm adherence was reduced by 75%. Thus, the continuous production of dextranase is expected to prevent the accumulation of firmly adherent glucan and bacteria. The water-insoluble but not the firmly adherent glucan would be easily removed with mouthwash. Although some oral bacteria, such as *Streptococcus salivarius*, secrete dextranase (27), they do not easily degrade insoluble glucans on the outside surface. On the other hand, *S. gordonii* cells secreting *Arthrobacter* dextranase adhere to the same dental plaque, competing with *S. sobrinus* 6715, and thus are expected to prevent the production and accumulation of firmly adherent glucans on the tooth surface.

Water-insoluble glucans have many 1,3-α-glucosyl linkages. These linkages may contribute to the water insolubility of the glucan, whereas 1,6-α-linkages may endow the glucan with adhesive properties. For complete release of glucans from the tooth surface, another enzyme, mutanase, which hydrolyzes 1,3-α-glucosyl linkages, is also necessary. As de novo formation of glucan film was significantly inhibited by the presence of a low concentration of mutanase in vitro (30), mutanase should also be expressed continuously from *S. gordonii* transformed with the mutanase gene.

The 430-bp fragment encoding the *Arthrobacter* signal peptide sequence and dextranase gene was introduced into *E. coli* and *S. gordonii* cells. In *E. coli*, the gene is expressed but the dextranase is not secreted (26). On the other hand, *S. gordonii* transformed with the same DNA expresses dextranase at a very low level, but the dextranase is not secreted. For secretion of dextranase, we needed to join the promoter and signal peptide sequence of the *gtfI* gene of *S. downei*, which is a close relative of *S. gordonii*, upstream of the *Arthrobacter* dextranase gene. Our construct made it possible to express and secrete mature dextranase. The *Arthrobacter* signal peptide (49 amino acids) is longer and not so rich in basic amino acids as that of *S. sobrinus* (38 amino acids) (Fig. 3). These differences would explain the ability to recognize the fused gene product and cleave it at the site expected for removal of a secretion signal peptide in *S. gordonii* (16, 31). For more efficient secretion of the dextranase, we should clone and sequence the secreting polypeptide, such as glucosyltransferase, from *S. gordonii* itself.

Recombinant DNA-containing bacteria raise issues of health, safety, and administrative restrictions. But in this case, the bacterium used as the vector is the oral bacterium *S. gordonii*, which is very populous in the natural flora and does not grow in other environments. Furthermore, the introduced plasmid contains the dextranase gene, which produces the enzyme but not the small peptide with physiological activity that is easily absorbed from the digestive organs. *S. gordonii* is the major bacterium in dental plaque, and we did not find growth differences between the transformed and untransformed cells. Therefore, they persist in the oral cavity, but repeated inoculation will give better results. Integration of the dextranase gene in *S. gordonii* should be a better strategy to consider for elimination of the resistance markers. Administrative restrictions will be resolved by determination of the safety of these bacteria and the therapeutic efficiency of this method.

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