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Sequence and Phylogenetic Analyses of Novel Glucosyltransferase Genes of Mutans Streptococci Isolated from Pig Oral Cavity

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Nucleotide sequences of water-insoluble glucan-producing glucosyltransferase (*gtf*) genes of new mutans streptococci isolated from pig oral cavity, *Streptococcus orisuis* JCM14035, and of *Streptococcus criceti* HS-6 were determined. The *gtf* gene of *S. orisuis* JCM14035 consisted of a 4,401 bp ORF encoding for a 1,466 amino acids, and was revealed to belong to the *gtfI* group. The percent homology of amino acid sequence of the GTF-I from *S. orisuis* and *S. criceti* are 95.0%, however, this score ranges from 77.0% to 78.0% when compared to *Streptococcus sobrinus* 6715. The deduced N-terminal amino acid sequence was considered responsible for the secretion of GTF-I in *S. orisuis* JCM14035 and *S. criceti* HS-6 with high similarity to known GTF proteins from other streptococci. In addition, two other conserved regions, i.e., N-terminal putative catalytic-site and C-terminal glucan binding domain, were also found in GTF-Is of *S. orisuis* JCM14035 and *S. criceti* HS-6. Phylogenetic analysis suggested that *S. orisuis* JCM14035 and *S. criceti* HS-6, closely related to each other, resemble *S. sobrinus* and *S. downei* based on the amino acid sequences of the GTFs.

***Keywords*:** glucosyltransferase, *Strptococcus orisuis*, *Streptococcus criceti*, phylogenetic tree

Among the oral mutans streptococci, *Streptococcus mutans* and *Streptococcus sobrinus* are generally accepted to be the principal etiological agent of human dental caries. These bacteria can secrete glucosyltransferases (GTFs), which catalyze the formation of water-insoluble and water-soluble glucans (WIG and WSG, respectively). It has been proposed that the function of WIG-producing enzymes is the most important virulent factors contributing to the development of caries formation in human (Hamada and Slade, 1980). *S. mutans* and *S. sobrinus* possess different WIG-producing enzymes, GTF-B and GTF-I, which are encoded by *gtfB* and *gtfI* genes, respectively (Shiroza *et al*., 1987; Abo *et al*., 1991).

Recently, a novel *S. mutans*-like species, *Streptococcus orisuis* JCM14035 was isolated from pig oral cavity, and characterized (Takada and Hirasawa, 2007). The serological characteristic of *S. orisuis* JCM14035 was serotype *d*, which is the same as that of *S. sobrinus* 6715. However, genetic analyses such as DNA-DNA hybridization, GC contents, and 16S rRNA gene sequence indicated closer relationship to *Streptococcus criceti* HS-6 (serotype *a*). The production of WIG-synthesizing GTF from pig strain was also confirmed.

In *S. criceti*, nucleotide sequence of two WSG-producing *gtf* genes were determined and characterized previously (Inoue *et al*., 2000), on the other hand, one WIG-producing GTF enzyme was reported (Tsumori *et al.,* 1985), though WIG-producing *gtf* gene has not yet been isolated.

In this communication, we present the nucleotide sequence of the WIG-producing *gtf* gene of *S. orisuis* JCM14035 and *S. criceti* HS-6. Also we attempted the enzymatic and antigenic analysis of WIG-producing *gtf* gene products and construction of phylogenetic tree based on deduced amino acid sequences from other streptococcal GTFs.

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Materials and Methods

Bacterial strains and medium

S. orisuis JCM14035 (Takada and Hirasawa, 2007) and *S. criceti* HS-6 (GTC242) were used in this study. The streptococci were maintained on Brain Heart Infusion agar (BHI, Difco Laboratories, USA). Partially defined medium, M4 (Fukushima *et al.*, 1981), was used for the preparation of the GTF proteins.

PCR experiments

The sequences of PCR primers used in this study are listed in Table 1. In the first PCR for the detection of WIG-producing *gtf* gene, two sets of primers, GTFB-F and GTFB-R, and GTFI-F and GTFI-R, were used (Oho *et al.*, 2000). Chromosomal DNA was isolated using the commercial Wizard® Genomic DNA Purification Kit (Promega, Co., USA). PCR amplification was carried out employing the GeneAmp® PCR system 9700 (PE Applied Biosystems, Ltd., USA), and the target region of the gene was amplified using the Ex *Taq* polymerase (TaKaRa Bio Inc., Japan) under the following conditions: (i) initial denaturation, 94°C for 1 min; (ii) 30 cycles of amplification, denaturation at 95°C for 30 sec, primer annealing at 59°C for 30 sec, and extension at 72°C for 1 min; (iii) final extension, at 72°C for 10 min. A final amplicon containing the entire *gtfI*-coding se-

Table 1. Used primers in this study

Primers	Sequence	Location	Reference
GTFB-F	5'-ACTACACTTTCGGGTGGCTTGG-3'	793-814[a]	Oho *et al.* (2000)
GTFB-R	5'-CAGTATAAGCGCCAGTTTCATC-3'	1228-1309[a]	Oho *et al.* (2000)
GTFI-F	5'-GATAACTACCTGACAGCTGACT-3'	871-892[b]	Oho *et al.* (2000)
GTFI-R	5'-AAGCTGCCTTAAGGTAATCACT-3'	1561-1582[b]	Oho *et al.* (2000)
GTFI-up	5'-GTCCTCAACAGAAATTGCCT-3'	9-28[b]	Shinozaki-Kuwahara *et al.* (2005)
GTFI-down	5'-AATGGACTTAAAGCGTAAACC-3'	5021-5042[b]	Shinozaki-Kuwahara *et al.* (2005)
gtfI-1235F	5'-ACGGTGCTCTCAAATTCGAC-3'	1547-1566[c]	This study
gtfI-1760F	5'-CAGTGTGGTTGATCGTGAAG-3'	2091-2110[c]	This study
gtfI-2262F	5'-TATCCCTCCACCAAGATGCA-3'	2933-2952[c]	This study
gtfI-3177F	5'-TTCTACCGGTCAGGCAATAGATCC-3'	3489-3512[c]	This study
gtfI-1187R	5'-GGTTGGGTCTTAACAAAGGC-3'	1480-1499[c]	This study
gtfI-1723R	5'-CCAGTGACCAAAGCATTGAC-3'	2025-2044[c]	This study
gtfI-2188R	5'-AATTTTGCATAGCTTGTCCG-3'	2481-2500[c]	This study
gtfI-2820R	5'- ATCAAGGAAGGTACCATCTG-3'	3113-3132[c]	This study
gtfI-5'F	5'-TACCCTCTTCGCTAAGGTGAAG-3'	1-22[c]	This study
gtfI-3'R	5'-GGCTTCCCCAAGTGTGAGGAAGTC-3'	4961-4984[c]	This study

[a] The nucleotide sequence from GenBank accession no. M17361 (*S. mutans gtfB*).
[b] The nucleotide sequence from GenBank accession no. D90213 (*S. sobrinus* 6715 *gtfI*).
[c] The nucleotide sequence from DDBJ accession no. AB272987 (*S. orisuis gtfI*).

quence was prepared using other primers, gtfI-5'F and gtfI-3'R, under the same conditions of inverse PCR (see below).

Inverse PCR and DNA sequencing

An inverse PCR method was utilized to determine unknown regions flanking the known sequence of the *gtf* genes. The purified chromosomal DNA was digested with appropriate restriction enzymes, re-circularized by ligation, and subjected to the template for inverse PCR with suitable primer sets. The target region of the gene was amplified using the LA PCR kit (TaKaRa) under the following conditions: (i) initial denaturation, 94°C for 1 min; (ii) 30 cycle of amplification, denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 3 min; (iii) final extension, at 72°C for 10 min. Amplified PCR products were purified and sequenced with ABI PRISM 310 Genetic analyzer (PE Applied Biosystems) using a Big Dye Terminator v1.1 Cycle Sequencing Kit (PE Applied Biosystems).

Sequence and phylogenetic analyses

Sequence data were analyzed using DNASIS sequence analysis software (Hitachi Software Engineering Co., Ltd., Japan). Multiple-alignment and phylogenetic analyses were performed by using the CLUSTAL W program (Thompson *et al*., 1994). Tree topology and evolutionary distances were calculated by Kimura (1983). Phylogenetic trees were constructed by neighbor-joining method (Saito and Nei, 1987), and were displayed with the TreeView program (Page, 1996).

Enzymatic and antigenic activities of GTFs prepared from *S. orisuis* and *S. criceti*

All centrifugation was carried out at 4°C. The streptococci were cultured in 5 ml of M4 broth at 37°C for 18 h under anaerobic condition. To detect the enzymatic activities of the GTF present in the culture supernatants, the cells were removed by centrifugation (8,000×g for 10 min). To 5 ml supernatant was added an equal volume of chilled ethanol, and the mixture was kept on ice for 30 min. Following centrifugation (20,000×g for 20 min), precipitates were dissolved in 100 μl of distilled water. Five microliter aliquots were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and the gels were allowed to soak in a sucrose solution (50 mM phosphate buffer; pH 6.0, 2% sucrose, and 1% Triton X-100). Following incubation at 37°C for 16 h, the presence of WIG in a gel could be detected as white bands on a black background. Western blotting was carried out following SDS-PAGE, and proteins were transferred onto a nitrocellulose membrane and allowed to react with anti-GTF-I mouse monoclonal antibody (MAb), which was prepared previously (Nanbu *et al*., 2000). Immuno-positive protein bands were visualized using the standard procedure employing horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Bio-Rad Laboratories, USA).

Nucleotide sequence accession numbers

The DDBJ/EMBL/GenBank accession numbers for *gtfI* genes of *S. orisuis* JCM14035 and *S. criceti* HS-6 in this paper are AB272987 and AB273728, respectively.

Results

Detection of *gtf* genes

To examine the presence of *gtf* genes in *S. orisuis* JCM 14035 and *S. criceti* HS-6 strains, a PCR experiment was

performed using the two sets of primers capable of detecting and identifying a difference of *S. mutans gtfB* and *S. sobrinus gtfI* genes (Oho *et al*., 2000). As shown in Fig. 1, the 712 bp fragment was observed in lanes 4 to 6, while no 517 bp fragment was amplified, except for the positive control (lane 1). It was suggested that *gtfI*-like gene was maintained in these strains. The 712-bp fragments were purified, and sequencing results revealed 80.0 to 82.0% homology compared to the corresponding region of *gtfI* gene from *S. sobrinus* 6715.

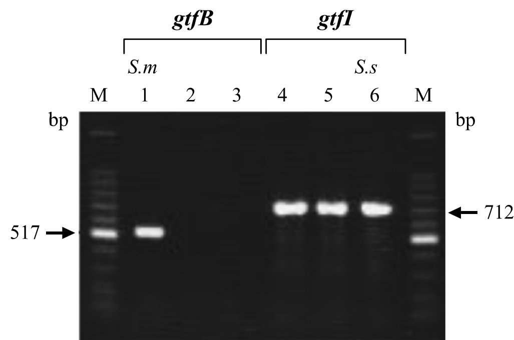

Fig. 1. Agarose gel electrophoresis of PCR products of the *gtfB* or *gtfI* sequences in chromosomal DNA from mutans streptococci using primers GTFB-F, -R (lanes 1 to 3), and GTFI-F, -R (lanes 4 to 6). Lanes 1, *S. mutans* GS5; 2 and 4, *S. orisuis* JCM14035; 3 and 5, *S. criceti* HS-6; 6, *S. sobrinus* 6715; M, 100-base ladder for size marker.

Determination of *S. orisuis gtf* gene by inverse PCR

Because GTFI-up and GTFI-down primers, designed previously based on the *gtfI* gene from *S. sobrinus* 6715 (Shinozaki-Kuwahara *et al*., 2005; see Table 1), were unable to amplify the counterpart of *S. orisuis* JCM14035 and *S. criceti* HS-6, an inverse PCR method was utilized to determine the unknown regions adjacent to the known sequence of the *gtf* genes in *S. orisuis* JCM14035 (Fig. 2). As shown in Fig. 2A, a set of other primers, gtfI-1235F and gtfI-1187R, was designed based on the sequenced 712-bp fragments, and used in *Bam*HI-digested inverse PCR. The resulting product (line a) was directly sequenced utilizing the gtfI-1235F and gtfI-1187R primers, respectively. The *Bam*HI-digested inverse PCR allowed to determine the sequence from position 1 (528 bp upstream of the start codon) to position 1,652, thus covering approximately one-fourth of the 5'-end of the *gtf* gene. To further examine the nucleotide sequence around the 3'-region of the *gtf* gene, the 0.9-, 1.1-, 0.7-, and 1.5-kb fragments were obtained by subsequent *Pst*I-, *Hin*dIII-, *Cla*I-, and *Pst*I-digested inverse PCRs, respectively (lines b to e, respectively). Utilizing these fragments, the whole *gtfI*-like gene of *S. orisuis* JCM14035 was determined. To amplify the entire *gtf*-coding region of *S. criceti* HS-6 by PCR, primers, gtfI-5'F and gtfI-3'R, were designed, 5.0 kb PCR prod-

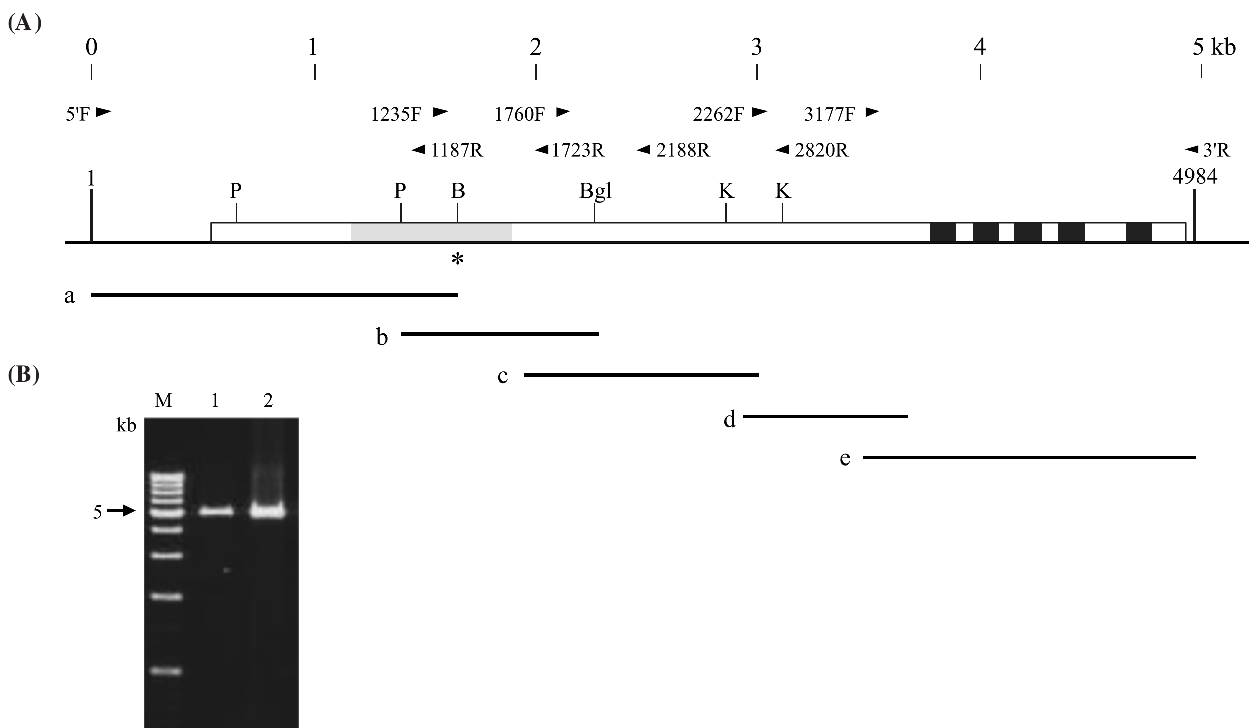

Fig. 2. (A) Restriction map of *S. orisuis* JCM14035 *gtfI* gene. Shaded bar presents the 712 bp region amplified by GTFI-F and GTFI-R primers. Solid bars show the amino acid sequences of repeating units. Asterisk indicates putative catalytic-site. Arrowheads denote the primers designed in this study (see Table 1). B, *Bam*HI; Bgl, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I. Solid lines a, *Bam*HI-digested; b and e, *Pst*I-digested; c, *Hin*dIII-digested; d, *Cla*I-digested inverse PCR products for determination of nucleotide sequences of *S. orisuis gtfI* gene. For details, see text. (B) Agarose gel electrophoresis of PCR products amplified by gtfI-5'F and gtfI-3'R primers. Lanes 1, *S. orisuis* JCM14035; 2, *S criceti* HS-6; and M, 1 kb ladder for size marker.

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So;MEKNVHYKMH KVKKRWVTIS VASATMLASA LGASVANADT DAAAADANNA ANAAVAEDQA ASSQDNQAAP LPATATSEDT GS----TEEV AVNQDAQV-- --------AN TDTQEAKWVP
Sc;·········· KVKKRWV··· ·········· ······S··· ·········· ······G··· ·········· ·········· ··----···· ········-- --------·· ·D········
Ss;·····RF··· KVKKRWV·L· ·········· ······S··· ·TASD·S·-- -QTV·TG··T TNN·ATDQTS IA······QS A·TDAA·DQA SAAEQT·GTT ASTDTAAQTT ·NAN······

So;NTGNNSEISD AATPQA-AVA ATDAQSVPQA LTNLSNVKQV DGKYYYYDAD GNVKKNFAVS VGDAFYFDET GAYKDTSKVD ADKTSSSVNQ TTETFAANNR AYSTAAENFE AIDNYLTADS
Sc;··D······· ------·--- ·A·V······ ·········· ·········· ········I· ·········· ·········G ·······A·· ··A······· ·········· ··········
Ss;·ENE·QGFT· EMLAE·KN·· TAESD·I·SD ·AKM······ ·········· ·········· ···KY····· ·········· ···S··A·S· NATI······ ····S·K··· ·V········

So;WYRPKSILKD GTTWTESTKD DFRPLLMAWW PDTETKRNYV NYMNKVVGID KTYTAETSQA DLTAAAELVQ ARIEQKITSE KNTKWLREAI SAFVKTQPQW NGESEKPYDD HLQNGALKF-
Sc;·········· ·K········ ·········· ·········· ·········· ·········· ·········· ·····R···· ·········· ·········· ·········· ·········-
Ss;·········· ·K····G··· ·········· ·········· ·········· ·········· ·········· ·····R···· N········· ·········· ·········· ·······L·I

So;DNETSLTPDT QSGYRILNRT PTNQTGSLDP RFTFNQNDPL GGYEYLLAND VDNSNPVVQA ESLNWLHYLL NFGSIYANDP EANFDSIRVD AVDNVDADLL QISSDYLKSA YKIDKNNKNA
Sc;·········· ·········· ·········· ·········· ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
Ss;··Q·D····· ··N······· ·········S ···Y·P···· ···DF····· ·········· ·Q········ ·········A ·········· ·E······Q· ········A· ··········

So;NDHVSIVEAW SDNDTPYLND DGDNLMNMDN KFRLSMLWSL AKPTNVRSGL NPLIHNSVVD REVDDREVEA TPNYSFARAH DSEVQDLIRD IIKAEINPNS FGYSFTQEEI DQAFKIYNED
Sc;·········· ········H· E········· ·······R·· ···LDK···· ·········· ·········E I·S······· ·········· ·········· ·········· ··········
Ss;·········· ········H· ·········· ·········· ···TDV···· ·······L·· ·········T V·S······· ·····I···· ·········· ·········· ··········

So;LKKTNKKYTH YNVPLSYTLL LTNKGSIPRI YYGDMFTDDG QYMANKTVNY DAIESLLKAR MKYVSGGQAM QNYNIGNGEI LTSVRYGKGA LKQSDKGDAT TRTSGIGVVM GNQPNFSLEG
Sc;·········· ·········· ·········· ·········· ·········· N········· ·········· ···Q······ ·········· ·········· ·······I·· ··········
Ss;···SDK···· ·········· ·········V ·········· ·········· ·········· ····A····· ···Q······ ·········· ·········· ·····V···· ········D·

So;KVVALNMGAT HTKQKYRALM VSTETGVAIY NSDEEAEAAG LIKTTDENGY LYFLNDDLKG VANPQVSGFL QVWVPVGAPA DQDIRVAATD AASTDGISLH QDAALDSRVM FEGFSNFQSF
Sc;·········· ·AN·E····· ···KD····· AT·AD·S··· MT·R······ ·········· ·····I···· ·········· ········TN A·····K··· ····M····· ········A·
Ss;·········A ·AN·E····· ···KD····· AT·AD·S··· ·V·R······ ·········· ·····V···· ········AD ········SD T·····K··· ····M····· ········S·

So;ATKEEEYTNV VIAKNVDKFV SWGITDFEMA PQYVSSTDGT FLDSVIQNGY AFTDRYDLGM SKANKYGTAD QLVAAIKALH AKGLRVMADW VPDQMYTFPK KEVVTVTRTD KFGNPVAGSQ
Sc;····D··A·· V········· ·········· ···T··D··Q ·········· ·········· ·········E H··K······ KA··K····· ·········· ·········· ··········
Ss;·········· V··N······ ·········· ·········Q ·Q········ ·········· ·········· ···K······ ····K····· ·········· Q········· ·····I····

So;INHTLYVTDT KGSGDDYQAK YGGAFLDELK EKYPELFTKK QISTGQAIDP SVKIKQWSAK YFNGSNILGR GANYVLSDQA SNKYFNVAEG KVFLPAAMLG KVVESGIRFD GKGYIYNSST
Sc;·········· ·········· ·········· ·········· ·········· ·········· ·········· ·········· ·········· ·····G···· ·········· ··········
Ss;···S······ ·S········ ·········· ·········· ·M········ ·········· ·········· ··D······V ········SD TL···SSL·· ·········· ·········A

So;TGEQVKDSFI TEAGNLYYFG KDGYMVMGAQ NIQGANYYFL ANGAALRNSI LTDQDGKSHY YANDGKRY-- ENGYYQFGND SWRYFENGVM AVGLTRVAGH DQYFDKDGIQ AKNKIIVTRD
Sc;·········· ·········· ·········· ·········· ·········· ·········· ········-- ·········· ·········· ···V······ ·········· ··········
Ss;··D···A··· ·········· ······T··· T·N····F·· E··T····T· Y··AQ·N··· ········EN ····Q····· -····KD·N· ·····T·D·N V·······V· ··D·······

So;GKVRYFDEHN GNAATNTFIS DQAGHWYYLG KDGVAVTGAQ TVGKQHLYFE ANGQQVKGDF VTAKDGKLYF FDGDSGDMWT DTFVQDKAGH WFYLGKDGAA VTGAQTVRGQ KLYFKANGQQ
Sc;·········· ···V······ ·········· ·········· ·········· ·········· ·········· ·········· ·········· ·········· ·········· ··········
Ss;·······Q·· ···V·····A ·KT······· ·········· ·····K···· ·········· ··SDE····· Y·V······· ···IE····N ·········· ······I··· ··········

So;VKGDIVKGAD GKIRYYDANS GDQVYNRTVK GSDGKTYIIG NDGVAITQTI ---------- ---------- ---------- ---------- ---------- ---------- ----------
Sc;·········· ·········· ·········· ·········· K········· ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ss;········T· ········K· ······K··· AA·····V·· ·····VDPSV VKGQTFKDAS GALRFYNLKG QLVTGSGWYE TANHDWVYIQ SGKALTGEQT INGQHLYFKK DGHQVKGQLV

So;---------- ---------- ---------- ---------- ---AKGQTIK DGSVLRFYSM EGQLVTGSGW YSNAKGQWLY VKNGQVLTGL QTVGSQRVYF DANGIQAKGK AVRTSDGKLR
Sc;---------- ---------- ---------- ---------- ---······· ·········· ·········· ·········· ·········· ·········· ·········· ··········
Ss;TGTDGKVRYY DANSGDQAFN KSVTVNGKTY YFGNDGTAQT AGNP······ ···DI····· ·········· ···Q······ ····K····· ·········· ·E········ ········I·

So;YFDANSGSMI TNQWKEVNGQ YYYFDNNGVA IYRGWN
Sc;·········· ·········· ·········· ······
Ss;···E······ ·········R ····G·D·AR ······
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Fig. 3. Alignment of the amino acid sequences of GTF-Is. Identical residues are shown by dots (·) while gaps (-) are introduced to maximize the homology. Putative active-site is under lined. The C-terminals of repeat regions are indicated by boxes according to Abo *et al*. (1991). So, *S. orisuis* JCM14035; Sc, *S. criceti* HS-6; Ss, *S. sobrinus* 6715.

ucts from *S. orisuis* JCM14035 and *S. criceti* HS-6 were amplified (Fig. 2B), and sequenced.

Sequence analysis

The determined nucleotide sequences of *S. orisuis* JCM 14035 *gtf* gene contain a 4,401 bp open reading frame (ORF) and the primary gene products are composed of 1466 amino acids with a molecular mass of ca.162 kDa. The ORF of this gene is preceded by a potential ribosome-binding site, AGGAGG (Shine and Dalgarno, 1974) that is complementary to the 3'-end of the 16S rRNA gene of *S. orisuis* JCM14035. In addition, several promoter-like sequences, i.e., -10 (ATTAAA) and -35 (TTGACG) region, can also be found, which resemble the *S. sobrinus* 6715 *gtfI* sequence (Abo *et al*., 1991). The percent homology of the nucleotide sequence of the *gtf* gene from *S. orisuis* JCM14035 was 74.0% when compared with that of *S. sobrinus* 6715. Therefore, *gtf* gene from *S. orisuis* JCM14035 seems to correspond to the *S. sobrinus gtfI* gene. The amino acid sequences of *S. orisuis* JCM14035, *S. criceti* HS-6, and *S. sobrinus* 6715 GTF-I were examined and aligned (Fig. 3). The percent homology of the GTF-I amino acid sequences from *S. orisuis* JCM 14035 and *S. criceti* HS-6 was 95.0%. The score of these two strains is ranging from 77.0 to 78.0% compared with

that of *S. sobrinus* 6715. The first 38 amino acids contained the signal sequence of GTF proteins, and these amino acids were similar to those by previously reported GTFs (Ferretti *et al*., 1987; Shiroza *et al*., 1987; Abo *et al*., 1991; Sato *et al*., 1993). Then, Mooser *et al*. (1991) reported the putative active-site sequence (DSIRDAVD) from GTF-I enzyme of *S. sobrinus* 6715, and the corresponding sequence was identified in the amino acid sequence of *S. orisuis* JCM14035. Near the C-terminus of the GTF molecule are the direct repeating units consisting of 35 amino acids responsible for glucan binding. However, the numbers of repeating units of these domains were different in *S. orisuis* JCM14035 and *S. criceti* HS-6 compared with that of *S. sobrinus* 6715.

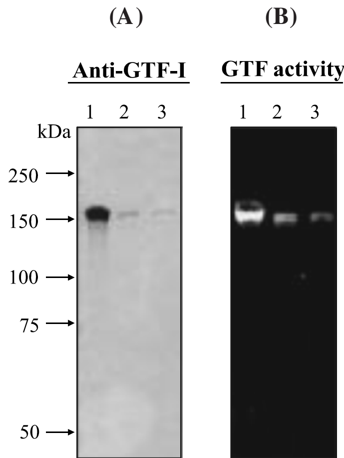

Fig. 4. Western blotting (A) and zymograph (B) of the crude GTF-I enzyme preparations following SDS-PAGE. Lanes: 1, *S. sobrinus* 6715; 2, *S. orisuis* JCM14035; and 3, *S criceti* HS-6, respectively. Molecular size markers are shown at the left in panel A.

Confirmation of WIG-producing GTFs from *S. orisuis* and *S. criceti*

To confirm the secretion of WIG-producing GTF in *S. orisuis* JCM14035 and *S. criceti* HS-6, extracellular crude enzymes were prepared from these streptococci (see 'Materials and Methods'). As shown in Fig. 4, both immunologically (A) and enzymatically (B) active GTF-I proteins were found in the preparations from supernatant of *S. sobrinus* 6715, *S. orisuis* JCM14035 and *S. criceti* HS-6 following SDS-PAGE experiments (lanes 1 to 3, respectively).

Phylogenetic analysis

To study the relationship among various the deduced amino acid sequences of the GTFs, a phylogenetic tree was constructed based on GTFs including WIG- and WSG-producing enzymes from oral streptococci. In this tree, one dextransucrase (DSRA) sequence from *Leuconostoc mesenteroides* was added. As shown in Fig. 5, WIG-producing enzymes from mutans streptococci (GTF-I, GTF-B, and GTF-C) were positioned in the same group. The two GTF-Is of *S. orisuis* JCM14035 and *S. criceti* HS-6 were closely related to that of *S. sobrinus* and *S downei*.

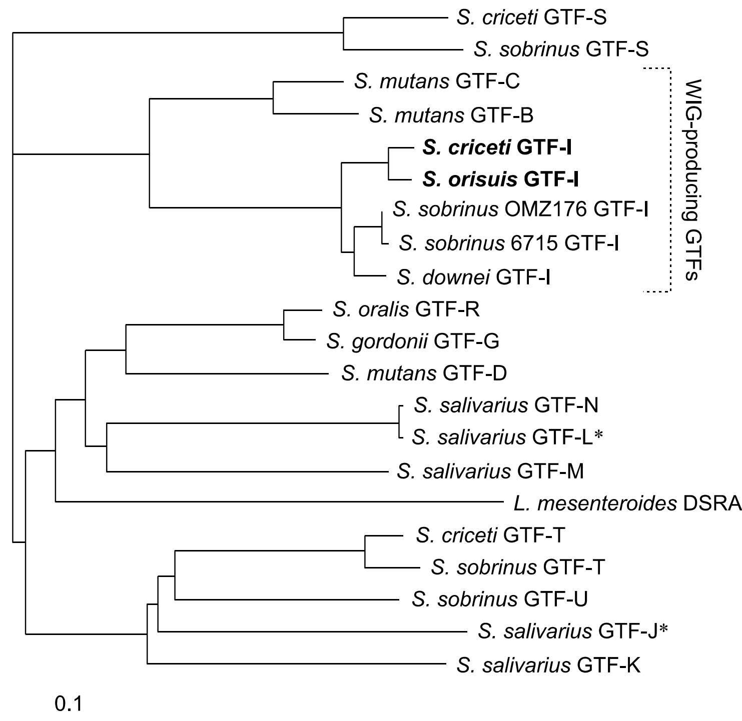

Fig. 5. Phylogenetic tree constructed from 20 GTF amino acid sequences of oral streptococci and from one dextransucrase of *L. mesenteroides* DSRA. Asterisks indicate the exceptional WIG-producing GTFs (for details, see text). The length of the connecting lines indicates relative phylogenetic distances.

Discussion

In human, the mutans streptococci have been implicated as etiologic factors in the development of dental caries. In these bacteria, *S. mutnas* and *S. sobrinus* have the ability to secrete GTF-B or GTF-I, which are different WIG-producing enzymes, encoded by *gtfB* or *gtfI* gene, respectively. It has been proposed that the functions of these GTFs are the principal virulence factor contributing to caries formation (Hamada and Slade, 1980).

Initially, PCR was carried out using the primer sets capable of amplifying and detecting the WIG-producing *gtf* gene in the present study, because the presence of WIG-producing *gtf* was focused on our attention. Since it is recognized that the homologies of 3'-end of both WIG and WSG-producing *gtf* genes were very high, it will be possible to amplify both WIG- and WSG-producing *gtf* genes by PCR using the primer sets designed by these region of the *gtf* genes.

Previously, it was reported that among six repeating units in the C-terminal of the *S. sobrinus* 6715 and *S. downei* GTF-I, the first two of these units are essential for GTF-I activity as well as for binding of glucans (Ferretti *et al*., 1987; Abo *et al*., 1991). In this study, it turned out that, in the C-terminal region, one repeating unit is deficient in *S. orisuis* JCM14035 and *S. criceti* compared to *S. sobrinus* 6715 GTF-I (Fig. 3), however, the first two of repeating units of GTF-I were preserved each two strains. It is difficult to explain this difference of the number of repeating units. Since *S. mutans* is known to be amenable to transformation, deletion of a repeating unit might arise by genetic recombination during transmission of mutans streptococci from animal to human

To confirm the secretion of other GTF proteins from *S. orisuis* JCM14035 and *S. criceti* in culture supernatant, immunostaining with MAbs anti-GTF-U and anti-T purified from *S. sobrinus* B13N (Nanbu *et al*., 2000) was performed. The prepared protein sample of *S. criceti* was only recognized with the ant-GTF-T MAb (data not shown). This result coincides with the previous study showing that *S. criceti* has *gtfS* and *gtfT* genes (Inoue *et al*., 2000). Protein from *S. orisuis* was not detected by anti-GTF-U and anti-GTF-T MAbs (data not shown). However, *S. orisuis* JCM14035 was able to secrete the enzymatically active WIG-producing GTF (Fig. 4B), further experiment would be necessary to identify the properties of this GTF-I enzyme.

Takada and Hirasawa (2007) reported that *S. orisuis* JCM 14035 is very closely related species to *S. criceti* HS-6. In accordance with this, each cluster was joined by *S. sobrinus* JCM14035 and *S. downei* based on the phylogenetic analysis using 16S rRNA gene sequences. In the present study, when GTF-based phylogenetic tree was constructed, the similar results could be drawn (Fig. 5). *S. sobrinus* and *S. downei* are very closely related species, and their amino acid sequences of GTF-I show a high degree of homology (Ferretti *et al*., 1987; Abo *et al*., 1991). Therefore, it was guessed that *S. orisuis* JCM 14035 and *S. criceti* positioned almost the same distances from *S. sobrinus* and *S. downei*, respectively. The WIG-producing GTFs group thought to have branched from WSG-producing GTFs except that *S. salivarius* GTF-L and GTF-J (Fig. 5, asterisks), and then GTF-B and GTF-Is was considered to have divided. *L. mesenteroides* is a free-living soil organism and secretes glucansucrases, which have common structural features, i.e., N-terminal catalytic site and C-terminal repeating units, against GTFs. Interestingly, there is no conservation of primary amino acid sequences between *L. mesenteroides* glucansucrases and streptococcal GTFs (Monchois *et al*., 1999). Therefore, DSRA of *L. mesenteroides* might poor relationships to other GTFs.

The ultimate goal of this ongoing work will be to identify the origin of cariogenic factor in human. In this study, two novel nucleotide sequences of *gtfI* gene were determined from pigs and hamsters, and a GTF-based phylogenetic tree was constructed indicating close relationships between mutans streptococci from animals and *S. sobrinus*.

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