Gene Encoding a Dextranucrase-like Protein in *Leuconostoc mesenteroides* NRRL B-512F*

Kazumi Funane, Kouichi Mizuno, Hidenari Takahara and Mikihiro Kobayashi

Carbohydrate Science Laboratory, National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

1Department of Resource Biology, Faculty of Agriculture, Ibaraki University, Ami, Ibaraki 300-0393, Japan

2Akita Research Institute of Food and Brewing, 4-26 Sanuki, Araya, Akita-shi 010-1623, Japan

Received June 29, 1999; Accepted August 23, 1999

A gene, *dsrT*, encoding a dextranucrase-like protein was isolated from the genomic DNA libraries of *Leuconostoc mesenteroides* NRRL B-512F dextranucrase-like gene. The gene was similar to the intact open reading frames of the dextranucrase gene *dsrS* of *L. mesenteroides* NRRL B-512F, dextranucrase genes of strain NRRL B-1299 and streptococcal glycosyltransferase genes, but was truncated after the catalytic domain, apparently by the deletion of five nucleotides. *dsrT* mRNA was produced in this strain *L. mesenteroides* when cells were grown in a sucrose medium, but at a level of 20% of that of *dsrS* mRNA. The molecular weight of the *dsrT* gene product was 150,000 by SDS-PAGE. The product did not synthesize dextran, but had weak sucrose cleaving activity. The insertion of five nucleotides at the putative deletion point in *dsrT* resulted in an enzyme with a molecular weight of 210,000 and with dextranucrase activity.

**Key words:** dextranucrase; glycosyltransferase; dextran; *Leuconostoc mesenteroides*

Dextranucrase (EC 2.4.1.5) is produced by several *Leuconostoc* and *Streptococcus* spp., and catalyzes the transfer of α-D-glucosyl groups from sucrose to form dextran. The catalytic domain for the cleavage of sucrose seems to occupy the N-terminal two-thirds of dextranucrases and streptococcal glycosyltransferases, and the C-terminal one-third contains the glucan-binding domain and a series of direct repeating units. MacGregor et al. concluded that glycosyltransferases form an (α/β)_{n} barrel-containing protein family from results of amino acid sequence alignment of glycosyltransferases and enzymes of the α-amylase family.

Streptococcal glucosyltransferases are classified into three groups: glucosyltransferase-S, which produces a water-soluble glucan with mostly α-1,6-linkages, glucosyltransferase-I, which produces a water-insoluble glucan with mostly α-1,3-linkages and glucosyltransferase SI, which produces a combination of soluble and insoluble glucans. These glucans are important in the formation of dental plaque by streptococcal cells. Oral streptococci express more than one glucosyltransferase. Both kinds of enzymes are required for maximum dental caries in experimental animals with *Streptococcus mutans*. The function of the dextran of *Leuconostoc mesenteroides* is not clear. *Leuconostoc mesenteroides* produce dextran with α-1,6-linked and α-1,3-linked D-glucopyranosyl residues, also. Many dextrins from the genus *Leuconostoc* have 1,2- or 1,4-linked residues. Many *Leuconostoc mesenteroides* strains produce more than one kind of dextranucrase. In contrast, *L. mesenteroides* NRRL B-512F produces only one kind of dextranucrase, one that synthesizes water-soluble α-1,6-D-glucan.

Many streptococcal genes encoding glucosyltransferase have been cloned and sequenced. Three *Leuconostoc* dextranucrase genes, *dsrS* from *L. mesenteroides* NRRL B-512F and *dsrA* and *dsrB* from strain NRRL B-1299, have been cloned. The gene *dsrS* encodes a dextranucrase that produces α-1,6 linkages, *dsrA* encodes a dextranucrase that produces α-1,6 and α-1,3 linkages, and *dsrA* encodes a dextranucrase that produce α-1,6 linkages. The nucleotide sequence of the dextranucrase gene from *L. mesenteroides* NRRL B-512F was registered as an unknown gene by Wilke-Douglas et al., and later, Monchois et al. identified it as a...
dextranucrase gene and named it *dsrS*.

*L. mesenteroides* NRRL B-512F was thought to have only one kind of dextranucrase, but we isolated another gene encoding a truncated dextranucrase that lacks all of the C-terminal glucan-binding domain by screening of a genomic libraries of strain NRRL B-512F. We characterized the gene product.

**Materials and Methods**

**Oligonucleotides.** Oligonucleotides used for the primers are listed in Table 1.

**Construction of genomic libraries from L. mesenteroides.** *L. mesenteroides* cells were cultured in the medium described before\(^2\) with 2% glucose overnight at 30°C without aeration. Chromosomal DNA of *L. mesenteroides* was prepared as described by Wilson.\(^3\) The chromosomal DNA was completely digested with EcoRI, ligated into λgt10/EcoRI arms (Stratagene), and packaged with Gigapack II Gold packaging extract (Stratagene). The resulting bacteriophages were used to infect *Escherichia coli* NM514. Plaques were transferred onto nylon membranes (Hybond-N\(^+\), Amersham). Screening was done with digoxigenin-labeled DNA probes with a Dig DNA labeling and detection kit (Boehringer Mannheim).

**DNA sequencing.** DNA fragments of clones with positive signals were subcloned into pBluescript SK\(^+\) (Stratagene). Nucleotides were sequenced with an ABI Prism\(^TM\) 310 genetic analyzer and a rhodamine terminator cycle sequencing ready reaction kit (both, Applied Biosystems). Analysis of nucleotide and protein sequences was done with the GENETYX program (version 9.0) (Software Development Co., Tokyo).

**Preparation of RNA from L. mesenteroides, northern blotting, and RT-PCR.** *L. mesenteroides* was cultured with the media described before\(^2\) with 2% glucose or sucrose overnight at 30°C without aeration. Two media was used because dextranucrases are induced by sucrose. RNA was prepared as described by Shaw and Clewell.\(^2\) Northern blotting was done as described by McMaster and Brownlee.\(^2\) RNA (20 mg) was denatured with glyoxal and transferred from 1.2% agarose gels onto positively charged nylon membranes (Hybond N\(^+\), Amersham). The probe DNA fragments were prepared from the cloned *dsrF* or *dsrS* DNAs, labeled with \(\alpha\)-\(\text{P}\) dCTP with a BcaBEST labeling kit (Takarashuzo), and used at the concentration of 2 ng/ml. The probed blots on the membrane were analyzed by an imaging analyzer (BAS3000, Fuji Film). RT-PCR was done for estimation of the size of mRNA with synthesized cDNA prepared from *L. mesenteroides* RNA with a first-strand cDNA synthesis kit (Amersham Pharmacia) as the template.

### Table 1. Oligonucleotides Used for the Experiments

<table>
<thead>
<tr>
<th>Name</th>
<th>5'-3' Sequence</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS1-dir</td>
<td>GT/G(C)CC(T/C)GCCGCTCATAT/CTATGG</td>
<td>Used to make a probe to clone dextranucrase gene</td>
</tr>
<tr>
<td>DS2-inv</td>
<td>G(A)/CC(T/C)TTTACG(A/G)GTA/CA(G/A)/C/GC</td>
<td>Used to make a probe to clone dextranucrase gene</td>
</tr>
<tr>
<td>DS3-dir</td>
<td>GAATGG(T/C)TIAAAGATGCATGGGC</td>
<td>Used to make a probe to clone dextranucrase gene</td>
</tr>
<tr>
<td>DS4-inv</td>
<td>CTGTACAGGCAGCCATACTA</td>
<td>Used to make a probe to clone dextranucrase gene and for RT-PCR</td>
</tr>
<tr>
<td>DS5-inv</td>
<td>GTACTTGGGAATGTATATTGTTG</td>
<td>Used to make a probe to clone dextranucrase gene</td>
</tr>
<tr>
<td>DS6-dir</td>
<td>AGATGCTAACTTGGATA</td>
<td>Used for genome PCR</td>
</tr>
<tr>
<td>DS7-inv</td>
<td>TACCATAACGAACTGATGT</td>
<td>Used for genome PCR</td>
</tr>
<tr>
<td>DS8-dir</td>
<td>GATACCTCAAGTCCGGCAACTG</td>
<td>Used for genome PCR</td>
</tr>
<tr>
<td>DS9-inv</td>
<td>TGTCATCCCACTCATCGTTGA</td>
<td>Used for genome PCR</td>
</tr>
<tr>
<td>RP1-dir</td>
<td>GTACACAGATGCTAACCAG</td>
<td>Used for RT-PCR</td>
</tr>
<tr>
<td>RP2-inv</td>
<td>GTCAACCACCTCAGTGTG</td>
<td>Used for RT-PCR</td>
</tr>
<tr>
<td>RP3-dir</td>
<td>CCATCTTGAAGACTGAGTAC</td>
<td>Used for RT-PCR</td>
</tr>
<tr>
<td>RP4-dir</td>
<td>GATCTTGGATCAATAC</td>
<td>Used for RT-PCR</td>
</tr>
<tr>
<td>RP5-inv</td>
<td>TTATGCTATCAATCCAG</td>
<td>Used for RT-PCR</td>
</tr>
<tr>
<td>RS1-dir</td>
<td>GTCATGAGATTGACATAGTATG</td>
<td>Used to introduce an NcoI site for construction of pDSRS</td>
</tr>
<tr>
<td>RS2-inv</td>
<td>GACTGTTGACATAGTATG</td>
<td>Used to construct pDSRS</td>
</tr>
<tr>
<td>RS3-dir</td>
<td>GCCATCGTCGACTGTTCC</td>
<td>Used to construct pDSRS</td>
</tr>
<tr>
<td>RS4-inv</td>
<td>ATTCGAGAAAGTCTATGCTGAC</td>
<td>Used to introduce an XhoI site for construction of pDSRS</td>
</tr>
<tr>
<td>RT1-dir</td>
<td>AACCATGGATAAATCTGGGA</td>
<td>Used to introduce an NcoI site for construction of pDSRT</td>
</tr>
<tr>
<td>RT2-inv</td>
<td>AAGCCTTACTGCTATCCTATC</td>
<td>Used to construct pDSRT</td>
</tr>
<tr>
<td>RT3-dir</td>
<td>AGCCTGAGCTTCTGGCTAACA</td>
<td>Used to construct pDSRT</td>
</tr>
<tr>
<td>RT4-inv</td>
<td>GTTCGAGGCTAAATCGG</td>
<td>Used to introduce an XhoI site for construction of pDSRT</td>
</tr>
<tr>
<td>RT5-inv</td>
<td>GATTGGGATCCAGATCAGATTTAATTTGAAAGG</td>
<td>Used to insert (CAGAT) into pDSRT for construction of pDSRT5</td>
</tr>
</tbody>
</table>

Primer sequences were designed on the basis of results of the alignment of amino acid sequences of strain B-512F dextranucrase and glucosyltransferases (DS1-dir, DS2-inv, DS3-dir), from *dsrF* (DS4-inv, DS5-inv, DS6-dir, DS7-inv, DS8-inv, DS9-inv, RP3-dir, RP4-dir, RP5-inv, RT1-dir, RT2-inv, RT3-dir, RT4-inv, RT5-dir), and from *dsrS* (RP1-dir, RP2-inv, RS1-dir, RS2-inv, RS3-dir, RS4-inv).
Enzyme assay and protein concentration. The sucrase activity was assayed in terms of the release of reducing sugar from sucrose as described previously. The protein concentration was measured with BCA protein assay reagent (Pierce) with bovine serum albumin as the standard.

SDS-PAGE, western blotting, and periodic acid-Schiff (PAS) staining. SDS-PAGE was done as described by Laemmli. A kit of molecular weight standards was purchased from Pharmacia. The gels were used for western blotting or PAS staining or else stained with Coomassie brilliant blue (CBB). Western blotting was done as described by Towbin et al. The primary antibody, mouse anti-glucosyltransferase was a gift from Professor Fukushima, Nihon University (Matsudo, Chiba, Japan). The antigen-antibody complex was detected with peroxidase-conjugated anti-mouse IgG (conjugate of goat-mouse IgG with (γ-horseradish peroxidase purchased from BRL) and an ECL random-primer-labeling detection system (Amersham Pharmacia) and used to expose Biomax film (Kodak) for 2 h at room temperature. Synthesized glucan was detected by PAS staining.

Construction of plasmids carrying dss, dssT, and dssT5 into pET23d. A plasmid, pDSRT, was constructed from two DNA fragments in phages that contained the complete dssT gene, which encoded the truncated dextranase of our strain. The NcoI-PstI fragment from the PCR product from genomic DNA of L. mesenteroides NRRL B-512F with primers RT1 and RT2 was put into dssT to introduce an NcoI-site just before the deduced starting codon at nt 1054, which would change the second amino acid of the product from tyrosine to aspartic acid. The SphI-XhoI fragment from the RT3-RT4 PCR product was put into dssT to introduce an XhoI-site at the 3′-end. The XhoI-site was introduced not at the actual end but at nt 5559, which is 13 bp downstream from the hypothetical end if the frame shift at nt 4098 did not occur. The resultant NcoI-XhoI fragment containing the complete dssT gene was cloned into pET23d and the new plasmid was named pDSRT. The five nucleotides CAGAT were introduced into the SphI-AflII fragment (nt 4056 to 4397) from dssT between nt 4098 and 4099 of that gene with the mutagenic oligonucleotide RT5 with a Mutan-super express km kit (Takara). The mutated fragment was substituted into pDSRT (plasmid pET23d [Novagen] carrying dssT) for construction of pDSRT5. pDSRTS was constructed with the DNA fragment, containing dssS that we isolated from genomic DNA libraries. The NcoI-SpeI fragment from the RS1-RS2 PCR product was put into dssS to introduce an NcoI-site just before the start codon, which would change the second amino acid of the product from proline to alanine. The EcoRI-XhoI fragment from the RS3-RS4 product was filled in at its 3′-end to introduce an XhoI-site after the termination codon. The resultant NcoI-XhoI fragment containing the complete dssS gene was cloned into pET23d and the new plasmid was named pDSRS.

Expression of gene products DSS, DSRT, and DSRT5 proteins in E. coli BL21(DE3). pET23d as a control, the plasmids carrying dssS, dssT, or dssT5, named pDSRS, pDSRT, and pDSRT5, respectively, were used to transform E. coli BL21(DE3) cells. Fresh single colonies grown on Luria-Bertani plates containing 200 µg/ml ampicillin were used to inoculate 3 ml of Luria-Bertani broth containing 200 µg/ml ampicillin and the broth was cultured at 37°C overnight with shaking. These cultures were used to inoculate 150 ml of the same broth at the ratio of 1:60 and the broth was incubated for 2 h at 37°C. Then 0.5 mM isopropyl-β-D-thiogalactopyranoside was added and the cells were cultivated for 8 h at 30°C. The cells were then pelleted by centrifugation at 1,500 × g for 10 min at 4°C, suspended in 30 ml of 20 mM sodium acetate buffer (pH 5.2) containing 30% glycerol (buffer A) and centrifuged again at 6,000 × g for 10 min at 4°C. Cells were resuspended in 16 ml of buffer A and lysed by sonication. The supernatant was then obtained by centrifugation at 10,000 × g for 10 min and used in SDS-PAGE followed by immunoblotting.

Results

Cloning of the gene encoding dextranase
The amino acid sequences of several peptides isolated from the dextranase prepared from L. mesenteroides NRRL B-512F culture supernatant and the deduced amino acid sequences of several streptococcal glucosyltransferases (accession numbers M30943, M17391, D90125, and M17361) were aligned for identification of the positions of the peptide fragments. A pair of degenerate primers, DS1 and DS2, were designed from the amino acid sequences. The DS1-DS2 PCR product obtained with genomic DNA of L. mesenteroides NRRL B-512F as the template was 918 bp long and it was about 60% identical to known gtf or dss DNAs. The product was used as a probe for screening of L. mesenteroides NRRL B-512F genomic DNA libra-
ries. A recombinant phage with an insert of a 5.2-kb genomic DNA fragment was obtained. The inserted DNA was subcloned into pBluescript SK⁺. The nucleotide sequence was resembled sequences of *Leuconostoc mesenteroides* *dsr* genes or streptococcal *gfp* genes but was not similar to other genes in the GenBank and EMBL data bases. The 5.2-kb DNA lacked the N-terminal region of dextranucrase. To obtain the rest of the gene, we prepared another probe by PCR with the primers DS3 and DS4. The probe was 585 bp long and was about 60% identical to known *gfp* and *dsr* DNAs. We isolated a recombinant phage containing a 4.3-kb genomic DNA fragment using this probe. The 4.3-kb genomic DNA fragment was subcloned into pBluescript SK⁺. Both strands of the 5.2-kb and 4.3-kb fragments were sequenced. To ensure that the 4.3-kb DNA was linked to the 5.5-kb DNA without gaps, a translation of the DNA was completed using *L. mesenteroides* NRRL B-512F genomic DNA as the template with primer DS6 from the 4.3-kb fragment and primer DS7 from the complementary sequence of the 5.2-kb fragment. The sequence of the PCR product showed that the 4.3-kb and 5.2-kb genomic DNA fragments overlapped and there was only one EcoRI site within the two fragments.

We designated the *dsr*-like gene on these two DNA fragments *dsrT*. So that the *dsrS* gene could be used as a control, a DNA fragment containing this gene was isolated from *L. mesenteroides* NRRL B-512F genomic DNA libraries with DS3-DS5 PCR product as the probe.

**Nucleotide sequence of dsrT**

The open reading frame (ORF) of the *dsrT* gene was 3148 bp long and it encoded the protein of 1015 amino acids with a deduced molecular weight of 112,000 indicated by the roman letters below the

---

**Fig. 1.** Nucleotide Sequence of the *dsrT* Gene and the Deduced Amino Acid Sequence of Its Product.

Positions 900, 930, and 1046, which are possible -35 and -10 promoter regions and a Shine-Dalgarno (SD) sequence, respectively, are shown by single underlining. The deduced signal peptide is indicated by a broken underline. The positions of binding with primers DS1, DS2, DS3, and DS4, which were used for preparation of probes for cloning of the *dsrT* gene, are indicated by double underlining. Conserved regions of the α-amylase family are enclosed by boxes. The catalytic and the second essential aspartates are indicated by asterisks and double asterisks, respectively. The vertical arrow indicates the deduced deletion point. Stop codon is indicated END. The inverted repeated sequence is indicated by horizontal arrows. The deduced amino acid sequence after the frame shift of the *dsrT* gene is repaired is given in italics. The amino acid sequences of DSRT, DSRS, (accession number 109598), DSRA (U38181), and DSRB (AF030129) around the deduced deletion point in DSRT are aligned in the brackets. Asterisks here show conserved residues.
nucleotide sequence in Fig. 1. A putative Shine-Dalgarno (SD) sequence\textsuperscript{30} was found five bases upstream from nt 1054, which probably is the start codon. An inverted repeated sequence, which could be involved in termination of transcription, was 1364 bp downstream of the termination codon. The identity of the nucleotide sequences of the ORFs of \textit{dssT} and \textit{dssS} was about 55% and their deduced amino acid sequences from \textit{dssT} and \textit{dssS} were about 50% identical.

The molecular weight of \textit{L. mesenteroides} NRRL B-512F dextranase is 170,000,\textsuperscript{22} and other dextranases and glucosyltransferases are similar in size. The deduced molecular weight of 112,000 for the truncated gene product, DSRT, was much smaller than those of other dextranases. Alignment of the amino acid sequences of DSRT and some other dextranases and glucosyltransferases showed that the DSRT protein has only the N-terminal catalytic region, completely lacking the C-terminal glucan-binding region (Fig. 1). Three out of the four conserved regions of the \textit{\alpha}-amylase family,\textsuperscript{32} the catalytic aspartate,\textsuperscript{29} and the second essential aspartate,\textsuperscript{19,26} which exist in known dextranases and glucosyltransferases, were found in DSRT. If two nucleotides were added or one nucleotide were removed at the termination of the \textit{dssT} gene, the ORF would reach nt 5543 at 19 bp upstream of the inverted repeat, and encode the C-terminal of dextranase, including the glucan-binding YG-repeat\textsuperscript{34} as indicated by italics in Fig. 1. By comparison of the amino acid sequences of DSRT and known dextranases, we concluded that five nucleotides were deleted between nt 4098 and 4099 in \textit{dssT}, because two amino acids, probably glutamine and isoleucine, seemed to be missing in DSRT. A stop codon appears only three bases downstream of the deduced deletion point. The \textit{dssT} clone we selected might have mutated during manipulation, so the genomic \textit{dssT} DNA fragment from \textit{L. mesenteroides} NRRL B-512F in the recombinant phage was sequenced directly. Genomic PCR of \textit{L. mesenteroides} NRRL B-512F was done with primers DS8 and DS9, and the amplified DNA fragments were expected to contain the deletion point. Eight \textit{E. coli} DH5\textsubscript{ax} recombinants carrying pT7-BlueT plasmid (Novagen) containing DS8-DS9 PCR fragments were selected and the inserted DNAs of the PCR product were sequenced. We found the five-base deletion both in the genomic DNA libraries and the genomic PCR products.

\textit{Northern blotting and RT-PCR}

\textit{dssT} may be a pseudogene not actually expressed in \textit{L. mesenteroides}. Northern blotting analysis was done to find whether \textit{dssT} mRNA was expressed in \textit{L. mesenteroides} NRRL B-512F. The probe DNA for \textit{dssT} was less than 50% identical to \textit{dssS}, and the probe for \textit{dssS} also was less than 50% identical to \textit{dssT}. Both probes reacted with RNA from cells grown with sucrose, but did not react with cells from glucose cultures, even when the imaging plate was overexposed (Fig. 2a). The level of \textit{dssT} mRNA measured by densitometry was 20% that of \textit{dssS} mRNA. Although the amount of \textit{dssT} mRNA was small, it probably was synthesized in \textit{L. mesenteroides} NRRL B-512F cells and is likely to be induced by sucrose, as are known \textit{Leuconostoc} dextranases. The distance between the stop codon and the transcription termination signal in the \textit{dssT} gene was extremely long about 1500 bases. If transcription ended at this termination signal, the \textit{dssT} mRNA should be about 4500 bases, about the same size as \textit{dssS} mRNA. The exact size of \textit{dssS} mRNA and \textit{dssT} mRNA was not found by northern blotting. Dextranase is induced in \textit{L. mesenteroides} only when growth is in sucrose. It was difficult to prevent detection of mRNAs under conditions when dextranase was being induced. Both \textit{dssT} and \textit{dssS} mRNAs looked broad shadows with two pale areas at 16S and 23S where ribosomes were.

The positions of the primers for RT-PCR and the PCR products are indicated in Fig. 2b (top). Products of the expected sizes were detected with the three sets of primers with cDNAs synthesized from RNAs from cells cultured with sucrose medium. There was little if any amplification when RNAs were from cells cultured with glucose. Both \textit{dssT} and \textit{dssS} were induced by sucrose. The three bands of amplified products were extracted and the products were sequenced. The sequences of the 0.6-kb and 1.2-kb products were exactly the same as the sequence of \textit{dssT}, and that of the 0.8-kb product was exactly the same as that of \textit{dssS}. The 1.2-kb product covered from 100 bp upstream of the end point of \textit{dssT} to 1050 bp downstream. The transcription of \textit{dssT} probably was not interrupted by the deletion.

\textit{Expression of gene products DSRT, DSRS, and DSRT5 protein}

The molecular weight of dextranase purified from \textit{L. mesenteroides} NRRL B-512F culture supernatant was 170,000, that of DSRS was about 200,000, that of DSRT was 150,000, and that of DSRT5 was 210,000 by staining with CBB and by western blotting (Fig. 3a and b). DSRT did not cause dextran production, but DSRT5 formed dextran (Fig. 3c, lanes 11 and 12). Two smaller forms of DSRS, probably degraded products that still synthesized dextran, were observed (lane 10). Both DSRS and DSRT5 synthesized dextran without additional primer.

DSRT had weak activity, 20% that of DSRS and 9% that of DSRT5 after 5 h of incubation (Fig. 3d).
Fig. 2. Northern Blotting and RT-PCR of \textit{dsrT} and \textit{dsrS}.

\textit{L. mesenteroides} NRRL B-512F was cultured with glucose (odd numbered lanes) or sucrose (even numbered lanes). The RNA was isolated and used in northern blotting and RT-PCR as described in Materials and Methods. (a) In lanes 1, 2, 5, and 6, the \textit{SalI-PstI} fragment (nt 1180–1712) from \textit{dsrT} DNA was used as the probes. In lanes 3, 4, 7, and 8, \textit{HindIII-TaqI} fragment (nt 271–587) from \textit{dsrS} DNA was used as the probe. The exposure time of the imaging plate was 2 h (lanes 1-4) or 6 h (lanes 5-8). (b) The positions of primers used for RT-PCR are shown at the top. In lanes 1 and 2, the primers were RP4 and RP5. In lanes 3 and 4, the primers were RP3 and DS4. In lanes 5 and 6, the primers were RP1 and RP2.

Fig. 3. Expression of pDSRS, pDSRT, and pDSRT5 in \textit{E. coli} BL21(DE3).

\textit{E. coli} BL21(DE3) cells carrying pET23d as control, pDSRS, pDSRT, or pDSRT5 were lysed by sonication. A 40-\mu g protein of a cell extract and 3 \mu g of purified dextran transferase prepared from the culture supernatant of \textit{L. mesenteroides} NRRL B-512F as described elsewhere were respectively denatured with 1% SDS and 0.5% mercaptoethanol at 37°C for 3 h and separated by 7.5% SDS-PAGE. (a) CBB staining. Arrowheads indicate purified native dextran transferase and the other expressed proteins. (b) Western blotting with anti-glucosyltransferase (see Materials and Methods). (c) Incubation with sucrose followed by PAS staining (see Materials and Methods). Lanes 1, 6, and 11: dextran transferase from \textit{L. mesenteroides} NRRL B-512F. Lanes 2, 7, and 12: extract prepared from cells carrying pET23d. Lanes 3, 8, and 13: extract prepared from cells carrying pDSRS. Lanes 4, 9, and 14: extract prepared from cells carrying pDSRT. Lanes 5, 10, and 15: extract prepared from cells carrying pDSRT5. (d) DSRS, DSRT, and DSRT5 were partially purified from cell extracts as described before and used. Sucrase activity was assayed as described in Materials and Methods. The enzymes were incubated with 7.5% sucrose in 20 mm sodium acetate buffer (pH 5.2) without additional primers. The activity in 1 ml of reaction mixture (1.8 \mu g of enzyme protein) is given. □, DSRS; △, DSRT; ▽, DSRT5.
Discussion

DSRT was a truncated dextranucrase with only the catalytic domain expressed in \textit{L. mesenteroides} NRRL B-512F. The molecular weight of DSRT5 was 60 kDa larger than DSRT. The molecular weight of native dextranucrase of \textit{L. mesenteroides} NRRL B-512F was 170,000 and that of DSRS also was 170,000 as deduced from the nucleotides of \textit{dsr}{\textit{S}}, but the molecular weight of DSRS was 30,000 higher by SDS-PAGE. Monchois \textit{et al.} \cite{19} earlier reported that DSRS protein expressed in \textit{E. coli} is about 200,000 by SDS-PAGE. The DSRT and DSRT5 expressed in \textit{E. coli} also seem to migrate during SDS-PAGE as species larger than predicted from the sequence data. The deduced molecular weight of DSRT was 112,000 and if there were no deletion, it should be 164,000 from the \textit{dsr}{\textit{T}} sequence. mRNA from \textit{dsr}{\textit{T}} may have been produced beyond the putative deletion point, but translation probably stopped at the predicted stop codon and produced truncated dextranucrase. For \textit{dsr}{\textit{T}}5, the frame shift seemed to have been repaired and the translation would in that case continue until the hypothetical end point, and producing a larger protein. \textit{C}-terminal deletions in DSRS\textsuperscript{5} and glucosyltransferases\textsuperscript{14,15} have been studied. Monchois \textit{et al.} \cite{5} reported lower in activities of fructose release and dextran synthesis of DSRS proteins with the \textit{C}-terminal deleted. Ferretti \textit{et al.}\textsuperscript{1} reported sucrase activity is absent when the glucan-binding domain containing direct repeat sequences is deleted from glucosyltransferase-I from \textit{S. downei} (former \textit{sobrinus})\textsuperscript{15} but Abo \textit{et al.}\textsuperscript{14} found sucrase activity even when all repeating units were completely deleted in glucosyltransferase-I from \textit{S. sobrinus}.\textsuperscript{14} The truncated dextranucrase, encoded by \textit{dsr}{\textit{T}} of \textit{L. mesenteroides} NRRL B-512F, lacked all of the repeating units and did not synthesize glucan. A spontaneous-mutant glucosyltransferase that had lost three out of the six internal direct repeats has 15\% of the parental activity.\textsuperscript{9} However, our report of \textit{dsr}{\textit{T}} is the first of a truncated dextranucrase gene present naturally in a type culture strain.

\textit{L. mesenteroides} NRRL B-512F has only one kind of dextranucrase, the extracellular enzyme DSRS.\textsuperscript{15,19} The \textit{dsr}{\textit{T}} sequence suggested that \textit{dsr}{\textit{T}} is an exocellular rather than an intracellular protein. Intracellular dextranucrase DSRA of \textit{L. mesenteroides} NRRL B-1299 has no signal peptide and the \textit{N}-terminal 100 amino acids are absent.\textsuperscript{20} The deduced \textit{N}-terminal sequence of DSRT started with a few hydrophilic amino acids followed by about 20 hydrophobic amino acids, showing DSRT had a signal peptide.\textsuperscript{15,25} The length of the \textit{N}-terminal region was similar to the length of other extracellular dextranucrases and glucosyltransferases.

The DNA sequence near the end of \textit{dsr}{\textit{T}} is TGGGTACCAGATTATAATTGG, encoding Trp-Val-Pro-Asp-Leu. The amino acid sequence of this region of other \textit{Leuconostoc} dextranucrase is Trp-Val-Pro-Asp-Gln-Ile-Tyr-Asn-Leu. The amino acid sequence from tryptophan to aspartic acid residues is identical to the deduced DSRT sequence. We assumed that a five-base deletion occurred just downstream of the aspartic acid codon in \textit{dsr}{\textit{T}}. The 5 bases including the aspartic acid codon were CAGAT in \textit{dsr}{\textit{T}}. If the same bases were added again after this sequence, the sequence of this region would be TGGGTACCAGAT CAGAT TTATAATTGG, encoding exactly the same amino acid sequence as other dextranucrases. If the \textit{dsr}{\textit{T}} sequence were this, \textit{dsr}{\textit{T}} would encode 484 more amino acids starting from Asp1015. The original \textit{dsr}{\textit{T}} gene in \textit{L. mesenteroides} NRRL B-512F probably had two CAGAT repeats next to each other, and during duplication, homologous recombination may have occurred between the tandem CAGAT sequence, resulting in a five-base deletion. Moreover, stop codons appeared frequently downstream of this frame shift and probably caused DSRT protein to be truncated and to lose its dextranucrase activity. As expected, addition of the five bases just upstream of the end point of \textit{dsr}{\textit{T}} resulted in production of active dextranucrase. The sequence of the corresponding region of \textit{dsr}{\textit{S}} is TGGGTGCCGGACCAAT-TTATAATTGG, which does not have striking repeats. Holt and Cote found that many polymer-producing \textit{Leuconostoc} strains produce more than one kind of dextranucrase,\textsuperscript{12} and even in \textit{L. mesenteroides} NRRL B-512F, they detected DNA polymorphism using primers from \textit{dsr}{\textit{S}} and \textit{dsr}{\textit{A}}. However, only one kind of dextranucrase was found in \textit{L. mesenteroides} NRRL B-512F. This strain probably had at least two dextranucrases, DSRS and DSRT in addition, but a mutation must have occurred in \textit{dsr}{\textit{T}} so that DSRT lost its dextranucrase activity. A self-derived peptide of the glucosyltransferase of \textit{Streptococcus mutans} inhibits both sucrose hydrolysis and glucosyl transfer to glucan by glucosyltransferase-I, and the peptide is tightly bound to the enzyme once in a complex, even in the presence of SDS.\textsuperscript{30} Although DSRT was a truncated, inactive dextranucrase, it may interact with DSRS. DSRS synthesizes \(\alpha\)-1,6 linkages, but 5\% of the linkages in the dextran produced by \textit{L. mesenteroides} NRRL B-512F are \(\alpha\)-1,3 linkages. In addition, the structure of produced dextran changed when subcultivation was repeated. In this study, only \textit{dsr}{\textit{T}} mRNA was looked for. We have not yet detected the protein in \textit{L. mesenteroides} NRRL B-512F culture.

In an examination of the evolutionary history of DSRT, evolutionary trees were made from results of the alignment of amino acid sequences deduced from the putative original \textit{dsr}{\textit{T}} (\textit{dsr}{\textit{T}}5), and the available
information on three *Leuconostoc dsr* genes and 12 streptococcal *gif* genes. Six of the *gif* genes (*gifS*<sup>13</sup>, *gifM*<sup>36</sup>, *gifG*<sup>37</sup>, *gifD*<sup>10</sup>, *gifK*<sup>21</sup> and *gifT*<sup>39</sup>) encode glucosyltransferase-Ss, five of the *gif* genes (*gifL*<sup>37</sup>, *gifB*<sup>17</sup>, *gifI* (from *S. downei*),<sup>1</sup> *gifI* (from *S. sobrinus*)<sup>13</sup> and *gifJ*<sup>40</sup>) encode glucosyltransferase-I-s, and *gifC*<sup>41</sup> encodes glucosyltransferase-SI. Four *Leuconostoc* dextransucrases, including DSRT5, formed a group separate from the streptococcal glucosyltransferases (Fig. 4). In glucosyltransferases, with some exceptions, glucosyltransferase-I-s and glucosyltransferase-SI are close to one another. Some glucosyltransferase-Ss are close to each other but glucosyltransferase-Ss are separated roughly into two groups. In the *Leuconostoc* dextranucrase group, *L. mesenteroides* NRRL B-512F DSRS and B-1299 DSRB, both of which produce only α-1,6 linkages, formed one group, and DSRT5 formed another group with B-1299 DSRA, which produces α-1,6 and α-1,3 linkages. The structure of dextran produced by the original of the *L. mesenteroides* NRRL B-512F strain must also have been different from the present NRRL B-512F product. DSRS or DSRT5 was incubated with 10% sucrose in sodium acetate buffer (pH 5.2) at 30°C for 8 h, and DSRS produced soluble dextran but the dextran produced
by DSRT5 was about 90% water-insoluble, suggesting that DSRT5 dextran includes 1,3-linkages.

The insoluble glucans produced by glycosyltransferase-I are essential for attachment of the cells to smooth surfaces. L. mesenteroides NRRL B-512F produces only soluble glucans. However, the existence of the dsrT gene suggested that L. mesenteroides NRRL B-512F was not the exception but like many other Leuconostoc or Streptococcus bacteria that produce glucan, it originally had different kinds of dextranucrases. The original DSRT (DSRT5) might have produced insoluble dextran and it might have helped in bacterial colonization. By loss of DSRT5 activity and retaining only α-1,6 dextranucrase DSRS, L. mesenteroides NRRL B-512F cells may have lost the ability to aggregate and happened to gain the industrially favorable property of producing a high percentage 95% of α-1,6 dextran.

Acknowledgments

We thank Professor Kazuo Fukushima of the School of Density, Nihon University for his gift of anti-glycosyltransferase antibody. We also thank Ms. Kazue Terasawa and Ms. Mayumi Matsushita for technical assistance. This research was supported by Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency, Japan.

References

thesizing only α(1, 6) and α(1, 3) linkages. Gene, 182, 23–32 (1996).