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Molecular Characterization of DSR-E, an α-1,2 Linkage-Synthesizing Dextransucrase with Two Catalytic Domains

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A novel *Leuconostoc mesenteroides* NRRL B-1299 dextransucrase gene, *dsrE*, was isolated, sequenced, and cloned in *Escherichia coli*, and the recombinant enzyme was shown to be an original glucansucrase which catalyses the synthesis of α-1,6 and α-1,2 linkages. The nucleotide sequence of the *dsrE* gene consists of an open reading frame of 8,508 bp coding for a 2,835-amino-acid protein with a molecular mass of 313,267 Da. This is twice the average mass of the glucosyltransferases (GTFs) known so far, which is consistent with the presence of an additional catalytic domain located at the carboxy terminus of the protein and of a central glucan-binding domain, which is also significantly longer than in other glucansucrases. From sequence comparison with family 70 and α-amylase enzymes, crucial amino acids involved in the catalytic mechanism were identified, and several original sequences located at some highly conserved regions in GTFs were observed in the second catalytic domain.

Glucansucrase enzymes from oral streptococci, *Leuconostoc mesenteroides* strains, and some *Lactobacillus* and *Neisseria* sp. catalyze the transfer of glucosyl residues from sucrose to synthesize α-D-glucopyranosyl homopolymers and oligomers. When sucrose is the sole substrate, high-molecular-weight polymers are obtained. Depending on the glucansucrase-producing strain, the synthesized glucans differ in size and structure. When efficient acceptors, such as maltose or isomaltose, are added to the reaction medium, glucansucrases catalyze the synthesis of low-molecular-weight oligosaccharides and the regiospecificity of several dextransucrases (type of linkages) from the *Leuconostoc* genus is conserved in oligosaccharide synthesis (8, 13, 41, 45).

To date, 17 glucosyltransferase (GTF)-encoding genes from *Streptococcus* spp., 8 glucansucrase-encoding genes from *L. mesenteroides*, and 1 gene from *Lactobacillus reuteri* have been cloned (for reviews, see references 3, 16, 32, and 59). Sequence information shows that they are closely related and share a common structure. These genes code for large enzymes with an average molecular mass of 160,000 Da composed of two main domains: an N-terminal conserved catalytic core of about 900 amino acids and a C-terminal domain covering 300 to 400 residues thought to be responsible for glucan binding and constituted by a series of repeated units (32). In addition, biochemical studies revealed that glucansucrases share many mechanistic features with amylolytic enzymes (15). Structural homologies were later confirmed by amino acid sequence comparison with glucoside hydrolases from family 13 (20). In family 13, the catalytic domain is formed of eight β-sheets alternating with eight α-helices, conferring a $(\beta/\alpha)_8$ barrel structure (55). Two structure predictions (9, 26) concluded that glucansucrase enzymes also possess a catalytic $(\beta/\alpha)_8$ barrel domain. However, MacGregor et al. (26) observed that well-recognized sequence segments appear in a different order, which tends to show that the β/α barrel elements are circularly permutated. This led to a new classification of the glucansucrase enzymes in family 70 of the glycoside hydrolases on the CAZy website (http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html). The only exception is amylosucrase from *Neisseria polysaccharea*, which belongs to family 13 since its structure is not circularly permutated and is closely related to the α-amylase structure with a much lower molecular size than that of the glucansucrase enzymes (40, 52).

Throughout family 13, sequence alignment and site-directed mutagenesis enabled the location of several residues known to be involved in the formation of the glucosyl enzyme intermediates and structural data confirmed the involvement of these amino acids in the α-retaining catalytic process of glycoside hydrolases (57).

Nevertheless, key elements involved in the selectivity towards an acceptor molecule or in the enzyme regiospecificity still remain unclear. Thus, cloning and sequencing of new and original dextransucrase genes, as well as studies of the reaction products and catalytic parameters of the enzymes expressed, are necessary to improve the understanding of the various specificities.

Among glucansucrases, *L. mesenteroides* NRRL B-1299 is known to produce a highly branched dextran, which is very unusual since it contains 27 to 35% α-1,2 branch linkages as well as a limited amount of α-1,3 branch linkages (24, 48). The dextransucrase activity of *L. mesenteroides* NRRL B-1299 was also used in acceptor reactions with maltose in order to obtain oligosaccharides with α-1,2 osidic bonds (39, 42). These α-1,2 gluco-oligosaccharides resist hydrolysis by digestive enzymes in

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TABLE 1. Oligoprimers used for PCR amplification

Designation	Description[a]	Sequence (5′–3′)[b]
29-dir	FYFESGK	TT(C/T)TA(C/T)TT(C/T)GA(A/G)TCAGG(C/G)AA(A/G)
24-inv	FESQNNNP	(T/G)GG(G/A)TT(G/A)TT(G/A)TTTTGTGA(T/C)TCAAA
IPCR-rev	nt 6334–6363	CCCTTTACAAGCTGATTTTGCTTATCTGCG
IPCR-dir	nt 8876–8907	GGGTCAAATCCTTACTATACATTGTCACACGG
pBAD-PS/ZV-dir	nt 677–706	GCCATGGCAAATACGATTGCAGTTGACACG
pBAD-ent-rev	nt 8482–8505	AATTTGAGGTAATGTTGATTTATC

[a] nt, nucleotides.
[b] Primer sequences are designed from the noncoding strand (IPCR-dir or ECHO-dir) or from the complementary strand (IPCR-rev or ECHO-inv).

animals and humans because of the configuration of their osidic bonds and selectively stimulate intestinal microflora such as *Bifidobacterium* sp., *Lactobacillus* sp., or *Bacteroides* sp. (10, 58). Thus, these molecules correspond to the definition of prebiotic agents which are food ingredients that are potentially beneficial to the health of consumers (44). This prebiotic effect is already exploited in animal and human nutrition as well as in dermocosmetic applications (37).

According to previous observations describing the existence of more than one glucansucrase in *L. mesenteroides* NRRL B-1299 (12, 23, 24), two dextransucrase genes from *L. mesenteroides* NRRL B-1299 have already been cloned. The *dsrA* gene encodes an intracellular dextransucrase DSR-A, which synthesizes a dextran containing 87% α-1,6 linkages and 13% α-1,3 linkages (29). The second gene, *dsrB*, corresponds to an enzyme responsible for α-1,6 bond synthesis only (31). However, strategies based on the design of degenerate oligonucleotides from highly conserved sequences in GTF-encoding genes did not permit the gene encoding the α-1,2 synthesizing dextransucrase to be cloned. Screening of the proteins can be successfully applied to isolate new original dextransucrase genes, as it was shown for the alternansucrase of *L. mesenteroides* NRRL B-1355 (3).

Using this strategy, we report here the cloning and sequencing of *dsrE*, a new dextransucrase gene encoding an enzyme catalyzing the synthesis of dextran and oligosaccharides containing α-1,2 linkages. The original structure of DSR-E, exhibiting two catalytical domains, is discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All strains were stored at −80°C in 15% glycerol.

L. mesenteroides NRRL B-1299 (National Center for Agricultural Utilization Research, Peoria, Ill.) was grown in a rotary shaker at 27°C at 200 rpm in standard medium (40 g of sucrose per liter, 20 g of potassium hydrogen phosphate per liter, 20 g of yeast extract per liter, 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter, 0.01 g of $MnSO_4 \cdot H_2O$ per liter, 0.01 g of NaCl per liter, 0.02 g of $CaCl_2$ per liter, 0.01 g of $FeSO_4 \cdot 7H_2O$ per liter) with an adjusted pH of 6.9.

Escherichia coli strains DH5α and JM109 were grown in Luria-Bertani (LB) medium. Selection of strains with cloned inserts in pUC18 or pGEM-T Easy was done on agar plates with 100 μg of ampicillin per ml, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml.

Plasmid pBAD/TOPO Thiofusion (Invitrogen) was used for the cloning and expression of *dsrE* in *E. coli* TOP10 cells grown in LB medium supplemented with 50 μg of ampicillin per ml.

Digested and dephosphorylated pUC18 plasmid was purchased from Amersham Biosciences and used for standard cloning. PCR product cloning requires the pGEM-T Easy plasmid (Promega) for DNA fragments less than 2 kbp and the TOPO-XL plasmid (Invitrogen) for larger fragments.

Gel electrophoresis and enzyme detection. After a 7-h *L. mesenteroides* NRRL B-1299 culture, the broth was centrifuged (4,000 × *g*, 4°C, 30 min). Extracellular insoluble dextransucrase activity probably bound to the cell wall was recovered in the cell pellet (11), concentrated 10-fold in sodium acetate buffer (20 mM, pH 5.4), and heated for 5 min at 95°C with the loading buffer (62.5 mM Tris HCl, 4% sodium dodecyl sulfate [SDS], 6 M urea, 0.01% bromophenol blue, and 200 mM β-mercaptoethanol). Three hundred microliters of the mixture was loaded onto a 7% (mass/vol) polyacrylamide gel. After separation, total proteins were stained with amido black, and dextransucrase enzymes, which remain active after a short renaturation in 20 mM sodium acetate buffer, pH 5.4, were detected in situ following dextran synthesis and a polymer stain reaction with periodic acid-Schiff base (15). Bands corresponding to active dextransucrase were excised and incubated separately in a 2-ml reaction mixture containing 100 g of sucrose per liter, 50 g of maltose per liter, and 20 mM sodium acetate buffer. After a 24-h reaction corresponding to sucrose exhaustion, the reaction was stopped by heating the mixture at 95°C for 5 min and the medium was centrifuged for 5 min at 15,000 × *g* to eliminate the insoluble dextran. Reverse-phase chromatography (C_{18} column, Ultrasep 100, 6-μm particle size, 5 by 300 mm; Bishoff Chromatography) was carried out with ultrapure water as the eluant at a constant flow rate of 0.5 $ml \cdot min^{-1}$. Each sample was analyzed within 30 min at room temperature, with oligosaccharides detected by refractometry. Protein sequencing on the selected band was performed by the Laboratoire de Microséquençage, Institut Pasteur, Paris, France.

Nucleic acid isolation and manipulation. *E. coli* plasmid isolation and *L. mesenteroides* genomic DNA purification were done with the QiaPrep Spin Plasmid kit and the Blood and Cell Culture DNA Maxi kit (Qiagen), respectively. DNA manipulation used standard methods (28). Restriction and modification enzymes were purchased from New England Biolabs or Gibco BRL and used according to the manufacturer's recommendations.

PCR amplification of homologous probe. Following protein sequencing, two selected peptides (29-FYFESGK and 24-FESQNNNP) were used to synthesize degenerate oligonucleotides (Isoprim) (Table 1).

A 666-bp fragment was generated by PCR with a Perkin-Elmer thermal cycler model 2400 and 50 ng of genomic DNA, 10 μM of primer 29-dir, 10 μM of 24-inv, 250 μM concentrations of each deoxynucleoside triphosphate, and *Taq* polymerase (Sigma) (25 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 5 min).

Southern hybridization and genomic libraries of *L. mesenteroides* NRRL B-1299. Chromosomal DNA from *L. mesenteroides* NRRL B-1299 was digested to completion with several restriction enzymes and separated on agarose gels, and genomic libraries were transferred to Hybond N+ nylon membranes (Amersham Biosciences). Hybridization was performed with the radiolabeled ([α-^{32}P]dATP; Amersham Biosciences) 666-bp PCR fragment. The labeling reaction was performed with the Mega-Prime DNA labeling system kit (Amersham Biosciences) followed by purification of the probe on MicroSpin columns S-200 HR (Amersham Biosciences). Prehybridization and hybridization were performed with high stringency conditions (65°C, overnight) according to standard methods (28).

Inverse PCR. *L. mesenteroides* NRRL B-1299 genomic DNA was digested by *Bcl*I or *Eco*RV under the conditions recommended by the supplier and, after recircularization, used as a template in an inverse PCR (Extrapol II DNA polymerase [Eurobio]) (25 cycles of 94°C for 30 s, 51°C for 30 s, and 72°C for 3 min). The two primers were designed according to the pSB2 insert sequence (Table 1).

DNA sequencing and sequence analysis. DNA fragment sequencing was carried out by Genome Express in both directions. Nucleotide sequence analyses were performed by using the Open Reading Frame (ORF) Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), Blast (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) (2), ClustalW (http://www2.ebi.ac.uk/clustalw) (56), PRODOM (http://protein.toulouse.inra.fr/prodom.html) (7), PFAM (http://pfam.wustl.edu/hmmsearch.shtml) (5), and SAPS (http://bioweb.pasteur.fr/seqanal/interfaces/saps.html) (6) internet programs.

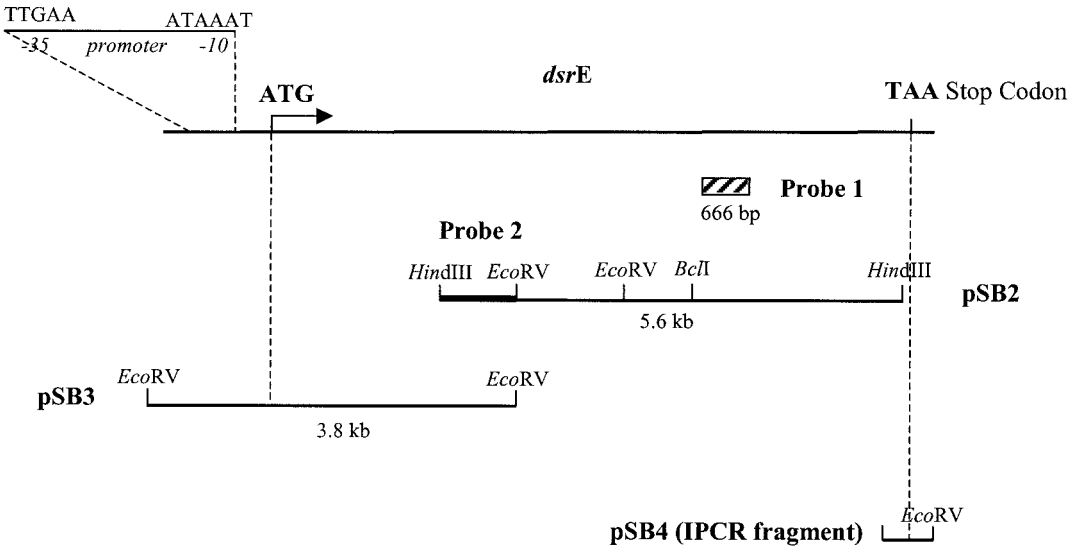

FIG. 1. Cloning of the *dsrE* gene from *L. mesenteroides* NRRL B-1299. Plasmids pSB2 and pSB3 were isolated by screening of genomic libraries with probes 1 and 2, respectively. Plasmid pSB4 contains the inverted PCR fragment obtained with primers IPCR-rev and IPCR-dir.

Protein expression with the pBAD cloning system (Invitrogen). An 8,385-bp fragment, containing the *dsrE* gene deleted from the sequence encoding the signal peptide, was generated by PCR with primers pBAD-PS/ZV-dir and pBAD-rev (Table 1). After purification, the PCR product was directly ligated into the pBAD/TOPO Thiofusion vector. Transformed *E. coli* TOP10 clones integrating a plasmid with the *dsrE* gene cloned into the proper orientation, that is, downstream of the arabinose promoter and in frame with the thioredoxin tag (N-terminal) and the V5 epitope and histidine tag (C-terminal), were selected. Correct construction of the plasmid containing the *dsrE* gene was confirmed by sequence analysis, showing that a single mutation occurred, which resulted in a D10R mutation in the variable domain.

Cells of *E. coli* TOP10 harboring pBAD-*dsrE* were grown in LB medium for 4 h after induction with 0.002% (mass/vol) arabinose. The pellet was resuspended at a final optical density at 600 nm (OD_{600}) of 80 in 20 mM sodium acetate buffer (pH 5.4) and 1% (vol/vol) Triton X-100 with 1 mM phenylmethylsulfonyl fluoride to prevent proteolysis in the cell extract after cell disruption by sonication.

Enzyme assays. Enzyme reactions were assayed under standard conditions at 30°C, in 20 mM sodium acetate buffer (pH 5.4), 0.05 g of $CaCl_2$ per liter, 1 g of NaN_3 per liter, and 100 g of sucrose per liter.

The DSR-E activity was determined by measuring the release of reducing sugars with the di-nitro-salicylic (DNS) assay (54), with 1 U defined as the amount of enzyme which catalyzes the formation of 1 μmol of fructose · min^{-1} under standard conditions.

Oligosaccharides were synthesized in reaction medium containing 100 g of maltose per liter, 200 g of sucrose per liter, and either 0.5 U of recombinant DSR-E from raw cellular extract per liter or 0.5 U of wild-type extracellular cell wall-associated glucansucrase from *L. mesenteroides* NRRL B-1299 per liter.

Nucleotide sequence accession number. The nucleotide and deduced amino acid sequences of *dsrE* have been submitted to the EMBL nucleotide sequence database under accession number AJ430204.

RESULTS

Isolation, micropurification, and peptide microsequencing of an active α-1,2 linkage-synthesizing dextransucrase. SDS-polyacrylamide gel electrophoresis (PAGE) analysis of cell wall-associated proteins of *L. mesenteroides* NRRL B-1299 revealed the presence of four main bands exhibiting GTF activity with molecular masses between 195 and 283 kDa.

Oligosaccharide syntheses were then performed with the excised active bands, and with the tested enzymes, all exhibited the ability to synthesize α-1,2 linkages. In order to ensure no contamination with forms that could be degraded by proteolysis, the upper band corresponding to the protein at 283 kDa was selected.

This band was then dried and used for protein trypsin digestion and peptide microsequencing.

Cloning and sequencing of the *dsrE* gene. Following peptide sequencing, degenerate primers (Table 1), designed according to the codon frequency table of *dsr* genes from *L. mesenteroides* NRRL B-1299, were synthesized. They allowed the amplification of a 666-bp DNA fragment named probe 1 (Fig. 1). After DNA sequencing, the corresponding 222-amino-acid sequence was shown to belong to a new glucansucrase, the peptide sharing 35% identity with DSR-S from *L. mesenteroides* NRRL B-512F and 36% identity with DSR-B from *L. mesenteroides* NRRL B-1299. Sequence comparison located the peptide at the junction between the highly variable region and the catalytic domain of glucansucrases.

A first *Hin*dIII genomic library was therefore screened with probe 1, and one recombinant plasmid, pSB2, containing a 5.6-kbp insert was purified. Sequence analysis of the *Hin*dIII insert revealed an ORF covering the whole fragment.

Then an *Eco*RV genomic library was screened with a *Hin*dIII-*Eco*RV probe (probe 2), which was isolated from the N terminus of the 5.6-kbp pSB2 insert (Fig. 1). The recombinant plasmid pSB3, positively tested by dot blot, carried a 3.8-kbp insert which, after sequencing, was shown to contain the initiation codon and the promoter region of the new dextransucrase gene, designated *dsrE*.

Inverse PCR was performed with divergent oligonucleotide primers designed from the previously described sequence of the pSB2 insert on self-ligated *L. mesenteroides* NRRL B-1299 genomic DNA digested either by *Bcl*I or by *Eco*RV. A unique expected 1-kbp fragment was amplified on the *Eco*RV recircularized libraries and was then cloned in pGEM-T Easy, leading to pSB4, prior to sequencing. The amplified sequence lo-

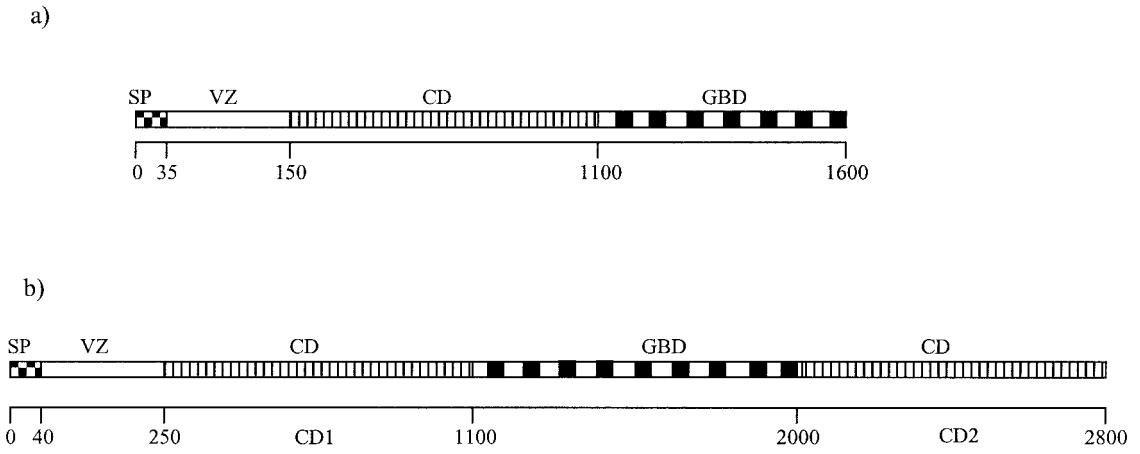

FIG. 2. Schematic general structure of GTFs and DSRs (a) and DSR-E (b). Abbreviations: SP, N-terminal signal sequence; VZ, variable region; CD, catalytic domain. Amino acid numbering from the N-terminal end is shown.

cated downstream of the *Hin*dIII site was 221 bases long and contained the termination codon of the *dsrE* ORF situated 30 nucleotides downstream of the *Hin*dIII restriction site. Each insert was sequenced in both directions.

Nucleotide sequence analysis. The combined nucleotide sequence of the different inserts (pSB2 and pSB3) and the inverted PCR product stretch over 9,264 bp. The 8,508-nucleotide ORF starts with an ATG codon at position 566 and terminates with a TAA stop codon at position 9071.

By analogy with promoter sequences from GTFs (34), the putative −35 TTGAAT and −10 ATAAAT sequences are located at positions 415 and 433. They share 67 and 50% identity, respectively, with the −35 TTGACA and −10 TAT AAT consensus sequences from *E. coli* (25). A putative ribosome-binding site is located 9 bp upstream of the start codon, with a hexanucleotide sequence AGGAGC that is 84% identical to the Shine-Dalgarno proposed consensus sequence AGGAGG (50). No putative transcription termination sequences can be identified downstream of the stop codon.

The ORF search on the entire nucleotide sequence reveals several other ORFs, the most interesting being situated upstream of *dsrE* on the opposite strand. This ORF is preceded by a putative ribosome-binding site (GGAGAC), and the corresponding translated 111-amino-acid peptide was submitted to a Blastp search and shown to share homologies with the transcription regulator PlcR from *Streptococcus pneumoniae*, *Bacillus anthracis*, and *Bacillus thuringiensis* and a bacteriocin gene (*mesF*) from *L. mesenteroides* Y105 (19).

Amino acid sequence analysis. The 8,508-nucleotide sequence of *dsrE* encodes a 2,835-amino-acid protein with a predicted pI of 4.88 and a theoretical molecular mass of 313,267 Da. Despite strong homologies with already known dextransucrases, DSR-E is characterized by a unique and very unusual structure involving the presence of two catalytic domains.

Indeed, alignment of the deduced amino acid sequence of DSR-E with available GTF and DSR sequences shows the presence of (i) a signal sequence, (ii) a poorly conserved region, (iii) a highly conserved catalytic domain, and (iv) a glucan-binding domain (GBD). Following this domain, the analysis reveals the presence of an extra catalytic domain at the carboxy terminus of DSR-E, as confirmed by PRODOM and Blast analysis (Fig. 2).

Signal peptide. Consistent with the extracellular location of the protein, the sequence encodes a typical gram-positive 40-amino-acid signal peptide, highly conserved with other signal peptides from DSRs of *L. mesenteroides*. It consists of a positively charged N-terminal part followed by a hydrophobic central region and a more-polar C-terminal region (60). The predicted cleavage site is located between amino acids 40 and 41 according to the algorithm of Nielsen et al. (38).

Variable region. Then, following the signal peptide, DSR-E displays a 207-amino-acid highly variable domain. Sequence analysis revealed the presence of a new 14-amino-acid repeated motif named S, which is rich in alanine, threonine, and aspartic acid residues and is found very conserved seven times and more-diverging four times (Fig. 3). Its consensus sequence shares some homology with a repeated unit that we also observed in the sequence of DSR-T from *L. mesenteroides* B-512F (16) and that we designated motif T (Table 2).

Catalytic domains. The first catalytic domain (CD1) ranges from amino acids 248 to 1141, whereas the second one (CD2) is located at the C terminus of the protein, between amino acids 1980 and 2835. CD1 and CD2 have 45% identity and 65% similarity with each other. Both domains contain the conserved amino acids already identified in GTFs or DSRs as being essential for enzymatic activity (Fig. 4).

By analogy with α-amylase family enzymes (9, 24), Asp527

^{95}PAADKAVDTTSSTT
^{109}PATDKAVDTTP-TT
^{122}PAADKAVDTTP-TT
^{135}PAADKAVDTTP-TT
^{148}PAANKAVDTTP-AT
^{161}AATDKAV-ATP-AT
^{173}PAADKLANTT—AT

FIG. 3. Alignment of the seven most-conserved S repeats found in the DSR-E variable domain.

TABLE 2. Sequence comparison of motif S from *L. mesenteroides* NRRL B-1229 DSR-E and motif T from *L. mesenteroides* NRRL B-512F DSR-T

L. mesenteroides strain	Enzyme	Motif	Consensus sequence of repeated motif[a]
NRRL B-512F	DSR-T	T	-TD**DKA**$\frac{A}{T}$-**TT**A$\frac{A}{D}$**T**S
NRRL B-1299	DSR-E	S	PA$\frac{A}{T}$**DKA**V**D**T**T**P$\frac{A}{T}$**T**

[a] Boldface type indicates conserved amino acids.

(CD1) and Asp2210 (CD2) can be proposed to play the role of nucleophile. Glu565 (CD1) and Glu2248 (CD2) would be the general acid-base catalysts. Finally, Asp638 (CD1) and Asp2322 (CD2) would correspond to the second aspartic acid of the catalytic triad, always conserved in the active site of α-amylase. Transition state stabilization also requires the interaction with two histidines in α-amylases. These residues correspond to a His and a Gln in glucansucrases of family 70 (30, 36, 47) and are conserved in both catalytic domains of DSR-E: it concerns His637 and Gln1019 for CD1 and His2321 and Gln2694 for CD2. In addition, three conserved amino acid residues essential for the function of *Streptococcus downei* GTF-I (Trp344, Glu349, and His355) also occur in the one-third of the core region assumed to be outside the $(\beta/\alpha)_8$ barrel (35). Among them, Trp and Glu residues which appear to be involved in the reaction mechanism are present in both catalytic domains, at positions 425 and 430 for CD1 and positions 2122 and 2127 for CD2. However, in the same block, only the CD1 His436 is conserved when compared to GTF-I His355, whereas Trp2135 is found at this position in the second catalytic domain.

Nevertheless, though there is an overall good conservation of key amino acids, some stretches of sequence located in usually conserved regions of GTFs and DSRs differ clearly in CD2. Thus, as shown in Fig. 4, ^{2214}FIHNDTI and ^{2323}KGV QEKV diverged from the corresponding consensus sequences, NVDADLL and SEVQTVI , respectively, in DSRs.

GBD. Finally, between the two catalytic domains, the GBD can be found. Usually around 500 amino acids long in GTFs and DSRs, it covers 839 amino acids in DSR-E. Many studies have shown that the GBD of glucansucrases is composed of a series of repeated units divided into 4 classes: A, B, C, and D repeats. According to the consensus sequences proposed by Russell (46), regularly alternating A and C repeats are found in the DSR-E GBD in which three putative cell wall binding domains (CW units) can be localized (Fig. 5). These triplets show homology with the two CW unit triplets forming the C-terminal choline binding domain of the autolysin LytA from *S. pneumoniae* (C-LytA). The three-dimensional structure of this domain was recently solved (14). It revealed a new solenoid fold formed by 6 CW units. Each CW unit constitutes an independent β-hairpin consisting of two antiparallel β-strands connected by a short internal loop region. Three consecutive hairpins (i.e., 3 CW units) form a complete turn of a left-handed superhelix, conferring the solenoid fold. In DSR-E GBD, two β-strands can also be predicted in the CW units identified by using the secondary structure prediction tool Predator (Fig. 5). This new alignment enables three more-divergent new C repeats to be isolated. One A and one C repeat did not match the newly proposed tandem model.

Expression of *dsrE* in *E. coli*. The DSR-E-encoding gene was cloned in an *E. coli* expression vector under the control of the araBAD promoter (P_{BAD}), leading to plasmid pBAD-*dsrE*. After optimization of the expression conditions, the highest dextransucrase activity shown by DNS measurements was obtained when *E. coli* TOP10(pBAD-*dsrE*) cells were incubated for 4 h with 0.002% arabinose (mass/vol), added at an OD_{600} of 0.5. Using these conditions, the activity reached 1,063 U/liter of culture. As shown in Fig. 6, SDS-PAGE gels of crude cellular extracts of recombinant cultures revealed that the protein undergoes proteolytic degradation. However, the main band at 320 kDa corresponds to the deduced molecular mass of DSR-E.

Characterization of enzyme activity. DSR-E was characterized by oligosaccharide synthesis with maltose as the acceptor molecule (Fig. 7). Comparisons of the chromatograms of the DSR-E reaction products with those produced by the native enzymes from *L. mesenteroides* NRRL B-1299 revealed the presence of (i) oligosaccharides from the OD series and (ii) oligosaccharide R5, which was previously shown to be an α-D-glucopyranosyl-(1 → 2)-α-D-glucopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 4)-α-D-glucopy-

		I		II		III		IV		V		VI
		■ ■ ■		•		⩛		⇓		◆◆ Δ		◆
AS			247	WTTFNSFQWDLNYSNPWVFRAMAG	278	LGVDILRMDAVAFIWKQMGTS	323	VFFKSEAIVHPD	386	VNYVRSHDDIGWTFA	182	DFIFNHTS
GTF-A	393	PMWNKDSENVNF-SGIQFQTG	445	RGQEFLLANDIDNSNPVVQAEQLN	487	ANFDSVRVDAPDNIDADLMNI	526	HINILEDWNHAD	596	YSFVRAHDNNSQDQI	979	DWVPDQIY
GTF-B	341	SAWNSDSEK----PFDDHLQN	402	GGYEFLLANDVDNSNPVVQAEQLN	443	ANFDSIRVDAVDNVDADLLQI	484	HLSILEAWSDND	555	YSFIRAHDSEVQDLI	928	DWVPDQMY
GTF-I	341	PQWNGESEK----PYDDHLQN	404	GGYELLLANDVDNSNPIVQAEQLN	445	ANFDSIRVDAVDNVDADLLQI	486	HVSIVEAWSDND	557	YSFARAHDSEVQDLI	932	DWVPDQMY
GTF-S	327	NQWSIASENETVYPNQDHMQG	388	AGYELLLANDVDNSNPVVQAEQLN	429	ANFDGVRVDAVDNVNADLLQI	470	HLSILEAWSGND	540	YVFIRAHDSEVQTRI	915	DLVPNQLY
DSR-S	444	PQWNETSED----MSNDHLQN	502	GGFELLLANDVDNSNPVVQAEQLN	543	ANFDGIRVDAVDNVDADLLQI	584	HLSILEDWSHND	655	YSFVRAHDSEVQTVI	1024	DWVPDQIY
DSR-A	181	PNWNIDSEA----KGDDHLQG	237	GGFELLLANDVDNSNPVVQAEQLN	278	ANFDGYRVDAVDNVDADLLQI	319	IYQFWKTGEMKI	390	YSFIRAHDSEVQTII	765	DWVPDQIY
DSR-B	426	PQWNMSSED----PKNDHLQN	484	GGFELLLANDVDNSNPVVQSEQLN	525	ANFDGIRVDAVDNVDADLLQI	566	HLSILEDWSHND	637	YSFVRAHDSEVQTVI	1005	DWVPDQIY
ASR	525	ANWNKQTEDEAF-DGLQWLQG	585	KGSEFLLANDIDNSNPIVQAEQLN	626	ANFDGIRVDAVDNVDADLLKI	667	HLSILEDWNGKD	759	YSFVRAHDYDAQDPI	1168	DWVPDQIY
CD1	423	ANWNIDSES----KGNDHLQG	478	GGYEMLLANDVDNSNPVVQAEQLN	519	ANFDGYRVDAVDNVDADLLQI	560	HISILEDWDNND	631	YAFIRAHDSEVQTVI	1014	DWVPDQIY
CD2	2120	FIWNKDSEYHG--GGDAWFQG	2161	NAFDFLLANDVDNSNPVVQAENLN	2202	ANFDSIRIDAVD*FIHNDTIQR*	2243	HISLVEAG*LDAG*	2315	YSIIHAHD*KGVQEKV*	2689	DVVDNQVY

FIG. 4. Alignment of highly conserved sequences in glucansucrase catalytic domains. AS, *N. polysaccharea* (40); GTF-A, *L. reuteri* (59); GTF-B, *Streptococcus mutans* GS5 (51); GTF-I, *S. downei* Mfe28 (15); GTF-S, *S. downei* Mfe28 (18); DSR-S, *L. mesenteroides* NRRL B-512F (61); DSR-A, *L. mesenteroides* NRRL B-1299 (29); DSR-B, *L. mesenteroides* NRRL B-1299 (31); ASR, *L. mesenteroides* NRRL B-1355 (3); CD1 and CD2, catalytic domains of DSR-E, *L. mesenteroides* NRRL B-1299 (this study); —, gap in the sequence; ■, key amino acid residues of the N-terminal end of the catalytic domain (35); ⇓, putative acid catalyst (9, 26); ⩛, putative nucleophile (9, 26); ●, putative calcium binding site (9); ◆, putative residues stabilizing the transition state (26, 35); Δ, residue involved in glucan structure determination (34, 43, 49). Underlined sequences in italics are DSR-E sequences which diverge from consensus sequences.

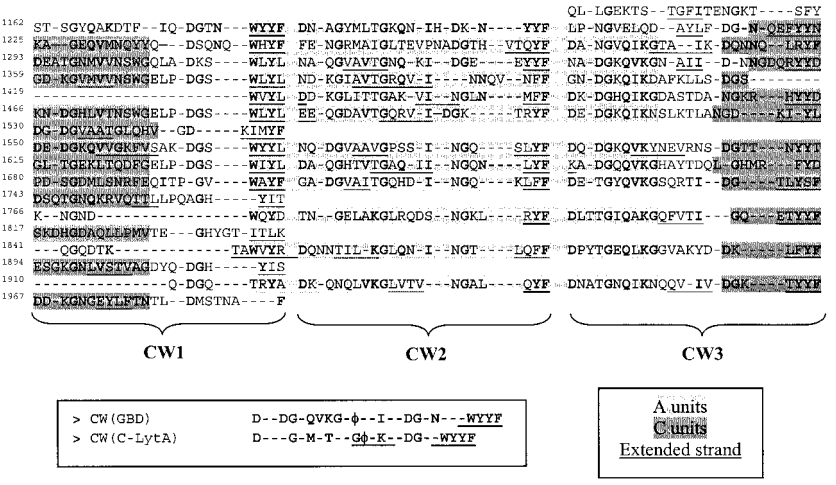

FIG. 5. Alignments of CW unit triplets found in the DSR-E GBD. Also shown is the repetitive presence of consecutive A and C motifs, as well as putative extended strands, analogous to those participating in the solenoid fold of C-LytA (14). Strongly conserved residues are printed in boldface type.

ranose (13). The presence of R5 clearly shows that DSR-E is the enzyme responsible for α-1,2 linkage synthesis.

DISCUSSION

The data reported here describe the cloning of a new gene, named *dsrE*, obtained from *L. mesenteroides* strain NRRL B-1299. As confirmed by glucan synthesis and periodic acid-Schiff activity staining, cultures of *E. coli* clones harboring the plasmid carrying the *dsrE* gene produced an active dextransucrase. Recombinant DSR-E was demonstrated to produce, with maltose as the acceptor, ODi and Ri oligosaccharides which contain α-1,2 linkages.

In addition to its unique regiospecificity, DSR-E possesses a very original structure, never observed before, characterized by the presence of an additional catalytic domain at the carboxy terminus. With a calculated molecular mass of 313,267 Da, DSR-E has twice the average mass of GTFs and DSRs (32).

The role and significance of the variable nonconserved region located downstream of the signal peptide remain unclear. Several studies showed that its deletion does not affect the enzyme activity (1, 33). However, in DSR-E we observed the presence of a 14-amino-acid repeated unit never identified before. That repeat, named S, could thus play a possible role in the enzymatic activity and/or specificity of DSR-E. Similarly, some glucansucrases with unusual specificities, for example,

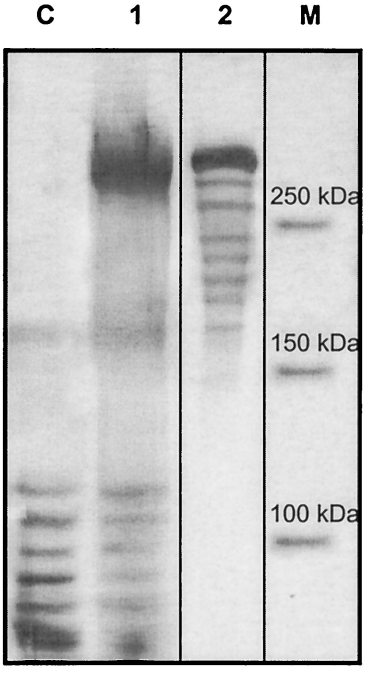

FIG. 6. SDS-PAGE profiles and zymogram of recombinant DSR-E produced by *E. coli* TOP10. Lanes: C, cellular extract, pBAD/TOPO Thiofusion, and Coomassie staining (negative control); 1, cellular extract, pBAD-*dsrE*, and Coomassie staining; 2, cellular extract, pBAD-*dsrE*, and periodic acid-Schiff staining (loading 5 mU of activity); M, broad-range prestained precision protein standard (Bio-Rad).

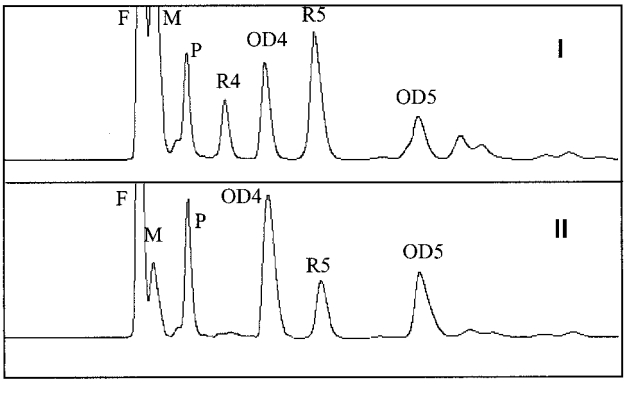

FIG. 7. Characterization of the products synthesized by the recombinant DSR-E. High-performance liquid chromatography analysis of gluco-oligosaccharides obtained with native DSRs from *L. mesenteroides* NRRL B-1299 (I) and the recombinant DSR-E (II). Peak identification: F, fructose; M, maltose; P, panose.

alternansucrase and DSR-T, also possess interesting repeats in the variable domain. So, the influence of this particular repeat on the catalytic properties of DSR-E must be evaluated with deletion experiments.

DSR-E is also remarkable because of the presence of a long GBD, ensuring the junction between the two catalytic domains CD1 and CD2. Along the GBD sequence, regularly alternating A and C repeats are found in which three CW units can be localized. From these observations, it can be suggested that the structure of GBD resembles that of the C-terminal choline binding domain of the autolysin LytA from *S. pneumoniae*. Thus, A-C tandem repeats could be due to the recurrence of specific duplication events of an ancestral CW unit triplet, corresponding to a complete turn of an original superhelix. This hypothesis corroborates (i) the initial suggestions of Giffard and Jacques (17), who proposed a definition of a fundamental repeating unit from which all classes of repeats (A, B, C, and D) are derived, and (ii) several studies that describe the presence of tandem repeats (4, 18, 22, 46).

Attention has been focused recently on repeated elements in the variable one-third of glucansucrases from *L. mesenteroides* (21). Unlike DSR-S, DSR-B, and ASR (alternansucrase), no A and C motifs can be found in this region. However, DSR-E is the very first and sole glucansucrase in which a catalytic domain is located after the GBD. Such a structure can be related to the presence of repeated units upstream of the catalytic domain in other DSRs. Either DSR-E might be the product of gene fusion caused by the recombination of two *dsr* genes, or recombination events between two *dsrE* genes have led to the presence of repeated elements usually found in the GBD in the N terminus of glucansucrases from *L. mesenteroides*.

The third and fifth domains correspond to two potential catalytic domains (CD1 and CD2), conferring to DSR-E a unique structure never observed before by analogy to glycoside hydrolase family 70 enzymes. Both domains cover about 900 amino acids, as in other GTFs (32), and thus, because of the presence of all the amino acids thought to play key roles in catalysis, DSR-E seems to possess a double catalysis system. CD1 and CD2 share 44% identity with each other and an average identity with other GTF and DSR catalytic domains of 53 and 44% for CD1 and CD2, respectively. The lower similarity of CD2 can be explained by several regions that diverge from consensus sequences. A tryptophan residue at position 2135 stands for a usually conserved histidine residue. This amino acid is thought to play a role in glucan and oligosaccharide binding (35). Peptide ^{2210}DAVDFIHNDTIQR in block C, the block containing the putative nucleophile, is very different from the highly conserved DAVDNVDADLLQI peptide found in all GTFs and DSRs. The usually conserved residues located just downstream of the first catalytic Asp in glucansucrases could constitute part of the subsite +1, which is involved in the acceptor binding (27). The structure of this site determines the positioning of the acceptor molecule and thus the type of glucosidic bond formed. All glucansucrase enzymes from family 70 have an Asn residue at a position equivalent to N555 of DSR-S. In CD2, the corresponding dipeptide NV is replaced with ^{2214}FI, which can also be found in the amylosucrase sequence. Structural data obtained for this enzyme (52) suggest that the Phe residue could be engaged in the specificity towards the fructo-furanosyl ring of sucrose (53). Moreover, the Ile residue at position 2215 is also found in GTF-A from *L. reuteri* (59) and is strongly conserved in the α-amylase family.

Likewise, in block E, the ^{2315}KGVQEKV peptide from CD2, following the second Asp of the catalytic triad, differs from the consensus sequence SEVQTVI found in most GTFs and DSRs. Besides, Arguello-Morales et al. (3) also observed in the ASR sequence a specific tripeptide located at the same position, and it can also be noticed that GTF-A from *L. reuteri* exhibits an original tripeptide just downstream of the third carboxylic acid of the triad (59). In this conserved area, Glu2327 can be found in a position usually involved in the glucan structure determination. Indeed, the corresponding mutant T667R in DSR-S was found to synthesize a glucan with 13% α-1,3 linkages compared to less than 5% for the wild-type enzyme (43). Consistent with this result, it appears that the presence of a carboxylic acid instead of a neutral amino acid (threonine) at the corresponding position of GTF-S increased the synthesis of α-1,3 glucosidic bonds by 30% (49). In addition, concerning GTF-I from *S. downei*, Monchois et al. (34) also attribute an influence of D569 on oligosaccharide synthesis trough interaction with the acceptor molecule. Thus, DSR-E possesses, because of its two catalytic domains, both a neutral residue in CD1, Thr643, and an acidic residue in CD2, Glu2327.

Moreover, when aligning the DSR-E catalytic domain sequences with other central regions of glucansucrases, stretches of sequence are significantly longer, for example, between the general acid catalyst and the second aspartic acid residue of the catalytic triad in CD2, or shorter, as shown by a 16-amino-acid gap located upstream of the Ca^{2+} binding site of CD2 (data not shown).

In addition to the attentive study of both catalytic domain sequences, the question is, as no glucansucrase with two catalytic domains has been previously observed, how does such a molecule work? DSR-E possesses two nucleophiles (D527 and D2210) and all the conserved residues required for the formation of two glucosyl enzyme complexes. Thus, DSR-E seems to possess two fully active catalytic domains, and we can assume that the specificity in synthesizing α-1,2 linkages is related to one of the two domains—most probably CD2, which presents a distinctive stretch of sequence compared to CD1. One domain (CD1) would catalyze the transfer of a glucose moiety from sucrose to a maltose residue to give panose, which in turn would be glucosylated, either in CD1, resulting in the addition of a new α-1,6 glucosidic bond, or in CD2, specific for the α-1,2 linkage. It can be suggested that the presence of the GBD enables CD1 and CD2 to be maintained in close proximity for an optimal branching of the polymer.

To determine whether both domains are active, deletion studies and site-directed mutagenesis must be performed to evaluate the influence of each catalytic domain on the activity and specificity of DSR-E.

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