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Cloning and Sequencing of the α-1→6 Dextransucrase Gene from *Leuconostoc mensenteroides* B-742CB

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Abstract A dextransucrase gene (*dsrB*742) that expresses a dextransucrase to synthesize mostly α-1→6 linked dextran with a low amount (3–5%) of α-1→3 branching was cloned and sequenced from *Leuconostoc mesenteroides* B-742CB. The 6.1-kb *Pst*I fragments were ligated with pGEM-3Zf(-) and transformed into *E. coli* DH5α. The recombinant clone (pDSRB742) synthesized dextran on an agar plate containing 2% (w/v) sucrose. The dextran synthesized was hydrolyzed with *Penicillium* endo-dextranase. The hydrolyzate was composed of glucose, isomaltose, isomaltotriose, and branched pentasaccharide. The nucleotide sequence of *dsrB*742 showed one open reading frame (ORF) composed of 4,524 bp encoding dextransucrase. The deduced amino acid sequence revealed a calculated molecular mass of 168.6 kDa. It also showed an activity band of 184 kDa on a non-denaturing SDS-PAGE (10%). The amino acid sequence of DSRB742 exhibited a 50% similarity with DSRA from *L. mesenteroides* B-1299, a 70% similarity with DSRS from *L. mesenteroides* B-512 (F, FMCM) and a 45–56% similarity with *Streptococcal* GTFs.

Key words: Dextransucrase, *Leuconostoc mesenteroides*, dextran, clone

Dextran is a generic term applied to bacterial polysaccharides that are synthesized from sucrose and composed of chains of D-glucose units connected by α-1→6 linkages. Dextrans can be composed of various amounts and arrangements of branches: for example, α-1→2, α-1→3, and α-1→4 to the main chains [17]. The enzymes that synthesize dextrans are known as dextransucrases (EC 2.4.1.5). Dextransucrases are extracellular glucansucrases that are produced by various species of *Leuconostoc* and *Streptococcus*. Different kinds of glucansucrases, synthesizing different kinds of glucans, are produced by various strains or species of *Leuconostoc* and *Streptococcus* [8, 10, 17]. The glucansucrases of *Streptococcus* sp. are produced constitutively, whereas the *L. mesenteroides* glucansucrases can only be produced by sucrose induction, with the exception of the constitutive mutants produced from *Leuconostoc* sp. [10, 11]. It has been previously reported that *L. mesenteroides* B-742 produces two kinds of dextransucrases with sucrose. One enzyme synthesizes a dextran containing α-1→4 branch linkages and the other synthesizes a dextran with a very high degree of single α-1→3 branched glucose residues [10, 17]. From *L. mensenteriodes* B-742, Kim and Robyt [10] isolated a dextransucrase constitutive mutant, *L. mesenteriodes* B-742C. Following further mutation of B-742C, two different constitutive mutants, B-742CA and B-742CB, were isolated. B-742CB produced extracellular dextransucrases on 2% glucose with higher activities than that produced by B-742 produced on 2% sucrose. The B-742CB dextransucrase synthesized both a soluble and an insoluble dextran. In this study, a gene was cloned from *L. mesenteroides* B-742CB that expressed a dextransucrase (*dsr*B742) forming only a soluble glucan that had glucose units connected mostly by α-1→6 glucosidic linkages. Accordingly, this report describes the isolation and expression in *E. coli* of the B-742CB dextransucrase gene and compares the homology characteristics with the reported sequences of other *L. mesenteroides* dextransucrase and *Streptococcus* sp. GTFs genes.

L. mesenteroides B-742CB was grown on an LM medium containing 2% (w/v) glucose [6]. *E. coli* DH5α, TOP10F', and BL21(DE3)pLysS (Invitrogen, The Netherlands) were maintained and grown on Luria-Bertani agar plates [12] containing the indicated antibiotics. pGEM3Zf(-) (Promega, U.S.A.) and pRSET (Invitrogen, The Netherlands) plasmids were used for cloning and expressing the dextransucrase gene.

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The genomic DNA of *L. mesenteroides* B-742CB was extracted as described by Haj-Ahmad *et al.* [5] with some modifications. The cells were harvested from a culture broth and the pellet was washed twice with a 50 mM Tris buffer (pH 8.0). Next, the cells were incubated with a lysozyme solution (4 mg/ml lysozyme, 50 mM Tris-HCl, 50 mM glucose, pH 8.0) at 37°C for 1 h in 25% sucrose, a TES buffer (100 mM Tris-HCl, 50 mM EDTA, 800 mM NaCl, pH 8.0), and sodium dodecyl sulfate (final conc. 1%) was then added and the solution gently agitated. Ribonuclease A (5 mg/ml, Sigma R-6513) was added and the mixture incubated at 37°C for 15 min. Proteinase K (5 mg/ml, Sigma P-6556) was added and the mixture was incubated at 50°C for 90 min. After phenol extraction, the DNA was precipitated with 2 volumes of cold ethanol, and then dissolved in distilled water. The plasmid DNA was isolated by an alkaline lysis method [12]. The endonuclease restriction, ligation, and transformation of the *E. coli* cells were carried out as described by Maniatis *et al.* [12]. The *E. coli* cells harboring pDSRB742 were cultured in 100 ml of LB broth with ampicillin (50 μg/ml) at 37°C for 18 h. The cells were collected by centrifugation, suspended in 2 ml of a 20 mM sodium acetate buffer (pH 5.2), and sonicated on ice. To express the *dsrB*742, the DNA insert of pDSRB742 was ligated into a pRSET vector and transformed into *E. coli* BL21(DE3)pLysS. In this plasmid, since the gene is under the control of the T7 promoter, it would be expected that the gene would be induced by isopropyl α-D-thiogalactoside (IPTG). The expression of *dsrB*742 was performed as described in the Invitrogen Manual [*A Manual of Methods for Expression of Polyhistidine-Containing Recombinant Proteins using the pRSET Vector System.* pp. 1-21] and confirmed by the appearance of fructose after a reaction with 50 mM sucrose. SDS-PAGE and dextransucrase activity detection were conducted as previously described [18].

The acceptor reaction of DSRB742 was performed at 30°C with final concentrations of 50 mM sucrose, 100 mM maltose, and 0.2 IU/ml of dextransucrase in a 20 mM sodium acetate buffer (pH 5.2) containing 0.01% sodium azide, 2 mM calcium chloride, and Tween 80 [9, 10]. The reaction was allowed to continue for 16 h and the reaction products were analyzed by TLC with two ascents of 2:5:1.5 (v/v/v) nitromethane: 1-propanol: water. The carbohydrates were visualized by dipping the plate into 5% (v/v) H_2SO_4 in methanol containing 0.3% (w/v) N-(1-naphthyl) ethylenediamine, followed by drying and heating for 10 min at 120°C [7, 10]. The sequencing of the *dsrB*742 was performed using a standard sequencing strategy with an ABI PRISM Cycle Sequencing Kit (Perkin Elmer) in a GeneAmp 9600 thermal cycler and a DNA sequencing system (model 373-18, Applied Biosystems). The nucleotide sequence similarity with other *L. mesenteroides* dextransucrases and *dsrB*742 was investigated using BLAST.

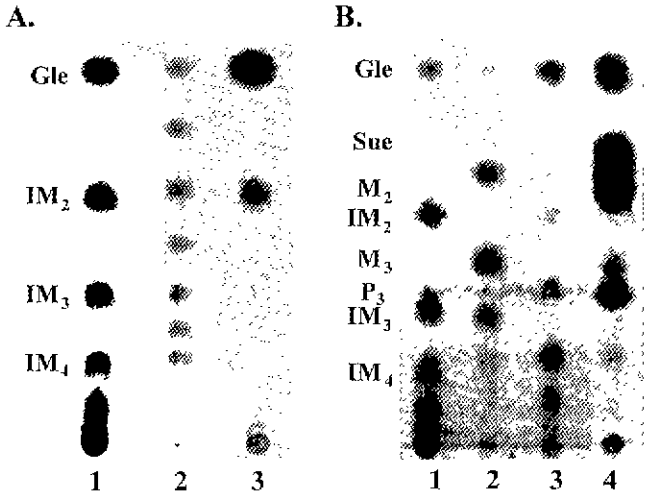

Fig. 1. Dextranase hydrolysis product (A) and maltose acceptor product prepared from DSRB742.
(A) Glc, glucose, IM2, isomaltose M3, isomaltotriose; IM4, isomaltotetraose; 1, isomaltooligosaccharide standard, 2, maltooligosaccharide standard; 3, dextranase hydrolysis products. There was no small saccharide contamination in the DSRB742 dextran preparation (B): Glc, glucose· Suc, sucrose; M2, maltose; IM2, isomaltose; M3, maltotriose; P3, panose; IM3, isomaltotriose; IM4, isomaltotetraose; 1, isomaltooligosaccharide standards; 2, maltooligosaccharide standards, 3, maltose acceptor product by B-512F dextransucrase; 4, maltose acceptor product.

The dextran producing recombinant clone (pDSRB742) was screened on LB plates containing 2% (w/v) sucrose. The dextran synthesized by DSRB742 was hydrolyzed with *Penicillium* endo-dextranase. The hydrolyzate was composed of glucose, isomaltose, isomaltotriose, and branched pentasaccharide (Fig. 1A). DSRB742 was able to transfer the glucosyl residue of sucrose to maltose, and formed panose (6^2-α-D-glucopyranosyl maltose) (Fig. 1B).

To characterize the dextransucrase, it was necessary to increase the expression level of the enzyme. For this purpose, the pDSRB742 DNA insert was ligated into a pRSET vector and transformed into BL21(DE3)pLysS. The isolated clone was named pDSRB742C. The cell lysates from *E. coli* BL21 (pDSRB742C) showed a maximal activity 2 h post-incubation. A non-denaturing SDS-PAGE (10%) showed an activity band of 184 kDa (Fig. 2).

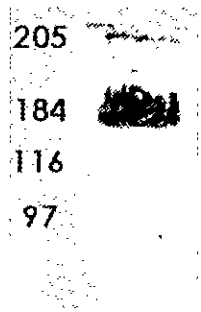

Fig. 2. Non-denaturing SDS-PAGE activity staining of dextransucrase from pDSRB742C.

The 6.1 kbp DNA fragment carrying *dsrB742* was sequenced (Fig. 3). One ORF composed of 4,524 bp was identified. The deduced amino acid sequence showed that it began from the start codon (ATG) at position 698 and extended to the termination codon (TAA) at position 5,221. The enzyme consisted of 1,508 amino acids and had

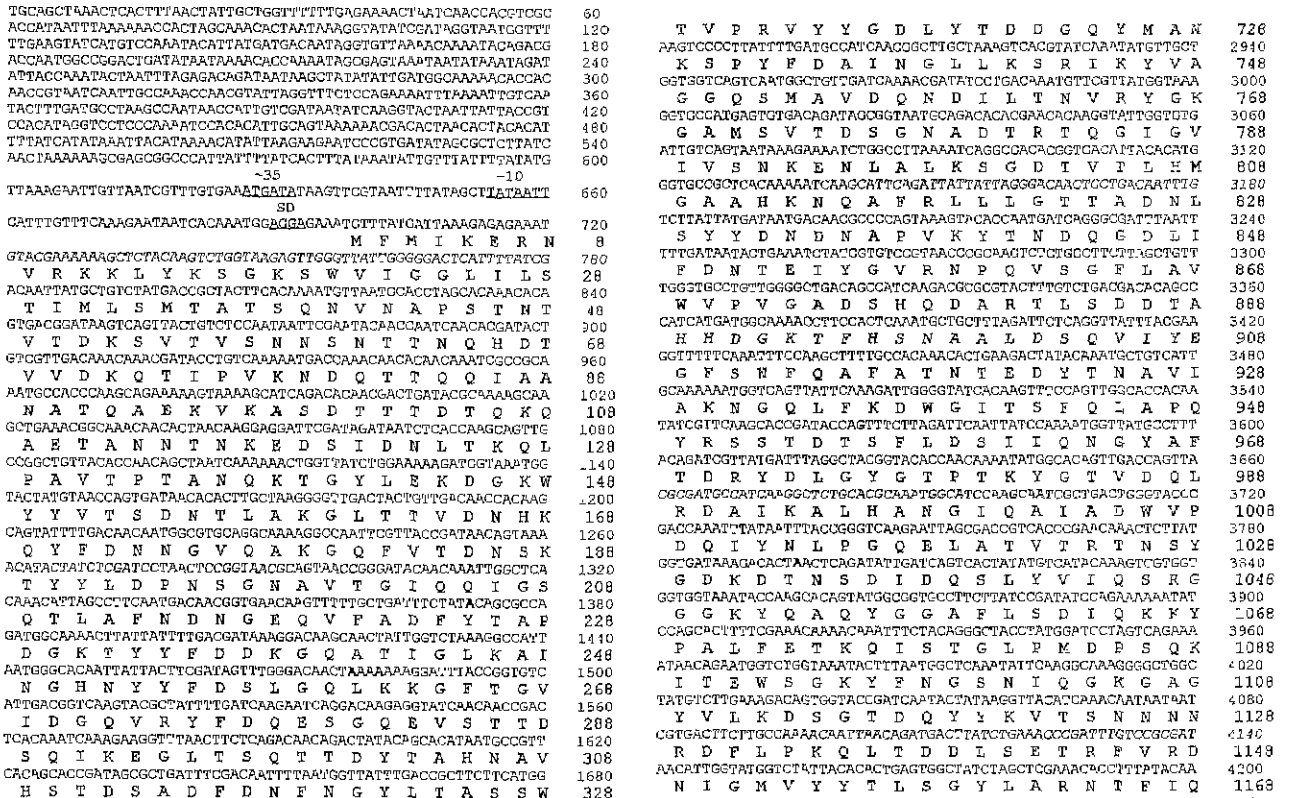

Fig. 3. Nucleotide and deduced amino acid sequence of *dsrB742*.
The underlined regions indicate the putative promoter, SD sequence. Bold letters indicate the YG repeats. The right-hand margin represents the nucleotide and amino acid number.

a calculated molecular mass of 168.6 kDa. A putative ribosome-binding site sequence (AGGA) was located five nucleotides upstream from the translation initiation codon. A potential promoter was identified with a -10 sequence (TATAAT) at 654 to 661 nt and a -35 sequence (ATGATA) at 627 to 637 nt (Fig. 3).

L. mensenteriodes B-742CB synthesized either a soluble or an insoluble dextran. The *dsrB*742 from *L. mensenteriodes* B-742CB was isolated and it was found that it encoded a dextransucrase that synthesized mostly α-1→6 linked dextran. The direct repeats in the C-terminal were composed of the structural element known as YG repeats that have been previously reported [1, 2, 13, 14, 21]. The DSRB742 amino acid sequence showed a 45% to 56% similarity with *Streptococcal* GTFs (2–4), a 50% similarity with DSRA [15], and a 70% similarity with DSRS [13]. DSRB742 included a putative sucrose binding site [ANFDGIRVDAVDNVD-ADLLQI] [16]. The Asp534 of DSRB742 was homologous to the Asp453 identified by Mooser *et al.* [16] as part of the active site of *S. sobrinus* GTF and to the Asp451 of *S. mutans* GTF [3]. A sequence homologous to the glucosyl-transfer site (GGFELLLANDVDNSNPVVQAEQLN) proposed by Funane *et al.* [4] was also identified at positions 484 to 507 of DSRB742 (Fig. 4). Asp493, Asp495, and His643 were all homologous to the conserved residues identified as essential amino acids for enzyme activity in GTFC [20] or DSRS [14]. The Thr649 residue, which has been suggested to greatly influence the structure of the glucan from *S. mutans* GTFs [19], was also identified in DSRB742. There were similar, yet not identical, α-1→6 glucan synthesizing genes for different *L. mesenteroides*.

In summary, the dextransucrase gene from the constituent mutant, *L. mesenteroides* B-742CB, was cloned and expressed. It was found that the expressed dextransucrase (DSRB742) synthesized a dextran with a high number of α-1→6 linkages. The amino acid sequence of DSRB742 had a 50% and 95% similarity with DSRA and DSRB, respectively, from *L. mesenteroides* B-1299, a 70% similarity with DSRS from *L. mesenteroides* B-512F [22] and B-512FMCM [18], and a 45–56% similarity with *Streptococcal* GTFs.

The *E. coli* cloned and expressed dextransucrases, DSRA, DSRB, and DSRS from *L. mesenteroides* strains also synthesized dextrans that contained very high amounts of α-1→6 linkages. The synthesis of almost exclusive α-1→6 linkages by DSRB742 was surprising as the gene was obtained from *L. mesenteroides* B-742 that elaborates two dextransucrases, one of which synthesizes a dextran with 13% α-1→4 branch linkages, whereas the other synthesizes a dextran with a very high degree (50%) of α-1→3 branch linkages [17].

Accordingly, it would appear that during the course of evolution, the *L. mesenteroides* dextransucrase genes have slowly changed and diverged from the ancestral gene that primarily synthesized α-1→6 linked glucans (dextrans). These changes have resulted in specific changes in and around the active site to give *L. mesenteroides* strains that synthesize dextrans with altered structures. These structures differ from the kind of branch linkages, for example, α-1→2, α-1→3, or α-1→4, and in the frequency of branching [17]. All the cloned genes from *L. mesenteroides* B-512F, B-1299, and B-742CB express the ancestral dextransucrase in *E. coli* that primarily synthesizes α-1→6 linkages. The dextransucrases from the *L. mesenteroides* wild-types and *L. mesenteroides* constitutive mutants that synthesize dextrans with more complex structures must either have undergone subtle changes in their amino acid sequences within and around the active sites, or, when the cloned genes were expressed in *E. coli*, changes occurred so that only the ancestral dextransucrase was elaborated.

Accordingly, to understand the molecular differences in the dextransucrases that produce different kinds of dextrans with different kinds and/or amounts of branch linkages, studies of the isolation and expression in *Leuconostoc* of the gene for dextransucrase that synthesizes the highly α-

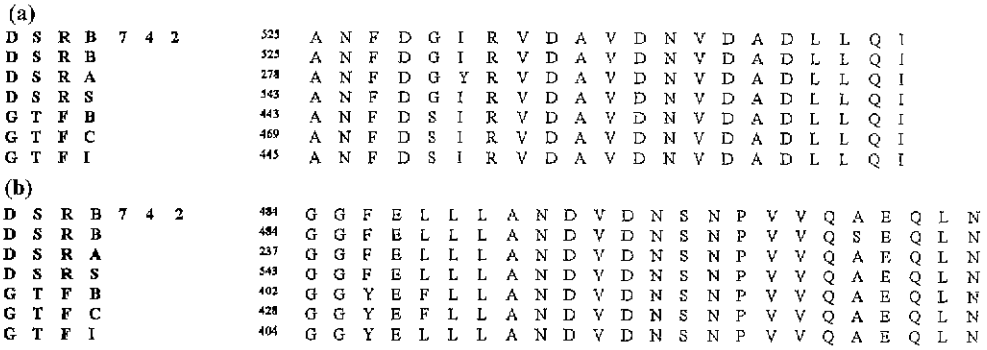

Fig. 4. Alignment of the deduced partial amino acid sequences of DSRS, *L. mesenteroides* NRRL B-512F dextransucrase gene, DSRA, DSRB, GTFB, GTFC, and GTFI.

(a) DSRB742 sequence homologous to the sucrose binding sites identified by Mooser *et al.* [16]. The active aspartic acid residue is in bold type. (b) DSRB742 sequence homologous to the active sites identified by Funane *et al.* [4].

1→3 branched dextran from *L. mesenteroides* B-742 [regular comb dextran, 17] is currently in progress.

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