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# Bioengineering of *Leuconostoc mesenteroides* Glucansucrases That Gives Selected Bond Formation for Glucan Synthesis and/or Acceptor-Product Synthesis

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**ABSTRACT:** The variations in glucosidic linkage specificity observed in products of different glucansucrases appear to be based on relatively small differences in amino acid sequences in their sugar-binding acceptor subsites. Various amino acid mutations near active sites of DSRBCB4 dextransucrase from *Leuconostoc mesenteroides* B-1299CB4 were constructed. A triple amino acid mutation (S642N/E643N/V644S) immediately next to the catalytic D641 (putative transition state stabilizing residue) converted DSRBCB4 enzyme from the synthesis of mainly α-(1→6) dextran to the synthesis of α-(1→6) glucan containing branches of α-(1→3) and α-(1→4) glucosidic linkages. The subsequent introduction of mutation V532P/V535I, located next to the catalytic D530 (nucleophile), resulted in the synthesis of an α-glucan containing increased branched α-(1→4) glucosidic linkages (approximately 11%). The results indicate that mutagenesis can guide glucansucrase toward the synthesis of various oligosaccharides or novel polysaccharides with completely altered linkages without compromising high transglycosylation activity and efficiency.

**KEYWORDS:** glucansucrase, oligosaccharides, mutation, glucan, *Leuconostoc mesenteroides*

## INTRODUCTION

The synthesis of oligosaccharides containing polysaccharides is a challenging goal for modern science. Despite the great importance and the enormous potential of oligosaccharides, their technical and pharmaceutical uses remain limited because of the high complexity of these molecules, which cause enormous problems in classical chemical synthesis.[1] An established industrial process is the fermentative production of polysaccharides (α-glucans) by lactic acid bacteria. Such polymers are used in the food industry as additives for dyes and in health care.[2,3] Differences in the glucosidic linkage type, the degree and type of branching, and molecular mass of glucans available show promising variations in functional properties.

Dextransucrases (EC 2.4.1.5) catalyze two different reactions: the synthesis of glucan from sucrose and the acceptor reaction. Glucansucrase can transfer an α-D-glucopyranosyl moiety from sucrose onto a hydroxyl group of mono- and disaccharides or noncarbohydrates.[4] Previously, a gene encoding a dextransucrase (DSRBCB4) that synthesized only α-(1→6) linked dextran was cloned from *Leuconostoc mesenteroides* B-1299CB4.[5] Analysis and comparison of the different protein sequences showed that dextransucrases were closely related and had a common structure: their N-terminal end started with a signal peptide, followed by a highly variable stretch, a highly conserved catalytic or sucrose binding domain, and a C-terminal domain composed of a series of tandem repeats.[6] On the basis of a comparison with sugar-binding subsites in the GH13 family of enzymes,[7] the locations of three regions putatively involved in acceptor and substrate binding in glucansucrases were identified: two are a C-terminal extension of the catalytic residues D530 (amino acid number of DSRBCB4, region II in Figure 1) and E568 (region III in Figure 1). The third (putative) acceptor substrate binding region was identified on the basis of earlier mutagenesis studies with different glucansucrases and is located at the C-terminus of the catalytic residue D641 (region IV in Figure 1), determining the solubility of the glucan products and the ratio of [α-(1→3) versus α-(1→6)] glucosidic linkages (Figure 1).[8−10]

The C-terminal region of the catalytic D530 (region II) contains the conserved amino acids D530-A-V(P)-D-N-V(I)535 (DSRBCB4 numbering). In the second catalytic domain (CD2) of dextransucrase from *L. mesenteroides* B-1299 (DSRE) [responsible for α-(1→6) and α-(1→2) linkage synthesis], the second Val residue is replaced by an Ile residue (I2215) (Figure 1). The P1026 and I1029 combination is present only in reuteransucrase from *L. reuteri* 121 (GTFA) and reuteransucrase from *L. reuteri* ATCC 55730 (GTFO), strikingly the only glucansucrases that synthesize reuterans [α-(1→4) polymers.[11,12] The region following D641 (transition state stabilizer)[7] in DSRBCB4 differs from the sequence D1133-N-N-S-Q-D-Q-I1139, conserved in reuteransucrases from *L. reuteri* ATCC 55730 and 121 (Figure 1, GTFO and GTFA), respectively.[11,12] In many dextransucrases the conserved tripeptide S-E-V is present immediately downstream of this catalytic D residue. The dextransucrases from *L. reuteri* 180 (GTF180) and mutansucrase from *L. reuteri* ML1 (GTFML1) contain a unique tripeptide at this position: S-N-A and N-G-S, respectively.[13,14] Also, both alternansucrase species of *L. mesenteroides* B-1355 and CD2 of DSRE contain an original tripeptide at this position: Y-D-A and K-G-V, respectively (Figure 1).[15,16]

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| Enzyme | Main α-linkages in glucan | Region II | | Region III | | Region IV |
|---|---|---|---|---|---|---|
| | | | | | | +1 +2 +3 +4 |
| AS | 1→4 | 281 | DLLRM**D**AVAFIWKQMGTS | 323 | VFFKS**E**AIVHPD | 386 | VNYVRSH**D**DIGWTFA |
| DSRBCB4 | 1→6 | 525 | DGIRV**D**AVDNVDADLLQI | 563 | HLSIL**E**DWSHND | 634 | YSFVRAH**D**SEVQTVI |
| DSRS | 1→6 | 546 | DGIRV**D**AVDNVDADLLQI | 584 | HLSIL**E**DWSHND | 655 | YSFVRAH**D**SEVQTVI |
| DSRE CD1 | 1→6 | 522 | DGYRV**D**AVDNVDADLLQI | 560 | HLSIL**E**DWDNND | 631 | YAFIRAH**D**SEVQTVI |
| DSRE CD2 | 1→2 | 2212 | DSIRI**D**AVDFIHNDTTQR | 2250 | HLSLV**E**AGLDAG | 2322 | YSIIHAH**D**KGVQEKV |
| DSRB742 | 1→6 | 528 | DGIRV**D**AVDNVDADLLQI | 566 | HLSIL**E**DWSHND | 637 | YSFVRAH**D**SEVQTVI |
| ASR | 1→6/1→3 | 630 | DGIRV**D**AVDNVDADLLKI | 668 | HLSIL**E**DWNGKD | 760 | YSFVRAH**D**YDAQDPI |
| GTFB | 1→3 | 446 | DSIRV**D**AVDNVDADLLQI | 484 | HLSIL**E**AWSDND | 555 | YSFIRAH**D**SEVQDLI |
| GTFD | 1→6 | 460 | DGVRV**D**AVDNVNADLLQI | 498 | HLSIL**E**AWSDND | 577 | YIFIRAH**D**SEVQTLI |
| GTFI | 1→3 | 448 | DSIRV**D**AVDNVDADLLQI | 486 | HVSIV**E**AWSDND | 557 | YSFARAH**D**SEVQDLI |
| GTF180 | 1→6 | 1020 | DGIRV**D**AVDNVDVDLLSI | 1058 | HINIL**E**DWGWDD | 1129 | YNFVRAH**D**SNAQDQI |
| GTFML1 | 1→3 | 1020 | DSIRV**D**AVDNVDADLLDI | 1058 | HINIL**E**DWGGQD | 1125 | YSFIRAH**D**NGSQDDI |
| GTFO | 1→4 | 1019 | DSVRV**D**APDNIDADLMNI | 1056 | HINIL**E**DWNSSD | 1126 | YSFIRAH**D**NNSQDQI |
| GTFA | 1→4/1→6 | 1019 | DSVRV**D**APDN**I**DADLMNI | 1056 | HINIL**E**DWNHAD | 1126 | YSFVRAH**D**N**N**S**S**QDQI |

**Figure 1.** Sequence alignment of regions flanking the catalytic residues of glucansucrases of various linkange specificities. DSRBCB4 (*L. mesenteroides* B-1299CB4), DSRS (*L. mesenteroides* B-512F),[41] DSRE CD1 (first catalytic domain of *L. mesenteroides* B-1299),[16] DSRB742 (*L. mesenteroides* B-742),[46] GTFD (*S. mutans* GS5),[47] and GTF180 (*L. reuteri* 180)[14] are specific for α-(1→6) linkages. GTFB (*S. mutans* GS5), GTFI (*S. downei* MFe28),[48] and GTFML1 (*L. reuteri* ML1)[14] are specific for α-(1→3) linkages. DSRE CD2 (second catalytic domain of *L. mesenteroides* B-1299)[49] is specific for α-(1→2) linkages. GTFO (*L. reuteri* ATCC 55730)[12] is specific for α-(1→4) linkages. ASR (*L. mesenteroides* B-1355)[15] is specific for alternating α-(1→6) and α-(1→3) linkages. GTFA (*L. reuteri* 121)[11] catalyzes the synthesis of a glucan composed of about 50% of α-(1→4) linkages. AS (*N. polysaccharea*) is specific for α-(1→4) linkages and is the only glucansucrase classified in GH family 13. +1 → +4 represent AS subsites shown to accept the molecules to be elongated.[50] Catalytic amino acids are in bold. DSRBCB4 amino acid residues targeted in this study are shown in bold and underlined. Bold and underlined letters in GTFA are amino acids corresponding to mutated residues in DSRBCB4.

Oligosaccharides with specific structures are currently needed. Oligosaccharides currently produced for commercial markets,[17] including isomaltooligosaccharides,[18] leucrose,[19] and palatinose,[20] are of interest in food, pharmacy, and cosmetics applications because of their ability to prevent and treat diseases of various biological origins. For the synthesis of oligosaccharides, the glycosynthase concept is very promising, where the hydrolytic activity of glycosidases has been suppressed by rational site-directed mutagenesis, allowing transglycosylation to occur instead.[21]

In this study, DSRBCB4 residues were substituted by double (V532P/V535I) and triple (S642N/E643N/V644S) mutagenesis of amino acids. Combinations of the mutations made in the separate acceptor and substrate binding regions were constructed, and the transfer activities of the mutated enzymes were characterized. The results indicated that specific amino acid residues were crucial for glucosidic linkage type specificity with various short-chain oligosaccharides synthesis and or novel polysaccharides synthesis with completely altered linkages.

## MATERIALS AND METHODS

**Mutant Construction, Enzyme Expression, and Purification.** Plasmid DSRBCB4-pRSETB was used as the template for mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).[5] The V532P/V535I mutant was constructed using $^{2188}$CGTGTCGATGCTCCCGATAATATTGACGCTG$^{2218}$, the S642N/E643N/V644S mutant using $^{2508}$CGCGCACACGATAATAATTCACAAACAGTCATTGCTC$^{2544}$, and mutant V532P/V535I/S642N/E643N/V644S using V532P/V535I primer and S642N/E643N/V644S mutant plasmid as a template for polymerase chain reaction (PCR). After successful mutagenesis (confirmed by nucleotide sequencing), DSRBCB4-pRSETB, V532P/V535I-pRSETB, S642N/E643N/V644S-pRSETB, and V532P/V535I/S642N/E643N/V644S-pRSETB were overexpressed in *Escherichia coli* BL21(DE3)pLysS. DSRBCB4 and mutant proteins were produced and purified as described previously.[5]

**Enzyme Assay.** All reactions were performed at 28 °C in 20 mM sodium acetate buffer, pH 5.2, containing 1 mM $CaCl_2$ and 1.14 U/mg purified DSRBCB4 or each variant glucansucrase. The DSRBCB4 and each variant glucansucrase activities were determined as initial rates by measuring fructose release (enzymatically) from sucrose conversion (six data points over a period of 6 min, using 15 different sucrose concentrations ranging from 0.25 to 100 mM), and the enzyme reaction was stopped by adjusting the pH to 10.5 with 25 mM NaOH. The fructose concentration liberated from sucrose was determined using thin layer chromatography (TLC) with the Public Domain NIH Image Program.[22] One unit was defined as the amount of enzyme that catalyzed the formation of 1 μmol of fructose per minute at 28 °C.

**Electrophoresis and Detection of Active Glucansucrase.** Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described.[23] The resolved proteins were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St. Louis, MO), and the dextransucrase activities were detected by incubating the gels in 200 mM sucrose overnight, followed by staining using a periodic acid−Schiff (PAS) procedure.[24] The protein concentration was determined[25] with crystalline bovine serum albumin (Sigma-Aldrich) as the standard.

**Products Synthesized from Sucrose and Acceptor Reactions.** After complete depletion of sucrose (100 mM, 18 h at 28 °C and pH 5.2) by DSRBCB4 and variant glucansucrases (1.14 U/mg), the concentrations of fructose, glucose, and leucrose in the reaction of mixture were determined using TLC and high-pressure liquid chromatography (HPLC) analyses. Acceptor reactions were performed following

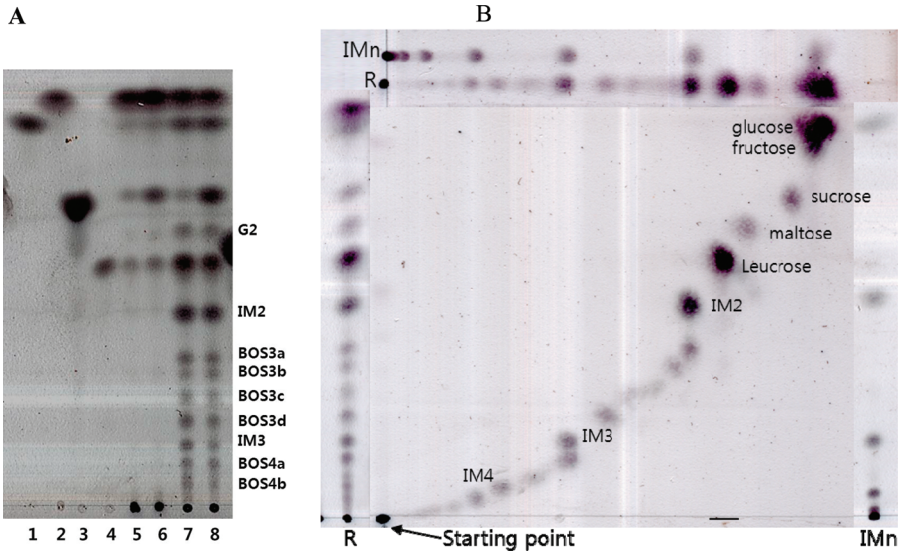


**Figure 2.** TLC of sucrose reaction products of DSRBCB4 and mutants. (A) The reaction was allowed to continue for 18 h, and then the reaction products were analyzed by TLC with four ascents of 85:15 (v/v) acetonitrile/water. Lanes: 1, glucose; 2, fructose; 3; sucrose; 4, leucrose; 5−8, reaction products of rDSRBCB4 and derived site-directed mutants with sucrose. Enzymes are DSRBCB4 (lane 5), V532P/V535I (lane 6), S642N/E643N/V644S (lane 7), and V532P/V535I/S642N/E643N/V644S (lane 8), respectively. G2, maltose; IM2, isomaltose; IM3, isomaltotriose; BOS series, branched oligosaccharides (DP = 3−4). (B) Two-dimensional TLC of sucrose reaction products of V532P/V535I/S642N/E643N/V644S enzyme (R). Composition of the first ascent developing solvent was 85:15 (v/v) acetonitrile/water, and then the second ascent developing solvent was 2:5:1.5 (v/v/v) nitromethane/1-propanol/water.

the same conditions, except that 100 mM maltose was added as acceptor. The maltose acceptor reaction products (10 mL) were fractionated on a Bio-Gel P-2 column (3 × 130 cm; Bio-Rad, Hercules, CA) by gel permeation chromatography. The fractions including the acceptor products were concentrated at 35 °C using a rotary evaporator and subjected to HPLC. A model LC-6AD HPLC system (Shimadzu, Kyoto, Japan), connected to a 7.8 × 300 mm internal diameter Shodex Asahipak $NH_2$ column (Showa Denko, Tokyo, Japan), and a model RI detector (RID-10A; Shimadzu, Kyoto, Japan) were used to isolate maltose acceptor products. The mobile phase consisted of 75% acetonitrile, and the flow rate was 3 mL/min by the isocratic method. The purified oligosaccharides (maltose acceptor products) were characterized by enzymatic degradation using Dextranase from *Penicillium* sp. (EC 3.2.1.11; Sigma-Aldrich), which hydrolyzes only α-(1→6) glucosidic bonds;[26] amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3; Sigma-Aldrich), which hydrolyzes α-(1→4), α-(1→3), and α-(1→6) linkages at decreasing rates, respectively, to produce glucose from the nonreducing end of linear oligosaccharides;[27,28] and α-glucosidase from *Geobacillus stearothermophilus* (EC 3.1.2.20; Sigma-Aldrich), which hydrolyzes terminal α-(1→4) linkages from the nonreducing end of oligosaccharides to produce glucose.[29] Oligosaccharides (1 g/L) were incubated with 30 units/mL Dextranase, 4 units/mL amyloglucosidase, and 20 U/mL α-glucosidase. After 30 min and 3 and 18 h of incubation at 40 °C and pH 6.5, samples were withdrawn and the products formed analyzed by TLC.

**Glucan Production.** Purified DSRBCB4 or each variant glucansucrase (0.11 U/mL) was added to 20 mM sodium acetate buffer (pH 5.2) containing 100 mM sucrose. The reaction was allowed to proceed at 28 °C until the sucrose was completely consumed. Glucan was prepared from the reaction mixture by ethanol precipitation (up to 67% of the original volume, v/v). The final glucan precipitates were dried and redissolved in a dimethyl sulfoxide (DMSO; AMRESCO, St. Louis, MO).

**Nuclear Magnetic Resonance (NMR) Spectroscopy.** $^1H$ NMR spectra were recorded on a 500 MHz DXR NMR spectrometer (Bruker, Billerica, MA) at a probe temperature of 353 K. Samples were dissolved in 0.4 mL of 99.8% ($^2H_6$) DMSO (Merck, Dorset, U.K.). $^1H$ chemical shifts ($\delta$) were expressed in parts per million (ppm) by reference to internal acetone ($\delta$ 2.225). $^1H$ NMR spectra were recorded with a spectral width of 5000 Hz in a 16K complex data set and zero-filled to 32K. A WEFT-pulse sequence was applied.[30] When necessary, a fifth-order polynomial baseline correction was applied.

**Methylation Analysis.** To analyze the structure of the glucan synthesized by purified DSRBCB4 or its variant glucansucrases, these glucans were methylated using a Hakomori reagent followed by acetic hydrolysis with 2 M trifluoroacetic acid (Acros, Morris Plains, NJ) and analyzed for methylated glucan using TLC.[31] The methylation products were separated on Whatman K5F TLC plates (Whatman Ltd., Maidstone, U.K.); the plate was irrigated with two ascents of 3:9:1 (v/v/v) acetonitrile/chloroform/methanol (Duksan, Korea).

## RESULTS

**Amino Acid Substitutions on DSRBCB4.** In vitro site-directed mutagenesis was employed to identify regions and specific amino acid residues crucial for the specificity of glucosidic linkage type in the synthesized glucans and oligosaccharides. On the basis of the reuteransucrase sequences, a double mutant (V532P/V535I), triple mutant (S642N/E643N/V644S), and combinative mutant (V532P/V535I/S642N/E643N/V644S) were constructed to introduce the activity of reuteran-like polysaccharides synthesis. Mutated enzymes were purified, and SDS-PAGE analysis showed a molecular mass corresponding to 163 kDa with yields of 33 mg/L for DSRBCB4, 51 mg/L for V532P/V535I, 39 mg/L for S642N/E643N/V644S, and 41 mg/L for V532P/V535I/S642N/E643N/V644S (data not shown). Glucansucrase activities of three mutant enzymes were compared to that of the DSRBCB4 (13.9 U/mg) on 100 mM sucrose. Variant glucansucrases showed a drastic loss of activity, 52.7% residual activity for V532P/V535I (7.32 U/mg), 11.5% residual activity for S642N/E643N/V644S (1.60 U/mg), and 8.21% residual activity for V532P/V535I/S642N/E643N/V644S (1.14 U/mg).

**Table 1. Products of DSRBCB4 and Mutants Incubated with 100 mM Sucrose[a]**

| enzyme | glucose (%) | maltose (%) | leucrose (%) | isomaltose (%) | BOS series[b] (%) | glucan (%) |
|---|---|---|---|---|---|---|
| DSRBCB4 | 6.1 ± 0.2 | –[c] | 10.4 ± 0.1 | – | – | 83.5 ± 0.4 |
| V532P/V535I | 11.1 ± 0.5 | – | 12.3 ± 0.2 | – | – | 76.6 ± 0.3 |
| S642N/E643N/V644S | 11.4 ± 1.3 | 4.7 ± 0.3 | 21.6 ± 0.3 | 15.0 ± 0.4 | 27.4 ± 0.6 | 19.9 ± 0.1 |
| V532P/V535I/S642N/E643N/V644S | 13.3 ± 1.5 | 3.8 ± 0.1 | 22.6 ± 0.5 | 17.1 ± 1.0 | 22.2 ± 0.5 | 21.1 ± 0.2 |

[a] Percentages indicate the relative conversion of sucrose into glucan, oligosaccharides (leucrose, isomaltose, maltose, and BOS series), and glucose (hydrolysis). Results are given as mean ± standard deviation ($n$ = 3). [b] Branched oligosaccharides (DP3−DP4) [c] –, not detectable.

**Glucan Synthesized with Sucrose.** After the complete depletion of sucrose, DSRBCB4 showed the following product distribution: dextran synthesis, 83.5%, and leucrose [α-D-glucopyranosyl-(1→5)-β-D-fructofuranose] synthesis, 10.4% (Figure 2; Table 1). Leucrose was synthesized from sucrose (donor substrate) and fructose (acceptor substrate). Fructose was derived from sucrose, accumulating in increasing amounts during the reaction. Isomaltose formation was not detected. The V532P/V535I double-mutant enzyme showed the same pattern in glucan and leucrose production as DSRBCB4 (Figure 3; Table 1). With sucrose, the S642N/E643N/V644S triple-mutant enzyme synthesized isomaltose in 15% yield; also, the extent of leucrose synthesis (21.6%) increased. Additional products, such as several unknown compounds (branched DP3 and DP4 oligosaccharides), were formed as well. The amount of polysaccharides synthesized by the triple mutant was reduced to 4.2-fold, compared to that of DSRBCB4 wild type (Figure 2; Table 1). The combinative mutant (V532P/V535I/S642N/E643N/V644S) enzyme also displayed a product profile similar to that of the triple-mutant enzyme (Figure 2; Table 1). The triple and combinative mutants clearly produced enhanced amounts of less water-soluble polysaccharides. Therefore, these polymers were characterized in more detail (see Composition of Glucan Produced by Wild Type and Each Variant Glucansucrase).

**Enzyme Reaction Product Profile of Variant Mutants with both Sucrose and Maltose.** In the presence of maltose, DSRBCB4 formed panose [MP1, α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-D-glucopyranose] as the most abundant acceptor reaction product (approximately 33% panose from 100 mM sucrose and 100 mM maltose), indicating that an α-1,6 linkage was formed at the nonreducing end of maltose (Table 2). Also, DSRBCB4 synthesized acceptor products such as MP2 [α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-D-glucopyranose], MP3 [α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-D-glucopyranose], and MP4 [α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-D-glucopyranose]. In addition, the two enzymes with the common S642N/E643N/V644S mutation synthesized unknown oligosaccharides (MP1a and MP2a) from sucrose and maltose as acceptor reaction substrate (Table 2).

Acceptor reactions confirmed that linkage specificity was altered. The S642N/E643N/V644S and V532P/V535I/S642N/E643N/V644S mutants did not produce oligosaccharides of DP higher than 4 but produced more dextran [α-(1→6)-linked glucosyl residues onto maltose]. The mutant was still able to synthesize MP2a (oligosaccharides of DP4, $6^{I}$-α-glucosylpanose), representing 20.4% of the total oligosaccharides produced (Table 2). The structuresd of MP1, MP2, MP1a, and

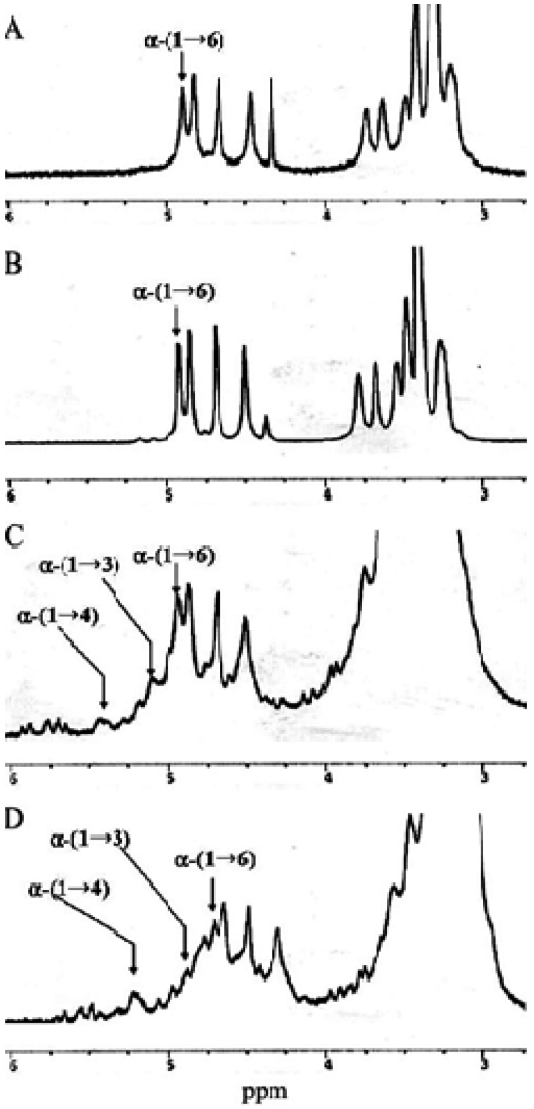


**Figure 3.** $^{1}$H NMR spectra of glucan synthesized by DSRBCB4 and mutants: (A) DSRBCB4 soluble dextran; (B) V532P/V535I soluble glucan; (C) S642N/E643N/V644S less-soluble glucan; (D) V532P/V535I/S642N/E643N/V644S less-soluble glucan. 5.0 ppm, α-(1→6) linkages; 5.1 ppm, α-(1→3) linkages; 5.4 ppm, α-(1→4) linkages.

MP2a were identified by the enzymatic method. MP1 produced maltose and glucose from its enzymatic degradation by Dextranase, amyloglucosidase, and α-glucosidase, confirming its identity as panose (Table 3). In the case of MP1a, amyloglucosidase and α-glucosidase degraded this sugar (DP3) into isomaltose and glucose. Therefore, MP1a was an

**Table 2. Products of DSRBCB4 and Mutants Incubation with 100 mM Sucrose and 100 mM Maltose[a]**

| enzyme | MP1a (%) | MP1 (%) | MP2a (%) | MP2 (%) | MP3 (%) | MP4 (%) | OS (%) |
|---|---|---|---|---|---|---|---|
| DSRBCB4 | −[b] | 34.8 ± 0.5 | − | 38.9 ± 0.1 | 14.2 ± 0.1 | 2.8 ± 0.2 | 9.3 ± 0.3 |
| V532P/V535I | − | 36.1 ± 0.1 | − | 39.8 ± 0.3 | 14.5 ± 0.1 | 2.7 ± 0.3 | 6.9 ± 0.4 |
| S642N/E643N/V644S | 7.8 ± 0.1 | 50.2 ± 0.6 | 26.0 ± 0.5 | 11.5 ± 0.5 | 1.4 ± 0.2 | − | 3.2 ± 0.1 |
| V532P/V535I/S642N/E643N/V644S | 6.8 ± 0.8 | 57.0 ± 0.8 | 20.4 ± 1.0 | 11.3 ± 0.7 | 1.1 ± 0.1 | − | 3.4 ± 0.1 |

[a] The total and individual oligosaccharide yields indicate the amount of maltose consumed as a percentages of the total amount of maltose initially present in the incubation. Results are given as mean ± standard deviation ($n$ = 3). MP1, panose; MP2, $6^{II}$-α-isomaltosyl maltose; MP3, $6^{II}$-α-isomaltotriosyl maltose; MP4, $6^{II}$-α-isomaltotetraosyl maltose; MP1a and MP2a, unknown linkaged oligosaccharides; OS, oligosaccharides. [b] −, not detectable.

**Table 3. Identification of Major Oligosaccharides Formed by the Two Enzymes Containing the Triple Amino Acid Mutation of S642N/E643N/V644S from Sucrose and Maltose, Determined by Enzymatic Degradation Products**

| | degradation product(s)[a] by | | | |
|---|---|---|---|---|
| degree of polymerization of oligosaccharide | dextranase | amyloglucosidase | α-glucosidase | identification of oligosaccharide structure[a] |
| 3 | −[b] | G + G2[c] | G[c] | MP1 |
| 3 | − | G + IM2[d] | G+IM2[d] | MP1a |
| 4 | G2 + IM2 | G + MP1[c] | G + MP1[c] | MP2 |
| 4 | − | G + IM2 + MP1a[d] | G[c] | MP2a |

[a] G, glucose; G2, maltose; IM2, isomaltose; MP1, panose; MP2, $6^{III}$-α-glucosylpanose; MP1a, isopanose; MP2a, $6^{I}$-α-glucosylpanose. [b] −, no degradation observed. [c] Relatively slow degradation. [d] Relatively fast degradation.

isopanose [α-D-glucopyranosyl-(1→4)-{α-D-glucopyranosyl-(1→6)}-D-glucopyranose]. In the case of MP2 (DP4), dextranase degraded this oligosaccharide into maltose (G2) and isomaltose (IM2). Amyloglucosidase and α-glucosidase hydrolyzed MP2 slowly to glucose and panose. In the case of MP2a, degradation of this tetraoligosaccahride by Dextranase was not possible. It was degraded quickly by amyloglucosidase into glucose, isomaltose, and MP1a, whereas α-glucosidase slowly degraded MP2a into glucose (Table 3). Therefore, MP2a was $6^{I}$-α-glucosylpanose. The two enzymes with the S642N/E643N/V644S mutation displayed up to a 1.6-fold increased level of panose synthesis compared to DSRBCB4 and the double-mutant enzyme (Table 3).

**Composition of Glucan Produced by Wild Type and Each Variant Glucansucrase.** DSRBCB4 and its mutants, V532P/V535I, S642N/E643N/V644S, and V532P/V535I/S642N/E643N/V644S, were incubated with sucrose, and the subsequent glucan formed was purified. The reaction products produced by DSRBCB4 and V532P/V535I made clear solutions, and the purified glucan was water-soluble. However, the products produced by S642N/E643N/V644S and V532P/V535I/S642N/E643N/V644S resulted in a very cloudy suspension, and the purified polysaccharides was less water-soluble. Multiangle laser light diffraction (MALLD) experiments showed that triple and combinatorial mutant polysaccharides have molecular masses comparable to that of the soluble wild type polysaccharide ($2 \times 10^6$ Da), ranging from $10^4$ to $10^5$ Da, with a significant change in size distribution (data not shown). The ethanol precipitate (up to 67% of the original volume, v/v) of S642N/E643N/V644S and the V532P/V535I/S642N/E643N/V644S polysaccharides exhibited an adhesive nature. The precipitate did not dissolve completely in DMSO by sonication at 50 °C and remained self-adhesive. These characteristics were different from the dextrans produced by DSRBCB4 and V532P/V535I.

**NMR Analysis.** To analyze the structure of the glucans produced by DSRBCB4 and each variant glucansucrase, $^1$H NMR spectra were obtained (Figure 3). In the spectra, the anomeric signals of α-(1→6) and α-(1→4) linkages in the main chain appeared at 5.0 and 5.4 ppm, respectively.[11] Also, the signal of α-(1→3) linkage was apparent at 5.1 ppm.[32] The dextran synthesized by DSRBCB4 or V532P/V535I was linear, as supported by the sole $^1$H NMR signal of an α-(1→6) linkage at 5.0 ppm. $^1$H NMR spectroscopy of S642N/E643N/V644S and V532P/V535I/S642N/E643N/V644S glucan indicated an α-anomeric configuration for all glucose residues. Integration of anomeric signals revealed α-(1→6) linked D-glucopyranose (5.0 ppm), α-(1→3) linked D-glucopyranose (5.1 ppm), and α-(1→4) linked D-glucopyranose residues (5.4 ppm), which is agreement with the overall substitution pattern found by methylation analysis (Figure 3). It should be noted that the low solubility of S642N/E643N/V644S and V532P/V535I/S642N/E643N/V644S glucan influenced the quality of the $^1$H NMR spectrum, whereas the broader peaks, compared to that of DSRBCB4 glucan, may be the result of more structural diversity.

**Methylation Analysis.** TLC analyses of *O*-methylated monosaccharides obtained from methylation and acid hydrolysis of glucans synthesized by DSRBCB4 and each variant are shown in Table 4. DSRBCB4 dextran had α-(1→6) glucosidic linkages in the main chains. The major methylated product was 2,3,4-tri-*O*-methyl-D-glucose with minor amounts of 2,3,4,6-tetra-*O*-methyl-D-glucose from the nonreducing ends of branched chains and 2,4-di-*O*-methyl-D-glucose from the branched glucose residues, but only a small fraction compared with the linearly linked compounds. The V532P/V535I double-mutant glucan showed the same pattern compared to the DSRBCB4 enzyme. The less water-soluble glucan of S642N/E643N/V644S possessed predominantly α-(1→6) glucosidic linkages (52%), together with approximately 11% α-(1→3) glucosidic linkages, α-(1→4) glucosidic linkages (2%), and 23.5% α-(1→3/6) or α-(1→4/6) branching points. The V532P/V535I/S642N/E643N/V644S glucan showed the same pattern compared to the S642N/E643N/V644S. The difference between the two less water-slouble

**Table 4. Proportions of the Partially Methylated D-Glucose in Methylation Analysis of Glucan Synthesized by DSRBCB4 and Mutants**

| | methylation (%) for glucosyl unit type | | | | |
|---|---|---|---|---|---|
| sample | 2,3,4,6-tetra-*O*-Me | 2,3,6-tri-*O*-Me | 2,4,6-tri-*O*-Me | 2,3,4-tri-*O*-Me | 2,3-di-*O*-Me and/or 2,4-di-*O*-Me |
| DSRBCB4 | 5.40 | 0 | 0 | 79.3 | 15.3 |
| V532P/V535I | 7.70 | 0 | 0 | 75.8 | 16.5 |
| S642N/E643N/V644S | 12.0 | 1.5 | 10.6 | 52.4 | 23.5 |
| V532P/V535I/S642N/E643N/V644S | 13.9 | 10.9 | 10.4 | 43.7 | 21.1 |

glucans was α-(1→4) linkage percentages. The less water-soluble glucan of S642N/E643N/V644S contained 1.5% α-(1→4) linkage. The glucan synthesized by V532P/V535I/S642N/E643N/V644S contained 10.9% α-(1→4) linkage.

## DISCUSSION

In the case of the DSRBCB4 dextransucrase, divergent amino acids are located near the catalytic residues of the triad (D530, E568, and D641 for DSRBCB4) and in an area proposed to be in contact with substrates and products.[5] The crystal structure of amylosucrase soaked with maltoheptaose allowed the mapping of the subsites from −1 to +5[33] and revealed interactions of the D394, T398, and F399 located just after the acid/base general catalyst with glucosyl units in positions +1 (D393) and +2 (D394), +3 (T398), and +4 (F399) (Figure 1).[33] Comparing the sequence and functional similarities between GH family 13 amylosucrases and GH family 70 glucansucrases, we propose that residues located immediately downstream from acid/base catalyst of GH family 70 glucansucrases would be important in forming the subsite +1 to +*n*, which influenced the glucansucrase linkage specificity. This region should, thus, have a particular influence on the glucansucrase region specificity.[34] Changing the tripeptide located in the C-terminus of the catalytic D641 in DSRBCB4 to S642N/E643N/V644S resulted in a drastic reduction in the glucansucrase activity for sucrose and changes in the type of glucosidic linkages in the oligosaccharide and glucan production, probably resulting from the transfer of glucosyl onto sucrose (Figure 2).[4,5] In the case of the combinative mutant, sucrose hydrolysis appeared to be largely favored; glucosyl residues were transferred onto water. Part of the glucose released then acted as an acceptor, resulting in the major synthesis of isomaltose (Figure 2; Table 1). This indicates that the conserved region IV in DSRBCB4 is important in sucrose binding/processing, consistent with the suggested role of D641 as a transition state stabilizer (Figure 1).[7,35]

To mimic the reuteransucrase sequences of DSRBCB4, the double-mutant V532P/V535I was constructed. Testing of the purified enzyme revealed that the mutation resulted in a drastic loss of activity, with only 53% residual activity. V532P/V535I displayed the same pattern of oligosaccharide and glucan production as DSRBCB4, with formation of an α-(1→6) (Figure 3). However, the combination of V532P/V535I with S642N/E643N/V644S showed a clear difference in the type and percentage of oligosaccharide and glucan synthesized, with an increase of α-(1→3) and α-(1→4) glucosidic linkages and a decrease of α-(1→6) glucosidic linkages. The glucan of the triple mutant showed the most extensive changes in linkage distribution compared to the DSRBCB4 glucan. Besides the native α-(1→6) and α-(1→3) linkages, as present in DSRBCB4, a significant amount of α-(1→4) linkages is introduced by the triple-mutant enzyme. The triple-mutant polysaccharide is less water-soluble than wild type DSRBCB4 glucan. The lower solubility is probably the result of more rigid structure, because α-(1→6) linkages are flexible, whereas α-(1→3) and α-(1→4) are more rigid.[36,37]

In CGTase, the corresponding region constitutes part of the sugar-binding acceptor subsites +1 and +2 [residues D229-(−1)-A-(+1)-V-K-(+2)-H-(+1), in *Bacillus circulans* 251 CGTase][38] responsible for the stereospecific positioning of the molecule accepting the glucosyl units.[39] Conserved region II in GH13 (sugar-binding acceptor site) that was previously suggested[7] was also present at a similar position in GH70, but it was different from our mutation data, which did not influence the acceptor binding and glucosyl transfer in region II.

DSRS synthesizes panose and α-(1→6) isomaltooligosaccharide-linked panose from sucrose as well as maltose.[40,41] DSRBCB4 only synthesized panose (DP3, $6^{II}$-α-D-glucopyranosylmaltose) and its derivatives such as $6^{II}$-α-isomaltosylmaltose (DP4), $6^{II}$-α-isomaltotriosylmaltose (DP5), and $6^{II}$-α-isomaltotetraosylmaltose (DP6). However, both mutant enzymes containing the common amino acid mutation S642N/E643N/V644S synthesized panose, $6^{II}$-α-isomaltosylmaltose, and branched oligosaccharides (Table 1). The data clearly indicate that, with respect to oligosaccharides synthesized from maltose, the DSRBCB4 mutants in conserved region IV increase the branched acceptor reaction products.

When a D-glucose was transferred to the nonreducing residue, the first product was also an acceptor, which produced the second reaction product, forming a homologous series. When D-glucose was transferred to the reducing residue, the first product did not become an acceptor for further reaction or became only a very poor acceptor to yield a small amount of the next product.[42] Panose or isopanose structures, for example, were found in dextransucrase acceptor reaction by transferring of a D-glucose residue to 6-OH groups of the nonreducing or reducing residue of the maltose, respectively. Panose (MP1) served further as an acceptor to give $6^{II}$-α-isomaltosylmaltose (MP2): this sequential reaction synthesized isomaltooligosaccharide-linked maltose. Isopanose (MP1a), however, did not readily serve further as an acceptor or was only a poor acceptor to give a small amount of 4-α-maltosyl-(6-α-glucosyl)glucose (MP2a). The MP2a was not recognized as an acceptor and, consequently, MP2a accumulated, almost no oligosaccharides of higher DP were synthesized, and more oligosaccharides of glucan series were produced (Table 2). The combinative mutant was able to synthesize MP2 and MP2a, representing 11.3 and 20.4% of the total oligosaccharides, respectively (Table 2). The mutants including S642N/E643N/V644S exhibited considerably higher transferring activity of glucose unit to the 6-OH position of the reducing end of the acceptor than the nonreducing end. A significant change in the specificity of glucosidic bond formation was

also observed in the glucan synthesized by the triple mutant (S642N/E643N/V644S)-containing mutation, with reduction of the quantity of α-(1→6) linkages to 40% and increased amounts of α-(1→3) and α-(1→4) linkages (Table 4; Figure 3). The less water-soluble glucans of S642N/E643N/V644S and V532P/V535I/S642N/E643N/V644S contained relatively high proportions of nonreducing ends (12.0 and 13.9%, respectively), due to branching through both the α-(1→3) and α-(1→4) linkages. The data suggest that both mutations in conserved regions II and IV of DSRBCB4 transformed the DSRBCB4 dextransucrase from linear dextran formation into a branched and less water-slouble glucan formation.[43] A similar result was reported with amylosucrase. In amylosucrase, the D394, next to the catalytically important H392 and D393 (transition state stabilizer), was shown to be involved in the correct positioning of the glucosyl residue at this site.[44] Mutant D394A displayed changes in the product formation specificity (mono- and oligosaccharides) from sucrose.[45]

The significant changes observed in the triple and combinative mutant enzymes, with respect to activity for sucrose, linkages, and solubility of oligosaccharide and glucan synthesized, strongly support the hypothesis that residues in conserved region IV are involved in acceptor substrate binding in glucansucrases. Many mutansucrases possess the same triplet amino acids SEV in region IV as DSRBCB4 mutant S642N/E643N/V644S, and instead of synthesizing dextran, mutansucrase synthesizes mutans. Furthermore, the higher amount of α-(1→3) glucosidic linkages synthesized by mutated DSRBCB4 could also be explained by the region IV replaced in this study.

In summary, we demonstrate that the oligosaccharide product and glycosidic linkage specificity of DSRBCB4 can be modified by rational site-directed mutagenesis, while maintaining high transglycosylation efficiency, resulting in excellent product yields. Enzyme engineering allowing the synthesis of tailor-made polysaccharides and oligosaccharides for a range of applications is in progress.

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