

## ACCEPTOR REACTIONS OF ALTERNANSUCRASE\* FROM *Leuconostoc mesenteroides* NRRL B-1355†

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### ABSTRACT

Extracellular glucansucrases from various bacterial sources, including *Leuconostoc mesenteroides*, have been shown to catalyze the transfer of glucosyl groups from sucrose to low-molecular-weight acceptor sugars, forming a series of oligosaccharides. An extracellular glucansucrase recently isolated from *Leuconostoc mesenteroides* NRRL B-1355 synthesizes a polysaccharide consisting of alternating  $\alpha$ -(1→6)- and  $\alpha$ -(1→3)-linked D-glucose residues. We have found that this enzyme preparation is capable of forming both  $\alpha$ -(1→6)- and  $\alpha$ -(1→3)-linked acceptor-products in the presence of a number of low-molecular-weight acceptor sugars. D-Glucose gave isomaltose only, no nigerose being formed. Similarly, methyl  $\alpha$ -D-glucoside, methyl  $\beta$ -D-glucoside, maltose, and nigerose gave methyl  $\alpha$ -isomaltoside, methyl  $\beta$ -isomaltoside, panose, and 6<sup>2</sup>-O- $\alpha$ -D-glucosylnigerose, respectively. However, isomaltose gave both isomaltotriose and 3<sup>2</sup>-O- $\alpha$ -D-glucosylisomaltose. These initial acceptor-products may also act as acceptors, and the structures of the products of higher d.p. show that a (1→3)- $\alpha$ -D-glucosidic bond is formed only when the non-reducing glucose acceptor group is linked through an  $\alpha$ -(1→6) bond to another glucose residue. Experiments using [<sup>14</sup>C]sucrose and unlabeled acceptors showed that acceptor reactions occur by transfer of a glucosyl group from sucrose to O-3 or O-6 of the glucosyl group at the nonreducing end of the acceptor saccharide. If a “good” acceptor is defined as one that gives rise to a greater amount of oligosaccharide and less polysaccharide, then the acceptors tested may be listed as follows: maltose ≥ nigerose > methyl  $\alpha$ -D-glucoside > isomaltose > D-glucose > methyl  $\beta$ -D-glucoside.

### INTRODUCTION

Koepsell *et al.*<sup>2</sup> showed that dextransucrase from *Leuconostoc mesenteroides*

\*Alternansucrase is an enzyme that synthesizes an alternating  $\alpha$ -(1→6)- $\alpha$ -(1→3)-D-glucan from sucrose<sup>1</sup>.

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NRRL B-512, in the presence of low-molecular-weight sugars, catalyzed the transfer of D-glucosyl groups from sucrose to these "acceptor" sugars, forming a series of oligosaccharide acceptor-products. The acceptor reaction competes with the normal formation of high-molecular-weight glucan, and different sugars compete to different degrees. Maltose and isomaltose served as the best acceptors, giving large proportions of oligosaccharide products, and only a small fraction of the dextran was formed as compared with the proportion formed when acceptors were absent. Yamauchi and Ohwada<sup>3</sup> studied the formation of acceptor products from all eleven glucobioses, and showed that, for D-glucose-containing mono- and di-saccharides in general, B-512F dextranucrase effected acceptor reactions by transferring a glucosyl group from sucrose to O-6 of the nonreducing end of the acceptor sugar. A notable exception to this general observation was the formation of 2<sup>1</sup>-O- $\alpha$ -D-glucosylcellobiose from cellobiose.

Recently, we have described<sup>1</sup> the separation of two different, extracellular glucansucrases produced by *Leuconostoc mesenteroides* NRRL B-1355. One of these produces a glucan consisting of alternating  $\alpha$ -(1 $\rightarrow$ 6)- and  $\alpha$ -(1 $\rightarrow$ 3)-linked D-glucose residues. This glucan has commonly been referred to as B-1355 fraction S dextran, but from its unusual structure and properties, we have proposed the name alternan for this glucan and alternansucrase for the enzyme that synthesizes it from sucrose<sup>1</sup>. We have studied the structures of a number of oligosaccharide acceptor-products formed by alternansucrase in the presence of sucrose and acceptors, and show that this enzyme forms, via acceptor reactions, both  $\alpha$ -(1 $\rightarrow$ 6) and  $\alpha$ -(1 $\rightarrow$ 3) linkages to a variety of acceptor sugars. We have also determined the relative abilities of some of these acceptors to divert glucosyl groups away from formation of alternan and into the formation of oligosaccharide acceptor-products.

## MATERIALS AND METHODS

*Enzymes and substrates.* — Dextranucrase from *Leuconostoc mesenteroides* B-512F was prepared as described by Robyt and Walseth<sup>4</sup>, and was used to prepare reference oligosaccharides. Alternansucrase was prepared from the culture fluid of *Leuconostoc mesenteroides* B-1355 and was separated from dextranucrase as previously described<sup>1</sup>. Exo-isomaltodextranase was prepared from *Arthrobacter globiformis* T6 by the method of Sawai *et al.*<sup>5</sup>.

<sup>14</sup>C-Uniformly labeled sucrose was obtained from New England Nuclear, Boston, MA. Reagent-grade maltose was purchased from EM Laboratories, Elmsford, N.Y. Methyl  $\alpha$ -D-glucoside was purchased from Eastman Kodak Co., Rochester, N.Y., and was recrystallized from ethanol before use. Methyl  $\beta$ -D-glucoside was purchased from Sigma Chemical Co., St. Louis, MO. Panose was kindly provided by Dr. Dexter French. Isomaltose was isolated, from a partial acid-hydrolyzate of commercial B-512F dextran, by column chromatography on silica gel with 80% acetonitrile in water. Nigerose and kojibiose were prepared by acetolysis of alternan and B-1299 dextran, respectively, followed by deacetylation and subsequent purifica-

tion by charcoal-column chromatography<sup>6</sup>. D-Allose was prepared by oxidation of 1,2:5,6-di-*O*-isopropylidene- $\alpha$ -D-glucofuranose with chromium trioxide<sup>7</sup>, followed by sodium borohydride reduction and acid-catalyzed removal of the acetal protective groups<sup>8</sup>. 3-Deoxy-3-fluoro-D-glucose was prepared as previously described<sup>1</sup>. All other materials were commercially available preparations.

*Chromatography.* — Descending paper-chromatography was performed on Whatman 3 MM paper with either 10:4:3 (v/v/v) ethyl acetate-pyridine-water or 7:3 (v/v) 1-propanol-water. Irrigation was carried out at 37° for 20–40 h, depending on the compounds being separated. For preparative paper-chromatography, the sample was streaked along the origin of a 23 × 56-cm sheet of paper. After irrigation, guide strips (1 cm) were cut from each edge and developed by the alkaline silver nitrate dip-method<sup>9</sup>; corresponding bands were cut out and the compound was eluted with water. For radioactive compounds, autoradiographic detection was used. T.l.c. was performed on either Whatman K5 0.25-mm silica gel plates, or on E. Merck plates (0.25-mm) of 1:1 silica gel 60-kieselguhr. Multiple ascents were made in one of the following solvent-mixtures: *A*, 4:1 (v/v) acetonitrile-water; *B*, 9:1 (v/v) acetonitrile-water; *C*, 4:1:3:2 (v/v/v/v) acetonitrile-nitroethane-ethanol-water; and *D*, 5:2:4:4 (v/v/v/v) 1-propanol-nitromethane-acetonitrile-water. Detection was by 20% sulfuric acid in methanol, 5% orthonilic acid in pyridine<sup>10</sup>, or 0.2% 1-(1-naphthyl)ethylenediamine dihydrochloride (Marshall's reagent) in 3% sulfuric acid in methanol<sup>11</sup>, with heating for 10–15 min at 100–110°.

*Acceptor-reaction conditions.* — Acceptor reactions were performed at room temperature, with final concentrations of 42mM sucrose, 100mM acceptor, and 0.13 IU/mL of alternansucrase. The reactions were allowed to proceed until sucrose had disappeared (~48 h), as judged by t.l.c. with two ascents of solvent *A*. When acceptor products were to be isolated, an equal volume of ethanol was added to precipitate the polysaccharide. Enzymic reactions were conducted in 20mM pyridinium acetate buffer (pH 5.3) containing 0.01% sodium azide and 2mM calcium chloride. Control mixtures containing glucansucrase and acceptor sugars, but no sucrose, showed no reaction, indicating that acceptors do not serve as glycosyl donors.

*Determination of relative strengths of acceptors.* — The relative strengths of the acceptors were determined by treating alternansucrase with 42mM [U-<sup>14</sup>C]sucrose (460 c.p.m./ $\mu$ g) and 100mM acceptor as already described until all sucrose had been consumed. Aliquots (20  $\mu$ L) of the reaction were spotted at the origin of a 23 × 56 cm paper chromatogram, which was irrigated with 7:3 (v/v) 1-propanol-water, for 24 h at 37°. Channels 3 cm wide were cut into 1-cm segments from the origin to the end of the paper, and each segment was counted for <sup>14</sup>C radioactivity by liquid scintillation in toluene cocktails. This procedure is similar to that described by Mayer *et al.*<sup>12</sup>, but the use of <sup>14</sup>C-labeled sucrose permits application to any acceptor, not just radioactive ones.

*Structural analyses of oligosaccharide acceptor-products.* — The structures of acceptor products isolated by paper chromatography, were determined by chemical and physical means. For some products, comparison by t.l.c. with known compounds

was sufficient. When the degree of polymerization (d.p.) of a sample was unknown, it was determined in one of two ways: the reducing value of unlabeled compounds was determined by the Somogyi–Nelson method<sup>13</sup> and the amount of total carbohydrate was determined by the phenol–sulfuric acid method<sup>14</sup>; alternatively the specific activity of <sup>14</sup>C-labeled compounds were determined and compared with the specific activity of the [<sup>14</sup>C]sucrose used in the acceptor reaction.

The products from hydrolysis<sup>15</sup> of trisaccharides and larger oligosaccharides by isomaltodextranase<sup>5</sup> were examined by t.l.c. with three ascents of solvent *A*. As the specificity of this exolytic enzyme<sup>5,15–17</sup> has been well studied, we were able to use it in the determination of the structures of a number of oligosaccharides.

When partial acid-hydrolysis was used to study the composition of an oligosaccharide, 0.2 mL of a solution of the sugar (~0.5–1.0 mg/mL) was added to 0.8 mL of 0.4M trifluoroacetic acid in a glass ampule. The ampule was then flushed with nitrogen, sealed, and heated for 40 min at 90°. The cooled samples were evaporated to solids under diminished pressure at 45°, and the residues redissolved in 0.2 mL of water. A 5–10-μL sample of this hydrolyzate was chromatographed by t.l.c. with three ascents in solvent *A*.

<sup>13</sup>C-N.m.r. spectrometry was used to aid in the determination of the structure of a tetrasaccharide acceptor-product from maltose (see Results). The spectrum was recorded with 2 mL of a 0.15M solution of the tetrasaccharide in D<sub>2</sub>O in a 10-mm tube, using a JEOL FX 90Q <sup>13</sup>C-n.m.r. spectrometer in the Fourier-transform, proton-decoupled mode at 22.5 MHz, with 1,4-dioxane as the internal reference standard.

## RESULTS

When dextranucrase from *L. mesenteroides* B-512F reacts with sucrose, dextran is formed and D-fructose is released. Once its concentration becomes high enough, fructopyranose acts as an acceptor, and the disaccharide leucrose [*O*-α-D-glucopyranosyl-(1→5)-D-fructopyranose] is formed<sup>18</sup>. We have also found leucrose to be formed when alternansucrase reacts with sucrose. Leucrose was identified by its *R*<sub>Glc</sub> value in two different t.l.c. systems (2 ascents with solvent *A* or 4 ascents with solvent *B*) and by its color reaction when orthonilic acid<sup>10</sup> was used for t.l.c. detection. D-Glucose, D-fructose, and sucrose all gave orange-brown spots with this reagent, whereas leucrose gave a brownish to olive-brown spot.

When D-glucose was added as the acceptor with alternansucrase, the only disaccharide product detected by t.l.c. (other than leucrose) was isomaltose. No spots corresponding to nigerose, maltose, or kojibiose were observed. A trace of leucrose was seen, as in all reactions where sucrose was a substrate. Products of higher d.p. were also observed with D-glucose, and they corresponded to the same products as when isomaltose was the added acceptor. These arise from the isomaltose, formed from D-glucose, also acting as an acceptor.

The first alternansucrase acceptor-product from methyl α-D-glucoside had the

same chromatographic mobility as the first product formed by B-512F dextran-sucrase, namely methyl  $\alpha$ -isomaltoside<sup>19</sup>. In paper chromatography with 10:4:3 (v/v/v) ethyl acetate-pyridine-water, this product had  $R_{Glc}$  0.62; in t.l.c., with four ascents in solvent *B*, its  $R_{Glc}$  value was 0.88. Orthanic acid did not react with this compound, indicating that it was nonreducing. Prolonged treatment with isomaltodextranase gave a small but significant amount of isomaltose. These results show that the alternansucrase acceptor-product from methyl  $\alpha$ -D-glucoside is methyl  $\alpha$ -isomaltoside. Products of higher d.p. were also formed, but their structures were not studied. It was found that methyl  $\beta$ -D-glucoside could also serve as an acceptor for both B-1355 alternansucrase and B-512F dextran-sucrase. As with methyl  $\alpha$ -D-glucoside, the disaccharide products from both enzymes had the same  $R_{Glc}$  (0.9) in t.l.c., and are presumed to be methyl  $\beta$ -isomaltoside. Again products of higher d.p. were formed but were not studied; presumably they have the same linkages as the products from D-glucose and isomaltose acceptors.

One of the best acceptors was maltose. Only one trisaccharide was isolated from the acceptor reaction of alternansucrase with sucrose and maltose (see Fig. 1). This trisaccharide had the same  $R_{Glc}$  in t.l.c. as panose (6<sup>2</sup>-*O*- $\alpha$ -D-glucosylmaltose), which was produced by the acceptor reaction of B-512F dextran-sucrase with sucrose and maltose<sup>2,3</sup>. Isomaltodextranase acted on this compound to give D-glucose and isomaltose (Fig. 2), confirming that this acceptor product was panose. No other trisaccharides were found in the acceptor reaction with maltose. Two tetrasaccharides were produced from maltose. The first of these (Fig. 1) migrated between panose and 6<sup>2</sup>-*O*- $\alpha$ -isomaltosylmaltose<sup>20</sup> in t.l.c., and was found to be a reducing tetrasaccharide. As it has been shown that acceptor products of higher d.p. arise from the lower-d.p. precursors<sup>12,20</sup>, this tetrasaccharide must contain panose as part of its structure. Isomaltodextranase converted this material into D-glucose and a trisaccharide having an  $R_{Glc}$  value similar to, but not identical to, that of panose (Fig. 2). Partial acid-hydrolysis yielded D-glucose, plus a spot corresponding to either nigerose or maltose, isomaltose, a trisaccharide, and starting material. No kojibiose was found. Of particular importance in the <sup>13</sup>C-n.m.r. spectrum of this tetrasaccharide (Table I) is the peak at 80.8 p.p.m. Only four types of glucobiose linkages give a peak in this region<sup>21</sup>, namely: C-2 in a  $\beta$ -(1 $\rightarrow$ 2) linkage, C-3 in a  $\beta$ -(1 $\rightarrow$ 3) linkage, C-4 in a  $\beta$ -(1 $\rightarrow$ 4) linkage and C-3 in an  $\alpha$ -(1 $\rightarrow$ 3) linkage<sup>21</sup>. The possibility of  $\beta$ -linkages in this tetrasaccharide can be ruled out, as isomaltodextranase would not act on such a compound, even if alternansucrase were capable of forming  $\beta$ -linked products. This means that the first tetrasaccharide acceptor-product from maltose contains a D-glucosyl group linked  $\alpha$ -(1 $\rightarrow$ 3) to panose, and must therefore have the structure *O*- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 3)-*O*- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 6)-*O*- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose.

The second, slower-migrating tetrasaccharide, had the same  $R_{Glc}$  value in t.l.c. as the tetrasaccharide (6<sup>2</sup>-*O*- $\alpha$ -isomaltosylmaltose<sup>20</sup>) produced by the B-512F acceptor-reaction with maltose (Fig. 1). Treatment with isomaltodextranase, converted this alternansucrase product into maltose and isomaltose (Fig. 2), indicating that alternansucrase also produces 6<sup>2</sup>-*O*- $\alpha$ -isomaltosylmaltose from maltose in the

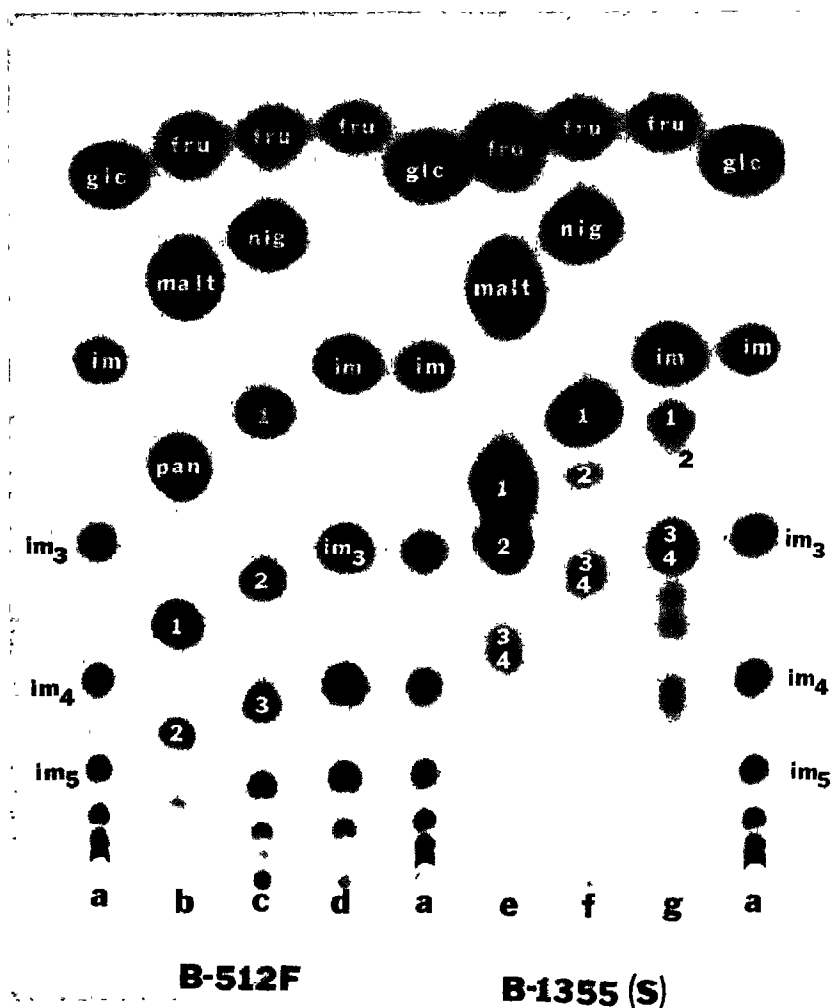


Fig. 1. Thin-layer chromatogram of various acceptor-reaction mixtures, 50% ethanol-soluble fractions. Whatman K5 0.25-mm silica gel, 2 ascents at 37° with solvent *D*, detection by sulfuric acid. (a) Isomaltooligosaccharide standards; (b-d) B-512F dextranucrase acceptor-reactions in the presence of sucrose; (b) maltose acceptor-reaction: 1 = 6<sup>2</sup>-*O*- $\alpha$ -isomaltosylmaltose, 2 = 6<sup>2</sup>-*O*- $\alpha$ -isomaltotriosylmaltose; (c) nigerose acceptor-reaction: 1 = 6<sup>2</sup>-*O*- $\alpha$ -D-glucosylnigerose, 2 = 6<sup>2</sup>-*O*- $\alpha$ -isomaltosylnigerose, 3 = 6<sup>2</sup>-*O*- $\alpha$ -isomaltotriosylnigerose; (d) isomaltose acceptor-reaction; (e-g) B-1355 alternansucrase acceptor-reactions in the presence of sucrose: (e) maltose acceptor-reaction: 1 = panose, 2 = 6<sup>2</sup>-*O*- $\alpha$ -nigerosylmaltose, 3 = 6<sup>2</sup>-*O*- $\alpha$ -isomaltosylmaltose, 4 = structure not determined; (f) nigerose acceptor-products: 1 = 6<sup>2</sup>-*O*- $\alpha$ -D-glucosylnigerose, 2 = 6<sup>2</sup>-*O*- $\alpha$ -nigerosylnigerose, 3 = 6<sup>2</sup>-*O*- $\alpha$ -isomaltosylnigerose, 4 = structure not determined; (g) isomaltose acceptor-products: 1 = 3<sup>2</sup>-*O*- $\alpha$ -D-glucosylisomaltose, 2 = structure not known, 3 = isomaltotriose, 4 = structure not known. (Abbreviations used: Glc = D-glucose, Fru = D-fructose, Im = isomaltose, Im<sub>n</sub> = isomaltose oligosaccharide of d.p. = *n*, Mal = maltose, Nig = nigerose, and Pan = panose.)



Fig. 2. Isomaltodextranase degradation-products of alternansucrase-maltose acceptor-products. Thin-layer chromatogram on Whatman K5 0.25-mm silica gel, 3 ascents in solvent *A* at 25°; detection by sulfuric acid. (a) Standards (top to bottom): fructose, glucose, sucrose, nigerose, isomaltose, and panose; (b) first maltose acceptor-product, d.p. 3; (c) isomaltodextranase hydrolyzate of b; (d) second maltose acceptor-product, d.p. 4; (e) isomaltodextranase hydrolyzate of d; (f) standards (top to bottom): fructose, glucose, sucrose, nigerose, and isomaltose; (g) standards: maltose (faster) and kojibiose (slower); (h) third maltose acceptor product, d.p. 4; (i) isomaltodextranase hydrolyzate of h; (j) fourth maltose acceptor-product(s); (k) isomaltodextranase hydrolyzate of j. (Abbreviations used: Suc = Sucrose, Koj = kojibiose; see also Fig. 1).

presence of sucrose. The fourth acceptor-product from maltose has not been structurally identified, but isomaltodextranase hydrolyzed it to D-glucose and two oligosaccharides having d.p. 3 or 4 (see Fig. 2), whose identities have not been established. This pattern of products does not support any of the possible structures consisting of linear, consecutive, D-glucose residues, and so it may be a "branched" oligosaccharide acceptor-product.

Nigerose was approximately as good an acceptor as maltose, and the pattern of products from the two appears similar (Fig. 1). Hydrolysis of the first nigerose acceptor-product with isomaltodextranase gave D-glucose and isomaltose (Fig. 3). Partial acid-hydrolysis gave isomaltose, nigerose, and D-glucose, indicating that the nigerose acceptor-product is 6<sup>2</sup>-O- $\alpha$ -D-glucosylnigerose, which is the same trisaccharide as that formed from nigerose by B-512F dextransucrase<sup>3</sup>.

The second alternansucrase product from nigerose is a tetrasaccharide having considerably greater mobility in t.l.c. (Fig. 1) than the tetrasaccharide produced by B-512F dextransucrase. This alternansucrase product was hydrolyzed rather slowly by isomaltodextranase to give D-glucose and a trisaccharide having the same

TABLE I

$^1\text{H}$ -DECOUPLED  $^{13}\text{C}$ -N.M.R. SPECTRUM OF THE FASTER-MIGRATING TETRASACCHARIDE ALTERNANSUCRASE-MALTOSE ACCEPTOR-PRODUCT

Chemical shift (p.p.m. relative to $\text{Me}_4\text{Si}$ )	Intensity	Carbon atom <sup>a</sup>
100.6	1029	1 <sup>2</sup>
100.1	1498	1 <sup>4</sup>
98.9	1244	1 <sup>3</sup>
96.6	838	1 $\beta$
92.7	384	1 $\alpha$
80.8	1077	3 <sup>3</sup>
78.2	607 <sup>b</sup>	
78.0	888 <sup>b</sup>	
77.0	863 <sup>b</sup>	
75.4	883 <sup>b</sup>	
74.8	1017 <sup>b</sup>	
74.0	1761 <sup>b</sup>	
73.7	1737 <sup>b</sup>	
72.5	3864 <sup>b</sup>	
72.1	1842 <sup>b</sup>	
70.8	2150 <sup>b</sup>	
70.2	2637 <sup>b</sup>	
69.9	327 <sup>b</sup>	
67.4 <sup>c</sup>	6969	
66.6	854	
61.5	906	} 6 <sup>2</sup> 6 <sup>1</sup> , 6 <sup>3</sup> , 6 <sup>4</sup>
61.1	1955	

<sup>a</sup>Assigned on the basis of resonances observed for panose and nigerose (ref. 22). <sup>b</sup>Not assigned.

<sup>c</sup>1,4-Dioxane internal standard.

$R_{\text{Glc}}$  value as the first nigerose acceptor product. This result indicates that the acceptor product must be 6<sup>2</sup>-*O*- $\alpha$ -nigerosylnigerose. The third nigerose acceptor-product from alternansucrase had the same  $R_{\text{Glc}}$  value as the second nigerose acceptor-product from B-512F dextranucrase (Fig. 1), and, on treatment with isomaltodextranase, gave nigerose and isomaltose (Fig. 3). Thus, the third acceptor-product produced from nigerose by alternansucrase is the tetrasaccharide, 6<sup>2</sup>-*O*- $\alpha$ -isomaltosylnigerose. Also produced in the alternansucrase reaction with sucrose and nigerose was a saccharide of higher d.p. and unknown structure. No nigerotriose was formed.

The acceptor products arising from the reaction of alternansucrase with isomaltose and sucrose were more difficult to analyze, partly because of difficulties in separation. For example, the chromatogram in Fig. 1 shows two compounds migrating between isomaltose and isomaltotriose (g, spots 1 and 2), but these could not be resolved by paper chromatography, the method used to prepare the other pure acceptor-products. The only isomaltose acceptor-product thus far isolated pure has been a trisaccharide having the same  $R_{\text{Glc}}$  value as isomaltotriose (Fig. 1). A sample



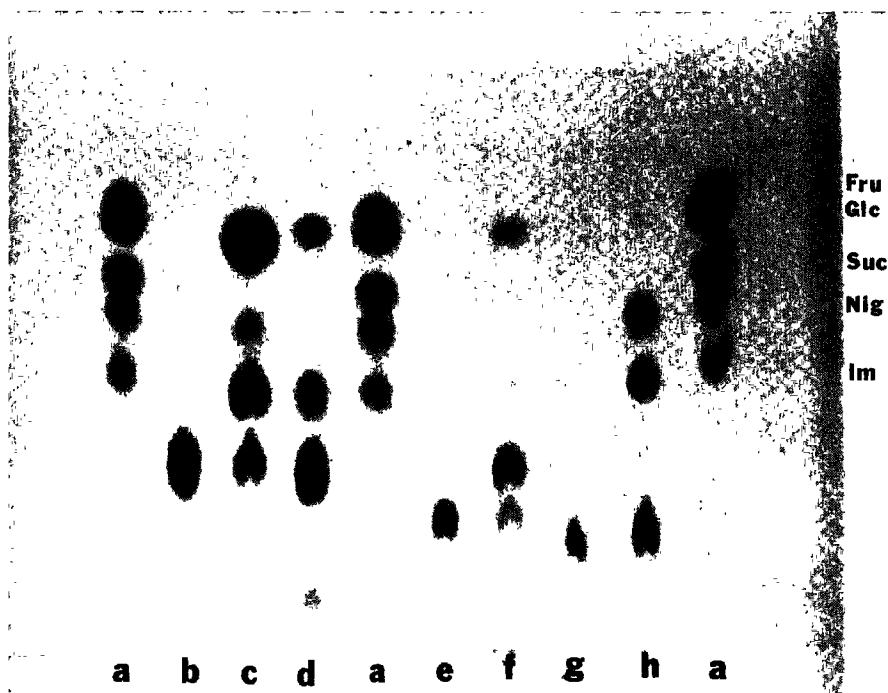


Fig. 3. Hydrolysis products of alternansucrase-nigerose acceptor-products. Thin-layer chromatogram on Merck 0.25-mm 50% silica gel 60 + 50% kieselguhr plate, 3 ascents in solvent *A* at 25°, detection by sulfuric acid. (a) Standards (top to bottom): fructose, glucose, sucrose, nigerose, and isomaltose; (b) first nigerose acceptor-product, d.p. 3; (c) partial acid hydrolyzate of *b*; (d) isomaltodextranase hydrolyzate of *b*; (e) second nigerose acceptor product, d.p. 4; (f) isomaltodextranase hydrolyzate of *e*; (g) third nigerose acceptor-product, d.p. 4; (h) isomaltodextranase hydrolyzate of *g*. (Abbreviations as in Figs. 1 and 2.)

of this product, labeled with  $^{14}\text{C}$  by using  $[\text{U-}^{14}\text{C}]$ sucrose as the D-glucosyl donor, was subjected to hydrolysis by isomaltodextranase. Fig. 4 shows that both isomaltose and D-glucose were produced by this enzymic hydrolysis, but that only the isomaltose was  $^{14}\text{C}$ -labeled. It was concluded that this acceptor product is isomaltotriose, which has arisen from transfer of a D-glucosyl group from sucrose to O-6 of the nonreducing end of isomaltose. Of the other major acceptor-products from isomaltose, the one having the highest mobility in t.l.c. has the same  $R_{\text{Glc}}$  value as an authentic sample of 3<sup>2</sup>-O- $\alpha$ -D-glucosylisomaltose, a trisaccharide also produced by the action of isomaltodextranase on alternan<sup>16</sup>. T.l.c. with solvent *D* shows this trisaccharide to have a slightly different  $R_{\text{Glc}}$  value from 6<sup>2</sup>-O- $\alpha$ -D-glucosylnigerose, the first nigerose acceptor acceptor-product. Thus, it would be reasonable to assume that this product is 3<sup>2</sup>-O- $\alpha$ -D-glucosylisomaltose, and arises from the transfer of a D-glucosyl group from sucrose to O-3 of the nonreducing end of isomaltose. The identity of the minor compound migrating just behind this product (Fig. 1) is not known, nor are the identities of any of the compounds in Fig. 1 which arise from isomaltose and have  $R_{\text{Glc}}$  values less than that of isomaltotriose.

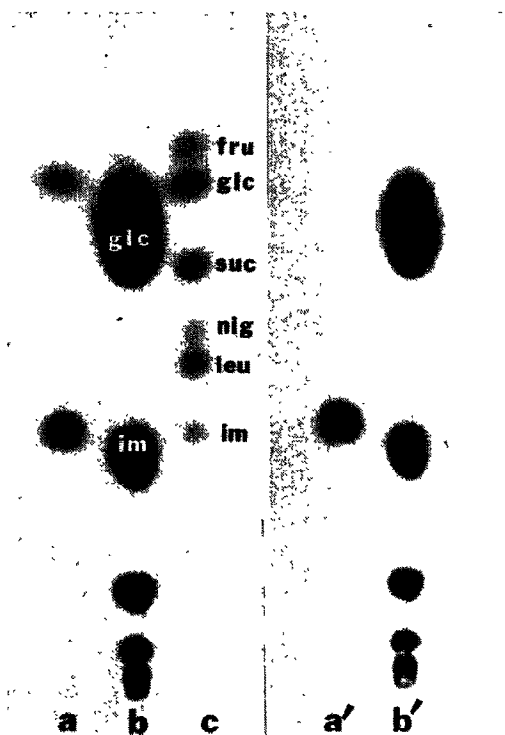


Fig. 4. Isomaltotriose produced by alternansucrose acceptor-reaction with isomaltose and [ $^{14}\text{C}$ ]-sucrose. Left: thin-layer chromatogram, Whatman K5 0.25-mm silica gel, 3 ascents in solvent *A* at  $25^\circ$ , detection by sulfuric acid. (*a, a'*) isomaltodextranase hydrolyzate of [ $^{14}\text{C}$ ]isomaltotriose; (*b, b'*) [ $^{14}\text{C}$ ]-labeled isomaltooligosaccharide standards; (*c*) unlabeled standards (top to bottom): fructose, glucose, sucrose, nigerose, leucrose, isomaltose; Right: Autoradiogram of t.l.c. plate at left. (Abbreviations used: Leu = leucrose, all others as in Figs. 1 and 2.)

D-Allose, D-mannose, D-galactose, cellobiose, melibiose, raffinose, 3-deoxy-3-fluoro-D-glucose, D-xylose, and L-sorbose were also found to be acceptors, but the structures of the products were not determined. The last two were only weak acceptors. D-Arabinose was not an acceptor.

The relative activities of five acceptors were determined by adding acceptor to alternansucrase and [ $^{14}\text{C}$ ]sucrose, and measuring the amounts of [ $^{14}\text{C}$ ]alternan and [ $^{14}\text{C}$ ]oligosaccharide acceptor-products formed. Table III gives the percentages of D-[ $^{14}\text{C}$ ]glucose incorporated after [ $^{14}\text{C}$ ]sucrose had all reacted. The percent of D-[ $^{14}\text{C}$ ]glucose from [ $\text{U-}^{14}\text{C}$ ]sucrose incorporated into each product was calculated as follows:

For products containing glucose only,  
percent of D-glucose incorporated =

$$= \frac{\text{c.p.m. of product} \times 100}{\text{Total c.p.m.} - \text{c.p.m. in fructose} - \frac{1}{2}\text{c.p.m. in leucrose}}$$

For leucrose,

percent of D-glucose incorporated =

$$= \frac{\frac{1}{2}\text{c.p.m. of leucrose} \times 100}{\text{total c.p.m.} - \text{c.p.m. in fructose} - \frac{1}{2}\text{c.p.m. in leucrose}}$$

The relative activities of these acceptors, in increasing order of percent of the D-[ $^{14}\text{C}$ ]glucose incorporated into oligosaccharide acceptor-products, are methyl  $\beta$ -D-glucoside (19%) < D-glucose (29%) < isomaltose (41%) < methyl  $\alpha$ -D-glucoside (49%) < nigerose (80%)  $\simeq$  maltose (84%).

## DISCUSSION

Alternansucrase is elaborated, together with dextransucrase, by *Leuconostoc mesenteroides* NRRL B-1355. We have recently separated alternansucrase from dextransucrase, and showed that it synthesizes a glucan (alternan<sup>1</sup>) having alternating  $\alpha$ -(1 $\rightarrow$ 6) and  $\alpha$ -(1 $\rightarrow$ 3) linkages. Alternan differs significantly from other glucans that contain similarly high amounts of 1,3-disubstituted  $\alpha$ -D-glucopyranose residues in that very few, if any, of these residues are adjacent or consecutively linked<sup>22</sup>. Furthermore, the majority of (1 $\rightarrow$ 3) linkages of alternan do not comprise branch points as they do in many other glucans synthesized by glucansucrases from sucrose.

Acceptor reactions occur with glucansucrases when monosaccharides, disaccharides, or oligosaccharides are added to the sucrose digests. The products are oligosaccharides containing one, two, three or more D-glucopyranosyl groups more than the acceptor. The structures of the products resulting from the reaction of acceptors with sucrose and B-512F dextransucrase have been previously determined<sup>2,3,19</sup>. Robyt and Walseth<sup>20</sup> studied the mechanism of formation of the acceptor products and showed that a specific hydroxyl group on the acceptor acted as a nucleophile and attacked C-1 of an enzyme-glucosyl complex to give an  $\alpha$ -D-glucosidic bond between glucose and the acceptor. Robyt and Taniguchi<sup>25</sup> showed that, when the acceptor is dextran, a 3-OH group acts as a nucleophile and an  $\alpha$ -(1 $\rightarrow$ 3) branch-linkage is formed.

Sidebotham<sup>23</sup> has stated that the specificity of acceptor reactions may account for the type and distribution of the linkages in the different glucans. This is true for the type and distribution of branch linkages, but not for the type and distribution of the linkages found in the main chains. Robyt *et al.*<sup>24</sup> have shown that the mechanism of elongation of the main chain of B-512F dextran is from the reducing end and that, even though the same enzyme is involved, the formation of linkages by the acceptor reaction (namely, branching) is not the same as elongation<sup>20,25</sup>.

In the present study, we found, as with other glucansucrases<sup>18,26</sup>, that D-fructopyranose serves an acceptor with alternansucrase and gives the disaccharide leucrose. D-Fructopyranose acceptor-products of higher d.p. were not detected, indicating that leucrose itself is a poor acceptor.

TABLE II

ALTERNANSUCRASE ACCEPTORS AND ACCEPTOR PRODUCTS

Acceptor	Acceptor products		
	d.p. 2	d.p. 3	d.p. 4
D-Fructose	leucrose	—	—
D-Glucose	isomaltose	(a) $\alpha$ -D-Glc-(1 $\rightarrow$ 3)- $\alpha$ -D-Glc-(1 $\rightarrow$ 6)-D-Glc (b) isomaltotriose (c) minor comp. <sup>a</sup>	<sup>a</sup>
Methyl $\alpha$ -D-glucoside	methyl $\alpha$ -isomaltoside	<sup>a</sup>	<sup>a</sup>
Methyl $\beta$ -D-glucoside	methyl $\beta$ -isomaltoside	<sup>a</sup>	<sup>a</sup>
Maltose	—	panose	(a) $\alpha$ -D-Glc-(1 $\rightarrow$ 3)- $\alpha$ -D-Glc-(1 $\rightarrow$ 6)- $\alpha$ -D-Glc-(1 $\rightarrow$ 4)-D-Glc (b) $\alpha$ -D-Glc-(1 $\rightarrow$ 6)- $\alpha$ -D-Glc-(1 $\rightarrow$ 6)- $\alpha$ -D-Glc-(1 $\rightarrow$ 4)-D-Glc
Nigerose	—	$\alpha$ -D-Glc-(1 $\rightarrow$ 6)- $\alpha$ -D-Glc-(1 $\rightarrow$ 3)-D-Glc	(a) $\alpha$ -D-Glc-(1 $\rightarrow$ 3)- $\alpha$ -D-Glc-(1 $\rightarrow$ 6)- $\alpha$ -D-Glc-(1 $\rightarrow$ 3)-D-Glc (b) $\alpha$ -D-Glc-(1 $\rightarrow$ 6)- $\alpha$ -D-Glc-(1 $\rightarrow$ 6)- $\alpha$ -D-Glc-(1 $\rightarrow$ 3)-D-Glc
Isomaltose	—	(a) $\alpha$ -D-Glc-(1 $\rightarrow$ 3)- $\alpha$ -D-Glc-(1 $\rightarrow$ 6)-D-Glc (b) isomaltotriose (c) minor comp. <sup>a</sup>	<sup>a</sup>

<sup>a</sup>Structure(s) not determined.

The fact that methyl  $\beta$ -D-glucoside acts as an acceptor for both alternansucrase and dextransucrase is interesting. This, along with the observation by Yamauchi and Ohwada<sup>3</sup> that  $\beta$ , $\beta$ -trehalose acts as an acceptor for dextransucrase, refutes the statement by Sidebotham<sup>23</sup> that "dextransucrases do not transfer  $\alpha$ -D-glucopyranosyl groups to  $\beta$ -D-glucopyranosyl residues". An  $\alpha$ -D-glucopyranosyl-type configuration is not required for a sugar residue to act as an acceptor.

The present work shows that the acceptor reactions of alternansucrase thus far studied proceed by transfer of an  $\alpha$ -D-glucopyranosyl group onto the nonreducing end-group of the di- and tri-saccharide acceptors. Whether or not the transfer to the nonreducing end occurs for higher oligosaccharide acceptors remains to be seen. Walker<sup>27</sup> reported that the glucansucrase of *Streptococcus mutans* transfers D-glucosyl groups onto O-3 of the D-glucose residue penultimate to the reducing end of isomaltooligosaccharide acceptors of d.p. 7 and higher.

A significant result of the present study is the observation that  $\alpha$ -(1 $\rightarrow$ 3) linkages are formed in oligosaccharide acceptor-products when the acceptor has the nonreducing D-glucose group linked by an  $\alpha$ -(1 $\rightarrow$ 6) linkage to another D-glucose residue. If the  $\alpha$ -(1 $\rightarrow$ 6) linkage is not present at the nonreducing end of the acceptor, the  $\alpha$ -(1 $\rightarrow$ 3) linkage is not formed. Thus, D-glucose gives isomaltose, and not to nigerose, maltose gives only panose but not 3<sup>2</sup>-O- $\alpha$ -D-glucosylmaltose, and so forth; isomaltose, however, gives both isomaltotriose and 3<sup>2</sup>-O- $\alpha$ -D-glucosylisomaltose. The patterns may be clearly seen in Table II. In addition, once an  $\alpha$ -(1 $\rightarrow$ 6) linkage is present, two acceptor products are formed in approximately equal amounts, resulting from the transfer of D-glucose to either O-3 or O-6 of the nonreducing D-glucosyl group of the acceptor. This result suggests that acceptors having the nonreducing unit linked  $\alpha$ -(1 $\rightarrow$ 6) are capable of binding in two equally favored ways.

It has been recognized that different acceptors give rise to different amounts of products<sup>2,3,12</sup>. A "good" acceptor is one that will give rise to a greater amount of oligosaccharide acceptor-product(s) and less polysaccharide. For B-512F dextransucrase, Koepsell *et al.*<sup>2</sup> and Yamauchi and Ohwada<sup>3</sup> found isomaltose to be the best acceptor. Maltose was found to be the second best<sup>2,3</sup>, followed by methyl  $\alpha$ -D-glucoside. Leucrose was a very weak acceptor<sup>2</sup>, and nigerose was moderate to weak<sup>3</sup>. Mayer *et al.*<sup>12</sup> reported that, for a glucansucrase from *Streptococcus sanguis* ATCC 10558, maltose was a much better acceptor than isomaltose. Curiously, they also reported D-fructose to be a good acceptor, but identified the product as sucrose, rather than leucrose or isomaltulose. They did not identify the structures of most of their other acceptor-products except to show that the glucosyl group of sucrose was transferred onto the nonreducing end of the acceptor.

Our results with alternansucrase differ significantly from those reported for B-512F dextransucrase in two ways. One difference is in the structures of the products formed. The second is in the relative strengths of the acceptors with respect to their abilities to divert D-glucosyl groups from glucan synthesis into synthesis of oligosaccharides. Like the results of Mayer *et al.*<sup>12</sup> with *S. sanguis* glucansucrase, and in contrast to the results of Koepsell *et al.*<sup>2</sup> and Yamauchi and Ohwada<sup>3</sup> with *L. mesen-*

TABLE III

DISTRIBUTION AND AMOUNTS OF ACCEPTOR PRODUCTS

Acceptor	D-[ <sup>14</sup> C]Glucose incorporated (%) into products from [(U) <sup>14</sup> C]sucrose		
	Alternan <sup>a</sup>	Leucrose <sup>b</sup>	Oligosaccharide acceptor-products <sup>a</sup>
None	90	10	0
Methyl β-D-glucoside	75	6	19
Methyl α-D-glucoside	45	6	49
D-Glucose	66	5	29 (22% isomaltose) 7% d.p. ≥ 3
Isomaltose	52	7	41 (13% 3 <sup>2</sup> -O-α-D-glucosylisomaltose) 12% isomaltotriose 14% d.p. 4 2% d.p. ≥ 4
Nigerose	15	5	80 (69% d.p. 3) 11% d.p. ≥ 4
Maltose	11	5	84 (68% panose) 16% d.p. ≥ 4

$$^a \text{For alternan and oligosaccharides, percent} = \frac{\text{CPM}_{\text{Product}} (\times 100)}{\text{CPM}_{\text{total}} - \text{CPM}_{\text{Fru}} - \frac{1}{2}\text{CPM}_{\text{Leu}}}$$

$$^b \text{For leucrose, percent} = \frac{\frac{1}{2}\text{CPM}_{\text{Leu}} (\times 100)}{\text{CPM}_{\text{total}} - \text{CPM}_{\text{Fru}} - \frac{1}{2}\text{CPM}_{\text{Leu}}}$$

*teroides* B-512F dextranucrase, we have found that maltose serves as a better acceptor for alternansucrase than isomaltose. Nigerose is also a much better acceptor than isomaltose (Table III). These observations, together with the differences in the structures of the various acceptor-products between dextranucrase and alternansucrase, clearly indicate that acceptors do not react with all glucanases in the same manner.

D-Glucose is intermediate in acceptor strength between methyl α-D-glucoside and methyl β-D-glucoside. Considering that D-glucose in solution exists ~37% in the α-pyranose form and 62% in the β-pyranose form, and taking the percentage of acceptor products obtained for methyl α-D-glucoside (49%) and methyl β-D-glucoside (19%) from Table III, the weighted average, (49%) (0.37) + (19%) (0.62) = 30%, is very close to the experimentally determined value of 29% for D-glucose. These results suggest that the acceptor reactivity of D-glucose may be a weighted average of the reactivities of the two anomeric forms present, and that the anomeric methyl groups of the D-glucosides have little effect on the reactivity of these acceptors.

Another phenomenon which has been observed by others<sup>12,28</sup>, and which we have also observed for alternansucrase, is that at higher acceptor-to-sucrose ratios, most of the acceptor product results from addition of a single D-glucosyl group to the

acceptor, whereas at lower ratios of acceptor to sucrose, relatively greater amounts of higher-d.p. acceptor-products are formed. This observation may be of use for determining the conditions to be used when acceptor reactions are used to prepare unusual saccharides. One unusual saccharide that may be prepared by these reactions is *O*- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 3)-*O*- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 6)-*O*- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose (6<sup>2</sup>-*O*- $\alpha$ -nigerosylmaltose), the faster-migrating alternansucrase acceptor-product arising from panose (Table II). This interesting tetrasaccharide, containing a sequence of three different  $\alpha$ -linkages (namely, 1 $\rightarrow$ 3, 1 $\rightarrow$ 6, and 1 $\rightarrow$ 4), has not previously been reported.

In summary, we have described some of the acceptor reactions of alternansucrase from *Leuconostoc mesenteroides* B-1355, and have shown that both  $\alpha$ -(1 $\rightarrow$ 6) and  $\alpha$ -(1 $\rightarrow$ 3) linkages are formed, although an  $\alpha$ -(1 $\rightarrow$ 6) bond must be present for an  $\alpha$ -(1 $\rightarrow$ 3) bond to be formed. We have also compared some of the acceptor reactions of alternansucrase with those of other bacterial glucansucrases.

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