Journal of Industrial Microbiology & Biotechnology (1998) 21, 37–45

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A mutant strain of *Leuconostoc mesenteroides* B-1355 producing a glucosyltransferase synthesizing α(1→2) glucosidic linkages

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A mutant strain (R1510) of *Leuconostoc mesenteroides* B-1355 was isolated which synthesized primarily an insoluble polysaccharide and little soluble polysaccharide when grown in sucrose-containing medium. Glucose or sucrose cultures of this strain produced a single intense band of GTF-1 activity of 240 kDa on SDS gels, and a number of faint, smaller bands. Oligosaccharides synthesized by strain R1510 from methyl-α-D-glucoside and sucrose included a trisaccharide whose structure contained an α(1→2) glucosidic linkage. This type of linkage has not been seen before in any products from strain B-1355 or its mutant derivatives. The structure of the purified trisaccharide was confirmed by ^{13}C-nuclear magnetic resonance. The insoluble polysaccharide also contained α(1→2) branch linkages, as determined by methylation analysis, showing that synthesis of the linkages was not peculiar to methyl-α-D-glucoside. GTF-1, which had been excised with a razor blade from an SDS gel of a culture of the parent strain B-1355, produced the same trisaccharides as strain R1510, showing that GTF-1 from the wild-type strain was the same as GTF-1 from strain R1510. Mutant strains resembling strain R1510, but producing a single intense band of alternansucrase (200 kDa) instead of GTF-1 were also isolated, suggesting that mutations may be generated which diminished the activities for any two of the three GTFs of strain B1355 relative to the third. Strain R1554 produced a soluble form of alternansucrase, while strain R1588 produced a cell-associated form. The mechanism(s) by which specific GTFs become associated with the cells of *L. mesenteroides* was not explored.

Keywords: glucosyltransferase; dextransucrase; alternansucrase; *Leuconostoc mesenteroides*; mutant; glucan; dextran; polysaccharide

Introduction

Strains of *Leuconostoc mesenteroides* produce extracellular glucosyltransferases (GTFs) which synthesize dextrans and related polymers by splitting sucrose and transferring the glucosyl group to nascent polymer chains [23]. Fructose is a byproduct of polymer synthesis. The GTFs are large (140–240 kDa) monomeric proteins [4,18,19], which are normally synthesized at high levels only when sucrose is present [23], but mutant strains producing the enzymes at high levels in media with other sugars have been reported [6–9,29]. GTFs of different types synthesize dextrans with different structures [23], and the number and types of GTFs and dextrans produced are dependent on the strain of *L. mesenteroides*. Some GTFs, such as dextransucrase (sucrose-1,6-α-glucan glucosyltransferase, E.C. 2.4.1.5) from strain B-512F, produce linear dextrans containing mostly α(1→6) linkages, while other types synthesize branched dextrans, containing α(1→2), α(1→3) or α(1→4) branch linkages in addition to the linear α(1→6) linkages. Other GTFs, such as alternansucrase (sucrose-1,6(3)-α-glucan-6(3)-α glucosyltransferase, E.C. 2.4.1.140) from strain B-1355 or GTFs from strain B-523, synthesize dextran-like polymers containing significant percentages of α(1→3) linkages in their primary chains [16,23]. Differences in the structures of the dextran products are accompanied by marked differences in their properties, such as solubility in water, viscosity of solutions, and resistance to hydrolysis by glucosidases. Some strains produce multiple types of GTFs and dextrans.

Recently, research on dextran production by *L. mesenteroides* has focused on strain development, using mutagenic or other techniques to obtain strains which might be beneficial to industry [6–9,12,13,28,29]. To determine the effects that mutations have on the GTFs produced, it is necessary to enumerate and quantify the GTFs produced by the parent and mutant strains. Enumeration of the GTFs has relied partly on the analysis of electrophoretic bands produced on SDS gels by cultures of the mutant strains after staining the gels for GTF activity [15]. Interpretation of the SDS gels has been a problem, however, because different laboratories sometimes reported different results using the same strains [6,12,27], because a number of faint, unexplained activity bands were sometimes seen on the gels, and because there is no general method for identifying the GTFs associated with each of the bands seen on the gels.

Strain B-1355 is one of the most intensively-studied strains of the multiple dextran-producing varieties of *L. mesenteroides*. It produced two GTFs and two easily distinguishable, water-soluble glucans (alternan and dextran) [14,16,23]. Measurement of the ratio of alternan to dextran

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Received 12 May 1998; accepted 16 July 1998

produced in cultures has relied heavily on methods which employ a glucosidase (dextranase, 1,6-α-d-glucan 6-glucanhydrolase, E.C. 3.2.1.11) which hydrolyzed the dextran while leaving the alternan intact [13]. We previously reported that, contrary to expectation, strain B-1355 produced a third type of GTF [28], which synthesized an insoluble dextran whose sensitivity to hydrolysis by dextranase has not been reported. However, work in other laboratories has called into question some of our findings [6–9,13].

In this article, we confirm our previous interpretations by describing a mutant strain derived from *L. mesenteroides* B-1355 (strain R1510), which synthesizes dextrans and oligosaccharides containing $\alpha(1\rightarrow2)$ glucosidic linkages, a type of linkage not previously seen in strain B-1355 or any of its mutant derivatives. We will show that strain R1510 is more easily explained as a result of mutation(s) which caused a drop in the production of alternansucrase and dextransucrase relative to GTF-1, than as a result of mutation(s) in a GTF structural gene which caused alternansucrase or dextransucrase to synthesize products with new types of linkages.

Materials and methods

Bacterial strains

L. mesenteroides NRRL B-1355, B-1299, and B-512F were obtained from the National Center for Agricultural Utilization Research (NCAUR, USDA-ARS, Peoria, IL, USA) culture collection. Strain B-512F produces commercial dextran which contains 95% $\alpha(1\rightarrow6)$ linkages and 5% $\alpha(1\rightarrow3)$ branch linkages, and strain B-1299 produces highly branched dextrans containing $\alpha(1\rightarrow2)$ linkages. Strain SL-1 (NRRL B-23183) was isolated and described previously [28]; it was used as a control which produced B-1355 dextransucrase, but not alternansucrase.

Strains R1588, R1554 and R1510 are described here for the first time and were obtained by mutagenizing strain B-1355 with UV, followed by mutagenizing suspensions of cells with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. Colonies which developed on 10% (wt/vol) sucrose agar plates, after appropriate dilution, and which exhibited changes from wild-type morphology were electrophoretically screened to detect changes from wild-type in the pattern of activity bands produced on SDS gels. Strain R1510 produced extremely rough, dry colonies on sucrose agar, which were difficult to cut through with an inoculating loop. Strain R1554 and R1588 produced smooth colonies. All strains were repeatedly transferred from single colonies to insure purity and phenotypic stability. Cultures were grown in GTF medium [33] at 30°C for the production of enzymes.

Chemicals

3,5-Dinitrosalicylic acid, crude (10–25 units mg^{-1}) and partially purified (400–800 units mg^{-1}) dextranase (from *Penicillium* sp, EC 3.2.1.11), amyloglucosidase (50 units mg^{-1} protein) (exo-1,4-α-glucosidase, EC 3.2.1.3, from *Aspergillus niger*), dextransucrase (EC 2.4.1.5), isomaltose, isomaltotriose, nigerose, panose and bovine serum albumin (fraction V) were purchased from Sigma Chemical Company, St Louis, MO, USA. Protein reagent for the Bradford [1] dye-binding assay was purchased from Bio-Rad, Richmond, CA, USA. Methyl-α-d-glucoside was purchased from Aldrich Chemical Company, Milwaukee, WI, USA. Kojibiose was purchased from WAKO Pure Chemical Industries, Richmond, VA, USA.

Assays

Assays for GTF activity depended on the release of reducing sugar, as measured by the 3,5-dinitrosalicylic acid (DNS) assay [30], as previously described [28,29]. One unit of GTF activity was defined as the amount of enzyme activity which released 1 μmole of reducing sugar (fructose) per min from sucrose at 30°C.

Proteins were assayed by the method of Bradford [1], using Bio-Rad dye-binding protein reagent, and bovine serum albumin (Sigma, Fraction V) as the standard. Proteins in whole cultures were assayed after adding NaOH to the cultures to 1 N concentration, then boiling the cultures for 5 min. The supernatant fractions obtained by centrifuging the suspensions were assayed by the Bradford method, using 1 N NaOH as diluent for the standards.

Enzyme preparations

Supernatant fractions containing GTFs were prepared by centrifuging ($13\,000 \times g$ for 30 min) cultures (100 ml) grown overnight (30°C) in GTF medium containing 2% (wt/vol) sucrose or 2% (wt/vol) glucose.

Polyacrylamide gel electrophoresis

SDS-PAGE was performed on samples using the Bio-Rad mini-Protean II slab gel system, gels of 0.75 mm thickness and the Laemmli [11] buffer system as described previously [29]. Samples containing suspended cells (cultures) were centrifuged at $15\,000 \times g$ in a microcentrifuge before applying them to the gels. Approximately 1.0–1.5 milliunits of activity (approximately 90 ng of protein), as measured by DNS assay, were loaded onto the gels and electrophoresis was carried out at 200 volts for 1 h. The gels were then stained for GTF activity by incubating them overnight with a solution containing 50 mM sodium acetate, pH 5.6, 2 mM $CaCl_2$, and 5% (wt/vol) sucrose, then washing and staining them for carbohydrate using the periodic acid-Schiff method, as previously described [15,33].

Oligosaccharides from methyl-α-D-glucoside

We synthesized oligosaccharides from methyl-α-d-glucoside and sucrose by incubating 300 μl of supernatant fractions or whole cultures in a solution (3.0 ml final volume) containing (final concentrations): 50 mM sodium acetate, 2 mM $CaCl_2$, 200 mM sucrose, 100 mM α-methyl-d-glucoside and 0.02% (wt/vol) sodium azide (to inhibit the growth of cells or molds). The reaction mixtures were incubated at 30°C for periods up to 18 days and sampled daily. Samples (100 μl) were injected into a high pressure liquid chromatograph (HPLC) and the oligosaccharides which were synthesized were separated using an ISCO HPLC system, a 20-cm Dextropak C_{18} column (Waters, Milford, MA, USA), and a mobile phase consisting of water at a flow rate of 1.0 ml min^{-1} [14,21,22]. The column was at ambient temperature. Peaks were detected using a Waters model 410 differential refractometer set to a constant temperature of 40°C. The scaling factor of the detector was set to measure

concentrations of trisaccharides ranging to approximately 50 mM. The limit of detection was approximately 50–100 μM. Concentrations were estimated using calibration curves with methyl-α-d-glucoside as a standard and peak areas were estimated by triangulation.

Peaks from methyl-α-d-glucoside, sucrose, and fructose were identified by comparing their retention times to those of known standards and by mixing standards with the samples before injecting them into the HPLC to determine if peaks co-eluted. Peaks corresponding to components of the culture medium, metabolic products and buffers were identified by injecting samples containing boiled cultures or supernatant fractions added to the solution used for generating the oligosaccharides. The oligosaccharides were identified by their retention times relative to the standards and by their susceptibility to hydrolysis with 10 units ml^{-1} (final concentration) of dextranase or amyloglucosidase (boiled samples were incubated at 30°C for 24–48 h). Dextranase hydrolyzed peaks from oligosaccharides containing α(1→6) linkages only, while amyloglucosidase hydrolyzes oligosaccharides containing α(1→4), or α(1→3), or α(1→6) linkages (in order of preference), but not α(1→2) linkages [22].

^{13}C nuclear magnetic resonance spectroscopy

Oligosaccharides were purified by preparative high pressure liquid chromatography (HPLC) to at least 97% purity (as estimated by peak areas), and concentrated by evaporation using a Buchi rotary evaporator. Nuclear magnetic resonance (NMR) spectra were obtained at 298°K from samples in deuterium oxide with sodium 3-(trimethylsilyl) 3,3,2,2-tetradeutero propionate (TSP) as an internal standard, using a Bruker Model ARX400 NMR Spectrometer (Bruker Instruments, Billerica, MA, USA) at a frequency of 100.62 MHz for carbon and 400.13 MHz for protons. One-pulse experiments were run for both nuclei. A 30-degree pulse and a 2.3-second repetition rate were used for carbon, and a 90-degree pulse and a 7-to-8-second repetition rate were used for protons. NMR spectra were also obtained from authentic samples of nigerose, kojibiose, maltose, panose, isomaltose, isomaltotriose and methyl-α-d-glucoside and compared with values reported by Usui *et al* [31] to confirm the resonance assignments.

Characterization of the R1510 polysaccharide by gas chromatography/mass spectrometry (GC/MS)

To prepare a sample of the R1510 polysaccharide for GC/MS analysis, a 10-ml suspension of the insoluble polysaccharide, obtained by low-speed centrifugation of a 24-h culture of strain R1510, was added to 35 ml of 20% (wt/vol) sucrose solution and incubated at ambient temperature for 48 h. The insoluble fraction was recovered by centrifugation and washed with water several times to extract soluble material. A sample of the washed, water-insoluble polysaccharide was permethylated [27] with sodium methylsulfinylmethanide and methyl iodide in dimethyl sulfoxide. The methylated polysaccharide was then hydrolyzed at 120°C for 1 h with 2 N trifluoroacetic acid and the sugars converted to peracetylated aldononitrile (PAAN) derivatives [27]. The derivatives were analyzed by GC/MS on a methylsilicone column (25 m × 0.022 i.d. × 0.1 μm thickness; Hewlett-Packard, Wilmington, DE, USA). The column temperature was held for 3 min at 130°C, increased at 5°C min^{-1} to 165°C, then held at 165°C for 10 min; helium was used as the carrier gas.

Results

SDS-PAGE of GTFs from strain B-1355 and mutants

Cultures of strain B-1355 generated three principal bands of GTF activity on SDS gels [28], corresponding to GTF-1 (approximately 240 kDa), alternansucrase (approximately 200 kDa) and dextransucrase (approximately 170 kDa). Figure 1 shows that mutant strains (R1510, R1554 and R1588) could be isolated whose cultures generated primarily single bands on SDS gels. The banding patterns for the parent strain B-1355 and SL-1 cultures were described elsewhere [28], and were included in Figure 1 for reference. Cultures of strain R1510 produced an activity band at 240 kDa, while strains R1554 and R1588 each generated a single intense band of alternansucrase (200 kDa) activity. The heavy band at 170 kDa, seen with cultures of SL-1, was produced by fraction L dextransucrase, which synthesizes a dextran having a structure similar to that of commercial dextran [23].

As previously mentioned [28,29], we saw minor bands of activity on the SDS gels in addition to the principal band. Almost all of the minor bands from strain R1510 were larger than 170 kDa, half were larger than 200 kDa, and the minor bands disappeared when gels were treated with dextranase before staining, indicating that they did not contain alternansucrase. They collectively represented a significant fraction (up to 70%) of the total GTF activity, as measured by densitometry, but their individual intensities were low. For reasons given below, we believe that most of these minor bands were derived from GTF-1, and that they might arise during translocation of the GTF across the cell membrane.

GTF activities in sucrose or glucose medium

We confirmed that strains produced high or low ratios of alternansucrase to dextransucrase by measuring the retention of GTF activity in supernatant fractions from cultures which had been heated to 45°C for 40 min to inactivate

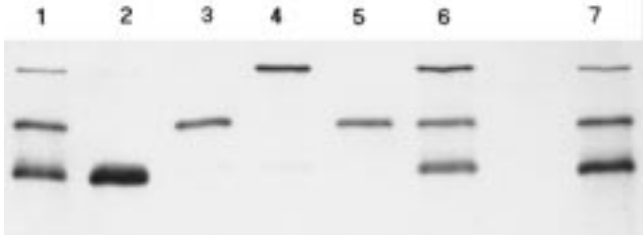

Figure 1 GTFs of strains B-1355, SL-1, R1588, R1510, and R1554 visualized by SDS-PAGE. Cultures (100 ml) in GTF medium containing 2% (wt/vol) sucrose were incubated 24 h at 30°C. Samples (3 μl) were then subjected to SDS-PAGE followed by staining for GTF activity. Protein concentrations (<30 μg ml^{-1}) were not sufficiently high to produce bands. Lanes 1, 6 and 7, strain B-1355; Lane 2, strain SL-1; Lane 3, strain R1588; Lane 4, strain R1510; Lane 5, strain R1554.

dextransucrase (Table 1). Alternansucrase was resistant to thermal inactivation [14]. Strains R1588 and R1554 produced mostly alternansucrase, as indicated by the nearly equal specific activities of heated *vs* unheated supernatant fractions. Supernatant fractions from cultures of strain R1510, like those from the SL-1 and B-1355 controls [29], showed marked (50% or more) loss of activity on heating, indicating a low ratio of alternansucrase to dextransucrase [14].

Strain R1554 apparently contained a mutation causing changes in the cellular association of alternansucrase, because the alternansucrase from strain R1554 was mostly in a soluble form (90% of the specific activity of the cultures resided in the supernatant fraction), while strain R1588 produced mostly a cell-associated form of alternansucrase, with only 10% of the specific activity of the cultures in the supernatant fraction. These results were confirmed by SDS-PAGE. The parent strain B-1355 also produces a cell-associated form of alternansucrase [33]. GTF-1 from strain R1510 was distributed more equally between the cells and supernatant fraction than alternansucrase, with 40% of the specific activity of cultures residing in the supernatant fraction.

Strain R1510 also produced GTFs in GTF medium containing 2% (wt/vol) glucose instead of sucrose. Specific activities in glucose medium were usually about 3.5 units mg^{-1} protein, and the pattern of activity bands produced on SDS gels after electrophoresis was identical to patterns produced in sucrose medium.

Oligosaccharides synthesized from methyl-α-D-glucoside

GTFs can transfer glucosyl groups from sucrose to other carbohydrates through so-called acceptor reactions, which compete with dextran synthesis [23,24]. A range of substrates can serve as glucosyl acceptors in acceptor reactions [24], resulting in the synthesis of a mixture of oligosaccharides whose length varies from two to eight or more glucose units. By a judicious choice of acceptor substrate, the types of linkages synthesized by single GTFs in mixtures of GTFs can be visualized and quantified from the oligosaccharides that are synthesized [14]. The ratios of different GTFs present in the mixtures can then be deduced. We had previously reported that GTF-1, alternansucrase and dextransucrase each synthesized maltosides from sucrose and maltose that were not produced by the other two GTFs [28], but we did not identify the structure of the unique maltoside produced by GTF-1. The maltosides consisted of mixed anomers which resolved on HPLC columns to give extra peaks which complicated interpretation of the results. To circumvent the problem, we repeated the experiments using methyl-α-d-glucoside instead of maltose as an acceptor molecule, because the oligosaccharides synthesized from methyl-α-d-glucoside and sucrose consisted of a single type of anomer.

Table 1 Distribution of GTF activity in cultures of strain B-1355 and mutants[a]

Strain	Specific activity (units mg^{-1} protein)[b]		
	Culture	Supernatant fraction	
		Unheated	Heated[c]
R1554	1.5	1.3	1.5
R1588	1.2	0.11	0.11
R1510	3.2	1.3	0.39
SL-1	9.1	6.9	0.13
B-1355	2.8	0.94	0.23

[a]Cultures were grown in 100 ml of GTF medium containing 2% (wt/vol) sucrose at 30°C for 24 h with shaking at 100 rpm. GTF activity was assayed by the DNS assay procedure.
[b]Specific activities were calculated from the protein concentrations for whole cultures.
[c]Samples (1.5 ml) of supernatant fraction were heated in a water bath at 45°C for 40 min to destroy dextransucrase activity.

We synthesized oligosaccharides from methyl-α-d-glucoside and sucrose by incubating cultures of strain B-1355, R1510, R1554, and R1588 for up to 18 days at 30°C in a solution containing 100 mM methyl-α-d-glucoside and 200 mM sucrose. To aid in identifying the oligosaccharides produced, we also synthesized oligosaccharides using control GTFs, which included commercial B-512F dextransucrase, alternansucrase (prepared by thermal inactivation of the supernatant fraction of a culture of strain B-1355), and the supernatant fraction from a culture of strain B-1299, whose GTFs are known to synthesize small oligosaccharides containing α(1→2) linkages [19]. The acceptor reaction products from strain B-512F dextransucrase [24] and alternansucrase [2] have been previously identified. The oligosaccharides produced from methyl-α-d-glucoside by alternansucrase were identified by Pelenc *et al* [21].

Cultures of strain B-1355 (Figure 2a) synthesized a mixture of oligosaccharides corresponding to the products of alternansucrase and dextransucrase. These we identified as methyl-α-isomaltoside (peak No. 2), 1-*O*-methyl-α-glucopyranosyl-α(1→3)isomaltoside (peak No. 4), and methyl-α-isomaltotrioside (peak No. 5). Peaks eluting earlier than 20 min were produced by the reactants (sucrose and methyl-α-d-glucoside (peak No. 1)), acetate, glucose, fructose, leucrose, culture medium components and buffers. A small peak (peak No. 3), from a trisaccharide which we identify below as 1-*O*-methyl-α-glucopyranosyl-(1→2)isomaltoside, was also produced.

Cultures of strain R1510 (Figure 2b) synthesized large amounts of methyl-α-isomaltoside (7 mM final concentration), 1-*O*-methyl-α-glucopyranosyl-α(1→2) isomaltoside (3 mM), and smaller amounts of 1-*O*-methyl-α-glucopyranosyl-α(1→3)isomaltoside (0.6 mM), methyl-α-isomaltotrioside (2 mM), and larger unidentified oligosaccharides, as indicated by the relative areas of their peaks. The molar ratio (1.5) of 1-*O*-methyl-α-glucopyranosyl-α(1→2)isomaltoside to methyl-α-isomaltotriose suggested that GTF-1 comprised at least 60% of the total GTF activity, with the remaining activity due to dextransucrase.

Strain R1554 and R1588 (Figure 2c and d) synthesized oligosaccharides which were the same as those produced by alternansucrase, and consisted of methyl-α-isomaltoside (29–39 mM), 1-*O*-methyl-α-glucopyranosyl-α(1→3) isomaltoside (21 and 15 mM), and methyl-α-isomaltotrioside (4 and 3 mM). The molar ratios of 1-*O*-methyl-α-glucosypyranosyl-α(1→3)isomaltoside to methyl-α-isomaltotriose

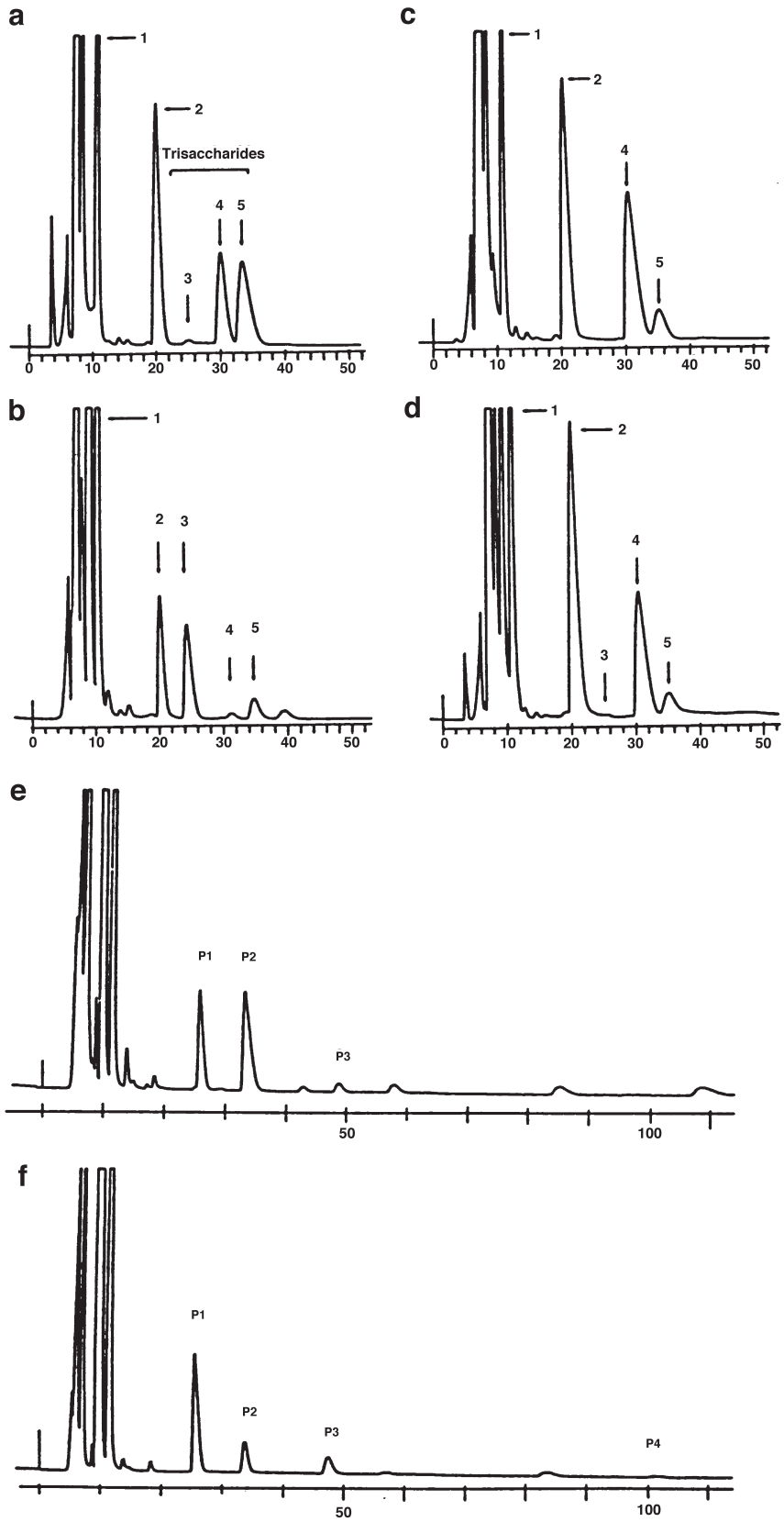

Figure 2 Oligosaccharides produced from methyl-α-d-glucoside and sucrose. Samples from 24-h cultures were incubated with 100 mM methyl-α-d-glucoside and 200 mM sucrose. The oligosaccharides that were synthesized were separated by HPLC. The horizontal axes show the retention times in minutes. Peak identifications: 1. 1-*O*-Methyl-α-d-glucoside, 2 and P1. 1-*O*-Methyl-α-isomaltoside, 3 and P2. 1-*O*-Methyl-α-glucopyranosyl-α(1→2)isomaltoside, 4. 1-*O*-Methyl-α-glucopyranosyl-α(1→3)isomaltoside, 5 and P3. 1-*O*-Methyl-α-isomaltotrioside. P4. 1-*O*-Methyl-α-isomaltotetraoside. (a) B-1355, (b) R1510, (c) R1554, (d) R1588, (e) R1510 at high resolution, (f) GTF-1 (excised from an SDS gel) from strain B-1355 at high resolution.

(5.0) suggested that the GTFs from strain R1554 and R1588 contained at least 85% alternansucrase.

To confirm that GTF-1 from the parent strain B-1355 synthesized the same products from methyl-α-d-glucoside and sucrose as GTF-1 from strain R1510, we banded GTF-1 from strain B-1355 on SDS gels, excised the bands with a razor blade, and incubated them for up to 18 days in our solution containing sucrose and methyl-α-d-glucoside (Figure 2e and f). The di- and trisacccharides synthesized by GTF-1 (oligosaccharides P1 and P2) from strain B-1355, after separation by HPLC at high resolution, co-eluted with those produced by cultures of strain R1510. Cultures of strain B-1299 (not shown) also synthesized oligosaccharides P1 and P2 from methyl-α-d-glucoside and sucrose, as evidenced by their coelution from HPLC columns with oligosaccharides P1 and P2 from strain R1510.

Structure of oligosaccharides P1 and P2

To be certain of the identity of key oligosaccharides synthesized by strain GTF-1 from strain R1510, we purified oligosaccharides P1 and P2 to at least 97% purity (based on HPLC peak areas) and obtained their ^{13}C-NMR spectra in deuterium oxide (Figure 3). Table 2 shows the assignments of the ^{13}C chemical shifts for oligosaccharides P1 and P2 and our reference standards (methyl-α-d-glucoside, α,β-kojibiose, α,β-nigerose, α,β-maltose, α,β-isomaltose, and α,β-panose), on which we based our proposed structures. Our conditions, internal standard and instrumentation resulted in resonances for methyl-α-d-glucoside which were shifted 1.0–1.6 ppm downfield from values reported by Usui *et al* [31], but peak assignments agreed with literature values after correction on the assumption that the literature values were referenced to dioxane at 67.4 ppm. C1 carbons produced anomeric resonances in the region 90.0–102.7 ppm downfield from TSP, while unsubstituted C2, C3, C4 carbon atoms, and C5 carbon atoms resonated in the region 70.0–79.0 ppm. C6 carbon atoms produced shifts in the region 63.0–69.0 ppm.

The linkages in oligosaccharides P1 and P2 were all α-glucosidic linkages, as indicated by the absence of chemical shifts downfield of 102.7 ppm. Linkages of the type α(1→3) or α(1→4) were absent, because chemical shifts between 79.0 and 86.0 ppm, as observed with maltose, panose and nigerose, were absent. Oligosaccharides P1 and P2 possessed the structure of methyl-α-d-glucoside at the reducing ends of their molecules, as indicated by shifts at 102.2 and 58.0 ppm, which were close to those produced by authentic methyl-α-d-glucoside (102.1 ppm and 57.9 ppm). Oliogosaccharides P1 and P2 were not reducing sugars in our DNS assays, confirming that all of the C1 carbons were involved in glucosidic linkages or contained substituents. Therefore, all of the chemical shifts in P1 and P2 were from the α anomers.

The ^{13}C-NMR spectrum of oligosaccharide P1 indicated that its structure was that of methyl-α-isomaltoside (Figure 3a). It produced two anomeric resonances (100.7 ppm and 102.2 ppm) and thirteen total resonances, indicating that P1 was a disaccharide. The C1′ shift at 100.7 ppm and C6 shift at 68.3 ppm showed that P1 possessed an α(1→6) glucosidic linkage, also seen in panose (100.9 and 68.8 ppm) and

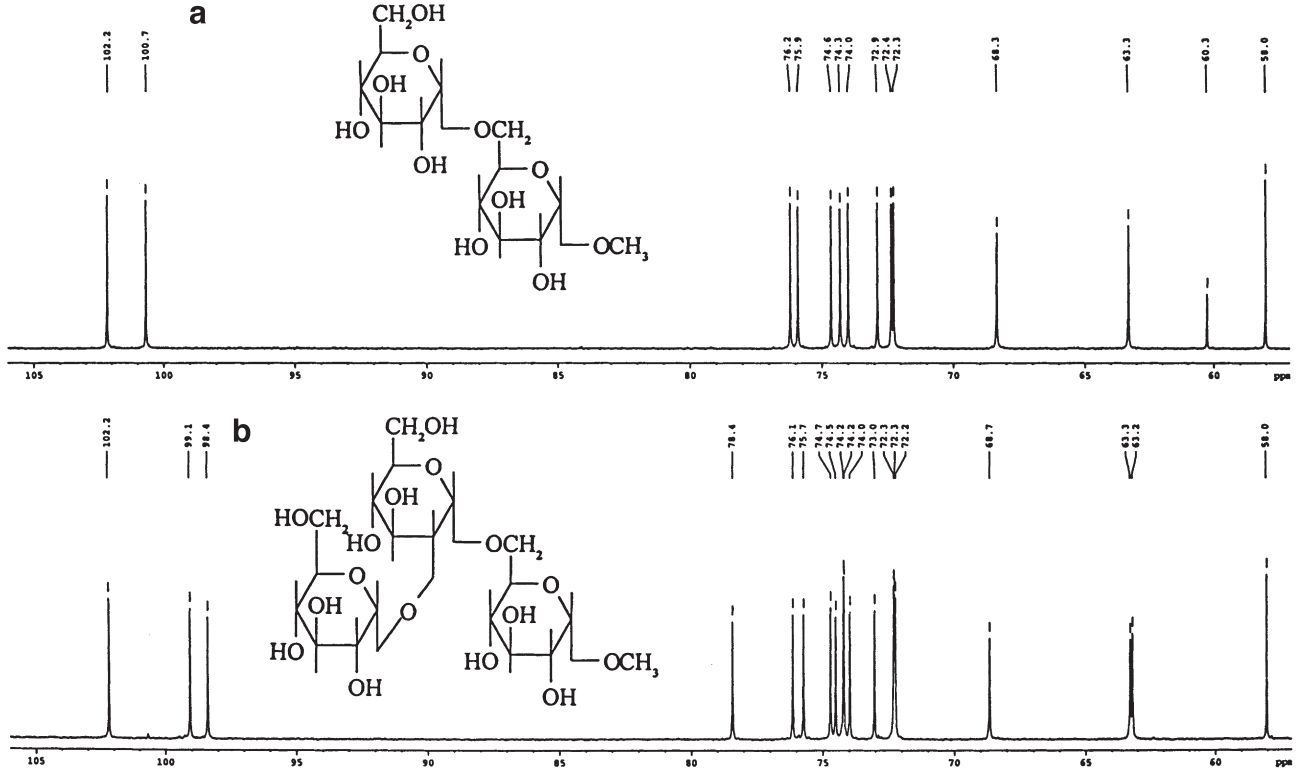

Figure 3 ^{13}C-Nuclear magnetic resonance spectra of oligosaccharides P1 and P2. The structures of oligosaccharides, P1, and P2, from Figure 3 were confirmed by purifying peaks P1 and P2, then obtaining their ^{13}C-NMR spectra in deuterium oxide. Their proposed structures are shown in the insets. (a) Oligosaccharide P1. (b) Oligosaccharide P2.

Table 2 ^{13}C Chemical shift assignments[a]

	P1[b]	P2[c]	AMG	KOJ	KOJ	NIG	NIG	MAL	MAL	IM	IM	PAN	PAN
Linkage:			C1-OMe	α(1→2)	α(1→2)	α(1→3)	α(1→3)	α(1→4)	α(1→4)	α(1→6)	α(1→6)	α(1→4) α(1→6)	α(1→4) α(1→6)
Anomer:	α	α	α	α	β	α	β	α	β	α	β	α	β
C1	102.2	102.2	102.1	92.3	99.1	95.1	98.8	94.7	98.6	95.1	98.9	94.8	98.6
C2	–	–	74.4	78.8	81.4	72.9	75.7	74.1	76.8	–	–	–	–
C3	–	–	75.9	–	77.3	82.6	85.1	–	78.8	–	78.8	–	–
C4	–	–	72.4	–	–	–	–	79.6	79.8	–	–	80.0	80.3
C5	–	–	74.1	–	78.6	74.0	78.5	72.8	77.4	–	–	–	–
C6[d]	68.3	68.7	63.4	63.2	63.6	63.2	63.4	63.3	63.6	68.6	68.7	63.3	63.7
C1′	100.7	98.4	–	99.1	100.5	101.8	101.9	102.3	102.4	100.8	100.9	102.6	102.6
C2′	–	78.4	–	–	–	–	–	–	–	–	–	–	–
C6′[d]	63.3	63.2	–	63.1	63.4	63.1	63.2	63.3	63.4	63.3	63.3	68.8	68.8
C1″	–	99.1	–	–	–	–	–	–	–	–	–	100.9	100.9
C6″[d]	–	63.3	–	–	–	–	–	–	–	–	–	63.3	63.5
–OCH_3	58.0	58.0	57.9	–	–	–	–	–	–	–	–	–	–

[a]Shifts (ppm) are reported downfield from TSP. Abbreviations; AMG, methyl-α-d-glucoside; KOJ, kojibiose; NIG, nigerose; MAL, maltose; IM, isomaltose; PAN, panose.
[b]Tentative P1 assignments (ppm); C2,C2′ (74.7, 74.3); C3,C3′ (76.2, 75.9); C4,C4′ (72.3, 72.4); C5,C5′ (72.9, 74.0).
[c]Tentative P2 assignments (ppm); C2,C2″ (74.5, 74.7); C3,C3′,C3″ (76.1, 74.2, 75.7); C4,C4′,C4″ (72.3, 72.2, 72.3); C5,C5′,C5″ (73.0, 74.0, 74.2).
[d]C6, C6′ and C6″ resonances: Values may be interchanged where they are nearly the same (as in kojibiose, nigerose and maltose), because we arbitrarily assigned the higher values to the β resonances and to the C6 carbons nearest the reducing end.

α,β-isomaltose (100.8, 68.6 and 68.7 ppm). An unlinked C6′ carbon was also present, as indicated by the chemical shift at 63.3 ppm. We also observed weak resonances at 60.3 and 19.6 ppm, which were produced by traces of ethanol in our samples, a solvent we used in preparing our samples.

The structure of oligosaccharide P2 was confirmed as 1-*O*-methyl-α-glucopyranosyl α(1→2)glucopyranosyl α(1→6) glucopyranoside (Figure 3b). The three anomeric shifts (98.4, 99.1 and 102.2 ppm) and nineteen total shifts indicated that oligosaccharide P2 was a trisaccharide. The C1-OMe shift at 102.2 ppm from methyl-α-d-glucoside has already been mentioned. The shift at 99.1 ppm indicated the presence of an α(1→2) glucosidic linkage involving the C1″carbon, as in kojibiose (C1′ at 99.1 ppm). The α(1→2) linkage was confirmed by the C2′ resonance at 78.4 ppm, also seen with kojibiose (C2 at 78.8 ppm). The α(1→2) linkage was assigned to the C1″ and C2′ carbons, rather than the C1′ and C2 carbons, because the C1-OMe resonance at the reducing end would otherwise have been shifted with respect to α-methyl-d-glucoside because of the linkage at C2. The only other reference sugars tested which exhibited shifts near 78.4 ppm were the β anomers of kojibiose (C5β at 78.6 ppm), isomaltose (C3β at 78.8) and nigerose (C5β at 78.4 ppm). However, as stated above, β-anomers were absent in oligosaccharides P1 and P2. The remaining C1 shift (98.4 ppm) was assigned to the C1′ carbon which was involved in an α(1→6) linkage, because the resonance at 68.7 ppm indicated that one of the three C6 carbons was involved in a linkage. The resonance at 98.4 ppm was shifted 2.4 ppm upfield of the C-1→6′ resonance of isomaltose (100.8 ppm) as a result of the linkage at the C2′ carbon, reflecting the expected γ shift effect. A shift of similar magnitude was reported for the C-1→6 resonances of dextran from *L. mesenteroides* strain B-1299 [25,26] and tetrasaccharide B4 [22], which also contained α(1→2) and α(1→6) linkages. Two unlinked C6 carbons were present in trisaccharide P2, as indicated by resonances at 63.2 and 63.3 ppm. Our proposed structure for trisaccharide P2 (Figure 3b) is analogous to the structure proposed by Remaud-Simeon *et al* [22] for tetrasaccharide B4, which was synthesized from maltose and sucrose by dextransucrase from strain B-1299. We concluded that GTF-1 synthesized trisaccharide P2 by adding a glucosyl residue to disaccharide P1 via formation of an α(1→2) linkage.

Confirmation of (1→2) linkages in the polysaccharide from strain R1510

The presence of (1→2) linkages in the polysaccharide synthesized by cultures of strain R1510 was confirmed by GC/MS analysis (Figure 4). Our results show that the (1→2) linkage always occurred as a branch point, because it was only found in glucose units also containing a (1→6) linkage. It was not detected in glucose units containing a (1→3) linkage. Also present in the analysis were the (1→6), (1→3) and (1→6,1→3) linkages which are found in alter-

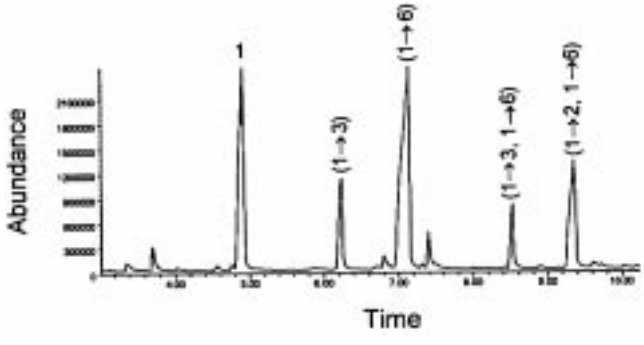

Figure 4 GC/MS of peracetylated aldononitrile (PAAN) derivatives of the polysaccharide from strain R1510. Peak identifications: (1) methyl-2,3,4,6-tri-*O*-methyl-d-glucose PAAN, the non-reducing end group sugar; (1→3) methyl-2,4,6-tri-*O*-methyl-d-glucose PAAN; (1→6) methyl-2,3,4-tri-*O*-methyl-d-glucose PAAN; (1→3, 1→6) methyl-2,4-di-*O*-methyl-d-glucose PAAN; (1→2, 1→6) methyl-3,4-di-*O*-methyl-d-glucose PAAN.

nan. There was a higher proportion of (1→6) linkages to (1→3) linkages in the polysaccharide from strain R1510 than in alternan, where the ratio of (1→6) to (1→3) linkages is closer to unity. A more complete description of the polysaccharide will be given elsewhere (J Ahlgren, G Cote and M Smith, manuscript in preparation).

Discussion

L. mesenteroides strain R1510 is a most unusual derivative of strain B-1355 because it produces a significant amount of an insoluble polysaccharide and little soluble polysaccharide, and the structure of the insoluble polysaccharide contains (1→2) glucosidic linkages, which have not been seen before in strain B-1355 or its derivative strains. Its principal GTF (GTF-1) resembles the GTFs from strain B-1299 in size and structures of the oligosaccharides and dextrans synthesized [29]. Possible explanations for strain R1510 are: (i) that strain R1510 produces a mutant form of dextransucrase or alternansucrase which synthesizes products whose structures are different from products made by the wild-type GTF; (ii) that strain R1510 expresses a latent GTF gene not expressed by the parent strain; or (iii) that strain R1510 produces the same GTFs as the parent strain, but that two of the GTFs (alternansucrase and dextransucrase) are produced with diminished activity relative to GTF-1. We believe our results indicate that the last mentioned possibility (iii) is the most likely, because GTF-1 from strain B-1355 and strain R1510 appeared to be identical in size (240 kDa) and in the α(1→2)-linked products which they synthesized from methyl-α-d-glucoside and sucrose. The essential identity of B-1355 and R1510 GTF-1 was also suggested by the synthesis by cultures of the parent strain B-1355 and its derivative strain R1510 of an insoluble polysaccharide containing (1→2) glucosidic linkages.

We were able to isolate other mutants (SL-1, R1588, R1554) in which activity from two GTFs was diminished relative to the third, suggesting that mutants of strain B-1355 could be isolated in which the activity of any two of its three GTFs was diminished relative to the third. These mutant strains suggest that alternansucrase, dextransucrase and GTF-1 from strain B-1355 are each synthesized by a different structural gene, and that all three genes are expressed in sucrose cultures of strain B-1355 (although each GTF is present at a different level of activity).

Latent GTFs have been described in strain B-1299 in which the GTFs were not detected in sucrose cultures, but were detected by cloning experiments or by their production by cultures only in media containing sugars other than sucrose [3,17,19]. A latent GTF resembling B-1355 GTF-1 in size (240 kDa) and in synthesizing a polysaccharide containing α(1→2) linkages might be present in strain B-742, because a mutant derivative (B-742CB) of strain B-742 synthesized the GTF, while the parent strain and other mutants did not [8,10]. The presence of 240 kDa activity bands on SDS gels, as seen in strain B-1355, B-742, and B-1299, might be a general indication of the presence of GTFs synthesizing α(1→2) linkages. However, it appears that GTF-1 from strain B-1355 is not a latent GTF, because we were able to detect the synthesis of α(1→2) linkages by cultures of strain B-1355.

We previously reported [33] the novel finding that alternansucrase from strain B-1355 was almost entirely cell-associated, while GTF-1 and dextransucrase were distributed more abundantly in the supernatant fractions of cultures. Strain R1554 and R1588 were unusual compared to other mutants reported to produce GTFs enriched for alternansucrase [12], because strain R1554 produced a supernatant form of alternansucrase, while strain R1588, like the parent strain B-1355, produced a cell-associated form of alternansucrase. Extracellular enzymes from Gram-positive bacteria are sometimes covalently anchored to the cell wall by their carboxyl-terminal amino acids, and changes in or deletions of those amino acids can result in the production of enzymes which are not cell-associated [32]. We do not know if a similar mechanism operates in *L. mesenteroides* to cause specific GTFs to become cell-associated, or if the cell-associated GTFs are attached at the cell membrane, but cell wall anchoring and mutational changes in the carboxyl-terminal amino acids of alternansucrase might account for the difference in cell association of alternansucrase between strain R1554 and R1588.

The GTFs from *L. mesenteroides*, particularly those which synthesize small sugars containing α(1→2) linkages, have applications in the cosmetics and other industries [21,22], where they are used to synthesize carbohydrates which are useful in a variety of applications. They may also have more general applications for carbohydrate engineering, because their acceptor reactions can be used to modify the structures of existing carbohydrates, such as starch [20]. However, research has tended to focus more on the structures of the dextrans produced than on the different types of GTFs produced by the different strains of *L. mesenteroides*. The methods [5,25–27] used to identify the polymers produced were probably too insensitive to detect dextrans representing less than about 10% of the total dextran, and the dextran fractions obtained might or might not represent the products of single GTFs. As a result, GTFs might have been overlooked, as had GTF-1 from strain B-1355. Only five (B-512F, B-1355, B-1299, B-742, and B-1142) of the more than 96 dextran-producing strains described [5] have so far been examined for the number and types of GTFs produced [3,6–9,12]. The dextran-producing strains of *L. mesenteroides* need to be resurveyed and classified according to the number and types of GTFs produced if the full potential value of their glucosyltransferases is to be realized.

Acknowledgements

We thank Denyse Goff (WRRC, Albany, CA) for expert technical assistance and James J Nicholson (NCAUR, Peoria, IL) for assistance with the methylation analysis of the polysaccharide from strain R1510.

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