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ORIGINAL PAPER

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A method for surveying and classifying *Leuconostoc* spp. glucansucrases according to strain-dependent acceptor product patterns

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Abstract A number of *Leuconostoc* spp. strains were screened for their ability to produce glucansucrases and carry out acceptor reactions with maltose. Acceptor products were analyzed by thin-layer chromatography (TLC) and it was discovered that they could be grouped into four distinct categories based on oligosaccharide product patterns. These patterns corresponded with structural features of the dextrans each strain is reported to produce. Strains that produced a typical dextran—characterized by a predominantly linear $\alpha(1 \rightarrow 6)$-linked D-glucan chain with a low to moderate degree of branching—produced a homologous series of isomaltooligosaccharides via acceptor reactions. Strains that produced dextrans with moderate to high levels of $\alpha(1 \rightarrow 2)$ branch points, exemplified by NRRL B-1299, synthesized the same isomaltodextrins as well as another series of oligosaccharides migrating slightly faster in our TLC system. Strains that produced dextrans with higher levels of $\alpha(1 \rightarrow 3)$-branches, such as NRRL B-742, synthesized isomaltodextrins plus a series of oligosaccharides that migrated slightly more slowly on TLC. And finally, strains known to produce alternansucrase produced isomaltodextrins plus oligoalternans. Within a given type, variability exists in the relative proportions of each product. The data presented here may be useful in selecting strains for the production of specific types of oligosaccharides, for example as prebiotics.

Keywords Dextransucrase · Oligosaccharides · Acceptor reactions · Alternansucrase · Prebiotics

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Introduction

Interest in oligosaccharides as functional food ingredients has increased sharply within the past decade. Our work focuses in particular on the use of glucansucrases to produce functional oligosaccharides from sucrose and other agricultural commodities. Glucansucrases are enzymes that synthesize glucans by glucosyltransferase reaction, utilizing sucrose as the glucosyl donor. In 1954, Jeanes et al. [10] published the results of a survey of 96 strains of glucan-producing bacteria. All produced dextran from sucrose, and most were *Leuconostoc mesenteroides* or closely related species. At that time their goal was to classify the dextrans by structure and properties and find a suitable strain for production of dextran as a blood plasma expander. Current research on the use of oligosaccharides as prebiotics has caused us to reexamine the use of glucansucrases for the production of novel carbohydrates. Prebiotics are substances that enhance the growth of beneficial bacteria—typically in the gastrointestinal tract—usually by serving as selective growth substrates. Most known prebiotics are oligosaccharides. To facilitate our search for novel prebiotic oligosaccharides, we have developed a protocol for examining oligosaccharide production by glucansucrase-producing bacteria. In the presence of suitable acceptor sugars, glucansucrases transfer glucosyl units from sucrose to the acceptor, resulting in the formation of glucosyl-oligosaccharides [13]. This paper describes a convenient protocol for the examination of glucansucrase acceptor products and proposes a classification scheme for glucansucrases from *Leuconostoc* and other bacteria.

Materials and methods

Chemicals

Polypeptone, beef extract, yeast extract, and agar were from Becton Dickinson (Sparks, Md.). Other chemicals were reagent grade from Sigma (St. Louis, Mo.).

Cultures

Leuconostoc spp. strains were obtained from the Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research (Peoria, Ill.; strain numbers with NRRL prefix) or from the American Type Culture Collection (Manassas, Va.; strain numbers with ATCC prefix). Most of these strains are considered to be *L. mesenteroides* subsp. *mesenteroides* (Table 1). Strains NRRL B-1121 and NRRL B-1420 are *L. mesenteroides* subsp. *dextranicum*. Strains NRRL B-742 and ATTC 49370 are now considered to be *L. citreum*, while NRRL B-1804 and ATCC 21436 are undetermined species of *Leuconostoc*. *Streptobacterium dextranicum* strain NRRL B-1254 was included in the landmark 1954 study by Jeanes et al. of dextran-producing bacteria [10].

Growth and production of acceptor products

Cultures were maintained on solid medium containing a modified MRS basal broth (1.5 g polypeptone, 1.5 g beef extract, 1.5 g yeast extract, 1.0 g Tween 80, 2.0 g ammonium citrate, 5.0 g sodium acetate, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot H_2O$, and 2.0 g K_2HPO_4 per liter, pH 6.5) amended with 2.0% (w/v) sucrose and 1.5% (w/v) agar. Liquid medium contained the same modified MRS basal broth amended with 5.0% (w/v) sucrose and 1.0% (w/v) maltose, which had been filter sterilized. Single colonies were used to inoculate liquid medium preinocula (10 ml in capped 50-ml Erlenmeyer flasks), which were incubated at 28°C under room air with shaking at 100 rpm for 2 days. Liquid cultures for production of acceptor products (same medium and

Table 1 Strains

Strain number	Species	Synonyms	Equivalent numbers	Isolated from
ATCC 14430	*Leuconostoc mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		Grass silage
ATCC 21436	*Leuconostoc* sp.			
ATCC 49370	*L. citreum*		NCDO 1837	Rye ear
NRRL B-512F	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*	ATCC 10830A	Slimy root beer
NRRL B-523	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*	ATCC 14935 NCIB 9317 OSU 535	
NRRL B-742	*L. citreum*	*L. amelibiosum* *L. mesenteroides* subsp. *mesenteroides*	ATCC 13145 DSM 20188 NRIC 1772	
NRRL B-1118	*L. mesenteroides* subsp. *mesenteroides* (type strain)	*L. mesenteroides*	ATCC 8293 DSM 20343 NCIB 8023 NCDO 523	Olives
NRRL B-1121	*L. mesenteroides* subsp. *dextranicum*	*L. dextranicum*	ATCC 8359	
NRRL B-1149	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*	NCIB 6109	
NRRL B-1254	*Streptobacterium dextranicum*		ATCC 13134	
NRRL B-1297	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*	ATCC 6025	
NRRL B-1298	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		Cane juice
NRRL B-1299	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*	ATTC 11449	
NRRL B-1355	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		
NRRL B-1374	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		
NRRL B-1375	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		
NRRL B-1377	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		
NRRL B-1397	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		Sugar cane
NRRL B-1399	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*	NCIB 9620	Cane stubble
NRRL B-1402	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		Orange concentrate
NRRL B-1420	*L. mesenteroides* subsp. *dextranicum*	*L. dextranicum*		Mill slime
NRRL B-1422	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		Orange concentrate
NRRL B-1424	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		Refined sugar
NRRL B-1431	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		Cane juice
NRRL B-1433	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		Cane juice
NRRL B-1804	*Leuconostoc* sp.			Spruce sap
NRRL B-3060	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		
NRRL B-3618	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		Dough product
NRRL B-3619	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		Dough product
NRRL B-3620	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		Dough product
NRRL B-21297	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*		Mutant of NRRL B-1355
NRRL B-23185	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*	R-1535	Mutant of NRRL B-1355
NRRL B-23186	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*	R-1579	Mutant of NRRL B-1355
NRRL B-23188	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*	R-1622	Mutant of NRRL B-1355
NRRL B-23311	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides*	R-1510	Mutant of NRRL B-1355

flasks) were inoculated with 0.1 ml preinocula and incubated at 28°C and 100 rpm until growth ceased as judged by optical density (600 nm). Appropriate dilutions were made in water if the optical density was too high to be reliably measured.

Analysis of acceptor products

Aliquots of cultures were centrifuged in micro-centrifuge tubes at 16,060 *g* for 3 min and the supernatant fluid was withdrawn by pipette. A 200-µl aliquot of culture supernatant fluid was pipetted into a micro-centrifuge tube and mixed with 300 µl ethanol in order to precipitate polysaccharide that may have formed. After mixing, the tubes were centrifuged at 16,060 *g* for 10 min. The supernatant liquid was then analyzed by thin-layer chromatography (TLC) on Whatman K5F silica plates, 250-µm thickness (Whatman, Kent, UK).

Samples of 1 µl were absorbed onto the plates on a line about 1 inch (~2.5 cm) above the lower plate edge. After drying with a hairdryer, the plate was irrigated for three ascents in a solvent mixture composed of ethyl acetate:acetonitrile:H_2O:1-propanol (2:7:5.5:5, v/v) [18]. To render sugars visible, plates were sprayed to saturation with the reagent described by Bounias [1]. This reagent consists of 0.2% *N**(1-naphthyl)ethylenediamine dihydrochloride in methanol with 3% sulfuric acid. The plates were then heated in a 120–140°C oven (vented to exhaust hood) until spots were visible (approximately 8–10 min).

For archiving and later analysis, plates were scanned on a flatbed scanner in reflectance mode. Original TLC plates may be covered with a clean glass plate and stored in the dark, but images degrade after several days.

Results and discussion

In preliminary experiments, more than 100 culture collection and new environmental isolates were screened for their ability to produce mucoid colonies on sucrose-containing solid medium. Those exhibiting this property were subsequently cultured in liquid medium containing sucrose plus maltose for the production of oligosaccharides as glucansucrase acceptor products. A TLC plate with the products of representative strains is shown in Fig. 1. The simplest pattern of products to arise is exemplified by *L. mesenteroides* strains NRRL B-512F and NRRL B-1118 (lanes N and O). Strain NRRL B-512F, which is used for the commercial production of dextran and is the most extensively studied [14], is known to produce a series of isomaltodextrin analogs [11]. In this case, each product contains a maltose unit at the reducing end and α(1 → 6)-linked D-glucopyranosyl units added to the non-reducing ends (Fig. 2) [12]. The spots in lane O represent products ranging from the trisaccharide α-D-Glc *p*(1 → 6)-α-D-Glc *p*(1 → 4)-D-Glc (panose) up to traces of the homologous octasaccharide.

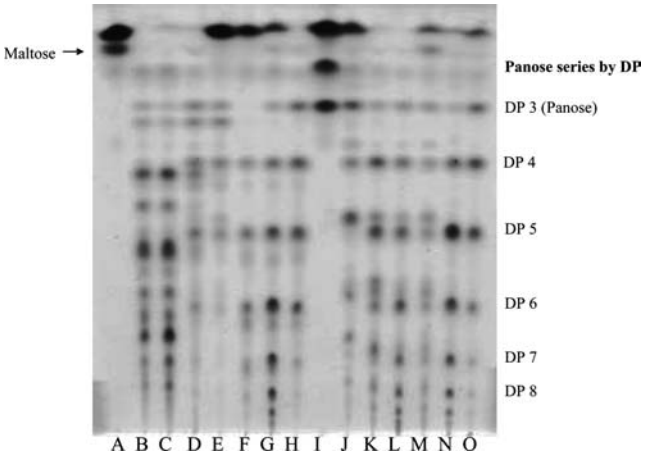


Fig. 1 Thin-layer chromatogram of representative types of acceptor reaction mixtures. Lanes (numbers refer to NRRL culture collection numbers): *A* Sterile medium (sucrose and maltose), *B* B-23188, *C* B-21297, *D* B-1355, *E* B-23311, *F* B-1377, *G* B-1375, *H* B-742, *I* standards (*top to bottom*: fructose, leucrose and panose), *J* B-1297, *K* B-1298, *L* B-1399, *M* B-1299, *N* B-1118, *O* B-512F. Fructose, glucose, and sucrose are not resolved from one another by the solvent system, and all migrate just behind the solvent front and just ahead of maltose. The panose series (see Fig. 2) is represented by lane *O*, starting with low degree of polymerization (DP) at the top and higher DP at the bottom (origin)

Strain B-1118 (lane N), the type strain for *L. mesenteroides*, produces the same oligosaccharides as B-512F. It is evident however that B-1118 produced less panose and degree of polymerization (DP) 4 product and greater amounts of the higher DP oligomers than did B-512F under identical conditions. Whether this is a consequence of different enzyme action and specificity or different amounts of bacterial growth and enzyme levels is not known, but results suggest that NRRL B-1118 could be used for the production of higher oligosaccharides.

Strains NRRL B-21297 and B-23188 (Fig. 1, lanes B, C) produce similar patterns of oligosaccharides that are markedly different from those of other strains. These strains are mutants derived from NRRL B-1355 that produce alternansucrase [15, 20]. The acceptor products of alternansucrase are known to contain both α(1 → 6)-linked and α(1 → 3)-linked D-glucopyranosyl units in alternating patterns (Fig. 3) [2, 5]. Other alternan-producing strains exhibited similar patterns. Strain NRRL B-1355, a wild strain that secretes both alternansucrase and dextransucrase [4], produced both alternansucrase-type products similar to those produced by B-21297 and B-23188, and dextransucrase-type products similar to those produced by B-1118 and B-512F. This is especially evident in the DP 4–5 range. Strain B-23311, another mutant of B-1355, is a bit unusual but the products seem to be similar to both B-1355 and B-1299. Little or no higher-DP products were observed, but a large amount of sucrose remained unused, so this may be a result of low enzyme levels. This strain is known to make relatively low amounts of an insoluble glucan with mixed

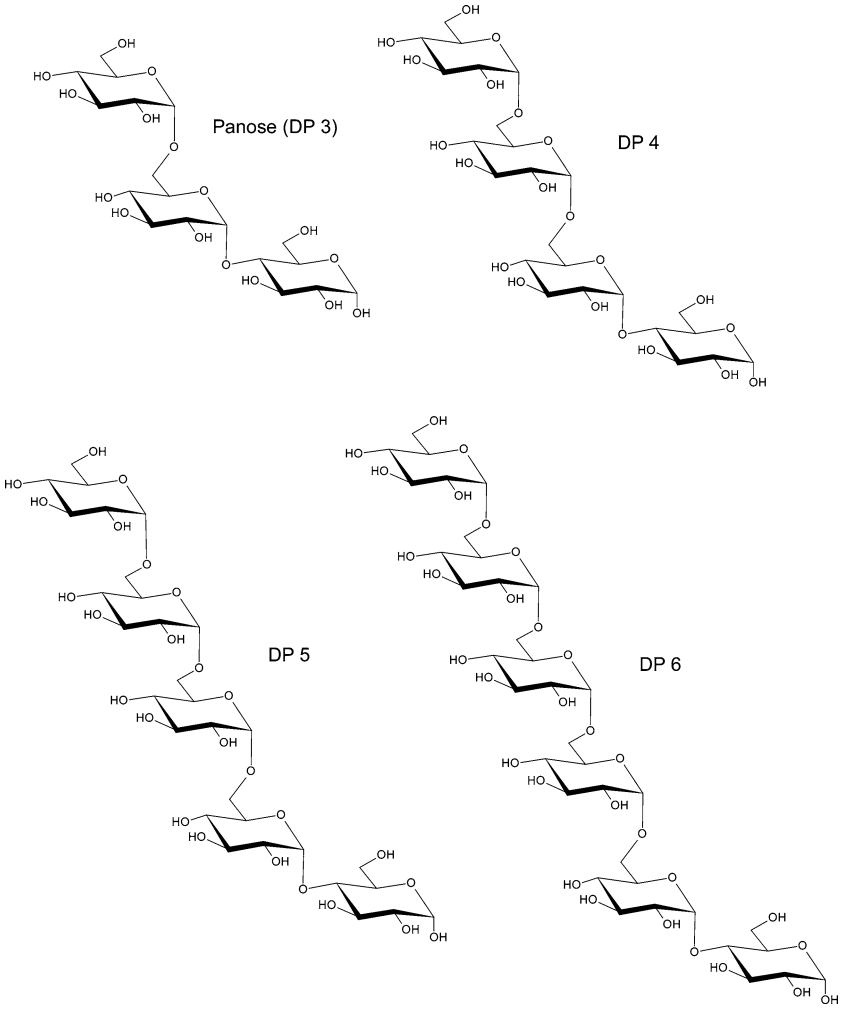


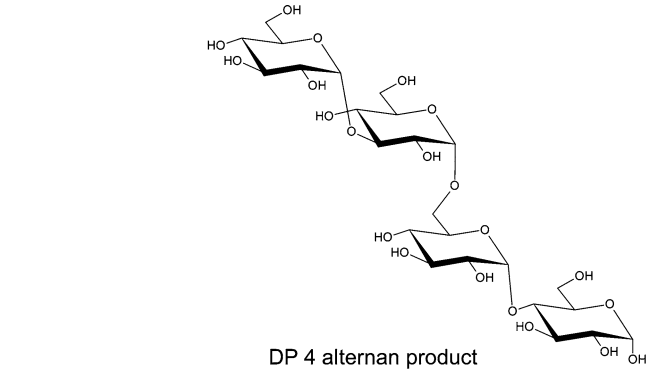
Fig. 2 Structures of panose series of oligosaccharides formed by strain B-512F action on sucrose plus maltose

linkages, including α(1 → 2) linkages, similar to B-1299 dextrans [6, 21].

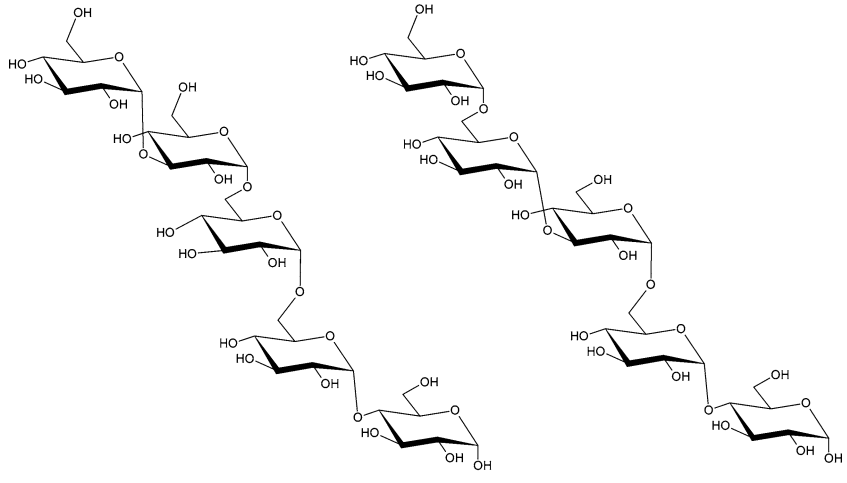
Strains B-1377, B-1375, and B-742 (Fig. 1, lanes F–H) produced mainly isomaltodextrin analogues identical to those produced by B-1118 and B-512F. However, each also produced small amounts of a second series of oligosaccharides that migrated slightly behind the linear isomaltodextrinyl series. The products from one of these strains, NRRL B-742, have been isolated and characterized by Remaud et al. [17]. They contain α(1 → 3)-linked branch points as well as the linear α(1 → 6)-linked D-glucopyranosyl units (Fig. 4). As expected, some strains produced more of the higher DP oligomers than others, or a greater proportion of branched products, but the overall patterns were qualitatively similar.

Several strains produced patterns of oligosaccharide products similar to B-1299 (Fig. 1, lanes J–M), which is used commercially in Europe for the production of oligosaccharides [16]. This strain, in addition to the typical isomaltodextrinyl products, also makes a series of oligosaccharides that migrate slightly ahead of the linear oligosaccharides. These represent branched oligosaccharides in which the branching occurs through α(1 → 2)-linkages (Fig. 5) [2, 9]. A couple of unusual features of individual members of this group may be seen in Fig. 1. Strain NRRL B-1297, which produces mainly linear products up to DP 4, produces virtually none of the linear products above DP 4. The main products of DP ≥5 appear to be only of the branched series. At the other extreme, we note that strain B-1399 produces only small amounts of the branched oligosaccharide series but larger amounts of the linear isomaltodextrinyl series. Finally, strain B-1298 seems to produce two separate branched oligomers migrating just ahead of the DP 6 linear product and little or no linear product above DP 6. Moreover, the branched products above DP 6 appear to differ from those produced by B-1297.

For our own convenience in the laboratory, we have placed most of the strains studied into one of four different classes, although it is clear that the actual situation is more complex than that. Most of the wild strains we have isolated in our laboratory clearly fall into the same class as strains NRRL B-512F and B-1118. These strains produce only the linear isomaltodextrinyl series of oligosaccharides. By reference to the analytical data published by Jeanes et al. [10] for 96 strains of dextran-producing bacteria, it is probably safe to conclude that



DP 4 alternan product



DP 5 alternan products

Fig. 3 Alternansucrase acceptor products arising from maltose plus sucrose, which are formed in addition to those shown in Fig. 2

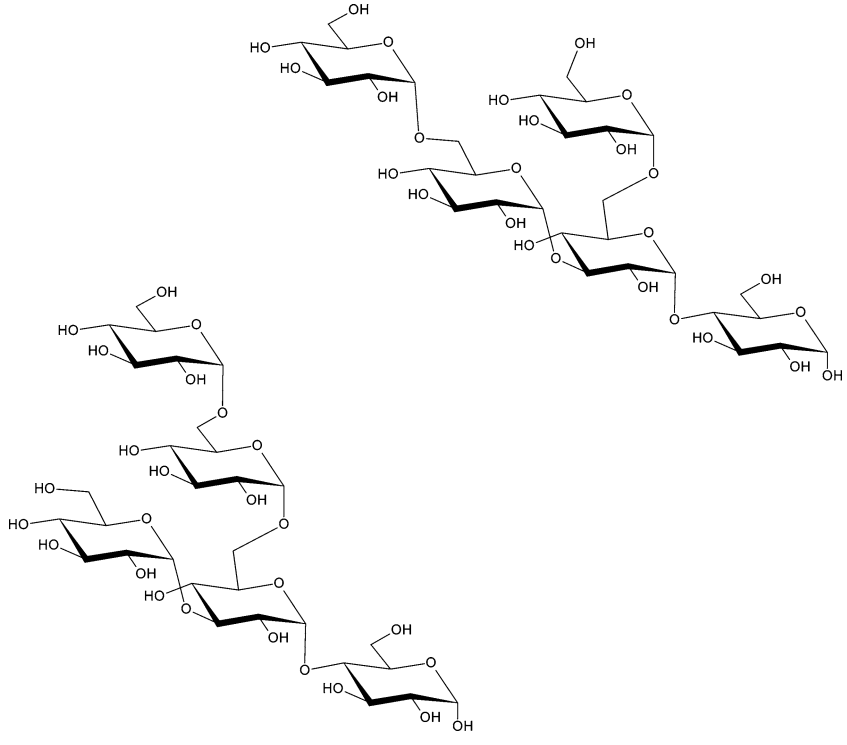


Fig. 4 Branched DP 5 acceptor products arising from B-742 dextransucrase action on maltose plus sucrose, which are formed in addition to those shown in Fig. 2

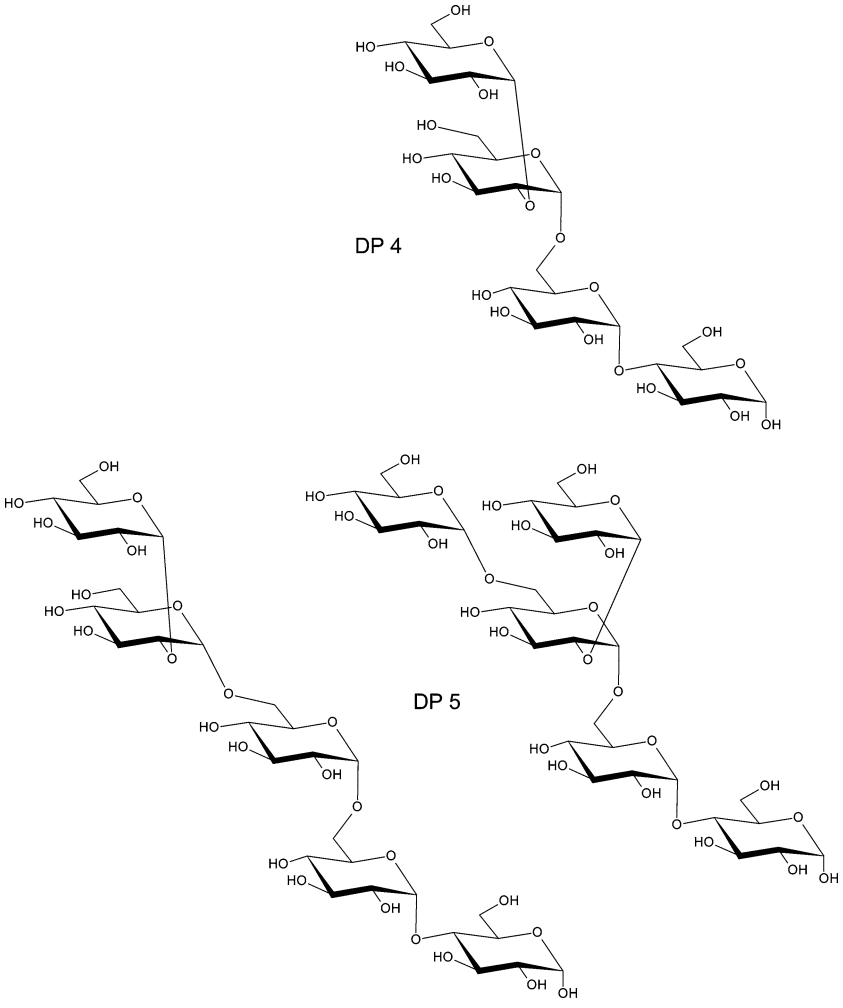


Fig. 5 Branched products formed by B-1299 dextransucrases from maltose plus sucrose in addition to those shown in Fig. 2

these strains also produce "typical" dextrans similar to the commercial product, with a predominantly α(1 → 6)-linked D-glucan main chain and a low degree (< 10–12%) of branching. We will refer to these as Category I glucansucrase-producers. Some of the strains we have studied are listed in Table 2 according to category of oligosaccharide products.

Another group yielded oligosaccharide products similar to NRRL B-1298 and B-1299. These strains produce, in addition to linear isomaltodextrins, a series of oligomers with α(1 → 2)-linked branches. These are listed in Table 2 as Category II glucansucrase-producers. These oligosaccharides are used as prebiotics and functional components of foods and cosmetics [16].

A third category of glucansucrase producers, Category III in Table 2, includes those that exhibit product patterns similar to strains NRRL B-742 and B-1375, namely linear isomaltodextrins plus branched oligosaccharides, probably with α(1 → 3)-linked branch points but possibly also α(1 → 4)-linked branches. The branched oligosaccharides produced by these strains are similar to those arising from dextranase hydrolysis of their respective branched dextrans, which have been put forth as potential prebiotics [3, 8].

Finally, we refer to alternan-oligosaccharide-producing strains as Category IV. These are clearly discernable from the other types and are exemplified by strains NRRL B-21297 and B-23188. Strain NRRL B-23311, which is a mutant of B-1355 lacking alternansucrase, is clearly different from the strains in Category IV. Alternan oligosaccharides show some promise as prebiotics [7], and are actively under investigation in our laboratory for this application.

No correlations were evident between acceptor product categories and the currently accepted taxonomic designations of the strains tested. *L. citreum* strains NRRL B-742 and ATCC 49370 appear in Categories III and I, respectively, and *L. mesenteroides* subsp. *dextranicum* strains NRRL B-1121 and NRRL B-1420 appear in Categories I and II, respectively. *S. dextranicum* strain NRRL B-1254 occurs as a member of Category III. Apart from species names, it is interesting to note that sugar cane isolates appear to predominate in Category II.

The method proposed here is useful for the characterization of glucansucrase-producing bacteria according to the types of oligosaccharide acceptor products they accumulate. We used 10-ml cultures, but it would

Table 2 Types of glucan produced by strains in each acceptor product category

Strain number	Type of glucan(s) produced
Category I—isomaltodextrins only	
NRRL B-512F	1,3-Branched dextran [10]
NRRL B-1121	1,3-Branched dextran [10]
NRRL B-523	1,3-Branched dextran w/1,3-linear segments [19]
NRRL B-1118	1,3-Branched dextran w/1,3-linear segments [19]
NRRL B-1804	Unknown
NRRL B-3618	Unknown
NRRL B-3619	Unknown
NRRL B-3620	Unknown
ATCC 14430	Unknown
ATCC 49370	Unknown
Category II—isomaltodextrins plus faster-migrating branched products	
NRRL B-1298	1,2-Branched dextran [19]
NRRL B-1299	1,2-Branched dextrans [19]
NRRL B-1397	1,2-Branched dextran [19]
NRRL B-1399	1,2-Branched dextrans [19]
NRRL B-1422	1,2-Branched dextran [19]
NRRL B-1424	1,2-Branched dextran [19]
NRRL B-1297	1,2- Or 1,4-branched dextran [10]
NRRL B-1402	1,2- Or 1,4-branched dextran [10]
NRRL B-1431	1,2- Or 1,4-branched dextran [10]
NRRL B-1433	1,2- Or 1,4-branched dextran [10]
NRRL B-1149	1,3- And 1,2- or 1,4-branched dextran [10]
NRRL B-1420	1,4-Branched dextran [19]
NRRL B-3060	Unknown
ATCC 21436	Unknown
Category III—isomaltodextrins plus slower-migrating branched products	
NRRL B-742	1,3-Branched + 1,4-branched dextran fractions [19]
NRRL B-1254	1,3-Branched + 1,4-branched dextran fractions [19]
NRRL B-1375	1,3-Branched + 1,2- or 1,4-branched dextran [10]
NRRL B-1377	1,3-Branched + 1,2- or 1,4-branched dextran [10]
NRRL B-1374	1,3-Branched dextran [19]
Category IV—alternan oligosaccharides (with or without isomaltodextrins)	
NRRL B-1355	Alternan + dextran [19]
NRRL B-21297	Alternan only [15]
NRRL B-23185	Alternan (+ dextran?) [20]
NRRL B-23186	Alternan (+ dextran?) [20]
NRRL B-23188	Alternan (+ dextran?) [20]

be feasible to use much smaller volumes. Liquid culture volumes of less than 1 ml could be analyzed using this procedure, and the method could be adapted to a microtiter plate format. The use of TLC for analysis of the culture fluids means that many samples can be analyzed simultaneously. We typically run 19 samples on a 20 cm square plate, spaced 1 cm apart. Tanks with ribbed separators are capable of handling five plates each, meaning up to 95 cultures can be analyzed in a single tank at one time. The use of multiple tanks increases the number of simultaneous analyses that can be performed. The protocol described here was optimized for the analysis of maltose acceptor products in the DP 3–10 range, but any acceptor reaction mixture can be analyzed in like manner, and reactant concentrations, TLC solvent composition, and other factors can be changed to suit particular needs.

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