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The production of glucans via glucansucrases from *Lactobacillus satsumensis* isolated from a fermented beverage starter culture

Gregory L. Côté · Christopher D. Skory · Suzanne M. Unser · Joseph O. Rich

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Abstract Several starter cultures used in the production of fermented beverages were screened for lactic acid bacteria that produced water-insoluble polysaccharides from sucrose. The strain producing the greatest amount was identified as *Lactobacillus satsumensis* by its 16S RNA sequence and was deposited in the ARS culture collection as NRRL B-59839. This strain produced at least two α-D-glucans from sucrose. One was a water-soluble dextran, consisting of predominantly α-(1→6)-linked D-glucose units, and the other was a water-insoluble glucan containing both α-(1→6)-linked and α-(1→3)-linked D-glucose units. The culture fluid was found to contain glucansucrases responsible for the two glucans, and no significant level of fructansucrase was detected. Glucansucrase activity was not present in the culture fluid when the bacteria were grown on glucose, fructose, or raffinose as the carbon source. Although the water-soluble glucans produced by cell-free enzyme and by cell suspensions were essentially identical, the same was not true for the water-insoluble glucans. The water-insoluble glucan produced by cell-free culture fluid contained a higher proportion of α-(1→3)-linked D-glucose units than the water-insoluble glucan produced by cell suspensions.

Keywords Kefir · Dextran · Mutan · Glucansucrase · Dextransucrase · Lactobacillus

G. L. Côté (✉) · C. D. Skory · S. M. Unser · J. O. Rich
Renewable Product Technology Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture,
1815 North University Street,
Peoria, IL 61604, USA
e-mail: greg.cote@ars.usda.gov

Introduction

A number of fermented beverages that rely on mixed microbial cultures to ferment sugar solutions are known throughout the world. Similar in some respects to dairy kefir, but fermenting sucrose instead of lactose, they are known variously as water kefir, sugary kefir, ginger beer, or tibi. The cultures are characterized by the presence of alcohol-producing yeasts and lactic acid bacteria (Gulitz et al. 2011; Miguel et al. 2011; Magalhães et al. 2011). Much variation is observed from one source to another and depends on the geographical origin, method of growth and storage, plant source, etc. Typically, the bacteria produce exopolysaccharides from the sucrose, which serve to bind the microbial cells together in an insoluble, particulate matrix that is readily separated from the liquid beverage by settling or crude filtration. These insoluble particles are often referred to as "tibi grains." A limited number of studies have addressed the nature of these polysaccharides, and most of them have been shown to be α-D-glucans (Daker and Stacey 1938; Horisberger 1969; Pidoux et al. 1988; Pidoux 1989; Pidoux et al. 1990). Unlike the heteropolysaccharides produced by dairy kefir bacteria, the glucans are synthesized by extracellular enzymes directly from sucrose (Waldherr et al. 2010). The enzymes, known as glucansucrases, are well-known for their ability to produce such water-soluble glucans as dextran (Leathers 2002), alternan (Côté 2002), and reuteran (Kralj et al. 2004). Dextran and its water-insoluble analog, mutan (Guggenheim and Newbrun 1969), are implicated in the etiology of dental caries, and many studies have looked at the glucansucrases responsible for their biosynthesis (Montville et al. 1978). However, very little is known about the water-insoluble glucans of water kefir, despite the fact that the insolubility and gel-like nature of the granules is a key characteristic of these fermentations.

It was recognized early on that the polysaccharides involved in the formation of tibi grains were structurally similar to dextrans, despite their limited solubility in water (Daker and Stacey 1938). Horisberger (1969) showed that the tibi grains from a native, mixed-culture fermentation contained a water-insoluble glucan consisting of α-(1→3)- and α-(1→6)-linked glucose units with branching through 3,6-disubstituted glucose units. However, no attempt was made to study the product from pure bacterial cultures. Pidoux et al. (1988) isolated and characterized two polysaccharide fractions from kefir grains and from an isolated strain of *Lactobacillus brevis*. The soluble, non-gelling polysaccharide was a dextran-like glucan with branching through 3,6-disubstituted glucose residues, along with a trace amount of branching through 2,6-disubstituted glucopyranosyl units. The less-soluble, gelling polysaccharide was similar but also contained a small percentage of 3-monosubstituted D-glucose residues. At that time, the difference in solubility was attributed to differences in the overall content of α-(1→3)-linkages, especially the branch points. Later studies on gelling polysaccharides from *Lactobacillus hilgardii* (Pidoux 1989; Pidoux et al. 1990) suggested that perhaps the gelling ability might be due to the 3-monosubstituted glucose residues. More recently, a glucansucrase from a kefir-derived *L. hilgardii* strain has been cloned and expressed in *Escherichia coli* (Waldherr et al. 2010). The glucan structure was not studied in detail, but susceptibility to dextranase hydrolysis suggested a dextran-like structure.

Our recent studies on water-insoluble glucans from *Leuconostoc mesenteroides* and *Leuconostoc citreum* (Côté and Skory 2012; Côté and Leathers 2009) have suggested a similarity with the glucans found in water kefir. In an attempt to discover a wider variety of glucansucrases that produce water-insoluble glucans from sucrose, we have undertaken a long-term search for novel food-grade bacteria that produce insoluble or difficultly-soluble gel-like masses. To this end, we have screened a number of lactic acid bacteria isolated from commercially available water kefir starter cultures, and describe here the enzymatic synthesis of glucans by one particularly productive strain. Potential applications of these water-insoluble polymers are discussed.

Materials and methods

Materials

Water kefir grains and ginger beer plant grains were obtained from a variety of commercial and private sources for screening. Bacterial strains NRRL B-523, B-1064, B-1118, B-1127, B-1139, B-1254, B-1255, and B-1843 were obtained from the ARS culture collection (Peoria, IL, USA). The type strain of *Lactobacillus satsumensis* was obtained from the German Collection of Microorganisms and Cell Cultures in Braunschweig, Germany.

Media components were obtained from the following: agar, polypeptone, and beef and yeast extracts from BD and Co. (Franklin Lakes, NJ, USA); Tween80, magnesium sulfate, and potassium phosphate from Fisher Chemical Co. (Pittsburgh, PA); ammonium citrate, sodium acetate, manganese sulfate, fructose, raffinose, and glucose from Sigma-Aldrich Chemical Co. (St. Louis, MO); and sucrose obtained from local grocer. Schiff's fuchsin-sulfite reagent was obtained from Sigma-Aldrich Co., and SYPRO Ruby stain from Invitrogen (Grand Island, NY).

A modified MRS medium was used for growth of all liquid cultures. It contained (listed as grams for a 1-L solution): 1.5 g each of polypeptone, beef extract, and yeast extract, 20 g of sugar source (sucrose, glucose, or equal parts glucose and fructose), 0.5 g Tween80, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulfate heptahydrate, 0.05 g manganese sulfate monohydrate, and 2 g potassium phosphate dibasic. Agar was added to the modified MRS (15 g per L of medium) as needed for making culture plates. Cultures were grown at 28 °C for 48 h, shaken at 100 rpm for liquid cultures.

Screening and identification of bacteria

Aqueous suspensions of starter cultures were plated onto both modified MRS with sucrose as carbon source and modified MRS with glucose as carbon source, and ten colonies from each were picked to include as many different morphological types as possible for a second and then a third round of isolation and colony purification. Isolated colonies were assigned identification numbers based on culture source and isolation medium.

All surviving isolates were processed for taxonomic identification based on 16S rDNA. DNA was extracted using either the Qiagen DNeasy Blood & Tissue Kit Gram-positive instructions (Qiagen, Valencia, CA, USA) or the Epicentre Ready-Lyse Lysozyme (Epicentre, Madison, WI, USA). 16S rRNA PCR utilized the HotMaster Taq DNA Polymerase Kit purchased from 5Prime (Gaithersburg, MD, USA), followed by the 5Prime PCR Extract MiniKit. In all cases, instructions provided by the supplier were followed. Primers were U1 (5′-CCA GCA GCC GCG GTA ATA CG-3′, corresponding to nucleotides 518–537 of the *E. coli* 16S rRNA gene) and U2 (5′-ATC GGY TAC CTT GTT ACG ACT TC-3′, corresponding to nucleotides 1,513–1,491 of the same gene), as described by Lu et al. (2000). Sequencing reaction followed, using ABI BigDye Terminator v. 3.1 (Applied Biosystems, Foster City, CA, USA). Sequences were compared with those in GenBank using BLAST available at the National Center for Biotechnology

Information (www.ncbi.nlm.nih.gov). Putative species identifications were based on similar strains with the highest BLAST scores, which in all cases shared greater than 98 % identity to a known species. The sequences were deposited with GenBank and are listed in Table 1.

Based both on genomic DNA identification results and the appearance of colony morphology, culture isolates were chosen for screening of insoluble glucan production, making sure to include all isolates identified as lactic acid bacteria. Single colonies were picked from isolate plates and inoculated into 100 mL of each MRS-Suc and MRS-Glc. After 48-h growth, cells were harvested by centrifugation and dried in a vacuum oven at approximately 50 °C. Insoluble glucan production was estimated by subtracting glucose-grown dry pellet weight from the sucrose-grown dry pellet weight. Soluble glucan was measured by mixing the cell-free culture fluid with two volumes of 95 % ethanol, followed by chilling to 4 °C. The resulting precipitate was pelleted by centrifugation and redissolved in 15 mL of water. Insoluble matter was removed by centrifugation. The soluble glucan was precipitated a second time with two volumes of 95 % ethanol, redissolved in water, and freeze-dried, then oven-dried in vacuum as above, and weighed.

Enzyme and polysaccharide preparations

Cell-free enzyme was prepared by removing cells from 1 L of sucrose-grown liquid culture via centrifugation at

Table 1 Screening of beverage inocula for bacteria producing water-insoluble polysaccharide from sucrose

Isolate	16S[a]	Identity	Net Dry Weight (g)[b]
GBPG-1	JX967007	*Lactobacillus hilgardii*	≤0.00
GBPG-2	JX967008	*Lactobacillus hilgardii*	≤0.00
GBPG-3	JX967009	*Lactobacillus hilgardii*	0.20
GBPG-6A	JX967010	*Lactobacillus hilgardii*	0.02
GBPG-7	JX967011	*Lactobacillus hilgardii*	≤0.00
GBPG-9	JX967012	*Lactobacillus hilgardii*	0.04
GBPS-3	JX967013	*Lactobacillus hilgardii*	≤0.00
GBPS-5	JX967014	*Lactobacillus hilgardii*	≤0.00
WK1G-1	JX967015	*Acetobacter malorum*	0.01
WK1G-2	JX967016	*Gluconobacter japonicus OR frateurii OR oxydans*	≤0.00
WK1G-3	JX967017	*Gluconobacter japonicus OR frateurii OR oxydans*	0.48
WK1G-4	JX967018	*Gluconobacter japonicus OR frateurii OR oxydans*	0.12
WK1G-5	JX967019	*Leuconostoc mesenteroides subsp. mesenteroides*	0.52
WK1G-6	JX967020	*Gluconobacter japonicus OR frateurii OR oxydans*	≤0.00
WK1G-8	JX967021	*Gluconobacter japonicus OR frateurii OR oxydans*	≤0.00
WK1G-10	JX967022	*Leuconostoc mesenteroides subsp. mesenteroides*	0.50
WK1S-4	JX967023	*Lactobacillus fermentum*	0.06
WK2G-3A	JX967024	*Lactobacillus casei*	≤0.00
WK2G-9B	JX967025	***Lactobacillus satsumensis NRRL B-59839***	**0.90**
WK2S-3A	JX967026	*Lactobacillus satsumensis*	0.00
WK2S-6A	JX967027	*Lactobacillus satsumensis*	0.57
WK2S-6B	JX967028	*Lactobacillus rhamnosus OR casei OR paracasei*	0.00
WK2S-10A	JX967029	*Lactobacillus satsumensis*	0.22
WK4LS-1	JX967030	*Lactobacillus nagelii*	0.08
WK4LS-3	JX967031	*Lactobacillus nagelii*	0.05
B-1843		*Lactobacillus hilgardii*	≤0.00
B-1064		*Weissella confusa*	≤0.00
B-1139		*Lactobacillus brevis*	≤0.00
B-1254		*Lactobacillus satsumensis*	0.09
B-1255		*Lactobacillus casei*	≤0.00
B-1127		*Lactobacillus brevis*	≤0.00
B-1118		*Leuconostoc mesenteroides*	1.39
B-523		*Leuconostoc mesenteroides*	0.20
DSM-16230		*Lactobacillus satsumensis*	0.25

[a]GenBank accession number for 16S sequence used for strain identification

[b]Net dry weight=dry weight of sucrose culture pellet−dry weight of glucose culture pellet

20,000×*g* for 20 min. The cell-free culture fluid was concentrated by ultrafiltration to approximately 200 mL, using a Millipore (Billerica, MA) Minitan apparatus with a 100,000-MW cutoff membrane packet. The concentrate was diafiltered vs. 20 mM pH 5.6 sodium acetate buffer containing 2 mM $CaCl_2$ and 0.01 % NaN_3 (hereafter referred to as acetate buffer). This concentrate was used as crude cell-free enzyme without further treatment. To produce glucans using the cell-free enzyme, 70 mL of the enzyme solution was mixed with 30 mL of 1 M sucrose in acetate buffer and allowed to stand for 3 days at room temperature. The reaction mixture was then centrifuged. The insoluble glucan was washed several times with water, followed by 50 % ethanol and finally absolute ethanol, and dried in a vacuum oven at 50 °C overnight. This represents insoluble glucan from cell-free enzyme. The supernatant solution from the enzyme reaction was mixed with two volumes of 95 % ethanol, and the resulting precipitate was removed by centrifugation. The sticky white precipitate was redissolved in water, precipitated a second time, then redissolved and freeze-dried. It is referred to as the soluble glucan from cell-free enzyme.

Cell-associated enzymes were also used to synthesize glucans, in a manner similar to that previously described (Cote et al. 1999; Padmanabhan and Kim 2002). The cell pellet from the 1-L culture, which contained large amounts of insoluble polysaccharide, was washed by suspending it in 250 mL of acetate buffer and centrifuging it again. The insoluble mass was then incubated with approximately 250 mL of 0.3 M sucrose in acetate buffer for approximately 48 h, with mixing by rocking. The suspension was then centrifuged and the pelleted material was washed with 250 mL of water. The combined water-soluble portions were mixed with two volumes of 95 % ethanol, and the resulting precipitate was redissolved in water and precipitated a second time. This material was dissolved in a small volume of water and freeze-dried; it is referred to as the soluble glucan from cell-associated enzyme. The insoluble material after sucrose incubation was mixed with 250 mL of 1 M NaOH and stirred until a viscous solution resulted. This was centrifuged as above to remove insoluble cell debris. The soluble portion was neutralized with 4 M HCl, and dialyzed against water. The resulting insoluble product was washed and dried as above, and represents the insoluble glucan from cell-associated enzyme.

Analytical methods

Gel electrophoresis was carried out, with staining for both enzyme activity and proteins present. Invitrogen NuPage 4–12 % Bis-Tris precast gels were run in MOPS-SDS running buffer, along with Hi-Mark protein standards from the same source; manufacturer protocols were followed for running of gels. Samples for all gels were mixed 1:1 with sample buffer (pH 6.8) consisting of 0.55 M Tris, 2 % SDS, 7 % glycerol, 4.3 % mercaptoethanol, and 5 M urea. Samples to be used for protein staining were then placed in a boiling water bath for 5 min; samples for enzyme activity staining not boiled prior to electrophoresis.

Protein gels were placed in a water wash for up to 1 h, then stained using Invitrogen SYPRO Ruby stain for 4–24 h, then destained with water and visualized under UV light. Enzyme activity periodic acid-Schiff gel staining procedure was adapted from Miller and Robyt (1986) and Kapitany and Zebrowski (1973). At room temperature, gels were incubated for 1 h in 50 mL 20 mM sodium acetate buffer containing 0.01 % sodium azide (pH 5.4), 10 mg/mL Tween80, and 2 mM $CaCl_2$. Gels were then incubated for 1–2 h in the same buffer with the addition of 50 mg/mL sucrose, then washed for 1 h to overnight in acetic acid/2-propanol/water (10/25/65). Gels were further treated by incubating in 1 % periodic acid for 1 h, then washed in 15 % acetic acid for 1 h, and finally placed in Schiff's reagent and stored in a refrigerator for 1 h to stain. Gels were destained with 7 % acetic acid, changing the solution at least three times over 24 h.

Enzyme assays were based on the incorporation of ^{14}C-labeled glucose from ^{14}C-(U)-labeled sucrose (PerkinElmer-NEN, Waltham, MA, USA) into alcohol-insoluble polysaccharide, according to a modification of the method described by Germaine et al. (1974). In a typical example, 40 μL of 0.3 M ^{14}C-sucrose in acetate buffer was incubated with 80 μL of enzyme solution at 30 °C. At timed intervals, 20 μL aliquots were withdrawn and absorbed into 1.5 cm squares of Whatman 3MM chromatography/filter paper. The squares were immediately dropped into a beaker containing approximately 150–200 mL of stirred methanol. A metal screen was used to protect the paper squares from maceration by the stir bar. After washing with three changes of methanol for 10 min per wash, they were dried under a heat lamp and counted for ^{14}C content in Ecolume cocktails (MPBio, Solon, OH, USA) using a Beckman-Coulter (Brea, CA, USA) LSC-6500 liquid scintillation counter. Although one of the glucans is essentially insoluble in water, we found that it is sufficiently soluble during the early stages of biosynthesis to give a linear assay plot using this method. An enzyme unit is defined here as the amount of enzyme activity that incorporates 1 μmol of glucose into glucan in 1 min at 30 °C. For differential determination of glucansucrase and fructansucrase activities, ^{14}C-(Glc)-sucrose and ^{14}C-(Fru)-sucrose were used.

For structural analysis, the glucans were subjected to methylation analysis and to ^{13}C NMR spectroscopy. Methylation, hydrolysis, and gas–liquid chromatography of the peracetylated aldononitrile derivatives were performed as described by Seymour et al. (1977). For NMR spectroscopy, a 50-mg sample of the water-insoluble glucan

was suspended in approximately 2 mL of a 1 M solution of sodium deuteroxide in D_2O and mixed until a viscous solution resulted. Any undissolved material was removed by centrifugation prior to NMR spectroscopy. For water-soluble glucan, the solvent was simply D_2O. ^{13}C NMR spectra were collected on a Bruker Avance 500 spectrometer using a 5 mM BBO probe with Z-gradient and Topspin 1.3. Spectra were acquired at 300.0 K. Chemical shifts are reported as parts per million from tetramethylsilane based on the lock solvent.

Resistance to endodextranase (EC 3.2.1.11) hydrolysis was measured by suspending a weighed amount (~20–400 mg) of water-insoluble glucan in 8 mL of sodium acetate buffer and adding 200 μL of *Chaetomium* sp. endodextranase (30,000 U/mL, Amano Enzyme USA, Elgin, IL). The reaction tubes were mixed by rocking at room temperature for 1–2 days and then centrifuged to sediment the material remaining insoluble. In the case of larger amounts of starting material (>100 mg), a second dextranase treatment was carried out. The sediment was washed three times by resuspending in water and centrifugation, then dehydrated by washing once in 50 % ethanol and once in absolute ethanol, and dried at 50 °C in a vacuum oven. The amount remaining insoluble was determined by weighing.

Where indicated, means, standard deviations, standard errors, probability (P), and significance levels were calculated using the one-way ANOVA utility in SigmaPlot software (ver. 11.0, SyStat Software, San Jose, CA). At least three replicate analyses were performed whenever statistical values are given.

Results

As expected, given our culture conditions, most of the bacteria isolated from the beverage starters were either lactic acid bacteria or members of the Acetobacter/Gluconobacter group. Of the many strains of bacteria isolated from the starter cultures, only those that appeared to produce polysaccharide on sucrose plates were selected for quantitative measurement of water-insoluble glucan (Table 1). Although many of the cultures produced visible amounts of polysaccharide on sucrose-containing plates, only a few produced significant amounts of water-insoluble polysaccharide in liquid cultures. It is presumed that the polysaccharides produced by most of the others were mainly water-soluble. Isolate WK2G9B produced the largest amount of insoluble polysaccharide (Table 1). It was identified as *L. satsumensis,* and has been deposited in the USDA-ARS culture collection as strain NRRL B-59839. When grown in liquid medium with sucrose as the carbon source, NRRL B-59839 produced soluble glucansucrases which could be measured in the cell-free culture fluid. Using the ^{14}C-sucrose method for measurement of enzyme activity, no measurable levels of glucansucrases were detected when this strain was grown on raffinose, glucose, or fructose as the main carbon source. Furthermore, no measurable level of fructansucrase activity was detected in the sucrose-grown culture fluid. When the concentrated culture fluid was analyzed by SDS-PAGE, at least two glucansucrase bands were detected, a major one at 208,000 Da and a minor one at 259,000 Da (Fig. 1).

When compared with two strains of *L. mesenteroides* that are known to produce water-insoluble glucans (Table 2), *L. satsumensis* NRRL B-59839 produced significantly more insoluble product than *L. mesenteroides* NRRL B-523, and a similar amount to *L. mesenteroides* NRRL B-1118. Moreover, *L. satsumensis* NRRL B-59839 produced significantly more soluble dextran than either *L. mesenteroides* strain ($P<0.05$), which did not differ significantly from each other in this regard ($P=0.79$). Also compared were the levels of soluble glucansucrase activity in the culture fluids after 48 h. As Table 2 shows, *L. satsumensis* NRRL B-59839 produced somewhat less soluble glucansucrase activity than NRRL B-1118 ($P=0.03$), but a similar amount to NRRL strain B-523 ($P=0.13$) (Table 2). The soluble enzymes present in the concentrated and dialyzed culture fluid from *L. satsumensis* NRRL B-59839 produced much more soluble glucan (2.75±0.19 g) than insoluble glucan (0.210 ±0.018 g) in a 100-mL reaction mixture with 0.3 M sucrose.

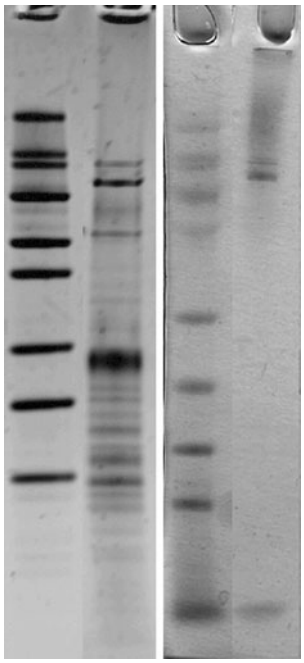

Fig. 1 SDS-PAGE of culture fluid, concentrated and dialyzed, from *L. satsumensis* NRRL B-59839 grown on sucrose + maltose medium. *Left*: Stained for protein. Standards, *top to bottom* (kDa): 500, 290, 240, 160, 116, 97, 66, 55, and 40. *Right*: stained for glucansucrase activity. Standards, *top to bottom* (kDa): 460, 265, 238, 171, 117, 71, 55, 41, and 31. *Band at bottom of activity gel* is tracking dye

Table 2 Soluble glucansucrase and soluble and insoluble glucan production by *L. satsumensis* NRRL B-59839 cultures grown on 2 % (*w*/*v*) sucrose compared to two strains of *L. mesenteroides*

Strain	Soluble glucan (g/100 mL ± SD)	Insoluble glucan (g/100 mL ± SD)	Soluble glucansucrase (IU/mL ± SD)
Lactobacillus satsumensis NRRL B-59839	0.36±0.12	0.59±0.26	0.046±0.014
Leuconostoc mesenteroides NRRL B-523	0.13±0.06	0.29±0.017	0.030±0.007
Leuconostoc mesenteroides NRRL B-1118	0.16±0.08	0.55±0.17	0.069±0.008

Two glucan fractions could be isolated from the reaction of resting cells with buffered sucrose, and two from reaction of concentrated cell-free culture fluid with buffered sucrose. In each case, one product was water-insoluble and one water-soluble. Structural analysis by methylation linkage analysis and by NMR spectroscopy showed that the water-soluble fraction from both preparations consisted mainly of α-(1→6)-linked glucose units (66 %) with approximately 10–20 % branching through 3,6-disubstituted glucose units. Both fractions also contained a trace (~5 %) of α-(1→3)-linked glucose units (Table 3) which may or may not be significant. A water-insoluble glucan fraction was also produced by both resting cells and cell-free enzyme. The water-insoluble glucan isolated from resting cells after incubation with sucrose contained 20 % of linear 3-linked glucose units and 55 % of 6-linked glucose units, whereas the water-insoluble glucan produced by cell-free enzyme contained 37 % of α-(1→3)-linked and 44 % of α-(1→6)-linked glucosyl residues (Table 3).

Both of the water-insoluble glucans were treated with endodextranase, and the amount remaining water insoluble after complete hydrolysis was determined. The insoluble glucan from cell-free enzyme was more resistant to endodextranase hydrolysis (22 %±2.5 remained water-insoluble) than the glucan produced by cell-associated enzyme (12.8 %±0.4 remained water insoluble).

Discussion

According to the original species description, *L. satsumensis* characteristically produces "dextran" from sucrose (Endo and Okada 2005). Although no reports have been published which describe the dextran(s) produced by this species per se, it must be kept in mind that bacteria identified as other species prior to 2005 may actually be *L. satsumensis*. In fact, one such example was recently described. A bacterium originally identified as *Streptobacterium dextranicum* nov. spec., strain H2, (Perquin 1940) and accessioned in our culture collection as NRRL strain B-1254 has now been identified as *L. satsumensis* (Leathers and Bischoff 2011). This strain produces at least two different water-soluble dextrans (Jeanes et al. 1954; Wilham et al. 1955), but is not known to produce water-insoluble glucans, at least under the conditions used in those studies. Thus, the present study is the first description of a water-insoluble glucan from *L. satsumensis*. Both examples of *L. satsumensis* whose dextrans have been studied, namely NRRL B-1254 and NRRL B-59839, are now known to produce at least two glucansucrases each.

The system described here differs in one significant respect from the water kefir dextrans previously described. In those cases where linkage analyses were performed, the water-insoluble glucans contained a relatively low amount (<10 mole %) of linear α-(1→3)-linkages as indicated by 3-monosubstituted glucosyl residues (Horisberger 1969; Pidoux et al. 1988; Pidoux et al. 1990). In the present system, the water-insoluble glucan produced by cell-free enzyme solutions contained nearly 40 mole% of 3-monosubstituted glucosyl residues. This is comparable to the insoluble glucan produced by a cloned enzyme from *L. mesenteroides* NRRL B-1118 (Côté and Skory 2012).

When compared with two strains of *L. mesenteroides* that are known to produce water-insoluble glucans, *L. satsumensis* seems to be an attractive option for further study, based on enzyme and glucan yields (Table 2). It produced more soluble and insoluble glucan than either strain, and a comparable level of soluble glucansucrase activity. We are currently cloning the glucansucrase genes from this strain.

In addition to soluble glucansucrases, the resting cell suspensions contained significant amounts of bound glucansucrase

Table 3 Methylation structural analysis of soluble and insoluble glucans from *L. satsumensis* NRRL B-59839

Percentages are mole percent of each per-*O*-acetylated aldononitrile derivative ± std. deviation

Sample	2,3,4,6-tetra-*O*-Me	2,4,6-tri-*O*-Me	2,3,4-tri-*O*-Me	2,4-di-*O*-Me
Cell-free enzymic soluble	17±4	5±5	66±2	12±1
Cell-associated soluble	23±8	4±4	66±6	9±9
Cell-free enzymic insoluble	14±2	37±3	44±2	5±3
Cell-associated insoluble	16±4	20±3	55±2	9±4

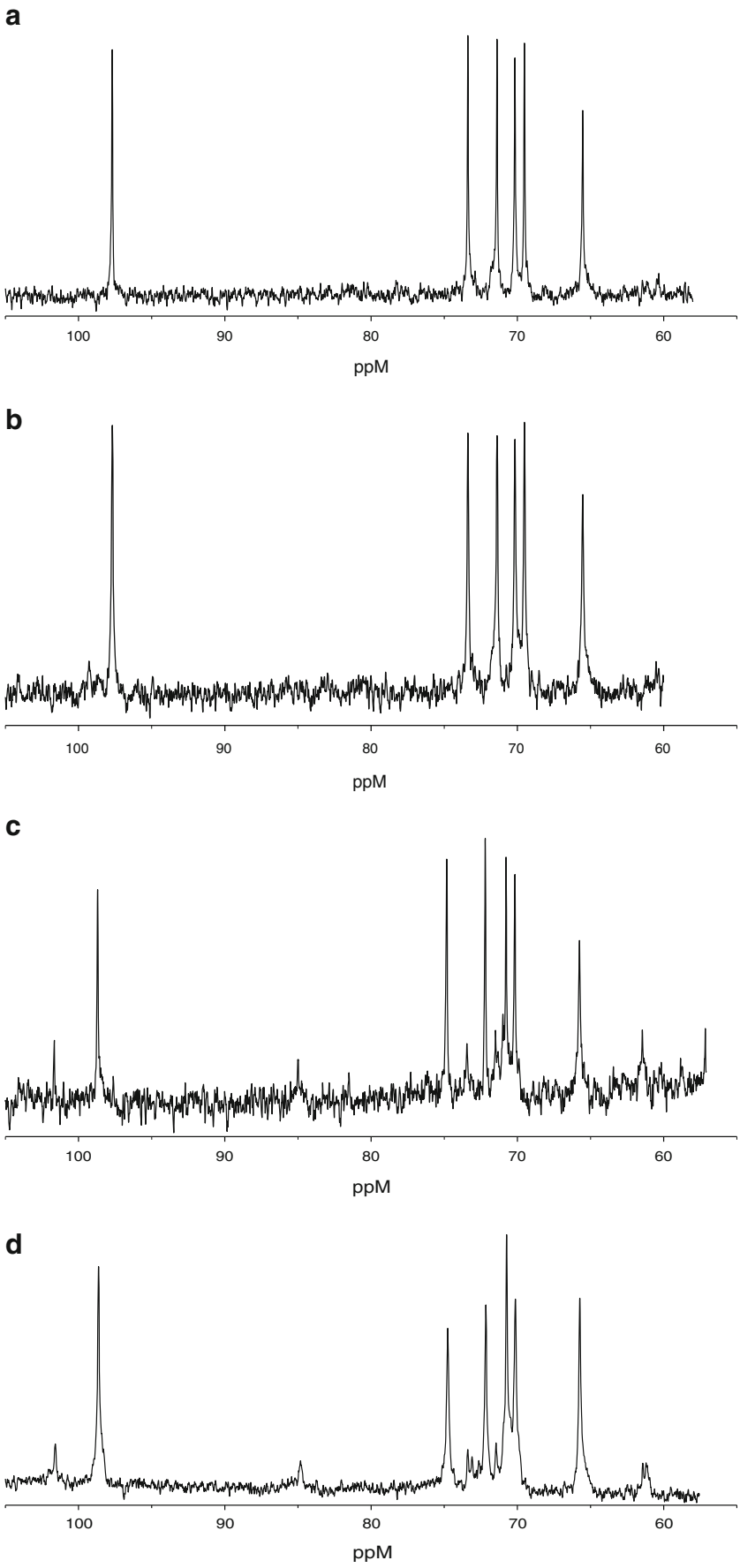

Fig. 2 **a** ^{13}C NMR spectrum of water-soluble glucan from cell-free enzyme. The single peak at ~98 ppm arises from anomeric carbon (C-1) in an α(1→6) linkage. The single peak at ~66 ppm arises from C-6 in the same linkage. **b** ^{13}C NMR spectrum of water-soluble glucan from cell suspension. The single peak at ~98 ppm arises from anomeric carbon (C-1) in an α(1→6) linkage. The single peak at ~66 ppm arises from C-6 in the same linkage. **c** ^{13}C NMR spectrum of water-insoluble glucan from cell-free enzyme. The large peak at ~98 ppm arises from anomeric carbon (C-1) in an α(1→6) linkage. The peak at ~66 ppm arises from C-6 in the same linkage. The smaller peak at ~102 ppm arise from anomeric carbon (C-1) in an α(1→3) linkage, and the smaller peak at ~85 ppm arises from C-3 in the latter linkage. **d** ^{13}C NMR spectrum of water-insoluble glucan from cell suspension. The large peak at ~98 ppm arises from anomeric carbon (C-1) in an α(1→6) linkage. The peak at ~66 ppm arises from C-6 in the same linkage. The smaller peak at ~102 ppm arise from anomeric carbon (C-1) in an α(1→3) linkage, and the smaller peak at ~85 ppm arises from C-3 in the latter linkage

activity, as evidenced by their production of both soluble and insoluble glucan. The water-soluble fractions were both similar to typical dextrans, having mainly α-(1→6)-linkages with a small but significant degree of branching through 3,6-disubstituted glucosyl residues. The branching was determined by methylation analysis to be on the order of approximately 10–20 %, with a fairly large degree of error. Branching was not detected by ^{13}C-NMR spectroscopy (Fig. 2a, b), owing to the relatively low occurrence of branch residues, but was detectable by ^{1}H-NMR as minor peaks at 5.27 ppm (spectra not shown).

Although the water-soluble dextran produced by resting cells did not differ markedly from the water-soluble dextran produced by cell-free enzyme (Table 3; Fig. 2a, b), the same was not true for the water-insoluble glucans. As Table 3 shows, the cell-associated insoluble glucan contained a higher percentage of linear α-(1→6)-linkages than that produced by cell-free enzyme, and a correspondingly lower percentage of linear α-(1→3)-linkages. The percent branching was not significantly different. Although the ^{13}C-NMR spectra (Fig. 2c, d) show the presence of both linkage types, the signals from the anomeric and C-3 carbons involved in these linkages are not sensitive enough to demonstrate the differences. It must be borne in mind that NMR spectroscopy is not as reliably quantitative as methylation analysis, due to differences in sensitivity of different nuclei, depending on degree of solvation and other variables. Therefore, because of this and because only methylation analysis can differentiate between 1,3-linkages at branch points and in linear sequences, we chose to use the methylation data as the primary structural method here. However, due to the tedious nature of methylation analysis and its dependence on painstaking analytical technique, NMR does provide a useful complement to methylation and a good basis for comparison of similar samples. Also providing evidence for different structures was the difference in dextranase susceptibility. Whereas 22 % of the glucan from cell-free enzyme remained water insoluble after dextranase hydrolysis, only 12.8 % of the glucan from cell-associated enzyme remained water-insoluble after identical treatment. This is an indication that the sequences of α-(1→6)-linked glucose units in the cell-associated glucan are longer and/or more frequently occurring than in the insoluble glucan from cell-free enzyme. We are currently investigating how this difference might arise in mixed-enzyme systems, using *L. mesenteroides* NRRL B-1118 and cloned enzymes. Although it is generally known that sequences of α-(1→3)-linked D-glucose units render a glucan water-insoluble, it is not known how long these sequences must be in order to adopt a water-insoluble conformation. Furthermore, the terms "soluble" and "insoluble" are, of course, relative terms. It is our experience that some of the so-called water-insoluble glucans are slightly water-soluble, so that they will eventually dissolve in water at very low concentrations. This is an important point when washing water-insoluble glucans to remove more soluble contaminants. The complex structures and physical properties of these systems containing both "water-soluble" and "water-insoluble glucans" require much more study on a biosynthetic level.

Polyacrylamide gel electrophoresis (Fig. 1) indicates two high-MW glucansucrase bands at approximately 208 and 259 kDa. Although it is tempting to attribute soluble glucan production to the major enzyme band and insoluble glucan production to the minor one, based on the relative proportion of each produced by the culture fluid, we do not have any definitive proof that this is the case.

The practical applications of water-insoluble α-D-glucans have not been explored to any great extent, but uses have been suggested. Pidoux (1989) observed that the insoluble tibi grains may represent an alternative to alginate or other gelling systems for cell immobilization. The use of water-insoluble α-D-glucans as spun hydrophilic fibers has been patented (Novak and Hogue 1956; Hiler 1961; O'Brien 2006), as have other potential applications (Novak 1962; Schwartz and Bodie 1983). However, except for the water-insoluble "mutan" from cariogenic streptococci, very little is known about the biosynthesis and structural details of these polymers.

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Conflict of interest Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

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