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Biochemical and Structural Characterization of the Glucan and Fructan Exopolysaccharides Synthesized by the *Lactobacillus reuteri* Wild-Type Strain and by Mutant Strains

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Lactobacillus reuteri **LB 121 cells growing on sucrose synthesize large amounts of a glucan (D-glucose) and a fructan (D-fructose) with molecular masses of 3,500 and 150 kDa, respectively. Methylation studies and ^{13}C or ^{1}H nuclear magnetic resonance analysis showed that the glucan has a unique structure consisting of terminal, 4-substituted, 6-substituted, and 4,6-disubstituted α-glucose in a molar ratio of 1.1:2.7:1.5:1.0. The fructan was identified as a (2→6)-β-D-fructofuranan or levan, the first example of levan synthesis by a *Lactobacillus* species. Strain LB 121 possesses glucansucrase and levansucrase enzymes that occur in a cell-associated and a cell-free state after growth on sucrose, raffinose, or maltose but remain cell associated during growth on glucose. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sucrose culture supernatants, followed by staining of gels for polysaccharide synthesizing activity with sucrose as a substrate, revealed the presence of a single glucansucrase protein of 146 kDa. Growth of strain LB 121 in chemostat cultures resulted in rapid accumulation of spontaneous exopolysaccharide-negative mutants that had lost both glucansucrase and levansucrase (e.g., strain K-24). Mutants lacking all levansucrase activity specifically emerged following a pH shiftdown (e.g., strain 35-5). Strain 35-5 still possessed glucansucrase and synthesized wild-type glucan.**

A variety of high-molecular-weight polysaccharides produced by plants (cellulose, pectin, and starch), seaweeds (alginate and carrageenan), and bacteria (alginate, gellan, and xanthan) find applications as viscosifying, stabilizing, emulsifying, gelling, or water-binding agents in food and nonfood industries (43, 44, 48). All of these polysaccharides are additives, however, and therefore they are considered less desirable in the food industry.

Lactic acid bacteria are food-grade organisms that possess GRAS (generally recognized as safe) status and are known to produce an abundant variety of exopolysaccharide (EPS) molecules (4, 9, 37), which contribute to the texture of fermented milk. EPS from these bacteria may allow development of a new generation of food-grade polysaccharides. Lactic acid bacteria often also contribute positively to the taste, smell, or preservation of the final product.

Synthesis of heteropolysaccharides by lactic acid bacteria, including lactobacilli, is currently being studied intensively (3, 4, 12, 18, 34, 42, 45, 47). Synthesis of homopolysaccharides (e.g., dextran and levan) has been studied mainly in *Leuconostoc mesenteroides* and in streptococci (4, 31, 32, 35). Limited information is available about homopolysaccharide biosynthesis in lactobacilli (9, 33, 38).

Recently, we have screened a large collection of lactobacilli for strains producing EPS from sucrose. One of these strains, identified as *Lactobacillus reuteri* LB 121, synthesized large amounts of water-soluble EPS material with both glucose and fructose as constituents (46). The present study reports the biochemical and mutational identification of the biosynthetic enzymes involved and provides a structural characterization of the glucan and fructan synthesized by strain LB 121.

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MATERIALS AND METHODS

Strains, media, and growth conditions. *L. reuteri* LB 121 (LMG 18388) and mutants derived from it, strain 35-5 (LMG 18390) and strain K-24 (LMG 18391), were grown anaerobically at 37°C in MRS medium (7). Modified MRS media, containing 100 g of raffinose (MRS-r) or sucrose (MRS-s) liter^{-1}, instead of the 20 g of glucose liter^{-1} normally present in MRS medium, was used for EPS production under cultivation conditions with or without pH control (46). When appropriate, media were solidified with 20 g of agar liter^{-1}. All media were autoclaved for 15 min at 121°C. Sugars were autoclaved separately.

Chemostat cultivation (Bioflow III fermentors; working volume, 1.5 liter) was performed in 0.5× MRS-s medium flushed with nitrogen. The pH was kept automatically at 5.5 with 4 M NaOH. After ca. 5 h of growth, fresh medium was pumped into the fermentor at a dilution rate of 0.05, 0.1, 0.2, or 0.4 h^{-1}.

Identification of spontaneous mutants in EPS biosynthesis. Samples from chemostat cultures were appropriately diluted and spread onto MRS agar plates. A number of colonies from each plate were picked randomly and grown anaerobically in culture tubes containing 10 ml of MRS-s. After 3 days of growth, EPS was isolated and determined as described below. Mutant strains producing either no EPS or EPS with a different appearance when purified and dried were selected for further studies.

Enzyme assays. Glucansucrase (EC 2.4.1.5) and levansucrase (EC 2.4.1.10) activities were measured at 37°C by monitoring the release of fructose and glucose, respectively, from sucrose. Reaction mixtures (1 ml) contained $CaCl_2$ (50 mg · liter^{-1}), acetate buffer (200 mM, pH 5.5), sucrose (50 mM), and appropriately diluted enzyme. Samples (100:1) were withdrawn at regular intervals, and 5:1 2 M NaOH was added to stop the reactions. Glucose and fructose formed were quantified enzymatically by monitoring the reduction of NADP as described previously (29). Glucose was measured first in a reaction mixture containing Tris-HCl (50 mM, pH 7.6), ATP (2.5 mM), NADP (1 mM), $MgSO_4$ (10 mM), hexokinase (3,000 U · liter^{-1}), and glucose-6-phosphate dehydrogenase (1,500 U · liter^{-1}). Fructose concentrations were measured in the same

reaction mixture but with the addition of phosphoglucoisomerase (7,000 U · liter^{-1}). One glucansucrase or levansucrase activity unit is defined as the amount of enzyme producing 1 μmol of monosaccharide per min. All enzyme assays were performed in triplicate; data presented are averages with a standard deviation of less than 10%.

Activity staining of EPS synthesizing enzymes. MRS, MRS-2, and MRS-4 (10 ml) media were inoculated with 200:1 dilutions of overnight cultures of strains LB 121, 35-5, and K-24 and incubated at 37°C for 8 h. Cells were removed by centrifugation, and proteins in the supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see below). After electrophoresis the gels were washed three times with demineralized water and incubated overnight at 37°C in acetate buffer (pH 5.5; 50 mM hydrogen acetate, 1% [vol/vol] Tween 80, 1 mM $CaCl_2$) with 1% (wt/vol) sucrose. Glucansucrase activities were detected by staining gels for polysaccharides by a periodic acid-Schiff (PAS) procedure (50). Gels loaded with the same supernatants and incubated without sucrose were used as controls.

Enzyme localization studies. Cells of strain LB 121 and of mutant strain 35-5, in the exponential phase of growth on MRS with 30 g of glucose, maltose, raffinose, or sucrose liter^{-1}, were harvested by centrifugation (25 min for 2,500 × *g*). The cells were washed twice with 0.05 M citric acid–0.1 M Na_2HPO_4 (pH 5.5) and resuspended in the same buffer containing 50 g of sucrose liter^{-1} and 50 μg of chloramphenicol ml^{-1}. Cell suspensions were incubated anaerobically at 37°C and sampled at regular intervals to determine the amount and monosaccharide composition (see below) of the EPS produced. Supernatants were filtered through a 0.2-μm-pore-size filter (Millipore), diluted 1:1 with 0.1 M citric acid–0.2 M Na_2HPO_4 (pH 5.5) with 50 g of sucrose liter^{-1}, and treated in the same way as the cell suspensions.

Isolation and purification of EPS. Cells were harvested by centrifugation (10 min for 11,000 × *g*). Two volumes of cold ethanol were added to culture supernatants, and the mixtures stored overnight at 4°C. Precipitated material was collected by centrifugation (20 min at 2,500 × *g*), resuspended in demineralized water, and mixed with 2 volumes of cold ethanol. Samples were centrifuged (20 min at 2,500 × *g*), and the pellets were dried at 100°C. EPS amounts were determined by measuring final dry weights.

HP-GPC, GLC, and MS. High-performance gel permeation chromatography (HP-GPC) analysis was carried out at room temperature by using a Progel TSK guard column, followed by a Progel TSK G6000 PW column, and a refractive index detector (Erna ERC-7510). Samples were eluted at a flow rate of 0.6 ml · min^{-1} with 0.1 M $NaNO_3$ as a mobile phase. Gas-liquid chromatography (GLC) measurements were performed on a Chrompack CP9002 gas chromatograph equipped with a CP-Sil 5CB fused silica capillary column (25 m by 0.32 mm; Chrompack) with a temperature program of 120 to 240°C at 4°C/min. GLC data were collected and processed by using Maestro Chromatography Software. GLC-mass spectrometry (MS) analysis was carried out on a MD800/8060 system (electron energy, 70 eV; Fisons Instruments) by using a DB-1 fused silica capillary column (30 m by 0.32 mm; J&W Scientific). A temperature program of 140 to 240°C at 4°C/min was used.

Molecular mass determination. The average molecular mass of the polysaccharides was determined by size exclusion chromatography. To determine the size distribution of the polysaccharides, EPS produced after 2 days of growth on MRS-s or MRS-r was isolated as described above. Instead of drying, EPS was dialyzed (cellulose dialysis tube [Sigma D-9777]; cutoff, 12 kDa) at 4°C against water for 3 days. Lyophilized EPS was dissolved in 0.1 M $NaNO_3$, filtered over a 0.45-μm filter (Millipore), and analyzed by HP-GPC (46).

Monosaccharide analysis. After complete hydrolysis of EPS (2 h in 1 M H_2SO_4 at 100°C), glucose was determined by HPLC (46), and fructose was measured by using an improved resorcinol reagent (49). The absolute configurations of the monosaccharides were determined by GLC analysis of the trimethylsilylated (−)-2-*butyl*-glycosides (13, 14) on CP-Sil 5CB.

Methylation analysis. Polysaccharides were permethylated by using methyl iodide and solid sodium hydroxide in methyl sulfoxide (6). After hydrolysis with 2 M trifluoroacetic acid (2 h, 120°C), the partially methylated monosaccharides were reduced with $NaBD_4$. After neutralization, removal of boric acid by coevaporation with methanol, and acetylation with acetic acid anhydride (3 h, 120°C), the mixtures of partially methylated alditol acetates obtained were analyzed by GLC on CP-Sil 43CB and by GLC-MS on DB-1 (23, 24).

NMR spectroscopy. Prior to nuclear magnetic resonance (NMR) spectroscopic analysis (Bijvoet Center Department of NMR Spectroscopy), samples were exchanged twice in 99.9 atom% D_2O (Isotec) with intermediate lyophilization and finally dissolved in 99.96 atom% D_2O (Isotec). Proton-decoupled 75.469-MHz [^{13}C]NMR spectra were recorded on a Bruker AC-300 spectrometer (probe temperature, 80°C). One-dimensional [1D] ^{1}H NMR spectra were recorded on a Bruker AMX-500 spectrometer (probe temperature, 80°C). The HOD signal was suppressed by applying a WEFT pulse sequence (19). Chemical shifts are expressed in parts per million by reference to internal acetone (δ = 2.225) for ^{1}H or to external methanol (δ = 49.00) for ^{13}C. Proton spectra were recorded in 16K data sets, with a spectral width of 5,000 Hz. Resolution enhancement of the spectra was performed by a Lorentzian-to-Gaussian transformation; when necessary, a fourth-order polynomial baseline correction was performed.

Gel electrophoresis. SDS-PAGE was performed according to Laemmli (27) by using the Phast System from Pharmacia with 10 to 15% polyacrylamide gels. After activity staining, the gels were silver stained (21). Lysozyme (molecular mass, 14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), and phosphorylase *b* (97,400) were used as molecular mass references.

Other assays. Fermentation patterns of the bacterial strains were established by using API CHL 50 tests (bioMérieux, Marcy l'Etoile, France). Protein was determined according to the method of Lowry et al. (28) with bovine serum albumin as a standard. Intact cells were first boiled for 20 min in 1 M NaOH.

Chemicals. All biochemicals were obtained from Boehringer Mannheim.

TABLE 1. Amount and composition of EPS synthesized by *L. reuteri* strains grown on MRS-s and MRS-r at 37°C for 3 days

Strain	Sugar in medium	EPS concn (g · liter^{-1})	% D-Glucose in EPS (wt/wt)	% D-Fructose in EPS (wt/wt)
LB 121	Sucrose	9.8	32	68
	Raffinose	7.3	0	100
35-5	Sucrose	9.7	100	0
	Raffinose	0	0	0
K-24	Sucrose	0		
	Raffinose	0		

RESULTS

EPS synthesis by strain LB 121. Strain LB 121 grown in batch cultures, with or without pH control, produced large amounts of nonropy EPS on both MRS-s and MRS-r media (Table 1). Monosaccharide analysis, including determination of absolute configurations, revealed the presence of both D-glucose and D-fructose (in a 1:2 ratio) in the EPS material synthesized by strain LB 121 grown on MRS-s; on MRS-r a polymer with only D-fructose was produced (Table 1). Repeated subculturing (ca. 350 generations) of strain LB 121 on MRS-s in batch culture did not affect EPS levels.

Isolation of spontaneous mutants in EPS biosynthesis. During growth of strain LB 121 in chemostat cultures the amounts of EPS synthesized varied strongly, and no stable and reproducible steady states were obtained. This was due to accumulation of spontaneous mutants in chemostat cultures. Continuous cultivation of strain LB 121 at pH 5.5 resulted in a rapidly decreasing EPS production in time: EPS concentrations dropped from 10 g · liter^{-1} in batch cultures on MRS-s to 1.5 and 2.5 g · liter^{-1} after 20 generations of growth on 0.5× MRS-s at dilution rates of 0.05 and 0.2 h^{-1}, respectively. In view of the nonropy character of strain LB 121 EPS, samples from these cultures were spread first onto MRS agar (no EPS production), and individual colonies were checked for the ability to synthesize EPS during growth in batch culture in MRS-s liquid medium. After 20 generations of growth, 25 individual colonies were examined for EPS production. Of these colonies, 21 produced no EPS at all (e.g., mutant strain K-24) or less than 1 g · liter^{-1}; only 4 colonies produced the same amount of EPS as strain LB 121 (about 10 g · liter^{-1}). Strain K-24 produced no EPS on either MRS-s or MRS-r (Table 1); no EPS synthesizing revertants were observed during further studies.

Strain LB 121 cells growing in chemostat cultures at pH 5.5 and dilution rates of 0.05 or 0.2 h^{-1} were also subjected to a shiftdown to pH 4.5. Within 10 generations of growth at either dilution rate, numerous mutants (e.g., strain 35-5) were identified to be producing EPS material that, when dried, had a different appearance from that of strain LB 121 EPS and was composed of D-glucose only. All mutants tested (>25) in these experiments synthesized EPS material with D-glucose only. Interestingly, strain 35-5 grown in batch culture on MRS-s produced the same amount of EPS as strain LB 121 (about 10 g · liter^{-1}), but this was now composed of glucose only. No EPS was synthesized by strain 35-5 in MRS-r medium (Table 1).

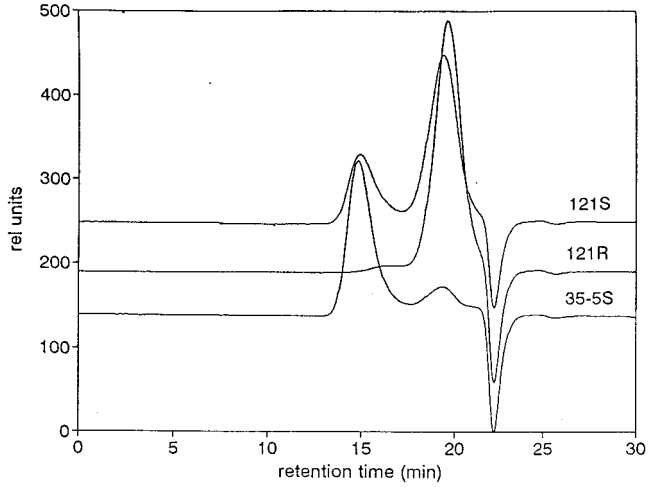

FIG. 1. HP-GPC elution patterns of native EPS from *Lactobacillus reuteri* strain LB 121 grown on sucrose (121S), strain LB 121 grown on raffinose (121R), and mutant strain 35-5 grown on sucrose (35-5S).

TABLE 2. ^{1}H and ^{13}C NMR chemical shifts of the fructan recorded in D_2O at 80°C

^{1}H and ^{13}C NMR	Chemical shift[a] (coupling constant [Hz])
Proton	
H-1a	3.750 (12.3)
H-1b	3.704 (12.1)
H-3	4.176 (8.2)
H-4	4.090 (7.9)
H-5	3.941 (3.3)
H-6a	3.891 (10.6)
H-6b	3.631 (7.7)
Carbon	
C-1	61.7
C-2	105.0
C-3	78.1
C-4	76.6
C-5	81.2
C-6	64.3

[a] Given in parts per million relative to the signal of internal acetone (δ = 2.225) for proton and relative to the α-anomeric signal of external glucose (δ = 49.00) for carbon. Coupling constants (Hz) are included in parentheses.

Mutant strain 35-5 turned out to be very stable during further studies. Mutants producing EPS composed of fructose only were not detected. Nonproducing mutants similar to strain K-24 did not appear in chemostat cultures run at pH 4.5 for prolonged periods of time. After a switch of cultures back to pH 5.5, nonproducing mutants similar to strain K-24 started to accumulate again.

Strains 35-5 and K-24 showed the same fermentation profiles as strain LB 121 in API50 CHL tests, including the ability to ferment sucrose and raffinose, confirming the identity of the mutant strains as derivatives of strain LB 121. The mutations resulting in loss of EPS synthesizing activity apparently have not affected the ability of these strains to grow on the various sugar (mono- and disaccharides) substrates tested. Strains K-24 and 35-5 were selected for further characterization.

EPS size and monosaccharide analysis. The HP-GPC elution patterns and size distribution analysis of the different EPS species synthesized by strains LB 121 and 35-5 were studied (Fig. 1). On MRS-s, strain LB 121 produced EPS with two size distributions (at 15.0 and 19.4 min). Strain LB 121 on MRS-r (19.4 min) and strain 35-5 on MRS-s (15.0 min) synthesized EPS with one size distribution. Monosaccharide analysis of HP-GPC fractions revealed that the polymer eluting at 19.4 min consisted solely of fructose (fructan), whereas the polymer eluting at 15.0 min consisted solely of glucose (glucan). Molecular masses of 3,500 and 150 kDa were determined for the glucan and fructan, respectively. Strain LB 121 thus synthesizes both a glucan and a fructan on sucrose. On raffinose, strain LB 121 produces the fructan only. Mutant strain 35-5 synthesizes only the glucan on sucrose and has lost the ability to produce the fructan (Table 1 and Fig. 1).

Methylation analysis. Methylation analysis showed that the EPS synthesized by strain LB 121 on MRS-s consists of terminal, 4-substituted, 6-substituted, and 4,6-disubstituted glucose in a molar ratio of 1.1:2.7:1.5:1.0, together with a large amount of 6-substituted fructose. These results, in combination with data presented above, indicate the presence of a branched glucan and a uniformly linked fructan. Methylation analysis of the EPS synthesized by strain LB 121 on MRS-r revealed the presence of merely 6-substituted fructose, indicating a uniformly linked fructan (levan). Methylation analysis of the EPS synthesized by mutant strain 35-5 on MRS-s revealed the presence of the same four glucose derivatives, in a comparable molar ratio, as identified in the strain LB 121 EPS produced on MRS-s.

NMR spectroscopy. In the 1D ^{13}C NMR spectrum of the fructan synthesized by strain LB 121 on MRS-r (spectrum not shown), six carbon signals are observed (Table 2). The C-2 resonance (δ = 105.0) indicates the occurrence of β-fructofuranose. Comparison of the ^{13}C chemical shifts of the fructan with published chemical shifts of Me-β-D-Fru*f* (1) and *Zymomonas mobilis* levan (2) demonstrates the fructan to be: $[\rightarrow 6)$-β-D-Fru*f*-$(2\rightarrow]_n$. In the 1D ^{1}H NMR spectrum of the fructan (Fig. 2B and Table 2), no signals in the anomeric region (δ = 5.3 to 4.3) were found, confirming the absence of anomeric protons. The observed peak pattern fits the fructofuranose configuration.

The 1D ^{1}H NMR spectrum of the glucan synthesized by strain 35-5 grown on MRS-s (Fig. 2C) showed two broad signals in the anomeric region (δ = ~4.97 and ~5.35). Comparison of the spectrum with ^{1}H NMR data of potato starch (15) demonstrates that the glucan consists of (1→4)- and (1→6)-linked α-glucopyranose residues. Due to poor resolution of the spectrum, it is not possible to trace the terminal and the (1→4,6)-linked residues as indicated by the methylation analysis. The line shape of the anomeric signals is characteristic for a glucan with various glucosyl linkages.

Comparison of the 1D ^{1}H NMR spectrum of the polysaccharide material synthesized by strain LB 121 grown on MRS-s (Fig. 2A) with the spectra of the fructan and the glucan demonstrates that both the fructan and the glucan are synthesized by strain LB 121 grown on MRS-s.

EPS biosynthetic enzymes. High activities of both glucansucrase (5.7 U mg of protein^{-1}) and levansucrase (6.9 U mg of protein^{-1}) were detected in supernatants of strain LB 121 cultures grown on MRS-s. Supernatants of mutant strain 35-5 cultures grown on MRS-s only contained glucansucrase activity (4.4 U mg of protein^{-1}); strain 35-5 had lost all levansucrase activity. Mutant strain K-24 had completely lost both glucansucrase and levansucrase activities. In strain LB 121 both sucrase enzymes showed maximum activity in the stationary phase of growth. In contrast, glucansucrase of strain 35-5

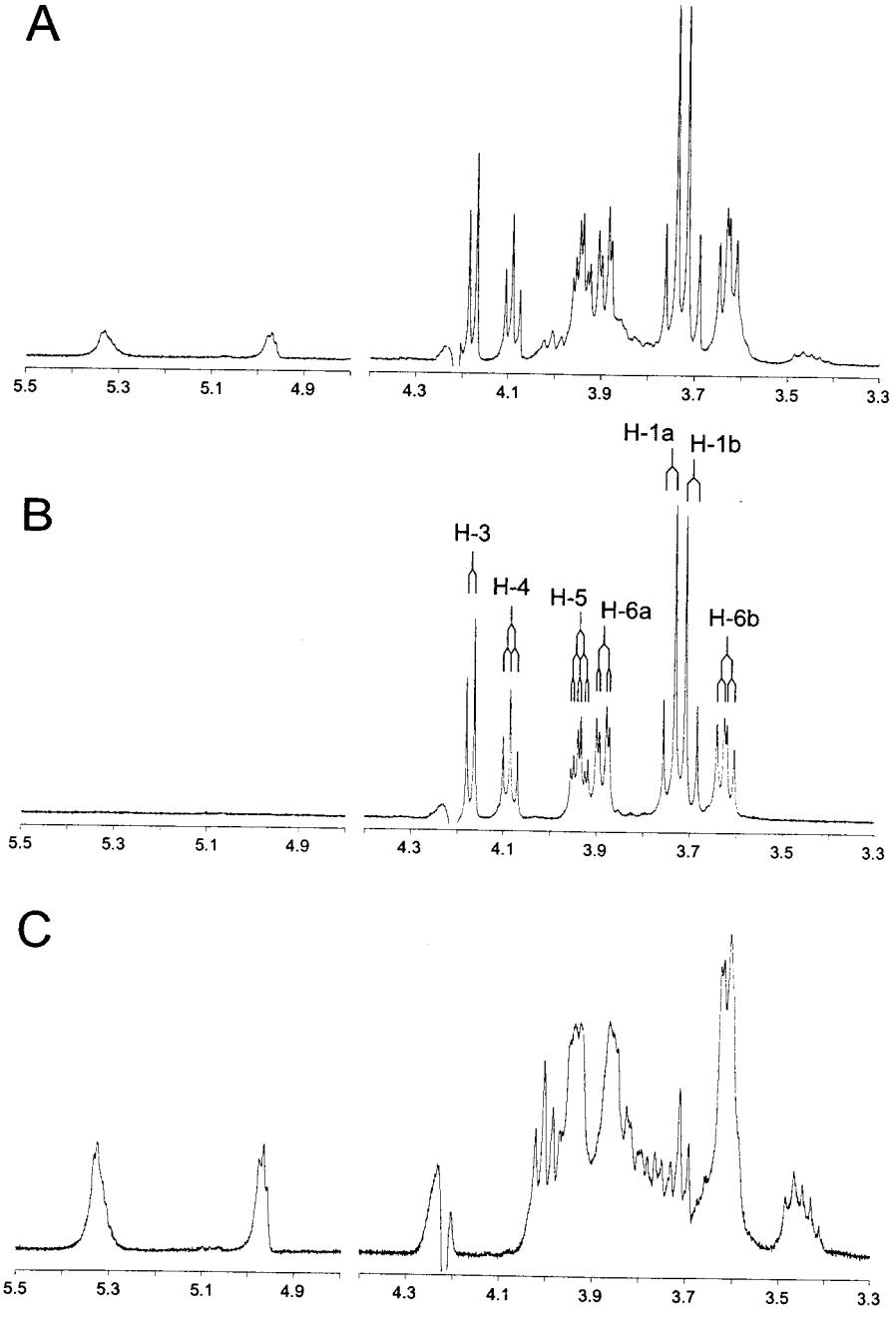

FIG. 2. A 500-MHz ^{1}H NMR spectra of EPS produced by *L. reuteri* LB 121 grown on sucrose (mixture of glucan and fructan) (A), strain LB 121 grown on raffinose (fructan) (B), and mutant strain 35-5 grown on sucrose (glucan) (C) recorded in D_2O at 80°C.

reached maximum activity during exponential growth and declined drastically at the end of the growth phase, reaching a fairly low activity level (0.6 U mg of protein^{-1}) in supernatants of overnight cultures.

Activity staining of EPS biosynthetic enzymes. After SDS-PAGE of proteins in supernatants of cultures of strains LB 121, 35-5, and K-24 grown on various sugars, gels were incubated with sucrose. Proteins able to synthesize polysaccharides from sucrose were visualized by PAS staining (Fig. 3). Supernatants of strain LB 121 grown on sucrose or on raffinose and of mutant strain 35-5 grown on sucrose each showed a single activity band on the gels corresponding to enzymes with a molecular mass of 146 kDa. Supernatants of these strains grown on glucose did not show any activity bands with sucrose (see below). No activity bands were observed with mutant strain K-24. Control gels loaded with supernatant samples, but incubated without sucrose, did not show any bands after PAS staining. Incubation of the SDS-PAGE gels with raffinose followed by PAS staining did not reveal positive bands. Apparently, only glucan(sucrase activity) and not levan(sucrase activity) can be detected by PAS staining. The data also show that after SDS-PAGE, the single glucansucrase enzyme present is free of polysaccharide and has remained active, able to synthesize glucan when incubated with sucrose.

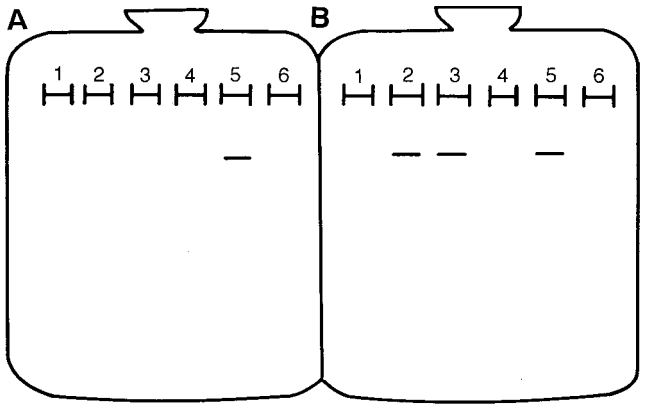

FIG. 3. Diagram of bands of proteins with glucansucrase activity present in supernatants of *L. reuteri* strains grown on MRS, MRS-s, or MRS-r and identified in SDS-PAGE gels by polysaccharide staining (PAS) after incubation with sucrose. After activity staining, the gels were silver stained (results not shown). (A) Lane 1, strain K-24 grown on MRS-r; lane 2, strain K-24 grown on MRS; lane 3, strain K-24 grown on MRS-s; lane 4, strain 35-5 grown on MRS-r; lane 5, strain LB 121 grown on MRS-s; lane 6, marker proteins. (B) Lane 1, strain 35-5 grown on MRS; lane 2, strain 35-5 grown on MRS-s; lane 3, strain LB 121 grown on MRS-r; lane 4, strain LB 121 grown on MRS; lane 5, strain LB 121 grown on MRS-s; lane 6, marker proteins.

Localization of EPS biosynthetic enzymes. Washed cells and supernatants of strain LB 121 cultures grown on MRS-s synthesized glucan as well as fructan when incubated with sucrose (Table 3). Washed cells and supernatants of strain 35-5 cultures grown on MRS-s synthesized only the glucan from sucrose. Similar observations were made with raffinose and maltose grown cells of strain LB 121 and strain 35-5 when incubated with sucrose (data not shown). The glucansucrase (strains LB 121 and 35-5) and levansucrase (strain LB 121) enzymes thus are synthesized during growth on various sugars; these enzymes occur both in a cell-associated and in a cell-free state after growth on sucrose, raffinose, or maltose. In contrast, no EPS synthesis was observed with supernatants of glucose-grown cells of strains LB 121 and 35-5 when incubated with sucrose. Washed cells of glucose grown cultures, however, clearly synthesized both glucan and fructan (strain LB 121) and glucan only (mutant strain 35-5) from sucrose. The EPS-synthesizing enzymes thus remain cell associated during growth on glucose (Table 3). Also, during incubations of washed cells in buffer (pH 5.5) with sucrose no release of EPS synthesizing enzymes was observed.

DISCUSSION

Lactic acid bacteria, e.g., *L. mesenteroides* strains and *Streptococcus* species, synthesize glucans with structures different from that of sucrose. Examples include dextrans with contiguous α-(1→6)-linked glucose residues, mutants with contiguous α-(1→3)-linked glucose residues, or alternans with alternating α-(1→6)- and α-(1→3)-linked glucose residues in the main chains. Different dextrans with various degrees of α-(1→2), α-(1→3), or α-(1→4) branching have been described (35). The present study shows that *L. reuteri* LB 121 synthesizes a high-molecular-mass branched α-glucan with terminal, 4-substituted, 6-substituted, and 4,6-disubstituted α-glucose residues. To the best of our knowledge such a glucan structure has not been described previously. The properties and possible industrial applications of this unique glucan, already produced in large amounts by wild-type *L. reuteri* LB 121 (reference 46 and this study), are currently under investigation.

L. reuteri LB 121 also synthesizes a low-molecular-mass (2→6)-β-D-fructofuranan (levan). Levan synthesis in lactic acid bacteria has been reported only for *Streptococcus* species (30, 40); the present study is the first report of the synthesis of a levan type of polysaccharide in the genus *Lactobacillus*.

The biochemical and mutant data presented here show that strain LB 121 employs glucansucrase and levansucrase enzymes to synthesize the glucan and levan from sucrose. Also, raffinose is a substrate for levan synthesis by the action of the levansucrase; raffinose is not a substrate for glucansucrase (36). Both enzymes are synthesized during growth on various sugars and occur in a cell-bound state and in a cell-free state in sucrose, raffinose, and maltose cultures, but only in a cell-bound state in glucose cultures.

The chemostat cultivation technique is a convenient tool for studying the effects of various environmental parameters on the physiology of microbial cells (8, 20). Attempts to study the physiology of EPS synthesis by strain LB 121 in chemostat

TABLE 3. EPS production by supernatants and washed cells of *L. reuteri* LB 121 and mutant strain 35-5 incubated at 37°C in buffer with sucrose as the substrate[a]

Sugar in growth medium	Washed cells or supernatant	Incubation time (h)	Glucan produced by strain 35-5 (g · liter^{-1})	Glucan produced by strain LB 121 (g · liter^{-1})	Fructan produced by strain LB 121 (g · liter^{-1})
Sucrose	Washed cells	0	0.28	ND	ND
		2	0.60	0.43	0.41
		10	3.12	1.19	0.92
		22	4.52	1.87	1.57
Sucrose	Supernatant	0	0.36	ND	ND
		2	0.44	0.37	0.55
		10	0.52	0.64	1.06
		22	1.00	0.85	1.70
Glucose	Washed cells	0	0.32	ND	ND
		2	1.24	0.89	0.27
		10	3.76	3.01	0.97
		22	5.72	4.35	1.51
Glucose	Supernatant	0	0.16	ND	ND
		2	0.08	ND	ND
		10	0.04	ND	ND
		22	0.12	ND	ND

[a] Cultures were harvested in the exponential phase of growth and incubated with sucrose plus 50 μg of chloramphenicol to block de novo enzyme synthesis. ND, not detected.

cultures failed, mainly because no stable steady-state conditions could be established due to the rapid accumulation of mutants. Instability of EPS production in lactic acid bacteria has been observed before during repeated transfer of cells in batch cultures (4, 5, 12, 25). Repeated subculturing (ca. 350 generations) of strain LB 121 on MRS-s in batch culture, however, did not affect EPS production. It remains unclear why EPS synthesis in strain LB 121 is unstable in chemostat cultures but not in batch cultures. Also, the nature of the (stable) mutations in strains 35-5 and K-24 remains to be characterized. Interestingly, although mutant strain 35-5 has lost all levansucrase activity, it still synthesizes the same total amount of EPS material as strain LB 121 when grown on sucrose, but this is now composed of the glucan only. Incubation of washed cells harvested from exponential-phase cultures of strains LB 121 and 35-5 with sucrose also resulted in the synthesis of similar amounts of EPS material.

Supernatants of sucrose-grown cultures of strain LB 121 possess both glucansucrase and levansucrase activities, but PAS staining of SDS-PAGE gels loaded with these supernatants and incubated with sucrose identified only glucan and not the levan. Incubation of these SDS-PAGE gels with raffinose followed by PAS staining did not reveal positive bands. Apparently, levan synthesis cannot be detected by PAS staining, or else the levansucrase enzyme is inactivated during SDS-PAGE. Accordingly, supernatants of strain LB 121 grown on raffinose only possessing both glucansucrase and levansucrase activity and of mutant strain 35-5 grown on sucrose possessing only the glucansucrase each displayed a single activity band with sucrose at the same 146-kDa position as in strain LB 121. The data thus indicate that the glucansucrase enzyme present in strains LB 121 and 35-5 is a monomeric enzyme with a molecular mass of 146 kDa. Glucosyltransferase proteins of *S. mutans* (22, 26, 39), *L. mesenteroides* (11), *S. downei* (17), *S. sobrinus* (10), and *S. salivarius* (16) have molecular masses of 130 to 180 kDa. The levansucrase protein of strain LB 121 remains to be identified and characterized with respect to its molecular mass and other properties. Fructosyltransferase enzymes studied in various bacteria have molecular masses between 50 and 100 kDa (see, for example, references 40, 41, and 43), whereas a 140-kDa enzyme was reported in *S. salivarius* (30).

The glucansucrase and levansucrase enzymes of *L. reuteri* LB 121, as well as the corresponding genes, will be characterized in more detail in further work.

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