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Levan-Producing *Leuconostoc citreum* Strain BD1707 and Its Growth in Tomato Juice Supplemented with Sucrose

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A levan-producing strain, BD1707, was isolated from Tibetan kefir and identified as *Leuconostoc citreum*. The effects of carbon sources on the growth of *L. citreum* BD1707 and levan production in tomato juice were measured. The changes in pH, viable cell count, sugar content, and levan yield in the cultured tomato juice supplemented with 15% (wt/vol) sucrose were also assayed. *L. citreum* BD1707 could synthesize more than 28 g/liter of levan in the tomato juice-sucrose medium when cultured at 30°C for 96 h. Based on the monosaccharide composition, molecular mass distribution, Fourier transform infrared (FTIR) spectra, and nuclear magnetic resonance (NMR) spectra, the levan synthesized by *L. citreum* BD1707 was composed of a linear backbone consisting of consecutive β-(2→6) linked D-fructofuranosyl units, with an estimated average molecular mass of 4.3×10^6 Da.

Levan is a natural fructan consisting of D-fructofuranosyl residues linked predominantly by β-(2,6) linkage in the backbone with occasional β-(2,1) branch chains. Sucrose is considered the sole carbon source for microbial levan synthesis. Levansucrase (sucrose:2,6-β-D-fructan 6-β-D-fructosyltransferase; EC 2.4.1.10), belonging to the glycoside hydrolase family 68 (GH68), is responsible for the catalysis of two reactions in the formation of levan: (i) transglycosylation, using the growing fructan chain or sucrose as the acceptor substrate, and (ii) hydrolysis of sucrose, in which water is used as the acceptor (1). First, sucrose is bound to the active center of the levansucrase and cleaved, which results in the release of glucose (Fig. 1A). Then, the remaining fructose moiety is transferred to the lengthening polyfructose chain (Fig. 1C). Alternatively, water reaches the catalytic center of levansucrase after cleavage of donor sucrose, serving as an alternative acceptor, which leads to the release of initially bound fructose and the complete hydrolysis of sucrose (Fig. 1B) (2). In nature, levan exists in a limited number of plant species and a broad range of microbial products. Levans from distinct sources differ in molecular weight and degree of branching. For example, low-molecular-weight levans are found mainly in plants, whereas levans of high molecular weight are primarily synthesized by various microbial species (3).

Microbial levans are preferred over plant levans for commercial use in the food, pharmaceutical, and cosmetic industries due to their high yield as well as better solubility in water. In the food industry, levan can act as an emulsifier, stabilizer, thickener, encapsulating agent, surface-finishing agent, carrier for flavor and fragrances (4), and prebiotic ingredient (5). For pharmaceutical application, levan has been used as a blood plasma volume extender (6), anti-obesity agent, hypocholesterolemic agent, antitumor agent, hypolipidemic agent, antidiabetic agent, antiviral agent, and antipathogenic agent (7). Owing to its positive properties in terms of moisturizing effect, cell cytotoxicity, cell proliferation effect, and anti-inflammation, levan can also be utilized in cosmetic products (8).

So far, a variety of microorganisms, most of them belonging to *Bacillus* and *Pseudomonas* and a few lactic acid bacteria (LAB) belonging to *Streptococcus* and *Lactobacillus* as well as *Leuconostoc mesenteroides*, are well known to synthesize levan (4). However, no study on the synthesis of levan by *Leuconostoc citreum* has been reported.

In the present work, a levan-producing *L. citreum* strain, BD1707 (CGMCC 6431), was screened out of Tibetan kefir and its growth characteristics in tomato juice supplemented with sucrose (tomato juice-sucrose medium) were determined, including changes in the viable cell count, pH, and the contents of sucrose, fructose, glucose, and levan during the cultivation. The composition and chemical structure of the levan were determined by analysis of monosaccharide composition, molecular mass distribution, and Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectra.

MATERIALS AND METHODS

Screening of EPS-producing strain. Kefir grains collected from Tibet, China, were used to screen for exopolysaccharide (EPS)-producing microorganisms. Five grams of grains was homogenized in 45 ml of sterile saline solution (8.5 g/liter of NaCl) in a stomacher (BagMixer; Interscience, France) for 10 min. Suspensions were serially diluted, and aliquots (100 μl) were spread on M17 agar (Merck KGaA, Darmstadt, Germany) containing 50 g/liter of sucrose as well as 0.08 g/liter of ruthenium red (Sigma-Aldrich Co. LLC, St. Louis, MO) and incubated at 30°C aerobically for 48 h. Nonropy strains form red colonies for the bacterial cell wall being stained by ruthenium red, while ropy strains form white colonies on the same plate for the capsular exopolysaccharide, which would prevent the cells from being stained (9). An isolate designated BD1707, which presented a ropy phenotype in this assay, was chosen for further study.

Strain identification. Physiological tests were conducted to confirm isolate identity. Gram staining was examined after 24 h of cultivation on M17 agar at 30°C aerobically. Catalase and oxidase activities were deter-

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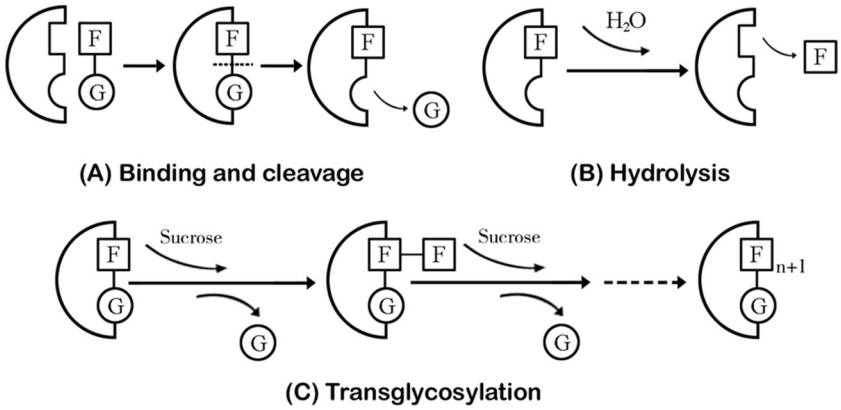

FIG 1 Schematic diagram of the levansucrase-catalyzed reactions in levan synthesis (based on work by Ozimek et al. [1]).

mined by the methods of Guo et al. (10). Growth at different temperatures was observed in M17 broth (Merck KGaA, Darmstadt, Germany) at 10 and 45°C for 7 days. Growth at pHs 4.5 and 9.5 was measured in M17 broth at 30°C for 7 days. Salt tolerance of the strain was tested in M17 broth containing 6.5% NaCl. Carbohydrate fermentation tests were carried out with API 50 CH strips (bioMérieux, Marcy l'Etoile, France).

Strain identification was also conducted by 16S rRNA sequence analysis. Briefly, cells grown in M17 broth at 30°C on a rotary shaker at 200 rpm for 12 h were collected to extract genomic DNA using a DNeasy blood and tissue kit (Qiagen, Valencia, CA). The 16S rRNA gene was amplified using universal primers (27F, 5′-AGAGTTTGATCCTGGCTCAG-3′, and 1492R, 5′-GGTTACCTTGTTACGACTT-3′) (11) for bacteria and a PCR thermal cycler (GenAmp PCR System 9700; PE Applied Biosystems, Foster City, CA) with a TaKaRa *Taq* PCR kit (TaKaRa Shuzo Co. Ltd., Otsu, Japan) by using the following program: 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 2 min and an additional extension step at 72°C for 10 min. Amplified 16S rRNA genes were purified using a QIAquick gel extraction kit (Qiagen) and sequenced with an automated DNA analyzer (Applied Biosystems). 16S rRNA gene sequence similarity searches were performed in the GenBank data library using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (12).

Preparation of the carbohydrate-blended tomato juice medium. The tomato juice medium used in this study was described previously (13). In brief, tomatoes were cut into small cubes, ground in a pulper, and filtered through cotton gauze to remove the majority cake containing peel and seeds. The crude tomato juice was further centrifuged and the supernatant was collected as tomato juice. Glucose, sucrose, arabinose, D-xylose, trehalose, cellobiose, amygdalin, maltose, fructose, mannitose, mannitol, and esculin were added individually as an additional carbon source to the tomato juice at a concentration of 5% (wt/vol); the pH of the mixture was adjusted to 6.5 by adding 5.0 M NaOH drop by drop. After being sterilized at 121°C for 20 min and cooling to ambient temperature, the carbohydrate-blended tomato juice medium was utilized for *L. citreum* BD1707 cultivation.

Cultivation conditions. A loop of fresh culture of BD1707 on M17 agar was inoculated into 10 ml of sterile M17 broth and cultivated at 30°C on a rotary shaker at 200 rpm for 24 h. The activation procedure in M17 broth was carried out twice. The cells were collected by centrifugation at 15,000 × *g* for 5 min at 4°C and washed twice with 20 mM sterile sodium phosphate buffer (pH 6.5). Subsequently, washed cells were resuspended in sodium phosphate buffer (pH 6.5) and adjusted to the initial volume. For analysis of the impact of individual carbon sources on the biomass and exopolysaccharide, 1 ml of the resuspended bacterial cells was inoculated into 100-ml Erlenmeyer flasks containing 50 ml of the sterile tomato juice supplemented with the corresponding carbohydrate and cultivated at 30°C on a shaker at 200 rpm for 72 h. To measure the kinetic change in viable cell count, pH value, and carbohydrate content in the sucrose-supplemented tomato juice medium, the resuspended bacterial cells were inoculated at a 2% (vol/vol) ratio into 250-ml Erlenmeyer flasks containing 150 ml of the broth. The cultivation was carried out at 30°C on a shaker at 200 rpm. During this procedure, 5 ml of sample was withdrawn at 0, 3, 6, 12, 24, 48, 72, and 96 h for further assays.

Preparation and purification of levan. The cells in the cultivation broth were removed by centrifugation at 15,000 × *g* for 5 min at 4°C. After 4 volumes of prechilled ethanol were added to the supernatant with gentle stirring, the mixture was stored at 4°C overnight. The precipitate was collected by centrifugation at 15,000 × *g* at 4°C for 20 min and redissolved in deionized water. This process was repeated twice to isolate the exopolysaccharides. The final aqueous solutions were dialyzed (molecular mass cutoff, 14,000 Da) against distilled water at 4°C for 72 h, then freeze-dried using FreeZone12 (Labconco Corporation, KS), and weighed for the exopolysaccharide yield. Since there was hardly detectable exopolysaccharides in the cultured broth with additional carbon sources such as glucose and arabinose, only the EPS (levan) produced by BD1707 in the tomato juice supplemented with sucrose was furtherly characterized for its composition as well as chemical structure.

Before characterization of the structure of the BD1707 levan, e.g., analysis of the monosaccharide composition and the molecular mass distribution, the residual protein content of the obtained BD1707 EPS solution (5 mg/ml) was determined by the Bradford assay using bovine serum albumin (Sigma-Aldrich Co. LLC, St. Louis, MO) as a standard (14).

Analytical methods. The viable cell counts of BD1707 in cultured broths were determined by plate counting. Aliquots of cultured broth samples at 0, 3, 6, 12, 24, 48, 72, and 96 h after 10-fold serial dilution in sterilized physiological saline were plated onto M17 agar and cultivated aerobically at 30°C for 48 h. The viable cell counts of BD1707 in the samples were expressed as CFU per milliliter.

The biomass was determined by measuring the dry weight of the cells collected in the 5-ml sample, and the pH value was measured by a pH meter (model PB-10, Sartorius AG, Goettingen, Germany).

Sucrose, glucose, and fructose contents. The sucrose, glucose, and fructose contents of the samples at various intervals were determined by a high-performance liquid chromatography (HPLC) method described previously (15).

Monosaccharide composition. The monosaccharide composition of BD1707 EPS was measured according the procedure described by Wood et al. (16). Rhamnose, arabinose, galactose, glucose, xylose, mannose, and fructose (Sigma-Aldrich Co. LLC, St. Louis, MO) were used as references. The percentages of monosaccharides were calculated from the peak areas using a response factor.

Molecular mass distribution. The molecular mass of the levan produced by BD1707 was determined by high-performance size exclusion chromatography (HPSEC) using a Perkin-Elmer series 200 liquid chro-

matograph (PerkinElmer, Waltham, MA) equipped with a Perkin-Elmer series 200 refractive index detector. Two TSK-GEL columns (G6000PW$_{XL}$ and G4000PW$_{XL}$; 7.8 by 300 mm) were maintained in series, utilizing 0.05 M $NaNO_3$ as the eluent at a flow rate of 0.6 ml/min. The columns and guard columns were maintained at 25°C, and 5 mg/ml of BD1707 levan dissolved in the 0.05 M $NaNO_3$ was filtered through a 0.22-μm filter (Sartorius AG) before injection. Commercial pullulans with molecular masses ranging from 6,000 to 2,560,000 Da (Sigma-Aldrich Co. LLC) were used as standards.

FTIR spectroscopic analysis. The FTIR spectrum of the BD1707 levan was obtained using a Nicolet Nexus 470 spectrometer (Nicolet Co., Waltham, MA). A sample was prepared as a KBr pellet and scanned against a blank KBr pellet background at wavelength range of 4,000 to 500 cm^{-1} with a resolution of 4.0 cm^{-1}.

^{1}H and ^{13}C NMR spectroscopy analysis. Ten milligrams of BD1707 levan from the cultured tomato juice-sucrose medium was dissolved in 0.5 ml of pure D_2O and then placed into 5-mm NMR tubes. One-dimensional ^{1}H NMR spectra utilizing tetramethoxysilane as an internal standard and ^{13}C NMR spectra were obtained on a Varian Mercury Plus 400 spectrometer (Varian Medical Systems, Inc., CA) at 25°C. The ^{1}H NMR data collection consisted of 256 acquisitions.

Statistical analysis. All experiments and analysis at every time point from each experiment were performed at least in triplicate. The means, standard errors, and standard deviation were calculated from replicates with the experiments and analyzed using OriginPro 8.0.

Nucleotide sequence accession number. The full-length 16S rRNA sequence of BD1707 has been deposited in GenBank under accession no. KT626384.

RESULTS

Isolation and identification of an EPS-producing strain. Strain BD1707 formed mucous, opaque, slightly convex, and irregularly edged colonies on M17 agar with 5% sucrose. When the colony was picked up with a loop, a sticky string could be observed, indicating that strain BD1707 had the capability to synthesize capsular and/or ropy exopolysaccharides (17). Cells of strain BD1707 are Gram-positive and catalase- and oxidase-negative cocci, visible predominantly in chains consisting of 3 to 8 cells under a light microscope. The strain is able to grow at 10°C and at pH 4.0 but unable to grow at 45°C, at pH 9.5, or in 6.5% (wt/vol) NaCl. Carbohydrate fermentation analysis by API 50 CH strips suggested that the new isolate could produce acid from glucose, sucrose, arabinose, D-xylose, trehalose, cellobiose, amygdalin, maltose, fructose, mannitose, mannitol, and esculin, which indicated a 98.6% similarity between this strain and the reported strains of *L. citreum*. Genetic analysis further confirmed the taxonomic status of strain BD1707. The 16S rRNA gene sequencing analysis showed that BD1707 had over 99% similarity to most of the reported strains belonging to *L. citreum* (99 to 100% similarity to more than 60 strains). Accordingly, the new isolate was designated *L. citreum* BD1707.

Effects of carbon sources on growth and EPS production. To determine the effects of different carbon sources on growth and EPS synthesis, *L. citreum* BD1707 was inoculated into tomato juice containing 12 individual carbon sources, which were chosen from the carbohydrates known to be metabolizable by this strain. As shown in Table 1, all the carbon sources tested could enhance the growth of *L. citreum* BD1707, as reflected by the obvious increase in bacterial biomass. The highest EPS production, 15.12 g/liter, was attained by BD1707 cells grown in the presence of 5% (wt/vol) sucrose. When cellobiose was utilized as an additional carbon source besides those already existing in the original tomato juice (13), the EPS synthesized could hardly be detected (although the observed biomass was only slightly less than that with sucrose). The same phenomenon was also observed in the other 10 carbon sources. Consequently, sucrose was supposed to be the optimal carbon source for the growth and EPS synthesis of BD1707, which was in good agreement with the results obtained by other researchers (1).

TABLE 1 Effects of carbon sources on BD1707 growth and EPS production in tomato juice containing 5% (wt/vol) individual carbon sources

Carbon source	Biomass (g/liter)	EPS concn (g/liter)
Amygdalin	4.85	0.37
Arabinose	3.49	0.17
Cellobiose	5.58	0.46
Esculin	2.81	0.14
Fructose	4.06	0.33
Glucose	3.67	0.25
Maltose	3.98	0.26
Mannitol	4.11	0.22
Mannitose	4.21	0.23
Sucrose	6.12	15.12
Trehalose	3.32	0.20
D-Xylose	2.53	0.19

Growth characteristics of *L. citreum* BD1707 in tomato juice-sucrose medium. The changes in the viable cell counts and the pH during the growth of *L. citreum* BD1707 in the tomato juice-sucrose medium are shown in Fig. 2A. *L. citreum* BD1707 grew well in the tomato juice-sucrose medium, with a significant ($P < 0.05$) increase in the viable cell count, from an initial value of 7.57 $\log_{10}$ to 9.40 $\log_{10}$ CFU/ml, while the pH value of the medium decreased dramatically, from 6.07 to as low as 4.20, during the first 24 h of cultivation. The fast growth of BD1707 cells in the medium might be partially attributed to the initial pH value of 6.07, detected in the inoculated tomato juice-sucrose medium, which was approximate to the optimal pH value reported for the growth of *L. citreum* (18, 19). However, the viable cell counts were found to decrease slowly after 24 h ofcultivation, which might have resulted from the decrease in the pH value and depletion of nutrients, as well as the accumulation of organic acid in the medium (20).

During the 96 h of cultivation, large amount of EPS (levan) accumulated in the cultured broth by *L. citreum* BD1707, with a climatic yield of 28.70 g/liter. Meanwhile, sucrose decreased from the initial level of 15.00% (wt/vol) to 7.27% (wt/vol) at 96 h, whereas glucose accumulated from the initial level of 1.27% (wt/vol) to a final level of 3.92% (wt/vol), as shown in Fig. 2B. No obvious change in the concentration of fructose, which fluctuated around 1% (wt/vol), was observed.

Molecular mass distribution. A sharp peak was observed at the retention time of 18.14 min, reflecting the high homogeneity or purity of the prepared EPS. Accordingly, based on the equation of the molecular masses of standards and their corresponding retention times, the average molecular mass of EPS synthesized by *L. citreum* BD1707 in the tomato juice-sucrose medium was estimated to exceed 4.3×10^6 Da (Fig. 3), which was much higher than those of many other bacterial sources documented before (21, 22).

Monosaccharide composition. To avoid the possible interference of the mono- and disaccharide residues on the monosaccha-

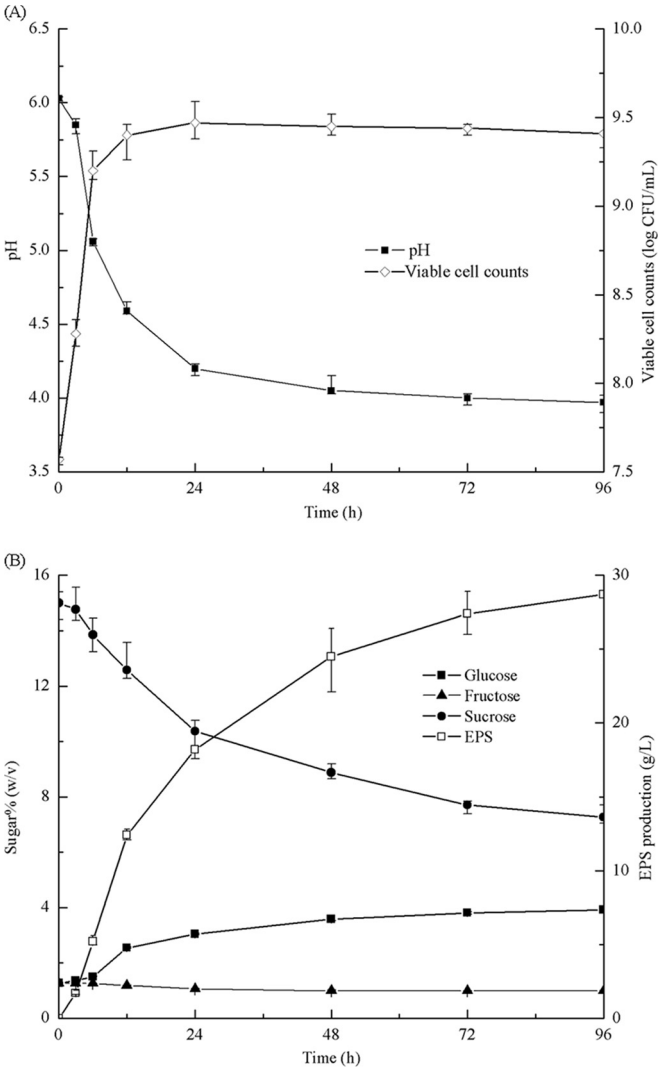

FIG 2 Changes in viable cell counts and pH (A) and sucrose, glucose, and fructose contents and EPS yields (B) during cultivation of *L. citreum* BD1707 in tomato juice-sucrose medium.

ride analysis of the prepared EPS, a solution of 5 mg/ml of the EPS sample was first subjected to the HPLC method mentioned in Materials and Methods to determine the residual sucrose, fructose, and glucose contents. None of the mentioned mono- or disaccharides was detectable in the prepared EPS by means of ethanol precipitation and washing repeatedly, indicating that the EPS sample was suitable for further monosaccharide analysis.

The retention time of the monosaccharide from the digested sample was 14.50 min, which accurately matched that of fructose, indicating that *L. citreum* BD1707 synthesized a fructan-type polysaccharide in tomato juice-sucrose medium. Inulin and levan are two basic types of fructan, and only the former had been reported for the *L. citreum* species until now (23). It was obviously of importance to ascertain whether the *L. citreum* BD1707 fructan shared a structure similar to that of fructans from other *L. citreum* strains. Therefore, the structure of *L. citreum* BD1707 fructan, such as the type of glycosidic linkages, was further characterized by FTIR spectroscopy analysis and nuclear magnetic resonance (NMR).

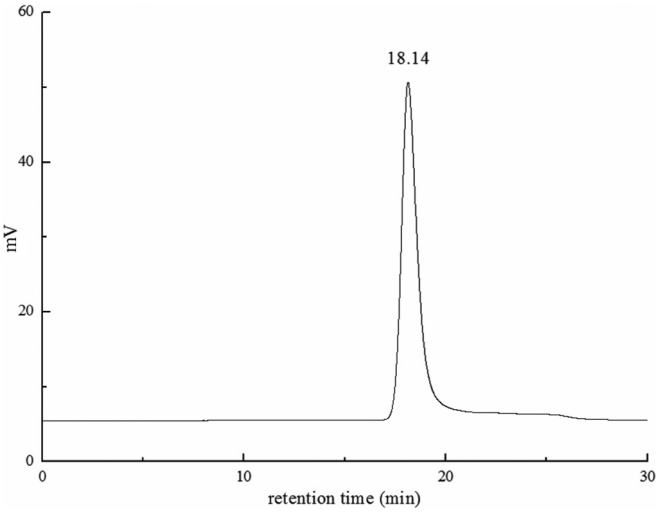

FIG 3 HPSEC profile of EPS produced by *L. citreum* BD1707 in tomato juice-sucrose medium.

FTIR spectroscopy analysis. FTIR spectroscopy analysis was performed to determine the type of the glycosidic linkages and the functional groups of levan produced by *L. citreum* BD1707. As shown in Fig. 4, the band in the region of 3,306 cm^{-1} was relevant to the hydroxyl (O—H) stretching vibration, and the two bands at 2,930 and 2,884 cm^{-1} arose from carbon-hydrogen (C—H) stretching vibration. The band observed at 1,634 cm^{-1} was due to bound water (24). Vibrations of the C—H plane deformation and aromatic skeleton caused the bands in the region of 1,412 and 1,217 cm^{-1} (24, 25). The bands between 1,120 and 1,008 cm^{-1} reflected the stretching vibration of C—O—C and C—O—H glycosidic linkages (26). The characteristic absorption at 923 and 810 cm^{-1} indicated the presence of the furanoid ring of the sugar units (27). The FTIR spectrum of BD1707 fructan well matched the characteristic peaks in the spectra of levan derived from *Halomonas* sp. strain AAD6 (28), indicating that the two polysaccharides shared similar structures. The β-(2→6) linkages in BD1707 fructan were further confirmed by ^{1}H and ^{13}C NMR analysis.

^{1}H and ^{13}C NMR spectroscopy analysis. The structure of *L.*

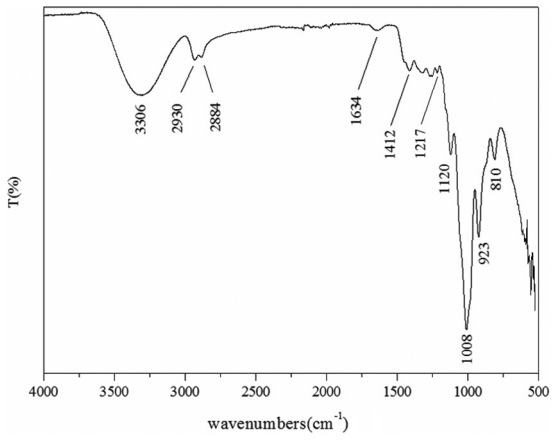

FIG 4 FTIR spectrum for levan synthesized by *L. citreum* BD1707 in tomato juice-sucrose medium. T, transmission.

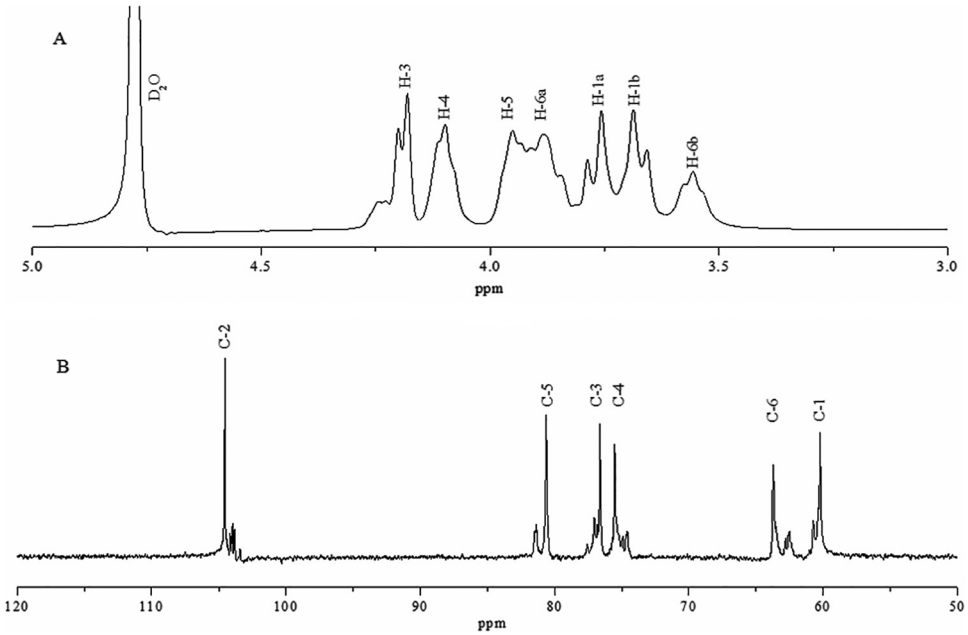

FIG 5 ^{1}H (A) and ^{13}C (B) NMR (400 MHz, D_2O) spectra for levan synthesized by *L. citreum* BD1707 in tomato juice-sucrose medium.

citreum BD1707 fructan was revealed by the ^{1}H and ^{13}C NMR spectra shown in Fig. 5. Seven protons corresponding to β-fructofuranosyl units were observed between 3.4 and 4.2 ppm in the ^{1}H NMR spectrum (Fig. 5A), indicating that BD1707 fructan was of the levan type with the linkage of (2→6) fructofuranoside, which is in good agreement with the absorption peak at 923 cm^{-1} in the FTIR spectrum (Fig. 4). No peaks were found in the anomeric region (5.3 to 4.3 ppm), reflecting the absence of anomeric protons (29). These results provided strong evidence that the polysaccharide from BD1707 is a homopolymer of fructose, which is in accordance with the result of monosaccharide analysis. On the whole, the ^{1}H NMR spectrum of the BD1707 fructan displayed high similarity to that of the levan from *Zymomonas mobilis* (ZAG-12L) (29).

The six major resonances at 104.537 (C-2), 80.622 (C-5), 76.628 (C-3), 75.532 (C-4), 63.716 (C-6) and 60.234 (C-1) ppm in the ^{13}C-NMR of BD1707 fructan (Fig. 5B) were assigned to the individual carbon atoms associated with the β-configurated fructofuranose units, via comparison with the published data for *B. subtilis* levan (30). Furthermore, the downfield shifted signal at C-6 confirmed a β-2,6 backbone structure of levan-type fructan (31). The assignments for the individual signals of levan from BD1707 and several published microorganisms (32–34) in D_2O are listed in Table 2.

As shown in Table 2, the primary carbons (C-1 and C-6) of BD1707 levan were not grouped so closely as that of inulin and the three ring carbons (C-3, C-4, and C-5) were more closely grouped in the case of inulin, a characteristic discrepancy in the ^{13}C-NMR spectra between inulin and levan (35).

DISCUSSION

Kefir is a natural source rich in LAB species, including *Leuconostoc* spp. (36). In this study, a levan-producing strain, BD1707, was screened out from Tibetan kefir grains on M17 agar containing 5% sucrose by the method of ruthenium red staining. The isolate was identified as *L. citreum* based on phenotypic and genotyping analysis.

Among the 12 different carbon sources tested, sucrose turned out to be the sole carbon source suitable for levan synthesis, which is in accordance with numerous reports suggesting that microbial levans are extracellularly biosynthesized from sucrose-based medium by transfructosylation of levansucrase (37). A likely expla-

TABLE 2 Assignments of individual signals in ^{13}C NMR spectra of BD1707 levan and bacterial levan from previous studies as well as inulin

Carbon atom	Chemical shift (ppm)				
	L. citreum BD1707 levan	*L. reuteri* 121 levan[a]	*B. subtilis* Takahashi levan[b]	*B. polymyxa* levan[c]	Inulin[c]
C-1	60.234	61.7	59.9	60.7	60.9
C-2	104.537	105.0	104.3	104.4	103.3
C-3	76.628	78.1	76.3	77.0	77.0
C-4	75.532	76.6	75.2	75.7	74.3
C-5	80.622	81.2	80.3	80.5	81.1
C-6	63.716	64.3	63.5	63.6	62.2

[a] Data cited from reference 32.
[b] Data cited from reference 33.
[c] Data cited from reference 34.

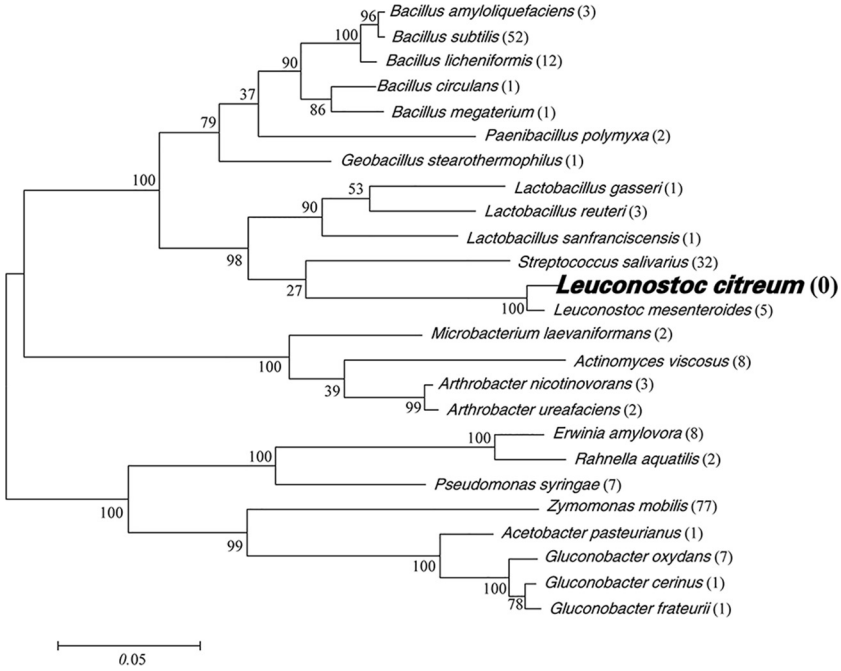

FIG 6 Phylogenetic tree of *Leuconostoc citreum* and reported levan-producing strains calculated on the basis of 16S rRNA gene sequences. Numbers in parentheses are numbers of published studies reporting levan synthesized by members of the species. The scale bar indicates number of changes per nucleotide.

nation for the substrate specificity of levansucrase is that sucrose, because of its small size, easily entered the active site and bound to the levansucrase, resulting in a progressive reaction of polymerization (Fig. 1). Therefore, the disaccharide sucrose was an indispensable fructosyl donor substrate for fructan polymer synthesis (1).

A remarkable increase in the viable cell count and decrease in the pH value were observed during the first 24 h of cultivation. Similar viable cell counts and pH trends were also noted for *L. mesenteroides* BD1710 growth in tomato juice-sucrose medium for dextran biosynthesis (13), which indicated that tomato juice-sucrose medium could be a potential culture medium for EPS biosynthesis and growth of *Leuconostoc* species. During the cultivation of strain BD1707, a steady increase in the concentration of levan with a constant decrease in the sucrose concentration was observed, and similar phenomena have also been reported in *L. citreum* strains able to synthesize other exopolysaccharides. *L. citreum* had been reported to be capable of synthesizing a variety of homopolysaccharides from sucrose. For example, *L. citreum* S5 could biosynthesize dextran from sucrose as the sole substrate by dextransucrase (EC 2.4.1.5) (38). Inulosucrase (EC 2.4.1.9) from *L. citreum* CW28 was capable of hydrolyzing sucrose to synthesize inulin (23). Although a higher concentration of sucrose seemed in favor of the accumulation of levan in the cultured broth of *L. citreum* BD1707, the sucrose was not completely utilized in the tomato juice supplemented at a 15% (wt/vol) level (Fig. 2). Similar findings were also obtained for other levan-producing strains, such as *Z. mobilis* (39). The initial amount of glucose in the tomato juice-sucrose medium originated from the tomato juice (13), while the increase in glucose during the fermentation probably derived from two factors: (i) release from sucrose continuously by levansucrase and (ii) insufficient metabolism of the bacterial cells in stationary phase.

A comparatively high yield of levan (28.70 g/liter) was obtained after 96 h of cultivation of *L. citreum* BD1707, which offered a way to prepare levan efficiently that is different from that utilized by some other LAB, such as *Streptococcus salivarius*. *S. salivarius* synthesized only 3 g/liter levan in the medium (40). The efficiency (mass ratio of produced levan to initial sucrose, 19.1%) and conversion yield value (mass ratio of produced levan to consumed sucrose, 37.1%) obtained in present study were much higher than those of other levan-producing strains. Viikari reported that the amount of levan synthesized by *Z. mobilis* was equivalent to almost 10% of the original sucrose (41). Silbir et al. also found that about 10.3% of initial sucrose and 21.7% of consumed sucrose were utilized by *Z. mobilis* to biosynthesize levan (39). The high efficiency of *L. citreum* BD1707 in converting sucrose into levan might be derived from the strong levansucrase activity expressed as well as the fulfillment by the tomato juice of the stringent nutrition requirement by the producer. Tomato juice has been proved to be a suitable medium for many species of lactic acid bacteria, e.g., *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus delbrueckii* (42) and *Leuconostoc mesenteroides* (13), to produce probiotic beverages or functional products.

As evidenced by its monosaccharide composition and structural characteristics (via FTIR and ^{1}H and ^{13}C NMR analysis), the levan synthesized by *L. citreum* BD1707 in the tomato juice-sucrose medium consisted of consecutive β-(2→6)-fructofuranosyl units, highly similar in structure to those produced by other bacterial species, although the former had a much higher molecular mass (the estimated mass of the former exceeded 4.3×10^{6} Da). Compared with the levan derived from other bacterial species or plants, the huge molecule of BD1707 levan might endow this polymer with an enhanced ability to perform its functions. It had been reported that the diversity in structure as well as functions of mi-

crobial exopolysaccharides was greatly influenced by their molecular masses, monosaccharide compositions, and molecular conformations (degree of branching as well as type of glycosidic or fructosidic linkages) (43). For instance, levans with high molecular masses (>300 kDa) demonstrated antitumor activity (44),while low-molecular-mass ones (<100 kDa) have been employed as blood plasma volume extenders (6) and antiviral agents (45).

Microbial levans were biosynthesized from sucrose-based substrates by microorganisms secreting levansucrase; most of them were nonfood bacteria, such as *Bacillius* spp. and *Zymomona* spp. (Fig. 6). Only a handful LAB species, including *Streptococcus* spp., *L. mesenteroides*, and *Lactobacillus* spp., had been reported to show levan synthesis. Among the published studies regarding LAB levan, most of them (32 of 38) were focused on the physico-chemical and biological characteristics of *S. salivarius* levan (40, 46).

In conclusion, *L. citreum* strain BD1707 is able to synthesize levan in tomato juice-sucrose medium, which is available worldwide. So far, microbial levans have been primarily derived from nonfood microorganisms, which might confer a safety risk for the biopolymer to be applied commercially. In contrast, *L. citreum* BD1707 offers an alternative to produce levan in a more safe and low-cost way.

Considering the potential of levan as a functional agent with wide application, further study is necessitated to elucidate the mechanism involved in levan synthesis by BD1707 as well as cultivation parameters affecting the yield.

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