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Screening and Selection of 2-Branched (1,3)-β-D-Glucan Producing Lactic Acid Bacteria and Exopolysaccharide Characterization⊥

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The ability to produce a 2-branched (1,3)-β-D-glucan was screened in 147 lactic acid bacteria strains recovered from cider. Among them, 32 identified as *Pediococcus parvulus* exhibited a ropy character and were PCR positive for the presence of the *gtf* gene, related to the synthesis of the β-glucan. Half of the strains produced more than 100 mg L^{-1} of exopolysaccharide (EPS). ^{1}H NMR spectra of the crude EPSs were identical to that previously described for *P. parvulus* 2.6, indicating that all are 2-branched (1,3)-β-D-glucans. The EPSs from two of the isolates were subjected to acid hydrolysis and methylation analysis, confirming the NMR results. Size exclusion chromatography (SEC) showed in all crude EPSs the presence of two different molecular mass fractions of about 10^7 Da and 10^4 Da, whose relative proportions varied among strains. EPS amounts and concentrations of high molecular fraction are linearly correlated. Intraspecific diversity of isolates was determined by RAPD profiles. Based on genotypic and phenotypic characteristics, two strains were selected to be further studied as probiotics.

KEYWORDS: (1,3)(1,2)-β-D-Glucans; *Pediococcus parvulus*; screening; exopolysaccharide characterization

INTRODUCTION

Exopolysaccharide (EPS) producing lactic acid bacteria (LAB) are becoming industrially important microorganisms for elaboration of functional food products. Most of them are heteropolysaccharide (HePS) producers and are being used as natural biothickeners to improve the texture and stability of some dairy products, such as fermented milks and cheese. In addition, health benefits have been claimed for EPS synthesized by LAB (*1*). In contrast, homopolysaccharide (HoPS) producing LAB strains have been rarely evaluated and used only for fermentation of nondairy products (*2*).

(1,3)-β-D-Glucans from several bacteria and fungi constitute a group of natural polysaccharides with a main chain of (1,3)-linked β-glucopyranosyl units. It can be linear or branched with either (1,6)- or (1,2)-linked side chains of varying length and distribution. In plants, (1,3)-β-D-glucans exhibit a linear structure with mixed β-(1,3) and β-(1,4) glycosidic bonds (*3*). These polysaccharides have been extensively studied in the past decade, and most of these polymers, such as Curdlan and scleroglucan, exhibit interesting physicochemical properties, especially gelling capability. Moreover, (1,3)-β-D-glucans are considered as biological response modifiers and numerous publications describe their biological activities and therapeutic uses (*4*). Their biological effects are influenced by their degree of branching, chain length, and tertiary structure (*5*).

In this research field, synthesis of (1,3)-β-D-glucans by LAB represents a promising alternative to diversify and extend the number and applications of EPS producing LAB in the development of new functional foods. Thus, *P. parvulus* 2.6 isolated from a ropy cider synthesizes a 2-branched (1,3)-β-D-glucan and the analysis of its rheological properties showed that it has potential utility as a biothickener (*6*). In addition, human ingestion of an oat-based food elaborated with *P. parvulus* 2.6 resulted in a decrease of serum cholesterol levels, boosting the effect previously demonstrated for (1,3)(1,4)-β-D-glucans in oats (*7*). The potential of *P. parvulus* 2.6 as probiotic strain has been examined, and it resists gastrointestinal stress, adheres to Caco-2 cells and induces the production of inflammation-related cytokines by polarized macrophages (*8*).

Synthesis of the 2-branched (1,3)-β-D-glucan by *P. parvulus* 2.6 is controlled by a single transmembrane glucosyltransferase (GTF), which polymerizes glucosyl residues from UDP glucose (*9*). For detection of (1,3)-β-D-glucan producing LAB, a glycosyltransferase gene (*gtf*) based PCR method was developed on *P. parvulus* 2.6 (*10*). The *gtf* gene was also detected in other EPS producing LAB, such as *Lactobacillus diolivorans* G-77 and *Oenococcus oeni* I4 (*10*, *11*), which besides the β-glucan produced a α-glucan and a heteropolysaccharide, respectively.

⊥This paper is dedicated to Javier Areizaga, who passed away April 2, 2010.

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The aim of this work was to screen the ability to produce (1,3)-β-D-glucans in a large number of LAB, isolated from ropy and nonaltered cider. We found that 28 *P. parvulus* isolates synthesize a single type of exopolysaccharide, a 2-branched (1,3)-β-D-glucan. Intraspecific diversity of these strains was also examined by randomly amplified polymorphic DNA (RAPD) with three separate random primers. Based on their EPS yields, high molecular masses of β-glucans, and RADP profiles, two of them were selected to be further studied as probiotics.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. A total of 147 LAB strains belonging to the culture collection of the UPV/EHU (Donostia-San Sebastián, Spain) were used in this study for screening of EPS production. They were previously isolated from both ropy and nonaltered natural ciders between 1993 and 2005. Strains were stored at −80 °C in Man Rogosa Sharpe (MRS) broth (Pronadisa, Madrid, Spain), containing 20% (v/v) glycerol. Before experimental use, bacteria were propagated in MRS broth supplemented with 0.05% (wt/v) L-cysteine hydrochloride (Merck, Darmstad, Germany) and 0.1% (wt/v) Tween 80 (Pronadisa, Barcelona, Spain) at 28 °C in an atmosphere containing 5% CO_2.

For EPS isolation and quantification, strains were grown in MST broth containing (per liter): 50 g of glucose, 0.06 g of L-alanine, 0.2 g of L-serine, 0.03 g of L-tryptophan, 0.2 g of L-asparagine, 0.5 g of L-cysteine, 0.01 g of reduced glutathione, 1 mL of Tween 80 (Merck), 0.06 g of uracil, 0.03 g of adenine, 0.03 g of guanine, 5 mg of Mn, 5% (v/v) of ethanol, and vitamins and trace elements according to Velasco et al. (*12*). Batch fermentations without pH control were carried out in fully filled 250 mL screw-cap flasks for 96 h at 28 °C in an atmosphere containing 5% CO_2. Growth was monitored spectrophotometrically at 600 nm (OD_{600}).

Rapid Screening of EPS-Producing LAB Strains. Screening for EPS-producing LAB was performed in MRS broth (pH 4.8) supplemented with 20% (v/v) tomato juice and 5 g L^{-1} fructose (MRS-T-F) at 28 °C in an atmosphere containing 5% CO_2 for 48 h. The EPS-producing ability was evaluated by visual observation of the culture viscosity. EPS positive strains showed a ropy liquid culture and upon agitation, the cell deposit formed a long string.

Carbohydrate Fermentation. Bacteria were grown in MRS broth (without glucose) containing chlorophenol red (0.04 g L^{-1}), as pH indicator and supplemented with 2% (wt/v) of the following carbohydrates: glucose, fructose, galactose, lactose, sucrose, maltose, sorbitol, raffinose, mannitol, rhamnose, trehalose, or xylose. Incubation was performed for 7 days, at 28 °C in an atmosphere containing 5% CO_2,

β-D-Glucan Agglutination Test. Agglutination tests were performed using *Streptococcus pneumoniae* type 37-specific antisera as previously reported by Werning et al. (*10*). Each culture (10 μL) was mixed with 10 μL of the antisera (Statens Seruminstitut, Copenhagen, Denmark) and incubated for 2 h at 4 °C. The preparations were analyzed by phase-contrast microscopy. The 2-branched (1,3)-β-D-glucan producer *P. parvulus* 2.6 was used as positive control, and *L. reuteri* CECT 925^T as negative control.

DNA Extraction, Identification of EPS Producers, and PCR Detection of the gtf Region. One-milliliter aliquots of MRS cultures were used for DNA extraction. Following centrifugation at 13000*g* for 3 min, pellets were washed in 0.5 mL of TE (10 mM Tris HCl; 1 mM EDTA, pH 8) and centrifuged at 13000*g* for 3 min. For DNA extraction, the resulting cell pellet was resuspended in 180 μL of an enzymatic lysis buffer, containing 20 mM Tris-HCl (pH 8), 2 mM sodium EDTA, 1,2% TritonX-100 and 20 mg mL^{-1} lysozyme, and incubated for 30 min at 37 °C. DNA was purified by DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. DNA samples were stored at −20 °C until use. Strains were identified by 16S rRNA sequence analysis. A fragment of 530 bp of the 16S rRNA was amplified with primers PA (5′-AGAGTTTGATCCTGG CTCAG-3′) and UP1R (5′-TCACGCGGCTGCTGGCAC-3′) corresponding to positions 9 to 539 in the *Escherichia coli* numbering system. Each 50 μL PCR reaction was carried out with 1.25 U of BIOTAQ DNA polymerase (Bioline, Luckenwalde, Germany) and contained 1 μM of each primer, 0.75 mM $MgCl_2$, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 200 μM of each dNTP and 5 μL of DNA template. Cycling conditions were 1× (94 °C, 5 min), 25× (94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min), and 1× (72 °C, 10 min). The PCR products were separated on a 0.8% (wt/v) agarose gel, and the amplicons were purified with the QIAquick Gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 16S rRNA sequences were identified with nucleotide identity at the species level higher than 98% by using the BLAST tool of the GenBank DNA database (http://www.ncbi.nlm.nih.gov/).

For detection of the *gtf* gene, specific primers (GTFF and GTFR) were used for PCR amplification (*10*). These primers were located in the coding sequence of the putative carboxyl glycosyltransferase domain and amplified a 417 bp fragment of the gene. Each 50 μL PCR reaction was carried out with 1.25 U of BIOTAQ DNA polymerase (Bioline, Luckenwalde, Germany) and contained 0.2 μM of each primer, 3.5 mM $MgCl_2$, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 25 μM of each dNTP and 5 μL of DNA template. Cycling conditions were 1× (94 °C, 5 min), 25× (94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min), and 1× (72 °C, 10 min).

RAPD-PCR. Total genomic DNA was isolated as described above, and the genomic diversity of the strains was analyzed by randomly amplified polymorphic DNA (RAPD) method using universal primers M13 (5′-GAG GGT GGC GGT TCT-3′) (*13*), P1 (5′-ACG CGC CCT-3′) and P2 (5′-ATG TAA CGC C-3′) (*14*) (synthesized by Eurofins MWG Operon, Germany). PCR amplifications were conducted in a solution containing 1× PCR buffer (Bioline), 5 μM of primer, 1 U of BIOTAQ DNA polymerase (Bioline), 5 mM $MgCl_2$ (Bioline) for P1 and P2 or 2.5 mM for M13, 100 μM of each dNTP (Bioline) and 200 ng of DNA template in a total volume of 50 μL. Amplification conditions were one cycle of 94 °C for 5 min, 40 °C for 5 min and 72 °C for 5 min. This was followed by 33 cycles of 94 °C for 30 s (M13) or 1 min (P1 and P2), 40 °C for 1 min and 72 °C for 1 min. Twenty microliters of the amplification product were electrophoresed on 1.8% agarose gel in TAE buffer at 80 V for 90 min. Gels were stained with ethidium bromide and photographed under UV light. Gel images were recorded using a Gel-Doc (Bio-Rad) and stored as TIFF files.

Banding Pattern Analysis. Digitized images were converted, normalized, analyzed and combined with the software package BioNumerics 2.5 (Applied Maths, St-Martens-Latem, Belgium). In order to normalize the banding patterns, molecular markers were included every seven tracks. The levels of similarity between pairs of traces were computed using the Pearson product-moment correlation coefficient that provides similarity based upon densitometric curves. Data obtained from RAPD profiles (M13, P1 and P2, on separate and combined) were clustered using the unweighted pair group method with arithmetic averaging (UPGMA) algorithm (*15*) using the corresponding tools in BioNumerics. The consistence of the clusters obtained was estimated by calculating the cophenetic correlation values corresponding to the dendrograms. This method calculates the correlation between the dendrogram-derived similarities and the matrix similarities.

Clustering of Multiple Data Sets. Clustering of multiple data sets, combining banding patterns (M13, P1, P2) and adding specific PCR result (gtf-PCR) and phenotypic traits (sugar fermentations, EPS quantification values and agglutination results), was calculated taking averaged similarity values from the individual experiments with the aid of the "Composite data set" tool in BioNumerics. The similarity between the strains based on specific PCR or phenotypic profiles was calculated by the binary coefficient of Jaccard (*15*). The consistency of the clusters obtained was estimated by calculating the cophenetic correlation values corresponding to the dendrograms.

EPS Isolation and Quantification. Bacterial cells were removed from fermented media by centrifugation (16000*g*, 4 °C, 30 min). The clear supernatant was collected and the EPS precipitated by adding two volumes of cold acetone (100% v/v) and maintained overnight at 4 °C. The precipitate was recovered by centrifugation at 14000*g* for 10 min at 4 °C, washed 3 times with 70% (v/v) acetone and centrifuged. Finally, precipitated polysaccharide was dissolved in distilled water and the total sugar content was determined by the phenol−sulfuric acid method (*16*) using glucose as standard as described by Ibarburu et al. (*17*). All determinations were made in triplicate. For EPS characterization, the final precipitate was dissolved in and dialyzed against deionized water, using a dialysis membrane (Medicell International, Ltd., London, U.K.) having a cutoff of 3.5 kDa, for 2−3 days (changed twice each day). After dialysis, the precipitate was lyophilized.

EPS Characterization. NMR analyses of the EPSs were performed using a spectrometer Avance 500 (Bruker Instruments Inc.). 0.6 mg of

each EPS was resuspended in 0.6 mL of deuterated water (deuterium oxide, 99 atom % D, Aldrich) and the solutions were stirred until complete homogenization. ^{1}H monodimensional and 2D-COSY analyses were carried out at 30 °C, according to the conditions described by Dueñas et al. (*18*). The molecular masses of the polysaccharides were determined by high-performance size exclusion chromatography (HP-SEC, GPCV 2000, Waters), which included a differential RI detector as described by Velasco et al. (*6*).

Monosaccharide composition was determined and quantified by gas chromatography after hydrolysis of the polysaccharides with 3 M trifluoroacetic acid (TFA) as previously described (*19*). For linkage type analysis, the samples were methylated according to Ciucanu and Kerek (*20*). The permethylated samples were hydrolyzed with 3 M TFA (1 h, 120 °C) and then reduced, acetylated and analyzed by gas chromatography−mass spectrometry (GC−MS) as reported by Leal et al. (*19*).

RESULTS

Screening of EPS-Producing LAB Strains and Identification. A total of 147 strains of lactic acid bacteria, previously isolated from both ropy and nonaltered natural cider, were screened for their EPS-producing ability in MRS-T-F broth. After incubation, the ropy character of culture broth was visually detected in 32 strains (**Table 1**). These strains were identified at species level by PCR amplification of a 530 bp fragment of the 16S rRNA gene. After sequencing, all strains were assigned with nucleotide identity higher than 98% to *Pediococcus parvulus* species.

To assess the ability of EPS-positive strains to ferment different raw materials for alimentary use, sugar fermentation patters were studied. All strains were able to grow on, and ferment, glucose, fructose and trehalose. Maltose was also fermented by the majority of strains (75%), as shown for the β-glucan producer *P. parvulus* 2.6, whereas galactose was used in a minor proportion (56%) (**Figure 1**).

EPS Production. The EPS producing ability of the 32 EPS-producing LAB strains was analyzed in a MST semidefined medium to avoid the problem of interference associated with complex medium ingredients, such as mannans in yeast extract. Most of the strains are able to synthesize EPS with yields ranging from 18 to 243 mg L^{-1} (**Table 1**). Thirteen strains yielded relatively large amounts of EPS, between 104 and 243 mg L^{-1}, 7 between 50 and 100 mg L^{-1} and 5 produced < 50 mg L^{-1}. Strains CUPV1, 22, and 23 produced the highest EPS concentration (about 250 mg L^{-1}). In contrast, four strains (CUPV14, 19, 20, and 21) were very poor producers or nonproducers. For this reason, they were excluded from further EPS characterization.

PCR and Immunological Detection of 2-Branched (1,3)-β-D-Glucan Producing Strains. PCR was used to detect the presence of the *gtf* gene in EPS-positive strains. This gene encodes for the GTF glycosiyltransferase involved in the synthesis of the 2-branched (1,3)-β-D-glucan by *P. parvulus* 2.6. The expected amplicon of 417 bp was detected in all strains (**Figure 1**), suggesting that they were (1,3)(1,2)-β-D-glucan producers. In addition, we used an agglutination test with antibodies against the capsular polysaccharide of *S. pneumoniae* serotype 37. Immunoprecipitation assays showed that most (25 strains) of the *gtf*-positive strains agglutinated in the presence of type 37-specific antiserum (**Figure 1**) and two kinds of agglutination were observed (**Figure 2**). When EPS production in MST medium was higher than 100 mg L^{-1}, huge cell aggregates were detected, and cell agglutination led to smaller aggregates when EPS yields ranged from 30 to 100 mg L^{-1}. However, no agglutination was found in seven *gtf*$^+$ strains (CUPV15, 16, 17, 18, 19, 20, and 21), which were very poor producers or nonproducers.

Structural and Molecular Mass Determination of EPSs. Structural characterization of the 28 crude EPSs was performed by ^{1}H NMR analysis. The chemical shifts for the ^{1}H resonances of all polysaccharides were identical to those found for the β-glucan produced by *P. parvulus* 2.6 (*18*), and **Figure 3** corresponds to a representative ^{1}H and 2D-COSY NMR spectrum. From these results, we concluded that all ropy *P. parvulus* strains synthesize a β-glucan with the same primary structure: a trisaccharide repeating unit, with two (1,3)-β linked residues in the main chain, one of which is branched in position 2 by a terminal glucose residue (**Figure 4**).

Table 1. Growth and EPS Production by *P. parvulus* Strains in MST Medium and Characterization of Their Polymers

			av mol wt (M_w)			
strain	OD_{600}[a]	EPS (mg L^{-1})	M_w F1[c] (10^6 Da)	rel proportion of F1 (%)	M_w F2[d] (10^4 Da)	rel proportion of F2 (%)
1	2.04	233	10.2	95.8	3.0	4.2
2	2.06	124	8.2	87.5	3.0	12.5
3	1.60	149	9.3	95.3	2.7	4.7
4	0.87	44	8.6	38.6	4.9	61.4
5	0.96	42	10.3	31.4	5.3	68.6
6	1.84	127	9.4	81.8	3.1	18.2
7	1.61	106	8.8	88.9	3.4	11.1
8	2.02	125	9.7	89.4	3.8	10.6
9	1.91	119	9.4	86.4	3.9	13.9
10	1.81	121	9.3	91.0	3.4	9.0
11	1.87	102	7.4	85.9	3.4	14.1
12	1.95	44	8.6	70.9	3.3	29.1
13	1.07	49	7.1	74.6	3.0	25.4
14	1.49	11	nd[b]	nd	nd	nd
15	1.50	22	9.5	49.5	2.8	50.5
16	1.63	25	9.1	42.3	2.8	57.7
17	1.48	18	9.6	54.6	2.8	45.4
18	1.51	21	9.3	51.7	3.5	48.3
19	1.40	nd	nd	nd	nd	nd
20	1.65	6	nd	nd	nd	nd
21	1.47	2	nd	nd	nd	nd
22	2.37	228	10.5	91.2	3.1	8.8
23	2.29	243	9.9	94.2	2.9	5.8
24	2.37	140	10.1	94.9	3.1	5.1
25	1.59	93	8.4	66.4	2.9	33.6
26	2.38	135	10.2	90.5	3.1	9.5
27	1.64	58	11.1	60.1	3.2	39.9
28	2.20	62	10.0	68.3	3.4	31.7
29	2.19	63	9.9	79.0	3.5	21.0
30	2.21	79	8.6	62.7	3.4	37.3
31	2.30	75	10.0	62.6	3.4	37.4
32	2.21	75	10.3	70.1	3.4	29.9
P. parvulus 2.6	1.92	193	9.3	90.6	4.1	9.4

[a] Optical density at 600 nm. [b] Not determined. [c] F1, high molecular mass fraction. [d] F2, low molecular mass fraction.

The EPSs from strains CUPV1 and CUPV22, which were the maximum EPS producers, were additionally analyzed by chemical means in order to confirm the NMR results. Acid hydrolysis of these polysaccharides yielded glucose as their unique component. Methylation analysis gave partially methylated alditol acetates corresponding to terminal, 3-*O*-substituted and 2,3-di-*O*-substituted glucopyranose in relative proportions 1:1:1. These data are in agreement with those expected for the trisaccharide repeating unit (**Table 2**).

Molecular weight determination of the β-D-glucans was carried out by size exclusion chromatography (SEC), using different dextrans as molecular weight standards. The analysis revealed in all cases the presence of two different molecular mass fractions (**Figure 5**). The high- and low-molecular-mass polymers ranged between 1.1×10^7 and 7.1×10^6 Da, and 5.3×10^4 and 2.7×10^4 Da, respectively, and most of them (22 out of the 28 strains) produced mainly the high-molecular-mass polymer. It is worth noting that amounts of EPS and concentrations of the high molecular fraction are linearly correlated (R^2 0.799, $P < 0.001$). Thus, strains

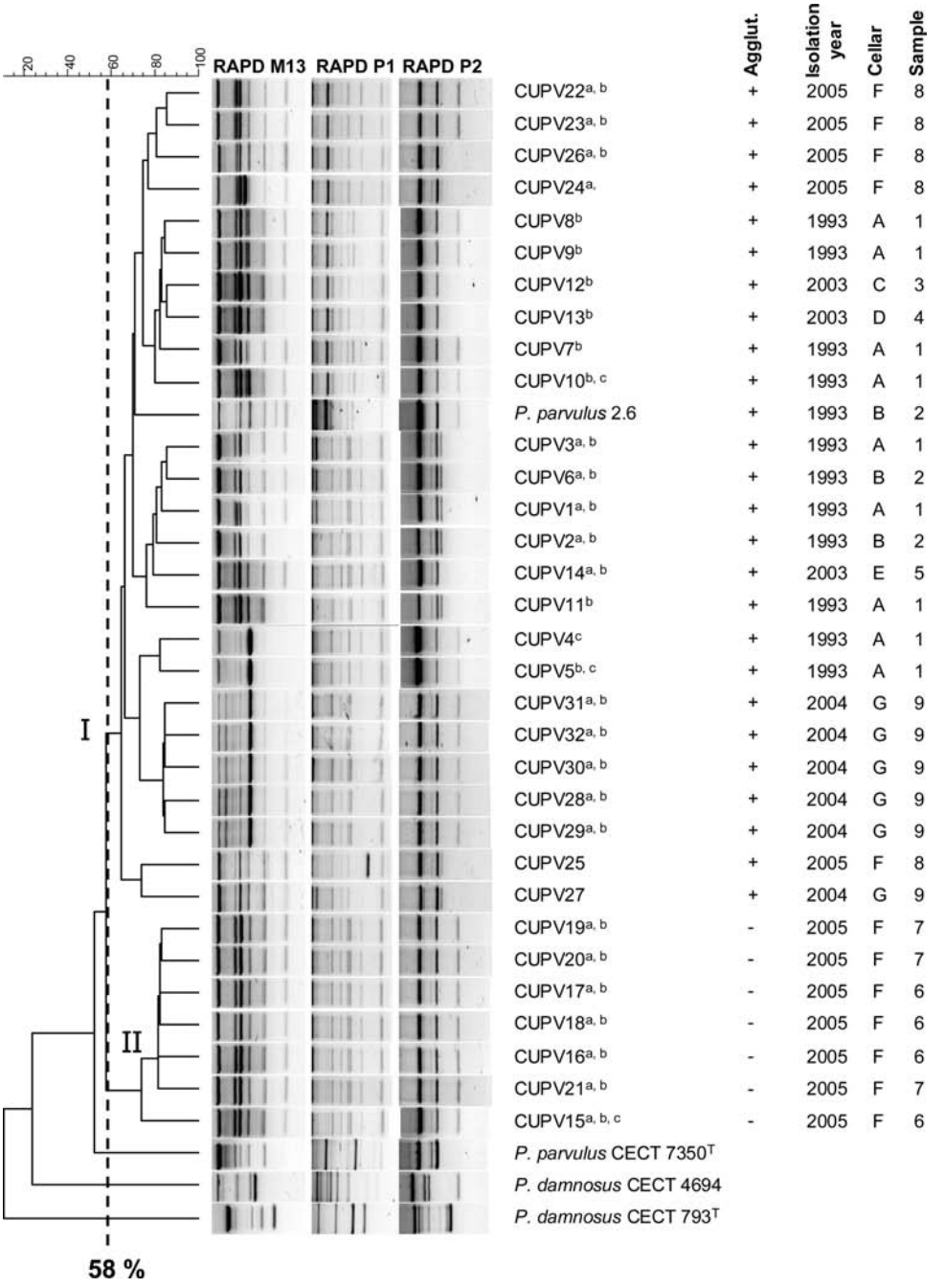

Figure 1. Dendrogram derived from UPGMA cluster analysis based on the combined similarity matrix obtained from the M13, P1 and P2 RAPD fingerprinting, gtf-PCR, sugar fermentation, agglutination test and EPS quantification of the 34 *P. parvulus* (1 reference strain and 33 cider isolates) and 2 *P. damnosus* reference strains. The scale measures the percentage of similarity. Clusters are numbered. The tracks show the processed band patterns corresponding to RAPD M13, P1, and P2 after conversion, normalization, and subtraction of the background. Columns on the right show data corresponding to isolation year, cider making cellar, sample number and agglutination test. Differences in sugar fermentation patterns are indicated as follows: [a]galactose; [b]maltose; [c]xylose. *P. parvulus* 2.6 is an EPS producing cider isolate previously described (*10*). CECT, Spanish Type Culture Collection, type strain.

yielding more than 100 mg L^{-1} of crude EPS produced almost exclusively the high-molecular-mass fraction (between 96% and 82%), while those that synthesized between 100 mg L^{-1} and 40 mg L^{-1} produced mainly high-molecular-mass EPS (79%−60%). In contrast, poor EPS producers (< 25 mg L^{-1}) synthesized both fractions in similar amounts (between 55% and 42%) (**Figure 5**).

RAPD Profile Analysis. The clustering analysis including reference strains and cider isolates was calculated individually and in combination for the RAPD-M13, RAPD-P1 and RAPD-P2 profiles. In all cases, *P. damnosus* strains were separated from *P. parvulus*. At least four patterns were observed with a single primer. Of them, P1 showed the lowest intraspecies differentiation, i.e., one pattern was shared by 21 out of the 34 strains. Cophenetic correlation values were 90, 96 and 77% for the clustering with RAPD-M13, RAPD-P1 and RAPD-P2 profiles, respectively, and 90% for the global analysis combining the three RAPD profiles. The highest intraspecies differentiation was obtained with the combined RAPD M13−P1−P2 profile showing up to 17 patterns within the strains.

Combination of Phenotypic and Genotypic Data. Figure 1 shows the dendrogram derived from UPGMA clustering based on the

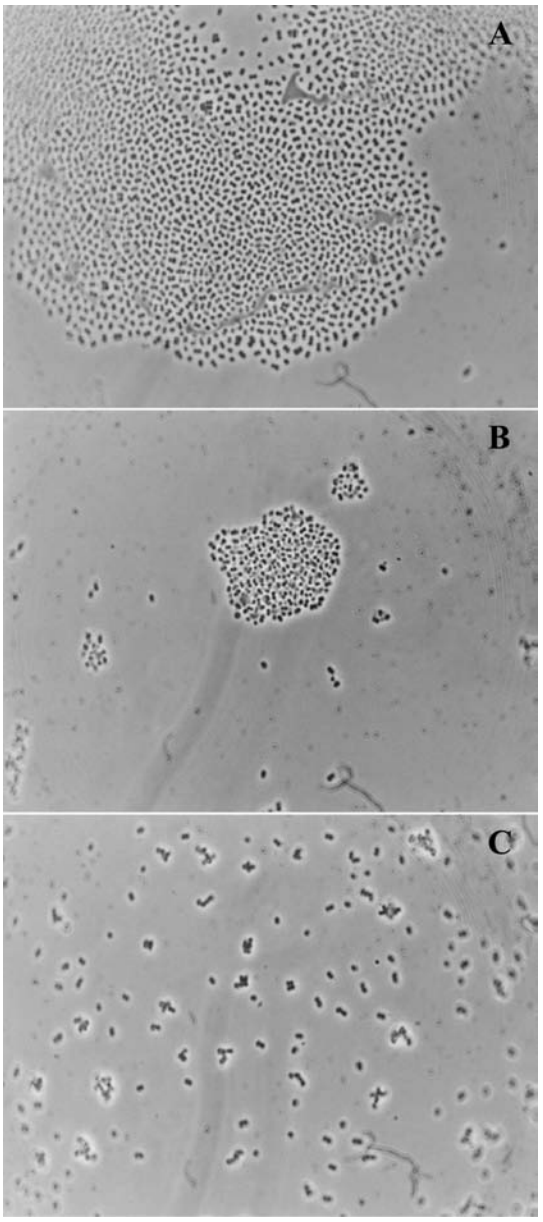

Figure 2. Immunoagglutination in presence of anti-type 37 antibody on (1,3)(1,2)-β-D-glucan producing *P. parvulus* strains CUPV22 (**A**) and CUPV25 (**B**). No agglutination with CUPV17 strain (**C**).

combined similarity matrix obtained from the M13, P1 and P2 RAPD fingerprinting, *gtf*-PCR amplification, sugar fermentation, agglutination test and EPS quantification. The total analyzed strains (34 *P. parvulus* and 2 *P. damnosus*) clustered at 10.5% similarity, with a cophenetic correlation value of 97%. At 58% similarity, two clusters were formed grouping all *P. parvulus* except the type strain CECT 7350^T that joined them at 52.5%. Cluster I grouped strain *P. parvulus* 2.6 together with 25 cider isolates. All isolates included in this cluster were positive for the agglutination test and exhibited the 417 bp amplicon (*gtf*-PCR positive). They also showed the highest EPS production values. Within this cluster, isolates CUPV28 to CUPV32 joined at 85% similarity. All of them were recovered the same year, from the same cellar and sample, which indicates their clonality. In other instances, isolates collected with 10 years of difference, i.e., CUPV7 to CUPV10, recovered in 1993 from cellar A, together with CUPV12 and CUPV13, recovered in 2003 from cellars C and D, respectively, joined at the same similarity. This finding might indicate a clonality of EPS producing strains linked to cider production. Strain *P. parvulus* 2.6 is clearly separated from other strains within the cluster. Cluster II grouped 7 cider isolates which were *gft*-PCR positive but negative for the agglutination test, and they showed the lowest EPS production levels. All isolates were recovered in 2005 from the same cellar, and 6 of them joined at 85% similarity while CUPV15 was clearly different.

DISCUSSION

EPS-producing lactic acid bacteria are being used as starter cultures or coadjutants for elaboration of fermented foods such as yogurt, cheese and cereal based products (*2*, *21*, *22*) because of their biothickening properties. In addition, the possible benefits for human health of exopolysaccharides produced by LAB are currently being studied (*1*). In this context, the 2-branched (1,3)-β-D-glucan producers may represent interesting strains for the food industry because of the widely described positive effects of the (1,3)-β-D-glucans as biological response modifiers (*4*). We have previously found that, besides the biothickener properties of the 2-branched (1,3)-β-D-glucan of *P. parvulus* 2.6 (*6*), this strain displayed immunomodulating activities and probiotic properties (*7*, *8*, *23*). In the present work, in order to identify new (1,3)-β-glucan producing strains, a screening among 147 LAB strains, previously isolated from ropy and nonaltered natural ciders, was performed.

Thirty-two isolates showed a ropy phenotype when they were grown in MRS broth, and all of them were identified as belonging to *P. parvulus*. As previously reported (*24*) for other homo- or heteropolysaccharide producing LAB strains, EPS production by these ropy *P. parvulus* varied widely among isolates. Interestingly, almost half of them synthesized large amounts of EPS (between 100 and 250 mg L^{-1}). These levels are relatively higher than those reported for other EPS^+ LAB strains isolated from natural food environments (*24*, *25*), with the exception of the huge amounts of dextrans or fructans produced by some *Leuconostoc* strains (*26*).

When molecular tools were used for β-D-glucan producing bacteria detection, it is worth noting that the 32 *P. parvulus* EPS^+ strains amplified in the PCR reaction with specific primers for the *gtf* gene, suggesting its β-D-glucan producing ability (*10*). In addition, the agglutination test with the specific antiserum against the capsular β-glucan of *S. pneumoniae* type 37 was assayed as supplementary method for rapid screening of the 2-branched (1,3)-β-D-glucan producers. Its capsular envelope is constituted by a branched polysaccharide composed of a backbone of →3)-β-D-Glc*p*-(1→ units with monosaccharide side chains of β-D-Glc-(1→ linked to C2 of each Glc residue (sophorosyl subunits). The anti-type 37 serum preferentially recognizes the branched part of this capsular polysaccharide (*27*), and, as a consequence, it reacts with *P. parvulus* 2.6 (*28*) and other 2-branched (1,3)-β-D-glucan producers (*11*, *29*). From our results, we can conclude that this agglutination assay could be successfully used as a rapid screening method when β-glucan levels are not very low (> 20−30 mg L^{-1}), in agreement with that reported by Dols-Lafargue et al. (*11*).

The synthesis of the 2-branched (1,3)-β-D-glucan by *gtf* and agglutination positive strains (25 strains) was confirmed by ^1H NMR analysis of the purified exopolysaccharides. In addition, methylation analysis data for the EPSs from strains CUPV1 and 22 (which were selected for producing high amounts of high-molecular mass EPSs) clearly showed that the crude EPS was only constituted by this kind of polysaccharide, in contrast to that previously described for *Lactobacillus sp.* G-77 (*30*) and *Oenococcus oeni* (*11*, *17*), which synthesize, besides the β-glucan, an α-glucan and heteropolysaccharides, respectively. This fact represents an

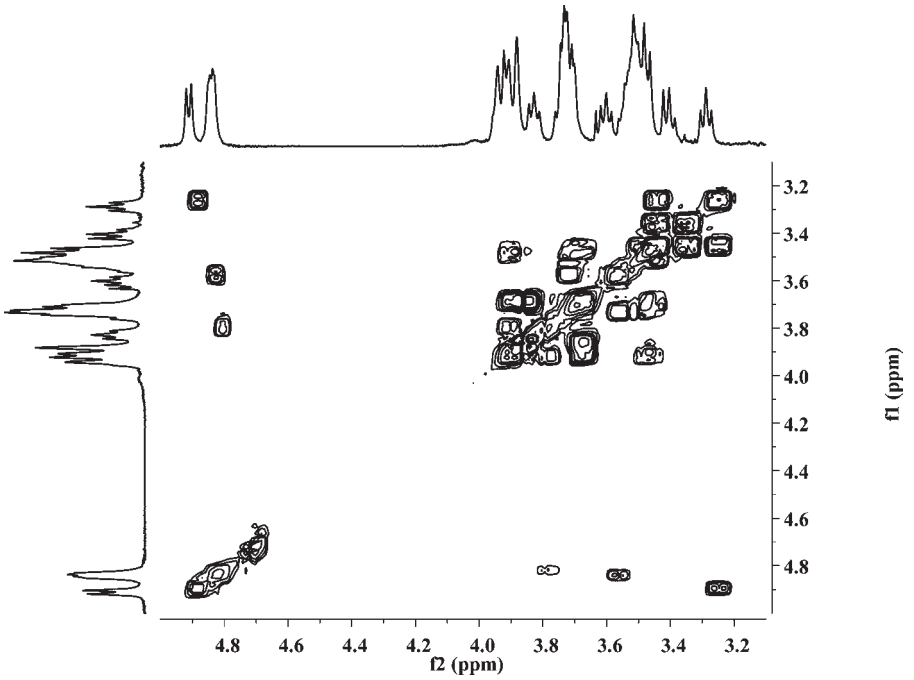

Figure 3. 500 MHz ^{1}H NMR spectrum of a representative β-glucan was recorded in D_2O at 30 °C, and the 500 MHz 2D homonuclear proton double-band filtered correlation spectrum (DBF-COSY) was obtained by double band selective excitation of the 3−5 ppm region.

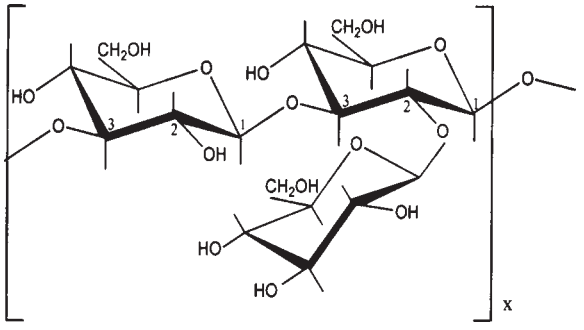

Figure 4. The trisaccharide repeating unit of the 2-branched-(1,3)-β-D-glucan synthesized by *P. parvulus*.

interesting characteristic in order to assess the biological activities or the prebiotic potential of these β-glucans.

In addition to primary structure and solution conformation, molecular weight may play a role on biological activity of the (1,3)-β-D-glucans, determining whether and with what affinity these polysaccharides bind to receptor(s) and modulate immune function (*4*). In this context, as found by Velasco et al. (*6*), culture medium composition decisively influences the molecular weight of the 2-branched (1,3)-β-D-glucan synthesized by *P. parvulus* 2.6, being glucose the sugar source in which molecular weight and EPS yield were highest.

For this reason, all strains in this study were grown on glucose and, similarly to that reported for other *P. parvulus* strains (2.6 and IOE 8801) (*6*, *11*), all β-glucans were constituted by two fractions, a high-molecular-mass polymer (close to 1×10^7 Da) and a low-molecular one (about 10^4 Da). The relative proportions of the two fractions varied widely between strains and were significantly correlated with EPS yield. As described by de Vuyst et al. (*31*), the molecular mass of an EPS is of primary importance for the intrinsic viscosity of the polymer, and, hence, some of our β-glucan producers could be considered as promising biothickening agents, such as strains CUPV1 and 22, because of the high molecular masses of their EPSs. For both strains, the molecular masses of their high-molecular fractions (**Table 1**) are higher than those reported for other (1,3)-β-D-glucans of commercial use, such as scleroglucan and Curdlan, which ranged from 1.3×10^5 to 6×10^6 Da (*32*) and from 5.3×10^4 to 2.0×10^6 Da (*33*), respectively. However, other factors such as EPS conformation or interactions between EPS and growth media microstructure could also contribute to the rheological features of the fermented media (*25*); further research is needed to asses its viscosifying properties in particular food matrices.

Regarding genotypic characterization, the EPS-producing *P. parvulus* cider isolates exhibited highly similar single RAPD profiles even though they had been recovered over a thirteen year period. However, the combined analysis of the three RAPD profiles and phenotypic traits grouped cider isolates together and apart from the type strain of the species, *P. parvulus* CECT 7350^T, isolated from silage. In addition, RAPD profiles distinguish clone isolates from singular strains such as *P. parvulus* 2.6, a previously characterized cider isolate (*10*), and they have been applied for strain selection. Besides, RAPD profiles obtained in the present study clearly allowed differentiation between *P. parvulus* and *P. damnosus*, two closely related species. It is worthwhile to mention that *P. parvulus* 2.6 was first described as belonging to the species *P. damnosus* (*18*, *34*) and later on assigned to *P. parvulus* following 16S rRNA sequencing analysis (*10*).

To conclude, in this study a screening of 2-branched (1,3)-β-D-glucan producers has been performed. All β-glucan positive strains belonged to *P. parvulus*, and 2 of them (CUPV 1 and 22) have been selected on the basis of their high EPS yields, molecular masses, and high proportion of the 10^7 Da fraction. They exhibit the ability to ferment several sugar sources, including glucose, galactose, and maltose, making them suitable for its use as starter or coadjuvant cultures in dairy and nondairy product fermentations. In addition, they are not of clonal origin as assessed by RAPD profiles and both strains can be easily differentiated by RAPD PCR with P1 or P2 primers. Currently, research on the probiotic and biothickening potential of these two strains is in progress.

Table 2. Percentages of the Linkage Types Deduced From Methylation Analysis of the EPS Produced by Strains 1 and 22 of *P. parvulus*

RT[a] (min)	deduced linkage type	characteristic fragments (*m*/*z*)	rel proportion	
			CUPV1	CUPV22
6.701	Glc*p*-(1→	87, 102, 118, 129, 145, 161, 162, 205	1	1
8.716	→3)-Glc*p*-(1→	101, 118, 129, 161, 173, 233	1	1
10.958	→2,3)-Glc*p*-(1→	87, 101, 129, 161, 202, 262	1	1

[a] Retention time.

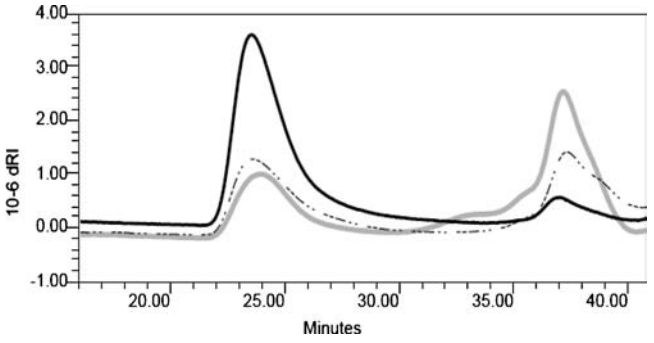

Figure 5. Size exclusion chromatograms of the β-glucans synthesized by *P. parvulus* CUPV22 (continuous line), *P. parvulus* CUPV15 (dashed lines) and *P. parvulus* CUPV5 (gray line) are depicted.

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