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Screening of lactic acid bacteria isolates from dairy and cereal products for exopolysaccharide production and genes involved

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Abstract

A total of 174 lactic acid bacteria (LAB) strains isolated from dairy and cereal products were screened for the production of exopolysaccharides (EPS). Therefore, a rapid screening method was developed based on ultrafiltration and gel permeation chromatography. Furthermore, a screening through the polymerase chain reaction (PCR) was performed with primer pairs targeting different genes involved in EPS production. Nine isolates produced a homopolysaccharide of the glucan type, whereas only one strain produced a heteropolysaccharide. The production of a glucan by a strain of *Lactococcus lactis* and the production of a heteropolysaccharide by a strain of *Lactobacillus curvatus* are reported for the first time. The PCR screening revealed many positive strains. For three of the ten EPS-producing strains, no corresponding genes could be detected. Furthermore, a lot of strains possessed one or more *eps* genes but did not produce an EPS. Therefore, a screening on the molecular level should always be accompanied by another screening method that is able to distinguish true EPS producer strains from non-producing ones. Statistical analysis did not reveal any relationship between the type and origin of the strains, the presence or absence of a capsular polysaccharide or EPS, and the presence or absence of *eps* genes.

Keywords: Lactic acid bacteria; Exopolysaccharides; Screening; Ultrafiltration

1. Introduction

The production of exopolysaccharides (EPS) by lactic acid bacteria (LAB) has been studied extensively during the last decade (De Vuyst and Degeest, 1999; De Vuyst et al., 2001; Welman and Maddox, 2003). The Generally Recognized As Safe (GRAS) status of LAB allows the *in situ* production of EPS in different food products (*e.g.* yoghurt and cheese), which meets the consumer's demand for products with low levels of additives (De Vuyst et al., 2001; Jolly et al., 2002). The increasing interest of the food industry in these so-called biothickeners results in an increasing demand for tailor-made polysaccharides (Duboc and Mollet, 2001). Although genetically modified organisms have the potential to fulfil these requests, their use is limited due to consumer's distrust and current legislations in many European countries (European Council Directive, 1990, 2001). As LAB are able to produce a great variety of both homopolysaccharides (HoPS) and heteropolysaccharides (HePS), the exploration of the biodiversity of wild LAB strains concerning their EPS production seems to be the most suitable approach (De Vuyst et al., 2001; Monsan et al., 2001; Vaningelgem et al., 2004a; Ruas-Madiedo and de los Reyes-Gavilán, 2005; Mozzi et al., 2006). Besides EPS, certain LAB produce capsular polysaccharides (CPS), which remain attached to the cell and are not excreted as slime, like EPS (De Vuyst et al., 2001; Mozzi et al., 2006).

Different EPS screening methods have already been described for LAB. The visual inspection of bacterial colonies

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on agar plates is most probably the easiest method, but unfortunately very insensitive and only indicative (Smitinont et al., 1999; Welman et al., 2003). This method is unable to detect LAB strains that produce low amounts of EPS, unless they are very ropy. The viscometric analysis of a culture medium during or after fermentation has also been applied to screen for EPS production (Vandenberg et al., 1993; Sanni et al., 2002). However, this is valid as far as the increase in viscosity can be ascribed to the production of EPS solely and not to other effects such as an increase in biomass. The partial purification of EPS through precipitation with ethanol or acetone and its spectrophotometrical or gravimetrical quantification is another screening method (van Geel-Schutten et al., 1998; Welman et al., 2003; Vaningelgem et al., 2004a). However, with this laborious technique the presence of contaminating polysaccharides in the culture medium, which may interfere with EPS quantification and characterization, cannot be neglected (Torino et al., 2000; Laws and Marshall, 2001; Vaningelgem et al., 2004a). Therefore, expensive semi-defined media, free of interfering polysaccharides, have been designed to study EPS production (Kimmel and Roberts, 1998; Torino et al., 2000; Bergmaier et al., 2001; Levander et al., 2001a). As an alternative, milk is often used with or without the addition of certain nutrients (Vaningelgem et al., 2004b; Mozzi et al., 2006). Ultrafiltration and gel permeation chromatography are interesting alternatives for rapid EPS screening (Toba et al., 1992; Levander et al., 2001a; Tieking et al., 2003). The increasing knowledge of the genetic structure and regulation of EPS production has recently led to the development of screening methods on the molecular level, both for HoPS and HePS (Kralj et al., 2003; Provencher et al., 2003; Tieking et al., 2003).

HoPS are synthesized by extracellular or cell-wall bound glycansucrases using sucrose as the glycosyl donor (Monchois et al., 1999; Monsan et al., 2001; van Hijum et al., 2006). HoPS of LAB can be subdivided into two major groups, *i.e.* those composed of only glucose (glucans) and those composed of only fructose (fructans). The production of various types of oligosaccharides is also possible, depending on the type of acceptor molecule (Monchois et al., 1998; Monsan et al., 2001; Demuth et al., 2002; Tieking and Gänzle, 2005). Glucansucrases and fructansucrases display a similar protein domain organization (van Hijum et al., 2006). Primers targeting the conserved catalytic domain have already been developed to screen both for genes encoding glucansucrases or fructansucrases in LAB (Kralj et al., 2003; Tieking et al., 2003). The biosynthesis mechanism of HePS is more complex. The repeating unit of HePS is produced intracellularly using sugar nucleotides as precursor molecules, then translocated across the cell-membrane, polymerized, and finally released into the medium (De Vuyst and Degeest, 1999; Welman and Maddox, 2003). The similarity between the HePS gene clusters from different LAB is most remarkable (Jolly and Stingele, 2001). These gene clusters, which are well conserved at the 5′ region, code for regulation, chain length determination, biosynthesis of the repeating unit, polymerization, and export (Jolly and Stingele, 2001; Broadbent et al., 2003). Several primer pairs have already been described, such as primers targeting regulatory genes (Low et al., 1998), genes involved in chain length determination (Deveau and Moineau, 2003), and genes coding for glycosyltransferases (van Kranenburg et al., 1999; Lamothe et al., 2002; Provencher et al., 2003).

The aim of this study was to systematically screen a large number of LAB isolates from dairy and cereal products as to their capacity to produce EPS. Therefore, a new, rapid method was developed to screen both for HoPS and HePS production. This method was compared with a screening for HoPS and HePS genes, using primers already described in the literature. Finally, we wanted to investigate possible relationships between strain characteristics and EPS production to find an explanation why LAB produce EPS in various food matrices (*e.g.* sourdough and dairy products).

2. Materials and methods

2.1. Microorganisms and media

The 174 LAB used throughout this study (*Enterococcus durans*, 10; *E. faecalis*, 1; *E. faecium*, 2; *E. saccharominimus*, 2; *Lactobacillus acidifarinae*, 1; *Lb. brevis*, 25; *Lb. curvatus*, 4; *Lb. farciminis/mindensis*, 3; *Lb. helveticus*, 4; *Lb. paracasei*, 2; *Lb. paralimentarius*, 9; *Lb. plantarum*, 28; *Lb. sakei*, 5; *Lb. sanfranciscensis*, 10; *Lb. zymae*, 1; *Lc. lactis*, 40; *Lc. garviae*, 1; *Leuconostoc citreum*, 7; *Leuc. mesenteroides*, 4; *Leuc. pseudomesenteroides*, 7; *Pediococcus pentosaceus*, 6; *P. acidilactici*, 1; *Weissella confusa/cibaria*, 1) were isolated from Belgian and Romanian dairy and cereal products, including raw milk (23 strains), fermented milk (14 strains), sour cream (47 strains), cheese (5 strains), and type I sourdough (85 strains). Strains were isolated on MRS agar (Oxoid, Basingstoke, United Kingdom), modified MRS agar with 50 g l^{-1} of sucrose instead of glucose, sourdough simulation medium (Messens et al., 2002), modified *Streptococcus thermophilus* (ST) agar (Dave and Shah, 1996) with 10 g l^{-1} of lactose instead of sucrose, or modified ST agar with 50 g l^{-1} of sucrose instead of lactose. All strains were stored at −80 °C in their corresponding isolation medium, containing 25% (vol/vol) glycerol as a cryoprotectant. Besides these isolates, five other strains were used as control strains for the different screening trials (see below): *Leuc. mesenteroides* subsp. *dextranicum* LMG 7939 (BCCM/LMG Bacteria Collection, Ghent, Belgium), *Lb. sanfranciscensis* 30, *Lc. lactis* subsp. *lactis* 11.2, *Streptococcus thermophilus* ST 111, and *S. thermophilus* LY03.

Filtrated MRS with sucrose (MRS_F-suc) or filtrated MRS with a mixture of maltose, fructose, and sucrose (MRS_F-mix) were used to screen for EPS (see below). Filtration aimed at the removal of glucomannans that could interfere with EPS screening. Therefore, ultrafiltration of the yeast extract (VWR International, Darmstadt, Germany), the bacteriological pepton (Oxoid), and the Lab Lemco powder (Oxoid), was performed, using a Vivaflow 200 module (Sartorius, Goettingen, Germany) with a 5-kDa molecular mass (MM) cut-off. This filtrate was then sterilized separately (210 kPa, 121 °C, 15 min). The energy

source (sucrose or a mixture of maltose, fructose, and sucrose) was also sterilized separately (210 kPa, 121 °C, 15 min) to prevent the interference of Maillard reaction products during EPS screening. After sterilization, all medium components were mixed aseptically.

To obtain fresh cultures, the strains were initially grown in their corresponding isolation medium at 30 °C (isolates from cereal products) or 37 °C (isolates from dairy products). Afterwards, they were propagated twice (1% [vol/vol]) in MRS_F-suc or MRS_F-mix to minimize carry-over of interfering polysaccharides from the initial medium. Cells were removed from 12-h old cultures by centrifugation (16,060 *g* for 15 min) and the cell-free culture supernatant was used to screen for EPS production.

2.2. Screening for CPS (staining) or EPS (gel permeation chromatography)

CPS formation was evaluated by the Indian ink negative staining technique (Mozzi et al., 2001) on cells from LAB strains grown in modified MRS with 50 g l^{-1} sucrose instead of glucose as the sole energy source. To screen for EPS production, isolates from dairy products were grown in modified MRS medium with 50 g l^{-1} of sucrose instead of glucose, and isolates from sourdough were grown in modified MRS medium with 10 g l^{-1} of maltose, 10 g l^{-1} of fructose, and 50 g l^{-1} of sucrose instead of glucose, and a pH adjusted to 5.40.

Screening for EPS through gel permeation chromatography (GPC) was performed using a Waters chromatograph (Waters Corp., Milford, MA), equipped with an Ultrahydrogel™ Linear column (Waters Corp.), kept at 35 °C, and coupled to a 2410 refractive index detector (sensitivity of 16 and scale factor of 25; Waters Corp.). The EPS were eluted with 0.1 M $NaNO_3$ at a flow rate of 0.6 ml l^{-1}. Dextran standards (ranging from 80 kDa to 4.9 MDa; Sigma-Aldrich, St-Louis, MO) were used to estimate the MM of the EPS.

To remove the proteins, 250 µl of Carrez I solution (3.6 g of $K_4(Fe(CN)_6).3H_2O$ in 100 ml of ultrapure water) and 250 µl of Carrez II solution (7.2 g of $ZnSO_4.7H_2O$ in 100 ml of ultrapure water) were added to 500 µl of cell-free culture supernatant. Samples were centrifuged (16,060 *g* for 15 min) and the supernatant, to which 3 ml of ultrapure water was added, was transferred to a Vivaspin 6 ultrafiltration module with a 10-kDa MM cut-off (Sartorius). After centrifugation (1250 *g* for 20 min), the retentate (usually <200 µl), to which 3 ml of ultrapure water was added again, was transferred to a new Vivaspin 6 module (Sartorius). In some samples the amounts of EPS were too high, causing blockage of the filter and resulting in a large retentate volume (>500 µl). If this was the case, 3 ml of ultrapure water was added to only part (500 µl) of the retentate, transferred to a new Vivaspin 6 module, and centrifuged (1250 *g* for 20 min). Finally, the retentate obtained after the second ultrafiltration (<200 µl) was adjusted to 500 µl with ultrapure water, filtered (0.2 µm), and 100 µl of this solution was injected onto the GPC column.

To confirm the results obtained through GPC screening, EPS from positive strains were isolated through acetone precipitation as described before (De Vuyst et al., 1998). To distinguish between HoPS and HePS, a monomer analysis was performed as described previously (Vaningelgem et al., 2004a), except that 8 N of hydrochloric acid was used instead of 6 N of trifluoracetic acid to hydrolyze EPS.

2.3. PCR screening for eps genes

A screening for *eps* genes was performed using different primers targeting HoPS and HePS genes (Table 1). Therefore, DNA from the 179 LAB strains was extracted as described before (Gevers et al., 2001). In total, five different PCR amplifications with the five pairs of primers were performed, using the same conditions as described by the corresponding authors (Table 1). Different strains were used as controls: *S. thermophilus* ST111 and *S. thermophilus* LY03 (*eps*A and priming glycosyltransferase genes), *Lc. lactis* subsp. *lactis* 11.2 (*eps*B gene), *Leuc. mesenteroides* subsp. *dextranicum* LMG 7939 (glucansucrase genes), and *Lb. sanfranciscensis* 30 (fructansucrase genes). The PCR amplicons were visualized through gel electrophoresis on 1% (wt/vol) agarose gels in 1×TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0), except for the PCR products of Provencher's primer pair, which were visualized on 2% (wt/vol) agarose gels. A 100-bp ladder (Invitrogen, Carlsbad, CA) was used to estimate the size of the bands. The bands obtained for the control strains as well as randomly chosen bands were purified using a GFX™ PCR, DNA, and gel band purification kit (Amersham Biosciences, Huckinghamshire, United Kingdom) according to

Table 1
Overview of the different primer pairs used to screen for *eps* genes involved in HoPS and HePS production

References	Sequence (5′-3′ orientation)	Target
Kralj et al. (2003)	F: GAYAAYWSIAAYCCIRYIGTIC R: ADRTCICCRTARTAIAVIYKIG	Glucansucrase genes
Tieking et al. (2003)	F: GAYGTITGGGAYWSITGGC R: TCITYYTCRTCISWIRMCAT	Fructansucrase genes
Low et al. (1998)	F: TAGTGACAACGGTTGTACTG R: GATCATTATGGACTGTCAC	*eps*A
Deveau and Moineau (2003)	F: CGTACGATTCGTACGACCAT R: TGACCAGTGACACTTGAAGC	*eps*B
Provencher et al. (2003)	F: TCATTTTATTCGTAAAACCTCAATTGAYGARYTNCC R: AATATTATTACGACCTSWNAYYTGCCA	Priming glycosyltransferase

F: forward, R: reverse.

the manufacturer's instructions. Sequencing was carried out using an ABI Prism®3100 Genetic Analyser (Applied Biosystems, Foster City, CA). Nucleotide and amino acid sequence analyses and translation of nucleotide sequences were performed with the BioEdit software version 7.0.5.2 (Hall, 1999). Similarity searches were performed with the advanced BLAST algorithm (Altschul et al., 1990, 1997) available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Sequence alignments were conducted with the BioEdit software or with the ClustalW algorithm (Thompson et al., 1994) as implemented in the BioEdit software. Comparison of putative conserved domains was performed using the conserved domain (CD) server (Marchler-Bauer and Bryant, 2004).

2.4. Nucleotide sequence accession numbers

The sequences obtained in this study are available under GenBank accession numbers DQ249312–DQ249319 and DQ873501–DQ873512.

2.5. Statistical analysis

All information about the strains (species and origin) and the data obtained throughout this study (presence/absence of *eps* genes, presence/absence of CPS, presence/absence of EPS) was merged into one database using the BioNumerics software Version 4.5 (Applied Maths N.V., Sint-Martens-Latem, Belgium). Similarity analysis was performed on the data obtained using the simple matching binary coefficient and clustering was performed with the unweighted pair group method with arithmetic averages (UPGMA), both as embedded in the BioNumerics software. Also, the BioNumerics software was used to perform a principal component analysis (PCA) to visualize possible relationships within the data matrix.

3. Results

3.1. Screening for CPS or EPS

The combination of ultrafiltration and GPC allowed the detection of EPS in cell-free culture supernatants down to a concentration of 20 mg l^{-1}. In total, ten out of 174 isolates were found to produce EPS in MRS_F-mix (sourdough isolates) or MRS_F-suc (all other isolates) (Table 2). A total of 27 out of 174 strains were found to produce CPS, including four out of the ten EPS positive strains (Table 2). The production of EPS by the control strains *Leuc. mesenteroides* subsp. *dextranicum* LMG 7937 and *S. thermophilus* ST111 was confirmed using the GPC method. The use of commercially available dextran standards did not allow an exact estimation of the MM of the EPS, as all EPS eluted earlier than the highest dextran standard available (MM of ± 5.0 MDa), indicating that the MM of all EPS exceeded 5.0 MDa. EPS-negative strains did not show any peak in the GPC chromatograms, thereby proving that contaminants were removed from the medium through ultrafiltration (Fig. 1A). For most EPS-positive strains, a second peak was observed in the GPC chromatograms. This peak always corresponded with a high retention time, indicating the presence of low MM compounds of less than 10 kDa (Fig. 1B), and thus not seen as interfering with EPS screening.

From all strains that were positive through GPC screening, EPS could be isolated, confirming the efficacy of this method. Furthermore, the MM of the isolated EPS corresponded with the MM obtained through GPC screening (Fig. 1C). Besides the GPC-positive strains, isolations from non-inoculated MRS_F-suc and MRS_F-mix were performed. As no pellet was retrieved from both media, contaminating polysaccharides were removed through ultrafiltration. As a final test, ten out of 164 strains, negative for GPC screening, were randomly selected for EPS isolation. No EPS could be retrieved in the case of all negative strains tested, which was in accordance with the results from GPC screening.

Monomer analysis of the isolated EPS revealed that nine out of ten EPS were glucans, produced in amounts of 0.8 to 17 g l^{-1} (Table 2). The only HePS obtained was produced by *Lb. curvatus* 10 in amounts of 22 mg l^{-1}. This HePS possessed a monomer ratio of 2:3:1 for galactosamine:galactose:glucose.

3.2. PCR screening for eps genes

All control strains displayed amplicons of the expected band size after PCR amplification with the corresponding primer pairs. The control fragments were sequenced and BLAST analysis indicated that all control bands corresponded with the expected *eps* genes (GenBank database). These control strains were used in each PCR experiment further on to check if PCR amplification was successful.

Screening for HoPS genes revealed that 29 out of 174 strains possessed genes coding for a glucan- or fructansucrase.

Table 2
Origin of EPS-producing strains and characteristics of the EPS produced

Strain	Origin	EPS		CPS[b]
		Yield (g l^{-1})[a]	Type	
Lc. lactis 1.8	Raw milk	0.8	Glucan	+
Leuc. citreum 1.10	Raw milk	7.0	Glucan	−
Leuc. citreum 1.11	Raw milk	5.0	Glucan	−
Leuc. citreum 1.12	Raw milk	16.7	Glucan	−
Leuc. citreum 2.8	Fermented milk	16.7	Glucan	−
Leuc. citreum 4.11	Raw milk	17.2	Glucan	−
Leuc. pseudomesenteroides 20.6	Raw milk	2.3	Glucan	+
Leuc. mesenteroides 21.2	Raw milk	0.9	Glucan	+
Weissella confusa/cibaria 38.2	Sourcream	8.6	Glucan	+
Lb. curvatus 10	Sourdough	0.02	2:3:1 GalN:Gal:Glc[c]	−

[a]Indicative EPS yield after growth of the strain in milk supplemented with 50 g l^{-1} sucrose (dairy origin) or in MRS_F-mix (sourdough origin); [b]presence (+) or absence (−) of a capsular polysaccharide; [c]HePS of which the repeating unit consisted of galactosamine (GalN), galactose (Gal), and glucose (Glc) in a ratio of 2:3:1, respectively.

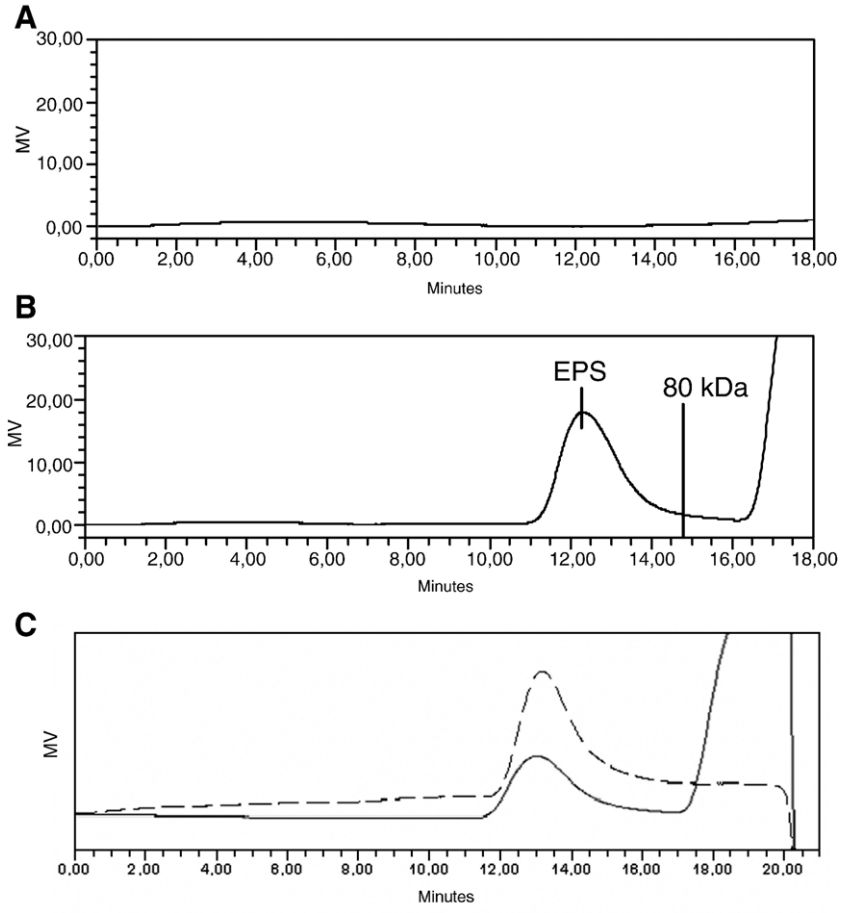

Fig. 1. (A) Gel permeation chromatography analysis of *Lactobacillus plantarum* strain 44.2 (EPS negative); (B) Gel permeation chromatography analysis of *Leuconostoc citreum* strain 2.8 (EPS positive). The vertical line corresponds with the retention time of an 80-kDa dextran standard; (C) Gel permeation chromatography analysis of *Leuc. citreum* 1.10. The dashed line corresponds with the chromatogram obtained for the isolated and purified EPS, whereas the full line corresponds with the analysis of the culture supernatants. Both peaks have the same retention time, indicating that the MM of the purified EPS matches the MM of the EPS produced in the culture medium. The scale on the *Y*-axis is different for both chromatograms to better visualize the similarity between the retention time of both peaks.

Sequencing of randomly selected strains proved that a partial sequence of the gene, coding for a glycansucrase, was amplified (Accession numbers DQ873509–DQ873511). The screening for possible glucansucrase genes revealed only ten positive strains. These belonged to the species *Lb. brevis* (1/25 strains), *Lb. plantarum* (1/28 strains), *Lc. lactis* (1/40 strains), *Leuc. citreum* (5/7 strains), and *Leuc. mesenteroides* (2/4 strains). Six of these ten positive strains were isolated from raw milk. The screening for putative fructansucrase genes revealed 21 positive strains, belonging to the species *E. faecalis* (1/1 strain), *Lb. brevis* (2/25 strains), *Lb. curvatus* (1/4 strains), *Lb. farciminis/mindensis* (1/3 strains), *Lb. paralimentarius* (2/9 strains), *Lb. plantarum* (2/28 strains), *Lb. sakei* (1/5 strains), *Lc. lactis* (5/40 strains), *Leuc. citreum* (1/7 strains), *Leuc. mesenteroides* (1/4 strains), *Leuc. pseudomesenteroides* (1/7 strains), and *P. pentosaceus* (3/6 strains). Two strains, *Lb. plantarum* 84 and *Leuc. citreum* 1.10, apparently possessed both a gene coding for a glucansucrase and one coding for a fructansucrase. From a culture of the latter strain a glucan could be isolated, for the former one no EPS production could be detected. For both the *Leuc. pseudomesenteroides* 20.6 and *W. cibaria/confusa* 38.2 strains, no HoPS gene could be detected, although GPC screening and EPS isolation revealed that both strains produced a glucan. For the *Leuc. mesenteroides* 21.2 strain, a positive signal was obtained for a gene coding for a fructansucrase, although a glucan was isolated.

The screening for HePS genes revealed that 75 out of 174 strains possessed one or more genes coding for enzymes involved in HePS biosynthesis. PCR screening with the primer pair of Low et al. (1998) for *eps*A revealed 15 positive strains. Analysis of several randomly selected sequences showed that this primer pair was not specific, because random sequences were amplified too. An example was the amplification of a sequence in *Leuc. mesenteroides* 19863 that encoded a cell-wall associated hydrolase (conserved domain accession no. COG0791) and a thymidine kinase (conserved domain accession no. COG1435), proving that sequences of the expected size but others than those of *eps* genes can be amplified with this primer pair. Screening with the primer pair of Deveau and Moineau (2003) for *eps*B revealed 44 positive strains, belonging to the species *Lb. brevis* (5/25), *Lb. plantarum* (2/28), *Lb. sakei* (1/5), *Lc. lactis* (31/40), *Leuc.*

Fig. 2. Multiple alignment of the partial priming glycosyltransferase sequences of *S. thermophilus* ST111 (ST111), *Lb. plantarum* 26.1 (26.1), *Leuc. citreum* 2.8 (2.8), *Leuc. mesenteroides* 19863 (19863), and *Lb. curvatus* 10 (10). Identical amino acids are shaded black, functionally similar ones are shaded gray.

citreum (3/7), *Leuc. pseudomesenteroides* (1/7), and *W. confusa/cibaria* (1/1). Randomly sequenced genes corresponded with the *eps*B gene. Finally, screening with the primer pair of Provencher et al. (2003) for the priming glycosyltransferase revealed 28 positive strains, belonging to the species *E. saccharominimus* (2/2), *Lb. brevis* (7/25), *Lb. curvatus* (3/4), *Lb. plantarum* (4/28), *Lb. sakei* (3/5), *Lc. lactis* (1/40), *Leuc. citreum* (4/7), *Leuc. mesenteroides* (3/4), and *P. acidilactici* (1/1). Not all randomly sequenced genes matched with the targeted gene on the nucleotide level. However, after translation of the non-matching sequences, a conserved bacterial sugar transferase region (conserved domain accession n°pfam02397) was found in the amino acid sequences, proving that the correct gene was amplified during PCR. As an example, an alignment of four randomly sequenced glycosyltransferases and that of the control strain *S. thermophilus* ST111 is represented in Fig. 2. The alignment illustrates that this part of the genes, coding for the priming glycosyltransferases, is indeed well-conserved among different LAB species.

3.3. Statistical analysis

All information about the strains (CPS positive/negative, EPS positive/negative, *eps* gene present/absent) was merged as binary data (absent/negative = 0, present/positive = 1) into one database (174 samples × 6 parameters). The data obtained with the primer pair of Low were not incorporated, as too many false positives were obtained for species other than streptococci (*i.e.* a band with the expected size was found, of which the sequence did not correspond with the *eps*A gene).

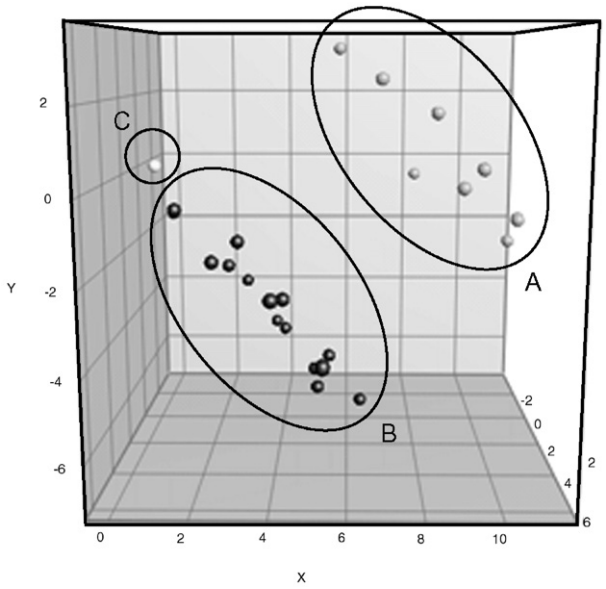

Fig. 3. 3D-plot of the principal component analysis, with PC1 (*X*-axis) representing 35.7% of the total variation, PC2 (*Y*-axis) representing 21.7% of the total variation, and PC3 (*Z*-axis) representing 16.1% of the total variation. Group A — strains positive for *eps*B gene; group B — strains positive for at least one characteristic (CPS, EPS, or *eps* gene other than the *eps*B gene); group C— strains negative for all characteristics studied. Principal component analysis did not reveal any relationship between type and origin of the strains, EPS or CPS production, and presence or absence of *eps* genes.

Cluster analysis of the 174 LAB isolates did not reveal any specific relationship between type and origin of the strains, presence or absence of CPS or EPS, and the presence or absence of *eps* genes. Therefore, a PCA was performed on the whole data set. The three principal components accounted for 73.5% of the total variation (PC1 = 35.7%, PC2 = 21.7%, and PC3 = 16.1%). The LAB isolates could be divided into three distinct groups (Fig. 3). The first group (group A) of 44 isolates included all strains that were positive for the *eps*B gene. Further analysis of this subgroup (cluster analysis and PCA) did not reveal any further information or relationship between type and origin of the strains and the data obtained. A second group (group B) comprised 52 isolates that were positive for at least one characteristic (CPS, EPS, or *eps* gene other than the *eps*B gene). Again, further analysis of this subgroup did not reveal any relationship between strain type and origin, presence of CPS or EPS, and presence of certain *eps* genes. A third and last group (group C) accounted for 78 isolates that were negative for all characteristics studied (points at origin). All ten *Lb. sanfransiscensis* strains belonged to the latter group.

4. Discussion

The application of EPS-producing LAB as functional starter cultures for the production of fermented foods such as yoghurt or cheese has gained much interest, because of the interesting technological properties of EPS (Jolly et al., 2002; Ruas-Madiedo et al., 2002). Although genetic modifications have been shown to be successful in enhancing the production of EPS (Boels et al., 2001; Levander et al., 2001b; Levander and Rådström, 2001), the public opinion and countries legislations restrict the use of GMOs for food applications (European Council Directive, 1990, 2001; Welman and Maddox, 2003). Therefore, exploration of the biodiversity of wild LAB strains, concerning their EPS production, seems the most suitable approach (Ruas-Madiedo and de los Reyes-Gavilán, 2005). The latter creates the need for rapid and reliable screening techniques to be able to screen in a fast and elegant way for EPS-producing LAB.

In the past, several screening methods have been applied that raised diverse problems during EPS determination and quantification, such as the presence of other polysaccharides in the media used (Kimmel and Roberts, 1998; Torino et al., 2000; Tieking et al., 2003). The latter problem was counteracted

in the present screening protocol through removal of interfering compounds by ultrafiltration. A second ultrafiltration procedure was included to partially purify EPS after fermentation, a method that was already applied previously (Toba et al., 1992; Bergmaier et al., 2001; Levander et al., 2001a). In general, the applied GPC screening method allowed the detection of EPS down to a concentration of 20 mg l^{-1} in cell-free culture supernatants in less than 2 h, which makes it a suitable method for a rapid screening of LAB isolates for EPS production. Also, the use of GPC as screening technique allowed an immediate estimation of the MM of the EPS. This information can be important when a selection of EPS-producing strains has to be made, which should be in favour of high-MM EPS, as in general these EPS give good results concerning viscosity-enhancing properties (Tuinier et al., 2001; Jolly et al., 2002).

Only ten out of 174 LAB isolates were found to produce EPS, which indicates a low frequency, in agreement with other studies (Vandenberg et al., 1993; van Geel-Schutten et al., 1998; Sanni et al., 2002). Four out of these ten EPS-producing strains showed a clear capsule. When LAB of the gastro-intestinal tract are screened, usually higher percentages of EPS-producing strains are found (van Geel-Schutten et al., 1998; Tieking et al., 2005). Nine of the positive isolates produced a HoPS (glucan), among which a strain of *Lc. lactis*, while only one strain, belonging to *Lb. curvatus*, produced a HePS. This is the first paper reporting on the production of a glucan polymer and a HePS by a strain of *Lc. lactis* and *Lb. curvatus*, respectively.

For seven of the ten EPS-producing strains, the presence of relevant genes was confirmed by PCR screening. For three others, a corresponding gene could not be detected, probably because the gene was less conserved in these strains. The presence of these false negatives is inevitable and has been reported previously (Tieking et al., 2003, 2005; Mozzi et al., 2006). Remarkably, a gene coding for a fructansucrase was found in *Leuc. mesenteroides* 21.2, although this strain produced a glucan under the conditions tested. This indicates dual activities of certain glycansucrases. For instance, Olivares-Illana et al. (2003) have recently characterized an inulosucrase with glucansucrase activity in a *Leuc. citreum* strain. Whether this is also the case for this *Leuc. mesenteroides* 21.2 strain has to be confirmed.

As observed during this study, a genetic screening also generates false positives that can be divided into two groups. The first group includes those strains that generate a positive PCR signal, but the sequence of the amplified fragment does not correspond with the targeted gene. This was the case when the primer pair of Low was used. The latter primer pair was designed on the sequence of *S. thermophilus* Sfi6 (Low et al., 1998). It worked perfectly for the two control strains used, both of them streptococci, but false positives were obtained with other LAB isolates. The second group of false positives includes strains that possess *eps* genes but do not produce EPS. This is to be explained by the non-expression of the genes. Although numerous in the present study, Kralj et al. (2003) and Tieking et al. (2003) did not report on the presence of such false positives. Therefore, a genetic screening method should always be accompanied with another method that distinguishes real EPS-producing strains from others (Mozzi et al., 2006). A genetic screening can, however, be a useful tool to make a first selection among a large number of isolates. With respect to this, the primer pairs of Kralj et al. (2003), Tieking et al. (2003), Deveau and Moineau (2003), and Provencher et al. (2003) have proven their specificity in this study to target the correct gene in several species of LAB, even in other species than those they were initially designed for.

Further, the screening revealed that several strains possessed more than one gene coding for a glycansucrase, which has been reported before (Li and Burne, 2001; van Hijum et al., 2002). Also, strains that possessed genes involved in both HoPS and HePs biosynthesis have been detected in this study. Strains capable of producing two types of EPS can be of interest from a commercial point of view, in particular when the EPS display a synergistic effect on the quality of the food product, *e.g.* one EPS that improves the rheology of the food product while the other may exert health benefits.

The reason why LAB produce EPS, if any general one exists, remains unclear. It is speculated that EPS production contributes to cell protection and cell adhesion (De Vuyst et al., 2001; Jolly et al., 2002). In an attempt to solve a piece of the puzzle, a statistical analysis of all data obtained throughout this study was performed. Unfortunately, cluster analysis and PCA did not reveal any relationship between type and origin of the strains, EPS or CPS production, and presence or absence of *eps* genes. Therefore, it might be interesting to increase the number of isolates or to take into account other characteristics, such as cultivation conditions (*e.g.* temperature, pH, energy source, *etc.*) or EPS characteristics (MM, monomer composition, *etc.*).

To conclude, a new screening method was developed that allows high-throughput screening of LAB isolates for their ability to produce EPS. Such screening method will allow a fast and rational selection of appropriate starter cultures for their implementation in value-added food fermentation processes.

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