APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2006, p. 4431–4435 Vol. 72, No. 6
0099-2240/06/$08.00+0 doi:10.1128/AEM.02780-05

Diversity of Heteropolysaccharide-Producing Lactic Acid Bacterium Strains and Their Biopolymers

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Received 24 November 2005/Accepted 27 March 2006

Thirty-one lactic acid bacterial strains from different species were evaluated for exopolysaccharide (EPS) production in milk. Thermophilic strains produced more EPS than mesophilic ones, but EPS yields were generally low. Ropiness or capsular polysaccharide formation was strain dependent. Six strains produced high-molecular-mass EPS. Polymers were classified into nine groups on the basis of their monomer composition. EPS from *Enterococcus* strains were isolated and characterized.

Certain lactic acid bacteria (LAB) produce exopolysaccharides (EPS), either capsular polysaccharides (CPS) that are tightly associated with the cell surface or slime EPS that are secreted into the extracellular environment. EPS from LAB can be divided into homopolysaccharides, which are polymers composed of one type of monosaccharide, and heteropolysaccharides (HePS), which are polymers of repeating units that are composed of two or more types of monosaccharides (5, 6, 7, 11). A large biodiversity of HePS from LAB exists regarding their composition and structure, molecular mass (MM), yield, and functionalities (5, 7, 35). Further, polymer formation is strongly influenced by culture conditions (1, 7, 22, 32). Recently, the molecular genetics of HePS biosynthesis have been studied for different LAB species (6, 25). Several glycosyltransferases involved in the assemblage of the HePS repeating units have been discovered (17, 18, 35). EPS can act as viscosifying, stabilizing, gel-forming, and/or water-binding agents in various foods (6, 10). Additionally, they have been claimed to display properties beneficial to health (6, 29). Little attention has been paid to CPS formation by food grade LAB. Exploration of the biodiversity of wild LAB strains is the most suitable approach to search for a desired EPS phenotype (28, 33, 34). The aims of this study were to seek new EPS-producing LAB strains, to characterize their biopolymers, and to explore their diversity to find novel or interesting HePS or HePS-producing strains.

EPS screening, isolation, and characterization. Two hundred one thermophilic (11 strains of *Lactobacillus acidophilus*, 79 of *Lactobacillus delbrueckii* subsp. *bulgaricus*, 1 of *L. delbrueckii* subsp. *lactis*, 1 of *Streptococcus macedonicus*, and 42 of *Streptococcus thermophilus*) and mesophilic (23 strains of *Enterococcus faecalis*, 1 of *Enterococcus faecium*, 5 of *Lactobacillus casei*, 29 of *Lactobacillus paracasei*, and 9 of *Lactobacillus rhamnosus*) LAB strains (CERELA Culture Collection, Tucumán, Argentina) were used throughout this study. All bacteria were stored as previously described (22). Before experimental use, cultures were propagated twice in MRS (Britania, Buenos Aires, Argentina) for lactobacilli or in LAPTg (27) for streptococci and enterococci.

Screening for EPS-producing LAB was performed with reconstituted skim milk (10%, wt/vol) at 37°C for 16 h using cultures propagated in milk as the inoculum (1%, vol/vol). Noninoculated medium was used as a control. Cell counts, expressed as numbers of CFU per milliliter, were determined by pour plating in MRS agar (MRS plus 15 g liter^{-1} agar) after 48 h at 37°C. The final pH of the milk cultures was measured as well. Milk cultures were evaluated for ropiness (R) as described by Mozzi et al. (23). CPS formation was determined by the India ink negative-staining technique (23), and slime EPS was isolated and quantified as described previously (1, 8), by using ethanol instead of acetone for EPS precipitation. EPS production was expressed as milligrams of polymer dry mass (PDM) per liter. EPS were purified by dialysis as described previously (34). The EPS isolations were performed in two independent experiments.

The MM of pure, freeze-dried EPS (10 mg ml^{-1}) was determined by gel permeation chromatography with a Waters Chromatograph (Waters Corp., Milford, Mass.) equipped with a Waters Ultrahydrogel column (500 to 8.0×10^6 Da) that was calibrated with dextran standards (ranging from 4.9×10^6 to 8.0×10^4 Da; Sigma, St. Louis, Mo.) with 0.1 M $NaNO_3$ as the eluent at a flow rate of 0.6 ml min^{-1}. Polysaccharides were detected with a Waters refractive index detector. High-MM EPS (HMM-EPS, arbitrarily defined as $>10^6$ Da) were distinguished from low-MM EPS (LMM-EPS, $<10^6$ Da). The MM determinations were performed in duplicate.

Purified, freeze-dried EPS (1.0 mg ml^{-1}) were hydrolyzed in 4 N (final concentration) HCl at 100°C for 3 h, freeze-dried, and redissolved in ultrapure water (1.0 mg ml^{-1}). Monomer analysis was carried out by high-performance anion-exchange chromatography with pulsed amperometric detection as described previously (34). EPS monomer analysis was performed in triplicate.

PCR conditions, DNA sequencing, and analyses. Genomic DNA from LAB was isolated as described previously (24). The presence of *eps* genes (HePS) was verified by using four primer

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pairs (Invitrogen Life Technologies, Karlsruhe, Germany) designed from *eps* genes described before (Table 1). Strains harboring these genes were used as positive controls after confirmation of the DNA sequences of the amplified genes. The PCR mixtures (25 μl) contained 15 ng of DNA, 2.5 mM $MgCl_2$, the four deoxynucleoside triphosphates at 100 μM each, each primer at 1 μM in *Taq* buffer, and 2.5 U of *Taq* polymerase. The PCRs were performed with a programmable heating incubator (Perkin-Elmer Corp., Norwalk, Conn.) by following the programs detailed in Table 1. Amplicons were analyzed by electrophoresis in 1% (wt/vol) agarose gels in 1× Tris-acetate-EDTA buffer. The 200-bp PCR products obtained with the *epsD/E* primers were run in 2% (wt/vol) agarose gels. A 100-bp ladder (Invitrogen Life Technologies) was used to identify the molecular sizes of the bands. PCR products were purified with the GFX PCR, DNA/gel band purification kit (Amersham Biosciences, Buckinghamshire, United Kingdom). Sequencing was performed with an ABI Prism 3100 genetic analyzer. Nucleotide and amino acid sequence analyses were performed as described recently (25).

Diversity of HePS from LAB. This is the first report on an extensive phenotypic and genotypic screening for EPS-producing strains from different LAB species. Thirty-one out of 201 LAB strains belonging to different species were capable of producing HePS (EPS^+) (Tables 2 and 3). No HePS were found for *L. acidophilus* and *S. macedonicus* strains. The growth and acidification capacity in milk of the LAB assayed was strain dependent. Vaningelgem et al. (34) investigated the HePS biodiversity of *S. thermophilus* strains, while other authors screened a large number of lactobacilli for homopolysaccharide production (33).

In general, the HePS yields were low, varying from 10 to 166 mg of PDM $liter^{-1}$. Seven out of 31 strains synthesized HePS in large amounts (>100 mg of PDM $liter^{-1}$). Also, Vaningelgem et al. (34) found a limited number of *S. thermophilus* strains producing HePS in large amounts. All thermophilic LAB strains produced HePS in larger amounts than mesophilic ones (this study), in contrast to other reports (5, 6). This indicates that culture conditions influence HePS formation for both thermophilic and mesophilic LAB.

The ability to synthesize CPS (CPS^+) or to display ropiness (R^+) by LAB was variable and strain dependent (Tables 2 and 3). Although CPS or ropy phenotypes are not usually sought in LAB, 14 LAB strains were CPS^+. Only two thermophilic LAB strains were both CPS^+ and R^+, while most of the mesophilic HePS-producing LAB strains were CPS^+ and R^+. *E. faecium* CRL210 was both CPS^+ and R^+. Two LAB strains (*L. delbrueckii* subsp. *bulgaricus* CRL861 and *L. paracasei* CRL208) that were ropy synthesized less than 10 mg of PDM $liter^{-1}$. In contrast, *S. thermophilus* CRL638 was the only R^+ strain among the LAB producing large amounts of HePS. Thus, ropiness was not correlated with the amount of EPS produced. Whether ropiness is associated with CPS or slime-EPS formation is still unclear. Hassan et al. (15) have classified EPS^+ LAB strains into three groups, CPS^+ R^+, CPS^+ R^-, and CPS^- R^-; no report of CPS^- R^+ strains, as was the case for *S. thermophilus* CRL863 (this study), was made. The ability to form CPS was much more widespread among the mesophilic HePS-producing LAB strains than among the thermophilic HePS producers. A positive effect of CPS formation on the

TABLE 1. Oligonucleotide primers used to detect *eps* genes containing sequences similar to the primers used

Primer	Sequence (5′–3′)	Gene target	Expected fragment size (bp)	PCR conditions	Positive control strain[a]	Reference(s)
epsEFG fw[b]	GAYGARYTNCCNCARYTNWKNAAYGT	Priming glycosyltransferase (*L. delbrueckii* subsp. *bulgaricus*)	1,600	30 cycles of 94°C (30 s), 49°C (45 s), 72°C (1 min)	*L. delbrueckii* subsp. *bulgaricus* CRL870	19, this work
epsEFG rev[c]	TGCAGCYTCWGCCACATG	Priming glycosyltransferase (*L. helveticus*)	300		*L. helveticus* CRL1176	19, this work
epsD/E fw	TCATTTTATTCGTAAAACCTCAATTGAY GARYTNCC	Priming glycosyltransferase (*L. casei* group and *S. thermophilus*)	200	5 cycles of 94°C (30 s), 62°C (30 s), 72°C (30 s); 40 cycles of 94°C (30 s), 52°C (30 s), 72°C (30 s)	*S. thermophilus* ST111 and LY03	25
epsD/E rev	AATATTATTACGACCTSWNAYYTGCCA					
epsA fw	TAGTGACAACGGTTGTACTG	EPS regulation (*S. thermophilus*)	800	35 cycles of 94°C (15 s), 40°C (30 s), 72°C (1 min)	*S. thermophilus* ST111 and LY03	20
epsA rev	GATCATTATGGACTGTCAC					
epsB fw	CGTACGATTCGTACGACCAT	EPS chain length determination (*L. lactis*)	1,150	35 cycles of 94°C (45 s), 46°C (1 min), 72°C (1 min)	*L. lactis* subsp. *lactis* 11.2	3
epsB rev	TGACCAGTGACACTTGAAGC					

[a] The presence and sequence analysis of the corresponding genes were confirmed by DNA sequencing.
[b] fw, forward.
[c] rev, reverse.

TABLE 2. HePS production by thermophilic LAB strains in milk and characterization of their polymers

Strain	Source	Cell count (CFU ml^{-1})	Final pH	PDM (mg liter^{-1})	R	CPS	MM (kDa)		Monomer ratio[a]					Group[b]
							High	Low	Glc	Gal	Rha	GlcN	GalN	
L. delbrueckii subsp. *bulgaricus*														
CRL142	Yoghurt	1.0×10^7	3.94	21	−	+	None	946		1.0				IX
CRL406	Yoghurt	3.8×10^8	3.95	83	−	−	None	642		1.0				IX
CRL553	Yoghurt	4.1×10^8	3.83	47	−	−	None	324	1.0	6.0				I
CRL563	Yoghurt	1.1×10^9	4.02	105	−	+	None	270	1.0	3.5				I
CRL852	Yoghurt	1.6×10^7	3.90	150	−	−	None	10	1.5	3.0	1.0			IV
CRL861	Yoghurt	1.4×10^9	4.56	<10	+	+	None	203	1.0	1.5				I
CRL863	Yoghurt	3.4×10^8	4.63	10	+	−	2,203	None	1.0	1.5				I
CRL865	Yoghurt	5.0×10^6	3.85	120	−	−	None	15	1.5	2.0	1.0			IV
CRL870	Yoghurt	4.0×10^7	4.19	126	−	−	>5,000	None	1.0	2.0				I
CRL874	Yoghurt	8.7×10^8	3.97	24	−	+	None	8	1.5	2.0	1.0			IV
L. debrueckii subsp. *lactis* CRL564	Yoghurt	9.6×10^8	3.95	55	+	−	None	314	1.0	1.5				I
L. helveticus CRL1176		3.9×10^9	4.38	41	+	−	1,800	None	2.0	1.0				I
S. thermophilus														
CRL419	Cheese	1.0×10^7	4.99	<10	−	−	None	186	2.0	1.0		1.0		V
CRL638	Yoghurt	1.3×10^9	4.60	118	+	−	1,190	None	1.0	2.0			2.0	III
CRL804	Yoghurt	2.1×10^8	4.50	166	−	−	None	95		2.5	1.0			II
CRL810	Yoghurt	4.1×10^8	4.30	56	−	−	None	123	1.0	2.5		1.0	2.0	VII
CRL815	Yoghurt	5.0×10^8	4.85	17	−	+	None	39		4.0	1.0	1.0	1.0	VIII
CRL817	Yoghurt	3.9×10^8	4.74	53	−	−	None	34	1.0	2.0		1.0	1.5	VII
CRL821	Yoghurt	3.0×10^8	4.69	<10	−	+	None	11	2.5	2.5		1.0	1.5	VII
CRL1190	Yoghurt	1.6×10^7	4.50	39	+	+	1,782	None	1.0	1.5				I

[a] Relative to other monomers in compound (on a molar basis).
[b] Classified according to the monomers present in the HePS.

viscoelastic properties of yoghurt has been shown before (15, 16). Moreover, the large CPS production by *S. thermophilus* MR-1C was responsible for the improved melting properties of low-fat mozzarella cheese (20).

The structural characteristics of the HePS analyzed in this study revealed a large diversity. A broad MM range, from 8 to >5,000 kDa, was found (Tables 2 and 3). LMM-EPS were more likely to occur than HMM-EPS. Only six LAB strains

TABLE 3. HePS production by mesophilic LAB strains in milk and characterization of their polymers

Strain	Source	Cell count (CFU ml^{-1})	Final pH	PDM (mg liter^{-1})	R	CPS	MM (kDa)		Monomer ratio[a]					Group[b]
							High	Low	Glc	Gal	Rha	GlcN	GalN	
E. faecalis														
CRL316	Cheese	6.7×10^8	5.80	110	−	−	None	280		2.0		1.0	2.0	VI
CRL434	Cheese	1.6×10^9	4.96	66	−	−	None	260	1.0	1.5		2.0		V
E. faecium CRL210	Cheese	4.2×10^8	5.14	27	+	+	2,160	None	3.0	1.5		1.0	2.0	VII
L. casei														
CRL87	Cheese	1.7×10^9	5.04	25	+	+	None	800	2.0	1.0	4.0			IV
CRL162	Cheese	5.1×10^8	4.88	23	−	−	None	180	1.0	2.0				I
CRL1425	Goat's milk	3.0×10^7	3.70	14	+	+	None	39	1.0	3.0				I
L. paracasei														
CRL72	Yoghurt	4.6×10^8	4.95	11	−	−	None	95	1.0	1.5		1.0	1.5	VII
CRL206	Yoghurt	6.6×10^8	5.43	18	+	+	None	110		1.5		1.0	3.0	VI
CRL208	Fermented milk	5.7×10^8	5.30	<10	+	+	None	64		2.0		1.0	3.0	VI
CRL717	Sausage	1.7×10^9	4.08	26	+	+	None	230	1.0	3.0				I
L. rhamnosus														
CRL627	Cheese	1.0×10^9	4.99	<10	−	+	None	78	3.0	1.0	1.0			IV

[a] Relative to other monomers in compound (on a molar basis).
[b] Classified according to the monomers present in the HePS.

produced HMM-EPS, including *E. faecium* CRL210. In contrast, Vaningelgem et al. (34) showed that HePS-producing *S. thermophilus* strains synthesized HMM-EPS or both HMM-EPS and LMM-EPS. *L. delbrueckii* subsp. *bulgaricus* CRL870 produced an HMM-EPS of >5,000 kDa, which could be of interest to improve the rheology of fermented milks, as the MM positively influences the rheological properties of a polysaccharide solution (9, 30). Five out of six HMM-EPS-producing strains were R^+; hence, ropiness is correlated with the size (HMM) of the HePS. However, the rheology of fermented milks with EPS^+ strains is affected not only by polymer structure, MM, and yields but also by EPS-protein interactions (6, 29).

Concerning the HePS monomer composition, galactose, glucose, galactosamine, glucosamine, and rhamnose were present but in different ratios (Tables 2 and 3). The isolated HePS were classified into nine groups, the number of different monomers varying from one to four, and each group harboring strains of different LAB species. Four of these groups were previously described for HePS from *S. thermophilus* strains (34). In our work, rhamnose was the least common monomer found, in contrast to other reports (7, 34). The presence of rhamnose could play a role in the bacteriophage sensitivity of the strains (12, 26). Galactose was the only monosaccharide present in all of the HePS. Also, galactose was present in all of the HePS produced by 26 *S. thermophilus* strains (34). Galactose incorporation could be due to poor expression of genes for galactose breakdown (4). The HMM-EPS produced by *E. faecium* CRL210 belonged to group VII, and the LMM-EPS produced by *E. faecalis* CRL316 and *E. faecalis* CRL434 belonged to groups VI and V, respectively. This is the first report on two strains of *L. delbrueckii* subsp. *bulgaricus* (CRL406 and CRL142) secreting polysaccharides composed solely of galactose. These unusual EPS were synthesized as HePS as no sucrose was added to the medium (5). To date, *L. lactis* strains have been shown to synthesize HePS with the same chemical composition (14, 35). In general, the biodiversity of HePS may be ascribed to the sugar nucleotide biosynthesis routes and the genetic potential of the strains (7). A correlation between the HePS monomers and the activity of glycosyltransferases (17, 18, 35) and enzymes for sugar nucleotide biosynthesis has been reported (2, 21, 22, 32).

The sequences of the *eps* PCR products of the genomic DNA of the control strains showed identities (61 to 97%) with the corresponding *eps* genes (GenBank database). With the *epsEFG* primers, a 300-bp PCR product was obtained with the DNA of *L. helveticus* CRL1176, while a 1,600-bp band was obtained with the DNA of *L. delbrueckii* subsp. *bulgaricus* CRL870. These differences were attributed to the absence of the *epsF* gene in the *eps* cluster of *L. helveticus* (18, 19, and GenBank accession no DQ222973). Most of the EPS^+ LAB strains possessed *eps* genes, as verified with the primers used. DNA of nine *L. delbrueckii* subsp. *bulgaricus* strains gave 1,600-bp PCR products with the *epsEFG* primers, while a 300-bp amplicon was obtained with the DNA of *L. delbrueckii* subsp. *bulgaricus* CRL861. All *S. thermophilus* strains exhibited 800-bp PCR products with the *epsA* primers. With the *epsD*/*E* primers, all of *S. thermophilus* strains displayed positive results, except for two strains (CRL815 and CRL638). Regarding the *epsB* primers, only DNA of *S. thermophilus* CRL815 yielded a PCR product. PCR products of 200 bp were amplified with the *epsD*/*E* primers for the *L. casei* group strains. No PCR products were obtained with the DNA from *L. delbrueckii* subsp. *lactis* CRL564 and from the enterococci tested with any of the primers used; only DNA of *E. faecalis* CRL434 gave an amplicon with the *epsB* primers. A good correlation between the presence of *eps* genes with the specific primers described for HePS and the HePS phenotype was observed. Specific primers to detect *eps* genes in the DNA of *L. delbrueckii* subsp. *lactis* CRL564 and enterococcal strains remain to be designed. Screening with different sets of *eps* primers could be a rapid alternative way to seek HePS-producing strains, although the HePS phenotype has to be confirmed afterwards.

For the first time, it was shown that enterococcal strains are able to produce HePS. Although enterococci are not widespread in the dairy industry, they are usually present in artisanal Argentinean cheeses (Tafí) and are often part of the dominant microbiota of ripened Mediterranean cheeses (13, 31). Safe HePS-producing enterococcal strains could improve the textural characteristics of cheeses, as long as they are not involved in virulence (13).

This extensive and systematic study showed a wide biodiversity of HePS from LAB. Furthermore, novel polysaccharides and new HePS-producing LAB species were found which will be of further interest for technological exploitation.

Nucleotide sequence accession numbers. The sequences obtained in this study are available under GenBank accession no. DQ222973, DQ249312 to DQ249314, DQ249316, DQ249317, and DQ249319.

We thank CONICET, ANPCyT, CIUNT, and SECyT of Argentina, FWO-Flanders of Belgium (bilateral scientific cooperation project FW/PA/02-BIX/011), and the academic cooperation agreement between VUB and CONICET for financial support.

We are very grateful to Tom Adriany, Cecilia Rodríguez, and Micaela Pescuma for assistance.

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