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Comparison of exopolysaccharide production by strains of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* grown in chemically defined medium and milk

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Exopolysaccharide (EPS) production was compared among three strains of lactobacilli. *Lactobacillus rhamnosus* strain 9595M can be classified among the highest EPS-producing strains of lactic acid bacteria reported to date with a maximum EPS production of 1275 mg L^{-1}. Under controlled pH, no significant differences in the quantity of EPS produced could be detected between carbon source (glucose or lactose) or fermentation temperature (32 or 37°C). In milk, strains ATCC 9595M and R produced more than 280 mg L^{-1} EPS whereas strain Type V produced less than 80 mg L^{-1} EPS. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 251–255.

Keywords: lactobacilli; exopolysaccharide; productivity; milk viscosity

Introduction

Exopolysaccharides (EPS) produced by mesophilic and thermophilic lactic acid bacteria play an important role in the improvement of the texture and viscosity of fermented milk [2]. A number of physiological conditions (medium composition, physico-chemical and kinetic parameters) have been described and are known to influence EPS production by lactic acid bacteria. However, few studies have been conducted on EPS production by *Lactobacillus casei* and *L. rhamnosus* [3,5,13]. However, strains of these species of lactobacilli have become important as adjunct cultures for the production of fermented milks [17]. Indeed, *L. casei*, *L. paracasei* and *L. rhamnosus*, which are used as probiotics in man and animals, are members of the *Lactobacillus casei* group [9].

Kojic *et al* [10] described an EPS-producing strain, *Lactobacillus casei* CG11, which was isolated from soft, white, home-made cheese. Plasmid curing experiments revealed that EPS production by this strain seems to be linked to a plasmid approximately 30 kb in size. *L. casei* CG11 was grown in basal minimal medium (BMM) which allows a more precise determination of EPS production because it does not contain any of the polysaccharides present in complex media. An industrial strain of *L. rhamnosus* isolated from fresh fermented milk which efficiently produces EPS in a synthetic medium was selected by Gamar *et al* [5]. However, the quantity of EPS produced has always been fairly modest and more productive strains as well as optimal conditions must be found for viable industrial use.

The objective of the present study was to characterize EPS production and viscosity in a chemically-defined medium under pH-controlled conditions and in milk by selected strains with different genomic profiles. This will allow starter cultures to be selected on the basis of improved knowledge of technological performance.

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Materials and methods

Bacterial strains and culture conditions

The exopolysaccharide (EPS)-producing strains of lactobacilli were isolated from reference cultures and commercially-available starter cultures (Table 1). All strains were subcultured in 20 ml of Lactobacilli MRS broth, incubated anaerobically for 48 h at 37°C. Stock cultures were kept frozen in brain heart infusion (BHI) with 15% glycerol.

Carbohydrate fermentation patterns and detection of mucoid phenotype

The carbohydrate fermentation profiles of isolates were determined with the micromethod according to Roy and Ward [15]. At the end of incubation (37°C for 48 h), lactobacilli were selected for ropiness by touching colonies on MRS agar with a sterile inoculating loop, and drawing the loop away from the colony until rupture of the resulting filament.

DNA extraction and determination of plasmid profiles

Genomic DNA was prepared according to Vincent *et al* [19] from stationary-phase cultures in MRS broth. Plasmid DNA was isolated according to Klaenhammer [8]. Agarose gel electrophoresis and staining was carried out with standard procedures [18]. Gels were photographed under UV transillumination with the Gel Print 2000i System (Bio/Can Scientific, Mississauga, ON, Canada).

Table 1 Designation, origin and characteristics of exopolysaccharide-producing strains used in this study

Designation	Isolated from	Identified as[b]	Name	Presence of plasmid
Isolate 1	Original culture of *L.* rhamnosus ATCC 9595[a]	*L. casei*/*L. rhamnosus*	*L. rhamnosus* 9595M	–
Isolate 2	Commercial culture, Rosell Institute[a], Canada	*L. casei*/*L. rhamnosus*	*L. rhamnosus* R	–
Isolate 3	Commercial culture, Rosell Institute, Canada	*L. paracasei*	*L. paracasei* Type V	–

[a]ATCC = American Type Culture Collection, Manassas, VA, USA; Rosell Institute, Montréal, PQ, Canada.
[b]Identified by dot blot hybridization, using specific 23S rDNA probes [17]. Lber (5′-GCAGGCAATACACTGATG) is specific for *Lactobacillus casei*, *Lactobacillus rhamnosus* and Lbpa (5′-CACTGACAAGCAATACAC) is specific for *Lactobacillus paracasei*.

Pulsed-field gel electrophoresis

Preparation of cells and genomic DNA was performed as described by Roy *et al* [16].

Dot-blot hybridization with 23S rDNA probes

Dot blot hybridization, using 23S rDNA probes designed by Hertel *et al* [6], was used for identification to the species level of the *L. casei*-related group. The Lbcr and Lbpa oligonucleotides were synthesized and labeled with digoxygenin by GSD (General Synthesis and Diagnostics, Centre for Biomaterials, Toronto, ON, Canada). Denatured genomic DNA (1 μg) was deposited on nylon membranes using a Bio-dot apparatus. Hybridization was carried out for 2 h at 42°C for Lbcr and at 40°C for Lbpa. Membranes were then washed twice for 5 min in 2 × SSC, 1% SDS at 53°C for Lbcr and at 49°C for Lbpa. Signals were detected by autoradiography of chemiluminescence with CSPD® substrate according to the manufacturer's instructions (Roche Diagnostics, Laval, Québec, Canada).

Production and extraction of EPS and viscosity of fermented milk

Strains were subcultured twice in MRS broth before inoculating them at 10% v/v in BMM medium [12] containing 10 g L^{-1} glucose, followed by anaerobic incubation for 48 h at 37°C. Kinetic studies were performed in BMM medium containing 20 g L^{-1} glucose or lactose in 6-L working volume bioreactors (Chemapec, Woodbury, NY, USA) inoculated at 1% (v/v) at a controlled temperature of 32 or 37°C and an agitation speed of 100 rpm. The pH was maintained at 6.0 by automatic addition of 7 N NH_4OH. Fermentations were carried out in triplicate. Two samples (2 × 50 ml) were collected at the beginning of fermentation, then at eight intervals during the 72 h of incubation. Culture supernatant was heated in a boiling water bath for 15 min and the EPS were isolated by ethanol precipitation as described by Cerning *et al* [3]. Total sugar concentration of purified EPS preparations was determined by the phenol-sulfuric acid method [4]. Results are expressed in milligrams of glucose per liter.

For milk fermentation, 1 L of reconstituted skim milk (10% w/v) was sterilized at 110°C for 10 min and inoculated with 3% (v/v) overnight culture, equally distributed in 100-ml screw cap bottles and incubated at 37°C for 24 h. Glucono-delta-lactone (GDL) was used to acidify milk as a control without bacterial EPS. Samples were withdrawn after 0, 12, 15, 18, 21, 24 and 36 h of incubation and stirred before measurement of viscosity. Rotational viscosity measurements were made with a coaxial cylinder (Rotovisco RV20, Haake M5-OSC, Haake, Karlsruhe, Germany) at a steady shear rate of 173 s^{-1} using an MK 50 rotor with an NVST sensor system operating at room temperature. For EPS extraction, 100-ml samples of fermented milk were first heated at 90°C for 10 min. The proteins were then digested with pronase (enzyme-to-substrate ratio of 1:68.5) at pH 7.5, 40°C for 20 h in the presence of 0.05% w/v merthiolate. After incubation, the mixture was heated at 90°C for 15 min, and the cells were removed by centrifugation. The EPS were isolated by ethanol precipitation. Viable counts of lactobacilli were estimated using MRS agar (Difco Laboratories, Detroit, MI, USA), for 48 h at 37°C under anaerobic conditions. Sugars were determined by HPLC (Bio-Rad Laboratories, Richmond, CA, USA) using an Ion-300 column (Mandel Scientific, Rockwood, ON, Canada) with 0.02 N H_2SO_4 as mobile phase at a flow rate of 0.4 ml min^{-1}.

Statistical analysis was carried out using the General Linear Models Procedure of SAS (SAS Institute, Cary, NC, USA).

Result and discussion

All three isolates were identified as *Lactobacillus* sp by carbohydrate fermentation patterns (data not shown). Pulsed-field gel electrophoretic restriction analysis revealed different profiles for the three isolates (results not shown). The oligonucleotide probe specific for *L. casei*/*L. rhamnosus* hybridized with DNA extracted from isolates 1 and 2 while the *L. paracasei*-specific probe reacted with DNA from isolate 3 (Table 1). Evidence accumulating from molecular typing and phylogenetic studies show that *L. paracasei* is highly related to *L. casei* ATCC 334 [11]. However, the classification approved by the Judicial Commission of the International Committee on Systematic Bacteriology [20] has been maintained for the purpose of the present study.

No plasmid DNA was detected for any of the three isolates (Table 1). For strain CRL 87 of *L. casei*, the absence of plasmids also suggests that the determinants of EPS synthesis are chromosomal [13], while EPS production by *L. casei* strain CG11 was linked to a 30-kb plasmid [10]. As no plasmids were found, the genes required for EPS might be associated with the chromosome in the strains currently under examination. For industrial use, strains with a more

Table 2 Maximum quantity of exopolysaccharides (EPS) produced, EPS yield and EPS productivity for three strains of *Lactobacillus* sp according to incubation temperature and carbon source

Strain	Carbon source	EPS produced[a] (mg L^{-1}) ± SE[d] at		EPS yield[b] (%) ± SE[d] at		EPS productivity[c] (mg L^{-1} h^{-1}) ± SE[d] at	
		32°C	37°C	32°C	37°C	32°C	37°C
L. rhamnosus 9595M	glucose	1138 ± 89	933 ± 96	4.89 ± 0.88	4.28 ± 0.83	40.73 ± 7.35	40.71 ± 7.93
	lactose	1275 ± 41	1031 ± 104	6.09 ± 0.71	4.85 ± 0.60	36.16 ± 5.21	40.38 ± 5.03
L. rhamnosus R	glucose	498 ± 88	438 ± 45	1.33 ± 0.40	2.19 ± 0.23	12.29 ± 2.30	12.17 ± 1.25
	lactose	601 ± 153	495 ± 33	3.31 ± 0.82	2.52 ± 0.19	22.26 ± 5.66	20.64 ± 1.37
L. paracasei Type V	glucose	85 ± 36	93 ± 36	0.64 ± 0.28	0.46 ± 0.18	1.42 ± 0.61	1.54 ± 0.59
	lactose	85 ± 16	67 ± 6	0.43 ± 0.13	0.33 ± 0.03	1.02 ± 0.31	0.93 ± 0.09

[a]EPS = exopolysaccharides expressed as mg glucose L^{-1} produced in basal minimum medium (BMM) under pH-controlled conditions. The values are the means of three experiments.
[b]EPS yield = defined as (EPS produced (mg L^{-1})/source of carbon used (mg L^{-1})) × 100.
[c]EPS productivity = defined as maximum slope of the tangent of the EPS concentration curve drawing from the point of initial EPS concentration.
[d]SE = Standard error.

stable mucoid phenotype would be preferred, so the chromosomal location may be advantageous. Such stability must, however, be tested experimentally.

The influence of carbon source (20 g L^{-1} glucose or lactose) and incubation temperature (32°C and 37°C) on EPS production were studied in chemically defined BMM medium with pH maintained at 6.0. The EPS production varied more among the three strains studied than between incubation temperatures or between carbon sources within strains (Table 2). There was no significant difference in the quantity of EPS produced between glucose and lactose as carbon source, except for *L. rhamnosus* R which gave higher yield, productivity and specific yield of EPS production when cultured with lactose. For the optimal growth temperature of 37°C and a sub-optimal temperature of 32°C, there was no significant difference in the quantity of EPS produced. Maximum production was reached in stationary growth phase after 48 h with *L. rhamnosus* 9595M, 27 h with *L. rhamnosus* R and 60 h (glucose) or 72 h (lactose) with *L. paracasei* Type V. Under the conditions used in this study, *L. rhamnosus* 9595M had the highest EPS production (1275 mg L^{-1}) reported to date for lactobacilli. *L. rhamnosus* R should also be classified among the best strains with a maximum EPS production of 601 mg L^{-1} (Table 2). In comparison, *L. casei* strain CRL 87 produced 488 mg L^{-1} of EPS when cultivated in culture medium at a constant pH of 6.0 for 24 h [14] and *L. delbrueckii* subsp *bulgaricus* RR synthesized 354 mg L^{-1} of EPS under similar conditions (synthetic medium with pH control) [7]. *L. rhamnosus* C83 produced a maximum of 132 mg L^{-1} polysaccharide in a synthetic medium without pH control [5].

The productivity, defined as the maximum production of EPS in a minimum of time, is an important variable from the industrial point of view [7]. Although the maximum EPS production was attained only between 36 and 48 h (data not shown), EPS productivity (Table 2) for *L. rhamnosus* 9595M was double that reported in the literature for *L. casei* CRL 87 (approximately 20.33 mg L^{-1} h^{-1} after 24 h [14]). The EPS productivity for *L. rhamnosus* R was only slightly higher after 27 h of incubation (Table 2) than that of *L. paracasei* CRL 87 and was very similar to that

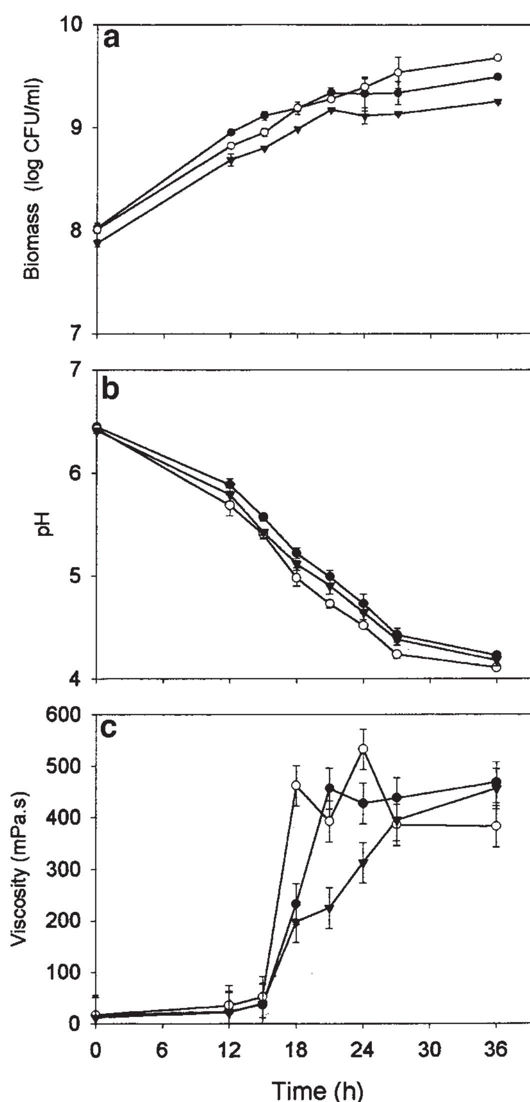

Figure 1 Growth (a), pH (b) and viscosity (c) during fermentation of milk by *Lactobacillus rhamnosus* ATCC 9595M (●), *L. rhamnosus* R (○) and *L. paracasei* Type V (▼). The values are the means of three experiments. Bar = standard error.

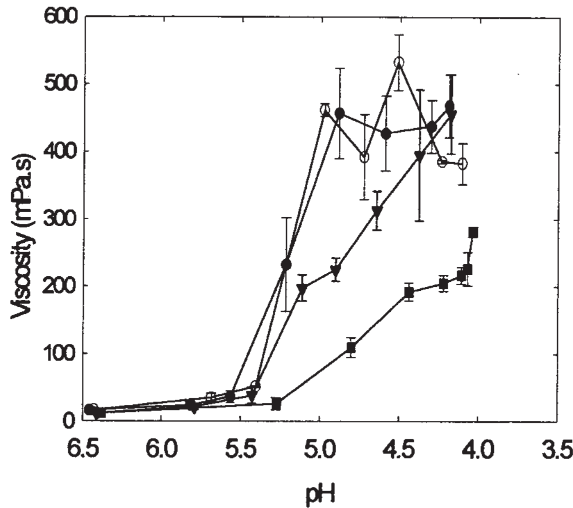

Figure 2 Viscosity *vs* pH for milk fermented with *Lactobacillus rhamnosus* ATCC 9595M (●), *L. rhamnosus* R (○), *L. paracasei* Type V (▼) or glucono-delta-lactone (■). The values are the means of three experiments. Bar = standard error.

of *L. delbrueckii* subsp *bulgaricus* RR, which was 23.35 mg L^{-1} h^{-1} after 15 h of fermentation [7]. Finally, the low EPS productivity of *L. paracasei* Type V (1.54 mg L^{-1} h^{-1}) was close to that reported for *L. rhamnosus* C 83 (2.84 mg L^{-1} h^{-1}) [5].

Growth and reduction of milk pH by the two *L. rhamnosus* strains were similar to that of *L. paracasei* Type V (Figure 1a,b). However, milk viscosity increased more rapidly with the two strains of *L. rhamnosus* than with *L. paracasei* Type V (Figure 1c), although viscosity was not significantly different ($P > 0.05$) among strains after 24 h of fermentation. In addition, pH has a considerable influence on the structure of the coagulum formed by milk proteins. Acidification of milk by glucono-delta-lactone (GDL) used as a control was faster than that obtained with bacterial fermentation (results not shown). Viscosity of both fermented and acidified milk progressively increased as pH decreased (Figure 2). At pH 5.0, which corresponds to the destabilization of casein micelles due to total solubilization of micellar calcium and phosphorus at 20°C, the viscosity in milk obtained with *L. rhamnosus* ATCC 9595M and *L. rhamnosus* R was significantly ($P \leqslant 0.05$) higher than that obtained with *L. paracasei* Type V, while GDL-acidified milk gave the lowest viscosity at this pH. The higher viscosity obtained when EPS-producing strains were added to ferment milk instead of GDL suggests that EPS production affects the viscosity of fermented milk, in agreement with Bouzar *et al* [1]. Below pH 4.5, viscosity of fermented milk was not significantly different among the strains, while still remaining significantly higher than that obtained in GDL-acidified milk.

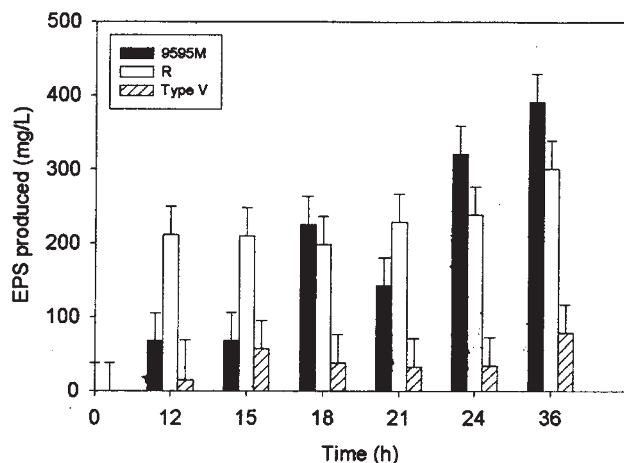

Figure 3 Exopolysaccharides (EPS) produced by *Lactobacillus rhamnosus* ATCC 9595M, *L rhamnosus* R and *L. paracasei* Type V during fermentation of milk. The results are expressed in milligrams of glucose per liter. The values are the means of two experiments. Bar = standard error.

EPS production for the two strains of *L. rhamnosus* was significantly higher than that of *L. paracasei* Type V (Figure 3). The time of maximal EPS production also varied among the strains tested. The EPS produced by *L. paracasei* Type V attained only 79 mg L^{-1} after 36 h of incubation while the *L. rhamnosus* strains (ATCC 9595M and R) produced more than 280 mg L^{-1} of EPS in milk. These *L. rhamnosus* strains are good candidates for EPS production in fermentation processes, showing higher yields than those obtained with *L. casei* CG11 in milk ultrafiltrate (35–40 mg L^{-1} [3]) or by *L. casei* CRL 87 growing in milk (121 mg L^{-1} [13]). Finally, *L. paracasei* V produced a lower level of EPS, but resulted in similar viscosity effects in comparison to ATCC 9595M and R strains after 48 h of culture growth in milk. Viscosity depends not only on the quantity of EPS present, but also on the structure and apparent molecular mass of the EPS which was different for that produced by *L. paracasei* V as compared with the *L. rhamnosus* strains (results not shown).

Conclusion

According to our knowledge of the literature, *L. rhamnosus* 9595M can be classified among the highest EPS-producing strains of lactic acid bacteria reported to date. Initial characterization revealed the technological potential of EPS-producing isolates of *L. paracasei* and *L. rhamnosus* to contribute to the viscosity of fermented milks. Further studies must be conducted in order to determine the influence of culture conditions (pH, temperature, carbon source) on EPS structure and yield by these strains.

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