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Isolation and characterization of exocellular polysaccharides produced by *Bifidobacterium longum*

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Abstract When grown anaerobically at pH values above 5.0, on ultrafiltered complex media containing excess lactose, *Bifidobacterium longum* formed up to 140 mg l^{-1} (glucose equiv.) exopolysaccharides. The highest yield was obtained when the cells were cultivated in a peptone/yeast extract medium with pH controlled by additions of NH_4OH. Whatever the conditions under study, exopolysaccharides represented about 30% of the polysaccharides produced by *B. longum* after 48 h of culture. Crude pronase-treated exopolysaccharide preparations were adsorbed on ion-exchange chromatographic resin to yield an anionic heteropolysaccharide fraction. Two subfractions with apparent molecular masses of 1.2 MDa and 0.36 MDa respectively were subsequently recovered after gel filtration on Sepharose 4B. In both subfractions, glucose, galactose and small amounts of uronic acids and hexosamines were present in similar molar proportions, suggesting that the excreted polymers may be synthesized from the same base unit and may have a structure resulting from repeating subunits.

Introduction

It has been demonstrated that lactic acid bacteria strains used in manufacturing fermented milk products can form exocellular polysaccharides (EPS) under certain conditions (Cerning et al. 1992). The intrinsic viscosity of the EPS produced by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, the traditional microflora of yoghurt, reflects the thickening property of the polymers (Doco et al. 1989; Cerning et al. 1990). Since 1986, this microflora has been enriched by a third bacterium belonging to the genus *Bifidobacterium*, sometimes associated with *Lactobacillus acidophilus* in fermentation processes (Ballongue 1993).

Bifidobacteria have long been recognized as bacteria with probiotic, nutritive and therapeutic properties (Hughes and Hoover 1991). Their adhesion to intestinal host cells is promoted by a high level of fatty acids in the lipoteichoic acids. The strong electrostatic charge of the polysaccharides of these gram-positive bacteria also favours their adhesion to the cell wall (Savage 1984; Op Den Camp et al. 1985).

Bifidobacteria are used as viable cells (more than 10^6 cfu/ml) (Driessen and DeBoers 1989) in commercial preparations such as milk products. The most commonly used species are *Bifidobacterium longum* and *Bifidobacterium bifidum*. Products fermented by these bacteria have a mild, acidic flavour similar to that of yoghurt (Rasic and Kurmann 1983).

The key to an effective fermentation of bifidobacteria in commercial dairy products is the maintenance of an anaerobic environment. Since bifidobacteria are obligate anaerobes, certain precautions are required to prevent the toxic effects of oxygen when they are cultivated for industrial applications (Rasic and Kurmann 1983), and it is because of these restrictions that no data are available yet on the production of EPS by these bacteria. In lactic acid bacteria, the quantities of EPS produced by different species vary according to the strains. For example, the amounts range from 45 mg/l to 350 mg/l in *S. thermophilus* (Cerning et al. 1988; Doco et al. 1989) and from 55 mg/l to 150 mg/l in *Lb. delbruckii* ssp. *bulgaricus* (Cerning et al. 1986). Moreover, the effects of environmental conditions on the production of polysaccharides by several microbial species have been studied both with growing cultures and washed cell suspensions. Traditional strains differ

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in their responses to changes in the environment such as carbon source utilization, mineral requirements and temperature (Sutherland 1972).

The purpose of this research was to quantify EPS production in skim milk and other media by *B. longum* and to determine the monomer composition of the isolated polymers.

Materials and methods

Microorganism and culture conditions

Bifidobacterium longum, strain ATCC 15707, isolated from the human intestine, was purchased from the American Type Culture Collection. Stock cultures were maintained at −20 °C in Rosenow medium (Institut Pasteur, Paris, France).

Two media were used: (a) skim milk at 10% and (b) peptone/yeast (PY) medium (Taniguchi et al. 1987) containing 5% lactose. The media were ultrafiltered (Miniset Filtron cassette, 8-kDa cut-off) and were inoculated with 10% subculture grown overnight at 37 °C. The pH was maintained above 5.0 by the initial addition of 15 g/l $CaCO_3$ or by successive additions of 3M NH_4OH. The growth of the bacteria was estimated by measuring the absorbance of the culture at 600 nm with a Shimadzu (Kyoto, Japan) 150-02 double-beam spectrophotometer.

EPS isolation and characterization

The culture was diluted with an equal volume of distilled water, and the proteins were digested with pronase (enzyme-to-substrate ratio 1:100, proteinase from *Streptomyces griseus*, Bœhringer, Mannheim, Germany) at pH 7.5, 30 °C, for 16 h. Merthiolate (1:1000) was added during incubation to inhibit cell growth. After incubation, the culture was heated at 100 °C for 5 min to inactivate the enzyme, and the cells were removed by centrifugation (6000 *g* for 30 min, 4 °C). The supernatant fluid was concentrated by ultrafiltration (cut-off molecular mass of 8 kDa) and the EPS were precipitated by adding three volumes of cold 95% ethanol. The precipitated material was collected by centrifugation (6000 *g* for 30 min, 4 °C), dissolved in distilled water, dialysed against distilled water for 48 h and finally freeze-dried.

The EPS were separated by chromatography on an anion-exchange DEAE-cellulose (Whatman 52) column with 0.02 M TRIS/HCl, pH 8.8 as the solvent. The elution was performed using a continuous (0–0.5 M) NaCl gradient. Apparent molecular masses were estimated by gel filtration on Sepharose Cl-4B (Pharmacia).

General analytical procedures

Lactose, glucose, galactose, lactic and acetic acids were estimated by HPLC using a Polyspher column (Merck OAKC, Darmstadt, Germany) and a Spectra Physics (Darmstadt, Germany) model 8430 refractive-index detector, with deionized water as solvent. Proteins were estimated by measuring the absorbance at 280 nm. Total carbohydrate content was determined by the phenol/sulphuric acid method of Dubois et al. (1956), using glucose as a standard.

The concentration of free amino groups was determined using the 2,4-dinitrofluorobenzene reagent according to the method of Ghuysen et al. (1965). Phosphorus was determined, after mineralisation at 210 °C, by the method of McClare (1971), using the ammonium molybdate/ascorbic acid reagent.

Freeze-dried samples (10 mg) were hydrolysed by heating to 105 °C for 2 h with 2 M HCl in a sealed tube. The resulting hydrolysates were used for the identification of hexose content by gas-liquid chromatography, as alditol acetate derivatives (Sawardeker et al. 1965), at 210 °C with a 180-cm column of 3% Sp 2340 on chromosorb WAW-DMCS (100–120 mesh). The determination of glucose and galactose concentrations was also performed by enzymatic assays using kits 716.260 and 176.303 (Bœhringer, Germany) respectively. The uronic acids were measured using the *m*-hydroxydiphenyl reagent of Blumenkrantz and Absoe-Hansen (1973) and the hexosamines were measured, using the *p*-dimethylaminobenzaldehyde reagent of Elson and Morgan (1933), by the micromethod described by Ghuysen et al. (1966) after hydrolysis of the samples under vacuum with 6 M HCl at 105 °C for 8 h in a sealed tube.

Results

Optimisation of metabolite production

B. longum is a strict anaerobic bacterium and does not evolve gases; growth was performed on ultrafiltered PY medium in cap-sealed bottles.

As shown by the curves of Fig. 1, the final lactose uptake and acid formation increased as a function of the initial concentration of $CaCO_3$ in the medium. Both values increased in parallel between 3 g/l and 15 g/l $CaCO_3$. Under the latter conditions, most of the acidity was neutralized as the pH was maintained near 5.0 after 48 h of culture. Less than 5 g/l glucides was excreted in the medium whereas more than 30 g/l lactose was metabolized.

Beyond 15 g/l $CaCO_3$, cells metabolized lactose at a lower level although the rate of galactose excretion was the same. Consequently, the initial concentration of 15 g/l $CaCO_3$ was retained as reference for further studies of the influence of medium and pH regulation on the production of metabolites.

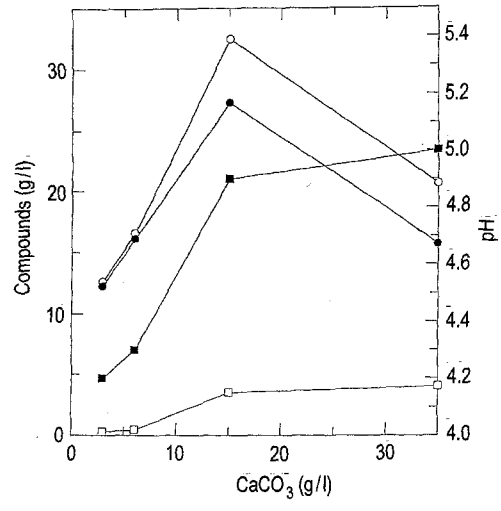

Fig. 1 Influence of $CaCO_3$ concentration on the fermentation of lactose by *B. longum* ATCC 15707. ○ Lactose uptake, ● acid excretion, □ glucide excretion, ■ pH variation during the growth

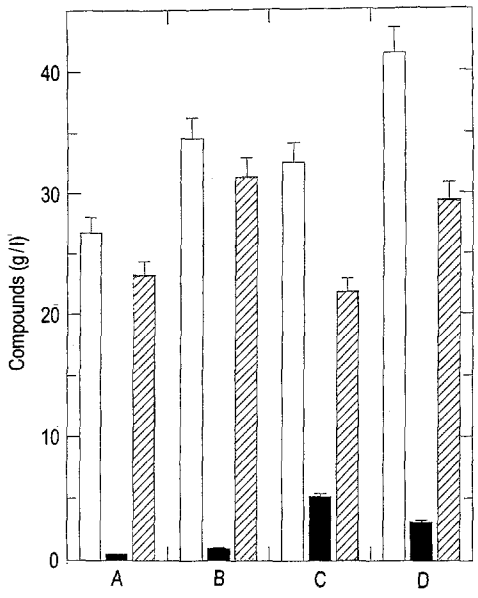

Fig. 2 Influence of medium composition and the mode of pH regulation on the fermentation of lactose by *B. longum* ATCC 15707. *A* Skim milk/$CaCO_3$, *B* skim milk/NH_4OH, *C* peptone/yeast (PY)/$CaCO_3$, *D* PY/NH_4OH. □ Lactose uptake, ■ glucide excretion, ▨ acid formation

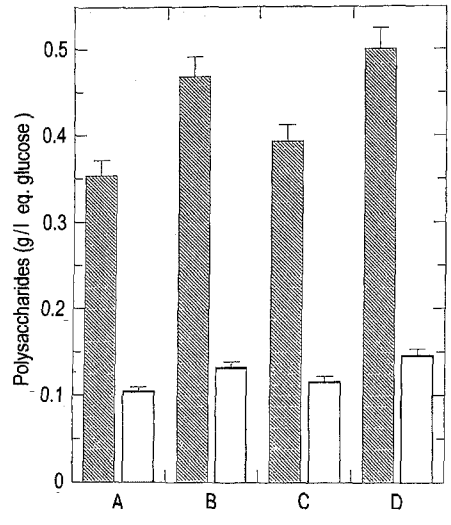

Fig. 3 Effect of medium and mode of regulation of pH on polysaccharides production by *B. longum* ATCC 15707. *A* Skim milk/$CaCO_3$, *B* skim milk/NH_4OH, *C* PY/$CaCO_3$, *D* PY/NH_4OH. ▩ Total polysaccharides, □ EPS

As shown in Fig. 2, the regulation of pH by successive additions of NH_4OH allowed, from the same carbon source, higher yields of acids and higher lactose uptake. Indeed, both concentrations increased by 30% under these conditions. No limitation by the carbon source was observed as, after 48 h of culture, 10 g lactose still remained in the culture. As similar results were obtained from both media it can be suggested that the successive additions of NH_4OH during the fermentation were more efficient than addition of initial concentrations of $CaCO_3$. Growth of *B. longum* was also enhanced by 20% on PY compared to skim milk when the pH was regulated with NH_4OH.

Figure 3 shows that the production of EPS varied according to the culture medium. *B. longum* synthesized 115–145 mg EPS/l in PY medium and 105–132 mg/l in skim milk. The highest production rate was obtained under the same conditions that favour both biomass and metabolite production by *B. longum*. The percentage of EPS compared to total polysaccharides was constant (approx. 30%) whatever the growth conditions of *B. longum*.

Comparison of EPS produced from PY and from skim milk showed that EPS obtained from PY (as a percentage of the freeze-dried supernatant sample) was about double that obtained from skim milk (data not shown). The difference could probably result from the presence of high casein and calcium contents in skim milk.

Therefore, the best production rate for EPS can be obtained from PY medium with pH regulation by NH_4OH. Moreover, these conditions allow EPS to be purified with higher yields because of smaller amounts of contaminating material in crude samples. Cultures in PY medium, by contrast with skim milk, should also allow the use of other carbon sources in order to study their influence on the production rate and the composition of EPS.

Kinetics of metabolite production

Batch growth of *B. longum* gave a mean generation time of 124 min on PY medium and this value doubled when this bacterium was grown on skim milk (data not shown). The same difference was obtained with *B. bifidum* grown on Man, Rogosa and Sharpe (MRS) and skim milk (Ventling and Mistry 1993).

The evolution of biomass and metabolite production by *B. longum* were therefore studied on PY medium with regulation of pH by NH_4OH. It appeared that biomass, lactic and acetic acid and polysaccharide production closely depended on lactose uptake by *B. longum*, as patterns of fermentation shown in Fig. 4 showed parallel curves for end-product synthesis and growth rate.

Total polysaccharides (cell wall polymers and EPS) should therefore be considered as minor products diverted away from glycolysis rather than secondary metabolites, since neither production nor utilization of polysaccharides was observed shortly after growth ceased. No carbon source limitation was involved since about 10 g/l lactose remained in the culture medium after 48 h of fermentation. In fact, limitation of cell growth occurred as soon as acids accumulated in the medium. At the end of the fermentation, the acid produced was 28 g/l, representing only 73% of the total

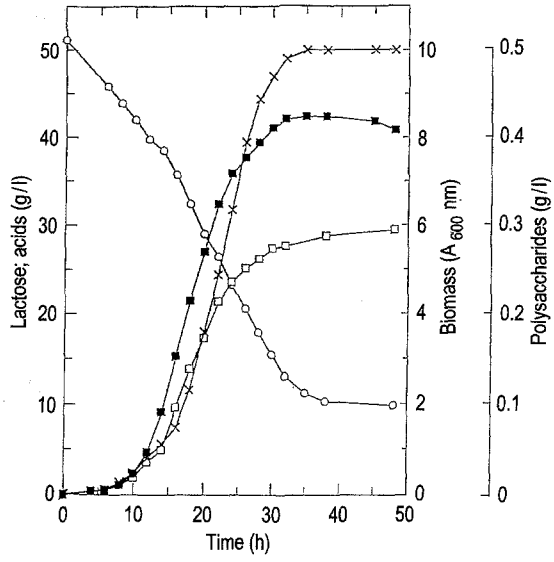

Fig. 4 Kinetics of batch lactose fermentation by *B. longum* on PY medium (regulation of pH with NH_4OH). ○ Lactose, □ acids, ■ biomass, * polysaccharides

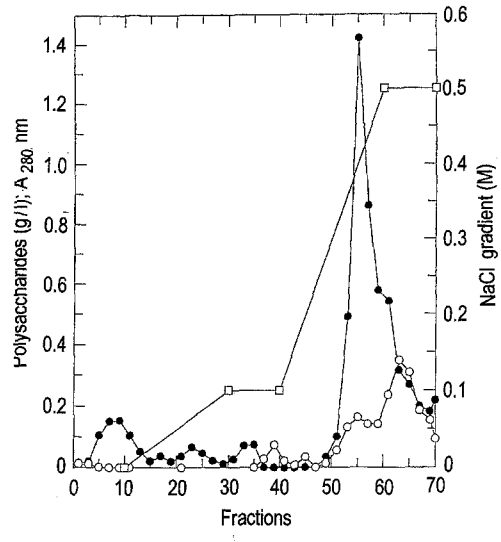

Fig. 5 Ion-exchange chromatography of crude EPS with increasing NaCl gradient. ● Polysaccharides, ○ proteins, □ gradient NaCl

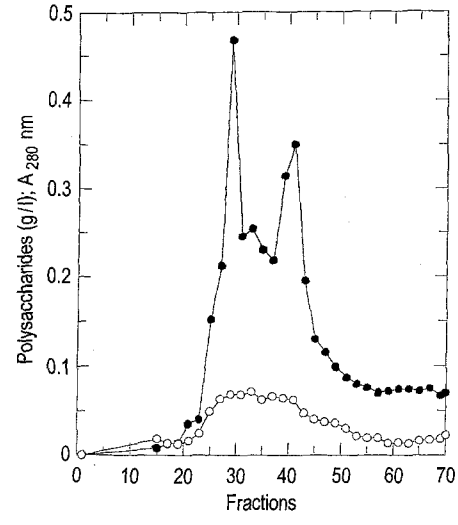

Fig. 6 Gel filtration chromatography of soluble exopolysaccharides isolated from the ion-exchange chromatography pool. Peaks were identified by colorimetric analysis (Dubois) of every two fractions. Each fraction was 3.3 ml. ● Polysaccharides, ○ proteins

lactose consumption, while the total polysaccharide production was 0.5 g/l.

Isolation and purification of EPS

The EPS were isolated from the supernatant fluid after a 48-h culture on PY medium and represented the crude fraction. The average quantity was estimated to be 140 mg/l ($\pm$ 10%; $n = 5$, glucose equiv.). The EPS were then submitted to ion-exchange chromatography and resolved in two peaks (Fig. 5). A minor peak was directly eluted with the washing buffer. The major peak of polysaccharides retained on DEAE-cellulose was eluted with the same buffer supplemented with 0.3–0.4 M NaCl. In this peak, which represented more than 80% of the initial sample, it was possible to distinguish two small peptide subfractions. The total yield of polysaccharides recovered was near 100%.

The fractions corresponding to the second peak were pooled and submitted to gel filtration on Sepharose 4B. As shown in Fig. 6, two peaks were resolved (GF1 and GF2) with elution volumes corresponding to apparent molecular masses of 1.2 MDa and 0.36 MDa respectively. The relative weight ratio of GF1 to GF2 was about 4:3. The total yield of recovered polysaccharides was only 60%.

Chemical composition of EPS

Analyses were performed on polysaccharide fractions isolated from culture media collected at the final stage of fermentations. They were tentatively purified by ion-exchange chromatography followed by gel filtration as presented in Figs. 5, 6.

The results of the Table 1 show that the EPS product excreted by *B. longum* grown on PY medium has a complex structure made of neutral sugars, uronic acid, phosphorus and nitrogen derivatives. As all these compounds are recovered together in all the fractions after ion-exchange chromatography and gel filtration, they must be associated by strong linkages. We can, however, observe that after ion-exchange chromatography, EPS was enriched in carbohydrate and phosphate by 21% and 13% respectively, while the levels of amino acids, uronic acids and hexosamines decreased by 17%, 29% and 50% respectively. In all cases, the

Table 1 Chemical composition of the different exopolysaccharide fractions isolated from *B. longum* cultivated on peptone/yeast medium (NH_4OH). Results are expressed as percentages and are the mean values of five assays. The standard deviations are shown in parentheses. *IE* fraction obtained by ion-exchange chromatography, *GF*1 and *GF*2 fractions obtained by gel filtration

Product	Composition (%) of fractions			
	Crude	IE	GF1	GF2
Neutral sugars	42.6 (± 2.2)	51.6 (± 3.5)	59.8 (± 4.5)	36.6 (± 3.3)
Glc/Gal ratio	1/1	1/1	1/1	1/1
Amino acids	45.1 (± 1.4)	37.7 (± 0.9)	33.6 (± 1.8)	56.3 (± 1.3)
Phosphorus	5.4 (± 0.4)	6.1 (± 0.5)	1.6 (± 0.1)	3.3 (± 0.2)
Uronic acid	5.5 (± 0.9)	3.9 (± 0.4)	4.4 (± 0.6)	3.5 (± 0.5)
Hexosamine	1.4 (± 0.3)	0.7 (± 0.1)	0.6 (± 0.1)	0.3 (± 0.1)

Table 2 Influence of medium composition and mode of pH regulation on the relative composition of neutral sugars in exopolysaccharides. Results are expressed as percentages and are the mean values of the differents assays

Carbohydrates	Exopolysaccharide composition (%)			
	Skim milk		PY medium	
	$CaCO_3$	NH_4OH	$CaCO_3$	NH_4OH
Glucose	47.6	41.2	51.9	49.3
Galactose	52.4	58.8	48.1	50.7
Neutral sugars/ total sugars	86.6	89.4	83.2	86.1

determination of carbohydrates in lyophilised samples indicated the presence of galactose and glucose in equimolecular ratios.

Indeed, Table 2 shows that the neutral sugars represented, in all cases, more than 80% of the total, whereas uronic acids were present in small amounts. The proportions of galactose, glucose and uronic acids were slightly altered by the culture conditions; the molar ratio was 4:4:1 in PY medium and 4:3:1 in skim milk.

The regulation of pH by successive additions of NH_4OH seemed to favour the incorporation of galactose in EPS at the expense of uronic acids. When the bacteria were grown in skim milk, the proportion of galactose was higher than that of glucose by 10%–30%.

Discussion

This paper demonstrates for the first time that *B. longum*, when cultivated on PY ultrafiltered medium under strict anaerobic conditions, can produce specific EPS in small amounts, these have been characterized in a similar manner to that previously described for traditional bacteria of yoghurt.

It is well-known that gram-positive bacteria cannot produce *N*-glycosidic or *O*-glycosidic glycoproteins, and the constant presence of amino acids associated with carbohydrates may be due to cell wall fragments that are liberated in the medium during the first steps EPS isolation.

Only cultures in a large-scale airtight fermentor would allow us to obtain sufficient EPS material to determine whether the nitrogen derivatives must be regarded as contaminants or as a significant part of the isolated polymers. Since *B. longum* is a strict anaerobic bacterium, difficult to handle on a large scale, an alternative way would be to determine culture conditions that induce EPS production up to a sufficient level to make further purification steps-possible.

The complete elucidation of the structure of the exopolymers isolated from *B. longum* requires further study; nevertheless this work allowed us to determine their constituant neutral sugars and sugar derivatives and to compare the results to those obtained in a similar way from other lactic acid bacteria.

The lactic acid bacteria synthesize, from milk or other complex media, exopolysaccharides that mainly contain glucose, galactose and mannose as neutral sugars (Cerning et al. 1986, 1988, 1992; Doco et al. 1989) and small amounts of hexosamines or uronic acids. The polysaccharides isolated from *B. longum* differ from those previously analysed by the absence of mannose and an average molar ratio of glucose, galactose and uronic acids of 4:4:1.

The production rates of EPS in *B. longum*, from 105 mg/ml to 145 mg/ml (glucose equiv.) are, however, similar to those reported in traditional bacteria used in yoghurt since the latter may vary between 50 ml/l to 350 ml/l according to the strain (Cerning et al. 1986.)

The production of EPS and the growth of the cells are parallel and both cease to increase as soon as acids accumulate in the medium. The excreted acids represented only 73% of the total lactose consumption at the end of the fermentation, also revealing a partial deviation of the carbohydrate flow from glycolysis and growth to anabolic pathways such as polysaccharide synthesis. The same results were observed in *Streptococcus thermophilus* (Petit et al. 1991).

At the end of the fermentation, the EPS represented 30% of the total polysaccharides. Further study on EPS formation during the growth of the bacterium in an airtight fermentor should allow us to determine whether this biosynthesis is simply a part of the total polysaccharide production or whether it belongs to a more specific pathway.

B. longum produces EPS that have apparent molecular masses of 0.36–1.2 MDa. Such values have already been reported for EPS produced by lactic acid bacteria such as *S. thermophilus* and *Lb. lactis* ssp. *cremoris*, which also synthesize high-molecular-mass polymers of 1–2 MDa (Cerning et al. 1986; Doco et al. 1989; Nakajima et al. 1992). The polymers produced by *B. longum*, despite their dispersed molecular masses, appear to have the same general composition, and especially an equimolar ratio of galactose and glucose. These data suggest that these polymers could be synthesized at different polymerization levels from the same base unit with a structure resulting from repeating subunits.

Further characterization is now required to clarify the role of these anionic heteropolysaccharides in host/bacterium relationships.

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