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Effect of galactose and glucose on the exopolysaccharide production and the activities of biosynthetic enzymes in *Lactobacillus casei* CRL 87

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Aims: The objective of this work was to study the influence of the sugar source on exopolysaccharide (EPS) production and the activities of the enzymes involved in the synthesis of sugar nucleotides in *Lactobacillus casei* CRL 87. The relationship between these enzymes and EPS formation was determined.
Methods and Results: The concentration of EPS was estimated by the phenol/sulphuric acid method while the chemical composition of purified EPS was investigated using gas-liquid chromatography. Biosynthetic enzyme activities were determined spectrophotometrically by measuring the formation or disappearance of NAD(P)H at 340 nm. Polysaccharide production by *Lb. casei* CRL 87 was 1·7 times greater on galactose than on glucose. The isolated polymer was composed of rhamnose, glucose and galactose. The activities of uridine-diphosphate (UDP)-glucose-pyrophosphorylase, thymidine-diphosphate (dTDP)-glucose-pyrophosphorylase and the dTDP-rhamnose-synthetic enzyme system were higher in galactose-grown than in glucose-grown cells. When an EPS^- mutant strain was used, galactokinase activity was not detected on galactose, this sugar not being available for the formation of sugar nucleotides for further EPS production. dTDP-glucose-pyrophosphorylase and dTDP-rhamnose-synthetic enzyme system activities were lower than the values found for the wild type strain.
Conclusions: The carbon source present in the culture medium affects EPS production by *Lb. casei* CRL 87. The greater polymer synthesis by galactose-grown cells is correlated with the higher UDP-glucose-pyrophosphorylase, dTDP-glucose-pyrophosphorylase and dTDP-rhamnose-synthetic enzyme system activities. Initial sugar metabolism is also an important step for the synthesis of EPS precursors by this strain.
Significance and Impact of the Study: Knowledge of the effect of the sugar source on EPS production and the activities of biosynthetic enzymes provides information about the mechanisms of regulation of the synthesis of EPS which can contribute to improving polymer production.

INTRODUCTION

The production of microbial exopolysaccharides (EPS) has been identified as an attractive and growing area for the fermentation industry. Among the wide variety of EPS-producing micro-organisms, lactic acid bacteria (LAB) have gained attention because of the interesting properties of these polymers and the GRAS (Generally Recognized as Safe) status of the group. Polysaccharides produced by LAB play an important role in the rheology, texture and body of fermented milks (De Vuyst and Degeest 1999). More recently, EPS^+ starter cultures have been used in the manufacture of low-fat cheeses (Mozzarella) to increase moisture retention (Perry *et al.* 1997).

Most LAB synthesize heteropolysaccharides which are composed of repeating units of neutral sugars. Although

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different types of EPS are secreted, D-galactose, D-glucose and L-rhamnose are usually present (De Vuyst and Degeest 1999). Contradictory results have been reported about the influence of the carbon source present in the culture medium on the chemical composition of EPS. *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 synthesizes different EPS when grown on glucose or fructose (Grobben *et al.* 1996). However, Degeest and De Vuyst (2000) and Escalante *et al.* (1998) found no variation in EPS composition when strains of *Streptococcus thermophilus* were grown on different carbohydrates as energy source.

The carbon source clearly affects the total amount of polysaccharides produced. Degeest and De Vuyst (2000) found a greater production of EPS with lactose than with glucose in *Strep. thermophilus* LY03. *Lactococcus lactis* subsp. *cremoris* NIZO B40 produces more EPS with glucose than with fructose as the sugar source (Looijesteijn and Hugenholtz 1999).

Heteropolysaccharides are made by polymerization of repeating unit precursors formed in the cytoplasm. The sugar nucleotides derived from sugar-1-phosphates play an essential role activating the carbohydrates, necessary for monosaccharide polymerization as well as interconversions. Several enzymes are involved in the biosynthesis and secretion of EPS which are not necessarily unique to EPS formation (De Vuyst and Degeest 1999). Sutherland (1972) classified these enzymes into four groups: (i) responsible for the initial metabolism of a carbohydrate; (ii) involved in the sugar nucleotide synthesis and interconversion; (iii) glycosyltransferases that form the repeating unit attached to the glycosyl carrier lipid and (iv) translocases and polymerases that form the polysaccharide.

Investigations of the enzymes involved in the biosynthesis of EPS by LAB have been reported by a few researchers. Escalante *et al.* (1998) and Grobben *et al.* (1996) found a correlation between the enzyme UDP-glucose pyrophosphorylase and EPS production by strains of *Strep. thermophilus* and *Lact. bulgaricus*, respectively. More recently, Degeest and De Vuyst (2000) determined a correlation between α-phosphoglucomutase, UDP-galactose 4-epimerase and UDP-glucose-pyrophosphorylase and EPS synthesis by *Strep. thermophilus* LY03.

We have previously reported that *Lb. casei* CRL 87 was able to synthesize EPS from different carbohydrates (Mozzi *et al.* 1995). Measurements of EPS production carried out in milk and medium for the synthesis of exopolysaccharides (MSE) medium, containing galactose as carbon source, showed that the largest amount of polysaccharide was obtained when the micro-organism grew on this latter medium (Mozzi *et al.* 1997). Regarding the chemical composition, the polymer synthesized by *Lb. casei* CRL 87 in MSE medium was composed of rhamnose, glucose and galactose in a ratio of 2·5 : 1·7 : 1.

In this work the effect of the carbon source, galactose and glucose, on EPS production and the activities of the enzymes involved in the synthesis of sugar nucleotides in *Lb. casei* CRL 87 was investigated.

MATERIALS AND METHODS

Micro-organism and growth conditions

Lactobacillus casei CRL 87 was obtained from the CERELA Culture Collection (Tucumán, Argentina) having previously been isolated from a semihard regional cheese. Growth experiments were performed in MSE medium using galactose or glucose (2% w/v) as carbon source as described previously (Mozzi *et al.* 1997). The micro-organism was subcultured on each carbohydrate at least twice just prior to experimental use. Fermentations were performed statically in sealed bottles containing 200 ml culture medium; a 16-h active culture (4% v/v) was used as inoculum. Cell growth and EPS production were carried out at 30°C (optimum temperature for EPS production; Mozzi *et al.* 1996).

Isolation, purification and characterization of exopolysaccharides

The EPS were isolated by precipitation in ethanol according to Mozzi *et al.* (1997). The amount of polysaccharides was estimated by the phenol/sulphuric acid method (Dubois *et al.* 1956). The EPS were purified by gel filtration chromatography on Sepharose 4B (Sigma) using 0·05 mol l^{-1} sodium phosphate (pH 7·0) as elution buffer. Purified samples were hydrolysed with 2 N trifluoroacetic acid in sealed tubes at 105°C for 3 h. Acid was removed by steam evaporation and samples neutralized to pH 7–7·5 with 0·1 mol l^{-1} NaOH. The chemical composition of the EPS was determined by gas-liquid chromatography (GLC). Polysaccharides were converted to their alditol acetates by using $NaBH_4$ and acetic anhidride : pyridine (1 : 1). Derivatized samples were analysed on a gas-liquid chromatograph (model 5840; Hewlett-Packard, Wilmington, DE, USA; flame ionization detection) fitted with a capillary column (SP-2340; Supelco Chromatography Products, Bellefonte, PA, USA; 30 m ×0·2 μm). The column temperature was 220°C and the injector and detector temperatures 250°C. Peaks were identified on the basis of retention times using inositol as an internal standard.

Preparation of cell-free extracts and permeabilized cells

Cultures of *Lb. casei* CRL 87 grown in MSE medium for 16 h at 30°C (as stated above) were harvested by centrifugation at 20 000 **g** for 10 min at 4°C and cells washed

twice and resuspended in cold 0·01 mol l^{-1} potassium phosphate buffer (pH 6·8) containing 5 mmol l^{-1} $MgCl_2$. Cells were disrupted by French-Press, unbroken cells and cell debris were removed by centrifugation (20 000 **g** for 20 min at 4°C) and the supernatant fluid was used as the cell-free extract.

An exponential phase culture (A_{560} 0·4–0·7) was used for galactose- and glucose-phosphotransferase reactions as well as glucokinase and galactokinase assays. Cells were washed, resuspended and preincubated in the above-mentioned buffer for 10 min at 37°C to reduce endogenous levels of carbohydrate. Cell suspension (1 ml) was permeabilized with 50 μl toluene : acetone solution (1 : 9 v/v) and vortexed for 5 min at room temperature (25°C). The toluene-treated cells were prepared immediately before use.

Enzyme assays

All *in vitro* assays were performed in a volume of 1·0 ml in 1-ml polystyrene or quartz cuvettes at 37°C in a spectrophotometer (model 2021 series 2000; Cecil Instruments Ltd, Cambridge, UK) with freshly prepared cell-free extracts or toluene-treated cells. The formation or disappearance of NAD(P)H was monitored by measuring the absorbance at 340 nm. In cell suspensions, the protein content was performed after extraction with one volume 1 mol l^{-1} NaOH at 100°C for 30 min. The protein content of both cell-free extracts and permeabilized cells was determined using the method of Lowry *et al.* (1951). The enzyme activities were expressed in nmol min^{-1} mg cell $protein^{-1}$.

The phosphoenolpyruvate (PEP)-galactose-phosphotransferase (PTS) and PEP-glucose-PTS systems were determined according to the method of Hickey *et al.* (1986). The reaction was started by adding galactose (for PEP-galactose-PTS) or glucose (for PEP-glucose-PTS) to a final concentration of 1 mmol l^{-1}. The same assay conditions were used for the measurement of galactokinase and glucokinase enzymes, except that PEP was replaced by ATP.

Galactose 1-phosphate-uridyltransferase activity was determined by the method of Kuruhashi and Anderson (1958). Concentrations of phosphoglucomutase and glucose-6-phosphate dehydrogenase had to be increased by a factor of 10 to give detectable enzyme activities (Bettenbrock and Alpert 1998).

The uridine-diphosphate (UDP)-glucose-pyrophosphorylase, thymidine-diphosphate (dTDP)-glucose 4,6-dehydratase and UDP-galactose 4-epimerase assays were determined as described by Grobben *et al.* (1996). The formation of dTDP-4-oxo-6-deoxyglucose for the dTDP-glucose 4,6-dehydratase reaction was measured at 320 nm.

The dTDP-glucose-pyrophosphorylase assay was measured according to Robbins and Bernstein (1966).

The dTDP-rhamnose-synthetic enzyme system that includes dTDP-glucose 4,6-dehydratase and the complex of dTDP-4-dehydrorhamnose epimerase and dTDP-4-dehydrorhamnose reductase (dTDP-rhamnose-synthetic enzyme system) was determined following the technique of Martins and Sá-Correia (1993).

The α-phosphoglucomutase assay was performed as described by Looijesteijn *et al.* (1999).

Isolation of a non-ropy exopolysaccharide-negative strain

Cells of *Lb. casei* CRL 87 grown in 10 ml MSE broth at 37°C (A_{560} 0·7–0·8) were harvested by centrifugation at 20 000 *g* for 10 min at 4°C, resuspended in fresh MSE broth containing nitrosoguanidine (400 μg ml^{-1}) and caffeine (0·1% w/v) and incubated for two generation times. Nitrosoguanidine was used as a mutagenic agent and caffeine as a DNA repairing system inhibitor. Diluted samples were spread on MSE agar (MSE broth with 13 g l^{-1} agar) and incubated at 30°C for 48 h. Individual colonies were screened for ropiness by touching with sterile toothpicks. The apparent EPS-negative mutants were grown in MSE broth to check the ropy character and EPS production.

Statistical evaluation

All experiments were performed in triplicate and results are the mean of triplicate measurements.

RESULTS AND DISCUSSION

Figure 1 shows the effect of galactose and glucose on growth and EPS production by *Lb. casei* CRL 87. This microorganism grew well on galactose (μ_{max} 0·39 h^{-1}) and glucose (μ_{max} 0·33 h^{-1}) reaching similar viability and $O.D._{560}$ values after 24 h of incubation ($2{\cdot}9 \times 10^9$ cfu ml^{-1} and 4·3 on galactose and $2{\cdot}2 \times 10^9$ cfu ml^{-1} and 4·1 on glucose, respectively). Despite this, EPS production was 1·7 times greater on galactose than on glucose. In both cases the polysaccharide synthesis started after 6 h of incubation at 30°C.

The monomeric composition of the EPS produced on galactose or glucose as sugar source was determined using GLC. On galactose, *Lb. casei* CRL 87 synthesized an unusual polysaccharide composed of a high content of rhamnose, glucose and galactose in a ratio of 2·5 : 1·7 : 1, a little change was observed in the monomeric composition of the EPS produced on glucose since rhamnose, glucose, galactose and a small amount of mannose was also present (3·0 : 1·7 : 1 : 0·6). Kojic *et al.* (1992) showed that the sugar composition of the EPS produced by *Lb. casei* CG11

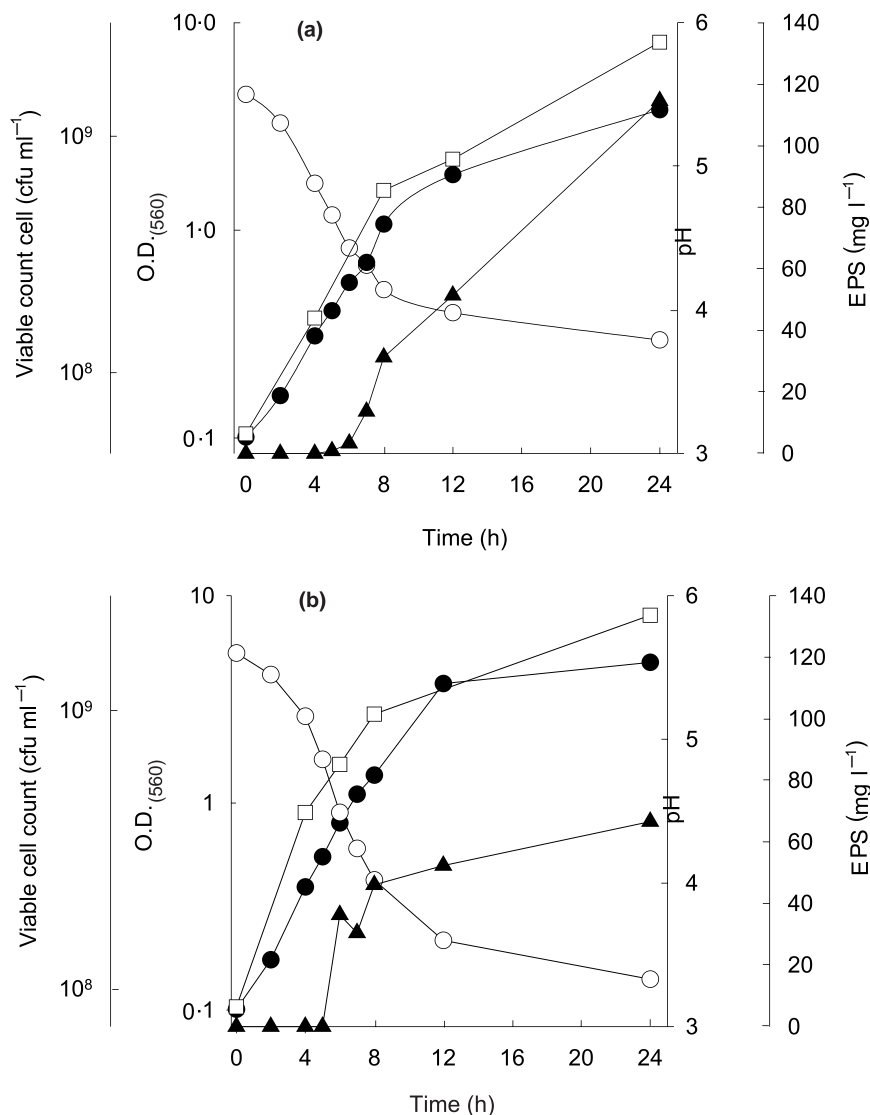

Fig. 1 Effect of (a) galactose and (b) glucose on growth and exopolysaccharide (EPS) production by *Lactobacillus casei* CRL 87. □, Viable cell count; ●, O.D.; ▲, EPS; ○, pH

depended on the complexity of the medium on which the strain grew. However, no variation in the monomeric composition of EPS was observed using different carbohydrates as energy source in strains of *Strep. thermophilus* (Escalante *et al.* 1998; Degeest and De Vuyst 2000).

To determine whether the difference in EPS production by *Lb. casei* CRL 87 grown on galactose or glucose could be related to the sugar metabolism and/or the biosynthesis of sugar nucleotides (dTDP-rhamnose, UDP-glucose and UDP-galactose), the enzymes involved in these pathways were investigated (Fig. 2).

In *Lb. casei* galactose and glucose can enter the cell via an ATP-energized permease system and/or via a sugar-specific phosphotransferase system (Chassy and Thompson 1983). This micro-organism has two alternative pathways for galactose metabolism: the Leloir pathway and the tagatose 1,6-biphosphate pathway. In the former case, exogenous galactose is transported via an ATP-energized permease system, activated by an ATP-dependent galactokinase. In the second case, galactose is transported via a galactose-specific phosphotransferase system (PEP-gal-PTS) which releases galactose 6-phosphate into the cytoplasm (Fig. 2). In the presence of glucose, this sugar can be activated by the PEP-glu-PTS or by a glucokinase, glucose 6-phosphate being released into the cell in both cases. With these possibilities in mind, the enzyme systems for galactose and glucose in *Lb. casei* CRL 87 were examined.

Table 1 shows the effect of galactose and glucose on the activities of the enzymes involved in sugar metabolism and formation of sugar nucleotides. Both PEP-gal-PTS and galactokinase activities were detected in the presence of galactose, which confirmed that galactose uptake is mediated

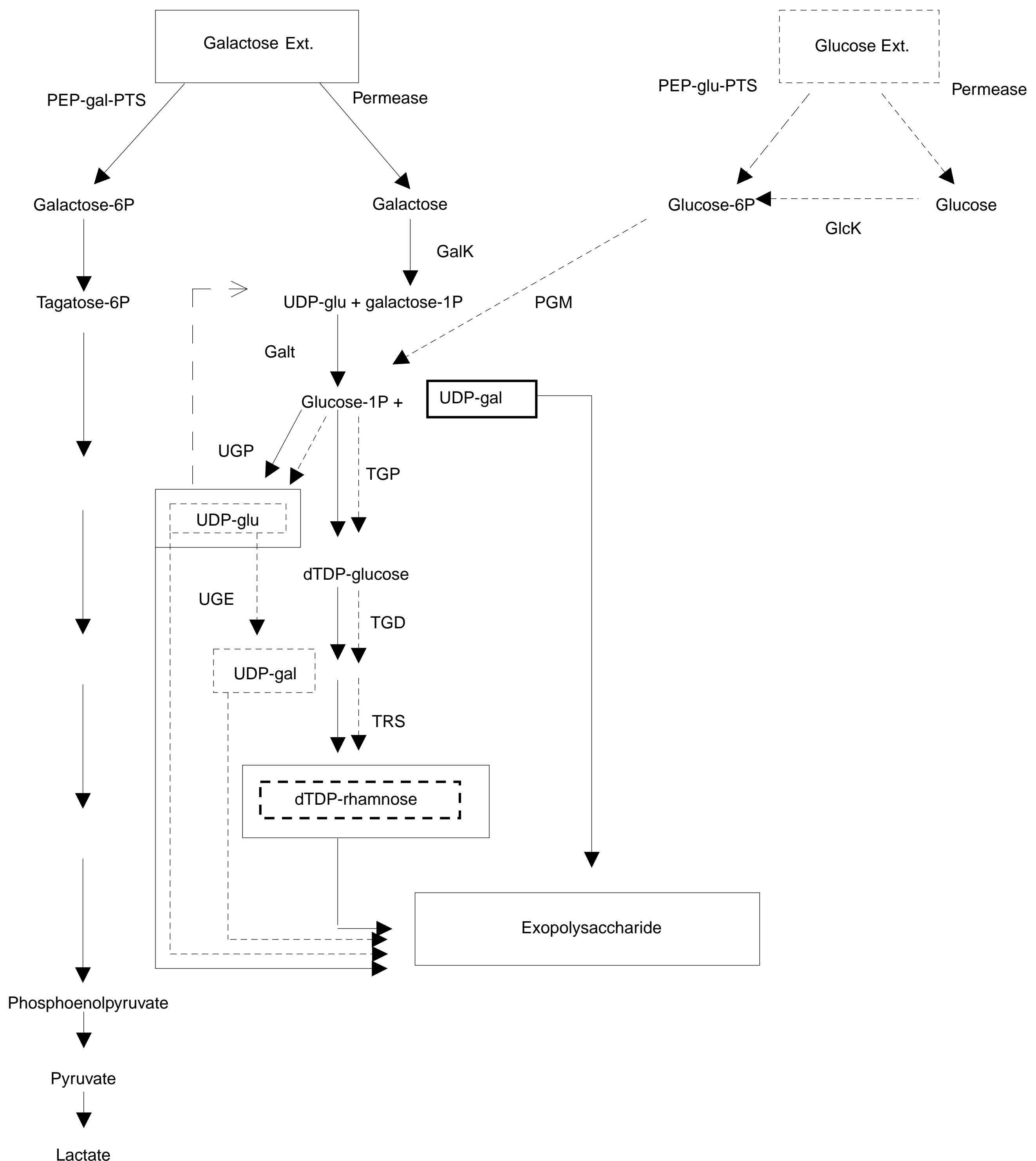

Fig. 2 Proposed pathways for the metabolism of galactose (—) and glucose (– –) and sugar nucleotide biosynthesis in *Lactobacillus casei* CRL 87. PEP-gal-PTS, phosphoenolpyruvate-galactose-phosphotransferase; GalK, galactokinase; GalT, galactose 1-phosphate-uridyltransferase; UGP, uridine-diphosphate (UDP)-glucose-pyrophosphorylase; UGE, UDP-galactose 4-epimerase; TGP, thymidine-diphosphate (dTDP)-glucose-pyrophosphorylase; TGD, dTDP-glucose 4,6-dehydratase; TRS, dTDP-rhamnose-synthetic enzyme system; PEP-glu-PTS, phosphoenolpyruvate-glucose-phosphotransferase; GlcK, glucokinase; PGM, α-phosphoglucomutase

Table 1 Effect of galactose and glucose on the enzymes involved in the sugar metabolism and biosynthesis of sugar nucleotides in *Lactobacillus casei* CRL 87*

Enzyme	Activity (nmol min^{-1} mg cell protein^{-1})†	
	Galactose	Glucose
PEP-gal-PTS	140·8 ± 28·6	NT
PEP-glu-PTS	NT	61·7 ± 6·7
Galactokinase	643 ± 21·2	NT
Glucokinase	NT	77·2 ±6·2
UDP-glucose-pyrophosphorylase	12·7 ± 0·8	7·10 ± 0·01
dTDP-glucose-pyrophosphorylase	56·1 ± 2·3	29·6 ± 5·7
dTDP-rhamnose-synthetic enzyme system	20 ± 2·8	5·7 ± 2·7
dTDP-glucose 4,6-dehydratase	10·8 ± 5·2	18·2 ± 1·3
Galactose 1-phosphate-uridyltransferase	60·2 ± 9·0	NT
α-phosphoglucomutase	NT	44·6 ± 3·9
UDP-galactose 4-epimerase	0·4 ± 0·2	1·6 ± 0·7

*The micro-organism was grown in MSE with galactose or glucose at 30°C.
†Each value represents the average of at least three measurements. NT, Not tested; PEP-gal-PTS, phosphoenolpyruvate-galactose-phosphotransferase; PEP-glu-PTS, phosphoenolpyruvate-glucose-phosphotransferase; UDP, uridine-diphosphate; dTDP, thymidine-diphosphate.

by both systems in *Lb. casei* CRL 87. Galactokinase activity was 4·6 times higher than PEP-gal-PTS, indicating that a large amount of galactose was metabolized by the Leloir pathway for the synthesis of sugar nucleotides. When the micro-organism was grown on glucose, similar values of glucokinase and PEP-glu-PTS activities (61·7 and 77·2 nmol min^{-1} mg cell protein^{-1}, respectively) were detected.

The activities of the enzymes leading to the formation of sugar nucleotides, UDP-glucose-pyrophosphorylase, dTDP-glucose-pyrophosphorylase and the dTDP-rhamnose-synthetic enzyme system were higher (1·7, 1·9 and 3·5 times, respectively) in cell-free extracts of galactose-grown cells than in glucose-grown cells. These results would explain the higher EPS synthesis observed on galactose. The high dTDP-glucose-pyrophosphorylase and dTDP-rhamnose-synthetic enzyme system activities found in *Lb. casei* CRL 87, with respect to the values in the literature, are correlated with the high content of rhamnose present in the EPS produced by this strain. *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 produced more EPS when grown on glucose than on fructose. Analysis of the enzymes involved in the synthesis of sugar nucleotides in this micro-organism showed a higher activity of UDP-glucose-pyrophosphorylase in cell-free extracts of glucose-grown cells than in fructose-grown cells. Both dTDP-glucose-pyrophosphorylase and dTDP-rhamnose-synthetic enzyme system activities were detected on glucose but not on fructose (Grobben *et al.* 1996). The activity of UDP-glucose-pyrophosphorylase was also linked to EPS production in a strain of *Strep. thermophilus* (Escalante *et al.* 1998). Degeest and De Vuyst (2000) showed a correlation between UDP-glucose-pyrophosphorylase, α-phosphoglucomutase and UDP-galactose 4-epimerase with EPS biosynthesis by *Strep. thermophilus* LY03. In *Lact. casei* CRL 87 low activity values of UDP-galactose 4-epimerase were detected in both galactose- and glucose-grown cells.

In order to determine the role of the enzymes involved in the synthesis of EPS, their activities were measured in an isogenic non-ropy EPS$^-$ mutant strain (frequency of non-ropy mutants 3·8%). Taxonomic assays (API 50 CH; BioMerieux, Marcy l'Etoile, France) were carried out on the EPS$^-$ strain (named M7) to confirm that it derives from *Lb. casei* CRL 87 (Hammes *et al.* 1992). The non-ropy phenotype of strain M7 was stable in MSE agar and MSE broth after periodical transfers and it was not able to synthesize EPS either on galactose or on glucose.

The activities of the enzymes of galactose- and glucose-grown cells of the mutant strain are shown in Table 2. On galactose, galactokinase activity was not detected. This would indicate that galactose is catabolized by the alternative pathway (tagatose pathway) and would not be available for

Table 2 Effect of galactose and glucose on the enzymes involved in the sugar metabolism and biosynthesis of sugar nucleotides in exopolysaccharide-negative mutant strain M7*

Enzyme	Activity (nmol min^{-1} mg cell protein^{-1})†	
	Galactose	Glucose
PEP-gal-PTS	252 ± 17·0	NT
PEP-glu-PTS	NT	67·3 ± 6·1
Galactokinase	0	NT
Glucokinase	NT	0
UDP-glucose-pyrophosphorylase	5·4 ± 0·3	10·5 ± 3·2
dTDP-glucose-pyrophosphorylase	45·9 ± 3·7	6·6 ± 2·9
dTDP-rhamnose-synthetic enzyme system	15 ± 2·2	1·5 ± 0·2
dTDP-glucose 4,6-dehydratase	13 ± 1·7	17·8 ± 9·7
Galactose 1-phosphate-uridyltransferase	57·8 ± 4·8	NT
α-phosphoglucomutase	NT	130·2 ± 11·2
UDP-galactose 4-epimerase	0·7 ± 0·1	0

*The micro-organism was grown in MSE broth at 30°C.
†Each value represents the average of at least three measurements. NT, Not tested; PEP-gal-PTS, phosphoenolpyruvate-galactose-phosphotransferase; PEP-glu-PTS, phosphoenolpyruvate-glucose-phosphotransferase; UDP, uridine-diphosphate; dTDP, thymidine-diphosphate.

the synthesis of EPS. The lack of galactokinase activity and the higher value (1·8 times) of PEP-gal-PTS found in M7, with respect to the wild type, could explain the non-ropy phenotype of the mutant. The UDP-glucose-pyrophosphorylase, dTDP-glucose-pyrophosphorylase and dTDP-rhamnose-synthetic enzyme system activities were lower than those found in the wild type. When glucose was used as carbon source, glucokinase activity was not detected, indicating that glucose enters into the cell by the PEP-glu-PTS system. Despite the fact that α-phosphoglucomutase activity was 2·9 times larger in M7 than in the wild type strain, the activities of dTDP-glucose-pyrophosphorylase and dTDP-rhamnose-synthetic enzyme system were markedly lower (4·5 and 3·8 times, respectively). Uridine-diphosphate-galactose 4-epimerase activity was not detected in glucose-grown cells of the mutant strain. Sutherland (1972) stated that possibilities for exerting control over polysaccharide synthesis exist at any enzyme level and mutants lacking enzymes of any group fail to synthesize EPS. Breedveld *et al.* (1998) isolated a random set of EPS-negative mutants of *Lb. sakei* 0-1 that lacked activity for different EPS biosynthetic enzymes.

From this work we conclude that the carbon source present in the culture medium affects EPS production by *Lb. casei* CRL 87. The larger polymer synthesis on galactose-grown cells is correlated with higher UDP-glucose-pyrophosphorylase, dTDP-glucose-pyrophosphorylase and dTDP-rhamnose-synthetic enzyme system activities. In the case of *Lb. casei*, which has two alternative pathways for galactose, the initial sugar metabolism is a very important step for the synthesis of EPS precursors.

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