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ORIGINAL PAPER

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The effect of cations on the hydrolysis of lactose and the transferase reactions catalysed by β-galactosidase from six strains of lactic acid bacteria

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Abstract β-Galactosidases from *Lactobacillus delbruekii* subsp. *bulgaricus* 20056, *Lb. casei* 20094, *Lactococcus lactis* subsp. *lactis* 7962, *Streptococcus thermophilus* TS2, *Pediococcus pentosaceus* PE39 and *Bifidobacterium bifidum* 1901 were partially purified. The rate of hydrolysis of lactose given by the predominant β-galactosidase activity from each of the bacteria studied was in all cases enhanced by Mg^{2+}, while the effect of K^+ and Na^+ differed from strain to strain. The β-galactosidases from all strains also catalysed transgalactosylation reactions. The types of oligosaccharides produced appeared to be very similar in each case, but the rates of their production differed. All the β-galactosidases were also capable of hydrolysing galactosyl-lactose although, unlike the other bacteria studied, *Lb. delbruekii* subsp. *bulgaricus* 20056 and *Lc. lactis* subsp. *lactis* 7962 were unable to utilise galactosyl-lactose as a carbon source for growth.

Introduction

The main catalytic activity of β-galactosidase (lactase, β-D-galactohydrolase EC 3.2.1.23) is the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides. However, the enzyme is also capable of catalysing transferase reactions, which can result in both internal rearrangement of the lactose molecule and transgalactosylation (first described by Wallenfels 1951). Both the hydrolase and transferase activities have industrial applications. The hydrolysis reaction can be applied to the production of lactose-hydrolysed dairy products, which have potential commercial advantage in populations where there is a high prevalence of lactose intolerance and/or a preference for a sweeter product. The transferase reaction can produce a range of oligosaccharides, which may confer functional properties in cultured dairy products related to their purported role as "bifidus growth factors" that promote the growth of bifidobacteria in the intestine (Smart 1991). It is of interest to determine whether β-galactosidases from sources other than those traditionally used in the dairy industry may have unique properties or characteristics that could be of commercial significance. In a previous paper the biochemical and genetic diversity amongst β-galactosidases obtained from different bacterial sources was described (Smart et al. 1993). This paper describes the effect of various cations on the activity of partially purified β-galactosidases from a selection of these bacteria.

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Materials and methods

Materials

A lactose-derived trisaccharide preparation was obtained from Yakult Honsha Company, Tokyo, Japan (fully described by Smart 1991). This preparation is referred to as galactosyl-lactose in this study. All other biochemicals were of the highest analytical purity grade from Sigma Chemical Co. (St Louis, MO 63178, USA).

Organisms and culture conditions

Bacterial strains were from the New Zealand Dairy Research Institute culture collection. *Lactobacillus delbruekii* subsp. *bulgaricus* 20056, *Lb. casei* 20094, *Pediococcus pentosaceus* PE39 and *Bifidobacterium bifidum* 1901 were grown in PP complex broth (Thomas et al. 1985) containing 2% (w/v) lactose. *Lactococcus lactis* subsp. *lactis* 7962 and *Streptococcus thermophilus* TS2 were grown in T5 complex broth (Thomas et al. 1974) containing 2% (w/v) lactose and J8 complex broth (Thomas and Crow 1984) containing 2% (w/v) lactose respectively. *S. thermophilus* and *Lb. delbruekii* subsp. *bulgaricus* were grown at 37°C; all other strains were grown at 30°C. Cells were grown in a 1.5-l fermenter (Series III Fermenter, LH Engineering, Stoke Poges, England) sparged with N_2/CO_2 (95:5)

and pH-controlled at 7.0 with automatic addition of 4 M NaOH. Growth was measured turbidimetrically at 600 nm and cells were harvested when the A_{600} of a sample (diluted 1:9 with water) was between 0.5 and 1.0. For studies of growth on galactosyl-lactose, cultures were grown statically for 2 days in the appropriate broths containing 2% (w/v) galactosyl-lactose as the carbon source.

Preparation of β-galactosidases

Harvested cells (10 000 *g*, 10 min, 4°C) were washed once with 0.05 M potassium phosphate buffer, pH 7, containing 2 mM $MgCl_2$ and 20% (v/v) glycerol, resuspended in 40 ml buffer and passed twice through a French pressure cell (55 mPa). The homogenate was treated with DNase then cell debris was removed by centrifugation (27 000 *g*, 30 min, 4°C). Protein precipitated from the supernatant with ammonium sulphate at 40% saturation was discarded. The resulting supernatant was taken to 75% saturation with ammonium sulphate and the precipitate obtained was resuspended in 0.05 M bis-Tris propane, pH 8, containing 2 mM $MgCl_2$ (BTP buffer) and desalted (FDS HR 10/10; Pharmacia, Uppsala, Sweden). The enzyme was partially purified with anion-exchange chromatography (Mono Q HR 10/10; Pharmacia) eluted with a gradient from 0 to 1 M NaCl in BTP buffer. Fractions containing activity were combined and concentrated by ultrafiltration (Diaflo YM10, Amicon, USA). Glycerol was added to a final concentration of 10% (v/v) and the samples were stored at − 20°C in 1-ml lots for kinetic studies.

Protein was estimated by the method of Bradford (1976) using BioRad protein assay reagent (BioRad Laboratories, Richmond, CA 94804, USA) with bovine serum albumin as the protein standard. A unit of enzyme activity was defined as the amount of enzyme required to hydrolyse 1 μmol *o*-nitrophenyl-β-D-galactopyranoside ONPG/min at 30°C as described by Yu et al. (1987).

Kinetic studies

For K_m determination, samples containing 4 units of activity in 6 ml 0.1 M potassium phosphate buffer, pH 7, containing 2 mM $MgCl_2$ (phosphate buffer) and 1–100 mM lactose were incubated at 30°C. Samples (0.9 ml) were removed at 0, 5, 10 and 15 min and 50 μl 4 M HCl added to stop the reaction. After cooling on ice for 20 min the samples were neutralised with 50 μl 4 M NaOH and assayed for glucose with Peridochrom reagent (Boehringer Mannheim, Germany). The rates of reaction obtained were used to derive K_m values. To determine the effects of Na^+, K^+ and Mg^{2+}, incubations (performed as above) were in 0.1 M bis-Tris propane, pH 7, containing 70 mM lactose and the various ions in the range 0–100 mM.

To determine the effects of Na^+ and K^+ on enzyme activity with ONPG as the enzyme substrate 2 units enzyme was incubated in 1 ml 0.1 M bis-Tris propane, pH 7, containing 2 mM $MgCl_2$, 2 mM ONPG, and the various ions in the range 0–100 mM. The release of *o*-nitrophenol was followed spectrophotometrically at 410 nm at 30°C (50°C for the *S. thermophilus* enzyme).

Transferase/hydrolysis reaction assay

Solutions of 40% (w/v) lactose and 24% (w/v) galactosyl-lactose (in phosphate buffer) were incubated at 30°C (50°C for the *S. thermophilus* enzyme) with 5 units/ml enzyme. Samples (0.9 ml) were taken at various assay times up to 24 h and the reaction was stopped as described previously. Analysis was performed on a high-pH anion-exchange column (CarboPac PA-100, 4 × 250 mm, with a CarboPac PA-100 guard column) using a Dionex LC carbohydrate system (Dionex Corp, Sunnyvale, California, USA). Samples were eluted isocratically for 12 min with 20 mM NaOH, followed by a continuous linear gradient from 40 mM to 150 mM NaOH over 43 min. The column eluent was monitored with pulsed amperometric detection (Pulsed Electrochemical Detector; Dionex). Identification and quantification of glucose, galactose and lactose was by comparison with standard solutions.

Results

Enzyme preparation

On anion-exchange chromatography preparations from *Lb. delbruekii* subsp. *bulgaricus* 20056, *Lc. lactis* subsp. *lactis* 7962 and *P. pentosaceus* PE39 gave a single peak of enzyme activity eluting at 0.50 M, 0.60 M and 0.49 M NaCl, respectively. Preparations from *Lb. casei* 20094 and *S. thermophilus* TS2 each gave a major peak of enzyme activity eluting at 0.66 M and 0.64 M NaCl, respectively, and a second, minor peak at 0.63 M and 0.56 M NaCl, respectively. Preparations of *B. bifidum* 1901 gave a major peak of enzyme activity eluting at 0.69 M NaCl with minor peaks at 0.54 M and 0.59 M NaCl.

K_m values for the enzymes on lactose

The K_m values obtained for the major β-galactosidase from *S. thermophilus* TS2, *Lb. delbruekii* subsp. *bulgaricus* 20056, *Lb. casei* 20094, *P. pentosaceus* PE39, *Lc. lactis* subsp. *lactis* 7962, *B. bifidum* 1901 on lactose were 6, 79, 4, 7, 2 and 2 mM, respectively. With the exception of the enzyme from *Lb. delbruekii* subsp. *bulgaricus* 20056 these values were similar to values previously reported for the *S. thermophilus* enzyme (Smart and Richardson 1987).

The effect of K^+, Na^+ and Mg^{2+} on the hydrolysis of lactose

The effects of Na^+, K^+ and Mg^{2+} on enzyme activity are shown in Table 1. In the absence of added ions no activity was detectable for the enzyme from *Lc. lactis* subsp. *lactis* 7962. The addition of 2 mM Mg^{2+} increased the activity of all the enzymes. The further addition of either 100 mM K^+ or 100 mM Na^+ led to an increase in activity in all cases with the exception that the enzyme from *S. thermophilus* TS2 was not affected by the addition of Na^+.

A more detailed study of the effects of Na^+ and K^+ on enzyme activity in the presence of Mg^{2+} showed different responses from some strains under certain conditions (Fig. 1). In the presence of 2 mM Mg^{2+}, increasing concentrations of K^+ up to 20 mM resulted in increased activity of the enzymes from all strains except that from *Lb. delbruekii* subsp. *bulgaricus* 20056,

Table 1 Synergistic effect of cations on the activity of β-galactosidases from different microbial species. Assays were performed in 0.1 M bis-tris propane pH 7.0, and activity is defined as mmol glucose produced min^{-1} mg $protein^{-1}$. 2 mM Mg^{2+} was also included in the buffer used for the enzyme purification and the consequent carry over of Mg^{2+} into these reactions gave the following final concentrations: *S. thermophilus* TS2 50 µM, *Lb. bulgaricus* 25 µM. *Lb. casei* 12 µM, *P. pentosaceus* 3 µM, *Lc. lactis* 25 µM, *B. bifidum* 6 µM. *ND* not detected

Species	Combined activation of cations; activity (mmol min^{-1} mg^{-1}) in the presence of				
	No ions	2 mM Mg^{2+}	100 mM Na^{+}	100 mM K^{+}	100 mM Na^{+}, 100 mM K^{+}
S. thermophilus TS2	14	40	39	122	59
Lb. delbruekii subsp. *bulgaricus* 20056	2.67	3.34	5.89	3.65	5.09
Lb. casei 20094	1.45	1.74	3.34	3.05	2.95
P. pentosaceus PE39	0.04	0.09	0.12	0.21	0.17
Lc. lactis subsp. *lactis* 7962	ND	0.12	0.37	0.52	0.26
B. bifidum 1901	0.25	0.34	1.01	0.51	0.55

which showed a decline in activity between 0 and 5 mM. Above this concentration activities were independent of ion concentration in the case of all the enzymes except those from *P. pentosaceus* PE39, where the activity decreased at concentrations above 50 mM, and from *S. thermophilus* TS2, where the activity continued to increase with higher concentrations of K^{+} (Fig. 1a). In the presence of both 2 mM Mg^{2+} and 100 mM Na^{+}, increasing concentrations of K^{+} initially resulted in increased activity for the enzymes from *Lb. delbruekii* subsp. *bulgaricus* 20056 and *P. pentosaceus* PE39. However, above 15 mM K^{+} the activity of the enzyme from *Lb. delbruekii* subsp. *bulgaricus* 20056 declined, while the activity of the enzyme from *P. pentosaceus* PE39 was not affected further (Fig. 1b). The activity of the enzyme from *Lc. lactis* subsp. *lactis* 7962 decreased at concentrations of K^{+} below 20 mM but increased at higher concentrations. All other enzymes showed decreased activities with increased concentrations of K^{+}. Increasing the concentration of Na^{+} in the presence of 2 mM Mg^{2+} resulted in increased activity of all enzymes to a lesser or greater extent (Fig. 1c). Increasing the concentration of Na^{+} in the presence of 2 mM Mg^{2+} and 100 mM K^{+} resulted in increased activity of the enzymes from *Lb. delbruekii* subsp. *bulgaricus* 20056 and *B. bifidum* 1901, while the enzymes from *Lb. casei* 20094, *S. thermophilus* TS2, *Lc. lactis* subsp. *lactis* 7962 and *P. pentosaceus* PE39 showed a decrease in activity (Fig. 1d).

When ONPG was used as the substrate in the place of lactose, the addition of K^{+} activated the β-galactosidase from all strains. Maximum activity was achieved in the presence of Na^{+} alone for the enzymes from *B. bifidum* 1901, *P. pentosaceus* PE39 and *Lb. casei* 20094 and in the presence of both Na^{+} and K^{+} for the enzymes from *S. thermophilus* TS2, *Lb. delbruekii* subsp. *bulgaricus* 20056 and *Lc. lactis* subsp. *lactis* 7962 (data not shown).

The transgalactosylation of lactose

The β-galactosidases from all species studied were able to catalyse transgalactosylation reactions with lactose as the substrate. While the products formed were similar (Fig. 2), in all cases differences were observed between the enzymes in the rate of product formation. The relative proportion of the amount of galactose and glucose released gives an indication of the extent of transgalactosylation. For the enzymes from all strains except *Lc. lactis* subsp. *lactis* 7962, the rate of production of free galactose increased with time. This indicated that, while transgalactosylation occurs early in the reaction, the galacto-oligosaccharide products formed are subsequently hydrolysed. Over the experimental period used here (24 h) no increase was observed with the enzyme from *Lc. lactis* subsp. *lactis* 7962 (data not shown).

Hydrolysis of galactosyl-lactose by β-galactosidases from different species

All species used in this study except *Lb. delbruekii* subsp. *bulgaricus* 20056 and *Lc. lactis* subsp. *lactis* 7962 were able to grow on galactosyl-lactose rather than lactose. However, the major β-galactosidases from all the different species were able to hydrolyse galactosyl-lactose to various extents (Table 2). The combination of intermediate compounds formed and removed during hydrolysis was enzyme specific.

Discussion

The β-galactosidases from a range of lactic acid bacteria, pediococci and bifidobacteria were all found to

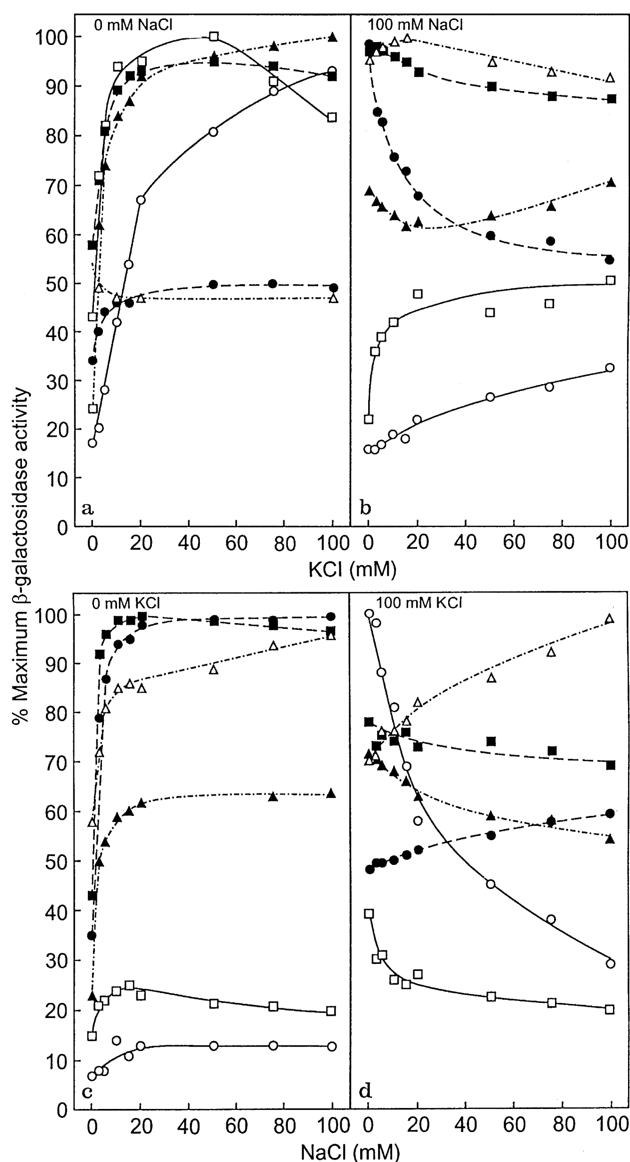

Fig. 1a–d The effect of Na^+ and K^+ on the activity of β-galactosidases, from six different species, on lactose. Assays were performed in 0.1 M bis-Tris propane buffer, pH 7.0, containing 2 mM $MgCl_2$ with 70 mM lactose as enzyme substrate and (**a**) 0–100 mM KCl, (**b**) 0–100 mM KCl in the presence of 100 mM NaCl, (**c**) 0–100 mM NaCl, (**d**) 0–100 mM NaCl in the presence of 100 mM KCl. The carryover NaCl from the enzyme preparation occurred at a level of 4.2 mM for *Lb. casei* 20094, 3 mM for *Lb. delbruekii* subsp. *bulgaricus* 20056, 2.3 mM for *B. bifidum* 1901, 0.4 mM for *S. thermophilus* TS2 and 0 mM for *P. pentosaceus* PE39 and *Lc. lactis* subsp. *lactis* 7962. ○ *S. thermophilus* TS2, □ *P. pentosaceus* PE39, △ *Lb. delbruekii* subsp. *bulgaricus* 22056, ● *B. bifidum* 1901, ■, *Lb. casei* 20094, ▲ *Lc. lactis* subsp. *lactis* 7962

catalyse both hydrolysis and transgalactosylation reactions, yet were also found to be kinetically diverse.

During chromatographic purification of the β-galactosidases in this study, more than one form of β-galactosidase was found within some strains. The presence of multiple β-galactosidases during chromatographic separation could be due either to the presence of distinct enzymes (as found by Dumortier et al. 1994; Matsumoto and Okonogi 1990) or to the ability of some β-galactosidases to remain active after subunit dissociation (Chang and Mahoney 1994; McFeters et al. 1969; Smart and Richardson 1987). In the present study, only the predominant forms of the β-galactosidases were investigated.

The activation or inhibition of β-galactosidase from different sources by mono- and divalent cations has been well documented (Cohn and Monod 1951; Smart and Richardson 1987). Mg^{2+} enhanced activity of the β-galactosidases from all species and, in the case of *Lc. lactis* subsp. *lactis* 7962, was shown to be essential for hydrolysis. Mg^{2+} has been found to be a major activator of β-galactosidase hydrolytic activity in *S. thermophilus* (Greenberg and Mahoney 1982; Smart and Richardson 1987), Lactobacilli (Cesca et al. 1984) and bifidobacteria (Matsumoto and Okonogi 1990). The effects of K^+ and Na^+ on enzyme activity varied greatly between species. Becker and Evans (1969) suggested that association of monovalent cations with a β-galactosidase was on the basis of ionic radius with Na^+ being more tightly bound than K^+, and that both ions affected activity by inducing conformational changes in the enzyme structure.

In dairy fluids the levels of Mg^{2+}, Na^+ and K^+ vary between processes and during processing (Matthews 1978) and therefore the different effects of the cations on the catalytic activities of the β-galactosidases observed in this study are likely to have important ramifications in specific industrial applications. The effect of Ca^{2+} ions on enzyme activity was not determined in this study. The calcium content of dairy fluids is reasonably constant and, although Ca^{2+} is a known inhibitor of β-galactosidases (Itoh et al. 1980; Itoh et al. 1992; Smart et al. 1985), almost all of the calcium in milk is bound to casein and therefore not free in solution. This has been shown by Smart et al. (1985), who found that for the hydrolysis of lactose in milk with the β-galactosidase from *S. thermophilus* no modifications of the milk were required while, in contrast, lactic casein whey (where free Ca^{2+} is present at inhibitory levels) required demineralisation, neutralisation (with KOH) and the addition of Mg^{2+} for the enzyme to be functional. Another important factor in commercial applications, which is affected by the presence of cations, is enzyme stability. The stability of β-galactosidases is improved by Mg^{2+} and Mn^{2+} for some strains (Macias et al. 1983; Lu 1992) but not others (Greenberg and Mahoney 1982).

Transgalactosylation reactions resulting in the formation of a range of oligosaccharides from lactose have been previously shown to be catalysed by the β-galactosidases from *Lactobacillus* species (Toba et al. 1981), *S. thermophilus* (Toba et al. 1981; Smart 1991), *Bacillus circulans* (Yanahira et al. 1995) and *E. coli* (Huber et al. 1976). However, direct comparisons

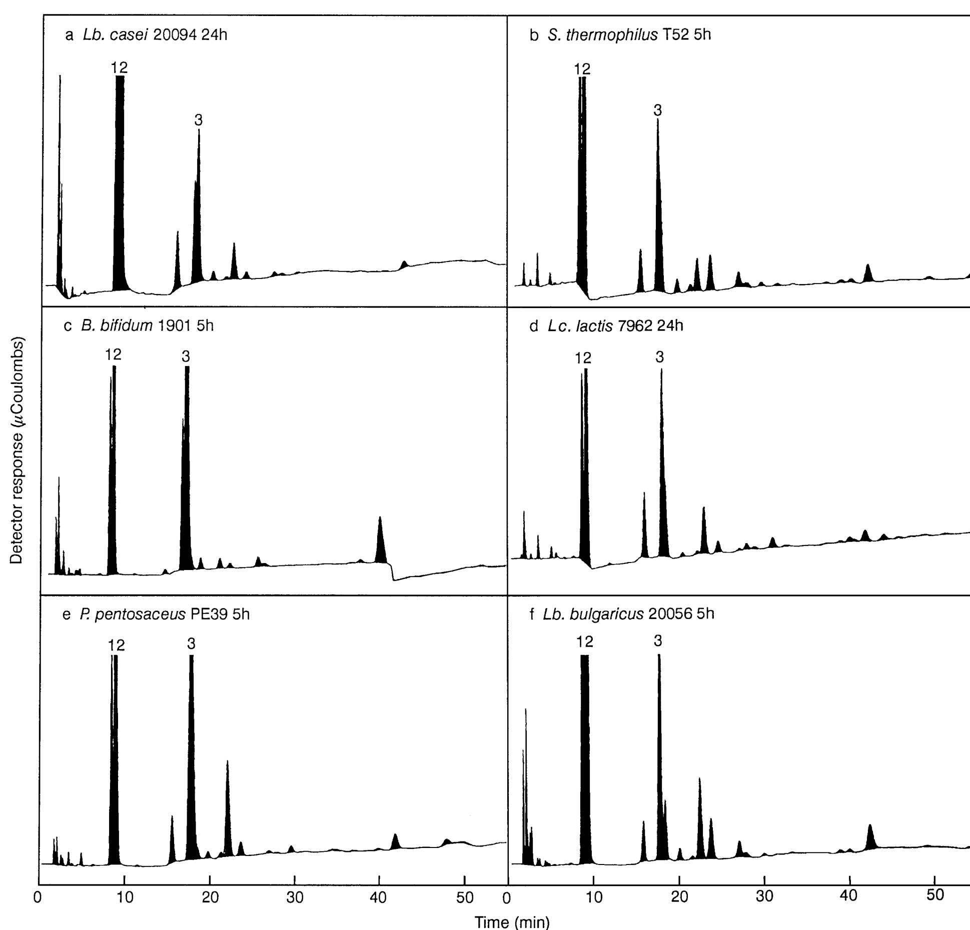

Fig 2a–f Analysis of samples taken from reactions of β-galactosidases from different microbial species with 40% w/v lactose using Dionex LC carbohydrate chromatography with pulsed amperometric detection. Incubation times were chosen so that the amount of lactose remaining approximated the amount of galactose-glucose produced. Peaks: *1* galactose, *2* glucose, *3* lactose. In all cases the amount of enzyme added was equivalent to 5 units (measured using *o*-nitrophenyl-β-D-galactopyranoside as a substrate)/ml reaction mix

Table 2 The relationship of the ability to use galactosyl-lactose as a substrate for growth and the ability to hydrolyse galactosyl-lactose. Growth was assessed after 2 days by measurement of culture turbidity, pH, disappearance of the carbon substrate and the appearance of the fermentation end-products L- and D-lactate. + growth, – no growth. Hydrolysis products were measured by the peak area (arbitrary area units) obtained through Dionex analysis after incubation of galactosyl-lactose (24% w/v) with β-galactosidase prepared from each species (5 units/ml) for 1 h.

Species	Growth on galactosyl-lactose	β-Galactosidase hydrolysis products	
		Galactose	Glucose
S. thermophilus TS2	+	181	33
Lb. delbruekii subsp. *bulgaricus* 20056	–	144	28
Lb. casei 20094	+	23	5
P. pentosaceus PE39	+	12	3
Lc. lactis subsp. *lactis* 7962	–	10	< 1
B. bifidum 1901	+	7	1

between the oligosaccharides found in these studies and those observed here are not possible because of the different methods used and the lack of positive identification of most of the products. In the present study it was found that there was very little difference in the types of oligosaccharides produced by the β-galactosidases from the different species but the amount and rate of production varied. Toba et al. (1981) have grouped strains according to the type of oligosaccharides produced as detected by paper chromatography. Since this is a relatively insensitive method the groupings may have reflected the level of transgalactosylation activity rather than differences in the types of oligosaccharides produced. In the present study, the β-galactosidases from *S. thermophilus* and *Lb. delbruekii* subsp. *bulgaricus* appeared to have significantly higher levels of transgalactosylation activity than those from the other species, this being consistent with the findings of Toba et al. (1981). The relative rates of transgalactosylation ascribed to the various β-galactosidases may have been affected by the use of ONPG to standardise enzyme activity, since the reaction rate of a β-galactosidase is substrate-specific (Premi et al. 1972; Itoh et al. 1980; Greenberg and Mahoney 1982; Smart and Richardson 1987).

A further point of interest is the finding that, while the various strains studied all possessed β-galactosidases capable of hydrolysing galactosyl-lactose, this did not correlate with the ability of a species to use galactosyl-lactose as a carbon source for growth. This

indicates that other factors are involved in the uptake and metabolism of these compounds.

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