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# Properties of a *Streptococcus lactis* Strain That Ferments Lactose Slowly

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*Streptococcus lactis* 7962, which ferments lactose slowly, has a lactose phosphoenolpyruvate-dependent phosphotransferase system and low phospho-β-galactosidase activity, in addition to high β-galactosidase activity. Lactose 6′-phosphate accumulated to a high concentration (>100 mM) in cells growing on lactose. In contrast, lactic streptococci, which ferment lactose rapidly and use only the lactose-phosphotransferase system for uptake, contained high phospho-β-galactosidase activity and low concentrations (0.9 to 1.6 mM) of lactose 6′-phosphate. It is concluded that rate-limiting phospho-β-galactosidase activity is primarily responsible for defective lactose metabolism in *S. lactis* 7962.

The principal requirement of lactic streptococci (*Streptococcus cremoris* and *Streptococcus lactis*) used as starters in milk fermentations is the rapid and homolactic fermentation of lactose. Starter strains, which have doubling times of 32 to 44 min when growing on lactose in complex broth (22), transport lactose via a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) (16, 26). The lactose-phosphate (Lac-6′P) formed is hydrolyzed by phospho-β-galactosidase (P-β-Gal) (12), giving D-glucose and D-galactose 6-phosphate (Gal-6P) (26). Gal-6P is further metabolized to triose phosphates by the three enzymes (Fig. 1) of the D-tagatose 6-phosphate (Tag-6P) pathway (2). The lactose-specific components of the PTS, P-β-Gal (17), together with the enzymes of the Tag-6P pathway (4), are coded on a single plasmid in some starter strains.

In contrast to starter strains, some *S. lactis* strains ferment lactose slowly and produce a variety of end products (6). These strains, which have been designated wild types (6), are often associated with plant material in which fast fermentation of lactose (a mammalian sugar) would not be a selective advantage. Wild-type strains contain both P-β-Gal and β-galactosidase (β-Gal) (6), whereas the activities of the lactose-PTS and the Tag-6P pathway enzymes have not been determined. DNA hybridization data suggest that wild- and starter-type strains are indistinguishable (9) and thus may differ only in their pathway for lactose metabolism. It has been suggested that starter-type organisms are of recent origin (11) and that their evolution has involved acquisition of the lactose plasmid by wild-type strains (17), thus allowing the adaptation to milk fermentation.

The best-studied strain that ferments lactose slowly is *S. lactis* 7962, which was originally isolated from milk (21). This strain appears to take up lactose as the free sugar (17), has high β-Gal activity (6, 16), and contains the enzymes of both the Tag-6P and Leloir pathways (2). It has a doubling time of ~100 min when growing on lactose in complex broth, and only ~15% of the lactose fermented appears as lactic acid (22). In the present study, the defective nature of lactose fermentation in *S. lactis* 7962 was investigated.

## MATERIALS AND METHODS

**Organisms and culture conditions.** All strains were from the collection held at the New Zealand Dairy Research Institute. *S. lactis* 7962 was originally obtained from the American Type Culture Collection, Rockville, Md. *S. lactis* 133 was received from the National Institute for Research in Dairying, Reading, England, as NCDO 496 (= ATCC 11454).

Static batch cultures were grown at 30°C in $T_5$ complex broth (24) which contained either 28 mM galactose, 28 mM glucose, or 14 mM lactose, and the initial pH was 7.2. Standardized carbohydrate solutions were filter sterilized before being added to autoclaved broth. Cells were grown for at least 20 generations on the appropriate carbohydrate before use in experiments.

**Enzyme assays.** Cells growing exponentially were harvested when the residual carbohydrate in the medium was half the initial concentration (pH ~6.5). Cells were disrupted in 20 mM phosphate buffer (pH 6.5) containing 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithioerythritol by shaking for 2 min at 0 to 5°C with glass beads in a Mickle disintegrator. Debris was removed by centrifugation at 35,000 × *g* for 5 min. Cell extracts were stored on ice, and enzyme assays were completed within 4 h after preparation of extracts. All enzymes assayed were stable (>90%) for at least 4 h.

P-β-Gal and β-Gal activities were assayed by using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) or ONPG-6-phosphate (ONPG-6P) as described previously (4), and calibrations with standard amounts of *o*-nitrophenyl (ONP) were carried out when different assay conditions were used. The standard assay (1 ml) for phosphatase activity contained 100 mM triethanolamine hydrochloride buffer (pH 7.5), 10 mM *p*-nitrophenyl phosphate (PNPP), 10 mM $MgCl_2$, and 0.1 ml of cell extract. The reaction (at 25°C) was stopped by the addition of an equal volume of 0.5 M $Na_2CO_3$, and the amount of PNPP hydrolyzed was determined by measuring the absorbance at 405 nm (1). One unit of phosphatase activity was defined as the amount of enzyme that released 1 nm of *p*-nitrophenol per min. The three enzymes of the Tag-6P pathway were assayed as described previously (4).

The procedure for assaying PTS activities in permeabilized cells by coupling to NADH oxidation has been described previously (4). The only modification was the use of 50 μl of toluene-acetone (1:9, vol/vol) per ml of cell suspension when permeabilizing *S. lactis* 7962, because the use of the larger amount (100 μl) gave lower PTS activities with this strain. To measure the product of PTS activities in permeabilized cells of *S. lactis* 7962, the PTS incubation mixture contained (in a total volume of 6 ml): 5 mM PEP, 10 mM NaF, 50 mM sodium-potassium phosphate buffer (pH 7.2), 5

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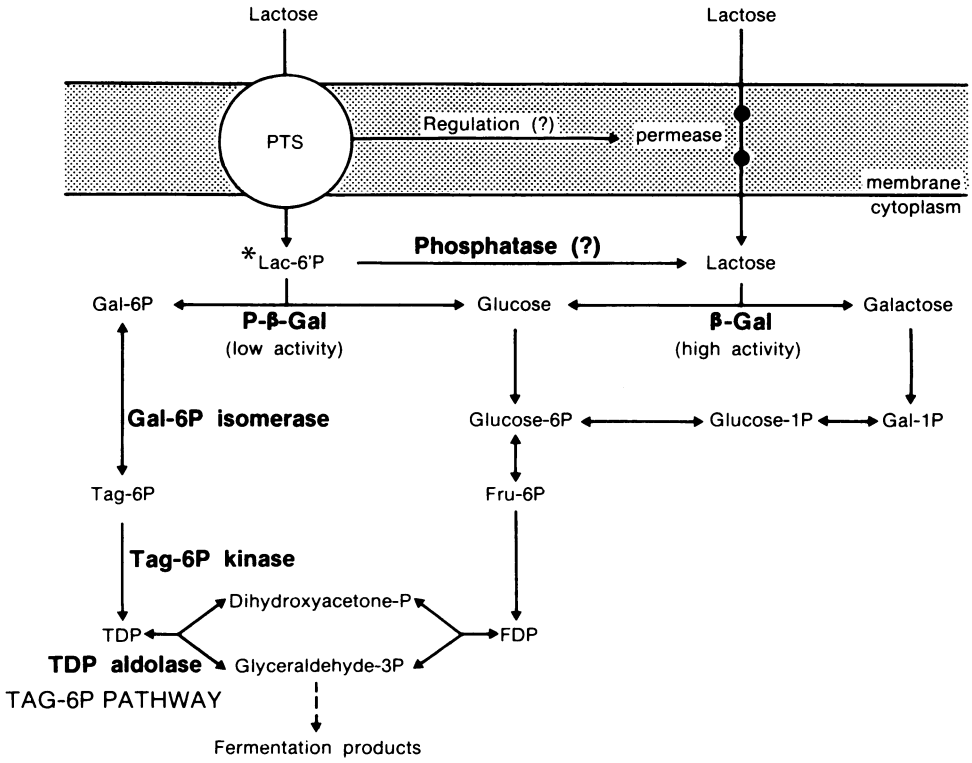


FIG. 1. Lactose metabolism by growing cells of *S. lactis* 7962 in which Lac-6'P (*) accumulates to a high concentration. The role of the PTS in regulating permease activity and the amount of Lac-6'P that is hydrolyzed by a phosphatase may be important, but their contributions to lactose metabolism are unknown.

mM $MgCl_2$, 600 μl of permeabilized cell suspension (5 to 7 mg [dry weight] of cells per ml), and 5 mM lactose. At 0, 1, 10, 20, and 30 min, 1-ml samples were removed and placed in a boiling-water bath for 1 min. After centrifugation at 13,500 × *g* for 5 min, the supernatants were removed and stored at −70°C.

**Extraction and assay of intermediates.** Cells growing exponentially were extracted with perchloric acid, and the intermediates were assayed enzymatically in neutralized extracts, using a fluorescence spectrophotometer (15, 25). Gal-1P and Gal-6P were assayed as described previously (25). In one series of experiments, Lac-6'P was extracted from resting cells (see Fig. 3). An exponentially growing culture (30 ml) was membrane filtered, and the cells were washed rapidly (10 s maximum) with 4 ml of 20 mM phosphate buffer (pH 6.5) containing 50 mM NaCl and 10 mM $MgCl_2$ at 0°C. Cells were then resuspended in 30 ml of the same buffer (but preincubated at 25°C) and held at 25°C. At the appropriate time, a 25-ml portion was filtered and extracted with perchloric acid (25).

The Lac-6'P concentration was determined by measuring the release of glucose and Gal-6P after P-β-Gal treatment and the release of lactose after alkaline phosphatase treatment. The appropriate corrections were made for free glucose, glucose 6-phosphate, lactose, galactose, and Gal-6P. Samples for Lac-6'P analysis (200 μl each) were added to 100 μl of 1 M triethanolamine hydrochloride buffer (pH 7.5) and incubated at 37°C for 2 h with either 0.5 U (50 μl) of P-β-Gal (*Streptococcus mutans*), 25 U (50 μl) of alkaline phosphatase (bovine intestine), or 50 μl water as a control. A sample (100 μl) which had been treated with P-β-Gal was further incubated for 2 h at 37°C with 10 U of alkaline phosphatase, whereas a sample (100 μl) which had been treated only with alkaline phosphatase was incubated for 2 h at 37°C with 2 U of β-Gal (*Escherichia coli*) and 5 mM $MgCl_2$. Glucose 6-phosphate, fructose 6-phosphate, and glucose were assayed, using a fluorescence spectrophotometer, by the sequential addition of glucose 6-phosphate dehydrogenase (bakers' yeast, 5 U), phosphoglucose isomerase (yeast, 4 U), and hexokinase (bakers' yeast, 10 U) to the same cuvette containing 100 mM imidazole hydrochloride buffer (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, 1 mM $NADP^+$, and up to 100 μl of sample. In addition, when the Lac-6'P concentration was sufficiently high, glucose was determined with Glucostat reagents (Worthington Diagnostics, Freehold, N.J.), whereas for lactose measurements the Glucostat reagents were modified by the addition of $MgCl_2$ final (concentration, 5 mM) and 0.8 U of β-Gal per ml of reconstituted Glucostat reagent.

**Partial purification of Lac-6'P.** Lac-6'P was extracted from *S. lactis* 7962 cells growing exponentially in $T_5$ broth (28 mM lactose). Cells were harvested from 50-ml culture samples (0.7 to 0.8 mg [dry weight] of bacteria per ml; ~18 mM residual lactose) by rapid filtration with membrane filters (47-mm diameter; 0.8-μm pore diameter) and then placed in 5 ml of 0.6 M $HClO_4$ at 0°C. A total of 850 ml of culture was treated in this way, the combined extracts were neutralized with 6 M KOH, and the precipitate was removed by centrifugation. Free lactose was removed by passage through a column (12 by 105 mm) containing a strong base ion-exchange resin (AG1-X4; Bio-Rad Laboratories, Richmond, Calif.) and by elution with 2 mM imidazole hydrochloride buffer (pH 7.5). The absorbed Lac-6'P was then eluted with 0.01 M HCl and 0.1 M NaCl. The Lac-6'P fraction (100 ml) was adjusted to pH 8.2 with 6 M KOH and maintained at this pH while 7.5 ml of barium acetate (25% [wt/vol]) was added. After 30 min at 0°C, the insoluble barium salts were removed

by centrifugation. The barium salt of Lac-6'P was precipitated from the supernatant by the addition of 4 volumes of cold 95% ethanol. After 2 h at 0°C, the precipitate was collected by centrifugation and dissolved in the minimum volume of water (9.6 ml). Resin (0.48 g of Bio-Rad AG 50W-X2 hydrogen form) was then added to remove the barium. The resin was removed by filtration, and the filtrate was adjusted to pH 7.1 with 1 M NaOH. This partially purified Lac-6'P solution (65 μmol) was stored at −70°C, a temperature at which it is stable for at least 6 months. This procedure gave 60 to 70% recovery of Lac-6'P. Previously, Lac-6'P has been purified from *S. lactis* only in small amounts (80 nmol; 26).

**Other procedures.** Protein was determined by a modification (10) of the Lowry method, using bovine serum albumin as the standard. The bacterial dry weight of permeabilized cells was determined directly, using membrane filters (23).

**Materials.** All biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo., and were grades with the highest analytical purity. The highly purified P-β-Gal, prepared from *S. mutans*, was from Robert Calmes, University of Kentucky, Lexington.

## RESULTS

**β-Gal and P-β-Gal activities.** By using ONP derivatives as substrates, high β-Gal specific activity and low P-β-Gal specific activity have been reported in *S. lactis* 7962 (6, 7, 19). It has been suggested that the apparent P-β-Gal activity is due to ONPG contamination of ONPG-6P (19), so that *S. lactis* 7962 actually possesses only β-Gal activity (17). However, in this strain, the P-β-Gal activity was more stable than the β-Gal activity, which suggests that a P-β-Gal exists in *S. lactis* 7962 (6) and should rule out a combination of phosphatase and β-Gal activities giving an apparent P-β-Gal activity. The existence of even low P-β-Gal activity could be of importance, so we assayed β-Gal, P-β-Gal, and phosphatase activities in *S. lactis* 7962 under a variety of conditions (Table 1) to determine the nature of the P-β-Gal activity. With standard assay conditions, the specific activities of P-β-Gal were low as compared with β-Gal activities in *S. lactis* 7962 cells grown on lactose or galactose. The P-β-Gal specific activity in a typical strain which grew fast on lactose (*S. lactis* 133) was 16 times greater than that in *S. lactis* 7962. The P-β-Gal from both *S. lactis* 7962 and 133 was more active in trienthanolamine buffer than in the standard phosphate buffer, whereas the β-Gal activity from *S. lactis* 7962 was the same in both buffers (data not shown). EDTA strongly inhibited the β-Gal activity of *S. lactis* 7962 (Table 1) but did not affect the P-β-Gal activity (data not shown). The addition of 5 mM $MgCl_2$ or 10 mM NaF did not alter the activity of either P-β-Gal or β-Gal, whereas phosphatase activity was 40% lower when assayed in the absence of added $MgCl_2$ and 90% lower in the presence of 4 mM NaF (data not shown). The different responses to the above-mentioned effectors, together with the differential induction of P-β-Gal and β-Gal during growth on galactose and lactose (Table 1), suggest that there is a low P-β-Gal activity (with ONPG-6P as the substrate) in strain 7962. ONPG was not detectable (using purified β-Gal from *E. coli*) in the ONPG-6P substrate.

TABLE 1. Specific activities of P-β-Gal, β-Gal, and phosphatase in cell extracts from *S. lactis* 7962 and 133[a]

| Strain | Sugar in growth medium | Sp act of: | | |
|---|---|---|---|---|
| | | P-β-Gal[b] | β-Gal[b] | Phosphatase[c] |
| 7962 | Galactose | 0.012 (0.031[d]) | 0.66 (0.06[e]) | 4.9 |
| 7962 | Lactose | 0.041 (0.104[d]) | 0.49 (0.03[e]) | 3.8 |
| 133 | Lactose | 0.67 (1.04[d]) | ND[f] | 5.5 |

[a] Culture conditions were 30°C in $T_5$ medium containing 28 mM galactose or 14 mM lactose. Exponentially growing cells were harvested when ~60% of the sugar had been fermented.

[b] Specific activities are expressed as micromoles of ONP released per minute per milligram of protein.

[c] Specific activities are expressed as nanomoles of PNP released per minute per milligram of protein.

[d] The standard assay buffer was replaced by 50 mM triethanolamine hydrochloride buffer (pH 7.2).

[e] EDTA (final concentration, 5 mM) was added to the standard assay mixture.

[f] ND, Not detectable.

TABLE 2. Specific activities of sugar-PTSs, β-Gal, and P-β-Gal in permeabilized cells of *S. lactis* 7962[a]

| Sugar in growth medium | Sugar-PTS sp act[b] | | | Galactosidase sp act[c] | |
|---|---|---|---|---|---|
| | Lactose | Glucose | Galactose | β-Gal | P-β-Gal |
| Lactose | 98.0 (178.1) | 148.3 | 12.0 | 278.0 | 11.4 |
| Galactose | 31.8 (64.3) | 26.3 | 4.2 | 321.0 | 3.4 |
| Glucose | 0.3 | 29.7 | 1.3 | 0.9 | 0.2 |

[a] Culture conditions, preparation of permeabilized cells, and enzyme assay procedures are described in the text.

[b] Specific activities are expressed as nanomoles of NADH oxidized (PEP and carbohydrate dependent) per minute per milligram (dry weight) of cells and are an average of at least three separate experiments. Values in parentheses are specific activities determined after preincubation of permeabilized cells with lactose for 4.5 min before the reaction was initiated with PEP.

[c] Specific activities are expressed as nanomoles of ONP released per minute per milligram (dry weight) of cells.

Although the presence of Tag-6P pathway enzymes in *S. lactis* 7962 has been demonstrated (2), we assayed these activities in view of the low P-β-Gal activity present in strain 7962. The specific activities (micromoles of substrate utilized per min per milligram of protein) of Gal-6P isomerase, Tag-6P kinase, and tagatose 1,6-diphosphate aldolase were, respectively, 0.54, 0.25, and 0.48 when strain 7962 was grown on galactose and 0.76, 0.27, and 0.72 when strain 7962 was grown on lactose.

**PTS activities.** The specific activity of the lactose-PTS in *S. lactis* 7962 (Table 2) was comparable to that found in *S. lactis* strains $C_{10}$ and 133 (4). However, unlike these two strains (4), *S. lactis* 7962 has high β-Gal activity (Tables 1 and 2), so it is possible that a combination of β-Gal activity and glucose-PTS (or galactose-PTS) (or both) activity could give an apparent lactose-PTS activity. The $K_m$ values for the lactose-, glucose-, and galactose-PTSs in *S. lactis* 7962 grown on lactose were 0.7, 0.023, and 21.7 mM, respectively (data not shown). Cells grown on galactose gave similar values, and the specific activity of the PTS was high with glucose and low with galactose as the substrates. This indicates that only the combined action of β-Gal and the glucose-PTS is likely to contribute apparent lactose-PTS activity. Preincubation of cells with lactose for 4.5 min before the addition of PEP gave higher lactose-PTS activity (Table 2) than that in the normal assay in which lactose was added last and the reaction rate was recorded 14 s later. This lactose-PTS activity was also significantly higher than the glucose-PTS activity, which was at a maximum with the standard glucose concentration (5 mM). Thus, the higher

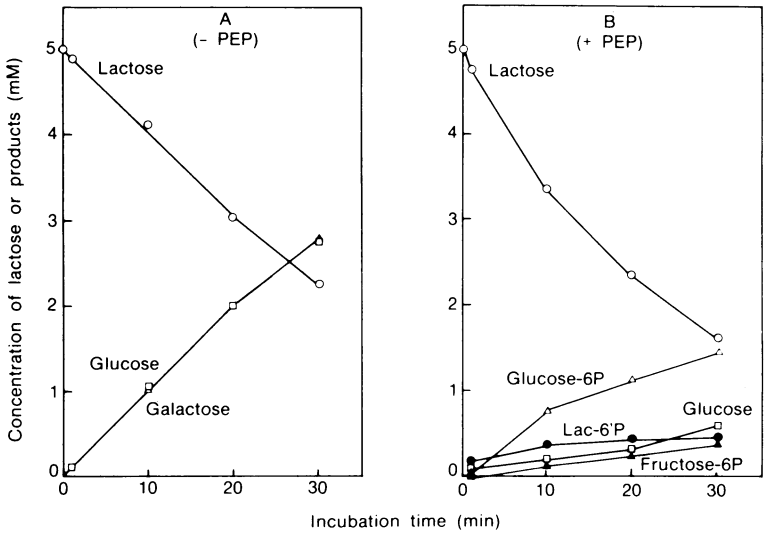


FIG. 2. Products formed by permeabilized cells (lactose grown) of *S. lactis* 7962 incubated with lactose (5 mM) in the absence (A) and presence (B) of PEP. Glucose-6P, glucose 6-phosphate. Fructose-6P, fructose 6-phosphate.

lactose-PTS activity resulting from preincubation of permeabilized cells with lactose was probably due to a combination of lactose-PTS and glucose-PTS activities. From kinetic considerations (data not shown), glucose-PTS activity would contribute an estimated 50 and 30%, respectively, of the lactose-PTS of lactose- and galactose-grown cells (Table 2).

To confirm the presence of a lactose-PTS reaction, the phosphorylated sugar products were analyzed in a reaction mixture in which both NADH and the coupling enzymes were absent (Fig. 2b). In the presence of PEP, permeabilized cells of *S. lactis* 7962 had formed 0.42 mM Lac-6'P after a 30-min incubation, confirming lactose-PTS activity. The initial rate of Lac-6'P formation was 70 to 80 nmol/min per mg (dry weight) of cells, which is comparable to the lactose-PTS activity measured indirectly in lactose-grown cells (Table 2). Some of the glucose 6-phosphate formed (Fig. 2b) was isomerized to fructose 6-phosphate by the enzyme glucose 6-phosphate isomerase (EC 5.3.1.9) which requires no cofactors. No other significant reactions occurred since >96% of the initial glucose moiety of lactose was accounted for, after 30 min of incubation, by the products shown in Fig. 2b. The galactose moiety of lactose was completely accounted for after 30 min of incubation (1.6 mM lactose, 2.95 mM free galactose, 0.42 mM Lac-6'P, and 0.045 mM Gal-6P). The low concentration of Gal-6P could be due to the limited hydrolysis of Lac-6'P by P-β-Gal (Tables 1 and 2) or to the low galactose-PTS activity. Although Gal-6P isomerase does not require any cofactors, no Tag-6P would be expected to be detected because of the kinetics of this enzyme (3). In a control experiment, permeabilized *S. lactis* 7962 cells were incubated for 30 min (Fig. 2a) except that lactose was replaced by 10 mM Gal-6P. With or without NaF, no free galactose nor any significant decrease in Gal-6P was detected. This suggests that both sugar phosphate phosphatase and Gal-6P isomerase activities were minimal under standard assay conditions. In another control (Fig. 2a), no phosphorylated sugars were detected when lactose was hydrolyzed. The initial rate of lactose hydrolysis by permeabilized cells was increased from 148 to 237 nmol/min per mg (dry weight) of cells by the addition of PEP (Fig. 2a and b).

**Intracellular concentrations of key metabolites of lactose metabolism.** The intracellular concentration of Lac-6'P in strain 7962 growing on lactose was >65 times that found in strains $E_8$, $ML_3$, and $ML_8$ (Table 3). In contrast to strain 7962, strains $E_8$, $ML_3$, and $ML_8$ had high P-β-Gal activities (respectively, 123.6, 84.9, and 60.3 nmol of ONP released per min per mg [dry weight] of cells) and no detectable β-Gal activity (<0.8 nmol of ONP released per min per mg [dry weight] of cells). The intracellular concentration of Gal-6P was lowest in strain 7962 (Table 3). This compound could arise from either lactose-PTS and P-β-Gal activities or from β-Gal and galactose-PTS activities. In strains $E_8$, $ML_3$, and $ML_8$, Gal-6P can only arise from lactose-PTS and P-β-Gal activities. The presence of Gal-1P in strain 7962 (Table 3) is consistent with the operation of the Leloir pathway during lactose metabolism.

**Metabolism of Lac-6'P by *S. lactis* 7962.** The intracellular Lac-6'P was metabolized when intact cells were incubated in buffer (half-life [$t_{1/2}$] = 1.4 min) (Fig. 3). By using this value, the initial rate of Lac-6'P removal was calculated to be 62.6 nmol/min per mg (dry weight) of cells in which the in vivo concentration of Lac-6'P was 105 mM and 1 mg (dry weight) of cells had a volume of 1.67 μl (25).

The products of Lac-6'P metabolism were examined by incubating permeabilized cells with partially purified Lac-6'P (Fig. 4). The rate of Lac-6'P decrease was 24 nmol/min per mg (dry weight) of cells, which was similar to the rate of Gal-6P and glucose increase (respectively, 22.4 and 23.2

TABLE 3. Intracellular concentrations of Lac-6'P, Gal-6P, and Gal-1P in lactic streptococci growing on lactose[a]

| Strain | Intracellular concn (mM) | | |
|---|---|---|---|
| | Lac-6'P | Gal-6P | Gal-1P |
| *S. lactis* 7962 | 105 | 9.1 | 0.5 |
| *S. lactis* $ML_3$ and $ML_8$ *S. cremoris* $E_8$ | 0.9–1.6 | 14.0–19.0 | ND[b] |

[a] Culture conditions and measurement of intracellular intermediate concentrations are described in the text. For *S. lactis* 7962, mean values from at least three separate experiments are given; for the other three strains, the ranges of mean values are given. At sampling, the lactose concentration in the medium was 6 to 8 mM.

[b] ND, Not detectable.

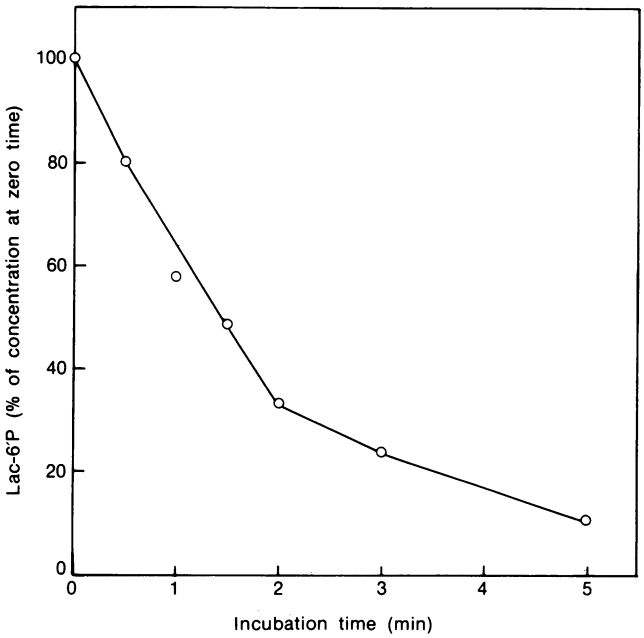


FIG. 3. Rate of Lac-6'P disappearance in *S. lactis* 7962. Cells growing on lactose were quickly harvested, washed, and incubated in buffer at 25°C as described in the text. There was an 18 to 20% drop in Lac-6'P concentration during the washing step (i.e., 100% value is 85 mM).

nmol/min per mg [dry weight] of cells). (Some Gal-6P [0.07 mM] was introduced with the partially purified Lac-6'P preparation.) These rates were not altered when NaF was omitted from the reaction mixture. The glucose 6-phosphate concentration (0.1 mM; introduced with the Lac-6'P preparation) did not increase, and no free galactose or lactose was detected during the incubation period. No PEP, ADP, or ATP was detected (<0.005 mM) in the reaction mixture after Lac-6'P was added. When permeabilized cells were incubated with either 5 mM Gal-6P or 5 mM glucose-6P, no decrease (with or without 10 mM NaF) was observed in the concentration of either metabolite during a 1-h incubation (data not shown). These results indicate that P-β-Gal activity was responsible for the degradation of Lac-6'P under the assay conditions used and rule out the possibility of involvement of a sugar phosphate phosphatase activity coupled with β-Gal and galactose-PTS activities.

**Specific rate of lactose utilization.** Strain 7962 was grown in batch cultures (initial lactose concentration, 17 mM), both at a constant pH (6.5 ± 0.05) and without pH control. The specific rate of lactose utilization during exponential growth (28) was 68.0 to 71.1 nmol/min per mg (dry weight) of cells.

## DISCUSSION

Although *S. lactis* 7962 is the best studied of the lactic streptococci that ferment lactose slowly, lactose transport has only been indirectly examined with a non-metabolizable sugar analog (thiomethyl-β-D-galactopyranoside). Thiomethyl-β-D-galactopyranoside was reported to accumulate unchanged (13), whereas in another study (18) the phosphate derivative accumulated at a slow rate. We have demonstrated, using two assay methods and lactose as the substrate, that the lactose-PTS activity is present in strain 7962. Furthermore, the level of activity is comparable to that found in *S. lactis* strains which ferment lactose rapidly (4). In one assay method, PEP utilization was measured; in strains with no β-Gal activity, the utilization of PEP is lactose dependent. However, in a strain such as *S. lactis* 7962 in which β-Gal activity is present, the PEP utilization could be glucose- or galactose-dependent or both if the initial substrate (lactose) is hydrolyzed quickly by the permeabilized cells. We concluded that glucose-PTS contributed up to 50% of the activity described as lactose-PTS, by using the assay which measured PEP utilization (Table 2). It was therefore important to confirm lactose-PTS activity by another method. We have demonstrated the PEP-dependent formation of Lac-6'P, the unique product of lactose-PTS activity, with permeabilized cells of strain 7962.

There has been some doubt as to whether the low P-β-Gal activity in *S. lactis* 7962 (16, 19) is real. However, it is apparent from the study by Farrow (6), and further supported by our study, that this strain does possess low P-β-Gal specific activity, in addition to high β-Gal activity. All published assays for P-β-Gal activity, except a radioactive assay (26), and even most assays for β-Gal are carried out with ONPG-6P and ONPG, respectively, and not with the natural substrates. In view of a report that a mutant of strain 7962 had a β-Gal with no affinity for ONPG but still had activity with lactose (16), a critical assessment of both β-Gal and P-β-Gal activities should be made with the natural substrates. Lac-6'P has not been used routinely for P-β-Gal assay, presumably because it is not commercially available. A useful spin-off from our study is the demonstration that *S. lactis* 7962 is potentially a good source of Lac-6'P.

We prepared partially purified Lac-6'P and demonstrated that permeabilized cells of strain 7962 hydrolyze this compound to glucose and Gal-6P, i.e., the expected products from P-β-Gal hydrolysis. The initial rate of Lac-6'P hydrolysis was 23 nmol/min per mg (dry weight) of cells when the initial Lac-6'P concentration was 4.94 mM. With 5 mM lactose, the initial rate of hydrolysis was much faster (139 nmol/min per mg [dry weight] of cells). Thus, in strain 7962, β-Gal and P-β-Gal hydrolyze both the natural and synthetic substrates. The other unique enzymes necessary for metabolism of Lac-6'P are the three Tag-6P pathway enzymes. These enzymes are present in cell extracts of strain 7962 (2; this study) at levels comparable to those in *S. lactis* strains which ferment lactose rapidly (2, 4).

Possession of the lactose-PTS, P-β-Gal, and the three

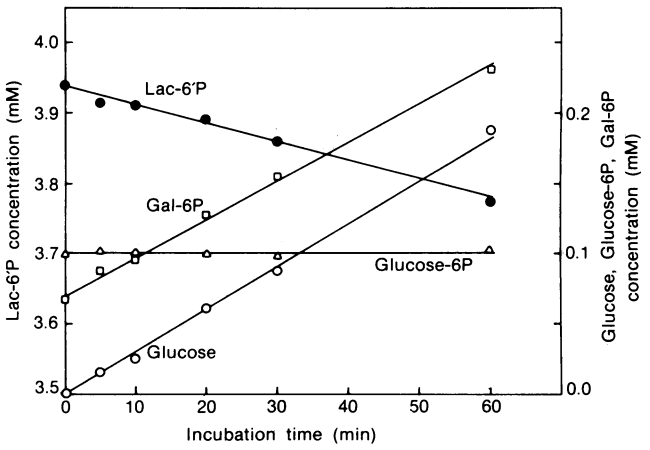


FIG. 4. Metabolism of partially purified Lac-6'P by permeabilized cells of *S. lactis* 7962. Experimental conditions and assay procedures are described in the text.

enzymes of the TAG-6P pathway are thought to be prerequisites for rapid, homolactic fermentation of lactose by group N streptococci (14). The full complement of enzymes to generate and metabolize Lac-6'P are present in *S. lactis* 7962. Of these enzymes, only P-β-Gal has low specific activity relative to that of the strains which ferment lactose rapidly. We conclude that rate-limiting P-β-Gal activity is primarily responsible for the slow metabolism of lactose by *S. lactis* 7962.

Lactose is transported in *S. lactis* 7962 by both a PTS and a permease (Fig. 1). Since P-β-Gal activity appears to limit the rate of lactose metabolism, comparison of the rate of Lac-6'P removal in intact washed cells with the overall rate of lactose utilization in growing cells (68 to 71 nmol of lactose per min per mg [dry weight] of cells) should indicate the relative contribution of the two transport systems in vivo. The initial rate of Lac-6'P removal in washed cells was 62.6 nmol/min per mg (dry weight) of cells. Therefore, up to 90% of the lactose could be taken up by the lactose-PTS in strain 7962. Lactose uptake by the permease, hydrolysis by β-Gal, and metabolism via the Leloir pathway may be limited by interference from the PTS, as in enteric bacteria in which the transport of non-PTS sugars is regulated by the PTS sugars (for reviews, see references 5 and 20). High intracellular concentrations of Lac-6'P may also inhibit these activities. In this regard, lactose-negative ($Lac^-$), galactose-defective ($Gal^d$) mutants of *S. lactis* growing on galactose contained high intracellular concentrations of Gal-6P and had reduced growth rates compared with the rates of the parent strains (4).

It was suggested in 1969 that lactose may be accumulated as Lac-6'P via the PEP-dependent system in *S. lactis* 7962 and then cleaved by a specific phosphatase (18). In strain 7962, the phosphatase activity with PNPP as the substrate and the sugar phosphate phosphatase in *S. lactis* 133 (27) are $Mg^{2+}$ dependent and sensitive to both fluoride and EDTA. We do not know whether the phosphatase assayed via PNPP or any other phosphatases in strain 7962 hydrolyze Lac-6'P under in vivo conditions. When partially purified Lac-6'P is used as a substrate at low concentrations (Fig. 4), the products formed rule out sugar phosphate phosphatase activity. However, as the phosphatase activity with glucose 6'-phosphate in strain 133 has a high $K_m$ and low $V_{max}$ (27), any potential phosphatase activity with Lac-6'P may only be detectable in strain 7962 by using high concentrations of radioactive Lac-6'P. The sensitivity required for detection may imply that the contribution of phosphatase to Lac-6'P metabolism in growing *S. lactis* 7962 cells is small. Sugar phosphate phosphatases may have the potential to maintain the concentration of some intracellular sugar phosphates within physiologically acceptable limits (8). However, it appears that in at least two examples of *S. lactis* the capability of the enzyme(s) is limited, as in both strain 7962 and strain 133 $Lac^-$ $Gal^d$ (4) growing on lactose and galactose, respectively, the sugar phosphates are at abnormally high concentrations (106 mM Lac-6'P and 49 mM Gal-6P, respectively).

Based on lactose fermentation rates and distribution of P-β-Gal and β-Gal, 11 strains of lactic streptococci, including strain 7962, were grouped as wild type (slow fermentation) as opposed to starter type (rapid fermentation) (6). From our studies, it is evident that strain 7962 may have originally been a fast fermenter of lactose that lost most of its P-β-Gal activity and subsequently acquired or expressed a permease system and β-Gal to partially compensate. Thus, strain 7962, and similar lactic streptococci, should not be classified as wild-type strains. Partial lactose-fermenting revertants arose from $Lac^-$ mutants of the fast lactose-fermenting strains *S. lactis* $C_2$ and *S. cremoris* $B_1$ (for a review, see reference 17). The origin of *S. lactis* 7962 could be similar to these revertants which partially ferment lactose. Isolation of an *S. lactis* 7962 mutant with no lactose-PTS, or a transconjugant with P-β-Gal from the lactose plasmid of another strain, would be logical approaches to try to convert strain 7962 to a fast fermenter of lactose.

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## LITERATURE CITED

1. **Bergmeyer, H. U., K. Gawehn, and M. Grassl.** 1974. Phosphatase, alkaline, p. 496–497. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, 2nd ed., vol. 1. Verlag Chemie, Weinheim, West Germany.
2. **Bissett, D. L., and R. L. Anderson.** 1974. Lactose and D-galactose metabolism in group N streptococci: presence of enzymes for both the D-galactose 1-phosphate and D-tagatose 6-phosphate pathways. J. Bacteriol. **117**:318–320.
3. **Bissett, D. L., W. C. Wenger, and R. L. Anderson.** 1980. Lactose and D-galactose metabolism in *Staphylococcus aureus*. II. Isomerization of D-galactose 6-phosphate to D-tagatose 6-phosphate by a specific D-galactose 6-phosphate isomerase. J. Biol. Chem. **255**:8740–8744.
4. **Crow, V. L., G. P. Davey, L. E. Pearce, and T. D. Thomas.** 1983. Plasmid linkage of the D-tagatose 6-phosphate pathway in *Streptococcus lactis*: effect on lactose and galactose metabolism. J. Bacteriol. **153**:76–83.
5. **Dills, S. S., A. Apperson, M. R. Schmidt, and M. H. Saier, Jr.** 1980. Carbohydrate transport in bacteria. Microbiol. Rev. **44**:385–418.
6. **Farrow, J. A. E.** 1980. Lactose hydrolysing enzymes in *Streptococcus lactis* and *Streptococcus cremoris* and also in some other species of streptococci. J. Appl. Bacteriol. **49**:493–503.
7. **Farrow, J. A. E., and E. I. Garvie.** 1979. Strains of *Streptococcus lactis* which contain β-galactosidase. J. Dairy Res. **46**:121–125.
8. **Fraenkel, D. G.** 1968. The accumulation of glucose 6-phosphate from glucose and its effect in a *Escherichia coli* mutant lacking phosphoglucose isomerase and glucose 6-phosphate dehydrogenase. J. Biol. Chem. **243**:6451–6457.
9. **Garvie, E. I., J. A. E. Farrow, and B. A. Phillips.** 1981. A taxonomic study of some strains of streptococci which grow at 10°C but not at 45°C including *Streptococcus lactis* and *Streptococcus cremoris*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **C2**:151–165.
10. **Hartree, E. F.** 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem. **48**:422–427.
11. **Hirsch, A.** 1952. The evolution of the lactic streptococci. J. Dairy Res. **19**:290–293.
12. **Johnson, K. G., and I. J. McDonald.** 1974. β-D-Phosphogalactoside galactohydrolase from *Streptococcus cremoris* HP: purification and enzyme properties. J. Bacteriol. **117**:667–674.
13. **Kashket, E. R., and T. H. Wilson.** 1972. Role of metabolic energy in the transport of β-galactosides by *Streptococcus lactis*. J. Bacteriol. **109**:784–789.
14. **Lawrence, R. C., T. D. Thomas, and B. E. Terzaghi.** 1976. Reviews of the progress in dairy science: cheese starters. J. Dairy Res. **43**:141–193.
15. **Lowry, O. H., and J. V. Passonneau.** 1972. A flexible system of enzymatic analysis, p. 168–170. Academic Press, Inc., New York.
16. **McKay, L., A. Miller III, W. E. Sandine, and P. R. Elliker.** 1970. Mechanisms of lactose utilization by lactic acid streptococci: enzymatic and genetic analyses. J. Bacteriol. **102**:804–809.
17. **McKay, L. L.** 1982. Regulation of lactose metabolism in dairy streptococci, p. 153–182. *In* R. Davies (ed.), Developments in

food microbiology, vol. 1. Applied Science Publishers, London.
18. **McKay, L. L., L. A. Walter, W. E. Sandine, and P. R. Elliker.** 1969. Involvement of phosphoenolpyruvate in lactose utilization by group N streptococci. J. Bacteriol. **99**:603–610.
19. **Molskness, T. A., D. R. Lee, W. E. Sandine, and P. R. Elliker.** 1973. β-D-Phosphogalactoside galactohydrolase of lactic streptococci. Appl. Microbiol. **25**:373–380.
20. **Postma, P. W., and S. Roseman.** 1976. The bacterial phosphoenolpyruvate: sugar phosphotransferase system. Biochim. Biophys. Acta **457**:213–257.
21. **Rahn, O., C. P. Hegarty, and R. E. Deuel.** 1938. Factors influencing the rate of fermentation of *Streptococcus lactis*. J. Bacteriol. **35**:547–558.
22. **Thomas, T. D.** 1976. Regulation of lactose fermentation in group N streptococci. Appl. Environ. Microbiol. **32**:474–478.
23. **Thomas, T. D., D. C. Ellwood, and V. M. C. Longyear.** 1979. Change from homo- to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures. J. Bacteriol. **138**:109–117.
24. **Thomas, T. D., B. D. W. Jarvis, and N. A. Skipper.** 1974. Localization of proteinase(s) near the cell surface of *Streptococcus lactis*. J. Bacteriol. **118**:329–333.
25. **Thomas, T. D., K. W. Turner, and V. L. Crow.** 1980. Galactose fermentation by *Streptococcus lactis* and *Streptococcus cremoris*: pathways, products, and regulation. J. Bacteriol. **144**:672–682.
26. **Thompson, J.** 1979. Lactose metabolism in *Streptococcus lactis*: phosphorylation of galactose and glucose moieties in vivo. J. Bacteriol. **140**:774–785.
27. **Thompson, J., and B. M. Chassy.** 1982. Novel phosphoenolpyruvate-dependent futile cycle in *Streptococcus lactis*: 2-deoxy-D-glucose uncouples energy production from growth. J. Bacteriol. **151**:1454–1465.
28. **Turner, K. W., and T. D. Thomas.** 1975. Uncoupling of growth and acid production in lactic streptococci. N.Z. J. Dairy Sci. Technol. **10**:162–167.