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Involvement of Phosphoenolpyruvate in Lactose Utilization by Group N Streptococci[1]

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The effect of sodium fluoride on lactose metabolism and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysis by *Streptococcus lactis* strains 7962 and C_2F suggested that different mechanisms of lactose utilization existed in the two strains. Sodium fluoride prevented lactose utilization and ONPG hydrolysis by whole cells of *S. lactis* C_2F but had no effect on *S. lactis* 7962. Although hydrolysis of ONPG by toluene-treated cells of *S. lactis* 7962 occurred without addition of phosphoenolpyruvate (PEP), toluene-treated cells of *S. lactis* C_2F required the presence of this cofactor. Concentrated cell extracts of *S. lactis* C_2F hydrolyzed ONPG; this hydrolysis was inhibited by NaF, but the addition of PEP, in the presence of NaF, restored maximal activity. Addition of acetyl-phosphate, carbamyl-phosphate, adenosine-5'-triphosphate, guanosine-5'-triphosphate, or uridine-5'-triphosphate did not stimulate activity. The presence of cofactors did not stimulate and NaF did not inhibit the hydrolysis in extracts of *S. lactis* 7962. To confirm the operation of two mechanisms, *S. lactis* 7962 was shown to hydrolyze lactose to glucose and galactose, whereas *S. lactis* C_2F was unable to split the disaccharide. In addition, whole cells of *S. lactis* C_2F rapidly accumulated a phosphorylated derivative of thiomethyl-β-D-galactoside (TMG) which behaved chromatographically and electrophoretically like TMG-PO_4. Unexpectedly, *S. lactis* 7962 also accumulated a TMG derivative, although the rate was extremely low. These data indicate that different mechanisms of lactose utilization exist in the two strains, with a phosphorylation step dependent on PEP involved in *S. lactis* C_2F.

Utilization of lactose by the lactic acid bacteria has been exploited by man for thousands of years in preservation of milk products, yet the control of this process by these microorganisms as related to enzymatic utilization of lactose has received little attention. Two recent reports (2, 23) have shown that β-galactosidase (EC 3.2.1.23, β-D-galactoside galactohydrolase) of *Streptococcus lactis* 7962 was stable to sonic disruption and toluene treatment; other strains of lactic streptococci revealed no enzyme activity. An analogous situation was observed (6, 22) when cell-free extracts of *Staphylococcus aureus* were tested for the enzyme. An explanation for these negative results subsequently was provided by Hengstenberg et al. (14, 15), who found that staphylococci could not hydrolyze *o*-nitrophenyl-β-D-galactopyranoside (ONPG) or free lactose but contained an enzyme which hydrolyzed the phosphorylated derivatives. A similar conclusion was made by Kennedy and Scarborough (17), who presented evidence that ONPG and lactose were phosphorylated by a phosphoenolpyruvate (PEP)-linked phosphotransferase system similar to that reported for certain sugars other than lactose in *Escherichia coli* (19). The same workers also could find no evidence for the phosphorylation of lactose by *E. coli* which is known to possess a typical β-galactosidase. Experiments reported here present evidence that the "β-galactosidaseless" lactic streptococci utilize lactose via the PEP-dependent system, whereas strain 7962, containing β-galactosidase, hydrolyzes lactose in the same way as does *E. coli* (18).

[1] Technical paper no. 2496, Oregon Agricultural Experiment Station.

MATERIALS AND METHODS

Organisms. All streptococci used were obtained from the stock culture collection maintained by the Department of Microbiology, Oregon State University.

Media and growth conditions. Cultures were routinely propagated in a liquid medium which consisted of the following: lactose, 5 g; glucose, 5 g; sucrose, 5 g; tryptone (Difco), 10 g; Yeast Extract (Difco), 5 g; gelatin, 2.5 g; sodium acetate, 1.5 g; ascorbic acid, 0.5

g; and distilled water to 1.0 liter (9). The *p*H of this medium was adjusted to 7.0 prior to autoclaving. For induction experiments, the medium was prepared without the sugars, autoclaved, and cooled. A sterile solution of lactose, the preferential inducer for *S. lactis* 7962 β-galactosidase (2), was then added to a concentration of 0.01 g/ml. Cultures were incubated at 32 C for 8 to 10 hr (*S. lactis* 7962) or 4 to 6 hr (*S. lactis* C_2F) after recieving a 1% inoculum.

Harvesting cells. Cells grown in the lactose broth were harvested using an RC-2 Sorvall refrigerated centrifuge (1 C) at 3,000 × g. The cells were washed twice with the appropriate suspending medium and resuspended to obtain approximately 1.2×10^{10} cells per ml.

Lactose fermentation studies. Two ml of cells plus 1.0 ml of lactose (3 mg/ml) were mixed and incubated at 37 C. At appropriate time intervals, 0.4-ml samples were removed and diluted 1:10 in ice water and centrifuged at 4,300 × *g* for 5 min to remove the cells. Supernatant fluids were assayed in duplicate for lactose by modification of the anthrone test (7) as follows: an appropriate dilution of the sample in a total volume of 1.2 ml was chilled, and 2.4 ml of cold anthrone reagent (0.2% anthrone in 95% H_2SO_4, prepared fresh daily) was added by layering over the surface of the solution. The reaction mixture was vigorously shaken and then heated in a boiling water bath for 10 min, after which the tubes were chilled in a slurry of ice. The optical density was read on a Spectronic-20 (Bausch & Lomb Inc., Rochester, N.Y.) at 625 nm. The weight in micrograms of lactose per tube was determined by referring to a standard curve prepared by carrying out the above procedure with known concentrations of lactose. Zero time readings were obtained by carrying out the above dilutions with 2.0 ml of phosphate buffer instead of cells. The number of cells was obtained from a standard curve relating cell suspension optical density at 650 nm to viable cell count as determined by the plate count.

β-Galactosidase assays. The preparation of toluene-treated cells and the assay of β-galactosidase were carried out as previously reported for *S. lactis* 7962 (2).

Hydrolysis of lactose by cell-free extracts. One liter of *S. lactis* 7962 or *S. lactis* C_2F was grown in lactose broth for 10 hr. Cells were harvested and suspended in 50 ml of 0.05 M Na_2HPO_4 buffer (*p*H 7.0) and ruptured in a Raytheon 10-kc sonic oscillator for 30 min. The cell debris was removed by centrifugation at 12,000 × *g* for 10 min at 1 C. The supernatant fluid was used as the cell-free extract; 4 ml of extract plus 4.0 ml of lactose (3.0 mg/ml) were mixed and incubated at 37 C for 1 hr. At the end of this period, samples were placed in an ice bath. The extracts were spotted on thin-layer chromatographic (TLC) plates prepared by dissolving 30 g of Silica Gel G in 60 ml of 0.067 M phosphate buffer at *p*H 8.0. Plates were developed in *n*-butanol–dioxane–water (4:5:1; v/v) for 3 hr (12). Carbohydrates were detected by the periodate-benzidine method (4).

Studies using labeled lactose and TMG. *S. lactis* C_2F was grown at 32 C for 4 hr in the presence of 1% galactose to induce for lactose utilization. Cells were harvested and washed twice with 0.05 M (*p*H 7.0) sodium phosphate buffer, containing 100 μg of chloramphenicol per ml, and resuspended in the same buffer. For measurement of accumulation of lactose, the assay consisted of 1.62 mg of cells (dry weight) per ml and 5.2×10^{-4} M lactose-*1*-^{14}C in a total volume of 1 ml. In studies on the accumulation of TMG, the system consisted of 2.00 mg (dry weight) of cells per ml and approximately 6×10^{-4} M ^{14}C-TMG in a total volume of one ml. Both samples were incubated in a water bath at 37 C for 1 hr, after which they were centrifuged, and the supernatant fluid was removed. The cells were then treated with boiling 80% ethyl alcohol for 5 min, the debris was sedimented by centrifugation, and the extracts were removed for further analysis.

To examine the distribution of ^{14}C-label from accumulated lactose, a portion of the ethyl alcohol extract was spotted on 1-inch (2.54 cm) strips of Whatman no. 1 paper, approximately 29 cm in length. The chromatogram was developed by ascending chromatography for 5 hr in 1 M sodium acetate buffer (*p*H 3.8) -95% ethyl alcohol (2:5). TMG accumulation was studied in the same manner except chromatograms were developed in ethyl acetate–pyridine–water (12:5:3) for 2.5 hr. After development chromatograms were scanned with a Packard Radiochromatogram Scanner model 7201.

Electrophoresis of the ^{14}C-TMG derivative. Portions of the ethyl alcohol extracts containing ^{14}C-TMG and the TMG derivative were spotted on cellulose acetate strips. Electrophoretic characteristics were determined employing 0.05 sodium phosphate buffer, *p*H 7.3, at 300 v and 15 ma.

Concentration of the ^{14}C-TMG derivative and treatment with phosphatase. The derivative was separated from contaminating ^{14}C-TMG by chromatography as previously described for the TMG system. A portion of the strip containing only the derivative was then eluted with water for 16 hr. The collected fraction (2.7 ml) was lyophilized and suspended in 0.4 ml of water.

Highly purified alkaline phosphatase (Mann Research Laboratories) was dissolved in 0.2 M glycine buffer, *p*H 8.8, containing 0.014 M $MgCl_2$ at a concentration of 1 mg per ml. Acid phosphatase (Calbiochem) was prepared in 0.15 M sodium acetate buffer, *p*H 5.0, at a concentration of 0.2 mg of protein per ml. For the test, 0.1 ml of substrate (^{14}C-TMG derivative) and 0.1 ml of the appropriate enzyme were mixed and incubated at 30 C for 1 hr. The entire 0.2 ml from each system was then spotted on separate chromatogram strips and developed in the TMG solvent system. The distribution of the label was determined by using the strip scanner.

Measurement of TMG uptake. ^{14}C-TMG uptake was measured at 37 C in 4- to 6-ml cell suspensions containing 1.0 to 2.0 mg (dry weight) per ml. At zero time the radioactive substrate was added, and samples were collected at intervals by filtration through membrane filters (Schleicher & Schuell Co., Keene, N.H.; 0.45 μm pore size) to stop the reaction quickly. The filters were washed with 4.0 ml of ice cold 0.05 M sodium phosphate buffer (*p*H 7.0) and dried at 60 C for 3 hr. The radioactivity accumulated by the cells was determined in a liquid scintillation counter employing 4.0 g

of 2,5-diphenyloxazole (PPO) and 0.05 g of 1,4-bis-2-(5-phenyloxazolyl-benzene) per liter of toluene as the fluor solution.

Effect of cofactors on the hydrolysis of ONPG in cell-free extracts. Cell-free extracts from galactose-grown cells of *S. lactis* C_2F were prepared by breaking the cells in a Eaton press (8). The frozen mixture was removed from the press, thawed, and centrifuged at 20,000 × *g* for 20 min. The supernatant fluid was diluted to contain 10 to 15 mg of protein per ml as measured by the method of Lowry et al. (21). Cell-free extracts from *S. lactis* 7962 were prepared from lactose-grown cells by sonic oscillation. The debris was sedimented by centrifugation, and the supernatant fluid was diluted to contain 100 to 200 μg of protein per ml.

The test system contained 11.4 mg of soluble protein for strain C_2F (128 μg of soluble protein in the case of strain 7962), 10 μmoles of ONPG, 20 μmoles of cofactor, and, when used, 20 μmoles of NaF in a total volume of 2.0 ml. After incubation at 37 C for 10 min, the reaction was stopped by the addition of 2.0 ml of 0.5 M Na_2CO_3, and the absorbancies were recorded at 420 nm.

RESULTS

Effect of inhibitors. Sodium azide did not significantly affect lactose utilization in *S. lactis* C_2F or *S. lactis* 7962 (Table 1). This was expected since lactic streptococci, unlike *E. coli*, lack a functional citric acid cycle and terminal respiration.

In the presence of phosphate and NaF, *S. lactis* C_2F was unable to utilize lactose, whereas the conrol cells fermented 2,475 μg of substrate in the presence of phosphate alone. *S. lactis* 7962, on the other hand, was not inhibited by NaF (Table 1). Assuming that PEP generation was inhibited by NaF, these results suggested that lactose metabolism in *S. lactis* C_2F must be dependent upon PEP, whereas *S. lactis* 7962 was independent of its formation.

TABLE 1. *Effect of different inhibitors on lactose utilization by two strains of S. lactis*[a]

Inhibitor system	Lactose fermented (μg) per 2.4 × 10^{10} cells	
	S. lactis C_2F	*S. lactis* 7962
0.05 M Na_2HPO_4 (control)	2,475[b]	1,250[c]
0.05 M Na_2HPO_4 + 0.01 M NaN_3	2,213	1,500
0.05 M Na_2HPO_4 + 0.03 M NaF	0	1,000

[a] Cells were washed and suspended in the appropriate buffer system. The reaction mixture contained 2.4 × 10^{10} cells and 1 mg of lactose per ml in total volume of 3.0 ml. Lactose utilization was measured by disappearance of substrate from the supernatant fluid. Results represent the total micrograms of lactose utilized.

[b] After 15 min of incubation.

[c] After 60 min of incubation.

These observations, together with the fact that the ability of over 50 strains of *S. lactis* (other than *S. lactis* 7962) to hydrolyze ONPG was destroyed during the preparation of cell-free extracts, led us to consider that the hydrolysis of ONPG in *S. lactis* C_2F was similar to that described for *S. aureus* by Kennedy and Scarborough (17). This mechanism involves the PEP-dependent phosphotransferase system of Kundig et al. (19), in which ONPG was phosphorylated and then hydrolyzed. Subsequently it was found that intact cells of *S. lactis* C_2F, induced by growth in galactose broth, hydrolyzed ONPG; the reaction was inhibited by 0.005 M NaF. Lactose-grown cells of *S. lactis* 7962 also catalyzed the hydrolysis of ONPG; but the reaction was not inhibited by NaF, supporting the idea that a continuous supply of PEP, via the enolase reaction, was needed only in *S. lactis* C_2F.

Effect of cofactors on ONPG hydrolysis. As previously observed, β-galactosidase could not be demonstrated in toluene-treated cells of *S. lactis* C_2F. Table 2 illustrates that when PEP was added to toluene-treated cells of strain C_2F, ONPG hydrolysis was restored. Adenosine triphosphate (ATP) could not replace the PEP requirement. In *S. lactis* 7962, toluene treatment did not destroy the ability to hydrolyze ONPG nor did PEP stimulate the hydrolysis.

In confirmation of a previous report (2), cell-free extracts of C_2F were defective in their ability to hydrolyze ONPG. However, such preparations did hydrolyze ONPG, if highly concentrated cell-free extracts (5.7 mg of protein/ml) were used (Table 3). This basal level of enzyme activity was enhanced only by the addition of PEP. In addition, this PEP-dependent, elevated ONPG hydrolysis was not affected by the presence of 0.01 M

TABLE 2. *Effect of toluene treatment on ONPG hydrolysis by two strains of S. lactis*

Assay system[a]	*S. lactis* C_2F	*S. lactis* 7962
Cells + ONPG + water	0	5,400
Cells + ONPG + PEP	2,190	5,400
Cells + ONPG + PEP + Mg^{2+}	2,400	5,400
Cells + ONPG + ATP + Mg^{2+}	0	6,600

[a] Incubation mixture consisted of toluene-treated cells, 5 mM ONPG, 2 mM PEP or ATP, and, when used, $MgCl_2$ in a total volume of 3.0 ml. Reaction was stopped after 15 min for *S. lactis* 7962 and 30 min for *S. lactis* C_2F by the addition of 2.5 ml of 0.5 M Na_2CO_3. Results were expressed as micromolecules of ONP liberated from ONPG per 30 min per g (dry weight) of cells.

TABLE 3. *Effect of NaF on ONPG hydrolysis in cell-free extracts of S. lactis C_2F and S. lactis 7962*[a]

Cofactor	*S. lactis* C_2F		*S. lactis* 7962	
	−NaF	+NaF	−NaF	+ NaF
None	573[b]	168	1,131	1,083
Acetyl-phosphate	558	171	1,065	1,080
Carbamyl-phosphate	579	168	1,086	1,092
ATP	516	162	1,005	762
PEP	840	885	1,083	1,104

[a] Assay mixture contained 11.4 mg of soluble protein for *S. lactis* C_2F and 128 μg of protein in the case of *S. lactis* 7962, 10 μmoles ONPG, 20 μmoles cofactor, and, when used, 20 μmoles NaF in a total volume of 2.0 ml. After incubation at 37 C for 10 min the reaction was stopped by the addition of 2.0 ml of 0.5 M Na_2CO_3.

[b] All results are expressed as nanomoles of ONP formed in 30 min.

NaF; however, in the absence of PEP, NaF inhibited ONPG hydrolysis. Guanosine-5-triphosphate (GTP) and uridine-5-triphosphate (UTP) were also unable to stimulate ONPG hydrolysis. When the above experiments were conducted employing cell-free extracts from *S. lactis* 7962, the addition of cofactors did not stimulate ONPG hydrolysis, nor did NaF inhibit the reaction (Table 3).

Hydrolysis of lactose by crude cell-free extracts. Although preliminary in nature, Fig. 1 shows typical results from a TLC plate. The extract from *S. lactis* 7962 hydrolyzed lactose to glucose and galactose, whereas the extract from *S. lactis* C_2F was unable to hydrolyze the disaccharide. Paper chromatography also supported the above conclusions; however, glucose and galactose could not be separated by the solvent systems employed. Cell-free extracts from each organism in which lactose was not added did not reveal any of the above sugars when chromatographed. The inability to demonstrate lactose hydrolysis in extracts of *S. lactis* C_2F may have been due to the insensitivity of the assay method and to the use of unconcentrated cell-free extracts. Nevertheless, the results showed that the β-galactosidase from *S. lactis* 7962 readily hydrolyzed lactose to glucose and galactose, whereas *S. lactis* C_2F was unable to carry out the reaction under the conditions employed.

Studies using labeled lactose and TMG. If *S. lactis* C_2F metabolized lactose via the PEP-dependent phosphotransferase system, the inability to demonstrate lactose or ONPG hydrolysis in dilute cell-free extracts would be expected, since the proper substrate for the "β-galactosidase"

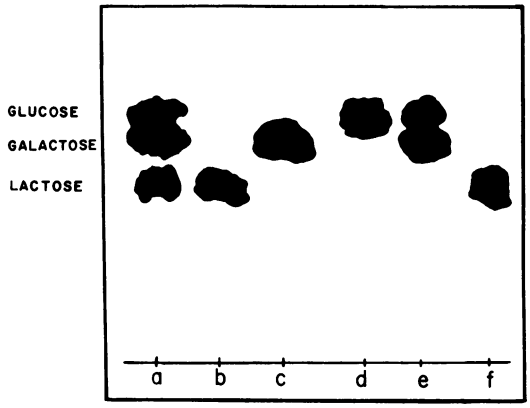

FIG. 1. *Effect of cell-free extracts on the hydrolysis of lactose by lactic streptococci. The figure is a reproduction of a typical thin-layer chromatogram resulting when cell-free extracts were incubated with lactose for 1 hr at 36 C, chromatographed, and tested for hydrolytic breakdown products. As shown, the spots from left to right represent (a) control mixture of glucose, galactose, and lactose; (b) lactose alone; (c) galactose alone; (d) glucose alone; (e) spots resulting when lactose was incubated with 7962 extract; and (f) spot resulting when lactose was incubated with C_2F extract.*

would be the phosphorylated derivatives (14, 17). Concentrated cell extracts, however, unlike the dilute extracts, probably contain the necessary components of the phosphotransferase system to phosphorylate lactose or ONPG and allow hydrolysis to proceed as was observed for ONPG in concentrated cell extracts from *S. lactis* C_2F. To determine whether lactose phosphate accumulated in whole cells of *S. lactis* C_2F, the uptake of lactose-*1*-^{14}C was measured. The results suggested the sugar was being metabolized, since the labeled substrate did not accumulate as a distinct peak but rather as several peaks along the chromatogram (Fig. 2A). Although lactose negative mutants have been isolated from both strains (C_2F and 7962), a mutant which accumulates lactose without subsequent metabolism of the sugar, i.e. lacking β-galactosidase, has not been isolated. To overcome this difficulty, the accumulation of TMG, a nonmetabolizable structural analogue of lactose, was examined; Fig. 3 illustrates the time course of ^{14}C-TMG uptake in *S. lactis* C_2F and 7962. *S. lactis* C_2F accumulated the analogue at a rapid rate, in comparison to the slow uptake by *S. lactis* 7962. Since both strains accumulated TMG, it was necessary to determine if ^{14}C-TMG accumulated as a derivative or as free TMG.

Fig. 2B, which shows the chromatographic behavior of radioactive compounds extracted from cells of *S. lactis* C_2F previously exposed to ^{14}C-TMG, shows that a labeled derivative traveled as

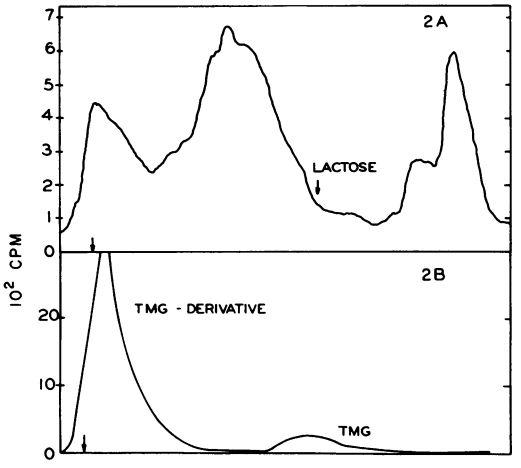

FIG. 2. *Accumulation of lactose-1-^{14}C and ^{14}C-TMG by S. lactis C_2F. Fig. 2A represents the distribution of the labeled material extracted from whole cells previously exposed to lactose-1-^{14}C. The position marked lactose indicates the location of lactose-1-^{14}C when co-chromatographed with the extract. Fig. 2B represents the distribution of the labeled material extracted from whole cells previously exposed to ^{14}C-TMG. Arrows mark the origin of the chromatogram.*

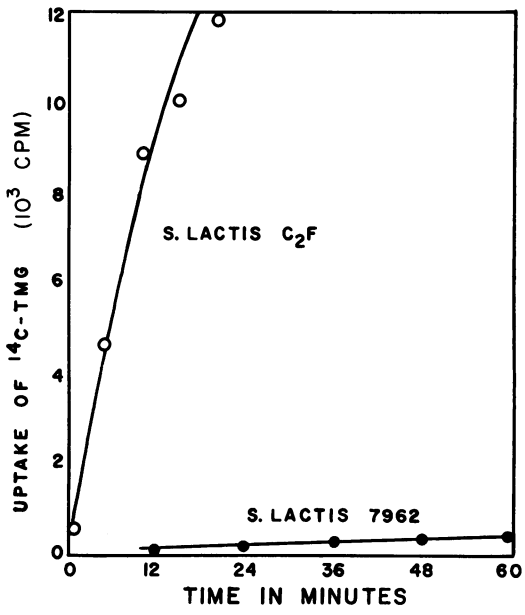

FIG. 3. *Intracellular accumulation of ^{14}C-TMG by resting-cell suspensions of S. lactis C_2F and S. lactis 7962 previously induced for lactose utilization. Incubation mixture consisted of about 0.92 mg (dry weight) of cells per ml (S. lactis C_2F) or about 1.39 mg (dry weight) of cells per milliliter in the case of S. lactis 7962 and approximately 33.3 μM ^{14}C-TMG in a total volume of 4.0 ml. Samples were collected by membrane filtration and the radioactivity retained by the cells was determined in a liquid scintillation counter.*

a peak distinct from TMG; the labeled material (small peak) corresponding to TMG probably resulted from carry-over of substrate from the supernatant fluid. This accumulation of derivative is similar to that reported recently for TMG-exposed cells of *S. aureus* (20).

Electrophoretic treatment (Fig. 4) of portions of the extract revealed that the derivative was more acidic and moved toward the anode at a faster rate than TMG. Since in *S. aureus* it has been shown that lactose and TMG accumulate as phosphorylated derivatives (14, 15, 20), these results suggested that *S. lactis* C_2F also accumulated TMG by phosphorylation. To obtain proof of this possibility, the derivative was purified, concentrated, and treated with alkaline and acid phosphatase. Figure 5 illustrates that alkaline phosphatase hydrolyzed the derivative liberating a compound which corresponded to TMG on the chromatograph. Acid phosphatase also hydrolyzed the derivative in a similar manner, and these data indicated that the derivative was TMG-PO_4.

Surprisingly, when *S. lactis* 7962 was examined for the intracellular state of the accumulated ^{14}C-TMG, it also was found to accumulate as a derivative. Experiments performed at 0 C did not reveal any TMG-derivative or TMG when the extracts were chromatographed.

Induction of lactose utilization. Lactose utilization in *S. lactis* 7962 was previously shown to be preferentially induced by lactose; galactose was only a partial inducer (2). However, in the present study, when whole cells of *S. lactis* C_2F were tested, it was found that galactose was a better inducer than lactose. Several other "β-galactosidase-less" lactic streptococci were also examined and found to be preferentially induced by galactose; lactose was only a partial inducer (Table 4).

DISCUSSION

Enzymes of lactose metabolism in *S. lactis* were initially studied by Novikova (Chem. Abstr. p. 2936, 1957); hydrolysis of substrate was determined by using *Saccharomyces globosus*, which ferments glucose and galactose but not lactose. With this technique, three of four strains tested were found to hydrolyze lactose to glucose and galactose, whereas the fourth was unable to cleave the disaccharide. The same author (Chem. Abstr., p. 2935, 1957) also tested *S. lactis* for hydrolysis of lactose-1-phosphate an intermediate in the synthesis of lactose. Neither whole cells nor extracts were able to hydrolyze the derivative, and it was assumed that *S. lactis* did not utilize lactose by way of direct phosphorylation.

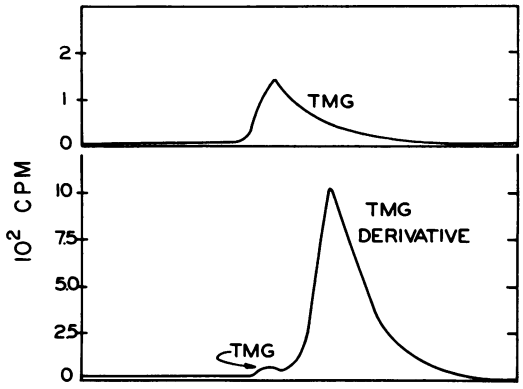

FIG. 4. *Electrophoresis of TMG and TMG derivative. Labeled TMG and ethyl alcohol extracts of S. lactis C_2F cells previously exposed to ^{14}C-TMG were spotted on cellulose acetate strips. Electrophoretic mobilities of the labeled compounds were determined at 300v and 15 ma for 20 min. Distribution of the label was determined by the Radiochromatogram Scanner. The anode was located to the right of the figure.*

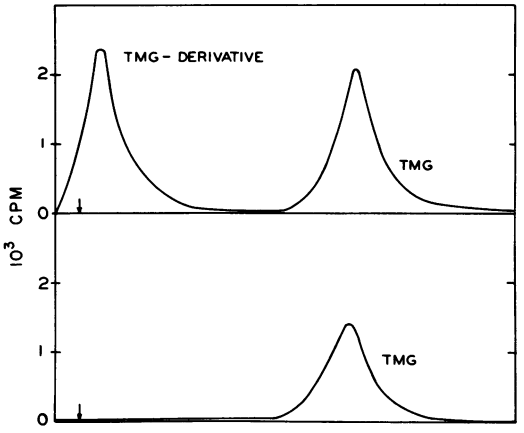

FIG. 5. *Treatment of the TMG derivative with alkaline phosphatase. The upper curve represents co-chromatography of ^{14}C-TMG and ^{14}C-TMG derivative. Bottom curve represents the location of the label from TMG after treatment of the ^{14}C-TMG derivative with alkaline phosphatase. The arrows mark the origin of the chromatograms.*

More recently Vakil and Shahani (29) described lactose utilization in *S. lactis* UN. They detected β-galactosidase and lactose dehydrogenase activity, but lactose phosphorylase could not be found. They also demonstrated that lactose-1-phosphate was not an intermediate in the metabolism of lactose. An alternative possibility, that lactose was utilized by the PEP phosphotransferase system described for *S. aureus* (14, 15, 17), was not considered.

TABLE 4. *Effect of inducer on ONPG hydrolysis in the lactic streptococci*

Organism	Inducer	
	Lactose	Galactose
Streptococcus lactis C_2F	63	122
S. lactis 11454	174	254
S. lactis 7963	56	114
S. lactis E	70	200
S. lactis b	63	128
S. cremoris 144F	210	224
S. lactis C10	72	101
S. lactis 7962	476	249

[a] Results are expressed as micromoles of ONP released per 30 min per g (dry weight) of cells. Assay system consisted of 1.0 ml of whole cells, 1.0 ml of sodium phosphate buffer (*p*H 7.0), and 1.0 ml of 0.033 M ONPG. Tubes were incubated at 37 C for 30 min, and reaction was stopped by addition of 3.0 ml of 0.5 M sodium carbonate. Cells were removed by centrifugation before reading absorbancy at 420 nm.

Vakil and Shahani (29) also described the conversion by *S. lactis* of lactose to lactobionic acid which was then cleaved to gluconate and galactose (Proceedings of Eighth International Congress of Microbiology, p. 46, 1962). *Pseudomonas* species also have been reported to produce lactobionate from lactose (1) but no evidence has been presented to indicate that the lactobionic acid is further metabolized. Furthermore, the evidence for utilization of this acid in *Penicillium chrysogenum* is only presumptive (5).

The results presented here indicate that lactic streptococci may possess different mechanisms for lactose utilization, functioning separately or together in a given strain. One involves a PEP-dependent phosphotransferase system, whereas another appears to be dependent on β-galactosidase. These conclusions were supported by the observation that NaF prevented lactose fermentation and ONPG hydrolysis in whole cells of *S. lactis* C_2F, but had no affect on *S. lactis* 7962. Moreover, only the addition of PEP to toluene-treated cells of strain C_2F restored its ability to hydrolyze ONPG. Concentrated cell-free extracts from this organism, which presumably contained the three soluble components of the phosphotransferase system in addition to the membrane-bound enzyme (16, 28), revealed ONPG hydrolytic activity stimulated only by PEP. NaF inhibited the hydrolysis of ONPG, yet only the addition of PEP in the presence of NaF restored maximal activity. This indicated that the lactose hydrolyzing enzyme of *S. lactis* C_2F was not the

fluoride-sensitive component of the system, since maximal ONPG hydrolysis occurred in the presence of NaF when PEP was present.

ONPG hydrolysis by strain 7962 was not stimulated by the addition of cofactors nor did NaF inhibit the process. In this respect, *S. lactis* 7962 resembled *E. coli*, since the latter organism hydrolyzed ONPG in the presence of NaF and did not appear to phosphorylate lactose (17). *S. lactis* 7962 also contained a typical β-galactosidase as measured by its ability to cleave lactose to glucose and galactose, whereas *S. lactis* C_2F did not contain an enzyme capable of hydrolyzing free lactose under the conditions employed. It therefore appeared that the appropriate substrate for the lactose-catabolizing enzyme from the C_2F strain would be a phosphorylated derivative of lactose. Experiments using concentrated cell extracts should yield fruitful results in future studies, since the extracts apparently contain the components of the phosphotransferase system (Table 3).

Experiments testing for the presence of phosphorylated lactose in *S. lactis* C_2F using the labeled carbohydrate were unsuccessful due to rapid metabolism of the sugar. However, when a nonmetabolizable analogue of lactose (TMG) was used, a derivative (TMG-PO_4) was shown to accumulate, supporting the hypothesis that lactose fermentation in *S. lactis* C_2F requires substrate phosphorylation by the PEP-dependent system.

S. lactis 7962 also accumulated TMG as a derivative, which was unexpected, since the organism hydrolyzed lactose to glucose and galactose. However, this result could explain the inability of TMG, a potent inducer of β-galactosidase in *E. coli*, to induce the enzyme in *S. lactis* 7962 (2). Since TMG appeared to accumulate as the phosphate derivative, it may no longer function as inducer. The possibility that lactose is accumulated via the PEP-dependent system in *S. lactis* 7962 as lactose-P and then cleaved by a specific phosphatase cannot be ruled out. On the other hand, the inability of TMG to induce *S. lactis* C_2F (*unpublished results*) could reflect the inability of the organism to hydrolyze the TMG-PO_4 to galactose-6-phosphate, which may function as the true inducer as in the case of *S. aureus* (24, 28).

It was found that the rate of uptake of TMG into cells of *S. lactis* 7962 was slow, in comparison to the rapid rate observed in cells of *S. lactis* C_2F. The low rate observed for *S. lactis* 7962 confirms a previous report from this laboratory (3). Subsequent experiments on the TMG transport system of *S. lactis* C_2F indicated all of the added TMG was accumulated by the cells, rapidly establishing a concentration gradient (*in preparation*).

The rapid rate of TMG uptake by whole cells of *S. lactis* C_2F raises the question of the possible energy source for this purpose. Since these organisms lack both a citric acid cycle and terminal respiration, it was assumed that anaerobic endogenous metabolism was supplying the necessary energy. In this regard, endogenous metabolism of *S. faecalis* has been reported to provide necessary energy to maintain a constant level of activity in the glycolytic enzymes for several hours after the removal of exogenous energy sources (10, 11). Since the TMG uptake experiments were conducted with freshly washed cells, endogenous metabolism could account for the necessary energy.

It also was interesting to note that lactose was better than galactose for induction of lactose utilization in *S. lactis* 7962. In *S. lactis* C_2F and the other lactic strains tested, however, galactose was the better inducer. This is identical to findings with *S. aureus* in which galactose was shown to be the inducer for lactose utilization; later experiments have shown, however, that galactose-6-phosphate is the true inducer (6, 24). This may also be true for *S. lactis* C_2F and other strains of lactic streptococci lacking β-galactosidase.

The fact that *S. lactis* 7962 was only one of many strains of lactic streptococci examined in our laboratory which possessed the classical β-galactosidase raises doubt regarding its classification. This organism was first described by Rahn et al. (25) as an isolate from milk typical for the species. Hegarty (13) used the strain in certain physiological studies and presumably deposited the bacterium with the American Type Culture Collection. Repeated attempts in our laboratory over the past 2 years to confirm the positive precipitin reaction between commercially avilable group N antiserum and extracts of *S. lactis* 7962 as reported earlier (26) have been negative; *S. lactis* C_2F gave a positive reaction. The response of *S. lactis* 7962 to other streptococcal antisera also gave negative results. When this finding was brought to the attention of the American Type Culture Collection, they confirmed that *S. lactis* 7962 did not give a positive group N precipitin reaction; however, physiological tests carried out by them indicated it was a lactic streptococcus (C. K. Mills, *personal communication*). Previous physiological tests carried out in our laboratory also indicated it was a lactic streptococcus (26, 27).

Apparently, *S. lactis* 7962 is not the only lactic streptococcus containing β-galactosidase. Vakil and Shahani (29) reported that *S. lactis* UN possessed β-galactosidase, which also was preferentially induced by lactose. Novikova (Chem.

Abstr., p. 2936, 1957) also reported on three strains which were assumed to contain β-galactosidase. It would be interesting to determine whether these strains, like *S. lactis* 7962, also fail to give a positive group N precipitin reaction.

From the above results, it may be concluded that the apparent lability of β-galactosidase activity in *S. lactis* C_2F and other strains tested is due to the absence of β-galactosidase and to the presence of a PEP-dependent system in which lactose like TMG, accumulates as the phosphorylated derivative; the lactose-PO_4 would then be hydrolyzed by a different enzyme. This enzyme may be analogous to the one found in staphylococci where the apparent in vitro lability of the β-galactosidase was also shown to be an artifact, since the proper substrate was lactose-PO_4 and not the free disaccharide (14, 15, 17). Experiments are now underway to further characterize the lactose-utilizing enzyme systems of *S. lactis* C_2F, *S. lactis* 7962, and other lactic acid bacteria. Attempts are also being made to fractionate the ONPG hydrolysis system of *S. lactis* C_2F into the component enzymes of the PEP phosphotransferase system.

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