JOURNAL OF BACTERIOLOGY, June 1983, p. 1204–1214
0021-9193/83/061204-11$02.00/0
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Vol. 154, No. 3

Regulation and Characterization of the Galactose-Phosphoenolpyruvate-Dependent Phosphotransferase System in *Lactobacillus casei*

BRUCE M. CHASSY* AND JOHN THOMPSON

Microbiology Section, Laboratory of Microbiology and Immunology, National Institute of Dental Research, Bethesda, Maryland 20205

Received 8 November 1982/Accepted 5 March 1983

Cells of *Lactobacillus casei* grown in media containing galactose or a metabolizable β-galactoside (lactose, lactulose, or arabinosyl-β-D-galactoside) were induced for a galactose-phosphoenolpyruvate-dependent phosphotransferase system (gal-PTS). This high-affinity system (K_m for galactose, 11 μM) was inducible in eight strains examined, which were representative of all five subspecies of *L. casei*. The gal-PTS was also induced in strains defective in glucose- and lactose-phosphoenolpyruvate-dependent phosphotransferase systems during growth on galactose. Galactose 6-phosphate appeared to be the intracellular inducer of the gal-PTS. The gal-PTS was quite specific for D-galactose, and neither glucose, lactose, nor a variety of structural analogs of galactose caused significant inhibition of phosphotransferase system-mediated galactose transport in intact cells. The phosphoenolpyruvate-dependent phosphorylation of galactose in vitro required specific membrane and cytoplasmic components (including enzyme III^{gal}), which were induced only by growth of the cells on galactose or β-galactosides. Extracts prepared from such cells also contained an ATP-dependent galactokinase which converted galactose to galactose 1-phosphate. Our results demonstrate the separate identities of the gal-PTS and the lactose-phosphoenolpyruvate-dependent phosphotransferase system in *L. casei*.

Gram-positive bacteria have been shown to transport and phosphorylate many sugars by the PEP-dependent PTS; however, a PTS specific for the transport of galactose has yet to be characterized (6, 9, 13, 15, 16, 18, 19). Galactose is a substrate of the lac-PTS in *Staphylococcus aureus* (23), but no attempt has been made to determine whether a separate gal-PTS is present in this organism. The biochemical studies of Thompson (28) and Thomas et al. (26) and the genetic data obtained recently by Park and McKay (17) provide evidence in favor of a separate gal-PTS and lac-PTS in *Streptococcus lactis*. In a preliminary study, Chassy reached similar conclusions from an analysis of PTS activities in *Lactobacillus casei* (B. M. Chassy, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, K203, p. 171). The product of PTS transport in *Staphylococcus aureus* (1) and *Streptococcus lactis* (2), gal-6P, is metabolized to triose phosphates by the action of the D-tagatose 6-phosphate pathway. In this study we used permeabilized cells, intact cells, and cell-free extracts to examine the gal-PTS of *L. casei*. In this paper we describe (i) induction of the gal-PTS, (ii) a galactose-specific enzyme III, and (iii) the characteristics and substrate specificity of the gal-PTS. Our data confirm the separate identities of the gal-PTS and lac-PTS in *L. casei*.

MATERIALS AND METHODS

Abbreviations. PEP, Phosphoenolpyruvate; PTS, phosphotransferase system; lac-PTS, lactose-phosphoenolpyruvate-dependent phosphotransferase system; gal-PTS, galactose-phosphoenolpyruvate-dependent phosphotransferase system; glu-PTS, glucose-phosphoenolpyruvate-dependent phosphotransferase system; gal-1P, galactose 1-phosphate; gal-6P, galactose 6-phosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; TMG, methyl-β-D-thiogalactopyranoside; α-MG, α-methylglucoside.

Bacterial strains and growth conditions. Bacterial strains were purchased from the American Type Culture Collection, Rockville, Md., or were taken from the culture collection of the National Institute of Dental Research (OC and CI series). *L. casei* 61BG and 64H were kindly supplied by Francis Gasser, Institut Pasteur, Paris, France. *manA* mutants (i.e., mutants lacking enzyme II^{man} of the mannose-PEP-dependent PTS) were prepared as described by Thompson and Chassy (30); 2-deoxy-D-glucose-resistant colonies were isolated, and strains displaying the *manA* genotype were selected from these colonies. Like *Streptococcus lactis*, *L. casei* does not transport

α-MEG and lacks a second "glu-PTS" (common in the enteric bacteria) (18). We refer to this single activity as the glu-PTS. Bacteria were grown in litmus milk (Difco Laboratories, Detroit, Mich.) fortified with 0.5% glucose and buffered with $CaCO_3$. Cells for experiments were cultivated in *Lactobacillus* carrying medium (8) after a single adaptive subculture in the same medium containing the appropriate carbohydrate at a concentration of 1% (wt/vol).

Transport experiments and analytical methods. Studies of sugar transport by starved cells and spectrophotometric assays (12) of PTS activities in permeabilized cells were conducted as described previously for the lac-PTS of *L. casei* (5). The in vitro assay used for gal-PTS activity was that described by Thompson and Chassy (30) for the glu-PTS of *Streptococcus lactis*, except that [^{14}C]galactose was substituted for radiolabeled glucose or 2-deoxy-D-glucose. Protein was determined by the Coomassie brillant blue dye-binding procedure of Bradford (3). gal-1P and gal-6P were separated by Dowex-1–borate ion-exchange chromatography and were detected by the anthrone method described previously (27). Gas chromatography-mass spectral analyses were performed with a model 9000 GC/MS system (LKB Instruments Inc., Rockville, Md.), using an SE-30 column to separate the sugar trimethylsilyl derivatives.

In vitro PTS complementation assay. Cells of *L. casei* 64H *lac* were grown in *Lactobacillus* carrying medium containing 1% galactose. The cells were harvested, washed, and broken in a Bead-Beater (Biospec Products, Bartlesville, Okla.) as described previously (29). Cell debris and residual whole cells were removed by centrifugation at 42,000 × *g* for 30 min, and the supernatant was further centrifuged for 120 min at 250,000 × *g*. The high-speed supernatant was concentrated to 20 ml by using a pressure ultrafiltration cell (Amicon Corp., Lexington, Mass.) and a type UM-05 membrane. The pellet obtained by high-speed centrifugation was suspended in 10 ml of 0.1 M HEPES buffer (pH 7.5) containing 1 mM dithiothreitol. The formation of [^{14}C]galactose phosphate was followed by binding this compound to DEAE-cellulose (type DE-81) filter circles by the method of Sherman (22). The PTS assay mixture contained 100 mM HEPES buffer (pH 7.5), 1 mM dithiothreitol, 5 mM PEP, 10 mM $MgCl_2$, 10 mM NaF, 0.25 mM [^{14}C]galactose (specific activity, 0.2 μCi/μmol), 0 to 25 μl of high-speed supernatant, and 0 to 50 μl of membranes (pellet obtained by high-speed centrifugation) in a final reaction volume of 100 μl. Incubation was at 37°C for 20 min. Samples (30 to 50 μl) of the reaction mixture were transferred to type DE-81 filter circles, washed free of unreacted [^{14}C]galactose, and air dried, and the residual radioactive [^{14}C]galactose phosphate on each filter was determined. In the galactokinase assay, 5 mM ATP was substituted for PEP, and the high-speed pellet fraction was omitted from the reaction system.

[^{14}C]galactose uptake by *L. casei* 64H and identification of intracellular derivatives. Starved cells (dry weight, 45 mg) of *L. casei* 64H grown previously on galactose were suspended at 37°C in 1.6 ml of 50 mM potassium phosphate buffer (pH 7) containing 1 mM $MgCl_2$, and 0.4 ml of 10 mM [^{14}C]galactose (specific activity, 0.2 μCi/μmol) was added. Within 10 s, further transport and metabolism of sugar were halted by adding 2 ml of ice-cold 4 N formic acid, and the suspension was clarified by centrifugation at 27,000 × *g* for 30 min. The supernatant containing the radiolabeled metabolites and free [^{14}C]galactose was then frozen, lyophilized, and finally reconstituted to 200 μl with distilled water. The reconstituted cell extract was applied to the origin of a sheet of Whatman 3M filter paper (20 by 50 cm), and free and phosphorylated galactose derivatives were separated by descending paper chromatography in a solvent containing 1-butanol, acetic acid, and water (5:2:3, vol/vol). The chromatogram was dried, and a 0.5-cm-wide strip running the length of the paper was cut into 0.5-cm portions. Radioactivity was determined by counting each portion (0.5 by 0.5 cm) of the chromatogram with 10 ml of Hydrofluor liquid scintillation cocktail. The radioactive areas (parallel to the origin) were then eluted, concentrated, and identified by cochromatography with standards, using the solvent described above. The intracellular concentrations of the radiolabeled intermediates were calculated from the total radioactivity, the original cell density, and the specific activity of [^{14}C]galactose, assuming an intracellular (protoplast) fluid volume of 1.67 ml/g (dry weight) of cells (5). The purified ^{14}C-labeled sugar derivatives were eluted and treated with alkaline phosphatase at 37°C for 1 h in 0.1 M Tris-hydrochloride buffer (pH 8) containing 10 mM $MgCl_2$ and 20 U of enzyme (total reaction volume, 100 μl). Free sugars were identified by paper chromatography in a solvent system containing pyridine, ethanol, butanol, and water (2:2:8:1, vol/vol).

Sephacryl S-200 chromatography. Galactokinase and galactose-specific enzyme III were separated and partially purified by molecular exclusion chromatography. A cell extract was prepared from galactose-grown cells of *L. casei* 64H *lac* as described by Thompson and Chassy for *Streptococcus lactis* (29). A cell-free high-speed supernatant was obtained by ultracentrifuging the extract at 250,000 × *g* for 2 h. The supernatant was concentrated 10-fold, and 5 ml was applied to a Sephacryl-S-200 column (2.5 by 100 cm). The proteins were eluted with 0.1 M HEPES buffer (pH 7.5) containing 1 mM dithiothreitol and 0.1 M NaCl at a flow rate of 0.5 ml/min.

Reagents. Chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Radioactive sugars were obtained from New England Nuclear Corp., Boston, Mass., and galactose analogs were purchased from Vega Biochemicals, Tucson, Ariz.

RESULTS

gal-PTS activity in permeabilized cells of *L. casei* 64H. We performed a number of assays to demonstrate PEP-dependent PTS-mediated transport of galactose by *L. casei* 64H. When assayed by the pyruvate formation method of Kornberg and Reeves (12), permeabilized cells of *L. casei* 64H (grown previously on galactose) rapidly oxidized NADH when PEP and galactose were both present in the complete assay (Table 1). The omission of either PEP or galactose reduced the rate of NADH oxidation by 90%. Although not replaceable by ATP, the PEP requirement was partially spared by 2-phosphoglyceric acid because this glycolytic intermedi-

TABLE 1. Demonstration of gal-PTS activity in permeabilized cells of *L. casei* 64H

Component(s) in assay mixture	Pyruvate formed (nmol/mg of protein per min)
Complete system[a]	105.4
−Galactose	11.4
−PEP (5 mM)	6.7
−PEP + ATP	5.4
+ATP	101.7
+NaF (10 mM)	106.1
−PEP + 2-phosphoglyceric acid (5 mM)	58.5
−PEP + 2-phosphoglyceric acid + NaF	13.4

[a] For the exact assay procedure used, see the text.

ate can be converted to PEP by the fluoride-sensitive enolase reaction. The data in Table 1 are consistent with the presence of a gal-PTS in permeabilized cells of *L. casei* 64H.

Inducibility of the gal-PTS. Since the experiments described above provided evidence for PEP-dependent phosphorylation of galactose, it was possible to use the permeabilized cell assay to study induction of the gal-PTS in wild-type and various PTS-defective strains of *L. casei* 64H (Table 2). gal-PTS activity was not detectable in cells of *L. casei* grown on glucose or on glucose plus TMG. However, after growth on galactose and other metabolizable β-galactosides (lactose, lactulose, or arabinosyl-β-D-galactoside), cells of *L. casei* 64H contained high levels of gal-PTS activity. Strains of *L. casei* 64H defective in the glu-PTS (strain 64H *manA*), the lac-PTS (strain 64H *lac*), or both systems (strain 64H *manA lac*) continued to grow well on galactose, and all three strains exhibited gal-PTS activity after growth on this sugar (Table 2). Therefore, the PEP-dependent phosphorylation of galactose by *L. casei* 64H was mediated by an inducible system which was separate from the glu-PTS or the lac-PTS. A survey of *L. casei* strains representative of all five subspecies (Table 3) revealed that all were inducible for the gal-PTS, and the levels of activity were generally 60 to 80% of the levels of the glu-PTS activities in the same strains.

PEP-dependent PTS activities in starved cells of *L. casei*. The permeabilized cell assay indicated the presence of an inducible gal-PTS in *L. casei* 64H. We wanted to demonstrate directly this PEP-dependent transport in physiologically intact cells to corroborate the data obtained with permeabilized cells. Starved cells of *L. casei* can accumulate PTS sugars at the expense of an intracellular reserve of PEP (5); we chose to use this assay system to evaluate in vivo galactose transport. Glucose-grown, starved cells of *L. casei* 64H rapidly accumulated 2-deoxy-D-glucose (as 2-deoxy-D-glucose 6-phosphate) via the glu-PTS to an intracellular concentration of approximately 16 mM within 15 s (Fig. 1A). The extent of 2-deoxy-D-glucose transport was used as an indicator of the amount of available PEP in the starved cells, as well as to demonstrate the ability of the cells to carry out PTS-mediated transport. By comparison, the rates of accumulation of lactose, TMG, and galactose were relatively low, indicating low levels of expression of the lac-PTS and gal-PTS. Starved cells of *L. casei* 64H grown previously on galactose accumulated 2-deoxy-D-glucose and galactose by PEP-dependent PTS activities, but these cells were not induced for the lac-PTS (Fig. 1B). The apparent accumulation of galactose (Fig. 1B) greatly exceeded the apparent accumulation of

TABLE 2. Induction of gal-PTS activity in wild-type and PTS-defective strains of *L. casei* 64H

Strain	Growth sugar(s)	Sp act[a]	
		glu-PTS	gal-PTS
64H	Glucose	112.8	0.0
	Galactose	103.4	66.9
	Glucose + TMG	165.1	0.0
	Lactose	77.5	28.4
	3-*O*-β-D-galacto-pyranosyl-D-arabinose	41.1	58.8
	4-*O*-β-D-galacto-pyranosyl-D-fructofuranose	68.1	44.8
	Lactobionic acid	71.4	88.3
64H *lac*	Glucose	130.1	0.0
	Galactose	124.1	86.3
64H *manA*	Glucose	4.1	0.0
	Galactose	13.1	111.4
	Glucose + TMG	8.6	3.6
	Lactose	12.9	33.9
64H *lac manA*	Glucose	0.0	0.0
	Galactose	5.6	79.3

[a] Expressed as nanomoles of sugar phosphorylated per minute per milligram of protein.

TABLE 3. Distribution and inducibility of the gal-PTS in subspecies of *L. casei*

Organism	Strain	glu-PTS activity of cells grown on:[a]		gal-PTS activity of cells grown on:[a]	
		Glucose	Galactose	Glucose	Galactose
L. casei subsp. *casei*	ATCC 393	103.3	68.4	6.8	74.1
	ATCC 4646	45.8	100.7	5.6	6.6
	64H	112.8	103.4	0.0	66.9
	61BG	43.8	83.3	0.0	77.8
L. casei subsp. *pseudoplantarum*	ATCC 25598	110.5	151.5	3.0	59.3
L. casei subsp. *tolerans*	ATCC 25599	72.4	51.6	4.7	61.8
L. casei subsp. *rhamnosus*	OC91	81.1	113.4	0.0	72.4
L. casei subsp. *alactosus*	Cl-16	91.2	103.8	0.0	73.1

[a] Expressed as nanomoles of glucose or galactose phosphorylated per milligram (dry weight) of cells per minute.

2-deoxy-D-glucose, but this was a consequence of increased PEP production resulting from the glycolytic metabolism of galactose. Although galactose was rapidly metabolized, a subsequent analysis (see below) showed that ^{14}C-labeled gal-6P was the predominant intracellular metabolite formed within 5 to 10 s of exposure of starved cells to radiolabeled sugar. The accumulation of galactose by starved cells exhibited saturation kinetics, and the gal-PTS had a high affinity for its substrate (K_m, 11 μM; V_{max}, 78 μmol of gal 6-P accumulated per g [dry weight] of cells per min).

Specificity of the gal-PTS. The sugar specificity of the gal-PTS was determined from a study of the effects of various galactose analogs (at 10- and 50-fold-greater concentrations than galactose) upon the initial rate of [^{14}C]galactose accumulation by starved cells of *L. casei* 64H (Table 4). With the exception of D-galactosamine, *o*-aminophenyl-β-D-galactopyranoside, and unlabeled galactose, the presence of 10-fold-greater concentrations of the analogs did not significantly reduce the rate of PEP-dependent accumulation of [^{14}C]galactose. Significant inhibition was observed in the presence of 50-fold-greater concentrations of 2-deoxy-D-galactopyranose, TMG, isopropyl-β-D-thiogalactopyranoside, phenylethyl-β-D-thiogalactopyranoside, D-talose, and 6-deoxy-D-galactopyranose. However, the results obtained from a gas chromatographic analysis of trimethylsilyl derivatives showed that many of these compounds contained 1 to 2% galactose or 1-thiogalactopyranose as contaminants. When the analogs were present at final concentrations of 5 mM, these levels of contamination (0.05 and 0.1 mM) were sufficient to reduce the rate of [^{14}C]galactose uptake by 33 and 50%, respectively. Disregarding inhibition due to contaminating galactose and thiogalactose, the data in Table 4 indicate that the gal-PTS is quite specific for D-galactose. The group translocation system may also have a low affinity for *o*-aminophenyl-β-D-galactopyranoside and the corresponding C-2 analogs (galactosamine and D-talose).

Intracellular products formed during [^{14}C]galactose accumulation by *L. casei* 64H. Our results indicated that galactose could be transported and phosphorylated by a highly specific system at the expense of intracellular PEP. It seemed reasonable to determine whether the gal-PTS or galactokinase (acting in concert with the galactose permease) was utilized for galactose phosphorylation in whole cells. These two possibilities could be distinguished since the PTS-

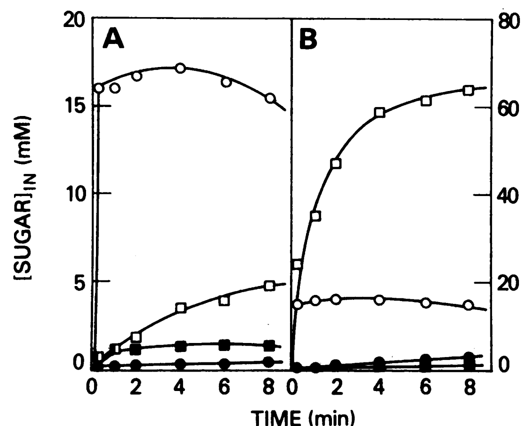

FIG. 1. Accumulation of sugars by starved cells of *L. casei* 64H grown previously on glucose (A) and galactose (B). The cells were suspended at a density of 200 μg (dry weight) per ml in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$. Radiolabeled sugar (0.2 mM; 0.2 μCi/μmol) was added to the appropriate cell suspension at 37°C. Symbols: ○, 2-Deoxy-D-glucose; □, galactose; ■, lactose; ●, TMG. Note the difference in ordinal concentrations. [SUGAR]$_{IN}$, Intracellular sugar concentration.

TABLE 4. Specificity of the gal-PTS in *L. casei 64H*: effect of galactose analogs upon transport of [^{14}C]galactose[a]

Sugar tested	Significant structural change	% Inhibition of [^{14}C]galactose uptake by analogs at final concentrations of:	
		1 mM	5 mM
D-Galactopyranose	None (unlabeled sugar)	88.8[b]	98.1[b]
1-*O*-methyl-β-D-galactopyranoside	C-1, $-OCH_3$ (β anomer)	5.6	16.0
1-*O*-methyl-α-D-galactopyranoside	C-1, $-OCH_3$ (α anomer)	4.0	9.6
Methyl-1-thio-β-D-galactopyranoside (TMG)	C-1, $-SCH_3$	10.3	49.5
Isopropyl-1-thio-β-D-galactopyranoside	C-1, $-SCH(CH_3)_2$	6.6	39.3
Phenylethyl-1-thio-β-D-galactopyranoside	C-1, -*S*-phenylethyl	13.1	55.4
Phenyl-β-D-galactopyranoside	C-1, -*O*-phenyl	2.3	30.8
o-Aminophenyl-β-D-galactopyranoside	C-1, -*O*-aminophenyl	26.9	84.6
2-Deoxy-D-galactopyranose	C-2, deoxy	8.4	67.3
2-Amino-2-deoxy-D-galactopyranose (galactosamine)	C-2, $-NH_2$	21.6	36.0
N-acetyl-D-galactosamine	C-2, $-NHCOCH_3$	3.9	7.7
D-Talose	C-2, epimer	12.0	55.9
D-Gulose	C-3, epimer	0.0	24.0
D-Glucopyranose[c]	C-4, epimer	7.7[c]	10.0[c]
D-Arabinose	C-5, dehydroxymethyl	0.0	0.0
6-Deoxy-D-galactopyranose	C-6, deoxy	1.0	62.6
β-D-Galactopyranosyl-1-thio-β-D-galactopyranoside (TDG)		1.5	0.0
4-*O*-β-D-galactopyranosyl-D-glucopyranose (lactose)		2.5	3.8
4-*O*-β-D-galactopyranosyl-D-fructofuranose (lactulose)		9.2	4.6
6-*O*-β-D-galactopyranosyl-D-glucopyranose (melibiose)		5.4	3.8

[a] The initial rate of [^{14}C]galactose uptake by the gal-PTS in *L. casei* 64H *manA* was 83.5 μmol/g (dry weight) of cells per min (defined as 100%). The concentration of [^{14}C]galactose used in all assays was 0.1 mM.

[b] The theoretical inhibition values expected for unlabeled galactose at concentrations of 1 and 5 mM are 90 and 98%, respectively.

[c] This experiment was performed with a glu-PTS-defective mutant (*L. casei* 64H *manA*).

mediated reaction produced gal-6P, whereas galactokinase produced gal-1P (see below) (28). To identify the product(s) of sugar transport, starved galactose-grown cells of *L. casei* 64H were incubated with [^{14}C]galactose for 10 s. Further transport and metabolism of the sugar were halted by adding formic acid to the reaction mixture. The cell extract and medium were lyophilized, and after reconstitution with water, samples were analyzed by paper chromatography and autoradiography (Fig. 2). Five major areas of radioactivity were detected; one of these, area V, was shown to be [^{14}C]galactose from the medium. The identities of the materials present in areas I, II, and III were determined by elution of a fraction from each area and further chromatography against various standards. The radiolabeled derivatives in the fractions from areas I, II, and III (fractions I, II, and III, respectively) cochromatographed with fructose 1,6-diphosphate (and tagatose 1,6-diphosphate), gal-6P, and gal-1P (and glucose 6-phosphate), respectively (Fig. 3A). These radioactive compounds were eluted and dephosphorylated with alkaline phosphatase. The free sugars formed were identified by paper chromatography (Fig. 3B).

From the data presented in Fig. 3, we concluded that fraction I (Fig. 2) contained approximately equal parts of fructose 1,6-diphosphate and tagatose 1,6-diphosphate, that fraction II (Fig. 2) was gal-6P, and that fraction III (Fig. 2) was primarily gal-1P (but also contained 10 to 20% glucose 6-phosphate). Fraction IV (from area IV) was not conclusively identified, but the radioactive material cochromatographed with 2- and 3-phosphoglyceric acids. From the total radioactivity present in each fraction (Fig. 2), we calculated (Table 5) that after 10 s of incubation approximately 63% of the radiolabeled intracellular material was present as gal-6P and 12% was present as gal-1P. The presence of high levels of gal-6P confirmed the operation of the gal-PTS, whereas the formation of gal-1P and glucose 6-phosphate confirmed the operation of the Leloir pathway (Fig. 4) in *L. casei* 64H.

Phosphorylation of galactose in vitro. Below we describe a preliminary in vitro characterization of the gal-PTS and demonstrate the similarity of this system to the lac-PTS. Cells of *L. casei* 64H *lac* (i.e., cells cured of the lactose plasmid [4]) were grown on galactose, and cell-free extracts were prepared from them. The PEP-dependent phosphorylation of galactose in

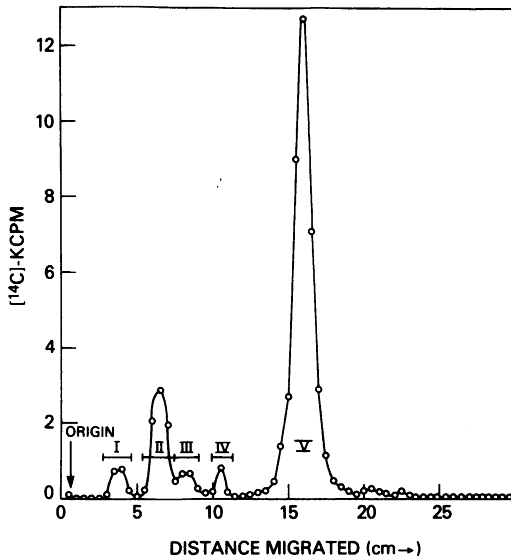

FIG. 2. Identification of radiolabeled intracellular metabolites formed in the initial 10 s of [^{14}C]galactose transport by *L. casei* 64H. The experimental protocol and chromatographic procedures are described in the text. Areas I through IV represent phosphorylated derivatives subsequently identified (see Fig. 3) as follows: area I, tagatose 1,6-diphosphate and fructose 1,6-diphosphate; area II, gal-6P; area III, mixture of gal-1P and glucose 6-phosphate; and area IV, probably 2- and 3-phosphoglyceric acids. Fraction V (from area V) was [^{14}C]galactose derived from the incubation medium. The five radioactive areas were eluted separately, and total radioactivity was measured.

vitro required cell membranes, high-speed soluble cytoplasmic components, PEP, and Mg^{2+} ions (Table 6). The phosphorylation process involved a factor (HPr) which was absorbed by charcoal, and heat treatment of either of the cellular fractions abolished activity. Complementation data (Table 7) showed that the PEP-dependent phosphorylation of galactose required a galactose-specific membrane component (enzyme II^{gal}) and a galactose-specific soluble component (enzyme III^{gal}). These components were present in cells grown on galactose, but were not detectable in the cell membrane or cytoplasmic fractions of glucose-grown organisms (Table 7). The rate of galactose phosphorylation was fourfold faster when ATP was substituted for PEP (Table 6). Phosphorylation by ATP did not require a membrane component or the charcoal-absorbed factor (data not shown), and the enzyme responsible for the reaction, galactokinase (molecular weight, 45,000), was separated from the galactose-specific enzyme III^{gal} (molecular weight, 35,000) by steric exclusion chromatography (Fig. 5). Although the major enzyme III^{gal} activity was present in the fractions centered around fraction 52, smaller amounts of two higher-molecular-weight forms, possibly enzyme III^{gal} aggregates, were also observed.

In vitro products of galactokinase and gal-PTS reactions. The product of galactose phosphorylation via the PEP-dependent PTS cochromatographed with authentic gal-6P (Dowex-1–borate ion-exchange chromatography [Fig. 6A]), and like gal-6P, the derivative was stable to boiling in 0.1 N HCl for 10 min at 100°C. The product of the ATP-dependent galactokinase reaction cochromatographed with authentic gal-1P (Fig. 6B), and like gal-1P, the compound was completely hydrolyzed to free galactose by exposure to dilute acid at 100°C.

DISCUSSION

Identification of the gal-PTS in *L. casei*. Galactose-grown cells of group N streptococci contain enzymes of both the D-tagatose-6-phosphate

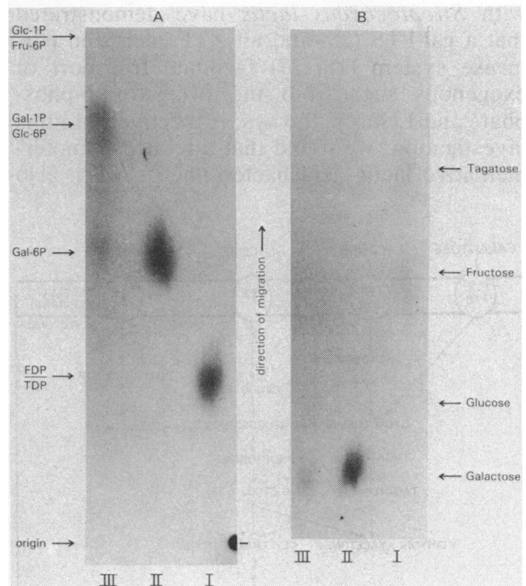

FIG. 3. Fluorographic identification of the radiolabeled intermediates comprising areas I, II, and III (see Fig. 2). The intermediates were eluted from the chromatogram, and after concentration, samples were chromatographed with phosphorylated standards (markers on the left) in order to determine purity and identity. The phosphorylated derivatives (A) were eluted and treated with alkaline phosphatase, and free ^{14}C-labeled sugars (B) were identified by paper chromatography, using standards (markers on the right). Glc-1P, Glucose 1-phosphate; Fru-6P, fructose 6-phosphate; Glc-6P, glucose 6-phosphate; FDP, fructose 1,6-diphosphate; TDP, tagatose 1,6-diphosphate.

TABLE 5. Intracellular radiolabeled metabolites present in starved cells of *L. casei* 64H after 10 s of exposure to [^{14}C]galactose

Radioactive area on chromatogram[a]	Compound	^{14}C radioactivity (dpm, $\times 10^3$)[b]	Intracellular concn (mM)[c]	% Of total radioactivity
I	{Tagatose 1,6-diphosphate / Fructose 1,6-diphosphate}	42.5	0.86[d]	14.1
II	Galactose 6-phosphate	190.2	4.10	63.2
III	{Galactose 6-phosphate / Glucose 6-phosphate}	40.8	0.82	13.5
IV	Unknown[e]	27.7	0.58	9.2

[a] See Fig. 3.
[b] Determined after separation of metabolites by paper chromatography (see Fig. 3), followed by elution and counting of samples by the liquid scintillation procedure.
[c] Assuming an intracellular (protoplast) fluid volume of 1.67 ml/g (dry weight) of cells.
[d] The phosphorylated derivatives were not separated by the chromatographic procedure, but hydrolysis and subsequent chromatography of the free sugar indicated approximately 50% of each component.
[e] Unidentified, but similar in chromatographic mobility to 2-phosphoglyceric and 3-phosphoglyceric acids.

pathway and the Leloir pathway for the metabolism of the sugar (Fig. 4). The relative contribution of each pathway to overall galactose metabolism may depend upon the strain and the conditions of growth (26). Transport studies with *Streptococcus lactis* have demonstrated that a gal-PTS (28) and an ATP-energized permease system (11, 27) facilitate transport of exogenous sugar into the D-tagatose-6-phosphate and Leloir pathways, respectively. In this investigation, we found that a related homofermentative lactic acid bacterium, *L. casei*, also has the capacity to metabolize galactose via pathways similar to those described for the group N streptococci (Fig. 4). When starved cells of *L. casei* 64H were incubated for 10 s with [^{14}C]galactose, approximately 63% of the intracellular radiolabeled material was recovered as gal-6P, and only 12% was recovered as gal-1P (Table 5). It should be emphasized that

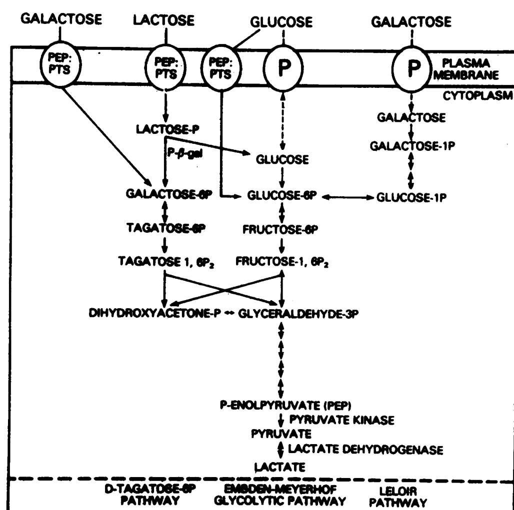

FIG. 4. Pathways for the transport and metabolism of glucose, galactose, and lactose by *L. casei* 64H. Abbreviations: PEP:PTS, PEP-dependent PTS; P, active transport (permease) systems for galactose and glucose; P-β-gal, β-D-phosphogalactoside galactohydrolase.

TABLE 6. Requirements for reconstitution of gal-PTS activity in vitro

Assay system	Galactose phosphate formed (nmol/mg of protein per min)
Complete assay system[a]	119
−High-speed supernatant	4
−High-speed membrane pellet	7
−PEP	23
$-MgCl_2$	45
−PEP + ATP	424
Heat-treated, high-speed supernatant[b]	2
Heat-treated, high-speed pellet[b]	19
Charcoal-absorbed, high-speed supernatant[c]	25

[a] The complete assay system contained 120 μg of a high-speed membrane pellet and 210 μg of a high-speed supernatant prepared as described in the text. Incubation was for 30 min at 37°C, and samples were removed for assay of [^{14}C]galactose phosphate at 5-min intervals.
[b] The fractions were heated at 100°C for 3 min, and denatured proteins were removed by centrifugation.
[c] In the charcoal-absorption procedure 1 g of acid-washed Norite A was added to 1 ml of high-speed supernatant, and the mixture was stirred gently for 15 min. Charcoal and absorbed materials were removed by centrifugation.

TABLE 7. Reconstitution of PTS activities in vitro: requirement of a soluble, galactose-specific factor for PEP-dependent phosphorylation of galactose

Fraction used for PTS complementation		Sp act[a]	
High-speed membrane pellet	High-speed super-natant	glu-PTS	gal-PTS
Glucose[b]	Glucose	211	7
Glucose	Galactose	174	13
Galactose	Glucose	154	22
Galactose	Galactose	147	145

[a] Specific activities are expressed as nanomoles of sugar phosphate formed per milligram of protein per minute.

[b] Cells of *L. casei* 64H were grown on the sugars indicated and then were disrupted by mechanical disintegration in a Bead-Beater. High-speed membrane pellet and high-speed supernatant fractions were obtained by high-speed centrifugation of the cell extracts.

the starved cells are initially primed with a large endogenous reserve of PEP (5, 31) and are virtually devoid of ATP. Under such conditions gal-6P would be formed preferentially within the first few seconds of exposure of the cells to the radiolabeled sugar. Because these results arise from initial uptake by starved cells, we cannot conclude from these data that the steady-state metabolism of galactose takes place primarily through the pathway initiated by the gal-PTS during growth.

The four major findings of this investigation are as follows: (i) PEP-primed starved cells of *L. casei* 64H grown on galactose rapidly accumulated galactose, but not lactose, via a high-affinity PEP-dependent PTS; (ii) lactose did not significantly inhibit the PEP-dependent translocation of galactose in starved cells (Table 4); (iii) cells cured of the lactose plasmid (strain *L. casei* 64H *lac*) grew well on galactose and possessed gal-PTS activity (Table 2); and (iv) galactose-specific membrane (enzyme II^{gal}) and cytoplasmic (enzyme III^{gal}) components were induced in wild-type and lactose plasmid-cured strains during growth on galactose (Table 7 and Fig. 5). We believe that these observations confirm the separate identities of the gal-PTS and the lac-PTS in *L. casei*. The components of the gal-PTS (and lac-PTS) in *L. casei* have not been characterized genetically or biochemically as completely as the components of the lac-PTS in *Staphylococcus aureus* (7, 9, 10, 20, 21, 23–25). However, it is interesting that the approximate molecular weight (35,000) of enzyme III^{gal} in *L. casei* is similar to the values reported (33,000 to 35,000) for purified enzyme III^{lac} isolated from *Staphylococcus aureus* (7, 10, 21).

Induction of the gal-PTS. The gal-PTS was inducible in all *L. casei* subspecies examined (Table 3). We suggest that gal-6P may serve as the inducer, since growth of the cells on β-galactosides (which give rise to intracellular gal-6P) also promoted full expression of the PTS (Table 2). TMG did not induce the gal-PTS in wild-type or *manA*-defective cells of *L. casei* 64H during growth on glucose (Table 2). In the wild-type organism, TMG is excluded from cells by PTS-mediated transport of glucose (5). However, the non-metabolizable analog can enter cells of *L. casei* 64H *manA* during growth on glucose, and the accumulation of methyl-β-D-thiogalactopyranoside 6-phosphate results in expression of the lac-PTS, but not the gal-PTS (5) (Table 2). gal-6P differs in structure from noninducers (e.g., methyl-β-D-thiogalactopyranoside 6-phosphate or glucose 6-phosphate) in that it possesses an axial hydroxyl group at C-4 and a free hydroxyl group at C-1 (anomeric

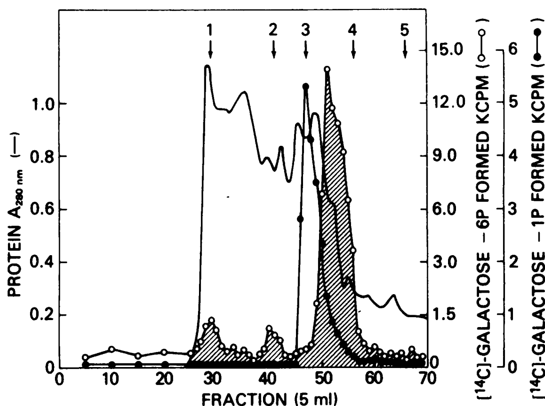

FIG. 5. Separation, identification, and partial purification of galactokinase, (●) and the galactose-specific enzyme III^{gal} (O) present in an extract prepared from galactose-grown cells of *L. casei* 64H *lac*. The conditions used for ATP-dependent phosphorylation of galactose by galactokinase are described in the text. In the complementation assay for enzyme III^{gal}, samples of each fraction were added to a mixture containing (i) membranes (112 μg of protein) prepared from galactose-grown cells, which provided enzyme II^{gal}, and (ii) a high-speed soluble supernatant (180 μg of protein) fraction prepared from glucose-grown cells, which provided the generalized PTS components enzyme I and HPr. Each assay mixture contained PEP and Mg^{2+}, and the formation of ^{14}C-labeled gal-6P was determined by standard procedures. The arrows indicate the elution positions of the protein standards used to estimate the molecular weights of galactokinase and enzyme III^{gal}. The following molecular weight markers were used: 1, ferritin (molecular weight, 540,000); 2, bovine serum albumin (68,000); 3, ovalbumin (43,000); 4, chymotrypsinogen (24,000); and 5, RNase (12,000). A_{280nm}, Absorbance at 280 nm.

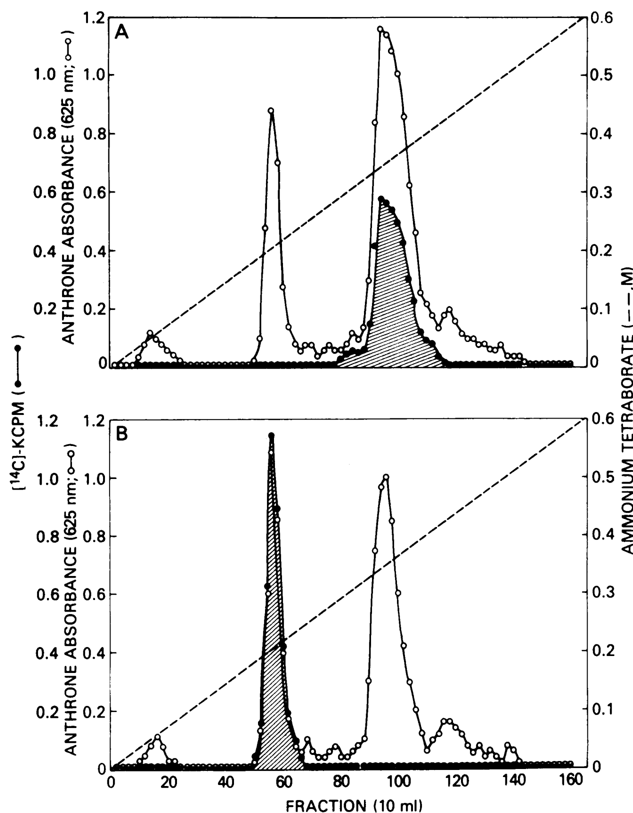

FIG. 6. Identification of the products formed by the gal-PTS (A) and ATP-dependent galactokinase (B) from *L. casei* 64H. The products of the two in vitro reactions were purified of [^{14}C]galactose by paper chromatography as described in the legend to Fig. 3. After elution from the chromatogram, each derivative was loaded separately onto a Dowex-1–borate column (1.5 by 90 cm) together with 25 mg each of gal-1P and gal-6P. The galactose phosphate standards (○) were eluted with an increasing concentration gradient (dashed line) of freshly prepared ammonium tetraborate (0 to 0.6 M) (27). Samples of each fraction were assayed for radioactive [^{14}C]galactose phosphate (●, shaded area), and the elution positions of standard gal-1P (fractions 52 through 64) and gal-6P (fractions 90 through 110) were determined by the anthrone method (27).

center) of the galactopyranose molecule. These two features may be structural requisites for an inducer of the gal-PTS operon. The equatorial hydroxyl group at C-2 may also be structurally important (Table 4), and this could be examined by using the C-2 analogs 2-deoxy-D-galactose 6-phosphate and D-talose 6-phosphate as potential inducers for gal-PTS in *L. casei* 64H. The gal-PTS of *L. casei* exhibited a much greater affinity for its substrate than the gal-PTS of *Streptococcus lactis* (reported K_m values, 1.1 mM [28] and 15 to 27 mM [13]). The system was quite specific for D-galactose, and neither glucose (a C-4 epimer) nor lactose (which contains a galactosyl moiety) was a substrate of the gal-PTS in *L. casei* (Table 4). It should be noted that, although our data show that lactose is not a substrate of the gal-PTS in *L. casei*, we cannot exclude the possibility that galactose may be a poor substrate of the lac-PTS.

Probable chromosomal location of the gal-PTS genes in *L. casei*. It is clear that *L. casei* and *Streptococcus lactis* use similar pathways for the transport and metabolism of galactose and lactose. However, there appear to be significant differences between the two organisms with respect to the genetic location and modes of regulation of PTS gene expression. For example, growth of *Streptococcus lactis* on galactose induces both the gal-PTS and the lac-PTS, but only the gal-PTS is expressed during growth of *L. casei* 64H on this sugar. In *Streptococcus lactis* the genes for lactose metabolism are plasmid encoded (13, 15). Cured strains, which are defective in lac-PTS activity (and gal-PTS activity) fail to grow on lactose and exhibit a slower growth rate on galactose (17). These strains (which display a Lac$^-$ Gal^d phenotype) continue to grow on galactose by using the Leloir pathway for sugar metabolism (Fig. 4). The genetic

studies of Park and McKay (17) indicate that the structural genes for both the lac-PTS and the gal-PTS are located on the same plasmid (i.e., the lactose plasmid) in *Streptococcus lactis*. In *L. casei* 64H there is only a single (23-megadalton) plasmid that encodes the *lac* genes (4, 5, 14). Plasmid-cured cells (*L. casei* 64H *lac*) are fully induced for the gal-PTS after growth on galactose (Table 2). These findings are consistent with a chromosomal locus for gal-PTS genes in *L. casei* 64H. It should be noted that the data do not exclude the possibility that the lac-PTS and gal-PTS utilize the same soluble enzyme III. Both enzyme III^{gal} and enzyme III^{lac} must be further purified and characterized to resolve this point.

The overall pathways of lactose and galactose metabolism in *L. casei* 64H are summarized in Fig. 4. Data in this and the accompanying paper (5) provide evidence for each of these pathways. The two most significant findings of the present work are (i) although a distinct gal-PTS has not been demonstrated directly in other organisms, it has been possible to characterize biochemically a gal-PTS in *L. casei*, and (ii) the components of the gal-PTS functionally resemble those of the lac-PTS. We are presently isolating these components and the genes that encode them in order to allow a direct comparison of relatedness and function.

ACKNOWLEDGMENTS

We thank Alfred S. Giuffrida and Lee M. Chassy for expert technical assistance in the early stages of this study. Jack London and Charles Wittenberger offered helpful comments during the preparation of the manuscript, and Laura Howell prepared the manuscript.

ADDENDUM IN PROOF

Since submission of this paper, Crow et al. (V. L. Crow, G. P. Davey, L. E. Pearce, and T. D. Thomas, J. Bacteriol. **153**:76–83, 1983) have shown that some strains of *S. lactis* of the Lac^- Gal^d phenotype exhibit gal-PTS activity. The authors attribute the phenotype to loss of the plasmid-associated enzymes of the D-tagatose 6-phosphate pathway.

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