Carbohydrate Utilization in *Lactobacillus sake*†

ROBERTE LAURET,¹ FRANÇOISE MOREL-DEVILLE,¹ FRANÇOISE BERTHIER,¹
MARIE CHAMPOMIER-VERGES,¹ PIETER POSTMA,² S. DUSKO EHRLICH,³
AND MONIQUE ZAGOREC^{1*}

Laboratoire de Recherches sur la Viande¹ and Laboratoire de Génétique Microbienne,³
Institut National de la Recherche Agronomique, Domaine de Vilvert, 78350 Jouy
en Josas, France, and E. C. Slater Institute, University of Amsterdam,
1018 TV Amsterdam, The Netherlands²

Received 22 November 1995/Accepted 12 March 1996

The ability of *Lactobacillus sake* to use various carbon sources was investigated. For this purpose we developed a chemically defined medium allowing growth of *L. sake* and some related lactobacilli. This medium was used to determine growth rates on various carbohydrates and some nutritional requirements of *L. sake*. Mutants resistant to 2-deoxy-D-glucose (a nonmetabolizable glucose analog) were isolated. One mutant unable to grow on mannose and one mutant deficient in growth on mannose, fructose, and sucrose were studied by determining growth characteristics and carbohydrate uptake and phosphorylation rates. We show here that sucrose, fructose, mannose, *N*-acetylglucosamine, and glucose are transported and phosphorylated by the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS). The PTS permease specific for mannose, enzyme II^{Man}, was shown to be responsible for mannose, glucose, and *N*-acetylglucosamine transport. A second, non-PTS system, which was responsible for glucose transport, was demonstrated. Subsequent glucose metabolism involved an ATP-dependent phosphorylation. Ribose and gluconate were transported by PTS-independent permeases.

Among lactic acid bacteria which develop on meat and fermented meat products, *Lactobacillus sake* is often dominant, suggesting that this species is particularly well adapted to such environments (11, 14). For this reason *L. sake* is often used, in combination with other microorganisms such as staphylococci and micrococci, in starter cultures for industrial production of raw sausages. The main role of *L. sake* seems to be in the production of lactate resulting from the consumption of carbohydrates added at the beginning of the process. It has been shown that the types of carbohydrates and microorganisms present are important for sausage manufacture, since the pH drop caused by lactic acid is a key step which affects the firmness, color, aroma, and flavor development of the product (2, 16). In addition, the pH drop contributes to food preservation. The capability of the lactic acid bacteria to grow at the expense of carbohydrates and fast production of lactic acid are two of the criteria for the selection of starter strains (3, 12).

In bacteria, carbohydrates are transported by various mechanisms involving permeases that use chemical energy or an electrochemical gradient. Carbohydrates translocated by permeases are subsequently phosphorylated by ATP-dependent kinases. Another pathway for carbohydrate utilization is the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS), which catalyzes concomitant internalization and phosphorylation of various carbohydrates (referred to as PTS carbohydrates). A phosphorylation cascade is driven from PEP through enzyme I and HPr and then to carbohydrate-specific permeases (enzymes II^{carbohydrate}). The PTS proteins are generally phosphoproteins in which the phospho group is covalently bound to histidine residues. The PTS is also involved

in catabolite repression, regulating various functions in both gram-negative and gram-positive bacteria. The involvement of the PTS in such regulation is well documented for members of the family *Enterobacteriaceae* (for a review, see reference 25). Recent studies with *Bacillus subtilis* and other gram-positive bacteria suggested a role for HPr as a central regulatory molecule in catabolite repression (7, 13).

Most of the studies concerning carbohydrate metabolism in lactic acid bacteria have been focused on dairy lactococci and lactobacilli and for this reason have concerned mainly lactose catabolism. The recent development of molecular genetic techniques for dairy microorganisms has resulted in a better knowledge of the PTS and its involvement in regulatory phenomena. The existence of a PTS in lactic acid bacteria has been described (for a review, see reference 23). However, evidence for the presence of active enzyme I in some heterofermentative lactobacilli remains unclear (27, 30). In *B. subtilis*, the phosphorylation of HPr on serine 46, by an ATP-dependent kinase, plays an important role in catabolite repression (7). In several gram-positive bacteria, including lactic acid bacteria, a regulatory mechanism involving a similar phosphorylation of HPr is suspected (27, 28, 30, 31). For *L. sake* the knowledge about carbohydrate utilization is scarce (4, 21), and the metabolic pathways which this bacterium utilizes for growth on meat are poorly understood. The presence of β -galactosidase and phospho- β -galactosidase involved in lactose utilization was described for some strains (21), and catabolism of arginine, leading to ATP synthesis via the arginine deiminase pathway, has been suggested (18). In both reports, evidence for regulatory processes resembling glucose repression was shown.

The aim of the present study was to obtain more complete information on carbohydrate metabolism in *L. sake*. We report here the development of a synthetic medium, designated MCD, and its use to investigate the growth of *L. sake* on various carbon sources. MCD was also used for the isolation and characterization of mutants, allowing us to determine which PTS

* Corresponding author. Phone: 33 1 34 65 22 89. Fax: 33 1 34 65 21 05. Electronic mail address: zagorec@biotec.jouy.inra.fr.

† This paper is dedicated to the memory of Michel Steinmetz, who introduced M.Z. to the phosphoenolpyruvate:carbohydrate phosphotransferase system and who died on 7 February 1995.

carbohydrates are utilized by *L. sake* and to postulate a putative involvement of some PTS components in regulatory mechanisms.

MATERIALS AND METHODS

Strains. The nine strains, *L. sake* 23, *L. sake* 55, *L. sake* 64, *L. sake* 112, *L. sake* 179, *L. sake* 160*1, *L. sake* 207, *L. sake* GV1, and *L. sake* G3, which were used in this study were from our laboratory stock, and all were isolated from meat and fermented meat products. *L. sake* 23K, *L. sake* 64F and *L. sake* 160*1K were plasmid-cured derivatives of *L. sake* 23, *L. sake* 64, and *L. sake* 160*1, respectively (2a). *L. sake* RV37 and *L. sake* RV43 were isolated as 2-deoxy-D-glucose (2DG)-resistant mutants of *L. sake* 160*1K.

Growth conditions. MRS medium (Difco) containing glucose (20 g · liter⁻¹) or reconstituted MRS medium (when specified) was used for strain propagation (6). A chemically defined medium (MCD) was developed (see below) and was used for liquid cultures or as a solid medium, containing 12 g of agar (Biteck; Difco) · liter⁻¹. Optimization of MCD was carried out on plates. When growth was observed, liquid medium was tested. The optical density at 600 nm (OD₆₀₀) was monitored during a preculture and a following culture. Bacteria were plated and checked to verify that no contaminant strains or prototrophic mutants appeared. Cells were grown at 30°C. Bacteria derived from a single clone on MRS plates were first grown in liquid MRS medium. A 1-ml sample of MRS exponential culture was centrifuged, and bacteria were washed once with 1 ml of MCD medium and resuspended at 0.1 OD₆₀₀ unit · ml⁻¹. The suspension was diluted 1,000 times in fresh MCD medium and grown overnight. In these conditions the cultures were in exponential growth after an overnight incubation. This culture was centrifuged, and the bacteria were inoculated at an initial concentration of 0.05 OD₆₀₀ unit · ml⁻¹ in fresh MCD medium. To monitor the growth rate, flasks containing the cell cultures were shaken moderately (120 rpm) to keep the bacteria in suspension.

Isolation of mutants. Spontaneous mutants resistant to 2DG were isolated by plating 0.1 ml of an exponential MRS culture on reconstituted MRS plates containing 10 g of gluconate · liter⁻¹ as a carbon source and 10 or 50 mM 2DG. Resistant clones were purified by streaking two times on the same selective medium and were then streaked on nonselective MRS medium before analysis.

Uptake of ¹⁴C-labelled carbohydrates. Bacteria were grown in 250 ml of MCD medium containing 5 g of a carbon source · liter⁻¹ and harvested in the exponential phase. After centrifugation, cells were washed twice in medium A [1 g of (NH₄)₂SO₄, 10.5 g of K₂HPO₄, 4.5 g of KH₂PO₄, and 1 g of MgSO₄ per liter of demineralized water, final pH 7.0] and resuspended in 3 ml of the same medium. Bacteria were incubated in medium A with ¹⁴C-labelled carbohydrates (final concentration, 0.5 mM; specific activity, 200 cpm · nmol⁻¹) at room temperature, and aliquots were taken at 15, 30, 60, and 120 s. The uptake of ¹⁴C-labelled carbohydrates was determined by the standard method of rapid cell filtration (24).

Preparation of toluenized cells. Bacterial cultures were grown as described above for uptake experiments. Bacteria were washed two times in 0.9% NaCl and resuspended in 25 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA and 0.5 mM dithiothreitol to a cell concentration of approximately 35 OD₆₀₀ units · ml⁻¹. Cells were permeabilized by addition of toluene-acetone (1:9, vol/vol) and vortexed at maximum speed for 5 min. Twenty microliters (for ATP-dependent phosphorylation) or 30 µl (for PEP-dependent phosphorylation) of toluene-acetone was used per ml of cell suspension. Toluenized cells were kept on ice until use.

PEP- and ATP-dependent carbohydrate phosphorylation assay. Phosphorylation of ¹⁴C-labelled compounds was determined in the presence of PEP or ATP as described by Postma and Stock (26) with the following modifications. Five to 20 µl of toluenized cells was incubated in a final volume of 100 µl of buffer containing KF (12.5 mM), MgCl₂ (5 mM), dithiothreitol (2.5 mM), PEP or ATP (pH 7.0) (10 mM), ¹⁴C-labelled carbohydrate (10 mM; specific activity, 200 cpm · nmol⁻¹), and potassium phosphate buffer (pH 7.5) (50 mM). After incubation for 30 min at 30°C, the phosphorylated carbohydrates were separated on Dowex AG 1-X2 columns as described previously (24).

Radiochemicals. [U-¹⁴C]methyl α-D-glucopyranoside (5.68 GBq · mmol⁻¹), D-[U-¹⁴C]glucose (10.8 GBq · mmol⁻¹), D-[U-¹⁴C]fructose (10.2 GBq · mmol⁻¹), D-[U-¹⁴C]mannose (10.7 GBq · mmol⁻¹), and N-acetyl-D-[U-¹⁴C]glucosamine (2.11 GBq · mmol⁻¹) were purchased from the Radiochemical Centre, Amersham, England; 2-deoxy-D-[U-¹⁴C]glucose (10.8 GBq · mmol⁻¹) was purchased from NEN Research Products, Du Pont, Boston, Mass.; [U-¹⁴C]gluconate was obtained from the Commissariat à l'Energie Atomique, Saclay, France; and [U-¹⁴C]ribose (8.14 GBq · mmol⁻¹) was a gift from Jean Labarre, Service de Biochimie et Génétique, Commissariat à l'Energie Atomique.

RESULTS

Development of a chemically defined growth medium. Nine strains from various meat origins previously isolated in our laboratory and characterized as *L. sake* were studied. *L. sake* has been reported to ferment D-ribose, D-galactose, D-glucose,

D-fructose, D-mannose, N-acetylglucosamine, D-melibiose, D-trehalose, sucrose, and gluconate (4). In order to determine the growth characteristics on various carbon sources, several media were tested. Residual growth was observed in the complex MRS medium in the absence of an added carbohydrate. This prevented us from carefully analyzing the carbohydrate-dependent growth. Various components of MRS were removed without successfully suppressing this residual growth. This prompted us to test chemically defined media reported to allow growth of lactobacilli. Neither the medium described by Ledesma et al. (15) nor the medium described by Montel and Labadie (19) gave satisfactory growth rates for *L. sake*. However, a rich but chemically defined medium, developed by F. Bringel (2b) (Table 1), allowed growth of *L. sake* with a doubling time of 60 min in the presence of 1% glucose. This basal medium was further simplified, as described below.

Amino acid requirements were tested by single or multiple omission. No growth was observed with any strains when glycine, phenylalanine, histidine, isoleucine, leucine, lysine, methionine, valine, proline, threonine, tyrosine, or arginine was removed, suggesting that these amino acids were essential. Aspartic and glutamic acids were not required for most strains, and either cysteine or cystine was necessary. Only cysteine was therefore used routinely. The presence of serine or tryptophan strongly stimulated growth of all strains. Some strain variations were observed with other amino acids. Asparagine and glutamine were essential for growth of *L. sake* 23, *L. sake* 64, and *L. sake* 179 and stimulated the growth of the other strains. Alanine was required for *L. sake* G3, *L. sake* 55, *L. sake* 64, and *L. sake* 179. The medium which allowed growth of all strains, and which was used in further experiments, contained 18 amino acids at a concentration of 50 mg · liter⁻¹ (Table 1).

Vitamins or other specific growth requirements were tested in a medium containing glucose as a carbon source. D-Biotin, cyanocobalamin, thiamine, L-ascorbic acid, oleic acid, and spermin could be omitted, with only a small effect (10 to 15%) on the final cell density. Thiamine was not required in the presence of glucose but was essential for growth on some carbohydrates (see below) and stimulated growth on others. Thiamine was routinely added in all further experiments unless specified otherwise. Tween 80 could be replaced by oleic acid, but no growth was observed when both Tween 80 and oleic acid were omitted. For practical purposes, Tween 80 was preferred to oleic acid in further experiments. Six vitamins were essential (Table 1).

The requirement for the various bases was tested in a medium containing 18 amino acids and six vitamins. Guanine, adenine, and uracil were essential, whereas omission of deoxyguanosine, hypoxanthine, and xanthine did not affect growth. The amounts of the remaining bases could be decreased to 5 mg · liter⁻¹ (adenine) or 10 mg · liter⁻¹ (guanine and uracil). *L. sake* 64 was found to be prototrophic for adenine and guanine and could grow at a low rate in the absence of uracil.

The salt content in the medium was adjusted by decreasing the amount of sodium acetate from 10 to 2 g · liter⁻¹ and that of potassium chloride from 3 to 0.75 g · liter⁻¹. Finally, the concentration of the carbon source was routinely kept at 5 g · liter⁻¹ in liquid cultures and at 1 g · liter⁻¹ on plates.

The composition of the final chemically defined medium, designated MCD, is summarized in Table 1. This simplified medium allowed growth of *L. sake* with only a slightly longer doubling time and a slightly lower final cell density than for growth in the initial medium (Fig. 1). On MCD medium containing glucose, the stationary phase was reached after 8 to 10 h, at a final OD of 0.85 to 0.9.

The growth of various lactobacilli in MCD medium was

TABLE 1. Compositions of the initial rich chemically defined medium and the newly formulated MCD medium

Compound	Concn (mg · liter ⁻¹) in:	
	Initial medium	MCD medium
Sodium acetate	10,000	2,000
Tween 80	1,000	1,000
Na ₂ HPO ₄ · H ₂ O	1,750	1,750
KCl	3,000	750
MnSO ₄	7.6	7.6
MgSO ₄	200	200
Glycine	200	50
L-Asparagine	250	50
L-Tryptophan	200	50
L-Serine	200	50
L-Alanine	200	50
L-Phenylalanine	200	50
L-Histidine	500	50
L-Isoleucine	500	50
L-Leucine	200	50
L-Lysine	50	50
L-Methionine	500	50
L-Proline	40	50
L-Threonine	50	50
L-Valine	500	50
L-Arginine	50	50
L-Tyrosine	200	50
L-Cysteine	400	50
Cystine	100	
L-Glutamine	300	50
L-Aspartic acid	2,000	
L-Glutamic acid	1,000	
<i>p</i> -Aminobenzoic acid	0.4	0.4
Pyridoxal	0.5	0.5
Nicotinic acid	1.7	1
Folic acid	0.32	0.2
Calcium pantothenate	1	1
Riboflavin	1	1
L-Ascorbic acid	500	
Thiamine	1	1
Oleic acid	10	
Spermin	1	
Pyridoxamine	0.0167	
D-Biotin	0.01	
Cyanocobalamin	0.005	
Adenine	83	5
Guanine	50	10
Uracil	50	10
Deoxyguanosine	50	
Hypoxanthine	33	
Xanthine	5	

investigated. We observed that the *L. sake*-related species *Lactobacillus curvatus* and *Lactobacillus bavaricus* grew at the same rate as *L. sake* but that *Lactobacillus pentosus* and *Lactobacillus plantarum* grew poorly (data not shown). Thus, MCD medium is suitable for several but not all lactobacilli.

Growth on different carbon sources. MCD medium was used to determine the growth rates of *L. sake* on various carbohydrates. No differences between wild-type strains and their plasmid-cured derivatives were observed. This suggests that plasmids present in the original strains do not encode enzymes required for carbohydrate catabolism, as was described for lactose or maltose utilization in several lactic acid bacteria (5, 8, 17). The growth rates of *L. sake* 64F, *L. sake* 160*1K, and *L.*

sake 23K are shown in Table 2. Thiamine was required for growth on ribose, gluconate, and arabinose, and, with two exceptions (*L. sake* 23K on glucose and *L. sake* 64F on *N*-acetylglucosamine), it stimulated the growth of all strains on all other carbon sources tested.

Isolation and characterization of 2DG-resistant mutants. To study the permeases involved in carbohydrate uptake, we attempted to isolate mutants affected in carbohydrate utilization. Methyl-D- α -glucoside (α MG) and 2DG are two nonmetabolizable glucose analogs which are specific, in *Escherichia coli*, for the PTS enzyme II^{Glc} and enzyme II^{Man}, respectively. *L. sake* 23K, *L. sake* 64F, and *L. sake* 160*1K were sensitive to 2DG but not to α MG. This suggests that a PTS-dependent mannose permease may be present in *L. sake*, whereas a glucose-specific enzyme II might be absent. Spontaneous 2DG-resistant mutants of *L. sake* 160*1K were isolated on MRS plates containing gluconate as a carbon source and 2DG. Twenty mutants were tested for growth on MCD medium containing various carbohydrates.

The *L. sake* RV43 mutant did not grow on mannose, *N*-acetylglucosamine, fructose, or sucrose and had a doubling time of more than 200 min on glucose, which is much longer than that observed with the parental strain. Furthermore, the OD reached after exponential growth was very low, suggesting that the bacteria stopped growing after only a few generations. The growth on MRS or MCD plates, supplemented with gluconate or ribose, was strongly impaired. The pleiotropic phenotype of this mutant suggested a mutation in the PTS inactivating either the *ptsH* or *ptsI* gene, which encode the general enzymes of the PTS (HPr and enzyme I, respectively), or a gene which regulates PTS functions.

The other 2DG-resistant mutants were unable to grow on mannose and *N*-acetylglucosamine but grew normally on the other carbon sources listed in Table 2. They were therefore probably mutated in enzyme II^{Man}, which was reported to be responsible for mannose and *N*-acetylglucosamine transport and phosphorylation in numerous bacteria (reference 25 and references therein). One of these mutants, *L. sake* RV37, was studied further. Its doubling time on fructose or sucrose was similar to that of the parent, whereas the doubling time was two times longer on glucose. The maximum OD reached by *L. sake* RV37 on glucose was 0.25, compared with 0.85 for the

FIG. 1. Growth curves for *L. sake* GV1 in MRS medium (squares), the initial chemically defined medium (circles), and MCD medium (triangles) containing 5 g of glucose · liter⁻¹.

TABLE 2. Doubling times of three *L. sake* strains in MCD medium supplemented with different carbon sources in the presence or absence of thiamine^a

Carbon source	Doubling time (min) of strain:					
	160*1K		23K		64F	
	Without thiamine	With thiamine	Without thiamine	With thiamine	Without thiamine	With thiamine
D(+)-Glucose	90	60	80	84	72	65
L-(+)-Arabinose	∞ ^b	∞	∞	≥360	∞	≥360
D(-)-Ribose	∞	≥360	∞	≥360	∞	140
D(+)-Galactose	300	240	≥360	≥360	∞	204
D(+)-Mannose	162	96	300	96	165	90
D(-)-Fructose	185	100	360	108	180	84
Sucrose	170	96	168	96	132	100
D-Gluconate	∞	≥360	∞	≥360	∞	≥360
N-Acetyl-D-glucosamine	125	84	108	84	84	85

^a The carbon source and thiamine concentrations were 5 g · liter⁻¹ and 1 mg · liter⁻¹, respectively.^b ∞, no detectable growth.

parent. These results suggest that glucose may be transported by both enzyme II^{Man} and a second, unknown system. This second system may involve a non-PTS permease, since it was still active in the mutant *L. sake* RV43, but may be regulated by the PTS, since growth on glucose ceased earlier in both mutants.

Carbohydrate uptake. Uptake of the two glucose analogs, αMG and 2DG, and of fructose, gluconate, glucose, mannose, N-acetylglucosamine, and ribose was determined with bacteria grown on various carbon sources. In the wild-type strains *L. sake* 23K, *L. sake* 64F, and *L. sake* 160*1K, αMG was not taken up, whereas 2DG was accumulated into the bacteria at a constant rate (5 nmol · min⁻¹ · mg [dry weight]⁻¹). In the three wild-type strains, the rates of ribose and gluconate uptake were linear and the uptake could be detected only when the bacteria were grown on ribose or gluconate (at rates of 28 to 30 nmol · min⁻¹ · mg [dry weight]⁻¹ or 1.3 to 3 nmol · min⁻¹ · mg [dry weight]⁻¹, respectively). These results suggest that the enzymes involved in ribose and gluconate utilization were inducible by the corresponding compounds. The rates of uptake of fructose, glucose, mannose, and N-acetylglucosamine are shown in Table 3. In the wild-type strains, the rates of uptake of glucose, fructose, mannose, and N-acetylglucosamine were decreased when bacteria were grown on glucose, suggesting a

repression by glucose of the enzymes involved in their utilization. In *L. sake* 160*1K, the highest fructose uptake rate was observed with fructose-grown cells, suggesting an induction by growth on fructose. Such an induction by mannose was not detected when uptake of mannose or N-acetylglucosamine was measured. The rates of fructose, mannose, and N-acetylglucosamine uptake were higher in *L. sake* 64F and lower in *L. sake* 23K, whereas the growth rates of these three strains were similar. In *L. sake* RV37 grown on glucose, the uptake of mannose and N-acetylglucosamine decreased strongly compared with the rates measured in *L. sake* 160*1K. In *L. sake* RV37 grown on fructose, the rates of uptake of fructose and glucose were slightly lower, while the rates of mannose and N-acetylglucosamine uptake were strongly decreased compared with the rates in *L. sake* 160*1K grown under the same conditions. This result confirmed that the mutant *L. sake* RV37 was deficient in both mannose and N-acetylglucosamine uptake and was partially affected in glucose uptake.

ATP- and PEP-dependent carbohydrate phosphorylation.

The PTS permeases are responsible for concomitant transport and PEP-dependent phosphorylation of carbohydrates, whereas the carbohydrates transported by non-PTS systems are subsequently phosphorylated by ATP-dependent kinases. To determine whether a particular carbohydrate is transported by the PTS, we measured the rates of phosphorylation of glucose, fructose, mannose, and ribose in the presence of ATP or PEP in permeabilized cells of three *L. sake* wild-type strains and in the mutants *L. sake* RV37 and *L. sake* RV43. The results are presented in Fig. 2. No significant phosphorylation could be detected in the absence of ATP and PEP (not shown). Ribose phosphorylation was observed in the presence of ATP, suggesting that ribose was not transported via the PTS. However, PEP-dependent phosphorylation was also observed with *L. sake* 64F and *L. sake* 23K. Its level was lower with diluted samples (Fig. 2, *L. sake* 64F), suggesting that it may be due to ATP synthesis by pyruvate kinase present in the bacteria, at the expense of PEP. Fructose and mannose are PTS carbohydrates, since phosphorylation was detected only in the presence of PEP. Glucose phosphorylation was mostly ATP dependent in glucose-grown wild-type cells, whereas PEP was mainly responsible for glucose phosphorylation when bacteria were grown on mannose. These results suggest that a PTS permease was mainly involved in glucose uptake, in the presence of mannose, whereas in glucose-grown cells, the transport of glucose was mainly due to a non-PTS permease. In *L. sake* RV43 no PEP-dependent phosphorylation could be detected for ei-

TABLE 3. Rates of carbohydrate uptake by different *L. sake* strains grown on various carbon sources

Carbon source used for growth	Strain	Initial rate of uptake (nmol · min ⁻¹ · mg [dry wt] ⁻¹) ^a of:			
		Glucose	Fructose	Mannose	NAG ^b
Glucose	23K	2.2 ± 0.10	— ^c	8.3 ± 0.45	10.6 ± 0.46
	64F	4.0 ± 0.17	6.1 ± 0.53	11.3 ± 1.18	16.1 ± 1.02
	160*1K	5.0 ± 0.10	9.6 ± 0.31	12.9 ± 1.26	9.2 ± 0.83
	RV37	1.0 ± 0.02	—	0.8 ± 0.14	2.5 ± 0.10
Fructose	23K	9.5 ± 0.30	62.5 ± 3.78	28.8 ± 1.52	24.4 ± 0.21
	64F	16.7 ± 0.93	49.0 ± 2.51	88.6 ± 1.85	109.3 ± 2.42
	160*1K	13.7 ± 0.45	95.8 ± 2.40	38.6 ± 0.94	53.2 ± 1.04
	RV37	8.6 ± 0.24	61.1 ± 0.49	6.6 ± 0.24	4.1 ± 0.06
Mannose	23K	17.3 ± 0.39	—	27.5 ± 3.64	31.1 ± 0.70
	64F	17.6 ± 1.05	21.1 ± 1.49	65.2 ± 4.52	46.0 ± 1.21

^a Values are the means from two separate experiments ± standard deviations.^b NAG, N-acetylglucosamine.^c —, not determined.

FIG. 2. PEP-dependent (open bars) and ATP-dependent (hatched bars) phosphorylation of glucose, ribose, mannose, and fructose. Experiments were carried out with *L. sake* 23K, *L. sake* 64F, *L. sake* 160*1K, *L. sake* RV37, and *L. sake* RV43 grown on glucose (g), mannose (m), ribose (r) [with a 10- μ l sample (a) or a 20- μ l sample (b)], or fructose (f). Phosphorylation rates are expressed in nanomoles $\cdot$ minute $^{-1}$ $\cdot$ OD $_{600}$ unit $^{-1}$. Values are the means $\pm$ standard deviations (error bars) for two separate experiments.

ther glucose or fructose, suggesting that this mutant was affected in PEP phosphorylation of these carbohydrates. We conclude that the mutant *L. sake* RV43 was deficient in a general function of the PTS.

DISCUSSION

We formulated a new synthetic medium, MCD, which allowed the growth of *L. sake* and related lactobacilli. The main difference between our new MCD medium and media previously reported is the amino acid composition. Several studies indicate that lactobacilli are prototrophic for asparagine and glutamine and auxotrophic for aspartate and glutamate. With *Lactobacillus casei* and *Lactobacillus helveticus*, Morishita et al. (20) isolated mutants which became prototrophic for aspartate, and they suggested that in the wild-type strains, the genes involved in aspartate synthesis are present but not functional. However, glutamate auxotrophy could not be reverted to prototrophy in *L. plantarum*, *L. casei*, *L. helveticus* or *Lactobacillus acidophilus*. The glutamate auxotrophy was described by Ledesma et al. (15) for other lactobacilli, including *Lactobacillus delbrueckii*, *Lactobacillus leichmanii*, *Lactobacillus buch-*

neri, and *Lactobacillus fermentum*. It appears that *L. sake* strains are different, i.e., that they are prototrophic for aspartate and glutamate but auxotrophic for asparagine and glutamine. The glutamine and asparagine auxotrophies could be the results of mutations in glutamine and asparagine synthetase genes, resulting in lowered activities, since the lack of these two amino acids impaired growth to some extent in different strains.

In most of the synthetic media described in the literature, ferrous sulfate is present. We did not observe such a requirement for growth of *L. sake*, in agreement with the results of Pandey et al. (22) for numerous lactic acid bacteria.

Thiamine was required for growth on arabinose, ribose, and gluconate, suggesting that *L. sake* strains could not synthesize this vitamin, which is a cofactor of phosphoketolase, an enzyme involved in the pentose phosphate pathway. The observation that thiamine stimulated growth on other carbohydrates, such as fructose, mannose, and sucrose, suggests that these carbohydrates were not exclusively catabolized via the Embden-Meyerhof pathway. Other pathways involving thiamine-dependent enzymes might thus be responsible for an efficient

catabolism of carbohydrates. Further experiments will be necessary to extend these studies to various metabolic pathways.

Mutants resistant to 2DG fell into two classes. The majority resembled *L. sake* RV37 and could be mutated in the PTS mannose permease. Another type of mutant, represented by *L. sake* RV43, was defective in the general enzymes of the PTS or in a regulatory gene of the PTS. These observations correlate with results described for other bacteria. For *Streptococcus salivarius*, Gauthier et al. (10) have shown that many classes of 2DG-resistant mutants could be isolated, including those with *ptsH* and *ptsI* mutations. The pleiotropic effect on carbohydrate utilization in the mutant *L. sake* RV43 strongly suggests such a Pts^- phenotype. This phenotype was confirmed for glucose and fructose by the results obtained from carbohydrate phosphorylation experiments. We conclude that glucose, mannose, *N*-acetylglucosamine, fructose, and sucrose were transported by the PTS. In *L. sake* RV37, growth on mannose and *N*-acetylglucosamine was abolished, as was uptake of these carbohydrates. Enzyme II^{Man} would thus be responsible for the transport of both carbohydrates, as is the case in *E. coli* (9). Another permease specific for *N*-acetylglucosamine might be lacking in *L. sake*. Both the *L. sake* RV37 and *L. sake* RV43 mutants were impaired in glucose utilization, suggesting that glucose was transported by an enzyme II^{Man} and by a second, PTS-independent permease, as shown for other lactic acid bacteria (1, 29). For both mutants, the doubling time on glucose was increased and the growth yield was lowered, possibly because of a regulatory effect of the PTS. Transport of gluconate and ribose was inducible by gluconate and ribose, respectively. Growth on these two carbon sources was impaired in *L. sake* RV43, although phosphorylation experiments showed that ribose was not a PTS carbohydrate. The effect of the PTS on ribose and gluconate catabolism would thus be the consequence of regulation by some PTS component.

Further characterization of the PTS components, enzyme I and HPr, and of the catabolic enzymes involved in carbohydrate utilization in *L. sake* will be required to unravel the intricacies of these regulatory processes.

ACKNOWLEDGMENTS

We thank Françoise Bringel for the communication of the composition of the initial chemically defined medium, Hélène Cordier for help with mutant selection, Jean Labarre for the gift of labelled ribose, and Christian Valin for his constant encouragement.

REFERENCES

- Abe, K., and K. Uchida. 1990. Non-PTS uptake and subsequent metabolism of glucose in *Pediococcus halophilus* as demonstrated with a double mutant defective in phosphoenolpyruvate:mannose phosphotransferase system and in phosphofructokinase. *Arch. Microbiol.* **153**:537–540.
- Acton, J. C., R. L. Dick, and E. L. Norris. 1977. Utilization of various carbohydrates in fermented sausage. *J. Food Sci.* **42**:174–178.
- Berthier, F., M. Zagorec, M. Champomier-Vergès, S. D. Ehrlich, and F. Morel-Deville. 1996. Efficient transformation of *Lactobacillus sake* electroporation. *Microbiology* **142**:1181–1190.
- Bringel, F. Personal communication.
- Buckenhüskes, H. J. 1993. Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. *FEMS Microbiol. Rev.* **12**:253–272.
- Champomier, M. C., M. C. Montel, F. Grimont, and P. A. D. Grimont. 1987. Genomic identification of meat lactobacilli as *Lactobacillus sake*. *Ann. Inst. Pasteur/Microbiol. (Paris)* **138**:751–758.
- Chassy, B. M., and C. A. Alpert. 1989. Molecular characterization of the plasmid-encoded lactose-PTS of *Lactobacillus casei*. *FEMS Microbiol. Rev.* **63**:157–166.
- De Man, J. C., M. Rogosa, and M. E. Sharpe. 1960. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* **23**:130–135.
- Deutscher, J., J. Reizer, C. Fischer, A. Gallinier, M. H. Saier, Jr., and M. Steinmetz. 1994. Loss of protein kinase-catalyzed phosphorylation of HPr, a phosphocarrier protein of the phosphotransferase system, by mutation of the *ptsH* gene confers catabolite repression resistance to several catabolic genes of *Bacillus subtilis*. *J. Bacteriol.* **176**:3336–3344.
- de Vos, W. M., I. Boerrigter, R. J. van Rooyen, B. Reich, and W. Hengstenberg. 1990. Characterization of the lactose-specific enzymes of the phosphotransferase system in *Lactococcus lactis*. *J. Biol. Chem.* **265**:22554–22560.
- Erni, B., and B. Zanolari. 1985. The mannose-permease of the bacterial phosphotransferase system: gene cloning and purification of the enzyme II^{Man}/III^{Man} complex of *Escherichia coli*. *J. Biol. Chem.* **261**:15495–15503.
- Gauthier, L., S. Thomas, G. Gagnon, M. Frenette, L. Trahan, and C. Vadeboncoeur. 1994. Positive selection for resistance to 2-deoxyglucose gives rise, in *Streptococcus salivarius*, to seven classes of pleiotropic mutants, including *ptsH* and *ptsI* missense mutants. *Mol. Microbiol.* **13**:1101–1109.
- Grant, I. R., and M. F. Patterson. 1991. A numerical taxonomy study of lactic acid bacteria isolated from irradiated pork and chicken packaged under various gas atmospheres. *J. Appl. Bacteriol.* **70**:302–307.
- Hammes, W. P., A. Bantleon, and S. Min. 1990. Lactic acid bacteria in meat fermentation. *FEMS Microbiol. Rev.* **87**:165–174.
- Hueck, J. H., and W. Hillen. 1995. Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for the Gram-positive bacteria? *Mol. Microbiol.* **15**:395–401.
- Hugas, M., M. Garriga, T. Aymerich, and J. M. Monfort. 1993. Biochemical characterization of lactobacilli from dry fermented sausages. *Int. J. Food Microbiol.* **18**:107–113.
- Ledesma, O. V., A. P. De Ruiz, G. Oliver, G. S. De Giori, P. Raibaud, and J. V. Galpin. 1977. A synthetic medium for comparative nutritional studies of lactobacilli. *J. Appl. Bacteriol.* **42**:123–133.
- Liepe, H. P., E. Pfeil, and R. Porobic. 1990. Influence of sugar and bacteria on dry sausage souring. *Fleischwirtschaft* **70**:189–192.
- Liu, M. L., J. K. Kondo, M. B. Barnes, and D. T. Bartholomew. 1988. Plasmid-linked maltose utilization in *Lactobacillus* spp. *Biochimie* **70**:351–355.
- Montel, M. C., and M. C. Champomier. 1987. Arginine catabolism in *Lactobacillus sake* isolated from meat. *Appl. Environ. Microbiol.* **53**:2683–2685.
- Montel, M. C., and J. Labadie. 1984. Specific nutritional requirements of lactobacilli spp. from meat. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe B* **183**:1–7.
- Morishita, T., Y. Deguchi, M. Yajima, T. Sakurai, and T. Yura. 1981. Multiple requirements of lactobacilli: genetic lesions affecting amino acid biosynthetic pathways. *J. Bacteriol.* **148**:64–71.
- Obst, M., R. Hehn, R. F. Vogel, and W. P. Hammes. 1992. Lactose metabolism in *Lactobacillus curvatus* and *Lactobacillus sake*. *FEMS Microbiol. Lett.* **97**:209–214.
- Pandey, A., F. Bringel, and J. M. Meyer. 1994. Iron requirement and search for siderophores in lactic acid bacteria. *Appl. Microbiol. Biotechnol.* **40**:735–739.
- Poolman, B. 1993. Energy transduction in lactic acid bacteria. *FEMS Microbiol. Rev.* **12**:125–148.
- Postma, P. W. 1977. Galactose transport in *Salmonella typhimurium*. *J. Bacteriol.* **129**:630–639.
- Postma, P. W., J. W. Lengeler, and G. R. Jacobson. 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol. Rev.* **57**:543–594.
- Postma, P. W., and J. B. Stock. 1980. Enzymes II of the phosphotransferase system do not catalyze sugar transport in the absence of phosphorylation. *J. Bacteriol.* **141**:476–484.
- Reizer, J., A. Peterkovsky, and A. H. Romano. 1988. Evidence for the presence of heat-stable protein (HPr) and ATP-dependent HPr kinase in heterofermentative lactobacilli lacking phosphoenolpyruvate:glycose phosphotransferase activity. *Proc. Natl. Acad. Sci. USA* **85**:2041–2045.
- Vadeboncoeur, C., L. Gauthier, G. Gagnon, A. Leduc, D. Brochu, R. Lapointe, B. Desjardins, and M. Frenette. 1994. Properties of a *Streptococcus salivarius* spontaneous mutant in which the methionine at position 48 in the protein HPr has been replaced by a valine. *J. Bacteriol.* **176**:524–527.
- Veyrat, A., V. Monedero, and G. Pérez-Martínez. 1994. Glucose transport by the phosphoenolpyruvate:mannose phosphotransferase system in *Lactobacillus casei* ATCC 393 and its role in carbon catabolite repression. *Microbiology* **140**:1141–1149.
- Ye, J. J., J. W. Neal, X. Cui, J. Reizer, and M. H. Saier, Jr. 1994. Regulation of the glucose: H^+ symporter by metabolite-activated ATP-dependent phosphorylation of HPr in *Lactobacillus brevis*. *J. Bacteriol.* **176**:3484–3492.
- Ye, J. J., J. Reizer, X. Cui, and M. H. Saier, Jr. 1994. Inhibition of the phosphoenolpyruvate:lactose phosphotransferase system and activation of a cytoplasmic sugar-phosphate phosphatase in *Lactococcus lactis* by ATP-dependent metabolite-activated phosphorylation of serine 46 in the phosphocarrier protein HPr. *J. Biol. Chem.* **269**:11837–11844.