JOURNAL OF BACTERIOLOGY, July 1993, p. 3941–3948
0021-9193/93/133941-08$02.00/0

Vol. 175, No. 13

Pathway and Regulation of Erythritol Formation in *Leuconostoc oenos*

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Received 16 February 1993/Accepted 25 April 1993

It was recently observed that *Leuconostoc oenos* GM, a wine lactic acid bacterium, produced erythritol anaerobically from glucose but not from fructose or ribose and that this production was almost absent in the presence of O_2. In this study, the pathway of formation of erythritol from glucose in *L. oenos* was shown to involve the isomerization of glucose 6-phosphate to fructose 6-phosphate by a phosphoglucose isomerase, the cleavage of fructose 6-phosphate by a phosphoketolase, the reduction of erythrose 4-phosphate by an erythritol 4-phosphate dehydrogenase and, finally, the hydrolysis of erythritol 4-phosphate to erythritol by a phosphatase. Fructose 6-phosphate phosphoketolase was copurified with xylulose 5-phosphate phosphoketolase, and the activity of the latter was competitively inhibited by fructose 6-phosphate, with a K_i of 26 mM, corresponding to the K_m of fructose 6-phosphate phosphoketolase (22 mM). These results suggest that the two phosphoketolase activities are borne by a single enzyme. Extracts of *L. oenos* were also found to contain NAD(P)H oxidase, which must be largely responsible for the reoxidation of NADPH and NADH in cells incubated in the presence of O_2. In cells incubated with glucose, the concentrations of glucose 6-phosphate and of fructose 6-phosphate were higher in the absence of O_2 than in its presence, explaining the stimulation by anaerobiosis of erythritol production. The increase in the hexose 6-phosphate concentration is presumably the result of a functional inhibition of glucose 6-phosphate dehydrogenase because of a reduction in the availability of NADP.

The metabolism of glucose in heterolactic acid bacteria is described as fermentation initiated by the oxidation of glucose 6-phosphate to gluconate 6-phosphate (13, 31). Following oxidative decarboxylation of the latter, ribulose 5-phosphate is converted to xylulose 5-phosphate, which is then split into acetyl phosphate and glyceraldehyde 3-phosphate by a pentose-phosphate phosphoketolase present in all heterolactic acid bacteria (7). Subsequently, acetyl phosphate is converted to ethanol and/or acetate, whereas glyceraldehyde 3-phosphate is metabolized to lactate via pyruvate.

There are, however, considerable variations in the end products obtained from the metabolism of glucose in the presence of O_2 (3, 20, 23, 32) or other external electron acceptors (16, 19), indicating differences in the pathways and/or control of sugar metabolism among the heterolactic acid bacteria themselves. As confirmation of the extent of this diversity, it was observed that *Leuconostoc oenos* GM, a wine lactic acid bacterium described as metabolizing glucose heterofermentatively (7, 17), produced erythritol and glycerol in addition to the end products mentioned above (25, 28, 33). Moreover, it was found that the production of erythritol occurred anaerobically from glucose but not from fructose or ribose and that it was almost absent under aerobic conditions. ^{13}C nuclear magnetic resonance (NMR) experiments showed, as expected, that *L. oenos* formed ethanol and acetate from C-2 and C-3 of glucose when this sugar was metabolized in the presence of oxygen but that substantial proportions of these glycolytic products came from C-1 or C-2 under anaerobic conditions. In addition, erythritol was found to be derived from C-3 to C-6 of glucose. These results therefore indicated that, in the absence of oxygen, the C6 molecule was cleaved between carbons C-2 and C-3 as well as between carbons C-3 and C-4. For explanation of the former type of cleavage, the existence of a fructose 6-phosphate phosphoketolase in *L. oenos* was suggested (33).

The purposes of the present study were to establish the enzymatic pathway involved in the formation of erythritol in *L. oenos* and to explain the effect of oxygen on the regulation of the production of this polyol from glucose.

MATERIALS AND METHODS

Materials. 3-(*N*-morpholino)propanesulfonic acid (MOPS), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and Tris were from Sigma (St. Louis, Mo.), as were the substrates for all enzyme activities measured. DEAE-Sepharose Fast Flow was from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). Auxiliary enzymes and the remaining biochemical agents were from Boehringer (Mannheim, Germany). Chemicals were from Merck (Darmstadt, Germany) and were of analytical grade.

Commercial erythrose 4-phosphate was found to be contaminated with approximately 10% glucose 6-phosphate, which interfered in the assay of erythritol 4-phosphate dehydrogenase. This contaminant was eliminated as follows. A 1-ml solution containing 20 mM erythrose 4-phosphate was incubated at 30°C in the presence of 50 mM HEPES, (pH 7.1)–5 mM $MgCl_2$–10 mM NADP–10 U of yeast glucose 6-phosphate dehydrogenase. For removal of the nucleotides, activated charcoal was added to a final concentration of 2% (wt/vol) after 90 min; these preparations were then incubated

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for 20 min at 30°C and centrifuged twice at 10,000 × *g* for 5 min to remove the charcoal.

Organisms and growth conditions. *L. oenos* GM was obtained from Microlife Techniques, Sarasota, Fla., and *L. oenos* ATCC 23277 was obtained from the American Type Culture Collection, Rockville, Md. *L. oenos* 8A and *L. oenos* CTQB M3 were isolated from Portuguese table wines; the former was supplied by A. Mendes Faia (Universidade de Vila Real e Tras-os-Montes, Vila Real, Portugal), and the latter was supplied by V. San Romão (Centro de Tecnologia Quimica e Biológica, Oeiras, Portugal). *Leuconostoc lactis* NCW1 was received from T. Cogan (National Dairy Products Research Center, Fermoy, Ireland). *Lactobacillus buchneri* B190 and *Lactobacillus brevis* B22 were both received from F. Radler (Institüt für Mikrobiologie und Weinforschung der Johannes Guttenberg, University of Mainz, Mainz, Germany).

All strains were grown at 30°C in static 2-liter glass bottles. *L. oenos* strains were grown in modified (33) FT 80 medium (2), (pH 5.0). *L. lactis* NCW1 was grown under the same conditions but without malic acid. *L. brevis* B22 and *L. buchneri* B190 were grown in a similar way with a complex medium containing yeast extract (15.0 g · liter^{-1}), triammonium citrate (4.8 g · liter^{-1}), and $MgSO_4 \cdot 7H_2O$ (0.1 g · liter^{-1}) in 0.1 M potassium phosphate buffer (pH 6.5). Glucose (10 g · liter^{-1}) was autoclaved separately and added to the above-mentioned medium before inoculation. $MnSO_4 \cdot 4H_2O$ was added to the growth medium for the cultures grown for the ^{13}C NMR experiments and for the enzyme assays at concentrations of 1.0 and 3.0 mg · liter^{-1}, respectively. The cells used for ^{31}P NMR analysis were grown without added manganese.

Preparation of culture supernatants for the quantification of erythritol, glycerol, and ethanol by ^{13}C NMR analysis. Fresh cell suspensions were prepared for each experiment. Cultures in the mid-logarithmic phase (three 2-liter cultures) were harvested by centrifugation (20 min at 3,000 × *g*) at 4°C, and the pellet was washed twice with 0.2 M potassium phosphate (pH 5.0) and suspended in the same buffer to a final volume of approximately 8 ml. This cell suspension (two 4-ml aliquots, each corresponding to 0.4 to 0.5 g [dry weight]) was incubated at 30°C in the presence of 100 μmol of glucose under O_2 or N_2 flushing. After approximately 2.5 to 3 h, once the substrate was exhausted, the cells were removed by centrifugation at 18,000 × *g* for 20 min and the liquid supernatant was transferred to a 10-mm NMR tube. ^2H_2O was added to a final concentration of approximately 5% (vol/vol) to provide a lock signal. The end products formed were quantified by ^1H NMR and ^{13}C NMR as previously described (33).

Preparation of perchloric acid extracts. The bacterial strains were grown as described above (6 to 8 liters of culture per extract) and harvested as for the ^{13}C NMR experiments by use of 50 mM potassium phthalate buffer at pH 5.0. After being washed, the cells were resuspended in a final volume of 6 to 7 ml of buffer, and the suspension was transferred to a test tube placed in a water bath at 30°C. After the addition of an adequate amount of fermentation substrate (300 μmol), the cell suspension was flushed with O_2 or N_2 for approximately 45 min to allow only part of the sugar to be metabolized. For minimization of the changes in intracellular metabolites, the cells were frozen immediately by dispersal of the suspension in liquid N_2 with a syringe. These aggregates of frozen cells were collected and used for $HClO_4$ extraction as described previously (14). The extract (approximately 100 ml) was lyophilized, resuspended in 3.0 ml of H_2O containing 5% (vol/vol) ^2H_2O, and centrifuged to remove the precipitated $KClO_4$. The supernatant was removed, and EDTA was added to a final concentration of 4 mM to chelate any paramagnetic ions. The pH was adjusted to exactly 8.0, and the extract was analyzed by ^{31}P NMR. The same extract was also used for the enzymatic quantification of intermediary metabolites. Approximately 1.0 g (dry weight) of cells was used per extract.

NMR spectroscopy. ^{13}C NMR spectra of the supernatant were recorded at 30°C in a Bruker AMX-300 spectrometer operating at 75.47 MHz. They were run with a 45° pulse, a 13-s repetition time, and 32,000 acquisition datum points; a 10-mm broad-band probe was used. Proton broad-band decoupling was applied during the acquisition time only. ^1H NMR spectra were recorded at 25°C in a Bruker AMX-500 spectrometer operating at 500.13 MHz, with H_2O presaturation, a 45° pulse, and a repetion time of 9.5 s. ^{31}P NMR spectra were acquired at 30°C in the same spectrometer with a 45° pulse, a repetition time of 8.8 s, and proton broad-band decoupling during the acquisition time (0.8 s).

Enzyme assays. *L. oenos* GM cells were collected as described above from 3- to 4-liter cultures, washed twice with 100 mM MOPS (pH 6.5), and kept at −70°C until used. For preparation of the extract, the pellet was resuspended in the same buffer at a concentration of approximately 0.06 g (dry weight) · ml^{-1}. The cells were disrupted by ultrasonication, and debris was removed by centrifugation before the enzymes in the extract were assayed.

Enzyme activities were measured spectrophotometrically at 30°C in the presence of 25 mM HEPES (pH 7.1) with 3 mM $MgCl_2$. Unless otherwise stated, the concentrations of substrate and, if required, of auxiliary enzymes were as follows: for glucose 6-phosphate dehydrogenase, 5 mM glucose 6-phosphate and 1 mM NAD(P); for 6-phosphogluconate dehydrogenase, 5 mM 6-phosphogluconate and 1 mM NAD(P); for phosphoglucose isomerase, 0.01 to 0.2 mM fructose 6-phosphate, 2 U of yeast glucose 6-phosphate dehydrogenase, and 1 mM NADP; for triose phosphate isomerase, 0.05 to 0.8 mM glyceraldehyde 3-phosphate, 2 U of rabbit muscle glycerol 3-phosphate dehydrogenase, and 0.17 mM NADH; for glycerol 3-phosphate dehydrogenase, 0.1 to 5.0 mM dihydroxyacetone phosphate, 10 U of rabbit muscle triose phosphate isomerase, and 0.17 mM NAD(P)H; for erythritol 4-phosphate dehydrogenase, 0.13 to 1.0 mM erythrose 4-phosphate and 0.17 mM NAD(P)H; for lactate dehydrogenase, 0.1 to 1.5 mM pyruvate and 0.17 mM NAD(P)H; for mannitol dehydrogenase, 50 mM fructose and 0.17 mM NAD(P)H; for alcohol dehydrogenase, 0.001 to 0.3 mM acetaldehyde and 0.17 mM NAD(P)H; and for acetaldehyde dehydrogenase (acylating), 0.25 to 3.0 mM acetyl coenzyme A and 0.17 mM NAD(P)H. When required, appropriate blanks with pyridine nucleotides but without substrate were subtracted.

The activities of the phosphatases were determined by measuring the P_i formed during incubation (30°C) of the cell extract (approximately 0.3 mg of protein · ml^{-1}) with various phosphorylated substrates at a concentration of 4 mM and in the presence of 25 mM HEPES (pH 7.1) containing 5 mM $MgCl_2$. The reaction was stopped (after 30, 60, and 90 min of incubation) by mixing 0.25-ml samples with 0.5 ml of 10% trichloroacetic acid. The phosphate formed was measured colorimetrically as described previously (6).

Xylulose 5-phosphate phosphoketolase activity was measured by assay of glyceraldehyde 3-phosphate or acetyl phosphate formed. Except as otherwise indicated, the concentrations of substrates and the cofactor were 10 mM for

TABLE 1. Purification of xylulose 5-phosphate phosphoketolase from *L. oenos*[a]

Purification step	Total activity (μmol · min^{-1}) with:		Sp act (μmol · min^{-1} · mg of protein^{-1}) with:		Activity ratio (%)
	Xylulose 5-phosphate	Fructose 6-phosphate	Xylulose 5-phosphate	Fructose 6-phosphate	
Crude cell extract	113	8.1	0.50	0.030	6.0
Polyethylene glycol	103	6.1	0.75	0.044	5.9
DEAE-Sepharose	60	3.6	2.3	0.144	6.2

[a] The activity with both substrates was measured with 10 mM substrate. The DEAE-Sepharose fractions were preincubated for 30 min at 30°C with 1 mM thiamine PP_i and 2 mM P_i before the assays.

xylulose 5-phosphate, 50 mM for P_i, and 1 mM for thiamine PP_i. Glyceraldehyde 3-phosphate production was measured spectrophotometrically by isomerization to dihydroxyacetone phosphate; this step was followed by the NADH-coupled reduction of the latter to glycerol 3-phosphate. The reaction mixture was as described for method B in reference 5 and also contained 0.17 mM NADH, 2 U of triose phosphate isomerase, and 10 U of glycerol 3-phosphate dehydrogenase, both from rabbit muscle. The reaction was started by the addition of the extract. The acetyl phosphate formed was assayed by the ferric acetyl hydroxamate method (8) with exactly the same reaction mixture as that used above but without NADH and auxiliary enzymes. The production of acetyl phosphate was stopped after 15 min by the addition of hydroxylamine. This second method was always used to determine the activity of fructose 6-phosphate phosphoketolase, while the first one was preferentially used to measure the reaction with xylulose 5-phosphate. The concentration of fructose 6-phosphate in the assay mixture was 10 mM.

NAD(P)H oxidase activity was determined by measuring the change in the A_{340} with a conventional spectrophotometer or by measuring the consumption of O_2 with an oxygen electrode. The assay conditions chosen were the same those described previously (15).

Except for NAD(P)H oxidase, which had to be measured in freshly prepared extracts, all enzyme activities mentioned above withstood storage at −20°C. One unit of enzyme activity was defined as the amount catalyzing the formation of 1 μmol of product · min^{-1} under the assay conditions stated. For comparison of rates measured in vivo and in vitro, the following correlations were used: 1 g of cells (wet weight) corresponds to 100 mg of soluble protein and 0.39 g (dry weight).

Measurement of intermediary metabolites. The following intermediary metabolites were quantified by enzymatic methods: glucose 6-phosphate and fructose 6-phosphate (21), xylulose 5-phosphate (27), dihydroxyacetone phosphate (22), L-(−)-glycerol 3-phosphate (18), and erythrose 4-phosphate (24).

Other assays. Protein was determined as described previously (1) with bovine gamma globulin as a standard.

Purification of pentose 5-phosphate phosphoketolase. A cell extract (from 2.5 g [wet weight] of cells) was prepared in 100 mM MOPS as described above. Following the addition of KCl (150 mM final) and adjustment of the pH to 6.0, the extract (8.5 ml; 236 mg of protein) was gently mixed with 10% (wt/vol) polyethylene glycol 6000 for 20 min at 4°C. The resulting preparation was centrifuged for 15 min at 10,000 × *g*. The pellet was discarded, and polyethylene glycol was added to the supernatant to a final concentration of 20% (wt/vol). After mixing and centrifugation were done as described above, the final pellet was dissolved in 4.3 ml of 25 mM Tris-HCl buffer (pH 7.5) and applied to a DEAE-Sepharose Fast Flow column (1.6 by 14 cm) equilibrated with the same buffer. The column was washed with 20 ml of buffer, and the retained protein was eluted with a KCl gradient (50 to 500 mM in two 50-ml portions of the equilibration buffer).

RESULTS

Phosphoketolase activities in *L. oenos*. Extracts of *L. oenos* were found to catalyze the formation of acetyl phosphate from 10 mM fructose 6-phosphate and 10 mM xylulose 5-phosphate at rates of 0.03 and 0.5 μmol · min^{-1} · mg of protein^{-1}, respectively; both activities were dependent on the presence of P_i and thiamine PP_i. Furthermore, they were copurified upon polyethylene glycol fractionation and chromatography on DEAE-Sepharose, their ratio remaining constant (Table 1). Pooled fractions from the DEAE-Sepharose step (10 ml with 2.6 mg of protein ml^{-1}) contained pentose phosphoketolase purified approximately fivefold. This preparation was free of glucose 6-phosphate dehydrogenase and phosphoglucose isomerase.

The activity of purified xylulose 5-phosphate phosphoketolase could be determined by measurement of the formation of acetyl phosphate or glyceraldehyde 3-phosphate, with identical results. The K_m values of the partially purified preparation were 22 mM for fructose 6-phosphate and 1.6 mM for xylulose 5-phosphate, and the respective V_{max} values were 0.48 and 2.33 U · mg of protein^{-1}.

No acetyl phosphate was formed when the purified enzyme was incubated with 10 mM glucose 6-phosphate or 6-phosphogluconate, and neither of these sugar phosphates inhibited the activity measured with xylulose 5-phosphate as the substrate. However, fructose 6-phosphate was found to be a competitive inhibitor for xylulose 5-phosphate phosphoketolase activity, with a K_i of 26 mM.

Characterization of other enzymes of glucose catabolism. Table 2 lists the activities and some kinetic properties of various enzymes involved in the metabolism of glucose in *L. oenos*. Glucose 6-phosphate dehydrogenase displayed an 80-fold lower K_m for NADP than for NAD, whereas 6-phosphogluconate dehydrogenase was NADP specific. The former enzyme was approximately fivefold more active than the latter. Alcohol dehydrogenase displayed a much higher affinity for NADPH than for NADH, and acetaldehyde dehydrogenase (acylating) was NADPH specific and approximately 100-fold less active than alcohol dehydrogenase. Mannitol dehydrogenase displayed a sevenfold higher affinity for NADH than for NADPH, with similar V_{max} values with the two nucleotides. In contrast, glyceraldehyde

TABLE 2. Activities of some enzymes involved in sugar metabolism by *L. oenos*[a]

Enzyme	Substrate	Cofactor	K_m for substrate (mM)	V_{max} (μmol · min^{-1} · mg^{-1})	K_m for cofactor (μM)
Glucose 6-phosphate dehydrogenase	Glucose 6-phosphate	NAD	1.60	1.27	500
		NADP	0.09	0.53	6.5
6-Phosphogluconate dehydrogenase	6-Phosphogluconate	NAD		No reaction	
		NADP	0.16	0.13	13
Phosphoglucose isomerase	Fructose 6-phosphate		0.10	0.46	
Triose phosphate isomerase	Glyceraldehyde 3-phosphate		0.56	0.33	
Glycerol 3-phosphate dehydrogenase	Dihydroxyacetone phosphate	NADPH	4.0	0.42	34
		NADH		No reaction	
Erythritol 4-phosphate dehydrogenase	Erythrose 4-phosphate	NADPH	0.56	0.025	
		NADH		No reaction	
Lactate dehydrogenase	Pyruvate	NADPH		No reaction	
		NADH	1.25	10.9	
Mannitol dehydrogenase	Fructose	NADPH	20	0.010	40
		NADH	21	0.012	6
Alcohol dehydrogenase	Acetaldehyde	NADPH	<0.01	0.82	
		NADH	0.077	0.23	
Acetaldehyde dehydrogenase	Acetyl coenzyme A	NADPH	2.5	0.007	
		NADH		No reaction	

[a] The measurements were made with crude cell extracts. All determinations were made in duplicate with at least two different extracts.

3-phosphate dehydrogenase (not shown) and lactate dehydrogenase were NAD specific.

The extracts also catalyzed the oxidation of NADPH, although not that of NADH, in the presence of dihydroxyacetone phosphate, indicating the presence of an NADPH-specific glycerol 3-phosphate dehydrogenase. Five- to sixfold lower activity was observed when glyceraldehyde 3-phosphate was used, but this result was presumably due to the conversion of this substrate to dihydroxyacetone phosphate by triose phosphate isomerase, which was also present in the extracts. These were also found to contain an enzyme able to oxidize NADPH, although not NADH, in the presence of erythrose 4-phosphate. The activity of this enzyme was linearly related to the concentrations of the extracts in the assay (not shown), and the oxidation of NADH was also found to occur without delay (<10 s) following the addition of erythrose 4-phosphate, indicating that the latter did not need to be isomerized to erythrulose 4-phosphate before being reduced. Fivefold lower NADPH-dependent dehydrogenase activity with approximately 30-fold higher K_m was observed when 1 mM erythrose was used as the substrate.

Extracts were also found to catalyze the hydrolysis of the following phosphate esters (all tested at a concentration of 4 mM): erythritol 4-phosphate (7.3 nmol · min^{-1} · mg of protein^{-1}), erythrose 4-phosphate (3.1 nmol · min^{-1} · mg of protein^{-1}), DL-glyceraldehyde 3-phosphate (12.7 nmol · min^{-1} · mg of protein^{-1}), and L-(+)-glycerol 3-phosphate (1.2 nmol · min^{-1} · mg of protein^{-1}). No activity was observed with glucose 6-phosphate, fructose 6-phosphate, or sorbitol 6-phosphate.

NADH oxidase activity in cell extracts of *L. oenos*. The oxidation of NADH and NADPH by NAD(P)H oxidase was also determined spectrophotometrically in freshly prepared cell extracts by measuring a decrease in the A_{340} and polarographically by measuring the consumption of O_2, with identical results. Activities of 21, 22, and 22 nmol · min^{-1} · mg of protein^{-1} were recorded at 0.1, 0.5, and 1 mM NADH, respectively, and activities of 5, 12, and 13 nmol · min^{-1} · mg of protein^{-1} were recorded at the same concentrations of NADPH. Thus, the enzyme was saturated with the lowest concentration of NADH (0.1 mM) used, whereas the K_m for NADPH was estimated to be approximately 0.3 mM. The calculated V_{max} values were similar for the two nucleotides, and the activity was lost after storage of the extracts at −20°C.

Concentrations of phosphorylated intermediates during sugar catabolism. The concentrations of several metabolites were determined enzymatically with neutralized perchloric acid extracts obtained from cell suspensions metabolizing glucose under continuous flushing with N_2 or O_2 and fructose or ribose under N_2 flushing (Table 3). During glucose metabolism, the concentrations of glucose 6-phosphate and fructose 6-phosphate were approximately 10-fold higher anaerobically than in the presence of O_2. In contrast, the concentration of xylulose 5-phosphate was about threefold higher aerobically than anaerobically.

The replacement of glucose by fructose or ribose in the anaerobic cell suspensions resulted in a decrease in the intracellular levels of hexose 6-phosphates and in the disappearance of glycerol 3-phosphate. The concentration of xylulose 5-phosphate was higher in the presence of ribose than in the presence of fructose or glucose, at least during O_2 deprivation. Dihydroxyacetone phosphate and erythrose 4-phosphate were not detected in these extracts, possibly because of their lability.

TABLE 3. Concentrations of hexose 6-phosphates, xylulose 5-phosphate, and glycerol 3-phosphate in *L. oenos* maintained under erythritol-producing and non-erythritol-producing conditions

Phosphorylated metabolite	Concn (μmol · g [dry weight]$^{-1}$) under the following conditions:			
	Glucose and O_2	Glucose and N_2	Fructose and N_2	Ribose and N_2
Glucose 6-phosphate	0.36	3.71	0.24	0.15
Fructose 6-phosphate	0.10	0.85	0.03	0.02
Xylulose 5-phosphate	0.33	0.10	0.03	0.27
Glycerol 3-phosphate	0.85	0.55	0.00	0.00

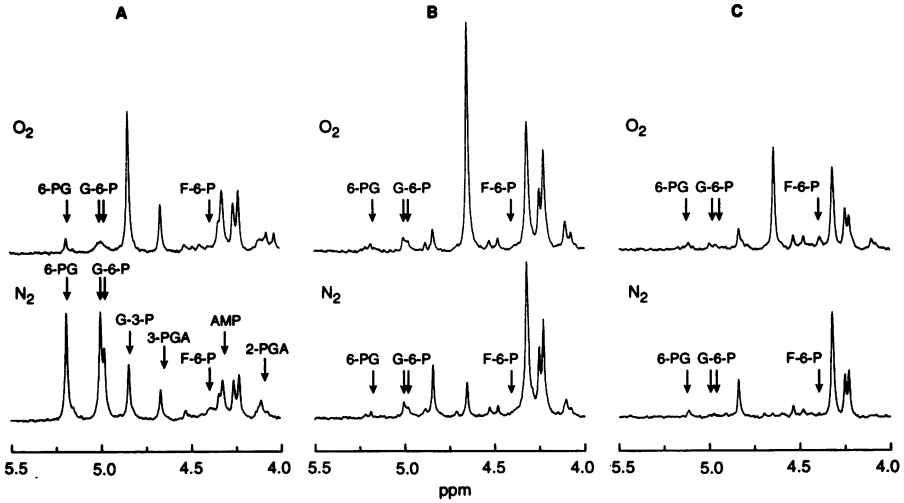

FIG. 1. ^{31}P NMR spectra of perchloric acid extracts of cell suspensions of *L. oenos* ATCC 23277 (A), *L. buchneri* B190 (B), and *L. lactis* NCW1 (C) metabolizing glucose under an O_2 or an N_2 atmosphere. The assigned resonances, 6-phosphogluconate (6-PG) at 5.19 ppm, glucose 6-phosphate (G-6-P) at 4.99 and 4.97 ppm, glycerol 3-phosphate (G-3-P) at 4.83 ppm, 3-phosphoglycerate (3-PGA) at 4.66 ppm, fructose 6-phosphate (F-6-P) at 4.39 ppm, AMP at 4.32 ppm, and 2-phosphoglycerate (2-PGA) at 4.10 ppm, were made by adding the pure compounds to the extracts. The unassigned resonances did not correspond to erythrose 4-phosphate at 5.11 ppm, xylulose 5-phosphate at 4.78 ppm, ribulose 5-phosphate at 4.81 ppm, acetyl phosphate at −1.55 ppm, or phosphoenolpyruvate at 4.23 ppm.

^{31}P NMR analysis of the same extracts confirmed the effect of O_2 deprivation on the concentrations of glucose 6-phosphate and fructose 6-phosphate and, in addition, revealed a clear accumulation of 6-phosphogluconate during the anaerobic (but not during the aerobic) metabolism of glucose (Fig. 1A). In the absence of O_2, 6-phosphogluconate was not detected during the metabolism of fructose or ribose (results not shown).

Ability of various heterolactic acid bacteria to produce erythritol and their intracellular levels of glucose 6-phosphate and fructose 6-phosphate. Natural-abundance ^{13}C NMR was used to investigate the influence of aeration on the production of erythritol and/or glycerol from glucose by three other strains of *L. oenos* (*L. oenos* ATCC 23277, *L. oenos* CTQB M3, and *L. oenos* 8A), one strain of *L. lactis* (NCW1), and two *Lactobacillus* strains (*L. brevis* B22 and *L. buchneri* B190). The spectra of the end products formed anaerobically from glucose (100 μmol) by these organisms showed that *L. oenos* was the only one that produced erythritol (Table 4). In all cases, erythritol production from glucose was stimulated by the absence of oxygen, whereas the production of glycerol was reduced. It is also apparent that the strains that did not produce erythritol formed up to eightfold more ethanol than the erythritol-producing strains under anaerobic conditions.

Perchloric acid extracts of the same bacteria, obtained during the metabolism of glucose in the presence or in the absence of oxygen, were analyzed by ^{31}P NMR (Fig. 1). Inspection of the spectra corresponding to *L. oenos* ATCC 23277 (Fig. 1A), GM, 8A, or CTQB M3 indicated that the concentrations of glucose 6-phosphate, fructose 6-phosphate, and 6-phosphogluconate were severalfold higher in the absence of O_2 than in its presence. In contrast, the resonances corresponding to the above-listed metabolites were very small, if at all detectable, in other species, such as *L. buchneri* B190 (Fig. 1B) and *L. lactis* NCW1 (Fig. 1C), which did not form erythritol.

DISCUSSION

Pathway of erythritol formation. Our results show that *L. oenos* is able to cleave fructose 6-phosphate via phosphoketolase, resulting in the production of acetyl phosphate and erythrose 4-phosphate. Fructose 6-phosphate phosphoketolase was copurified with xylulose 5-phosphate phosphoketolase. Furthermore, the activity of the latter was competi-

TABLE 4. Aerobic and anaerobic production of erythritol, glycerol, and ethanol from glucose by nongrowing cultures of various lactic acid bacteria[a]

Bacterial strain	Aeration conditions	Glycerol (μmol)	Erythritol (μmol)	Ethanol (μmol)
L. oenos GM[b]	O_2	37	0	1
	N_2	12	37	10
L. oenos 8A	O_2	10	5	8
	N_2	0	30	45
L. oenos ATCC 23277	O_2	27	0	0
	N_2	16	14	22
L. oenos CTQB M3	O_2	35	5	8
	N_2	5	16	38
L. lactis NCW1	O_2	7	0	0
	N_2	7	0	72
L. buchneri B190	O_2	0	0	0
	N_2	0	0	77
L. brevis B22	O_2	0	0	0
	N_2	0	0	80

[a] After glucose (100 μmol) consumption, the cells were removed from the suspension and the end products were analyzed by ^{13}C NMR spectroscopy as described in Materials and Methods.

[b] Data are from reference 33.

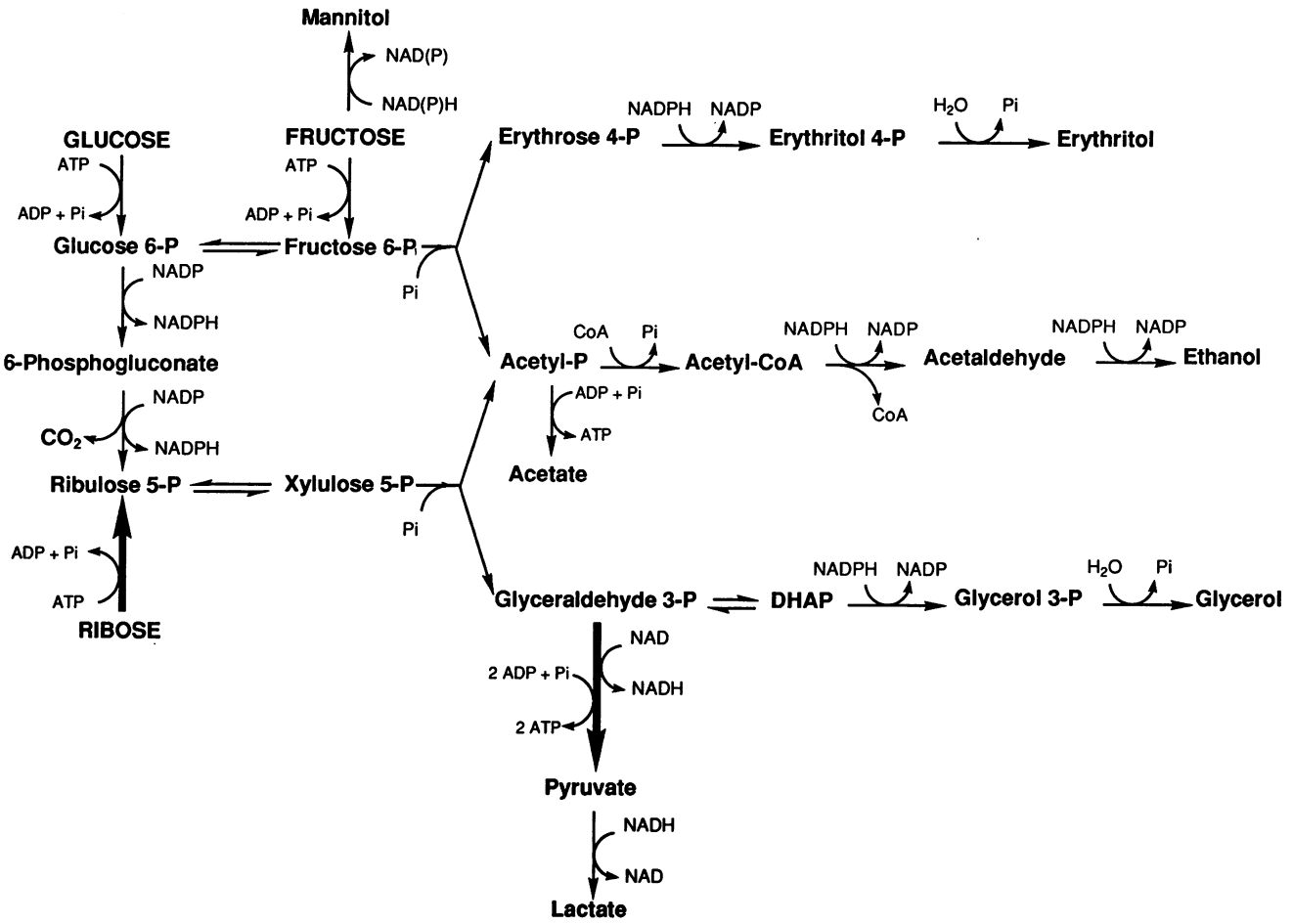

FIG. 2. Pathway of glucose metabolism and erythritol formation in *L. oenos*.

tively inhibited by fructose 6-phosphate, with a K_i corresponding to the K_m of fructose 6-phosphate phosphoketolase. These results suggest but do not prove that the two phosphoketolase activities are borne by a single enzyme with a much higher affinity for pentulose phosphate than for hexulose phosphate. The existence of a nonspecific phosphoketolase has been described for *Leuconostoc mesenteroides* (8), *Lactobacillus plantarum* (11), *Acetobacter xylinum* (26, 29), *Bifidobacterium bifidum* (4), and *Bifidobacterium globosum* (30), whereas separate xylulose 5-phosphate and fructose 6-phosphate phosphoketolases have been described for *L. mesenteroides* (12), *Bifidobacterium dentium* (30), *L. plantarum* (10), *Thiobacillus novellus* (9), and various yeast species (5, 34).

If we assume that the fructose 6-phosphate phosphoketolase activity measured with 10 mM substrate is 0.032 $\mu mol \cdot min^{-1} \cdot mg$ of $protein^{-1}$ in cell extracts, we can calculate that the V_{max} is close to 0.1 $\mu mol \cdot min^{-1} \cdot mg$ of $protein^{-1}$, which corresponds to 10 $\mu mol \cdot min^{-1} \cdot g$ (wet weight)$^{-1}$. For *L. oenos* GM, in the absence of oxygen, the rate of erythritol production represents 37% of the rate of glucose consumption (1.4 $\mu mol \cdot min^{-1} \cdot g$ [wet weight]$^{-1}$) (33), i.e., 0.51 $\mu mol \cdot min^{-1} \cdot g$ (wet weight)$^{-1}$. Therefore, the rate of erythritol production measured in whole cells can account for erythritol biosynthesis in vivo.

On the basis of these results, we propose the pathway shown in Fig. 2 for the formation of erythritol from glucose 6-phosphate by *L. oenos*. It involves the isomerization of glucose 6-phosphate to fructose 6-phosphate by a phosphoglucose isomerase, the cleavage of fructose 6-phosphate by a phosphoketolase, the reduction of erythrose 4-phosphate by an erythritol 4-phosphate dehydrogenase and, finally, the hydrolysis of erythritol 4-phosphate to erythritol by a phosphatase. Although we cannot exclude the possibility that some erythrose 4-phosphate is dephosphorylated before it is reduced, the results obtained indicate that the sequence of reactions proposed in Fig. 2 is the main one. In fact, (i) the rate of erythritol 4-phosphate hydrolysis in extracts was more than twice as high as the rate of erythrose 4-phosphate hydrolysis, and (ii) the dehydrogenase acting on erythrose 4-phosphate was much more active than the enzyme acting on erythrose. If we assume that the acetyl phosphate formed during fructose 6-phosphate phosphoketolase cleavage is converted to ethanol and P_i, this pathway allows for the disposal of 3 mol of NADPH per mol of glucose consumed, at the expense of one high-energy bond used for the phosphorylation of glucose. If instead acetate is produced, only 1 mol of NADPH is consumed, and the ATP used for the phosphorylation of glucose is regenerated by acetate kinase.

Mechanism of electron disposal. On the basis of the specificity of dehydrogenases, it appears that 1 mol of NADH and 2 mol of NADPH are produced when 1 mol of glucose is converted to CO_2, acetyl phosphate, and pyruvate. In theory, the NADPH formed during the initial steps of glucose

oxidation could be recycled via the reduction of acetyl coenzyme A to ethanol, while the NADH formed by glyceraldehyde 3-phosphate dehydrogenase could be reoxidized by lactate dehydrogenase. In both cases, a perfect balance between the production and the consumption of reducing equivalents would be expected. However, the activity of acetaldehyde dehydrogenase (acylating) is more than 20 times lower than those of the NADPH-producing enzymes, and it is likely that this low activity prevents NADPH from being readily recycled by this pathway.

Other enzymes involved in the recycling of NADPH are (i) NAD(P)H oxidase; (ii) mannitol dehydrogenase, which converts fructose (if added to the medium) to mannitol; (iii) glycerol 3-phosphate dehydrogenase; and (iv) erythrose 4-phosphate dehydrogenase. In the presence of oxygen, the oxidation of NADPH by molecular oxygen is an important route for the disposal of NADPH, as indicated by the imbalance between the reduced and the oxidized products of glucose metabolism. Thus, Veiga-da-Cunha et al. (33) showed that in the presence of oxygen, only 0.37 mol of glycerol and 0.01 mol of ethanol were formed per mol of glucose, accounting for the recycling of only about 20% of the NADPH formed during glucose dissimilation. The remaining NADPH was presumably reoxidized by NAD(P)H oxidase. The activity of the latter (13 nmol · min^{-1} · mg of $protein^{-1}$) entirely accounted for the rate of NADH oxidation (10.3 nmol · min^{-1} · mg of $protein^{-1}$) required to explain the consumption of glucose by the aerated cell suspensions (6.3 nmol · min^{-1} · mg of $protein^{-1}$).

In the presence of fructose, reducing equivalents can be transferred to a large extent to this substrate, as indicated by the fact that mannitol is the major end product of fructose metabolism in *L. oenos* (33).

Control of erythritol production. The effect of anaerobiosis on the production of erythritol can be explained as follows. When *L. oenos* metabolizes glucose in the absence of O_2, the oxidation of NADPH is difficult because of the low activity of acetaldehyde dehydrogenase (acylating). As a result, the NADPH/NADP ratio is high, leading to the inhibition of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. This inhibition in turn results in the intracellular accumulation of glucose 6-phosphate and fructose 6-phosphate and in a decrease in the concentrations of later intermediates, such as xylulose 5-phosphate. The increase in the concentration of fructose 6-phosphate and the decrease in that of xylulose 5-phosphate allow phosphoketolase to cleave the former, leading to the formation of erythrose 4-phosphate and ultimately to erythritol biosynthesis. This explanation is consistent with the observation that the erythritol-producing bacteria were the only ones in which the concentration of hexose 6-phosphate increased in the absence of O_2. In this respect, it is interesting to note that the non-erythritol- and non-glycerol-producing lactic acid strains formed more ethanol under anaerobic conditions than the erythritol-producing strains, possibly because the former have a more active acetaldehyde dehydrogenase (acylating).

This interpretation of the results also accounts for the fact that erythritol is not formed when fructose is the fermentation substrate, even under anaerobic conditions, because fructose can serve as an effective electron acceptor for NADPH, hence preventing an increase in the hexose 6-phosphate concentration. The lack of erythritol production from ribose is explained by the fact that this sugar is a pentose and therefore does not have to go through the oxidation-decarboxylation steps to become a suitable substrate for xylulose 5-phosphate phosphoketolase.

ACKNOWLEDGMENTS

We are grateful to H. G. Hers for helpful suggestions during the preparation of the manuscript.

This work was supported by the Biotechnology (BRIDGE) Program, contract BIOT-CT91-0263, of the Commission of the European Communities; by the Junta Nacional de Investigação Científica e Tecnológica, Lisbon, Portugal; by the Fonds de la Recherche Scientifique Médicale; and by the Prime Minister's Office Science Policy Programming. E. Van Schaftingen is Maître de Recherches of the Fonds National de la Recherche Scientifique.

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