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Biochemistry

New pathway for the metabolism of pentitols

(pentitol phosphate/lactobacilli)

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ABSTRACT **Certain strains of *Lactobacillus casei* can grow at the expense of one or more pentitols. These microorganisms possess a pentitol phosphate pathway that appears to be analogous to the hexitol phosphate pathway found in many facultatively anaerobic bacteria. Pentitol is transported into the cell by a phospho*enol*pyruvate phosphotransferase system that converts it to pentitol phosphate, whereupon a specific dehydrogenase oxidizes the intermediate product to ketopentose phosphate. The ketopentose phosphate is subsequently converted to xylulose-5-*P* and enters one of the pathways of central metabolism.**

Only a limited number of bacteria and fungi are able to utilize naturally occurring and rare pentitols as growth substrates. In a recent review, Mortlock described the catabolic pathways by which these microorganisms dissimilate pentoses and pentitols (1). Although the dehydrogenases and kinases that participate in pentitol catabolism differ from species to species in their specificity for substrate or substrate and cofactor, respectively, the sequence of enzymatic steps is the same (1–3). Pentitols are transported into the cells and oxidized to their corresponding 2-ketopentoses by an NAD- or NADP-dependent dehydrogenase. An appropriate kinase phosphorylates the ketopentose, producing ketopentose 5-phosphate, and, when the original substrate is ribitol or L-arabitol, the phosphorylated ketopentose undergoes one or more epimerization reactions whereby xylulose-5-*P* is formed. At this point, pentitol carbon enters one of the major carbohydrate dissimilitory pathways.

Recently, Rogosa (4) observed that certain homofermentative lactobacilli are capable of growing at the expense of one or two of the following four polyhydric alcohols: xylitol, ribitol, D-arabitol, and L-arabitol. A cursory examination of the pentitol dehydrogenase activities in cell-free extracts of xylitol- or ribitol-grown cells of *Lactobacillus casei* strains revealed that these enzymes were markedly different from those previously studied. This report describes a novel pathway for the metabolism of ribitol and xylitol that is, as far as is presently known, unique to the lactic acid bacteria.

MATERIALS AND METHODS

Bacteria. *Lactobacillus casei* strains 64H (ribitol-utilizing), CL-16 (ribitol-utilizing), and CL-83 (xylitol- and D-arabitol-utilizing) from the National Institute of Dental Research culture collection were grown in screwcap bottles containing 400 ml of *Lactobacillus* carrying medium (5) supplemented with 0.5% (wt/vol) ribitol or xylitol, respectively; in several experiments, 0.5% D-arabitol was substituted for xylitol. Following 20- to 24-hr incubation at 37°, the cultures were harvested and washed twice with 0.02 M sodium-phosphate-buffered normal saline, pH 7.0. Cell pellets to be used for intact cell transport studies were adjusted to a density equivalent to 15–20 mg of protein per ml with 0.01 M potassium phosphate buffer, pH 7.0. Protein contents of such suspensions were determined by the method of La Rivière (6). Cell extracts for enzyme assays were prepared by suspending cell pastes (2–4 g wet weight) in 10 ml of potassium phosphate buffer containing 10 mM 2-mercaptoethanol and treating them with an icewater-cooled Branson model 350 ultrasonic cell disrupter operating at 175 W for 10 min. The crude extract was centrifuged for 20 min at 20,000 × *g* and the clarified supernatant fluid was decanted and assayed for enzyme activity. Protein content of cell extracts was measured by the biuret technique (7).

Enzyme Assays. Pentitol phosphate dehydrogenase activity was measured by following the rate of NADH oxidation at 340 nm in a Gilford model 2400S recording spectrophotometer using xylulose- or ribulose-5-*P* (Xu5*P* or Ru5*P*) as substrate. The reaction mixture contained the following: potassium phosphate buffer, pH 6.5, 50 mM; 2-mercaptoethanol, 5 mM; NADH, 0.1 mM; $MgCl_2$, 50 mM; Xu5*P*, 2 mM, or Ru5*P*, 5 mM; sufficient extract to give a rate of 0.2–0.4 absorbance units/min; and water to 1 ml. Endogenous NADH oxidation was measured for 2 min in the absence of substrate to obtain a basal value and the reaction was initiated by the addition of Ru5*P* or Xu5*P*. All dehydrogenase values are corrected for NADH oxidase activity.

Activity of the phospho*enol*pyruvate phosphotransferase system (PTS) was measured by the Maryanski and Wittenberger (8) modification of a previously published procedure (9). Duplicate 10-μl samples were withdrawn at 5-min intervals over a 30-min period from a reaction mixture containing Tris·HCl buffer, pH 7.5, 100 mM; phospho*enol*pyruvate (PEP), 10 mM; $MgCl_2$, 5 mM; 2-mercaptoethanol, 1 mM; [*U*-^{14}C]-xylitol (Amersham/Searle), 5 mM (5×10^5 cpm) or [*1*-^{14}C]-ribitol (New England Nuclear), 5 mM (4×10^5 cpm); extract, 4–6 mg of protein; and distilled water to 0.5 ml; the incubation temperature was 30°. The samples were applied over the surface of DE81 filter paper pads (Whatman, diameter 2.5 cm), quickly dried with a stream of air, and washed twice with 5 ml of distilled water. After complete drying, the amount of radiolabeled pentitol phosphate absorbed to the pad was measured in a Beckman model LS-350 liquid scintillation counter. The remainder of the PTS reaction mixture was incubated an additional 4 hr, after which time the solution was deproteinized by the addition of 100 μl of 10% trichloroacetic acid. The solution was clarified by centrifugation, neutralized with 0.5 M NaOH, and stored at −40° until used for thin-layer chromatographic separation of the reaction products. Ribitol kinase assays were performed in the same assay system used to measure PTS activity except that 10 mM ATP was substituted for PEP

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Abbreviations: Xu5*P*, xylulose 5-phosphate; Ru5*P*, ribulose 5-phosphate; PTS, phospho*enol*pyruvate phosphotransferase system; PEP, phospho*enol*pyruvate; TLC, thin-layer chromatography; RPDH, ribitol-phosphate dehydrogenase; XPDH, xylitol-phosphate dehydrogenase.

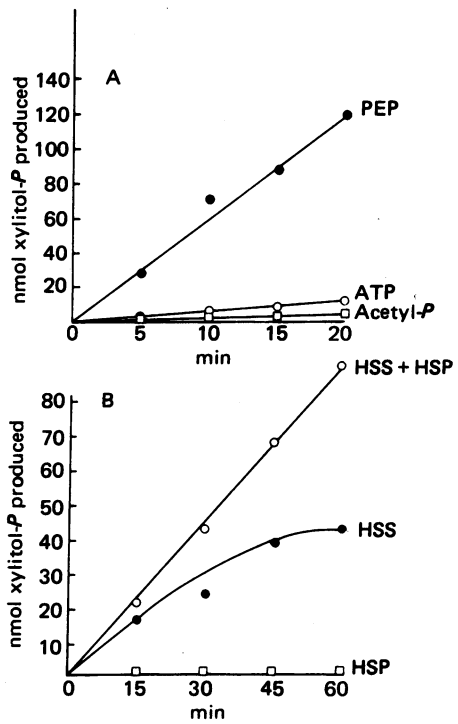

FIG. 1. Phosphorylation of [U-^{14}C]xylitol by cell extracts of *L. casei* CL-83. (*A*) Efficacy of PEP, ATP, or acetyl-*P* (each at 10 mM) as phosphate donors. Reaction mixtures contained 6 mg of extract protein. (*B*) Localization of PTS activity in subcellular components of the cell extract following centrifugational fractionation (centrifugation at 160,000 × *g* for 90 min). High-speed pellet (HSP) contained 15.2 mg of protein per ml; high-speed supernatant (HSS) contained 8.4 mg of protein per ml; reaction mixtures contained 0.2 ml of HSP, 0.4 ml of HSS, or 0.1 ml of HSP + 0.4 ml of HSS.

and the amount of extract was reduced by half.

Ribitol and xylitol transport in resting cells was measured by diluting the cell suspension described above by half with 0.01 M potassium phosphate buffer containing 15 mM radiolabeled pentitol (10^6 cpm). Duplicate 0.5-ml samples were removed at 5-min intervals, filtered through a Millipore membrane (0.45 μm pores), and washed twice with 5 ml of phosphate-buffered saline and once with 5 ml of 15 mM nonradioactive pentitol. The filters were dried and the intracellular levels of radioactive pentitol were measured as described above.

Radioactive products from PTS and ribitol kinase reaction mixtures, as well as unlabeled products from preparative scale dehydrogenase assays, were separated and identified by cellulose thin-layer chromatography (TLC). Replicate thin-layer plates were developed in the following three solvent systems, 1-butanol/propionic acid/water (100:50:70 by volume) (10); 1-butanol/acetic acid/water (5:3:2 by volume) (11); and 1-butanol/ethanol/water (4:1:5 by volume) (11). Areas containing pentitol or ketopentose were visualized by spraying the dried plates with ammoniacal $AgNO_3$ (12) and heating at 80° for 5 min, while pentose and pentitol phosphates were localized with a perchloric acid/molybdate spray (12). Substrates and products from isotopic experiments were identified by removing 5- × 20-mm sections of the cellulose layer between the origin and solvent front of the plate and determining their level of radioactivity in a liquid scintillation counter. Internal standards of pentitol, ketopentose, and ketopentose phosphate were included on all plates.

Exceptions to the above protocols and special procedures are cited in the *text*. All reagents were of the highest quality available.

Table 1. Levels of pentitol-dissimilating enzymes in fully induced strains of *L. casei*

Strain	Growth substrate	Specific activity: PTS*	Ribitol kinase*	RPDH†	XPDH†
CL-83	Xylitol	0.74	0	0.13	1.02
	D-Arabitol	0.51‡	0	0.11	0.95
CL-16	Ribitol	0.13	0.58	0.29	0.15

* nmol of phosphate ester formed per min/mg of protein.
† μmol of substrate utilized per min/mg of protein.
‡ Value for phosphorylation of [^{14}C]xylitol.

RESULTS

Pentitol Phosphorylating Systems. Evidence for the existence of a xylitol PTS system in *L. casei* CL-83 is presented in Fig. 1 *A* and *B*. In cell extracts of the xylitol- or D-arabitol-grown strain of *L. casei*, PEP appears to be the primary phosphate donor for pentitol. Acetyl-*P* is almost completely inactive as a phosphate donor and the low level of ATP activity shown in Fig. 1 is inhibited rather than enhanced by the addition of the phosphatase inhibitor NaF (data not shown). This suggests that some ancillary reaction other than kinase activity is responsible for the observed ATP-dependent activity. As for other PTS systems (13), a linear and constant rate of activity requires the presence of both soluble (histidine-containing protein and enzyme I) and membrane-bound (enzyme II) proteins in cell extracts (Fig. 1*B*). The PTS enzyme complex was surprisingly specific for its substrate. [U-^{14}C]Mannitol could not be substituted for xylitol, and the addition of equimolar amounts of mannitol or glucose did not inhibit the reaction rate. In contrast to the strain of *L. casei* capable of growing on xylitol, cell extracts of *L. casei* strains 64H and CL16, which grow only in ribitol media, possess an ATP-dependent ribitol kinase in addition to a ribitol PTS system. The PTS system is similar to that described for xylitol with the exception that its specific activity is significantly lower than the xylitol system (see Table 1). Kinase activity in extracts of ribitol-grown *L. casei* is enhanced by the addition of 0.01 M NaF (Fig. 2). In contrast to the PTS, all of the ribitol kinase activity remained in the supernatant fraction following 3 hr of centrifugation at 160,000 × *g*.

Transport Studies. The uptake of [U-^{14}C]xylitol by resting cells of *L. casei* CL-83 was linear over a 30-min incubation period. In three separate experiments, the rate of xylitol transport ranged between 1 and 2 nmol/min per mg of bacterial protein; this value is comparable to the rate observed with the cell-free PTS system. Resting cell suspensions prepared from ribitol-grown cultures of *L. casei* CL-16 give similar uptake rates with [1-^{14}C]ribitol.

Pentitol Dehydrogenase Activity. Growth of *L. casei* CL-83 at the expense of xylitol or D-arabitol induces both a ribitol-phosphate dehydrogenase (RPDH) activity and a xylitol-phosphate dehydrogenase (XPDH) activity. Extracts of this strain catalyze an NADH-dependent reduction of Ru5*P* at roughly one eighth the rate observed with Xu5*P* (Table 1). Similarly, extracts prepared from ribitol-grown cells of *L. casei* CL-16 also exhibit activity with both substrates; the level of RPDH is twice that of XPDH (Table 1). However, the specific activity of RPDH in strain CL-83 is only one-half that found in fully induced ribitol-grown cells of strain CL-16. Cell extracts of *L. casei* 64H contain levels of RPDH and XPDH activity that

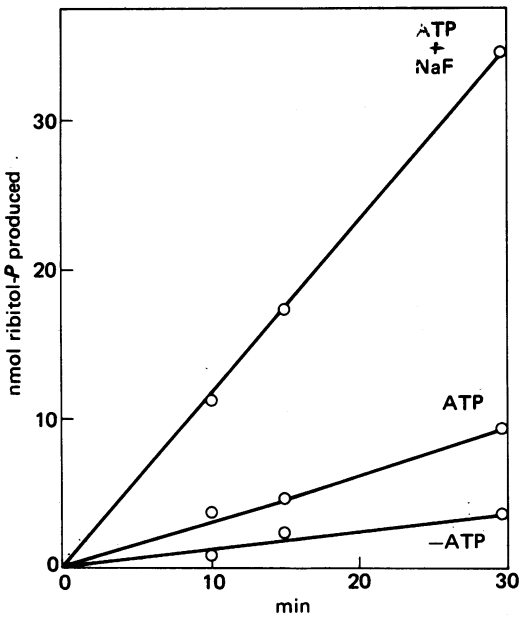

FIG. 2. Phosphorylation of [1-^{14}C]ribitol by ATP-dependent kinase activity in cell extracts of *L. casei* strain 64H. Reaction mixtures contained 2.05 mg of extract protein and 0.01 M NaF where added.

are the same as those reported for CL-16 and are, therefore, not shown here. The dehydrogenase activities of these organisms exhibit an almost absolute specificity for the phosphate ester of ketopentose as substrate. Ribulose prepared from the nitrophenylhydrazone derivative was inactive as a substrate, as were stock solutions of Ru5*P* and Xu5*P* (50 and 20 mM, respectively) treated with alkaline phosphatase for 3 hr at 37°; controls containing boiled alkaline phosphatase remained suitable substrates. Extracts of the two strains containing RPDH and XPDH activity catalyzed the forward reaction of mannitol-1-*P* dehydrogenase in the presence of 5 mM mannitol-1-*P* (8) very slowly; the rates were from $\frac{1}{20}$ to $\frac{1}{100}$ the rate observed for the major pentitol activity. Neither ribulose nor mannitol-1-*P* inhibited the RPDH or XPDH to any significant extent when present at a concentration twice that of the substrate. The two dehydrogenase activities from both strains show a near-absolute requirement for NADH; in extracts of strain CL-83, the substitution of NADPH for NADH produced reaction rates that were $\frac{1}{50}$ and $\frac{1}{127}$ the rates for the RPDH and XPDH, respectively.

DEAE-cellulose chromatography of an extract from ribitol-grown *L. casei* 64H partially separated the RPDH from XPDH activity (Fig. 3), demonstrating that each activity was carried by a discrete dehydrogenase. The same treatment of an extract prepared from xylitol-grown cells of *L. casei* CL-83 appeared to separate the two activities also; however, the RPDH was almost totally inactivated by the separation procedure and thus the existence of two distinct enzymes is not yet proven. The K_m values for substrate and cofactor were determined for the purified RPDH (specific activity = 12.2 μmol of ribitol-*P* oxidized per min/mg of protein) and XPDH (specific activity = 3.02 μmol/min per mg) from *L. casei* strain 64H. K_m values of 0.4 and 0.9 mM were found for Ru5*P* and Xu5*P*, respectively; the respective K_m values of RPDH and XPDH for NADH were 0.023 and 0.027 mM.

Identification of Pentitol Phosphate Intermediate Products. Radiolabeled xylitol phosphate and ribitol phosphate were identified in PTS reaction mixtures allowed to incubate for 4–6 hr at 30°. Deproteinized solutions were applied to TLC plates and the plates were developed in solvents that separated substrate from intermediate products. In the three solvent systems used, the pentitols migrated slower than the ketopentoses and the phosphorylated ketopentoses travelled well behind the pentitols. The radioactive product of the xylitol/PEP reaction mixture trailed the Xu5*P* standard (Fig. 4). Treatment with

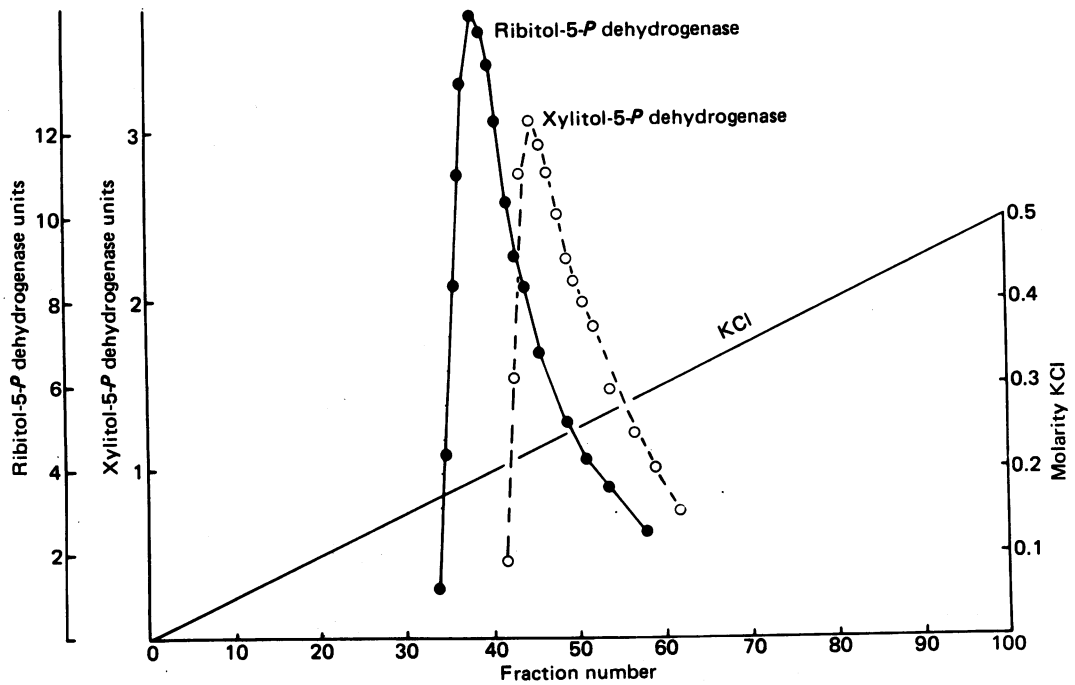

FIG. 3. DEAE-cellulose chromatographic separation of RPDH and XPDH activities from an extract of *L. casei* 64H. A 252-mg protein preparation containing 158 international units of RPDH and 93 international units of XPDH was applied to a 2.5- × 40-cm DEAE-cellulose column and subsequently eluted with an 0-0.5 M KCl gradient in 0.05 M Tris·HCl buffer, pH 7.5 at 4° (one international unit catalyzes conversion of 1 μmol/min).

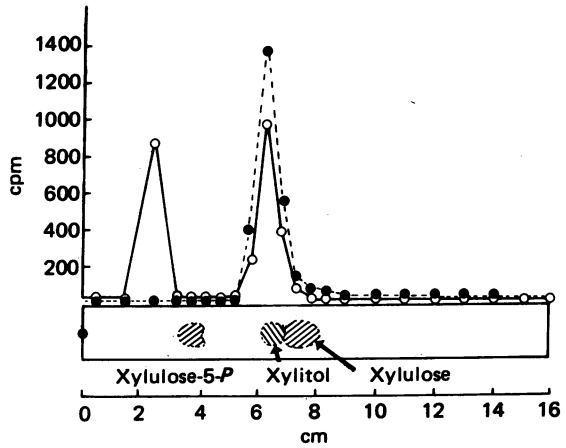

FIG. 4. TLC separation of ^{14}C-labeled intermediate product from xylitol/PEP reaction mixture. After receiving applications of 0.2 μmol each of Xu5*P* and xylulose, 2 μmol of xylitol, and 20 μl of reaction mixture, the TLC plate was developed in 1-butanol/acetic acid/H_2O (5:3:2 by volume). O — O, Untreated reaction mixture; ● - - - ●, alkaline phosphatase-treated reaction mixture.

alkaline phosphatase converted the slowly migrating radioactive product to xylitol (Fig. 4) thereby indicating that the intermediate was, in fact, xylitol-*P*. Ribitol/PEP and ribitol/ATP reaction mixtures contained a product with the same mobility as the xylitol-*P*. In both instances, treatment with alkaline phosphatase converted all of the trailing radioactive product to a compound with the same R_F as ribitol; Fig. 5 presents a set of data obtained from a ribitol/ATP reaction mixture.

Larger amounts of the phosphorylated intermediate product were prepared by increasing the scale of dehydrogenase reaction that catalyzed the reduction of ketopentose-5-*P* to pentitol-*P*. Results from the chromatography of the products of such reaction mixtures are shown in Fig. 6. Although the substrates are not completely utilized in the reaction, a major portion of Xu5*P* and Ru5*P* were converted to phosphate esters, which travel behind the respective pentulose phosphate substrates with an R_F corresponding to the migration rate of the radioactive products from the pentitol/PEP and pentitol/ATP reaction mixtures.

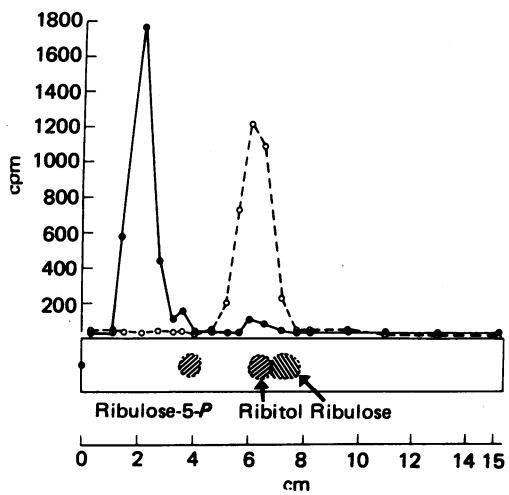

FIG. 5. TLC separation of ^{14}C-labeled intermediate product of ribitol–ATP reaction mixture. Applications of 0.2 μmol each Ru5*P* and ribulose, 2 μmol of ribitol, and 20 μl of reaction mixture were made on the plate; plates were then developed as per Fig. 4. ● — ●, Untreated reaction mixture; O- - - O, alkaline phosphatase-treated reaction mixture.

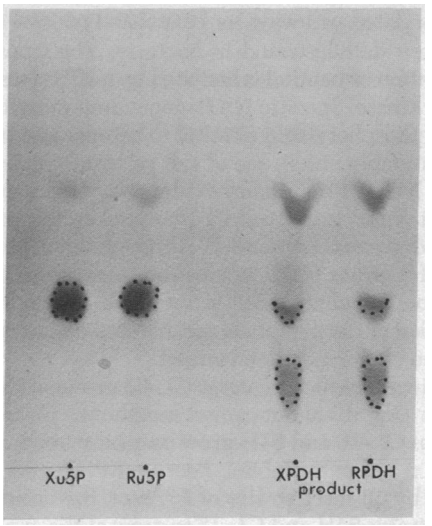

FIG. 6. TLC separation of intermediate products from RPDH and XPDH reaction mixtures. Scale of dehydrogenase assay was increased 5-fold using DEAE-cellulose-purified RPDH (0.76 mg of protein) and XPDH (0.90 mg of protein). 0.2 μmol of Xu5*P* and Ru5*P* were applied as standards; 5 μl of deproteinized reaction mixture was applied. The TLC plate was developed in the solvent system described below Fig. 4 and visualized with perchlorate/molybdate spray.

DISCUSSION

The ability to grow at the expense of pentitols, is, as far as is presently known, a unique metabolic property among Gram-positive bacteria. Certain species of *Sarcina* grown on either mannitol or sorbitol metabolize xylitol; however, the pentitol was never tested for its ability to support growth (14). *Bacillus subtilis* grows on xylitol only if the medium is supplemented with low levels of sorbitol; the inducible sorbitol dehydrogenase is sufficiently nonspecific for its substrate to metabolize the pentitol at a rate capable of sustaining growth (15). Thus, among the Gram-positive organisms tested, only strains of *L. casei* are able to grow solely at the expense of ribitol or xylitol and D-arabitol.

The novelty of the observations reported here extends beyond the abilities of certain Gram-positive bacteria to metabolize pentitols, inasmuch as the initial steps of the pathway appear to be totally distinct from the nonphosphorylated pathway reported for Gram-negative bacteria (1). As can be seen from the scheme in Fig. 7, the pentitol phosphate pathway is analogous to the hexitol phosphate pathway found in strict or facultatively anaerobic bacteria (8, 16–18) and its description completes the symmetry in the evolution of polyol dissimilating pathways in that the existence of both phosphorylated and

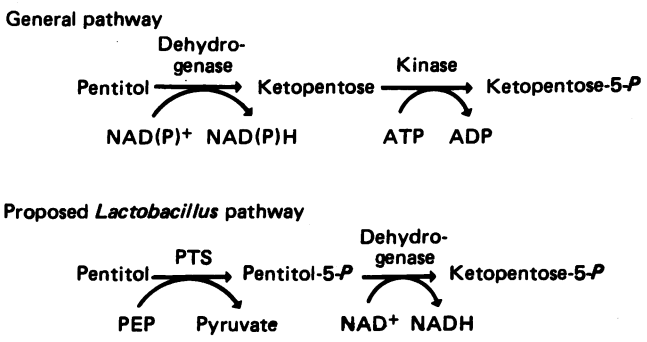

FIG. 7. A comparison of the pathways of pentitol metabolism by lactobacilli and Gram-negative bacteria (general pathway).

nonphosphorylated pathways for hexitol and pentitol utilization has now been demonstrated in bacteria. The transport and phosphorylation of pentitol is facilated by a PTS system or PTS system and kinase. Specific NAD-dependent dehydrogenases oxidize the phosphorylated pentitol to ketopentose phosphate, which presumably enters one of the pathways of central metabolism as Xu5*P*. Preliminary evidence not reported here indicates that Xu5*P* is cleaved by phosphoketolase to produce acetyl-*P* and glyceraldehyde-3-*P*. Although the dehydrogenase specificity for either Ru5*P* as substrates strongly suggests that the corresponding pentitol-5-*P* is the intermediate product, the actual position of the phosphate moiety on the sugar alcohol has not yet been chemically determined.

As mentioned earlier, *L. casei* CL-83 grows at the expense of xylitol or D-arabitol but cannot metabolize ribitol, while *L. casei* strains CL-16 and 64H grow on ribitol but are unable to dissimilate xylitol or D-arabitol. Because RPDH and XPDH are coinduced by all three strains of *L. casei*, the failure of strain CL-83 or strains 64H and CL-16 to grow at the expense of xylitol and ribitol, respectively, may be attributable to a metabolic block at the level of transport, phosphorylation, or induction of either process. If the PTS system is, in fact, responsible for both transporting and phosphorylating pentitols, it should not be difficult to isolate a mutant of either strain with an altered specificity for the nonmetabolizable pentitol. It is curious, therefore, that repeated attempts to isolate a xylitol-utilizing mutant of *L. casei* 64H have been unsuccessful (E. St. Martin, personal communication).

The highly specific nature of the PTS system operating in tandem with an equally specific dehydrogenase probably makes this pathway a very efficient metabolic system (19). However, efficiency of pentitol utilization may have been attained at the expense of metabolic or nutritional diversity as expressed in those Gram-negative bacteria possessing polyol dehydrogenases whose substrate specificity can readily be altered (20). Such alterations do not appear to be easily achieved in the *Lactobacillus* pentitol-metabolizing system.

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