

Purification and Characterization of Ribitol-5-Phosphate and Xylitol-5-Phosphate Dehydrogenases from Strains of *Lactobacillus casei*

SALLY Z. HAUSMAN AND JACK LONDON*

Laboratory of Microbiology and Immunology, National Institute of Dental Research, Bethesda, Maryland 20892

Received 14 October 1986/Accepted 6 January 1987

A simple three-step procedure is described which yields electrophoretically homogeneous preparations of ribitol-5-phosphate dehydrogenase and xylitol-5-phosphate dehydrogenase. The former enzyme is a 115,000-molecular-weight protein composed of two subunits of identical size and is specific for its substrate, ribitol. The xylitol-5-phosphate dehydrogenase exists as a tetrameric protein with a molecular weight of 180,000; this enzyme oxidizes the phosphate esters of both xylitol and D-arabitol. Characterization of the physical, kinetic, and immunological properties of the two enzymes suggests that the functionally similar enzymes may not be structurally related.

Only two species of lactic-acid bacteria, *Streptococcus avium* and *Lactobacillus casei*, have strains that are capable of growing at the expense of one or more five-carbon polyalcohols (9, 16). These bacteria utilize pentitols via a pathway not found in eucaryotes or other procaryotes (9, 10). The sugar alcohol is transported into the cell as pentitol-5-phosphate by a phosphoenolpyruvate-dependent phosphotransferase system in which a NAD-linked dehydrogenase oxidizes the phosphate ester to xylulose-5-phosphate, an intermediate of the pentose phosphate pathway. The transport systems and dehydrogenases are substrate specific, recognizing either the three- (ribitol pathway) or erythro- (xylitol and D-arabitol pathway) configuration of the sugar phosphates and their corresponding phosphate esters. The xylitol pathway of *L. casei* C183 consists of only three novel components, a membrane-bound EII^{xtl}, a soluble III^{xtl}, and a soluble xylitol-5-phosphate dehydrogenase; the ribitol pathway appears to be similarly constituted (10). III^{xtl} of *L. casei* C183 has been purified, and its physical and biochemical properties have been described (12). In this report, we describe the purification and properties of the xylitol-5-phosphate and ribitol-5-phosphate dehydrogenases from *L. casei* C183 and C116, respectively.

MATERIALS AND METHODS

Cultivation of bacteria. *L. casei* C183 and C116 (National Institutes of Health, Bethesda, Md.) were grown in 1-liter screw-cap bottles or 20-liter carboys containing *Lactobacillus* carrying medium (6) supplemented with filter-sterilized ribitol or xylitol to a final concentration of 33 mM under static conditions at 37°C. When the cultures reached an optical density of 240 Klett units (Klett Summerson colorimeter, red filter), the cells were harvested, washed once with 0.02 M potassium phosphate buffer (pH 7.2) containing 0.9% NaCl, divided into 15- to 20-g batches, and stored at -40°C until used. *S. avium* 559 was cultivated in the same medium under the same conditions. When substrates other than the pentitols were used as energy sources, they were added to the medium as sterile solutions to a final concentration of 20 mM.

Purification of xylitol-5-phosphate and ribitol-5-phosphate dehydrogenases. Between 18 and 20 g (wet weight) of *L. casei* C183 or C116 cells was suspended in 60 ml of 0.02 M potassium phosphate buffer (pH 7.2) containing 10 mM 2-mercaptoethanol (PBME). Cells were disrupted by two 6-min treatments in an ice-water-cooled chamber of a sonifier (model 350; Branson Sonic Power Co., Danbury, Conn.) operating at 70% of maximum power. Intact cells were removed from the extract by centrifugation at 13,200 × g for 30 min. Comminuted membranes were removed from the cell-free preparation by centrifugation at 104,000 × g for 120 min in an ultracentrifuge (model L2-65B; Ivan Sorvall, Inc., Norwalk, Conn.).

The supernatant fluid was decanted from the membrane pellet and applied to a DE52 column (15 by 2.5 cm; DEAE-cellulose [Whatman, Inc., Clifton, N.J.]) equilibrated with PBME. After the column was washed with 100 ml of PBME, dehydrogenase activity was eluted with a 0 to 0.4 M KCl gradient in PBME, and 10-ml fractions were collected. Xylitol-5-phosphate dehydrogenase from extracts of *L. casei* C183 appeared as two peaks; the first, containing 32% of the recoverable activity (as measured in the reverse direction; see below), was eluted at a KCl concentration of 0.10 M and the major activity peak was eluted at KCl concentration of 0.14 M. Ribitol-5-phosphate dehydrogenase activity found in extracts of *L. casei* C116 eluted as a single activity peak (as measured in the reverse direction) from DE52 columns at a KCl concentration of 0.06 M. Fractions containing peak activity were pooled and concentrated to a volume of roughly 5 ml in an Amicon cell (Amicon Corp., Lexington, Mass.) with a PM-10 filter. The respective preparations were applied separately to a 5'AMP Sepharose 4B column (22 by 1.5 cm; Pharmacia, Inc., Piscataway, N.J.) equilibrated with PBME, and the column was washed with 2 volumes of PBME. Enzyme activity was eluted with a 0 to 3 mM NAD⁺ gradient prepared in 0.01 M potassium phosphate buffer (pH 7.2) containing 10 mM 2-mercaptoethanol; 5-ml fractions were collected. When the minor xylitol-5-phosphate dehydrogenase activity was applied to the affinity column, a significant loss of enzyme activity occurred (Table 1). Both the major and minor xylitol-5-phosphate dehydrogenase activities eluted at a concentration of 0.08 mM NAD⁺, while ribitol-5-phosphate dehydrogenase activity eluted at 1.2 mM

* Corresponding author.

TABLE 1. Purification procedure for pentitol-5-phosphate dehydrogenases

Dehydrogenase	Total vol (ml)	Total protein (mg)	Total activity (U) ^a	Sp act
Xylitol-5-phosphate				
Crude HSS ^b	43	997	1,806	1.88
DEAE-52				
peak 1	4.7	82	1,034	12.6
peak 2	5.1	191	2,960	15.5
Affinity column				
peak 1	5.0	22	610	27.7 ^c
peak 2	5.4	41	2,484	60.5 ^d
Ribitol-5-phosphate				
Crude HSS	11	330	297	0.90
DEAE-52	8	40	360	9.1
Affinity column	6	11	154	14.2 ^e

^a Units reported as micromoles of substrate utilized per minute.

^b HSS, High-speed supernatant fluid (104,000 × g).

^c Recovery of 58%.

^d Recovery of 84%.

^e Recovery of 52%.

NAD⁺. Peak-activity fractions were pooled and concentrated to a volume of 4 to 5 ml by ultrafiltration. With the exception of DE52 peak 1 activity, the enzyme preparations could be stored as solutions or lyophilized and stored as desiccated powders at -20°C without significant loss of activity for 2 years. Protein was determined by the biuret method (7) or the Bio-Rad protein assay kit (Bio-Rad Laboratories, Rockville Center, N.Y.).

Enzyme assays. Dehydrogenase activity in the reverse (reductive) direction was measured by a previously described procedure with ribulose-5-phosphate or xylulose-5-phosphate as the substrate and NADH as the reductant (10). Enzyme activity in the forward (oxidative) direction was measured in the presence of NAD⁺ with xylitol-5-phosphate or ribitol-5-phosphate as substrate. The pentitol phosphates were synthesized by NaBH₄ reduction of their corresponding pentulose-5-phosphate esters (11). D-Arabitol-5-phosphate was isolated from scaled-up xylitol-specific phosphotransferase assay mixtures (9). The assay mixture was deproteinized by passage over a PD-10 column (Pharmacia), and the portion of the eluate containing the low-molecular-weight components was reduced in volume by lyophilization. The concentration of D-arabitol-5-phosphate was estimated by determining the organic phosphate level of the powder (15); however, this value was probably high owing to contaminating phosphate esters in the extract. For this reason, the preparation was used only for qualitative determinations to learn whether the respective enzyme preparations could use D-arabitol-5-phosphate as a substrate. The forward-direction reaction was measured in an assay mixture containing 25 mM Tris hydrochloride buffer (pH 8.5), 1 mM NAD⁺, 10 mM MnCl₂ (for xylitol-5-phosphate dehydrogenase), 10 mM pentitol-5-phosphate, and sufficient enzyme to give a rate of roughly 0.2 μmol of pentitol-5-phosphate oxidized per min. All measurements were made with a model 2400S recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) set at a wavelength of 340 nm.

Electrophoresis and isoelectric focusing. Anionic polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Davis (3) in cylindrical glass tubes (diameter, 5 mm) or 1.5-mm thick slabs (Hoefer Scientific Instruments, San Francisco, Calif.). Samples containing between 50 and

150 μg of protein were loaded onto the gels. Protein was detected by the Coomassie blue stain (19), and dehydrogenase activity was visualized by staining unfixed gels in 10 to 20 ml of a solution containing 100 mM Tris hydrochloride buffer (pH 9.0), 24 mM ribitol-5-phosphate or xylitol-5-phosphate, 5 mM NAD⁺, 0.0013 mM phenazine methosulfate, and 0.004 mM Nitro Blue Tetrazolium. Denaturing (sodium dodecyl sulfate) PAGE was performed by the method of Weber and Osborne (19). Enzyme was extracted from anionic gels by excising the appropriate unstained portions of the gel and macerating the polyacrylamide cylinder with a tissue homogenizer containing 1 to 2 ml of PBME. The gel beads were allowed to settle, and the PBME was decanted.

Isoelectric focusing was carried out on thin-layer 7.5% polyacrylamide gel slabs containing 2% ampholines; the model 2117 Multiphor (LKB Instruments, Inc., Rockville, Md.) was used as specified by the manufacturer (LKB bulletin 1-2117-EOL). Gel slabs were fixed overnight in 12% trichloroacetic acid, rinsed with distilled H₂O, and stained with Coomassie blue. The pI of the dehydrogenase was estimated by comparing the migration position of the enzyme with those of standard proteins.

Molecular weight determinations. The molecular weights of the native dehydrogenases were estimated by sieve chromatography on calibrated Sephacryl 200 columns (Pharmacia) by the method of Andrews (1). Subunit sizes of the respective enzymes were estimated from their migration rates on denaturing polyacrylamide gels (see above) with standard protein solutions as molecular weight references.

Immunological procedures. Anti-*L. casei* Cl83 xylitol-5-phosphate dehydrogenase sera were prepared by injecting two 4-month-old male New Zealand White rabbits with a total of 2.04 mg of protein each by a previously published protocol (13). Immunodiffusion assays were performed by the method of Stollar and Levine (17). The procedure of Towbin et al. (18) as modified by Batteiger et al. (2) was used for immunoblot analysis.

RESULTS

Enzyme purification. Anionic PAGE of the affinity column eluates indicated that ribitol-5-phosphate dehydrogenase was electrophoretically homogeneous (Fig. 1, lane 3), while the xylitol-5-phosphate dehydrogenase preparation exhibiting bidirectional activity migrated as a single discrete band preceded by an area of diffusely staining proteinaceous material (Fig. 2, lane 2). The amount of diffuse material varied from preparation to preparation. A stain that detected enzyme activity in the forward direction produced a single sharp band that corresponded to the discrete xylitol-5-phosphate dehydrogenase band in the Coomassie blue-stained gels (Fig. 2, lane 1). Both areas were extracted from unstained companion gels and tested for enzyme activity; the material extracted from the area of the well-defined protein band exhibited activity in both the forward and reverse directions. Eluate from the diffuse region of the gel possessed activity only in the reverse direction. When each fraction was subjected to another round of gel electrophoresis, the eluate with bidirectional activity migrated as a well-defined band again (Fig. 2, lane 3) and stained positively for enzyme activity. In contrast, the material exhibiting only reverse-direction activity could no longer be visualized by the protein stain.

The nature of the diffusely staining material observed in the anionic polyacrylamide gels of the purified peak 2

material is similar to that isolated as peak 1 preparation from the ion-exchange column. Namely, the peak 1 enzyme also shows no forward-direction activity and much of its reverse direction activity is lost following its application to an affinity column. However, the peak 1 enzyme does elute at the same NAD^+ concentration as the peak 2 preparation.

Physical properties of the dehydrogenases. The molecular weights of the native forms of ribitol-5-phosphate and xylitol-5-phosphate dehydrogenases (DE-52 peak 2 activity) were estimated to be 115,000 and 180,000, respectively, by molecular sieve chromatography. Denaturing PAGE resolved the ribitol-5-phosphate dehydrogenase into a single polypeptide band with an estimated molecular weight of 49,000, while xylitol-5-phosphate dehydrogenase (peak 2 activity) migrated as a single band with a molecular weight of approximately 42,000 (data not shown). Thus, in their native states, the ribitol-5-phosphate dehydrogenase appears to be a dimer, while the xylitol-5-phosphate dehydrogenase exists as a tetramer. DE-52 peak 1 activity (xylitol-5-phosphate dehydrogenase) had a native molecular weight of 110,000. However, when this preparation was subjected to denaturing PAGE, no Coomassie blue-staining material could be detected in or on top of the gels. Furthermore, the relatively benign treatment of sieving resulted in a 50% loss of activity although it caused no loss in activity of the ribitol-5-phosphate and peak 2 xylitol-5-phosphate dehydrogenases. On slab isoelectric focusing gels, the xylitol-5-phosphate dehydrogenase migrated like standard proteins with a pI of 5.3 to 5.5.

Kinetic parameters of the dehydrogenases. Table 2 summarizes several catalytic properties of the two dehydrogenases. An accurate determination of the forward-direction kinetic characteristics of the two dehydrogenases was difficult because the initial rates of catalysis were exceedingly rapid and nonlinear. The reaction ceased after 0.2 to 0.4 μmol of

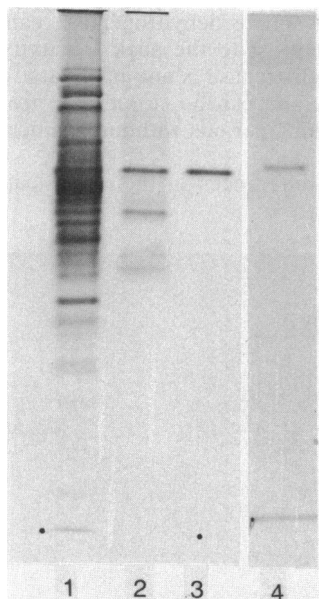


FIG. 1. Efficacy of ribitol-5-phosphate dehydrogenase purification procedure. Lanes 1, 2, and 3: protein stains of anionic polyacrylamide gels containing high-speed supernatant extract (23 μg of protein), DEAE column eluate (23 μg of protein) and 5'AMP column eluate (8 μg of protein), respectively. Lane 4: activity stain of the 5'AMP column eluate (8 μg of protein).

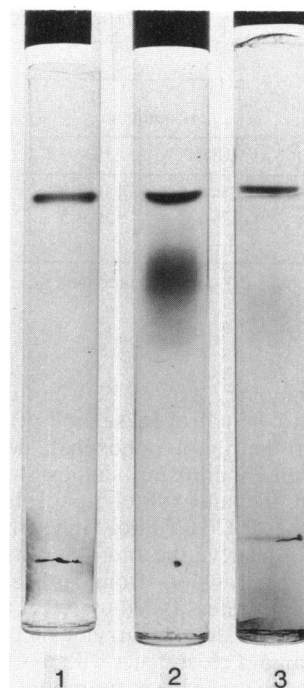


FIG. 2. Electrophoretic profile of xylitol-5-phosphate dehydrogenase following 5'AMP column chromatography. Lanes: 1, activity stain on anionic gel (17.5 μg of protein); 2, protein stain on anionic gel (15 μg of protein); 3, protein stain of enzyme extracted from well-defined region of anionic gel and rechromatographed on anionic gel (15 μg of protein). Note the faintly staining diffuse region below the discrete band in lane 3.

substrate had been oxidized; addition of more pure enzyme to the reaction mixture resulted in a marginal increase in activity. The nonlinearity of the reactions was attributed to a product inhibition resulting from the accumulation of reduced pyridine nucleotide, pentulose-5-phosphate, or both (data not shown). Both enzymes exhibited an absolute specificity for NAD^+ as cofactor. The ribitol-5-phosphate dehydrogenase utilized only ribitol-5-phosphate as a substrate, while xylitol-5-phosphate dehydrogenase oxidized both xylitol-5-phosphate and D-arabitol-5-phosphate. Fructose-6-phosphate, fructose-1-phosphate, mannitol-1-phosphate, and sorbitol-6-phosphate did not serve as substrates for either enzyme.

The following intermediate products of glycolysis were tested as potential inhibitors of the two dehydrogenases, in both the forward and reverse directions, at final concentrations between 1 and 10 mM: fructose-6-phosphate, glucose-6-phosphate, fructose-1,6-bisphosphate, ribose-5-phosphate, glyceraldehyde-3-phosphate, 3-phosphoglycerate, phosphoenolpyruvate, pyruvate, acetylphosphate, and mono-, di-, and triphosphate esters of guanosine and adenosine. With the exception of ATP, none of the compounds tested inhibited either oxidative or reductive dehydrogenase activity at 10 mM. At 10 mM, ATP produced a 45% inhibition in both the forward and reverse directions of the xylitol-5-phosphate dehydrogenase. However, the inhibition may be due to the ability of ATP to chelate the divalent cation Mn^{2+} , which is required by the enzyme for optimal activity (4).

Immunological studies. The rabbit anti-xylitol-5-phosphate dehydrogenase serum was used to determine whether any

TABLE 2. Catalytic properties of the ribitol-5-phosphate and xylitol-5-phosphate dehydrogenases

Dehydrogenase	Property							
	pH optimum		Substrate specificity	Substrate K_m (mM)		Cofactor k_m (mM)		Metal requirement
	RD ^a	FD ^a		RD	FD	RD	FD	
Ribitol-5-phosphate	5.5-6.5	8.0-9.0	Ribitol-5-phosphate	0.66	ND ^b	0.025	ND	None
Xylitol-5-phosphate	5.5-6.0	8.0-9.5	Xylitol-5-phosphate, D-arabitol-5-phosphate	0.72	2.4	0.04	0.50	Mn ²⁺ , Mg ²⁺

^a RD, Reverse direction; FD, forward direction.

^b ND, Not done.

immunological homology existed between the lactobacillus xylitol-5-phosphate dehydrogenase and (i) that found in streptococci or (ii) the ribitol-5-phosphate dehydrogenase of *L. casei* Cl16. Immunodiffusion studies showed no cross-reaction between the anti-xylitol-5-phosphate dehydrogenase and the *L. casei* Cl16 ribitol-5-phosphate dehydrogenase (Fig. 3A). Similarly, the more sensitive immunoblot technique, in which transfers from both anionic gels and denaturing gels were tested, failed to detect any immunological homology between the two enzymes (not shown). However, the antiserum did cross-react with extracts of *S. avium* 559 cells grown in the presence of D-arabitol and xylitol (data not shown). The antiserum, which was prepared against the affinity-purified DEAE peak 2 activity, failed to react with (i) the peak 1 material isolated from the DE-52 column or (ii) the diffuse Coomassie blue-staining protein associated with the purified DE-52 peak 2 enzyme preparation. The failure to react with the antiserum occurred although the latter was present in the xylitol-5-phosphate dehydrogenase preparation used to immunize the rabbit (Fig. 3B).

In other immunodiffusion studies, the monospecific antiserum was used to determine whether xylitol-5-phosphate dehydrogenase was synthesized by *L. casei* Cl83 during growth on other sugar substrates. The enzyme was present, albeit at low concentrations, when the organism was grown

at the expense of lactose, galactose, and glucose but not mannitol (Fig. 4).

DISCUSSION

A simple three-step procedure is described for the purification of xylitol-5-phosphate and ribitol-5-phosphate dehydrogenases from *L. casei* Cl16 and Cl83, respectively. When assayed in the reverse (reductive) direction, two xylitol-5-phosphate dehydrogenase activities were identified in ion-exchange column eluates. The enzyme activity eluted as peak 1 was unable to catalyze the forward (oxidative) reaction and proved to be unstable during purification and on storage. Passage through an affinity and sieving column reduced the recovered activity by 75 to 80%. The activity resembles the unstable material that migrates as a diffuse protein smear in anionic gels containing the purified and stable DE-52 peak 2 enzyme activity. The peak 1 reverse-direction activity appears to be a breakdown product of the more stable peak 2 (bidirectional) enzyme activity that is produced during the early stages of purification, perhaps by ultrasonic disruption. However, the possibility that the 110-kilodalton protein is an unstable xylulose-5-phosphate reductase which is distinct from and completely unrelated to the xylitol-5-phosphate dehydrogenase, cannot be entirely excluded. In contrast to the peak 1 activity, the xylitol-5-phosphate dehydrogenase is not inactivated during the purification process and can be stored as a frozen solution or lyophilized for long periods without significant loss of activity.

The marked differences in their physical characteristics

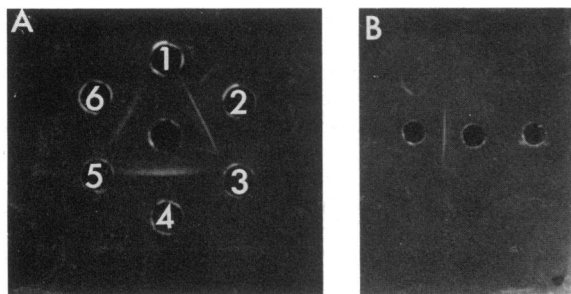


FIG. 3. Immunodiffusion experiments with rabbit anti-xylitol-5-phosphate dehydrogenase. (A) Absence of immunological cross-reactivity between anti-xylitol-5-phosphate dehydrogenase and ribitol-5-phosphate dehydrogenase. Wells 1, 3, and 5 contain ribitol-5-phosphate dehydrogenase as 5' AMP preparation (8 μ g of protein), DEAE eluate (20 μ g of protein), and crude extract (26 μ g of protein), respectively. Wells 2, 4, and 6 contain, in that order, amounts of xylitol-5-phosphate dehydrogenase of 2, 15, and 20 μ g of protein, respectively. The center well contains 15 μ l of anti-xylitol-5-phosphate dehydrogenase. (B) Comparison of purified DEAE peak 1 and peak 2 enzyme activities. The left well contains 8 μ g of peak 2 material, and the right well contains 6 μ g of peak 1 material. The center well contains 10 μ l of anti-xylitol-5-phosphate dehydrogenase.

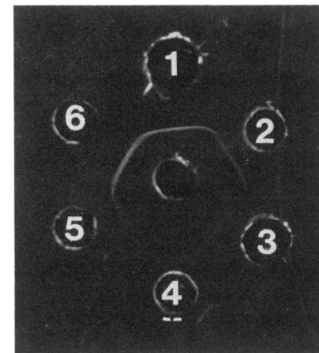


FIG. 4. Detection of anti-xylitol-5-phosphate dehydrogenase-cross-reacting material in extracts from *L. casei* CL83 grown on various substrates. Each well received between 75 and 85 μ g of crude extract protein prepared from cells grown at the expense of (1) D-arabitol, (2) glucose, (3) galactose, (4) mannitol, (5) lactose, and (6) xylitol.

and the absence of immunological homology suggest that the ribitol-5-phosphate and xylitol-5-phosphate dehydrogenases are not derived from a recent common ancestral gene. While it may be argued that relatively large, inducible proteins might be expected to evolve at a more rapid rate than smaller, constitutively synthesized proteins (8, 13), immunological homology was readily demonstrated among the inducible malic enzymes isolated from strains of *L. casei* and *S. faecalis* (14). However, a final decision regarding the presence or absence of structural relatedness between the ribitol-5-phosphate and xylitol-5-phosphate dehydrogenases must await a comparison of the relevant gene sequences. The anti-*L. casei* Cl83 xylitol-5-phosphate dehydrogenase sera reacted with extracts of *S. avium* extracts that contained xylitol-5-phosphate dehydrogenase activity, suggesting that these two disparate microorganisms possess structurally related enzymes. The failure to detect immunological cross-reactivity with the mannitol-metabolizing enzymes present in extracts of mannitol-grown *L. casei* Cl83 cells and the inability to demonstrate reverse-direction activity with fructose-1-phosphate and forward-direction activity with mannitol-1-phosphate suggest that neither of the dehydrogenases is closely related to the mannitol-1-phosphate dehydrogenase. These observations compound the enigma regarding the origin of the two dehydrogenases described here. Like the xylitol-metabolizing enzymes found in certain strains of *Erwinia uredovora* (5), it is not clear which extant enzymes might have served as progenitors for the proteins in question.

Finally, like the III^{xil} of the *L. casei* Cl83 xylitol phosphotransferase system (12), synthesis of the xylitol-5-phosphate dehydrogenase of this organism does not appear to be rigorously regulated. The dehydrogenase was readily detected when the bacterium was grown at the expense of a number of sugars, including glucose.

LITERATURE CITED

1. Andrews, P. 1964. Estimation of molecular weight of proteins by Sephadex gel filtration. *Biochem. J.* **91**:222-233.
2. Batteiger, B., W. J. Newhall, and R. B. Jones. 1982. The use of Tween-20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. *J. Immunol. Methods* **55**:297-307.
3. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum. *Ann. N.Y. Acad. Sci.* **121**:404-427.
4. Dixon, M. E., and E. C. Webb. 1964. *Enzymes*, p. 346-347. Academic Press, Inc., New York.
5. Doten, R. C., and R. P. Mortlock. 1985. Characterization of xylitol-utilizing mutants of *Erwinia uredovora*. *J. Bacteriol.* **161**:529-533.
6. Efthymiou, C., and C. A. Hansen. 1962. An antigenic analysis of *Lactobacillus acidophilus*. *J. Infect. Dis.* **110**:258-267.
7. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**:751-766.
8. London, J. 1977. Evolution of proteins in prokaryotes and bacterial phylogeny. *Trends Biochem. Sci.* **2**:256-258.
9. London, J., and N. M. Chace. 1977. New pathway for the metabolism of pentitols. *Proc. Natl. Acad. Sci. USA* **74**:4296-4300.
10. London, J., and N. M. Chace. 1979. Pentitol metabolism in *Lactobacillus casei*. *J. Bacteriol.* **140**:949-954.
11. London, J., and S. Z. Hausman. 1982. Xylitol-mediated transient inhibition of ribitol utilization by *Lactobacillus casei*. *J. Bacteriol.* **150**:657-661.
12. London, J., and S. Z. Hausman. 1983. Purification and characterization of the III^{xil} phospho-carrier protein of the phosphoenolpyruvate-dependent xylitol:phosphotransferase found in *Lactobacillus casei*. *J. Bacteriol.* **156**:611-619.
13. London, J., and K. Kline. 1973. Aldolase of lactic acid bacteria: a case history in the use of an enzyme as an evolutionary marker. *Bacteriol. Rev.* **37**:453-478.
14. London, J., E. Y. Meyer, and S. R. Kulczyk. 1971. Detection of relationships between *Streptococcus faecalis* and *Lactobacillus casei* by immunological studies with two forms of malic enzyme. *J. Bacteriol.* **108**:196-201.
15. Munn, D. H. C., and K. C. Smith. 1968. A micro phosphate method. *Anal. Biochem.* **26**:364-368.
16. Nowlan, S. S., and R. H. Deibel. 1967. Group Q streptococci. I. Ecology, serology, physiology, and relationships to established enterococci. *J. Bacteriol.* **156**:291-296.
17. Stollar, D., and L. Levine. 1963. Two dimensional immunodiffusion. *Methods Enzymol.* **6**:848-854.
18. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.
19. Weber, K., and M. Osborne. 1969. The reliability of molecular weight determinations by sodium dodecyl sulfate polyacrylamide gel electrophoresis. *J. Biol. Chem.* **224**:4406-4412.