JOURNAL OF BACTERIOLOGY, Nov. 1983, p. 611–619
0021-9193/83/110611-09$02.00/0

Vol. 156, No. 2

Purification and Characterization of the III^{Xtl} Phospho-Carrier Protein of the Phosphoenolpyruvate-Dependent Xylitol:Phosphotransferase Found in *Lactobacillus casei* C183

JACK LONDON* AND SALLY Z. HAUSMAN

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

Received 5 May 1983/Accepted 10 August 1983

The phosphoenolpyruvate-dependent xylitol:phosphotransferase system of *Lactobacillus casei* strain C183 requires a small, soluble, substrate-specific protein for catalytic activity. Designated enzyme III^{Xtl} (or III^{Xtl}), the protein was purified to electrophoretic homogeneity and characterized. III^{Xtl}, as purified, is a single polypeptide composed of 109 amino acid residues. It has an estimated molecular weight of 12,000 and is hydrophobic in nature. The hydrophobicity of III^{Xtl} is apparently due to the fact that the enzyme was isolated as the phosphorylated phosphocarrier protein. Removal of the phosphate group with alkaline phosphatase results in the loss of immunological cross-reactivity with anti-P-III^{Xtl} and an alteration in charge. The *L. casei* C183 III^{Xtl} is antigenically related to enzymes III^{Xtl} in *Streptococcus avium* and other, genetically distinct strains of *L. casei*.

Certain strains of lactic acid bacteria are readily induced to grow at the expense of ribitol, xylitol and D-arabitol, or all three polyhydric alcohols (12). In marked contrast to the gram-negative enteric bacteria which transport the five carbon polyols via specific permeases (18), the gram-positive lactic acid bacteria fulfill that function with phosphoenolpyruvate-dependent phosphotransferases (PTS) (12). In a recent report (14) we presented preliminary evidence that, in addition to the two soluble, general-purpose PTS proteins, enzymes I and HPr, the xylitol PTS contains a substrate-specific soluble component and at least one membrane-associated recognition component (EII^{Xtl}). Because of its soluble nature, the substrate-specific component was thought to be functionally analogous to the III^{Lac} of *Staphylococcus aureus* (9) or III^{Glu} of *Salmonella typhimurium* (17, 23) and was tentatively designated III^{Xtl}.

The five carbon polyalcohols constitute a group of unique carbon sources that are metabolized by relatively few microorganisms (18). It is precisely because of this trait that one of them, xylitol, is now being used as a sweetener in a variety of foods. However, virtually nothing is known about the mechanism(s) by which either gram-positive or gram-negative bacteria transport these substances. Indeed, within PTS systems in general, little is known of the physical nature of the enzymes III or the manner in which they interact with their respective membrane bound counterparts, the enzymes II. For these reasons, III^{Xtl} of *Lactobacillus casei* C183 was purified to electrophoretic homogeneity and characterized. In this report we describe the biochemical and immunological properties of P-III^{Xtl} from *L. casei* and compare the characteristics of the enzyme with those of other enzymes III.

MATERIALS AND METHODS

Cultivation and maintainance of bacterial strains. *L. casei* strain C183, *L. casei* strain ATCC 393 (C128), and *Streptococcus avium* strain 559 (from Robert Deibel, University of Wisconsin) were maintained on NIH litmus milk medium (15). Cell pastes to be used for all biochemical and immunological assays and starting material for the isolation of specific enzymes were harvested from *Lactobacillus* carrying medium (8a) supplemented with filter-sterilized solutions of glucose or the appropriate pentitol to a final concentration of 20 or 50 mM, respectively. Cultures were grown without mixing at 37°C and harvested after a 16-h incubation period.

Purification of enzyme P-III^{Xtl}. A paste containing between 16 and 22 g (wet weight) of cells was suspended in 0.02 M potassium phosphate buffer (pH 7.0) containing 10 mM β-mercaptoethanol (KPME) to a final volume of 75 ml. The suspension was disrupted by two 6-min exposures to a Branson model 350 sonifier operating at 75% of maximum power with a jacketed cup cooled by a constant flow of ice water. The extract was centrifuged at 25,000 × *g* for 20 min,

and the supernatant was decanted and centrifuged at 220,000 × *g* in a Beckman model L2-65B ultracentrifuge for 2 h to separate the soluble cytosol from the membrane particles. The high-speed supernatant (HSS) was applied to a 20- by 2.5-cm DEAE-52 column equilibrated with KPME buffer (pH 7.2), and the column was washed with 75 ml of KPME buffer. Most of the P-III^{Xtl} activity was washed through the column, whereas 85% or more of the total soluble protein was retained on the column. The DEAE 52 washout was concentrated to a volume of 5 ml with an Amicon concentrating device with a YM-10 filter. The concentrated preparation was applied to a 90- by 2.5-cm Sephadex G50 column equilibrated with KPME containing 0.1 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (Calbiochem) and eluted with the same buffer in 5-ml fractions. Peak activity fractions were pooled and concentrated as described above to a volume of 4 to 5 ml. The concentrated G-50 eluate was made up to 1 M NaCl and applied to a 35- by 1.5-cm octyl-Sepharose CL-4B column equilibrated with KPME buffer containing 0.5 M NaCl. After the column was washed with 100 ml of KPME containing 0.1% Lubrol-PX (Sigma Chemical Co.), P-III^{Xtl} activity was eluted with KPME and a 0.1 to 0.2% gradient of Lubrol-PX. Because Lubrol-PX reduces surface tension of the buffer, as the concentration of the detergent increased the drop size and hence the fraction volume decreased. To compensate for the decreasing fraction volumes and to facilitate the assay procedure, 100-drop fractions were collected, every five fractions were pooled, and the volume of each pool was determined. P-III^{Xtl} activity was eluted at a Lubrol-PX concentration of between 0.15 and 0.17%. Since Lubrol-PX interferes with the Bio-Rad protein determination, values are corrected by using comparable controls containing only the detergent. The complete purification procedure which resulted in a recovery of 53% of the activity is summarized in Table 1. Purified P-III^{Xtl} was stable for several months when stored at −20°C in Lubrol-PX. Evidence demonstrating that the enzyme was in the phosphorylated form and the probable reasons for its existence in that form will be presented later.

PTS assays. Phosphorylation assays for cell-free systems were carried out in reaction mixtures containing the following (final concentrations in a final volume of 125 μl): potassium phosphate buffer (pH 7.0), 10 mM; $MgCl_2$, 1 mM; NaF, 5 mM; phosphoenolpyruvate, 10 mM; β-mercaptoethanol, 5 mM; xylitol (carrier) 1 mM; [*U*-C^{14}]xylitol (0.01 μCi or 10 μCi/mmol of xylitol; Amersham, England). An amount of extract of xylitol-grown *L. casei* Cl83 sufficient to produce a linear rate of xylitol 5-phosphate production over a 30-min period was used to start the reaction. Galactose PTS assays were carried out in an identical system employing extracts of galactose-grown *L. casei* Cl83, 1 mM D-galactose (carrier), and 0.02 μCi of [*U*-^{14}C]galactose (20 μCi/mmol of galactose). Reactions were carried out in 12- by 75-mm glass tubes incubated at 37°C; 10-μl samples were withdrawn at 10- or 15-min intervals, spotted on DE81 filter paper circles (Whatman, England), and washed twice with 5 ml of distilled water. Extracts of *L. casei* were added to reaction mixtures at protein levels ranging from 400 to 600 μg. When extracts were fractionated into their cytosol (HSS) and membrane components (high-speed pellet [HSP]) by centrifugation at 220,000 × *g* for 120 min in the Beckman model L2-65B centrifuge the PTS system was reconstituted by combining approximately 400 μg of protein HSS with 400 to 600 μg of HSP. Before use in the assays, the HSP was washed twice with KPME and subjected to high-speed centrifugation to free it of adsorbed HSS components.

TABLE 1. Procedure for the purification[a] of P-III^{Xtl}

Purification step	Total vol (ml)	Total protein (mg)	Total activity (μmol of Xtl[b] per min)	Sp act (nmol of Xtl per min per mg of protein)
Crude extract (HSS)	65	2,015	34.2	16
DE52 wash	7.0	33.6	25.2	750
Sephadex G-50	4.4	5.7	14.1	2,473
Octyl Sepharose	16.9	4.3[b]	17.3	4,032

[a] The starting material was 17.2 g (wet weight) of cells.

[b] Xtl, Xylitol 5-phosphate.

[c] This protein value was corrected for the interference produced by Lubrol-PX.

To monitor P-III^{Xtl} activity during the purification procedure, HSP from xylitol-grown cells of *L. casei* Cl83 was initially mixed with HSS from glucose-grown strain Cl83 cells. However, this mixture generated a low constitutive level of activity equal to roughly 20% of that observed with the homologous xylitol system. To circumvent this problem, the HSS fraction from *L. casei* Cl28, a close genetic relative of *L. casei* Cl83 lacking the xylitol transport system, was used instead of the strain Cl83 HSS. This material produced a baseline rate of xylitol phosphorylation of 0.02 nmol/min per mg of protein or less. PTS activity with the latter system exhibited a strict dependence on the addition of P-III^{Xtl}. P-III^{Xtl} activity is reported as nanomoles xylitol 5-phosphate produced per minute per milligram of protein in a P-III^{Xtl} limiting assay system.

PAGE. Anionic polyacrylamide gel electrophoresis (PAGE) was carried out by the procedure of Davis (5) with a 7.5% gel system. Protein was detected by staining gels with Coomassie blue. The Schiff-periodate stain was used to detect associated polysaccharide (16). Sodium dodecyl sulfate-PAGE was performed by the method of Weber and Osborne (26) or King and Laemmli (11).

Isoelectric focusing in a thin layer of 7.5% polyacrylamide containing a 2% solution of the appropriate ampholines was carried out with the LKB 2117 multiphor by following the manufacturer's instructions (LKB bulletin 1-2117-EOL). Gels were fixed overnight in 12% trichloroacetic acid, rinsed with several changes of distilled water, and stained with Coomassie blue R-250 (7). The pI of P-III^{Xtl} was determined by comparing the protein's migration rate with those of standard proteins of known pI.

Amino acid analysis. The four-buffer procedure of Piez et al. (22) was used to estimate the amino acid content of P-III^{Xtl}. P-III^{Xtl} samples containing 100 μg of protein were hydrolyzed in constant boiling 6 N HCl

(Pierce Chemical Co.) at 108°C for 24 h. Hydrolysates were brought to dryness with a rotovac, washed with distilled deionized water, dried again, and suspended to a concentration of 1 mg/ml in 1 M citrate buffer (pH 2.2). Analyses were performed with a Durrum (Dionex) B55 amino acid analyzer.

Immunological procedures. Anti-PIII^{Xtl} sera were raised in 3- to 4-month-old white male New Zealand rabbits with the following injection schedule. Four intradermal injections containing 80 μg of P-III^{Xtl}, 5 μg of methylated bovine serum albumin (fraction V), and 0.3 ml of complete Freund adjuvant were administered to each rabbit at weekly intervals. Those were followed by three intravenous injections containing 100 μg of P-III^{Xtl} plus 5 μg of methylated bovine serum albumin. Rabbits were bled 2 weeks after the last injection and at 9-day intervals thereafter for the next month. The blood was allowed to clot overnight at 4°C, and the serum was decanted and clarified by centrifugation at 27,000 × *g* for 15 min.

Immunodiffusion was carried out by the Ouchterlony technique as modified by Stollar and Levine (24). The procedure for the quantitative precipitation of III^{Xtl} from HSS fractions has been described previously for another enzyme (21).

Molecular weight determinations. The M_r of enzyme III^{Xtl} was estimated by molecular sieve chromatography with 90- by 2.5-cm columns containing either Sephacryl S-200 or Sephadex G50 by the method of Andrews (1). The S-200 column was standardized with ferritin (M_r, 440,000), rabbit heart muscle aldolase (M_r, 158,000), bovine serum albumin (M_r, 67,000), ovalbumin (M_r, 43,000), chymotrypsinogen A (M_r, 25,000), and RNase (M_r, 13,700), whereas the G50 column was standardized with ferritin, carbonic anhydrase (M_r, 30,000), chymotrypsinogen A, and RNase. Protein was determined by the biuret method or with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

Fatty acid analysis. A modification of the method of Kenyon and Stanier (10) was used to determine whether P-III^{Xtl} contained associated lipid. Approximately 10 mg of P-III^{Xtl} was dialyzed overnight against 1 liter of distilled water and then lyophilized. Methylation of the lyophilized samples was carried out by (i) treating III^{Xtl} with 0.5 to 1.0 ml of methanolic base (Supelco) for 15 min and acidification with 0.5 M HCl to pH 2 to 3 or (ii) saponifying protein samples with a 5-ml mixture of 50% KOH, methanol, and water in a ratio of 1:1:1 for 1 h at 65°C. Samples were then acidified with concentrated HCl to a pH of 2. The acidified preparations were extracted several times with equal volumes of heptane, and extracts were pooled and brought to dryness with a stream of N_2. After drying, the samples were treated with diazomethane prepared by the method of Fales and Jaouni (8) and analyzed with a programmable Packard model 429 gas chromatograph equipped with either an OV17 Chromosorb WHP or SE30 column. Lipid standards were purchased from Sigma or Supelco.

Dephosphorylation of P-III^{Xtl}. Before the treatment of P-III^{Xtl} with swine mucosal alkaline phosphatase (Sigma) or calf intestinal alkaline phosphatase (Miles Laboratories), 1 to 2 ml of HSS from xylitol-grown cells or P-III^{Xtl} purified through the G-50 step was dialyzed against 0.05 M Tris-hydrochloride buffer (pH 8.5) containing 10 mM β-mercaptoethanol for 24 h at 4°C to remove residual phosphate and adjust the pH of the preparations to 8.0 or greater. Approximately 200 μg of dialyzed HSS or 10 μg of the dialyzed G-50 preparation was treated with between 10 and 30 μl of alkaline phosphatase (30 to 50 U) in a buffered solution containing 50 mM Tris-hydrochloride (pH 9.0), 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$ made up to a final volume of 60 μl. At hourly intervals over a 6- to 8-h period, 3 μl of the reaction mixture was removed and tested for the loss of immunological cross-reactivity in conventional immunodiffusion plates containing 20 μl of anti-P-III^{Xtl} in the center well. Larger quantities of the reaction mixture were used to detect charge shifts by PAGE. Protease activity in the alkaline phosphatase was determined by measuring dye released from the protease substrate Azacol according to the manufacturer's (Sigma) instructions with a Gilford model 2400S spectrophotometer at a wavelength of 530 nm.

RESULTS

Detection of P-III^{Xtl}. The existence of a soluble, substrate-specific component of the xylitol PTS was discovered from the ability to reconstitute the activity by combining the HSP fraction derived from xylitol-grown *L. casei* Cl83 cells with an HSS fraction from glucose-grown cells of the same organism (Table 2). In the homologous system, maximum activity was observed only when HSS and HSP fractions of xylitol-grown cells were recombined; recombining the HSS and HSP fractions from glucose-grown cells or the HSS from xylitol-grown cells with the HSP from glucose-grown cells produced no measurable xylitol PTS activity. However, the combination of HSS and HSP derived from extracts of glucose and xylitol-grown cells, respectively, consistently produced a low level of PTS activity; the reason for this will be discussed later. Fractions derived from D-arabitol-

TABLE 2. Requirement of soluble, substrate-specific component in xylitol PTS and interchangability of components between heterologous transport systems

Complementations	Source of PTS components		Sp act (nmol/min per mg of protein)
	HSS	HSP	
Homologous[a]	Xtl	Xtl	1.1
	Glu	Glu	0
	Glu	Xtl	0.09
	Xtl	Glu	0.02
Heterologous[b]	*S. avium* 559	*S. avium* 559	1.5
	S. avium 559	*L. casei* Cl83	0.5
	L. casei Cl83	*S. avium* Cl83	2.2

[a] All components were derived from cells of *L. casei* Cl83 grown on the respective substrates.

[b] All components were derived from *S. avium* 559 cells or *L. casei* Cl83 cells grown at the expense of xylitol.

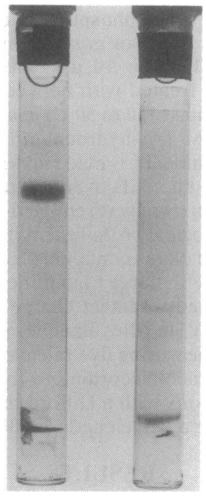

FIG. 1. PAGE of purified III^{Xtl}. (A) Anionic 7.5% acrylamide gel loaded with 40 μg of protein. (B) Sodium dodecyl sulfate–7.5% acrylamide gel loaded with 25 μg of protein.

grown cells gave rates of xylitol PTS activity identical to those of the xylitol cell-free system (data not shown) when recombined together or complemented with fractions from xylitol-grown cells. Extracts prepared from gluconate- or ribose-grown cells produced lower rates of PTS activity under the same conditions, rates were similar or identical to those reported earlier (13).

Complementation of heterologous HSS and HSP fractions obtained from xylitol-grown cells of *L. casei* C183 and *S. avium* (Table 2) or *L. casei* C183 and *L. casei* C116 (data not shown) also produced xylitol PTS activity. These observations indicated that the respective components were functionally compatible and that they might possibly share some degree of structural homology.

Physical and biochemical properties of P-III^{Xtl}. The state of purity of P-III^{Xtl} was ascertained by PAGE. Purified P-III^{Xtl} migrated as a single protein band in 7.5% anionic polyacrylamide gels or sodium dodecyl sulfate-polyacrylamide gels (Fig. 1). The faint upper bands with the light area beneath them that migrated in the area of proteins with M_r of 35,000 and 24,000, respectively, appear to be an artifact produced by the Lubrol-PX. However, if the two faint bands do, in fact, represent contaminating proteins, they comprise less than 3% of the total protein in the preparation as determined by a comparison of the areas under the peaks of a gel scan performed at 600 nm (data not shown). In a calibrated Sephadex G-50 column, P-III^{Xtl} migrated like a protein with a M_r of 12,000 ± 2,000 when eluted with a low-ionic-strength buffer (data not shown; see Fig. 3). The subunit molecular weight was estimated to be between 10,000 to 11,000 by sodium dodecyl sulfate-PAGE, indicating that, in the form isolated, P-III^{Xtl} exists as a single polypeptide chain. In isoelectric focusing experiments, III^{Xtl} exhibited a pI of 6.3 to 6.4. Fatty acid analysis indicated that the enzyme contained no associated lipid, and Schiff-periodate stains of anionic polyacrylamide gels gave no indication of associated polysaccharide.

In PTS assay systems employing an HSS fraction from glucose-grown cells of *L. casei* C128 and a HSP fraction from xylitol-grown cells of *L. casei* C183, a complete dependence of transport activity upon III^{Xtl} was easily demonstrable (Fig. 2).

Hydrophobic nature of P-III^{Xtl}. The fact that P-III^{Xtl} readily absorbed to octyl Sepharose suggested that the protein was, by nature, hydrophobic. Hydrophobicity was expressed in yet another fashion. When the partially purified protein (before contact with Lubrol-PX) was applied to a Sephacryl S-200 column equilibrated with KPME buffer containing 0.1 M KCl, all P-III^{Xtl} activity was eluted with the void volume and exhibited an apparent M_r of greater than 200,000 (Fig. 3); this form of the enzyme lost 80 to 90% of its catalytic activity within 24 h when stored at room temperature or 4°C. When the ionic concentration of the S-200 column was reduced by equilibration with only KPME, P-III^{Xtl} migrated with the RNase standard (Fig. 3).

Amino acid analysis. P-III^{Xtl} is composed of 109 amino acid residues (Table 3). The only sulfur-containing amino acid found was methionine. If the enzyme functions like other enzymes

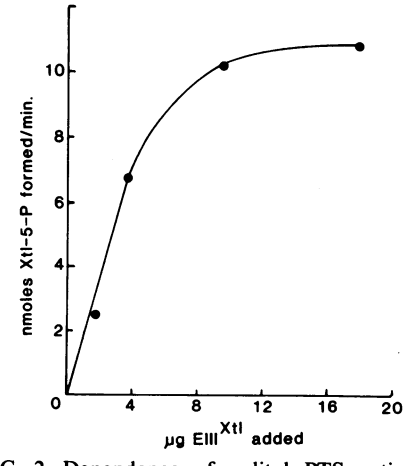

FIG. 2. Dependence of xylitol PTS activity on III^{Xtl}. The assay system contained the HSS fraction from glucose-grown *L. casei* C128 containing 475 μg of protein and the HSP fraction from xylitol-grown *L. casei* C183 containing 430 μg of protein. III^{Xtl} was added at protein concentrations of between 1.8 mg and 18 μg.

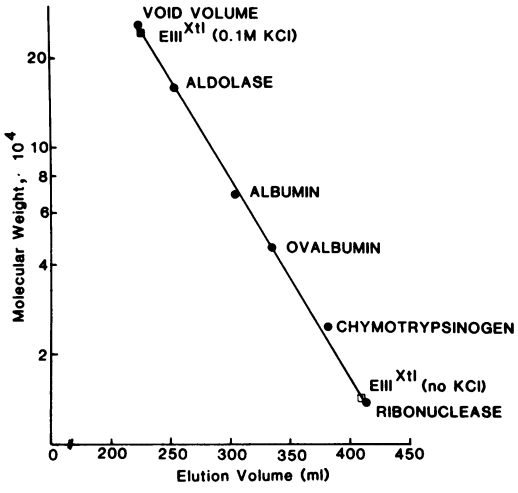

FIG. 3. Effect of KCl on the migration rate of III^{Xtl} on a Sephacryl S-200 column. A diethylaminoethyl eluate was divided equally to give identical III^{Xtl} fractions containing 40 μg of protein each. The migration rate of each was determined in a Sephacryl S-200 column equilibrated with KPME to which 0.1 M KCl had been added in one instance.

III, one of the five histidine residues probably acts as the phosphoryl acceptor (6, 9).

Immunological experiments. Structural similarities among the III^{Xtl} of lactic acid bacteria were demonstrated by immunodiffusion experiments in which extracts of ribitol-grown *L. casei* Cl16 (under these conditions the organism possesses a xylitol PTS [15]) and xylitol- or D-arabitol-grown *S. avium* 559 were tested against anti-*L. casei* Cl83 P-III^{Xtl} (Fig. 4). Extracts of *L. casei* Cl16 and *S. avium* 559 were tested against each other or with the reference strain. The streptococcal III^{Xtl} was the protein least related to the *L. casei* Cl83 P-III^{Xtl}.

Extracts prepared from *L. casei* Cl83 cells grown on lactose, galactose, glucose, and mannitol were also tested against anti-P-III^{Xtl}. The extracts produced fused lines of precipitation when compared with one another and with extracts of xylitol-grown *L. casei* Cl83 (Fig. 5); extracts of mannitol- and glucose-grown cells produced the faintest spurs. Unfortunately, the immunodiffusion experiments did not clearly differentiate between (i) the presence of low constitutive levels of P-III^{Xtl} in the respective extracts or (ii) possible cross-reactions of anti P-III^{Xtl} with soluble III components of other PT systems. If anti-P-III^{Xtl} was reacting with soluble components of other transport systems, it should be possible to inhibit such PTS reactions with the antiserum. However, should P-III^{Xtl} be synthesized during growth on other substrates, the antibody would have no effect on the activity of other PTS systems. If the latter supposition is true, HSS fractions obtained from cells grown on putatively noninducing substrates could be used to complement the HSP fraction from xylitol-grown cells to produce significant levels of PTS activity. Since Chassy and Thompson (3, 4) presented preliminary evidence identifying III-like components associated with both the lactose and galactose PTS of *L. casei*, the latter system was used as a control in the following studies.

Direct assays which incorporated anti-P-III^{Xtl} in the PTS reaction mixture could not be used because nonimmune sera produced a lower, but significant, level of inhibition (data not shown); the reason for the inhibition is not yet known. Thus, the indirect procedure described above was used to lower the actual serum concentrations below the inhibitory threshold in the assays. Anti-P-III^{Xtl} removed essentially all P-III^{Xtl} activity from HSS fractions, whereas normal sera had no effect on III^{Xtl} activity (Fig. 6A). In contrast, the same levels of anti-P-III^{Xtl} had no effect on the galactose PTS (Fig. 6B) even though copious amounts of immune precipitate were produced during the overnight incubation of HSS with anti-P-III^{Xtl}.

The finding that P-III^{Xtl} was synthesized during growth at the expense of noninducing substrates was supported by complementation experiments. In these experiments, HSS fractions derived from glucose- or galactose-grown cells were combined with an HSP fraction obtained from xylitol-grown cells of *L. casei* Cl83 (Fig. 7). At comparable protein concentrations, HSS derived from galactose- and glucose-grown cells supported a rate of xylitol PTS activity equivalent to 38 and 20%, respectively, of that observed with the homologous system. Finally, it

TABLE 3. Amino acid analysis of enzyme III^{Xtl}

Amino acid	Residues per 1,000 residues	Mol/mol of protein[a]
Asp	84	9
Thr	88	10
Ser	57	6
Glu	78	9
Pro	19	2
Gly	64	7
Ala	93	10
Val	67	7
Met	43	5
Ileu	93	10
Leu	89	10
Tyr	17	2
Phe	21	2
His	42	5
Lys	132	14
Arg	12	1

[a] Based on a molecular weight of 12,000.

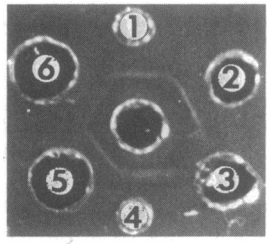

FIG. 4. Demonstration of immunological homology among the enzymes III^{Xtl} from several lactic acid bacteria. In clockwise order starting with the uppermost well, each well received extracts of the following: 1, xylitol-grown *L. casei* Cl83 (35 μg of protein); 2, D-arabitol-grown *L. casei* Cl83 (25 μg of protein); 3, glucose-grown *L. casei* Cl28 (42 μg of protein); 4, xylitol-grown *L. casei* Cl83 (35 μg of protein); 5, ribitol-grown *L. casei* Cl16 (75 μg of protein); 6, xylitol-grown *S. avium* 559 (100 μg of protein). The center well contained 25 μl of anti-P-III^{Xtl}.

was possible to remove all III^{Xtl} activity from galactose HSS by treatment with anti-P-III^{Xtl} (data not shown).

Alterations in the conformation of P-III^{Xtl} as a consequence of dephosphorylation. According to Deutscher et al. (6), III^{Lac} of *S. aureus* undergoes a conformational change when phosphorylated which results in its conversion from a hydrophilic to a hydrophobic molecule. Two other consequences of this change in state were (i) a complete loss of immunological cross-reactivity with anti-III^{Lac} and (ii) a charge shift. Because preliminary attempts to phosphorylate III^{Xtl} were unsuccessful (data not shown) and the protein exhibited a distinctly hydrophobic character, it appeared likely that III^{Xtl} was actually isolated as P-III^{Xtl}. To remove the putative phosphate group from III^{Xtl}, HSS fractions or partially purified P-III^{Xtl} free of Lubrol-PX were treated with swine mucosal or calf intestinal alkaline phosphatase. Although P-III^{Xtl} was not

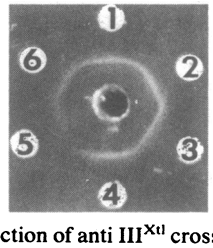

FIG. 5. Detection of anti III^{Xtl} cross-reacting material in extracts from cells of *L. casei* Cl83 grown on noninducing substrates. In clockwise order starting with the uppermost well, each received extracts of *L. casei* Cl83 prepared from cells grown on the following: 1, xylitol; 2, D-arabitol; 3, D-galactose; 4, lactose; 5, D-glucose; mannitol. Sample wells received between 30 and 35 μg of extract protein. The center well contained 20 μl of anti-P-III^{Xtl}.

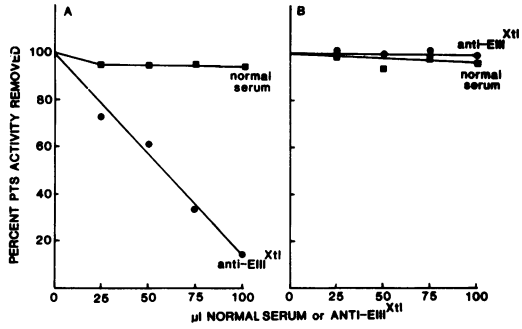

FIG. 6. Removal of P-III^{Xtl} from HSS fractions of xylitol-grown cells of *L. casei* Cl83 with anti-P-III^{Xtl}. (A) The xylitol PTS assay mixture contained the following: 50 μl of the HSS fraction from glucose-grown *L. casei* Cl28 (620 μg of protein); 10 μl of the HSP fraction from xylitol-grown *L. casei* Cl83 (460 μg of protein); 10 μl of the HSS fraction from xylitol-grown *L. casei* (120 μg of protein) with a 50-μl sample treated with 25, 50, 75, or 100 μl of anti-P-III^{Xtl}. (B) The galactose PTS assay mixture contained the following: 50 μl of the HSS fraction from glucose-grown *L. casei* Cl83 (620 μg of protein); 10 μl of the HSP fraction from galactose-grown *L. casei* Cl83 (940 μg of protein); 10 μl of the HSS fraction from galactose-grown *L. casei* Cl83 (140 μg of protein) with a 50-μl sample treated with anti-P-III^{Xtl} as in A. Rates were determined over a 15-min period.

the ideal substrate for the phosphatases, after 4 to 6 h of incubation the immunological reactivity between the purified enzyme preparation and anti-P-III^{Xtl} was markedly reduced as monitored by immunodiffusion (Fig. 8A). Identical results were obtained with HSS fractions (data not shown). Experiments repeated in the presence or absence of 0.01 M EDTA established that the loss of immunological reactivity was not the result of trace amounts of contaminating protease activity present in the phosphatase preparations (Fig. 8B and C). Phosphatase activity that is dependent on the presence of Zn^{2+} and Mg^{2+} was inhibited by EDTA, and immunological reactivity was not lost, whereas protease activity as measured by Azacol solubilization decreased only slightly from a rate of change of optical density at 530 nm of 0.016 to 0.013 per h. If III^{Xtl} had been subjected to the proteolytic action in the phosphatase preparation, its degradation should have produced spur formation and multiple bands in the Ouchterlony plates; none was observed. Therefore, the action of the phosphatase preparations on immunological reactivity can be attributed to their primary function of dephosphorylation. Analysis of the phosphatase-treated reaction mixture by PAGE revealed that the disappearance of the major band in the partially purified preparations of III^{Xtl} coincided with the appearance of a new, more basic pro-

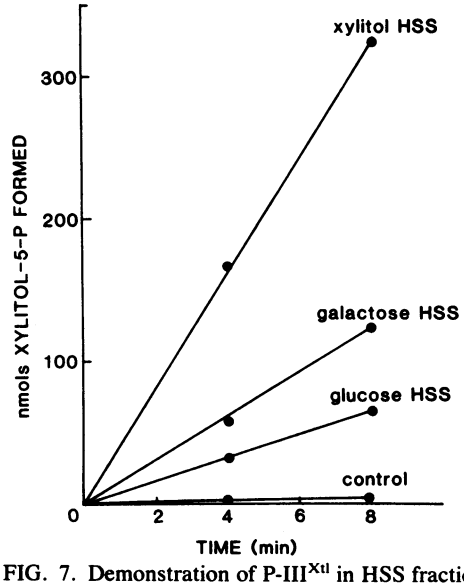

FIG. 7. Demonstration of P-III^{Xtl} in HSS fractions of glucose- and galactose-grown cells of *L. casei* Cl83. The xylitol PTS reaction mixture contained the following: 40 μl of the HSS fraction from glucose-grown *L. casei* Cl28 (490 μg of protein); 10 μl of the HSP fraction from xylitol-grown *L. casei* Cl83 (460 μg of protein); 10 μl of protein from *L. casei* Cl83 grown on xylitol (120 μg of protein), galactose (140 μg of protein), or glucose (124 μg of protein). The control contained 40 μl of the HSS from *L. casei* strain Cl28 (490 μg of protein) and 10 μl of the HSP from *L. casei* C183 (460 μg of protein).

tein band with a significantly altered R_f of 0.56 ± 0.02 (Fig. 9). To verify that the phosphatase-treated enzyme preparations had not been structurally altered, dephosphorylated III^{Xtl} obtained after phosphatase treatment of HSS fractions was tested for activity in the conventional PTS complementation assay system. The HSS fraction treated for 6 h with alkaline phosphatase gave a rate identical to that of the untreated control (data not shown).

DISCUSSION

Catalytic activity of the phosphoenolpyruvate-dependent xylitol PTS of *S. avium* and certain strains of *L. casei* depends upon a soluble, substrate-specific component similar to the enzymes III associated with the high-affinity glucose PTS of *Salmonella typhimurium* (17, 23), the lactose PTS of *S. aureus* (6, 9), and the gluconate PTS of *Streptococcus faecalis* (2). Because of its soluble nature, ability to exist in a phosphorylated state, and small size, this PTS component has been designated III^{Xtl}. With an estimated molecular weight (M_r) of 12,000, the *L. casei* P III^{Xtl} appears to be the smallest catalytically active enzyme III described thus far. However, III^{Lac} and III^{GluA} are composed of protomers having a molecular weight of 12,000, and it is possible that in the dephosphorylated state, III^{Xtl} exists as a stable aggregate with a higher molecular weight.

The intensely hydrophobic behavior of III^{Xtl} distinguished it from III^{Lac} (6) and III^{Glu} (17); the difference between the proteins can be attributed to the state in which III^{Xtl} was isolated, namely, as the phosphoprotein. Deutscher et al. (6) reported that phosphorylation of *S. aureus* III^{Lac} converted the protein to a more hydrophobic molecule with a concomitant loss of immunological reactivity and a charge shift (6). These workers proposed that III^{Lac} underwent a conformational shift as a result of the phosphorylation and that the resultant increase in hydrophobicity permitted the protein to interact with that part of the membrane containing EII^{Lac}. The action of alkaline phosphatase on P-III^{Xtl} appears to produce analogous changes in the enzyme's properties as manifested by a loss of immunological reactivity and a shift of the migration rate in anionic polyacrylamide gels. Nairn et al. (19) demonstrated that the presence or absence of a phosphate group on a synthetic heptapeptide representing one of the antigenic determinants of G substrate dictated antigenic specificity; that is, antibodies produced against

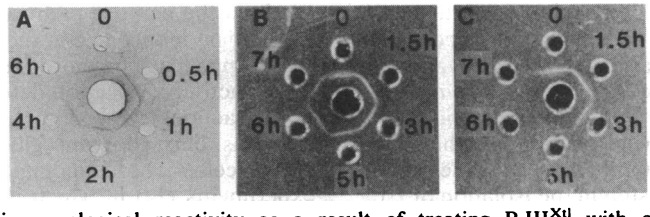

FIG. 8. Loss of immunological reactivity as a result of treating P-III^{Xtl} with calf intestine alkaline phosphatase. (A) Immunodiffusion plate (stained) charged with samples of a reaction mixture containing 10.5 μg of III^{Xtl} (G-50 preparation) and 42 μg of phosphatase (40 U). Samples were removed at the times indicated. (B) Immunodiffusion plate containing a reaction mixture consisting of 10.5 μg of III^{Xtl} (G-50 preparation), 70 μg of phosphatase (80 U) and EDTA to a final concentration of 0.01 M. (C) Same as B, except EDTA was omitted. The time sequence was as indicated.

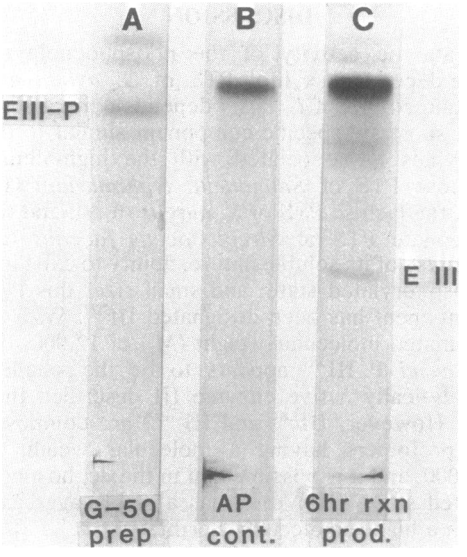

FIG. 9. Alteration of electrophoretic mobility of III^{Xtl} produced by alkaline phosphatase treatment. (A) G50 preparation control (20 μg of protein); (B) calf intestine alkaline phosphatase control (30 μg of protein); (C) 6-h reaction mixture (45 μg of protein; 7 μg of III^{Xtl} plus 38 μg of phosphatase).

one form (i.e., dephospho form) failed to recognize the other (i.e., phospho form) at equivalent antiserum concentrations. There is precedent, therefore, for localized conformational alterations resulting from phosphorylation or dephosphorylation of specific proteins. However, there appears to be no precedent in the literature which can be used to explain the sort of dislocation or masking of multiple antigenic determinants observed with III^{Lac} and III^{Xtl} by the simple addition or removal of a phosphate group.

The reason III^{Xtl} was isolated in the phosphorylated form, whereas III^{Lac} and III^{Glu} from *S. aureus* (6) and *S. typhimurium* (17, 23), respectively, were purified in the dephosphorylated forms, deserves some discussion. Chassy and Thompson (4, 26) have demonstrated that certain streptococci and lactobacilli maintain large intracellular pools of phosphoenolpyruvate and its triose phosphate precursors under conditions of growth or starvation. This observation has recently been extended to include *L. casei* strain Cl83 (Thompson, private communication). With a phosphoenolpyruvate potential of this magnitude (greater than 30 mM) and with the removal of EII^{Xtl} as the first step in the isolation of III^{Xtl}, it is little wonder that most, if not all, of the III^{Xtl} existed as $P\text{-}III^{Xtl}$. This suggestion is supported by the observation that little or no active dephosphorylated form of III^{Xtl} was recovered from the DE52 column eluted with 0.5 M KCl in KPME (data not shown). Since III^{Xtl} is found in the primed state as $P\text{-}III^{Xtl}$, it appears likely that the general PTS components, HPr and EI, will also exist in the phosphorylated forms in these organisms. The advantage of such a primed system to resting or starved cells is obvious. The interaction between the substrate recognition component, EII^{Xtl}, and xylitol would trigger an instantaneous response of the transport system resulting in the accumulation of intermediate products vital to the cell's survival.

The facility with which the enzymes III^{Xtl} and membrane-bound enzymes II^{Xtl} of *S. avium* and *L. casei* Cl83 or *L. casei* Cl83 and *L. casei* Cl16 were interchangeable attested to the functional homology of the respective transport systems. In this instance, functional homology reflected a relatively high degree of structural homology as demonstrated by the immunodiffusion experiments. Because ribitol-grown cells of *L. casei* Cl16 gratuitously induce a functional xylitol transport system, extracts of this organism react with anti-III^{Xtl}, as do extracts of both xylitol- and D-arabitol-grown cells of *S. avium*. If any structural homology exists between the III^{Xtl} and the III^{Lac} or III^{Gal} of *L. casei* described by Chassy and Thompson (3, 4), it is below the level of immunological detection.

The high levels of xylitol PTS activity in extracts prepared from cells of *L. casei* Cl83 grown at the expense of gluconate or ribose reported in earlier studies (13) was attributed to the production of an inducer molecule that served as a common intermediate product for the gluconate, pentose, and pentitol pathways. It was assumed, therefore, that the synthesis of the xylitol PTS components was relatively tightly controlled, because virtually no activity could be demonstrated in glucose-grown cells. However, the detection of (i) anti-$P\text{-}III^{Xtl}$ cross-reacting material in extracts of *L. casei* Cl83 cells grown on a variety of carbohydrate substrates and (ii) the demonstration of III^{Xtl} activity in several of the HSS fractions derived from these extracts indicate that III^{Xtl} is not stringently regulated in this stain. Since none of the carbohydrate substrates examined is metabolized via the pentose pathway, it is unlikely the III^{Xtl} was induced gratuitously. In contrast, EII^{Xtl} appears to be more rigorously controlled. EII^{Xtl} activity was not detected when xylitol HSS fractions were complemented with HSP fractions derived from glucose or galactose-grown cells. These findings agree with earlier experiments (14) in which it was demonstrated that total xylitol PTS activity of *L. casei* Cl83 was reduced 8- to 10-fold five generations after the addition of glucose to cultures growing at the expense of xylitol. It will be recalled that III^{Xtl} activity was decreased only fivefold in cultures

fully grown at the expense of glucose (Fig. 7). Future studies will concentrate on the regulation of the xylitol pathway components with specific emphasis on the arrangement of the respective genes within the organism's genome.

ACKNOWLEDGMENTS

We thank Vince Hascall and Guy Hawkins for their assistance with the amino acid analysis of III^{Xtl} and Henry Fales for his assistance with the fatty analysis. Thanks also go to John Thompson and Alan Peterkofsky for their critical evaluation of the manuscript.

LITERATURE CITED

1. **Andrews, P.** 1964. Estimation of the molecular weights of proteins by Sephadex gel filtration. Biochem. J. **91**:222–233.
2. **Bernsmann, P., C. A. Alpert, P. Muss, J. Deutscher, and W. Hengstenberg.** 1982. A bacterial PEP-dependent phosphotransferase system: mechanism of gluconate phosphorylation in *Streptococcus faecalis*. FEBS Lett. **138**:101–103.
3. **Chassy, B. M., and J. Thompson.** 1983. Regulation and characterization of the galactose-phosphoenolpyruvate-dependent phosphotransferase system in *Lactobacillus casei*. J. Bacteriol. **154**:1204–1214.
4. **Chassy, B. M., and J. Thompson.** 1983. Regulation of lactose-phosphoenolpyruvate-dependent phosphotransferase system and β-D-phosphogalactoside galactohydrolase activities in *Lactobacillus casei*. J. Bacteriol. **154**:1195–1203.
5. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum. Ann. N.Y. Acad. Sci. **121**:404–427.
6. **Deutscher, J., K. Beyreuther, H. M. Sobek, K. Stüber, and W. Hengstenberg.** 1982. Phosphoenolpyruvate-dependent phosphotransferase system of *Staphylococcus aureus*: factor III^{lac}, a trimeric phospho-carrier protein that also acts as a phase transfer catalyst. Biochemistry **21**:4867–4873.
7. **Fairbanks, G., T. L. Steck, and D. F. H. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**:2606–2617.
8. **Fales, H. M., and T. M. Jaouni.** 1973. Simple device for preparing ethereal diazomethane without resorting to redistillation. Anal. Chem. **45**:2302–2303.

8a. **Hansen, P. A. (ed.).** 1968. Type strains of *Lactobacillus* species, p. 16. American Type Culture Collection, Rockville, Md.

9. **Hays, J. B., R. O. Simoni, and S. Roseman.** 1973. Sugar transport. V. A trimeric lactose-specific phosphocarrier protein of the *Staphylococcus aureus* phosphotransferase system. J. Biol. Chem. **248**:941–956.
10. **Kenyon, C., and R. Y. Stanier.** 1970. Possible evolutionary significance of polyunsaturated fatty acids in blue-green algae. Nature (London) **227**:1164–1165.
11. **King, J., and U. K. Laemmli.** 1971. Polypeptides of the tail fibers of bacteriophage T4. J. Mol. Biol. **62**:465–477.
12. **London, J., and N. Chace.** 1977. New pathway for the metabolism of pentitols. Proc. Natl. Acad. Sci. U.S.A. **74**:4296–4300.
13. **London, J., and N. M. Chace.** 1979. Pentitol metabolism in *Lactobacillus casei*. J. Bacteriol. **140**:949–954.
14. **London, J., and S. Hausman.** 1982. Xylitol-mediated transient inhibition of ribitol utilization by *Lactobacillus casei*. J. Bacteriol. **150**:657–661.
15. **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.
16. **Maurer, H. R.** 1971. Methods of analytical disc electrophoresis, p. 76–78. *In* W. deCruyter (ed.), Disc electrophoresis and related techniques of polyacrylamide gel electrophoresis. Springer-Verlag, New York.
17. **Meadow, N. D., and S. Roseman.** 1982. Sugar transport by the bacterial phosphotransferase system. Isolation and characterization of a glucose-specific phosphocarrier protein (III^{Glu}) from *Salmonella typhimurium*. J. Biol. Chem. **257**:14526–14537.
18. **Mortlock, R. P.** 1976. Catabolism of unnatural carbohydrates by microorganisms. Adv. Microb. Physiol. **13**:1–58.
19. **Nairn, A. C., J. A. Detre, J. E. Casnellie, and P. Greengard.** 1982. Serum antibodies that distinguish between the phospho- and dephospho-forms of a phosphoprotein. Nature (London) **299**:734–736.
20. **Nowlan, S. S., and R. H. Deibel.** 1967. Group Q streptococci. I. Ecology, serology, physiology, and relationship to established enterococci. J. Bacteriol. **94**:291–296.
21. **Perrin, D.** 1963. Immunological studies with genetically altered β-galactosidases. Ann. N.Y. Acad. Sci. **103**:1058–1060.
22. **Piez, K. A., and L. Morris.** 1960. A modified procedure for the automatic analysis of amino acids. Anal. Biochem **1**:187–201.
23. **Scholte, B. J., A. R. Schuitema, and P. W. Postma.** 1981. Isolation of III^{Glu} of the phosphoenolpyruvate-dependent glucose phosphotransferase system of *Salmonella typhimurium*. J. Bacteriol. **148**:257–264.
24. **Stollar, D., and L. Levine.** 1963. Two dimensional immunodiffusion. Methods Enzymol. **6**:848–854.
25. **Thompson, J.** 1978. *In vivo* regulation of glycolysis and characterization of sugar:phosphotransferase system in *Streptococcus lactis*. J. Bacteriol. **136**:465–476.
26. **Weber, K., and M. Osborne.** 1969. The reliability of molecular weight determinations by sodium dodecylsulfate polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.