JOURNAL OF BACTERIOLOGY, Dec. 1982, p. 1138–1146
0021-9193/82/121138-09$02.00/0

Vol. 152, No. 3

Cloning and Expression of the β-D-Phosphogalactoside Galactohydrolase Gene of *Lactobacillus casei* in *Escherichia coli* K-12

LYANG-JA LEE,[1] JEFFREY B. HANSEN,[2] E. KATARZYNA JAGUSZTYN-KRYNICKA,[2]† AND BRUCE M. CHASSY[1]*

Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205,[1] and Department of Microbiology and Institute of Dental Research, University of Alabama in Birmingham, Birmingham, Alabama 35294[2]

Received 21 June 1982/Accepted 16 August 1982

Lactose metabolism in *Lactobacillus casei* 64H is associated with the presence of plasmid pLZ64. This plasmid determines both phosphoenolpyruvate-dependent phosphotransferase uptake of lactose and β-D-phosphogalactoside galactohydrolase. A shotgun clone bank of chimeric plasmids containing restriction enzyme digest fragments of pLZ64 DNA was constructed in *Escherichia coli* K-12. One clone contained the gene coding for β-D-phosphogalactoside galactohydrolase on a 7.9-kilobase *Pst*I fragment cloned into the vector pBR322 in *E. coli* strain χ1849. The β-D-phosphogalactoside galactohydrolase enzyme isolated from *E. coli* showed no difference from that isolated from *L. casei*, and specific activity of β-D-phosphogalactoside galactohydrolase was stimulated 1.8-fold in *E. coli* by growth in media containing β-galactosides. A restriction map of the recombinant plasmid was compiled, and with that information, a series of subclones was constructed. From an analysis of the proteins produced by minicells prepared from transformant *E. coli* cells containing each of the recombinant subclone plasmids, it was found that the gene for the 56-kilodalton β-D-phosphogalactoside galactohydrolase was transcribed from an *L. casei*-derived promoter. The gene for a second protein product (43 kilodaltons) was transcribed in the opposite direction, presumably under the control of a promoter in pBR322. The relationship of this second product to the lactose metabolism genes of *L. casei* is at present unknown.

Much is known about the structural and regulatory features of the *lac* biochemical pathway that is widespread in both procaryotic and eucaryotic cells (1). Certain gram-positive bacteria, however, have an alternate pathway of lactose metabolism, depicted schematically in Fig. 1, about which comparatively little is known. The uptake pathway consisting of a phosphoenolpyruvate-dependent phosphotransferase system (PEP:PTS) for lactose was first described in *Staphylococcus aureus* (27), as was the enzyme that cleaves intracellular lactose 6-phosphate, β-D-phosphogalactoside galactohydrolase (P-β-galactosidase, EC 3.2.1.85) (26). The galactose 6-phosphate thus formed is further metabolized by the tagatose 6-phosphate pathway described by Bissett and Anderson (3). It appears that the same pathway is used by *Streptococcus faecalis* (25), *Streptococcus mutans* (7), *Streptococcus salivarius* (23), *Streptococcus lactis* (38), and *Lactobacillus casei* (42). The majority of these organisms lack the better-known pathway of lactose metabolism encoded by the *lac* operon. The lactose PEP:PTS has been isolated from *S. aureus* (44) and is composed of a membrane-associated enzyme $II^{lactose}$ and a peripheral membrane protein, enzyme III (or factor $III)^{lactose}$. Enzyme II has a catalytic function (32), while the enzyme III serves as an intermediate phosphate donor (41); little more is known about these proteins. The P-β-galactosidase has been isolated from a number of different bacteria; it bears little resemblance to β-D-galactoside galactohydrolase (β-galactosidase), but is functionally analogous to it. The regulation of expression of these lactose metabolism genes in these bacteria appears quite complex. In *S. aureus*, galactose 6-phosphate has been reported to be the preferred inducer (40). In *S. lactis*, expression appears to be regulated more by a repression due to the growth substrate; for example, full expression is observed after growth on maltose (48) or ribose (34). In *L. casei*

† Present address: Institute of Microbiology, Warsaw University, 00-046 Warsaw, Novy Swiat 67, Poland.

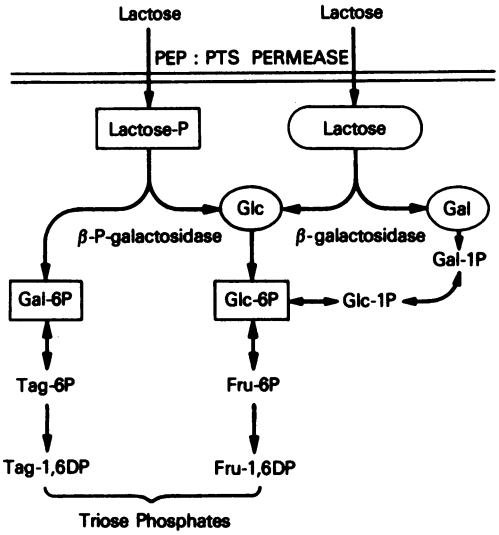


FIG. 1. Lactose metabolic pathways. This is a composite of pathways for lactose, galactose, and glucose metabolism in different bacteria. The entry forms of the sugars after transport across the cytoplasmic membrane via either the PEP:PTS or permease transport system are indicated by squares or circles, respectively. Phosphorylated sugars are indicated by P (or DP for diphosphate), with the position number given. Abbreviations are as follows: Gal, galactose; Glc, glucose; Fru, fructose; and Tag, tagatose. The triose phosphates are metabolized by the lower portion of the Embden-Meyerhoff glycolytic pathway.

64H, a β-galactoside is required for induction; neither galactose nor galactose 6-phosphate can induce expression (B. Chassy and J. Thompson, submitted for publication). In all of these organisms glucose at least partially represses the expression of lactose genes, even though the bacteria lack cyclic AMP (cAMP) (5). The repression is due to glucose PEP:PTS-mediated "inducer exclusion" as well as repressive effects exerted by functional PEP:PTS activities and glucose-derived metabolites (Chassy and Thompson, unpublished data).

Both the origin and the selective factors leading to this alternate pathway of lactose metabolism are unclear, as is the extent of its distribution. A possible mechanism for its dissemination among various bacteria may be indicated by the finding that in *S. lactis* (21, 45) and *L. casei* (11), the lactose metabolism genes are encoded by extrachromosomal elements. *L. casei* 64H contains a 35-kilobase (kb) plasmid, pLZ64, that determines both the lactose PEP:PTS and P-β-galactosidase activities of the strain; the biochemical and physiological characteristics of this strain are also well documented. Some of the lactose plasmids are capable of conjugal self-transfer (13, 21, 45). The degree of homology among the genes specifying lactose metabolic proteins in various gram-positive bacteria is unknown.

Molecular cloning of DNA has proven to be a powerful tool for studying the structure, function, regulation, and relatedness of genes. Cloning and many other genetic manipulations within the genus *Lactobacillus* are at present impossible due to the absence of a transformation system. The present report describes the molecular cloning of the gene determining the P-β-galactosidase of *L. casei* 64H into *Escherichia coli*. We would have preferred to use a gram-positive host-vector system, but neither that described for *Bacillus subtilis* (9) nor that described for *Streptococcus sanguis* (36) fully met our needs. The availability of well-defined hosts and multifunctional cloning vectors such as pBR322 (4) and pACYC184 (8) and the myriad of genetic tools available in *E. coli*, including the ability to use minicell (19) analysis of the products of cloned genes, prompted our choice of this host-vector system. Several drawbacks of this approach are immediately apparent however. In particular, *E. coli* PEP:PTS components do not complement those of gram-positive bacteria very well (43), and *E. coli* lacks the tagatose 6-phosphate pathway necessary for the further metabolism of galactose 6-phosphate (see Fig. 1). Expression of lactose metabolism or lactose PEP:PTS activity was therefore not anticipated; rather, it was decided to construct a clone bank of recombinant chimera-containing isolates, phenotypically identifiable by the insertional inactivation of one of the antibiotic resistance genes in the multifunctional cloning vector. We describe the strategy for construction and analysis of such a clone bank and our characterization of the products of cloned *L. casei* genes in *E. coli*.

MATERIALS AND METHODS

Bacterial strains and growth media. Bacterial strains used are listed in Table 1; all strains were grown at 37°C. *L. casei* was grown in *Lactobacillus* carrying medium (18). Liquid minimal media (ML [15] or M9 [39]) contained 0.5% glucose or 1% glycerol and the following supplements (per ml) as needed: 2 μg of DL-*meso*-diaminopimelic acid (DAP), 20 μg of L-isoleucine, 20 μg of DL-valine, 22 μg of L-histidine hydrochloride, 20 μg of DL-methionine, 40 μg of DL-threonine, 100 μg of L-proline, and 100 μg of L-leucine. To grow cells for minicell harvests, 0.5% Casamino Acids (Difco Laboratories) replaced individual amino acids except DAP. Complex media for *E. coli* growth were L-broth (35) or Penassay (antibiotic medium 2 [Difco] plus 0.5% NaCl) supplemented with DAP. Plate media contained 1.2% agar (Difco). Solid media used for antibiotic selection and screening contained 25 μg of chloramphenicol, 25 μg of ampicillin, or 10 μg of tetracycline per ml.

Plasmid DNA isolation, electrophoresis, restriction,

and transformation. *L. casei* DNA was isolated by the method of Chassy et al. (10, 11); various procedures were used to isolate plasmid DNA from *E. coli* (2, 22, 31). Also, a modification of the method of Holmes and Quigley (28) was used where RNase was included at the same concentration as lysozyme. Plasmid DNA was electrophoresed through 0.7% agarose gels and visualized by UV light fluorescence after staining with ethidium bromide (24). Digestion of DNA with restriction endonucleases used conditions described by the vendors (Bethesda Research Laboratories and New England Biolabs). Restricted DNA was ligated to vector plasmid DNA that was treated with bacterial alkaline phosphatase (49). Ligation conditions were as previously described (24), except that DNA concentrations were adjusted to optimize formation of recombinant circular molecules (17). The method of Tabak and Flavell (47) was used to recover individual restriction fragments after preparative agarose gel electrophoresis. Standard DNAs for size determinations included multiple plasmids from *E. coli* strain V517 (Table 1) or restriction digests of lambda phage DNA (Bethesda Research Laboratories). Transformation of recombinant plasmids into *E. coli* strains, with selection for acquisition of antibiotic resistances, was as previously described (24, 30).

Minicell analysis of plasmid gene products. Minicells of plasmid-containing derivatives of *E. coli* χ1849 were purified and incubated with a U-^{14}C-labeled amino acid mixture (ICN; 0.1 mCi/ml) as previously described (24), except that preincubation before labeling was extended to 60 min to permit degradation of stable mRNA. Lysis and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli and Favre (33) and fluorography were as previously described (24).

Enzyme assays and the isolation of P-β-galactosidase. Plasmid-containing *E. coli* cells for enzyme isolation were prepared by growth in L-broth supplemented with DAP (1 liter distributed among six 500-ml Erlenmeyer flasks) for 16 h at 37°C with continuous shaking at 120 rpm. The cells were harvested by centrifugation at 27,000 × *g* for 20 min and washed by centrifugation with 250 ml of 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer containing 1 mM dithiothreitol and 1 mM EDTA. This and all subsequent steps were carried out at 0 to 4°C. The washed cells were resuspended in 15 ml of the same buffer and broken by sonication at full power (185 W, Branson 185-D) for 6 min with continuous cooling. The cell debris and any unbroken whole cells were removed by centrifugation at 42,000 × *g* for 1 h. The resulting supernatant fluid was dialyzed for 18 h against 4 liters of 0.01 M HEPES containing 1 mM dithiothreitol and 1 mM EDTA. The cell extract was purified by DEAE ion-exchange and Ultrogel AcA 54 steric exclusion chromatography as described by Chassy and Porter (12) for the purification of sucrose 6-phosphate hydrolase. Fractions were assayed for P-β-galactosidase and β-galactosidase activity as previously described (27, 42) with the chromogenic substrates *o*-nitrophenyl-β-D-galactoside 6-phosphate (ONPG 6-phosphate; Sigma Chemical Co.) and *o*-nitrophenyl-β-D-galactoside (ONPG). Protein was determined by the Coomassie blue dye-binding assay described by Bradford (6). Purity was assessed by electrophoresis in 7.5% polyacrylamide gels with 0.188 M Tris-glycine (pH 8.7) as the electrophoresis buffer. P-β-galactosidase isolated from *L. casei* 64H was used as a protein and activity standard. Activity was localized by soaking the gels in 0.1 M sodium phosphate buffer, pH 6.8 (20 ml, one change of buffer), followed by incubation of the gels at 22°C for 1 h in the same buffer containing 10 mM ONPG 6-phosphate. An intense orange-yellow activity band developed; it was photographed immediately to minimize diffusion of the *o*-nitrophenol. The preparations were also subjected to SDS-polyacrylamide gel electrophoresis (33) to further assess purity and to allow an estimation of molecular weight.

To screen clones for P-β-galactosidase expression, aliquots of cells were lysed with toluene in microtiter tray wells and then incubated with 10 mM ONPG 6-phosphate in 0.1 M sodium phosphate buffer, pH 6.8.

Specific activity and induction of P-β-galactosidase in *E. coli*. *E. coli* cells containing recombinant plasmids were grown overnight in 30 ml of L-broth supplemented with DAP where necessary and containing 1% glucose or glycerol as the growth carbohydrate. Potential inducers, lactose, isopropylthio-β-D-galactoside, and thiomethyl-β-D-galactoside, were added at concentrations of 10, 2, and 5 mM, respectively. Cells were collected by centrifugation at 17,400 × *g* for 30 min and washed by centrifugation with 10 ml of 0.1 M

TABLE 1. Bacterial strains and plasmids

Strains and plasmids	Relevant genotype or phenotype	References
L. casei 64H	Chromosomal Lac$^-$; contains lactose utilization plasmid pLZ64	20
E. coli K-12		
χ1849	*minA1 minB2 hsdR2*	24
χ2359	*ΔlacZ39 hsdR2 recA56 relA1*	—[a]
V517	Contains multiple size reference plasmids	37
Plasmids		
pLZ64	Lac$^+$; 35 kb	This paper
pBR322	Ap^r Tc^r; 4.3 kb	4
pACYC184	Cm^r Tc^r; 4.0 kb	8

[a] Roy Curtiss III, University of Alabama in Birmingham.

TABLE 2. Cloning of pLZ64 restriction fragments into *E. coli* K-12

Digestion fragments of pLZ64			*E. coli* clones with pLZ64 fragments				
Restriction endonuclease	No. of fragments	Size range (kb)	Vector plasmid	Phenotype selection[a]		No. of clones screened	Proportion of fragments found
				Direct	Indirect		
*Bam*HI	3	9.7–14.6	pACYC184	Cm^r	Tc^s	32	2/3[b]
*Pst*I	4	5.5–17	pBR322	Tc^r	Ap^s	17	3/4[b]
*Hin*dIII	9	1– 8.8	pBR322	Ap^r	Tc^s	144	9/9
*Eco*RI	11	1–12.5	pACYC184	Tc^r	Cm^s	79	10/11[b]

[a] Phenotypes were screened on antibiotic media for sensitivity or resistance to chloramphenical (Cm), tetracycline (Tc), and ampicillin (Ap).

[b] In these cases, we failed to clone the largest-sized fragment.

sodium phosphate buffer, pH 6.8. The washed cells were resuspended in 2 ml of the same buffer and broken by 2 min of sonic disruption with a microtip Sonifier probe. Cell debris and insoluble matter were removed by centrifugation at 17,400 × *g* for 30 min, and the supernatant was used for the assay of P-β-galactosidase and protein.

RESULTS

Cloning fragments of plasmid pLZ64 into *E. coli*. Previous work had indicated that the restriction endonucleases *Bam*HI, *Eco*RI, *Pst*I, and *Hin*dIII would be useful in constructing a gene bank of plasmid pLZ64 in *E. coli*; the enzymes cut the plasmid into a limited number of fragments, some of which were fairly large (14). We chose the plasmids pBR322 and pACYC184 to use as vectors, since they had useful single restriction endonuclease sites in antibiotic resistance genes to permit indirect selection of cloned DNA fragments (4, 8). Appropriate restricted DNAs were ligated and transformed into χ1849; transformants were screened for insertional inactivation as shown in Table 2. By this shotgun method, 272 clones were obtained. All of these were screened by agarose gel electrophoresis to find the size of their chimeric plasmids; we determined that for restriction endonucleases *Bam*HI, *Pst*I, and *Eco*RI, we had cloned all of the possible restriction fragments of pLZ64 except the largest. For *Hin*dIII, all fragments were obtained (Table 2).

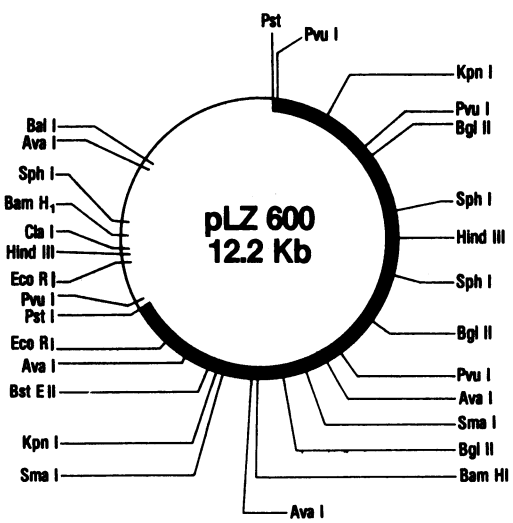
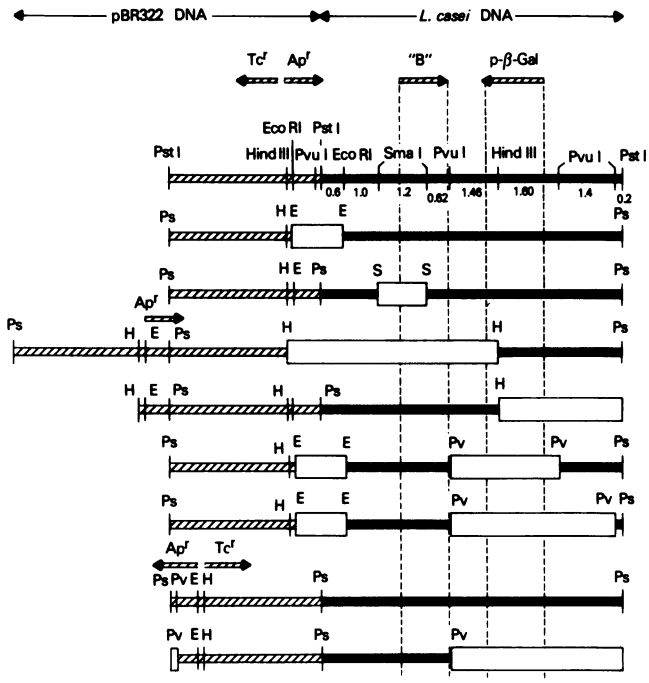


FIG. 2. Physical map of recombinant plasmid pLZ600. The 7.9-kb *Pst*I B fragment of pLZ64 is indicated by the heavy line and the vector plasmid pBR322 by the thin line. Sites where restriction endonucleases cut are shown, to scale.

Identification of the P-β-galactosidase clone. All *Pst*I-, *Bam*HI-, and *Eco*RI-generated clones and all *Hin*dIII-generated clones that had inserts larger than 1.4 kb (23 out of 144) were screened for evidence of P-β-galactosidase activity. Only one clone produced a strong yellow color from the ONPG 6-phosphate substrate; the recombinant plasmid in this clone was named pLZ600. This plasmid contained the 7.9-kb *Pst*I B fragment (i.e., second largest) of pLZ64 cloned into the pBR322 β-lactamase gene.

Physical maps of pLZ600 and derivative plasmids. By double digestion techniques, restriction endonuclease sites on the pLZ64 *Pst*I B fragment cloned in plasmid pLZ600 were mapped. We found single sites for *Eco*RI, *Bam*HI, *Hin*dIII, and *Bst*EII, and multiple sites for *Ava*I, *Bgl*II, *Kpn*I, *Pvu*I, *Sma*I, and *Sph*I (Fig. 2). We then constructed numerous subclones of pLZ600 using various approaches: restriction and random ligation, religation of a single purified restriction fragment, or recloning of a single isolated fragment. Maps of pLZ600 and eight derivative plasmids are shown on the left side of Fig. 3, with the sites where *Eco*RI, *Sma*I, *Pvu*I, and *Hin*dIII cut the *Pst*I B fragment indicated (physical characterization of the gene products, shown on the right side of Fig. 3, is discussed later in the text). The types of subclones included an intact *Pst*I B fragment in the opposite orientation (pLZ601) and derivatives with one or two deletions from the *Pst*I-B insert (pLZ602 through pLZ609). As shown in Fig. 3,



Plasmid	P-β-Gal Specific Activity (μmoles/min/mg)	P-β-Gal Size (Kdal)	"B" Size (Kdal)
pLZ600	0.027	56	43
pLZ602	0.028	56	43 (very strong)
pLZ605	0.028	56	–
pLZ604	–	46	–
pLZ603	–	–	43
pLZ608	–	–	43
pLZ609	–	–	43
pLZ601	0.045	56	43 (very faint)
pLZ607	–	–	43 (faint)

FIG. 3. Physical maps of P-β-galactosidase-positive and -negative recombinant plasmids. The construction and characterization of these plasmids are described in the text. Each plasmid is represented as a linear map, and deletions are indicated by open boxes. Distances between restriction endonuclease sites are indicated in kb. The position and direction of transcription of the pBR322 vector plasmid genes for tetracycline and ampicillin resistance and of the *L. casei* plasmid pLZ64 genes for P-β-galactosidase and B (function unknown, see the text) are shown. The sizes of the P-β-galactosidase and B gene products are given for each plasmid; a dash indicates that no product was observed (see Fig. 6). Note that pLZ601 and pLZ607 have the opposite orientation of the cloned insert DNA relative to the vector plasmid, as compared with the other seven recombinant plasmids.

some derivative plasmids contained deletions or duplications of vector DNA. When crude lysates of *E. coli* χ2359 strains with the nine different derivative plasmids were tested for P-β-galactosidase, only four (containing pLZ600, pLZ601, pLZ602, and pLZ605) showed activity. Orientation of the insert appeared to affect expression by about 40% (Fig. 3). For the other five plasmid-containing strains, deletions in the cloned fragment had apparently prevented expression of the gene. None of the nine plasmid-containing strains demonstrated PEP:PTS activity for lactose (data not shown).

Cloned P-β-galactosidase resembles that isolated from *L. casei*. To compare the P-β-galactosidase expressed by *E. coli* strains containing chimeric plasmid pLZ600 or its derivatives with that produced by *L. casei* 64H, the enzyme was isolated from a large batch of cells that contained derivative plasmid pLZ601. The purification strategy was the same as that used with *L. casei* to prepare homogeneous enzyme preparations. The enzyme emerged from a DEAE-sephacel column (Fig. 4) in the same fractions as that isolated from *L. casei* 64H (not shown) and was completely resolved from the β-galactosidase; this finding eliminated the possibility that cloned activity was due to the combined action of phosphatase and *E. coli* β-galactosidase acting on ONPG 6-phosphate. The active fractions were concentrated and further purified by gel filtration (Fig. 5). Once again, the enzyme activity emerged in the same fractions as observed with the preparation isolated from *L. casei* 64H. About 1.5% of the cellular soluble protein was isolated as P-β-galactosidase. The preparation was subjected to polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis: it appeared homogeneous and migrated in both systems with a mobility identical to that found for the *L. casei* 64H P-β-galactosidase. Duplicate gels containing the enzyme isolated from *L. casei* and *E. coli* were stained for activity; the activity-staining bands were coincident (data not shown).

Is the cloned enzyme inducible? Growth of *L. casei* strains on β-galactosides induces the lactose PEP:PTS and P-β-galactosidase activities, but the nature of the regulatory system is unknown. The inducibility of the enzyme cloned in

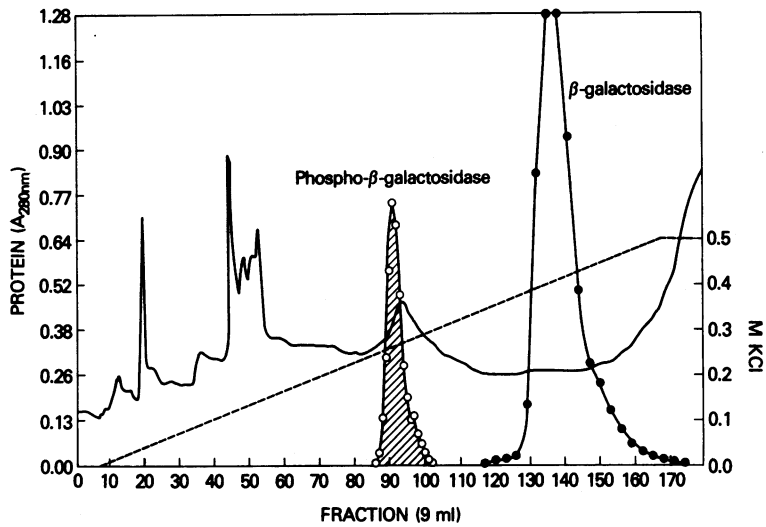


FIG. 4. DEAE-sephacel ion-exchange chromatography of intracellular extracts prepared from *E. coli* χ1849 carrying pLZ601. The solid line (—) represents protein (absorbance at 280 nm [A_{280}]), and the dashed line (---) represents the ascending KCl gradient. The fractions were assayed for P-β-galactosidase (○) and β-galactosidase (●), and the area representing P-β-galactosidase-containing fractions is shaded.

E. coli was examined by growing *E. coli* χ2359 containing either pLZ602 or pLZ605 in glycerol L-broth with and without potential inducers. Growth of these strains in the presence of thiomethyl-β-D-galactoside or lactose stimulated the production of P-β-galactosidase about 1.8-fold while isopropylthio-β-D-galactoside was without effect. By comparison, growth of *L. casei* 64H on lactose resulted in a 50-fold enhancement of P-β-galactosidase activity. While *L. casei* 64H produces little P-β-galactosidase without induction, *E. coli* strains carrying plasmid chimeras coding for this enzyme expressed P-β-galactosidase activity at about 10 to 20% the level

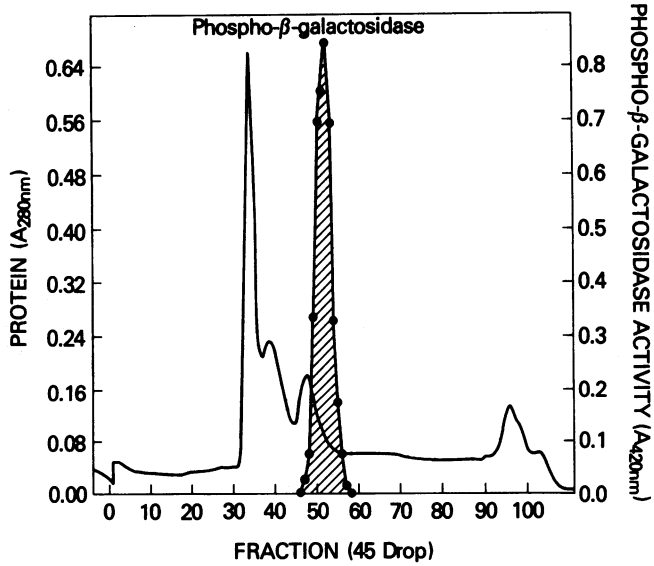


FIG. 5. Chromatography of P-β-galactosidase on Ultrogel AcA 54. Partially purified P-β-galactosidase from the ion-exchange chromatography step was further purified by gel filtration. The solid line represents protein concentration (absorbance at 280 nm [A_{280}]), and the P-β-galactosidase activity is represented by closed circles (●); the fractions containing P-β-galactosidase are shaded.

observed in fully induced *L. casei* 64H. However, this seems to be due to the increased copy number of recombinant plasmids in *E. coli* compared with pLZ64 (data not shown).

Location of two genes, P-β-galactosidase and "B", on the PstI B fragment. Plasmid-containing minicells of *E. coli* χ1849 were used to specifically label gene products encoded by the various recombinant plasmids so that they could be identified by SDS-polyacrylamide gel electrophoresis and fluorography. Such a gel is shown in Fig. 6, and the results are also tabulated in Fig. 3. The P-β-galactosidase-positive clones all showed a protein of about 56,000 subunit molecular weight (Fig. 6, lanes A through D), which migrated identically with purified *L. casei* 64H P-β-galactosidase visualized by Coomassie blue staining (data not shown). Of P-β-galactosidase-negative clones, strains with plasmids pLZ604, pLZ603, pLZ608, pLZ609, and pLZ607 lacked the 56,000-molecular-weight band (Fig. 6, lanes E through I), and the pLZ604 strain had a 46,000-molecular-weight band (Fig. 6, lane E). Under the assumption that this last protein represents premature termination of the P-β-galactosidase translation, we assigned the location and transcriptional direction of the P-β-galactosidase gene shown in Fig. 3. Several other bands are visible in Fig. 6. The major β-lactamase product (about 33,000 molecular weight) of pBR322 is seen for pBR322 and the two recombinant plasmids that contain intact β-lactamase genes, pLZ604 and pLZ603 (Fig. 6, lanes E, F, and J). Additionally, a 43,000-molecular-weight product of what we call the B gene appears in lysates of strains containing the plasmids pLZ600, pLZ602, pLZ603, pLZ608, and pLZ609 (Fig. 6, lanes A, C, F, G, and H). The band is also seen faintly with the pLZ601 and pLZ607 strains (lanes B and I). The function of this gene product is unknown. The levels of expression of B seen in Fig. 6 indicated that orientation of the gene in the vector plasmid affected its expression: in pLZ601 and pLZ607, expression was negligible or reduced (see Fig. 3 and 6). These data supported the location and transcriptional direction for B shown in Fig. 3. The tetracycline resistance gene product of pBR322 was too faint to be visible in Fig. 6.

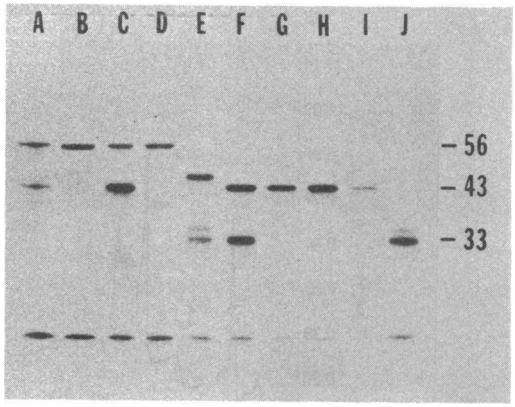


FIG. 6. Fluorograph, after SDS-polyacrylamide gel electrophoresis, of radioactively labeled proteins from *E. coli* χ1849 minicells containing the following various recombinant plasmids. Lane: A, pLZ600; B, pLZ601; C, pLZ602; D, pLZ605; E, pLZ604; F, pLZ603; G, pLZ608; H, pLZ609; I, pLZ607; J, vector plasmid pBR322. See Fig. 3 for descriptions of the plasmids. Sizes of proteins, in kilodaltons, are indicated to the right.

DISCUSSION

Data derived from curing (11) and conjugal transfer (13) support the hypothesis that lactose PEP:PTS and P-β-galactosidase are plasmid-associated products in *L. casei*. The results reported here directly demonstrate that the gene coding for P-β-galactosidase is an integral part of the *L. casei* lactose plasmid pLZ64, since a portion of that plasmid directs synthesis of the enzyme in *E. coli*. Of all of the fragments identified in the clone bank, the 7.9-kb *Pst*I B fragment is the only one that contains the entire P-β-galactosidase gene. A *Hin*dIII site (see Fig. 6) interrupts the structural gene, and clones containing the adjacent *Hin*dIII fragments did not express P-β-galactosidase activity. One of our deletion subclones produced a truncated gene product, presumably from the P-β-galactosidase gene (see pLZ604, Fig. 3 and 6); this allowed us to estimate the position of the gene in pLZ600. Using a physical map of pLZ64 (unpublished data), the gene can also be positioned on the parent plasmid. In pLZ64, the largest *Bam*HI and *Eco*RI fragments contain the P-β-galactosidase gene (unpublished data), but we did not obtain those fragments in our clone bank (Table 2).

Minicell analysis of our recombinant plasmids revealed the presence of a second cloned gene that encoded a 43-kilodalton product (Fig. 6). We do not know whether this gene is involved in lactose metabolism; the presence or absence of its gene product had little effect on P-β-galactosidase expression in *E. coli*. Other lactose metabolism genes of pLZ64 are apparently not in a single transcriptional unit with P-β-galactosidase and must map outside of the *Pst*I B fragment in pLZ64. This is consistent with the previously observed lack of coordinate regulation of P-β-galactosidase and the structural genes of the lactose PEP:PTS (J. Thompson and B. Chassy, unpublished results). Our attempts to screen our

clone bank for lactose PEP:PTS activity were unsuccessful; we also shotgun cloned into an *E. coli* lactose deletion strain that contained a functional tagatose 6-phosphate pathway (see Fig. 1) cloned from *S. mutans* (Hansen et al., unpublished data) but were unable to find a lactose-positive transformant. There may be various reasons for this: the genes may not be functional or may be lethal when expressed, lactose metabolism genes on pLZ64 may be dispersed so that one fragment will not contain them all, or the genes may be all contained on the largest *Bam*HI, *Eco*RI, and *Pst*I fragments of pLZ64 that we failed to clone (Fig. 2).

We do not know whether we have cloned a repressor or activator gene of the P-β-galactosidase gene. In *E. coli* clones, P-β-galactosidase comprises as much as 1.5% of the soluble cellular protein. However, if this activity is normalized with regard to the copy number of pLZ600, it is found to be about equal to the uninduced level produced by pLZ64 in *L. casei*, normalized for copy number. Thiomethyl-β-D-galactoside and lactose only slightly enhanced the expression of P-β-galactosidase by *E. coli* clones; however, the actual in vivo inducer may be a β-galactoside phosphate, which could only be produced by *E. coli* that contained a functional cloned lactose PEP:PTS. We do not know whether modulation of expression of P-β-galactosidase in *E. coli* by thiomethyl-β-D-galactoside and lactose is mediated through a repressor/activator system or post-transcriptional control such as that observed for inducible erythromycin resistance in gram-positive bacteria (29).

All of the foregoing discussion of levels of expression depends on our interpretation of the minicell experiments. We believe the data justify the conclusion that the B gene product is transcribed by read-through from promoters in pBR322; levels of expression are orientation dependent and are greatly enhanced by deletion of the portion of DNA between the β-lactamase promoters of pBR322 (P1 and P3 of Stüber and Bujard [46]) and the B structural gene (see Fig. 3 and 6). On the other hand, P-β-galactosidase seems to be expressed from its own functional promoter in *E. coli*; compared with the B product, its level of expression is little affected by the orientation or size of the insert.

With this initial study, we have started to define the molecular genetics of lactose metabolism in *L. casei*. Besides a preliminary report of a cloned dihydrofolate reductase gene (16), it is the only other example of expression of *L. casei* genes in *E. coli*. Future work to better define the system will be directed towards identifying the lactose PEP:PTS genes of pLZ64 and determining the mechanism of regulation of lactose metabolism genes by pLZ64.

ACKNOWLEDGMENTS

J.B.H. was supported in part by Public Health Service postdoctoral traineeship T32 AI 07041 from the National Institutes of Health. Other research support was from Public Health Service grant DEO2670 from the National Institute of Dental Research.

We thank Roy Curtiss III for his help in initiating and facilitating this work.

LITERATURE CITED

1. **Beckwith, J. R., and D. Zipser (ed.).** 1970. The lactose operon. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513–1523.
3. **Bissett, D. L., and R. L. Anderson.** 1973. Lactose and D-galactose metabolism in *Staphylococcus aureus*: pathway of D-galactose 6-phosphate degradation. Biochem. Biophys. Res. Commun. 52:641–647.
4. **Bolivar, F., R. L. Rodriquez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. 2. Multipurpose cloning system. Gene 2:95–113.
5. **Botsford, J. L.** 1981. Cyclic nucleotides in procaryotes. Microbiol. Rev. 45:620–642.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein, utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
7. **Calmes, R.** 1978. Involvement of phosphoenolpyruvate in the catabolism of caries-conducive disaccharides by *Streptococcus mutans*: lactose transport. Infect. Immun. 19:934–942.
8. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134:1141–1156.
9. **Chang, S., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. 168:111–115.
10. **Chassy, B. M., E. V. Gibson, and A. Giuffrida.** 1976. Evidence for extrachromosomal elements in lactobacilli. J. Bacteriol. 127:1576–1578.
11. **Chassy, B. M., E. V. Gibson, and A. Giuffrida.** 1978. Evidence for plasmid-associated lactose metabolism in *Lactobacillus casei* subsp. *casei*. Curr. Microbiol. 1:141–144.
12. **Chassy, B. M., and E. V. Porter.** 1979. Initial characterization of sucrose 6-phosphate hydrolase from *Streptococcus mutans* and its apparent identity with intracellular invertase. Biochem. Biophys. Res. Commun. 89:307–314.
13. **Chassy, B. M., and E. Rokow.** 1981. Conjugal transfer of lactose plasmids in *Lactobacillus casei*, p. 590. *In* S. Levy, R. Clowes, and E. Koenig (ed.), Molecular biology, pathogenesis and ecology of bacterial plasmids. Plenum Press, New York.
14. **Chassy, B. M., E. Rokow, and A. S. Giuffrida.** 1980. Studies on the function and structure of lactose plasmids in *Lactobacillus casei* subsp. *casei*. Plasmid 3:235.
15. **Curtiss, R., III.** 1965. Chromosomal aberrations associated with mutations to bacteriophage resistance in *Escherichia coli*. J. Bacteriol. 89:28–40.
16. **Davies, R. W., and A. M. Gronenborn.** 1982. Molecular cloning of the gene for dihydrofolate reductase from *Lactobacillus casei*. Gene 17:229–233.
17. **Dugaiczyk, A., H. W. Boyer, and H. M. Goodman.** 1975. Ligation of *Eco*RI endonuclease generated DNA fragments into linear and circular structures. J. Mol. Biol. 96:171–184.
18. **Efthymiou, C., and C. A. Hansen.** 1962. An antigenic analysis of *Lactobacillus acidophilus*. J. Infect. Dis. 110:258–267.

19. **Frazer, A. C., and R. Curtiss III.** 1975. Production, properties and utility of bacterial minicells. Curr. Top. Microbiol. Immunol. **69**:1–84.
20. **Gasser, F., and N. Mandel.** 1968. Deoxyribonucleic acid base composition of the genus lactobacillus. J. Bacteriol. **96**:580–588.
21. **Gasson, M. J., and F. L. Davies.** 1980. High-frequency conjugation associated with *Streptococcus lactis* donor cell aggregation. J. Bacteriol. **143**:1260–1264.
22. **Guerry, P., D. J. LeBlanc, and S. Falkow.** 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. **116**:1064–1066.
23. **Hamilton, I. R., and G. Y. C. Lo.** 1978. Co-induction of β-galactosidase and the lactose-P-enolpyruvate phosphotransferase system in *Streptococcus salivarius* and *Streptococcus mutans*. J. Bacteriol. **136**:900–908.
24. **Hansen, J. B., Y. Abiko, and R. Curtiss III.** 1981. Characterization of the *Streptococcus mutans* plasmid pVA318 cloned in *Escherichia coli*. Infect. Immun. **31**:1034–1043.
25. **Heller, K., and R. Röschenthaler.** 1978. β-D-phosphogalactoside galactohydrolase of *Streptococcus faecalis* and the inhibition of its synthesis by glucose. Can. J. Microbiol. **24**:512–519.
26. **Hengstenberg, W., J. B. Egan, and M. L. Morse.** 1967. Carbohydrate transport in *Staphylococcus aureus*. V. The accumulation of phosphorylated carbohydrate derivatives and evidence for a new enzyme splitting lactose phosphate. Proc. Natl. Sci. U.S.A. **58**:274–279.
27. **Hengstenberg, W., W. K. Penberthy, K. L. Hill, and M. L. Morse.** 1969. Phosphotransferase system of *Staphylococcus aureus*: its requirement for the accumulation and metabolism of galactosides. J. Bacteriol. **99**:383–388.
28. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
29. **Horinouchi, S., and B. Weisblum.** 1980. Posttranscriptional modification of mRNA conformation: mechanism that regulates erythromycin-induced resistance. Proc. Natl. Acad. Sci. U.S.A. **77**:7079–7083.
30. **Jagusztyn-Krynicka, E. K., M. Smorawinska, and R. Curtiss III.** 1982. Expression of *Streptococcus mutans* aspartate-semialdehyde dehydrogenase gene cloned into plasmid pBR322. J. Gen. Microbiol. **128**:1135–1145.
31. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1365–1373.
32. **Korte, T., and W. Hengstenberg.** 1971. Purification and characterization of the inducible lactose-specific membrane-bound component of the staphylococcal phosphoenolpyruvate-dependent phosphotransferase system. Eur. J. Biochem. **23**:295–302.
33. **Laemmli, U. K., and M. Favre.** 1973. Maturation of the head of bacteriophage T4. J. Mol. Biol. **80**:575–599.
34. **LeBlanc, D. J., V. L. Crow, L. N. Lee, and C. F. Garon.** 1979. Influence of the lactose plasmid on the metabolism of galactose by *Streptococcus lactis*. J. Bacteriol. **137**:878–884.
35. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
36. **Macrina, F. L., K. R. Jones, and P. H. Wood.** 1980. Chimeric streptococcal plasmids and their use as molecular cloning vehicles in *Streptococcus sanguis* (Challis). J. Bacteriol. **143**:1425–1435.
37. **Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers, and S. M. McCowen.** 1978. A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid **1**:417–420.
38. **McKay, L. L., A. Miller, W. E. Sandine, and P. R. Elliker.** 1970. Mechanisms of lactose utilization by lactic acid streptococci: enzymatic and genetic analyses. J. Bacteriol. **102**:804–809.
39. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 431. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Morse, M. L., K. L. Hill, J. B. Egan, and W. Hengstenberg.** 1968. Metabolism of lactose by *Staphylococcus aureus* and its genetic basis. J. Bacteriol. **95**:2270–2274.
41. **Nakazawa, T., R. D. Simoni, J. B. Hays, and S. Roseman.** 1971. Phosphorylation of a sugar-specific protein component of the lactose transport system in *Staphylococcus aureus*. Biochem. Biophys. Res. Commun. **42**:836–843.
42. **Premi, L., W. E. Sandine, and P. R. Elliker.** 1972. Lactose-hydrolyzing enzymes of *Lactobacillus* species. Appl. Microbiol. **24**:51–57.
43. **Simoni, R. D., T. Nakazawa, J. B. Hays, and S. Roseman.** 1973. Sugar transport. IV. Isolation and characterization of the lactose phosphotransferase system in *Staphylococcus aureus*. J. Biol. Chem. **248**:932–940.
44. **Simoni, R. D., M. F. Smith, and S. Roseman.** 1968. Resolution of a staphylococcal phosphotransferase system into four protein components and its relation to sugar transport. Biochem. Biophys. Res. Commun. **31**:804–811.
45. **Snook, R. J., and L. L. McKay.** 1981. Conjugal transfer of lactose-fermenting ability among *Streptococcus cremoris* and *Streptococcus lactis* strains. Appl. Environ. Microbiol. **42**:904–911.
46. **Stüber, D., and H. Bujard.** 1981. Organization of transcriptional signals in plasmids pBR322 and pACYC184. Proc. Natl. Acad. Sci. U.S.A. **78**:167–171.
47. **Tabak, H. F., and R. A. Flavell.** 1978. A method for the recovery of DNA from agarose gels. Nucleic Acids Res. **5**:2321–2332.
48. **Thompson, J., and M. H. Saier.** 1981. Regulation of methyl-β-D-thiogalactopyranoside-6-phosphate accumulation in *Streptococcus lactis* by exclusion and expulsion mechanisms. J. Bacteriol. **146**:885–894.
49. **Ullrich, A., J. Shine, J. Chirgwin, R. Pictet, E. Tischer, W. J. Rulter, and H. M. Goodman.** 1977. Rat insulin genes: construction of plasmids containing coding sequences. Science **196**:1312–1319.