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Plasmids, Loss of Lactose Metabolism, and Appearance of Partial and Full Lactose-Fermenting Revertants in *Streptococcus cremoris* B_1[1]

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The unstable ability to metabolize lactose (lac) via the phosphoenolpyruvate-phosphotransferase system (PTS) was examined in *Streptococcus cremoris* B_1. The presence of functional lactose-specific PTS enzymes was correlated with the presence of a distinct plasmid species. Characterization of deoxyribonucleic acid extracted from lactose-positive (Lac^+) *S. cremoris* B_1 revealed two plasmids having molecular weights of 9×10^6 and 36×10^6. An acriflavine (BC1)-induced, lactose-negative (Lac^-) mutant possessed no plasmids and was devoid of all three lac-specific PTS enzymes. A Lac^- mutant (DA2) isolated by growing at elevated temperatures only possessed the 9×10^6-dalton plasmid and also lacked the lac PTS enzymes. A spontaneous Lac^- mutant possessed both the 9×10^6- and 36×10^6-dalton plasmids. This mutant displayed FIII-lac and phospho-β-D-galactosidase (P-β-gal) activity but was deficient in EII-lac activity. The spontaneous Lac^- strain reverted to both full and partial lactose-fermenting phenotypes having FIII-lac, EII-lac, and P-β-gal activities. BC1 and DA2 Lac^- mutants reverted only to the partial lactose-fermenting phenotype having P-β-gal activity; EII-lac and FIII-lac activities were absent. The results indicate that the genetic determinants for EII-lac, FIII-lac, and P-β-gal are located on the 36×10^6-dalton plasmid in *S. cremoris* B_1. Evidence for a second chromosomally associated P-β-gal gene operating in the partial lactose-fermenting revertants is also presented.

The lactic streptococci utilize lactose via the phosphoenolpyruvate (PEP)-dependent phosphotransferase (PTS) system (15, 16). The reactions of this metabolic pathway are described below:

$$\text{PEP} + \text{HPr} \underset{Mg^{2+}}{\overset{EI}{\rightleftharpoons}} \text{P-HPr} + \text{pyruvate}$$

$$\text{FIII-lac} + 2\text{P-HPr} \longrightarrow \text{FIII-lac-2P} + 2\text{HPr}$$

$$\text{FIII-lac-2P} + 2 \text{ lactose} \xrightarrow[Mg^{2+}]{\text{EII-lac}} 2 \text{ lactose-P} + \text{FIII-lac}$$

$$\text{Lactose-P} \xrightarrow{\text{P-}\beta\text{-gal}} \text{glucose} + \text{galactose-6-phosphate}$$

HPr is a heat-stable soluble protein and EI (enzyme I) is also a soluble protein. Both are required for the metabolism of a number of carbohydrates via the PEP-PTS system and appear to be constitutively produced. EII-lac (enzyme II) is a lactose-specific, membrane-bound component and FIII-lac (factor III) is a lactose-specific factor found in the soluble fraction of the cell. P-β-gal refers to phospho-β-D-galactosidase. The lac-specific enzymes (EII-lac, FIII-lac, P-β-gal) are inducible by growing the cells on lactose or galactose. This metabolic system was first observed in *Staphylococcus aureus* (8, 18).

The ability of lactic streptococci to metabolize lactose is not a stable characteristic (9, 11, 19, 22). McKay et al. (14) also found that growing the cells in the presence of acriflavine increased the frequency of appearance of the Lac^- variants above the spontaneous rate. Enzymatic analysis of Lac^- *S. lactis* C2 mutants showed that they were deficient in EII-lac, FIII-lac, and P-β-gal (15). These Lac^- mutants, however, reverted to a partial lactose-fermenting phenotype that possessed P-β-gal activity but lacked EII-lac and FIII-lac activities (4). Thus, these partial revertants were defective in the PEP-PTS-mediated lactose translocation.

Recently, extrachromosomal elements were

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described in lactic streptococci (5). Since conditions selecting for the loss of plasmid deoxyribonucleic acid (DNA) have been observed to result also in loss of lactose metabolism, it has been postulated that the genetic determinant for lactose metabolism may be located on a plasmid. A correlation between the ability to metabolize lactose and the presence of a distinct plasmid species has been described in *Streptococcus lactis* C10, M18, and ML_3 (6a) and via transduction of lactose metabolism in *S. lactis* C2 (13a). The present study attempts to correlate the presence of plasmid DNA in *Streptococcus cremoris* B_1 with lactose fermentation in Lac^+, Lac^-, and lactose-fermenting revertant strains. A working model for the metabolism of lactose is presented.

MATERIALS AND METHODS

Bacterial strains and culturing conditions. *S. cremoris* B_1 was obtained from the stock culture collection maintained in our laboratory. This culture was transferred at biweekly intervals in sterile, reconstituted, nonfat dry milk. The organisms were incubated at 21°C until coagulation and then stored at 4°C. Lactose-negative (Lac^-) derivatives of *S. cremoris* B_1 were obtained spontaneously by treatment with acriflavine (14) and by propagating at 38°C. The Lac^- strains were detected by their appearance on bromocresol purple-0.5% lactose indicator agar (14). The isolation of lactose-fermenting revertants from lactic streptococci was previously described (14). The Lac^- mutants and revertants were maintained in Elliker broth and transferred at biweekly intervals as described above. The *S. cremoris* B_1 strains used in this study are shown in Table 1.

Lactose-negative mutants of *S. aureus*, defective in the components of the PTS system, were originally obtained from M. L. Morse, Department of Biophysics, University of Colorado Medical Center, Denver. The strains used were *S. aureus* 5714B (*lac*$^-$ *gal*$^-$, EII-lac deficient) and *S. aureus* 5714G (*lac*$^-$ *gal*$^-$, FIII-lac deficient) and were propagated in 2% peptone broth (Difco) at 37°C.

TABLE 1. *Strains of S. cremoris B_1 used in various experiments*

Strain	Derivation	Phenotype[a]
B_1	Wild type	Lac^+
BC1	Acriflavine treatment	Lac^-
BC1-1	Revertant of BC1	Partial Lac^+
DA1	Spontaneous	Lac^-
DA1-1	Revertant of DA1	Lac^+
DA1-2	Revertant of DA1	Partial Lac^+
DA2	High temperature	Lac^-
DA2-1	Revertant of DA2	Partial Lac^+

[a] Partial Lac^+ refers to a slow or defective fermentation of lactose as compared with the parent strain, *S. cremoris* B_1.

Escherichia coli K-12 strain W1485-1 (F$^-$ *thy*$^-$ *nal*^{r}), containing RSF1030 (ampicillin resistant [Ap^r]), was obtained from J. H. Crosa, Department of Microbiology, School of Medicine, University of Washington, Seattle. The Ap plasmid was used in this study as a sedimentation reference. The organism was grown in Trypticase soy broth (Difco) supplemented with 5 μg of thymine and 20 μg of ampicillin per ml.

Plasmid analysis. The labeling and extraction of DNA, preparation of cesium chloride (CsCl)-ethidium bromide (EB) gradients, and electron microscopy of plasmid DNA were described in an earlier paper (5). Molecular weights were calculated from the equivalence: 1.0 μm = 2.07×10^6 DNA (12).

The procedure used for the isolation of *E. coli* plasmid DNA was previously described by Guerry et al. (7).

Neutral sucrose gradient analysis. Neutral sucrose gradients (5 to 20%) having a volume of 11.3 ml were prepared using a Buchler apparatus connected to a peristaltic pump. The gradients were formed above a 0.2-ml shelf of 33% sucrose and were allowed to stabilize for 1 h at 25°C. Linearity of the gradients was confirmed by refractometer measurements of fractioned tubes. The sucrose buffer composition and sedimentation conditions were described by Crosa et al. (6). To the top of the stabilized gradients, 0.25 ml of ^{3}H-labeled streptococcal plasmid DNA and 0.05 ml of ^{14}C-labeled RSF 1030 DNA was added. Layered gradients were placed in a Beckman SW40 rotor and centrifuged at 36,000 rpm for 4 h at 15°C. Ten-drop fractions were collected directly on Whatman no. 3 filter disks. The disks were dried at 70°C and washed twice with cold 5% trichloroacetic acid containing 50 μg of thymine per ml, followed by two cold 95% ethanol washes. After drying, the disks were placed in scintillation vials containing 5 ml of scintillation fluor (47 ml of New England Nuclear Corp. Liquifluor in 1-liter Beckman scintillation toluene). Samples were counted in a Beckman scintillation spectrophotometer.

Complementation analysis. To determine the enzyme deficiencies in the Lac^- and revertant strains, their ability to complement enzymatic defects in two *S. aureus* mutants was examined. The conditions of strain propagation, washing of cells, preparation of extracts, and enzyme assays were previously described by Cords and McKay (4).

Assay of P-β-gal. Growth conditions and assay of P-β-gal in toluene-treated cells were similar to that described by Cords and McKay (4), except the cells were grown at 32°C for 10 to 12 h. When P-β-gal was measured in extracts, the enzyme preparations described for the complementation analysis were used. Concentrations of protein were estimated by the method of Lowry et al. (13).

RESULTS

Isolation and characterization of plasmid DNA from *S. cremoris* B_1. To characterize the plasmid DNA from *S. cremoris* B_1, cells grown to stationary phase in the presence of

[^{3}H]thymidine were treated with lysozyme and sodium lauryl sulfate to effect lysis. The cleared lysate was added to a CsCl-EB preparation and subjected to equilibrium centrifugation. The elution profile in Fig. 1 shows the presence of a dense satellite band characteristic of covalently closed circular (CCC) DNA, in addition to a larger, less dense band of chromosomal DNA. The degree of [^{3}H]thymidine incorporation in *S. cremoris* B_1 strains is low, and only about 0.5% of the radioactive label added was routinely recovered. Gradient fractions within the satellite band were pooled and prepared for electron microscopy.

Electron microscopy revealed the presence of two plasmid species in *S. cremoris* B_1. A distribution of contour-length measurements of open circular (OC) molecules is shown in Fig. 2. The plasmids had contour lengths of 4.5 and 18 μm, corresponding to molecular weights of approximately 9×10^6 and 36×10^6, and were designated pLM0901 and pLM3601, respectively. The CCC, OC, and linear forms were observed by using an electron microscope. Electron micrographs of the OC species are presented in Fig. 3.

To confirm the presence of the two plasmid species, a fresh plasmid DNA preparation was analyzed in a 5 to 20% neutral sucrose gradient. The ampicillin resistance factor RSF1030, isolated from *E. coli* K-12 strain W4185-1 (F^- *thy*$^-$ *nal*^{r}) and labeled with [^{14}C]thymine, was used as a sedimentation marker. The *S* values of the CCC and OC forms of the ampicillin plasmid

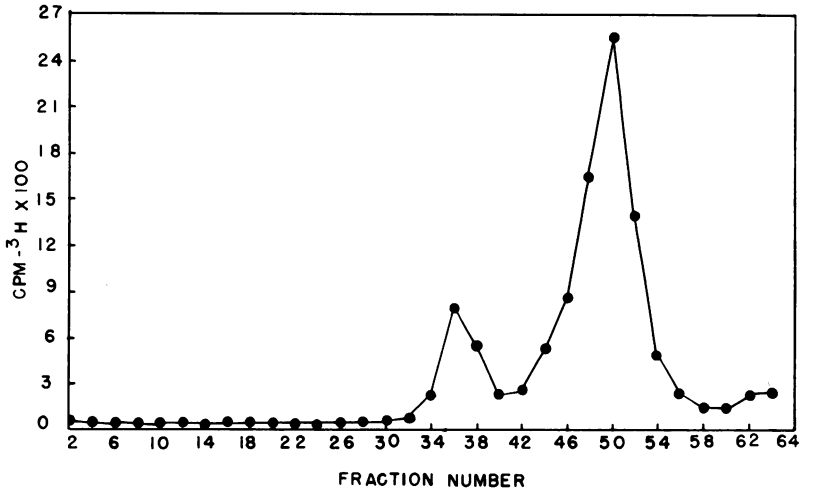

FIG. 1. *Elution profile of CsCl-EB gradients of DNA from cleared lysate material of Lac$^+$ S. cremoris B_1.*

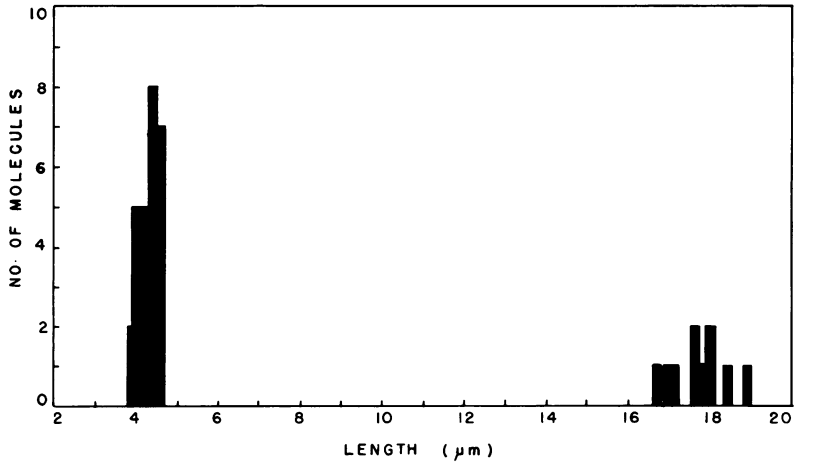

FIG. 2. *Distribution of contour lengths of circular molecules of DNA from Lac$^+$ S. cremoris B_1.*

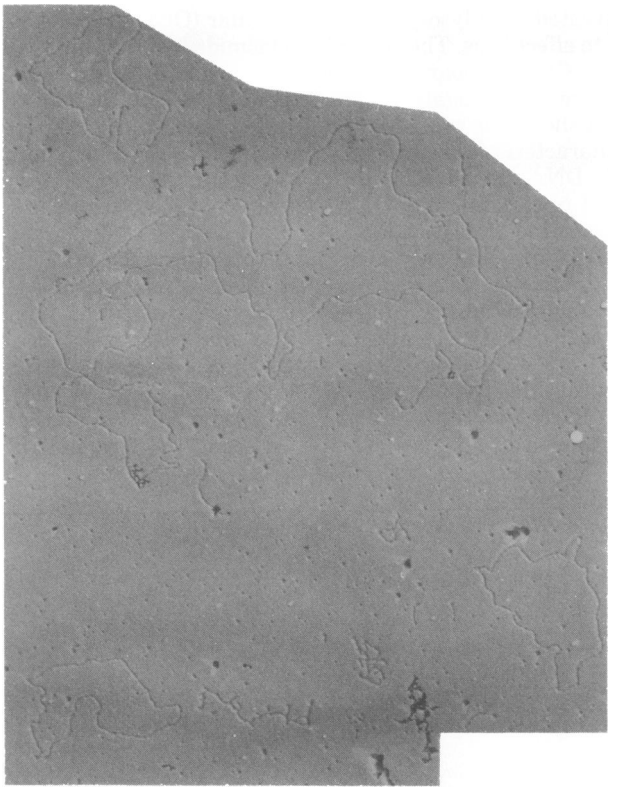

FIG. 3. *OC forms of the 9 × 10⁶ and 36 × 10⁶ plasmids isolated from Lac⁺ S. cremoris B₁. Magnification, ×17,000.*

were 26*S* and 19.2*S*, rspectively (J. H. Crosa, personal communication). Figure 4 shows the results of the neutral sucrose gradient sedimentation analysis. Significant peaks of 36*S* and 28*S* were observed, but peaks corresponding to pLM3601 were only suggested. According to the equation of Bazaral and Helinski (1), molecules with an *S* value of 36 correspond to the sedimentation behavior expected of DNA supercoils with a molecular weight of 9 × 10⁶. Similarly, molecules with an *S* value of 28 correspond to the sedimentation behavior expected of OC DNA with a molecular weight of 9 × 10⁶, according to the equation of Svedberg (10). The broad peak observed between fractions 44 and 52 was probably due to a variety of linear fragments of DNA. The apparent small amount of pLM3601 in the sucrose gradient may be explained by a combination of factors, including: (i) low efficiency of label incorporation; (ii) a small proportion of the plasmid population consisting of 37 × 10⁶-dalton plasmids; and (iii) the increased sensitivity of this molecule to mechanical forces encountered in routine handling.

Plasmid profile of BC₁. To correlate the presence of plasmid DNA with the functional characteristic of lactose metabolism, several Lac⁻ mutants were randomly isolated and characterized in terms of the plasmid species each mutant possessed.

When the DNA from an acriflavine-induced Lac⁻ mutant of *S. cremoris* B_1 was analyzed using a CsCl-EB density gradient, no satellite band of CCC DNA was observed (Fig. 5). The gradient fractions normally containing CCC DNA were pooled and prepared for electron microscope observations. No plasmid DNA was observed, indicating lactose metabolism may be plasmid linked.

Plasmid DNA from DA2. The DNA extracted from a Lac⁻ mutant of *S. cremoris* B_1, obtained by repetitive transfer in Elliker broth at 38°C and designated DA2, displayed the characteristic satellite peak of plasmid DNA after buoyant density centrifugation (Fig. 6). Electron microscopy of the pooled satellite fractions revealed the presence of a single plasmid species. The histogram in Fig. 7 indicated that only pLM0901 was present in DA2, suggesting

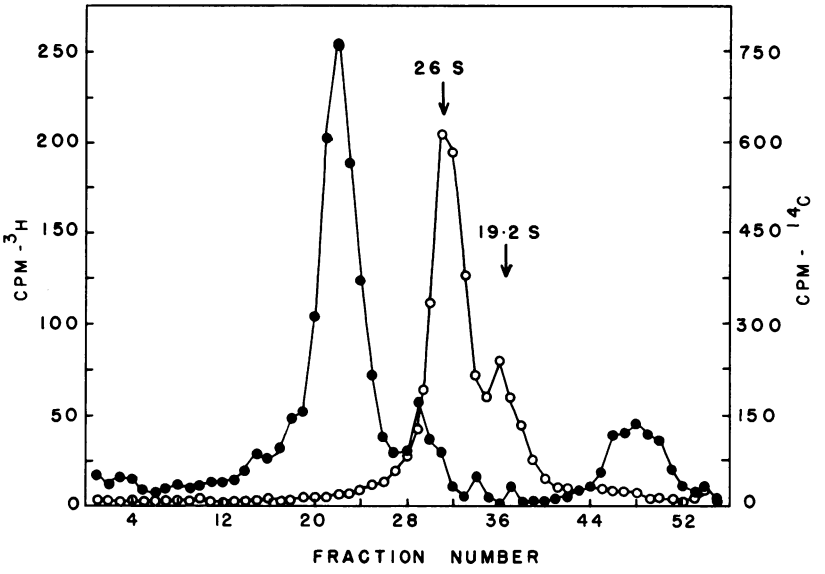

FIG. 4. *Neutral sucrose gradient centrifugation of plasmid DNA from S. cremoris B_1. Plasmid DNA was isolated from cleared lysates by CsCl-EB centrifugation. The fractions containing plasmid DNA were dialyzed against 50 mM tris(hydroxymethyl)aminomethane-hydrochloride, 0.55 M NaCl, and 5 mM ethylenediaminetetraacetic acid at pH 8.0. A 0.25-ml sample of ^{3}H-labeled streptococcal plasmid DNA (●) was mixed with 0.05 ml of ^{14}C-labeled RSF 1030 DNA (○), and the mixture was centrifuged in a Spinco SW40 rotor at 36,000 rpm and 15°C for 4 h.*

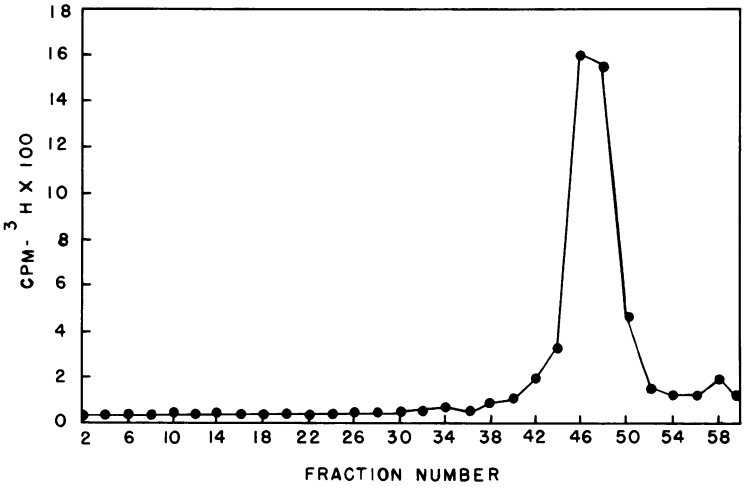

FIG. 5. *Profile of CsCl-EB gradients of DNA from cleared lysate material from an acriflavine-induced Lac$^-$ S. cremoris B_1.*

that a genetic determinant for lactose metabolism may be located on pLM3601.

Plasmid analysis of DA1. A spontaneous mutant of *S. cremoris* B_1, designated DA1, was isolated at 25°C, and the extracted DNA gradient profile of a CsCl-EB density gradient is shown in Fig. 8. Electron microscopy of DNA from the satellite peak revealed the presence of two plasmid species. The distribution of plasmid contour lengths shown in Fig. 9 indicates that the plasmid composition of DA1 is similar, if not identical, to the plasmid profile of the Lac$^+$ parent strain *S. cremoris* B_1, based on physical dimensions.

Reversion analysis. The reversion of Lac⁻ strains of *S. lactis* C2 to partial lactose-fermenting revertants has been reported (4, 14). When diluted cell suspensions of *S. cremoris* B_1 Lac⁻ mutants BC1, DA2, and DA1 were spread over the surface of lactose indicator agar plates and incubated at 21°C for 7 days, lactose-fermenting revertants were also observed.

The preliminary indication of the fermentative capabilities of the revertants was based on the degree of acid production, reflected by a color change in the indicator medium. After 3 days of incubation, revertants from DA1 appeared that were capable of rapid acid production. Comparative growth studies in both lactose broth and milk indicated that the lactose-fermenting ability of these DA1 revertants and that of the parent culture *S. cremoris* B_1 were identical. This revertant type was designated a "full" revertant (DA1-1). Continued incubation of DA1 resulted in the appearance of another and more predominant revertant type. This revertant type appears capable of fermenting lactose to lactic acid, but at a very slow rate. The colonial appearance of this revertant is a faint, pale-yellow colony surrounded by a minute yellow zone, in contrast to the bright-yellow colony and large yellow zone produced by *S. cremoris* B_1 or DA1-1. Growth studies of the second revertant type in lactose broth indicated that it utilized lactose, but at a rate lower than the parent strain, and the revertant was unable to coagulate milk. This type of revertant was termed a "pseudorevertant" (DA1-2). Thus, mutant DA1 exhibits reversion to two different phenotypes distinguishable on the basis of (i) colony appearance on the lactose indicator agar and (ii) characteristic growth behavior in lactose broth and milk.

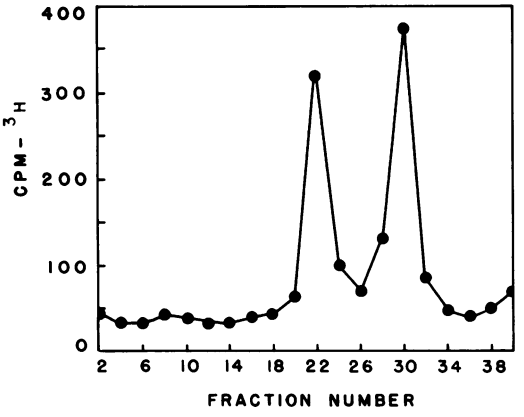

FIG. 6. *Profile of CsCl-EB gradients of DNA from cleared lysate material of a high-temperature-selected Lac⁻ mutant of S. cremoris* B_1.

Incubation of diluted cell suspensions from DA2 and BC1 required 5 to 7 days before revertants appeared. Characterization of DA2 and BC1 revertants by the parameters discussed above indicated that all revertants were of the partial lactose-fermenting phenotype. These revertants were designated DA2-1 and BC1-1. Extensive efforts to select a full lactose-fermenting revertant from DA2 and BC1 were unsuccessful. The partial lactose-fermenting revertants from DA1, DA2, and BC1 resembled those previously described by Cords and McKay (4) for *S. lactis* C2.

The partial lactose-fermenting phenotype may be a common phenomenon, since all pseudorevertants appear phenotypically similar. However, the reversion of DA1 to a full revertant phenotype is a previously unreported phe-

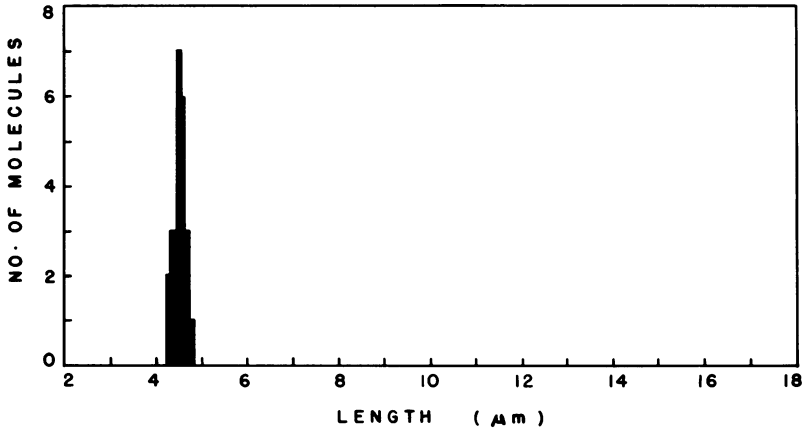

FIG. 7. *Distribution of contour lengths of circular molecules of DNA from a high-temperature-selected Lac⁻ mutant of S. cremoris* B_1.

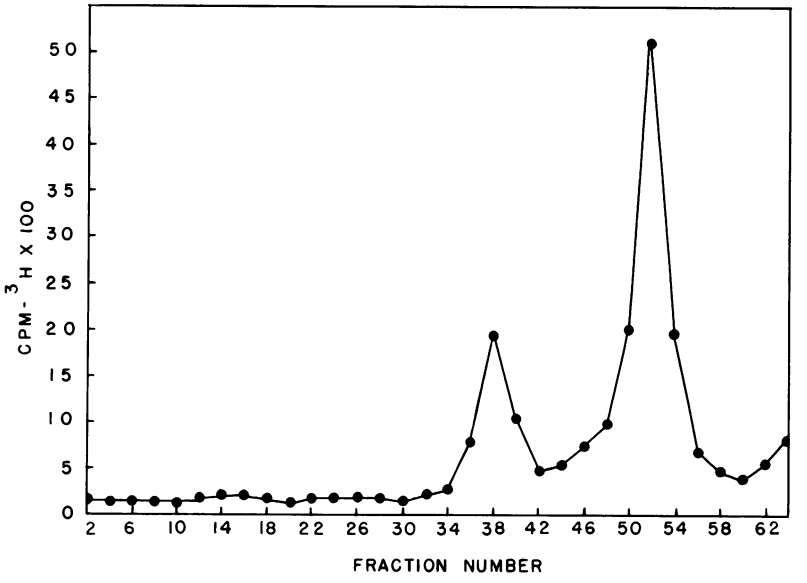

FIG. 8. *Elution profile of CsCl-EB gradients of DNA from cleared lysate material of a spontaneous Lac⁻ mutant of S. cremoris B_1.*

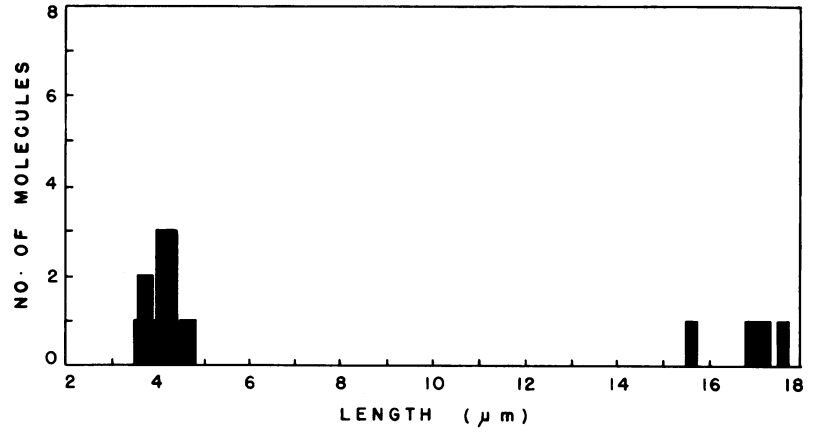

FIG. 9. *Distribution of contour lengths of circular molecules of DNA from a spontaneous Lac⁻ mutant of S. cremoris B_1.*

nomenon and occurred only in the Lac⁻ strain of B_1 shown to possess pLM3601.

EII-lac, FIII-lac, and P-β-gal analysis. To correlate the lactose-fermenting abilities of *S. cremoris* B_1, Lac⁻, and revertant derivatives with their respective lactose-specific enzymatic components, complementation tests were conducted (Table 2). The ability of crude extracts of the streptococcal strains to complement extracts from *S. aureus* 5714G (FIII-lac deficient) and 5714B (EII-lac deficient) and provide a complete in vitro PTS was measured. The P-β-gal activity of the extracts was also measured.

DA1 appeared to be deficient in EII-lac and had reduced FIII-lac and P-β-gal activity. The EII-lac defect present in Lac⁻ DA1 was restored to 0.77 in DA1-1. DA1-2 behaved as a pseudorevertant even though it possessed one-third the EII-lac and twice the P-β-gal activity. These results suggest that the Lac⁻ phenotype of DA1 was due to the EII-lac defect: the inability to catalyze the transfer of phosphate groups from FIII-lac to lactose with concomitant translocation of lactose into the cell.

BC1 and DA2 were uniformly devoid in all three lac-specific enzymes, which accounts for

TABLE 2. *Determination of lactose PTS components in mutants isolated from S. cremoris B_1*

Strain	Relative activities[a]		
	FIII-lac	EII-lac	P-β-gal
B_1	1.0	1.0	1.0
DA1	0.28	0.04	0.85
DA1-1	0.68	0.77	0.69
DA1-2	0.81	0.31	2.09
BC1	0.05	0.03	0.02
BC1-1	0.09	0.07	0.17
DA2	0.07	0.06	0.02
DA2-1	0.09	0.07	0.15

[a] The values represent specific activities determined with crude extracts of each of the strains. The value 1.0 signifies the following specific activities (nanomoles of *o*-nitrophenol formed from *o*-nitrophenyl-β-D-galactoside or *o*-nitrophenyl-β-D-galactoside-6-phosphate per minute per milligram of protein at 37°C): FIII-lac, 1.30; FII-lac, 1.68; P-β-gal, 73.9. The relative activities represent the average from two independently prepared crude extracts, each assayed in quadruplicate. The standard deviation was ±0.04.

TABLE 3. *Effect of lactose or galactose on production of P-β-gal by S. cremoris B_1 and Lac⁻ and revertant strains*

Strain	Carbohydrate source[a]	
	Lactose	Galactose
B_1	1.23	2.44
DA1	1.89	2.71
DA1-1	1.30	3.69
DA1-2	8.07	3.53
BC1	0	0
BC1-1	0.49	0
DA2	0	0
DA2-1	0.04	0

[a] Results are expressed in terms of nanomoles of *o*-nitrophenol released from *o*-nitrophenol-β-D-galactoside per minute per 1.0 ml of toluene-treated cells adjusted to an optical density of 0.5 at 500 nm. Incubation temperature was 37°C.

their Lac⁻ phenotype. The pseudorevertants BC1-1 and DA2-1 lacked EII-lac and FIII-lac but possessed definite P-β-gal activity. Due to the enzymatic deficiencies, it appears that a lactose-PTS could not operate in the pseudorevertants and that another lactose transport system might be operating to account for the strains' ability to ferment lactose.

Induction of P-β-gal. *S. cremoris* B_1 and the Lac⁻ and revertants were grown in lactose or galactose broth to determine the degree of induction of P-β-gal occurring on each carbohydrate source. The results are shown in Table 3. In *S. lactis* C2 galactose was reported to be a better inducer of P-β-gal than was lactose (15), and similar results were observed for *S. cremoris* B_1. The same results were also noted for DA1 and DA1-1, indicating that the same mechanism of induction may be operating. The pseudorevertant DA1-2, however, displayed a significantly higher degree of P-β-gal induction when grown on lactose. In BC1-1 and DA2-1, galactose was unable to induce P-β-gal, but activity was observed when the cells were grown on lactose. Since galactose was an ineffective inducer of P-β-gal in the pseudorevertants, lactose or lactose derivative must be postulated to be the inducer of P-β-gal in BC1-1 and DA2-1.

DISCUSSION

Presumptive evidence for the presence of plasmid DNA in Lac⁺ *S. cremoris* B_1 and the absence of plasmid DNA in an acriflavine-induced Lac⁻ variant (BC1) was reported by Cords et al. (5). Characterization of plasmid DNA of Lac⁺ *S. cremoris* B_1 by contour-length measurement and sedimentation analysis indicated the presence of two distinct plasmid species having molecular weights of 9×10^6 and 36×10^6. Analysis of mutant BC1 revealed the absence of plasmid DNA, suggesting that lactose metabolism may be plasmid associated. Characterization of plasmid DNA derived from a Lac⁻ mutant, DA2, isolated by growing at elevated temperatures indicated the presence of a single plasmid species with a molecular weight of 9×10^6. These data suggested that the genetic determinant for lactose metabolism may be located on the 36×10^6-dalton plasmid.

The analysis of plasmid DNA extracted from DA1, however, showed the presence of two plasmid species similar to those observed in Lac⁺ *S. cremoris* B_1 on the basis of contour-length measurements. The phenotypic difference observed between the two strains may be explained by a single deficiency in a lac-specific enzyme of the PTS. DA1 was defective in EII-lac and thus unable to catalyze the transport and phosphorylation of lactose. Because lactose-phosphate could not be formed, the formation of galactose-6-phosphate and glucose from cleavage of lactose-phosphate also failed to occur. Since galactose-6-phosphate is assumed to be the inducer of the "*lac* operon" (18) in lactic streptococci, the uninduced level of FIII-lac might be explained by inducer exclusion.

The mutational nature of the EII-lac defect is not known. However, the ability of DA1 to revert to a full lactose-metabolizing phenotype suggests a point mutation. The analysis of lac-

specific enzymes derived from DA1-1 indicates that an operational reactivation of the EII-lac defect has occurred and that all three lac-specific enzymes are being coordinately expressed. The presence of the three lac-specific enzymes in revertant derivatives of DA1 that possessed pLM3601 in conjunction with the comparative absence of these enzymes in Lac$^-$ and revertant strains not possessing this plasmid imply that the genetic determinants for all three lac-specific enzymes are located on the 36 $\times$ 10^6-dalton plasmid.

The site of inducer activity may also be correlated with the presence of pLM3601. Galactose was shown to be a better inducer of the lac enzymes than lactose in *S. lactis* C2 (15). The lactic streptococci also contain two different pathways for galactose metabolism. One involves the Leloir pathway and the other includes the D-tagatose-6-phosphate pathway, in which PEP serves as the phosphoryl donor and galactose-6-phosphate is the first intermediate (2). In *S. cremoris* B_1, galactose was a better inducer of P-β-gal than lactose. The latter was also observed in *S. aureus*, where it was ultimately shown that galactose-6-phosphate was the true inducer of the lac enzymes (18). Galactose-6-phosphate may also be the true inducer of the lac enzymes in lactic streptococci (15). The ability of galactose to induce P-β-gal in DA1 and its revertants while inducing absolutely no P-β-gal in BC1 or DA2 and their respective revertants suggests that the latter strains were utilizing galactose via the Leloir pathway and they did not use the lactose PEP-PTS, and thus galactose-6-phosphate, the inducer of the lac enzymes, was not formed. The above also suggests that the ability to form galactose-6-phosphate and the locus of its inducer action are associated with pLM3601. Since no model for the regulation of lactose metabolism in lactic streptococci has been proposed, this assertion is strictly speculative.

Growth on galactose was more effective in inducing P-β-gal in DA1-1 than lactose, but lactose was the better inducer in the partial Lac$^+$ revertants. This suggests that one P-β-gal gene exists and its activity is regulated by one of two inducers, or that two separate P-β-gal genes exist whose activity is independently regulated by a single inducer. The first possibility is unlikely, since partial Lac$^+$ revertants were observed in strains without the large plasmid. The analysis of a CsCl-EB elution profile of BC1-1 showed no plasmid peak, offering presumptive evidence that an integrated plasmid was not being excised and expressed.

The second possibility, two separate P-β-gal genes, is more likely. A plasmid locus was suggested for one P-β-gal gene and a chromosomal locus could be postulated for a second P-β-gal gene. This location would be required for Lac$^-$ mutants without pLM3601 to acquire a partial Lac$^+$ phenotype and coincident P-β-gal activity. The observation that all three independently isolated Lac$^-$ mutants reverted to the partial Lac$^+$ phenotype is additional evidence that the gene(s) enabling slow lactose metabolism is located on the only common genetic determinant, the bacterial chromosome. The reversion of Lac$^-$ mutants to a partial Lac$^+$ phenotype was also reported in *S. lactis* C2 (14), in which case no apparent differences were observed between the P-β-gal enzymes from the wild-type and revertant cells.

The ability of partial Lac$^+$ revertants to utilize lactose requires the presence of mechanisms for both the transport and phosphorylation of lactose. Evidence for the presence of P-β-gal is presented in Table 2. Although little is known about the lactose transport and phosphorylation process in the pseudorevertants, it is evident that both processes are not constitutively operating at basal levels. If this were the case, the P-β-gal activity of the DA1 shown in Table 2 would be sufficient for lactose utilization. A partial Lac$^+$ phenotype would be expected of DA1. This does not occur. Therefore, the ability to transport and/or phosphorylate lactose is acquired in the pseudorevertants. Lactose transport studies were not performed on the revertants isolated from Lac$^-$ *S. cremoris* B_1. However, transport studies on a Lac$^-$ and a pseudo-Lac$^+$ revertant of *S. lactis* C2 indicated that the partial revertant had a slight but definite ability to accumulate [^{14}C]thiomethyl-β-D-galactoside (4). It is not known whether the ability to utilize lactose in the pseudo-Lac$^+$ revertants is the expression of a limited lactose-specific transport system or the result of mutational change in a non-lactose-specific permease system allowing it to transport and phosphorylate lactose, but not galactose, with low efficiency. Evidence for lactose permeation via the arabinose transport system in *E. coli* has been reported by Messer (17).

A wide range of induced P-β-gal activity was observed by growing the cells on lactose. However, the growth characteristics of the three pseudo-Lac$^+$ revertants did not reflect their divergent P-β-gal activities. All three displayed similar growth rates. This implies that the amount of P-β-gal activity is not the rate-limiting factor of the pseudorevertants' slow growth on lactose. Either the transport or the phosphorylation of lactose may be postulated as the rate-limiting step.

On the basis of the information and interpre-

tations presented, a more complete model for the metabolism of lactose in *S. cremoris* B_1 can be proposed. The location of the genetic determinants for the lac-specific enzymes (EII-lac, FIII-lac, P-β-gal) of the PTS appear to be on pLM3601. The site of galactose-6-phosphate induction may also be located on the same plasmid. Loss of this extrachromosomal element results in the inability to metabolize lactose via the PTS system, with a phenotypic change from Lac^+ to Lac^-. A Lac^- strain may undergo a chromosomal alteration(s) of unknown nature, resulting in the ability to slowly metabolize lactose. The partial lactose-utilizing system requires lactose transport and phosphorylation steps as well as P-β-gal activity. Lactose, or a lactose derivative, is the inducer of the chromosomal P-β-gal gene. The rate of partial lactose metabolism is limited by lactose transport and/or the phosphorylation process.

There are instances of a second set of β-galactosidase genes in *E. coli*, which appear under prolonged intensive selection pressure and may arise through a sequence of mutations (3) or which appear without prolonged exposure to lactose and are not attributed to a set of evolved genes (21). In the case of lactic streptococci, this latter pattern would seem more likely, as the partial revertants appear much earlier than would be expected for an evolved system. Reeve and Braithwaite (20) have also shown that *Klebsiella aerogenes* V 9A carries two sets of *lac* genes (one plasmid associated and the other chromosomal linked). Two distinct systems of lactose metabolism may also exist in *S. cremoris* B_1, *S. lactis* C2, and other lactic streptococci.

The model presented in this paper is useful in explaining the lac-specific enzyme activities observed for DA1-2 shown in Table 2. Since DA1-2 is a partial revertant, lactose is transported and/or phosphorylated by a system other than the wild-type lactose-PTS. Intracellular lactose, or a derivative, induces the chromosomal P-β-gal gene. The chromosomal P-β-gal then cleaves the lactose-phosphate within the cell to glucose and galactose-6-phosphate. Since galactose-6-phosphate is now present within the cell, it is capable of inducing the plasmid-associated lac genes. Thus, it is conceivable that both the chromosomal and plasmid P-β-gal genes are being expressed in DA1-2, resulting in the elevated level of P-β-gal activity observed for lactose-grown cells (Tables 2 and 3).

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