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Distinct Galactose Phosphoenolpyruvate-Dependent Phosphotransferase System in *Streptococcus lactis*†

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Lactose-negative (Lac^-) mutants were isolated from a variant of *Streptococcus lactis* C2 in which the lactose plasmid had become integrated into the chromosome. These mutants retained their parental growth characteristics on galactose (Lac^- Gal^+). This is in contrast to the Lac^- variants obtained when the lactose plasmid is lost from *S. lactis*, which results in a slower growth rate on galactose (Lac^- Gal^+). The Lac^- Gal^+ mutants were defective in [^{14}C]thiomethyl-β-D-galactopyranoside accumulation, suggesting a defect in the lactose phosphoenolpyruvate-dependent phosphotransferase system, but still possessed the ability to form galactose-1-phosphate and galactose-6-phosphate from galactose in a ratio similar to that observed from the parental strain. The Lac^- Gal^d variant formed only galactose-1-phosphate. The results imply that galactose is not translocated via the lactose phosphoenolpyruvate-dependent phosphotransferase system, but rather by a specific galactose phosphoenolpyruvate-dependent phosphotransferase system for which the genetic locus is also found on the lactose plasmid in *S. lactis*.

Lactose utilization in group N streptococci (*Streptococcus cremoris*, *S. lactis*, and *S. lactis* subsp. *diacetylactis*) is mediated via a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) (18, 19). This system (designated herein as PEP-PTS) appears similar to that described for *Staphylococcus aureus* (7, 20). However, lactose-negative (Lac^-) mutants of *S. aureus* missing enzyme II-lac or factor III-lac of the lactose PEP-PTS are also galactose negative (Gal^-) (20), whereas Lac^- mutants of *S. lactis* missing the two enzymes are able to grow on galactose, but at a slower rate (2). This phenotype (Lac^- Gal^d) was due to the inability of *S. lactis* to utilize galactose via a PEP-PTS. The variants were only utilizing galactose by the Leloir pathway (2, 11). Thus, *S. lactis* has the ability to utilize galactose by two different pathways, and it has been assumed that one of these pathways, as in *S. aureus*, is the lactose PEP-PTS. The results presented in this communication support the recent suggestion of Thompson (24) that galactose is translocated into the *S. lactis* cell by a galactose PEP-PTS which is distinct from the lactose system.

MATERIALS AND METHODS

Bacteria and culture conditions. The *S. lactis* strains used in this study were obtained from our stock culture collection and are described in Table 1. Unless otherwise specified, all strains were propagated in Elliker broth (4) and incubated at 32°C.

Isolation of lactose-constitutive mutants. *S. lactis* KB21 was grown in Elliker broth, and the cells were centrifuged, suspended in 0.85% NaCl, and exposed to UV irradiation as previously described by McKay and Baldwin (14). The cells were irradiated to give 0.001% survival. After irradiation, a 1% inoculum was made into lactose broth (18), and tubes were incubated for 48 h at 32°C. Harvey-Collins medium (6) containing 0.02 M sodium lactobionate as a carbon source was inoculated and incubated at 32°C for 24 h. The cells were diluted and spread over the surface of lactic agar plates (4) for colony isolation. To confirm the constitutive phenotype of suspected isolates, the growth behavior in Harvey-Collins broth containing 0.02 M sodium lactobionate was determined (21).

Isolation of Lac^- derivatives. Lac^- variants were isolated from the constitutive strains by using UV irradiation or ethyl methane sulfonate (EMS). For UV mutagenesis the cells were treated as described above and plated on bromocresol purple-lactose indicator agar (17) for differentiation of Lac^+ and Lac^- colonies. For EMS treatment, a 16-h Elliker broth culture at 32°C was centrifuged, and the cells were washed twice with 0.05 M sodium phosphate buffer (pH 7.0). Cells were suspended in 0.5% dibasic potassium phosphate. The pH was adjusted to 8.0, and EMS was added to give a 3% (vol/vol) concentration. The cells were incubated at 37°C for 75 min, diluted, and spread over the surface of bromocresol purple-lactose indicator agar plates for mutant isolation.

Phospho-β-galactosidase assay and [^{14}C]-TMG uptake. The preparation of cell extracts and phospho-β-galactosidase assay were conducted as described by

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TABLE 1. Strains of *S. lactis* used

Strain	Phenotype	Derivation (reference)
KB21	Lac^+ Gal^+	Stabilized Lac^+ transductant of LM0220 (15)
YP2	Lac^+ Gal^+	UV-induced lactose constitutive mutant of KB21 (this paper)
YP2-1	Lac^- Gal^+	UV-induced mutant of YP2 (this paper)
YP2-2	Lac^- Gal^+	UV-induced mutant of YP2 (this paper)
YP2-3	Lac^- Gal^+	EMS-induced mutant of YP2 (this paper)
YP2-4	Lac^- Gal^+	EMS-induced mutant of YP2 (this paper)
YP2-5	Lac^- Gal^+	EMS-induced mutant of YP2 (this paper)
LM0220	Lac^- Gal^d	Spontaneous mutant of C2 originally designated 25Sp (2)

Schifsky and McKay (21). Protein concentrations of cell extracts were measured by the method of Lowry et al. (13). [^{14}C]thiomethyl-β-galactoside ([^{14}C]TMG) uptake was measured as described by McKay et al. (19).

Extraction and assay for Gal-1P and Gal-6P. Cultures (25 ml) were grown at 30°C in T_5 broth (22). The initial galactose concentration was 28 mM, and cells were harvested when the concentration reached 12 to 14 mM (pH 6.5) as described by Thomas et al. (23). Cells from the 25-ml culture were collected on membrane filters (Millipore Corp., 47-mm diameter, 0.8-μm pore size), and filters were added to 5 ml of 5% *n*-butanol and incubated at 37°C for 10 min. Extracts were centrifuged, and supernatants were stored at −20°C. Before assaying for galactose-1-phosphate (Gal-1P) and galactose-6-phosphate (Gal-6P), free galactose was removed by passing the supernatant through a column containing a strong base ion-exchange resin (AG1-X8; Bio-Rad Laboratories).

The difference in acid lability of Gal-1P and Gal-6P allows for the enzymatic analysis of both derivatives from the same sample (12). After hydrolysis in 0.1 N HCl at 100°C for 15 min, the sample was neutralized with 0.1 N NaOH, and Gal-1P was enzymatically determined as free galactose by the method of Kurz and Wallenfels (9). Gal-6P in the acid hydrolyzed sample was enzymatically measured as described by Grassl (5).

The intracellular concentrations of Gal-1P and Gal-6P were calculated on the assumption that 25 ml of a T_5 broth culture contains approximately 16 mg (dry weight) of cells (23) and that 1 g (dry weight) of cells is equivalent to 1.67 ml of intracellular fluid (25).

RESULTS

Growth response on lactobionic acid. Figure 1 shows the growth response of *S. lactis* KB21 and *S. lactis* YP2, a variant of KB21. The parental culture was unable to grow on lactobionate during a 48-h incubation period, whereas YP2 was capable of utilizing this carbon source, thus supporting the belief that YP2 is a constitutive mutant. Lactobionic acid does not induce β-galactosidase synthesis and is poorly bound and hydrolyzed by this enzyme. Hence, mutants which utilize lactobionate as a carbon source must form β-galactosidase constitutively (10). *S. lactis* YP2 was subsequently used to isolate Lac^- variants by UV irradiation (YP2-1, YP2-2) or EMS treatment (YP2-3, YP2-4, YP2-5).

Growth of Lac^- mutants on glucose, galactose, or lactose. Figure 2 shows the growth response of *S. lactis* YP2, YP2-1, and YP2-2 in a semisynthetic medium (6) containing 0.5% glucose, galactose, or lactose as the carbon source. All three strains grew equally well in the presence of glucose, and the Lac^- strains (YP2-1 and YP2-2) were unable to grow on lactose. An unexpected observation was the growth response of the Lac^- variants on galactose (Fig. 2B). They grew about the same rate as the parental culture, YP2. The EMS Lac^- isolates also exhibited similar growth responses on the same three sugars (data not shown).

This Lac^- Gal^+ phenotype has not previously been reported in *S. lactis*, except for *S. lactis* 7962 which is considered an atypical strain (19, 25). LeBlanc et al. (11) reported that Lac^- Gat^+ mutants were obtained from *S. lactis* DR1251; however, these mutants exhibited a slower growth response on galactose than did the parent culture. This phenotype has been described for other Lac^- mutants of *S. lactis* (2, 3), and we have designated it as Lac^- Gal^d. Figure 3 shows the growth patterns for the Lac^- Gal^+ (YP2-2)

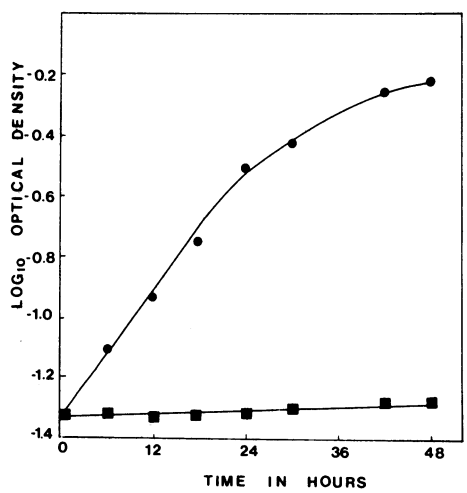

FIG. 1. Growth of *S. lactis* KB21 (■) and *S. lactis* YP2 (●) in Harvey-Collins medium containing 0.02 M sodium lactobionate as the carbohydrate source.

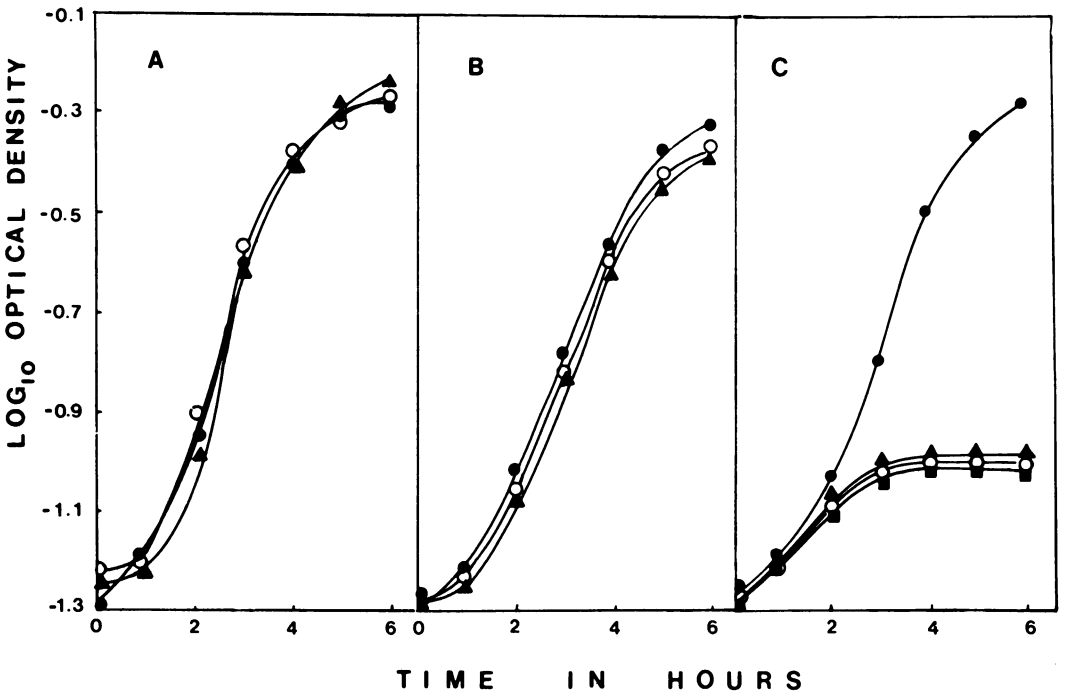

FIG. 2. Growth of Lac$^+$ Gal$^+$ *S. lactis* YP2 (●), Lac$^-$ Gal$^+$ *S. lactis* YP2-1 (○), and Lac$^-$ Gal$^+$ *S. lactis* YP2-2 (▲) in a semisynthetic medium containing 0.5% glucose (A), 0.5% galactose (B), or 0.5% lactose (C). The growth response of *S. lactis* YP2-2 (■) in the medium without added sugar is also shown (C).

and the Lac$^-$ Gal^d (LM0220) phenotype when the strains were grown in a medium containing galactose. The difference in growth response is quite evident.

[^{14}C]TMG uptake and phospho-β-galactosidase activity. The uptake of [^{14}C]TMG by *S. lactis* YP2, YP2-2, and YP2-5 indicated that in contrast to the parent, the Lac$^-$ variant YP2-2 was unable to accumulate [^{14}C]TMG. Although the Lac$^-$ YP2-5 did accumulate [^{14}C]TMG, the process was defective when compared with YP2 (Fig. 4). The uptake of [^{14}C]TMG by YP2-1, YP2-3, and YP2-4 resembled that for YP2-2 (data not shown). These results indicate that the Lac$^-$ variants isolated from YP2 were defective in one or more of the enzymes involved in the lactose PEP-PTS.

Table 2 shows the phospho-β-galactosidase activity of *S. lactis* KB21 and its phenotypic derivatives. The specific activity of the enzyme from YP2 was about 2.5 times higher than that observed for KB21. No phospho-β-galactosidase activity was observed in the Lac$^-$ variants YP2-2 and YP2-4. These two strains were also unable to accumulate [^{14}C]TMG. YP2-3 exhibited some enzyme activity, as did YP2-5. Even though the latter strain possessed low levels of phospho-β-galactosidase and galactoside transport activity, it still exhibited the Lac$^-$ phenotype on the lactose indicator agar and in the semisynthetic medium containing lactose as the carbon source. The Lac$^-$ strain YP2-1 contained substantial phospho-β-galactosidase activity, but was unable to accumulate [^{14}C]TMG.

Intracellular concentration of Gal-1P and Gal-6P. Galactose-grown cells of *S. lactis* can utilize the sugar via a PEP-PTS in which Gal-6P is an intermediate and via the Leloir pathway in which Gal-1P is an intermediate (1). Since Gal-1P and Gal-6P are intermediates of the two respective pathways, the presence of these compounds would indicate the participation of the two pathways in galactose metabolism (23). Therefore, the intracellular concentration of Gal-1P and Gal-6P was measured in galactose-grown cells of Lac$^+$ Gal$^+$ YP2, the Lac$^-$ Gal$^+$ YP2-2, and the Lac$^-$ Gal^d LM0220 to compare the route of galactose utilization in the Gal$^-$ and Gal^d phenotypes.

The concentration of Gal-1P (0.8 ± 0.2 mM) and Gal-6P (0.4 ± 0.2 mM) in the Lac$^-$ Gal$^+$ YP2-2 was similar to that observed in the Lac$^+$ Gal$^+$ parental culture YP2 (0.8 ± 0.1 mM Gal-1P and 0.5 ± 0.1 mM Gal-6P). The ratio of Gal-1P to Gal-6P in YP2 and YP2-2 resembled that demonstrated by Thomas et al. (23) in *S. lactis* ML3 and *S. lactis* 7962. The concentration of the two intermediates, however, was lower than that

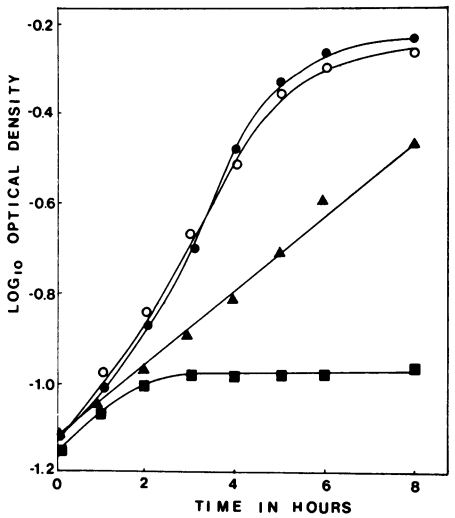

FIG. 3. Comparative growth of Lac$^+$ Gal$^+$ *S. lactis* YP2 (●), Lac$^-$ Gal$^+$ *S. lactis* YP2-2 (○), and Lac$^-$ Gal^d *S. lactis* LM0220 (▲) in a semisynthetic medium with 0.5% galactose as the carbon source. The growth response of *S. lactis* YP2-2 (■) without added carbohydrate is also shown.

reported by Thomas et al. (23). This difference could be due to the extraction methods employed. Gal-6P was not detected in the Lac$^-$ Gal^d LM0220, and the concentration of Gal-1P was observed to be 1.0 ± 0.1 mM, suggesting that only the Leloir pathway was involved in galactose utilization in this strain.

DISCUSSION

If the genetic regulation of lactose metabolism in *S. lactis* occurs as in *S. aureus* (20), Lac$^-$ mutants should be isolated from a lactose-constitutive mutant so that mutants defective in each of the lactose PEP-PTS components can be obtained. Langridge (10) described a lactobionic acid technique for the selection of *Escherichia coli* mutants constitutive for β-galactose synthesis. Lactobionic acid was useful in selecting for constitutive or high β-galactosidase producers, as it did not induce the enzyme and was poorly bound and hydrolyzed by β-galactosidase. Thus, mutants which grow in broth containing this compound as the carbon source must form β-galactosidase constitutively. Schifsky and McKay (21) showed that *S. lactis* C2 exhibited poor growth in broth containing lactobionic acid as the carbon source, whereas mutants possessing high phospho-β-galactosidase activity were capable of initiating growth. Therefore, this approach was used to select for lactose-constitutive strains from *S. lactis* KB21 for the isolation of Lac$^-$ variants.

McKay et al. previously isolated Lac$^-$ variants from *S. lactis* C2 (18). Although these mutants were incorrectly designated Lac$^-$ Gal$^-$, they were defined as being unable to ferment lactose and as being defective (slow fermentation) in galactose utilization. In a subsequent publication, Cords and McKay (2) showed that these Lac$^-$ cells contained about 13 times the galactokinase activity of the parent culture *S. lactis* C2, indicating that the Lac$^-$ cells were using the Leloir pathway for galactose metabolism. The fact that the change to a Lac$^-$ also resulted in a galactose-defective phenotype suggested that a common component was required for the metabolism of lactose and galactose. Other workers have also noted that Lac$^-$ variants of *S. lactis* exhibit a slower growth rate on galactose (Gal^d), but no Lac$^-$ Gal$^+$ mutants had been described. For example, Demko et al. (3) isolated Lac$^-$ derivatives of *S. lactis* ATCC 11454 which exhibited a slightly decreased growth rate on galactose. More recently, LeBlanc et al. (11) showed that Lac$^-$ isolates of *S. lactis* DR1251 were able to grow on galactose, but with a longer generation time than the Lac$^+$ strain.

This defect in galactose metabolism accompanying the Lac$^-$ phenotype was explained on the basis that *S. lactis* can utilize galactose by two different metabolic sequences. These included a

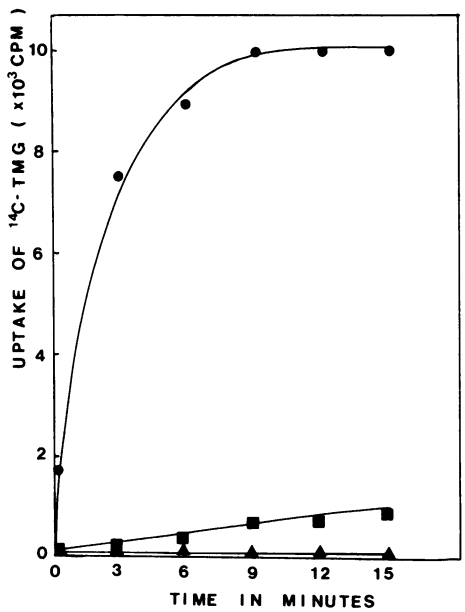

FIG. 4. Accumulation of [^{14}C]TMG by resting cell suspensions of Lac$^+$ Gal$^+$ *S. lactis* YP2 (●), Lac$^-$ Gal$^+$ *S. lactis* YP2-2 (▲), and Lac$^-$ Gal$^+$ YP2-5 (■) previously grown on galactose. The incubation mixture consisted of approximately 1.6 mg (dry weight) of cells per ml and 6.0 μM [^{14}C]TMG in a total volume of 5.0 ml.

PEP-PTS for the D-tagatose-6-phosphate pathway and an ATP permease system for the Leloir pathway (1). In the *S. lactis* C2 system (2) as well as in *S. lactis* DR1251 (11) it was assumed that galactose was metabolized primarily by the D-tagatose-6-phosphate pathway in which galactose was translocated into the cell via the lactose PEP-PTS to yield Gal-6P. Lac$^-$ Gal^d derivatives of C2 and DR1251 were deficient in the lactose PEP-PTS and utilized galactose by the Leloir pathway. Both derivatives possessed enhanced galactokinase activity which supported the use of this pathway. Recent data from Thomas et al. (23) indicate that the relative participation of alternate pathways for galactose metabolism for the Lac$^+$ Gal$^+$ phenotype varied widely with different *S. lactis* and *S. cremoris* strains.

Although evidence obtained from several laboratories (2, 3, 11, 26) suggested that the translocation of galactose into the cell to form Gal-6P was mediated via a lactose PEP-PTS, data have also been obtained for a distinct galactose PEP-PTS. First, Thomas et al. (23) suggested that *S. lactis* 7962 had a functional galactose PEP-PTS and that this system did not recognize TMG. This assumption was based on the fact that *S. lactis* 7962 does not utilize lactose via a PEP-PTS (18, 25), yet Gal-6P was found in 7962 at concentrations similar to that for *S. lactis* ML3 (23). Secondly, Thompson (24) showed that preincubation of starved *S. lactis* ML3 cells with *p*-chloromercuribenzoate reduced the PEP-dependent accumulation of lactose and TMG by 75 and 100%, respectively. The PEP-dependent accumulation of galactose, however, was not significantly inhibited by *p*-chloromercuribenzoate. These observations prompted Thompson (24) to suggest the presence of more than one PEP-PTS for galactose metabolism in *S. lactis*. The genetic results presented in this communication support the latter conclusion.

S. lactis KB21 is a Lac$^+$ transductant in which the lactose plasmid or a portion of it has become integrated into the host chromosome (15). This strain was being used to isolate a variety of mutants to examine the enzymatic and genetic regulation of lactose metabolism in *S. lactis*. The change from Lac$^+$ Gal$^+$ to a Lac$^-$ Gal^d phenotype in *S. lactis* C2 is due to loss of a lactose plasmid (8, 16). Since the ease of plasmid loss has made it difficult to isolate point mutations for lactose metabolism, KB21 provided a means for circumventing this problem. We observed that Lac$^-$ mutants isolated from KB21 grew as well in galactose as did KB21. In other words, instead of possessing the Lac$^-$ Gal^d phenotype such as occurs when the lactose plasmid is lost from *S. lactis* C2 (2) or *S. lactis* DR1251 (11), the Lac$^-$ mutants from KB21 were Lac$^-$ Gal$^+$. The Lac$^-$ Gal$^+$ phenotype in these mutants was due to the absence of phospho-β-galactosidase or (as determined by the uptake of TMG) to a defect in the lactose PEP-PTS or to both. The precise enzymatic defect in the lactose PEP-PTS is currently unknown. In any event, this defect presumably should have given the Lac$^-$ Gal^d phenotype in *S. lactis*. In *S. aureus*, the isolation of Lac$^-$ mutants defective in the lactose-specific PTS components would result in a Lac$^-$ Gal$^-$ phenotype (20). Since *S. lactis* can metabolize galactose by two separate pathways (1), the intracellular concentrations of Gal-1P (Leloir pathway) and Gal-6P (D-tagatose-6-phosphate pathway) were measured in the Lac$^-$ Gal$^+$ mutants to determine which pathway(s) was being used. The results suggested that the participation of the two pathways was similar in the Lac$^+$ Gal$^+$ parent strain and in YP2-2 (Lac$^-$ Gal$^+$). Gal-6P, however, was not detected in the Lac$^-$ Gal^d LM0220. These observations suggest that Gal-6P is not being formed via the lactose-PTS in the Lac$^-$ Gal$^+$ mutants and support the suggestion of Thompson (24) that a distinct galactose PEP-PTS, separate from the lactose-PTS, exists in *S. lactis* (Fig. 5). The possibility that Gal-6P is also formed via the lactose-PTS is not excluded, and the operation of both pathways could explain the slightly enhanced growth of Lac$^+$ Gal$^+$ strains over Lac$^-$ Gal$^+$ strains on galactose (Fig. 2B).

Since the loss of the lactose plasmid is accompanied by loss of the PTS for both lactose and galactose and gives rise to the Lac$^-$ Gal^d phenotype (Fig. 3), workers in several laboratories, including our own (2, 11, 26), assumed that Gal-6P was formed via the lactose-PTS. In this study, the evidence for a distinct galactose-PTS separate from the lactose-PTS was obtained by isolating Lac$^-$ mutants from a strain in which the lactose plasmid or a portion of it had become integrated into the chromosome. Such mutants, although defective in the lactose PEP-PTS, grew as well on galactose, as did the parental culture,

TABLE 2. Specific activity of phospho-β-galactosidase in cell extracts of *S. lactis* KB21 and its derivatives

Organism	Phenotype	Sp act[a]
S. lactis KB21	Lac$^+$ Gal$^+$	6.9
S. lactis YP2	Lac$^+$ Gal$^+$	18.0
S. lactis YP2-1	Lac$^-$ Gal$^+$	9.4
S. lactis YP2-2	Lac$^-$ Gal$^+$	0
S. lactis YP2-3	Lac$^-$ Gal$^+$	0.9
S. lactis YP2-4	Lac$^-$ Gal$^+$	0
S. lactis YP2-5	Lac$^-$ Gal$^+$	2.8

[a] Results are expressed as nanomoles of *o*-nitrophenol released from *o*-nitrophenyl-β-D-galactoside-6-phosphate per minute per milligram of protein.

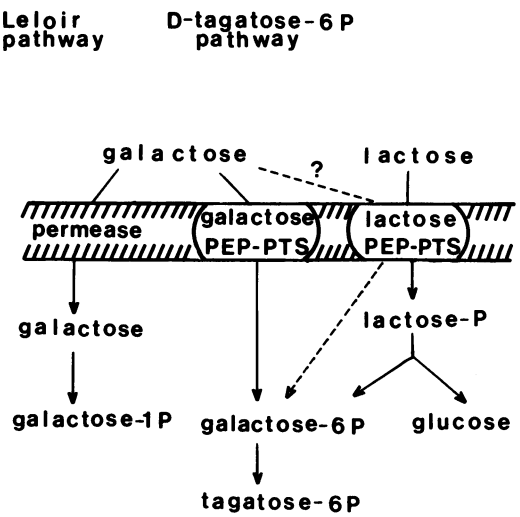

FIG. 5. Systems for galactoside transport in group N streptococci.

and utilized the sugar via a PEP-PTS as evidenced by the formation of Gal-6P.

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