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Mechanisms of Lactose Utilization by Lactic Acid Streptococci: Enzymatic and Genetic Analyses[1]

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The apparent instability of β-galactosidase in toluene-treated cells or cell-free extracts of lactic streptococci is explained by the fact that these organisms do not contain the expected enzyme. Instead, various strains of *Streptococcus lactis*, *S. cremoris*, and *S. diacetilactis* were shown to hydrolyze *o*-nitrophenyl-β-D-galactoside-6-phosphate (ONPG-6-P), indicating the presence of a different enzyme. In addition, lactose metabolism in *S. lactis* C_2F was found to involve enzyme I (EI), enzyme II (EII), factor III (FIII), and a heat-stable protein (HPr) of a phosphoenolpyruvate (PEP)-dependent phosphotransferase system analogous to that of *Staphylococcus aureus*. Mutants of *S. lactis* C_2F, defective in lactose metabolism, possessed the phenotype lac^- gal^-. These strains were unable to accumulate ^{14}C-thiomethyl-β-D-galactoside, to hydrolyze ONPG, or to utilize lactose when grown in lactose or galactose broth. In addition, these mutants contained EI and HPr, but lacked EII, FIII, and the ability to hydrolyze ONPG-6-P. This suggested that the defect was in the phosphorylation step. Lactose-negative mutants of *S. lactis* 7962, a strain containing β-galactosidase, could be separated into several classes, which indicated that this organism is not dependent upon the PEP-phosphotransferase system for lactose metabolism.

In a previous report (17), it was shown that the labile nature of β-galactosidase (EC 3.2.1.23, β-D-galactoside galactohydrolase) in *Streptococcus lactis* C_2F (2) is due to the absence of this enzyme and the presence of a hydrolyzing system dependent upon phosphoenolpyruvate (PEP). The data suggested the involvement of the PEP-dependent phosphotransferase system similar to that reported for galactoside accumulation and metabolism in *Staphylococcus aureus* (5, 6, 8, 9, 15, 16, 18, 19).

The hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) by *S. aureus* can be described as follows:

$$\text{PEP} + \text{HPr} \overset{\text{EI}}{\rightleftharpoons} \text{P-HPr} + \text{pyruvate}$$

$$\text{P-HPr} + \text{ONPG} \overset{\text{EII lac, FIII lac}}{\rightleftharpoons} \text{ONPG-P} + \text{HPr}$$

$$\text{ONPG-P} \xrightarrow{\text{phospho-β-galactosidase}} \text{ONP} + \text{galactose-6-phosphate}$$

HPr is a heat-stable soluble protein, EI (enzyme I) is a soluble protein, 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. EI and HPr are produced constitutively, whereas EII lac, FIII lac, and phospho-β-galactosidase are induced by growing cells in the presence of galactose or galactose-6-phosphate (18).

The present study shows that *S. lactis* C_2F hydrolyzes ONPG-6-phosphate (ONPG-6-P) and consequently metabolizes lactose by a PEP-dependent system which is similar to that found in *S. aureus*.

MATERIALS AND METHODS

Organisms and culturing procedures. All lactic streptococci, except *S. lactis* UN, were obtained from the stock culture collection maintained by the Department of Microbiology, Oregon State University. *S. lactis* UN was provided by K. M. Shahani, Department of Food Science and Technology, University of Nebraska, Lincoln. The cultures were routinely propagated in lactic broth (4). For induction experiments, the medium was prepared without the sugars, autoclaved, and cooled. A sterile solution of galactose, the preferential inducer of lactose utilization in group N streptococci (17), was then added to a concentration

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of 0.01 g/ml. *S. lactis* 7962 was induced by growing cells in 1% lactose broth (2). Unless otherwise specified, all cultures were grown at 32 C.

Constitutive lactose-negative mutants of *S. aureus*, defective in the components of the phosphotransferase system, were obtained through the courtesy of M. L. Morse, Department of Biophysics, University of Colorado Medical Center, Denver. These mutants were defective in a single component of the phosphotransferase system, yet they synthesized the remaining proteins constitutively. The strains with their respective defect were S305A (wild type), S710A (pleiotropic, EI$^-$), S797A (pleiotropic, HPr$^-$), S714B (lac$^-$ gal$^-$, EII lac$^-$) and S714G (lac$^-$ gal$^-$, FIII lac$^-$). Cultures were propagated at 37 C in 1% Bacto Peptone containing 1% glucose.

Isolation of lactose mutants from S. lactis. Lactic agar (4), containing 1% lactose or galactose as the sole carbon source and supplemented with 0.004% bromocresol purple, served as the indicator medium. Plates were incubated at 32 C for approximately 30 hr. On this medium, colonies producing acid were yellow, in contrast to the white non-acid-forming colonies. To avoid the isolation of contaminants, only catalase-negative mutants which were lysed by their parent phages were selected.

Mutations were induced with ultraviolet light (UV), acriflavine, or *N* methyl *N'*-nitro-*N*-nitrosoguanidine (NTG). The mutagenic treatments were as follows. For UV treatment, cells were grown in 100 ml of lactic broth at 32 C for 4 to 6 hr, collected, washed, and resuspended in 0.85% saline. A 40-ml amount of the cell suspension was then added to a sterile 6-inch (15 cm) petri dish and irradiated to obtain 99.999% kill. Portions of 1 ml were added to 10-ml amounts of lactic broth and were incubated at 21 C for 6 hr. Samples were then removed, diluted, and spread over the surface of indicator agar. For acriflavine treatment, cultures were grown at 32 C for 10 hr, after which a 5% inoculum was added to 10 ml of lactose broth. Acriflavine was added to give a final concentration of 10 or 20 μg/ml. Cultures were incubated at 40 C for 24 hr, and then 0.1-ml samples were removed and spread over the surface of indicator plates (10). For NTG treatment, *S. lactis* 7962 was grown for 4.5 hr and *S. lactis* C_2F for 3.5 hr at 32 C in glucose broth. A 20-ml portion of culture was then removed and filtered through a membrane filter (0.45 μm pore size; Millipore Corp., Bedford, Mass.). The cells were washed on the filter with 10 ml of tris(hydroxymethyl)-aminomethane (Tris)-maleate buffer [0.5 M Tris, 0.05 M maleic acid, 1.0 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 0.25 mg of $FeSO_4 \cdot 7H_2O$ per liter of distilled water, *p*H 7.0], and the filter was then placed in 18 ml of the buffer to remove the cells. The filter was removed, and NTG (1) was added to a final concentration of 200 μg/ml (*S. lactis* 7962) or 100 μg/ml (*S. lactis* C_2F). The flasks were incubated at 32 C for 2 hr (*S. lactis* 7962) or 30 min (*S. lactis* C_2F), and then samples were removed, diluted, and plated on indicator agar.

β-Galactosidase and 6-phospho-β-galactosidase assays. The preparation of toluene-treated cells and the assay of β-galactosidase were performed as previously described for *S. lactis* 7962 (2).

The assay of phosphogalactosidase was performed by incubating 1 ml of toluene-treated cells with 12 μmoles of ONPG-6-P in a total volume of 2 ml at 37 C. ONPG-6-P was obtained from Research Plus Laboratories, Jersey City, N.J. The reaction was terminated after the desired absorbancy was obtained by the addition of 2 ml of 0.5 M Na_2CO_3. The absorbancy at 420 nm was recorded after the cells had been removed by centrifugation.

Uptake of ^{14}C-thiomethyl-β-D-galactoside (^{14}C-TMG). ^{14}C-TMG uptake was measured in washed cell suspensions as previously described (17).

Fractionation of crude extracts from S. lactis C_2F. For preparation of a soluble fraction (charcoal-absorbed) and a membrane fraction, cells from 2 liters of the galactose induction medium incubated for 10 hr at 21 C were harvested and washed twice with 0.05 M Tris-hydrochloride buffer (*p*H 7.6) containing 0.1% dithiothreitol and 10^{-3} M ethylenediaminetetraacetate (EDTA). The cells were resuspended in 10 ml of the buffer, and cell extracts were prepared by use of an Eaton press (3). The frozen mixture was removed from the press, thawed, and diluted to 20 ml with buffer. This suspension was sonically disrupted by use of a Branson Sonifier at the maximum power setting for three 15-sec pulses. The extract was centrifuged at 10,000 $\times$ *g* for 20 min to remove cellular debris, and the supernatant fluid was centrifuged at 100,000 $\times$ *g* for 2 hr at 4 C. The pellet was resuspended in 10 ml of the buffer and washed once by a second high-speed centrifugation. The pellet, which constituted the membrane fraction, was then resuspended in 10 ml of buffer.

The supernatant fraction from the first ultracentrifugation was treated with Norit for 5 min. The mixture was centrifuged at 10,000 $\times$ *g* for 20 min, and the supernatant fluid was designated as the soluble fraction (charcoal-absorbed). The charcoal was prepared by centrifuging 15 ml of a 10% solution at 10,000 $\times$ *g* for 15 min. The resulting precipitate was then mixed with 15 ml of the extract.

For preparation of a heat-treated soluble fraction, 2 liters of galactose-grown cells were harvested, washed, and resuspended in 10 ml of 0.05 M Tris-hydrochloride buffer at *p*H 7.6. Crude extracts were prepared as above, and 5-ml samples in screw-cap test tubes (125 $\times$ 20 mm) were heated in a boiling-water bath for 10 min. The coagulated protein was removed by centrifugation at 10,000 $\times$ *g* for 20 min. The supernatant fluid served as the heat-treated fraction. The above fractionation procedures were similar to those previously described (13, 14, 19, 20).

Requirements for ONPG hydrolysis by S. lactis C_2F. The complete assay system consisted of 0.05 M Tris-hydrochloride buffer (*p*H 7.6) containing 0.05 M $MgCl_2$, 10 μmoles of PEP, 10 μmoles of ONPG, 20 μmoles of sodium fluoride, 1.32 mg of soluble fraction (charcoal-absorbed), 0.17 mg of soluble fraction (heat-treated), and 0.1 ml of membrane fraction in a total volume of 2 ml. After 1 hr of incubation at 37 C, the reaction was terminated by adding 2 ml of 0.5 M

Na_2CO_3. The absorbancy was recorded at 420 nm after any debris present had been removed by centrifugation.

Complementation experiments for components of the phosphotransferase system. *S. aureus* strains were grown for 15 hr in 1-liter quantities of 2% peptone at 37 C on a rotary shaker. Cells of *S. lactis* C_2F were grown in 1-liter quantities of 1% galactose broth at 21 C for 12 hr. The cells were centrifuged and washed twice with 0.05 M Tris-hydrochloride buffer (*p*H 7.6) containing 0.05 M NaCl, 0.05 M $MgCl_2$, and 0.001 M dithiothreitol. A Bronwill mechanical cell homogenizer (model MSK) was used to prepare cell extracts. The cell pellets were mixed with 6 g of glass beads (0.11 to 0.12 mm in diameter), and enough buffer was added to form a thick paste. The capsules were shaken for 2 min, with cooling effected by a jet of compressed carbon dioxide.

The complementation tests were based on the ability of the *S. lactis* C_2F strains to restore ONPG hydrolysis in crude extracts from *S. aureus* mutants (8, 18). The assay system contained 10 μmoles of ONPG, 10 μmoles of PEP, 0.5 ml of *S. aureus* extract, and 1 ml of extract of the complementation test strain in a total volume of 2 ml. Reaction tubes were incubated at 37 C and examined for ONPG hydrolysis.

RESULTS AND DISCUSSION

Selection of mutants from S. lactis. Table 1 lists the carbohydrate-negative mutants isolated from *S. lactis* 7962 and *S. lactis* C_2F. Only two mutants were isolated by use of UV light; however, NTG was an effective mutagenic agent. Although lac$^-$ mutants were not isolated from *S. lactis* 7962 when acriflavine was used, such mutants were isolated from *S. lactis* C_2F. In addition, a spontaneous lac$^-$ mutant was isolated from stock cultures of *S. lactis* C_2F after several months of daily transfer in lactic broth. The strain designated *S. lactis* C_2F-G2 was isolated by selecting for gal$^-$ mutants. Since these mutants grew as well as the wild type on glucose, maltose, mannose, and fructose, it was argued that the defect in the metabolism of lactose did not involve the glycolytic enzymes.

TABLE 1. *Method of isolation and phenotype designation for S. lactis mutants*

Mutant	Method of isolation	Phenotype[a]
S. lactis 7962-3	NTG	lac$^-$
S. lactis 7962-C	NTG	lac$^-$
S. lactis 7962-B	NTG	lac$^-$
S. lactis 7962-9	NTG	lac$^-$
S. lactis 7962-18	NTG	lac$^-$
S. lactis 7962-6	NTG	lac$^-$ gal$^-$
S. lactis 7962-5	UV	lac$^-$
S. lactis 7962-16	UV	lac$^-$
S. lactis C_2F-38-3	Acriflavine	lac$^-$ gal$^-$
S. lactis C_2F-C_2FW	Spontaneous	lac$^-$ gal$^-$
S. lactis C_2F-L13	NTG	lac$^-$ gal$^-$
S. lactis C_2F-F22	NTG	lac$^-$ gal$^-$
S. lactis C_2F-F7	NTG	lac$^-$ gal$^-$
S. lactis C_2F-G2	NTG	lac$^-$ gal$^-$

[a] The mutants designated lac$^-$ gal$^-$ were unable to ferment lactose and were defective in the fermentation of galactose.

Examination of S. lactis 7962 mutants. In an effort to learn the nature of the defective function in the lac$^-$ mutants, β-galactosidase activity and ^{14}C-TMG uptake were measured. The latter was used to measure β-galactoside permease activity in *S. lactis* 7962. This strain revealed β-galactosidase activity which was neither inhibited by NaF nor stimulated by PEP (17) in cell-free extracts and toluene-treated cells, suggesting that lactose metabolism occurred as in *Escherichia coli*. Table 2 indicates that a normal level of β-galactosidase was found in mutants C, 3, and 18, whereas mutant B consistently exhibited greater activity and mutant 9 less activity. No β-galactosidase, as measured by ONPG hydrolysis, could be detected in mutants 6, 5, and 16.

Figure 1 illustrates the uptake of ^{14}C-TMG by *S. lactis* 7962 and by the mutants 5, C, and 3; the uptake of ^{14}C-TMG by the mutants was defective in comparison with uptake by the parent strain. *S. lactis* 7962-5, however, accumulated some TMG. The uptake of ^{14}C-TMG by mutants 6 and 9 resembled that observed for strains 3 and C.

From these observations, it was concluded that the lac$^-$ mutants from *S. lactis* 7962 could be differentiated into several classes on the basis of ^{14}C-TMG uptake. The decreased uptake of analogue in several of the mutants suggested a defect in permease function, especially since the same mutants possessed full β-galactosidase activity. These results further indicated that galactoside permease and β-galactosidase were independent entities in this bacterium, as is true in *E. coli* (11).

TABLE 2. *Specific activity of β-galactosidase in toluene-treated cells of a wild-type and mutant strains of S. lactis 7962*

Organism	Specific activity
S. lactis 7962	0.164[a]
S. lactis 7962-C	0.189
S. lactis 7962-3	0.186
S. lactis 7962-B	0.244
S. lactis 7962-9	0.087
S. lactis 7962-18	0.190
S. lactis 7962-6	0
S. lactis 7962-5	0
S. lactis 7962-16	0

[a] Results are expressed as micromoles of ONP formed from ONPG per minute per milligram of cell dry weight.

Mutant 6 lacked both permease and β-galactosidase activity, which suggested that the two structural genes were closely linked; this mutant also was unable to ferment galactose. A third phenotype was observed in mutant 5. This organism lost the ability to hydrolyze ONPG, yet ^{14}C-TMG accumulated. In addition, lactose was fermented, suggesting that the mutation resulted in a loss in β-galactosidase affinity for ONPG, but not for the natural substrate lactose. Thus, these mutants are different from those observed in *S. lactis* C_2F, providing additional evidence (17) that *S. lactis* 7962 does not metabolize lactose via the PEP-phosphotransferase system.

Preliminary characterization of S. lactis C_2F mutants. Although induced whole cells of *S. lactis* C_2F hydrolyzed ONPG (2, 17), whole cells of all of the mutants were unable to hydrolyze the substrate. In addition, it can be seen from Fig. 2 that the uptake of ^{14}C-TMG by the mutants was negligible when compared with the uptake of this compound by the parent strain. These results suggested that the mutants were both permease- and β-galactosidase-negative. It seemed unusual, however, that the mutants, even though they were randomly isolated, would all have the same phenotype. This was clarified for us when Hengstenberg et al. (5) presented evidence that the apparent labile nature of β-galactosidase in *S. aureus* was due to the absence of this enzyme and the presence of a new enzyme that hydrolyzed the phosphorylated derivative of lactose. In addition, Kennedy and Scarborough (12) suggested that *S. aureus* metabolized lactose by the PEP-dependent phosphotransferase system. These findings suggested that in the lactic streptococci, which were reported to possess a labile β-galactosidase (2), metabolism of lactose may instead resemble that in *S. aureus*. This was found by McKay et al. (17) to be likely, and the data presented here, further characterizing lactose metabolism in the *S. lactis* C_2F mutants, provide confirming evidence.

Hydrolysis of ONPG-6-P by lactic streptococci. As previously observed (2), ONPG hydrolysis could not be demonstrated in toluene-treated cells or cell-free extracts of *S. lactis* C_2F. However, such preparations did hydrolyze the substrate when PEP was added to the reaction mixture (17). This result suggested that the proper substrate was ONPG-P and not ONPG, as phosphorylation of ONPG by the PEP-dependent system could occur in the presence of PEP. Table 3 shows the hydrolysis of ONPG and ONPG-6-P by toluene-treated cell suspensions of various lactic streptococci. Not only did strains of *S. lactis* hydrolyze ONPG-6-P, but strains of *S. cremoris* and *S. diacetilactis* were also active in this regard. The latter two organisms, like *S. lactis*, could not

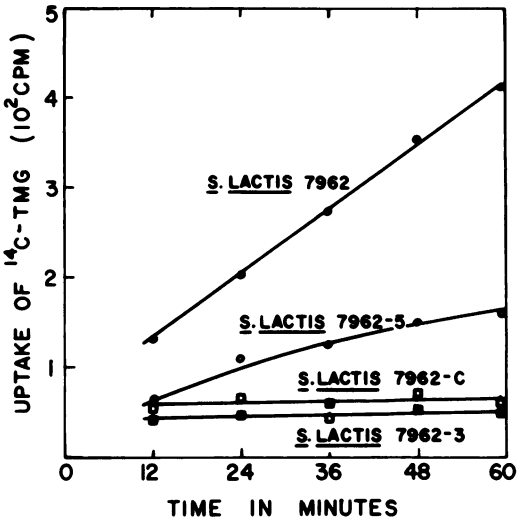

FIG. 1. *Uptake of ^{14}C-TMG by S. lactis 7962 and the lac$^-$ mutants. Incubation mixtures consisted of about 1.39 mg of cell dry weight per ml and approximately 33.3 μM ^{14}C-TMG in a total volume of 4.0 ml.*

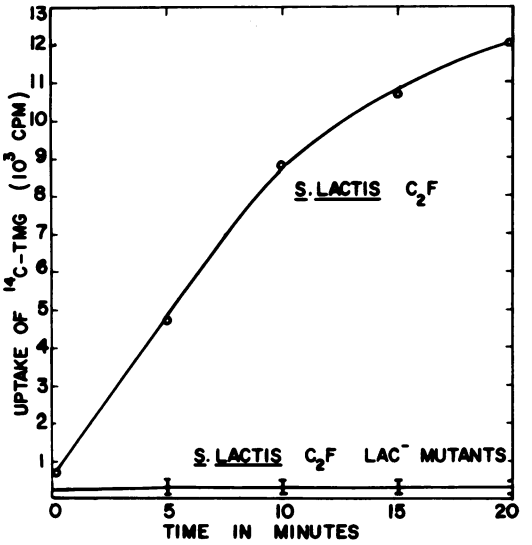

FIG. 2. *Uptake of ^{14}C-TMG by S. lactis C_2F and the lac$^-$ gal$^-$ mutants. Incubation mixtures consisted of about 0.92 mg of cell dry weight per ml and approximately 33.3 μM ^{14}C-TMG in a total volume of 4.0 ml.*

hydrolyze ONPG in cell-free extracts or in toluene-treated cell suspensions. The only exception was *S. lactis* 7962 which hydrolyzed ONPG and not ONPG-6-P; however, this strain was also the only lactic streptococcus of over 40 examined which contained β-galactosidase activity in toluene-treated cells or cell-free extracts (2). Therefore, it may be concluded that the apparent lability of β-galactosidase in *S. lactis* C_2F and the

other strains examined is due to the absence of this enzyme and the presence of 6-phospho-β-galactosidase, which hydrolyzes only the phosphorylated derivatives of lactose or ONPG. This enzyme appears to be analogous to the one found in *S. aureus* (5, 7). The β-galactosidase from *S. lactis* 7962 appears to be similar to the one found in *E. coli* (7), as it hydrolyzes ONPG and not ONPG-P.

Recently, Vakil and Shahani (21) described lactose utilization in *S. lactis* UN; they detected β-galactosidase and lactose dehydrogenase activities in this strain. The results presented here indicate that toluene-treated cells of *S. lactis* UN preferentially hydrolyze ONPG-6-P and not ONPG. In addition, it was found that galactose was better than lactose for induction of ONPG hydrolysis by whole cells and for ONPG-6-P hydrolysis by toluene-treated cells in this strain. These results suggest that *S. lactis* UN metabolizes lactose by the PEP-dependent system and not via β-galactosidase.

Requirements for the hydrolysis of ONPG in crude extracts. Simoni et al. (19) resolved the phosphotransferase system of *S. aureus* for lactose utilization into four protein components. These components were EI, HPr, EII, and FIII. Table 4 indicates the requirements for the hydrolysis of ONPG by crude extracts of *S. lactis* C_2F. As previously determined, PEP was an absolute requirement. When the soluble fraction was boiled for 10 min to remove heat-labile protein, it still retained a component necessary for the hydrolysis of ONPG. This component could be HPr. The soluble fraction also retained components necessary for ONPG hydrolysis after charcoal treatment to remove HPr. These proteins could be EI, FIII, and 6-phospho-β-galactosidase. The system was also dependent upon the membrane fraction which could contain EII.

TABLE 3. *Hydrolysis of ONPG and ONPG-P by toluene-treated cell suspensions of various lactic streptococci*

Organism	ONPG	ONPG-P
S. lactis C_2F	4[a]	59[a]
S. lactis C10	14	101
S. lactis 7963	19	80
S. lactis 11454	1	118
S. lactis 11955a	1	90
S. lactis E	1	129
S. lactis b	2	172
S. lactis UN	2	99
S. lactis 7962	73	5
S. cremoris 9596	3	246
S. cremoris M4W4-4-3	0	83
S. cremoris 144F	9	25
S. cremoris 9625	1	151
S. cremoris 3	5	232
S. diacetilactis 18-16	2	269
S. diacetilactis 26-2	13	180
S. diacetilactis DRC-3	3	254
S. diacetilactis 11007	1	71

[a] Results are expressed as nanomoles of ONP formed from ONPG or ONPG-P per minute per milligram of cell dry weight.

TABLE 4. *Requirements for the hydrolysis of ONPG by crude extracts of S. lactis C_2F*

System	Activity
Complete: PEP, soluble fraction (heat-treated), soluble fraction (charcoal-absorbed), and membrane fraction	0.823[a]
Complete minus PEP	0.077
Complete minus soluble fraction (heat-treated)	0.039
Complete minus soluble fraction (charcoal-absorbed)	0.011
Complete minus membrane fraction	0.005

[a] Results are expressed as the optical density obtained at 420 nm after a 1-hr incubation period at 37 C.

That *S. lactis* C_2F contained EI, EII, FIII, and HPr was confirmed for us by M. L. Morse (*personal communication*), who performed complementation experiments with mutants of *S. aureus* lacking each of the components. Not only did *S. lactis* C_2F contain these proteins, but they were interchangeable with the *S. aureus* components. Thus, the phosphotransferase system of *S. lactis* C_2F has been partially characterized and appears to be identical to that described for *S. aureus* (9, 19).

Biochemical analysis of S. lactis C_2F mutants. Table 5 shows the ability of the *S. lactis* C_2F mutants to restore ONPG hydrolysis in the *S. aureus* mutants. The positive controls were the wild-type (wt) cultures of *S. aureus* and *S. lactis* C_2F. No ONPG hydrolysis occurred unless the complementary strain contained the missing component of the *S. aureus* mutant. If it contained the missing protein, it completed the system and restored ONPG hydrolysis. From Table 5, it can be seen that *S. lactis* C_2F and *S. aureus* each contained the four essential components. *S. aureus* S714B, which lacks EII, restored ONPG hydrolysis in the other three *S. aureus* mutants. When mutants of *S. lactis* C_2F were examined, they were found to contain EI and HPr but not EII or FIII. In addition, the C_2F mutants lacked 6-phospho-β-galactosidase.

In *S. lactis* C_2F, galactose is a better inducer for lactose utilization than lactose (17). This is identical to findings with *S. aureus*, in which galactose was shown to be the inducer for lactose utilization; however, later experiments have shown that

TABLE 5. *Biochemical analysis of mutants from S. lactis defective in lactose utilization*

Complementation strains	Factors absent			
	S710A (EI⁻)	S714B (EII⁻)	S714G (FIII⁻)	S797A (HPr⁻)
S. aureus (wt)	+[a]	+	+	+
S. lactis C_2F (wt)	+	+	+	+
S. aureus S714B (EII⁻)	+	0	+	+
S. lactis C_2F-C_2FW	+	0	0	+
S. lactis C_2F-L13	+	0	0	+
S. lactis C_2F-38-3	+	0	0	+
S. lactis C_2F-F22	+	0	0	+
S. lactis C_2F-F7	+	0	0	+
S. lactis C_2F-G2	+	0	0	+

[a] Results indicate the ability of the complementation strain to restore the enzymatic defect in the *S. aureus* lactose-negative mutants.

galactose-6-phosphate is the true inducer (18). The mutants from C_2F could not be induced for lactose utilization by growing them in the presence of galactose or lactose, yet galactose was slowly fermented. These observations, coupled with the inability of C_2F mutants to accumulate ^{14}C-TMG and their lack of EII, FIII, and 6-phospho-β-galactosidase, suggested that the mutants were defective in the phosphorylation step. Thus, if these mutants were unable to phosphorylate lactose or galactose, and if galactose-6-phosphate is the true inducer as in *S. aureus*, then the defect in the induction of lactose metabolism in the *S. lactis* C_2F mutants is explained.

The results in Table 5 also indicate that EI and HPr are constitutive proteins in *S. lactis* C_2F as in *S. aureus* (19), because the mutants, although not induced for lactose utilization, still contained these two components.

In *S. aureus*, two types of enzymatic defects are known to give rise to a lac⁻ gal⁻ phenotype, namely, the lack of EII or FIII (8). Since all of the C_2F mutants possessed the lac⁻ gal⁻ phenotype, it is possible the genetic defect also occurs in either EII or FIII. This possibility cannot be examined until methods are developed to induce these mutants. Galactose-6-phosphate may serve as an inducer if a permeability barrier does not exist for this substrate.

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