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Biochemistry and Genetics of Galactose Metabolism in Group H *Streptococcus* Strain Challis

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Galactose-negative mutants of the group H *Streptococcus* strain Challis were obtained by treatment with nitrosoguanidine. Enzyme assays of extracts of these mutants revealed that 12 of the mutants were lacking one of the enzymes of the Leloir pathway. Thus, the Leloir pathway is the major means of galactose metabolism in strain Challis. In addition, uridyl diphosphate galactose pyrophosphorylase, a permease function, and at least one other function are required for the utilization of galactose. The enzymes of the Leloir pathway are induced by galactose and fucose; no compounds which act as repressors of these enzymes have been found, although the system appears to be sensitive to catabolite repression. Transformation was used to map the mutants. The genes for galactose-1-phosphate uridyl transferase and glucose-4-epimerase appear to be closely linked. Within the transferase gene, six mutations have been mapped. The permease function and the undetermined functions are not linked to the Leloir pathway.

In many organisms, galactose is metabolized by conversion to glucose via the Leloir pathway. This pathway involves the following three enzymes:

(1) galactokinase (EC 2.7.1.6):galactose + adenosine triphosphate (ATP) → galactose-1-phosphate + adenosine diphosphate (ADP)
(2) galactose-1-phosphate uridyl transferase (EC 2.7.7.10):galactose-1-phosphate + uridyl diphosphate (UDP)-glucose ↔UDP-galactose + glucose-1-phosphate
(3) UDP-glucose-4-epimerase (EC 5.1.3.2): UDP-galactose ↔ UDP-glucose

In *Escherichia coli* and in yeasts, the structural genes for these three enzymes are located adjacent to one another and their levels are coordinately controlled (3, 4, 8, 9, 15, 29). Evidence is accumulating that control in *E. coli* is via a repressor molecule whose gene maps away from the gal operon (28). In yeasts, it appears that galactose activates the product of one gene which in turn inactivates the repressor activity of a second gene product (9).

In addition to these three enzymes, at least three other presumed enzyme activities are required for a cell to be able to utilize galactose as a growth substrate. One of these activities is a permeability function. This may be a specific or general sugar permease or a protein involved in facilitated diffusion. Phosphoglucomutase (EC 2.7.5.1) catalyzes the conversion of glucose-1-phosphate to glucose-6-phosphate, and is required to connect the Leloir pathway to the Embden-Meyerhof pathway (14). UDP-glucose pyrophosphorylase (EC 2.7.7.9) has also been shown to be required for galactose utilization in other organisms which possess the Leloir pathway (12). This enzyme can serve to maintain adequate levels of UDP-glucose in the cell. It catalyzes the reaction: uridine triphosphate (UTP) + glucose-1-phosphate → UDP-glucose + pyrophosphate. In organisms in which genes for these last three functions have been mapped, these genes have been well separated on the genome from each other and from the *gal* operon (8, 11).

Among the streptococci, sugar metabolism has been studied in many species, but most extensively in *Streptococcus bovis* and *S. lactis* (6, 7, 22, 23, 26, 31; E. J. Buecher and T. D. Brock, Bacteriol. Proc., p. 105, 1962). Some of the enzymes involved in the utilization of different sugars and their inducibility have

been investigated. Such studies are of interest because the streptococci are fastidious organisms unable to grow without complex nutritional supplements. The metabolic pathways of such an organism, its metabolic control mechanisms, and the organization of its genome could be quite different from those found in the more commonly studied microorganisms which are capable of much greater nutritional independence.

Because genetic studies are possible in the group H *Streptococcus* strain Challis, we investigated the metabolism of galactose by this strain. We set out to determine the pathway by which galactose is utilized as an energy source, the manner in which this pathway is regulated, and whether the genetics of this pathway could be studied by transformation.

MATERIALS AND METHODS

Bacterial strain, growth conditions, and preparation of extracts. The group H *Streptococcus* strain Challis was used throughout these studies. Stocks were maintained at −20 C as stab cultures in the solidified synthetic medium.

The synthetic medium used for the growth and transformation of strain Challis was that of Lawson and Gooder (20). A filtered solution of galactose or glucose at 20% (w/v) was added to the autoclaved medium to provide a final concentration as indicated below. The galactose was "substantially glucose-free" galactose from Sigma Chemical Co., St. Louis, Mo. Solidified synthetic medium was prepared by the addition of 1.3% purified agar (Difco). Tryptone Soy Agar (TSA, Oxoid, Colab Laboratories, Inc., Chicago Heights, Ill.) with or without 250 μg of streptomycin (Chas. Pfizer & Co., New York, N.Y.) was used in the transformation experiments for the determination of the number of streptomycin-resistant transformants and the total viable count, respectively.

To obtain maximal activity of the galactose enzymes in mutants unable to utilize galactose, the cells were grown in 100 ml of the synthetic medium supplemented with 4×10^{-3} M glucose and 10^{-3} or 10^{-2} M fucose. In this "induced" condition, the concentration of glucose is growth-limiting, and growth as measured turbidimetrically stopped abruptly at a Klett reading of 60 or 70. Incubation was continued for 2 to 3 hr, during which time turbidity, as measured by a Klett colorimeter, dropped very slightly. The cells were collected by centrifugation at 12,000 × *g* for 10 min at 2 C. The cells were washed once and suspended in 10 ml of the growth medium lacking glucose and fucose. This cell suspension was transferred to a 12-ml rosette cell immersed in ice, and was sonically treated for 10 min in a Branson sonifier operated at maximal output. This treatment resulted in breakage of a majority of the cells accompanied by a 1,000-fold decrease in viable count. The sonically treated cell suspension was centrifuged at 29,000 × *g* for 15 min. Samples of the clarified supernatant fluid, essentially free from whole cells and debris, were distributed into 1-ml vials and frozen immediately in liquid nitrogen. Protein determinations were performed later on 2 ml of the supernatant fluid stored at −20 C. The Lowry method as modified by Neidhardt and Boyd was used to determine protein concentrations of the extracts (21, 25).

In the uninduced condition, cells were grown in the synthetic medium with 4×10^{-3} M glucose, but no fucose was added. In all other respects, the procedure was the same as in the induced condition. On some occasions, cells were grown in medium supplemented with 1% galactose.

In conditions of enzyme repression, cells were grown in 4×10^{-3} M glucose with 10^{-3} M fucose and 5×10^{-3} M suspected repressor. Suspected repressors were thiomethyl-β-D-thiogalactopyranoside (TMG) obtained from Mann Research Laboratories, (New York, N.Y.), and isopropyl-β-D-thiogalactopyranoside (IPTG) and phenyl-β-D-thiogalactopyranoside (PTG) obtained form Calbiochem (Los Angeles, Calif.).

Isolation and selection of mutants. Several modifications of the original description of nitrosoguanidine mutagenesis (1) were tried, and the procedure which proved to be the most successful is as follows. An overnight culture of wild-type Challis was inoculated into 10 ml of Todd-Hewitt broth (BBL) and incubated at 37 C until the middle of the logarithmic phase of growth (a Klett reading of approximately 70). The cells were centrifuged, washed once with 0.05 M tris(hydroxymethyl)aminomethane-maleate buffer, *p*H 5.6, and suspended in 5 ml of buffer. Nitrosoguanidine (Aldrich Chemical Co., Cedar Knolls, N.J.) solutions (1 mg/ml) were prepared immediately before use and diluted 1:1 by the addition of the cell suspensions. After incubation for 30 min at 37 C, the cells were centrifuged, washed once, and suspended in 2 ml of Todd-Hewitt broth. A 1:10 dilution of this cell suspension was grown overnight in Todd-Hewitt broth, and the entire procedure was then repeated with this culture. After three cycles of nitrosoguanidine treatment, the cells were grown overnight in Todd-Hewitt broth. Appropriate dilutions of the cells were plated on TSA. After 48 hr of incubation, individual colonies were picked with sterile toothpicks and streaked both to TSA and to solidified synthetic medium supplemented with either galactose or glucose. The plates were incubated for 48 to 72 hr at 37 C. Colonies were scored as mutants if they grew only on the TSA and as galactose mutants if they grew on the glucose but not on the galactose plates. This procedure evolved over the course of several different experiments with nitrosoguanidine. We found that several cycles of mutagenesis greatly facilitated the isolation of mutants. For example, in one preliminary experiment we found that after two cycles of nitrosoguanidine treatment 1% of the population was streptomycin resistant, after three cycles 14% was resistant, and after four cycles 37% was resistant. Only three cycles were routinely used because problems of contamination began to arise after further cycles.

Individual colonies were streaked onto variously

supplemented media because replica plating was unsatisfactory, possibly owing to the small size of the colonies. Attempts to select nonfermenting mutants on indicator plates, a successful technique with *S. lactis*, were also unsuccessful. Approximately 1 of 300 colonies streaked was unable to grow on the galactose plate. About one-half of these, or 0.2% of all the colonies streaked, were stable galactose-negative mutants.

Enzyme assays. Galactose-*1*-^{14}C (2.0 mCi/mmole), uniformly labeled ^{14}C-galactose-1-phosphate (5.0 mCi/mmole), nicotinamide adenine dinucleotide, UDP-galactose, UDP-glucose, ATP, and all amino acids were obtained from Calbiochem. UDP-glucose dehydrogenase, glucose-6-phosphate dehydrogenase, and phosphoglucomutase were obtained from Sigma Chemical Co

Galactokinase was assayed by the method described by Sherman and Adler (30). Transferase was assayed by Buttin's method (3), the product and substrate being separated by 14 hr of chromatography on strips of Whatman no. 1 paper in isobutyric acid-ammonium hydroxide-water (57:4:39). In both assays, chromatographic strips were cut at 2-cm intervals, placed in vials with 10 ml of scintillation fluid [4 mg of 2,5-diphenyloxazole and 0.5 mg of 1,4-bis-2-(5-phenyloxazolyl)benzene in 1 liter of toluene], and counted in a Packard Tri-Carb liquid scintillation counter.

The two-step procedure of Kalckar et al. (17) was used to determine epimerase activity. Each extract was incubated with the reaction mixture for 0, 2.5, and 5 min at 37 C. Extracts which showed no activity were also tested after incubation for 10 min.

Phosphoglucomutase was assayed by the method of Joshi and Handler (16). The disappearance of the substrate glucose-1-phosphate was observed as a decrease in acid-labile phosphate detected as described by Chen et al. (5).

Uridine diphosphoglucose pyrophosphorylase was detected by the method of Hansen et al. (14).

The ability of cells to concentrate labeled galactose was taken as a measure of their galactose permeability. Induced and uninduced cultures were grown to a Klett reading of 80 to 90, which corresponded to a viable count of approximately 5×10^8 colony-forming units (CFU) per ml. The cells were centrifuged in the cold, washed once with cold growth medium minus sugar, and resuspended in 2 ml of growth medium minus sugar (approximately 5×10^8 CFU/ml) with 5 μliters of ^{14}C-labeled galactose (diluted 1:20 with cold 24 mM galactose) and 22 μliters of 3.3 mg/ml chloramphenicol. After incubation for 5 min at 37 C, 0.5 ml of the mixture was filtered through a GS membrane filter (pore size, 0.22 μm; Millipore Corp). The filters were washed with 10 ml of cold growth medium, dried, and counted in a liquid scintillation counter. Cells were grown both with and without 10^{-3} M fucose. As a control, formaldehyde-treated cells were also included.

Extraction of DNA. To minimize breakage of the deoxyribonucleic acid (DNA), the cells were disrupted by the gentlest method available. This method involves treatment of the cells with lysozyme followed by sodium lauryl sulfate as described by S. E. Coleman, I. Van de Rign, and A. S. Bleiweis (Bacteriol. Proc., p. 48, 1970). After cell breakage, the method of Marmur (24) was followed through three deproteinizations. The DNA concentration was determined by the modified Burton method (2), described by Giles and Myers (13). The DNA preparations were adjusted to concentrations of 10 to 20 μg/ml.

Construction of strains for transformation. Before extracting DNA from the galactose-negative mutants, an outside marker had to be transformed into the mutants. For this reason, the galactose-negative mutants were transformed with DNA from a streptomycin-resistant donor. The transformed cells were plated on TSA with 250 μg of streptomycin/ml. To insure the selection of *gal*$^-$ *str*^{r} cells to be used as donors in the mapping experiments, 10 of the streptomycin-resistant transformants from each of the galactose-negative mutants were streaked onto galactose plates. All of the transformed mutants were tested in this way, and only an occasional doubly transformed colony (one which was galactose-positive as well as streptomycin-resistant) was found, indicating that the galactose mutations (those mutations leading to the galactose-negative phenotype) were not linked to the streptomycin-resistance mutation. Not all of the galactose-negative mutants were transformable. Mutants were considered nontransformable when no streptomycin-resistant colonies appeared in two separate transformation experiments. The transformation frequency usually obtained with the streptomycin marker was 1%.

RESULTS

Leloir pathway in strain Challis. The parent strain Challis was grown under several different conditions. Extracts were first prepared as described in Materials and Methods, with the following change. The cells were washed and suspended in 0.02 M potassium phosphate buffer containing 0.001 M ethylenediaminetetraacetic acid and 0.01 M mercaptoacetic acid at *p*H 7.0. This buffer had been used in previously published investigations of the Leloir pathway (3, 17), so we also used it when we began our studies. In our extracts made with this buffer, galactokinase and transferase activity could be demonstrated, but little or no epimerase activity could be detected. On the other hand, when the extracts were prepared in the growth medium, all three of the enzymes could be detected. The levels of enzyme activity in extracts prepared in the two different solutions are shown in Table 1. Since the specific activities of the kinase and transferase are not diminished in the extract prepared in the growth medium, and in fact are slightly higher, this medium was used for the preparation of all further extracts. The

high level of galactokinase obtained in extracts prepared in the growth medium is significant. In other organisms, this enzyme is unstable in the absence of mercaptoacetic acid or other similar reducing agents. In contrast to the buffer, which contains 0.01 M mercaptoacetic acid, the growth medium contains cysteine and cystine in a total concentration of 5×10^{-4} M. Thus, the streptococcal galactokinase appears to require less protection of sulfhydryl groups than the enzyme isolated from other microorganisms, and may in fact be slightly inhibited by the 20-fold greater concentration of reducing compound present in the buffer. There is a significant difference between the epimerase found in strain Challis and the epimerase found in other microorganisms. The lack of activity in the buffer may reflect an instability of this enzyme which is somehow protected in the growth medium. Some of the other components of the medium may help to stabilize this enzyme further, thereby leading to higher specific activity in the growth medium than in the buffer.

The parent strain Challis was grown under several different conditions, and extracts were prepared as described in Materials and Methods. The enzymes of the Leloir pathway, phosphoglucomutase and pyrophosphorylase, were assayed (Table 2). Extracts prepared from cells grown in galactose exhibited approximately 30 times more kinase and transferase activity than extracts prepared from cells grown in glucose. Increases in mutase and epimerase activities were twofold or less. Pyrophosphorylase activity could not be detected in glucose-grown cells, whereas substantial activity could be detected in the galactose-grown cells. In the extracts of cells grown in 4×10^{-3} M glucose with fucose, the activities of all of the enzymes were intermediate between the other two conditions. Kinase and transferase activities were 2- to 10-fold greater than in cells grown in glucose alone.

TABLE 1. *Specific activities of enzymes of galactose metabolism in extracts prepared in buffer or in synthetic medium (SM)*[a]

Prepn	Kinase	Transferase	Epimerase
Buffer	2.7	10	0.3
SM	3.6	16	3.7

[a] Specific activities are expressed as micromoles of substrate converted per hour per milligram of protein.

The effect of three compounds which repress the synthesis of the Leloir enzymes in *E. coli* was also examined in strain Challis. In Table 3 are shown the enzyme levels from cells grown in 4×10^{-3} M glucose, 10^{-3} or 10^{-2} M fucose, and TMG, IPTG, or PTG. The enzyme levels were not significantly affected by any of these compounds, even when TMG was present at 10 times the level of fucose.

The studies on the enzyme activities in the parent strain Challis indicated that galactose could be metabolized via the Leloir pathway in this *Streptococcus*. To determine whether this pathway is the major means by which galactose is utilized in Challis, mutants unable to grow on galactose were examined.

Characterization of galactose-negative mutants. Challis can utilize only a limited number of sugars as sources of energy. Therefore, of necessity, extracts of all of the galactose-negative mutants were prepared from cells grown on a growth-limiting concentration of glucose plus 10^{-3} M fucose. Cells were harvested 2 to 3 hr after the turbidity of the culture, as measured by a Klett colorimeter, leveled off. Mutants were first assayed for only the three enzymes of the Leloir pathway; however, a large proportion of the mutants contained these enzymes, so the other activities required for growth on galactose were assayed. The results of these assays have allowed us to present the classification scheme for the galactose-negative mutants which appears in Table 4. Once the enzyme block of most of the galac-

TABLE 2. *Specific activity of the enzymes involved in the utilization of galactose in wild-type extracts*[a]

Growth conditions	Kinase	Transferase	Epimerase	Mutase	Pyrophosphorylase
Glucose, 5×10^{-2} M	0.8	1.5	1.7	120	0
	0.6	7.4			
Galactose, 5×10^{-2} M	10.0	46.0	2.8	255	0.97
	13.8	31.6	1.5	220	0.40
Glucose, 4×10^{-3} M, + fucose, 10^{-3} M	1.0	8.7	1.7	172	0.25
	1.1	16.0	2.1	139	0.55

[a] Specific activities are expressed as micromoles of substrate transformed or product produced per hour per milligram of protein.

tose-negative mutants was identified, the designation for the mutants which appears in Table 4 was adopted. Those mutants in which specific enzyme deficits have yet to be established are called *gal-n*, where *n* is a different number for each mutant. Transferase mutants are called *gal-tn;* epimerase mutants, *gal-en;* permease mutants, *gal-pn;* and pyrophosphorylase mutants, *gal-un*. Each mutant was given the next available number as its defect was identified. There are eight transferase, four epimerase, three pyrophosphorylase, and four permease mutants. No mutants have been found lacking kinase or phosphoglucomutase activity. Conversely, there are seven mutants which are unable to grow on galactose but which nevertheless show activity for all six of the enzymes assayed.

Genetics of galactose-negative mutants. One of the purposes of this study was to compare the genetic organization of the genes affecting galactose metabolism in streptococci with the organization of these genes in the well studied microorganisms, especially *E. coli* and yeasts. The lack of galactokinase mutants makes this comparison incomplete. However, the transferase and epimerase mutants were investigated, and the positions of the mutations in the other mutants were tested for linkage to these genes and to each other. In *E. coli*, the transferase and epimerase genes are adjacent and lie within the galactose operon. Therefore, we had some reason to hope that linkage between these genes, if it existed in the streptococcus, could be demonstrated. Since transformation is the only means of genetic transfer available in group H streptococci, linkage can only be demonstrated between genes lying close together on the genome.

TABLE 3. *Effect of repressors of galactose enzymes in E. coli on galactose enzyme levels[a] in strain Challis*

Expt	Fucose (M)	Compound added[b]	Kinase	Transferase	Epimerase
1	10^{-3}	—	0.34	8.7	1.7
	10^{-2}	0.5×10^{-2} M TMG	0.34	8.7	1.9
	10^{-3}	0.5×10^{-2} M TMG	0.44	5.2	2.2
2	10^{-3}	—	0.64	18.2	18.0
	10^{-3}	10^{-3} M IPTG	0.82	21.7	23.7
	10^{-3}	10^{-3} M PTG	0.67	15.8	20.7

[a] Specific activities are expressed as micromoles of substrate transformed or product produced per hour per milligram protein.

[b] TMG = thiomethyl-β-D-thiogalactopyranoside; IPTG = isopropyl-β-D-thiogalactopyranoside; PTG = phenyl-β-D-thiogalactopyranoside.

The DNA used in the crosses was isolated from a streptomycin-resistant galactose-positive strain and from all of the transformable galactose-negative, streptomycin-resistant mutants which had been constructed as described in Materials and Methods. Three of the transferase mutants, *gal-t1*, *gal-t7*, and *gal-t4*, were transformable and were used as donors and recipients in the crosses. Three other transferase mutants, *gal-t5*, *gal-t3*, and *gal-t2*, could not be used as recipients because of their very low levels of competence for transformation; however, occasional streptomycin-resistant transformants could be obtained and used as sources of DNA. Only one of the epimerase mutants, *gal-e2*, was transformable. DNA from one permease mutant, *gal-p1*, and from *gal-1* and *gal-4*, all of which had been transformed to streptomycin resistance, were also used. Each genetically competent streptomycin-sensitive transferase and epimerase mutant was transformed with all of the above DNA preparations.

Mapping was carried out by computing the recombination index, RI (18), between mutants according to the following scheme:

Cross 1: gal^+, str^r DNA × gal_1^-, str^s cells

Cross 2: gal_2^-, str^r DNA × gal_1^-, str^s cells

TABLE 4. *Classification of galactose-negative mutants*

Class	Mutants	Kinase	Transferase	Epimerase	Permease	Pyrophosphorylase	Mutase
I	*gal-t1, -t2, -t3, -t4, -t5, -t6, -t7, -t8,*	+	−	+	+	+	+
II	*gal-e1, -e2, -e3, -e4*	+	+	−	+	+	+
III	*gal-p1, -p2, -p3, -p4*	+	+	+	−	+	+
IV	*gal-u1, -u2, -u3*	+	+	+	+	−	+
V	*gal-1, gal-2, gal-3, gal-4, gal-5, gal-6, gal-7*	+	+	+	+	+	+

RI = (no. of str^r transformants in cross 1/no. of gal^+ transformants in cross 1) × (no. of gal^+ transformants in cross 2/no. of str^r transformants in cross 2)

The designations gal_1^- and gal_2^- refer to any two different galactose-negative mutants; str^r refers to streptomycin resistance, the reference marker. The results of the transformation obtained with the $str^r gal^+$ DNA were compared one at a time with those obtained with each of the nine $str^r gal^-$ DNA preparations with the use of all of the $str^s gal^-$ mutants as recipients. If the gal^- loci of the donor and recipients are far apart, the number of gal^+ transformants from the gal^- donor will approach the number of gal^+ transformants from the gal^+ donor. The closer together these two loci are, the fewer will be the gal^+ transformants produced by the cross. The number of transformants to the reference marker, streptomycin resistance, is included in the computation to compensate for different transforming efficiencies of various DNA preparations, differences in the transformability of different strains, and the day-to-day variability of transformation.

The recombination indexes obtained between the various transferase and epimerase mutants are presented in Table 5. Reciprocal crosses yield very similar or identical values. The low recombination indexes indicate that the mutation sites leading to lack of transferase activity are all closely linked to each other and may represent alleles in a single gene. These recombination indexes reflect distances between mutation sites, and therefore they can be used to construct a genetic map. Such a map of the transferase region of the streptococcal genome appears in Fig. 1. Overall, the additive properties of the map are good although not precise.

The results obtained with the transformable epimerase mutant *gal-e2* were unexpected. Its recombination index with the various transferase mutants indicates that this epimerase mutation lies within the transferase region. The two most probable explanations for this phenomenon are either that one long polypeptide with multiple functions is produced, or that there are two transferase genes with an epimerase gene between them.

In Table 6, the recombination indexes computed from the transformation of the transferase mutant *gal-t1* and the epimerase mutant *gal-e2* by DNA from the permease mutant, *gal-p1*, and mutants *gal-1* and *gal-4* are presented. These recombination indices are all greater than one, indicating that the mutations in the permease and unidentified function(s) are not linked to the transferase-epimerase region of the genome.

DISCUSSION

Our investigations have shown that the Leloir pathway is operative in strain Challis, and is the major—possibly the only—means of galactose utilization in this strain. This we know because mutants which lack either galactose-1-phosphate uridyl transferase or glucose-4-epimerase, both enzymes of the Leloir pathway, are unable to grow on galactose. Besides these

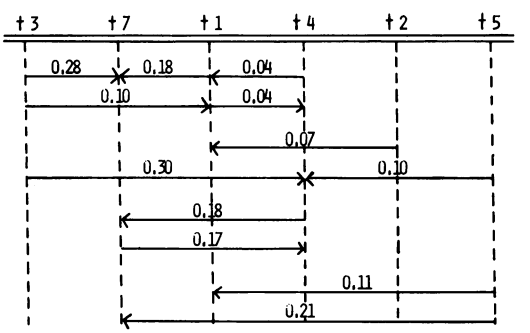

FIG. 1. *Map of transferase region. Arrows point to transformation recipient, and numbers above arrows refer to the recombination index obtained between the mutants.*

TABLE 5. *Recombination between the transferase mutants*[a]

DNA donor	Transformation recipients			
	gal-t1	*gal-t7*	*gal-t4*	*gal-e2*
gal-t1	0	0.18	0.04	0.08
gal-t7	NT	0	0.17	0.26
gal-t5	0.11	0.21	0.10	0.22
gal-t2	0.07	NT	NT	0.09
gal-t3	0.10	0.28	0.30	NT
gal-t4	0.04	0.18	0	0.04

[a] Results are given as the recombination index. NT = not tested.

TABLE 6. *Recombination between Leloir pathway mutants and other galactose-negative mutants*[a]

DNA donor	Transformation recipients	
	gal-t1	*gal-e2*
gal-e2	0.03	0
gal-4	1.3	1.4
gal-1	1.4	1.3
gal-p1	1.5	1.3

[a] Results are given as the recombination index.

enzymes, a function which serves to concentrate galactose intracellularly is also essential for growth on galactose. This function has been referred to as a permease, but no specific enzyme reaction is intended by this name. No attempt was made to quantitate the assay for the permease. The permease function measured in the assay is specifically induced by fucose. Little activity was associated with cells grown in glucose alone. Thus, this is an inducible function, and may be an enzyme comparable to the MG or gal permeases of *E. coli* (27), both of which are induced by fucose and transport galactose.

The three enzymes of the Leloir pathway, kinase, transferase and epimerase, are induced by galactose and less well by fucose. It is not known whether or not fucose is metabolized by strain Challis. Further studies are also needed on the inhibitors or repressors of the Leloir pathway enzymes as they exist in strain Challis. One possible explanation for the inactivity of TMG and the other compounds tested which repress the synthesis of the Leloir enzymes in *E. coli* (3) is that Challis is impermeable to these compounds. Radioactively labeled TMG could be used to test this possibility.

Studies on the regulation of the Leloir pathway have been hampered by the variability of the enzyme levels in different extracts of strain Challis. In the "induced" cultures (4×10^{-3} M glucose plus 10^{-3} M fucose), 2- to 10-fold differences in enzyme levels are observed, with the greatest fluctuation occurring in the epimerase levels. A definite explanation for this variation cannot be given, but the activity of a given extract does not diminish appreciably during routine storage in liquid nitrogen. Although the reasons for the degree of variability observed in the enzyme levels must be uncovered so that further work on the regulation of these enzymes can be undertaken, the variability does not affect the conclusions drawn from the present study. The main purpose of performing the enzyme assays was to identify the enzyme activities missing in the various galactose-negative mutants. The enzyme activities were above the lower limits of detection of the assays.

We have reported the existence of a number of mutants possessing all six of the activities surveyed. We do not know why these mutants are unable to grow on galactose, though many possibilities could be suggested. It is possible that, under the conditions of selection, some mutants were not able to grow into visible colonies on galactose even though they could slowly metabolize it. Alternatively, in vivo conditions may be considerably different from the enzyme assay conditions, and these mutants could have an altered enzyme which still possesses some activity in the assay system but not in the cell. It is likely that, as the conditions of mutagenesis which we employed were severe, many mutants possessed multiple genetic lesions. All of the mutants could grow when glucose was substituted for galactose, so the expression of any additional mutations only occurred when galactose was the growth substrate. Under these conditions, the combinations of deficiencies could result in a galactose-negative phenotype; for example, changed enzyme kinetics in another interconnected pathway could siphon off a substrate for one of the Leloir pathway reactions. The fate of labeled galactose added to growing cultures could be followed to determine whether galactose is being utilized through any pathways in addition to the Leloir pathway.

The genetic studies reveal that all of the mutations leading to loss of transferase activity (for convenience referred to as transferase mutations) are closely linked to each other, strongly suggesting that the region encompassed by the transferase mutations is the transferase gene. A recombination index of 0.21 was obtained between the two mutations farthest apart. The recombination index between two mutants on opposite ends of a gene is approximately 0.15 in *Bacillus subtilis* (10) and 0.25 in *Diplococcus* (*Streptococcus*) *pneumoniae* (19). The determination of this index can be affected by the cells' characteristics of DNA uptake and recombination frequency, as well as the length of the gene in nucleotides.

The lack of linkage between the transferase-epimerase region and the other *gal*$^-$ mutations also corresponds to the situation in *E. coli* (11) and *Saccharomyces cerevisiae* (8).

Our mapping of the galactose-negative mutants is rudimentary, but it represents the first attempt at mapping in strain Challis, and the first use of transformation for mapping in the streptococci. Most critical to the extension of this work is the isolation of a great many more galactose-negative mutants. These mutants are needed to extend and to delineate more precisely the transferase and epimerase regions of the genome. It is hoped that further study of this pathway will also lead to an understanding of the type of control mechanisms employed by the streptococcus, a fastidious pathogenic bacterium, in comparison with the primarily nonpathogenic organisms previously employed for such studies.

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