JOURNAL OF BACTERIOLOGY, June 1983, p. 1245–1251
0021-9193/83/061245-07$02.00/0

Vol. 154, No. 3

Characterization of Lactose Transport in *Kluyveromyces lactis*

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Received 8 November 1982/Accepted 21 March 1983

We have determined that lactose uptake in *Kluyveromyces lactis* is mediated by an inducible transport system. Induction, elicited by lactose or galactose, of the transporter required protein synthesis. Transport of lactose required an energy-generating system and occurred by an active process, since an intracellular lactose concentration 175 times greater than the extracellular concentration could be obtained. The K_m for lactose transport was about 2.8 mM in uninduced and lactose- or galactose-induced cells. The lactose transporters in *K. lactis* and *Escherichia coli* appear to be different since they respond uniquely to inhibition by substrate analogs.

Kluyveromyces lactis can utilize lactose as its sole carbon and energy source (10). Lactose utilization requires induction of both a β-galactosidase activity (EC 32.1.2.3) and a lactose transport system (3). Using genetic and biochemical techniques, we are examining the mechanism for regulating induction of these activities. Thus far, seven genes, defined by Lac^- mutations, have been identified and partially characterized (8). One of these genes, *LAC4*, codes for β-galactosidase (9); the function of the other six genes is unknown but some appear to regulate β-galactosidase activity (8; M. I. Riley and R. C. Dickson, unpublished data). Another gene, *LAC10*, identified by mutations that increase the basal level of β-galactosidase activity, *lac10*^{c}, has been identified and partially characterized (4). Although the function of regulatory genes identified to date is unknown, we do know that induction of β-galactosidase activity is regulated at the transcriptional level (7) and that mutations in *lac10*^{c} cause increased levels of β-galactosidase mRNA and enzyme activity. We have hypothesized that the product of *lac10*^{c} regulates transcription of the β-galactosidase gene in a negative manner (4). None of the genes identified to date are closely linked to *LAC4*, indicating that we are not dealing with promoter or operator mutations in *LAC4*.

To further elucidate the mechanism of β-galactosidase induction in *K. lactis*, we have examined lactose uptake. Our aims were to verify that the transport system is inducible, to determine whether induction requires protein synthesis, to examine the transport mechanism, and to determine whether any of our previously identified genes code for components of the transport system.

MATERIALS AND METHODS

Strains, media, and culture conditions. The wild-type strain Y1140, strain MS425 (*lac4-8 ade1-1*), and other strains used in this study have been described previously (8).

Minimal medium was yeast nitrogen base (Difco Laboratories, Detroit, Mich.) prepared at twice the manufacturer's recommended concentration. This medium was supplemented with 40 μg each of adenine and uracil per ml, and 20 mM sorbitol was the standard carbon source. During transport measurements this medium was buffered at pH 4.7 with sodium barbiturate-acetate (7 mM barbituric acid, 7 mM sodium acetate, titrated with HCl to pH 4.7). All cultures were grown at 30°C in a Gyrotory water bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.).

Measurement of transport. Cells in log phase, referred to as standard log-phase cells, were used in all transport measurements. They were prepared by growing a fresh stationary-phase culture from an absorbance at 600 nm (A_{600}) of 1 to an A_{600} of 2 units in minimal medium at 30°C. Transport activity was induced during this period by inclusion of 20 mM lactose or 40 mM galactose. After this period, cells were harvested by centrifuging for 3 min at 3,000 × *g* followed by washing four times with an equal volume of medium lacking the inducer (23°C) or by filtering on a filter (47 mm, type HA; 0.45-μm pore size; Millipore Corp., Bedford, Mass.) and washing six times with 10 ml of medium. Cells were resuspended in buffered medium lacking inducer and incubated for about 5 min at 30°C. An experiment was initiated by adding cells to a 25-ml DeLong flask containing an appropriate amount of [^{14}C]lactose (0.5 to 1.0 μCi per ml of culture), D-[^{14}C]galactose (4 μCi/ml), or D-[^{3}H]galactose (10 μCi/ml). Radioisotopes were diluted to contain 1 to 10 μCi/μl. The concentration of lactose or galactose used in each experiment is given. At the times indicated, the A_{600} of the culture was determined and 0.5-ml duplicate samples were filtered onto 25-mm-diameter Nuclepore filters (0.4-μm pore size; Nuclepore Corp., Pleasanton, Calif.). Filters were washed three times with 5 ml of ice-cold medium

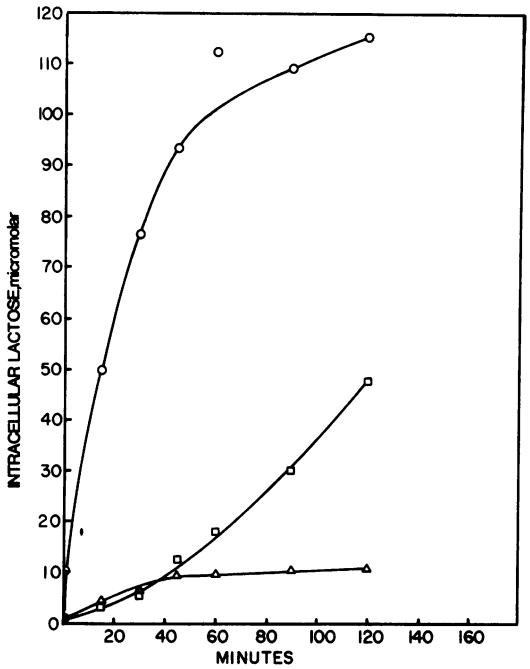

FIG. 1. Kinetics of lactose transport. Standard lactose-induced or uninduced log-phase wild-type cells were prepared after overnight growth in the presence or absence of 20 mM lactose, respectively. After washing, 1 mM [^{14}C]lactose was added to each culture and transport measurements were made at the times indicated. Cultures were: lactose-induced, ○; uninduced, □; uninduced plus 5 μg of trichodermin per ml, △.

containing nonradioactive inducer, dried, and counted in a liquid scintillation counter. More than 300 net cpm were obtained per filter for most experiments. Background radioactivity, 25 to 30 cpm, was determined by filtering 0.5 ml of medium lacking cells through a filter in the same manner as was done for transport measurements. Data were corrected to micromoles of lactose transported (intracellular lactose) during the specified uptake period. A correction factor of 4.67×10^{-7} liters of cells per A_{600} unit was used to convert A_{600} units to cell volume. This factor was obtained by measuring the volume of packed *K. lactis* cells per A_{600} unit, using a microhematocrit. Actual intracellular water volume was not determined. One A_{600} unit equaled 3.7×10^7 cells under the conditions used for these experiments.

Miscellaneous. D-Glucose-[^{14}C]lactose (CFA 278; 50 mCi/mmol) was from Amersham Corp., Lexington, Mass. D-[1-^{14}C]galactose (CMM-264; 45 mCi/mmol) was from Research Products International, Mount Prospect, Ill. and D-[^{3}H]galactose (NET-311; 1.64 Ci/mmol) was from New England Nuclear, Boston, Mass. All sugars listed in Table 3 were from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Induction of the lactose transport system. We had noted previously that lactose is transported at a higher rate in induced cells than in uninduced cells (3). The data in Fig. 1 verify this result since induced cells began to transport lactose immediately upon addition of [^{14}C]lactose, whereas uninduced cells did not. Instead, there was lag of 15 to 30 min before lactose transport began. In other experiments, using higher-specific-activity [^{14}C]lactose and more time points, a lag of 10 to 15 min was obtained (data not shown). This lag is similar to the 10- to 15-min lag noted previously for induction of β-galactosidase activity (3). The concentration of lactose, 1 mM, used for the experiment shown in Fig. 1 elicits the maximum rate of β-galactosidase induction (3), and we have assumed that the same is true for induction of the transporter since the two inducible activities are regulated by the same system (4; Riley and Dickson, unpublished data).

Since galactose is a good inducer of β-galactosidase activity (3), it should also induce lactose transport activity. In the presence of 20 mM galactose we found that lactose transport activity was induced in a manner similar to that shown in Fig. 1 (data not shown).

Next we determined whether induction of the lactose transport system requires protein synthesis. The data (Fig. 1) indicate that an inhibitor of protein synthesis, trichodermin, inhibits induction of lactose transport. The concentration of trichodermin used here blocks incorporation of amino acids into proteins by >95% (7).

In a control experiment the effect of trichodermin on lactose transport was examined to deter-

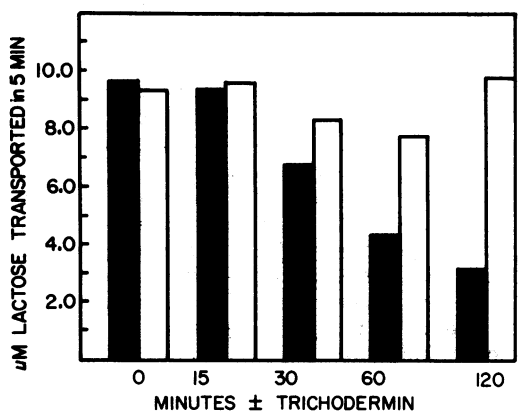

FIG. 2. Effect of trichodermin on lactose transport activity. Standard lactose-induced log-phase wild-type cells were prepared after overnight growth in the presence of 20 mM lactose. After washing, 1 mM lactose was added to the cells and they were divided into two parts. At time zero, one part received trichodermin, 5 μg/ml (closed bars), and the other served as a control (open bars). At the indicated times 1.5 ml of culture was removed into a flask containing [^{14}C]lactose, and after 5 min lactose transport was measured.

mine whether trichodermin blocks the appearance (synthesis) of transport activity or whether it inhibits activity of the transport system per se. As the data indicate (Fig. 2), transport activity per se was not immediately affected by trichodermin; only after 15 min of drug treatment did transport activity begin to decrease. The final drop in transport activity was not due solely to dilution by cell growth in the absence of synthesis of new transport activity. If this were the case, transport activity should have decreased from 9.7 to 7.2 instead of to 3.1. This unexpectedly large drop in transport activity could reflect turnover, but this possibility has not been examined. We conclude from these experiments that induction of the lactose transport system requires protein synthesis.

Effects of pH and energy inhibitors on lactose transport. The pH optimum for lactose transport was broad, with a peak at about 4.7 (Fig. 3). In all subsequent experiments, culture medium was buffered at pH 4.7 to promote optimal lactose transport.

The data in Table 1 demonstrate that lactose transport requires energy. Since 2,4-dinitrophenol, a proton ionophore, prevented transport, it would appear that transport is coupled to a proton motive force. The energy dependence of lactose transport was present in uninduced cells, as well as in either lactose- or galactose-induced cells, indicating that transport in uninduced cells is not occurring by passive diffusion.

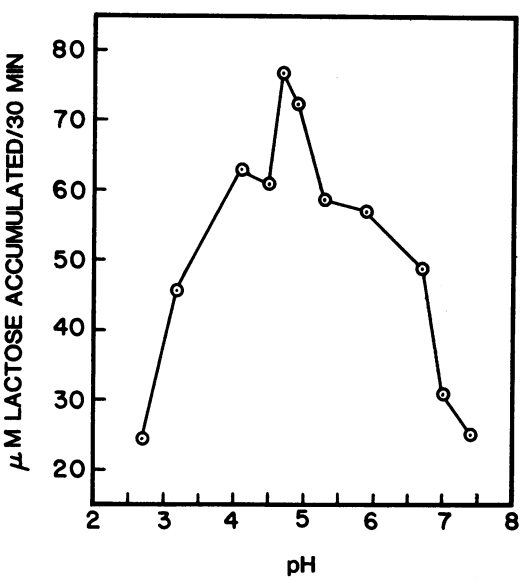

FIG. 3. Rate of lactose transport as a function of pH. Standard log-phase wild-type cells were induced for one generation with 20 mM lactose. After washing, cells were divided into samples and resuspended in medium buffered with barbiturate-acetate at the pH values indicated. [^{14}C]lactose (1 μCi/ml) was added to a final concentration of 1 mM, and intracellular accumulation was measured after 30 min as described in the text.

TABLE 1. Energy requirement for lactose transport in *K. lactis*

Inhibitor	% of transport inhibition[a]
None	0
1 mM dinitrophenol	95
5 mM sodium azide	91
5 mM potassium cyanide	78
5 mM sodium arsenate	49
50 mM sodium arsenate	66
50 mM sodium fluoride	10

[a] Standard lactose-induced log-phase cells, prepared from an overnight culture grown on 20 mM lactose, were used in these experiments. Before transport measurements, cells were washed and resuspended in fresh medium without lactose. The experiment was started by transferring cells to a flask containing the inhibitor and 1 mM [^{14}C]lactose. Thirty minutes later lactose transport was measured. The rate of transport was linear during this period in all experiments.

We also examined the effect of an energy inhibitor on lactose efflux from cells preloaded with lactose. A strain of *K. lactis* (MS425) defective in the β-galactosidase structural gene was used in these experiments so that intracellular lactose would not be metabolized (see below).

Cells were induced with galactose, allowed to accumulate [^{14}C]lactose for 40 min, washed free of lactose, resuspended in fresh medium, and incubated in the presence or absence of azide. As the data indicate (Fig. 4), there is an immediate efflux of lactose in the presence of azide. In the absence of azide there is little or no efflux even after 120 min of incubation. The efflux of lactose seems to occur in two steps. The first step is rapid (perhaps exponential) because the "zero" time point for the culture with azide already indicates that 10 to 15% of the lactose has escaped from cells. It took about 1 min to obtain the zero time sample. The second step involves a gradual linear efflux and begins at about 10 min. Thus, these data demonstrate that intracellular lactose is retained by an energy-dependent process. Since the extracellular concentration of lactose was near zero, these data imply that intracellular lactose was maintained against a concentration gradient. Further evidence for transport against a concentration gradient is presented below.

Accumulation of lactose against a concentration gradient. The data presented thus far suggest that lactose is transported by an active transport system. Active transport can be distinguished from facilitated or passive diffusion by demon-

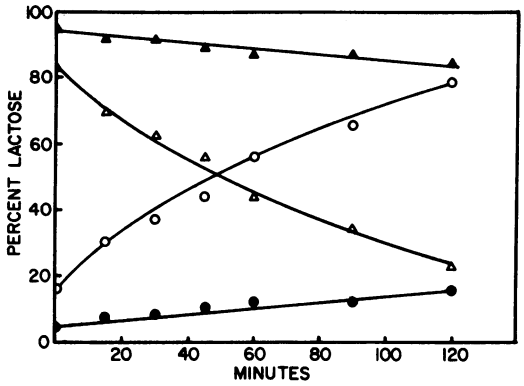

FIG. 4. Effect of energy inhibitor on efflux of lactose. Standard galactose-induced log-phase cells of strain MS425 were prepared after overnight growth in the presence of 20 mM galactose. Cells were preloaded with [^{14}C]lactose by washing out the galactose, resuspending in fresh medium containing 1 mM [^{14}C]lactose (1 μCi per ml of culture), and growing for 40 min. To examine lactose efflux, cells were resuspended in fresh medium without lactose and divided into two parts. At time zero one part received 5 mM sodium azide and the other did not. At each time point, duplicate 0.5-ml samples were centrifuged for 15 s in a Beckman Microfuge, and the radioactivity in the cell pellet (solubilized with Nuclear-Chicago solubilizer) and supernatant fluid was determined in a scintillation counter. Data for each time point are expressed as the percentage of counts in the supernatant fluid or pellet divided by the total counts (supernatant plus pellet). Data points are: (△) cell pellet, azide treated; (○) supernatant fluid, azide treated; (▲) cell pellet, no azide; (●) supernatant fluid, no azide.

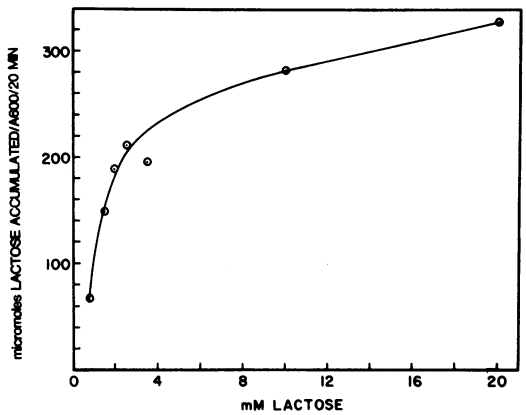

FIG. 5. Rate of lactose transport as a function of substrate concentration. Standard log-phase wild-type cells were induced for one generation in the presence of 20 mM lactose. Lactose was then removed by filtration. Cells were washed and resuspended in various concentrations of lactose (containing 1 μCi of [^{14}C]lactose per ml), and intracellular accumulation was determined after 20 min as described in the text.

strating substrate transport against a concentration gradient. To demonstrate transport of lactose against a concentration gradient, we used *K. lactis* strain MS425. This strain carries a mutation in the structural gene for β-galactosidase (9) and cannot grow on lactose. This strain should thus accumulate but not metabolize lactose. We verified this prediction as follows. Cells were allowed to transport [^{14}C]lactose for 50 min, using the procedure described in the legend to Fig. 4. Cells were washed by centrifugation to remove extracellular lactose, and the cell pellet was extracted with 70% ethanol (11). One hundred percent of the radioactivity in the cell pellet was extractable by this procedure. When a sample of the extract was chromatographed on Whatman 3 MM paper (5), >99% of the radioactivity chromatographed as lactose (data not shown), indicating that no detectable metabolism of lactose had occurred. At the end of the 50-min transport period the intracellular concentration of lactose was 175 mM or 175 times greater than the extracellular concentration. We conclude from these experiments that lactose is transported by an active transport process. The data also substantiate our previous contention that lactose is not transported by a phosphotransferase system since lactose is not phosphorylated during transport (2).

Lactose transport is mediated by a carrier. Results presented above suggest that lactose is transported into the cell by a carrier-mediated process. This possibility was verified by showing that the transport process can be saturated at high substrate concentrations (Fig. 5). The apparent K_m value for lactose transport (Table 2) was the same in uninduced and in lactose- or galactose-induced cells, indicating that the same transport system was present under these three growth conditions. The K_m for lactose transport was the same in wild-type cells as in the mutant (MS425) lacking β-galactosidase (Table 2). However, only galactose and not lactose was able to induce the transport system in this strain (data not shown), suggesting that lactose is not an

TABLE 2. Kinetic parameters of lactose transport[a]

Growth condition	Strain	K_m (mM)	
No inducer	Y1140 (wild type)	3.25 ± 1.3	(6)
No inducer	MS425 (*lac4-8*)	2.8	(1)
20 mM lactose	Y1140	2.77 ± 0.31	(6)
40 mM galactose	Y1140	2.28 ± 0.54	(2)
40 mM galactose	MS425	3.2	(1)

[a] The K_m values for lactose transport were determined by treating data obtained from the procedures described in the legend to Fig. 5 by the method of Cleland (1). The numbers in parentheses are the number of experimental replicas used to obtain standard deviations of the mean.

TABLE 3. Inhibitors of lactose transport

Compound	% Inhibition[a]		
	Experimental		Calculated
	Lactose induced	Galactose induced	
No addition	0	0	0
11 mM 4-*O*-β-D-galactosyl-D-glucose (lactose)	83.5	87.2	80
115 mM 4-*O*-β-D-galactosyl-D-glucose	97.5	48.1	97.5
11 mM D-galactose	17.5	21	80
115 mM D-galactose	61.8	62	97.5
11 mM 1-*O*-methyl-β-D-galactoside	12.4		80
115 mM 1-*O*-methyl-β-D-galactoside	64.6		97.5
11 mM 1-*O*-phenyl-β-D-galactoside	85.7		80
115 mM 1-*O*-phenyl-β-D-galactoside	93.7	93.4	97.5
11 mM isopropyl-1-thio-β-D-galactoside	13.1		80
115 mM isopropyl-1-thio-β-D-galactoside	26.6		97.5
7.5 mM D-galactosyl-1-thio-β-D-galactose	0		50
7.5 mM Stachyose[b]	0		50
7.5 mM 4-nitrophenyl-α-D-galactoside	7	0	50
7.5 mM 4-*O*-β-D-galactosyl-D-fructofuranose (lactulose)	65	30	50
7.5 mM 3-*O*-β-D-galactosyl-D-arabinose	56	25	50
7.5 mM 6-*O*-α-D-galactosyl-D-glucose (mellibiose)	10	14	50

[a] Inhibition of lactose transport was measured with standard log-phase cells prepared after overnight growth in the presence of 20 mM lactose (or 40 mM galactose for galactose-induced cells). Each culture contained the indicated compound and 0.55 mM [^{14}C]lactose. Lactose transport was determined after 36 min. Lactose transport was not actually inhibited by 11 or 115 mM lactose; however, intracellular accumulation of [^{14}C]lactose was reduced due to dilution of the specific radioactivity of the isotope. The percent inhibition in all cultures was calculated by using a lactose concentration of 0.55 mM. The calculated value is the level of inhibition expected for a compound having the same K_m value for transport as lactose.

[b] Stachyose: α-D-galactosyl-α-D-galactosyl-α-D-glucosyl-β-D-fructose.

inducer, but must be converted to the real inducer by a process requiring β-galactosidase activity.

Inhibitors of lactose transport. Characterization of the lactose transport system could be facilitated if nonmetabolized competitive inhibitors of lactose transport were available. In an initial screening for such inhibitors we chose inhibitor concentrations that would give 50, 80, or 97.5% inhibition if the inhibitor had the same apparent K_m value as lactose for transport (a K_m value of 2.4 mM was used in this calculation). As the data in Table 3 indicate, several inhibitors, including 1-*O*-phenyl-β-D-galactoside, 4-*O*-β-D-galactosyl-D-fructofuranoside, and 3-*O*-β-D-galactosyl-D-arabinoside, inhibited lactose transport to the value expected whereas the other compounds showed little or no inhibition. All inhibitors contained β-*O* linkages. A thio linkage would not substitute for the *O* linkage. Unfortunately, all compounds shown in Table 3 that have β-*O* linkages can be utilized by *K. lactis* as carbon sources (data not shown), and they are thus metabolized and not the type of compound we sought.

To determine whether lactose and galactose, which are strong inducers of β-galactosidase and the galactose catabolic enzymes, induce the same lactose transport system, we measured inhibition of lactose transport in galactose-induced cells. For all of the inhibitors examined, lactose transport was inhibited to the same extent in galactose-induced or lactose-induced cells except for lactulose and galactosyl-D-arabinose (Table 3). In galactose-induced cells these two compounds inhibited lactose transport by about half as much as in lactose-induced cells. This difference, although small, suggests that lactose may be transported by more than one transporter in galactose-induced cells.

Transport of lactose in Lac$^-$ strains of *K. lactis*. To determine whether any of the genes required for lactose utilization codes for a component of the transport system, we measured lactose transport in Lac$^-$ strains. The data (Table 4) indicate that all strains showed an increased rate of lactose transport after induction with either lactose or galactose or with both compounds except those defective in *lac9*, suggesting that *lac9* might be defective in lactose transport. However, strains defective in *lac9* also show very low, uninducible levels of β-galactosidase (8) and low, uninducible levels of other lactose-inducible enzyme including galactokinase and α-D-galactose-1-phosphate uridyl transferase (Riley and Dickson, unpublished data), suggesting

TABLE 4. Lactose transport in Lac⁻ mutants of *K. lactis*

Mutant (strain)	Phenotype	μmol of lactose accumulated/A_{600} per 20 min[a]		
		Uninduced	Induced with lactose	Induced with galactose
Wild type (Y1140)	Lac⁺ Gal⁺	1.7	16	10
lac3-4 (RS222)	Lac⁻ Gal⁻	3.0	5.6	7.6
lac4-8 (MS425)	Lac⁻ Gal⁺	2.6	3.3	47
lac5-10 (AS1D)	Lac⁻ Gal⁻	2.9	1.8	15
lac6-18 (MS93)	Lac⁻ Gal⁺	2.0	3.4	23
lac8-1 (MS12)	Lac⁻ Gal⁻	2.5	2.8	4.8
lac9-6 (MS114)	Lac⁻ Gal⁻	1.2	1.2	1.3

[a] Cells were grown overnight in minimal medium at 30°C, diluted into fresh medium without inducer or with 10 mM lactose or galactose, and grown for one generation. Accumulation of [^{14}C]lactose was measured as described in the text. The values shown represent the average of three or four determinations. The standard deviations from the mean were <±15%.

that *lac9* mutant strains are blocked in regulation (induction). These two possible explanations can be distinguished by determining whether the enzymes can be induced under conditions where inducer is introduced into cells without the need for a functional lactose transporter. To force inducer into cells, strain MS25 (*lac9*⁻) was grown to saturation in minimal medium containing 20 mM sorbitol as a carbon source and 100 mM galactose as inducer. The saturated culture was diluted into fresh medium, grown for one cell doubling, filtered, and resuspended in buffered medium containing sorbitol and galactose. Cells were then incubated for 6 h at 30°C in the same medium after addition of 1 μCi of [^{14}C]-galactose per ml. The apparent intracellular concentration of galactose was then measured by the procedures described in Materials and Methods and was found to be 430 mM. However, this value does not represent free intracellular galactose. To measure this entity, cells were extracted with 70% ethanol at 70°C (11) and the extracted products were chromatographed on Whatman 3 MM paper (2). About 55% of the radioactivity taken up by cells was extractable, and of this about 35% chromatographed as galactose. Thus, the actual intracellular concentration of galactose was 83 mM (430 mM × 0.55 × 0.35). At the end of the 6-h labeling period the levels of β-galactosidase, galactokinase, and α-D-galactose-1-phosphate uridyl transferase activities were the same as or slightly less than those in uninduced cells, indicating that no enzyme induction had occurred. We believe that 83 mM galactose should induce enzyme activity unless the induction mechanism is blocked. We know, for example, that galactose induces as well as lactose (3) and that induction in wild-type cells is initiated by 15 min or at a time when the intracellular concentration of lactose is <10 mM (Fig. 1). We conclude that *LAC9* is not the structural gene for the lactose transporter.

LAC8 is also not the transporter structural gene even though strains defective in this gene show reduced transport activity, because we have recently determined that *LAC8* codes for α-D-galactose-1-phosphate uridyl transferase (Riley and Dickson, unpublished data). *LAC5* has been shown to be the structural gene for galactokinase (Riley and Dickson, unpublished data). The Lac⁻ phenotype of these mutants results from hydrolysis of lactose and subsequent buildup of uridine diphosphate galactose or galactose-1-phosphate, respectively, which are growth inhibitory (Riley and Dickson, unpublished data).

Mutants defective in *LAC6* are interesting and could be blocked specifically in lactose transport. To be consistent with the data in Table 4 for cells induced with galactose we would have to assume that galactose induces a second transport system that can transport lactose, in addition to galactose. Data from inhibitor studies (Table 3) are consistent with this possibility. Inconsistent with this hypothesis, however, is that fact that β-galactosidase activity is only slightly (four- to sevenfold) inducible by galactose in strains defective for *lac6* (8). We would expect galactose to be a strong inducer of β-galactosidase activity.

DISCUSSION

The data presented here verify that lactose is transported into the cell by an inducible system. Induction requires protein synthesis since trichodermin, a powerful inhibitor of protein synthesis, blocks this process. We have not yet established whether induction of transport activity is regulated at the level of transcription. We presume that it is, since induction of β-galactosidase activity is regulated at this level (7) and mutants that perturb the regulation of β-galactosidase activity also do the same to transport activity (4, 8).

The data in Table 1 establish that lactose transport requires energy, and transport goes

against a concentration gradient of up to 175-fold. Thus, transport is an active rather than a passive process. Since transport is inhibited by the proton ionophore 2,4-dinitrophenol, it would appear that the lactose transporter operates, at least in part, by a proton symport mechanism, but this will have to be substantiated.

Our search for nonmetabolized substrate analogs that inhibit lactose transport (Table 3) did not identify such a compound. These results point to a considerable difference between the substrate binding site of the lactose transporter in *K. lactis* and that in *Escherichia coli* (the *lacY* gene product or permease). The *E. coli* permease is strongly inhibited by D-galactosyl-1-thio-β-D-galactose (thiodigalactoside) and by 4-nitrophenyl-β-D-galactoside (6), but neither compound effectively inhibits lactose transport in *K. lactis*.

The data in Table 4 suggest that *LAC6* codes for a component of the lactose transporter. This possibility is only tenable if we assume that lactose can be transported by more than one transporter. For example, lactose and galactose may induce both a *gal*- and a *lac*-specific transporter. In the absence of the *lac* transporter, the *gal* transporter might be able to transport enough lactose to allow cell growth. Our data would not distinguish between one or two transporters if they had similar K_m values for lactose or if one of them had a much lower K_m. Our inhibitor studies (Table 3) suggest that lactose transport in lactose-induced cells may be different than that in galactose-induced cells. Further studies are in progress to determine the function of *LAC6* and its possible role in lactose transport.

ACKNOWLEDGMENTS

We thank Li Cheng-Su, David Iovannisci, and Dennis Harrison for technical assistance.

This research was supported by Public Health Service grant GM-22749 from the National Institutes of Health. R.C.D. was supported by Faculty Research Award FRA-183 from the American Cancer Society.

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