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Binding-Protein-Dependent Lactose Transport in *Agrobacterium radiobacter*

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***Agrobacterium radiobacter* NCIB 11883 was grown in lactose-limited continuous culture at a dilution rate of 0.045/h. Washed cells transported [^{14}C]lactose and [*methyl*-^{14}C]β-D-thiogalactoside, a nonmetabolisable analog of lactose, at similar rates and with similar affinities (K_m for transport, <1 μM). Transport was inhibited to various extents by the uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, by unlabeled β-galactosides and D-galactose, and by osmotic shock. The accumulation ratio for methyl-β-D-thiogalactoside was ≥4,100. An abundant protein (molecular weight, 41,000) was purified from osmotic-shock fluid and shown by equilibrium dialysis to bind lactose and methyl-β-D-thiogalactoside, the former with very high affinity (binding constant, 0.14 μM). The N-terminal amino acid sequence of this lactose-binding protein exhibited some homology with several other sugar-binding proteins from bacteria. Antiserum raised against the lactose-binding protein did not cross-react with two glucose-binding proteins from *A. radiobacter* or with extracts of other bacteria grown under lactose limitation. Lactose transport and β-galactosidase were induced in batch cultures by lactose, melibiose [*O*-α-D-galactoside-(1→6)α-D-glucose], and isopropyl-β-D-thiogalactoside and were subject to catabolite repression by glucose, galactose, and succinate which was not alleviated by cyclic AMP. We conclude that lactose is transported into *A. radiobacter* via a binding protein-dependent active transport system (in contrast to the H^+ symport and phosphotransferase systems found in other bacteria) and that the expression of this transport system is closely linked to that of β-galactosidase.**

Agrobacterium radiobacter is a gram-negative aerobe that utilizes a wide range of sugars as sole carbon substrates for growth. Glucose is transported into the cell via two high-affinity active transport systems, which are synthesized maximally during growth under glucose limitation and require periplasmic glucose-binding proteins GBP1 and GBP2 (7). These GBP1- and GBP2-dependent systems, respectively, also transport galactose and xylose with high affinity, and their general properties indicate that they have much in common with the binding protein-dependent systems that transport sugars such as maltose, galactose, arabinose, and ribose into enteric bacteria (1, 5, 9, 15). These latter systems have generally been shown to consist of a periplasmic binding protein, two transmembrane proteins, and at least one protein that is associated with the cytoplasmic surface of the membrane and binds ATP. It is likely, although not proven, that the energy for transport is provided by the hydrolysis of ATP (1, 5, 15, 16).

Bacteria can also transport sugars by using proton- or cation-linked systems or via phosphoenolpyruvate-dependent phosphotransferase systems (5, 15, 23). Lactose transport has been extensively investigated in *Escherichia coli* and other enteric bacteria, in which it is catalyzed by a single transmembrane protein at the expense of the proton motive force generated by respiration or ATP hydrolysis (H^+-lactose symport) (15, 17, 22). The transported lactose is subsequently hydrolyzed to galactose and glucose by β-galactosidase. The genes coding for the transport protein (*lacY*) and β-galactosidase (*lacZ*) are part of the *lac* operon, which also codes for galactoside transacetylase (*lacA*) and the *lac* repressor (*lacI*). Transcription is induced by lactose, melibiose, and isopropyl-β-D-thiogalactoside (IPTG) (also by galactose in *Klebsiella aerogenes*) and is subject to catabolite repression by glucose which is alleviated by cyclic AMP (cAMP) (13).

In contrast, lactose transport by some species of bacteria, including the lactic acid bacteria *Lactobacillus casei* and *Streptococcus lactis*, has been shown to involve a phosphotransferase system. Lactose enters the cell as lactose 6-phosphate and is subsequently hydrolyzed to galactose 6-phosphate plus glucose by β-phosphogalactosidase (6, 23).

In this report we describe some of the physiological and biochemical properties of a binding-protein-dependent transport system for lactose in *A. radiobacter*. As far as we are aware, this is the first report of lactose transport in bacteria that does not involve either a proton-symport or phosphotransferase system.

MATERIALS AND METHODS

Bacteria. *A. radiobacter* NCIB 11883 and *Rhizobium meliloti* were obtained from Shell Research, Sittingbourne, Kent, United Kingdom. *Agrobacterium tumefaciens* T37 was obtained from J. Draper, Department of Botany, University of Leicester. *E. coli* K-12 and *Klebsiella pneumoniae* MSa1 were provided by R. A. Cooper, Department of Biochemistry, University of Leicester. *L. casei* subsp. *rhamnosus* NCDO 243 was obtained from A. Abbot, Department of Microbiology, University of Leicester.

Growth conditions. *A. radiobacter* was grown in lactose-limited continuous culture at 30°C (pH 7.0) at a dilution rate (*D*) of 0.045/h in a defined minimal medium (18) with an LH series 500 chemostat (L. H. Engineering, Stoke Poges, United Kingdom) with a 2-liter working volume as described previously (7, 10). The concentration of lactose in the inflowing medium was 1.9 g/liter, and the steady-state cell density was 0.82 g (dry weight) per liter. *A. radiobacter* was grown in batch culture at 30°C (pH 7.0) in 500-ml baffled flasks containing 150 ml of minimal medium (18) supple-

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mented with lactose or another carbon source (5 g/liter); cultures were harvested during the exponential phase of growth.

Other organisms were grown in lactose-limited fed-batch culture in a defined minimal medium (18) at 30°C (except for *E. coli*, which was grown at 37°C) at pH 7.0. The growth medium was inoculated with washed cells prepared from exponential-phase batch cultures grown on the same medium to give an initial cell density of approximately 0.1 g (dry weight) per liter. Sterile lactose (1.9 g/liter) was continuously pumped into the culture to give an initial specific growth rate (μ; μ = 2.303 $\log_2$/mean generation time; units of μ are per hour) of approximately 0.1/h. Cells were harvested when the cell density of the culture had increased approximately fourfold.

Preparation of cell suspensions and broken cells. Cells were harvested by centrifugation in an MSE High Speed 18 centrifuge at 10,000 × *g* for 15 min, washed in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–KOH buffer (pH 7.0), and suspended in the same buffer to a density of approximately 10 mg (dry weight) of cells per ml. Broken cells were prepared by disrupting washed-cell suspensions by using sonication (MSE sonicator; 4 times for 30 s each at full amplitude with cooling on ice).

Measurement of sugar uptake. Rates of lactose and methyl-β-D-thiogalactoside (MTG) uptake were determined by measuring the incorporation of 250 μM D-glucose-1-[^{14}C]lactose and [^{14}C]MTG into cells suspended in 20 mM HEPES–KOH buffer (pH 7.0) (1 mg [dry weight] of cells per ml) at 15-s intervals over a period of 1 min. Uptake ocurred at a linear rate over this 1-min period. The procedure was as described previously for [^{14}C]glucose uptake (7), except that the washed filters were immediately immersed in Optiphase T scintillant (LKB, Bromma, Sweden) before counting. Uptake rates at low concentrations of lactose and MTG (1 to 5 μM) were measured as described above, except that the specific activity of the radiolabeled sugars was increased 10-fold, the cell density was decreased 10-fold, and the length of the experiment was reduced to 35 s. The specific activity is expressed as units (micromoles of substrate transported per minute) per milligram (dry weight) of cells.

The substrate specificity of the lactose transport system was determined by measuring the extent to which unlabeled lactose analogs (10 mM) reduced the rate of uptake of [^{14}C]lactose and [^{14}C]MTG (250 μM) after a 5-s incubation of the cells with the unlabeled analog in the reaction mix before the addition of substrate.

The accumulation of lactose and MTG (50 μM) by whole cells was measured over periods of 10 s and 6 min, respectively, as described previously for [^{14}C]glucose (7), except that the [^{14}C]lactose and [^{14}C]MTG were separated from other labeled metabolites in the cell extracts by paper chromatography with a different solvent system (*n*-butanol–pyridine–water; 1:1:1, by volume). Samples of the filtrate plus washings were also counted to determine the extracellular concentration of the lactose and MTG at the time of sampling.

Measurement of pH changes during sugar uptake. Cells washed in 20 mM HEPES–KOH buffer (pH 7.0) were suspended in 2.3 mM HEPES–KOH buffer (pH 7.0) plus 150 mM KCl (1.5 mg (dry weight) of cells per ml; 5-ml total volume), allowed to stand for several minutes to attain anaerobiosis, and then placed in the chamber of a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, United Kingdom) at 30°C. A tightly fitting lid containing a combination pH electrode linked to a pH meter and thence to a potentiometric recorder was inserted into the top of the chamber, and dilute KOH was added through a narrow side port to bring the stirred suspension to pH 7.00. When a steady baseline had been obtained, anaerobic lactose or MTG (5 μmol) was added, and the pH change was measured. Some experiments were carried out under aerobic conditions by omitting the lid and inserting the pH electrode directly into the reaction mix.

Osmotic shock. The osmotic shock procedure was based on that of Neu and Heppel (21). After harvesting, cells were washed three times in 30 mM Tris hydrochloride buffer (pH 8.0) and finally suspended to a density of 10 to 15 mg (dry weight) per ml in 30 mM Tris hydrochloride buffer (pH 8.0) containing 20% (wt/vol) sucrose. The suspension was stirred at 30°C, and EDTA was added at intervals to a final concentration of 5 mM. The cells were then centrifuged at 20,000 × *g* for 20 min, and the pellet was suspended in ice-cold distilled water and stirred on ice for 10 min. The cells were again centrifuged at 20,000 × *g* for 20 min, and the supernatant (shock fluid) was removed.

Purification of lactose-binding protein (LBP). Periplasmic proteins released from whole cells by osmotic shock were separated by anion-exchange fast-protein liquid chromatography (FPLC). Samples of the shock fluid (up to 10 ml containing 5 to 10 mg of protein) were passed through an Acrodisc filter (0.45-μm pore size), loaded on to a Mono-Q column (Pharmacia) equilibrated with 20 mM bis-Tris (pH 6.8), and eluted by using a linear gradient of KCl (0 to 500 mM over 20 min at a flow rate of 1 ml/min). GBP1, GBP2, and BP3 eluted at their usual positions (8), but an additional protein of molecular weight 41,000, which was apparently absent from glucose-limited cultures, was eluted with 490 mM KCl. This protein, which was homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), was stored at −20°C until required. The molecular weight of the native protein was determined by gel filtration FPLC with a Superose column (8).

Equilibrium dialysis. Binding constants (K_D) for lactose and MTG were determined at 4°C by equilibrium dialysis essentially as described previously (7). Experiments were carried out in an eight-cell rotating module (Hoefer Scientific Instruments), which was divided into two chambers of 0.5-ml volume by a membrane with a 6,000- to 8,000-molecular-weight cutoff. Pure LBP was added to one chamber of each cell in 0.3 ml of bis-Tris (pH 6.8), and each of the opposing chambers was loaded with 0.3 ml of bis-Tris containing [^{14}C]lactose or [^{14}C]MTG, the concentration of which was varied over an appropriate range (when [^{14}C]lactose was the substrate the concentration of LBP was 0.97 μM and the lactose concentration was in the range 0.25 to 10.0 μM, and when [^{14}C]MTG was the substrate the concentration of LBP was 3.33 μM and the MTG concentration was in the range 10 to 100 μM). The module was rotated at 10 rpm for 30 h to attain equilibrium, and then 50-μl samples were taken in triplicate from each chamber, added to 3 ml of Optiphase T (LKB), and assayed for radioactivity by scintillation counting. The data were analyzed by the method of Scatchard (24). The effect of unlabeled sugars on binding was also measured by the same procedure but with 3.3 μM LBP and either 10 μM [^{14}C]lactose plus 400 μM unlabeled sugars or 50 μM [^{14}C]MTG plus 2 mM unlabeled sugars.

Amino acid sequencing. N-terminal amino acid sequencing of LBP (approximately 1 nmol loaded onto a polybrene disk)

was carried out with an Applied Biosystems model 470 gas-phase sequencer.

Preparation of antisera. Three samples of LBP (300 μg each) that had been purified by FPLC were injected into a New Zealand White rabbit at fortnightly intervals by using Freund complete adjuvant for the first injection and incomplete adjuvant thereafter. Blood samples were taken 2 weeks after each injection; erythrocytes were removed by centrifugation, and the resultant serum was stored at −20°C. This procedure was identical to that used previously to raise antibodies against GBP1 and GBP2 (9).

SDS-PAGE and Western blotting. Discontinuous SDS-PAGE was carried out with 12.5% (wt/vol) polyacrylamide slab gels (14). Cells, shock fluids, and LBP preparations were boiled for 4 min in dissolving buffer (19), and a volume containing 2 to 20 μg of protein was loaded onto each gel track. Molecular weight protein standards (Sigma Chemical Co., St. Louis, Mo.) were also used as described previously (7). After the gels were run, they were stained with Kenacid blue R (BDH, Poole, United Kingdom), then destained, and, if required, scanned at 633 nm with an LKB densitometer linked to a recording integrator as described previously (7). For Western blotting (immunoblotting), gels were run in duplicate; one was stained for protein using Kenacid blue, whereas the contents of the other gel were transferred onto nitrocellulose sheets (26) by using an LKB Novablot apparatus as recommended by the manufacturer. Molecular weight standards were located by using Ponceau red stain, which was subsequently washed off (9). Immunological staining for proteins that cross-reacted with antisera to LBP, GBP1, and GBP2 was carried out by using the horseradish peroxidase procedure (26).

Enzyme assays. The following enzymes were assayed in broken-cell suspensions at 30°C by using modifications of established procedures: hexokinase and β-galactosidase (4), glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase ($NADP^+$ linked) (3), and galactose dehydrogenase (NAD^+ linked) (20). One enzyme unit catalyzes the conversion of 1 μmol of substrate per min; specific activities are expressed as units per milligram (dry weight) of cells.

Chemicals. D-Glucose-1-[^{14}C]lactose (57 mCi/mmol [2.11 GBq/mmol]) was purchased from Amersham International, and [^{14}C]MTG (50 mCi/mmol [1.85 GBq/mmol]) was purchased from New England Nuclear Corp. Lactose analogs were obtained from Sigma. All other reagents were obtained from Fisons or BDH and were of the highest grade available.

RESULTS

Lactose transport by washed cells of *A. radiobacter*. Lactose transport by washed cells of *A. radiobacter* prepared from a lactose-limited continuous culture (*D*, 0.045/h) was linear for 2.5 min after the addition of [^{14}C]lactose (250 μM) (Fig. 1) and occurred at an average rate of 35 nmol/min per mg (dry weight) of cells (Table 1). This rate then fell by approximately 20% to a new linear rate, which was maintained until all of the lactose had been taken up and probably reflected the steady-state rate of lactose metabolism (rather than lactose transport) by the cells.

The rate of lactose transport was not significantly altered by preincubating the cells with 10 mM glucose or ethanol but was inhibited over 90% by the uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (20 μM). Since the rate of lactose transport did not change when the lactose concentration was decreased to 50, 5, 3, and

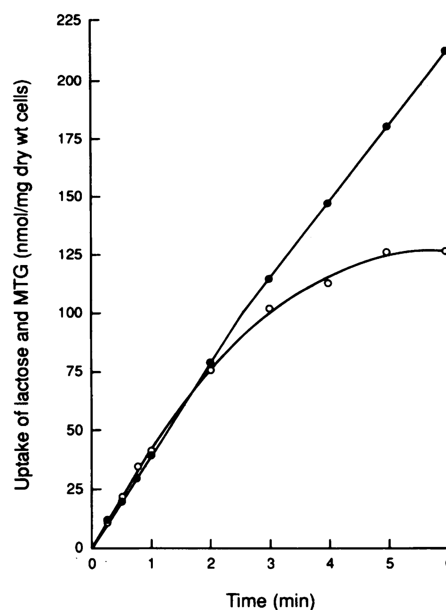

FIG. 1. Uptake of [^{14}C]lactose (●) and [^{14}C]MTG (○) by washed cells of *A. radiobacter* prepared from a lactose-limited continuous culture and suspended in 20 mM HEPES-KOH buffer (pH 7.0). Uptake was measured at 30°C in the same buffer by using 250 μM sugars (see Materials and Methods).

finally 1 μM, we concluded that the K_m for lactose transport (K_T) was <1 μM. These results indicated that lactose was taken up via a high-affinity active (energy-dependent) transport system, the energy requirement for which was satisfied by the oxidation of endogenous substrates. It is likely that the very low uptake rate in the presence of FCCP reflected either incomplete deenergization of the cells or a small amount of facilitated diffusion.

Chromatographic analysis of cell extracts prepared from washed cells that had been allowed to transport [^{14}C]lactose for 10 s showed that approximately 77% of the recovered radioactivity was in the form of glucose, compared with approximately 10% as an unidentified polar product (possibly a sugar phosphate or a nucleoside diphosphate sugar) and less than 4% as lactose. This indicated that the transported lactose was rapidly hydrolyzed to glucose plus galactose (although, since the galactose moiety of the original lactose was unlabeled, the presence of free galactose could

TABLE 1. Enzyme activities of *A. radiobacter* following growth in lactose-limited continuous culture (*D*, = 0.045/h)[a]

Enzyme activities	Sp act (mU/mg [dry weight] of cells)[b]
Lactose uptake (250 μM)	35
MTG uptake (250 μM)	39
β-Galactosidase	271
Galactose dehydrogenase	39
Hexokinase	58
Glucose-6-phosphate dehydrogenase	36
6-Phosphogluconate dehydrogenase	66

[a] Uptake rates were measured in whole cells, and other enzyme activities were measured in broken cells produced by sonication; binding protein concentrations were determined by SDS-PAGE and scanning densitometry (see Materials and Methods). Binding protein concentrations (percentage of cell protein) were as follows: LBP, 8%; GBP1, 6%; GBP2, <2%; BP3, 18%.

[b] The values quoted are the means of up to 10 independent determinations.

TABLE 2. Effect of unlabeled lactose analogs on the uptake of [^{14}C]lactose and [^{14}C]MTG by washed cells of *A. radiobacter* and on the binding of [^{14}C]lactose and [^{14}C]MTG to pure LBP from *A. radiobacter*[a]

Addition[b]	Uptake rate (% of control)		Binding (% of control)	
	[^{14}C]Lactose	[^{14}C]MTG	[^{14}C]Lactose	[^{14}C]MTG
None	100	100	100	100
Lactose	3	2	3	0
MTG	55	2	90	3
PTG	72	4	93	90
TDG	85	58	87	61
ONPG	54	33	88	2
D-Galactose	50	23	84	57

[a] The values quoted are the means of up to three independent determinations.

[b] Unlabeled sugars were present in 40-fold molar excess over labeled sugars (10 mM versus 250 μM in the uptake experiments, 400 μM versus 10 μM in the [^{14}C]lactose-binding experiments, and 2 mM versus 50 μM in the [^{14}C]MTG-binding experiments). Uptake was measured at 30°C in 20 mM HEPES-KOH buffer (pH 7.0), and binding was measured at 4°C in 20 mM bis-Tris (pH 6.8).

not be confirmed by this approach), and this was supported by the observation that broken cells exhibited a high β-galactosidase activity (Table 1). Galactose dehydrogenase, hexokinase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase activities were also detected. All of these enzyme activities were sufficient to satisfy the in situ rate of lactose utilization ($q_{lactose}$) by the growing culture (approximately 5.3 nmol/min per mg [dry weight] of cells at a dilution rate of 0.045/h).

MTG transport by washed cells of *A. radiobacter*. The failure to determine an accumulation ratio for lactose because of its very rapid further metabolism stimulated a search for a suitable nonmetabolizable analog that was transported via the same uptake system. This was carried out by measuring the effect of a 40-fold excess of unlabeled analogs of this type on the uptake of [^{14}C]lactose by washed cells. Lactose uptake was significantly inhibited by MTG, phenyl-β-D-thiogalactoside (PTG), and thiodigalactoside (TDG) in the order MTG > PTG >TDG (Table 2).

[^{14}C]MTG was therefore tested as a potential analog substrate for the lactose transport system. Washed cells took up [^{14}C]MTG (250 μM) at an average rate of 39 nmol/min per mg (dry weight) of cells (Table 1), which remained linear for almost 2 min but then tailed off rapidly and stopped after approximately 5 min, when about 50% of the substrate had been taken up (Fig. 1). MTG transport was virtually abolished by the uncoupling agent FCCP (20 μM) and was strongly inhibited by a 40-fold excess of unlabeled lactose, PTG, and TDG in the order lactose > PTG > TDG. Since the rate of MTG transport did not decrease when the [^{14}C]MTG concentration was decreased to 50, 5, 3, and finally 1 μM, we concluded that the K_T for MTG uptake was <1 μM. These results, together with the observations that both lactose and MTG transport were substantially inhibited by *o*-nitrophenyl-β-D-galactoside and D-galactose, strongly suggested that lactose and MTG were taken up via a common energy-dependent transport system.

Chromatographic analysis of cell extracts prepared from washed cells that had been allowed to take up [^{14}C]MTG (50 μM) for 6 min showed that 85% of the recovered radioactivity was in the form of MTG; the remainder of the radioactivity was smeared lightly over the chromatogram, and no other radiolabeled metabolites could be detected. The residual concentration of [^{14}C]MTG in the filtrate was 3.8 μM. The accumulation ratio for MTG ($[MTG]_{in}/[MTG]_{out}$) was ≥4,100, based on an intracellular volume of ≤3 μl/mg (dry weight) of cells. The addition of the uncoupling agent FCCP (20 μM) to cells that had been allowed to accumulate 50 μM [^{14}C]MTG for 6 min did not elicit the exit of any radioactivity from the cells. This experiment was not feasible with [^{14}C]lactose, since the latter was hydrolyzed too rapidly to glucose and galactose after entry into the cells.

The addition of lactose or MTG (1 mM) to weakly buffered, anaerobic cell suspensions (1.5 mg [dry weight] per ml in 150 mM KCl plus 2.3 mM HEPES buffer; adjusted to pH 7.00) caused no alkalinization of the external medium (the pH remained unchanged and decreased to 6.99, respectively). Under aerobic conditions, lactose elicited a rapid and prolonged acidification (0.05 pH units in 3 min), which presumably reflected its further metabolism; MTG caused only a very slight and transient acidification (to pH 6.99) under similar conditions (possibly caused by the oxidation of a very small amount of a contaminating sugar), which was commensurate with its nonmetabolizable nature. The rate of lactose and MTG transport was inhibited up to 30% when the cells were subjected to osmotic shock; control experiments showed that no β-galactosidase activity was detectable in the shock fluid (since the limit of detection of β-galactosidase activity was approximately 2 mU/mg [dry weight] of cells, this result indicates that <1% of the enzyme was released from the cells by osmotic shock).

These results suggested that lactose (and MTG) was transported by *A. radiobacter* by an osmotic-shock-sensitive, binding-protein-dependent active transport system (rather than a proton-symport or phosphotransferase system of the type found in other species of bacteria) similar to those previously reported for the uptake of glucose and galactose (GBP1 dependent) and glucose and xylose (GBP2 dependent) by this organism (7, 9).

Purification and properties of LBP. The above hypothesis was tested by preparing an osmotic-shock fluid from cells grown under lactose limitation (*D*, 0.045/h), and then analyzing the polypeptide profiles of the cells and shock fluid for a potential LBP by using SDS-PAGE. The gel showed the presence of three abundant proteins (Table 1), all of which were substantially released from the cells by osmotic shock (Fig. 2). Two of these proteins were identified from their molecular weights on SDS-PAGE as GBP1 and BP3 (molecular weights, 36,500 and 30,500, respectively), whereas the third protein was significantly larger (molecular weight, approximately 41,000) and, in contrast to the other two, was essentially absent from cells grown under glucose, galactose, or xylose limitation (9). Cells grown under lactose limitation contained only a very low concentration of GBP2.

This putative LBP was purified by using FPLC; it was shown to have a molecular weight of approximately 41,000 by using SDS-PAGE and of approximately 45,000 by using gel-filtration FPLC. The protein therefore exists as a monomer.

The ability of this protein to bind lactose was measured by using equilibrium dialysis; the resultant Scatchard plot showed that it bound [^{14}C]lactose with high affinity (K_D, 0.14 μM), using a single binding site (Fig. 3). Similar experiments showed that it also bound [^{14}C]MTG, using a single binding site, albeit with a much lower affinity (K_D, 26 μM). The large difference between the K_D for binding and the K_T for transport of [^{14}C]MTG but not [^{14}C]lactose cannot currently be explained.

Competitive binding assays with a 40-fold excess of unlabeled sugars showed that unlabelled MTG, PTG, TDG, *o*-nitrophenyl-β-D-galactoside, and D-galactose slightly in-

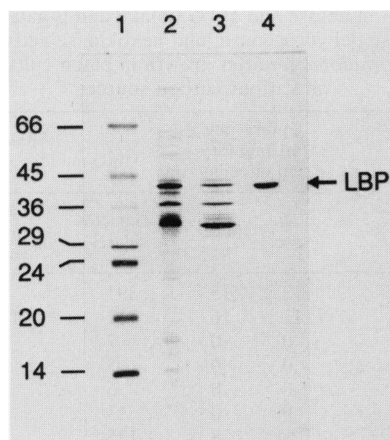

FIG. 2. Purification of a putative LBP from *A. radiobacter* grown in continuous culture under lactose limitation (*D*, 0.045/h). Samples were subjected to SDS-PAGE and then stained with Kenacid blue (14). Tracks: 1, molecular weight standards (in thousands); 2, washed cells; 3, osmotic shock fluid prepared from washed cells; 4, putative LBP purified from shock fluid using anion-exchange FPLC (see Materials and Methods).

hibited the binding of [^{14}C]lactose to LBP and that unlabelled lactose, PTG, TDG, *o*-nitrophenyl-β-D-galactoside, and D-galactose variably inhibited the binding of [^{14}C]MTG to LBP (Table 2). The inhibition of [^{14}C]MTG binding was generally higher (in the case of MTG and *o*-nitrophenyl-β-D-galactoside, much higher) than the inhibition of [^{14}C]lactose binding, as was also observed above for uptake. Although

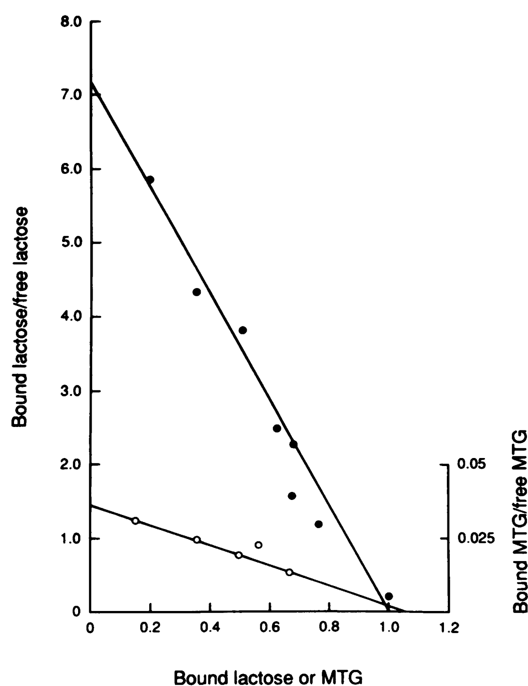

FIG. 3. Scatchard plot (24) of equilibrium dialysis of LBP in the presence of [^{14}C]lactose (●) and [^{14}C]MTG (○). Equilibrium dialysis was carried out at 4°C in 20 mM bis-Tris (pH 6.8) (see Materials and Methods). For [^{14}C]lactose the LBP concentration was 0.97 μM and the substrate concentration ranged from 0.25 to 10 μM, and for [^{14}C]MTG the LBP concentration was 3.33 μM and the substrate concentration ranged from 10 to 100 μM.

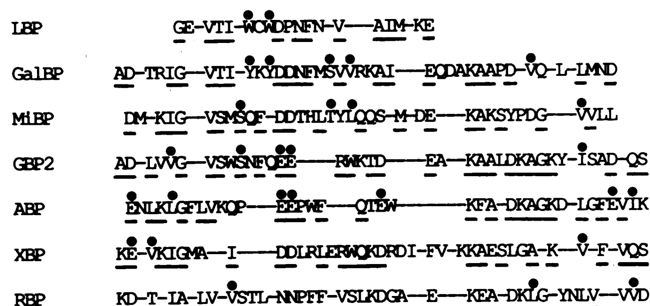

FIG. 4. Comparison of N-terminal sequences of the following: LBP and GBP2 from *A. radiobacter*; galactose-binding protein (GalBP), arabinose-binding protein (ABP), xylose-binding protein (XBP), and ribose-binding protein (RBP) from *E. coli*, and *myo*-inositol-binding protein (MiBP) from *Pseudomonas* sp. Sequences were taken from this paper (LBP) and from the literature (2, 9, 12, 25) and were aligned to give optimum fit. Identical residues that appear at the same aligned positions in any of the seven proteins are underlined, and conservative replacements (I/L/V; S/T; D/E; W/Y) are indicated (●).

these results probably in part reflected the different concentrations of [^{14}C]lactose and [^{14}C]MTG in the binding experiments, they were also compatible with the ability of LBP to bind lactose with a much higher affinity than MTG. Inhibition of binding was almost always less than inhibition of uptake, but as the two assays were carried out at different temperatures and pH values it would be unwise to read too much into these differences.

The binding of [^{14}C]lactose and [^{14}C]MTG to LBP was not significantly inhibited (<6%) by a 40-fold excess of melibiose, D-glucose, D-xylose, sucrose, and maltose, all of which also inhibited the uptake of [^{14}C]lactose and [^{14}C]MTG by washed cells by <5%. Both binding and uptake were inhibited exactly as expected (97 to 98%) by 40-fold excesses of unlabeled lactose and MTG, respectively. Overall, the results of these competition experiments support the view that lactose and MTG are taken up via a common transport system.

N-terminal amino acid sequence of LBP. The N-terminal amino acid sequence of LBP was determined by using an Applied Biosystems 470 gas-phase protein sequencer. The sequence was then compared with those of the aligned N-terminal regions of GBP2 from *A. radiobacter*, several different sugar-binding proteins from *E. coli*, and the *myo*-inositol-binding protein from *Pseudomonas* sp. (Fig. 4). Some regions of homology were observed between LBP and the other binding proteins, particularly the galactose-binding protein of *E. coli*. No significant homology was observed between LBP and the maltose-binding protein from *E. coli* (data not shown).

Immunoblotting of LBP. Western blotting of LBP, GBP1, and GBP2 with anti-LBP, and of LBP with anti-GBP1 and anti-GBP2, showed that cross-reaction only occurred between LBP and anti-LBP (Fig. 5). Since GBP1 only reacts with anti-GBP1, and GBP2 only reacts with anti-GBP2 (9), it would appear that the different binding proteins contain no common epitopes.

Anti-LBP was also used to screen other species of gram-negative bacteria to see whether they produced a protein similar to LBP after growth under lactose limitation in fed-batch culture (μ, 0.05 or 0.1/h), conditions which were chosen to maximize the production of a lactose transport system. Cross-reaction was observed with *A. radiobacter*

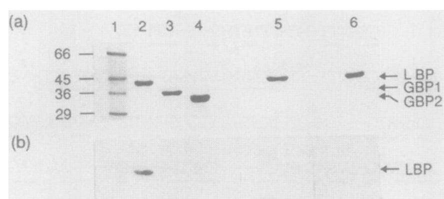

FIG. 5. Immunoblotting of LBP, GBP1, and GBP2 from *A. radiobacter*. Binding proteins were subjected to SDS-PAGE (approximately 5 μg of protein per track), then either (a) stained with Kenacid blue, or (b) transferred to nitrocellulose, and then cut into three sections and Western blotted with anti-LBP, anti-GBP1 and anti-GBP2 (see Materials and Methods). Tracks: 1, molecular weight standards (in thousands); 2, LBP; 3, GBP1; 4, GBP2; 5 and 6, LBP. Samples were blotted with anti-LBP (tracks 2, 3, and 4), anti-GBP1 (track 5), and anti-GBP2 (track 6).

but not, rather surprisingly, with its close relative *A. tumefaciens* (Fig. 6). No cross-reaction was observed with any of the other organisms (*K. pneumoniae*, *E. coli*, *R. meliloti*, or *L. casei*), all of which are known to transport lactose via proton-symport or phosphotransferase systems.

Lactose transport and metabolism by *A. radiobacter* grown in batch culture. After growth in batch culture with lactose as the sole carbon source, washed cells of *A. radiobacter* exhibited substantial lactose uptake, MTG uptake, β-galactosidase, galactose dehydrogenase, and hexokinase activities (Table 3). The lactose and MTG uptake rates were significantly lower than those of lactose-limited continuous cultures, whereas the β-galactosidase and galactose dehydrogenase activities were significantly higher and the hexokinase activity was unchanged.

Comparative enzymic and immunological analyses of cells grown in batch culture on various carbon sources (Table 3; Fig. 7) showed that the lactose (MTG) transport system and β-galactosidase were induced by lactose, melibiose, and IPTG and were repressed by glucose, galactose, and succinate (galactose and succinate more than glucose). Since the organism grew rapidly on all three of these latter substrates (μ_{max}, 0.38 ± 0.02/h) but only slowly on the nonrepressing substrate glycerol (μ_{max}, 0.25/h), we concluded that expression of the lactose (MTG) transport system and β-galactosidase was subject to catabolite repression. The latter was not alleviated by the inclusion of 3 mM cAMP or 2 mM dibutyryl-cAMP (a more easily transported form of cAMP) in the growth medium. The ratio of lactose (MTG) transport to β-galactosidase activity after growth under these various

TABLE 3. Lactose and MTG uptake and β-galactosidase, galactose dehydrogenase, and hexokinase activities of *A. radiobacter* after growth in batch culture on various carbon sources[a]

Carbon substrate[b]	Uptake rate (mU/mg [dry wt] of cells)		Sp act (mU/mg [dry wt] of cells)		
	Lactose	MTG	β-Galactosidase	Galactose dehydrogenase	Hexokinase
Lactose	12	15	395	77	58
Melibiose	12	16	117	123	71
Glucose	0	0	7	25	77
Galactose	0	0	5	89	41
Glycerol	0	0	6	29	60
Succinate	0	0	3	15	16
Glucose + IPTG	5	8	175	17	79
Galactose + IPTG	3	4	110	79	54
Glycerol + IPTG	12	13	191	27	54
Succinate + IPTG	5	6	98	15	25

[a] The results are the means of up to five independent determinations.
[b] The concentrations of carbon substrates and IPTG in the growth media were 5 g/liter and 1 mM, respectively.
[c] The concentrations of [^{14}C]lactose and [^{14}C]MTG in the uptake experiments were 250 μM.

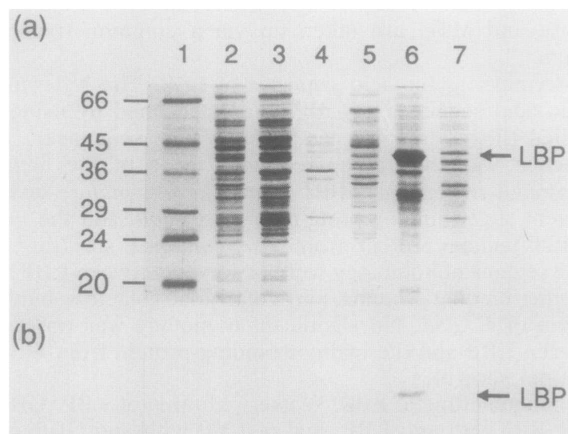

FIG. 6. Screening of gram-negative bacteria by using SDS-PAGE and Western blotting for a protein capable of cross-reacting with anti-LBP from *A. radiobacter*. Bacteria were grown in lactose-limited fed-batch culture (μ, 0.1/h) except for *A. radiobacter*, which was grown in lactose-limited continuous culture (*D*, 0.045/h). Cellular proteins were separated by using SDS-PAGE, then either (a) stained with Kenacid Blue or (b) transferred to nitrocellulose, and probed with anti-LBP. Tracks: 1, molecular weight standards (in thousands); 2, *K. pneumoniae*; 3, *E. coli*; 4, *R. meliloti*; 5, *L. casei*; 6, *A. radiobacter*; 7, *A. tumefaciens*. Note that *A. radiobacter* grown in lactose-limited fed batch culture (data not shown) also gave a positive reaction.

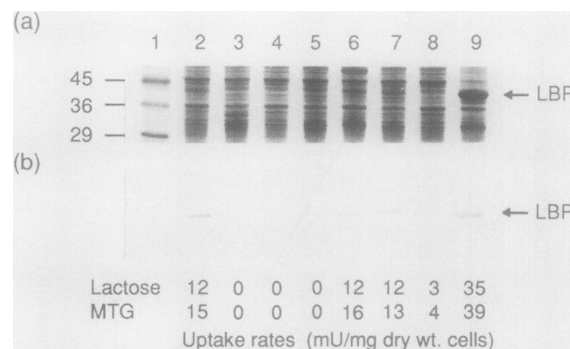

FIG. 7. Induction and repression of the lactose uptake system of *A. radiobacter* as revealed by SDS-PAGE and Western blotting. *A. radiobacter* was grown in lactose-limited continuous culture (*D*, 0.045/h) or at μ_{max} in batch culture on a minimal medium containing different carbon sources; μ_{max} values for batch cultures were 0.38 ± 0.02/h on all carbon sources except glycerol (0.25/h) and melibiose (0.16/h). Cellular proteins were separated by using SDS-PAGE, then either (a) stained with Kenacid blue or (b) transferred on to nitrocellulose, and then probed with anti-LBP (see Materials and Methods). Tracks: 1, molecular weight standards (in thousands); 2, lactose; 3, glucose; 4, galactose; 5, glycerol; 6, melibiose; 7, glycerol plus IPTG; 8, galactose plus IPTG; 9, lactose limited. Lactose and MTG uptake rates of washed cells measured using 250 μM substrates are shown below each track.

conditions ranged from 0.03 after batch growth on lactose to 0.14 after growth in continuous culture under lactose limitation. Galactose dehydrogenase was present at a significant basal level in all cultures and was further induced by lactose, melibiose, and galactose (melibiose > lactose > galactose). Hexokinase was present at a substantial level in all cultures except after growth on succinate.

These results with batch cultures, taken together with those from lactose-limited continuous culture, indicated that the four enzymes involved in the initial stages of lactose catabolism by *A. radiobacter* were not regulated coordinately.

DISCUSSION

The results described in this report show that *A. radiobacter* takes up lactose (and its nonmetabolizable analog MTG) via a binding-protein-dependent active-transport system. There is no evidence for the presence in *A. radiobacter* of a proton-symport or phosphotransferase system for lactose transport of the type found in other bacteria (5, 6, 15, 23). As far as we are aware, this work with *A. radiobacter* constitutes the first report of a binding-protein-dependent lactose transport system in bacteria.

The LBP from *A. radiobacter* has several properties in common with various sugar-binding proteins from *E. coli* as well as with the *myo*-inositol-binding protein from *Pseudomonas* sp. and with the two glucose-binding proteins (GBP1 and GBP2) from *A. radiobacter*. These properties include a periplasmic location, a high affinity for the natural substrate (K_D, ≤1.3 μM), and a molecular weight within the range of 30,000 to 41,000 (1, 5, 12, 15). Furthermore, the N-terminal amino acid sequence of LBP shows sufficient homology with all of the other binding proteins (except the maltose-binding protein and also possibly GBP1, for which no N-terminal sequence is available) to suggest that they are significantly related to each other and may have evolved from a common ancestor (1). It should be noted, however, that cysteine is absent from all of the other binding proteins except the arabinose-binding protein (2, 11), whereas LBP contains at least one cysteine residue.

The observation that *A. tumefaciens*, a close relative of *A. radiobacter*, does not react immunologically with anti-LBP from *A. radiobacter* suggests either that *A. tumefaciens* does not transport lactose by using a binding-protein-dependent system or that the two binding proteins have few, if any, common epitopes. However, since anti-GBP1 and anti-GBP2 prepared against GBP1 and GBP2 from *A. radiobacter* cross-reacted strongly with proteins corresponding to GBP1 and GBP2 in *A. tumefaciens* (9), the first explanation is probably more likely.

The ability of D-galactose and several β-galactosides (but not D-glucose or melibiose) to inhibit the binding and transport of lactose, together with the extent of homology that was observed between LBP and the galactose-binding protein of *E. coli*, suggests that the lactose might be sequestered mainly via its galactosyl rather than via its glucosyl moeity. After being transported into *A. radiobacter*, the lactose is hydrolyzed very rapidly to glucose and galactose by β-galactosidase. The presence of hexokinase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase (together with the Entner-Doudoroff enzymes [8]) indicates that the glucose is subsequently metabolized via the Entner-Doudoroff and hexose monophosphate pathways, whereas the presence of galactose dehydrogenase suggests that the galactose is metabolized via the De Ley-Doudoroff pathway (11).

Studies with batch cultures of *A. radiobacter* grown on different carbon sources indicated that the lactose uptake system and β-galactosidase are subject to similar physiological regulation, i.e., induced by α- and β-galactosides (melibiose, lactose, IPTG) and catabolite repressed by the products of lactose hydrolysis (glucose, galactose) and by rapidly utilized carbon substrates (succinate) but not by slowly utilized carbon substrates (glycerol). These general physiological properties are similar to those exhibited by the lactose-H^+ symport system of *E. coli*. However, in contrast to the latter, the ratio of lactose (MTG) transport to β-galactosidase activity in *A. radiobacter* varies significantly with the growth conditions, and catabolite repression of lactose transport and β-galactosidase is not alleviated by cAMP. It is likely, therefore, that the regulation and/or organization of the genes coding for the lactose uptake system and β-galactosidase in *A. radiobacter* differs from that of the *lac* operon in *E. coli* (13).

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