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DEFECTIVE LACTOSE UTILIZATION BY A MUTANT OF *ESCHERICHIA COLI* ENERGY-UNCOUPLED FOR LACTOSE TRANSPORT

THE ADVANTAGES OF ACTIVE TRANSPORT *VERSUS* FACILITATED DIFFUSION

MEREDITH KUSCH and T. HASTINGS WILSON

Department of Physiology, Harvard Medical School, Boston, Mass. 02115 (U.S.A.)

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SUMMARY

1. The lactose operon of an *Escherichia coli* mutant (X71-54), energy-uncoupled for lactose transport, was transduced into an inducible β-galactosidase negative recipient. A transductant was isolated which was inducible (i^+) and possessed both β-galactosidase (z^+) and the uncoupled transport property (y^{UN}).

2. This inducible energy-uncoupled transductant (54-41) and the constitutive mutant from which it was derived (X71-54) were defective in energy-dependent accumulation of thiogalactosides, but showed no loss of carrier-mediated transport, as compared to their respective parents.

3. A striking feature of the inducible mutant 54-41 was its inability to grow on lactose in minimal medium. The addition of the gratuitous inducer thioisopropyl-β-D-galactopyranoside permitted normal growth of these cells on lactose.

4. In the energy-uncoupled transductant the rate of induction of β-galactosidase by lactose and by low concentrations of thioisopropyl-β-D-galactopyranoside was much slower than that of the parental cell.

5. The constitutive mutant X71-54 grew poorly on melibiose at 37 °C. This was due to the inability of this sugar to induce α-galactosidase.

6. The data are consistent with the hypothesis that for normal growth of inducible cells on lactose or melibiose, carrier-mediated entry (facilitated diffusion) of substrates is inadequate; the membrane carriers must also be capable of accumulating the sugars.

INTRODUCTION

Escherichia coli possesses a specific membrane transport system for lactose[1]. When this disaccharide is transported into induced cells, it is rapidly hydrolyzed by

Abbreviations: TMG, thiomethyl-β-D-galactopyranoside; IPTG, thioisopropyl-β-D-galactopyranoside; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; TDG, D-galactopyranosyl-β-D-thiogalactopyranoside; PNPG, *p*-nitrophenyl-α-D-galactopyranoside; XG, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; y^{UN}, the gene coding for the membrane carrier which is defective in the energy-uncoupled mutant; K_t, concentration of substrate which gives one-half the maximum transport rate (analogous to K_m of enzyme kinetics).

β-galactosidase, and thus a low intracellular lactose concentration is maintained. On the other hand, when certain non-metabolizable lactose analogs are transported into such cells, the sugars are accumulated inside the cell to concentrations 100–200 times those in the external medium. This transport against a concentration gradient requires expenditure of energy and is blocked by a variety of metabolic inhibitors[2]. It is not clear, however, whether energy expenditure is needed when the external sugar concentration is high, or whether energy coupling is obligatory regardless of the direction of the concentration gradient.

In an attempt to study this and other questions related to the coupling of energy to transport, mutants were sought which retained carrier activity but had lost the ability to accumulate against a concentration gradient. Two spontaneous mutants, ML-308-22[3] and X71-54[4], were isolated and they each possessed a severe defect in accumulation of substrates of the lactose transport system. However, they showed normal or slightly increased carrier activity as assayed by *o*-nitrophenyl-β-D-galactopyranoside (ONPG) entry, counterflow and M protein assay [D-galactopyranosyl-β-D-thiogalactopyranoside (TDG) binding]. Entry of substrates on the uncoupled membrane carrier appeared normal while exit was abnormally rapid, presumably due to an elevated affinity of the carrier for exit[5]. Deletion mapping experiments located the defect in X71-54 within the y gene[6], which codes for the membrane protein involved in lactose transport.

A study of the properties of an inducible transductant of X71-54 indicates that energy coupling plays an important role in the process of induction of the lactose operon and in growth of cells on lactose.

MATERIALS AND METHODS

Chemicals

Thio[^{14}C]methyl-β-D-galactopyranoside ([^{14}C]TMG) was purchased from New England Nuclear Corp.; thio[^{14}C]isopropyl-β-D-galactopyranoside ([^{14}C]IPTG) was obtained from Schwartz Bioresearch. Lactose, *p*-nitrophenyl-α-D-galactopyranoside (PNPG) and non-radioactive ITPG were obtained from Sigma. Melibiose, TDG, and ONPG were purchased from Mann Research Laboratories. Non-radioactive TMG was obtained from Calbiochem Corp. and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XG) was obtained from Cyclo Chemical Co. Casein hydrolysate (Tryptone) was provided by Difco, and glycerol was purchased from Fisher Scientific Co. Glucostat reagents were purchased from Worthington Biochemical Corp.

Bacteria

The parent strain X71 ($i^-z^+y^+a^-$, $ProC^-$, try^-, $B_1{}^-$) was isolated from X5072 ($i^-z^+y^+a^+$, obtained from Dr J. Beckwith). The energy-uncoupled mutant X71-54 ($i^-z^+y^{UN}a^-$)[4] and a y^- strain, 316 ($i^-z^+y^-a^-$)[5], were derived from X71. X9003 ($i^+z^-y^+a^+$, $B_1{}^-$) was isolated by W. Epstein[7].

Growth of cells

For routine assays cells were grown in Medium 63 (ref. 8) supplemented with NaCl (0.29%) and casein hydrolysate (Tryptone) to a concentration of 1%. For X71 and its derivatives, the growth medium was supplemented with L-tryptophan

(10 μg/ml), L-proline (100 μg/ml) and thiamine (0.5 μg/ml). Thiamine (0.5 μg/ml) was the only growth supplement added to cultures of X9003 and its derivatives.

All induction experiments were carried out with cells in the exponential growth phase in 0.4% glycerol in Medium 63 (pH 5.8) unless otherwise noted. Since previous experiments[5] showed that the accumulation defect of the energy-uncoupled mutant X71-54 was most striking at pH 5.8, this pH was used for the experiments discussed here unless otherwise specified.

Assays

To determine β-galactosidase activity 1 ml of cells was treated with 2 drops toluene and 0.1 ml deoxycholate (100 μg/ml). The cells were vortexed for 10 s and left at room temperature for 20 min to 1 h. The assay was started by addition of various amounts (0.05–0.2 ml) of the toluenized cells to Medium 63 (pH 7.0) containing 2 mM ONPG to a final volume of 2 ml. The reaction was stopped by addition of 4 ml 0.6 M Na_2CO_3. A Klett Summerson colorimeter (No. 42 filter) was used to determine the amount of *o*-nitrophenol formed.

Carrier mediated transport was determined by the rate of entry of ONPG (1 mM) into washed cells at 37 °C in Medium 63, pH 7.0. The assay was stopped at 30 s and at later times by adding 2 ml of the reaction mixture to 4 ml 0.6 M Na_2CO_3. A Klett Summerson colorimeter (No. 42 filter) was used to determine *o*-nitrophenol production. The rate of entry *via* pathways other than the lactose transport system, was determined in control experiments in which TDG (10 mM) was used to inhibit ONPG entry. The TDG-sensitive entry was taken as a measure of carrier activity. In both the parent and the energy-uncoupled cells the rate of entry of ONPG (1 mM) was unchanged by shifting from pH 7.0 to pH 5.8.

Transport at pH 5.8 was measured by exposing cells to [^{14}C]TMG (0.1 mM) or [^{14}C]IPTG (0.008 mM) at 25 °C followed by filtration of 0.2 ml aliquots at various times through Millipore filters (0.6 μm pore size). The filters were washed once with 5 ml Medium 63, pH 5.8, containing 2-mercaptoethanol (1 mM) and then dissolved in 15 ml Bray's solution in liquid scintillation vials. Intracellular sugar concentration was calculated assuming that 1 ml of 100 Klett units (No. 42 filter) contained $6 \cdot 10^8$ cells which was equal to 0.6 μl cell water[9]. A 1-ml sample of 100 Klett units equaled 0.82 μg wet wt of cells.

Thiogalactoside transacetylase was determined by the method of Fox and Kennedy[10]. Heat-treated cells (70 °C for 5 min) were incubated with IPTG (50 mM) and [^{14}C]acetyl-CoA (0.05 mM, 0.7 μCi/ml). A Dowex-1 (acetate) column was used to separate [^{14}C]acetyl-IPTG from acetyl-CoA.

α-Galactosidase activity was determined by a modification of the procedures of Schmitt and Rotman[11] and Burstein and Kepes[12]. Cells were washed twice with Tris buffer (50 mM, pH 7.6) and then incubated at 37 °C in Tris buffer (50 mM, pH 7.6) containing 2-mercaptoethanol (50 mM), $MnSO_4$ (5 mM) and PNPG (10 mM) for various times. The reaction was stopped by addition of 4.5 ml ice-cold 0.6 M Na_2CO_3 and 0.1 ml 0.2 M potassium EDTA to 1.0 ml of cell suspension. The cells were centrifuged and the color of the supernatant determined with a Klett Summerson colorimeter (No. 42 filter). This assay was performed with intact cells because α-galactosidase is very unstable in broken cell preparations, especially in dilute solutions[11,12]. The high concentration of PNPG used was selected to minimize the

transport component of sugar entry. Thus, the rate of PNPG hydrolysis gives a reasonable estimate of α-galactosidase activity[12].

Transduction

In order to construct strains which were inducible for the lactose gene products, transduction was performed by modification of the procedures of Luria *et al.*[13] and Arber[14]. Lysates of X71 and X71-54 were prepared with the generalized transducing phage P_1 (kindly provided by Dr S. Kistler). The recipient strain for transduction was X9003 which is inducible, and β-galactosidase negative. Transductants were isolated and purified on 10 mM lactose–1 mM IPTG minimal medium agar plates. The purified clones were streaked onto XG plates; white and very light blue clones (i^+) were selected for further testing. Of 250 transductants of each cell type which grew on lactose, ten of X71-54 and eight of X71 were also inducible. The transport properties, β-galactosidase and transacetylase of several inducible transductants were tested carefully. The results obtained with a typical transductant of each type are given in Table I. One of the transductants of X71 is called X71-15, and one of X71-54 is called 54-41. The inducible transductants had the same levels of β-galactosidase activity and carrier mediated transport, measured by ONPG hydrolysis, as X71 and X71-54. The steady state levels of TMG accumulation were slightly higher in X71-15 and 54-41 than in the corresponding parents X71 and X71-54; however, the defect in TMG accumulation was clearly transmitted to the transductant 54-41. The defect in accumulation in 54-41 is illustrated in Fig. 1 with the non-metabolizable substrate IPTG at a concentration ($8 \cdot 10^{-6}$ M) often employed in induction experiments. The transductants possessed about 5% of the normal transacetylase activity, as did X71 and X71-54.

In the transductants of X71, 5% transacetylase activity was taken as an indication that the y gene had been transduced from X71 since the recipient strain, X9003, made the enzyme (Table I).

TABLE I

PROPERTIES OF PARENTAL CELLS AND TRANSDUCTANTS

Cells were grown to late exponential phase in Tryptone, harvested by centrifugation, washed and resuspended in Medium 63 *plus* 1 mM 2-mercaptoethanol. Assays were performed as indicated in Materials and Methods. The pH for the TMG accumulation assay was 5.8; that for the other assays was 7.0. Data for TMG, ONPG and β-galactosidase are expressed as per cent of X71 values. The transacetylase data are expressed as per cent of X9003 value.

Cell No.	*Genotype*	*TMG accumulation*	*ONPG entry*	*β-Galactosidase*	*Transacetylase*
X71	($i^-z^+y^+a^-$)	100	100	100	5.0
X71-54	($i^-z^+y^{UN}a^-$)	21	130	100	5.0
X71-15*	($i^+z^+y^+a^-$)	118	105	103	5.2
54-41*	($i^+z^+y^{UN}a^-$)	29	132	102	5.3
X9003*	($i^+z^-y^+a^+$)	110	0	0	100

* Cells grown in the presence of 0.5 mM IPTG.

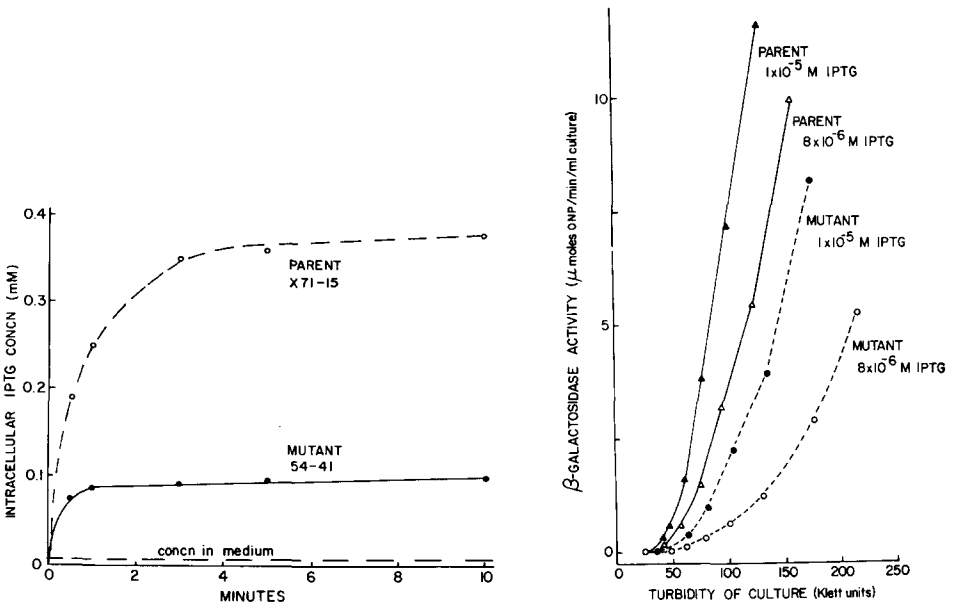


Fig. 1. Accumulation of IPTG by X71-15 and 54-41. Cells growing in logarithmic phase in Tryptone (30 ml) containing 0.5 mM IPTG were harvested by centrifugation at 4 °C and washed 4 times with 10 ml Medium 63 (pH 5.8) containing 2-mercaptoethanol (1 mM). Cells ($A = 830$) were incubated at 25 °C in Medium 63 (pH 5.8) in the presence of [^{14}C]IPTG ($8 \cdot 10^{-6}$ M, 0.2 μCi/ml). Cells were separated from the medium with a 0.6-μm filter, washed and counted.

Fig. 2. Induction of β-galactosidase by IPTG in X71-15 and 54-41. IPTG was added to final concentrations of $8 \cdot 10^{-6}$ M (open symbols) and $1 \cdot 10^{-5}$ M (closed symbols) to 30 ml of cells growing logarithmically on 0.4% glycerol in Medium 63 (pH 5.8). Samples (1.0 ml) were taken every 10–15 min for 4 h and immediately treated with toluene and deoxycholate. β-Galactosidase activity was determined at 37 °C by addition of 0.1 ml toluenized cells to 1.9 ml Medium 63, pH 7.0, containing 2 mM ONPG and 1 mM 2-mercaptoethanol. The reaction was stopped by addition of 4.0 ml 0.6 M Na_2CO_3 and yellow color was determined.

RESULTS

Induction of β-galactosidase

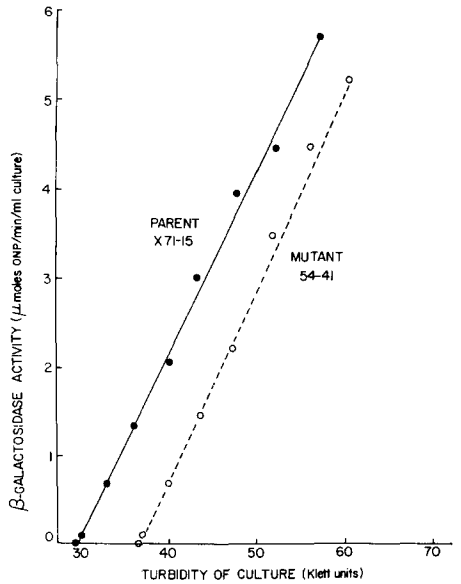
A remarkable feature of the inducible energy-uncoupled mutant was its inability to grow on lactose in a minimal salts medium. The possibility that an actively accumulating transport mechanism was necessary for normal induction was investigated. Induction of β-galactosidase by IPTG and lactose was studied in the inducible transductants X71-15 (parent) and 54-41 (mutant) growing logarithmically on glycerol. Both 54-41 and X71-15 grew with a doubling time of approximately 90 min during the induction experiments. In one series of experiments IPTG was added to final concentrations of $8 \cdot 10^{-6}$ M and $1 \cdot 10^{-5}$ M, and induction of β-galactosidase was followed (Fig. 2). β-Galactosidase was not induced as rapidly in 54-41 as in the parent strain. At these low inducer concentrations the rate of induction in both strains was dependent upon the external concentrations of inducer; however, the uncoupled mutant required a higher external IPTG concentration to achieve the same

rate of induction as the parent. Four similar experiments with low concentrations of IPTG confirmed the finding that 54-41 was induced at a slower rate than X71-15.

Normal rates of induction were obtained in the energy-uncloupled mutant when high inducer concentrations were used. Several experiments were performed in which IPTG was added to a final concentration of 0.5 mM to cells growing logarithmically on glycerol. Fig. 3 shows that under these conditions β-galactosidase was induced at the same rate in 54-41 as in X71-15.

Induction of β-galactosidase by addition of lactose to growing cells was also studied. An experiment in which lactose was added to a final concentration of 3 mM to cells growing exponentially on 0.4% glycerol is illustrated in Fig. 4. The enzyme was not induced as rapidly in the energy-uncoupled mutant as in the parent. Induction was somewhat faster in the mutant when 5 mM lactose was used; however, β-galactosidase was not induced in the mutant as rapidly as in X71-15. When 1 mM lactose was added as an inducer, there was an even greater difference in induction rates than is shown in the figure. When cells were grown on succinate (0.2%), the rates of induction of β-galactosidase in both X71-15 and 54-41 by 3 mM lactose were similar to those found in glycerol-grown cells.

Since induction of β-galactosidase by lactose and growth on lactose (see later section) were impaired in the inducible energy-uncoupled mutant, the possibility was considered that the mutant possessed an abnormal β-galactosidase. This hypothesis



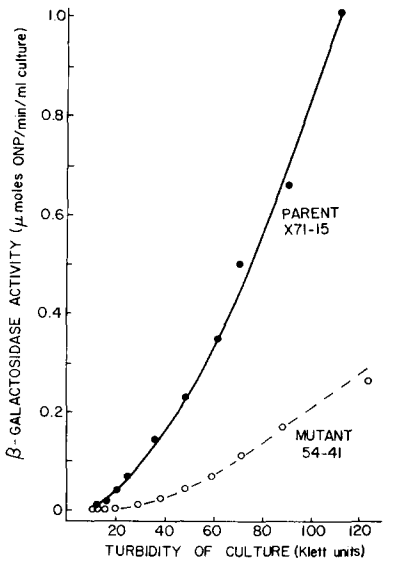


Fig. 3. Induction of β-galactosidase by 0.5 mM IPTG in X71-15 and 54-41. IPTG was added to 20 ml cells growing logarithmically on 0.4% glycerol in Medium 63 (pH 5.8). Samples (1.0 ml) were taken for assay of β-galactosidase every 10 min for 1.5 h. The cells were treated with toluene and enzyme activity determined as in Fig. 2.

Fig. 4. Induction of β-galactosidase by lactose in 54-41 and X71-15. Cells had been growing logarithmically for several generations on 0.4% glycerol in Medium 63, pH 5.8, (30 ml) when lactose was added to a final concentration of 3 mM. Samples (1.0 ml) were removed every 15–30 min for 5 h, toluenized and assayed for β-galactosidase as in Fig. 2.

was tested by measuring the affinity of β-galactosidase for lactose in both the mutant and parental cells. Fully induced, logarithmically-growing cells were washed and treated with toluene and used for assay of β-galactosidase by two methods. In one type of experiment the K_m of ONPG hydrolysis was determined and inhibition of ONPG hydrolysis by lactose was measured. In the second type of experiment lactose hydrolysis was measured directly by measuring glucose production. The results of these experiments are summarized in Table II and indicate that the β-galactosidase from the parental strain and the energy-uncoupled strain have similar properties. The K_m values for lactose agree well with the K_i values for lactose and indicate that about 5 mM lactose is necessary for half-maximal enzyme activity. From these measurements the enzyme appears to be the same in X71-15 and 54-41.

TABLE II

KINETIC PROPERTIES OF β-GALACTOSIDASE

Cells were grown to mid-logarithmic phase on Tryptone *plus* IPTG (0.5 mM) and prepared for β-galactosidase assay as in Materials and Methods. I. Cells treated with toluene were added to Medium 63, pH 7.0, (37 °C) containing 2-mercaptoethanol (1 mM) and ONPG (0.5 mM, 0.75 mM, 1.0 mM, 2.0 mM or 5.0 mM) with or without lactose (5 mM). The reaction was stopped by addition of 4 ml 0.6 M Na_2CO_3 to 2 ml assay mixture and yellow color measured. No more than 7% of the ONPG initially present in the assay was hydrolyzed. II. Cells treated with toluene were added to Medium 63, pH 7.0, (37 °C) containing 2-mercaptoethanol (1 mM) and lactose (1.25 mM, 2.5 mM, 5.0 mM or 10.0 mM). The reaction was stopped by addition of 1.5 ml cells to 0.6 ml 0.3 M $Ba(OH)_2$; assay tubes were immediately placed on ice and 0.6 ml 15% $ZnSO_4$ added 5 min later. The precipitate was removed by centrifugation and the supernatant used for glucose determination by the Glucostat assay. The cells treated with toluene did not metabolize glucose to a measurable extent and no more than 6% of the lactose was hydrolyzed.

Assay	*X71-15* ($i^+z^+y^+a^-$)	*54-41* ($i^+z^+y^{UN}a^-$)
I. ONPG as substrate		
V (mmoles/min per g)*	2.2	2.2
K_m (mM)	0.4	0.4
K_i of lactose (mM)	4.6	4.6
II. Lactose as substrate		
V (mmoles/min per g)*	0.31	0.31
K_m (mM)	4.9	5.1

* g wet wt.

Induction of α-galactosidase

E. coli K_{12} grows well on melibiose at 37 °C because the lactose membrane carrier transports this α-galactoside into the cell where it induces α-galactosidase which splits melibiose to form glucose and galactose[11,15] (the melibiose transport system is not active in *E. coli* K_{12} grown at 37 °C[16]). A striking finding was that the constitutive energy-uncoupled strain X71-54 did not grow as well as X71 on melibiose minimal medium agar plates[6]. In addition, X71-54 did not ferment melibiose on indicator plates[6] (see later section). The possibility was considered that the transport defect did not permit X71-54 to accumulate melibiose to a sufficiently high internal

concentration for full induction of α-galactosidase. This hypothesis was tested by studying the induction of α-galactosidase activity by melibiose in cells growing logarithmically on glycerol at 37 °C (Fig. 5). The energy-uncoupled mutant, X71-54, did not permit as rapid induction of α-galactosidase activity as the parent, X71.

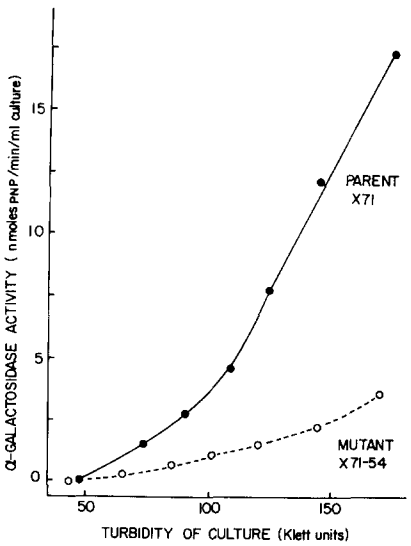


Fig. 5. Induction of α-galactosidase by X71 and X71-54 at 37 °C. Melibiose was added to a final concentration of 2 mM to cells growing logarithmically in 80 ml of 0.4% glycerol in Medium 63, pH 5.8. Samples (10 ml) were withdrawn every 30 min, centrifuged and washed twice with Tris buffer (50 mM, pH 7.6) containing chloramphenicol (40 μg/ml). The cells were resuspended in 1.5 ml Tris buffer (50 mM, pH 7.6) containing $MnSO_4$ (10 mM), 2-mercaptoethanol (0.1 M), and chloramphenicol (80 μg/ml). To 0.5 ml cells 0.5 ml PNPG (20 mM in Tris buffer) was added and the reaction allowed to proceed at 37 °C. The reaction was stopped by addition of 4.5 ml ice-cold 0.6 M Na_2CO_3 and 0.1 ml 200 mM EDTA. The cells were centrifuged and yellow color of the supernatant determined.

Melibiose did not induce α-galactosidase activity in a transport-negative strain, 316. It was possible to induce X71-54 at a somewhat faster rate by raising the external concentration of melibiose, but even at 25 mM melibiose, the highest concentration tested, the uncoupled mutant was unable to induce α-galactosidase as fully or as rapidly as the parent strain. By growing the cells on melibiose at 25 °C the specific melibiose transport system[16] could be induced and it was possible to induce α-galactosidase in X71-54 to 62% of the level present in X71. Full induction of X71-54 at 25 °C may have been prevented by exit of melibiose on the defective lactose transport system.

Growth properties

A previous communication[5] reported that the constitutive energy-uncoupled mutant X71-54 grew more slowly than X71 on low lactose concentrations, but both strains grew at the same rate on high concentrations. Similar growth experiments were performed with melibiose as the sole source of carbon and energy. The uncoupled mutant X71-54 grew more slowly than the parent strain on melibiose at sugar concentrations of 2 mM and 20 mM (Table III).

TABLE III

GROWTH RATE ON MELIBIOSE

The pH of the growth medium was 5.8.

Cell type	*Doubling time (min)*	
	2 mM melibiose	*20 mM melibiose*
X71	125	125
X71-54	900	540

A very striking finding was that the inducible energy-uncoupled mutant, 54-41, did not grow on lactose (5 M–50 mM) in liquid medium, but cultures of X71-15, the inducible parental strain, grew well. 2-day incubation of $4 \cdot 10^8$ cells of 54-41 in lactose led to the appearance of constitutive cells. When 0.5 mM IPTG was added to 5 mM lactose liquid medium, 54-41 grew at the same rate as the parental strain, and no constitutive cells appeared in the culture. The only conditions under which 54-41 could grow on lactose alone were those in which the cells were pre-induced with IPTG. Thus, cells grew fairly well if pre-grown to mid-exponential phase on 0.4% glycerol or 5 mM lactose minimal medium containing 0.5 mM IPTG, then washed and placed in 5 mM lactose. With this procedure the doubling time of the energy-uncoupled mutant varied from 90 to 120 min and the doubling time of X71-15 was about 65 min. An interesting finding was that the doubling time of the inducible uncoupled cells gradually became longer as the cells grew on lactose and eventually growth of 54-41 stopped altogether. In Fig. 6 is illustrated an experiment in which 54-41 and X71-15 were pre-induced on lactose with IPTG, washed, grown for 3–4 doublings on lactose alone and stored for 12–15 h at 4° C. The cells were then inoculated into fresh medium and growth followed. This procedure was repeated for a

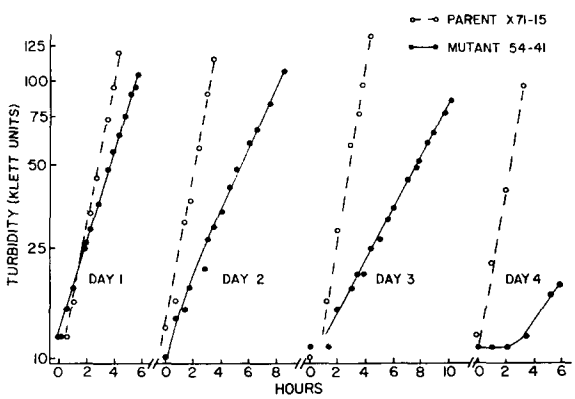


Fig. 6. Growth of X71-15 and 54-41 in lactose after pre-induction. Cells were grown to mid-logarithmic phase ($A = 140$) in 5 mM lactose *plus* 0.5 mM IPTG in Medium 63, pH 5.8 (10 ml), centrifuged, washed twice sterilly, and diluted into Medium 63 (pH 5.8) containing 5 mM lactose. Growth was followed for several doublings. The cells were placed at 4 °C overnight and the next day the cells were diluted into fresh medium and growth was again monitored. This procedure was followed for 4 days.

total of 4 days. With each succeeding day, the cultures of 54-41 grew more slowly while those of X71-15 continued growing at the same rate. By the fourth day there was no measurable growth of 54-41 for 3 h; when growth started again, it was found that 9% of the cells were constitutive. This growth regimen applied a very strong selective pressure for constituive cells and regardless of when measurable growth stopped (in some experiments after only 2 days) further incubation always led to the appearance of constitutive cells. In cultures of the parental strain constitutive cells never appeared.

The growth properties of the constitutive and inducible mutant and parental cells were tested on agar plates and are summarized in Table IV. The inducible energy-uncoupled strain grew very poorly on minimal medium lactose agar plates unless IPTG was present. Likewise, the MacConkey indicator plates showed that the inducible mutant fermented lactose very poorly unless IPTG was added. Both the constitutive and the inducible energy-uncoupled mutants fermented melibiose poorly. Glucose and galactose indicator plates showed normal fermentation for all the cells, indicating that glycolytic metabolism was not impaired in the energy-uncoupled mutants.

Since the energy-uncoupled mutant grew poorly on lactose and melibiose, it was possible that the mutant's membrane carrier had a greatly altered affinity for these sugars. This possibility was investigated by studying the kinetics of inhibition of ONPG entry by melibiose and lactose. Fully induced, growing cells were washed, resuspended in Medium 63 (pH 7.0) and exposed to various ONPG concentrations with or without melibiose, lactose or TDG. The results (Table V) indicate that the

TABLE IV

GROWTH OF CELLS ON AGAR PLATES

Minimal plates were incubated at 37 °C for 42 h. MacConkey plates were incubated for 15 h at 37 °C. Red clones indicate fermentation of the sugar.

Plate type	*X71* i^-y^+	*X71-54* i^-y^{UN}	*X71-15* i^+y^+	*54-41* i^+y^{UN}
I. Minimal medium plates (diameters in mm)				
Lactose (1 mM) pH 5.8	1.5	1.5	1.5	0.25
Lactose (10 mM) pH 5.8	1.5	1.5	1.5	0.5
Lactose (10 mM) pH 7.0	2	2	2	1
Lactose (10 mM) + IPTG (1 mM) pH 5.8	1.5	1.5	1.5	1.5
Melibiose (1 mM) pH 5.8	—	—	1	< 0.2
II. MacConkey indicator plates (clone color)				
Lactose, 0.2%	Red	Red	Red	Light pink
Lactose, 0.2% + IPTG, 0.5 mM	Red	Red	Red	Red
Melibiose, 0.2%	Red	Light pink	Red center	White
Glucose, 0.2%	Red	Red	Red	Red
Galactose, 0.2%	Red	Red	Red	Red

mutant's transport system has a somewhat higher affinity for melibiose and TDG than the parent's; the affinity of the uncoupled carrier for lactose is slightly lower than the affinity of the normal carrier.

TABLE V

INHIBITION OF ONPG ENTRY BY LACTOSE AND MELIBIOSE

Cells were grown to mid-exponential phase in Tryptone *plus* IPTG (0.5 mM) and assayed for entry of ONPG (0.75 mM, 1.0 mM, 1.5 mM, 2.0 mM, and 5 mM) as described in Materials and Methods. Inhibition of ONPG entry was determined using lactose (0.25 mM and 0.5 mM), melibiose (0.25 mM and 0.5 mM) and TDG (0.1 mM and 0.2 mM). TDG (10 mM) was used to determine non-carrier-mediated entry. The numbers in parentheses indicate the number of experiments and the data are expressed as the mean values ± S.E.

	X71-15 ($i^+z^+y^+a^-$)		*54-41* ($i^+z^+y^{UN}a^-$)	
V of ONPG (μmoles/min per g)*	231 ± 20	(4)	514 ± 52	(4)
K_t of ONPG (mM)	1.3 ± 0.1	(4)	2.5 ± 0.3	(4)
K_i of melibiose (mM)	0.3 ± 0.01	(3)	0.1 ± 0.007	(3)
K_i of lactose (mM)	0.24 ± 0.06	(3)	0.8 ± 0.2	(3)
K_i of TDG (mM)	0.06 ± 0.001	(3)	0.03 ± 0.002	(3)

* g wet wt.

DISCUSSION

Living cells have evolved two distinct types of membrane transport systems, (1) facilitated diffusion which catalyzes equilibration of the substrate concentrations on the two sides of the plasma membrane and requires no expenditure of energy by the cell, and (2) active transport which results in accumulation against an electrochemical gradient and requires metabolic energy. Facilitated diffusion is utilized by cells for substrates whose first intracellular enzyme possesses a relatively high affinity and whose extracellular substrate concentration is relatively high. Familiar examples of this type of transport include glucose uptake by most animal cells (*e.g.* muscle and red blood cells) and by yeast. Active transport, on the other hand, may be found in circumstances where the external substrate concentration is low. For maximum efficiency, the affinity for transport is greater than that of the first enzyme in the metabolic sequence (*e.g.* galactose in *E. coli*).

A well-known example of active transport is the lactose transport system of *E. coli*, which normally inhabits the mammalian lower intestine where the lactose concentration is probably low. Non-metabolizable thiogalactosides are accumulated to high levels in *E. coli* and lactose itself can be shown to accumulate in cells lacking β-galactosidase[1,2]. In normal cells, however, lactose is rapidly split after entry into the cell, and its intracellular concentration is assumed to be very low[2]. The advantages and disadvantages of facilitated diffusion *versus* active transport of lactose under physiological conditions have never been seriously considered. This question may now be approached experimentally since the energy-uncoupled mutants are severely defective in the capacity to accumulate while retaining the membrane carriers capable

of facilitated diffusion. Two important selective advantages of active transport have been clarified by this study. First, accumulation is required for the normal induction of the specific lactose metabolizing proteins and second, active transport is essential for normal growth on low concentrations of this disaccharide.

Induction

Induction of β-galactosidase in a normal, inducible *E. coli* is an autocatalytic process due to the concomitant induction of a membrane carrier capable of transporting inducer[1,2,17,18]. During induction with a low concentration of inducer, entry of a small amount of inducer into the uninduced cell permits binding of a small fraction of repressor to the inducer, enabling the cell to synthesize more molecules of β-galactosidase and membrane carrier proteins. Insertion of these new carrier proteins into the cell membrane enables the cell to accumulate more inducer which, in turn, will bind an even greater number of repressor molecules, permitting synthesis of more *lac* gene products. This cyclical process continues until the cell is making β-galactosidase and the lactose transport protein at the maximal rate[17].

During induction by sub-optimal concentrations of a gratuitous inducer, a fraction of the cell population synthesizes β-galactosidase at the maximal rate, while the rest of the cells remain uninduced[17,19]. Under these conditions, the chance entry of an inducer molecule into a cell begins the autocatalytic cycle resulting in rapid and full induction of that cell. During the same time period, inducer fails to enter other cells, and the transport protein and β-galactosidase are not produced. The concentration of inducer in the medium determines the probability of inducer's initially entering a cell and therefore determines the rate at which a particular growing culture will make β-galactosidase and the transport system[17]. On the other hand, when a growing culture of *E. coli* is exposed to high concentrations of a gratuitous inducer, such as IPTG, all cells are induced rapidly to the maximal rate of β-galactosidase synthesis, and this process has been found to be independent of membrane transport[18,20].

At low inducer concentrations the rate of induction of β-galactosidase is significantly slower in 54-41 than in its parent, presumably because the uncoupled cell cannot accumulate the inducer (Fig. 1). The rates of induction of both the parent and the energy-uncoupled mutant are dependent upon the external IPTG concentration; in order to achieve the same rate of induction as the parent, the uncoupled mutant requires a higher external IPTG concentration. These experiments suggest that induction with low IPTG concentrations is dependent upon the ability of the induced transport system to accumulate inducer.

When cells were growing in succinate or glycerol, the rate of induction by lactose (1–5 mM) was slower than that of the parent. These results may be due to a failure of the uncoupled cell to accumulate lactose or to the failure of the cells to retain the true inducer or both. This failure of induction had a dramatic effect on growth in lactose, as discussed in a later section.

The accumulation defect in the energy-uncoupled mutant had interesting consequences when melibiose was used as substrate. It is well known that in constitutive cells at 37 °C this α-galactoside enters the cell on the lactose transport system, a specific melibiose transport system being absent at this temperature[16]. Melibiose induces α-galactosidase which splits the disaccharide to glucose and galactose (β-galactosidase having no affinity for melibiose). Energy-uncoupled cells show a severe

defect in the induction of α-galactosidase by melibiose. This is presumably the explanation for the poor growth of such cells on this sugar. The failure of induction by melibiose is probably due to a defect in accumulation and not to a decreased affinity for uptake. Table V shows that the X71-54 transport system has a slightly increased affinity for melibiose.

Growth

Previous studies with energy-uncoupled cells constitutive for the lactose operon indicated that such cells were unable to grow normally at low external lactose concentrations[3,5]. One of these energy-uncoupled mutants, ML-308-22, failed to grow on lactose at a concentration of 0.25 mM while the parental cell (ML-308) grew normally[3]. Presumably when the external sugar concentration is very low, an active membrane transport system is necessary for accumulation of lactose to concentrations above the K_m of β-galactosidase (5 mM).

One dramatic consequence of defective active transport was the failure of inducible energy-uncoupled cells to grow on lactose at any concentration. This lack of growth is presumably due to failure of induction. When IPTG was added to the growth medium, however, the uncoupled cell grew at the same rate as the parent with 5–10 mM lactose. On lactose minimal plates 54-41 grew extremely slowly, and on MacConkey indicator plates the mutant showed almost no fermentation. These findings suggest that energy coupling is essential for an inducible cell to adapt to growth on lactose.

Induction by lactose is far more complex than that by IPTG. In 1965 Burstein *et al.*[21] showed that lactose was not an inducer of the lactose operon, but that several products of the action of β-galactosidase on lactose were very effective inducers. More recently it has been shown that *allo*-lactose, one of these products, is probably the true inducer when lactose is the substrate[22]. Since energy-uncoupled cells show an abnormally rapid rate of exit[3,4,9], it is possible that *allo*-lactose is not retained within the cell but leaves on the uncoupled membrane carrier. Once the inducer escaped from the energy-uncoupled cell, re-entry on the carrier would be blocked by lactose in the external medium. This loss of *allo*-lactose could account for the failure of the inducible energy-uncoupled cell to grow on lactose and for the impaired induction of β-galactosidase in 54-41 when lactose is added to cells growing in glycerol or succinate.

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