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ISOLATION AND PROPERTIES OF THIOGALACTOSIDE TRANSACETYLASE-NEGATIVE MUTANTS OF *ESCHERICHIA COLI*

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SUMMARY

Mutants which lacked thiogalactoside transacetylase were isolated from ML and K_{12} *Escherichia coli* strains. When the parental cells were incubated overnight on amino acid containing agar plates in the presence of thio[^{14}C]methyl-β-D-galactopyranoside ([^{14}C]TMG), they converted a considerable fraction of the external thiogalactoside to the acetyl derivative which diffused out of the cell into the incubation medium. The mutants, on the other hand, did not reduce the external TMG concentration by metabolism, and were consequently able to accumulate more radioactivity within the cells. ^{14}C content of each clone was determined by preparing an imprint of the clones on filter paper which, after drying, was exposed to photographic film. Several properties of these acetylase-negative mutants were studied.

INTRODUCTION

Preliminary to the study of mutants with abnormal membrane transport, a method was devised for assay of galactoside transport by cells in clones on agar plates. When mutagenized cells were spread on agar plates containing amino acids *plus* thio[^{14}C]methyl-β-galactoside ([^{14}C]TMG), a few of the resulting clones were found to accumulate abnormally large amounts of [^{14}C]TMG. These mutants were found to be of two types: the first of these accumulated large amounts of TMG phosphate (and will be discussed in a subsequent communication), and the second type consisted of cells lacking thiogalactoside transacetylase. The transacetylase-negative mutants exhibited several interesting features which will be the subject of this communication.

EXPERIMENTAL PROCEDURE

Bacteria

Two parental *Escherichia coli* strains were used in these studies. The first, isolated in the laboratory of Dr. J. MONOD, was ML-308 ($i^- z^+ y^+ a^+$) which is constitutive for β-galactosidase, β-galactoside transport and thiogalactoside transacetylase. The second, isolated by Dr. J. Beckwith, was X5072 ($i^- z^+ y^+ a^+$, $proC^-$, try^+, B_1^-)

Abbreviations: TMG, thiomethyl-β-D-galactopyranoside; IPTG, thioisopropyl-β-D-galactopyranoside; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

which was also constitutive for the lactose operon. Dr. J. Beckwith also kindly supplied a thiogalactoside transacetylase-negative strain lysogenic for acetylase-negative Φ80 transducing phages which were described by Fox *et al.*[1]. The mineral medium[2] used was Medium 63 *plus* NaCl which contains KH_2PO_4 (13.6 g), $(NH_4)_2SO_4$ (2.0 g), $MgSO_4 \cdot 7\ H_2O$ (0.2 g), $FeSO_4 \cdot 7\ H_2O$ (0.5 mg), as well as NaCl (2.9 g) per l of water and adjusted to pH 7.0 with KOH. The carbon and energy source for growth was casein hydrolysate (Tryptone, Difco) at a concentration of 1 %. In the case of X5072 cells and their mutants, L-tryptophan (10 μg/ml), L-proline (100 μg/ml) and thiamine (0.5 μg/ml) were also included in all growth media.

Logarithmically growing cells were obtained by transferring 0.5 ml of an overnight-grown cell suspension to a 250-ml sidearm flask containing 30 ml of fresh medium containing amino acids as carbon and energy source and growing on a rotary shaker at about 200 rev./min at 37° for several hours. These cells were then centrifuged at 4°, washed by suspending in mineral Medium 63, again centrifuged and then suspended to a suitable cell density in Medium 63 containing chloramphenicol (50 μg/ml) in the presence or absence of glucose.

Chemicals

Thio[^{14}C]methyl-β-D-galactopyranoside ([^{14}C]TMG) and [^{14}C]acetyl-1-coenzyme A were obtained from New England Nuclear Corp.; thio[^{14}C]isopropyl-β-D-galactopyranoside ([^{14}C]IPTG) was obtained from Schwarz Bioresearch. Nonradioactive TMG, IPTG, and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were obtained from Calbiochem Corp. Chloramphenicol was a gift from Parke, Davis and Co.

Mutagenesis and selection of acetylase-negative mutants

Washed cells from exponential phase, either ML-308 or X5072, were treated for 20 min at 37° with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at a concentration of 100 μg/ml in 0.02 M Tris–maleic acid buffer (pH 6.0), according to the method of ADELBERG, MANDEL AND CHEN[3]. After mutagenesis 0.5 ml of the above suspension was added to 25 ml of rich medium (Antibiotic Medium No. 3, Difco) and grown aerobically overnight for phenotypic expression. In the isolation of ML-308-831 and ML-308-1133 2 ml of cells from rich medium were added to a 50-ml screw-cap test tube and filled to the top with Medium 63 containing 0.4 % lactose. After growth overnight at 37°, 5 ml of the suspension were inoculated into a second tube containing similar medium and grown a second time overnight anaerobically at 37°. The same general procedure was used for the isolation of X5072-71 except that the growth on lactose was aerobic. Cells were then diluted appropriately in Medium 63 and spread on TMG transport assay plates (which consisted of 7.5 g agar, 2.5 g NaCl, 0.45 ml 1 M K_2HPO_4, 4 g Tryptone per 500 ml water, to which were added after autoclaving 2.5 ml of triphenyltetrazoliumchloride (10 mg/ml) and 2.5 ml of 5 mM [^{14}C]TMG at 10 μC/ml, both sterilized by Millipore filtration). After growth for 18 h at 37°, sterile Whatman No. 1 filter paper was pressed on the surface of the clones and a portion of each clone was removed onto the filter paper as a red spot. After drying, this paper was placed face down on X-ray film (Kodak medical X-ray "No Screen" film) and exposed for 1–2 days. On development of the film the wild type clones appeared as grey circles while acetylase-negative mutants appeared black (Fig. 1). These clones were identified on the original plate and recloned.

Assays

Thiogalactoside transacetylase was assayed by the method of FOX AND KENNEDY[4]. Heat treated cells (70° for 5 min) were incubated in the presence of IPTG and [^{14}C]acetyl-coenzyme A. [^{14}C]Acetyl-IPTG was measured after separation from the coenzyme A on a Dowex-1 (formate) column.

Active transport of TMG was measured[5] by exposing washed cells to [^{14}C]TMG for various periods of time at 25° followed by separation of the cells from the medium on Millipore filters placed in liquid scintillation vials for counting. Transport of ONPG was measured indirectly as the *o*-nitrophenol liberated following the incubation of intact cells in this β-galactoside.

Chromatography

Acetylated TMG was separated from TMG by descending chromatography on Whatman No. 1 paper with 2-propanol–H_2O (3:1, by vol.). The papers were dried and exposed to X-ray film (Kodak medical X-ray "No Screen" film) for several days. The developed film was used to identify the position of compounds on the paper chromatogram. The following R_F values were obtained: TMG, 0.62; acetylated TMG, 0.75; TMG phosphate, 0.20.

Quantitative determination of these substances was carried out by liquid-scintillation counting of the areas of the paper containing the radioactive material.

RESULTS

An assay was developed for TMG transport in clones growing on agar plates. The principle was to grow cells in the presence of [^{14}C]TMG with amino acids as the carbon and energy source. The extent of TMG accumulation by cells of an individual

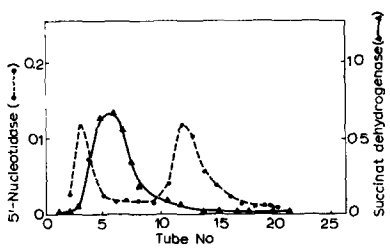

Fig. 1. Radioautograph of imprint of clones on filter paper. Mutagenized cells were spread on a rich plate containing triphenyltetrazolium *plus* 0.5 mM [^{14}C]TMG (0.05 μC/ml) final concn. Whatman No. 1 filter paper was pressed lightly on clones to make imprint. After drying, the paper was exposed to X-ray film for 2 days. Note dark clone at 12 o'clock.

Fig. 2. Accumulation of radioactivity within cells growing in the presence of [^{14}C]TMG. 2.0 ml of an overnight culture (2 mg dry wt./ml) were added to 10 ml of Medium 63 containing 1 % amino acids *plus* 0.5 mM [^{14}C]TMG. Samples were removed at intervals and filtered on Millipore filters, as described previously[5].

clone was determined by removing a portion of the clone on filter paper and after drying, determining radioactivity by exposure of filter paper to X-ray film. A similar technique has been recently used by ZWAIG AND LIN[6] to assay steps in glycerol metabolism. When transport positive (ML-308) and transport negative organisms (ML-35) were compared by this procedure, the clones of ML-35 were found to take up much less radioactivity than the clones of ML-308.

When mutagenized cells were grown twice on 0.4 % lactose and then assayed for TMG accumulation, several clones were discovered which contained more radioactivity than the parent. The appearance of one such clone is shown in Fig. 1.

These mutants showed a similar behavior in liquid medium. When stationary phase cells were grown in amino acids *plus* [^{14}C]TMG, increasing intracellular levels of radioactivity were found during the first 3 h in both the wild type (ML-308) and the two mutants until mid-exponential phase was reached (Fig. 2). Further incubation led to a marked fall in the intracellular level of radioactivity in the ML-308, no comparable fall being observed with the mutants. Cells grown in parallel without TMG were removed at the termination of this experiment, washed and assayed for transport capacity with ONPG and [^{14}C]TMG. There was no transport abnormality found in these mutants (Table I). The possibility was considered that the presence of amino acids or some condition related to growth might be involved in the differences. Therefore [^{14}C]TMG was added to late exponential phase cells in their growth medium and uptake assayed for a 10-min period. Again, no transport abnormality was found.

TABLE I

GALACTOSIDE TRANSPORT IN CELLS GROWN TO STATIONARY PHASE

Cells were grown under the same conditions (and on the same day) as those in Fig. 2 but without [^{14}C]TMG. After 7 h incubation the cells were washed and assayed for ONPG (1 mM) hydrolysis in intact cells and for [^{14}C]TMG (0.5 mM) accumulation.

Organism	*ONPG transport (μmoles/min per g wet cells)*	*TMG transport, intracellular concn after 15-min incubation (mM)*
ML-308	39	40
ML-308-831	37	37
ML-308-1133	39	37

Intracellular and extracellular derivatives of TMG

After growth of cells for 6 h in the presence of [^{14}C]TMG, cell extracts were prepared and examined by paper chromatography, followed by autoradiography. Two major metabolites of TMG were found in ML-308. First, a slow-moving spot ($R_F = 0.2$) which proved to be retainable on a Dowex (formate) column and was presumed to be TMG phosphate. In this solvent TMG migrated with an $R_F = 0.62$. The second metabolite was a fast-moving spot ($R_F = 0.75$). This was presumed to be acetylated TMG as it was not produced by a transacetylase-negative organism incubated under similar conditions. As shown in Fig. 3, 17 % of the radioactivity within the cell was this acetylated compound. In the growth medium from which these cells had been removed, 85 % of the radioactivity was in the acetylated form.

When the mutant ML-308-831 was examined in a similar manner, it was found to lack the acetylated compound both inside the cell and in the growth medium. About 10% of the intracellular radioactivity was in the form of the phosphate derivative.

In agreement with the previous finding of normal transport in the mutant, the steady state ratios of TMG concentration inside to outside of parent and mutant were the same (Fig. 3). Thus the level in the parent was low as a direct consequence of the low TMG concentration in the external medium.

The time course of the acetylation reaction was measured with washed cells of ML-308 and ML-308-831 in the presence of chloramphenicol (Fig. 4). In this experiment glucose was added to inhibit the phosphorylation of TMG and less than 1% of the radioactivity within the cells could be attributed to TMG phosphate. ML-308 converted 40% of the TMG in the growth medium to the acetylated compound, while ML-308-831 produce none. At the end of the 90 min incubation period the remaining

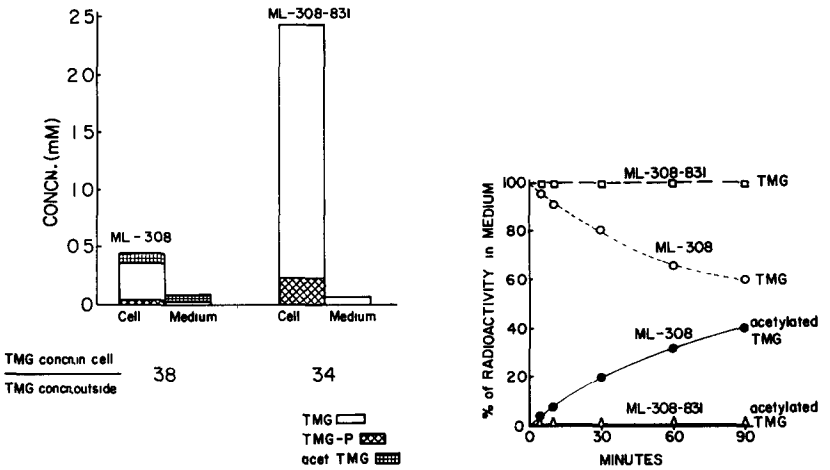

Fig. 3. Identification of intracellular and extracellular radioactive compounds following growth of cells in the presence of [^{14}C]TMG. 2.0 ml of stationary-phase cells (2 mg dry wt./ml) were added to 10 ml of Medium 63 containing 1% amino acids *plus* [^{14}C]TMG (0.083 mM, 0.24 μC/ml final concn.). The cultures were shaken in siderarm flasks for 6 h at 37°. The cells were centrifuged and supernatant removed. To the pellet was added 2 ml 10% trichloroacetic acid at room temp After centrifugation the supernatant fluid was extracted 3 times with diethyl ether until the pH reached 5 and chromatographed with 2-propanol–H_2O (3:1, by vol.) as solvent for 18 h (Whatman No. 1, descending). After drying, the chromatogram was exposed to X-ray film for 2 days. The radioactive spots corresponding to the dark areas on the film were cut from the paper and counted in a liquid scintillation counter. The compound running faster (R_F = 0.75) than TMG (R_F = 0.62) was not found in cells which were acetylase-negative and was therefore taken to be 6-*O*-acetyl-TMG. A slowly moving compound (R_F = 0.2) was retainable by Dowex (formate) columns at neutral pH and was assumed to be TMG phosphate.

Fig. 4. Acetylation of TMG by intact cells of ML-308 and ML-308-831. Cells grown to exponential phase were washed and resuspended to an absorbance of 600 Klett units (1.3 mg dry wt./ml) in 15 ml of Medium 63 containing chloramphenicol (50 μg/ml), glucose (5 mM) and [^{14}C]TMG (0.2 mM; 0.5 μC/ml final concn) The cells were incubated with shaking at 25°. At various time intervals 0.5 ml of the suspension was passed through a Millipore filter (0.45 μ pore size) and 0.1 ml of filtrate removed for chromatography. At the end of 90 min of incubation, the cells were centrifuged and the pellet extracted with 0.5 ml boiling water. The cell debris was centrifuged and a 0.1 ml of the supernatant fluid was chromatographed and exposed to X-ray film for 2 days, as described in Fig. 3. Paper chromatograms were cut out and counted in a liquid scintillation counter.

cells were centrifuged and the intracellular radioactivity chromatographed. While the intracellular TMG concentration in ML-308-831 was 16 mM compared with 9.8 mM for ML-308, the final concentration ratios (inside/outside) were similar (115 and 127, respectively). The concentration of acetylated TMG inside ML-308 was 40 times higher than outside, in this experiment.

A similar experiment was performed with [^{14}C]IPTG (Fig. 5). More than 70% of the sugar was acetylated by the parent strain. At the end of the 60 min incubation the remaining cells were separated from the medium. Extracts of these cells revealed that 80% of the intracellular radioactivity was acetylated IPTG and 20% free sugar. In agreement with previous findings ML-308-831 showed no acetylation

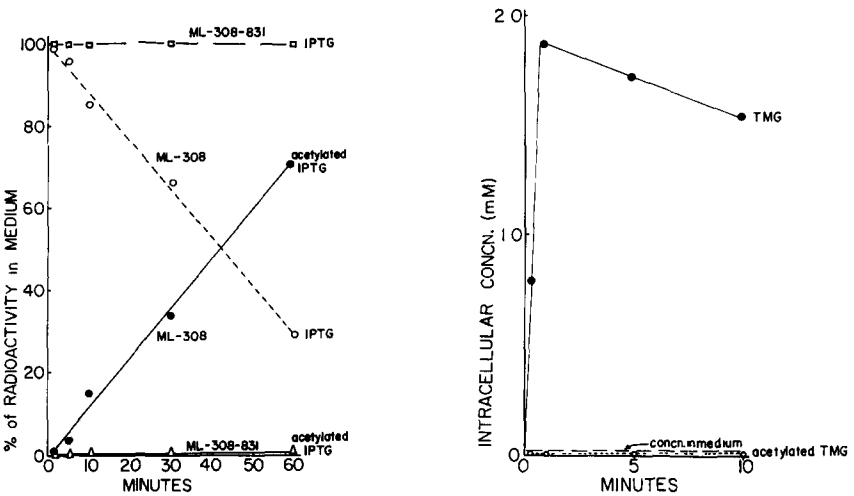

Fig. 5. Acetylation of IPTG by intact cells of ML-308 and ML-308-831 Cells grown to exponential phase were washed and exposed (at a concentration of 1.1 mg dry wt./ml) to chloramphenicol (50 μ/ml), glucose (5 mM) and IPTG (0.013 mM, 0.5 μC/ml final concn.). The general procedure is given in Fig. 4.

Fig. 6. The inability of ML-308-831 to actively transport acetylated TMG. Washed exponential phase cells were incubated at concentrations of 500 Klett units (1.1 mg dry wt./ml) in the presence of [^{14}C]TMG or acetylated [^{14}C]TMG at a concn. of 0.02 mM. The two radioactive sugars were prepared by incubating ML-308 cells in medium containing [^{14}C]TMG, removing the cells, chromatogramming the incubation medium, and eluting from the paper. Cell suspensions (0.20 ml) were filtered on a Millipore filter (0.65 μ pore size), rinsed with 5 ml Medium 63 and counted in a liquid scintillation counter.

Possible active transport of acetylated TMG

Radioactive acetylated TMG was prepared by elution from paper chromatograms of incubation medium from which ML-308 had been removed. When ML-308-831 was incubated in the presence of ^{14}C-labeled acetylated TMG, it entered the cells slowly and only reached diffusion equilibrium after 10 min (Fig. 6). No transport against a concentration gradient was observed in the three experiments performed. Under the same conditions, however, TMG similarly eluted from chromatograms was actively transported.

Acetylated IPTG was similarly tested. In three experiments this acetylated

sugar entered ML-308-831 only slowly and never reached an internal concentration higher than that in the external medium.

Assay for thiogalactoside transacetylase

Transacetylase was assayed in parental and mutant strains by the method of FOX AND KENNEDY[4]. Table II shows that ML-308-831 had no detectable enzyme activity while the other mutants had only very low activity.

TABLE II

THIOGALACTOSIDE TRANSACETYLASE ASSAY

Organism	*Transacetylase activity (nmoles IPTG acetylated/100 μg dry wt. per 30 min)*	*Genotype*
ML-strains		
ML308	3.7	(i^- z^+ y^+ a^+)
ML-308-225	5.2	(i^- z^- y^+ a^+)
ML-35	1 5	(i^- z^+ y^- a^+)
ML-308-831	<0.005	(i^- z^+ y^+ a^-)
ML-308-1181	0.04	(i^- z^+ y^+ a^-)
K_{12}-strains		
X5072	3.0	(i^- z^+ y^+ a^+)
X5072-71	0.1	(i^- z^+ y^+ a^-)

Isolation of transacetylase mutant from X5072

The general procedure was repeated with X5072 and a mutant (X5072-71) isolated. The mutant possessed normal β-galactosidase, normal transport capacity as measured by TMG accumulation (15 sec uptake) and ONPG hydrolysis, and 3 % of the normal transacetylase activity.

DISCUSSION

RICKENBERG *et al.*[7] discovered that during the transport of radioactive thiogalactosides by *E. coli* a small amount of radioactivity migrated with a R_F different from that of the parent compound. HERZENBERG[8] subsequently identified this metabolite as the 6-*O*-acetyl derivative of the parent thiogalactoside. The enzyme which carries out the acetylation has been designated thiogalactoside transacetylase and has been the subject of a variety of investigations[9–13]. The present study has confirmed the findings of FOX *et al.*[1] that the lack of this enzyme has no detectable effect on membrane transport and no effect on growth rate in 0.2 % lactose, 0.2 % glucose or 1 % amino acids.

The presence of thiogalactoside transacetylase can be troublesome when studying certain aspects of active transport of thiogalactosides in *E. coli*. While short incubations of cells with TMG lead to relatively little acetylation, prolonged exposure results in such extensive acetylation that considerable reduction in the extracellular

concentration of the original sugar may occur. This reduction of sugar concentration with the consequent fall in intracellular concentration of TMG was the basis on which acetylase-negative organisms were isolated in the present study. Another type of transport experiment in which long incubation times are required are those experiments in which cells are preloaded prior to measuring exit rates. Under these conditions 10% or more of the intracellular TMG would be in the form of the acetylated derivative. The addition of glucose increases the rate of acetylation[13]. For extensive studies of membrane transport with thiogalactosides it would appear to be advantageous to utilize a transacetylase-negative organism.

The loss of the acetylated derivative from the cell is interesting and provokes the question as to the route of exit. It is clear from the present work that the acetylated derivatives of TMG and IPTG cannot be actively transported by the lactose transport system. Presumably an alternative route of exit is used, although one cannot exclude a very poor affinity for the lactose carrier. As the concentration within the cell is always much higher than that in the external medium, the driving force for movement is probably the concentration gradient.

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