Roles of Individual mgl Gene Products in the \( \beta \)-Methylgalactoside Transport System of Escherichia coli K12

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Previous findings showed that galactose-binding protein defective mutants (mgl \( B^{-} \), \( A^{+} \), \( C^{+} \)) of Escherichia coli K12 are still capable of growth on methyl-\( \beta \)-d-galactopyranoside, while mgl \( A^{-} \) and mgl \( C^{-} \)mutants are not. When assayed by previous methods, none of these mutants exhibited methylgalactoside transport system activity. In this study, we present a modified assay developed for measuring low levels of transport. Using this assay, we found that mgl \( B^{-} \), \( A^{+} \), \( C^{+} \) mutants defective in galactose-binding protein accumulate methyl-\( \beta \)-d-galactopyranoside up to six times the concentration gradient while mgl \( A^{-} \) and mgl \( C^{-} \)mutants failed to accumulate this substrate. Similar results were obtained using D-galactose-\( \beta \)-d-galactopyranoside, another substrate of the methylgalactoside transport system. In contrast, all sugars tested which are not substrates of this system were transported equally by all mgl mutants.

The kinetic parameters of transport in mgl \( B^{-} \)mutants were compared to those of the isogenic mgl+ strain which accumulates methyl-\( \beta \)-d-galactopyranoside against a 10,000-fold concentration gradient. The apparent \( K_{m} \) of methyl-\( \beta \)-d-galactopyranoside influx was 1,000 times greater in mgl \( B^{-} \) than in mgl+ strains. In contrast, there was no significant difference between these strains in either the \( V_{\text{max}} \) of substrate influx or the rate of substrate exit. D-Galactose competitively inhibited methyl-\( \beta \)-d-galactopyranoside influx into both mgl \( B^{-} \) and mgl+ strains; the \( K_{i} \) of inhibition in mgl \( B^{-} \) cells was 2,000-fold greater than that in mgl+ cells.

The \( \beta \)-methylgalactoside permease of Escherichia coli K12 is a multicomponent transport system dependent on three genes, mgl \( A \), \( B \), and \( C \) (1, 2). Only the product of mgl \( B \), the galactose-binding protein, has been identified (3, 4). A galactose-binding protein independent activity which requires both mgl \( A \) and mgl \( C \) has been demonstrated; mgl \( B \) mutants were observed to grow on \( \beta \)-methylgalactoside while isogenic mgl \( A \) and mgl \( C \) mutants did not (5).

In this report we further characterize this galactose-binding protein independent transport activity using direct measurements of substrate flux.

Materials and Methods

Bacteria and Culture Media—With the following exceptions, the genotypes and derivation of all strains used here have been previously reported (5, 6). S185-27 [his, str, lac(Z,Y,A), ara(C,O), mgl D,B], S185-27T [his, str, lac(Z,Y,A)\( \beta \), ara(C,O)\( \beta \), mgl D,B,C], and S185-726 [his, str, lac(Z,Y,A)\( \beta \), ara(C,O)\( \beta \), mgl D] were constructed as follows. A deletion of ara \( C \) and \( O \) was introduced into S183 reported (5, 6). S185-27 [his, str, lac(Z,Y,A), ara(C,O), mgl D,B] were constructed as follows. A deletion of ara \( C \) and \( O \) was introduced into S183 reported (5, 6) to yield the corresponding derivatives of S185. Cells were grown at \( 37^\circ \)C in DM minimal medium (7) supplemented with 0.4% sodium lactate (Fisher Scientific Co., Fair Lawn, N. J.) and 0.5 pg/ml of vitamin B1. Required amino acids were supplied at a concentration of 10 \( \mu \)g/ml. For most experiments, cultures were grown in flasks which were shaken by gyration. Chemicals—Carrier-free D-galactose-\( \beta \)-d-galactopyranoside and \( \beta \)-d-galactopyranoside were synthesized in this laboratory by the method of Avigad (8). Briefly, this consists of oxidizing the 6-OH group of the galactose with galactose oxidase (EC 1.1.3.9, Worthington Corp., Freehold, N. J.) and then reducing it with carrier-free NaB\( \beta \)H\(_{4}\) (New England Nuclear Corp., Cambridge, Mass.) and purified as above. Unlabeled \( \beta \)-methylgalactoside was obtained from Nortok Assoc. (Lexington, Mass.).

Transport Assays—Intracellular accumulation of radioactive substrate was measured using cells harvested during exponential growth. Cells were centrifuged and resuspended with fresh growth medium before testing. The assay mixtures (4 ml final volume) for measuring the transport activity of mgl+ strains contained about 5 \( \times \) 10\(^{6} \) cells suspended in growth medium, 200 \( \mu \)g of chloramphenicol, and 1.39 nmoi of \( [\beta \]C]methyl-\( \beta \)-d-galactopyranoside (10 \( \mu \)Ci). After incubation at room temperature (22-24°C) with shaking, the mixtures were filtered through HA Millipore membranes. The radioactivity of the
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dried Millipore membranes was determined by liquid scintillation (efficiency for \({^3}C, 68\%\); for \({^3}H, 23\%\)). Blank values were obtained using formaldehyde-treated cells in the assay (9).

The transport activity of \(mgl\) mutants was measured by a modification of the above assay. This modified test, referred to as the "60x assay," was performed as follows: 0.08 ml of a washed cell suspension containing about \(1 \times 10^9\) cells was incubated with the radioactive substrate in a final volume of 0.1 ml which contained 2.77 nmol of \(\beta\)-methyl-D-galactopyranoside (2 x \(10^4\) cpm) and 0.05 mg of chloramphenicol. The assay was terminated by adding 2.9 ml of growth medium containing 33 ng/ml of chloramphenicol, filtering the mixture through a Millipore membrane, and washing the membrane (at room temperature) with 3 ml of growth medium. The radioactivity of the membranes was determined and corrected for blank values as indicated above. Unlike otherwise stated, cells were incubated with the substrate at 28\(^\circ\). In measurements of initial rate of uptake, the cells accumulated less than 5\% of the extracellular substrate.

RESULTS

An indirect assay, i.e. growth of bacteria on \(\beta\)-methy1galactoside, was previously used to demonstrate a transport function in \(mgl\) mutants which exhibit no transport activity in routine measurements of intracellular substrate accumulation. This partial transport function was found to depend on two (\(mgl\ A\) and \(mgl\ C\)) of the three genes required for \(\beta\)-methylgalactoside permease activity; no dependence on galactose-binding protein (the product of \(mgl\ B\)) was observed (5). A direct assay for the partial transport activity exhibited by \(Tra^+\) mutants (\(mgl\ A^+, B^- C^+\)) and the applications of this assay are described here.

Since growth of \(Tra^+\) mutants is observed only at or above 1 \(\mu\)M \(\beta\)-methylgalactoside (5), we tried to measure substrate flux into these mutants using elevated substrate concentrations. However, it was found that increasing the concentration of radioactive substrate in our routine permease assay caused a concomitant increase in the nonspecific radioactivity bound to Millipore membranes. For this reason the assay was modified such that the cells were exposed in a reduced volume to radioactive \(\beta\)-methylgalactoside, and intracellular uptake of substrate was terminated by diluting the assay mixture (see "Materials and Methods"). As shown in Table I, when \(Tra^+\) and \(Tra^-\) cells were compared using this modified assay (hereafter referred to as the "60x assay"), differences in transport activity greater than 4-fold were obtained. This difference increased when 5 \(\mu\)M unlabeled substrate was present in the assay. Washing the cells on the Millipore membranes did not significantly alter the relative transport activities of \(Tra^+\) and \(Tra^-\) cells. This procedure was adopted since assays incorporating washing of the cells lowered the blanks resulting in improved discrimination between the two classes of mutants.

The characteristics of the 60x assay of \(Tra^+\) activity were investigated. The intracellular concentration of \(\beta\)-methylgalactoside increased linearly with time from 0 to 5 min, reaching a steady state at about 20 min; the bulk of the intracellular radioactivity could be eliminated by a cold chase (Fig. 1). The steady state concentration of intracellular \(\beta\)-methylgalactoside was independent of the number of cells used in the assay over the range 4 \(\times\) \(10^6\) to 1.2 \(\times\) \(10^8\) cells (Fig. 2). No evidence of chemical alteration of the intracellular substrate (at the detection level of 1.0\%) was found by chromatographic analyses of the accumulated radioactivity. The optimal temperature for \(Tra^+\) activity was 28\(^\circ\) to 31\(^\circ\), as compared to 18\(^\circ\) to 21\(^\circ\) for the \(\beta\)-methylgalactoside permease in \(mgl\) cells.

\(Tra^+\) activity was also demonstrable using another substrate of the \(\beta\)-methylgalactoside permease, \(D\)-glyceryl-\(\beta\)-D-galactopyranoside, in the 60x assay (S185-27) measured by the 60x assay (Fig. 1). Cells were incubated with substrate for the indicated time. A value of 1000 cpm per \(10^8\) cells is equivalent to 154 pmol/\(10^8\) cells. At the time designated by the arrow, 80 \(\mu\)M unlabeled \(\beta\)-methylgalactoside was added to some of the assay mixtures (O). The results are corrected for blanks which ranged from 22 to 50 cpm/\(10^8\) cells. Blank values were not proportional to time of incubation.

The failure of \(Tra^-\) cells to accumulate substrates of the methylgalactoside permease in the 60x assay cannot be explained by a generalized defect in the permeability of these mutants, since both \(Tra^+\) and \(Tra^-\) cells exhibited significant uptake of radioactive 2-deoxyglucose and 3-O-methylglucose (Table II).
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Fig. 2. Effect of cell number in the 60x assay. The intracellular accumulation of $[^{14}C] \text{methylgalactoside}$ by the Tra+ mutant S185-27 (○) and the Tra- mutant S185-27T (□) was measured following a 3-min incubation in the presence of 5 mM unlabeled substrate. Blank values were independent of both strain and cell number; the range of blanks was from 10 to 20 cpm. A value of 10 nmol is equal to 400 cpm. The inset shows the results obtained with these strains in the absence of unlabeled substrate. The ordinate is in picomoles. A value of 50 pmol is equal to 373 cpm.

We compared the initial rates of $\beta$-methylgalactoside influx into Tra+ and isogenic mgl+ cells at different $\beta$-methylgalactoside concentrations. There was a 1000-fold difference between the apparent $K_m$ of influx measured with Tra+ mutants (Fig. 3A) and the $K_m$ of the mgl+ strain (Fig. 3B). In contrast, the maximal influx ($V_{max}$) of $\beta$-methylgalactoside of Tra+ cells was found to be comparable to that of mgl+ cells, 15 and 19 nmol/min/10^9 cells, respectively.

The apparent $K_m$ of Tra+ cells for D-galactose was measured

![Graph](image)

Fig. 3. Double reciprocal plots of initial rate of $\beta$-methylgalactoside uptake ($V_{max}$) versus extracellular substrate concentration ($C$). A, the Tra+ mutant S185-27 was tested using the 60x assay at 28°C with incubation times of 3 min. B, the isogenic mgl+ strain S185-726 was tested using the routine permease assay at 28°C with incubation times of 1 min. The concentration of substrate was adjusted by varying the amount of unlabeled $\beta$-methylgalactoside. Blank values were independent of concentration of unlabeled substrate; for A, they ranged from 34 to 46 cpm/10^9 cells, and for B, from 147 to 194 cpm/10^9 cells.

Table II

| Sugars (specific radioactivity) | Intracellular accumulation | Ratio $K_m^{+/}/K_m^{\text{-}}$ |
|-------------------------------|---------------------------|-------------------------------|
| [$^3H]$Methyl-$\beta$-D-galactopyranoside (4.78 mCi/mmol) | 955 (15) 109 (8) | 8.8 |
| D-Glyceryl-$\beta$-[6-$^3H$]galactopyranoside (4.8 mCi/mmol) | 732 (64) 180 (60) | 3.7 |
| [$^3C]$Methyl-$\beta$-D-galactopyranoside (1.85 mCi/mmol) | 183 (83) 142 (96) | 1.3 |
| [$^3C]$Methyl-$\beta$-D-galactopyranoside (8.7 mCi/mmol) | 210 (25) 171 (20) | 1.2 |
| [$^3C]$Methyl-$\beta$-D-galactopyranoside (10.3 C/mmol) | 156 (47) 202 (55) | 1.0 |
| [$^3C]$Methyl-$\beta$-D-galactopyranoside (0.86 C/mmol) | 118 (41) 99 (39) | 1.2 |
| [$^3C]$Methyl-$\beta$-D-galactopyranoside (7.5 C/mmol) | 17 (23) 14 (24) | 1.2 |
| 3-O-$[^3H]$Methylglucose (3.6 C/mmol) | 2519 (1029) 2697 (815) | 0.9 |
| 2-[3H(G)]Deoxyglucose (10.3 C/mmol) | 2519 (1029) 2697 (815) | 0.9 |

Fig. 2. Intracellular accumulation of various sugars in Tra+ and Tra- cells

The intracellular accumulation in S185-27 (Tra+) and S185-27T (Tra-) was measured using the 60x assay with the indicated radioactive sugars. The accumulation was determined after 30-min incubation at 28°C using 10^9 cells/assay. The results are corrected for the indicated blanks which were prepared by adding the cells after dilution of the substrate. The blanks were incubated 30 min at 20°C before filtration. Thus these blanks are equivalent to results from routine permease assays in which the concentration of radioactive substrate is doubled.
by D-galactose inhibition of β-[14C]methylgalactoside influx. This procedure was necessary since Escherichia coli possesses a number of transport systems with affinity for D-galactose (7). Our results indicate that D-galactose competitively inhibits influx of β-methylgalactoside with a $K_i$ of 6.3 mM (Fig. 4). The apparent $K_m$ of D-glyceryl-β-D-galactopyranoside accumulation, measured directly at the steady state, was 20 mM. The accumulation of D-glyceryl-β-D-galactopyranoside was inhibited competitively by β-methylgalactoside with a $K_i$ of 20 mM.

A series of independent, isogenic mgl- mutants were compared with respect to their transport of β-methylgalactoside at 27.7 μM and 5 mM external substrate; these concentrations are near the $K_m$ values of influx of mgl+ and Tra+ cells, respectively. As shown in Fig. 5, at 27.7 μM substrate all Tra+ mutants tested accumulated more than 0.1 nmol/10^9 cells; at 5 mM all transported more than 10 nmol/10^9 cells. In contrast, the accumulation in Tra- mutants at 27 μM substrate was about 33% of the average Tra+ accumulation with the exception of one mgl A- mutant (S183-10) which accumulated to levels comparable to those attained by Tra+ cells. However, at 5 mM none of the Tra- mutants (including S183-10) transported more than 2.4 nmol/10^9 cells, only 15% of the accumulation observed in Tra+ cells.

Efflux of β-methylgalactoside down a concentration gradient was also compared in Tra+ and mgl+ cells. Cells were incubated with substrate until they had attained a steady state of accumulation, then diluted 50-fold into prewarmed medium, and samples were measured at intervals in order to determine the remaining intracellular substrate concentration. As shown in Fig. 6, the rates of efflux measured for Tra+ and mgl+ cells were comparable, loss of half the intracellular substrate occurring in 4.3 and 5.6 min, respectively. Recapture of substrate under the conditions of these experiments was shown to be negligible since accumulation was reduced by 95% upon the 50-fold dilution of extracellular substrate.

**DISCUSSION**

Isogenic mgl+ and Tra+ (mgl- A+, C+) cells, which differ only in the presence or absence of functional galactose-binding protein, accumulate β-methylgalactoside intracellularly to maxima of 10,000 and 6 times the concentration gradient, respectively. The lower level of accumulation achieved by cells which lack the binding protein is shown to result from a decrease in their affinity for extracellular substrate. This conclusion is based on our findings that while the apparent $K_m$ values of substrate influx in mgl+ and Tra+ cells differ 1,000-fold, no significant differences are observed between
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FIG. 6. Efflux of β-methylgalactoside from Tra+ and mgl+ cells. The Tra+ mutant S185-27 ( —O ) was loaded with β-[14C]methylgalactoside under the conditions of the 80x assay for 30 min at 28°, then diluted 50-fold with medium prewarmed at 28°. Cells were filtered at the indicated times. The zero time value was 148 pmol/10^9 cells. Each value was corrected for a control in which cells were added to the assay after the 50-fold dilution of substrate. These controls were 1.2, 0.4, 2.1, and 2.2 pmol for the 0-, 2-, 6-, and 10-min samples, respectively. The mgl+ strain S185-726 (O——O ) was assayed as described for the Tra+ mutant except that loading with substrate was done by incubating for 15 min at 28° under the conditions of our routine assay. The zero time value was 116 pmol/10^9 cells. The controls were 1.6, 4.8, and 5.9 pmol for the 2-, 6-, and 10-min samples, respectively.

These cells with respect to either the maximal rate of substrate uptake or the efflux down a concentration gradient. Since the mgl+ and Tra+ strains examined here differ only in the structural gene mgl B, the greater affinity for substrate exhibited by mgl+ cells must be a function of the galactose-binding protein.

We also conclude that in the complete methylgalactoside transport system the gene products of mgl A and mgl C mediate substrate translocation. Moreover, it is the step of translocation which is rate-limiting in the overall process of transport at maximal influx. These conclusions stem from (a) mutants defective in mgl B exhibit substrate translocation while mgl A− and mgl C− mutants do not; (b) the maximal substrate influx measured in mgl B− mutants is similar to that measured in mgl+ cells. One apparently exceptional mgl A− mutant (S183-10), which at 28 μM substrate exhibited translocation similar to that of mgl B+ cells, was found to fall in the same category as the other mgl A− mutants when its maximal substrate influx was determined.

The substrate specificity of the galactose-binding protein in vitro is strikingly similar to that of the β-methylgalactoside transport system (10). In view of this correlation, it was necessary to determine the substrate specificity of translocation occurring in the absence of the binding protein. We found that specificity is retained in mgl B+ cells; i.e. sugars which are substrates of the complete transport system are translocated by mgl B+ cells and sugars excluded by the former are excluded by the latter.

In summary, through comparison of appropriate mutants, it is possible to assign discrete steps in the process of active transport to individual components of the β-methylgalactoside transport system. At this stage, we assign to the galactose-binding protein the function of an “affinity modulator,” and to the mgl A and mgl C products the function of substrate translocation.

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