Characterization of the Lactose Transport System in \textit{Citrobacter freundii}

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The lactose transport system of \textit{Citrobacter freundii} was characterized. Both the lactose transport system and \(\beta\)-galactosidase were induced with either lactose or isopropyl-\(\beta\)-d-thiogalactopyranoside (IPTG), the latter being the better inducer. The \(K_m\) values for methyl-\(\beta\)-d-thiogalactopyranoside (TMG) transport and lactose transport were 0.61 mM and 1.1 mM, respectively, and the \(V_{\text{max}}\) values were 53 nmol/min/mg cell protein and 12 nmol/min/mg cell protein, respectively. Thus, TMG is a better substrate than lactose. Thiodigalactopyranoside (TDG) was a very potent competitive inhibitor. Neither Na\(^+\) nor Li\(^+\) had a significant effect on the TMG transport or the lactose transport. Proton/substrate cotransport (symport) via this system was observed.

\textbf{Keywords} lactose transport; properties; cotransport; \textit{C. freundii}

\textit{Citrobacter freundii}, which belongs to \textit{Enterobacteriaceae}, is often found in clinical specimens as an opportunistic or secondary pathogen.\textsuperscript{11} \textit{C. freundii} shows the fermentation of lactose (red colony on the MacConkey/lactose plate) and grows on lactose as a sole source of carbon. This bacterium shows white colonies on MacConkey/melibiose plates, and fails to grow on melibiose. Thus, \textit{C. freundii} possesses a functional utilization system for lactose, but not for melibiose. The lactose utilization system of \textit{Escherichia coli} consists of a lactose transport system and a lactose degrading enzyme (\(\beta\)-galactosidase). The lactose transport system and the melibiose transport system of \textit{E. coli} share common substrates, such as methyl-\(\beta\)-d-thiogalactopyranoside (TMG), melibiose and lactose. Therefore, it is necessary to use mutants which lack one of the transport systems to characterize one of the systems in \textit{E. coli}. Since \textit{C. freundii} does not possess a functional melibiose system, the lactose transport system of \textit{C. freundii} can be characterized using wild type cells.

The lactose transport system of \textit{E. coli} is one of the best characterized transport systems. This system mediates the cotransport (symport) of H\(^+\) and a substrate.\textsuperscript{2} This type of ion/substrate cotransport is widely distributed in membranes, from bacterial cells to animal cells. Information obtained from this system is useful in understanding the structure-function relationships of other transport systems. Analyses of the \textit{E. coli} lactose system at the molecular level are underway in several laboratories. In addition to such approaches, a comparison of its properties and structure with the lactose transport systems of other microorganisms would be interesting. Thus, we analyzed the lactose transport system of \textit{C. freundii}.

\textbf{MATERIALS AND METHODS}

\textbf{Bacteria} \textit{C. freundii} ATCC8090 (kindly provided by Dr. N. Ishiguro, Obihiro University of Agriculture and Veterinary, Japan) and \textit{E. coli} K12 were used in this study.

\textbf{Assays} For the galactosidase assay, cells were grown for 4 h in LB medium\textsuperscript{3} in the absence or presence of either 10 mM lactose, melibiose or 1 mM isopropyl-\(\beta\)-d-thiogalactopyranoside (IPTG). \(\beta\)-Galactosidase activity was measured using \(p\)-nitophenyl-\(\beta\)-d-galactopyranoside as a substrate.\textsuperscript{4} \(\alpha\)-Galactosidase activity was measured using \(p\)-nitophenyl-\(\alpha\)-d-galactopyranoside as a substrate.\textsuperscript{5} One unit of enzyme activity is defined as that releasing 1 \(\mu\)mol of \(p\)-nitophenol per min.

For the transport assay, cells were grown in LB medium.\textsuperscript{3} For induction, either 10 mM lactose or 1 mM IPTG was added. Cells were harvested at the late exponential phase of growth, and washed with a modified Tanaka medium.\textsuperscript{6} Transport of \([\text{\textsuperscript{14}C}}\)TMG in such cells was measured as described previously.\textsuperscript{7} For \textit{H}\(^+\) uptake assay, cells were washed with 0.15 M KCl containing 2 mM MgSO\(_4\). H\(^+\) uptake was measured as described previously.\textsuperscript{8}

Protein was determined by the method of Lowry et al.\textsuperscript{9}

\textbf{RESULTS AND DISCUSSION}

We first tested whether the lactose utilization system in \textit{C. freundii} is inducible or constitutive. In \textit{E. coli}, at least two proteins are necessary for the utilization of lactose, a transport protein and \(\beta\)-galactosidase. We tested whether the cells of \textit{C. freundii} showed constitutive \(\beta\)-galactosidase activity or whether they are inducible. We detected only a very low level of \(\beta\)-galactosidase activity in cells grown in the absence of an inducer. On the other hand, high \(\beta\)-galactosidase activity (30-fold higher than the control) was observed in cells grown in the presence of 10 mM lactose (Table I). Much higher activity (50-fold higher) was observed when cells were grown in the presence of 1 mM IPTG. Thus, at least the gene encoding \(\beta\)-galactosidase of \textit{C. freundii} is inducible, and IPTG is a better inducer than lactose, as in the case of \textit{E. coli}.\textsuperscript{10} Although melibiose is an inducer of the lactose operon in \textit{E. coli}, it did not induce \(\beta\)-galactosidase in \textit{C. freundii}. The activity of \(\alpha\)-galactosidase in \textit{C. freundii} cells grown in the absence or presence of either lactose, IPTG or melibiose was also tested. We detected no \(\alpha\)-galactosidase activity in any of the cells tested (Table I). Thus, \textit{C. freundii} appears to possess no functional melibiose operon. This
TABLE I. Induction of β-Galactosidase in C. freundii

<table>
<thead>
<tr>
<th>Inducer*</th>
<th>β-Galactosidase (units/mg protein)</th>
<th>α-Galactosidase (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.65</td>
<td>0.00</td>
</tr>
<tr>
<td>IPTG</td>
<td>1.12</td>
<td>0.00</td>
</tr>
<tr>
<td>Melibiose</td>
<td>0.03</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* Lactose (10 mM), IPTG (1 mM) or melibiose (10 mM) was added to the culture medium.

Fig. 1. Induction of the Lactose Transport System of C. freundii

Cells of C. freundii were grown in LB medium in the absence (△) or presence of either 10 mM lactose (●) or 1 mM IPTG (○). Uptake of [14C]TMG (0.1 mM) by the cells was measured.

Fig. 2. Transport of Lactose (A) and TMG (B) in Cells of C. freundii and of E. coli

Cells of C. freundii (○) or E. coli (●) were grown in LB medium in the presence of 1 mM IPTG, and the uptake of [14C]lactose or [14C]TMG was measured. Concentration of the substrate was 0.1 mM.

Fig. 3. Effect of Galactosides on TMG Transport in C. freundii

Uptake of [14C]TMG (0.1 mM) in cells induced with IPTG was measured in the absence (○), or presence of either 2 mM lactose (●), 2 mM melibiose (△) or 0.2 mM TDG (△). The galactosides were added 30 s prior to the addition of [14C]TMG.

The notion is consistent with the fact that C. freundii showed white colonies on the MacConkey/melibiose plate, and failed to grow on melibiose as a sole source of carbon (data not shown).

We assumed that genes encoding β-galactosidase and lactose transport protein constitute one operon, as in E. coli10 and Klebsiella pneumoniae.11) If this is the case, then we should observe the induction of transport activity in parallel with β-galactosidase. In fact, we observed very high TMG transport activity with cells grown in the presence of IPTG, and fairly high activity with cells induced by lactose (Fig. 1). Low but significant TMG transport was detected with uninduced cells.

We then investigated substrate specificity in the lactose transport system of C. freundii. In E. coli, lactose and TMG (lactose > TMG) are very good substrates for the lactose transport system.10) We compared the lactose transport activity and TMG transport activity in both E. coli and C. freundii (Fig. 2). E. coli cells showed significantly higher lactose transport activity than C. freundii, the activity in C. freundii being about 7% (initial velocity) of that of E. coli. This result is consistent with the observation that E. coli showed much more rapid fermentation on MacConkey/lactose plates (colonies of E. coli were darker red than those of C. freundii). As for TMG transport, E. coli cells showed about twice the activity level of C. freundii cells. In C. freundii, the initial velocity of TMG transport was about 4-fold faster than that of lactose transport when measured at 0.1 mM substrate concentration. Thus, TMG seems to be a much better substrate than lactose. We tested the effect of a 20-fold excess of lactose and melibiose on the TMG transport (Fig. 3). Considerable inhibition by these two sugars was observed. Thiogalactopyranoside (TDG) showed very strong inhibition of the TMG transport at 20-fold excess concentration (2 mM). We then reduced the TDG concentration to 2-fold excess (0.2 mM) compared with TMG concentration (0.1 mM), and still observed very strong inhibition (Fig. 3). Judging from the extent of the inhibition, it is clear that TDG has a very high affinity for the lactose transport system, followed by TMG, melibiose and then lactose.

Kinetic parameters for the TMG transport and the
TABLE II. Kinetic Parameters for TMG Transport and Lactose Transport in the Lactose System of C. freundii

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mm)</th>
<th>$V_{max}$ (nmol/min/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMG</td>
<td>0.61</td>
<td>53</td>
</tr>
<tr>
<td>Lactose</td>
<td>1.1</td>
<td>12</td>
</tr>
</tbody>
</table>

a) Cells induced with IPTG were used.

lactose transport were determined (Table II). The $K_m$ value for TMG transport was 0.61 mm and that for lactose transport was 1.1 mm; the $V_{max}$ value for TMG transport was 53 nmol/min/mg cell protein and that for lactose transport was 12 nmol/min/mg cell protein. Since TDG showed very strong inhibition of the TMG transport, we tested the effect of TDG on the kinetics of the TMG transport (Fig. 4). As expected, TDG showed competitive inhibition. The $K_i$ value was 11 $\mu$m, much lower than the $K_m$ values for TMG and lactose.

Although we tested the effect of Na$^+$ and Li$^+$ on the TMG transport and on the lactose transport, no significant effect was observed (data not shown). Also, we detected no substrate-induced Na$^+$ uptake via the lactose transport system of C. freundii (data not shown). Therefore, we conclude that Na$^+$/substrate cotransport does not take place in this system.

We then tested whether protons are taken up when the substrate is added to the cell suspension, which is a good assay for H$^+$/substrate cotransport. As shown in Fig. 5, we observed a large uptake of H$^+$ elicited by the addition of TMG. We also observed the uptake of H$^+$ elicited by TDG and melibiose. In the case of lactose, significant H$^+$ uptake was observed, which was followed by the rapid acidification of the assay medium. This acidification is due to the production of organic acids as a result of the metabolism of lactose. Very little H$^+$ uptake was elicited by TMG in uninduced cells. Thus, we conclude that H$^+$/TMG, H$^+$/TDG, H$^+$/melibiose and H$^+$/lactose cotransport proceed via the lactose transport system of C. freundii. Although TDG showed very high affinity for the lactose transport system of C. freundii, it did not elicit very large H$^+$ uptake. This phenomenon is also seen in E. coli and is probably due to a lower $V_{max}$ of TDG compared with that of TMG.

The next step in our research is to clone the structural gene encoding the lactose transport system of C. freundii, and to sequence it. Very recently, we have succeeded in the cloning.

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