Lactose Permease of *Escherichia coli* Catalyzes Active β-Galactoside Transport in a Gram-Positive Bacterium

WERNER BRABETZ, WOLFGANG LIEBL,* and KARL-HEINZ SCHLEIFER

Lehrstuhl für Mikrobiologie, Technische Universität München, D-80290 Munich, Germany

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The following several lines of evidence demonstrate that lactose permease (LacY) of *Escherichia coli* is assembled into the cytoplasmic membrane of gram-positive *Corynebacterium glutamicum*, expressing the lacY gene, as a functional carrier protein. (i) LacY was detected immunologically in the cytoplasmic membrane fraction of the heterologous host. (ii) Recombinant *C. glutamicum* cells bearing the lacY gene displayed an increased influx of o-nitrophenyl-β-D-galactopyranoside, which was inhibited by N-ethylmaleimide. (iii) Washed cells were capable of accumulating methyl-β-D-thiogalactoside about 60-fold. (iv) The uptake of methyl-β-D-thiogalactoside was energy dependent and could be inhibited by the addition of 10 μM carbonyl cyanide-m-chlorophenylhydrazone. LacY of *E. coli* was active in the recombinant *C. glutamicum* cells despite the different membrane lipid compositions of these organisms.

The expression of genes of gram-negative origin in gram-positive hosts, e.g., *Bacillus subtilis*, is normally extremely poor. Problems may arise at the transcriptional or the translational level. For *B. subtilis*, more stringent requirements for transcription and translation initiation than those found in *Escherichia coli* have been proposed to be the major cause (2, 10, 16). On the other hand, some high-GC gram-positive bacteria seem to be less specific than *B. subtilis* in this respect. For example, *Corynebacterium glutamicum* ("Brevibacterium lactofermentum"), a gram-positive host with a high GC content (15), readily utilizes certain *E. coli* promoters (17). The expression of membrane proteins of gram-negative origin in gram-positive organisms has not been investigated at all to date. This scenario is even more complex than that with soluble proteins because, besides efficient transcription and translation, the heterologous polypeptide must also be targeted to the membrane of the host cell and assume correct overall folding and orientation of the transmembrane segments.

We have previously (4) found that the introduction of the *E. coli lac* operon into *C. glutamicum* conferred the ability to utilize lactose upon the recombinant organism. The presence of lacY in addition to lacZ was an absolute prerequisite for growth on lactose, whereas expression of lacZ alone was not sufficient. This work has provided genetic and phenotypic evidence for the functional expression of the lactose carrier (LacY) in *C. glutamicum*. We now show that LacY, an integral membrane protein with 12 hydrophobic membrane-spanning segments, is indeed integrated into the cytoplasmic membrane of *C. glutamicum*, and we provide biochemical evidence demonstrating that it catalyzes the active transport of β-galactosides in this heterologous membrane environment. The two *C. glutamicum* strains used in this study were R163(pECL1x) and R163(pECL3x), which bear recombinant plasmids with the entire *E. coli lac* operon (pECL1x) or only the lacZ gene (pECL3x) under control of a mutant lac promoter which works efficiently in *C. glutamicum* (4).

Localization and integrity of the *E. coli lac* gene products expressed in *C. glutamicum*. The cellular localization of the lacZ and lacY gene products expressed in *C. glutamicum* R163 was investigated. For this purpose, recombinant *C. glutamicum* R163 cells were disrupted by a twofold passage through a French pressure cell at 6.9 MPa at 4°C in the presence of DNase I, phenylmethylsulfonyl fluoride, MgSO₄, and dithiothreitol at final concentrations of 45 μg/ml, 150 μM, 7.5 mM, and 1.5 mM, respectively. The crude extract was freed of debris (two times, 20 min, 9,000 × g, 4°C) and fractionated with high-speed centrifugation (two times, 2 h, 105,000 × g, 2°C). The success of the fractionation procedure was verified by measuring the activities of the marker enzymes α-glutamate dehydrogenase and succinate dehydrogenase as described by others (3, 12) (data not shown). More than 95% of the β-galactosidase activity was found to be located in the cytoplasmic fraction of the LacZ-bearing strains, as expected. Additionally, immunoblot experiments demonstrated the cytoplasmic and membrane localization of β-galactosidase and lactose permease, respectively, in *C. glutamicum* R163 (pECL1x) and showed that the mobilities in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of both proteins when expressed in this host were the same as when produced by *E. coli* (not shown).

LacY-dependent hydrolysis of ONPG by whole cells of recombinant *C. glutamicum* strains. An indirect assay method for the determination of LacY activity with intact cells poisoned with sodium azide, originally developed for *E. coli* (8), 14, was used. Intact cells of *C. glutamicum* R163(pECL1x) took up o-nitrophenyl-β-D-galactopyranoside (ONPG) and hydrolyzed the substrate via intracellular β-galactosidase activity (not shown). Two lines of evidence prove that ONPG uptake was mediated by LacY in this strain. First, cells of *C. glutamicum* R163(pECL3x), which lack the lacY gene but have a level of cellular β-galactosidase activity similar to that of *C. glutamicum* R163(pECL1x) (about 3 U mg of protein⁻¹ in crude cell extracts), hydrolyzed ONPG only at about 15% of the extent found with *C. glutamicum* R163(pECL1x) cells. Second, by addition of N-ethylmaleimide (NEM), which is well known as an inhibitor of lactose permease activity in similar assays performed on *E. coli* cells (8), ONPG hydrolysis by *C. glutamicum* R163(pECL1x) cells was inhibited to the background (LacY-independent) level of *C. glutamicum* R163 (ECL3x) cells (data not shown). Half-maximal inhibition was observed at 1,6 × 10⁻⁴ M NEM.

Uptake of [14C]TMG by *C. glutamicum* R163(pECL1x) cells. Since the method of whole-cell ONPG hydrolysis does not

* Corresponding author.
supply information about the energy dependence of LacY activity (actually, merely the phenomenon of LacY-catalyzed facilitated diffusion is measured by this method), we sought to investigate whether LacY-mediated β-galactoside uptake by C. glutamicum was an active transport process. For this purpose, cells were inoculated into 200 ml of LB broth (4) supplemented with 5 μg of chloramphenicol per ml to an initial optical density at 600 nm (OD600) of about 0.6 and incubated with aeration at 30°C. After reaching an OD600 of about 1.2 to 1.3, the cells were harvested (3,500 × g, 10 min, 4°C), washed twice with 100 ml of ice-cold 50 mM Tris-HCl (pH 7.5)–10 mM NaCl–10 mM KCl containing 20 μg of kanamycin sulfate per ml, and resuspended in the same buffer. The cells were adjusted to an OD600 of 20 to 23 and diluted appropriately before use. When the cells were stored on ice, the properties of the cells relevant for this work remained stable for at least 4 h. A cell suspension of C. glutamicum R163 with an OD600 of 1 corresponded to 0.431 mg (dry weight) of cells ml⁻¹. The silicon oil layer centrifugation method (7) was used to measure the uptake of the nonmetabolizable substrate analog [¹⁴C]taurine-impermeable (['⁴C]TMG). Cells (final concentration in the reaction mixture, 0.8 to 3.5 mg [dry weight] of cells ml⁻¹, corresponding to an OD600 of 1.86 to 8.12) were preincubated at 30°C for 10 min before the addition of ['⁴C]TMG (10 to 56 Ci mol⁻¹). After various times within the first 8 min, aliquots of 100 to 170 μl were withdrawn, and uptake was terminated by spinning the cells through a silicone oil layer into 20% perchloric acid (13). The method was performed as described by Ebbighausen et al. (7), except that the uptake parameters were calculated on the basis of values of 1.31 ± 0.15 μg (dry weight) of cells⁻¹ for the cytoplasmic ([¹⁴C]taurine-impermeable) volume and 3.25 ± 0.65 mg (dry weight) of cells⁻¹ for the extracellular (taurine) space adherent to the cells during centrifugation through the silicon oil layer (our own determinations with cells of C. glutamicum R163). By using this method, ['⁴C]TMG uptake was detected with C. glutamicum R163(pECLlxx) cells (Fig. 1 and 2) but not with C. glutamicum R163(pECLlxx).

Uptake kinetics obtained with C. glutamicum R163 (pECLlxx) cells at low concentrations of ['⁴C]TMG are shown in Fig. 1. Irrespective of the substrate concentration in the range between 2.5 and 50 μM, the increase of ['⁴C]TMG internalization ceased after 150 to 200 s. The accumulation factors reached were calculated at equilibrium as the ratios of the cytoplasmic and external ['⁴C]TMG concentrations ([Ci]/[Ce]). For all substrate concentrations tested (2.5 to 50 μM), the accumulation factors were about the same, i.e., 58 to 64. This result is a clear indication of the occurrence of active transport. After maximum accumulation, the values measured remained constant for more than 300 s, demonstrating that there was no extrusion of label from the cells and therefore probably no deenergization of the membrane. After prolonged incubation of loaded cells, however, a loss of label from the cells did eventually occur (not shown).

['⁴C]TMG uptake was almost completely prevented in the presence of 10 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP; Fig. 2). Also, the addition of 10 μM CCCP to cells preloaded with ['⁴C]TMG resulted in the rapid efflux of the label from the cells (Fig. 2). These results clearly demonstrate that ['⁴C]TMG uptake by the recombinant C. glutamicum cells was dependent on an energized cytoplasmic membrane and thus provide additional proof that LacY catalyzes active transport in the heterologous host.

The kinetic parameters of ['⁴C]TMG uptake by C. glutamicum R163(pECLlxx) cells were determined (Fig. 3). It was not possible to address this question with the standard silicon oil centrifugation procedure since samples could not be drawn faster than every 20 to 30 s because of technical restrictions. To achieve an increased sampling frequency, the thiol-specific reagent HgCl₂ was used to rapidly stop further uptake of ['⁴C]TMG. The use of NEM for this purpose proved unsatisfactory because NEM displayed only very slow uptake inactivation kinetics, i.e., a 30-min incubation at 30°C with 0.05 M NEM was necessary to completely inhibit uptake (data not shown). Aliquots were drawn at various times within the first 2000 s.
FIG. 3. Determination of initial velocities of [14C]TMG uptake by C. glutamicum R163(pECL1x) cells at various concentrations of TMG (A) and Lineweaver-Burk (insert) and Eadie-Hofstee diagrams of the kinetic data obtained (B). dw, dry cell weight.

minute after [14C]TMG addition (0.5 to 56 Ci mol⁻¹) and injected into an HgCl₂ solution (final HgCl₂ concentration, 20 mM) containing nonlabeled TMG at a concentration equimolar to the initial substrate concentration. After all aliquots were taken, the standard silicon oil centrifugation technique was applied to the samples. Control experiments showed that, under these experimental conditions, the efflux of [14C]TMG from preloaded cells was very low for more than 10 min after poisoning with HgCl₂ (data not shown; see Fig. 4). By using this rapid method, samples could be drawn every 8 s (Fig. 3A). The uptake measured in the range of 0.1 to 8 mM [14C]TMG was typically Michaelis-Menten-like. The kinetic parameters were determined from an Eadie-Hofstee plot of the data (Fig. 3B). The apparent Kₘ was 930 μM, and the V_max was 43 nmol mg⁻¹ dry weight min⁻¹. However, it is difficult to compare the apparent substrate affinities and maximum uptake velocities measured for LacY expressed in C. glutamicum with the corresponding values determined for E. coli by others (1) because the cell walls (which may have influence on the uptake process) of these two organisms are completely different. In contrast to E. coli, C. glutamicum does not possess an outer membrane but has a mycolic acid layer as a part of its cell wall, which is absent in E. coli.

Finally, the effects of CCCP and HgCl₂ on [14C]TMG uptake were studied in more detail (Fig. 4). Twenty-millimolar HgCl₂ not only caused an immediate stop of [14C]TMG uptake by C. glutamicum R163(pECL1x) cells (see above; Fig. 4A), it also effectively blocked the CCCP-induced extrusion of the labeled substrate from preloaded cells (Fig. 4B). Obviously, HgCl₂ in both experiments reacted with the same thiol groups necessary for specific β-galactoside uptake, and consequently, the substrate efflux observed upon CCCP addition in the absence of HgCl₂ (Fig. 2 and 4B) is nonspecific but rather takes place via LacY in the membrane. These data provide indirect evidence for the reversibility (which is a typical characteristic of secondary transport carriers such as LacY) of [14C]TMG uptake by C. glutamicum R163(pECL1x) cells.

By genetic evidence (4) and our localization studies and uptake experiments (present study), we have demonstrated that the E. coli lactose permease is assembled into the C. glutamicum R163(pECL1x) cytoplasmic membrane to yield a functional protein capable of uphill β-galactoside transport. To

FIG. 4. Effect of HgCl₂ on [14C]TMG uptake (A) by C. glutamicum R163(pECL1x) cells and its efflux from preloaded cells (B). (A) Twenty-micromolar [14C]TMG was added at 0 s to cells either without HgCl₂ (○) (open circles) or simultaneously with 20 mM HgCl₂ (●) or to cells pretreated for 30 min at 30°C with 0.05 M NEM (□), and the uptake was monitored. (B) Five hundred microliters of cells at a concentration of about 3 mg (dry weight) of cells ml⁻¹ was preloaded with [14C]TMG (20 μM, 8 min, 30°C) and then mixed with 550 μl of an additive solution resulting in final concentrations of 10 μM CCCP (○), no CCCP (control; ○), 20 mM HgCl₂ (△), 10 μM CCCP-20 mM HgCl₂ (◇), or 10 μM CCCP-20 mM HgCl₂-20 μM cold TMG (to compensate for the dilution of the initial substrate concentration; ●), and the decrease in intracellular label was monitored by the silicone oil centrifugation method. dw, dry cell weight.
our knowledge, this is the first report demonstrating that an integral membrane protein of a gram-negative bacterium can be expressed and can operate correctly in a gram-positive organism. This result is not self-evident because there may be substantial differences between these groups of bacteria with regard to the mechanism(s) of membrane integration of such proteins. Furthermore, the chemical compositions of the cellular membranes are markedly different between gram-positive and gram-negative bacteria (and there is even considerable diversity within each group [9]), and it is known that the chemistry of bilayer lipids can drastically affect the activity of membrane enzymes (5). As for the case discussed in this study, the C. glutamicum membrane contains no phosphatidylethanolamine (11), while this is the main phospholipid in the natural lipid environment of LacY in E. coli (6). However, these differences do not seem to be of major importance for correct membrane assembly or function of the E. coli lactose carrier.

Finally, we believe that the expression of genes encoding integral membrane proteins in heterologous hosts may prove to be a valuable tool for studying the behavior of membrane proteins in different lipid surroundings and for constructing recombinant strains with new uptake or export capabilities.

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