Identification and Characterization of a Bacterial Transport System for the Uptake of Pyruvate, Propionate, and Acetate in Corynebacterium glutamicum

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The metabolism of monocarboxylic acids is of central importance for bacteria in their natural habitat as well as during technological production. Although biosynthesis and degradation are well understood, the transport of such compounds is still a matter of discussion. Here we present the identification and characterization of a new transport system in Corynebacterium glutamicum with high affinity for acetate and propionate and with lower affinity for pyruvate. Biochemical analysis of this monocarboxylic acid transporter (MctC) revealed for the first time a quantitative discrimination of passive diffusion and active transport of acetate by bacterial cells. MctC is a secondary transporter and belongs to the class of sodium solute symporters, but it is driven by the electrochemical proton potential. The mctC gene is preceded by and cotranscribed with cg0952, a locus encoding a small membrane protein, and the transcription of the cg0952-mctC operon is under the control of the transcriptional regulators RamA and RamB. Both of these proteins directly bind to the promoter region of the operon; RamA is essential for expression and RamB exerts a slightly negative control on expression of the cg0952-mctC operon. mctC expression is induced in the presence of pyruvate and beneficial under substrate-limiting conditions for C. glutamicum.

Corynebacterium glutamicum is one of the major work horses in industrial biotechnology. More than 1.5 million tons of glutamate as well as 0.75 million tons of lysine per year are produced with the use of this bacterium (25, 27). C. glutamicum is a nonmotile, gram-positive bacterium and is able to utilize a number of carbon sources, including sugars such as glucose, fructose, and sucrose, organic acids such as gluconate, acetate, propionate, pyruvate, and l-lactate, ethanol, and also amino acids such as glutamate and serine (9, 10, 30, 31). The utilization of tri- and dicarboxylates by C. glutamicum depends on the expression and/or activity of the relevant transport systems, as shown recently (37, 45). Monocarboxylic acids like acetate and propionate are abundant carbon sources in soil and also unwanted by-products in fermentation processes (44). The utilization of acetate by C. glutamicum involves the activation of acetate by the combined activities of acetate kinase and phosphotransacetylase and the subsequent flux into the tricarboxylic acid and glyoxylate cycles (17). However, the uptake of acetate by C. glutamicum cells is not yet understood. It has been proposed that a specific secondary uptake system is involved and that passive diffusion does not significantly contribute at pH 8 or with low substrate concentrations (14). On the other hand, a discrepancy between the relatively low uptake rates observed by Ebbringhausen et al. (14) and the much higher consumption rates of acetate during growth experiments, as well as the long lag phase after addition of higher (≥2%) concentrations of acetate, point to diffusion being relevant for the utilization of this particular substrate (17, 43). Also, for propionate, passive diffusion into the cell has been proposed (24) and so far, no transport system is known in bacteria. Propionate has been suggested to be activated in C. glutamicum also by acetate kinase and phosphotransacetylase, and subsequently propionyl coenzyme A (CoA) is converted to pyruvate via the 2-methylcitrate cycle (9). However, growth of C. glutamicum on propionate was observed after a long lag phase, indicating toxic effects on cell metabolism, as have been described for many bacteria (32). Pyruvate can also be utilized by C. glutamicum, and it has been proposed that this substrate cannot diffuse through cell membranes and, consequently, an uptake system would be required (31, 38). However, pyruvate transport, as well as the corresponding transport system, has not yet been unraveled for C. glutamicum.

In the present study, we identified a new transport system in C. glutamicum acting as a secondary carrier for pyruvate, acetate, and propionate. We addressed the biochemical properties of this new transporter, analyzed the transcriptional organization and expression control of the respective gene and operon, and investigated its function and physiological relevance.

MATERIALS AND METHODS

Media and growth conditions. Escherichia coli was grown at 37°C in LB medium (34). C. glutamicum was grown aerobically at 30°C either in complex brain heart infusion medium (BHI; Difco Laboratories), 2× tryptone-yeast (TY) medium (34), or in minimal MM1 medium (4) composed of 5 g (NH₄)₂SO₄, 5 g urea, 2 g KH₂PO₄, and 2 g K₂HPO₄ per liter of H₂O. The pH was adjusted to 7.0 with KOH, and the medium was supplemented with 0.25 g MgSO₄, 0.01 g CaCl₂,

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Transporter for monocarboxylic acids in C. glutamicum

Utilization and uptake of monocarboxylic acids in C. glutamicum.

In order to find conditions for the identification of uptake systems for monocarboxylic acids, growth experiments with C. glutamicum WT in liquid media as well as on agar plates with different substrates as sole carbon source were performed. A bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL) was used to determine protein concentrations, and bovine serum albumin was used as a standard.

**RNA techniques.** Total RNA was prepared after disruption of C. glutamicum cells with glass beads and using the NucleaseSpin RNA II kit as recommended by the supplier (Macherey-Nagel). The RNA was blotted on a positively charged nylon membrane (BioRad Sigma, Taufkirchen, Germany) using a Minifold I dot blotter (Schleicher & Schuell, Dassel, Germany). Hybridization of digoxigenin-labeled RNA probes was detected with the Fuji luminescence image analyzer LAS1000 (Raytest, Straubenhardt, Germany) using alkaline phosphatase-conjugated antidigoxigenin Fab fragments and CSPD as a light-emitting substrate as recommended by the supplier (Roche Diagnostics).

For the detection of the cg0952-mctC operon, total RNA was prepared as described above and DNA contamination was removed by treatment with DNase (New England Biolabs) and subsequent purification with NucleoSpin columns. Reverse transcription-PCR (RT-PCR) was carried out with the Revert Aid H Minus first-strand cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) as recommended by the supplier using random hexamers for the RT and combinations with the following primers: Reg0952-0953 forward, 5'-GCC GTC CGT TCA ACA GCA GCC TTT T3'; Reg0952, 5'-GCA TCC TGC AGC TGA CAT ATG AAT TCC AC-3' PCR and restriction analysis before transformation into C. glutamicum cells by electroporation (2.5 kV, 5 μF). The integration into the genome by homologous recombination was verified by PCR analysis, proving the integration of the plasmid into the cg0952 locus. Essentially by the same procedure, the genes cg0952 (CG11001), cg0953 (CG11002), cg0973 (CG11003), cg1419 (CG11004), cg1422 (CG11005), cg2457 (CG11006), and cg3371 were inactivated by insertion mutagenesis.

Bacterial strains, plasmids, and mutagenesis. The experiments were carried out with C. glutamicum wild type (WT; ATCC 13032), C. glutamicum RGI (RamB16) and RGG (RamA13), and C. glutamicum ΔaceE (35). Cells were precultivated for approximately 8 h in BHI medium and used to inoculate 20 ml of MM1 medium. After 16 h fresh MM1 medium was inoculated to an OD600 of 0.1 to 1.

The locus cg0953 of C. glutamicum was inactivated by insertion mutagenesis. For this purpose, a 1,312-bp internal fragment of the locus cg0953 was amplified using the primers cg0953-5' (5'-CAGAACGGCTTGCGATATC3'; P1 in Fig. 4, below) and cg0953-3' (5'-ATTGCCAGCTGCTGCACC3'; P2 in Fig. 4, below) and cloned into the vector pDrive (Qiagen, Hilden, Germany) according to the supplier's instructions. All cloning steps were performed in E. coli DH5α (19). The resulting plasmid (pDrive-cg0953) was isolated from E. coli by using the NucleoSpin plasmid DNA purification kit (Macherey-Nagel) and checked by PCR and restriction analysis before transformation into C. glutamicum cells by electroporation (2.5 kV, 5 μF). The integration into the genome by homologous recombination was verified by PCR analysis, proving the integration of the plasmid into the cg0953 locus. Essentially by the same procedure, the genes cg0952 (CG11001), cg0953 (CG11002), cg0973 (CG11003), cg1419 (CG11004), cg1422 (CG11005), cg2457 (CG11006), and cg3343 were inactivated.

For transcriptional analysis, promoter probe plasmid pET2(40) was employed.

**Transport measurements.** Cells were grown to mid-log-phase in MM1 supplemented with selected carbon sources, washed three times with 2-(N-morpholino)ethanesulfonic acid (MES)–Tris buffer (50 mM MES, 50 mM Tris, 10 mM NaCl, pH 7.2, 1 mM thiaminepyrophosphate, 5 mM pyruvate, 3 mM L-cysteine, 10 mM L-cysteine) and centrifugation (14,000 rpm, 4°C, 15 min). The enzyme assay was monitored photometrically by the optical density at 600 nm (OD600). Growth screening was performed on MM1 agar plates containing different carbon sources by spotting 50 μl of a bacterial culture with an adjusted OD600.

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performed. As expected, addition of 100 mM glucose promoted growth to a higher extent than the same concentration of acetate or pyruvate with respect to the final cell density, whereas the growth rate during the exponential phase was comparable (Fig. 1). After addition of propionate, no increase in biomass within 4 days was observed (data not shown). This is in agreement with data obtained recently (9) and the proposed toxic effect of propionate caused by the accumulation of 2-methylcitrate (32).

Subsequently, the uptake of pyruvate was analyzed by determination of the accumulation of 14C-labeled substrate in pyruvate-grown C. glutamicum WT cells at an external pH of 6 and various substrate concentrations. As shown in Fig. 2A, the measurements revealed saturation kinetics, which indicates that pyruvate is taken up by a carrier-mediated mechanism and not by diffusion. Plotting the data according to the Hill equation revealed a maximal activity of 5.6 ± 0.6 nmol/min/mg (dry weight), a $K_{0.5}$ of 250 ± 55 μM, and a Hill coefficient of 2.13 ± 0.60. Varying the pH in the assay buffer showed that carrier activity varies with different pH conditions in C. glutamicum WT cells. At pH 7, the pyruvate uptake rate was increased by 50%, and at higher pH values the activity was decreased in comparison to the rates observed at pH 6. At pH 9 only 15% of the maximum uptake rate was observed (data not shown).

To discriminate whether the uptake of pyruvate is catalyzed by a primary or secondary active carrier, the membrane potential was abolished by simultaneous addition of the ionophores valinomycin and nigericin. After collapse of the membrane potential, pyruvate uptake ceased completely (Table 1). This indicates that pyruvate transport occurs via a secondary active carrier. To identify the cotransported ion, uptake measurements were performed in Na+-depleted buffer. No impact on pyruvate uptake was observed (data not shown). In order to validate the electrochemical proton potential as the driving force, the membrane potential was disrupted by addition of valinomycin in the presence of 200 mM KCl and pyruvate uptake was measured at pH 6.0 and 7.5. Under these conditions, the membrane potential is strongly decreased and the pH gradient is the sole driving force for transport at an external pH of 6. At a pH of 7.5, the pH gradient is abolished also. C. glutamicum WT cells were able to import pyruvate after disruption of the membrane potential (Table 1). However, pyruvate import activity completely ceased when the pH gradient was abolished also (Table 1). These results strongly support the function of the pyruvate carrier as a proton substrate symporter.

In order to identify further possible substrates of the carrier, different monocarboxylic acids were tested for inhibition of pyruvate transport. In the presence of 600 μM pyruvate, 10-fold higher concentrations of lactate, alanine, and succinate had no inhibitory effect on pyruvate transport (Table 1).
TABLE 1. Driving force and specificity of pyruvate transport in C. glutamicum

<table>
<thead>
<tr>
<th>Parameter and condition(s)</th>
<th>Uptake rate (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transport energization</td>
<td></td>
</tr>
<tr>
<td>Control (ΔΨ, ΔpH)</td>
<td>100</td>
</tr>
<tr>
<td>Val/Nig</td>
<td>9 ± 9</td>
</tr>
<tr>
<td>pH 6 (ΔΨ, ΔpH)</td>
<td>102</td>
</tr>
<tr>
<td>pH 6 + Val/Val (ΔpH)</td>
<td>96 ± 11</td>
</tr>
<tr>
<td>pH 7.5 (ΔΨ, ΔpH)</td>
<td>90 ± 12</td>
</tr>
<tr>
<td>pH 7.5 + Val/Val (ΔpH)</td>
<td>90 ± 12</td>
</tr>
<tr>
<td></td>
<td>2 ± 21</td>
</tr>
<tr>
<td>Substrate specificity</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>Acetate</td>
<td>50 ± 18</td>
</tr>
<tr>
<td>Propionate</td>
<td>38 ± 30</td>
</tr>
<tr>
<td>Lactate</td>
<td>86 ± 18</td>
</tr>
<tr>
<td>Succinate</td>
<td>104 ± 3</td>
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</tbody>
</table>

a Uptake of pyruvate was determined under control conditions or in the presence of a membrane potential (ΔΨ) and a pH gradient (ΔpH), only ΔpH, or with neither ΔΨ nor ΔpH as driving forces for pyruvate uptake (see text for details). The substrate specificity was addressed by addition of acetate and propionate in 10-fold excesses and succinate and lactate in 100-fold excesses. The presented data are the averages of at least three measurements. Val, valinomycin; Nig, nigericin.

b Control values were 4.27 and 1.91 nmol/min/mg (dry weight) for transport driving force measurements and for substrate specificity measurements, respectively. All experiments were performed in the presence of 600 μM pyruvate.

To test the transport capacity of C. glutamicum WT for these substrates, cells were grown on acetate or acetate plus propionate as carbon source and uptake measurements were performed with 14C-labeled acetate or propionate, respectively. The measurements were performed at a high pH value of 8 in order to avoid significant rates of passive diffusion predicted by the results of Ebbighausen et al. (14). The data were plotted, and Vmax values of 143 ± 4 and 14 ± 0.8 nmol/min/mg (dry weight) and K0.5 values of 31 ± 2.1 and 9 ± 0.7 μM, as well as Hill coefficients of 1.30 ± 0.15 and 3.65 ± 1.07 for acetate and propionate, respectively, were determined by nonlinear regression according to the Hill equation (Fig. 2). Therefore, acetate is transported with higher activity than propionate and pyruvate. The highest affinity was found for propionate; it was slightly lower for acetate and significantly lower for pyruvate, leading to the conclusion that the transporter may represent an acetate/propionate uptake system with pyruvate being an additional substrate.

The uptake data for pyruvate, acetate, and propionate described so far were obtained from C. glutamicum WT cells grown in minimal medium containing pyruvate or acetate or a mixture of acetate and propionate as carbon and energy source in order to induce maximal uptake activities. To analyze the carbon source-dependent induction of the transport system we compared cells grown on glucose or pyruvate with respect to acetate uptake activity. Cultivation on glucose led to a maximal specific acetate uptake activity of 85 ± 19 nmol/min/mg (dry weight), whereas cultivation on pyruvate resulted in a maximal activity of 110 ± 5 nmol/min/mg (dry weight). Although this increase in transport activity was not very pronounced, the result suggests that the acetate/propionate/pyruvate uptake system is regulated by the carbon source in the growth medium.

Identification of the monocarboxylate importer gene mctC. For identification of the C. glutamicum gene encoding the transport system for pyruvate, a mutant library lacking putative secondary transport systems was constructed (see Materials and Methods) and screened for growth on solid minimal medium containing 50 mM pyruvate as the sole carbon source. Pyruvate was chosen due to the expected low permeability of the C. glutamicum membrane for this substrate. Only one strain lacking the gene product Cg0953 showed severely impaired growth on pyruvate in comparison to WT cells (data not shown). This particular mutant was subsequently analyzed for its growth phenotype in liquid medium (Fig. 1). Growth of mutant cg0953 on glucose as well as on acetate in minimal medium at pH 7 was indistinguishable from WT cells. On pyruvate, however, no growth was detectable, indicating the importance of Cg0953 for pyruvate utilization. In order to prove the direct participation of Cg0953 in pyruvate transport, uptake measurements were performed with the cg0953 mutant strain. As shown in Fig. 2A, at an external pH of 6, no pyruvate uptake was observed, irrespective of the substrate concentration.

To exclude the possibility that the inability to grow on pyruvate and to transport pyruvate is due to a defective pyruvate metabolism, the activity of the PDHC in protein extracts from WT and mutant cells was determined. For both cell lines comparable PDHC activities (0.32 ± 0.04 U/mg protein for WT and 0.40 ± 0.02 U/mg of protein for the mutant cg0953 strain) were observed, indicating that the PDHC was not affected. In addition we performed aerobic growth assays of the WT and mutant strain on lactate. After lactate uptake the lactate dehydrogenase (LldD) converts lactate into pyruvate and, subsequently, the same metabolic pathways as during pyruvate utilization are required. The mutant strain was able to grow on lactate like WT cells (data not shown). The findings that pyruvate metabolism was not affected, that growth on pyruvate or pyruvate uptake was not observed in the mutant cg0953 strain, and that Cg0953 is predicted to be a membrane protein belonging to the solute:sodium symporter (SSS) family (7) with 12 to 13 transmembrane domains (according to predictions by SOSUI or TMHMM), suggest that Cg0953 is the pyruvate importer in C. glutamicum. Cg0953 mutant cells were also strongly affected in acetate and propionate uptake, and the remaining transport capacity could be attributed to the diffusion of these particular substrates (Fig. 2). In conclusion, we propose the designation MctC (monocarboxylic acid transporter in C. glutamicum) for the newly identified transport system and mctC for the respective gene.

Discrimination of passive diffusion and active transport of acetate. In order to quantify the extent of diffusion of acetate and the contribution of MctC to acetate uptake in C. glutamicum, transport experiments at different external pH values were performed. For WT cells, we observed the highest acetate uptake activity at pH 6 in the presence of 300 μM acetate, which is in the Vmax range. At higher pH values, the uptake activity was reduced by a factor of 1.5 (Fig. 3). Acetate uptake by cells of the mctC mutant was found to be maximal at pH 6.
but decreased essentially to zero at pH 8 and 9 (Fig. 3). Consequently, at alkaline pH values the transporter is required for acetate uptake, whereas at pH 7, it confers about 70% and at pH 6 only about 35% to the total acetate uptake activity at the applied substrate concentration (Fig. 3). For propionate, a similar result was obtained. At pH 9.0, 95% of propionate uptake was due to active transport. This fraction decreased with decreasing pH to about 5% at pH 6.0 (data not shown). In conclusion, MctC represents the only transport system for pyruvate, acetate, and propionate in C. glutamicum which is essential for pyruvate utilization and of importance for uptake of acetate and propionate at alkaline conditions and/or low substrate concentrations.

Impact of MctC on growth under limiting conditions. To address the impact of MctC for growth of C. glutamicum under limiting conditions, we performed growth assays in microtiter plates in the presence of high and low concentrations of the substrates glucose and acetate at a high external pH. At the lowest concentration of 0.5 mM, no significant growth was observed for either strain. At a high substrate concentration of 50 mM as well as in the presence of 5 mM glucose, both strains did grow and no significant difference was obtained. However, in the presence of 5 mM acetate, growth of the MctC-deficient mutant was impaired in comparison to WT cells. These results indicate that under particular conditions, namely, the combination of low substrate concentration and alkaline pH, diffusion of substrates is not sufficient, while the active transporter facilitates uptake of nutrients and thereby allows growth under limiting conditions.

Expression and regulation of mctC. The mctC gene of C. glutamicum consists of 1,656 bp and is followed by a region of dyad symmetry (22 to 57 bp downstream of the stop codon), and the mRNA hairpin loop predicted from this sequence has a \( \Delta G^\circ \) value of \(-13.7\) kcal/mol at 25°C. This structure indicates transcriptional termination downstream of mctC. Upstream of mctC and separated by only 6 bp is another open reading frame (cg0952), encoding a small membrane protein of unknown function (Fig. 4A). At 552 bp upstream of cg0952 and divergently transcribed, we found a gene encoding a putative acyl-CoA carboxylase \( \beta \)-subunit (accD4; cg0951) (15). The close neighborhood of cg0952 and mctC suggests that both genes represent an operon. To test for cotranscription of cg0952 and mctC, we performed RT-PCRs (Fig. 4B). As a control, an internal fragment of mctC was also amplified. With total RNA as template, no PCR products were observed, indicating that the RNA preparation was free of DNA contamination. By application of genomic DNA as well as cDNA with all three primer combinations, amplicons of the expected sizes were obtained. These results showed that mctC and the adjacent locus cg0952 are cotranscribed as bicistronic mRNA.

To confirm the presence of a promoter upstream of cg0952 and to investigate transcriptional regulation of the cg0952-mctC operon, the upstream fragment mctCP was tested for activity. For this purpose, a transcriptional fusion between the putative promoter region (DNA fragment mctCP) (Fig. 5) and the promoterless CAT gene was constructed in the promoter test vector pET2. Plasmid pET2-mctCP was transformed into C. glutamicum WT, and the specific CAT activities in the resulting strain were determined during growth in minimal medium containing glucose, acetate, pyruvate, or acetate plus...
propionate. As shown in Table 2, C. glutamicum WT (pET2-
mctC
RG1(pET2-
mctC
P2) cells grown in either glucose or acetate medium. WT cells grown in medium containing pyruvate or acetate plus propionate, however, showed about twice as much activity (Table 2). These results confirm the presence of a promoter upstream of the cg0952 locus, and in accordance with the different specific transport activities of cells grown on glucose or pyruvate the results indicate transcriptional control of the cg0952-mctC operon by the carbon source in the growth medium, i.e., induction or derepression in the presence of pyruvate or propionate.

To test for a potential additional promoter directly upstream of mctC, we constructed plasmid pET2-mctC
P2, carrying a transcriptional fusion of the respective region (i.e., DNA fragment mctC
P2 in Fig. 5) with the CAT gene, transformed it into C. glutamicum WT, and determined the specific CAT activities. As shown in Table 2, no CAT activities were detected in C. glutamicum(pET2-mctC
P2) cells grown in either glucose or acetate medium. These results corroborate our finding that mctC is organized in an operon together with cg0952 and is transcribed from a single promoter located upstream of cg0952.

Recently, two novel regulatory proteins, designated as regulators of acetate metabolism A and B (RamA and RamB, respectively), were identified as transcriptional regulators of genes encoding enzymes involved in acetate metabolism of C. glutamicum (3, 13, 16). Interestingly, we observed a typical expression pattern of the cg0952-mctC operon by the carbon source in the growth medium, i.e., induction or derepression in the presence of pyruvate or propionate.

To test for a direct influence of RamA and RamB on cg0952-mctC promoter activity, the specific CAT activities were determined in the RamA- and RamB-deficient C. glutamicum strains RG2 and RG1 carrying plasmid pET2-mctC
P (Table 2). The RamA-negative strain did not grow in minimal medium with acetate, pyruvate, or acetate plus propionate; however, in minimal medium containing glucose, it showed normal growth (i.e., comparable to the WT strain carrying the former as an essential activator and the latter as a weak repressor in the presence of glucose, acetate, and pyruvate).

DISCUSSION

Knowledge on bacterial transport systems for the monocarboxylic acids pyruvate, acetate, and propionate is scarce. Only for the two proteins ActP of E. coli and MctP of Rhizobium leguminosarum has a function as such a transporter been proposed (18, 21). For MctP, alanine, lactate, and pyruvate transport was analyzed in detail, whereas for ActP kinetic data were...
obtained only for acetate transport, showing a relatively high affinity ($K_M = 5.4 \mu M$) (18). This is comparable to the affinity of MctC of *C. glutamicum* for the same substrate. MctP affinity for pyruvate was also very high ($K_M = 3.8 \mu M$), which is different from that of MctC of *C. glutamicum* ($K_M = 250 \mu M$). In addition, MctP of *R. leguminosarum* has a broader substrate spectrum, including alanine and lactate and probably also propionate, acetate, pyruvate, butyrate, and $\alpha$-hydroxybutyrate (21). MctC of *C. glutamicum* did not accept alanine and lactate as substrate (Table 1), and for ActP only propionate was found to inhibit acetate transport, pointing out the rather narrow substrate spectra of MctC and ActP in comparison to the MctP transporter of *R. leguminosarum*. An interesting new finding is the cooperative binding of pyruvate and propionate by MctC, as indicated by Hill coefficients of 2 and 3.7, respectively. For acetate the coefficient was only 1.3. This finding is in agreement with kinetic data obtained for acetate transport by ActP. Possible further substrates of ActP and MctP have not been analyzed kinetically, and the cooperativity of these transporters remains elusive.

Although all three transporters (MctC, MctP, and ActP) were defined as members of the SSS family, none of them was found to depend on the electrochemical sodium potential. We proved that the driving force for MctC is the proton potential, as suggested for ActP and MctC (18, 21). The transporter Jen1p of *Saccharomyces cerevisiae* was described as a lactate/acetate/pyruvate/propionate carrier, also dependent on the proton potential (36). However, this eukaryotic carrier belongs to the MFS transporter family and lacks significant similarity to proteins of *C. glutamicum*. Using the MctC sequence we compared bacterial protein sequences of SSS-type transporters (Fig. 7). Thereby, we found a cluster of carriers including the proline transporter PutP of *C. glutamicum* and *E. coli* and OpuE of *B. subtilis* (cluster II), another cluster III of so-far uncharacterized transporters, and a cluster I of (putative) monocarboxylic acid carriers including the MctC and ActP proteins (Fig. 7A). For all carriers, 12 to 13 transmembrane helices (TMH) were predicted. Since sodium-dependent proline transport by *E. coli* PutP was proven and was also proposed for OpuE (41), the clustering might reflect a particular mode of driving force for carriers of the particular clusters. The monocarboxylic acid transporters of cluster I seem to depend on the proton potential, whereas the proline carriers of cluster II are driven by the sodium potential. The MctP transporter of *R. leguminosarum* did not cluster in any of the identified SSS-type transporter subgroups.

Interestingly, a closer inspection of the genomic loci of all transporter-encoding genes revealed that all carrier genes of clusters I and III were found to be in close vicinity to a gene encoding a small membrane protein in the same orientation, in contrast to all genes encoding (putative) proline carriers of cluster II (Fig. 7B). In *E. coli* the actP and yjcH genes are cotranscribed like the mctC gene and cg0952 locus in *C. glutamicum* (18) (Fig. 6). These small membrane proteins might be involved in transport by these carrier systems. Secondary carrier systems with two membrane-bound subunits are known in bacteria, such as the BrnFE system for methionine excretion in *C. glutamicum* or the DctPQM TRAP-T-type dicarboxylate transporter of *Rhodobacter capsulatus* (33, 39). The small subunits of these transport systems, however, harbor four TMH, whereas the small proteins encoded adjacent to MctC or ActP harbor only two TMH. The only known transport systems with two subunits of such a different number of TMH are the eukaryotic amino acid transporters of the HAT family. Whereas a 12-TMH-containing subunit acts as a permease, a single-TMH-containing subunit mediates the substrate speci-
ficity (8). However, we could not find a significant similarity on the level of protein sequence between HAT proteins and MctC as well as Cg0952. Until now we could not discriminate whether there is direct participation in transport of these small subunits or a possible function as a membrane chaperone or as a regulatory unit. Further investigations are required to unravel their contributions in monocarboxylic acid transport.

The cg0952-mctC promoter activity in *C. glutamicum* was found to be higher in cells grown on pyruvate or on acetate plus propionate compared to glucose or acetate as sole carbon sources (Table 2). These results are in agreement with recent results obtained by transcriptome analyses of *C. glutamicum* cells grown in different media (17). Gerstmeir et al. (17) found almost the same cg0952-mctC mRNA levels in media containing glucose, acetate, or glucose plus acetate as carbon sources, indicating that the presence of acetate does not induce (or derepress) mctC expression. In contrast, Hüser et al. found the mRNA level of mctC to be about fourfold higher when the cells were grown in medium containing acetate plus propionate instead of acetate alone (22). These data and our findings that cg0952-mctC promoter activity and monocarboxylic acid transport were higher in cells grown on pyruvate than in cells grown on glucose indicate that pyruvate and propionate induce expression of the cg0952-mctC operon.

To our knowledge, nothing is known about pyruvate- or propionate-dependent transcriptional regulators in *C. glutamicum*. However, the results obtained in this study show that the regulators of acetate and ethanol metabolism, RamA and RamB, bind to the cg0952-mctC promoter region and both are involved in cg0952-mctC expression control. Both were previously shown to be transcriptional regulators of the pta-ack operon encoding acetate kinase and phosphotransacetylase, of the genes for the glyoxylate cycle enzymes isocitrate lyase and malate synthase (aceA and aceB, respectively), and of the alcohol dehydrogenase gene *adhA* (1, 13, 16), and both regulators have been shown to be subject to negative autoregulation (11, 12). Similar to the regulation of pta-ack, aceA, aceB, and *adhA* (2, 13), RamA activates the cg0952-mctC operon and, in fact, seems to be essential for its expression. This result explains the observation that the RamA-deficient mutant *C. glutamicum* Rg2 did not grow in medium containing pyruvate. The fact that mutant Rg2 also did not grow on acetate or acetate plus propionate cannot be explained by the lack of the MctC transport activity, since both acetate and propionate should enter the cell by diffusion at the pH values chosen for the growth experiments. In the case of acetate-containing medium, the nongrowth phenotype is due to the complete lack of isocitrate lyase and malate synthase activities (i.e., the glyoxylate cycle) (13), and in the case of the medium containing acetate plus propionate, it might be explained by a direct or indirect effect of RamA on expression of genes encoding enzymes of the methylcitrate cycle, including the *pta-ack* operon. However, this hypothesis has to be experimentally addressed.

In contrast to RamA, RamB obviously exerts a negative effect on cg0952-mctC expression, as it does on expression of the *pta-ack*, *aceA*, *aceB*, and *adhA* genes (2, 16). However, the cg0952-mctC operon was not completely derepressed in RamB-deficient cells (strain RG1) grown on glucose or acetate, indicating that RamB is not responsible for the carbon source-dependent expression control of this operon. Thus, the carbon source-dependent expression must be mediated by either RamA or a hitherto-unidentified additional regulator. A candidate for such a regulator might be GlxR, a cyclic AMP-dependent regulator previously suggested to be involved in carbon source-dependent expression control of *aceB* (26), of genes involved in gluconate metabolism (29) and glycolysis/glucconeogenesis (*gapA* and *pk*; 20), and of the resuscitation-promoting factor Rpf2 (23). By in silico identification of respective binding sites and by EMSA experiments, Kohl et al. (28) recently found strong evidence for a global regulatory role of GlxR in *C. glutamicum* (28). Indeed, two motifs with some similarity to recognition sites for GlxR can be found in the promoter region of the cg0952-mctC operon, i.e., TGTGATCgACAaCATA and TGTGAGTTAGGTAACAT (consistent nucleotides are shown by capital letters), centered 134 bp and 98 bp upstream of the start codon of cg0832. However, the functionality and the significance of these binding sites and the involvement of GlxR in expression control of the cg0952-mctC operon remain to be elucidated.

The diverse regulation might reflect the complexity of the conditions *C. glutamicum* encounters in natural environments. In addition, our results underline the impact of MctC for *C. glutamicum* not only in its natural habitat but also under biotechnologically relevant conditions. By determination of MctC transport activities and dependence on the external pH, we have demonstrated for the first time that (i) pyruvate does not diffuse through the cytoplasmic membrane of *C. glutamicum* and the transporter MctC is essential for pyruvate uptake, (ii) acetate and propionate diffuse at high substrate concentrations and neutral or acidic external pH and (iii) under alkaline conditions and/or at very low substrate concentrations MctC mediates the bulk acetate uptake. Moreover, by these results the extended lag phase of *C. glutamicum* inoculated in media containing higher concentrations of acetate or propionate can be explained by the uncontrolled influx of these particular substrates by diffusion and the challenge of the pH homeostasis (9, 42). However, we cannot exclude participation of a low-affinity carrier in acetate or propionate uptake at high substrate concentrations, although it seems to be very unlikely. The fact that pyruvate is virtually membrane impermeable allowed us to successfully apply a screening for pyruvate uptake mutants. In view of many biotechnological productions where pyruvate and acetate represent unwanted by-products, knowledge of the relevant transporter systems is of significance. During valine or lysine production by *C. glutamicum*, for example, pyruvate excretion was observed and lowered the product yield (5, 6). By overexpression of the cg0952-mctC operon, pyruvate reutilization could be achieved and thereby carbon loss prevented. In contrast, if pyruvate production were desired deletion of mctC would be indispensable in order to avoid reuptake of the product and to increase the efficiency of the process. In the natural habitat, the MctC transporter is important not only for pyruvate uptake but also for fast utilization of acetate. Only if cells are equipped with a functional MctC transporter can they compete with other cells at very low substrate concentrations. Under natural conditions when nutrients are limiting a carrier system for diffusible substrates enables efficient nutrient utilization and thereby growth and survival.