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Gram-positive soil bacterium Corynebacterium glutamicum uses the compatible solutes glycine betaine, proline, and ectoine for protection against hyperosmotic shock. Osmoregulated glycine betaine carrier BetP and proline permease PutP have been previously characterized; we have identified and characterized two additional osmoregulated secondary transporters for compatible solutes in C. glutamicum, namely, the proline/ectoine carrier, ProP, and the ectoine/glycine betaine/proline carrier, EctP. A ΔbetP ΔputP ΔproP ΔectP mutant was unable to respond to hyperosmotic stress, indicating that no additional uptake system for these compatible solutes is present. Osmoregulated ProP consists of 504 residues and preferred proline (\(K_m = 48 \, \mu M\)) to ectoine (\(K_m = 132 \, \mu M\)). The proP gene could not be expressed from its own promoter in C. glutamicum; however, expression was observed in Escherichia coli. ProP belongs to the major facilitator superfamily, whereas EctP, together with the betaine carrier, BetP, is a member of a newly established subfamily of the sodium/solute symporter superfamily. The constitutively expressed ectP codes for a 615-residue transporter. EctP preferred ectoine (\(K_m = 63 \, \mu M\)) to betaine (\(K_m = 333 \, \mu M\)) and proline (\(K_m = 1,200 \, \mu M\)). Its activity was regulated by the external osmolality. The related betaine transporter, BetP, could be activated directly by altering the membrane state with local anesthetics, but this was not the case for EctP. Furthermore, the onset of osmotic activation was virtually instantaneous for BetP, whereas it took about 10 s for EctP.

Cells exposed to a high osmolality have to solve the problem that membranes are permeable to water but constitute a barrier to other solutes. In order to prevent dehydration, effective adaption mechanisms are required. A general strategy of bacteria to overcome hyperosmotic stress is the accumulation of osmoprotective solutes such as glycine betaine, ectoine ([S]-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic-acid; C\(_6\)H\(_{10}\)N\(_2\)O\(_2\)), and proline (8, 9). Unlike other solutes, e.g., K\(^{+}\) ions, they do not interfere with vital cellular functions when present at high concentrations in the cytoplasm and are thus called compatible solutes.

Osmoregulation has been most intensively studied in Escherichia coli and Salmonella typhimurium (3, 4, 17, 31). Two uptake systems, secondary transporter ProP and ATP-binding cassette (ABC)-type carrier ProU, mediate the uptake of compatible solutes (2, 27, 47). The level of activity (4, 17, 30) and transcription (4, 27, 48, 49) of both are regulated by the external osmolality. In contrast, PutP, a specific proline uptake carrier, is not involved in osmoregulation but is responsible for proline utilization (47). A well-studied example of a gram-positive bacterium with low GC content is Bacillus subtilis. In this organism the uptake of glycine betaine is mediated by three osmoregulated uptake systems belonging either to the ABC type (OpuA and OpuC) or to the class of secondary carriers (OpuD) (18, 21).

Corynebacterium glutamicum, a GC-rich gram-positive soil bacterium, is extensively used in amino acid production (22). It is equipped with a set of osmoreponsive uptake systems for compatible solutes (13, 33, 34), as well as a mechanosensitive efflux channel(s) (41). The high-affinity glycine betaine uptake system, BetP, was kinetically analyzed (13), and the corresponding gene was cloned and sequenced (33). Secondary structure predictions identify BetP as a typical 12-transmembrane segment transporter additionally carrying cytoplasmic domains at its N- and C-terminal parts. Truncation of the C-terminal domain led to loss of the response to osmotic stress, i.e., to a permanently active carrier. This indicates that BetP, besides its transport function, is responsible for both osmosensing and osmoregulation (35). Additionally, we characterized a specific proline uptake system, PutP (34), which, similar to the corresponding system in E. coli, is not osmoregulated and provides proline for anabolism. In the present study, we showed that C. glutamicum possesses, besides BetP and PutP, two further uptake carriers for compatible solutes with broader substrate spectra. We identified the encoding genes, characterized their kinetics, and studied the regulatory properties of these transport systems for compatible solutes in C. glutamicum.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids are described in Table 1. E. coli strains were grown at 37°C either in Luria-Bertani medium (29) or in minimal medium (10, 11), supplemented as described earlier (10, 33–35). C. glutamicum strains were grown at 30°C in either...
brain heart infusion (BHI) medium (Difco, Detroit, Mich.) or minimal medium as described previously (20).

DNA techniques, manipulations, and sequence analyses. C. glutamicum genomic DNA was isolated as described previously (12). Plasmid DNA was isolated with the Qiagen (Hilden, Germany) plasmid kit. Genomic DNA was isolated as described previously (20).

**Construction of C. glutamicum genomic libraries.** For isolation of the prop gene a genomic DNA preparation from C. glutamicum DHPP was partially digested with Sau3A. The resulting DNA fragments were ligated with pUC19 plasmid DNA, digested with BamHI, and dephosphorylated with shrimp alkaline phosphatase (U.S. Biochemicals, Bad Homburg, Germany). Ligation products were transformed in restriction-deficient E. coli DH5αmcr. Plasmids were isolated and transformed for complementation into E. coli mutant strain WG389, which is unable to synthesize or transport proline (10). As a consequence, this strain is not able to grow in media containing 25 μM proline. After transformation, approximately 25,000 resulting clones were tested for growth on minimal medium containing 25 μM proline and 0.3 M NaCl. Plasmids were isolated from clones which were able to grow, tested for complementation, and analyzed by agarose gel electrophoresis. The smallest complementing plasmid was designated pH4A. The prop gene was isolated by a similar approach, except that a genomic DNA preparation from C. glutamicum DHPP was partially digested with Sau3A and the resulting DNA fragments were ligated with pUC19 plasmid DNA, digested with BamHI, and dephosphorylated with shrimp alkaline phosphatase. The complementation was carried out by transforming transport-deficient C. glutamicum NR2 with the genomic library of DHPP. Approximately 25,000 resulting clones were tested for growth in minimal medium containing 1.2 M NaCl and 100 μM proline. The smallest complementing plasmid, designated pBW1, carried an insert of 8 kb.

**Construction of C. glutamicum deletion strains.** The genes prop and ectP were deleted by the method of Schäfer et al. (44). For prop, DHPP (Δprop) was used as the parental strain. An internal prop gene fragment of 1,096 bp (EcoRI/SmI) was removed from plasmid pH4A. After religation of the remaining plasmid DNA, the Δprop fragment of 1,420 bp, which carried the DNA regions upstream and downstream of this gene, was isolated by a Scul/Nael digestion and ligated into plasmid pK19mobacB (pHP6). Plasmid pHP6 was transferred by conjugation into C. glutamicum DHPP. A deletion mutant (DHPP) resulting from a double-chromosomal recombination event was identified by PCR (data not shown). The resulting strain, DHPP, was used as the parental strain for the deletion of the ectP gene. An internal 700-bp ClaI fragment of ectP was removed from plasmid pBW2, and the remaining DNA was religated. A 1.8-kb BamHI/EcoRI fragment of this plasmid was subsequently ligated into pK19mobacB. The resulting plasmid, pBW3, was transferred via conjugation into C. glutamicum DHPP. After two chromosomal recombination events, deletion strain DHPE (Δprop Δprop Δprop Δprop Δprop) was isolated. The genomic deletion of ectP was verified by PCR.

**Isolation of a proline uptake-deficient mutant strain of C. glutamicum.** For the isolation of uptake-deficient mutants of C. glutamicum DHPP (Δprop Δprop Δprop Δprop Δprop), cells were grown overnight in BHI medium, harvested by centrifugation, and resuspended in 0.9% NaCl solution. The cell suspension was adjusted to a final optical density at 600 nm of 1. UV mutagenesis was carried out for 10 min, leading to a survival rate of <0.2%. Directly after the irradiation, the cells were incubated for 3 h in the dark in order to prevent light-dependent DNA repair. For the selection of proline uptake-deficient mutants, 100 μl of the cell suspension was spread onto agar plates containing minimal medium with 600 mM NaCl. A filter disc soaked in 5 M azetidine-2-carboxylic acid solution, a toxic proline analog, was placed in the middle of each plate. After 48 h of incubation at 30°C a growth inhibition zone around the filter disc was visible. Clones which showed significant growth in the inhibition zone were isolated and tested for growth on agar plates containing minimal medium with 1.2 M NaCl and 100 μM proline. Clones whose growth was not restored in this medium were tested in the transport assay to determine their deficiencies in glycine betaine, cysteine, and proline uptake. One clone with the desired phenotype was named NR2.

**Labeled transport substrates and transport assays.** Synthesis of [14C]glycine betaine by oxidation of [14C]choline with C. glutamicum DHPP was performed as described previously (25). Labeled [14C]choline was purchased from Amersham International (Buckinghamshire, United Kingdom). [14C]Ectoine was a gift from E. Galinski (University of Münster, Münster, Germany). The transport assay was carried out as described recently (34, 35).

**Computer-assisted analyses.** Computer-assisted nucleotide and protein sequence analyses were carried out with the PCGene program (release 6.26; Genofit, Geneva, Switzerland). For sequence similarity searches the EMBL (Heidelberg, Germany) data bank program BLASTX was used. Protein sequence alignments and the protein secondary structure analyses were carried out with the PHDtopology program (EMBL).

**Nucleotide sequence accession numbers.** The nucleotide sequence data reported here were submitted to GenBank (EMBL) and assigned accession no. Y12537 (prop) and AJ001436 (ectP).

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**TABLE 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Relevant genotype and/or characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. glutamicum ATCC 13032</td>
<td>Wild type</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>DHP1***</td>
<td>Δprop</td>
<td>This study</td>
</tr>
<tr>
<td>DHP8**</td>
<td>Δprop Δprop</td>
<td>This study</td>
</tr>
<tr>
<td>DHPP**</td>
<td>Δprop Δprop Δprop Δprop Δprop</td>
<td>This study</td>
</tr>
<tr>
<td>DHPE**</td>
<td>Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δprop Δpro...</td>
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</table>
RESULTS

Isolation and analysis of proP. BetP and PutP are specific uptake systems for glycine betaine and proline, respectively, in C. glutamicum. However, after deletion of betP and putP, strain DH8 still showed uptake of proline, ectoine, and betaine, although with relatively low affinity (34). We also observed that the uptake of any one of these compounds was inhibited by the others. This indicates the presence of at least one additional transport system for compatible solutes. For the isolation of the corresponding gene(s) a genomic library of C. glutamicum DH8 was transformed into E. coli mutant strain WG389, which lacks transport systems PutP, ProP, and ProU and is unable to synthesize proline (10). Heterologous complementation led to the isolation of a complementing plasmid (pHP4A) with an insert of 5.1 kb. A subcloned 2.9-kb fragment was sufficient for growth in minimal medium containing 25 μM proline and 0.3 M NaCl. Both strands of this fragment of the complementing plasmid were sequenced, and the sequencing revealed an open reading frame (positions 789 to 2318) with a single translation start site, a GTG codon at position 804, resulting in a protein of 504 residues. The gene was designated proP. The primary structure of the osmoregulated C. glutamicum proline permease, ProP, shows a high degree of identity to OusA of Erwinia chrysanthemi (39%) (15) and to ProP of E. coli (38%) (10). ProP of C. glutamicum is predicted to possess 10 transmembrane segments (program PHDTopology) (40). As a consequence, both the C- and N-terminal extensions of ProP of C. glutamicum are proposed to face the cytoplasm.

Kinetic properties and regulation of proP. In contrast to what was found for control strain MKH13/pEKEX2, E. coli MKH13/pHP5 carrying proP under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible tac promoter showed highly active proline and ectoine uptake, i.e., 71 and 129 nmol/min/mg (dry weight [dw]), respectively, after induction of transcription by IPTG. Ectoine was not accepted as a substrate, and unlabeled betaine in 50-fold excess did not inhibit the uptake of ectoine or proline. Thus, proP encodes a proline/ectoine uptake system. The transport affinity for proline (K_m of 48 μM) was higher than that for ectoine (132 μM) (data not shown). In contrast to the activity of PutP, which is not osmoregulated, ProP activity did not depend on the presence of Na^+ (data not shown). On the contrary, ProP was immediately and completely inhibited by the addition of the uncoupler carbonyl cyanide m-chlorophenyl hydrazone (data not shown). Thus, it is most likely that protons are the coupling ions of this secondary transporter. We therefore conclude that the proP gene does not code for the previously described low-affinity sodium-dependent proline uptake system of C. glutamicum observed in the ΔbetP ΔputP strain (34) but rather represents an additional uptake system for compatible solutes in C. glutamicum. Proline transport in strain MKH13/pHP5 increased in response to increasing external osmolality and reached its maximal activity at 400 mM external NaCl, which correlates to an overall osmolality of about 1.0 osmol/kg (Fig. 1).

For elucidating the role of ProP in solute uptake in C. glutamicum we constructed a ΔbetP ΔputP ΔproP strain (DHPP) based on strain DH83 (34). However, no change in the uptake of ectoine, proline, and betaine was observed in mutant DHPP (data not shown), although we varied the osmolality and composition of the buffer in the uptake assay and the culture medium. These results further indicate that the transport activity of an as yet unknown system, not related to ProP, has previously been observed in strain DH83 (34). Since ProP, when synthesized in E. coli, was activated by an increase in medium osmolality, the missing activity of ProP in C. glutamicum is obviously due to regulation of the level of expression rather than to activity regulation. To prove this hypothesis, plasmid pHP5 carrying the proP gene downstream of the tac promoter was transferred into strain DHPP. Osmoregulated proline uptake was measured in strain DHPP/pHP5 in the presence of betaine in 100-fold excess, which was added to inhibit proline uptake by the putative additional transport system. The observed osmoregulated activity in strain DHPP/pHP5 can thus be assigned to the ProP system (Fig. 1).

Isolation and analysis of EctP. BetP, PutP, and ProP were identified by heterologous complementation of E. coli strains deficient in the synthesis and transport of compatible solutes. Since we failed to isolate the gene of the putative fourth uptake system by using this approach, homologous complementation was applied. For isolation of an uptake-deficient mutant strain of C. glutamicum the toxic proline analog azetidinecarboxylate was used to provide selection pressure (17, 26, 37). Mutant NR2 was isolated as described in Materials and Methods. NR2 was unable to take up proline and ectoine, while betaine transport was reduced to 1.5 nmol/min/mg (dw) compared to 15 nmol/min/mg (dw) for the parental strain. This indicates that NR2 had lost all transport systems for these compatible solutes.

A genomic library of strain DHPP was then transformed into C. glutamicum mutant strain NR2. Clones which regained the ability to grow in media of high osmolality in the presence of 100 μM proline were isolated. They all harbored plasmid pBW1, which carried an insert of 8 kb. Subcloning led to a complementing plasmid (pBW2) with an insert of 2.7 kb. The sequence analysis revealed an open reading frame with a single translational start site, an ATG codon at position 405, resulting in a protein of 615 residues (EctP). EctP is highly similar to OpuD of B. subtilis (18), to a putative BetP protein of Mycobacterium tuberculosis (36), to BetP of C. glutamicum (33), to a putative choline permease of Haemophilus influenzae (14), and to BetT of E. coli (23). All proteins are predicted to have 12 transmembrane segments. In addition, EctP carries N- and C-terminal extensions predicted to face the cytoplasm. While
TABLE 2. EctP-mediated uptake of compatible solutes ectoine, glycine betaine, and proline in different C. glutamicum and E. coli strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Uptake rate (nmol/min/mg [dw]) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 mM proline</td>
</tr>
<tr>
<td>C. glutamicum</td>
<td></td>
</tr>
<tr>
<td>DHPP</td>
<td>19.2 ± 2.6</td>
</tr>
<tr>
<td>DHPE</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>DHPE/NACl</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>DHPE/pBW1</td>
<td>53.7 ± 0.0</td>
</tr>
<tr>
<td>E. coli MHK13/pBW1</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

* The measurements were carried out in triplicate in the presence of 600 mM NaCl.
* Below the detection limit.

Kinetic properties and functional analysis of EctP. The ectP gene was deleted in strain DHPP, leading to strain DHPE (ΔbetP ΔputP ΔproP ΔectP). In contrast to what was found for DHPP, the uptake of ectoine and proline in DHPE was below the detection limit and only a very low residual glycine betaine uptake rate of 1.4 nmol/min/mg (dw) was observed (Table 2). The kinetic properties of EctP could thus be determined in C. glutamicum DHPP (ΔbetP ΔputP ΔproP). EctP, does not carry any uptake system for compatible solutes. Vₘₐₓ values for ectoine, betaine, and proline, as well as for symport ion Na⁺, were determined in the presence of 600 mM NaCl or 600 mM sorbitol. Whereas the Vₘₐₓ values for all substrates were similar, the Kₘ values were strikingly different (Table 3). Ectoine, with a Vₘₐₓ/Kₘ ratio of 0.43, was the preferred substrate, followed by glycine betaine (Vₘₐₓ/Kₘ = 0.1 ml/min/mg [dw]) and proline (Vₘₐₓ/Kₘ = 0.03 ml/min/mg [dw]). At 9.1 mM, the Kₘ of Na⁺ was within the range observed for the betP protein (13).

Regulation of EctP activity. Whereas no uptake of ectoine, proline, or betaine was detected in E. coli MHK13/pBW1 carrying the ectP gene under the control of its own promoter, high uptake rates of these substrates were observed in C. glutamicum DHPE/pBW1 (Table 2). This result indicates that the C. glutamicum ectP promoter does not function in E. coli, as we determined by the functional expression of ectP in this host from pBW4, where it is under the control of the tac promoter (data not shown). We furthermore analyzed the effect of the regulation of EctP on the level of activity by using the same plasmid in strain DHPE. Similar to what was found for the regulation of BetP (35) and ProP (see above), betaine uptake by DHPE/pBW4 increased with an increasing concentration of either NaCl or sorbitol (Fig. 2).

For further elucidation, we modulated the state of the membrane without altering the transmembrane osmotic gradient. For this purpose, local anesthetics can be used (24, 35). Tetracaine is known to influence the physical state of the membrane and was used to test whether the activity of the respective carrier protein is influenced via the membrane directly (for further references, see reference 24) (Fig. 3). The external osmolality was adjusted with NaCl to final values of 0.4, 0.7, and 1.1 osmol/kg, and tetracaine was varied from 0 to 1.25 mM (24). As previously observed in the C. glutamicum wild-type strain (35), BetP was directly activated by the addition of tetracaine. Surprisingly, this was not the case for EctP (Fig. 3).

TABLE 3. Basic kinetic parameters of EctP in C. glutamicum DHPP

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kₘ (μM)</th>
<th>Vₘₐₓ (nmol/min/mg [dw])</th>
<th>Vₘₐₓ/Kₘ (ml/min/mg [dw])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectoine</td>
<td>63 ± 4.9</td>
<td>27</td>
<td>0.43</td>
</tr>
<tr>
<td>Glycine betaine</td>
<td>333 ± 45</td>
<td>34</td>
<td>0.10</td>
</tr>
<tr>
<td>Proline</td>
<td>1,200 ± 180</td>
<td>34</td>
<td>0.03</td>
</tr>
<tr>
<td>Sodium</td>
<td>9,100 ± 1,300</td>
<td>9.03</td>
<td></td>
</tr>
</tbody>
</table>

* Uptake of labeled ectoine, glycine betaine, and proline was measured in triplicate at a constant NaCl concentration of 600 mM. In those experiments in which the NaCl concentration was varied, 0.6 M sorbitol and 0.1 to 50 mM NaCl were used. As a monitor for sodium interaction with EctP, the uptake of glycine betaine was determined.

FIG. 2. Stimulation of glycine betaine uptake by C. glutamicum DHPE/pBW4 with NaCl and sorbitol. Cells were grown overnight in BHI medium supplemented with 0.2 mM IPTG. Uptake was started by the addition of 750 μM labeled glycine betaine. The osmolality of the uptake assay medium was increased by the addition of either NaCl or sorbitol in the presence of 50 mM NaCl (○). The uptake rates at an external osmolality of 1.4 or 1.1 osmol/kg were defined as 100% activity for stimulation by NaCl and sorbitol, respectively. The absolute rates were 42.8 nmol/min/mg (dw) at an osmolality of 1.4 osmol/kg (NaCl) and 21.8 nmol/min/mg (dw) at an osmolality of 1.1 osmol/kg (sorbitol).

FIG. 3. Stimulation of glycine betaine uptake in C. glutamicum DHPE/pBW4 and DHPE/pGTG by the local anesthetic tetracaine. Cells were grown overnight in BHI medium supplemented with 0.2 mM IPTG. Uptake was started by the addition of 750 μM labeled betaine. Betaine uptake in strain DHPE/pGTG (■) was determined at an external NaCl osmolality of 0.4 osmol/kg. The activity of DHPE/pBW4 (open symbols) was measured at values of external osmolality of 0.4 (○), 0.7 (□), and 1.1 osmol/kg (△).
The expression of BetP of *C. glutamicum* is strongly regulated by the osmolality and composition of the growth medium (13). Although the growth conditions of strain DHPP (ΔbetP ΔputP ΔproP) were varied extensively, we did not observe a significant change in the activity of EctP (data not shown).

**Regulation of the onset of the activity of BetP and EctP.** Since we observed differences in the responses of BetP and EctP to the change in the physical state of the membrane, we elucidated the time courses of the responses of these two transport systems to an instantaneous change in external osmolality. For this purpose, we analyzed the onset time of activity regulation, i.e., the period of time necessary to transform the carrier from an inactive to an active state (Fig. 4). For comparison, cells preadapted to these conditions, which should be active without a delay, were tested. Genes betP and ectP were expressed by using IPTG-inducible vector pEKEX2 in strain DHPE, which lacks all chromosomal genes coding for uptake systems of compatible solutes. Uptake was started by the addition of high-osmolality buffer together with labeled betaine (Fig. 4). For BetP, no lag time for the onset of activity was observed, irrespective of whether the cells were adapted to high osmolality or not (Fig. 4A). The observation of negative values for the extrapolated onset time may be explained by a small amount of label being instantaneously bound to binding sites after addition of the cells. In contrast, EctP, even in the activated state, showed a response which was delayed by about 10 s compared to that of BetP (Fig. 4B). This result, together with the different effects of tetracaine on BetP and EctP activity, is a strong indication that the mechanism of response to osmotic stress is different for these two carrier proteins.

**Physiological significance of the different uptake systems for compatible solutes.** For comparing the physiological significance of the three osmoregulated carriers, three different growth conditions in minimal media containing 1.2 M NaCl were tested, in either the absence or the presence (at 1 mM) of compatible solute betaine or proline (Fig. 5). Growth of ΔbetP strain DHPI, as well as that of the double and triple mutants, was more or less identical to the growth of the wild type, regardless of whether the hyperosmotic medium contained the compatible solute or not. In contrast, growth inhibition by osmotic stress of strain DHPE, which lacks all four uptake systems, was not restored by betaine or proline. This result indicates that no further uptake system for the compatible solutes is present in *C. glutamicum*. In all strains, glycine betaine proved to be more effective than proline in protecting *C. glutamicum* against osmotic stress. The results in Fig. 5 furthermore show that EctP is able to fully compensate for the uptake activity of the other systems under the conditions tested.

**The phylogenetic relationship of the osmoregulated transporters BetP, EctP, and ProP.** Prokaryotic and eukaryotic secondary transporters are phylogenetically divided into three superfamilies, namely, the major facilitator superfamily (MFS), the APC family (amino acids, polyamines, and cholines), and the sodium/solute symporter superfamily (SSSS) (38, 39, 42). BetP, ProP, and EctP were analyzed by the program ALLALL (ETH, Zürich, Switzerland). EctP and BetP are closely related to each other and to five other prokaryotic carriers for compatible solutes, all belonging to the SSSS, namely, OupD (*B. subtilis*), BetT (*E. coli*), CaiT (*E. coli*), and putative BetP proteins from *M. tuberculosis* and from *H. influenzae*. These proteins constitute a separate subfamily of betaine/choline/carnitine transporters (42a) or trimethylammonium transporters (18). In contrast to the other uptake systems for compatible solutes in *C. glutamicum*, ProP is closely related to the ProP of *E. coli* and the OusA of *E. chrysanthemi*, which are members of the MFS.

**DISCUSSION**

In its natural habitat, gram-positive soil bacterium *C. glutamicum* has to cope with dramatic changes of water availability. Consequently, it is equipped with a set of transport systems that respond to hyperosmotic stress. We identified two uptake systems, ProP and EctP, for compatible solutes in addition to those previously identified, the specific betaine uptake system, BetP, and the proline permease, PutP (33, 34). The gene of the secondary ectoine/proline uptake system, ProP, was isolated by heterologous complementation in *E. coli*, whereas the gene of the secondary ectoine/proline/betaine uptake system, EctP, was identified by homologous complementation of a specifically selected *C. glutamicum* mutant strain. ProP of *C. glutamicum* shows significant similarity to ProP of *E. coli* (10) and to OusA of *E. chrysanthemi* (15) and belongs to the MFS (38). From a computer-assisted analysis, 10 transmembrane segments are predicted for the ProP of *C. glutamicum*, with both the N- and C-terminal extensions located in the cytoplasm. It was suggested that the C-terminal domain of the *E. coli* ProP protein is involved in sensing osmotic stress (10), as has actu-
ally been shown for the C-terminal domain of BetP of *C. glutamicum* (35). Interestingly, the C-terminal extensions of OusA from *E. chrysanthemi* and of ProP from *E. coli* have 70% identical residues and are both predicted to form a coiled-coil structure, but, in contrast to what was found for ProP of *E. coli*, no activity regulation of OusA by a change in osmolality was observed (15). It is important to note, however, that OusA activity was measured in heterologous host *E. coli* by using a supraoptimal hyperosmotic shift of 0.5 M NaCl, which would inhibit ProP activity in *E. coli*. Note also that the C-terminal extension of the *C. glutamicum* ProP is not predicted to form a coiled-coil structure. The same is true for BetP and EctP of *C. glutamicum*.

Uptake system EctP has significant identity with BetP from *C. glutamicum*, OpuD from *B. subtilis*, BetT from *E. coli*, a putative BetP from *M. tuberculosis*, and putative BetT proteins from *M. tuberculosis* and *H. influenzae*. Together with these proteins, it belongs to the SSSS (39). This group obviously constitutes a new subfamily of trimethylammonium transporters (18) or betaine/choline/carnitine transporters (42a). A comparison of the EctP amino acid sequence with those of OpuD of *B. subtilis*, BetP of *M. tuberculosis*, and BetP of *C. glutamicum*, and BetT of *E. coli* revealed, besides the general secondary structure of 12 transmembrane segments, a highly conserved amino acid sequence between helices 8 and 9. The function of this domain is not known so far; however, it may be related to the fact that members of this family of carriers all accept substrates with a trimethylammonium group (18).

The fact that more than one system is available for a particular compatible solute emphasizes the physiological significance of osmoregulation for *C. glutamicum*, similar to that for *E. coli* and *B. subtilis*. Under the conditions tested, PutP and ProP do not seem to contribute to osmotic protection. The role of ProP is not clear so far, since we did not find conditions under which it is expressed in *C. glutamicum*. The growth response to a high salt concentration indicated that EctP alone is sufficient for osmotic protection, provided betaine or proline is available. EctP seems to be the emergency system, accepting all known compatible solutes in *C. glutamicum* but with a preference for ectoine. It needs to be established whether EctP is able to accept further possible compatible solutes as has been shown for the emergency system, OpuC, in *B. subtilis* (19).

The levels of expression of osmoregulated transporters BetP, ProP, and EctP of *C. glutamicum* differ. The activity of BetP is increased up to 15-fold by a change in medium osmolality (unpublished results). Under the same conditions, the activity of EctP, measured after full stimulation of activity by osmotic stress, was not observed to change. These results reflect the physiological significance of the two systems in *C. glutamicum*. The emergency system, EctP, is constitutively expressed from its own promoter in the heterologous host *E. coli*. However, although various conditions for the expression of ProP were tried, we did not find ProP-related activity in *C. glutamicum*. It can be speculated that proP is expressed under different stress conditions, e.g., heat stress, or, alternatively, that the gene is silent in *C. glutamicum*. In contrast to the genes for transporters BetP, PutP, and ProP of *C. glutamicum*, the ectP gene could not be expressed in *E. coli* from its own promoter, although the structures of *C. glutamicum* promoters seem to be very similar to those of *E. coli* promoters (32). However, there are also reports of promoters specific for corynebacteria which are not functional in *E. coli* (5).
The most surprising result of this investigation, however, concerns the regulation of the levels of activity of transporters BetP, ProP, and EctP. BetP, ProP, and EctP were all shown to be strictly regulated by the external osmolality. At isosmolar conditions or at low levels of hyperosmotic stress, i.e., below an external osmolality of about 0.5 osmol/kg, the carriers are not active at all. After a sharp rise in activity, they all reach a maximum of solute uptake at an external osmolality of 1.1 to 1.4 osmol/kg. In comparison, ProP from E. coli also shows an optimum of activation (17). Surprisingly, BetP and EctP from C. glutamicum, with 34% identical amino acids and belonging to the same transporter family, were significantly different with respect to the mechanism regulating the level of activity. Although the true mechanism by which mechanical stress due to a transmembrane osmotic gradient acts on carrier proteins remains to be elucidated at the molecular level (24), it would seem to be a general motif of osmoregulated membrane proteins, is a facilitator for glycerol uptake and efflux and is inactive under normal osmotic stress. EMBO J. 1994. Glycine betaine excretion in Corynebacterium glutamicum: molecular analysis of the ibkBbN-ibC operon. J. Bacteriol. 175:5595–5603.


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