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# Isolation of the *putP* gene of *Corynebacterium glutamicum* and characterization of a low-affinity uptake system for compatible solutes

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**Abstract** *Corynebacterium glutamicum* accumulates the compatible solutes proline, glycine betaine, and ectoine under conditions of high osmolality. Uptake of proline is mediated by both a high-affinity and a low-affinity secondary transport system. The low-affinity uptake system also accepts glycine betaine and ectoine as substrates. In the present study, the gene encoding the high-affinity proline uptake system PutP was isolated by heterologous complementation of *Escherichia coli* mutant strain WG389, which lacks the transport systems BetT, PutP, ProP, and ProU and is unable to synthesize proline and glycine betaine. This gene (*putP*) encodes a protein of 524 amino acids that shares identity with the proline transport systems PutP of *E. coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Haemophilus influenzae*, and *Klebsiella pneumoniae*. Functional studies of PutP synthesized in *E. coli* mutant strain MKH13, which also lacks the transport systems for compatible solutes and is unable to synthesize glycine betaine, revealed that this carrier system is not regulated by the external osmolality on the level of activity. $K_m$ values of 7.6 mM for proline and 1.3 mM for sodium as cotransported ion were determined. Deletion of the *putP* gene allowed the functional characterization of another proline uptake system with low affinity.

**Key words** *Corynebacterium glutamicum* · PutP · Proline transport · Compatible solutes · Osmoregulation

**Abbreviations** *PMBN* Polymyxin B nonapeptide · *CCCP* Carbonyl cyanide *m*-chlorophenylhydrazone · *IPTG* Isopropyl-β-D-thiogalactopyranoside

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## Introduction

The gram-positive bacterium *Corynebacterium glutamicum* is used in industrial fermentations for the production of amino acids such as glutamate, lysine, and isoleucine. During the fermentation process, the osmotic conditions change significantly. Also in its natural habitat, the soil, *C. glutamicum* requires effective adaptation mechanisms in order to prevent cell lysis or dehydration as a consequence of frequently changing osmotic conditions in its surroundings. A strategy of bacteria to overcome hyperosmotic stress is the accumulation of osmoprotective solutes such as glycine betaine and proline [for reviews, see Csonka (1989) and Csonka and Hanson (1991)]. Proline uptake of the well-studied gram-negative organisms *Escherichia coli* and *Salmonella typhimurium* is mediated by three transport systems: ProU, ProP, and PutP (Wood 1988; Booth and Higgins 1990; Lucht and Bremer 1994). ProU and ProP accept a broad range of substrates and are regulated by the external osmolality. While the binding-protein-dependent transport system ProU is regulated at the level of transcription and activity (Cairney et al. 1985b; Lucht and Bremer 1994), the secondary carrier ProP is mainly regulated on the level of activity (Cairney et al. 1985a; Grothe et al. 1986; Milner et al. 1988). PutP, the high-affinity proline uptake system, is not involved in osmoregulation, but functions in proline utilization (Wood 1988).

The situation in gram-positive bacteria is best understood in *Bacillus subtilis*. Similar to enterobacteria, *B. subtilis* accumulates glycine betaine and proline in response to hyperosmotic stress (Kappes et al. 1996). Uptake of glycine betaine in this organism is mediated by two binding protein-dependent transport systems, OpuA (Kempf and Bremer 1995) and OpuC, and by a secondary uptake system, OpuD (Kappes et al. 1996). Also *Staphyloccocus aureus* accumulates glycine betaine and proline as major osmoprotectants (Bae et al. 1993). Two feedback-regulated proline uptake systems, one with low affinity and one with high affinity (PutP), have been identified in

this organism (Pourkomailian and Booth 1994; Stimeling et al. 1994; Wengender and Miller 1995). *C. glutamicum* uses glycine betaine, proline, and ectoine as compatible solutes (Frings et al. 1993; Farwick et al. 1995; Guillouet and Engasser 1995). Glycine betaine is accumulated by the secondary transport system BetP, which is strongly regulated mainly at the level of activity (Peter et al. 1996). In the present communication, we describe the mechanisms of proline transport in *C. glutamicum*. Two proline uptake systems were characterized: a low-affinity one and a high-affinity one. The gene encoding the high-affinity proline transporter, *putP*, was isolated and analyzed.

## Materials and methods

### Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* strains were grown at 37°C either in Luria Bertani (LB) medium (Maniatis et al. 1982) or in minimal medium (Davis and Mingioli 1950) containing 0.5% glucose, 245 mM tryptophan, and 0.001% thiamine for growth of *E. coli* WG389, and 0.5% glucose, 0.004% arginine, 0.004% isoleucine, and 0.004% valine for growth of *E. coli* MKH13. *C. glutamicum* strains were grown in Brain Heart Infusion medium (Difco, Detroit, Mich., USA) at 30°C.

### General DNA techniques

Genomic DNA was isolated from *C. glutamicum* according to Eikmanns et al. (1994). Plasmid DNA was isolated using the Qiagen Plasmid Kit (Qiagen, Hilden, Germany). *E. coli* was transformed using standard methods (Chung et al. 1989). Conjugation between *E. coli* S17-1 and *C. glutamicum* ATCC 13032 was carried out as described by Schäfer et al. (1990). Transconjugants were selected on Brain Heart Infusion agar plates containing 25 mg kanamycin/l and 50 mg nalidixic acid/l.

### DNA manipulations and sequence analyses

Restriction enzymes, DNA polymerase I (Klenow fragment), proteinase K, RNase, and T4 DNA ligase were obtained from Boehringer (Mannheim, Germany) or from Promega (Heidelberg, Germany) and were used as recommended by the manufacturers. Restriction analyses were carried out by separation of the DNA fragments in 0.8% agarose gels. DNA fragments were isolated by use of QIAex (Qiagen, Hilden, Germany). DNA sequencing was performed using the A.L.F. DNA sequencer from Pharmacia (Freiburg, Germany). Sequence reactions were carried out using the AutoRead Sequencing Kit (Pharmacia, Freiburg, Germany) as described by the manufacturer. Additionally, some sequencing was performed by MediGene (Martinsried, Germany).

### Construction of a *C. glutamicum* genomic library

A genomic DNA preparation from *C. glutamicum* was partially digested with *Sau*3A. The resulting DNA fragments were ligated with pUC18 plasmid DNA digested with *Bam*HI and dephosphorylated with shrimp alkaline phosphatase (USB, Bad Homburg, Germany). Ligation products were transformed in the restriction-deficient strain of *E. coli* DH5amcr in order to circumvent DNA degradation by the wild-type *E. coli* restriction system. The resulting colonies were pooled in groups of approximately 5,000–10,000 clones; plasmids were isolated from these clones and were trans-

**Table 1** Strains and plasmids used in this study

| Strains or plasmids | Relevant genotype or characteristics | Source/reference |
|---|---|---|
| Strains | | |
| *Corynebacterium glutamicum* | | |
| ATCC 13032 | Wild-type | American Type Culture Collection |
| DHP1 | *betP* deletion strain | This study |
| DHP8 | *putP/betP* deletion strain | This study |
| *Escherichia coli* | | |
| DH5αmcr | *mcrA, Δ(mrr-hsdRMS-mcrBC)* | Grant et al. (1990) |
| MKH13 | MC4100 *Δ(putPA)101 Δ(proP)2 Δ(proU)* | Kempf and Bremer (1995) |
| WG389 | $F^-$ *trp rpsL thi Δ(putPA)101 Δ(proP mel)212 Δ(proU)600 Δ(brnQ phoA proC)* | Culham et al. (1993) |
| S17-1 | *thi1 endAR1 hsdR17* ($r^-m^+$) *supE44 pro* | Simon et al. (1983) |
| Plasmids | | |
| pUC18/19 | $Ap^R$, *ptac* | Veiira and Messing (1982) |
| pEKEX2 | $Km^R$, *lacI*$^q$, *ptac* | Eikmanns et al. (1994) |
| pK19mobsacB | $Km^R$, *mob, sacB* | Schäfer et al. (1994) |
| pAB1 | pUC19 containing a 3.8-kb *C. glutamicum* genomic DNA fragment | This study |
| pAB2 | pEKEX2 containing the *putP* gene of pAB1 | This study |
| pDN1 | pEKEX2 containing a 2.4-kb *Rca*I-*Sph*I fragment of *putP* | This study |
| pHP2 | pUC18 containing the *betP* gene | Peter et al. (1996) |
| pKA1 | pK19mobsacB containing a 2.7-kb gene fragment of pAB1 | This study |
| pKH1 | pK19mobsacB containing a 1,681-bp *betP* fragment | This study |

formed into *E. coli* mutant strain WG389 for the complementation assay.

### Determination of the translational start site of *putP*

For determination of the start codon of *putP*, all possible translation start sites other than the ATG at position 858 were deleted. For this purpose, an *Rca*I-*Sph*I fragment (2.4 kb) of plasmid pAB1 was isolated and ligated with plasmid pEKEX2. The resulting plasmid, pDN1, was transferred into the *E. coli* mutant strain MKH13 by transformation, and the proline uptake of this transformed strain was measured.

### Southern blot hybridization

Plasmid and genomic DNA were digested with restriction endonucleases; the resulting DNA fragments were separated by agarose gel electrophoresis and transferred to a nylon filter for hybridization with a digoxigenin-labeled, 963-bp *Sal*I-*Pst*I fragment. Hybridization and detection were performed using the Boehringer DIG DNA Labeling and Detection Kit (Mannheim, Germany) according to procedures described by the manufacturer.

### PCR

Deletion mutants were identified by PCR amplification using *Taq* polymerase (Boehringer, Mannheim, Germany) and a Thermo-Cycler 480 (Perkin-Elmer, Norwalk, Connecticut, USA). For PCR amplification of the deletion mutant DHP1 (Δ*betP*), the oligonucleotides 5′-GTGAGTTTTATGACTACATCTGAC-3′ (nt 457–480) and 5′-GCGTCGCTTTGCAGCCAG-3′ (nt 2214–2197) (each 10 pM) were used. A denaturating temperature of 94° C for 30 s, an annealing temperature of 62° C for 30 s, and an extension temperature of 72° C for 150 s was used for 30 cycles. PCR of the deletion mutant DHP8 (Δ*betP*Δ*putP*) was carried out with the oligonucleotides 5′-CAAGACAGAAAAATACGACG-3′ (nt 937–957) and 5′-GATTCATACAACGTATCG-3′ (nt 2255–2236) (each 10 pM). A denaturating temperature of 94° C for 30 s, an annealing temperature of 52° C for 30 s, and an extension temperature of 72° C for 90 s was used for 30 cycles. Heat-treated, whole-cell preparations were used as templates (Joshi et al. 1991).

### Synthesis of [$^{14}$C]glycine betaine

Synthesis of [$^{14}$C]glycine betaine by oxidation of [$^{14}$C]choline (specific activity 52 μCi/mmol) using choline oxidase was basically performed as described by Landfald and Strøm (1986). In order to dilute the ethanolic [$^{14}$C]choline solution, the volume was increased to 500 μl and 30 U choline oxidase was used. Radiolabeled [$^{14}$C]choline was purchased from Amersham (Buckinghamshire, UK).

### Transport assays

*C. glutamicum* cells were grown overnight in Brain Heart Infusion medium. The cells were washed with buffer containing 50 mM potassium phosphate (pH 7.5) and 10 mM NaCl, energized with 10 mM glucose and, if indicated, osmotically stressed by addition of NaCl for 3 min at 30° C. Uptake measurements were started by adding [$^{14}$C]proline (Hartmann, Braunschweig, Germany) or [$^{14}$C]glycine betaine (approximately 0.1 μCi/ml of reaction mix) at a final concentration of 100 or 200 μM. Aliquots of 200 μl were transferred to glass-fiber filters (type F, pore size 0.45 μm; Millipore, Eschborn, Germany) at time intervals of 15 or 30 s. The cells were washed twice with 2.5 ml 0.1 M LiCl solution, and the radioactivity retained on the filters was determined by liquid scintillation counting.

*E. coli* cells, that were grown overnight in minimal medium (Davis and Mingioli 1950) were inoculated in fresh medium to an $OD_{600}$ of 0.05. If appropriate, 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. Cells were harvested in the mid-exponential phase ($OD_{600}$ of 0.4–0.6), washed twice with medium, and suspended to an $OD_{600}$ of 1. Subsequently, the cells were equilibrated for 3 min at 37° C, and transport was initiated by adding [$^{14}$C]proline (approximately 0.1 μCi/ml of reaction mix) at a final concentration of 100 μM. For $K_m$ determination, the proline concentration was varied from 1 μM to 100 mM. The $K_m$ of the co-transported sodium ions was measured in 50 mM Mops-KOH (pH 7.0) containing 10 mM glucose and 0.1–25 mM NaCl. Uptake was terminated by rapid filtration through glass-fiber filters. The cells were washed twice with buffer containing 50 mM $MgCl_2$ and 0.5 M sucrose (Perroud and Le Rudelier 1985). Radioactivity was determined by liquid scintillation counting.

### Computer-assisted analyses

Computer-assisted nucleotide and protein sequence analyses were carried out using 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 using the PHDhtm program (EMBL, Heidelberg, Germany).

### Nucleotide sequence accession number

Nucleotide sequence data have been submitted to GenBank (EMBL, Heidelberg, Germany) under accession no. Y09163.

## Results

### Deletion of *betP* in *C. glutamicum* ATCC 13032

Deletion of the *betP* gene, which encodes the high-affinity glycine betaine uptake system of *C. glutamicum* (Peter et al. 1996), was carried out by using the method of Schäfer et al. (1994). For this purpose, an internal gene fragment of 661 bp (*Hin*dII-*Nru*I) of the plasmid pHP2 was removed. The resulting insert of 1,681 bp, which carried the DNA regions upstream and downstream of *betP*, was cloned into vector pK19mobsacB. The resulting plasmid pKH1 was transferred by conjugation into *C. glutamicum* wild-type strain ATCC 13032. A deletion mutant (DHP1) resulting from a double chromosomal recombination event was identified by PCR. In contrast to the 1.8-kb fragment obtained by PCR amplification of the wild-type DNA, the fragment obtained for the mutant DHP1 showed the expected size of 1.1 kb.

### Proline and glycine betaine uptake of *C. glutamicum* DHP1

Glycine betaine transport of *C. glutamicum* strain DHP1 was measured in the presence of high and low external osmolality (Table 2). Uptake at high osmolality (600 mM NaCl) was strongly decreased to about 12.5 nmol $min^{-1}$ (mg dry wt.)$^{-1}$ as compared to the wild-type strain [110 nmol $min^{-1}$ (mg dry wt.)$^{-1}$]. This result demonstrates the

**Table 2** Glycine betaine and proline uptake rates of strain *C. glutamicum* DHP1. Cells were grown in complex medium, washed once, and were allowed to equilibrate for 3 min at 30°C after addition of NaCl. The uptake measurements were started by the addition of labeled glycine betaine or proline at a final concentration of 100 μM

| Addition | Uptake rate [nmol $min^{-1}$ (mg dry wt.)$^{-1}$] | | | |
|---|---|---|---|---|
| | [$^{14}$C]glycine betaine | | [$^{14}$C]proline | |
| | 10 mM NaCl | 600 mM NaCl | 10 mM NaCl | 600 mM NaCl |
| None | < 0.5 | 12.5 | 8.6 | 18.5 |
| Glycine betaine (10 mM) | < 0.5 | < 0.5 | 9.1 | 5.1 |
| Ectoine (10 mM) | < 0.5 | < 0.5 | 6.3 | 5.8 |
| Proline (10 mM) | < 0.5 | < 0.5 | < 0.5 | < 0.5 |

loss of the major glycine betaine transport system but at the same time indicates the existence of other uptake system(s) for this compatible solute; other uptake systems have been previously observed in an insertion mutant of *betP* (Peter et al. 1996). In order to investigate the substrate specify of the additional glycine betaine uptake system(s), unlabeled ectoine and proline, and glycine betaine as a control, were added in 100-fold excess to the glycine betaine uptake assay (Table 2). Ectoine, proline, and glycine betaine inhibited uptake of the labeled substrate glycine betaine. At low osmolality, no glycine betaine uptake was detectable. These results indicate the existence of at least one additional uptake system that accepts glycine betaine, proline, and ectoine as substrates and is regulated at the level of activity dependent on dependence of the external osmolality.

For further characterization, the uptake of proline was measured (Table 2). Also proline uptake was influenced by the external osmolality. However, in contrast to glycine betaine, proline uptake was also active at low osmolality. Under conditions of low osmolality, addition of glycine betaine and ectoine did not compete with proline uptake; this is in contrast to conditions of high osmolality, where inhibition of approximately 70% was observed. These results indicate the existence of two different proline uptake systems. One of the systems accepts – besides proline – the substrates ectoine and glycine betaine and is stimulated by the external osmolality. The other transport system is specific for proline, i. e., it is not inhibited by ectoine and glycine betaine. Furthermore, it is not influenced by the external osmotic conditions. Addition of the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) immediately abolished both glycine betaine and proline uptake (data not shown), while the internal ATP concentration was not significantly decreased within the first minutes after application of the uncoupler, indicating that both substrates are transported by secondary carriers.

Furthermore, we determined the $K_m$ values for proline and glycine betaine uptake in the *betP* deletion strain DHP1. Whereas the kinetic analysis of glycine betaine uptake revealed only one $K_m$ value of approximately 400 μM, the kinetic analysis of proline uptake indicated the existence of two transport systems, i. e., both a low-affinity system and a high-affinity system were observed (Fig. 1). This result confirms the findings described above (Table 2). For isolation of the genes encoding the proline uptake systems, heterologous complementation of the *E. coli* mutant WG389 was performed.

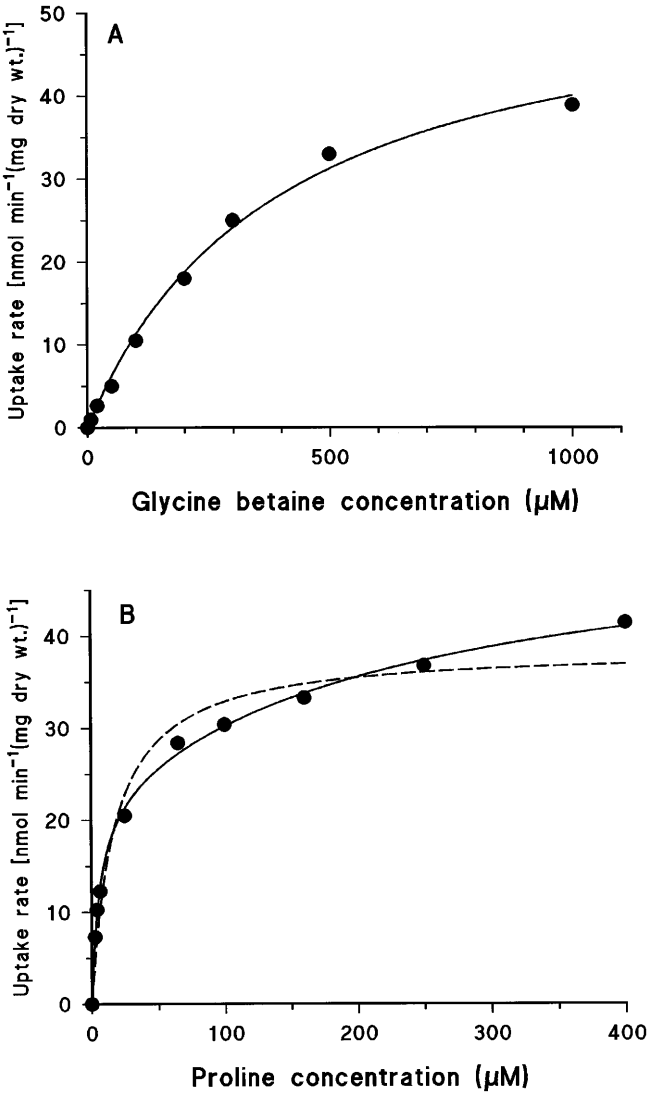


**Fig. 1** Kinetic analysis of (**A**) glycine betaine uptake and (**B**) proline uptake of *Corynebacterium glutamicum* Δ*betP*. Cells were grown in BHI medium and washed with potassium phosphate buffer. The osmolality in the uptake assays was adjusted with NaCl to a final value of 1,600 mosM (**A**) or 1,100 mosM (**B**). **A** The data were fitted by nonlinear regression and led to a $K_m$ value of 398 μM. **B** The proline uptake data were fitted by nonlinear regression assuming a single $K_m$ value (result: 17.5 μM, *broken line*) or by assuming the presence of two different uptake systems (*solid line*). The second alternative fitted the data significantly better and led to a $K_m$ value of 6 μM for the high-affinity system and a $K_m$ value of 260 μM for the low-affinity uptake system

## Complementation of *E. coli* WG389

A genomic DNA library of *C. glutamicum* was transferred by transformation into the *E. coli* mutant strain WG389,

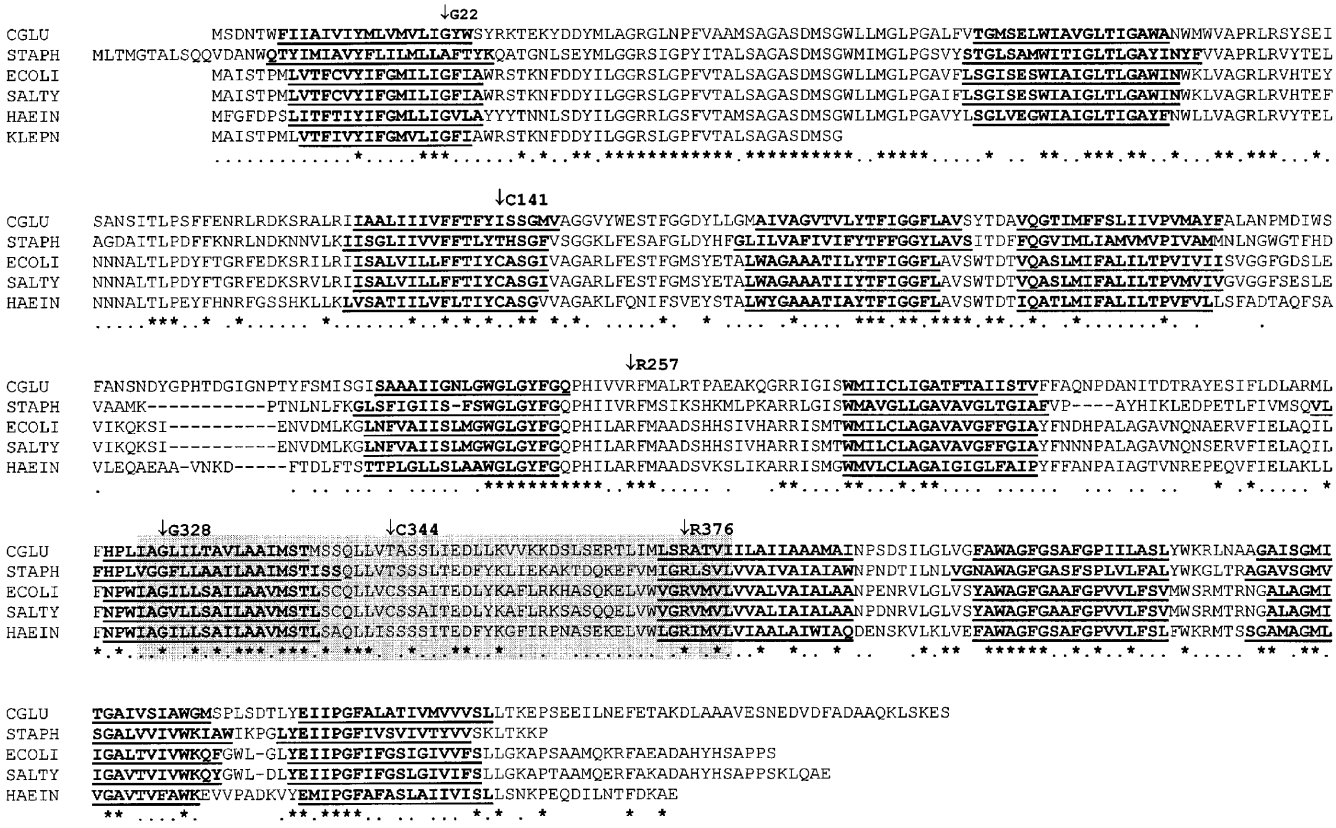


**Fig. 2** Amino acid alignment of *Corynebacterium glutamicum* PutP (*CGLU*) with PutP proteins from *Salmonella typhimurium* (*SALTY*), *Escherichia coli* (*ECOLI*), *Staphylococcus aureus* (*STAPH*), with a fragment from *Klebsiella pneumoniae* (*KLEPN*), and with a putative proline permease from *Haemophilus influenzae* (P45174) (*HAEIN*). Identical amino acids in five or six aligned proteins are indicated by *asterisks*, and identical amino acids in four or three proteins are indicated by *dots*. The position of predicted transmembrane helices are in *boldface* and *underlined*. The predicted sodium ion binding (SOB) domain (Deguchi et al. 1990) is *shadowed in grey*

which lacks the transport systems BetT, PutP, ProP, and ProU and which, in addition, is unable to synthesize proline and glycine betaine (Culham et al. 1993). As a consequence, in contrast to the parental strain, the mutant is not able to grow on 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 five clones that were able to grow, tested again for complementation, and analyzed by agarose gel electrophoresis. The smallest complementing plasmid, designated "pAB1", carried an insert of 3.8 kb. Southern hybridization experiments confirmed that the insert pAB1 was derived from *C. glutamicum* ATCC 13032 genomic DNA and that no chromosomal rearrangements in the cloned DNA fragment had occurred (data not shown).

## Sequence analysis

Both strands of the DNA insert of the complementing plasmid were sequenced. The computer-assisted analysis revealed an open reading frame with six possible translation start sites, three GTG codons (one each at positions 591, 609, and 741), and three ATG codons (one each at positions 750, 756, and 858). Depending on the translation start site, the predicted protein comprises 12–13 transmembrane helices. The predicted primary structure of the *C. glutamicum* proline permease reveals a high degree of identity to PutP from *S. typhimurium* (47%; Miller and Maloy 1990), from *E. coli* (47%; Nakao et al. 1987), and from *S. aureus* (48%; Wengender and Miller 1995) to a protein sequence from *Klebsiella pneumoniae* (52%; Chen and Maloy 1991) and to a putative proline permease from *Haemophilus influenzae* (P45174) (Fig. 2). Based on the multiple alignments, the last ATG codon was assumed to be the translation start site in vivo. To address this question in more detail, a deletion mutant was constructed carrying only the ATG start codon at position 858. Proline uptake of a strain carrying the corresponding plasmid was essentially identical to a strain carrying the complementing plasmid (data not shown). This result strongly favors this particular translation start site, which was expected on the basis of the multiple alignments (see above). The hydropathy analysis of PutP predicts 12 transmembrane helices (see Fig. 2). This structure represents a common feature of secondary transport systems (Henderson and Maiden 1989).

The PutP transport systems belong to the family of sodium solute symporters (SSF; Reizer et al. 1994). From a multiple alignment of four members of this family (the glutamate carrier GltS, the proline carrier PutP of *E. coli*, and the $Na^+$/glucose cotransporters of rabbit and human intestines), a conserved region, the proposed sodium ion binding (SOB) motif, was derived (Deguchi et al. 1990). This motif begins within the eighth putative transmembrane helix and consists of five conserved amino acids: $GX_nAX_4LX_3GR$ (Deguchi et al. 1990). However, out of these, only two amino acids, namely Gly-328 and Arg-376, are conserved in the *C. glutamicum* PutP (Fig. 2). In other studies (Yamato et al. 1994), Arg-376, the unique positively charged amino acid residue in the mid-membrane-spanning region of the *E. coli* PutP carrier, has been shown to have no influence on sodium binding. It has been concluded from additional site-directed mutagenesis experiments of PutP of *E. coli*, that Gly-22, Cys-141, and Arg-257 are involved in sodium binding (Yamato and Anraku 1988; Yamato et al. 1990). In the *C. glutamicum* PutP, only one of these three amino acids, Arg-257, is conserved. Additionally, Hanada and coworkers have performed studies with recombinant proteins of *E. coli* PutP and have proposed that Cys-344 has an important role in sodium binding (Hanada et al. 1992). In *C. glutamicum* PutP, Cys-344 is not conserved. In summary, no amino acid residues putatively involved in sodium binding were found in the derived PutP sequence.

However, two highly conserved regions, one between the first and the second transmembrane helix and another motif at the end of the sixth transmembrane helix, were identified. The function of these domains is so far unknown.

## Functional expression of *putP* in *E. coli* mutant strain MKH13

The ability of *E. coli* WG389 clones carrying *putP* on a plasmid to grow on minimal medium containing 25 mM proline was expected to depend on the uptake of this compound. We therefore measured [$^{14}$C]proline uptake activity of the clone *E. coli* MKH13(pAB1). However, proline uptake was found to be very low, i. e., around the detection limit (not shown). In order to enhance proline uptake, we cloned the *putP* gene into the expression vector pEKEX2 downstream of a *tac* promoter, thereby forming plasmid pAB2. *E. coli* MKH13(pAB2) was characterized by a proline uptake rate of 20 nmol $min^{-1}$ (mg dry wt.)$^{-1}$ under standard assay conditions (not shown).

For further characterization of PutP, we measured the characteristic kinetic parameters of the transport system. The $K_m$ value for proline at saturating $Na^+$ concentrations was 7.6 μM (data not shown). By variation of the external ion composition, we characterized the transport mechanism as being coupled to the cotransport of sodium ions. The $K_m$ value for $Na^+$ was determined to be 1.3 mM (data not shown). Proline uptake was inhibited by the addition of the uncoupler CCCP and by the $K^+$-ionophore valinomycin in the presence of high external $K^+$ concentrations, whereas no effect was observed when ectoine, glycine betaine, and carnitine were added in 100-fold excess (Table 3). The polycation polymyxin B nonapeptide (PMBN) was added in these experiments in order to sensitize *E. coli* to valinomycin. Otherwise the plasma membrane of the gram-negative *E. coli* cells is protected against valinomycin by the impermeability of the outer membrane to this ionophore (Alatossova et al. 1984). Addition of PMBN alone had no effect on proline uptake. In order to investigate whether PutP is activated by osmotic shifts at the level of activity, the proline uptake rate in the presence of various NaCl concentrations was measured (not shown). Proline uptake was unchanged over a wide range of external NaCl concentrations (5–400 mM).

**Table 3** [$^{14}$C]proline uptake mediated by expression of *putP* in *E. coli* MKH13. Cells were washed twice and were allowed to equilibrate for 3 min at 37°C. Proline uptake was started by the addition of [$^{14}$C]proline at a final concentration of 100 μM (*CCCP* carbonyl cyanide *m*-chlorophenylhydrazone, *PMBN* polymyxin B nonapeptide)

| Addition | Uptake rate [nmol $min^{-1}$(mg dry wt.)$^{-1}$] |
|---|---|
| None | 20.4 |
| PMBN (2–5 μg/ml) | 21.5 |
| Valinomycin (20 μM) + PMBN (2–5 μg/ml) | < 0.5 |
| CCCP (50 μM) | < 0.5 |
| Proline (10 mM) | < 0.5 |
| Ectoine (10 mM) | 18.5 |
| Glycine betaine (10 mM) | 21.0 |
| Carnitine (10 mM) | 22.5 |

## Proline uptake in the *putP* deletion strain DHP8

For the deletion of *putP*, an internal gene fragment of 558 bp was removed from plasmid pAB1 by digestion with *Nco*I. The resulting 2,698-bp insert was isolated by *Sph*I restriction and ligated with plasmid pK19mobsacB. The resulting plasmid, pKA1, was transferred into the *betP* deletion strain *C. glutamicum* DHP1. Deletion mutants resulting from a twofold chromosomal recombination event were selected by PCR. In contrast to the fragment obtained by PCR amplification of the wild-type DNA of 1,270 bp, the fragment of the mutant showed the expected size of 712 bp.

For further functional characterization of the remaining low-affinity proline transport system, proline uptake of the deletion strain DHP8 was measured (Fig. 3). At low osmolality, no proline uptake was detectable in this strain. In contrast, under the same conditions, the parental strain DHP1 showed a proline uptake rate of 8.5 nmol $min^{-1}$ (mg dry wt.)$^{-1}$, which is obviously mediated by the PutP system (Fig. 3). Proline transport of the strain DHP8 became significant at NaCl concentrations higher than 250 mM and reached its maximal value at 600 mM NaCl,

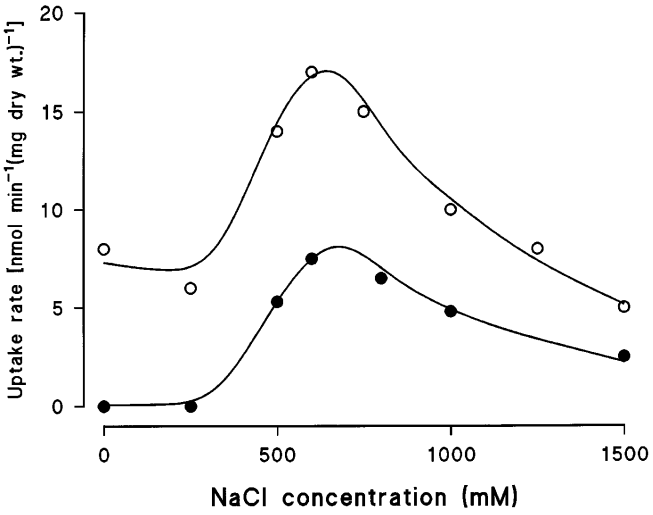


**Fig. 3** Proline uptake of the deletion mutant strain DHP8 (●) as compared to that of the parental strain DHP1 (○) dependent on the external $Na^+$ concentration. Cells were grown in BHI medium and washed with potassium phosphate buffer. Uptake measurements were started by the addition of labeled proline at a final concentration of 750 mM

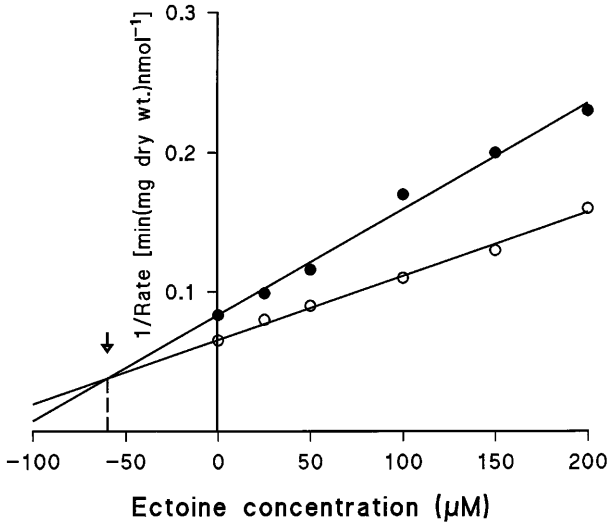


**Fig. 4** Dixon plot of glycine betaine uptake dependent on different ectoine concentrations of strain *Corynebacterium glutamicum* DHP8. Cells were grown in BHI medium and washed with potassium phosphate buffer. The osmolality in the uptake assays was adjusted with NaCl to a final value of 1,370 mosM. Uptake measurements were started by the addition of labeled glycine betaine at a final concentration of 200 μM (●) or 400 mM (○). The $K_i$ value of ectoine derived from the intersection point in the Dixon plot was 63 μM

which corresponded to a total osmolality of the medium of 1,370 mosM (Fig. 3). Whereas the determination of the $V_{max}$ of glycine betaine and proline uptake led to the same value of 34 nmol $min^{-1}$ (mg dry wt.)$^{-1}$ for both substrates, the $K_m$ values differed. The transport affinity for glycine betaine ($K_m$ = 333 ± 45 μM) was significantly higher than that for proline ($K_m$ = 1,200 ± 180 μM). The characteristic kinetic data of ectoine transport could not be determined directly because labeled ectoine is not commercially available. Thus, we measured the inhibition constant $K_i$ of ectoine with respect to the uptake of glycine betaine. A Dixon analysis of the glycine betaine uptake rate in the presence of different ectoine concentrations revealed a $K_i$ value of ectoine of 63 ± 5 μM (Fig. 4).

In order to investigate the physiological significance of the uptake systems BetP and PutP, we carried out growth experiments with the two strains DHP1 and DHP8 in comparison to the wild-type strain. We did not detect a significant difference in growth behavior when the cells were grown in minimal medium with 1 M NaCl complemented with glycine betaine (10 or 200 μM) or proline (10 mM) (data not shown). In addition, we performed shift experiments by adding salt to a high concentration (0.5 M NaCl) and by adding 200 μM glycine betaine to growing cells. In this case, a small but significantly longer lag phase of the mutants was observed after the hyperosmotic shock (results not shown). Obviously, uptake of compatible solutes is of utmost importance for *C. glutamicum*. Consequently, the uptake activity of BetP is fully replaceable by at least one further glycine betaine uptake system, presumably the one described above.

## Discussion

In previous studies, we have shown that glycine betaine, proline, and ectoine are effective osmoprotectants of *C. glutamicum* under conditions of hyperosmotic stress (Farwick et al. 1995). Glycine betaine uptake is mainly mediated by the high-affinity permease BetP (Peter et al. 1996). In the present communication, we report the identification of the gene *putP* that encodes the high-affinity proline uptake system, and the characterization of another transport system for the compatible solutes glycine betaine, proline, and ectoine.

The high-affinity proline transport system PutP shares a high degree of identity to other known bacterial PutP proteins. As in the case of the well-studied PutP of *E. coli*, proline uptake is coupled to sodium ions. No activation of proline transport by osmotic shift was observed. PutP is, thus, not involved in the proline accumulation as a response to osmotic stress, but provides the cells with proline for biosynthesis. A similar situation was observed in the gram-positive bacterium *S. aureus*, where the activity of the high-affinity proline transport system PutP is also not sensitive to changes in external osmolality (Bae et al. 1993; Wengender and Miller 1995). Osmotically regulated accumulation of glycine betaine and proline of *S. aureus* is mediated by a low-affinity uptake system (Pourkomailian and Booth 1994; Stimeling et al. 1994). *C. glutamicum* possesses at least two glycine betaine uptake systems, namely the previously described high-affinity permease BetP (Peter et al. 1996) and another, low-affinity glycine betaine uptake system that was also characterized in the present report. The low-affinity uptake system seems to prefer ectoine as the substrate ($K_i$ = 63 mM) and, in addition to glycine betaine ($K_m$ = 333 μM),

also mediates the transport of proline ($K_m$ = 1,200 μM). Similar to BetP, it is effectively regulated at the level of activity. Interestingly, both uptake systems have exactly the same optimum of osmotic stimulation at 0.6 M external NaCl, which corresponds to a total osmolality of 1,370 mosM. The closest functional equivalent of the low-affinity uptake system seems to be the ProP protein of *E. coli*, which is also regulated by osmotic shifts (Milner et al. 1988; Culham et al. 1993). However, the only known gene to date that encodes a protein sharing a high degree of identity to ProP (Culham et al. 1993), *ousA* of *Erwinia chrysanthemi*, is not osmoregulated at the level of activity (Gouesbet et al. 1996). The isolation of the gene encoding the low-affinity proline uptake system of *C. glutamicum* (research in progress) should provide a closer insight into common structural features of osmoregulated carrier proteins.

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