

Characterization of the Phosphoenolpyruvate Carboxykinase Gene from *Corynebacterium glutamicum* and Significance of the Enzyme for Growth and Amino Acid Production

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Abstract

Corynebacterium glutamicum possesses phosphoenolpyruvate (PEP) carboxykinase, oxaloacetate decarboxylase and malic enzyme, all three in principle being able to catalyze the first step in gluconeogenesis. To investigate the role of PEP carboxykinase for growth and amino acid production, the respective *pck* gene was isolated, characterized and used for construction and analysis of mutants and overexpressing strains. Sequence analysis of the *pck* gene predicts a polypeptide of 610 amino acids showing up to 64% identity with ITP-/ GTP-dependent PEP carboxykinases from other organisms. *C. glutamicum* cells harbouring *pck* on plasmid showed about tenfold higher specific PEP carboxykinase activities than the wildtype. Inactivation of the chromosomal *pck* gene led to the absence of PEP carboxykinase activity and the inability to grow on acetate or lactate indicating that the enzyme is essential for growth on these carbon sources and thus, for gluconeogenesis. The growth on glucose was not affected. Examination of glutamate production by the recombinant *C. glutamicum* strains revealed that the PEP carboxykinase-deficient mutant showed about fourfold higher, the *pck*-overexpressing strain two- to threefold lower glutamate production than the parental strain. Inactivation and overexpression of *pck* in a lysine-producer of *C. glutamicum* led to an only 20% higher and lower lysine accumulation, respectively. The results show that PEP carboxykinase activity in *C. glutamicum* is counteractive to the production of glutamate and lysine and indicate that the enzyme is an important target in the development of strains producing amino acids derived from citric acid cycle intermediates.

Introduction

Corynebacterium glutamicum is an aerobic, Gram-positive organism that grows on a variety of sugars and organic acids, and is widely used in the industrial production of amino acids, e.g., L-glutamate and L-lysine. For growth on organic acids such as acetate, gluconeogenic reactions are necessary in order to provide the cells with hexose and pentose sugars. The initial step in the gluconeogenic pathway is the conversion of tricarboxylic acid (TCA) cycle intermediates to phosphoenolpyruvate (PEP). In most organisms, this reaction is accomplished by a PEP carboxykinase which catalyzes the decarboxylation and simultaneous ATP- or GTP-dependent phosphorylation of oxaloacetate (Utter and Kolenbrander, 1972). In an alternative pathway, oxaloacetate or malate are decarboxylated to pyruvate by oxaloacetate decarboxylase or malic enzyme, respectively (Hansen and Juni, 1974). The pyruvate formed then is converted to PEP by PEP synthetase or pyruvate:orthophosphate dikinase (Cooper and Kornberg, 1967). *C. glutamicum* possesses PEP carboxykinase as well as oxaloacetate decarboxylase and malic enzyme (Figure 1) and all three enzymes have been (partially) purified and biochemically characterized (Jetten and Sinskey, 1993; Jetten and Sinskey, 1995; Gourdon *et al.*, 2000). *C. glutamicum* has also been postulated to possess PEP synthetase activity (Vallino and Stephanopoulos, 1993; Jetten *et al.*, 1994). However, the *in vivo* activity of this enzyme is probably very low (Peters-Wendisch *et al.*, 1998) and thus, a gluconeogenic function of oxaloacetate decarboxylase or malic enzyme together with PEP synthetase is uncertain.

PEP carboxykinases are present in human liver and kidney and in a variety of animals, plants, yeasts and microorganisms. The animal enzymes generally are GTP- and/or ITP-dependent (EC 4.1.1.32) whereas the plant, fungal and microbial enzymes in most cases use ATP as phosphate donor (EC 4.1.1.49) (Utter and Kolenbrander, 1972). Exceptions from this rule are the bacteria *Ruminococcus flavefaciens* (Schöcke and Weimer, 1997), *Ralstonia* (formerly *Alcaligenes*) *eutrophus* (Schobert and Bowien, 1984), *Arthrobacter globiformis* (Utter and Kolenbrander, 1972) and *C. glutamicum* (Peters-Wendisch *et al.*, 1993; Jetten and Sinskey, 1993) which have been shown to possess GTP-dependent PEP carboxykinase activity.

Genes coding for PEP carboxykinases have been isolated from a variety of pro- and eukaryotic organisms, e.g., those for ATP-dependent enzymes from *Escherichia coli* (Medina *et al.*, 1990) and *Rhizobium meliloti* (Østerås *et al.*, 1995) and those for GTP-dependent enzymes from

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Table 1. Specific activities of PEP carboxykinase in cell-free extracts of different *C. glutamicum* strains grown in complex (LB) medium or in minimal medium (MM) containing 4% glucose, 2% acetate, or 2% lactate as carbon source.

Strain	PEP carboxykinase [U / mg protein] ^a			
	LB medium	MM + glucose	MM + acetate	MM + lactate
<i>C. glut.</i> WT	0.132	0.044	0.072	0.135
<i>C. glut.</i> WT (pEK0)	0.117	0.040	0.066	0.099
<i>C. glut.</i> WT (pEK-pckA)	1.109	0.537	0.821	0.941
<i>C. glut.</i> WT (pEK-pckB)	1.299	0.413	0.648	1.082
<i>C. glut.</i> WT Δ pck	< 0.003 ^b	< 0.003	NG	NG
<i>C. glut.</i> MH20-22B	0.148	0.075	ND	ND
<i>C. glut.</i> MH20-22B(pEK-pckB)	1.423	0.680	ND	ND
<i>C. glut.</i> MH20-22B Δ pck	< 0.003 ^b	< 0.003	NG	NG

^a The values are means obtained from at least three independent cultivations and two determinations per experiment. The standard deviations were in all cases below 10%. NG, no growth; ND, not determined.

^b For better growth of the *pck* mutants, 1% (w/v) glucose was added to the LB medium.

important for *C. glutamicum* during growth on lactate or acetate than on glucose.

Isolation and Subcloning of the *pck* Gene from *C. glutamicum*

The *C. glutamicum pck* gene encoding PEP carboxykinase was isolated by heterologous complementation of *E. coli* mutant HG4 using a *C. glutamicum* WT cosmid gene library based on vector pHC79. Due to its PEP carboxykinase and PEP synthetase deficiency, strain HG4 is not able to grow on minimal medium containing succinate as sole carbon source (Goldie and Sanwal, 1980). Pooled recombinant cosmids were transformed into *E. coli* HG4 and, by testing about 1200 transformants for growth on succinate minimal medium, two clones were obtained which grew on succinate as sole carbon source. After isolation of the two cosmids and retransformation into *E. coli* HG4, the transformants again grew on succinate minimal medium suggesting that the cosmids carried the *C. glutamicum* WT

pck gene.

For subcloning purposes, one of the two complementing cosmids was digested with either *Xho*I, *Sca*I or *Pvu*II and fragments ≥ 3 kb were ligated into the *Sal*I (*Xho*I-fragments) or into the blunt-ended *Eco*RI site (*Sca*I and *Pvu*II-fragments) of the *E. coli*-*C. glutamicum* shuttle vector pEK0. The ligation mixtures were transformed into *E. coli* HG4 and kanamycin-resistant transformants were again screened for growth on succinate minimal medium. The transformation with the ligation mixture containing pEK0 and the *Pvu*II fragments of the complementing cosmid resulted in seven clones growing on this medium. The plasmids isolated from these clones all contained a 4.3 kb *Pvu*II fragment inserted in either one or the other direction and were designated pEK-pckA and pEK-pckB, respectively. The restriction map of the 4.3 kb *Pvu*II fragment is shown in Figure 2.

To confirm the origin of the cloned DNA fragment in pEK-pckA and pEK-pckB, genomic DNA from *C.*

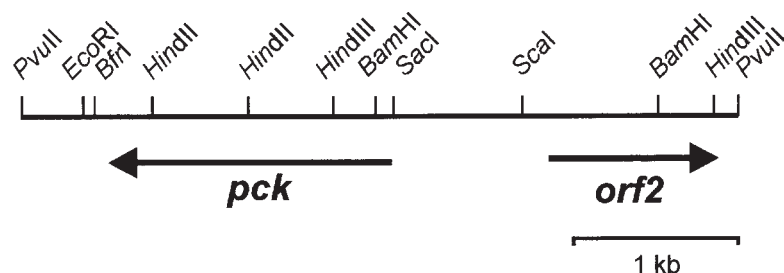


Figure 2. Restriction map of the 4.3 kb *C. glutamicum Pvu*II fragment carrying the *pck* gene. The *pck* gene and the orf with unknown function are indicated by black arrows.

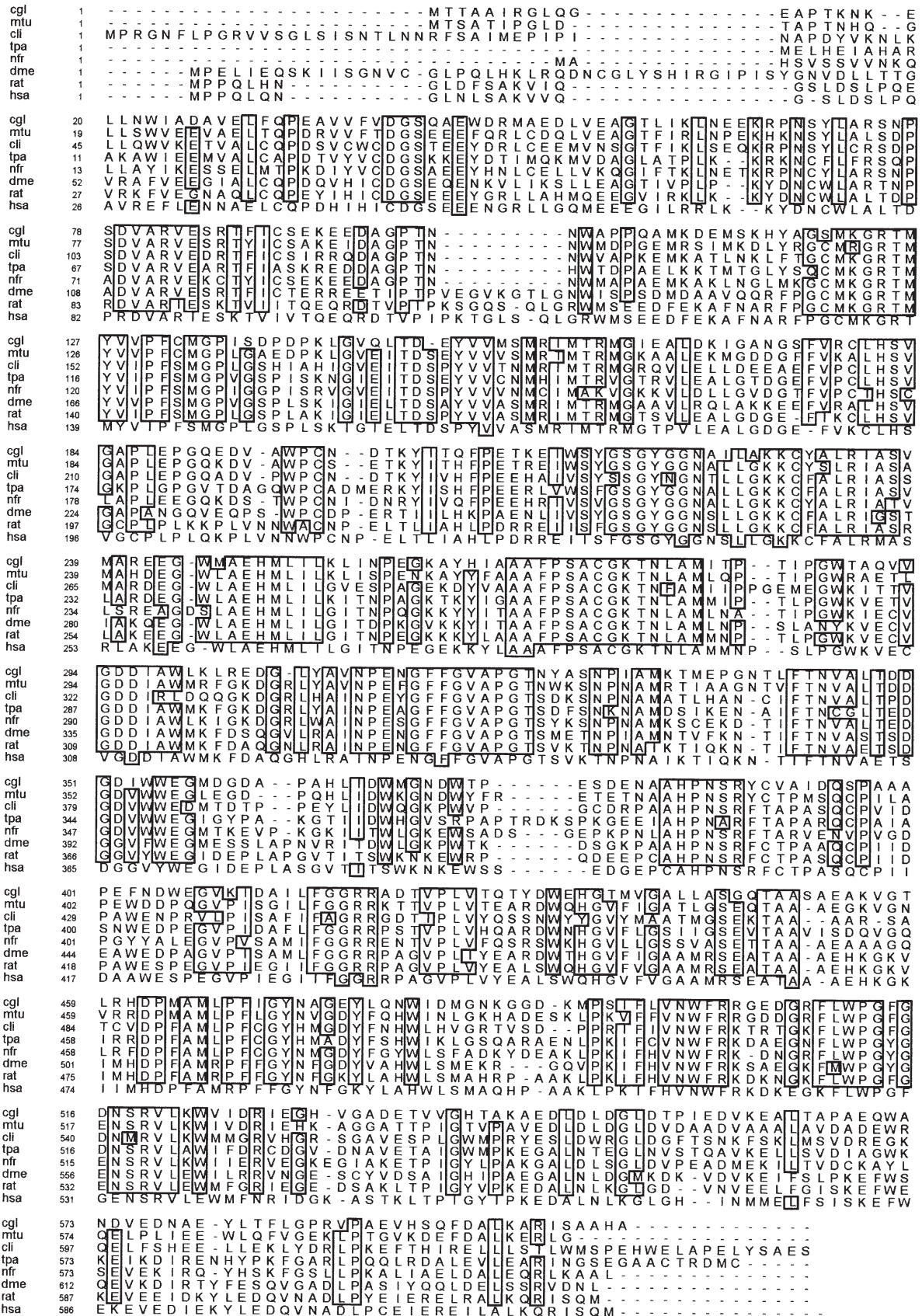


Figure 3. Alignment of the predicted PEP carboxykinase sequences from *C. glutamicum* (cgl), *Mycobacterium tuberculosis* (mtu), *Chlorobium limicola* (cli), *Treponema pallidum* (tpa), *Neocallimastix frontalis* (nfr), *Drosophila melanogaster* (dme), rat and human (hsa).

glutamicum WT was analyzed by Southern hybridization. For this purpose, the 1.49 kb *EcoRI-HindIII* fragment from pEK-pckA was isolated, labelled and hybridized to *PvuII*-restricted and size-fractionated chromosomal DNA from *C. glutamicum*. The hybridization resulted in a single signal at 4.3 kb (not shown). This result confirms that the isolated DNA fragment corresponds to a fragment within the genome of *C. glutamicum*.

Expression of the Cloned *pck* Gene

In order to assess the *pck*-complementing fragments for expression of a functional *pck* gene, plasmids pEK-pckA and pEK-pckB were transformed into *C. glutamicum* WT and the specific PEP carboxykinase activities in cell-free extracts of the resulting strains were determined after growth in different media. As shown in Table 1, *C. glutamicum* strains harbouring either of the two plasmids showed about eight- to thirteenfold higher specific activities of PEP carboxykinase than did the host strain or its derivative carrying the cloning vector pEK0. This result shows that the cloned fragment contains a functional *pck* gene which is expressed in *C. glutamicum*.

C. glutamicum WT and the strains carrying pEK-pckA and pEK-pckB grew equally well (doubling time and final optical density) on complex medium and on minimal medium containing glucose, acetate or lactate. This result indicates that *pck*-overexpression has neither a positive nor a negative effect on growth of *C. glutamicum*.

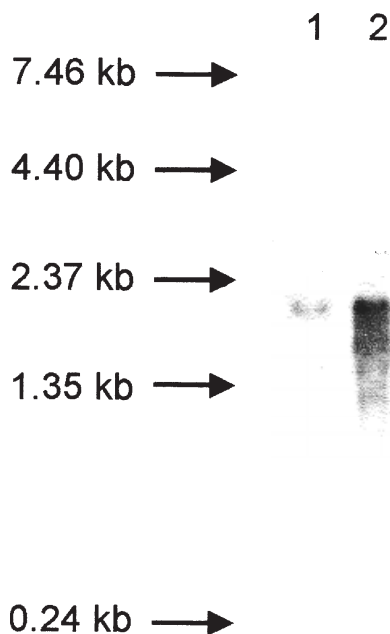


Figure 4. Northern-blot analysis of the *C. glutamicum pck* gene after growth of the cells on minimal medium containing glucose (lane 1) or lactate (lane 2) as a carbon source. Total RNA (10 µg) was electrophoresed and probed with a *pck*-specific probe. The positions of RNA standards are shown on the left.

Nucleotide Sequence of the *C. glutamicum pck* Gene and Analysis of the Deduced PEP Carboxykinase Amino Acid Sequence

The nucleotide sequence of a 3920 bp *EcoRI-PvuII* fragment from plasmid pEK-pckB was determined from both strands. Computer analysis revealed two divergently orientated open reading frames (orfs) of 1833 bp (orf1) and 1020 bp (orf2) which both exhibited codon usages corresponding to that of corynebacterial genes (Malumbres *et al.*, 1993). The deduced amino acid sequence of orf2 showed weak similarity to some transmembrane and transport proteins from various organisms. The amino acid sequence encoded by orf1 showed significant identity to PEP carboxykinases from other organisms (see below) indicating that this orf represents the *pck* gene of *C. glutamicum*. The predicted *pck* gene product consists of 610 amino acids with an M_r of 66,874. The gene is preceded by a typical ribosomal binding site (GGAGA). Centered 47 bp downstream of the *pck* stop codon, a region of dyad symmetry followed by several T residues similar to rho-independent transcription terminators was found. The mRNA hairpin loop predicted from this sequence has a ΔG (25°C) of -36.6 kcal/mol (153.8 kJ/mol). This result indicates transcriptional termination downstream of the *pck* gene.

An alignment of the deduced amino acid sequence of the *C. glutamicum pck* gene product with sequences from a variety of other organisms is shown in Figure 3. Homology analysis revealed that the *C. glutamicum* PEP carboxykinase shows a high degree of identity to putative PEP carboxykinase enzymes from the prokaryotic *M. tuberculosis* (64%; SWISS-PROT P96393), *M. leprae* (62%; TrEMBL O06084; not shown in Figure 3), *C. limicola* (52%; SWISS-PROT Q08262) and *T. pallidum* (51%; SWISS-PROT O83159). The *C. glutamicum* enzyme showed also relatively high identity to several eukaryotic GTP-dependent PEP carboxykinases, e.g., from *N. frontalis* (55%; SWISS-PROT P22130), *D. melanogaster* (49%; P20007), rat (48%; SWISS-PROT P07379) and human (47%; SWISS-PROT P35558) (Figure 3). In contrast, it showed only very weak similarity (<27%) with PEP carboxykinase sequences from *Bacillus subtilis* (SWISS-PROT P54418), *S. aureus* (SWISS-PROT P51065), *E. coli* (SWISS-PROT P22259), *Haemophilus influenzae* (SWISS-PROT P43923) and *Rhizobium sp.* (SWISS-PROT P43086).

Northern Analysis of the *C. glutamicum pck* Gene

Northern (RNA) hybridization experiments were performed to analyse the size of the *pck* transcript. For this purpose, total RNA from glucose- and from lactate-grown cells of *C. glutamicum* WT was isolated, size-fractionated, transferred onto a nylon membrane and hybridized to a *pck*-specific digoxigenin-dUTP-labelled probe. The hybridization revealed a signal at about 2.0 kb (Figure 4) which is approximately the size of the *pck* gene. The concentration of the *pck* transcript in lactate-grown cells of *C. glutamicum* was estimated to be sixfold higher than in glucose-grown cells. The difference is slightly higher than the difference observed in the specific activity of PEP carboxykinase on medium containing either glucose or lactate. However, the results indicate that the *C. glutamicum pck* transcript is

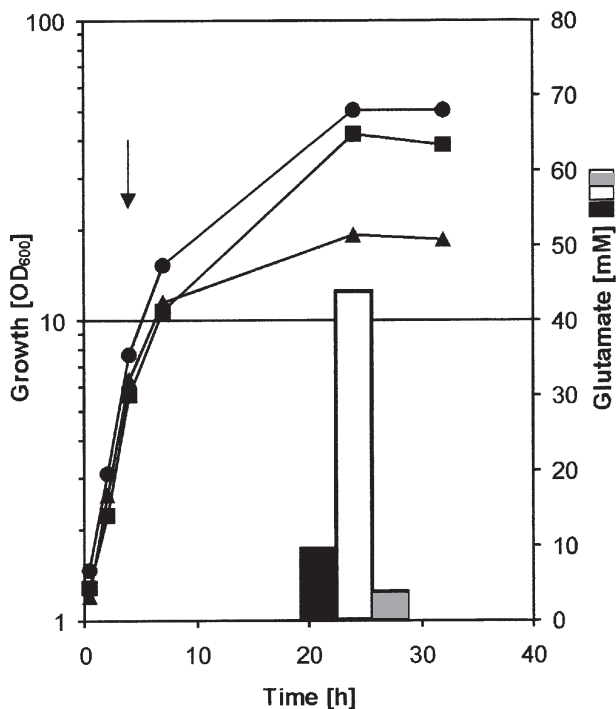


Figure 5. Growth (symbols) and glutamate formation (bars) of *C. glutamicum* WT (circle, black bar), WT Δ pck (triangle, white bar) and WT(pEK-pckB) (square, grey bar) on minimal medium containing glucose as carbon source. The arrow indicates the time when Tween 60 was added to the medium. The bars represent the glutamate concentrations determined at 24 hours after inoculation.

monocistronic and that the observed regulation of the enzyme occurs primarily at the mRNA level.

Inactivation of the Chromosomal *pck* Gene

In order to obtain a defined PEP carboxykinase-negative mutant of *C. glutamicum* WT, the chromosomal *pck* gene was replaced by a truncated gene. The resulting mutant *C. glutamicum* WT Δ pck was tested for PEP carboxykinase activity and for growth on different media. The specific activity was measured in cell-free extracts after growth on complex and minimal medium containing glucose. As shown in Table 1, the WT Δ pck mutant was devoid of any detectable PEP carboxykinase activity. The growth experiments revealed that *C. glutamicum* WT Δ pck grew significantly worse than *C. glutamicum* WT on LB complex medium (doubling times of 200 - 220 min and 130 - 135 min, and final OD₆₀₀ of 3.7 and 9.6, respectively) unless supplemented with glucose (doubling times of 70 - 80 min and final OD₆₀₀ of about 25 for both strains). On minimal medium with glucose as sole carbon source, the mutant WT Δ pck grew as well as the parental WT strain (doubling times of 95 - 105 min and final OD₆₀₀ of about 45) whereas, in contrast to the WT strain, the mutant did not grow on minimal medium containing either acetate or lactate as the sole carbon source. These results show that the PEP carboxykinase in *C. glutamicum* is essential for growth on acetate and lactate but not for growth on glucose and thus they indicate a gluconeogenic function of the enzyme.

Significance of PEP Carboxykinase for Glutamate Production

To analyze the influence of the lack of PEP carboxykinase activity and of elevated PEP carboxykinase activity on glutamate production by *C. glutamicum*, we performed detergent-dependent glutamate production assays. Minimal medium containing 5% (w/v) glucose was inoculated with cells of *C. glutamicum* WT, *C. glutamicum* WT(pEK-pckB), and *C. glutamicum* WT Δ pck, and growth of the cultures and the glutamate concentration in the culture fluid was analyzed. Without the addition of Tween 60, all three strains grew to about the same final optical density (maximal OD₆₀₀ = 40 to 50 after 24 hours) and all three strains showed no significant glutamate secretion in a time period of up to 48 hours (0.5 mM glutamate in the culture supernatant). When Tween 60 was added 4 hours after inoculation, the WT strain and the *pck*-overexpresser *C. glutamicum* WT(pEK-pckB) showed the same growth behaviour as in the absence of Tween 60 whereas the *pck*-mutant *C. glutamicum* WT Δ pck reproducibly stopped growth about four hours after addition of Tween 60 as exemplarily shown in Figure 5. As also exemplarily shown in Figure 5, *C. glutamicum* WT accumulated about 10 mM glutamate, the *pck*-mutant *C. glutamicum* WT Δ pck about 44 mM glutamate, and the *pck*-overexpresser *C. glutamicum* WT(pEK-pckB) about 4 mM glutamate within 20 hours after addition of Tween 60, i.e., 24 hours after inoculation. 32 hours after inoculation the glutamate concentrations in the respective cultures were about 13 mM, 49 mM and 5 mM. Taking into consideration that *C. glutamicum* WT Δ pck grew to a lower final optical density (OD₆₀₀ = 18 to 20) than *C. glutamicum* WT and WT(pEK-pckB) (OD₆₀₀ = 40 to 50), the difference in the glutamate productivity (glutamate produced per g of cells) between the *pck*-mutant strain and the two other strains is even higher. These results show that the capacity of *C. glutamicum* to produce glutamate is severely influenced by the presence and by the level of PEP carboxykinase activity.

Significance of PEP Carboxykinase for Lysine Production

To analyze the significance of PEP carboxykinase for lysine production by *C. glutamicum*, we constructed a *pck*-overexpressing strain and a *pck*-inactivation mutant of the lysine producer *C. glutamicum* MH20-22B. The overexpresser was obtained by transformation of this strain with plasmid pEK-pckB. The inactivation of the *pck* gene in the producer strain was performed in the same way as described for the construction of *C. glutamicum* WT Δ pck. As shown in Table 1, the specific PEP carboxykinase activity of MH20-22B(pEK-pckB) was about tenfold higher than that of the parental strain whereas the inactivation mutant MH20-22B Δ pck did not show any PEP carboxykinase activity.

C. glutamicum MH20-22B, MH20-22B(pEK-pckB) and MH20-22B Δ pck were grown in minimal medium plus glucose (5%, w/v) and the growth and the lysine concentration in the culture medium was analyzed. In contrast to the detergent-dependent glutamate production assay, the three MH20-22B strains showed identical growth behaviour. The Δ pck mutant accumulated approximately 20% more lysine (62 to 69 mM; values obtained from three

independent cultivations by two determinations per experiment) and the *pck*-overexpressing strain about 20% less lysine (42 to 45 mM) than the parental strain (about 50 to 58 mM) within 24 h of incubation. These results show that lysine production by *C. glutamicum* is weakly influenced by the presence and the level of PEP carboxykinase activity.

Discussion

The present study describes the genetic and functional characterization of the GTP-dependent PEP carboxykinase from *C. glutamicum*. In accordance with the GTP specificity of the *C. glutamicum* enzyme (Jetten *et al.*, 1993; Peters-Wendisch *et al.*, 1993), the overall amino acid sequence deduced from the *C. glutamicum pck* gene showed almost no similarity to known ATP-dependent but significant identity to GTP-dependent PEP carboxykinases from other organisms. The alignment of the *C. glutamicum* enzyme with other GTP-dependent PEP carboxykinases revealed highly conserved motifs, especially in those regions suggested to be important for the catalytic activity (Linss *et al.*, 1993; Matte *et al.*, 1997). These motifs, which can also be found with some variations and partially at different relative locations within the overall sequence of ATP-dependent PEP carboxykinases, include the 'PEP carboxykinase-specific domain' which binds oxaloacetate (GSGYGGNAILAKK; residues 218 - 230 in the *C. glutamicum* sequence), a typical PEP binding site (DGSQAE, residues 41 - 46), a phosphoryl-binding motif (AAFPSACGKTNLAM; residues 268 - 281), and possible binding sites for divalent metal ions (VGDD; residues 293 - 296 and GDIWWE; residues 351 - 357). These findings corroborate the previous theory (Matte *et al.*, 1997) that all PEP carboxykinases have similar active site organization and have retained the catalytically important motifs over a large evolutionary distance.

Different types of regulation have been observed for the expression of PEP carboxykinase genes in different bacteria. The *pckA* gene from *Rhodospseudomonas palustris* No. 7 is strongly induced in the exponential growth phase, irrespective of the carbon source in the growth medium (Inui *et al.*, 1999). In contrast, PEP carboxykinase gene expression in *E. coli* and *R. meliloti* is strongly induced in the stationary growth phase and additionally dependent on the carbon source present in the growth medium (Goldie, 1984; Goldie and Medina, 1990; Østerås *et al.*, 1995). Carbon source-dependent regulation of the *pck* expression has been also described for *S. aureus* (Scovill *et al.*, 1996) and *Rhizobium leguminosum* (McKay *et al.*, 1985). In all these cases gluconeogenic carbon sources induced (or derepressed) the *pck* expression whereas much lower expression was observed on glycolytic carbon sources. Similarly, the *C. glutamicum pck* gene expression is dependent on the carbon source in the medium as evidenced by the lower specific PEP carboxykinase activities in cells grown with glucose instead of acetate or lactate and by the different amounts of specific *pck* RNA in glucose- and lactate-grown cells. However, in contrast to the situation with *E. coli*, *R. meliloti* and *R. palustris* No. 7, growth phase dependent regulation of *pck* expression in *C. glutamicum* is very unlikely since we observed no

significant difference in the specific PEP carboxykinase activity throughout all stages of growth.

In most organisms, PEP carboxykinase is a gluconeogenic enzyme that converts oxaloacetate to PEP and CO₂ (Utter and Kolenbrander, 1972). Although, in some bacteria, e.g. *R. flavofaciens* and *R. eutrophus*, PEP carboxykinase has been shown to have anaplerotic function under glycolytic conditions and thus to catalyze the reverse reaction (Schöcke and Weimer, 1997; Schobert and Bowien, 1984). The gluconeogenic function of this enzyme in *C. glutamicum* was unequivocally shown by the fact that, in contrast to the original host strain, the PEP carboxykinase-deficient mutant was unable to grow on substrates which require gluconeogenesis. In addition, the inability of this mutant to grow on acetate and lactate indicates that PEP carboxykinase is the only enzyme responsible for PEP synthesis and that it cannot be functionally replaced by the combined activities of malic enzyme or oxaloacetate decarboxylase and PEP synthetase. Whereas the malic enzyme of *C. glutamicum* has recently been proposed to be involved in the generation of NADPH on substrates known to have a low flux through the pentose pathway (Gourdon *et al.*, 2000), the role of oxaloacetate decarboxylase in *C. glutamicum* is completely unclear and remains to be investigated.

Aside from PEP carboxykinase, oxaloacetate decarboxylase and malic enzyme, *C. glutamicum* possesses at the PEP/pyruvate/oxaloacetate node two further enzymes involved in the interconversion of C₃ (PEP/pyruvate) and C₄ metabolites (oxaloacetate/malate), i.e. PEP carboxylase (Mori *et al.*, 1985) and pyruvate carboxylase (Peters-Wendisch *et al.*, 1997) which form oxaloacetate from PEP and pyruvate, respectively (see Figure 1). During growth of *C. glutamicum* on carbohydrates these two enzymes are responsible for the replenishment of the TCA cycle and thus have anaplerotic function (Peters-Wendisch *et al.*, 1998). However, labelling experiments using ¹³C-glucose with subsequent nuclear magnetic resonance (NMR) analyses in combination with metabolite balancing repeatedly indicated that in addition to the PEP and/or pyruvate carboxylating forward flux a relatively strong backward flux from oxaloacetate to PEP and/or pyruvate takes place during growth on and during amino acid production from glucose (Sonntag *et al.*, 1995; Marx *et al.*, 1996; Marx *et al.*, 1999; Wendisch *et al.*, 2000). By additional isotopomer analyses, Petersen *et al.* (2000) recently were able to precisely quantify the individual (forward, back and parallel) carbon fluxes at the PEP/pyruvate/oxaloacetate node of *C. glutamicum*. Their results again indicated simultaneous bidirectional fluxes between C₃ and C₄ metabolites, with pyruvate carboxylase reaction constituting the principal route of anaplerotic C₃ carboxylation and PEP carboxykinase catalyzing more than 90% of the oxaloacetate decarboxylating flux. Thus, together with pyruvate kinase and pyruvate carboxylase, the PEP carboxykinase catalyzes an apparent futile cycle (see Figure 1) under glycolytic conditions. A similar futile cycling between PEP or pyruvate and oxaloacetate involving the PEP carboxykinase reaction has been found in *E. coli* (Sauer *et al.*, 1999) and in *B. subtilis* (Sauer *et al.*, 1997), however, in contrast to the situation in *C. glutamicum* only under glucose limitation. Although the

physiological function of a PEP/pyruvate/oxaloacetate cycling remains highly speculative, the results of the isotopomer analyses by Petersen *et al.* (2000) suggest that the PEP carboxykinase in *C. glutamicum* may have physiological significance under non-gluconeogenic conditions. However, as the growth of the PEP carboxykinase-defective mutants of *C. glutamicum* on glucose minimal medium was identical to that of the parental strains, the enzyme is certainly not essential under laboratory glycolytic growth conditions.

Recently, we showed that in *C. glutamicum* the pyruvate carboxylase is a major bottleneck for the overproduction of glutamate and lysine (Peters-Wendisch *et al.*, 2001). Increasing the pyruvate carboxylase activity led to an increase of glutamate and lysine production whereas abolition of the activity led to reduced productivity. In contrast, increasing the PEP carboxykinase activity led to a decreased glutamate and lysine productivity and abolition of this activity led to an increase in the productivity of these amino acids. Having in mind the bidirectional carbon fluxes between oxaloacetate and PEP/pyruvate involving pyruvate carboxylase and PEP carboxykinase (see above), the positive effect on glutamate and lysine production by abolition of PEP carboxykinase activity can be explained by just an increase in the net carbon flux towards oxaloacetate and thus by an increase in the precursor supply for both amino acids. Obviously, the *C. glutamicum* cell is able to modulate the net anaplerotic carbon flux not only by varying the activity of the anaplerotic pyruvate carboxylase itself but additionally by varying the activity of the carbon backflux, i.e. of PEP carboxykinase. However, this hypothesis has to be confirmed by comparative carbon flux measurements in *C. glutamicum* WT and derivatives with modified pyruvate carboxylase and PEP carboxykinase activities.

Our results indicate that PEP carboxykinase activity in *C. glutamicum* is an important factor for glutamate and lysine production by our model strains and thus an important target to improve industrial strains to be used in large scale fermentation processes. The effect of varying the PEP carboxykinase activity on lysine production by *C. glutamicum* was, however, less pronounced than the effect on glutamate production. Provided that PEP carboxykinase activity affects glutamate and lysine productivity by modulation of the net anaplerotic carbon flux, this result corroborates our previous hypothesis that in contrast to glutamate production, under our conditions the lysine production is not only limited by the oxaloacetate supply but additionally by other metabolic functions like the biosynthetic pathway or the export (Peters-Wendisch *et al.*, 2001).

Experimental Procedures

Bacteria, Plasmids, and Culture Conditions

The wildtype (WT) strain of *Corynebacterium glutamicum* ATCC 13032 and the lysine hyper-producer *C. glutamicum* MH20-22B (Schrumpf *et al.*, 1992) were employed in this study. For cloning purposes and plasmid constructions, *E. coli* DH5 α (*supE44*, *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*) (Hanahan, 1985) or S17-1 (*thi-1*, *endAR1*, *hsdR17*, *supE44*, *pro*) (Simon *et al.*, 1983) were used. *E. coli* strain

HG4 (*tonA22*, *galK35*, λ^- , *pyrD34*, *pps-3*, *edd-1*, *his-68*, *tyrA2*, *rpsL125*, *pck-2*, *malA1*, *xyl-7*, *mtl-2*) (Goldie and Sanwal, 1980) and a pHc79 based cosmid gene library from *C. glutamicum* WT (Börmann *et al.*, 1992) were used for the isolation of the *C. glutamicum* *pck* gene. The plasmids employed were the *E. coli*-*C. glutamicum* shuttle vector pEK0 (Eikmanns *et al.*, 1991a), plasmids pGEM-5Zf(+) and pGEM-7Zf(+) (Promega Corp., Madison, WI) and the mobilizable *E. coli* vector pk19mobsacB (Schäfer *et al.*, 1994). The minimal medium used for growth of and amino acid production by *C. glutamicum* was described previously (Eikmanns *et al.*, 1991b) and contained 4% or 5% (w/v) glucose (as indicated in the results section), 2% (w/v) sodium lactate or 2% (w/v) potassium acetate. For growth of *C. glutamicum* MH20-22B, 2 mM leucine was added to the medium. M9 medium (Sambrook *et al.*, 1989) containing 0.5% (w/v) glucose or 0.4% (w/v) sodium succinate was used as minimal medium for *E. coli*. LB medium (Sambrook *et al.*, 1989) was used as complex medium for *C. glutamicum* and *E. coli*. When appropriate, kanamycin (50 μ g/ml) or ampicillin (100 μ g/ml) was added to the medium. *C. glutamicum* was grown aerobically at 30°C and *E. coli* at 37°C as 60-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 140 rpm. In glutamate fermentation experiments, 1.5 g Tween 60 (Sigma-Aldrich, Deisenhofen, Germany) (25 mg/ml), prewarmed to 50°C, was added after 4 hours.

Preparation of DNA, Transformation, Conjugation, and DNA Manipulations

The isolation of chromosomal and plasmid DNA from *C. glutamicum* was performed as described previously (Eikmanns *et al.*, 1994). Plasmids from *E. coli* were isolated as described by Birnboim (1983). *E. coli* was transformed by the CaCl₂ method (Sambrook *et al.*, 1989), *C. glutamicum* by electroporation (van der Rest *et al.*, 1999). The conjugation between *E. coli* S17-1 and *C. glutamicum* was performed as described by Schäfer *et al.* (1990), the resulting transconjugants were selected on LB agar plates containing kanamycin (25 μ g/ml) and nalidixic acid (50 μ g/ml).

All restriction enzymes and T4 DNA ligase were obtained from MBI Fermentas (Leon-Rot, Germany), Klenow polymerase, calf intestine phosphatase, proteinase K, DNase I, RNase A and RNasin and Taq polymerase from Roche Diagnostics (Mannheim, Germany) and used as instructed by the manufacturer. Vent DNA polymerase was purchased from New England Biolabs (Schwalbach, Germany). Restriction-generated or PCR-generated DNA fragments were separated on 0.8% agarose gels and isolated and purified by using the NucleoTrap kit from Macherey und Nagel (Düren, Germany).

DNA hybridization experiments were performed as previously described (Eikmanns *et al.*, 1994). The 1.49-kb *EcoRI*-*HindIII* fragment isolated from plasmid pEK-pckB was labelled with digoxigenin-dUTP and used as a probe. Labelling, hybridization, washing and detection was performed using the non-radioactive 'DNA Labeling and Detection Kit' and the instructions from Roche Diagnostics.

For sequence analysis of the *pck* gene, the 3.9-kb *EcoRI*-*PvuII* fragment from plasmid pEK-pckB was isolated and ligated into plasmid pGEM-5Zf(+). Sequencing of the

insert was performed by primer walking with the dideoxy chain-termination method (Sanger *et al.*, 1977) using fluorescence-labelled nucleotides. The subsequent electrophoretic analysis was performed with an automatic DNA sequencer from Applied Biosystems by MediGene (Munich, Germany). Sequence data were compiled and analysed by the HUSAR program package from EMBL. The nucleotide sequence of the *pck* gene has been deposited at the EMBL Nucleotide Sequence Database under accession number AJ269506.

RNA Isolation and Northern Hybridization

Total RNA from *C. glutamicum* WT was isolated after growth of a 60-ml culture to an OD₆₀₀ of about 4. Cells from 20 ml culture were harvested, washed with 10 ml of 10 mM Tris/HCl, pH 6.6, resuspended in 2 ml of the same buffer and then, using glass beads (150 - 212 µm, Sigma, Deisenhofen, Germany), mechanically disrupted by incubating three times for 45 s at 4°C in a ribolyser (Hybaid, Munich, Germany). Subsequently, the RNA was purified using the 'RNeasy Kit' from Qiagen (Hilden, Germany). As a Northern (RNA) hybridization probe, an intragenic 0.88 kb *pck* fragment (from base 501 to 1380 in the sequence AJ269506, deposited in the EMBL Data Library) was amplified and digoxigenin-dUTP-labelled by PCR using plasmid pEK-pckB, the primers 5'-AGTACGTTGTCATGTCCA-3' and 5'-CATGGTTGGTGCCTGCT-3' and the 'PCR DIG Probe Synthesis Kit' from Roche Diagnostics. For hybridization, 10 µg of total RNA from *C. glutamicum* WT was separated on an agarose gel containing 17% (v/v) formaldehyde and transferred onto a nylon membrane (Eikmanns *et al.*, 1994). Hybridization (at 50°C, in the presence of 50% formamide, v/v), washing and detection were performed using the 'Nucleic Acid Detection Kit' according to the instructions from Roche Diagnostics. The size marker was the 0.24 - 9.5 kb RNA ladder from GibcoBRL (Karlsruhe, Germany).

Gene Inactivation

Inactivation of the *pck* gene was performed by the gene replacement method of Schäfer *et al.* (1994). For this purpose, the 1.83 kb *EcoRI-SacI* fragment from plasmid pEK-pckB (see Figure 2) was isolated and ligated into vector pGEM-7Zf(+). From the resulting plasmid, a *pck*-internal 1.07 kb *HindII-HindIII* fragment was excised and after religation of the vector, the truncated *pck* gene was isolated as 0.69 kb *BstI-SacI* fragment and ligated into the mobilizable *E. coli* vector pk19mobsacB which is non-replicative in *C. glutamicum*. Applying the method described by Peters-Wendisch *et al.* (1996) for inactivation of the *ppc* gene, the resulting vector pk19mobsacB-Δ*pck* was used to replace the intact chromosomal *pck* gene in *C. glutamicum* WT and MH20-22B by the truncated *pck* gene. The screening of the *pck* mutants was done on LB agar plates containing 0.5% (w/v) glucose and 10% (w/v) sucrose. A PCR using primers covering position 164 to 186 and 1843 to 1821 in the EMBL deposited *pck* sequence AJ269506 was performed to verify the replacement at the chromosomal locus. With template DNA from the original strains, we obtained PCR products of 1.68 kb whereas template DNA from the replacement mutants resulted in PCR products of about 0.6 kb (data not shown). The *pck*

replacement mutants were designated *C. glutamicum* WTΔ*pck* and MH20-22BΔ*pck*, respectively.

PEP Carboxykinase Assay

To determine PEP carboxykinase activity in cell-free extracts, *C. glutamicum* cells were grown to the exponential growth phase, washed twice in 20 ml 100 mM Tris/HCl buffer, pH 7, and resuspended in 1 ml of the same buffer containing 20 mM KCl, 5 mM MgSO₄, 0.1 mM EDTA and 2 mM dithiothreitol (DTT). After disruption of the cells by sonication (Eikmanns *et al.*, 1991b) and subsequent centrifugation for 30 min at 13 000 *g* and 4°C, the supernatant was used for the assays. The protein concentration was determined by the Biuret method (Gornall *et al.*, 1949) using bovine serum albumin as standard.

PEP carboxykinase was assayed photometrically as described by Bentle and Lardy (1976) in 1 ml 100 mM HEPES, pH 7, 10 mM MnCl₂, 100 mM KHCO₃, 2 mM glutathione, 0.2 mM NADH, 24 U malate dehydrogenase, 10 mM PEP and 2 mM GDP. The decrease of NADH was followed photometrically at 340 nm. One unit of PEP carboxykinase activity is defined as 1 µmol NADH consumed per min at 30°C.

Amino Acid Analysis

For the analysis of glutamate and lysine accumulation in the culture fluid, aliquots were withdrawn and the cells were removed by centrifugation (5 min at 13,000 x *g*). Amino acids were analyzed as *ortho*-phtaldialdehyde derivatives by reversed phase chromatography as described previously (Schrupf *et al.*, 1991).

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