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Phosphoenolpyruvate carboxylase in *Corynebacterium glutamicum* is dispensable for growth and lysine production

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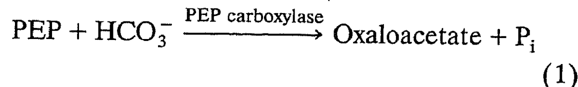
Abstract: Phosphoenolpyruvate (PEP) carboxylase is assumed to be of major importance as anaplerotic enzyme in the amino acid producing *Corynebacterium glutamicum*. We constructed PEP carboxylase-negative strains of the wild-type and of the L-lysine producer MH20–22B by disruption of the respective gene. Analysis of these strains and comparison to the parental strains revealed: (i) identical growth characteristics on all media tested; (ii) identical capacity for lysine production; and (iii) the presence of the alternative anaplerotic enzyme PEP carboxykinase in all four strains. These results show that PEP carboxylase is dispensable as anaplerotic enzyme in *C. glutamicum* and may indicate that PEP carboxykinase alone can fulfil the anaplerotic function required for growth on glucose and for lysine production.

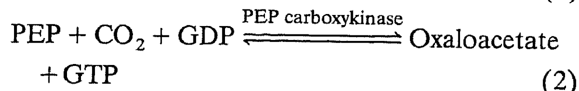
Key words: Corynebacterium glutamicum; Anaplerotic reaction; Phosphoenolpyruvate carboxylase; Phosphoenolpyruvate carboxykinase; Lysine production

Introduction

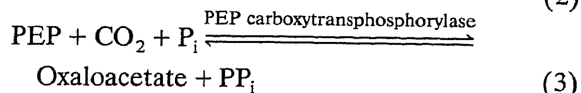
The tricarboxylic acid (TCA) cycle of growing cells requires a continuous replenishment of C4 molecules in order to replace the intermediates withdrawn for anabolic pathways. During growth on carbohydrates, these replenishing (anaplerotic) reactions can be catalyzed by irreversible phosphoenolpyruvate (PEP) carboxylase (EC 4.1.1.31) (reaction 1), by PEP carboxykinase (EC 4.1.1.32) (reaction 2), by PEP carboxytransphosphorylase (EC 4.1.1.38) (reaction 3) or by pyruvate carboxylase (EC 6.4.1.1) (reaction 4).



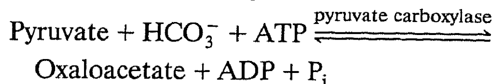
$$\text{PEP} + \text{HCO}_3^- \xrightarrow{\text{PEP carboxylase}} \text{Oxaloacetate} + \text{P}_i \quad (1)$$



$$\text{PEP} + \text{CO}_2 + \text{GDP} \xrightleftharpoons{\text{PEP carboxykinase}} \text{Oxaloacetate} + \text{GTP} \quad (2)$$



$$\text{PEP} + \text{CO}_2 + \text{P}_i \xrightleftharpoons{\text{PEP carboxytransphosphorylase}} \text{Oxaloacetate} + \text{PP}_i \quad (3)$$



$$\text{Pyruvate} + \text{HCO}_3^- + \text{ATP} \xrightleftharpoons{\text{pyruvate carboxylase}} \text{Oxaloacetate} + \text{ADP} + \text{P}_i$$

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However, although all these pathways are basically possible, the anaplerotic function during growth on carbohydrates is in general mediated by either PEP carboxylase or pyruvate carboxylase [1–4].

Corynebacterium glutamicum and its subspecies *Brevibacterium flavum* and *B. lactofermentum* are widely used in the industrial production of various amino acids [5]. As the carbon flux from carbohydrates to amino acids derived from the TCA cycle depends on anaplerotic reactions these coryneform bacteria have been intensively studied for anaplerotic enzymes. From these studies it is known that *C. glutamicum*, *B. flavum* and *B. lactofermentum* possess PEP carboxylase activity [e.g. 6–9]. *B. lactofermentum* was reported to additionally show significant pyruvate carboxylase activity when grown under biotin excess [7]. However, despite several attempts this enzyme could not be detected in the other two species [10,11]. Also, there are no reports on PEP carboxykinase or PEP carboxytransphosphorylase activities in either of the organisms. Therefore, it has been generally accepted that PEP carboxylase fulfils the anaplerotic function in coryneform bacteria [5,10–12]. In order to test this hypothesis, in this study we constructed defined PEP carboxylase-negative strains of *C. glutamicum* wild-type (WT) and of the L-lysine producer MH20–22B by the use of the respective *ppc* gene from *C. glutamicum* [8,9] and analyzed the recombinant strains with respect to growth, to lysine production and to alternative anaplerotic enzyme activities.

Materials and Methods

Bacteria, plasmids and culture conditions

C. glutamicum ATCC 13032 (wild-type), *C. glutamicum* MH20–22B [13] and *Escherichia coli* S17–1 (mobilizing donor strain) [14] were used. The plasmids employed in this study were pUC-ppcII (pUC8 containing the *C. glutamicum ppc* gene) [8] and the mobilizable vector pEM1 [15]. LB medium [16] was used as complex medium for both organisms. The minimal medium used for *C. glutamicum* has been described previously [17]. When appropriate, kanamycin (50 μg/ml) was added to the medium. For enzyme assays, growth experiments, and lysine production experiments *C. glutamicum* cells were grown aerobically as 60-ml cultures in 500-ml baffled Erlenmeyer flasks at 30°C on a rotary shaker at 140 rpm. Growth was followed by measurement of the optical density at 600 nm.

Preparation of DNA, transformation and DNA manipulations

Plasmid DNA from *E. coli* and *C. glutamicum* and chromosomal DNA from *C. glutamicum* was obtained as described previously [8,17]. *E. coli* was transformed using the $CaCl_2$ method [16]. Conjugation between *E. coli* S17–1 and *C. glutamicum* was performed as described by Schäfer et al. [18], transconjugants were selected on LB agar plates containing kanamycin (25 μg/ml) and nalidixic acid (50 μg/ml).

For Southern blot analysis genomic DNA (about 10 μg) of the *C. glutamicum* strains was restricted with *Sal*I, size-fractionated on a 0.8% agarose gel and transferred onto a Nytran filter (Schleicher und Schuell, Dassel, FRG). A 2.45-kb *Sal*I-*Bam*HI fragment, isolated from plasmid pUC-ppcII and labelled with digoxigenin-dUTP, was used as a probe. Labelling, hybridization, washing and detection were performed using the 'Nonradioactive DNA Labelling and Detection Kit' from Boehringer Mannheim.

Enzyme assays

To determine enzyme activities cells were harvested at exponential growth, washed twice in 20 ml 100 mM Tris/HCl buffer, pH 7.0, and resuspended in 1 ml of the same buffer containing 20% glycerol. The cells were disrupted by sonication [17] and after centrifugation (13 000 × *g*, 30 min) the supernatant was used for the assays. The protein concentration was determined by the Biuret method [19] using bovine serum albumin as standard.

PEP carboxylase was assayed photometrically as described previously [8]. PEP carboxykinase and PEP carboxytransphosphorylase were assayed as described by Bentle and Lardy [20] and by Willard et al. [21], respectively. Pyruvate car-

boxylase was assayed by measurement of oxaloacetate formation with malate dehydrogenase or with citrate synthase as described by Tosaka et al. [7]. The activities were monitored by measuring the decrease of absorbance at 340 nm with the exception of the citrate synthase-coupled pyruvate carboxylase assay, which was monitored by following the increase of absorbance at 412 nm.

Quantification of lysine

For analysis of lysine accumulation in the medium, aliquots of 1 ml culture were withdrawn and the cells were separated by centrifugation (13 000 × *g*, 5 min). Amino acids in the supernatant were analyzed as ortho-phthal-dialdehyde derivatives by reversed-phase chromatography as described previously [15].

Results and Discussion

Construction and assessment of PEP carboxylase-negative C. glutamicum strains

To obtain PEP carboxylase-negative mutants of *C. glutamicum* the *ppc* gene was disrupted by gene-directed mutagenesis [22]. For this purpose the *ppc* internal 0.65-kb *Xho*I fragment from plasmid pUC-ppcII was inserted into the *Sal*I site of the mobilizable *E. coli* vector pEM1, which is nonreplicative in *C. glutamicum*. The resulting plasmid pEM-PP was transferred via conjugation from the mobilizing donor strain *E. coli* S17–1 to *C. glutamicum* WT and to *C. glutamicum* MH20–22B and several transconjugants of both strains were obtained by selection on LB agar plates containing kanamycin. The kanamycin-resistant phenotype of these transconjugants indicated integration of the vector pEM-PP into the chromosomal *ppc* gene via homologous recombination between the plasmid-borne *ppc* fragment and the respective region on the corynebacterial chromosome. Thus, the transconjugants are expected to carry two truncated (non-functional) *ppc* genes separated by pEM-PP DNA on their chromosome. DNA from six transconjugants of both strains was analyzed by agarose gel electrophoresis confirming the absence of autonomous plasmids. One derivative of *C. glutamicum* WT, designated WT-PP, and one derivative of *C. glutamicum* MH20–22B, designated MH20–22B-PP, were further characterized by Southern analysis. *Sal*I-restricted chromosomal DNA of both strains and, as references, of the parental strains was hybridized to a digoxigenin-labelled 2.45-kb *Sal*I-*Bam*HI *ppc* fragment derived from plasmid pUC-ppcII. In the cases of WT and MH20–22B the *Sal*I-*Bam*HI fragment hybridized to chromosomal fragments of 3.4 kb (Fig. 1) corresponding to the chromosomal organization reported for the *C. glutamicum ppc* locus [9]. In the cases of WT-PP and MH20–22B-PP the probe hybridized to chromosomal fragments of 7.4 kb (Fig. 1). As the integrated vector contains no *Sal*I site the size of the chromosomal *Sal*I fragment in strains WT-PP and MH20–22B-PP was expected to be 7.4 kb, i.e. 3.4 kb of the original fragment plus 4 kb of pEM-PP. These results confirm that in *C.*

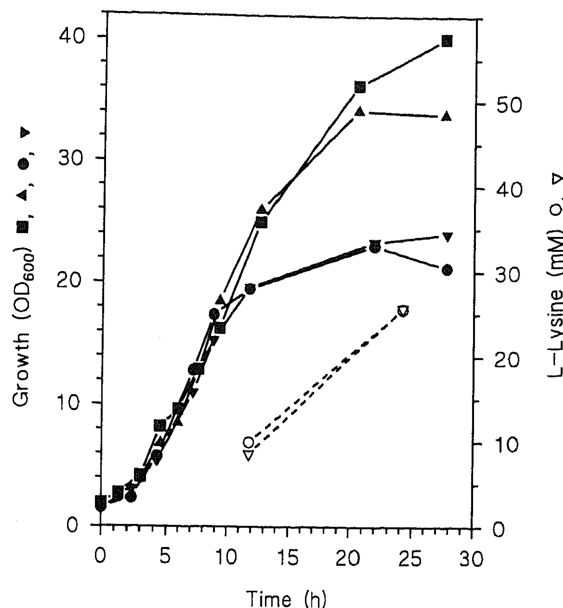

Fig. 1. Southern blot analysis of genomic DNA from *C. glutamicum* WT and MH20–22B (lane 1 and 3, respectively) and from the PEP carboxylase-negative strains WT-PP and MH20–22B-PP (lane 2 and 4, respectively). Chromosomal DNA was restricted with *Sal*I and probed with a digoxigenin-labelled *ppc*-specific probe. Size standards are shown on the right. Arrows on the left indicate the size of the observed bands.

Table 1
Specific activities of anaplerotic enzymes in cell-free extracts of *C. glutamicum* WT, MH20–22B and the PEP carboxylase-negative derivatives WT-PP and MH20–22B-PP. The cells were grown on minimal medium containing 4% glucose

C. glutamicum strain	Specific activities (mU/mg protein) [a]			
	PEP carboxylase	PEP carboxykinase	PEP carboxy-transphosphorylase	pyruvate carboxylase
WT	145 ± 11	65 ± 16	< 3	< 3
WT-PP	< 3	60 ± 17	< 3	< 3
MH20-22B	155 ± 10	75 ± 17	< 3	< 3
MH20-22B-PP	< 3	68 ± 20	< 3	< 3

[a] Values are means ± S.D. from at least six independent extracts.

glutamicum WT-PP and MH20–22B-PP the integration of pEM-PP occurred at the *ppc* locus.

To assess the inactivation of the chromosomal *ppc* gene in *C. glutamicum* WT-PP and MH20–22B-PP the specific PEP carboxylase activities were determined in cell-free extracts of both recombinant strains and of the respective parents (Table 1). Both WT-PP and MH20–22B-PP showed no detectable PEP carboxylase activity indicating that the *ppc* gene in both strains is in fact inactivated.

Comparative growth experiments and lysine production by the PEP carboxylase-negative C. glutamicum MH20–22B-PP

To study the consequences of the lack of PEP carboxylase activity on growth, *C. glutamicum* WT and MH20–22B and the respective PEP carboxylase-negative derivatives WT-PP and MH20–22B-PP were cultivated on minimal medium plus 4% glucose and growth was followed. As shown in Fig. 2, the recombinants showed the same growth compared to the respective parental strains. The same result was obtained on minimal medium containing acetate or lactate as carbon source. This indicates that the growth of *C. glutamicum* is not impaired by the lack of PEP carboxylase. Different results were previously obtained with PEP carboxylase-negative *E. coli* and *Salmonella typhimurium* strains, which were shown to grow on minimal medium plus glucose only when the medium is supplemented with utilizable intermediates of the TCA cycle or precursors thereof [3].

C. glutamicum MH20–22B is known to produce large amounts of lysine [13]. To study the effect of the lack of PEP carboxylase activity on the lysine production ability, the lysine concentration in the culture fluid of *C. glutamicum* MH20–22B and its derivative MH20–22B-PP was analyzed after 12 and 24 h (Fig. 2). Both strains accumulated approximately the same amount of lysine indicating that the capacity of *C. glutamicum* to produce lysine is not dependent on PEP carboxylase activity.

Alternative anaplerotic enzyme activities

The results obtained from comparative growth experiments and from lysine production imply that *C. glutamicum* must possess at least one

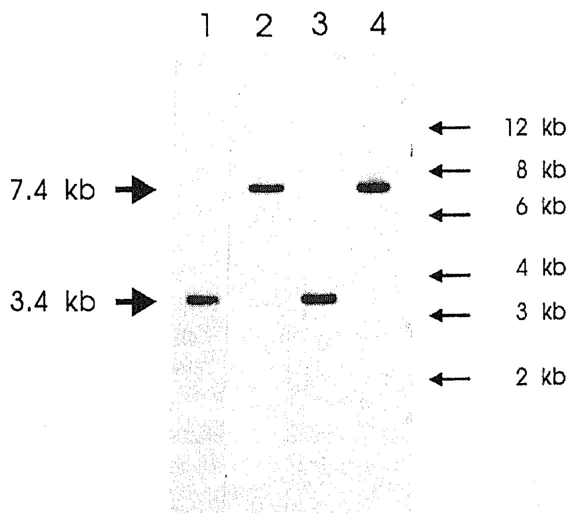

Fig. 2. Growth of *C. glutamicum* WT (■), WT-PP (▲), MH20–22B (●) and MH20–22B-PP (▼) on minimal medium containing 4% glucose as carbon source and lysine formation by MH20–22B (○) and MH20–22B-PP (▽).

other anaplerotic enzyme besides PEP carboxylase. To test for alternative anaplerotic pathways the specific activities of PEP carboxykinase, PEP carboxytransphosphorylase and pyruvate carboxylase were determined in *C. glutamicum* WT, MH20–22B and the PEP carboxylase-negative derivatives (Table 1). The results show that PEP carboxykinase is present in all strains with activities of 60 to 75 mU/mg protein. However, under a variety of assay conditions, PEP carboxytransphosphorylase and pyruvate carboxylase could not be detected in any of the strains. Characterization of the PEP carboxykinase activity revealed a pH optimum of 7.0, a strict dependence on Mg^{2+} or Mn^{2+} and a high specificity for IDP or GDP. The apparent K_m-values determined for PEP and IDP were 0.15 mM and 0.5 mM, respectively, which is in good agreement with data previously reported for PEP carboxykinases of other organisms [3]. The IDP/GDP specificity of the *C. glutamicum* enzyme is noteworthy since bacterial PEP carboxykinases are, with only very few exceptions (namely in *Arthrobacter globiformis* [3] and *Alcaligenes eutrophus* [23]), specific for ATP and only those from higher organisms are IDP- and/or GDP-specific [3]. The specific enzyme activity of PEP carboxykinase was essentially the same when *C. glutamicum* was grown on minimal medium with acetate or lactate as sole carbon source indicating that this enzyme is constitutively formed in this organism.

Conclusions

The data presented in this study show that *C. glutamicum* possesses PEP carboxylase and PEP carboxykinase which can both basically serve as anaplerotic enzymes. Analysis of PEP carboxylase-negative *C. glutamicum* strains revealed that this enzyme is dispensable for growth and for the lysine production capacity of this organism. These results suggest that PEP carboxykinase alone can replenish the TCA cycle to replace the intermediates withdrawn for growth and for lysine formation. However, our data do not exclude the possibility that the PEP carboxylase reaction is the primary anaplerotic pathway when it is present in the cells.

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References

1 Wood, H.D. and Utter, M.F. (1965) The role of CO_2 fixation in metabolism. In: Essays in Biochemistry (Campbell, P.N. and Greville, G.D., Eds.), Vol. 1, pp. 1–27. Academic Press, New York.

2 Kornberg H.L. (1966) Anaplerotic sequences and their role in metabolism. In: Essays in Biochemistry (Campbell, P.N. and Greville, G.D., Eds.), Vol. 2, pp. 1–31. Academic Press, New York.

3 Utter, M.F. and Kohlenbrander, H.M. (1972) Formation of oxaloacetate by CO_2 fixation on phosphoenolpyruvate. In: The Enzymes (Boyer, P.D., Ed.), Vol. 6, pp. 117–170. Academic Press, New York.

4 Gottschalk, G. (1986) Biosynthesis of *Escherichia coli* cells from glucose. In: Bacterial Metabolism, pp. 38–95. Springer Verlag, New York.

5 Liebl, W. (1991) The genus *Corynebacterium*-nonmedical. In: The Procaryotes (Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.H., Eds.), Vol. 2, pp. 1157–1171, Springer Verlag, New York.

6 Shiio, I., Otsuka, S. and Tsunoda, T. (1960) Glutamic acid formation from glucose by bacteria, IV. Carbon dioxide fixation and glutamate formation in *Brevibacterium flavum* No. 2247. J. Biochem. 48, 110–120.

7 Tosaka, O., Morioka, H. and Takinami, K. (1979) The role of biotin-dependent pyruvate carboxylase in L-lysine production. Agric. Biol. Chem. 43, 1513–1519.

8 Eikmanns, B.J., Follettie, M.T., Griot, M.U. and Sinskey, A.J. (1989) The phosphoenolpyruvate carboxylase gene of *Corynebacterium glutamicum*: Molecular cloning, nucleotide sequence, and expression. Mol. Gen. Genet. 218, 330–339.

9 O'Regan, M., Thierbach, G., Bachmann, B., Villeval, D., Lepage, P., Viret, J-F. and Lemoine, Y. (1989) Cloning and nucleotide sequence of the phosphoenolpyruvate carboxylase gene of *Corynebacterium glutamicum* ATCC13032. Gene 77, 237–251.

10 Ozaki, H. and Shiio, I. (1969) Regulation of the TCA and glyoxylate cycles in *Brevibacterium flavum*. J. Biochem. 66, 297–311.

11 Vallino, J.J. and Stephanopoulos, G. (1993) Metabolic flux distributions in *Corynebacterium glutamicum* during growth and lysine overproduction. Biotechnol. Bioeng. 41, 633–646.

12 Shiio, I. and Ujigawa, K. (1978) Enzymes of the glutamate and aspartate synthetic pathways in a glutamate-producing bacterium, *Brevibacterium flavum*. J. Biochem. 84, 647–657.

13 Schrumpf, B., Eggeling, L. and Sahm, H. (1992) Isolation and prominent characteristics of an L-lysine hyperproducing strain of *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. 37, 566–571.

14 Simon, R., Priefer, U. and Pühler, A. (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology 1, 784–791.

15 Schrumpf, B., Schwarzer, A., Kalinowski, J., Pühler, A., Eggeling, L. and Sahm, H. (1991) A functionally split pathway for lysine synthesis in *Corynebacterium glutamicum*. J. Bacteriol. 173, 4510–4516.

16 Sambrook, J., Fritsch, E.F. and Maniatis, J. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

17 Eikmanns, B.J., Metzger, M., Reinscheid, D., Kircher, M. and Sahm, H. (1991) Amplification of three threonine biosynthesis genes in *Corynebacterium glutamicum* and its influence on carbon flux in different strains. Appl. Microbiol. Biotechnol. 34, 617–622.

18 Schäfer, A., Kalinowski, J., Simon, R., Seep-Feldhaus, A.H. and Pühler, A. (1990) High frequency conjugal plasmid transfer from gram-negative *Escherichia coli* to various gram-positive coryneform bacteria. J. Bacteriol. 172, 1663–1666.

19 Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) Determination of serum proteins by means of the Biuret reaction. J. Biol. Chem. 177, 751–766.

20 Bentle, L.A. and Lardy, H.A. (1976) Interactions of anions and divalent metal ions with phosphoenolpyruvate carboxykinase. J. Biol. Chem. 251, 2916–2921.

21 Willard, J.M., Davies, J.J. and Wood, H.G. (1969) Phosphoenolpyruvate carboxytransphosphorylase. IV. Requirement for metal cations. Biochemistry 8, 3137–3144.

22 Schwarzer, A. and Pühler, A. (1991) Manipulation of *Corynebacterium glutamicum* by gene disruption and replacement. Bio/Technology 9, 84–87.

23 Schobert, P. and Bowien, B. (1984) Unusual C_3 and C_4 metabolism in the chemoautotroph *Alcaligenes eutrophus*. J. Bacteriol. 159, 167–172.