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Pyruvate carboxylase as an anaplerotic enzyme in *Corynebacterium glutamicum*

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The recent discovery that phosphoenolpyruvate carboxylase (PEPCx) is dispensable for growth and lysine production in *Corynebacterium glutamicum* implies that this organism possesses (an) alternative anaplerotic enzyme(s). In permeabilized cells of *C. glutamicum*, we detected pyruvate carboxylase (PCx) activity. This activity was effectively inhibited by low concentrations of ADP, AMP and acetyl-CoA. PCx activity was highest [45±5 nmol min^{-1} (mg dry wt)$^{-1}$] in cells grown on lactate or pyruvate, and was about two- to threefold lower when the cells were grown on glucose or acetate, suggesting that formation of PCx is regulated by the carbon source in the growth medium. In cells grown at low concentrations of biotin (< 5 μg l^{-1}), PCx activity was drastically reduced, indicating that the enzyme is a biotin protein. Growth experiments with the wild-type and a defined PEPCx-negative mutant of *C. glutamicum* on glucose showed that the mutant has a significantly higher demand for biotin than the wild-type, whereas both strains have the same high biotin requirement for growth on lactate and the same low biotin requirement for growth on acetate. These results indicate that (i) PCx is an essential anaplerotic enzyme for growth on glucose in the absence of PEPCx, (ii) PCx is an essential anaplerotic enzyme for growth on lactate even in the presence of PEPCx, and (iii) PCx has no anaplerotic significance for growth on acetate as the carbon source. In support of these conclusions, screening for clones unable to grow on a minimal medium containing lactate, but able to grow on a medium containing glucose or acetate, led to the isolation of PCx-defective mutants of *C. glutamicum*.

Keywords: *Corynebacterium glutamicum*, anaplerotic reactions, pyruvate carboxylase, phosphoenolpyruvate carboxylase

INTRODUCTION

Bacteria require anaplerotic reactions for continuous replenishment of the tricarboxylic acid (TCA) cycle with C_4-dicarboxylic acids used for anabolic purposes, for example amino acid biosynthesis (Kornberg, 1966). In most organisms, the anaplerotic function during growth on glucose is mediated by either phosphoenolpyruvate (PEP) carboxylase (PEPCx; reaction 1) or pyruvate carboxylase (PCx; reaction 2) (Wood & Utter, 1965; Kornberg, 1966). However, some bacteria use PEP carboxykinase (PEPCk) or PEP carboxytransphosphorylase as anaplerotic enzymes (Wood & Utter, 1965; Kornberg, 1966; Schobert & Bowien, 1984), and some *Arthrobacter* strains even use the glyoxylate cycle as an anaplerotic sequence for growth on glucose (Krulwich & Pelliccione, 1979).

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

$$\text{Pyruvate} + \text{HCO}_3^- + \text{ATP} \xrightleftharpoons{\text{PCx}} \text{Oxaloacetate} + \text{ADP} + \text{P}_i \quad (2)$$

Corynebacterium glutamicum is an aerobic, Gram-positive biotin auxotroph that grows on a variety of sugars and organic acids, and is widely used in the industrial production of amino acids, for example L-glutamate and L-lysine (Liebl, 1991). During growth on glucose, the anaplerotic function in *C. glutamicum* has

Abbreviations: CTAB, *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide; GOT, glutamate–oxaloacetate transaminase; PCx, pyruvate carboxylase; PEP, phosphoenolpyruvate; PEPCk, PEP carboxykinase; PEPCx, PEP carboxylase; TCA, tricarboxylic acid; WT, wild-type.

generally been attributed to PEPCx (Kinoshita, 1985; Liebl, 1991). This enzyme is present with relatively high specific activities in all *C. glutamicum* strains tested (Ozaki & Shiio, 1969; Mori & Shiio, 1985a; Eikmanns *et al.*, 1989; Jetten *et al.*, 1994; Gubler *et al.*, 1994). From *C. glutamicum* subsp. *flavum*, the enzyme was purified and shown to be activated by acetyl-CoA and fructose-1,6-bisphosphate and inhibited by aspartate and 2-oxoglutarate (Mori & Shiio, 1985a, b). These regulatory properties, as well as carbon flux studies (Vallino & Stephanopoulos, 1993), suggested a key role of PEPCx as an anaplerotic enzyme, and thus in carbon flow to amino acids derived from the TCA cycle. However, comparison of defined PEPCx-negative mutants with the parent strains of *C. glutamicum* showed identical growth characteristics on all media tested, and identical capacity for lysine production (Peters-Wendisch *et al.*, 1993; Gubler *et al.*, 1994). These surprising results showed that PEPCx is dispensable as an anaplerotic enzyme in *C. glutamicum*, and indicated that different anaplerotic enzymes operate in this organism.

Enzyme studies with cell-free extracts of *C. glutamicum* further revealed that, besides PEPCx, the organism possesses PEPCk (Jetten & Sinskey, 1993; Peters-Wendisch *et al.*, 1993). The activity of this enzyme in the direction of oxaloacetate synthesis was, however, completely inhibited by low concentrations of ATP (Jetten & Sinskey, 1993), which makes it likely that the PEPCk of *C. glutamicum* is involved in gluconeogenesis rather than in anaplerosis. Genetic experiments as well as *in vivo* ^{13}C-labelling experiments with *C. glutamicum* wild-type (WT) and the PEPCx-negative mutant, and subsequent ^{1}H-NMR analysis, excluded operation of the glyoxylate cycle as an anaplerotic sequence during growth on glucose, and identified the anaplerotic pathway in the presence and absence of PEPCx to involve carboxylation of PEP and/or pyruvate (Peters-Wendisch *et al.*, 1996). These findings prompted us to thoroughly test for the presence of PCx in *C. glutamicum*. During the course of these experiments, we detected PCx activity in permeabilized cells. Here, we characterize this activity and examine its significance for growth of *C. glutamicum* on different substrates in the presence and absence of PEPCx.

METHODS

Bacterial strains and growth conditions. We used *C. glutamicum* wild-type (ATCC 13032), the restriction-deficient *C. glutamicum* R127 (Liebl & Schein, 1990), the lysine producer *C. glutamicum* MH20-22B (Schrumpf *et al.*, 1992), the PEPCx-negative strain *C. glutamicum* WT-PP (Peters-Wendisch *et al.*, 1993) and *Bacillus subtilis* DB104 (Kawamura & Doi, 1984). Luria–Bertani (LB) medium or 2 × TY broth were used as complex media (Sambrook *et al.*, 1989). The minimal media for growth of *C. glutamicum* were described previously (Eikmanns *et al.*, 1991). They contained 4% (w/v) glucose, 2% (v/v) sodium D,L-lactate, 2% (w/v) sodium pyruvate or 2% (w/v) potassium acetate as carbon sources. When appropriate, kanamycin (50 μg ml^{-1}) was added to the medium. The cells were grown aerobically as 60 ml cultures in 500 ml baffled Erlenmeyer flasks at 30 °C. Growth was measured as increase in OD_{600}.

For isolation of PCx-defective mutants, *C. glutamicum* R127 cells grown overnight on 2 × TY broth were incubated at 25 °C with NMG (*N'*-methyl-*N'*-nitro-*N*-nitrosoguanidine) (0·25 mg ml^{-1}) for 15 min. The cells were then washed twice in 50 mM Tris/HCl buffer, pH 7, resuspended in the same buffer containing 30% (v/v) glycerol and stored at −20 °C until used. To screen for PCx-negative mutants, aliquots of the cell suspension were spread on LB agar and successively replica-plated on minimal medium agar containing glucose, lactate, pyruvate or acetate as carbon sources.

PCx assay with permeabilized cells. PCx activity in permeabilized cells of *C. glutamicum* was determined by the discontinuous glutamate–oxaloacetate transaminase- (GOT-) coupled assay described by Fisher & Magasanik (1984). For this assay, cells were grown in minimal medium to the exponential growth phase, washed once in 20 ml 50 mM Tris/HCl buffer, pH 6·3, containing 50 mM NaCl, resuspended to an OD_{600} of 150 in 100 mM HEPES buffer, pH 7·5, containing 20% (v/v) glycerol and then frozen at −20 °C. For permeabilization, the frozen cells were thawed slowly on ice and then mixed with a solution of 10% (w/v) CTAB (*N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide) to give a final concentration of 0·3%. This cell suspension was used directly to assay for PCx by the formation of oxaloacetate from pyruvate, HCO_3^- and ATP, and subsequent conversion of the oxaloacetate to aspartate with GOT. The standard reaction mixture consisted of 100 mM Tris/HCl, pH 7·3, 25 mM $NaHCO_3$, 20 mM pyruvate, 4 mM ATP, 2 mM glutamate, 20 μM pyridoxal phosphate and 2 units (U) of pig heart GOT in a final volume of 1 ml. The reaction was started by adding up to 50 μl of the cell suspension. After incubation of the reaction mixture at 30 °C for 60, 90 or 120 s, the reaction was terminated by boiling for 5 min. After incubation in ice-water for 5 min, the cell debris was removed by centrifugation for 20 min (13000 *g*, 4 °C) and aspartate was quantified as its *o*-phthaldialdehyde derivative by reversed-phase HPLC (Schrumpf *et al.*, 1992). One unit of PCx activity corresponds to 1 μmol aspartate formed per min. Cell mass was determined by measuring OD_{600} (OD_{600} = 1 corresponded to 0·3 mg dry wt ml^{-1}). Treatment of the cells with concentrations of CTAB higher or lower than 0·3% decreased the specific PCx activity, and cells not treated with CTAB showed no PCx activity.

In the ^{13}C-labelling experiments, $NaHCO_3$ in the PCx reaction mixture was replaced by $H^{13}CO_3^-$ (99% atom-enrichment; Cambridge Isotope Laboratories), or 70% of the unlabelled pyruvate was replaced with either [2-^{13}C]pyruvate or [3-^{13}C]pyruvate (each 99% atom-enrichment; Isotech). The aspartate formed in the assay was purified by cation-exchange chromatography on an Ultrapac 11 μ resin column (Pharmacia) using an FPLC system from Sykam as described previously (Peters-Wendisch *et al.*, 1996). Triethylamine (0·2 M) with a pH gradient from 3·2 to 10·5 was used for elution in 1 ml fractions. The fractions containing aspartate were combined, vacuum-dried, resuspended in 0·6 ml D_2O and then subjected to ^{1}H-NMR spectroscopy. As an internal standard, sodium 3-trimethylsilyl-[2,2',3,3'-D_4]propionate was included at 2 mM in each sample.

Enzyme assays with cell-free extracts. *C. glutamicum* cells were grown in minimal medium to the exponential phase, washed twice in 20 ml 100 mM Tris/HCl buffer, pH 7, and resuspended in 1 ml of the same buffer containing 20% (v/v) glycerol. After disruption of the cells by sonication (Eikmanns *et al.*, 1991) and subsequent centrifugation (13000 *g*, 30 min, 4 °C), the supernatant was used for the assays. Protein was

measured by the biuret method (Gornall *et al.*, 1949) with bovine serum albumin as standard.

PCx in cell-free extracts of *C. glutamicum* was assayed at 30 °C by three different methods. The first was that of Payne & Morris (1969) in which the oxaloacetate formed by PCx is converted to citrate by citrate synthase in the presence of acetyl-CoA and 5,5′-dithio-bis-2-nitrobenzoate. The activity in this assay was monitored by following the increase in A_{412} due to CoA-dependent formation of 5-thio-2-nitrobenzoate. The second method was that of Milrad de Forchetti & Cazzulo (1976), in which the oxaloacetate formed by PCx is converted to malate by malate dehydrogenase in the presence of NADH. The decrease of NADH was monitored at 340 nm. The third method was the discontinuous GOT-coupled assay (Fisher & Magasanik, 1984) described above for measuring the PCx activity in permeabilized cells. PCx from bovine liver (Sigma) and cell-free extracts prepared from *Bacillus subtilis* DB104 were used as positive controls.

PEPCx and PEPCk were assayed photometrically at 30 °C as described previously (Peters-Wendisch *et al.*, 1993).

NMR spectroscopy and determination of ^{13}C enrichments. High-resolution ^1H-NMR spectra of aspartate were obtained on a Bruker AMX-400 WB spectrometer operating at 400·13 MHz and equipped with a multichannel interface and a 5 mm inverse probe head. ^{13}C enrichments in C-2 and C-3 of aspartate were calculated from NMR spectra with and without ^{13}C decoupling using the parameters and the method described by Peters-Wendisch *et al.* (1996). ^{13}C enrichments in C-1 and C-4 of aspartate were quantified by spin echo ^1H-NMR difference spectroscopy with and without selective ^{13}C inversion also according to the method described previously (Peters-Wendisch *et al.*, 1996).

Western blot analysis. Cell-free extracts of *C. glutamicum* were prepared as described above. Equal amounts of heat-denatured cell extract protein were loaded and separated on an SDS-PAGE (7·5% or 10% acrylamide, w/v; 10 mA for 14 h) and electroblotted at 5 mA cm^{-2} for 30 min onto a polyvinylidene difluoride membrane (Millipore) using a Fast Blot 33 semidry transfer cell from Biometra. After blocking of the membrane with 1·5% (w/v) bovine serum albumin in 50 mM Tris/HCl, pH 7·4, 150 mM NaCl, 0·1% (v/v) Tween 20, the biotinylated proteins were detected using streptavidin-linked alkaline phosphatase from Boehringer Mannheim according to the instructions of the manufacturer.

RESULTS

PCx activity in *C. glutamicum*

Cell-free extracts of *C. glutamicum* grown on complex medium, and on minimal media containing glucose, lactate or acetate as carbon sources, were tested for PCx activity. Using the citrate-synthase-coupled and the malate-dehydrogenase-coupled assays and a variety of assay conditions, no PCx activity could be detected. Using the discontinuous GOT-coupled PCx assay, pyruvate- and ATP-dependent activity was observed; however, this activity decreased from about 40 nmol min^{-1} (mg protein)$^{-1}$ at the start of the reaction to about 4 nmol min^{-1} (mg protein)$^{-1}$ after 1 min and to <1 nmol min^{-1} (mg protein)$^{-1}$ after 2 min. In contrast, PCx activity could easily and reliably be demonstrated in cell-free extracts of *B. subtilis* with any of the three assays used (data not shown).

Table 1. ^{13}C enrichments in the C atoms of aspartate derived from different pyruvate carboxylase assays with permeabilized cells of *C. glutamicum*

Substrate	Percentage ^{13}C enrichments in aspartate*			
	C-1	C-2	C-3	C-4
HCO_3^- + [2-^{13}C]pyruvate (70%)	1·1	70·0	1·1	1·1
HCO_3^- + [3-^{13}C]pyruvate (70%)	1·1	1·1	68·0	1·1
$H^{13}CO_3^-$ (99%) + pyruvate	1·1	1·1	1·1	98·0

* Values are means of two determinations. Absolute errors are $<0·5$%.

In a further attempt to reliably demonstrate PCx activity in *C. glutamicum*, we tested permeabilized cells. For this purpose, *C. glutamicum* WT cells were suspended in HEPES buffer containing 0·3% CTAB and then subjected to the GOT-coupled PCx assay. *C. glutamicum* cells grown on minimal medium containing lactate as carbon source displayed specific aspartate-forming activity of 45 ± 5 nmol min^{-1} (mg dry wt)$^{-1}$. That the observed reaction represented PCx activity was indicated by its linear dependence on cell concentration (up to 2 mg dry wt per assay), incubation time (up to 5 min), and the presence of Mg^{2+} and the substrates pyruvate, ATP and HCO_3^-. The activity in either HEPES, Tris/HCl, MES or MOPS buffer was approximately the same and optimal over a pH range of 7·0 to 7·5. The reaction rates at different pyruvate concentrations followed Michaelis–Menten kinetics with a K_m value of 1·3 mM and a V_{max} of 50 nmol min^{-1} (mg dry wt)$^{-1}$. The K_m value for ATP was 0·2 mM. ADP, AMP and ITP inhibited the PCx activity with inhibition constants of 2·6 mM, 0·75 mM and 15 mM, respectively. Additionally, the *C. glutamicum* PCx activity was efficiently inhibited by acetyl-CoA with a K_i value of 110 μM.

To prove that the aspartate formed by the permeabilized cells originates from pyruvate and HCO_3^-, i.e. from carboxylation of the methyl group of pyruvate, we performed the GOT-coupled assay in the presence of either [2-^{13}C]pyruvate plus unlabelled HCO_3^-, [3-^{13}C]pyruvate plus unlabelled HCO_3^- or unlabelled pyruvate plus $H^{13}CO_3^-$, and analysed the purified aspartate by ^1H-NMR. As shown in Table 1, the assays with 70% enriched [2-^{13}C]pyruvate or [3-^{13}C]pyruvate resulted in about 70% ^{13}C enrichments in aspartate C-2 and C-3, respectively, and the assay with 99% $H^{13}CO_3^-$ resulted in 98% ^{13}C enrichment in the C-4 carboxyl group of aspartate. This labelling pattern was expected in the case of direct carboxylation of pyruvate to oxaloacetate and subsequent conversion to aspartate by GOT. Thus, we conclude that aspartate formation in permeabilized *C. glutamicum* cells was due to PCx activity.

To test whether PCx in *C. glutamicum* is regulated by

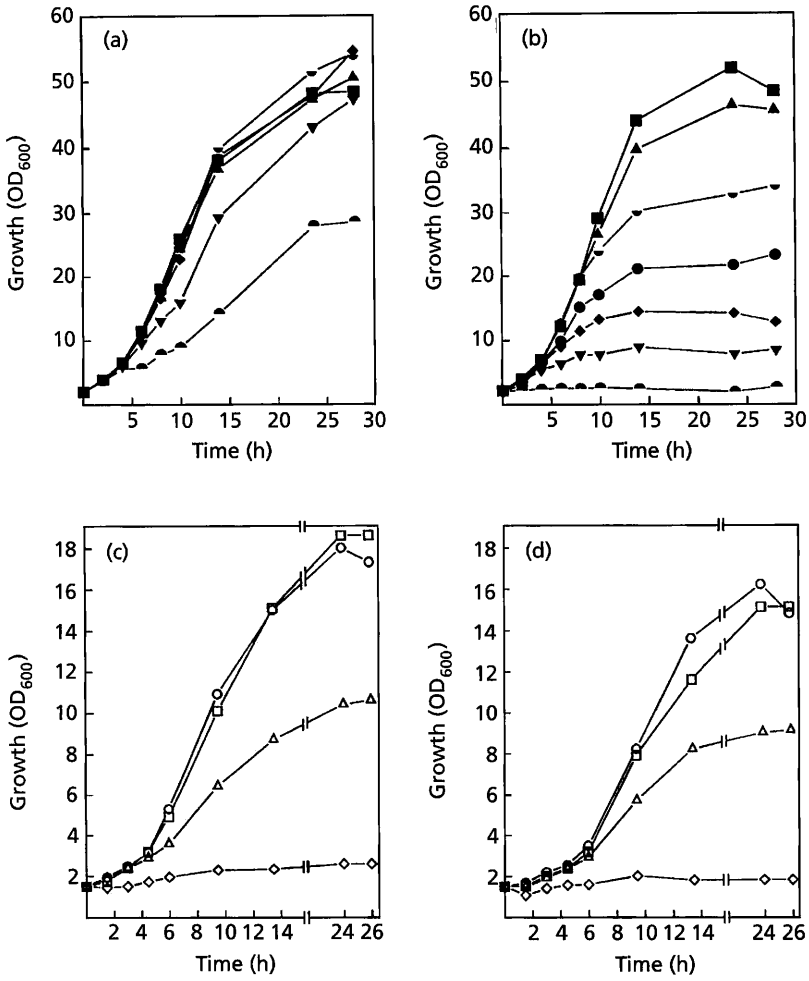

Fig. 1. Comparative growth experiments with *C. glutamicum* WT and the PEP carboxylase-negative mutant WT-PP on different minimal media. (a) Growth of the WT strain and (b) of strain WT-PP on glucose medium containing different concentrations of biotin: no biotin (▲); 5 μg biotin l^{-1} (▼); 10 μg biotin l^{-1} (◆); 15 μg biotin l^{-1} (●); 25 μg biotin l^{-1} (▬); 50 μg biotin l^{-1} (▲); 100 μg biotin l^{-1} (■). (c) Growth of the WT strain and (d) of strain WT-PP on medium containing lactate plus 100 μg biotin l^{-1} (□), lactate plus 5 μg biotin l^{-1} (△), lactate plus 5 μg biotin l^{-1} plus 35 mM fumarate (○) or 35 mM fumarate plus 100 μg biotin l^{-1} without lactate (◇).

the carbon source, the effect of different growth substrates on the specific activity of the enzyme was determined. The highest activity [45 ± 5 nmol min^{-1} (mg dry wt)$^{-1}$] was found in cells grown on lactate or pyruvate; it was significantly lower when the cells were grown on glucose [16 ± 4 nmol min^{-1} (mg dry wt)$^{-1}$] or acetate [19 ± 4 nmol min^{-1} (mg dry wt)$^{-1}$]. These results suggest that *C. glutamicum* PCx is regulated by the carbon source, and that the enzyme is more important for *C. glutamicum* during growth on lactate or pyruvate than on the other two substrates.

The PCx activity was also determined with cells of the PEPCx-negative mutant *C. glutamicum* WT-PP and of the L-lysine producer MH20-22B. Both strains showed the same specific PCx activities as *C. glutamicum* WT.

Effect of biotin on the growth of *C. glutamicum*

All PCx enzymes examined so far contain covalently bound biotin as a prosthetic group (Scrutton & Young, 1972). To study whether biotin has an effect on the growth of *C. glutamicum*, especially on that of the PEPCx-negative mutant WT-PP, we compared growth under biotin limitation and excess. As shown in Fig. 1(a, b), growth of the mutant strain on glucose was considerably more dependent on biotin (optimal at > 50 μg biotin l^{-1}) than growth of the WT (optimal at > 5 μg biotin l^{-1}). These results show that for growth on glucose the demand of *C. glutamicum* for biotin is significantly higher in the absence of PEPCx than in its presence. In contrast, with lactate as carbon source, the WT and the PEPCx-negative mutant showed about the same high demand for biotin (e.g. in Fig. 1c, d). However, when fumarate at 35 mM was added to the medium with growth-limiting concentrations of biotin, growth of both strains was completely restored (Fig. 1c, d). Since neither strain grew on fumarate as the only carbon source, these results indicate that a biotin-dependent reaction or pathway in *C. glutamicum* can be circumvented by supplying the cells with the TCA cycle intermediate fumarate. For optimal growth on acetate as carbon source, both the WT and the PEPCx-negative strain required less than 5 μg biotin l^{-1} (data not shown). Assuming that the *C. glutamicum* PCx is a biotin enzyme, these results indicate that (i) PCx is essential as an anaplerotic enzyme for growth on glucose in the absence of PEPCx, (ii) PCx is essential as an anaplerotic enzyme for growth on lactate even in the presence of PEPCx, and (iii) PCx has no anaplerotic function during growth on acetate.

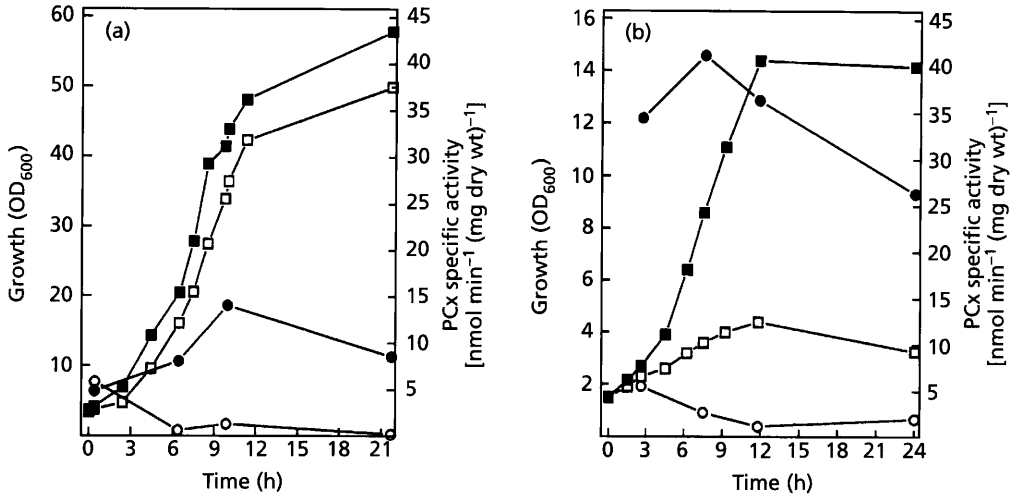

Fig. 2. Growth (squares) and specific pyruvate carboxylase (PCx) activity (circles) of *C. glutamicum* WT on glucose medium (a) or on lactate medium (b) containing 100 μg biotin l^{-1} (closed symbols) or 5 μg biotin l^{-1} (open symbols).

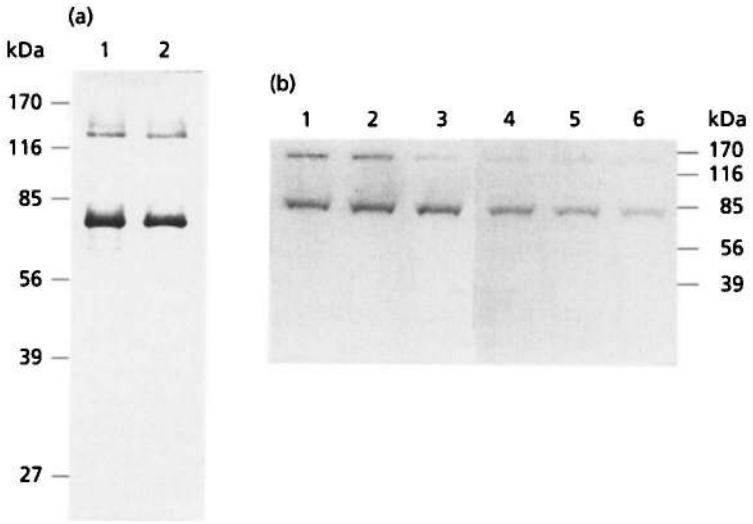

Fig. 3. (a) Western blot of SDS-PAGE of cell-free extracts (20 μg protein each) of *C. glutamicum* WT (lane 1) and of the PEP-carboxylase-negative mutant WT-PP (lane 2) grown on glucose medium. (b) Western blot of SDS-PAGE of cell-free extracts (10 μg protein each) of *C. glutamicum* WT grown on lactate medium containing 100 μg biotin l^{-1} (lanes 1–3) and 5 μg biotin l^{-1} (lanes 4–6). The extracts were prepared 5 h (lanes 1 and 4), 12 h (lanes 2 and 5) and 24 h (lanes 3 and 6) after start of the growth experiment. The sizes and positions of protein molecular mass markers are indicated.

Effect of biotin on the PCx activity of *C. glutamicum*

To investigate whether there is a correlation between the biotin concentration in the growth medium and the PCx activity of *C. glutamicum*, we cultured WT cells on glucose medium supplemented with 100 μg biotin l^{-1} or 5 μg biotin l^{-1}, and determined their specific PCx activity. The cells showed approximately the same growth on both media; however, the PCx activity of cells growing on the medium with 5 μg biotin l^{-1} was significantly lower than that of the cells growing in the presence of 100 μg biotin l^{-1} (Fig. 2a). The same experiments were performed on media containing lactate as the carbon source (Fig. 2b). Under biotin excess, i.e. when the growth of *C. glutamicum* was optimal, the specific PCx activity was relatively high. In contrast, under biotin limitation, i.e. when the growth of *C. glutamicum* was severely impaired, the specific PCx activity was drastically reduced. These data support the idea that PCx is a biotin protein and corroborate the conclusion that PCx is not essential as an anaplerotic enzyme for growth of *C. glutamicum* WT on glucose, but is essential for growth on lactate.

Identification of biotinylated proteins in *C. glutamicum*

Cell-free extracts of *C. glutamicum* WT and the PEP carboxylase-negative mutant WT-PP grown on glucose medium were assayed for biotinylated proteins by SDS-PAGE and Western blot analysis. Two biotin-containing proteins, with approximate molecular masses of 65 kDa and 125 kDa, were detected in both strains (Fig. 3a). Since the smaller protein represents the biotinylated subunit of an acyl-CoA carboxylase (Jäger *et al.*, 1996) the 125 kDa signal might correspond to the PCx.

To investigate whether there is a relation between

Table 2. Specific activities of pyruvate carboxylase (PCx), PEP carboxylase (PEPCx) and PEP carboxykinase (PEPCk) in *C. glutamicum* R127, R127-SP078 and R127-SP733

PCx was determined in permeabilized cells, PEPCx and PEPCk in cell-free extracts of the respective *C. glutamicum* strain grown on glucose medium.

Strain	Specific activity of:		
	PCx [nmol min^{-1} (mg dry wt)$^{-1}$]	PEPCx [nmol min^{-1} (mg protein)$^{-1}$]	PEPCk [nmol min^{-1} (mg protein)$^{-1}$]
R127	15	231	165
R127-SP078	1·6	256	159
R127-SP733	< 0·4	262	175

biotin-dependent growth, PCx activity and the 125 kDa biotin protein, we tested *C. glutamicum* WT cells grown on lactate medium containing 100 μg biotin l^{-1} or 5 μg biotin l^{-1} for their content of the 125 kDa biotin protein. Under biotin excess, when the growth of *C. glutamicum* was optimal and the specific PCx activity was high (see Fig. 2b), significant amounts of the 125 kDa biotin protein were present (Fig. 3b, lanes 1–3). In contrast, under biotin limitation, i.e. when the growth of *C. glutamicum* was severely impaired and PCx activity was almost not detectable, the 125 kDa biotin protein was essentially absent (Fig. 3b, lanes 4–6). When the same experiments were performed with cells grown on media containing glucose, the 125 kDa biotin protein was again present in the biotin-supplemented cells and not detectable in the biotin-limited cells. These results indicate that the biotinylated 125 kDa protein of *C. glutamicum* probably represents the PCx enzyme.

Isolation and characterization of PCx-negative *C. glutamicum* mutants

To isolate PCx-negative mutants of *C. glutamicum*, mutagenized cells of the restriction-deficient strain R127 were plated on complex medium and about 40000 clones were screened for their ability to grow on solid medium with glucose or acetate but not lactate or pyruvate as carbon source. After retesting twice, 15 clones showing this phenotype were obtained. Permeabilized cells of these mutants were then tested for their specific PCx activity. When compared to the parental strain R127, 13 of the 15 showed between 50% and 100% PCx activity [i.e. 7·5–14 nmol min^{-1} (mg dry wt)$^{-1}$]. One mutant, designated R127-SP078, showed approximately 10% PCx activity and one, designated R127-SP733, was devoid of any PCx activity (Table 2). These two strains were further tested for PEPCx and PEPCk activities and for their growth phenotype in liquid medium. Cell-free extracts of both mutants showed about the same levels of PEPCx and PEPCk as the parental strain (Table 2) and both mutants grew well on liquid medium with glucose or acetate but did not grow with lactate or pyruvate as carbon source. However, when lactate or pyruvate medium was supplemented with 35 mM fumarate, both mutants showed the same growth as the parental strain in the absence of fumarate. These results suggested that the two isolates are not able to replenish the TCA cycle from lactate and pyruvate and thus, that the phenotype of strains R127-SP733 and R127-SP078 is in fact due to the lack or drastic decrease in anaplerotic PCx activity.

DISCUSSION

Previous studies have shown that PEPCx is not essential as an anaplerotic enzyme for *C. glutamicum* (Peters-Wendisch *et al.*, 1993; Gubler *et al.*, 1994), and from ^{13}C-labelling experiments and NMR analysis it was concluded that *C. glutamicum* must possess alternative anaplerotic activity that carboxylates either PEP or pyruvate (Peters-Wendisch *et al.*, 1996). This conclusion has now been confirmed. The data reported here for the first time provide evidence that *C. glutamicum* possesses PCx as well as PEPCx, and they suggest that PCx functions exclusively as an anaplerotic enzyme during growth on lactate and pyruvate. In the PEPCx-negative mutant, PCx is obviously also responsible for the net synthesis of oxaloacetate during growth on glucose, and it might even be possible that PCx is the predominant anaplerotic enzyme in *C. glutamicum* WT during growth on glucose under biotin excess.

In having both PEPCx and PCx, *C. glutamicum* differs from *Escherichia coli*, *B. subtilis* and *B. stearothermophilus*. In *E. coli*, PEPCx represents the only anaplerotic enzyme during growth on glucose since a defined PEPCx-negative mutant is not able to grow on glucose as the sole carbon source and requires succinate as a supplement (Chao & Liao, 1993). In *B. subtilis* and *B. stearothermophilus*, only PCx is responsible for the net formation of oxaloacetate. Mutants blocked in this enzyme were also unable to grow on minimal glucose or lactate media unless supplemented with TCA cycle intermediates (Diesterhaft & Freese, 1973; Sundaram, 1973). Some bacteria, for example *Pseudomonas citronellolis*, *P. fluorescens*, *Azotobacter vinelandii* and *Thiobacillus novellus*, possess both PEPCx and PCx (O'Brien *et al.*, 1977; Scrutton & Taylor, 1974; Milrad de Forchetti & Cazzulo, 1976; Charles & Willer, 1984). However, to our knowledge it has not been determined whether in these bacteria one or the other of the two enzymes is essential under any given growth condition.

Previously, the presence of PCx has been reported in cell-free extracts of *C. glutamicum* subsp. *lactofermentum* (Tosaka *et al.*, 1979), and there has been recent speculation that this enzyme might function in other *C. glutamicum* strains as well (Cocaign-Bousquet *et al.*, 1996). However, in spite of intensive efforts, we and several other groups were unable to reliably measure PCx activity in cell-free extracts of this or any other *C. glutamicum* strain tested (Peters-Wendisch *et al.*, 1993; Jetten *et al.*, 1994; Gubler *et al.*, 1994; Cocaign-Bousquet & Lindley, 1995; Cocaign-Bousquet *et al.*,

1996). This surprising finding could be due to instability of the enzyme under the chosen conditions, to loss of an enzyme component, or to loss of an activator molecule during preparation of the extract. PCx from some organisms, for example *Arthrobacter globiformis*, *Aspergillus niger*, chicken and rat liver, is very unstable under certain conditions (at 0 °C, pH values > 8 or at low salt concentrations; in the case of *A. globiformis* due to structural changes in the native enzyme) (Gurr & Jones, 1977; Feir & Suzuki, 1969). However, the enzyme from other organisms is stable and easily measured (see, for example, Cazzulo *et al.*, 1970; Scrutton & Young, 1972; Scrutton & Taylor, 1974; Modak & Kelly, 1995). Biochemical and genetic approaches are now needed to find why PCx activity is difficult to measure in cell-free extracts of *C. glutamicum*.

The specific PCx activity in permeabilized cells of *C. glutamicum* depended on the growth medium and varied between 14–19 nmol min^{-1} (mg dry wt)$^{-1}$ during growth on acetate or glucose and about 45 nmol min^{-1} (mg dry wt)$^{-1}$ during growth on lactate or pyruvate. Assuming that approximately 50% of the dry weight consists of protein, these activities correspond to about 30 and 90 nmol min^{-1} (mg protein)$^{-1}$, respectively, and thus are comparable to those observed in cell-free extracts of other organisms such as *A. globiformis* [90 nmol min^{-1} (mg protein)$^{-1}$], *Rhizobium etli* [50 nmol min^{-1} (mg protein)$^{-1}$], *T. novellus* [25–100 nmol min^{-1} (mg protein)$^{-1}$] or *Rhodobacter capsulatus* [20–66 nmol min^{-1} (mg protein)$^{-1}$] (Gurr & Jones, 1977; Encarnacion *et al.*, 1995; Charles & Willer, 1984; Willison, 1988). As in *C. glutamicum*, the specific PCx activity of some other organisms, for example *A. vinelandii*, *R. sphaeroides*, *R. capsulatus* and *Saccharomyces cerevisiae*, was three- to fourfold higher when the cells were grown on lactate instead of substrates such as glucose, glycerol, sucrose or malate (Scrutton & Taylor, 1974; Payne & Morris, 1969; Willison, 1988; Young *et al.*, 1969) suggesting a pivotal anaplerotic role of PCx during growth on lactate. For *C. glutamicum*, this suggestion was substantiated by isolating a PCx-negative mutant due to its inability to grow on lactate (and pyruvate).

In its absolute requirement for Mg^{2+}, its apparent K_m values of 1·3 mM for pyruvate and 0·2 mM for ATP and its sensitivity to AMP and ADP, the *C. glutamicum* PCx is similar to the PCx enzymes from other organisms (Milrad de Forchetti & Cazzulo, 1976; Scrutton & Taylor, 1974; Cazzulo *et al.*, 1970; Gurr & Jones, 1977; Modak & Kelly, 1995). However, in contrast to all PCx enzymes studied so far, the *C. glutamicum* enzyme was inhibited by acetyl-CoA. Due to this feature, the *C. glutamicum* PCx does not conform to the classification of PCx enzymes by Scrutton & Young (1972), which is based on the degree of activation of the enzyme by acetyl-CoA. Some PCx enzymes, for example those from *B. subtilis*, *B. stearothermophilus* and *R. capsulatus*, are strictly dependent on the presence of acetyl-CoA (Cazzulo *et al.*, 1970; Diesterhaft & Freese, 1973; Modak & Kelly, 1995). The PCx enzymes of *A. globiformis*, *T. novellus* and *S. cerevisiae* are active without acetyl-CoA but they are stimulated significantly by it (Gurr & Jones, 1977; Charles & Willer, 1984; Ruiz-Amil *et al.*, 1965), whereas those of *P. fluorescens*, *P. citronellolis*, *A. vinelandii* and *A. niger* are completely independent of acetyl-CoA (Milrad de Forchetti & Cazzulo, 1976; Seubert & Weicker, 1969; Scrutton & Taylor, 1974; Feir & Suzuki, 1969). The effective inhibition of the *C. glutamicum* PCx by acetyl-CoA together with the strong inhibition of the *C. glutamicum* PEPCx by aspartate (Eikmanns *et al.*, 1989) might ensure that during growth of *C. glutamicum* on acetate or other precursors of acetyl-CoA the anaplerotic carboxylation reactions do not interfere with gluconeogenesis, i.e. with PEP and/or pyruvate formation from oxaloacetate by PEPCk and/or oxaloacetate decarboxylase. Both these enzymes have been found in *C. glutamicum* and shown to be formed under glycolytic as well as under gluconeogenetic conditions (Jetten & Sinskey, 1993; Peters-Wendisch *et al.*, 1993; Jetten & Sinskey, 1995; Jetten *et al.*, 1994). Since PEPCx and PCx are also formed under both conditions, a tight effector-modulated regulation of all the enzymes involved in interconverting PEP, pyruvate and oxaloacetate is required to properly adjust the carbon flux at this branch point within central metabolism, and to prevent extensive futile cycling. However, while the regulatory properties of the *C. glutamicum* PEPCx, PEPCk and oxaloacetate decarboxylase are well known [PEPCx is inhibited by aspartate and activated by acetyl-CoA and fructose-1,6-bisphosphate (Mori & Shiio, 1985a, b; Eikmanns *et al.*, 1989); PEPCk is inhibited by ADP and ATP (Jetten & Sinskey, 1993); oxaloacetate decarboxylase is inhibited by ADP, CoA and succinate (Jetten & Sinskey, 1995)], there are still some uncertainties about the regulation of PCx, since, for example, the effects of aspartate and 2-oxoglutarate, which both are potent inhibitors of PCx enzymes from some other organisms (e.g. Gurr & Jones, 1977; Modak & Kelly, 1995), could not be tested due to the principle of the GOT-coupled assay applied in this work.

Besides PEPCx, PEPCk, PCx and oxaloacetate decarboxylase, the decarboxylating/carboxylating enzymes located at the metabolic node around pyruvate in *C. glutamicum* include the NADP-dependent malic enzyme (Cocaign-Bousquet & Lindley, 1995; Vallino & Stephanopoulos, 1993). In principle, the reaction catalysed by this enzyme is reversible, but in most organisms the malic enzyme is assumed to catalyse the oxidative decarboxylation of malate rather than the reductive carboxylation of pyruvate (Kornberg, 1966). Although the role of the malic enzyme in *C. glutamicum* has never been clearly defined, Cocaign-Bousquet & Lindley (1995) deduced from kinetic data of steady-state chemostat and batch cultures growing on lactate that the enzyme is involved in a metabolic cycle generating NADPH from NADH and thus provides reducing equivalents for anabolic metabolism. In this cycle, malate is decarboxylated to pyruvate by the NADP-dependent malic enzyme, pyruvate is carboxylated to

oxaloacetate by PCx, and oxaloacetate is then reduced to malate by NADH-dependent malate dehydrogenase. Our results indicating that PCx is exclusively responsible for the net synthesis of oxaloacetate in *C. glutamicum* WT during growth on lactate and in the PEPCx-negative mutant during growth on glucose agree with this model. They indicate that the malic enzyme does not catalyse the reductive carboxylation of pyruvate, and thus does not have an anaplerotic function under the conditions employed in this study.

It is evident that there is still a lot to be learned about PCx, the anaplerotic carboxylation and the gluconeogenetic and/or NADPH-generating decarboxylation reactions in *C. glutamicum*. The isolation and characterization of the genes for PCx, PEPCk, oxaloacetate decarboxylase and malic enzyme, construction and analysis of defined mutants, and *in vivo* flux analyses are necessary to elucidate the regulatory network of the branchpoint between anaplerosis and gluconeogenesis in this industrially important organism.

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REFERENCES

Cazzulo, J. J., Sundaram, T. K. & Kornberg, H. L. (1970). Properties and regulation of pyruvate carboxylase from *Bacillus stearothermophilus*. *Proc R Soc Lond B* **16**, 1–19.

Chao, Y.-P. & Liao, J. C. (1993). Alteration of growth yield by overexpression of phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase in *Escherichia coli*. *Appl Environ Microbiol* **59**, 4261–4265.

Charles, A. M. & Willer, D. W. (1984). Pyruvate carboxylase from *Thiobacillus novellus*: properties and possible functions. *Can J Microbiol* **30**, 532–539.

Cocaign-Bousquet, M. & Lindley, N. D. (1995). Pyruvate overflow and carbon flux within the central metabolic pathways of *Corynebacterium glutamicum* during growth on lactate. *Enzyme Microb Technol* **17**, 260–267.

Cocaign-Bousquet, M., Guyonvarch, A. & Lindley, N. D. (1996). Growth rate-dependent modulation of carbon flux through central metabolism and the kinetic consequences for glucose-limited chemostat cultures of *Corynebacterium glutamicum*. *Appl Environ Microbiol* **62**, 429–436.

Diesterhaft, M. D. & Freese, E. (1973). Role of pyruvate carboxylase, phosphoenolpyruvate carboxykinase and malic enzyme during growth and sporulation of *Bacillus subtilis*. *J Biol Chem* **248**, 6062–6070.

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

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

Encarnacion, S., Dunn, M., Willms, K. & Mora, J. (1995). Fermentative and aerobic metabolism in *Rhizobium etli*. *J Bacteriol* **177**, 3058–3066.

Feir, H. A. & Suzuki, I. (1969). Pyruvate carboxylase of *Aspergillus niger*: kinetic study of a biotin-containing carboxylase. *Can J Biochem* **47**, 697–710.

Fisher, S. H. & Magasanik, B. (1984). Synthesis of oxaloacetate in *Bacillus subtilis* mutants lacking the 2-ketoglutarate dehydrogenase enzymatic complex. *J Bacteriol* **158**, 55–62.

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

Gubler, M., Park, S. M., Jetten, M., Stephanopoulos, G. & Sinskey, A. J. (1994). Effects of phosphoenolpyruvate carboxylase deficiency on metabolism and lysine production in *Corynebacterium glutamicum*. *Appl Microbiol Biotechnol* **40**, 857–863.

Gurr, J. A. & Jones, K. M. (1977). Purification and characterization of pyruvate carboxylase from *Arthrobacter globiformis*. *Arch Biochem Biophys* **179**, 444–455.

Jäger, W., Peters-Wendisch, P. G., Kalinowski, J. & Pühler, A. (1996). A *Corynebacterium glutamicum* gene encoding a two-domain protein similar to biotin carboxylases and biotin-carboxyl-carrier proteins. *Arch Microbiol* **166**, 76–82.

Jetten, M. S. M. & Sinskey, A. J. (1993). Characterization of phosphoenolpyruvate carboxykinase from *Corynebacterium glutamicum*. *FEMS Microbiol Lett* **111**, 183–188.

Jetten, M. S. M. & Sinskey, A. J. (1995). Purification and properties of oxaloacetate decarboxylase from *Corynebacterium glutamicum*. *Antonie Leeuwenhoek* **67**, 221–227.

Jetten, M. S. M., Pitoc, G. A., Follettie, M. T. & Sinskey, A. J. (1994). Regulation of phospho(enol)-pyruvate- and oxaloacetate-converting enzymes in *Corynebacterium glutamicum*. *Appl Microbiol Biotechnol* **41**, 47–52.

Kawamura, F. & Doi, R. H. (1984). Construction of a *Bacillus subtilis* double mutant deficient in extracellular alkaline and neutral proteases. *J Bacteriol* **160**, 442–444.

Kinoshita, S. (1985). Glutamic acid bacteria. In *Biology of Industrial Microorganisms*, pp. 115–142. Edited by A. Demain & N. Solomon. London: Benjamin/Cummings Publishing.

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

Krulwich, T. A. & Pelliccione, N. J. (1979). Catabolic pathways of coryneforms, nocardias and mycobacteria. *Ann Rev Microbiol* **33**, 95–111.

Liebl, W. (1991). The genus *Corynebacterium* – nonmedical. In *The Prokaryotes*, vol. II, pp. 1157–1171. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer-Verlag.

Liebl, W. & Schein, B. (1990). Isolation of restriction deficient mutants of *Corynebacterium glutamicum*. In *DECHEMA Biotechnology Conferences*, vol. 4, pp. 323–327. Edited by D. Behrens & P. Krämer. Weinheim: Verlag Chemie.

Milrad de Forchetti, S. R. & Cazzulo, J. J. (1976). Some properties of the pyruvate carboxylase from *Pseudomonas fluorescens*. *J Gen Microbiol* **93**, 75–81.

Modak, H. V. & Kelly, D. J. (1995). Acetyl-CoA-dependent pyruvate carboxylase from the photosynthetic bacterium *Rhodobacter capsulatus*: rapid and efficient purification using dye-ligand affinity chromatography. *Microbiology* **141**, 2619–2628.

Mori, M. & Shiio, I. (1985a). Purification and some properties of phosphoenolpyruvate carboxylase from *Brevibacterium flavum* and its aspartate-overproducing mutant. *J Biochem* **97**, 1119–1128.

Mori, M. & Shiio, I. (1985b). Synergistic inhibition of phosphoenolpyruvate carboxylase by aspartate and 2-oxoglutarate in *Brevibacterium flavum*. *J Biochem* **98**, 1621–1630.

O'Brien, R. W., Chuang, D. T., Taylor, B. L. & Utter, M. F. (1977). Novel enzymic machinery for the metabolism of oxaloacetate, phosphoenolpyruvate and pyruvate in *Pseudomonas citronellolis*. *J Biol Chem* **252**, 1257–1263.

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

Payne, J. & Morris, J. G. (1969). Pyruvate carboxylase in *Rhodopseudomonas sphaeroides*. *J Gen Microbiol* **59**, 97–101.

Peters-Wendisch, P. G., Eikmanns, B. J., Thierbach, G., Bachmann, B. & Sahm, H. (1993). Phosphoenolpyruvate carboxylase in *Corynebacterium glutamicum* is dispensable for growth and lysine production. *FEMS Microbiol Lett* **112**, 269–274.

Peters-Wendisch, P. G., Wendisch, V. F., de Graaf, A. A., Eikmanns, B. J. & Sahm, H. (1996). C_3-Carboxylation as an anaplerotic reaction in phosphoenolpyruvate carboxylase-deficient *Corynebacterium glutamicum*. *Arch Microbiol* **165**, 387–396.

Ruiz-Amil, M., deTorrontegui, G., Palacian, E., Catalina, L. & Losada, M. (1965). Properties and functions of yeast pyruvate carboxylase. *J Biol Chem* **240**, 3485–3492.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schobert, P. & Bowien, B. (1984). Unusual C3 and C4 metabolism in the chemoautotroph *Alcaligenes eutrophus*. *J Bacteriol* **159**, 167–172.

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

Scrutton, M. C. & Taylor, B. L. (1974). Isolation and characterization of pyruvate carboxylase from *Azotobacter vinelandii* OP. *Arch Biochem Biophys* **164**, 641–654.

Scrutton, M. C. & Young, M. R. (1972). Pyruvate carboxylase. In *The Enzymes*, vol. 6, pp. 1–35. Edited by P. D. Boyer. New York: Academic Press.

Seubert, W. & Weicker, H. (1969). Pyruvate carboxylase from *Pseudomonas*. *Methods Enzymol* **13**, 258–262.

Sundaram, T. K. (1973). Physiological role of pyruvate carboxylase in a thermophilic bacillus. *J Bacteriol* **113**, 549–557.

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

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

Willison, J. C. (1988). Pyruvate and acetate metabolism in the photosynthetic bacterium *Rhodobacter capsulatus*. *J Gen Microbiol* **134**, 2429–2439.

Wood, H. D. & Utter, M. F. (1965). The role of CO_2 fixation in metabolism. In *Essays in Biochemistry*, vol. 2, pp. 1–27. Edited by P. N. Campbell & G. D. Greville. New York: Academic Press.

Young, M. R., Tolbert, B. & Utter, M. F. (1969). Pyruvate carboxylase from *Saccharomyces cerevisiae*. *Methods Enzymol* **13**, 250–258.

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