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Pyruvate carboxylase from *Corynebacterium glutamicum*: characterization, expression and inactivation of the *pyc* gene

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In addition to phosphoenolpyruvate carboxylase (PEPCx), pyruvate carboxylase (PCx) has recently been found as an anaplerotic enzyme in the amino-acid-producing bacterium *Corynebacterium glutamicum*. Using oligonucleotides designed according to conserved regions of PCx amino acid sequences from other organisms, a 200 bp fragment central to the *C. glutamicum* PCx gene (*pyc*) was amplified from genomic DNA by PCR. This fragment was then used to identify and to subclone the entire *C. glutamicum pyc* gene. The cloned *pyc* gene was expressed in *C. glutamicum*, as cells harbouring the gene on plasmid showed four- to fivefold higher specific PCx activities when compared to the wild-type (WT). Moreover, increased PCx protein levels in the *pyc*-plasmid-carrying strain were readily detected after SDS-PAGE of cell-free extracts. DNA sequence analysis of the *pyc* gene, including its 5′ and 3′ flanking regions, and N-terminal sequencing of the *pyc* gene product predicts a PCx polypeptide of 1140 amino acids with an M_r of 123070. The amino acid sequence of this polypeptide shows between 62% and 45% identity when compared to PCx enzymes from other organisms. Transcriptional analyses revealed that the *pyc* gene from *C. glutamicum* is monocistronic (3·5 kb mRNA) and that its transcription is initiated at an A residue 55 bp upstream of the translational start. Inactivation of the chromosomal *pyc* gene in *C. glutamicum* WT led to the absence of PCx activity and to negligible growth on lactate, indicating that PCx is essential for growth on this carbon source. Inactivation of both the PCx gene and the PEPCx gene in *C. glutamicum* led additionally to the inability to grow on glucose, indicating that no further anaplerotic enzymes for growth on carbohydrates exist in this organism.

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

INTRODUCTION

Pyruvate carboxylase (PCx) [pyruvate:carbon dioxide ligase (ADP-forming), EC 6.4.1.1] is a biotin-containing enzyme which fulfils an important anaplerotic function in many organisms (for a review see Scrutton & Young, 1972). The enzyme catalyses the carboxylation of pyruvate to form oxaloacetate in two steps:

$$\text{Enzyme-biotin} + \text{ATP} + \text{HCO}_3^- \overset{Mg^{2+}}{\rightleftharpoons} \text{Enzyme-biotin-CO}_2^- + \text{ADP} + \text{P}_i \quad (1)$$

$$\text{Enzyme-biotin-CO}_2^- + \text{pyruvate} \rightarrow \text{Enzyme-biotin} + \text{oxaloacetate} \quad (2)$$

In the metabolism of carbohydrates, this two-step reaction is used for continuous replenishment of the

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Abbreviations: AC, accession number; PCx, pyruvate carboxylase; PEP, phosphoenolpyruvate; PEPCx, PEP carboxylase; TCA, tricarboxylic acid; WT, wild-type.

The EMBL accession number for the sequence reported in this paper is Y09548.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics*	Source/reference
Strains		
E. coli DH5α	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1985)
E. coli S17-1	Mobilizing donor strain	Simon *et al.* (1983)
C. glutamicum WT	Wild-type strain ATCC 13032	American Type Culture Collection
C. glutamicum WTΔpyc	Defined mutant of the WT strain, PCx-negative	This work
C. glutamicum WTΔpycΔppc	Defined double mutant of the WT, PCx- and PEPCx-negative	This work
Cosmids/plasmids		
pHC79-based gene library	*C. glutamicum* WT chromosomal DNA cloned in cosmid pHC79	Börmann *et al.* (1992)
pHC79-1	Recombinant pHC79 cosmid containing a 43 kb chromosomal *Sau*3A fragment from *C. glutamicum*	This work
pUC18	Ap^R	Vieira & Messing (1982)
pUCpyc	pUC18 containing a 17 kb *Hin*dIII fragment from pHC79-1	This work
pGEM-T	PCR cloning and transcription vector carrying the T7 and SP6 promoters, Ap^R	Promega
pK19mobsacB	Mobilizable vector, *oriT sacB* Km^R	Schäfer *et al.* (1994)
pK19mobsacBΔpyc	pK19mobsacB containing the 1 kb fragment of the deleted *pyc* gene	This work
pEK0	*E. coli–C. glutamicum* shuttle vector, Km^R	Eikmanns *et al.* (1991a)
pEK0pyc	pEK0 containing a 6·2 kb *Sca*I–*Ssp*I fragment from pUCpyc	This work

* Ap^R, ampicillin resistance; Km^R, kanamycin resistance.

tricarboxylic acid (TCA) cycle for anabolic purposes, such as amino acid synthesis. Whereas PCx plays the major anaplerotic role in vertebrate tissues, especially in liver and kidney (Kornberg, 1966; Scrutton & Young, 1972; Jitrapakdee *et al.*, 1996) and in yeast (Ruiz-Amil *et al.*, 1965; Stucka *et al.*, 1991), only a few prokaryotes, e.g. some *Bacillus* strains and *Rhodobacter capsulatus*, use PCx as the sole anaplerotic enzyme (Cazzulo *et al.*, 1970; Diesterhaft & Freese, 1973; Sundaram, 1973; Modak & Kelly, 1995). In many bacteria, e.g. enteric bacteria, cyanobacteria and streptomycetes, another oxaloacetate-forming enzyme, phosphoenolpyruvate carboxylase (PEPCx), is the only enzyme used for replenishing the TCA cycle during growth on glucose (Chao & Liao, 1993; Owttrim & Coleman, 1986; Dekleva & Strohl, 1988). In some other bacteria, such as *Pseudomonas citronellolis*, *P. fluorescens*, *Azotobacter vinelandii* and *Rhizobium etli*, both PCx and PEPCx have been detected (Higa *et al.*, 1976; O'Brien *et al.*, 1977; Liao & Atkinson, 1971; Scrutton & Taylor, 1974; Dunn *et al.*, 1996). However, so far the physiological role of the one anaplerotic enzyme or the other in these bacteria has not been studied in detail.

The Gram-positive, aerobic and biotin-auxotrophic *Corynebacterium glutamicum* is widely used in the industrial production of amino acids, particularly L-glutamate and L-lysine (Liebl, 1991; Eikmanns *et al.*, 1993). Due to their importance in amino acid production, the anaplerotic reactions in this organism and in the subspecies *C. glutamicum* subsp. *flavum* and subsp. *lactofermentum* have been intensively studied. Ozaki & Shiio (1969) and Mori & Shiio (1985a, b) purified a PEPCx from *C. glutamicum* subsp. *flavum* and showed that the enzyme is inhibited by aspartate and 2-oxoglutarate and activated by acetyl-CoA and fructose-1,6-bisphosphate. These properties, as well as carbon flux studies, suggested that PEPCx is the major if not the only anaplerotic enzyme for growth and amino acid production on glucose as the sole carbon source (Kinoshita, 1985; Liebl, 1991, Vallino & Stephanopoulos, 1993). However, recently it was shown that defined PEPCx-deficient mutants of *C. glutamicum* exhibit the same growth behaviour and the same lysine production capability as the PEPCx-positive parental strains (Peters-Wendisch *et al.*, 1993; Gubler *et al.*, 1994). These results indicated the presence of another anaplerotic enzyme in *C. glutamicum* apart from PEPCx. In fact, we have been able to detect PCx activity in permeabilized cells of *C. glutamicum* (Peters-Wendisch *et al.*, 1997). The characterization of this activity revealed that the enzyme is effectively inhibited by AMP, ADP and acetyl-CoA. In this communication we describe the isolation and sequence analysis of the PCx gene *pyc* of *C. glutamicum* as well as its transcriptional organization and its expression. Furthermore, to clarify the physiological role of PCx and PEPCx in *C. glutamicum*, we constructed and characterized a defined PCx-negative mu-

tant and a defined PCx-/PEPCx-negative double mutant.

METHODS

Bacteria, plasmids and culture conditions. The bacterial strains and plasmids used are listed in Table 1. The minimal medium for growth of *C. glutamicum* has been described previously (Eikmanns *et al.*, 1991b). Carbon sources were added to the minimal medium at final concentrations of 4% (w/v) glucose, 2% (v/v) sodium DL-lactate or 2% (w/v) potassium acetate. Luria–Bertani (LB) medium or 2 × TY broth (Sambrook *et al.*, 1989) were used as the complex medium for *Escherichia coli* and *C. glutamicum*. When appropriate, ampicillin (50 μg ml^{-1}) or kanamycin (50 μg ml^{-1}) was added to the medium. *C. glutamicum* cells were grown aerobically as 60 ml cultures in 500 ml baffled Erlenmeyer flasks at 30 °C; *E. coli* cells were grown under the same conditions at 37 °C. Growth was measured as increase in OD_{600}.

DNA preparation, transformation and conjugation. Plasmids from *E. coli* were isolated as described by Birnboim (1983); those from *C. glutamicum* were isolated by the same method with prior incubation (1 h, 37 °C) of the cells with lysozyme (15 mg ml^{-1}). Chromosomal DNA from *C. glutamicum* was isolated as described previously (Eikmanns *et al.*, 1994). *E. coli* was transformed by the $CaCl_2$ method (Sambrook *et al.*, 1989) and *C. glutamicum* was transformed by electroporation as described by Liebl *et al.* (1989). 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 μg ml^{-1}) and nalidixic acid (50 μg ml^{-1}).

Generation of a *pyc*-specific PCR product. To amplify a *pyc*-specific fragment from chromosomal *C. glutamicum* DNA, two PCR primers were designed according to highly conserved regions within the PCx enzymes from other organisms. The conserved regions were deduced from the PCx amino acid sequences so far known, i.e. from *Saccharomyces cerevisiae* (Lim *et al.*, 1988; EMBL accession number (AC) P11154), man (Wexler *et al.*, 1994; MacKay *et al.*, 1994; AC U04641), mouse (Zhang *et al.*, 1993; AC L09192), rat (Jitrapakdee *et al.*, 1996; AC U36585); *Aedes aegypti* (Z. Tu & H. H. Hagedorn, unpublished; AC L36530) and *Mycobacterium tuberculosis* (D. R. Smith, unpublished; AC U00024). The PCR primers chosen corresponded to the nucleotide sequence 810–831 (primer 1: 5′-CGCGGGCACCGTCGAGTTCCTG-3′) and 1037–1015 (primer 2: 5′-CCACGGTGGTGATCCGGCACTG-3′) of the putative *pyc* gene of *M. tuberculosis*. Using this primer pair and chromosomal DNA of *C. glutamicum*, a 0·2 kb DNA fragment was amplified by PCR performed according to the standard procedure given by Boehringer Mannheim. The PCR fragment was cloned into the *Sma*I restriction site of plasmid pK19mobsacB and its DNA sequence was determined. The deduced amino acid sequence was compared with the respective region of the PCx amino acid sequences mentioned above and an identity of up to 64% was found. On the basis of these comparisons, it was concluded that the cloned PCR fragment represented a central 0·2 kb fragment of the *pyc* gene from *C. glutamicum*. For identification of a chromosomal *C. glutamicum* DNA fragment carrying the entire *pyc* gene, the 0·2 kb fragment was amplified from genomic DNA using digoxigenin-labelled dUTP instead of dTTP, thus generating a digoxigenin-labelled *pyc*-specific DNA probe.

DNA manipulations. Standard protocols (Sambrook *et al.*, 1989) were used for the construction and analysis of plasmid DNA. All restriction enzymes, T4 DNA ligase, Klenow polymerase, calf intestine phosphatase and *Taq* polymerase were obtained from Boehringer Mannheim and used as instructed. Restriction-generated fragments were separated on 0·8% (w/v) agarose gels and isolated using the QiaexII kit (Qiagen).

DNA hybridization experiments were performed as described previously (Peters-Wendisch *et al.*, 1993). As a hybridization probe, the terminal 493 bp of the *pyc* gene (from base 3095 to 3587 in the sequence AC Y09548, deposited in the EMBL Data Library) were amplified and digoxigenin-dUTP-labelled by PCR (PCR DIG Probe Synthesis Kit from Boehringer Mannheim) using the plasmid pUCpyc as a template. The sequence of the primers used (primers 5 and 6) is given below.

Colony hybridization was performed according to a method of Boehringer Mannheim. *E. coli* cells were transformed with a pHC79-based *C. glutamicum* gene library and plated onto LB-agar plates. After overnight growth, the colonies were transferred onto nylon membranes (Schleicher & Schüll) which were then gently incubated (5 min) in a solution of 0·5 M NaOH and 1·5 M NaCl to lyse the cells. Afterwards the membranes were neutralized (5 min) with 1 M Tris/HCl, 1·5 M NaCl, pH 7·5, and washed (5 min) in 2 × SSC (0·3 M NaCl, 0·03 M sodium citrate, pH 7). The cross-linking of the DNA was performed by UV irradiation (3 min) at 366 nm. To remove the cell debris, the membranes were incubated at 50 °C for 3 h in 3 × SSC plus 0·1% SDS. The filters were then used for hybridization as described above.

For sequence analysis of the *pyc* gene, several overlapping subfragments of the 17 kb *Hin*dIII fragment in pUCpyc were isolated and ligated into pUC18. Sequencing of the fragments in pUC18 was performed by primer walking with the dideoxy chain-termination method (Sanger *et al.*, 1977) using fluorescently labelled nucleotides. The subsequent electrophoretic analysis was performed with an automatic DNA sequencer from Applied Biosystems by MediGene (Munich). Sequence data were compiled and analysed by the HUSAR program package from EMBL.

Gene inactivation. Inactivation of the *pyc* gene was performed by the gene replacement method of Schäfer *et al.* (1994). Plasmid pUCpyc was used for the construction of a truncated *pyc* gene. Based on the sequence of the *pyc* gene (AC Y09548 in the EMBL Data Library), four primers (primers 3, 4, 5 and 6) were designed with extensions containing a *Sal*I (primers 3 and 6) or a *Bam*HI restriction site (primers 4 and 5). Primers 3 and 4 correspond to the sequence 5′-AG**GTCGAC**TCACACATCTTCAACG-3′ (positions 167–188 in the sequence AC Y09548) and 5′-CC**GGATC**CACAAAGATGGGGTAAG-3′ (positions 665–649). Primers 5 and 6 correspond to the sequence 5′-CC**GGATCC**-GCCAACCGAAGAGTTC-3′ (positions 3095–3110) and 5′-CC**GTCGAC**TTAGGAAACGACGACG-3′ (positions 3587–3572). Underlined nucleotides derive from the *pyc* sequence; nucleotides in bold represent the *Bam*HI or *Sal*I restriction sites. The primer pairs were used to amplify a 499 bp fragment of the 5′ end and a 493 bp fragment of the 3′-end of the *pyc* gene by PCR. After restriction with *Sal*I and *Bam*HI, the two fragments were separately ligated into the vector pGEM-T (Promega), resulting in pGEMpycA and pGEMpycB, respectively. Afterwards, a 1·56 kb *Xmn*I–*Bam*HI fragment from vector pGEMpycA was ligated with a 2·46 kb *Bam*HI–*Xmn*I fragment from vector pGEMpycB, resulting in vector

pGEMpycAB. This vector contains the two PCR products, i.e. the 5′ end of the *pyc* gene directly followed by the 3′ end in the same orientation as indicated in Fig. 1(b). The truncated *pyc* gene was then isolated as a 1 kb *Sal*I 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), the resulting vector pK19mobsacBΔpyc was used to replace the intact chromosomal *pyc* gene in *C. glutamicum* WT by the truncated *pyc* gene. Southern blot hybridization was performed to verify the replacement at the chromosomal *pyc* locus (data not shown). The *pyc* mutant was designated *C. glutamicum* WTΔpyc.

Vector pK19mobsacBΔpyc was also employed to replace the intact chromosomal *pyc* gene in *C. glutamicum* KT (Peters-Wendisch *et al.*, 1996), which carries a PEPCx gene (*ppc*) disrupted by the chloramphenicol acetyltransferase gene (*cat*) in its chromosome. The chromosomal rearrangement at the *pyc* and *ppc* loci in the resulting *pyc-ppc* double mutant was verified by Southern blot hybridization (data not shown) and the strain was designated *C. glutamicum* WTΔpycΔppc.

RNA analysis. Total RNA from *C. glutamicum* WT was isolated according to a method described by Börmann *et al.* (1992). For Northern (RNA) hybridization, a *pyc*-antisense RNA probe was prepared using plasmid pGEMpycAB. After linearization with *Sph*I, digoxigenin-dUTP-labelled RNA was synthesized using SP6 RNA polymerase and the RNA Labelling Kit (SP6/T7) from Boehringer Mannheim. For hybridization, 10 μg total RNA from *C. glutamicum* WT was denatured in loading dye (Sambrook *et al.*, 1989) for 5 min at 95 °C, cooled in ice water and subjected to agarose gel electrophoresis (in 17 %, v/v, formaldehyde). The size-fractionated RNA was transferred onto a nylon membrane as described for DNA blotting (see above). Hybridization to the *pyc* antisense RNA probe (at 46 °C, in the presence of 50 % formamide), washing and detection were performed using the Nucleic Acid Detection Kit according to the instructions from Boehringer Mannheim. The size marker was the 0·24–9·5 kb RNA ladder from Gibco-BRL.

For primer extension experiments, 20 pmol primer (primer 7, 5′-TTTCAACAAGAGACCGCC-3′, complementary to the sequence from position 149 to 132 in the sequence AC Y09548; and primer 8, 5′-CGGTTTGCTACCAAGATC-3′, complementary to the sequence from position 223 to 206) was labelled by T4 polynucleotide kinase (10 U; Boehringer Mannheim) with 20 μCi (740 kBq) [γ-^{33}P]ATP (Amersham) using the ThermoSequenase Kit from Amersham. Thirty micrograms of vacuum-dried RNA from *C. glutamicum* was dissolved in 100 μl 40 mM PIPES buffer, pH 6·4, containing 1 mM EDTA, 0·4 M NaCl and 80 % (v/v) formamide. After adding 4 pmol labelled primer, denaturing at 95 °C (10 min) and annealing at 45 °C (16 h), the mixture was ethanol-precipitated for 1 h on ice, washed once with 70 % ethanol and vacuum-dried. The pellet was dissolved in 20 μl 50 mM Tris/HCl buffer, pH 7·6, containing 60 mM KCl, 10 mM $MgCl_2$, 500 μM dATP, dCTP, dGTP and dTTP, and 40 U RNasin. After incubation for 2 min at 42 °C, 5 U avian myeloblastosis virus (AMV) reverse transcriptase (Promega) was added. The mixture was incubated for 2 h at 42 °C and the reaction was stopped by adding 1 μl 0·5 mM EDTA, pH 8·0. RNA was subsequently removed by incubation with RNase A (0·1 mg ml^{-1}) for 30 min at 37 °C. After ethanol precipitation and vacuum drying, the primer extension product was dissolved in 3 μl TE buffer plus 3 μl formamide (80 %, v/v) containing 10 mM EDTA, 0·1 % xylene cyanole and 0·1 % bromphenol blue. Two microlitres of this mixture was denatured for 5 min at 95 °C and loaded onto a 6 % (w/v) polyacrylamide gel. For exact localization of the transcriptional start site, sequencing reactions using vector pUC18 carrying the 5′ region of the *pyc* gene on a 1·6 kb *Cla*I fragment and the primers 3 and 4 were co-electrophoresed.

Enzyme assays. PCx activity was determined in permeabilized cells of *C. glutamicum* using the glutamate–oxaloacetate-transaminase-coupled discontinuous assay described previously (Peters-Wendisch *et al.*, 1997). The aspartate formed was quantified by reversed-phase HPLC.

PEPCx activity was measured photometrically using a malate dehydrogenase coupled assay as described by Peters-Wendisch *et al.* (1993).

Detection of biotinylated proteins. To detect biotinylated proteins in *C. glutamicum*, crude extracts of different strains were separated by SDS-PAGE (7·5 %, w/v acrylamide). Proteins containing biotin were detected with a streptavidin–alkaline phosphatase conjugate (Boehringer Mannheim) as described by Peters-Wendisch *et al.* (1997), except that the detection and colour reaction were performed directly in the gel.

N-terminal sequence analysis. To determine the N-terminal amino acid sequence of PCx, the protein was isolated by avidin-affinity chromatography using a monomeric avidin resin (SoftLink Soft Release Avidin Resin, Promega). For this purpose, *C. glutamicum* WT(pEK0pyc) was cultivated in 1 l minimal medium with lactate as carbon source and 20 mg biotin l^{-1}. Exponentially growing cells were harvested, washed twice in 50 mM Tris/HCl, 50 mM NaCl, pH 6·3, and re-suspended in HEPES/glycerol buffer (100 mM HEPES, pH 7·3, 20 %, w/v, glycerol). After sonication, the cell debris was removed by centrifugation at 13000 ***g*** and 4 °C. Crude extract (10 ml, 36 mg total protein) was loaded onto a column with 5 ml bed volume of avidin resin which was prepared as described by Promega and equilibrated with HEPES/glycerol buffer, at a flow rate of about 1 ml min^{-1}. After complete saturation of the resin with crude extract, the flow was stopped for 14 h. Afterwards, the column was washed with 50 ml HEPES/glycerol buffer and then the biotinylated proteins were eluted with 15 ml HEPES/glycerol buffer containing 5 mM biotin and 1 mM Pefabloc (Boehringer Mannheim). Two 4 ml fractions were collected and concentrated threefold under vacuum. After diluting the samples with 1 vol. Laemmli buffer (Laemmli, 1970), the biotinylated proteins were size-fractionated by SDS-PAGE (7·5 %, w/v, acrylamide), blotted onto a polyvinylidene difluoride membrane (Millipore) using a Fast Blot 33 semidry transfer cell from Biometra and stained with amido black (Sigma). The 123 kDa band corresponding to the PCx was subjected to N-terminal sequencing by the method of Edman & Begg (1967) using a Knauer sequencer 27413.

RESULTS

Isolation of the *pyc* gene from *C. glutamicum*

To isolate the *pyc* gene from *C. glutamicum*, a pHC79-based cosmid gene library (Börmann *et al.*, 1992) was transformed into *E. coli* DH5α. Approximately 10^4 transformants were analysed by colony hybridization using a *pyc*-specific, digoxigenin-labelled PCR fragment (see Methods) as a probe. From the colonies tested, 20 hybridized to the *pyc* probe; one of these clones was

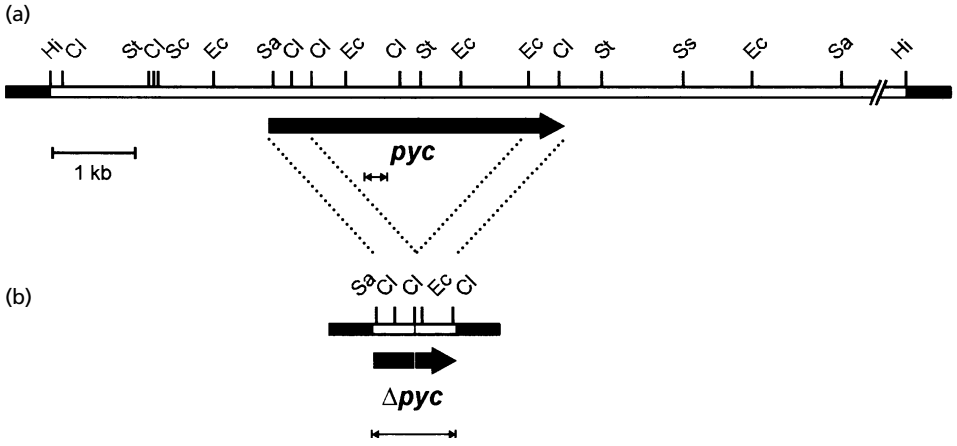

Fig. 1. Restriction map of the 17 kb *C. glutamicum* chromosomal *Hin*dIII fragment carrying the *pyc* gene (a) and of the truncated 1 kb Δ*pyc* fragment (b) flanked by vector DNA. The ORF of the *pyc* gene is indicated by a black arrow. The double-headed arrow in (a) represents the 200 bp PCR fragment used as a probe for identification of the *pyc* gene; the double-headed arrow in (b) represents the antisense RNA probe used for Northern hybridization. Cl, *Cla*I; Ec, *Eco*RI; Hi, *Hin*dIII; Sa, *Sal*I; Sc, *Sca*I; Ss, *Ssp*I; St, *Stu*I.

analysed further. It harboured cosmid pHC79-1, which contained an insert of about 43 kb. Restriction analysis of this cosmid and hybridization with the *pyc*-specific probe revealed signals of about 17 kb after restriction with *Hin*dIII, of 6·7 kb after restriction with *Sal*I and of 1·35 kb after restriction with *Eco*RI. The 17 kb *Hin*dIII fragment was cloned into vector pUC18, resulting in plasmid pUCpyc. The restriction map of this fragment is shown in Fig. 1.

DNA sequence analysis of the *pyc* gene

From pUCpyc, the 1·35 kb *Eco*RI fragment hybridizing to the *pyc*-specific probe was subcloned for sequencing. The nucleotide sequence analysis of this fragment revealed a continuous open reading frame (ORF) of 1353 bp corresponding to a sequence of 451 amino acids. Comparison of the deduced amino acid sequence with that of the PCx from *M. tuberculosis* revealed 60 % identity to an internal region. Thus, it could be concluded that the cloned 17 kb *Hin*dIII fragment in fact contains the *pyc* gene from *C. glutamicum*. The 0·85 kb *Sal*I–*Eco*RI fragment located upstream of the 1·35 kb *Eco*RI fragment (see Fig. 1) and the 1·6 kb *Eco*RI–*Eco*RI–*Stu*I fragment located downstream of it were isolated from pUCpyc and ligated into the vector pUC18. Sequence analysis of these fragments revealed one large ORF of 1139 codons beginning at the start of the 0·85 kb *Sal*I–*Eco*RI fragment. To obtain information about the 5′ region of the *pyc* gene, a 1·6 kb *Cla*I fragment partially overlapping the *Sal*I–*Eco*RI fragment (see Fig. 1) was subcloned and the terminal 381 bp containing the *Cla*I and *Sal*I restriction sites were sequenced. The nucleotide sequence assembled from all sequence reactions performed is deposited in the EMBL Data Library (AC Y09548). An ORF extending from nucleotide 108 to 3584 was observed. The first potential translational start codon in this ORF is a GTG in position 114. A second in-frame GTG codon at position 165 also represents a possible translational initiation site. All other potential translation start sites within the ORF are located downstream of regions which display a significant degree of identity to *pyc* gene products of other organisms (see below). Downstream of the *pyc* gene, at positions 3598–3639 in the sequence AC Y09548, a potential rho-independent termination structure was found. According to the rules of Tinoco *et al.* (1973), this palindromic structure should be capable of forming a stem–loop with a ΔG (25 °C) of -37 kcal mol^{-1} ($-155{\cdot}4$ kJ mol^{-1}).

N-terminal sequence analysis

As indicated above, two possible translational start sites were found (the GTG codons at positions 114 and 165 in the sequence AC Y09548). To identify the actual translational initiation site, the N-terminal amino acid sequence of the PCx protein was determined. For this purpose, the enzyme was isolated from crude extract of strain *C. glutamicum* WT(pEK0pyc), which carries the *pyc* gene on plasmid pEK0 (see below), by using avidin-affinity chromatography. This technique allows the isolation of biotin-containing proteins such as PCx (Thampy *et al.*, 1988). The N-terminal amino acid sequence of the *C. glutamicum* PCx was X-T-H-T-S-X-T-L-P-A (X representing amino acids which could not be unequivocally identified) and thus corresponds to the sequence S-T-H-T-S-S-T-L-P-A deduced from the nucleotide sequence of the *pyc* gene starting at position 165 in the sequence AC Y09548. The missing initial methionine might indicate that it is removed by processing. The start codon is preceded by a typical ribosome-binding site (GAA**AGGA**A), although the spacing of 14 bp between the translation start site and

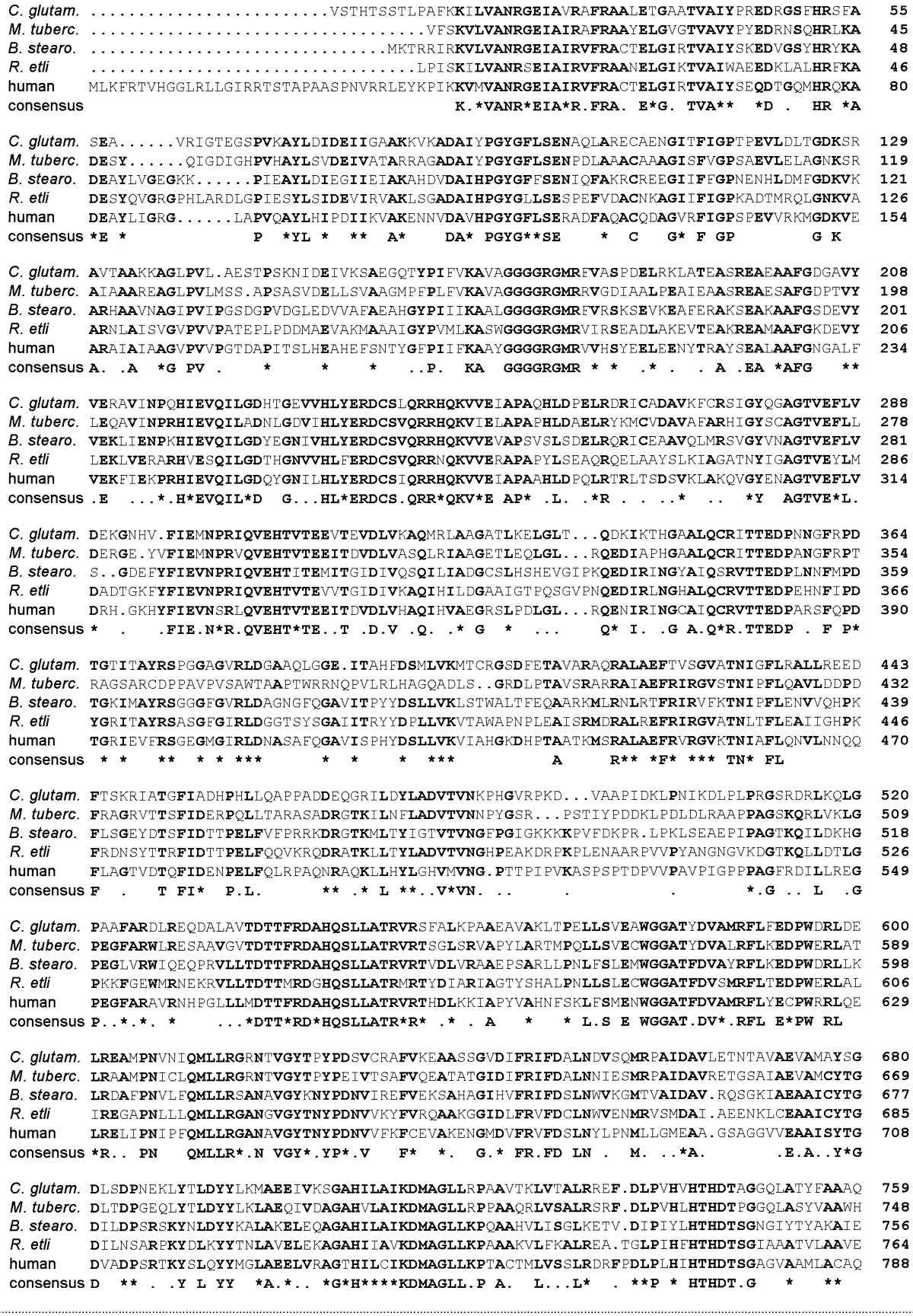

Fig. 2. For legend see facing page.

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C. glutam.  AGADAVDGASAPLSGTTSQPSLSAIVAAFAHTRRDTGLSLEAVSDLEPYWEAVRGLYLPFESGTP..GPTGRVYRHEIPG  837
M. tuberc.  AGADAVDGAAAPLAGTTSQPALSSIVAAAAHTEYDTGLSLSAVCALEPYWEALRKVYAPFESG..LPGPTGRVYHHEIPG  826
B. stearo.  AGVDIVDVAISSMAGLTSQPSANTLYYALEGTERAPEVDIYGLEQLARYWEDVRKFYQEFE..SGMNAPHTEVYMHEMPG  834
R. etli     AGVDAVDAAMDALSGNTSQPCLGSIVEALSGSERDPGLDPAWIRRISFYWEAVRNQYAAFE..SDLKGPASEVYLHEMPG  842
human       AGADVVDVAADSMSGMTSQPSMGALVACTRGTPLDTEVPMERVFDYSEYWEGARGLYAAFDCTATMKSGNSDVYENEIPG  868
consensus   AG.D.VD A   ..G TSQP..  .*.*  .*..*...    .  .  YWE..R  Y. F*      .*   VY *E.PG

C. glutam.  GQLSNLRAQATALGLADRFELIEDNYAAVNEMLGRPTKVTPSSKVVGDLALHLVGAGVDPADFAADPQKYDIPDSVIAFL  917
M. tuberc.  GQLSNLRQQAIALGLGDRFEEIEEAYAGADRVLGRLVKVTPTSKVVGDLALALVGAGVSADEFASDPARFGIPESVLGFL  906
B. stearo.  GQYSNLQQQAKAVGLGDRWDEVKEMYRRVNDLFGDIVKVTPSSKVVGDMALYMVQNNLTEQDIFERGETLNFPDSVVELF  914
R. etli     GQFTNLKEQARSLGLETRWHQVAQAYADANQMFGDIVKVTPSSKVVGDMALMMVSQDLTVADVVSPDREVSFPESVVSML  922
human       GQYTNLHFQAHSMGLGSKFKEVKKAYVEANQMLGDLIKVTPSSKIVGDLAQFMVQNGLSRAEAEAQAEELSFPRSVVEFL  948
consensus   GQ .NL  QA ..GL..*. ..  .Y. .* ..G. .KVTP*SK*VGD A* .V  ..  ..         .P SV. .*

C. glutam.  RGELGNPPGGWPEPLRTRALEGRSEGKAPLTEVPEEEQAHLDADDSKERR.......NSLNRLLFPKPTEEFLEHRRRFG  990
M. tuberc.  RGELGDPPGGWPEPLRTAALAGRGAARPTA.........QLAADDEIALSSVGAKRQATLNRLLFPSPTKEFNEHREAYG  977
B. stearo.  EGYLGQPHGGFPKELQRIILKGREPITVRPGELLEPVDFEQMKKELYDKLGREVTDFDAIAYALYPKVFLEYAETVEKYG  994
R. etli     KGDLGQPPSGWPEALQKKALKGEKPYTVRPGSLLKEADLDAERKVIEKKLEREVSDFEFASYLMYPKVFTDFALASDTYG 1002
human       QGYIGVPHGGFPEPFRSKVLKDLPRVEGRPGASLPPLDLQALEKELVDRHGEEVTPEDVLSAAMYPDVFAHFKDFTATFG 1028
consensus    G LG P.*G.P*.*.   L.*.     ...  .   .     .         ..     .  ...P..  .* .    .G

C. glutam.  NTSALDDREFFYGLVEGRETLIRLPDVRTPLLVRLDAISEPDDKGMRNVVANVNGQIRPMRVRDRSVES.VTATAEKADS 1069
M. tuberc.  DTSQLSANQFFYGLRQGEEHRVKLERGVE.LLIGLEAISEPDERGMRTVMCILNGQLRPVLVRDRSIASAVPAAE.KADR 1055
B. stearo.  DVSVLDTPTFLYGMRLGEEIEVEIERGKT.LIVKLVSIGQPQADGTRVVYFELNGQPREVVIRDESIKTAVVE.HIKADR 1072
R. etli     PVSVLPTPAYFYGLADGEELFADIEKGKT.LVIVNQAVSATDSQGMVTVFFELNGQPRRIKVPDRAHGATGAAVRRKAEP 1081
human       PLDSLNTRLFLQGPKIAEEFEVELERGKT.LHIKALAVSDLNRAGQRQVFFELNGQLRSILVKDTQAMKEMHFHP KALK 1106
consensus     * L .  *.*G   **E  . .*.*.* L .   *.* ..  G.* V ..*NGQ R   *.D..    .     KA.

C. glutam.  SNKGHVAAPFAG.VVTVTVAEGDEVKAGDAVAIIEAMKMEATITASVDGKIDRVVVPAATKVEGGDLIVVVS    1140
M. tuberc.  GNPGHIAAPFAG.VVTVGVCVGERVGAGQTIATIEAMKMEAPITAPVAGTVERVAVSDTAEWRAETCWWW    1124   (61.7 %)
B. stearo.  TNPNHIAATMPGTVVKVLVEKGEKVDKGDHLMVTEAMKMETTVQAPFAGVVKDIYVKSGDAIQAGDLLMELSK    1145   (47.4 %)
R. etli     GNAAHVGAPMPGVISRVFVSSGQAVNAGDVLVSIEAMKMETAIHAEKDGTIAEVLVKAGDQIDAKDLLAVYGG    1154   (46.3 %)
human       DVKGQIGAPMPGKVIDIKVVAGAKVAKGQPLCVLSAMKMETVVTSPMEGTVRKVHVTKDMTLEGDDLILEIE    1178   (47.0 %)
consensus   * .*..A*..G *. * V  G  V .G. .  .*AMKME. ..*.  G..  * V       . .*
```

Fig. 2. Alignment of the predicted PCx sequences from *C. glutamicum* (*C. glutam.*), *M. tuberculosis* (*M. tuberc.*; D. R. Smith, unpublished, AC U00024), *B. stearothermophilus* (*B. stearo.*, Kondo *et al.*, 1997), *R. etli* (Dunn *et al.*, 1996) and human (Wexler *et al.*, 1994). Conserved amino acids are in bold; identical amino acids in all five sequences are given as a consensus sequence below the alignment and identical amino acids in four or three sequences are marked with stars (*) and points (.), respectively.

the centre of the AGGA sequence is longer than usual (Vellanoweth & Rabinowitz, 1992). According to the determination of the N-terminus, the *pyc* gene encodes a PCx polypeptide of 1140 amino acids with an M_r of 123070.

Comparison of the deduced PCx amino acid sequence from *C. glutamicum* with those from other organisms

The amino acid sequence of the *C. glutamicum* PCx as deduced from the nucleotide sequence of the *pyc* gene was aligned and compared to those of PCx enzymes from other sources (Fig. 2). The highest identity (62%) was found to the putative PCx from *Mycobacterium tuberculosis* (Smith, 1994). Considering conservative exchanges, the similarity between the two PCx enzymes was as much as 76%. A comparison of the other two prokaryotic PCx sequences identified, i.e. from *Bacillus stearothermophilus* (Kondo *et al.*, 1997) and *Rhizobium etli* (Dunn *et al.*, 1996), revealed 47% and 46% identical and 65% and 64% similar amino acids, respectively. About the same percentage of identical and similar amino acids was found when the *C. glutamicum* PCx was compared to human PCx (Wexler *et al.*, 1994) and to all other known eukaryotic PCx enzymes (Zhang *et al.*, 1993; Jitrapakdee *et al.*, 1996; Z. Tu & H. H. Hagedorn, unpublished, AC L36530; Lim *et al.*, 1988; Stucka *et al.*, 1991).

Transcriptional analysis of the *pyc* gene

Northern (RNA) hybridization experiments were performed to analyse the size of the *pyc* transcript. For this purpose, total RNA from *C. glutamicum* WT was isolated, size-fractionated, transferred onto a nylon membrane and hybridized to a *pyc*-specific digoxigenin-dUTP-labelled antisense RNA probe. This probe was synthesized by *in vitro* transcription of the 1·0 kb Δ*pyc* insert of plasmid pGEMΔpycAB (Fig. 1b). The hybridization revealed a signal at about 3·5 kb (Fig. 3a), which

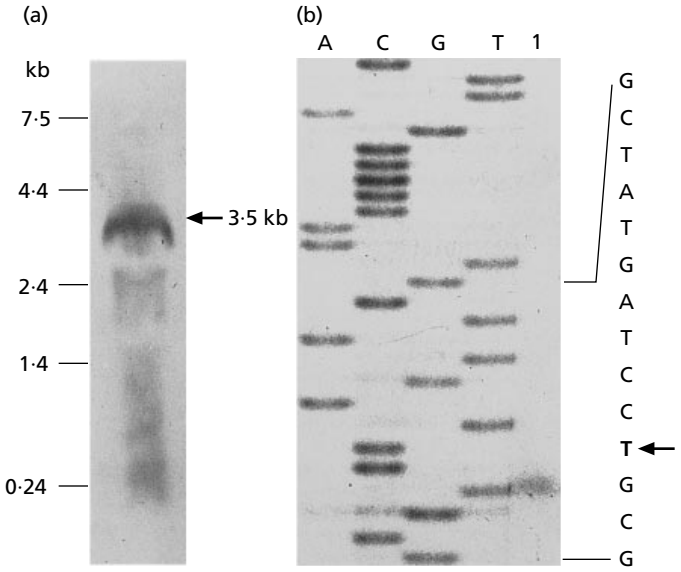

Fig. 3. Northern hybridization analysis of the *pyc* gene from *C. glutamicum* (a) and primer extension analysis of the transcriptional start site (b). RNA standards in the Northern experiment are shown on the left. The primer extension product is shown in lane 1. Lanes A, C, G and T represent the sequencing reaction using the same primer as for the primer extension. The relevant DNA sequence is shown on the right. Note that the sequence represents the coding strand and is thus complementary to that shown in Fig. 2.

is approximately the size of the *pyc* gene. Thus, the result indicates that the *C. glutamicum pyc* transcript is monocistronic.

To identify the transcriptional initiation site of the *C. glutamicum pyc* gene, primer extension experiments were performed. Total RNA of *C. glutamicum* WT was isolated from cells grown on lactate minimal medium and used for reverse transcription with the different *pyc*-specific primers 7 and 8 (see Methods). With both primers, signals were obtained corresponding to the A_{109} residue in in the sequence AC Y09548. The result of the primer extension experiment with primer 7 is shown in Fig. 3(b). Thus, the transcription of the *C. glutamicum pyc* gene starts 55 nucleotides upstream of the *pyc* translational start. The distance from this transcriptional start site to the predicted terminator structure downstream of the *pyc* gene is 3·54 kb, which is in complete agreement with the size of the transcript determined by Northern blot hybridization (see above).

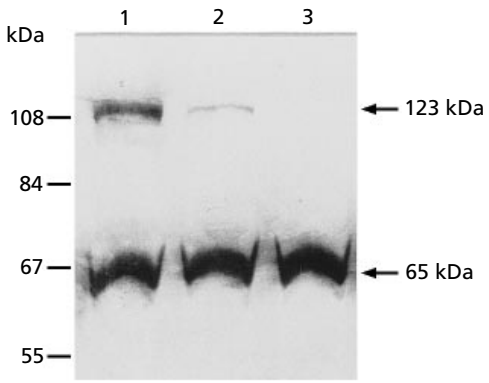

Fig. 4. Analysis of biotinylated proteins from *C. glutamicum*. Lanes 1, 2 and 3 represent cell-free extracts from *C. glutamicum* WT(pEK0pyc), WT and WTΔpyc, respectively. Sizes of biotinylated proteins and the positions of protein molecular mass markers (Sigma) are indicated.

Expression of the cloned *pyc* gene

To investigate whether the isolated *pyc* gene encodes a functional PCx, the gene was cloned as a 6·2 kb *Sca*I–*Ssp*I fragment into the *E. coli*–*C. glutamicum* shuttle vector pEK0, giving rise to the plasmid pEK0pyc. This plasmid was transformed into *C. glutamicum* R127-SP733, a PCx-negative mutant previously generated by chemical mutagenesis and isolated due to its inability to grow on lactate as the sole carbon source (Peters-Wendisch *et al.*, 1997). The resulting strain R127-SP733(pEK0pyc) was able to grow on lactate, suggesting that the *pyc* gene cloned was functional.

To test the overexpression of the *pyc* gene in *C. glutamicum*, the pEK0pyc was transformed into *C. glutamicum* WT and crude extract of the resulting strain, *C. glutamicum* WT(pEK0pyc), was tested for biotinylated proteins (Fig. 4). In extracts of *C. glutamicum* WT, two biotinylated proteins appeared: a 65 kDa protein corresponding to a subunit of an acyl-CoA carboxylase of *C. glutamicum* (Jäger *et al.*, 1996) and a 123 kDa protein representing the *pyc* gene product as shown by N-terminal sequence analysis. Whereas the 65 kDa protein was present in the same amounts in *C. glutamicum* WT and WT(pEK0pyc), a significantly higher amount of the 123 kDa protein was synthesized in strain WT(pEK0pyc) than in the original host strain WT.

The overexpression of the *pyc* gene was also tested by enzyme analysis. PCx activity of *C. glutamicum* WT(pEK0pyc) was measured in cells grown on complex medium as well as in cells grown on minimal medium with lactate, glucose or acetate as sole carbon source. As shown in Table 2, the specific activity of the PCx in

Table 2. Specific PCx and PEPCx activities in different strains of *C. glutamicum*

PCx was determined in permeabilized cells and PEPCx in crude extracts; the values are means ± SD obtained from at least two independent cultivations by two determinations per experiment. ND, Not determined; NG, no growth.

Strain	PCx [nmol min^{-1} (mg dry wt)$^{-1}$]				PEPCx [nmol min^{-1} (mg protein)$^{-1}$] (complex medium*)
	Minimal medium			Complex medium*	
	Lactate	Glucose	Acetate		
WT	37 ± 2	19 ± 4	27 ± 7	27 ± 3	69 ± 5
WT(pEK0pyc)	152 ± 11	75 ± 13	97 ± 4	127 ± 10	ND
WTΔpyc	NG	ND	ND	< 0·3	62 ± 4
WTΔpycΔppc	NG	NG	ND	< 0·3	< 0·3

* Complex medium with 0·5 % lactate.

WT(pEK0pyc) was four to five times higher than in the WT strain. PCx activity was highest in cells grown on lactate and two times lower in cells grown on acetate or glucose. This regulation was also observed in the WT strain and this result thus suggests that the cloned fragment contains not only the structural *pyc* gene but also the regulatory regions.

Inactivation of the chromosomal *pyc* gene

In order to obtain a defined PCx-negative mutant of *C. glutamicum*, the chromosomal *pyc* gene was replaced by a truncated gene. The resulting mutant *C. glutamicum* WTΔpyc was tested for PCx activity, for formation of the 123 kDa biotinylated protein and for growth on different media. The specific activity was measured in permeabilized cells after growth on complex medium containing lactate as a carbon source. As shown in Table 2 the mutant was devoid of any detectable PCx activity whereas PEPCx activity was measurable in the same amount as in the WT strain. Fig. 4 (lane 3) shows that the mutant was also devoid of the 123 kDa protein. The growth experiments revealed that *C. glutamicum* WTΔpyc grew almost as well as the parental strain *C. glutamicum* WT on minimal medium with glucose (Fig. 5a) or acetate (Fig. 5c), whereas the growth of the mutant was negligible on minimal medium containing lactate as the sole carbon source (Fig. 5b). These results indicate that the PCx in *C. glutamicum* is essential for growth on lactate but not for growth on glucose or acetate as the sole carbon source.

To test whether *C. glutamicum* possesses, apart from PCx and PEPCx, further enzymes which can fulfil the anaplerotic function during growth on glucose, a double mutant with truncated PCx and PEPCx genes was constructed and its growth behaviour analysed. This double mutant *C. glutamicum* WTΔpycΔppc showed neither PCx nor PEPCx activity (Table 2). The double mutant was able to grow in minimal medium containing acetate but it did not grow in medium containing lactate or glucose as the sole carbon sources (Fig. 5). These results indicate that PCx is an essential anaplerotic enzyme for growth on glucose in the absence of PEPCx and thus that during growth on glucose no further anaplerotic enzymes are functional in *C. glutamicum*.

DISCUSSION

Despite the importance of PCx in the central metabolism of many organisms, only a few genetic studies on this enzyme have been performed. So far, *pyc* genes have been isolated, sequenced and characterized only from the eukaryotic organisms man, mouse, rat and *S. cerevisiae*, and from the prokaryotes *R. etli* and *B. stearothermophilus* (Wexler *et al.*, 1994; Zhang *et al.*, 1993; Jitrapakdee *et al.*, 1996; Lim *et al.*, 1988; Stucka *et al.*, 1991; Dunn *et al.*, 1996; Kondo *et al.*, 1997). Additionally, the *pyc* sequences of the mosquito *Aedes aegypti* and of *M. tuberculosis* are available in the EMBL Data Library (Tu & Hagedorn, 1994; Smith, 1994). We have now isolated and characterized the *pyc* gene of *C. glutamicum*. Structural analysis of the gene and the N-terminal amino acid sequence analysis of the protein revealed a polypeptide of 1140 amino acids, corresponding to an M_r of 123070. With this size, the PCx of *C. glutamicum* is comparable to most other PCx enzymes, which were shown to be homotetramers consisting of four tetrahedrically arranged subunits with an M_r of about 120000 (Attwood, 1995). Some bacterial PCx enzymes have a different subunit structure and composition, e.g. *Pseudomonas citronellolis* possesses two different subunits, α and β, forming a native $\alpha_4\beta_4$ enzyme (Goss *et al.*, 1981; Fuchs *et al.*, 1988). The α subunit (M_r 65000) carries the biotin moiety and contains all catalytically active sites necessary for the PCx reaction, and the β subunit (M_r 54000) is responsible for the conformational stability in the core of the enzyme (Goss *et al.*, 1981).

The biotin-containing PCx enzymes belong to a family of enzymes which includes acyl-CoA carboxylases,

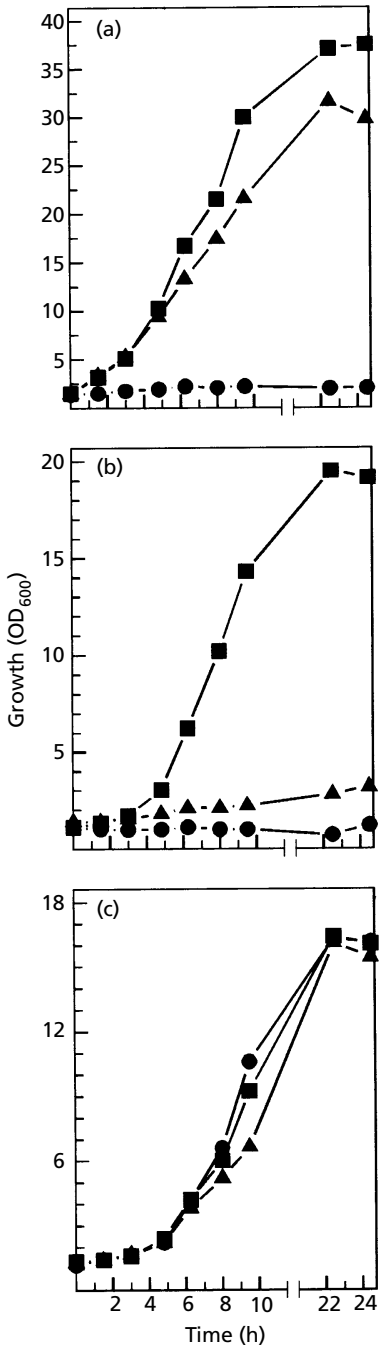

Fig. 5. Comparative growth experiments with *C. glutamicum* WT (■), the pyruvate carboxylase-negative mutant WTΔpyc (▲) and the pyruvate carboxylase- and PEP carboxylase-negative double mutant WTΔpycΔppc (●) on glucose (a), lactate (b) and acetate (c).

oxaloacetate decarboxylases and transcarboxylases (Samols *et al.*, 1988; Toh *et al.*, 1993). All the enzymes of this family show a common catalytic mechanism: the carboxylation of a covalently attached biotin moiety (reaction 1; see Introduction), and transcarboxylation from the carboxy-biotin to a specific acceptor molecule (exemplified by reaction 2). These common catalytic features correspond to conserved structural and sequence motifs (Knowles, 1989; Toh *et al.*, 1993), i.e. the biotin-carrier domain, the biotin carboxylase domain characterized by a typical ATP-binding site, and the carboxytransferase domain characterized by a specific substrate-binding site (for pyruvate in the case of PCx, oxaloacetate decarboxylase and transcarboxylase, and for acyl-CoA in the case of acyl-CoA carboxylases). Accordingly, a comparison of the PCx from *C. glutamicum* with PCx enzymes and biotin-dependent (de)carboxylases from other organisms revealed amino acid sequence motifs with a high degree of identity, especially in those regions suggested to be essential for the catalytic activity. Near the C-terminus of the *C. glutamicum* PCx there is the conserved sequence motif -A-M-K-M- (positions 1104–1107 in Fig. 2) representing the biotin-binding site (Samols *et al.*, 1988; Dunn *et al.*, 1996). Biotin is covalently attached to the central lysine residue within this sequence motif (Rylatt *et al.*, 1977). Apart from the -A-M-K-M- motif, the so-called proline hinge (position 1078 of the *C. glutamicum* PCx in Fig. 2), suggested to be necessary for the flexibility of the biotin-carrier domain (Leon-Del-Rio & Gravels, 1994), and three glycine residues (positions 1090, 1096 and 1117) involved in the biotinylation of the enzymes (Leon-Del-Rio & Gravels, 1994) can be found in the *C. glutamicum* PCx. A further highly conserved amino acid sequence (GGGGRG at position 173 in Fig. 2) was found at the N-terminus of the *C. glutamicum* PCx. This GGGG(R/K)G motif is present in all biotin-containing (de)carboxylases and is believed to correspond to the ATP-binding site (Lim *et al.*, 1988; Wexler *et al.*, 1994). In the central region of the *C. glutamicum* PCx protein, the motif -F-L-F-E-D-P-W-D-R- (positions 588–596 in Fig. 2) is found. The transcarboxylase from *Propionibacterium shermanii* contains a similar amino acid sequence (-F-L-N-E-D-P-W-E-R-) and tryptophan fluorescence analysis showed the tryptophan within this sequence to be involved in pyruvate binding in the transcarboxylation reaction (Kumar *et al.*, 1988). All these findings corroborate and extend the previous theory that the biotin-containing (de)carboxylases derive from a common ancestor protein and that these enzymes have retained the catalytically important motifs over a large evolutionary distance (Samols *et al.*, 1988; Kondo *et al.*, 1997).

The determination of the *C. glutamicum pyc* transcript length revealed a size of about 3·5 kb, indicating that the gene is transcribed as a monocistronic message and is not organized as an operon. This is the first time that the genomic organization of a *pyc* gene has been analysed in a prokaryotic organism. In addition, the transcriptional start site of the *pyc* gene was determined and found to be located at an A residue 55 bp in front of the translational start site. Upstream of the transcriptional start site, putative −10 and −35 regions could be found which are reasonably well-conserved in comparison to the predicted promoter consensus sequence from *C. glutamicum* (Pátek *et al.*, 1996). In the −10 region

(TACGAT) four of six bases are identical to the conserved sequence TA.aaT, whereas in the −35 region (TTGATT) three of six nucleotides are identical to the consensus hexamer ttGcca. The relatively low similarity to the consensus sequence might indicate that the *pyc* promoter mediates a moderate expression of the *pyc* gene and this corresponds to the relatively low amount of the PCx protein (123 kDa biotinylated protein, Fig. 4) present in cell-free extracts.

The characterization of the defined PCx-negative mutant *C. glutamicum* WTΔpyc corroborates our previous hypothesis that PCx is essential as an anaplerotic enzyme for growth on lactate but not essential for growth on glucose or acetate as carbon sources (Peters-Wendisch *et al.*, 1997). However, in comparison to the double mutant WTΔpycΔppc the single mutant WTΔpyc, which possesses the same PEPCx activity as the WT strain (Table 2), showed some, albeit slow, growth on lactate. This slow growth might be due to oxaloacetate formation from pyruvate via PEP (PEP synthase and PEPCx reactions). PEP synthase activity was previously proposed to be present in some *C. glutamicum* strains (Vallino & Stephanopoulos, 1993; Jetten *et al.*, 1994; Cocaign-Bousquet & Lindley, 1995), although, according to the results presented here, the *in vivo* activity of this enzyme is probably very low in our *C. glutamicum* strain.

In *Bacillus subtilis* and *B. stearothermophilus*, PCx is not only essential for growth on lactate but also for growth on glucose, as PCx-negative mutants were unable to grow on either substrate as the sole carbon source (Diesterhaft & Freese, 1973; Sundaram, 1973). In contrast to the two *Bacillus* species, *C. glutamicum* possesses PEPCx as an additional anaplerotic enzyme. As shown by the fact that the PCx- and PEPCx-negative double mutant *C. glutamicum* WTΔpycΔppc did not grow on glucose, the ability of *C. glutamicum* WTΔpyc to grow on this substrate is due to the presence of PEPCx. On the other hand, it has previously been shown by the analysis of a defined PEPCx-negative mutant of *C. glutamicum* that PEPCx is dispensable for growth of *C. glutamicum* on glucose and that this mutant possesses the same level of PCx activity as the WT strain (Peters-Wendisch *et al.*, 1993, 1997), and therefore our results indicate that PCx and PEPCx can basically replace each other as the anaplerotic enzyme in this organism. However, when compared to the WT strain, the PCx-negative mutant but not the PEPCx-negative mutant showed slightly slower growth on glucose (see Fig. 5a and Peters-Wendisch *et al.*, 1993), which might suggest a primary role of PCx. For *Rhizobium etli* and *R. tropici*, both also possessing PEPCx in addition to PCx (Dunn *et al.*, 1996), it has been shown that PCx-negative mutants grow on glucose much more slowly and have a much lower final cell yield than the parental strains (Dunn *et al.*, 1996). These results indicate that in *R. etli* and *R. tropici* the PEPCx can replace the PCx only to a minor extent and thus that PCx in these organisms is the predominant anaplerotic enzyme for growth on glucose. For a pyruvate-kinase-defective mutant of *C. glutamicum*, it has recently been shown by ^{13}C-NMR spectroscopy that during lysine production with pyruvate and gluconate as carbon sources (a) pyruvate-carboxylating pathway(s) contribute(s) approximately 90% of the total oxaloacetate synthesis (Park *et al.*, 1997). The extent to which the anaplerotic function during growth of *C. glutamicum* WT on glucose is accomplished by PCx and/or PEPCx is certainly dependent on the intracellular concentrations of the various effectors of the two enzymes [PCx is inhibited by acetyl-CoA, ADP and AMP (Peters-Wendisch *et al.*, 1997), and PEPCx is inhibited by aspartate and activated by acetyl-CoA and fructose-1,6-bisphosphate (Mori & Shiio, 1985a, b)]; this remains to be studied by appropriate ^{13}C-labelling experiments and carbon-flux analysis.

Apart from PEPCx and PCx, further enzymes have been discussed as possibly playing an anaplerotic role during growth of *C. glutamicum* on glucose, i.e. PEP carboxykinase (Gubler *et al.*, 1994), the glyoxylate cycle enzymes isocitrate lyase and malate synthase (de Hollander *et al.*, 1994) and the malic enzyme (Cocaign-Bousquet *et al.*, 1996). The fact that the defined PCx- and PEPCx-negative double mutant of *C. glutamicum* did not grow on glucose minimal medium excludes participation in replenishment of the TCA cycle by any of the other enzymes discussed as potential anaplerotic enzymes in *C. glutamicum* WT.

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