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Sequence of the *Corynebacterium glutamicum* pyruvate carboxylase gene

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Abstract Pyruvate carboxylase is an important anaplerotic enzyme replenishing oxaloacetate consumed for biosynthesis during growth, or lysine and glutamic acid production in industrial fermentations. We used regions of homology from pyruvate carboxylase sequences of 12 different species (corresponding to the ATP- and pyruvate-binding sites), to design polymerase chain reaction (PCR) primers for amplifying a fragment of the pyruvate carboxylase (*pc*) gene from *C. glutamicum* genomic DNA. This 850-base-pair fragment was used to probe a *C. glutamicum* cosmid library and four candidate *pc* cosmids were identified. The fragment was sequenced and the sequence of the complete gene was obtained by several rounds of primer synthesis, PCR on one of the positive cosmids, and sequencing. The *C. glutamicum pc* sequence shows 64% homology with the *pc* gene of *Mycobacterium tuberculosis* and 44% homology with the human *pc* gene. Regions of ATP, pyruvate and biotin binding have also been identified.

Introduction

Pyruvate carboxylase is a biotin-dependent enzyme that catalyzes the carboxylation of pyruvate to form oxaloacetate (Attwood 1995; Scrutton 1978). It has an anaplerotic function and helps replenish the oxaloacetate consumed for biosynthesis.

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The two-step reaction mechanism catalyzed by pyruvate carboxylase is shown below:

$$\mathrm{MgATP} + \mathrm{HCO_3^-} + \mathrm{ENZ\text{-}biotin} \xrightarrow{\mathrm{Mg^{2+}acetyl-CoA}} \mathrm{MgADP} + \mathrm{Pi} + \mathrm{ENZ\text{-}biotin\text{-}CO_2^-} \quad (1)$$

$$\mathrm{ENZ\text{-}biotin\text{-}CO_2^-} + \mathrm{Pyruvate} \longrightarrow \mathrm{ENZ\text{-}biotin} + \mathrm{oxaloacetate} \quad (2)$$

In reaction (1) the ATP-dependent biotin carboxylase domain carboxylates a biotin prosthetic group linked to a specific lysine residue in the biotin-carboxyl-carrier protein (BCCP) domain. Acetyl-coenzyme A activates reaction (1) by increasing the rate of bicarbonate-dependent ATP cleavage. In reaction (2), the BCCP domain donates the CO_2 to pyruvate in a reaction catalyzed by the transcarboxylase domain (Attwood 1995).

To date, pyruvate carboxylase genes have been cloned and sequenced from four prokaryotes: *Rhizobium etli* (Dunn et al. 1996), *Bacillus stearothermophilus* (Kondo et al. 1997), *Bacillus subtilis* (Genbank accession no.Z97025), and *Mycobacterium tuberculosis* (Genbank accession no.Z83018). Pyruvate carboxylase activity has been measured previously in *Brevibacterium lactofermentum* (Tosaka et al. 1979) and *Corynebacterium glutamicum* (Peters-Wendisch et al. 1997).

Previous research has indicated that the yield and productivity of the aspartate family of amino acids depends critically on the carbon flux through anaplerotic pathways (Vallino and Stephanopoulos 1993). On the basis of the metabolite balances, it can be shown that the rate of lysine production is less than or equal to the rate of oxaloacetate synthesis via the anaplerotic pathways. It has also been shown that lysine production is unaffected in a phospho*enol*pyruvate carboxylase (*ppc*) deletion mutant. However, lysine production is reduced in a pyruvate kinase (*pyk*) deletion mutant, consistent with the viewpoint that pyruvate carboxylation is a significant anaplerotic activity leading to oxaloacetate production. In a *ppc pyk* double mutant, lysine production

is further decreased to 25% of the wild-type levels. In this case, the only route for pyruvate formation is via the phospho*enol*pyruvate: glucose transferase system, which produces one molecule of pyruvate for every molecule of glucose imported. In the double mutant there is less pyruvate available for the synthesis of oxaloacetate via pyruvate carboxylation and this limits the production of lysine (Park et al. 1997a). The presence of pyruvate carboxylation activity was also strongly suggested by ^{13}C labeling studies in *C. glutamicum*. In these experiments, carried out with *C. glutamicum ppc*, *pyk* and *ppc pyk* mutants, [1-^{13}C]pyruvate or [2-^{13}C]pyruvate was added to the fermentor and the fractional ^{13}C enrichment at individual carbon positions of lysine was measured. The pattern that was observed cannot be explained by sole operation of phospho*enol*pyruvate carboxylation and supports the model that *C. glutamicum* has a pyruvate-carboxylating activity (Park et al. 1997b). In order to understand fully the role of carbon flux through the anaplerotic pathway, it is necessary to clone and sequence the *C. glutamicum* pyruvate carboxylase gene as it has potential importance in increasing lysine and glutamic acid production.

Materials and methods

Bacterial strains and plasmids

C. glutamicum 21253 (*hom*$^-$, lysine overproducer) was used for the preparation of chromosomal DNA. *Escherichia coli* DH5α (*hsdR*$^-$, *recA*$^-$) (Hanahan 1983) was used for transformations. Plasmid pCR2.1 TOPO (Invitrogen) was used for cloning polymerase chain reaction (PCR) products. The plasmid pRR850 was constructed in this study and contained an 850-bp PCR fragment cloned in the pCR2.1 TOPO plasmid.

Media and culture conditions

E. coli strains were grown in Luria-Bertani (LB) medium at 37 °C (Sambrook et al. 1989). *C. glutamicum* was grown in LB medium at 30 °C. Where noted, ampicillin was used at the following concentrations: 100 μg/ml in plates and 50 μg/ml in liquid culture.

DNA manipulations

Genomic DNA was isolated from *C. glutamicum* as described by Tomioka et al. (1981). PCR fragments were cloned into the pCR2.1 TOPO vector following the manufacturer's instructions. Cosmid and plasmid DNA were prepared using Qiaprep spin columns and DNA was extracted from agarose gels with the Qiaex kit (Qiagen). For large-scale high-purity preparation of cosmid DNA for sequencing, the Promega Wizard kit was used (Promega). Standard techniques were used for transformation of *E. coli* and agarose gel electrophoresis (Sambrook et al. 1989). Restriction enzymes were purchased from Boehringer Mannheim or New England Biolabs.

Cosmid library

The cosmid library used was kindly provided by Dr. Phil Lessard (Department of Biology, MIT) and was constructed by cloning *C. glutamicum* chromosomal DNA into the Supercos vector (Stratagene).

Polymerase Chain Reaction (PCR)

PCR was performed using the Boehringer Mannheim PCR core kit following the manufacturer's instructions. When PCR was performed on *Corynebacterium* chromosomal DNA, about 1 μg DNA was used in each reaction. The forward primer used was 5′GTCTTCATCGAGATGAATCCGCG3′ and the reverse primer used was 5′CGCAGCGCCACATCGTAAGTCGC3′ for the PCR reaction.

Dot-blot analysis

Dot blots containing DNA from cosmids identified in this study and the probe as a positive control were prepared using the S&S (Schleicher & Schüll) minifold apparatus. An 850-bp fragment encoding a portion of the *C. glutamicum* pyruvate carboxylase gene was used as the probe. The probe was labeled with digoxigenin-11-dUTP (Boehringer Mannheim) in a randomly primed DNA-labeling reaction as described by the manufacturer. Hybridization, washing and colorimetric detection of the dot blots were done with the Genius system from Boehringer following the protocols in their user's guide for filter hybridization. The initial hybridization with the 291 cosmids was carried out at 65 °C overnight and washes were performed at the hybridization temperature. For the 17 cosmids that were used in the second screen, the hybidization was carried out at 65 °C, but for only 8 h, and the time of exposure to the film was decreased.

Detection of biotin-containing proteins by Western blotting

Cell extracts from *C. glutamicum* were prepared as described by Jetten and Sinskey (1993). Proteins in cell extracts were separated in sodium dodecyl sulfate (SDS)/7.5% polyacrylamide gels in a BioRad mini gel apparatus and were electroblotted onto nitrocellusose, using the BioRad mini transblot apparatus described by Towbin et al. (1979). Biotinylated proteins were detected using avidin-conjugated alkaline phosphatase from BioRad and 5-bromo-4-chloro-3-indoylphosphate-p-toluidine salt)/nitroblue tetrazolium chloride from Schleicher & Schüll.

DNA sequencing

Automated DNA sequencing was performed by the MIT Biopolymers facility employing an ABI Prism 377 DNA sequencer.

Sequence analysis

The program DNA Strider Version 1.0 (Institut de Recherche Fondamentale, France) was used to invert, complement and translate the DNA sequence, and find open-reading frames in the sequence. The BLAST program (Altschul et al. 1990) from the National Center for Biotechnology Information (NCBI) was employed to compare protein and DNA sequences. Homology searches in proteins were done using the MACAW software (NCBI). PCR primers were designed with the aid of the Primer Premier software from Biosoft International. The compute pI/MW tool on the ExPasy molecular biology server (university of Geneva) was used to predict the molecular mass and pI of the deduced amino acid sequence.

Results

Western blotting to detect biotinylated enzymes

Since pyruvate carboxylase is known to contain biotin, we employed Western blotting to detect the production

of biotinylated proteins by *C. glutamicum*. We detected two biotinylated proteins in extracts prepared from cells grown in LB medium, (data not shown) consistent with previous reports. One band, located at approximately 80 kDa, has been identified as the biotin-carboxyl-carrier domain (BCCP) of the acetyl-CoA carboxylase (Jager et al. 1996). The second band, at 120 kDa, is believed to be the pyruvate carboxylase subunit, as these subunits are in the range 113–130 kDa (Attwood 1995).

PCR and cloning

We chose to clone the *C. glutamicum* pyruvate carboxylase gene on the basis of the homology of highly conserved regions in previously cloned genes. We examined pyruvate carboxylase genes from thirteen organisms and designed primers corresponding to an ATP-binding submotif conserved in pyruvate carboxylases and the region close to the pyruvate-binding motif (Table 1). Where the amino acids were different the primers were designed on the basis of *M. tuberculosis* because of its close relationship to *C. glutamicum*. An 850-bp fragment was amplified from *C. glutamicum* genomic DNA using the PCR and cloned in the pCR2.1 TOPO vector of Invitrogen to construct plasmid pRR850. We also designed primers based on the conserved biotin-binding site and pyruvate-binding site (data not shown) and tried to amplify this region from *C. glutamicum* chromosomal DNA but these attempts were unsuccessful.

Table 1 Pyruvate carboxylase sequences from 13 organisms (obtained from GenBank) were aligned using the MACAW software. Two highly conserved regions were selected and oligonucleotide primers were designed on the basis of the *Mycobacterium tuberculosis* DNA sequence corresponding to these regions. The forward primer was based on the DNA sequence corresponding to conserved region A and the reverse primer was based on the DNA sequence corresponding to conserved region B

Organism	Conserved region A	Conserved region B
Caenorhabditis elegans	YFIEVNAR	ATFDVSM
Aedes aegypti	YFIEVNAR	ATFDVAL
Mycobacterium tuberculosis	VFIEMNPR	ATYDVAL
Bacillus stearothermophilus	YFIEVNPR	ATFDVAY
Pichia pastoris	YFIEINPR	ATFDVSM
Mus musculus	YFIEVNSR	ATFDVAM
Rattus norvegicus	YFIEVNSR	ATFDVAM
Saccharomyces cerevisiae 1	YFIEINPR	ATFDVAM
Saccharomyces cerevisiae 2	YFIEINPR	ATFDVAM
Rhizobium etli	YFIEVNPR	ATFDVSM
Homo sapiens	YFIEVNSR	ATFDVAM
Schizosaccharomyces pombe	YFIEINPR	ATFDVSM

Isolating a cosmid containing the C. glutamicum pyruvate carboxylase gene

The 850-base-pair fragment containing a portion of the *C. glutamicum* pyruvate carboxylase gene was used to probe a *C. glutamicum* genomic library. In the first round of screening, 17 out of 291 cosmids in a dot blot appeared positive. A second round of screening was performed on these 17 cosmids, using the same probe but more stringent hybridization conditions, yielding four cosmids with a positive signal (Fig. 1). To confirm that these cosmids indeed contained the pyruvate carboxylase gene, we performed PCR using the four positive cosmids as templates and the same primers used to make the probe. An 850-bp fragment was amplified from all four positive cosmids, which we designated IIIF10, IIE9, IIIG7 and IIIB7.

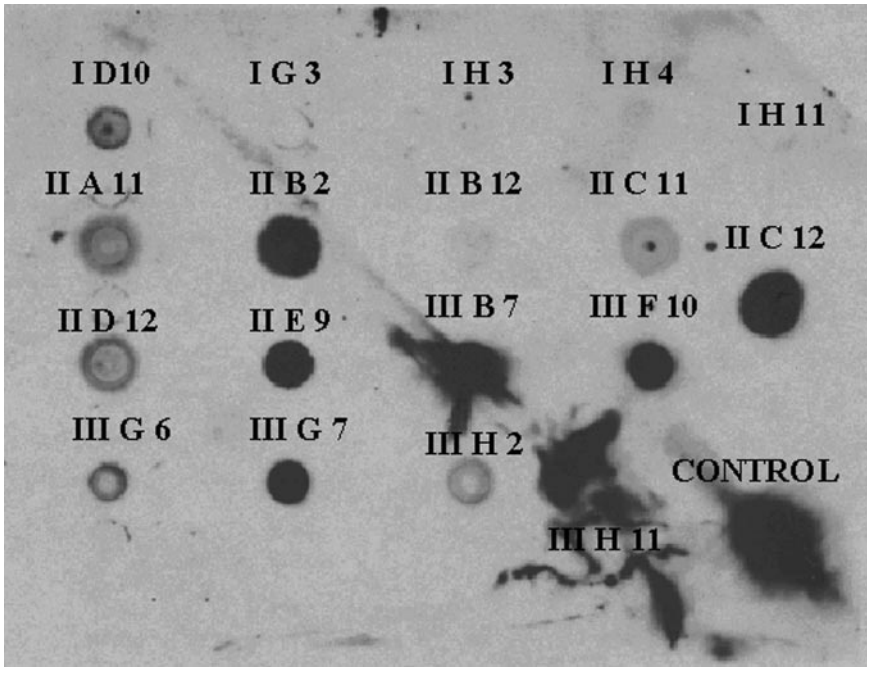

Fig. 1 Dot-blot hybridization results for the 17 cosmids that appeared positive in the first screen. The cosmids IIE9, IIIF10, IIIG7 and IIIB7 were chosen for further analysis. The probe was included as a positive control

Sequencing strategy

The 850-bp insert of plasmid pRR850 was sequenced using the M13 forward and M13 reverse primers. On the basis of this sequence, primers Begrev1 and Endfor1 were designed and used to sequence outwards from the beginning and the end of the 850-bp portion of the pyruvate carboxylase gene. Cosmid III F10 was used as the sequencing template. The sequencing was continued by designing new primers (Table 2) and "walking" across the gene as shown in Fig. 2.

Table 2 DNA sequences of the primers used to obtain the sequence of the pyruvate carboxylase gene in the cosmid IIIF10

Primer name	Primer sequence (5′–3′)
Begrev1	TTCACCAGGTCCACCTCG
Endfor1	CGTCGCAAAGCTGACTCC
Begrev2	GATGCTTCTGTTGCTAATTTGC
Endfor2	GGCCATTAAGGATATGGCTG
Begrev3	GCGGTGGAATGATCCCCGA
Endfor3	ACCGCACTGGGCCTTGCG
Endfor4	TCGCCGCTTCGGCAACAC

Sequence analysis

We sequenced 3637 bp of cosmid III F10 and identified a 3420-bp open reading frame, which is predicted to encode a protein of 1140 amino acids. The deduced protein is 63% identical to *M. tuberculosis* pyruvate carboxylase and 44% identical to human pyruvate carboxylase (Fig. 3), and we named the *C. glutamicum* gene *pc* on the basis of this homology. The deduced protein has a predicted pI of 5.4 and molecular mass of 123.6 kDa, which is similar to the subunit molecular mass of 120 kDa estimated by SDS/polyacrylamide gel electrophoresis. Upstream of the starting methionine there appears to be a consensus ribosome binding-site AAGGAA. The predicted translational start site, based on homology to the *M. tuberculosis* sequence, is a GTG codon, as has been observed in other bacterial sequences (Stryer 1988; Keilhauer et al. 1993). The DNA sequence has been submitted to GenBank and has been assigned the accession number AF038548.

The amino-terminal segment of the *C. glutamicum* pyruvate carboxylase contains the hexapeptide GGGGRG, which matches the GGGG(R/K)G sequence that is found in all biotin-binding proteins and is believed to be an ATP-binding site (Fry et al. 1986; Post et al. 1990). A second region that is proposed to be involved in ATP binding and is present in biotin-dependent carboxylases and carbamylphosphate synthetase (Lim et al. 1988) is conserved in the *C. glutamicum* sequence (Fig. 3). The predicted *C. glutamicum* pyruvate carboxylase protein also contains a putative pyruvate-binding motif, FLFEDPWDR, which is conserved in the transcarboxylase domains of *Mycobacterium*, *Rhizobium* and human pyruvate carboxylases (Dunn et al. 1996). Tryptophan fluorescence studies with transcarboxylase have shown that the Trp residue present in this motif is involved in pyruvate binding (Kumer et al. 1988). The carboxy-terminal segment of the enzyme contains a putative biotin-binding site, AMKM, which is identical to those found in other pyruvate carboxylases as well as the biotin-carboxyl-carrier protein (BCCP) domains of other biotin-dependent enzymes (Fig. 3).

Discussion

Previous studies have shown that phospho*enol*pyruvate carboxylase (*ppc*) is not the main anaplerotic enzyme for *C. glutamicum*, since its absence does not affect lysine production (Gubler et al. 1994; Peters-Wendisch et al. 1993). Moreover, a number of studies have indicated the presence of a pyruvate-carboxylating enzyme, employing ^{13}C-labeling experiments and NMR and GC-MS analysis (Park et al. 1997b; Peters-Wendisch et al. 1996), or enzymatic assays with cell free extracts (Tosaka et al. 1979) and permeable cells (Peters-Wendisch et al. 1997). We have detected a very low pyruvate carboxylation activity in cell-free extracts, but we have not been able to uncouple this activity from a very high ATP background. It is highly probable that the activity we have measured is due to reversible gluconeogenic enzymes, such as oxaloacetate decarboxylase and malic enzyme. In this report we have identified and sequenced a gene encoding pyruvate carboxylase, thus providing unequivocal evidence for the existence of this enzyme in *C. glutamicum*. The presence of pyruvate carboxylase makes it highly unlikely that the gluconeogenic enzymes

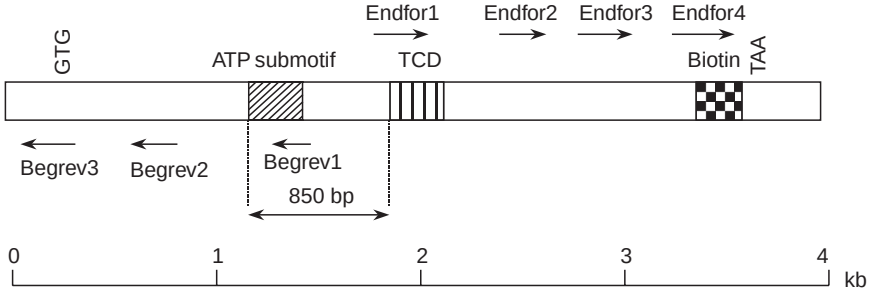

Fig. 2 The strategy used for sequencing cosmid IIIF10. The 850-bp fragment was first sequenced and primers Endfor1, Endfor2, Endfor3 and Endfor4 were used to obtain the sequence on one side of the 850-bp fragment and primers Begrev1, Begrev2 and Begrev3 were used to obtain the sequence on the other side of the 850-bp fragment

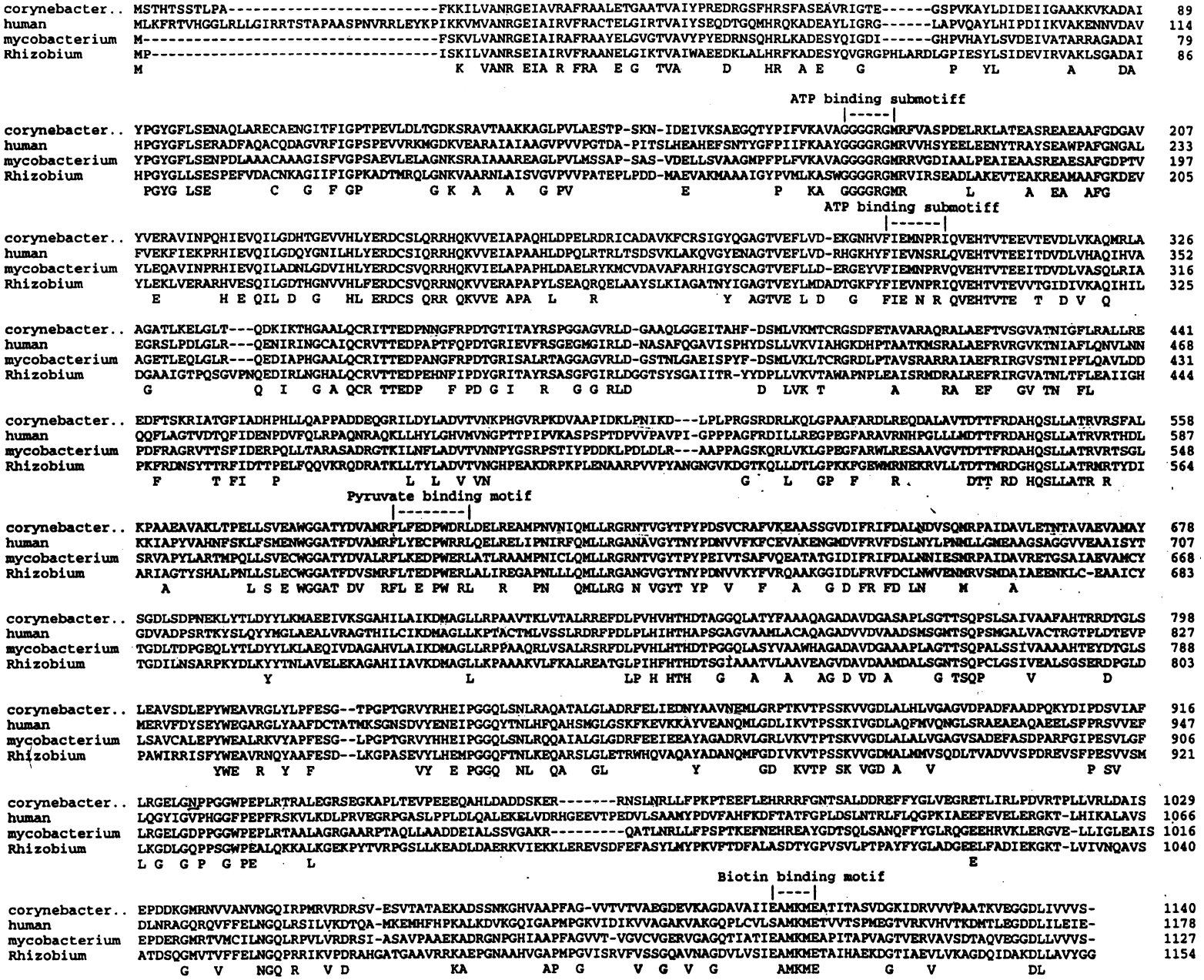

Fig. 3 Alignment of the protein sequences of *Corynebacterium glutamicum*, *Mycobacterium tuberculosis*, *Rhizobium etli* and human pyruvate carboxylase. The alignment was performed using the MACAW software package and the conserved residues are indicated below the four sequences. The two conserved ATP-binding submotifs, the pyruvate-binding motif and the biotin-binding motif are also shown indicated

mentioned above can serve the anaplerotic needs of this strain.

The deduced amino acid sequence of the *C. glutamicum* pyruvate carboxylase gene has significant similarity to the pyruvate carboxylase sequences from a diverse group of organisms. It contains a biotin carboxylase domain in its N-terminal region, a BCCP domain in its C-terminal region, and a transcarboxylase domain with a binding site specific for pyruvate in its central region. The *C. glutamicum* pyruvate carboxylase protein showed strong homology to *M. tuberculosis* and the human pyruvate carboxylase (Wexler et al. 1994). While this work was in progress the pyruvate carboxylase gene was cloned and sequenced from *C. glutamicum* strain ATCC 13032 (Peters-Wendisch et al. 1998).

There are precedents to our finding that *C. glutamicum* contains more than one enzyme to perform the anaplerotic function of regenerating oxaloacetate. *Pseudomonas citronellolis*, *Pseudomonas fluorescens*, *Azotobacter vinelandii* and *Thiobacillus novellus* contain both *ppc* and pyruvate carboxylase (O'Brien et al. 1977; Scrutton and Taylor 1974; Milrad de Forchetti and Cazzulo 1976; Charles and Willer 1984). *Zea mays* contains three isozymes of *ppc* (Toh et al. 1994) and *Saccharomyces cerevisiae* contains two isozymes of pyruvate carboxylase (Brewster et al. 1994), each differentially regulated. With the present discovery of the existence of a pyruvate carboxylase gene in *C. glutamicum*, the number of enzymes that can interconvert phospho*enol*pyruvate (PEP), oxaloacetate and pyruvate in this strain rises to six (Fig. 4). This presence of all six enzymes in one organism has not been reported previously. *P. citronellolis* contains a set of five enzymes that interconvert oxaloacetate, PEP and pyruvate, namely pyruvate kinase, PEP synthetase, PEP carboxylase, oxaloacetate decarboxylase and pyruvate carboxylase (O'Brien et al. 1977). *Azotobacter* contains all the above

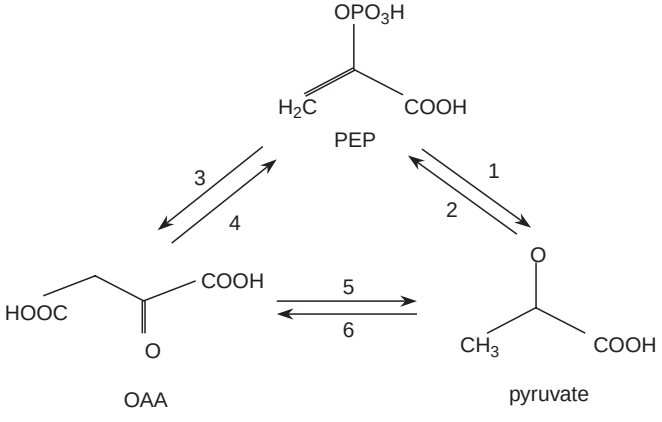

Fig. 4 The metabolic pathways for the interconversion of oxaloacetate (*OAA*), phospho*enol*pyruvate (*PEP*) and pyruvate in *C. glutamicum*. Enzymes: *1* pyruvate kinase, *2* phospho*enol*pyruvate carboxykinase, *3* phospho*enol*pyruvate carboxylase, *4* phospho*enol*pyruvate synthetase, *5* oxaloacetate decarboxylase, *6* pyruvate carboxylase

enzymes except PEP synthetase (Scruton and Taylor 1974).

The presence in *C. glutamicum* of the six metabolically related enzymes suggests that the regulation of these enzymes through effectors is important. Biochemical and genetic study of all six enzymes in coordination with other downstream activities may lead to the elucidation of the exact procedures necessary for maximizing the production of primary metabolites by this industrially important organism.

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