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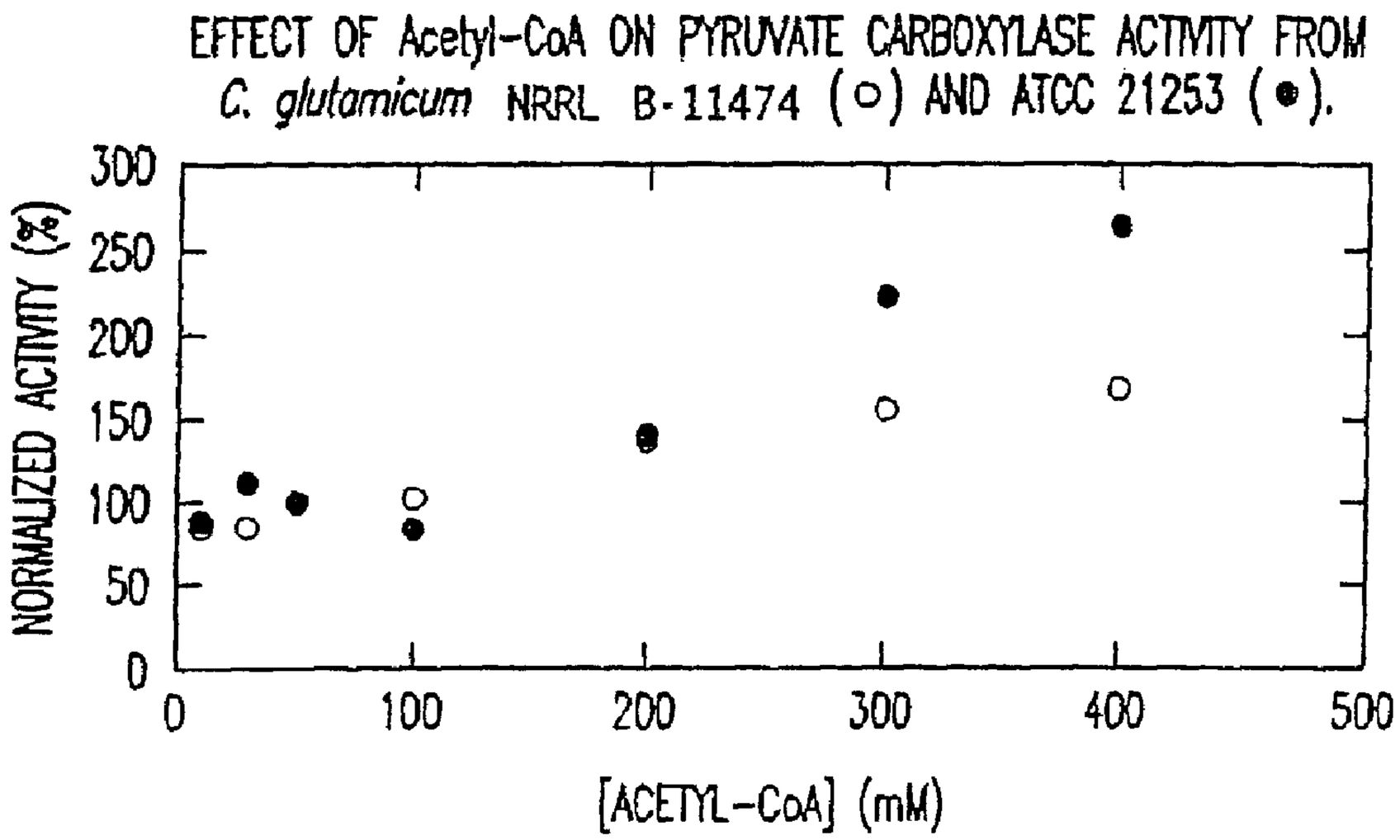
(54) **Titre : GENE DE PYRUVATE CARBOXYLASE RESISTANT A UNE RETROACTION PROVENANT DE CORYNEBACTERIUM**
(54) **Title: FEEDBACK-RESISTANT PYRUVATE CARBOXYLASE GENE FROM CORYNEBACTERIUM**



(57) **Abrégé/Abstract:**
The present invention relates to a mutated pyruvate carboxylase gene from Corynebacterium. The mutant pyruvate carboxylase gene encodes a pyruvate carboxylase enzyme which is resistant to feedback inhibition from aspartic acid. The present invention also relates to a method of replacing the wild-type pyruvate carboxylase gene in Corynebacterium with this feedback-resistant pyruvate carboxylase gene. The present invention further relates to methods of the production of amino acids, preferably lysine, comprising the use of this mutant pyruvate carboxylase enzyme in microorganisms.

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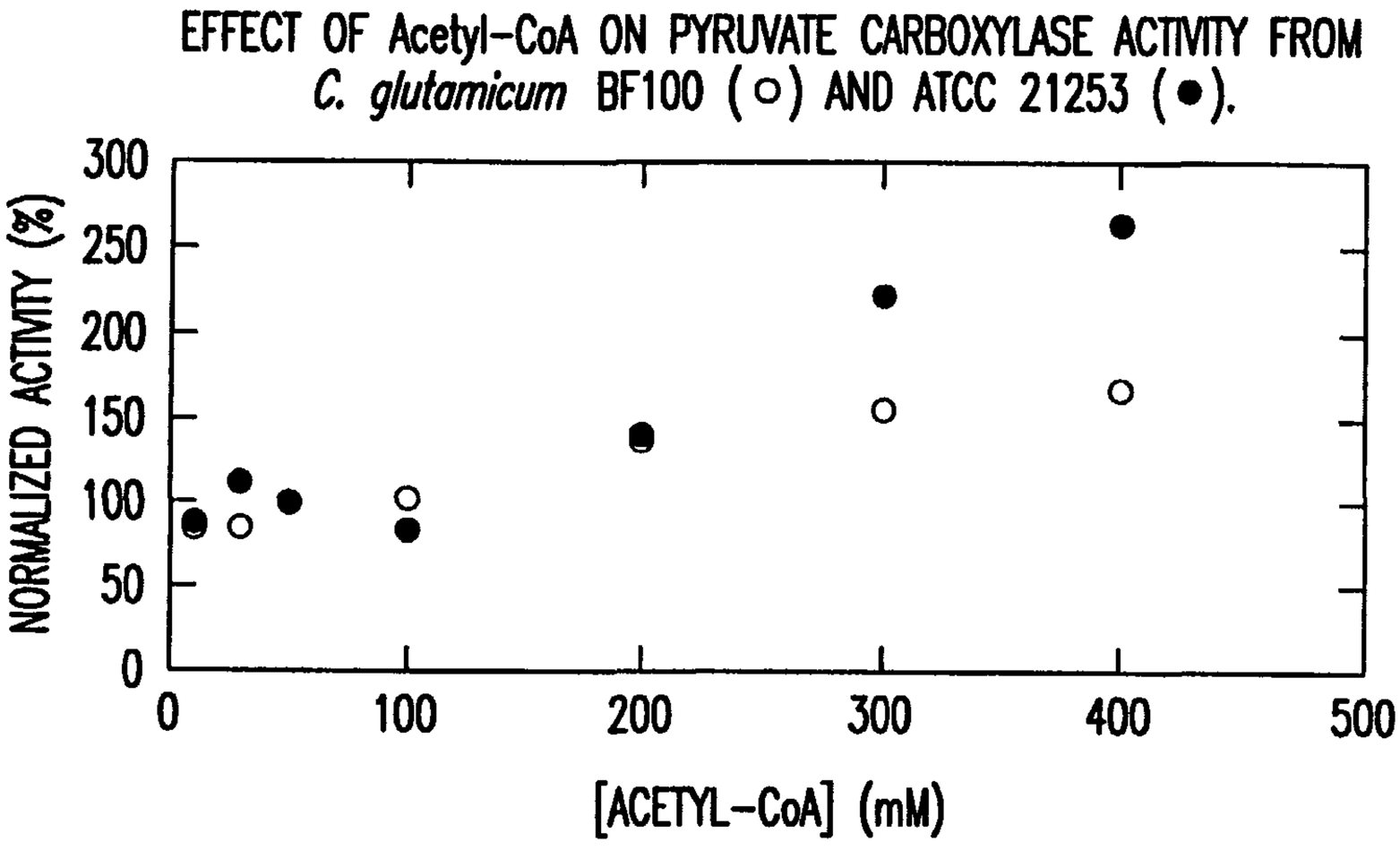
(54) Title: FEEDBACK-RESISTANT PYRUVATE CARBOXYLASE GENE FROM CORYNEBACTERIUM



(57) Abstract: The present invention relates to a mutated pyruvate carboxylase gene from *Corynebacterium*. The mutant pyruvate carboxylase gene encodes a pyruvate carboxylase enzyme which is resistant to feedback inhibition from aspartic acid. The present invention also relates to a method of replacing the wild-type pyruvate carboxylase gene in *Corynebacterium* with this feedback-resistant pyruvate carboxylase gene. The present invention further relates to methods of the production of amino acids, preferably lysine, comprising the use of this mutant pyruvate carboxylase enzyme in microorganisms.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Feedback-Resistant Pyruvate Carboxylase Gene from Corynebacterium

Background of the Invention

Field of the Invention

The present invention relates to a mutated pyruvate carboxylase gene from *Corynebacterium*. The mutant pyruvate carboxylase gene encodes a pyruvate carboxylase enzyme which is resistant to feedback inhibition from aspartic acid. The present invention also relates to a method of replacing the wild-type pyruvate carboxylase gene in *Corynebacterium* with this feedback-resistant pyruvate carboxylase gene. The present invention further relates to methods of the production of amino acids, preferably lysine, comprising the use of this mutant pyruvate carboxylase enzyme in microorganisms.

Background of the Invention

Pyruvate carboxylase is an important biotin-containing enzyme found in a variety of plants and animals, as well as some groups of bacteria (Modak, H.V. and Kelly, D.J., Microbiology 141:2619-2628 (1995)). In the presence of adenosine triphosphate (ATP) and magnesium ions, pyruvate carboxylase catalyzes the two-step carboxylation of pyruvate to form oxaloacetate, as shown in the equations below:

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

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

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, P.V., *Int. J. Biochem. Cell. Biol. 27*:231-249 (1995)).

In bacteria such as *Corynebacterium glutamicum*, pyruvate carboxylase is utilized during carbohydrate metabolism to form oxaloacetate, which is in turn used in the biosynthesis of amino acids, particularly L-lysine and L-glutamate. Furthermore, in response to a cell's metabolic needs and internal environment, the activity of pyruvate carboxylase is subject to both positive and negative feedback mechanisms, where the enzyme is activated by acetyl-CoA, and inhibited by aspartic acid. Based on its role in the pathway of amino acid synthesis, and its ability to be regulated, pyruvate carboxylase plays a vital role in the synthesis of amino acids.

Bacteria such as *C. glutamicum* and *E. coli* are widely used in industry for the production of amino acids such as L-glutamate and L-lysine. Because of the central importance of pyruvate carboxylase in the production of amino acids, particularly L-glutamate and L-lysine, the exploitation of pyruvate carboxylase to increase amino acid production is of great interest in an industrial setting. Thus, promoting the positive feedback mechanism of pyruvate carboxylase, or inhibiting its negative feedback mechanism, in *C. glutamicum* or could augment amino acid production on an industrial scale.

Summary of the Invention

One aspect of the present invention relates to a nucleic acid molecule comprising a nucleotide sequence which codes for a pyruvate carboxylase of SEQ ID NO:19, wherein this pyruvate carboxylase contains at least one mutation which desensitizes the pyruvate carboxylase to feedback inhibition by aspartic acid.

Another aspect of the present invention provides methods for using the nucleic acid of SEQ ID NO: 1, which encodes the amino acid sequence of a mutant pyruvate carboxylase. Such uses include the replacement of the wild-type pyruvate carboxylase with the feedback-resistant pyruvate carboxylase, and the production of amino acids. An additional aspect of the present invention provides a polypeptide comprising the amino acid sequence of SEQ ID NO:2. Still another aspect of the present invention provides a polypeptide comprising the amino acid sequence selected from the group comprising SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16 and SEQ ID NO: 18.

Another aspect of the present invention also relates to a nucleic acid molecule comprising a nucleotide sequence which encodes the amino acid sequence of SEQ ID NO: 2 or the amino acid sequence encoded by the DNA contained in Deposit Number NRRL B-11474. Another aspect of the present invention further relates to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 1.

BRIEF DESCRIPTION OF THE FIGURES

FIGURES 1A-1G show the full-length nucleotide sequence (SEQ ID NO:1) encoding the amino acid sequence of feedback-resistant pyruvate carboxylase, and the corresponding amino acid sequence (SEQ ID NO:2).

FIGURE 2 shows the amino acid sequence of the wild-type pyruvate carboxylase (SEQ ID NO: 19), isolated from *Corynebacterium glutamicum* ATCC 21253. The specific changes corresponding to the amino acid sequence of the feedback-resistant pyruvate carboxylase (SEQ ID NO: 2) ISOLATED FROM *Corynebacterium glutamicum* NRRL B-11474, are indicated.

FIGURE 3 shows the effects of various substrate concentrations on the pyruvate carboxylase activity in *C. glutamicum* ATCC 21253 and NRRL B-11474.

FIGURE 4 shows the effects of aspartate concentration on the activity of pyruvate carboxylase in *glutamicum* ATCC 21253 and NRRL B-11474.

FIGURE 5 shows the effects of acetyl-CoA concentration on the activity of pyruvate carboxylase in *C. glutamicum* ATCC 21253 and NRRL B-11474.

Detailed Description of the Preferred Embodiments

The present invention relates to variations of the polypeptide comprising the amino acid sequence which codes for the pyruvate carboxylase as shown in SEQ ID NO:19. Preferably, the variations of pyruvate carboxylase enzyme in the present invention contain at least one mutation which desensitizes the pyruvate carboxylase to feedback inhibition by aspartic acid. Such mutations may include deletions, insertions, inversions, repeats, and type substitutions. More preferably, the amino acid sequence mutation which desensitizes the wild-type pyruvate carboxylase enzyme (SEQ ID NO:19) to feedback inhibition comprises at least one substitution selected from the group consisting of (a) methionine at position 1 being replaced with a valine, (b) glutamic acid at position 153 being replaced with an aspartic acid, (c) alanine at position 182 being replaced with a serine, (d) alanine at position 206 being replaced with a serine, (e) histidine at position 227 being replaced with an arginine, (f) alanine at position 452 being replaced with a glycine, and (g) aspartic acid at position 1120 being replaced with a glutamic acid. Still more preferably, the variation of the polypeptide encoded by the amino acid sequence of SEQ ID NO:19 contains more than one of the above-mentioned mutations. Most preferably, the variation of the polypeptide encoded by the amino acid sequence of SEQ ID NO:19 contains all of the above-mentioned mutations.

As one of ordinary skill in the art would appreciate, the numbering of amino acid residues of a protein as used herein, begins at the amino

terminus (N-terminus) and proceeds towards the carboxy terminus (C-terminus), such that the first amino acid at the N-terminus is position 1.

An embodiment of the present invention relates to an isolated or purified nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of: (a) a nucleotide sequence which encodes the amino acid sequence of SEQ ID NO: 2; (b) a nucleotide sequence encoding the amino acid sequence encoded by the DNA contained in Deposit Number NRRL B-11474 or; (c) a nucleotide sequence complementary to any of the nucleotide sequences in (a) or (b).

Further embodiments of the invention include isolated nucleic acid molecules that comprise a polynucleotide having a nucleotide sequence at least 90% identical, and more preferably at least 95%, 97%, 98%, 99% or 100% identical, to any of the nucleotide sequences in (a), (b), (c) or (d) above, or a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide having a nucleotide sequence identical to a nucleotide sequence in (a), (b), (c) or (d) above. However, the polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide having a nucleotide sequence consisting of only A residues or of only T residues.

Another aspect of the invention is directed to nucleic acid molecules at least 90%, 95%, 97%, 98% or 99% identical to the nucleic acid sequence shown in FIG. 1 (SEQ ID NO: 1), or to the nucleic acid sequence of the deposited DNA (NRRL B-30293, deposited May 12, 2000).

A further aspect of the invention provides a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of: SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15 and SEQ ID NO:17.

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that

the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the pyruvate carboxylase polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in Figure 1 or to the nucleotide sequence of the deposited DNA can be determined conventionally using known computer programs such as the FastA program. FastA performs a Pearson and Lipman search for similarity between a query sequence and a group of sequences of the same type nucleic acid. Professor William Pearson of the University of Virginia Department of Biochemistry wrote the FASTA program family (FastA, TFastA, FastX, TFastX and SSearch). In collaboration with Dr. Pearson, the programs were modified and documented for distribution with GCG Version 6.1 by Mary Schultz and Irv Edelman, and for Versions 8 through 10 by Sue Olson.

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the ABI Prism 377). Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule.

Unless otherwise indicated, each "nucleotide sequence" set forth herein is presented as a sequence of deoxyribonucleotides (abbreviated A, G , C and T). However, by "nucleotide sequence" of a nucleic acid molecule or polynucleotide is intended, for a DNA molecule or polynucleotide, a sequence of

deoxyribonucleotides, and for an RNA molecule or polynucleotide, the corresponding sequence of ribonucleotides (A, G, C and U) where each thymidine deoxynucleotide (T) in the specified deoxynucleotide sequence in is replaced by the ribonucleotide uridine (U). For instance, reference to an RNA molecule having the sequence of SEQ ID NO:1 set forth using deoxyribonucleotide abbreviations is intended to indicate an RNA molecule having a sequence in which each deoxynucleotide A, G or C of SEQ ID NO:1 has been replaced by the corresponding ribonucleotide A, G or C, and each deoxynucleotide T has been replaced by a ribonucleotide U.

As indicated, nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, DNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described herein. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5x

Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C. By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers.

Of course, polynucleotides hybridizing to a larger portion of the reference polynucleotide (e.g., the deposited plasmid), for instance, a portion 25-750 nt in length, or even to the entire length of the reference polynucleotide, are also useful as probes according to the present invention, as are polynucleotides corresponding to most, if not all, of the nucleotide sequences of any of the nucleotide sequences included in the present intention. By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from any of the nucleotide sequences of the reference polynucleotides, (e.g., the deposited DNA or the nucleotide sequence as shown in any of the figures). As indicated, such portions are useful diagnostically either as a probe, according to conventional DNA hybridization techniques, or as primers for amplification of a target sequence by the polymerase chain reaction (PCR), as described, for instance, in *Molecular Cloning, A Laboratory Manual*, 2nd. edition, edited by Sambrook, J., Fritsch, E. F. and Maniatis, T., (1989), Cold Spring Harbor Laboratory Press, the entire disclosure of which is hereby incorporated herein by reference.

The nucleic acid molecules of the present invention are suitable for use in vectors. As such, polynucleotides of interest can be joined to the nucleic acid molecules of the present invention, which may optionally contain selectable markers. A preferred embodiment of the present invention is that the vector comprises a functional *Corynebacterium* replication origin. A replication origin is a nucleotide sequence, typically several hundred base pairs long, that is vital to the initiation of DNA replication.

The vectors can optionally contain an exogenous terminator of transcription; an exogenous promoter; and a discrete series of restriction endonuclease recognition sites, said series being between said promoter and said terminator. The vector can optionally contain their native expression vectors and/or expression vectors which include chromosomal-, and episomal-derived vectors, e.g., vectors derived from bacterial exogenous plasmids, bacteriophage, and vectors derived from combinations thereof, such as cosmids and phagemids.

A DNA insert of interest should be operatively linked to an appropriate promoter, such as its native promoter or a host-derived promoter, the phage lambda P_L promoter, the phage lambda P_R promoter, the *E. coli lac* promoters, such as the *lac*I and *lacZ* promoters, *trp* and *tac* promoters, the T3 and T7 promoters and the *gpt* promoter to name a few. Other suitable promoters will be known to the skilled artisan.

The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs can include a translation initiating codon at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

As indicated, the expression vectors will preferably include at least one selectable marker. Preferably the selection marker comprises a nucleotide sequence which confers antibiotic resistance in a host cell population. Such markers include amikacin, augmentin (amoxicillin plus clavulonic acid), ampicillin, cefazolin, cefoxitin, ceftazidime, ceftiofur, cephalothin, enrofloxacin, florfenicol, gentamicin, imipenem, kanamycin, penicillin, sarafloxicin, spectinomycin, streptomycin, tetracycline, ticarcillin, tilmicosin, or chloramphenicol resistance genes. Other suitable markers will be readily apparent to the skilled artisan.

The invention also provides for a method of producing a host cell where the expression vectors of the current invention have been introduced into the host

cell. Methods of introducing genetic material into host cells, such as those described in typical molecular biology laboratory manuals, for example J. Sambrook, E.F. Fritsch and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989), are well known to the skilled artisan. These methods include, but are not limited to, calcium phosphate transfection, DEAE-dextran mediated transfection, microinjection, lipid-mediated transfection, electroporation or infection. Accordingly, a preferred embodiment of the present invention provides a host cell comprising the vector of the present invention.

As used in the present invention, a host cell refers to any prokaryotic or eukaryotic cell where the desired nucleic acid sequence has been introduced into the cell. There are a variety of suitable host cells, including but not limited to bacterial, fungal, insect, mammalian and plant cells, that can be utilized in the present invention. Representative bacterial host cells include, but are not limited to, *Streptococci, Staphylococci, E. coli, Streptomyces, Bacillus* and *Corynebacterium*. Representative fungal cells include but are not limited to, yeast cells and *Aspergillus*. Insect cells include, but are not limited to, *Drosophila* S2 and *Spodoptera* Sf9 cells. Examples of mammalian cells include, but are not limited to, CHO, COS and Hela cells.

The present invention provides methods for utilizing the nucleic acid of SEQ ID NO:1, which encodes the amino acid sequence of a mutant pyruvate carboxylase. Such methods include the replacement of the wild-type pyruvate carboxylase with the feedback-resistant pyruvate carboxylase, and the production of amino acids. The method for replacement of a wild-type pyruvate carboxylase gene, with a feedback resistant pyruvate carboxylase gene, in a *Corynebacterium glutamicum* host cell comprises the steps of: (a) replacing a genomic copy of the wild-type pyruvate carboxylase gene with a selectable marker gene through homologous recombination to form a first recombinant strain; and (b) replacing the selectable marker gene of step (a) in the first recombinant strain, with the feedback resistant pyruvate carboxylase gene through

homologous recombination to form a second recombinant strain. The homologous recombination in steps (a) and (b) would occur between the genetic material of the host cell and any of the vectors of the present invention.

Homologous recombination is a technique that is used to disrupt endogenous nucleotide sequences in a host cell. Normally, when an exogenous nucleotide sequence is inserted into a host cell, this polynucleotide may randomly insert into any area of the host cell's genome, including endogenous plasmids. However, with homologous recombination, the exogenous nucleotide sequence contains sequences that are homologous to an endogenous nucleotide sequence within the host cell. Once introduced into the cell, for example by electroporation, the exogenous nucleotide sequence will preferentially recombine with and replace the endogenous nucleotide sequence with which it is homologous.

As used herein, an exogenous nucleotide sequence, is a nucleotide sequence which is not found in the host cell. Thus, the term exogenous nucleotide sequence is meant to encompass a nucleotide sequence that is foreign to the host cell, as well as a nucleotide sequence endogenous, or native, to the host cell that has been modified. Modification of the endogenous nucleotide sequence may include, for instance, mutation of the native nucleotide sequence or any of its regulatory elements. As used herein, mutation is defined as any change in the wild-type sequence of the host's genetic material, including plasmid DNA. An additional form of modification may also include fusion of the endogenous nucleotide sequence to a nucleotide sequence that is normally not present, in relation to the endogenous nucleotide sequence.

Host cells that have undergone homologous recombination are selected on the basis of antibiotic resistance through the use of, for example, the selectable markers mentioned above. The process of selecting cells that have undergone homologous recombination will be readily apparent to one skilled in the art.

Another aspect of the current invention is a method for producing amino acids. In the current context, production of amino acids is accomplished by

culturing host cells where a vector of the present invention has been introduced into the host cell, or culturing host cells where homologous recombination, involving a vector of the present invention, has taken place. Culturing of the host cells is performed in the appropriate culture media. Subsequent to culturing the host cells in culture media, the desired amino acids are separated from the culture media. Preferably, the amino acids produced by the methods described herein include L-lysine, L-threonine, L-methionine, L-isoleucine, L-glutamate, L-arginine and L-proline. More preferably, the present invention relates to the production of L-lysine.

The present invention provides an isolated or purified polypeptide encoded by the DNA plasmid encoding pyruvate carboxylase contained in Deposit Number NRRL B-30293, or the amino acid sequence of SEQ ID NO: 2. Still another aspect of the present invention provides a polypeptide comprising the amino acid sequence selected from the group consisting of SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16 and SEQ ID NO: 18.

Accordingly, SEQ ID NO: 6 corresponds to the amino acid sequence: PSKNIDDIVKSAE. SEQ ID NO:8 corresponds to the amino acid sequence: RGMRFVSSPDELR. SEQ ID NO: 10 corresponds to the amino acid sequence: AAFGDGSVYVERA. SEQ ID NO: 12 corresponds to the amino acid sequence: VQILGDRTGEVVH. SEQ ID NO: 14 corresponds to the amino acid sequence: IATGFIGDHPHLL. SEQ ID NO: 16 corresponds to the amino acid sequence: TITASYDGKIERV. SEQ ID NO: 18 corresponds to the amino acid sequence: MTAITLGGLLLKGIITLV.

All of the polypeptides of the present invention are preferably provided in an isolated form. As used herein, "isolated polypeptide" is intended to mean a polypeptide removed from its native environment. Thus, a polypeptide produced and/or contained within a recombinant host cell is considered isolated for purposes of the present invention. Also intended as an "isolated polypeptide" are polypeptides that have been purified, partially or substantially, from a

recombinant host. For example, a recombinantly produced version of the pyruvate carboxylase enzyme can be substantially purified by the one-step method described in Smith and Johnson, *Gene 67:*31-40 (1988).

One aspect of the present invention include the polypeptides which are at least 80% identical, more preferably at least 90%, 95% or 100% identical to the polypeptide encoded by the DNA plasmid encoding pyruvate carboxylase contained in Deposit Number NRRL B-30293, the polypeptide of SEQ ID NO:2.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to the amino acid sequence of SEQ ID NO:2, for example, it is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the amino acid sequence of SEQ ID NO:2, for example. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is, for instance, 95% identical to the amino acid sequence shown in SEQ ID NO:2,
or to the amino acid sequence encoded by deposited DNA clone can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). When using Bestfit or any other sequence alignment program to

determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

Another aspect of the present invention provides a nucleic acid molecule encoding the polypeptide comprising the amino acid sequence selected from the group consisting of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16 and SEQ ID NO:18. Preferably, the invention provides for nucleic acid molecules, which code for the aforementioned polypeptides, that are selected from the group consisting of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15 and SEQ ID NO:17.

Accordingly, SEQ ID NO:5 corresponds to the nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:6. SEQ ID NO:7 corresponds to the nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:8. SEQ ID NO:9 corresponds to the nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:10. SEQ ID NO:11 corresponds to the nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:12. SEQ ID NO:13 corresponds to the nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:14. SEQ ID NO:15 corresponds to the nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:16. SEQ ID NO:17 corresponds to the nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:18.

Methods used and described herein are well known in the art and are more particularly described, for example, in J.H. Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1972); J.H. Miller, *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1992); M. Singer and P. Berg, *Genes & Genomes*, University Science Books, Mill Valley, California (1991); J.

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Examples

The following examples are illustrative only and are not intended to limit the scope of the invention as defined by the appended claims.

Strains and Media

Bacterial strains used were *Corynebacterium glutamicum* ATCC 21253 and NRRL B-11474. These strains have an auxotrophy for homoserine (ATCC 21253) and for threonine, methionine and alanine (NRRL B-11474).

Defined medium for *Corynebacterium glutamicum* ATCC 21253 contained the following ingredients (per liter): glucose, 20 g; NaCl, 2 g; citrate (trisodium salt, dihydrate), 3 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $Na_2EDTA \cdot 2H_2O$, 75 mg; $FeSO_4 \cdot 7H_2O$, 50 mg; 100x salt solution, 20 ml; K_2HPO_4, 4 g; KH_2PO_4, 2 g; $(NH_4)_2SO_4$, 7.5 g; urea, 3.75 g; leucine, 0.1 g; threonine, 0.15 g; methionine, 0.05 g; thiamine, 0.45 mg; biotin, 0.45 mg; pantothenic acid, 4.5 mg (pH 7.0). The salt solution contained the following ingredients (per liter): $MnSO_4$, 200 mg; $Na_2B_4O_7 \cdot 10H_2O$, 20 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 10 mg; $FeCl_3 \cdot 6H_2O$, 200 mg; $ZnSO_4 \cdot 7H_2O$, 50 mg; $CuCl_2 \cdot 2H_2O$, 20 mg (pH 2.0).

Defined medium for *Corynebacterium glutamicum* NRRL B-11474 contained the following ingredients (per liter): glucose, 20 g; NaCl, 1 g, $MgSO_4 \cdot 7H_2O$, 0.4 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $MnSO_4 \cdot H_2O$, 0.01 g; KH_2PO_4, 1 g; $(NH_4)_2SO_4$, 10 g; urea, 2.5 g; alanine, 0.5 g; threonine, 0.25 g; methionine, 0.5 g; thiamine, 0.45 mg; biotin, 0.45 mg; niacinamide, 50 mg (pH 7.2).

Pyruvate Carboxylase and Phosphoenol Pyruvate Carboxylase Assay

Pyruvate carboxylate and phosphoenol pyruvate carboxylate assays were performed with permeabilized cells prepared by the following method. Log phase cells were harvested by centrifugation for 10 min at 5000 xg at 4°C and washed with 20 ml of the ice-cold washing buffer (50 mM Tris/HCl [pH 6.3] containing

50 mM NaCl). The cell pellet was resuspended in an ice-cold Hepes buffer (100 mM Hepes [pH 7.5] containing 20% Glycerol) to reach a final concentration of 25 g dry cell weight/liter. Resuspended cells were permeabilized by adding 30 µl of a 10% Hexadecyltrimethyl-ammonium bromide (CTAB) (w/v) solution to 1 ml of cells to give a final concentration of 0.3% (CTAB)(v/v).

For determination of pyruvate carboxylate activity, the assay mixture contained 10 mM pyruvic acid, 14 mM $KHCO_3$, 4 mM $MgCl_2$, 1.75 mM ATP, 50 µmole acetyl-CoA, 0.3 mg bovine serum albumin, 0.055 U citrate synthase and 50 mM sodium phosphate buffer ([pH 7.5] containing 0.1 mg 5, 5′-Dithio-bis(2-nitrobenzoic acid) (DTNB)) in a final volume of 1 ml. The reaction was started at 30°C with the addition of 10 µl of the permeabilized cell suspension, and the formation of DTNB-thiophenolate was followed over time at 412 nm. Relevant standards and controls were carried out in the same manner.

For determination of phosphoenol pyruvate carboxylase activity, the assay mixture contained 10 mM phosphoenol pyruvate, 14 mM $KHCO_3$, 4 mM $MgCl_2$, 50 µmole acetyl-CoA, 0.3 mg bovine serum albumin, 0.055 U citrate synthase and 50 mM sodium phosphate buffer ([pH 7.5] containing 0.1 mg 5, 5′-Dithio-bis(2-nitrobenzoic acid) (DTNB)) in a final volume of 1 ml. The reaction was carried out in the same conditions described for the pyruvate carboxylase assay.

The reproducibility for enzyme assays was typically 10%.

DNA Isolation and Purification

DNA was isolated from cultures of NRRL B-11474 cells. Defined media for NRRL B-11474 (CM media) contain the following ingredients, per liter: sucrose, 50 g; KH_2PO_4, 0.5 g; K_2HPO_4, 1.5 g; urea, 3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; polypeptone, 20 g; beef extract, 5 g; biotin, 12.5 ml (60 mg/L); thiamine, 25 ml (120 mg/L), niacinamide, 25 ml (5g/L); L-methionine, 0.5 g; L-threonine, 0.25 g; L-alanine, 0.5 g. NRRL B-11474 cells were harvested from CM media and suspended in 10 ml of TE, pH 8 (10 mM Tris*Cl, 1 mM EDTA). Forty

micrograms of RNase A and 10 milligrams of lysozyme were added per milliliter of suspension and the suspension was incubated at 37°C for 30 minutes. The suspension was made in 1.0% in sodiumdodecyl sulfate (SDS) and 0.1 mg/l proteinase K was added, and the cells were lysed by incubation at 37°C for 10 minutes. Nucleic acids were purified by three extractions with TE-saturated phenol (pH7), followed by ethanol precipitation. Nucleic acid precipitates were twice washed with 80% ethanol and redissolved in TE pH 8.

The concentrations of DNA were quantified spectrophotometrically at 260 nm. Purity of DNA preparations were determined spectrophotometrically (A260/A280 and A26JA230 ratios) and by agarose gel electrophoresis (0.8% agarose in 1x TAE).

Sequencing of the genomic DNA was performed, as is known by one of ordinary skill in the art, by creating libraries of plasmids and cosmids using pGEM3 and Lorist 6 respectively. Briefly, a Sau3AI digestion was performed on the genomic DNA and inserted into the BamHI site of pGEM3. The forward primer was used to generate a sequence, and primer walking generated the remainder of the sequence.

Activity of Pyruvate Carboxylase

Development of a Continuous Assay for Determining Pyruvate Carboxylase Activity

A discontinuous assay for determining pyruvate carboxylase from permeabilized cells has been previously described (Peters-Wendisch, P.G. *et al. Microbiology,* 143:1095-1103 (1997)). Because of the central location of OAA in the metabolism, it seemed to be that OAA would accumulate during the first reaction of the discontinuous assay. Most likely, OAA would be lost to other products, because of the competing enzymes that are still active. This depletion of OAA would inevitably lead to the underestimation of pyruvate carboxylase activity. To verify this assumption of decreasing OAA concentrations, a known

amount of OAA was added to the first reaction in presence of permeabilized and non-permeabilized cells. A significant loss of OAA was detected, demonstrating that permeabilized cells are capable of further transformation of OAA.

To account for the intrinsic loss of OAA during the experiment, a continuous assay was carried out by coupling the two-reaction assay to a one-reaction assay in presence of an excess of citrate synthase. The amount of permeabilized cells added in the assay was optimized to obtain a detectable activity, with the lowest possible background absorbency due to the presence of cells.

To confirm that the continuous assay specifically detected pyruvate carboxylase activity, controls were carried out by assaying for activity in absence of each reaction component (Table 1). Using these controls, the detected activity was determined to be a carboxylation reaction requiring pyruvate, Mg and ATP.

Table 1: Controls for the continuous pyruvate carboxylate assay.

Control	Detected Activity (Abs/min.mg DCW)
Complete mixture	0.30
Cells omitted	0
Pyruvate omitted	0.01
$KHCO_3$ omitted	0.03
$MgCl_2$ omitted	0.02
ATP omitted	0.03
Citrate synthase omitted	0.10
Complete + biotin	0.35
Complete + avidin	Not determined yet

To optimize the assay, the influence of the ratio of CTAB:cells was tested. Maximal activity was measured between 8 and 24 mg CTAB/mg dry cell weight (DCW). Pyruvate carboxylase activity was measured in cells incubated with CTAB with varying incubation times. The activity of pyruvate carboxylase remained constant within 0 and 5 minutes. Similarly, different concentrations of DTNB, within the range 0.1-0.3 g/l, gave identical pyruvate carboxylase activity.

To confirm the ability of the assay for determining pyruvate carboxylase activity in *Corynebacterium glutamicum*, different quantities of cells were used. Linearity between enzyme activity and quantity of cells was observed within the range 0-0.3 mg DCW.

Enzymology Study of Pyruvate Carboxylase from Corynebacterium glutamicum: Behavior of Pyruvate Carboxylase Towards its Substrates

Pyruvate carboxylase activity was determined as a function of various concentrations of its substrates: pyruvate, bicarbonate and ATP (Figure 3). Based on the data generated, the affinity constants of pyruvate carboxylase for its substrates were determined (Table 2). The pyruvate carboxylase from NRRL B-1474 (also known as BF 100) and ATCC21253 strains demonstrated a similar affinity for pyruvate and ATP. Pyruvate carboxylase activity in both strains were inhibited by ATP above a concentration of 2 mM. However pyruvate carboxylase in ATCC21253 had a higher affinity for bicarbonate than pyruvate carboxylase from NRRL B-11474 (BF 100).

Strain	$K_{M(pyruvate)}$[mM]	$K_{M(HCO3-)}$[mM]	$K_{M(ATP)}$[mM]
C. glutamicum			
Pyc	1.3 ± 0.3	14.4 ± 4	0.4 ± 0.1
Pyc ATCC 21253	0.3 ± 0.1	2.9 ± 0.8	0.3 ± 0.1

Table 2: *Comparison of affinity constants for substrates on pyruvate carboxylase from C. glutamicum, BF100 and ATCC 21253.*

Aspartate Inhibition of Pyruvate Carboxylase

Aspartate inhibits phosphoenol pyruvate carboxylase (PEPC) activity. To determine the effect of aspartate on the activity of pyruvate carboxylase, aspartate was added at different concentrations in the spectrophotometer cuvette and

enzyme activities were measured. As a comparison, the same experiment was carried out with PEPC in ATCC 21253 (Figure 4).

The PEPC of *Corynebacterium glutamicum* (ATCC 21253) was found to be strongly inhibited by aspartate. The enzyme was completely inhibited with a concentration of 5 mM aspartate. However, pyruvate carboxylase from the same strain was less sensitive to aspartate, i.e. it retained 35% of its original activity in the presence of 25 mM aspartate.

The pyruvate carboxylase activity in NRRL B-11474 showed a higher basal pyruvate carboxylase activity than ATCC 21253, i.e. the pyruvate carboxylase activity was about 5-times higher in NRRL B-11474 than in the ATCC 21253. Moreover, a dramatic difference in their aspartate inhibition patterns was found. Pyruvate carboxylase from NRRL B-11474 strain was activated by low aspartate concentrations within the range 0-30 mM and inhibited within the range 30-100 mM aspartate. Nevertheless it retained 50% of its original activity, even in the presence of 100 mM aspartate. Activity was maintained at 30% in the presence of 500 mM aspartate. On the other hand, Pyruvate carboxylase from ATCC 21253 was found to be more sensitive to aspartate than pyruvate carboxylase from NRRL B-11474. The pyruvate carboxylase from ATCC21253 lost 70% of its original activity at a concentration of 30 mM aspartate.

The feedback resistant pyruvate carboxylase gene of the present invention was isolated and cloned from NRRL B-11474. The isolated/closed pyruvate carboxylase gene has been deposited in an *E. coli* host cell under deposit NRRL B-30293. Deposit Number NRRL B-30293 was deposited on May 12, 2000 at the Agricultural Research Culture Collection (NRRL) International Depository Authority; 1815 North University Street, Peoria, Illinois, 61064 U.S.A. All strains were deposited under the terms of the Budapest Treaty.

Activation of Pyruvate Carboxylase by Acetyl-CoA

Pyruvate carboxylase activity was measured in the presence of different concentrations of acetyl-CoA (Figure 5). Pyruvate carboxylase activity in both strains increased with increasing acetyl-CoA concentrations. The effect of acetyl-CoA on citrate synthase itself was studied also. Acetyl-CoA had a Km of 10 μM, demonstrating that under our conditions, citrate synthase is saturated with acetyl-CoA. Therefore, the increasing activity of pyruvate carboxylase with increasing acetyl-CoA concentration is the result of acetyl-CoA activating pyruvate carboxylase.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

Applicant's or agent's file reference number 1533.123PC01	International application No. TBA

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 12 line 12.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depository institution
AGRICULTURAL RESEARCH SERVICE CULTURE COLLECTION (NRRL)

Address of depository institution *(including postal code and country)*

1815 North University Street
Peoria, Illinois 61604
United States of America

Date of deposit	Accession Number
30 May 2000 (30.05.00)	NRRL B-30293

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Escherichia coli DH5αMCR pBSII-PYCBF100

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
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cta gtg tcg act cac aca tct tca acg ctt cca gca ttc aaa aag atc        96
Leu Val Ser Thr His Thr Ser Ser Thr Leu Pro Ala Phe Lys Lys Ile
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        35                  40                  45

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Leu Glu Thr Gly Ala Ala Thr Val Ala Ile Tyr Pro Arg Glu Asp Arg
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gga tca ttc cac cgc tct ttt gct tct gaa gct gtc cgc att ggt act       240
Gly Ser Phe His Arg Ser Phe Ala Ser Glu Ala Val Arg Ile Gly Thr
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gaa ggc tca cca gtc aag gcg tac ctg gac atc gat gaa att atc ggt       288
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Leu Ser Glu Asn Ala Gln Leu Ala Arg Glu Cys Ala Glu Asn Gly Ile
        115                 120                 125

act ttt att ggc cca acc cca gag gtt ctt gat ctc acc ggt gat aag      432
Thr Phe Ile Gly Pro Thr Pro Glu Val Leu Asp Leu Thr Gly Asp Lys
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145                 150                 155                 160

gaa tcc acc ccg agc aaa aac atc gat gac atc gtt aaa agc gct gaa      528
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Gly Gln Thr Tyr Pro Ile Phe Val Lys Ala Val Ala Gly Gly Gly Gly
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cgc ggt atg cgc ttt gtt tct tca cct gat gag ctc cgc aaa ttg gca      624
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gtg aaa atg acc tgc cgt ggt tcc gac ttt gaa act gct gtt gct cgt      1296
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            420                 425                 430

gca cag cgc gcg ttg gct gag ttc acc gtg tct ggt gtt gca acc aac      1344
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        435                 440                 445

att ggt ttc ttg cgt gcg ttg ctg cgg gaa gag gac ttc act tcc aag      1392
Ile Gly Phe Leu Arg Ala Leu Leu Arg Glu Glu Asp Phe Thr Ser Lys
    450                 455                 460

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Arg Ile Ala Thr Gly Phe Ile Gly Asp His Pro His Leu Leu Gln Ala
465                 470                 475                 480

cca cct gcg gat gat gag cag gga cgc atc ctg gat tac ttg gca gat      1488
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cct gcg gca gag gcc gtc gca aag ctg act cct gag ctt ttg tcc gtg     1776
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        595                 600                 605

gag gat ccg tgg gac agg ctc gac gag ctg cgc gag gcg atg ccg aat     1872
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gta aac att cag atg ctg ctt cgc ggc cgc aac acc gtg gga tac acc     1920
Val Asn Ile Gln Met Leu Leu Arg Gly Arg Asn Thr Val Gly Tyr Thr
625                 630                 635                 640

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Pro Tyr Pro Asp Ser Val Cys Arg Ala Phe Val Lys Glu Ala Ala Ser
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tcc ggc gtg gac atc ttc cgc atc ttc gac gcg ctt aac gac gtc tcc     2016
Ser Gly Val Asp Ile Phe Arg Ile Phe Asp Ala Leu Asn Asp Val Ser
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cag atg cgt cca gca atc gac gca gtc ctg gag acc aac acc gcg gta     2064
Gln Met Arg Pro Ala Ile Asp Ala Val Leu Glu Thr Asn Thr Ala Val
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gcc gag gtg gct atg gct tat tct ggt gat ctc tct gat cca aat gaa     2112
Ala Glu Val Ala Met Ala Tyr Ser Gly Asp Leu Ser Asp Pro Asn Glu
    690                 695                 700

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Lys Leu Tyr Thr Leu Asp Tyr Tyr Leu Lys Met Ala Glu Glu Ile Val
705                 710                 715                 720

aag tct ggc gct cac att ctg gcc att aag gat atg gct ggt ctg ctt     2208
Lys Ser Gly Ala His Ile Leu Ala Ile Lys Asp Met Ala Gly Leu Leu
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cgc cca gct gcg gta acc aag ctg gtc acc gca ctg cgc cgt gaa ttc     2256
Arg Pro Ala Ala Val Thr Lys Leu Val Thr Ala Leu Arg Arg Glu Phe
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Asp Leu Pro Val His Val His Thr His Asp Thr Ala Gly Gly Gln Leu
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att gtt gct gca ttc gcg cac acc cgt cgc gat acc ggt ttg agc ctc      2448
Ile Val Ala Ala Phe Ala His Thr Arg Arg Asp Thr Gly Leu Ser Leu
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gag gct gtt tct gac ctc gag ccg tac tgg gaa gct gtg cgc gga ctg      2496
Glu Ala Val Ser Asp Leu Glu Pro Tyr Trp Glu Ala Val Arg Gly Leu
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Tyr Leu Pro Phe Glu Ser Gly Thr Pro Gly Pro Thr Gly Arg Val Tyr
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Ala Ser Ala Pro Leu Ser Gly Thr Thr Ser Gln Pro Ser Leu Ser Ala
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Ile Val Ala Ala Phe Ala His Thr Arg Arg Asp Thr Gly Leu Ser Leu
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Ser Lys Val Val Gly Asp Leu Ala Leu His Leu Val Gly Ala Gly Val
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995 1000 1005

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Lys Val Glu Gly Gly Asp Leu Ile Val Val Val Ser
1130 1135 1140

What Is Claimed Is :

1. An isolated or purified nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of :

(a) the nucleotide sequence encoding amino acids 1 to 1157 of SEQ ID NO : 2;

(b) a nucleotide sequence encoding a pyruvate carboxylase enzyme desensitized to feedback inhibition by aspartic acid, said enzyme consisting of an amino acid sequence that differs from SEQ ID NO: 19 by at least one but no more than six mutations, said at least one but not more than six mutations selected from the group consisting of:

(i) glutamic acid at position 153 is replaced with an aspartic acid;

(ii) alanine at position 182 is replaced with a serine;

(iii) alanine at position 206 is replaced with a serine;

(iv) histidine at position 227 is replaced with an arginine;

(v) alanine at position 455 is replaced with a glycine, and

(vi) aspartic acid at position 1120 is replaced with a glutamic acid; and

(c) a nucleotide sequence at least 95% identical to SEQ ID NO: 1 and which codes for a pyruvate carboxylase enzyme desensitized to feedback inhibition by aspartic acid, wherein said pyruvate carboxylase contains a mutation that corresponds in amino acid position to a mutation in SEQ ID NO: 19 where a:

(i) glutamic acid at position 153 is replaced with an aspartic acid;

(ii) alanine at position 182 is replaced with a serine;

(iii) alanine at position 206 is replaced with a serine;

(iv) histidine at position 227 is replaced with an arginine;

(v) alanine at position 455 is replaced with a glycine, or

(vi) aspartic acid at position 1120 is replaced with a glutamic acid.

2. The nucleic acid molecule of claim 1, comprising the nucleotide sequence of SEQ ID NO : 1.

3. A vector comprising: (a) the nucleic acid molecule of claim 1; and (b) at least one marker gene.

4. The vector of claim 3, further comprising a functional *Corynebacterium* replication origin.

5. A method for producing a recombinant cell comprising introducing the vector of claim 3 into a host cell.

6. A recombinant cell comprising the vector of claim 3.

7. A method of producing an amino acid, comprising: (a) culturing the recombinant cell of claim 6, in a suitable media; and (b) separating said amino acid from said medium, wherein said amino acid is selected from the group consisting of: L-lysine, L-threonine, L-methionine, L-isoleucine, L- glutamic acid, L-arginine and L-proline.

8. The method of claim 7, wherein said amino acid is L-lysine.

9. A method for replacement of a wild-type pyruvate carboxylase gene, with a feedback resistant pyruvate carboxylase gene, in a *Corynebacterium glutamicum* host cell comprising the steps of:

(a) replacing a genomic copy of said wild-type pyruvate carboxylase gene with a selectable marker gene through homologous recombination to form a first recombinant strain; and

(b) replacing said selectable marker gene of step (a) in said first recombinant strain, with said feedback resistant pyruvate carboxylase gene through homologous recombination to form a second recombinant strain;

wherein said homologous recombination in steps (a) and (b) occurs between a wild-type pyruvate carboxylase gene in said host cell and the vector of claim 3.

10. A recombinant *Corynebacterium glutamicum* strain produced by the method of claim 9.

11. A method of producing an amino acid, comprising:

(a) culturing the recombinant strain of *Corynebacterium* of claim 10 in a suitable medium; and

(b) separating said amino acid from said medium,

wherein said amino acid is selected from the group consisting of : L-lysine, L-threonine, L-methionine, L-isoleucine, L- glutamic acid, L-arginine and L-proline.

12. The method of claim 11, wherein said amino acid is L-lysine.

```
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1     ·····+·····+·····+·····+·····+·····+·····+ 60

       M  T  A  I  T  L  G  G  L  L  L  K  G  I  I  T  L  V  S  T

      CACACATCTTCAACGCTTCCAGCATTCAAAAAGATCTTGGTAGCAAACCGCGGCGAAATC
61    ·····+·····+·····+·····+·····+·····+·····+ 120

       H  T  S  S  T  L  P  A  F  K  K  I  L  V  A  N  R  G  E  I

      GCGGTCCGTGCTTTCCGTGCAGCACTCGAAACCGGTGCAGCCACGGTAGCTATTTACCCC
121   ·····+·····+·····+·····+·····+·····+·····+ 180

       A  V  R  A  F  R  A  A  L  E  T  G  A  A  T  V  A  I  Y  P

      CGTGAAGATCGGGGATCATTCCACCGCTCTTTTGCTTCTGAAGCTGTCCGCATTGGTACT
181   ·····+·····+·····+·····+·····+·····+·····+ 240

       R  E  D  R  G  S  G  H  R  S  F  A  S  E  A  V  R  I  G  T

      GAAGGCTCACCAGTCAAGGCGTACCTGGACATCGATGAAATTATCGGTGCAGCTAAAAAA
241   ·····+·····+·····+·····+·····+·····+·····+ 300

       E  G  S  P  V  K  A  Y  L  D  I  D  E  I  I  G  A  A  K  K

      GTTAAAGCAGATGCTATTTACCCGGGATATGGCTTCCTGTCTGAAAATGCCCAGCTTGCC
301   ·····+·····+·····+·····+·····+·····+·····+ 360

       V  K  A  D  A  I  Y  P  G  Y  G  F  L  S  E  N  A  Q  L  A

      CGCGAGTGCGCGGAAAACGGCATTACTTTTATTGGCCCAACCCCAGAGGTTCTTGATCTC
361   ·····+·····+·····+·····+·····+·····+·····+ 420

       R  E  C  A  E  N  G  I  T  F  I  G  P  T  P  E  V  L  D  L

      ACCGGTGATAAGTCTCGTGCGGTAACCGCCGCGAAGAAGGCTGGTCTGCCAGTTTTGGCG
421   ·····+·····+·····+·····+·····+·····+·····+ 480

       T  G  D  K  S  R  A  V  T  A  A  K  K  A  G  L  P  V  L  A

      GAATCCACCCCGAGCAAAAACATCGATGACATCGTTAAAAGCGCTGAAGGCCAGACTTAC
481   ·····+·····+·····+·····+·····+·····+·····+ 540

       E  S  T  P  S  K  N  I  D  D  I  V  K  S  A  E  G  Q  T  Y
```

FIG.1A

```
     CCCATCTTTGTAAAGGCAGTTGCCGGTGGTGGCGGACGCGGTATGCGCTTTGTTTCTTCA
541 ---------+---------+---------+---------+---------+---------+ 600

      P  I  F  V  K  A  V  A  G  G  G  G  R  G  M  R  F  V  S  S

     CCTGATGAGCTCCGCAAATTGGCAACAGAAGCATCTCGTGAAGCTGAAGCGGCATTCGGC
601 ---------+---------+---------+---------+---------+---------+ 660

      P  D  E  L  R  K  L  A  T  E  A  S  R  E  A  E  A  A  F  G

     GACGGTTCGGTATATGTCGAACGTGCTGTGATTAACCCCCAGCACATTGAAGTGCAGATC
661 ---------+---------+---------+---------+---------+---------+ 720

      D  G  S  V  Y  V  E  R  A  V  I  N  P  Q  H  I  E  V  Q  I

     CTTGGCGATCGCACTGGAGAAGTTGTACACCTTTATGAACGTGACTGCTCACTGCAGCGT
721 ---------+---------+---------+---------+---------+---------+ 780

      L  G  D  R  T  G  E  V  V  H  L  Y  E  R  D  C  S  L  Q  R

     CGTCACCAAAAAGTTGTCGAAATTGCGCCAGCACAGCATTTGGATCCAGAACTGCGTGAT
781 ---------+---------+---------+---------+---------+---------+ 840

      R  H  Q  K  V  V  E  I  A  P  A  Q  H  L  D  P  E  L  R  D

     CGCATTTGTGCGGATGCAGTAAAGTTCTGCCGCTCCATTGGTTACCAGGGCGCGGGAACC
841 ---------+---------+---------+---------+---------+---------+ 900

      R  I  C  A  D  A  V  K  F  C  R  S  I  G  Y  Q  G  A  G  T

     GTGGAATTCTTGGTCGATGAAAAGGGCAACCACGTTTTCATCGAAATGAACCCACGTATC
901 ---------+---------+---------+---------+---------+---------+ 960

      V  E  F  L  V  D  E  K  G  N  H  V  F  I  E  M  N  P  R  I

     CAGGTTGAGCACACCGTGACTGAAGAAGTCACCGAGGTGGACCTGGTGAAGGCGCAGATG
961 ---------+---------+---------+---------+---------+---------+ 1020

      Q  V  E  H  T  V  T  E  E  V  T  E  V  D  L  V  K  A  Q  M

     CGCTTGGCTGCTGGTGCAACCTTGAAGGAATTGGGTCTGACCCAAGATAAGATCAAGACC
1021 ---------+---------+---------+---------+---------+---------+ 1080

      R  L  A  A  G  A  T  L  K  E  L  G  L  T  Q  D  K  I  K  T
```

FIG.1B

```
     CACGGTGCAGCACTGCAGTGCCGCATCACCACGGAAGATCCAAACAACGGCTTCCGCCCA
1081 ---------+---------+---------+---------+---------+---------+ 1140

      H  G  A  A  L  Q  C  R  I  T  T  E  D  P  N  N  G  F  R  P

     GATACCGGAACTATCACCGCGTACCGCTCACCAGGCGGAGCTGGCGTTCGTCTTGACGGT
1141 ---------+---------+---------+---------+---------+---------+ 1200

      D  T  G  T  I  T  A  Y  R  S  P  G  G  A  G  V  R  L  D  G

     GCAGCTCAGCTCGGTGGCGAAATCACCGCACACTTTGACTCCATGCTGGTGAAAATGACC
1201 ---------+---------+---------+---------+---------+---------+ 1260

      A  A  Q  L  G  G  E  I  T  A  H  F  D  S  M  L  V  K  M  T

     TGCCGTGGTTCCGACTTTGAAACTGCTGTTGCTCGTGCACAGCGCGCGTTGGCTGAGTTC
1261 ---------+---------+---------+---------+---------+---------+ 1320

      C  R  G  S  D  F  E  T  A  V  A  R  A  Q  R  A  L  A  E  F

     ACCGTGTCTGGTGTTGCAACCAACATTGGTTTCTTGCGTGCGTTGCTGCGGGAAGAGGAC
1321 ---------+---------+---------+---------+---------+---------+ 1380

      T  V  S  G  V  A  T  N  I  G  F  L  R  A  L  L  R  E  E  D

     TTCACTTCCAAGCGCATCGCCACCGGATTTATCGGCGATCACCCACACCTCCTTCAGGCT
1381 ---------+---------+---------+---------+---------+---------+ 1440

      F  T  S  K  R  I  A  T  G  F  I  G  D  H  P  H  L  L  Q  A

     CCACCTGCGGATGATGAGCAGGGACGCATCCTGGATTACTTGGCAGATGTCACCGTGAAC
1441 ---------+---------+---------+---------+---------+---------+ 1500

      P  P  A  D  D  E  Q  G  R  I  L  D  Y  L  A  D  V  T  V  N

     AAGCCTCATGGTGTGCGTCCAAAGGATGTTGCAGCACCAATCGATAAGCTGCCCAACATC
1501 ---------+---------+---------+---------+---------+---------+ 1560

      K  P  H  G  V  R  P  K  D  V  A  A  P  I  D  K  L  P  N  I

     AAGGATCTGCCACTGCCACGCGGTTCCCGTGACCGCCTGAAGCAGCTTGGCCCAGCCGCG
1561 ---------+---------+---------+---------+---------+---------+ 1620

      K  D  L  P  L  P  R  G  S  R  D  R  L  K  Q  L  G  P  A  A
```

FIG.1C

```
     TTTGCTCGTGATCTCCGTGAGCAGGACGCACTGGCAGTTACTGATACCACCTTCCGCGAT
1621 ---------+---------+---------+---------+---------+---------+ 1680

      F  A  R  D  L  R  E  Q  D  A  L  A  V  T  D  T  T  F  R  D

     GCACACCAGTCTTTGCTTGCGACCCGAGTCCGCTCATTCGCACTGAAGCCTGCGGCAGAG
1681 ---------+---------+---------+---------+---------+---------+ 1740

      A  H  Q  S  L  L  A  T  R  V  R  S  F  A  L  K  P  A  A  E

     GCCGTCGCAAAGCTGACTCCTGAGCTTTTGTCCGTGGAGGCCTGGGGCGGCGCGACCTAC
1741 ---------+---------+---------+---------+---------+---------+ 1800

      A  V  A  K  L  T  P  E  L  L  S  V  E  A  W  G  G  A  T  Y

     GATGTGGCGATGCGTTTCCTCTTTGAGGATCCGTGGGACAGGCTCGACGAGCTGCGCGAG
1801 ---------+---------+---------+---------+---------+---------+ 1860

      D  V  A  M  R  F  L  F  E  D  P  W  D  R  L  D  E  L  R  E

     GCGATGCCGAATGTAAACATTCAGATGCTGCTTCGCGGCCGCAACACCGTGGGATACACC
1861 ---------+---------+---------+---------+---------+---------+ 1920

      A  M  P  N  V  N  I  Q  M  L  L  R  G  R  N  T  V  G  Y  T

     CCGTACCCAGACTCCGTCTGCCGCGCGTTTGTTAAGGAAGCTGCCAGCTCCGGCGTGGAC
1921 ---------+---------+---------+---------+---------+---------+ 1980

      P  Y  P  D  S  V  C  R  A  F  V  K  E  A  A  S  S  G  V  D

     ATCTTCCGCATCTTCGACGCGCTTAACGACGTCTCCCAGATGCGTCCAGCAATCGACGCA
1981 ---------+---------+---------+---------+---------+---------+ 2040

      I  F  R  I  F  D  A  L  N  D  V  S  Q  M  R  P  A  I  D  A

     GTCCTGGAGACCAACACCGCGGTAGCCGAGGTGGCTATGGCTTATTCTGGTGATCTCTCT
2041 ---------+---------+---------+---------+---------+---------+ 2100

      V  L  E  T  N  T  A  V  A  E  V  A  M  A  Y  S  G  D  L  S

     GATCCAAATGAAAAGCTCTACACCCTGGATTACTACCTAAAGATGGCAGAGGAGATCGTC
2101 ---------+---------+---------+---------+---------+---------+ 2160

      D  P  N  E  K  L  Y  T  L  D  Y  Y  L  K  M  A  E  E  I  V
```

FIG.1D

```
      AAGTCTGGCGCTCACATTCTGGCCATTAAGGATATGGCTGGTCTGCTTCGCCCAGCTGCG
2161  ---------+---------+---------+---------+---------+---------+ 2220

       K  S  G  A  H  I  L  A  I  K  D  M  A  G  L  L  R  P  A  A

      GTAACCAAGCTGGTCACCGCACTGCGCCGTGAATTCGATCTGCCAGTGCACGTGCACACC
2221  ---------+---------+---------+---------+---------+---------+ 2280

       V  T  K  L  V  T  A  L  R  R  E  F  D  L  P  V  H  V  H  T

      CACGACACTGCGGGTGGCCAGTTGGCTACCTACTTTGCTGCAGCTCAAGCTGGTGCAGAT
2281  ---------+---------+---------+---------+---------+---------+ 2340

       H  D  T  A  G  G  Q  L  A  T  Y  F  A  A  A  Q  A  G  A  D

      GCTGTTGACGGTGCTTCCGCACCACTGTCTGGCACCACCTCCCAGCCATCCCTGTCTGCC
2341  ---------+---------+---------+---------+---------+---------+ 2400

       A  V  D  G  A  S  A  P  L  S  G  T  T  S  Q  P  S  L  S  A

      ATTGTTGCTGCATTCGCGCACACCCGTCGCGATACCGGTTTGAGCCTCGAGGCTGTTTCT
2401  ---------+---------+---------+---------+---------+---------+ 2460

       I  V  A  A  F  A  H  T  R  R  D  T  G  L  S  L  E  A  V  S

      GACCTCGAGCCGTACTGGGAAGCTGTGCGCGGACTGTACCTGCCATTTGAGTCTGGAACC
2461  ---------+---------+---------+---------+---------+---------+ 2520

       D  L  E  P  Y  W  E  A  V  R  G  L  Y  L  P  F  E  S  G  T

      CCAGGCCCAACCGGTCGCGTCTACCGCCACGAAATCCCAGGCGGACAGTTGTCCAACCTG
2521  ---------+---------+---------+---------+---------+---------+ 2580

       P  G  P  T  G  R  V  Y  R  H  E  I  P  G  G  Q  L  S  N  L

      CGTGCACAGGCCACCGCACTGGGCCTTGCTGATCGCTTCGAGCTCATCGAAGACAACTAC
2581  ---------+---------+---------+---------+---------+---------+ 2640

       R  A  Q  A  T  A  L  G  L  A  D  R  F  E  L  I  E  D  N  Y

      GCAGCCGTTAATGAGATGCTGGGACGCCCAACCAAGGTCACCCCATCCTCCAAGGTTGTT
2641  ---------+---------+---------+---------+---------+---------+ 2700

       A  A  V  N  E  M  L  G  R  P  T  K  V  T  P  S  S  K  V  V
```

FIG.1E

```
     GGCGACCTCGCACTCCACCTGGTTGGTGCGGGTGTAGATCCAGCAGACTTTGCTGCAGAC
2701 .........+.........+.........+.........+.........+.........+ 2760

      G  D  L  A  L  H  L  V  G  A  G  V  D  P  A  D  F  A  A  D

     CCACAAAAGTACGACATCCCAGACTCTGTCATCGCGTTCCTGCGCGGCGAGCTTGGTAAC
2761 .........+.........+.........+.........+.........+.........+ 2820

      P  Q  K  Y  D  I  P  D  S  V  I  A  F  L  R  G  E  L  G  N

     CCTCCAGGTGGCTGGCCAGAACCACTGCGCACCCGCGCACTGGAAGGCCGCTCCGAAGGC
2821 .........+.........+.........+.........+.........+.........+ 2880

      P  P  G  G  W  P  E  P  L  R  T  R  A  L  E  G  R  S  E  G

     AAGGCACCTCTGACGGAAGTTCCTGAGGAAGAGCAGGCGCACCTCGACGCTGATGATTCC
2881 .........+.........+.........+.........+.........+.........+ 2940

      K  A  P  L  T  E  V  P  E  E  E  A  A  H  L  D  A  D  D  S

     AAGGAACGTCGCAACAGCCTCAACCGCCTGCTGTTCCCGAAGCCAACCGAAGAGTTCCTC
2941 .........+.........+.........+.........+.........+.........+ 3000

      K  E  R  R  N  S  L  N  R  L  L  F  P  K  P  T  E  E  F  L

     GAGCACCGTCGCCGCTTCGGCAACACCTCTGCGCTGGATGATCGTGAATTCTTCTACGGA
3001 .........+.........+.........+.........+.........+.........+ 3060

      E  H  R  R  R  F  G  N  T  S  A  L  D  D  R  E  F  F  Y  G

     CTGGTCGAGGGCCGCGAGACTTTGATCCGCCTGCCAGATGTGCGCACCCCACTGCTTGTT
3061 .........+.........+.........+.........+.........+.........+ 3120

      L  V  E  G  R  E  T  L  I  R  L  P  K  V  R  T  P  L  L  V

     CGCCTGGATGCGATCTCTGAGCCAGACGATAAGGGTATGCGCAATGTTGTGGCCAACGTC
3121 .........+.........+.........+.........+.........+.........+ 3180

      R  L  D  A  I  S  E  P  D  D  K  G  M  R  N  V  V  A  N  V

     AACGGCCAGATCCGCCCAATGCGTGTGCGTGACCGCTCCGTTGAGTCTGTCACCGCAACC
3181 .........+.........+.........+.........+.........+.........+ 3240

      N  G  Q  I  R  P  M  R  V  R  D  R  S  V  E  S  V  T  A  T
```

FIG.1F

```
      GCAGAAAAGGCAGATTCCTCCAACAAGGGCCATGTTGCTGCACCATTCGCTGGTGTTGTC
3241  ---------+---------+---------+---------+---------+---------+ 3300

       A  E  K  A  D  S  S  N  K  G  H  V  A  A  P  F  A  G  V  V

      ACTGTGACTGTTGCTGAAGGTGATGAGGTCAAGGCTGGAGATGCAGTCGCAATCATCGAG
3301  ---------+---------+---------+---------+---------+---------+ 3360

       T  V  T  V  A  E  G  D  E  V  K  A  G  D  A  V  A  I  I  E

      GCTATGAAGATGGAAGCAACAATCACTGCTTCTGTTGACGGCAAGATTGAACGCGTTGTG
3361  ---------+---------+---------+---------+---------+---------+ 3300

       A  M  K  M  E  A  T  I  T  A  S  V  D  G  K  I  E  R  V  V
      GTTCCTGCTGCAACGAAGGTGGAAGGTGGCGACTTGATCGTCGTCGTTTCCTAA
3421  ---------+---------+---------+---------+---------+---------+---- 3474

       V  P  A  A  T  K  V  E  G  G  D  L  I  V  V  V  S  *
```

FIG.1G

```
                                          10        20        30        40
ATCC 21523                          MSTHTSSTLPAFKKILVANRGEIAVRAFRAALETGAATVAIYP
NRRL-B11474   MTAITLGGLLLKGIITLV

                    50        60        70        80        90        100
ATCC 21523    REDRGSFHRSFASEAVRIGTEGSPVKAYLDIDEIIGAAKKVKADAIYPGYGFLSENAQLA
NRRL-B11474

                   110       120       130       140       150       160
ATCC 21523    RECAENGITFIGPTPEVLDLTGDKSRAVTAAKKAGLPVLAESTPSKNIDEIVKSAEGQTY
NRRL-B11474                                                   D

                   170       180       190       200       210       220
ATCC 21523    PIFVKAVAGGGGRGMRFVASPDELRKLATEASREAEAAFGDGAVYVERAVINPQHIEVQI
NRRL-B11474                     S                         S

                   230       240       250       260       270       280
ATCC 21523    LGDHTGEVVHLYERDCSLQRRHQKVVEIAPAAQHLDPELRDRICADAVKFCRSIGYQGAG
NRRL-B11474       R

                   290       300       310       320       330       340
ATCC 21523    VEFLVDEKGNHVFIEMNPRIQVEHTVTEEVTEVDLVKAQMRLAAGATLKELGLTQDKIKT
NRRL-B11474

                   350       360       370       380       390       400
ATCC 21523    HGAALQCRITTEDPNNGFRPDTGTITAYRSPGGAGVRLDGAAQLGGEITAHFDSMLVKMT
NRRL-B11474

                   410       420       430       440       450       460
ATCC 21523    CRGSDFETAVARAQRALAEFTVSGVATNIGFLRALLREEDFTSKRIATGFIADHPHLLQA
NRRL-B11474                                                   G

                   470       480       490       500       510       520
ATCC 21523    PPADDEQGRILDYLADVTVNKPHGVRPKDVAAPIDKLPNIKDLPLPRGSRDRLKQLGPAA
NRRL-B11474

                   530       540       550       560       570       580
ATCC 21523    FARDLREQDALAVTDTTFRDAHQSLLATRVRSFALKPAAEAVAKLTPELLSVEAWGGATY
NRRL-B11474

                   590       600       610       620       630       640
ATCC 21523    DVAMRFLFEDPWDRLDELREAMPNVNIQMLLRGRNTVGYTPYPDSVCRAFVKEAASSGVD
NRRL-B11474

                   650       660       670       680       690       700
ATCC 21523    IFRIFDALNDVSQMRPAIDAVLETNTAVAEVAMAYSGDLSDPNEKLYTLDYYLKMAEEIV
NRRL-B11474

                   710       720       730       740       750       760
ATCC 21523    KSGAHILAIKDMAGLLRPAAVTKLVTALRREFDLPVHVHTHDTAGGQLATYFAAAQAGAD
NRRL-B11474

                   770       780       790       800       810       820
ATCC 21523    AVDGASAPLSGTTSQPSLSAIVAAFAHTRRDTGLSLEAVSDLEPYWEAVRGLYLPFESGT
NRRL-B11474
```

FIG. 2A

```
                    830        840        850        860        870        880
ATCC 21523    PGPTGRVYRHEIPGGQLSNLRAQATALGLADRFELIEDNYAAVNEMLGRPTKVTPSSKVV
NRRL-B11474

                    890        900        910        920        930        940
ATCC 21523    GDLALHLVGAGVDPADFAADPQKYDIPDSVIAFLRGELGNPPGGWPEPLRTRALEGRSEG
NRRL-B11474

                    950        960        970        980        990       1000
ATCC 21523    KAPLTEVPEEEQAHLDADDSKERRNSLNRLLFPKPTEEFLEHRRRFGNTSALDDREFFYG
NRRL-B11474

                   1010       1020       1030       1040       1050       1060
ATCC 21523    LVEGRETLIRLPDVRTPLLVRLDAISEPDDKGMRNVVANVNGQIRPMRVRDRSVESVTAT
NRRL-B11474

                   1070       1080       1090       1100       1110       1120
ATCC 21523    AEKADSSNKGHVAAPFAGVVTVTVAEGDEVKAGDAVAIIEAMKMEATITASVDGKIDRVV
NRRL-B11474                                                              E

                   1130       1140
ATCC 21523    VPAATKVEGGDLIVVVS
NRRL-B11474
```

FIG. 2B

EFFECT OF VARIOUS SUBSTRATE CONCENTRATIONS ON PYRUVATE CARBOXYLASE ACTIVITY FROM *C. glutamicum* NRRL B-11474 (o) AND ATCC 21253 (•).

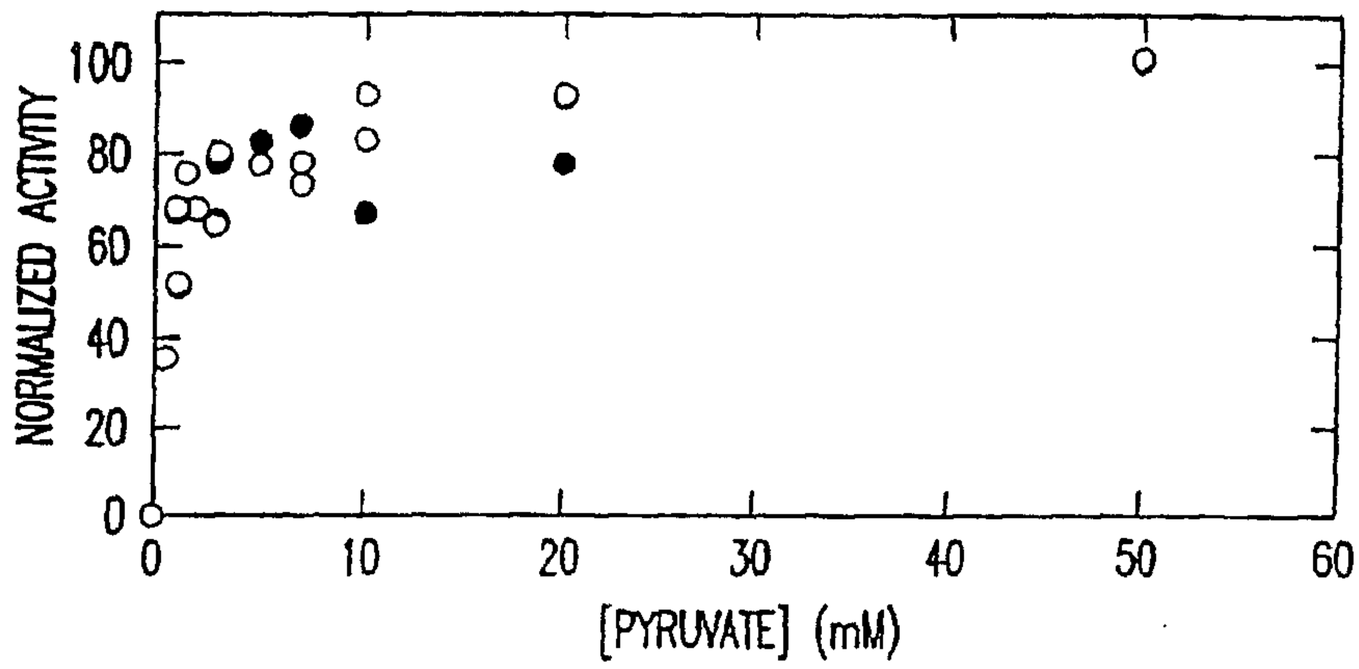

FIG. 3A

EFFECT OF VARIOUS SUBSTRATE CONCENTRATIONS ON PYRUVATE CARBOXYLASE ACTIVITY FROM *C. glutamicum* NRRL B-11474 (o) AND ATCC 21253 (•).

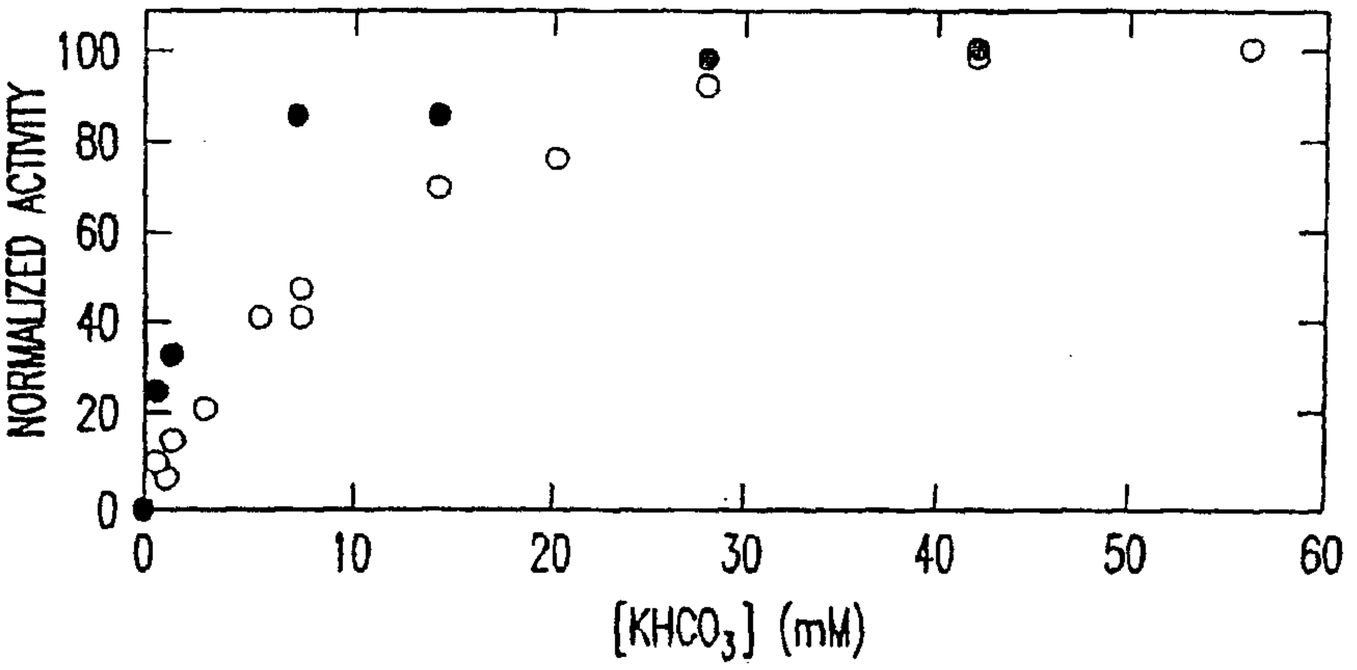

FIG. 3B

EFFECT OF VARIOUS SUBSTRATE CONCENTRATIONS ON PYRUVATE CARBOXYLASE ACTIVITY FROM *C. glutamicum* NRRL B-11474 (o) AND ATCC 21253 (•).

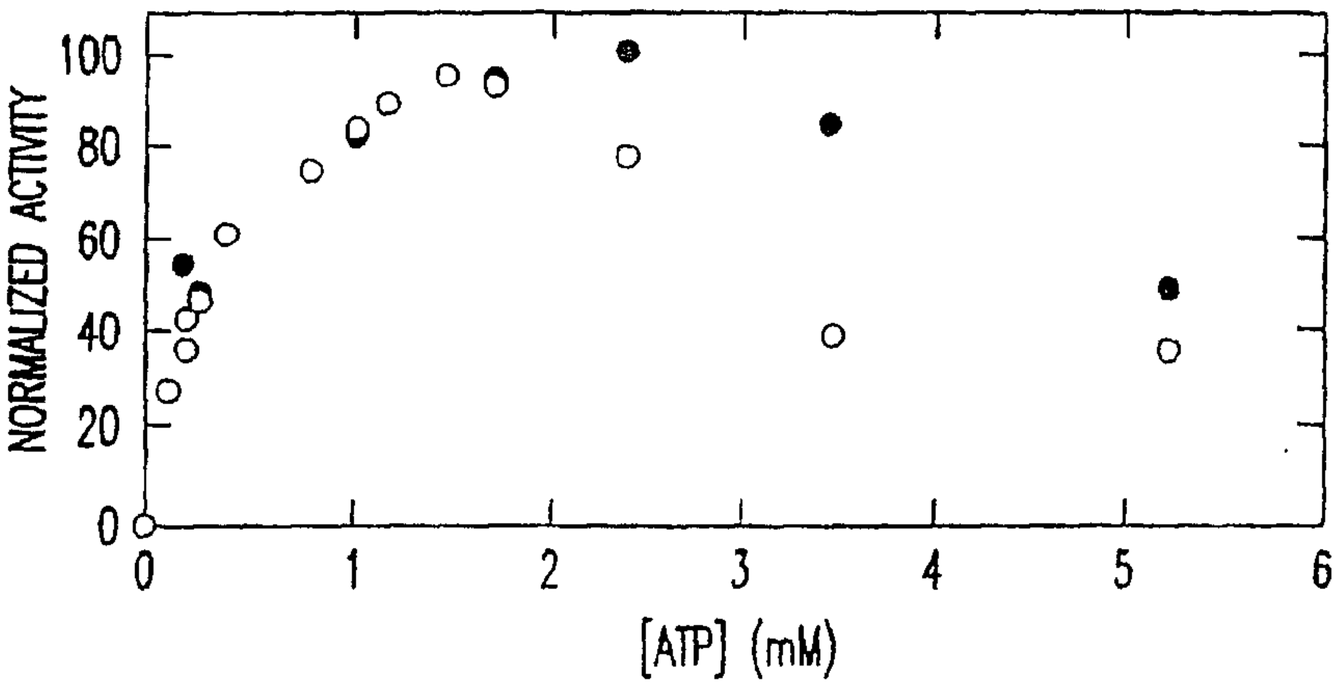

FIG. 3C

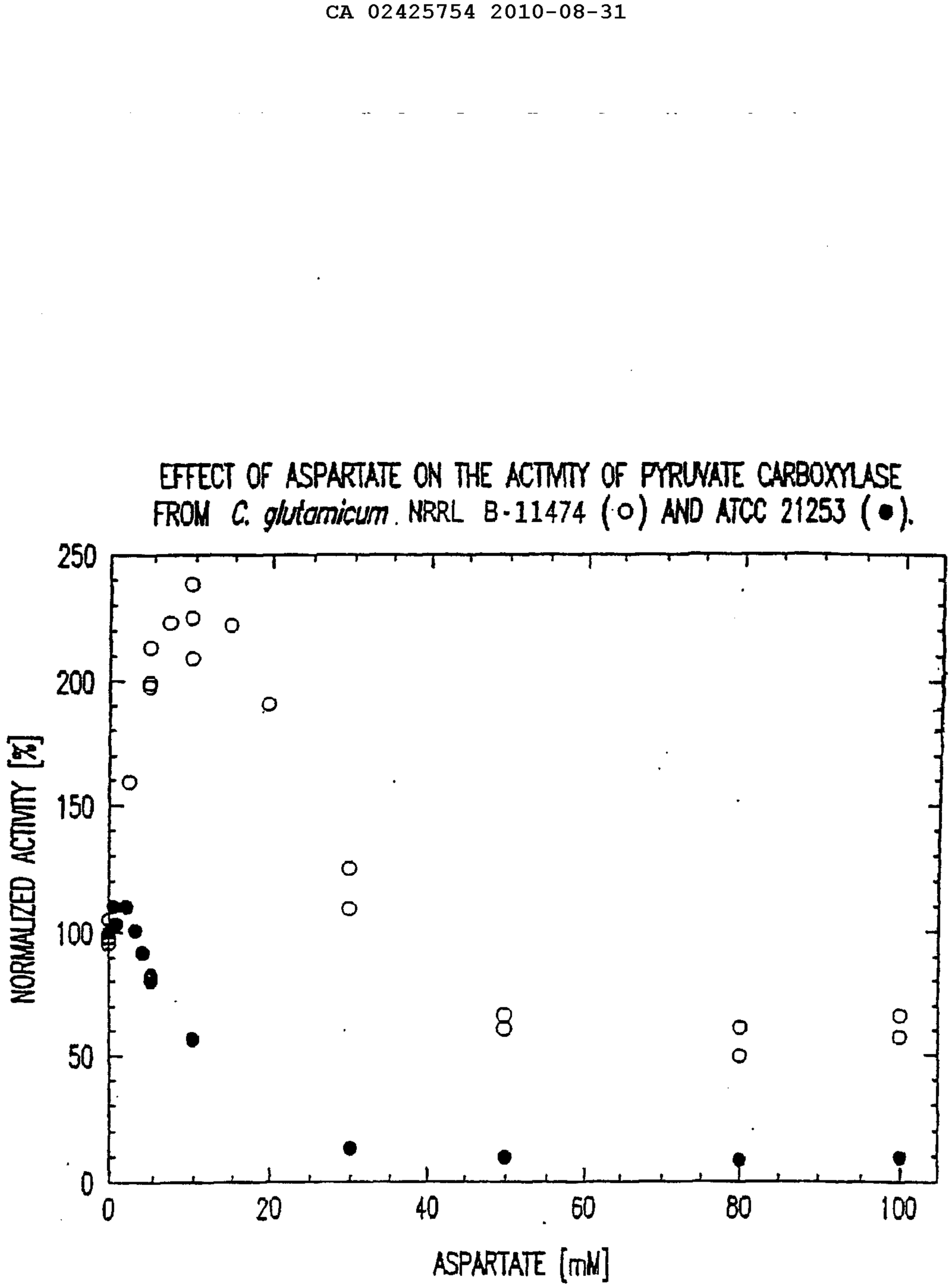
EFFECT OF ASPARTATE ON THE ACTIVITY OF PYRUVATE CARBOXYLASE
FROM C. glutamicum. NRRL B-11474 (○) AND ATCC 21253 (●).
NORMALIZED ACTIVITY [%]
250
200
150
100
50
0
0
20
40
60
80
100
ASPARTATE [mM]

FIG. 4

EFFECT OF Acetyl-CoA ON PYRUVATE CARBOXYLASE ACTIVITY FROM *C. glutamicum* NRRL B-11474 (○) AND ATCC 21253 (●).

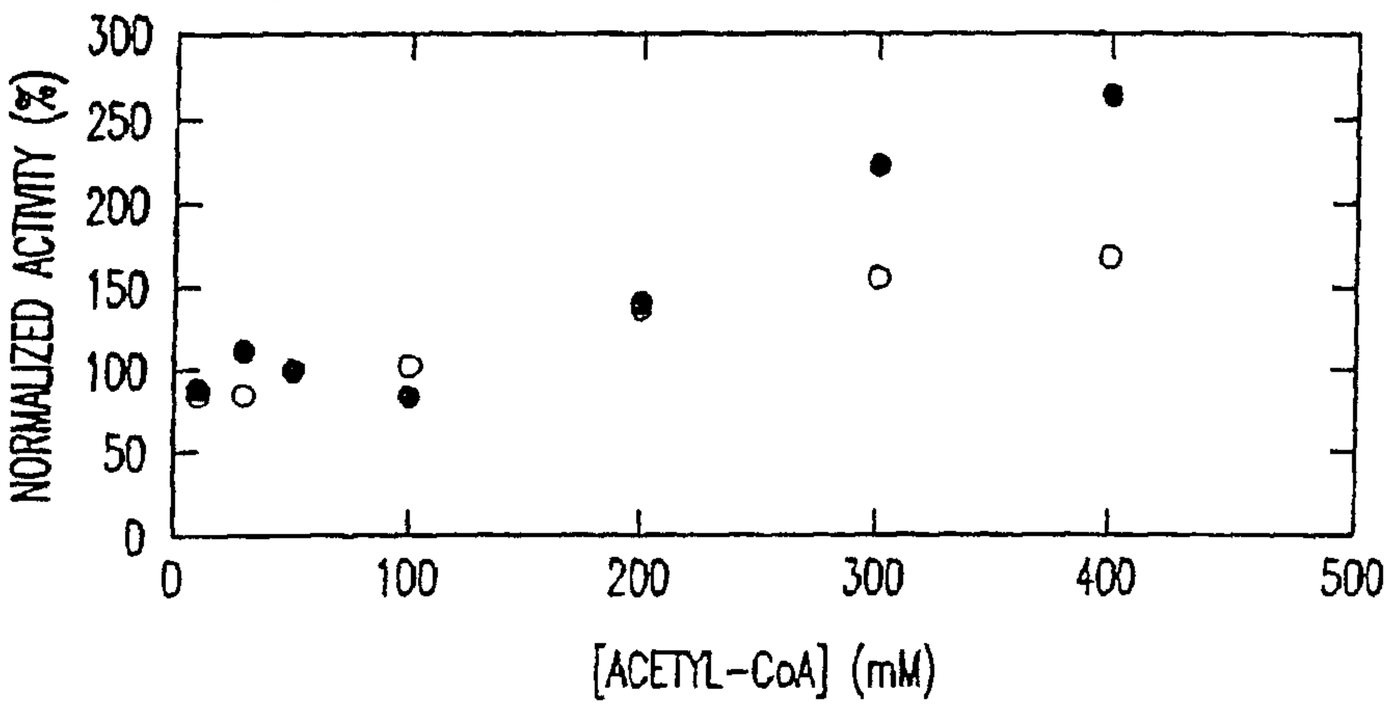

FIG. 5

EFFECT OF Acetyl-CoA ON PYRUVATE CARBOXYLASE ACTIVITY FROM *C. glutamicum* NRRL B-11474 (○) AND ATCC 21253 (●).