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Cloning of the Pyruvate Kinase Gene (*pyk*) of *Corynebacterium glutamicum* and Site-Specific Inactivation of *pyk* in a Lysine-Producing *Corynebacterium lactofermentum* Strain

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The pyruvate kinase gene *pyk* from *Corynebacterium glutamicum* was cloned by applying a combination of PCR, site-specific mutagenesis, and complementation. A 126-bp DNA fragment central to the *C. glutamicum pyk* gene was amplified from genomic DNA by PCR with degenerate oligonucleotides as primers. The cloned DNA fragment was used to inactivate the *pyk* gene in *C. glutamicum* by marker rescue mutagenesis via homologous recombination. The *C. glutamicum pyk* mutant obtained was unable to grow on minimal medium containing ribose as the sole carbon source. Complementation of this phenotype by a gene library resulted in the isolation of a 2.8-kb *PstI-BamHI* genomic DNA fragment harboring the *C. glutamicum pyk* gene. Multiple copies of plasmid-borne *pyk* caused a 20-fold increase of pyruvate kinase activity in *C. glutamicum* cell extracts. By using large internal fragments of the cloned *C. glutamicum* gene, *pyk* mutant derivatives of the lysine production strain *Corynebacterium lactofermentum* 21799 were generated by marker rescue mutagenesis. As determined in shake flask fermentations, lysine production in *pyk* mutants was 40% lower than that in the *pyk*$^+$ parent strain, indicating that pyruvate kinase is essential for high-level lysine production. This finding questions an earlier hypothesis postulating that redirection of carbon flow at the phosphoenol pyruvate branch point of glycolysis through elimination of pyruvate kinase activity results in an increase of lysine production in *C. glutamicum* and its close relatives.

The biosynthetic pathway for the aspartate-derived amino acids in *Corynebacterium glutamicum*, *Corynebacterium lactofermentum*, and the closely related *Brevibacterium flavum* is primarily controlled by feedback inhibition of a few key enzymes (9). Isolation of strains which overproduce these amino acids has been achieved in two ways: (i) by selecting for resistance against nonmetabolizable amino acid analogs to obtain mutants with feedback-insensitive biosynthetic enzymes (18) and (ii) by selecting for amino acid auxotrophs which lack a branch of the split biosynthetic pathway in order to eliminate competition for a common pathway intermediate (12, 24). *C. glutamicum* strains, for example, which possess feedback-insensitive aspartokinase or which exhibit homoserine or threonine auxotrophy produce lysine at elevated levels. To further improve lysine production in a *C. lactofermentum* strain with feedback-resistant aspartokinase, we attempted to optimize the carbon flow from glycolysis towards the lysine biosynthetic pathway.

The biosynthesis of lysine requires carbon which stems from pyruvate and oxaloacetate decarboxylase (OAA) in a 1:1 ratio (23). In *Corynebacterium* and *Brevibacterium* strains, the following enzyme reactions of the central carbon metabolism have been shown to result in the formation of pyruvate and OAA: (i) for the sugar phosphotransferase system (PTS), glucose + phosphoenol pyruvate (PEP) → glucose-6-phosphate + pyruvate; (ii) for pyruvate kinase (EC 2.7.1.40), PEP + ADP → pyruvate + ATP; (iii) for OAA decarboxylase (EC 4.1.1.3), OAA → pyruvate + CO_2; (iv) for the malic enzyme (EC 1.1.1.40), malate + $NADP^+$ → pyruvate + CO_2 + NADPH; (v) for PEP carboxylase (EC 4.1.1.31), PEP + CO_2 → OAA + P_i; (vi) for PEP carboxykinase (EC 4.1.1.32), PEP + CO_2 + GDP $\overset{(\rightarrow)}{\longleftarrow}$ OAA + GTP; and (vii) for the glyoxylate cycle, 2 acetate + 2 NAD^+ → → → → → → OAA + 2 NADH + H_2O.

During growth on glucose, formation of pyruvate and OAA via reactions (iv), (vi), and (vii) is negligible because the enzymes involved are repressed or the reaction is thermodynamically unfavorable (7, 10, 15). Although the decarboxylation reaction (iii) was found in *B. flavum* cell extracts, its in vivo contribution to the formation of pyruvate remained unclear (13). Consequently, pyruvate can be generated via reactions (i) and (ii), which are catalyzed by PTS and pyruvate kinase, respectively (14, 15), whereas OAA can be generated via reaction (v), which is catalyzed by PEP carboxylase (15).

Simultaneous action of PTS and pyruvate kinase results in almost complete conversion of glucose to pyruvate, which, after entering the tricarboxylic acid cycle as acetyl coenzyme A, is oxidized to CO_2. On the other hand, simultaneous action of PTS and PEP carboxylase yields equimolar amounts of pyruvate and OAA from glucose as the substrate. Thus, a 1:1 ratio in the generation of pyruvate and OAA may be achieved by selecting for *C. glutamicum* strains which lack pyruvate kinase activity.

In the present study, we describe the cloning of the pyruvate kinase gene *pyk* from *C. glutamicum* and its use to inactivate the pyruvate kinase gene of a lysine-producing *C. lactofermentum* strain by marker rescue mutagenesis. The results of fermentation experiments revealed that in *C. lactofermentum* elimination of pyruvate kinase activity leads to a decrease in lysine production and concomitant accumulation of glyceraldehyde and dihydroxyacetone. This finding indicates that the absence of pyruvate kinase activity causes an accumulation of

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
C. glutamicum		
AS019	Wild type, rifampicin-resistant derivative of ATCC 13059	25
E12	Restriction-deficient derivative of AS019	6
M1	Kn^r, *pyk* mutant of E12	This work
M2	Cm^r, *pyk* mutant of M1	This work
A124	Kn^r, *pyk* mutant of AS019	This work
A126	Kn^r, *pyk* mutant of AS019	This work
A127	Kn^r, *pyk* mutant of AS019	This work
C. lactofermentum		
21799	Lysine production strain, aminoethyl cysteine resistant	ATCC[a]
L124	Kn^r, *pyk* mutant of 21799	This work
L126	Kn^r, *pyk* mutant of 21799	This work
L127	Kn^r, *pyk* mutant of 21799	This work
E. coli		
DH5α	*lacZ*ΔM15 *hsdR recA*	8
S17-1	*hsdR recA*, *tra* from RP4-2 integrated in the chromosome	20
Plasmids		
pBLSCR SKII+	Ap^r, *lacZ*α	Stratagene
pSUP301	Kn^r Ap^r, *mob*	20
pMF1014α	Kn^r derivative of *C. glutamicum* plasmid pSR1	1
pMG114	Ap^r, *pyk* PCR fragment in pBLSCR SKII+	This work
pMG117	Kn^r Ap^s, *pyk* PCR fragment in pSUP301	This work
pMG118	Cm^r Kn^s derivative of pMG117	This work
pMG119	Kn^r, *Pst*I excision plasmid from the M1 genome containing the 3′ end of the disrupted *pyk* gene	This work
pMG123	Kn^r, 2.8-kb *Pst*I-*Bam*HI fragment containing the *pyk* gene from AS019 in pMF1014α	This work
pMG124	Kn^r, 1.1-kb *Eco*RI-*Sal*I *pyk* fragment in pSUP301	This work
pMG126	Kn^r, 0.45-kb *Bss*HII-*Sal*I *pyk* fragment in pSUP301	This work
pMG127	Kn^r, 0.65-kb *Eco*RI-*Bss*HII *pyk* fragment in pSUP301	This work

[a] ATCC, American Type Culture Collection.

glycolytic intermediates and does not favor the redirection of carbon flow towards the lysine biosynthetic pathway.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. All bacterial strains and plasmids used in this study are listed in Table 1. *Corynebacterium* strains were routinely grown at 30°C in TSY (per liter, 15 g of tryptone, 5 g of soytone, 5 g of yeast extract, 5 g of NaCl). Cells used for enzyme assays were grown in TSY supplemented with 10 g of glucose per liter. Minimal medium contained, per liter, 10 g of glucose, ribose, or gluconate; 10 g of $(NH_4)_2SO_4$; 2.5 g of urea; 1 g of NaCl; 1 g of KH_2PO_4; 0.4 g of $MgSO_4 \cdot 7H_2O$; 10 mg of $MnSO_4 \cdot 2H_2O$; 10 mg of $FeSO_4$; 0.5 g (each) of L-leucine, L-threonine, and L-methionine; and 0.4 mg (each) of biotin, thiamine, and calcium pantothenate. Fermentation medium contained, per liter, 80 g of glucose, 60 g of $(NH_4)_2SO_4$, 1.2 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 12 mg of $MnSO_4 \cdot 2H_2O$, 12 mg of $FeSO_4$, 2 mg of biotin, 2 mg of calcium pantothenate, and 120 ml of corn steep liquor (40% [dry weight] per liter). The pH was adjusted to 7.2 with KOH. If necessary, kanamycin and chloramphenicol were added to 25 and 10 mg/liter, respectively.

Escherichia coli strains were grown in LB (17) at 37°C. The following antibiotics were used at the concentrations indicated (per liter): ampicillin, 100 mg; kanamycin, 50 mg; and chloramphenicol, 20 mg.

Transconjugation and electroporation. Mobilization of plasmids from *E. coli* to *Corynebacterium* strains was done as described previously (19, 20). The donor-recipient ratio was 1:5, and heat treatment of recipients was done at 49°C for 9 min. *Corynebacterium* transconjugants were selected on TSY agar containing, per liter, 25 mg of nalidixic acid and 25 mg of kanamycin or 10 mg of chloramphenicol. Electroporation of *Corynebacterium* strains was done as described previously (5).

PCR. To amplify a *pyk* gene-specific fragment from genomic *Corynebacterium* or *E. coli* DNA, two degenerate 23-mer oligonucleotides were designed on the basis of the criteria used for PCR amplification of *recA* genes from gram-positive bacteria (4). The nucleotide sequences of the oligonucleotides were derived from the amino acid sequences MVARGD and ATQMLE, which are highly conserved in pyruvate kinase polypeptides. The DNA sequence of the coding strand primer is 5′-CCATCGATGGT(G,A,T,C)GC(G,A,T,C)GC(A,T,C)G G(G,A,T,C)GA-3′ (192-fold degeneracy); it has an extension of six nucleotides at the 5′ end, four of which complete a *Cla*I restriction site (underlined), and two C residues serve as a clamp for efficient cleavage by *Cla*I after PCR amplification. The DNA sequence of the complementary strand is 5′-CC GAATTC(G,A,T,C)AGCAT(T,C)TG(G,A,T,C)GT(G,A,T,C) GC-3′ (128-fold degeneracy); it also has an extension of six nucleotides at the 5′ end, four of which complete an *Eco*RI site (underlined), and two C residues serve as a clamp to facilitate cleavage by *Eco*RI. As a template, genomic DNA was heat denatured (5 min in a boiling-water bath) prior to amplification. PCR was performed with a 100-μl mixture of 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 2.5 mM $MgCl_2$; 200 μM (each) dGTP, dATP, dTTP, and dCTP; 100 pmol of each primer; 1 μg of template DNA; and 2.5 U of AmpliTaq polymerase (Perkin-

Elmer Cetus). The temperature profile of the first three PCR cycles was 1 min at 95°C, 1 min at 40°C, and 1 min at 72°C, with a slow ramping time of 2 min for the temperature adjustment between 40 and 72°C. After these initial cycles, the samples were subjected to another 30 cycles of 30 s at 95°C, 1 min at 55°C, and 1 min at 72°C, with fast ramping for all temperature adjustments.

To label the cloned *pyk*-specific PCR fragment with digoxigenin-11-dUTP (Boehringer Mannheim), 25 pg of *Pvu*II-digested pMG114 plasmid DNA was subjected to PCR amplification as described above, except that the reaction mixture contained dGTP, dATP, and dCTP at a concentration of 50 μM (each), dTTP at 32.5 μM, and digoxigenin-11-dUTP at 17.5 μM.

PCR products (5 μl of a 100-μl reaction mixture) were analyzed on 2.5% agarose gels. For cloning or use as a probe, PCR-amplified DNA was separated from excess nucleotides and primers by using cellulose filter microconcentrators (UFC3LTK; Millipore) with a cutoff of 30,000-kDa molecular mass.

DNA techniques. Standard protocols (17) were used for the construction, purification, and analysis of plasmid DNA. For the construction of plasmid pMG114, the PCR product obtained from *C. glutamicum* AS019 was digested with *Cla*I and *Eco*RI. The resulting 126-bp fragment was purified from an agarose gel and cloned in the polylinker cloning site of vector pBLSCR SKII+. Plasmid pMG117 was constructed as follows: the 126-bp *pyk*-specific PCR fragment was retrieved from pMG114 as a *Sal*I-*Pst*I cartridge and was cloned in the vector portion of plasmid pSME7. It is a derivative of the mobilizable vector pSUP301 harboring the *C. glutamicum ppc* gene, which can be excised by cleavage with *Sal*I and *Pst*I (7). Plasmid pMG118 was derived from pMG117 by replacing the kanamycin resistance marker (Km^r, *aph* from Tn*903*) with a chloramphenicol resistance marker (Cm^r, *cat* from Tn*9*) as follows: the *cat* gene was excised from plasmid pMG110 (7) as a 1.1-kb *Sal*I-*Xho*I fragment, and the *Sal*I ends were partially filled in with DNA polymerase I (Klenow fragment) and dTTP plus dCTP. The fragment was ligated into the *Bam*HI-*Xho*I backbone of pMG117 after partially filling in the *Bam*HI site with dGTP plus dATP. pMG119 and pMG123 were constructed as described in Results. To obtain pMG124, the 1.1-kb *Eco*RI-*Sal*I fragment from pMG123 was used to substitute for the 126-bp *pyk* PCR fragment in pMG117. Deletion derivatives of pMG124 were generated by cleavage of pMG124 with *Eco*RI and *Bss*HII (to yield pMG126) or with *Bss*HII and *Sal*I (to yield pMG127), by filling in all 5′ protruding ends and subsequent religation.

Isolation of genomic DNA from *Corynebacterium* strains was carried out as described previously (7), and Southern blot analysis was done by using the Genius System from Boehringer Mannheim following their users' guide for filter hybridization.

DNA sequence analysis was performed by the dideoxy chain termination method with the Sequenase kit from United States Biochemical Corp.

Preparation of cell extracts and enzyme assays. Cell extracts were prepared either from freshly grown cells or from cell pellets which had been stored at −70°C as described previously (10). Pyruvate kinase was assayed spectrophotometrically at room temperature by determining the decrease of the A_{340} of NADH, with lactate dehydrogenase as a coupling enzyme. The assay contained, in 1 ml, 80 μmol of Tris-HCl (pH 7.8), 10 μmol of $MgCl_2$, 10 μmol of PEP, 2 μmol of ADP 2, 0.4 μmol of NADH, 5 U of lactate dehydrogenase, and between 1 and 10 μl of cell extract. Specific activities are expressed in micromoles of NADH converted to NAD^+ per minute per milligram of protein.

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Ec1  241  MVARGDLGVEIPVEEVIFAQKMMIEKCIRARKVVITATQMLD
Ec2  247  --------V--GDPELVGI--AL-RRARQLNRA--T----ME
Bst  242  --------V--PAEEVPLI--LL-KKCNMLGKP--T----LD
Sce  261  --------I--PAPEVLAV--KL-AKSNLAGKP--C----LE
Ani  275  --------I--PAPKVFIA--MM-AKCNIKGKP--C----LE
Tre  284  --------I--PAAEVFAA--KM-AMCNIAGKP--C----LE
Hsa  290  --------I--PAEKVFLA--MM-GRCNRAGKP--C----LE
CON       MVARGD                              ATQMLE
Cgl  PCR  --------V-VPLEEVPLV--RA-QIARENAKP--V----LE
               **** * ***   **  *       ****
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FIG. 1. Alignment of the central segments of pyruvate kinase amino acid sequences of prokaryotic and eukaryotic origin. The sequences are from *E. coli* pyruvate kinase I (Ec1) (GenBank accession no. M24636), *E. coli* pyruvate kinase II (Ec2) (GenBank accession no. M63703), *Bacillus stearothermophilus* (Bst) (16), *Saccharomyces cerevisiae* (Sce) (2), *Aspergillus nidulans* (Ani) (3), *Trichoderma reesei* (Tre) (GenBank accession no. L07060), and *Homo sapiens* (Hsa) (22). The numbers on the left of the sequence segments designate the positions of the left-most amino acid residues in the complete pyruvate kinase amino acid sequences. Residues which are identical in all pyruvate kinase polypeptides are indicated by hyphens. The hexameric consensus sequences MVARGD and ATQMLE on which the design of the PCR primers was based are shown in bold. The bottom line represents the amino acid sequence deduced from the cloned PCR product, which was obtained from *C. glutamicum* genomic DNA. Asterisks indicate amino acid residues which are identical to the corresponding residues in at least three other pyruvate kinase polypeptides.

Fermentations and analysis of fermentation products. *C. lactofermentum* strains were cultivated in 250-ml baffled shake flasks containing 45 ml of medium. Inoculation was done with 5 ml of an overnight culture in TSY containing 1% glucose. Samples of 1 ml were taken every 12 h and analyzed for cell growth (optical density at 600 nm), glucose, amino acids, organic acids, and alcohols. Glucose was quantified enzymatically with the Trinder (Sigma Diagnostics) assay. Amino acids were analyzed as orthophthal-dialdehyde derivatives by reversed-phase chromatography by using the Aminoquant (Hewlett-Packard Analytical Division) method on a Hewlett-Packard 1050 high-performance liquid chromatography (HPLC) system. Organic acids and alcohols were determined by HPLC with a Bio-Rad HPX-87H column at 45°C, with a mobile phase consisting of 5 mM H_2SO_4. Compounds were detected by both A_{206} and refractive index.

RESULTS

PCR amplification and cloning of an internal fragment of the *C. glutamicum pyk* gene. The sequences of seven pyruvate kinase polypeptides of prokaryotic and eukaryotic origin were compared, and several regions of strong homology were identified. Figure 1 shows the central segment of the amino acid sequence alignment in which two hexameric amino acid sequences, MVARGD and ATQMLE, are highly conserved. On the basis of these amino acid residues, two degenerate oligonucleotides were designed in order to amplify by PCR a central fragment of the *C. glutamicum pyk* gene. As shown in Fig. 2, amplification with genomic DNA from *C. glutamicum*, *C. lactofermentum*, and *E. coli* as templates produced specific DNA fragments with identical sizes. In accordance with the known DNA sequences of pyruvate kinase genes, the length of the amplified DNA fragments was calculated to be 137 bp, including the extensions of the PCR primers. The PCR product obtained from *C. glutamicum* genomic DNA was cloned, and the DNA sequence was determined. The deduced amino

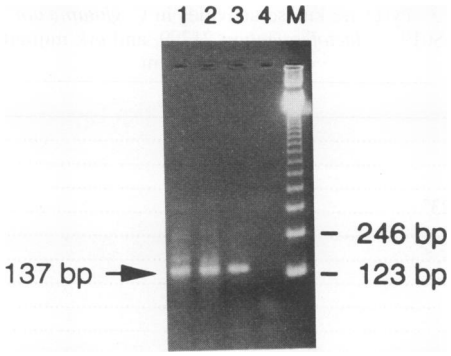

FIG. 2. Agarose gel electrophoresis of PCR products obtained with degenerate primers for the amplification of an internal segment of *pyk* genes. Genomic DNA from *C. glutamicum* (lane 1), *C. lactofermentum* (lane 2), and *E. coli* (lane 3) were used as templates. Lane M contains a 123-bp DNA ladder as a size marker (Gibco BRL).

acid sequence was compared with the central region of those pyruvate kinase amino acid sequences which were chosen for the design of the PCR primers (Fig. 1). From the 30 amino acid residues lying between the two conserved amino acid hexamers, 15 are identical to the corresponding residues in at least three other pyruvate kinase polypeptides. On the basis of this homology, we concluded that the cloned PCR fragment represents a central 126-bp fragment of the *pyk* gene from *C. glutamicum*.

Site specific disruption of the *pyk* gene in *C. glutamicum* E12. The 126-bp fragment of the *C. glutamicum pyk* gene was subcloned into a derivative of the mobilizable vector pSUP301, which resulted in plasmid pMG117. This plasmid was transferred by conjugation from *E. coli* to three different *Corynebacterium* recipient strains: *C. glutamicum* AS019 and E12 (restriction-deficient derivative of AS019) and *C. lactofermentum* 21799 (lysine production strain). Since plasmid pMG117 confers resistance against kanamycin but cannot replicate in *Corynebacterium* cells, plating on kanamycin selected for clones which had integrated pMG117 into the genome via a single crossover between the *pyk* DNA on the plasmid and the genomic *pyk* locus. Figure 3A schematically illustrates the recombination event and demonstrates how it leads to disruption of the *pyk* gene. Only a few kanamycin-resistant clones were isolated, all of which were derivatives of *C. glutamicum* E12, whereas no colonies were obtained from transconjugations with *C. glutamicum* AS019 and *C. lactofermentum* 21799. The low frequency of marker rescue may be due to the small DNA region offered for homologous recombination or incomplete heat inactivation of the DNA restriction systems in strains AS019 and 21799. Two of the isolated kanamycin-resistant derivatives of *C. glutamicum* E12 were assayed for pyruvate kinase activity in cell extracts after growth in complex medium. Both clones retained less than 0.04 U of pyruvate kinase activity, whereas the parent strain E12 exhibited 1.0 U of pyruvate kinase activity when grown under the same conditions. One of the clones, *C. glutamicum* M1, was further characterized for its ability to grow on minimal medium containing ribose or gluconate instead of glucose. Table 2 summarizes the growth behavior of *C. glutamicum* E12 and M1 on different carbon sources. On glucose minimal medium plates, the growth rates of both strains were identical. However, on ribose or gluconate minimal medium plates, strain M1 did not grow whereas the parent strain E12 grew. On ribose plus pyruvate and gluconate plus pyruvate, strains M1 and E12

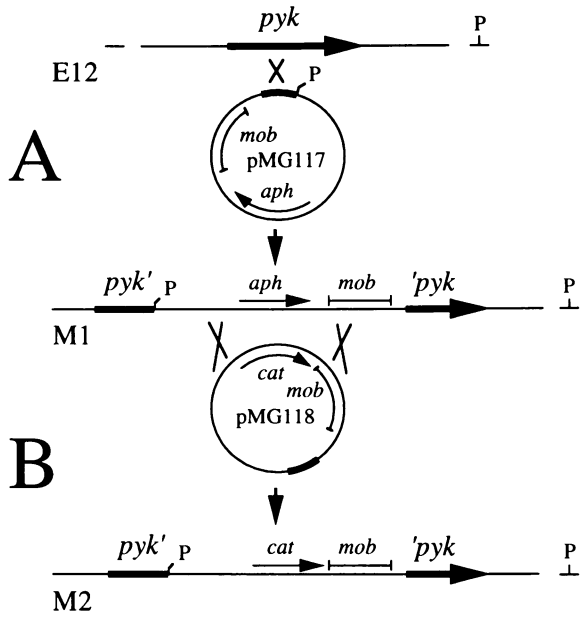

FIG. 3. Recombination events leading to site-specific disruption of the *C. glutamicum pyk* gene (A) and subsequent marker exchange of chloramphenicol resistance for kanamycin resistance (B). Single crossover between the 126-bp *pyk* gene fragment on the nonreplicative plasmid pMG117 and the genomic *C. glutamicum pyk* locus disrupted the *pyk* gene by plasmid integration and conferred resistance against kanamycin. The progeny clone, M1, was used in a second transconjugation experiment to eliminate the kanamycin resistance marker gene *aph*. Double crossover between homologous vector DNA of pMG118 and of integrated pMG117 produced the kanamycin-sensitive, chloramphenicol-resistant *C. glutamicum* clone, M2, with its *pyk* gene remaining inactivated.

grew at identical rates. These data suggested that growth on ribose or gluconate can be used as a criterion to select for *C. glutamicum* pyruvate kinase mutants and to clone the *pyk* gene in a *C. glutamicum pyk* mutant by complementation.

Mapping of the *pyk* locus in the *C. glutamicum* genome. The 126-bp *pyk*-specific PCR fragment was used as a probe for a Southern blot experiment to map the *pyk* gene to restriction fragments of *C. glutamicum* AS019 and *C. lactofermentum* 21799 genomic DNA. The probe hybridized to 10.0-kb *Bam*HI, 5.5-kb *Pst*I, 2.3-kb *Sal*I, and 1.7-kb *Hin*dIII fragments of both AS019 and 21799 DNA. To more precisely map the *pyk* gene within the 10.0-kb *Bam*HI and the 5.5-kb *Pst*I fragments of the *C. glutamicum* chromosome, genomic DNA of the *C. glutamicum pyk* mutant M1 was cleaved with *Pst*I. In this way, the integrated vector pMG117 fused to the 3′ half of the disrupted

TABLE 2. Growth of *C. glutamicum* E12 and *pyk* mutant M1 on minimal medium containing different carbon sources

Carbon source	Growth of[a]:	
	E12	M1
Glucose	++	++
Ribose	+	−
Gluconate	++	−
Ribose + pyruvate	++	++
Gluconate + pyruvate	++	++

[a] ++, normal growth; +, slow growth; −, no growth.

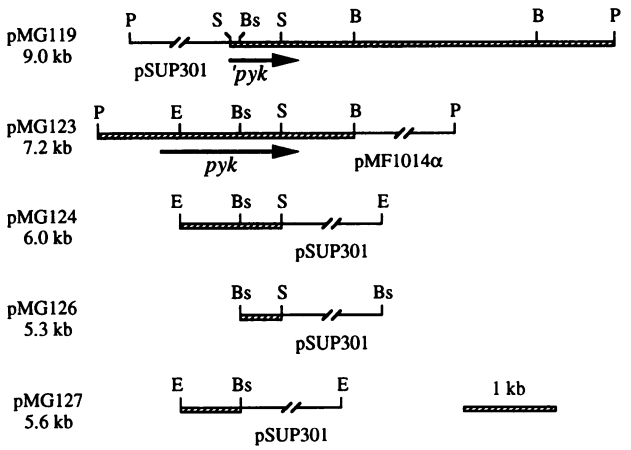

FIG. 4. Physical map of plasmids harboring *C. glutamicum pyk* DNA. Plasmid pMG119 was obtained from genomic DNA of the *C. glutamicum pyk* mutant M1 after excision with *Pst*I, religation, and transformation of *E. coli*. The location of the *Bam*HI and *Pst*I sites in pMG119 together with the data from Southern blot experiments predicted that the *pyk* gene lies on a 2.8-kb *Pst*I-*Bam*HI genomic DNA fragment. Plasmid pMG123 contains the 2.8-kb *Pst*I-*Bam*HI fragment which codes for pyruvate kinase. Plasmids pMG124, pMG126, and pMG127 contain internal fragments of the *pyk* gene cloned into the mobilizable *E. coli* plasmid pSUP301. The plasmids were used to generate the *C. glutamicum* and *C. lactofermentum pyk* mutants A124, A126, and A127 and L124, L126, and L127, respectively, by marker rescue mutagenesis.

pyk gene was released as a *Pst*I fragment (see the structure of the *pyk* locus of strain M1 in Fig. 3). *Pst*I-digested genomic M1 DNA was diluted, recircularized by ligation, and transformed into *E. coli*. All colonies which were obtained harbored the same plasmid, subsequently named pMG119, a restriction map of which is depicted in Fig. 4. Southern blot analysis (data not shown) with the *pyk* PCR fragment as a probe and restriction mapping indicated that a 2.8-kb overlap between the genomic *Bam*HI and *Pst*I DNA fragments contained the complete *pyk* gene.

Cloning of the *pyk* gene in *C. glutamicum*. The three findings described above were the necessary prerequisites to clone the *C. glutamicum pyk* gene by complementation: (i) *C. glutamicum* strain M1 lacks pyruvate kinase activity, (ii) growth on ribose minimal medium can be used to select for complementation of pyruvate kinase deficiency, and (iii) the *C. glutamicum pyk* gene is located on a 2.8-kb *Pst*I-*Bam*HI genomic DNA fragment. As a vector to clone the *pyk* gene, plasmid pMF1014α was used. It is a derivative of the cryptic *C. glutamicum* plasmid pSR1 and harbors the kanamycin resistance gene *aph* as a selective marker. Since the *C. glutamicum pyk* mutant M1 carries the same kanamycin resistance gene integrated at the *pyk* locus, it was not suitable as a host strain in conjunction with the cloning vector pMF1014α. A kanamycin-sensitive analog of strain M1 was generated by substituting the chloramphenicol resistance gene *cat* for the *aph* gene at the *pyk* locus of M1. For this purpose, the *aph* gene was excised from plasmid pMG117 and replaced by a *cat* cartridge, which resulted in pMG118 (Fig. 3B). Plasmid pMG118 was subsequently transconjugated from *E. coli* to *C. glutamicum* M1. Several hundred chloramphenicol-resistant colonies were obtained, five of which were found to be sensitive against kanamycin and at the same time remained unable to grow on ribose minimal medium plates. Figure 3B illustrates the double crossover between pMG118 DNA and integrated pMG117 DNA at the *pyk* locus of strain M1. The resulting *C. glutamicum* M2 was confirmed to lack pyruvate kinase activity in cell extracts. With the proper host strain available, cloning of the *pyk* gene was done as follows: genomic DNA from *C. glutamicum* AS019 was cleaved with *Pst*I and *Bam*HI, and fragments of approximately 2.8 kb were ligated with *Pst*I- and *Bam*HI-digested vector pMF1014α. After electroporation of *C. glutamicum* M2 with the ligation products, the transformants were selected on minimal medium containing ribose as the sole carbon source. Restriction analysis of the plasmids isolated from six clones revealed that they all contained the same 2.8-kb *Pst*I-*Bam*HI DNA fragment. The pyruvate kinase activity of one strain was assayed and found to be 19 U in cell extracts. This represents a 20-fold increase compared with the wild-type level, correlating well with the copy number of the cloning vector pMF1014α. One of the isolated plasmids was named pMG123 (Fig. 4). On the basis of the findings that it complemented the *pyk* mutation in strain M2 and caused an increase in pyruvate kinase activity (Table 3), we concluded that pMG123 harbors the *C. glutamicum pyk* gene.

TABLE 3. Pyruvate kinase activities in *C. glutamicum* E12 and AS019, *C. lactofermentum* 21799, and *pyk* mutants derived from them

Strain	Activity[a]
E12	0.96
M1	0.04
M2	0.01
M2 pMG123[b]	19.10
AS019	1.25
A124	0.02
A126	0.01
A127	0.02
21799	1.60
L124	0.02
L126	0.02
L127	0.02

[a] Activity is express in micromoles per minute per milligram of protein. Results are the mean values of three measurements. The standard deviations 1.8 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for strain M2 pMG123 and <0.1 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for all other strains.

[b] pMG123 harbors the cloned *pyk* gene from *C. glutamicum* AS019.

Inactivation of *pyk* in *C. glutamicum* AS019 and *C. lactofermentum* 21799. Three different fragments from plasmid pMG123 were subcloned in the mobilizable vector pSUP301 to obtain plasmids pMG124, pMG126, and pMG127 (Fig. 4). These plasmids were used to generate *pyk* mutants of the wild-type strain *C. glutamicum* AS019 and the lysine production strain *C. lactofermentum* 21799. Transconjugation of pMG124, pMG126, and pMG127 into AS019 and 21799 yielded kanamycin-resistant colonies in all cases. The corresponding derivatives of *C. glutamicum* AS019 (A124, A126, and A127) and those of *C. lactofermentum* 21799 (L124, L126, and L127) were deficient in pyruvate kinase activity (Table 3). These results suggest that (i) the *pyk* gene extends past the *Eco*RI and *Sal*I sites in plasmid pMG123 (otherwise mutagenesis with pMG124 would not have resulted in a Pyk^- phenotype) and (ii) genes isolated from *C. glutamicum* strains may be used for mutagenesis of *C. lactofermentum* strains.

Lysine fermentations with *pyk* mutant strains. Shake flask fermentations were carried out primarily to determine the impact of pyruvate kinase deficiency on lysine production but also to determine other fermentation products of pyk^+ and *pyk* mutant *C. lactofermentum* strains. Table 4 summarizes the

TABLE 4. Accumulation of fermentation products in cultures of *C. lactofermentum pyk*$^+$ and *pyk* mutant strains after 60 h

Strain	Fermentation product yield (g/liter)[a]					
	Lysine	Valine	Alanine	Acetate	Dihydroxy-acetone	Glyceral-dehyde
21799	26.3	3.2	5.4	6.1	0	0
L124	15.0	5.5	3.1	7.6	3.7	4.2
L126	15.0	3.6	2.6	14.4	4.1	3.5
L127	14.0	2.5	2.1	12.8	4.7	4.4

[a] Results are the mean values of two independent cultures; standard deviation was <5%.

yields of the compounds which accumulated to significant amounts in the culture media. After 60 h of fermentation, *C. lactofermentum* 21799 produced 26.3 g of lysine per liter from 80 g of glucose per liter. During the same time, the *C. lactofermentum pyk* mutants L124, L126, and L127 accumulated, per liter, only 15, 15, and 14 g of lysine, respectively, from 80 g of glucose per liter. In addition, all strains, including the parent strain, produced valine (between 2.5 and 5.5 g/liter) and alanine (between 2.1 and 5.4 g/liter). Acetate, an undesirable side product during lysine fermentation, also accumulated to relatively high levels, 6.1 g/liter in 21799 cultures and 7.6, 14.4, and 12.8 g/liter in L124, L126, and L127 cultures, respectively.

Decrease of lysine and increase of acetate levels were not the only shifts in fermentation products observed in cultures with *pyk* mutants. Two additional compounds were identified by HPLC analysis on the basis of the comparison of retention times and UV spectra with those of authentic compounds. All three *pyk* mutants excreted dihydroxyacetone and glyceraldehyde at concentrations of between 3.5 and 4.7 g/liter.

Surprisingly, we observed differences neither in the growth profile nor in the rate of glucose consumption during the fermentations with *pyk*$^+$ and *pyk* mutant *C. lactofermentum* strains. This indicated that in spite of causing perturbations in glycolysis, inactivation of pyruvate kinase did not impair the ability of *C. lactofermentum* to grow at high rates. To make sure, that the *C. lactofermentum pyk* mutants L124, L126, and L127 did not revert to *pyk*$^+$, pyruvate kinase activity was assayed in cell extracts at the end of the 60-h fermentation. All *pyk* mutants possessed less than 0.01 U of pyruvate kinase activity whereas *C. lactofermentum* 21799 exhibited 2.25 U, proving that the mutations were stable during the entire experiment.

DISCUSSION

On the basis of the observation that pyruvate kinase genes from a wide variety of organisms share extensive homology (11, 16), we have chosen a strategy involving PCR, marker rescue mutagenesis, and complementation in order to clone the *C. glutamicum pyk* gene and to obtain *pyk* mutants of the lysine production strain *C. lactofermentum* 21799. To investigate the impact of pyruvate kinase deficiency on lysine fermentation yields, we had previously undertaken several efforts to isolate *pyk* mutants of *C. lactofermentum* 21799. However, random mutagenesis by exposure of the cells to nitrosoguanidine or UV light, and subsequent screening of more than 60,000 individual colonies for mutants unable to grow on the non-PTS sugars ribose and gluconate, did not yield any pyruvate kinase mutants (data not shown).

By using large internal fragments of the cloned gene for marker rescue mutagenesis, *pyk* mutants of both the wild-type strain *C. glutamicum* AS019 and the lysine production strain *C. lactofermentum* 21799 were obtained. Phenotypically, *pyk* mutant strains did not differ from *pyk*$^+$ strains with respect to growth rate and glucose consumption in shake flask fermentations. This was an unexpected finding because the inactivation of pyruvate kinase was supposed to limit the amount of carbon that enters the tricarboxylic acid cycle. In fact, we hoped to control the metabolism in *pyk* mutants in such a way that (i) generation of energy from glucose remains low, (ii) cell mass accumulation is reduced, (iii) loss of carbon in the tricarboxylic acid cycle is minimized, and (iv) carbon flow will be directed towards the synthesis of OAA from large amounts of PEP. Thus, OAA would mostly be used as a precursor for lysine biosynthesis rather than being consumed in the tricarboxylic acid cycle. However, the lysine titers in cultures of all *C. lactofermentum pyk* mutants were substantially lower than those in the parent strain 21799. Obviously, anabolic reactions and energy production as well as the synthesis of lysine are all processes in competition for pyruvate, which, in pyruvate kinase-deficient strains, is present in limiting amounts only. While cell growth of the mutants was unaffected, lysine production decreased, showing that in these strains metabolic control mechanisms favored the use of pyruvate for growth rather than for lysine production. It is interesting to note that in spite of presumably low availability of pyruvate, the *pyk* mutants produced significant amounts of valine and alanine, which are comparable to those produced by the parent strain. Both amino acids are synthesized from pyruvate as a precursor. It appears that synthesis of valine and alanine is constitutive, whereas synthesis of lysine reaches maximal levels only when pyruvate is readily available. Although acetate accumulated in *C. lactofermentum* L126 and L127 to almost twice the level of that in *C. lactofermentum* 21799 cultures, it does not represent all the carbon which could potentially have been converted into lysine. In addition, dihydroxyacetone and glyceraldehyde, which may be derivatives of dihydroxyacetone phosphate and glyceraldehyde phosphate, respectively, after dephosphorylation either in vivo or in vitro, were formed. In the phosphorylated state, both compounds are metabolic intermediates which may have accumulated in *pyk* mutants because of blockage of the pyruvate kinase reaction. Release of these compounds into the medium may be indicative of an accumulation of glycolytic intermediates upstream of PEP. At this stage, it is not clear why carbon flow through glycolysis should stop at the PEP branch point in *pyk* mutants, because there are two other enzyme reactions which consume PEP besides the one catalyzed by pyruvate kinase: (i) PTS converts PEP into pyruvate, and (ii) PEP carboxylase condenses PEP and CO_2 to OAA. Both pyruvate and OAA represent the only carbon precursors required in equimolar amounts for the biosynthesis of lysine. Assuming that resting *C. lactofermentum* cells do not consume carbon for their own metabolism, the concerted action of the glycolysis and pentosephosphate cycle would provide enough energy and reduction equivalents to reach the theoretical limit of 75% moles per mole of glucose converted to lysine in *pyk* mutants (21). The results presented in this study, however, point out that the central carbon metabolism in amino acid-producing *Corynebacterium* strains is not well understood and that extensive analysis of specific mutations will be required before redirection of carbon flow can be used to optimize the substrate-product ratio in these organisms.

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REFERENCES

1. **Archer, J. A. C., M. T. Follettie, and A. J. Sinskey.** 1989. Biology of *Corynebacterium glutamicum*: a molecular approach, p. 27–33. *In* C. L. Hershberger, S. W. Queener, and G. Hegeman (ed.), Genetics and molecular biology of industrial microorganisms. American Society for Microbiology, Washington, D.C.
2. **Burke, R. L., P. Tekamp-Olson, and R. Najarian.** 1983. The isolation, characterization, and sequence of the pyruvate kinase gene of *Saccharomyces cerevisiae*. J. Biol. Chem. **58:**2193–2201.
3. **de Graaff, L., and J. Visser.** 1988. Structure of the *Aspergillus nidulans* pyruvate kinase gene. Curr. Genet. **14:**553–560.
4. **Duwat, P., S. D. Ehrlich, and A. Gruss.** 1992. Use of degenerate primers for polymerase chain reaction cloning and sequencing of the *Lactococcus lactis* subsp. *lactis recA* gene. Appl. Environ. Microbiol. **58:**2674–2678.
5. **Follettie, M. T., O. P. Peoples, C. Agoropoulou, and A. J. Sinskey.** 1993. Gene structure and expression of the *Corynebacterium flavum* N13 *ask-asd* operon. J. Bacteriol. **175:**4096–4103.
6. **Follettie, M. T., and A. J. Sinskey.** 1986. Recombinant DNA technology for *Corynebacterium glutamicum*. Food Technol. **40:** 88–94.
7. **Gubler, M., S. M. Park, M. Jetten, G. Stephanopoulos, and A. J. Sinskey.** 1993. Effects of phosphoenol pyruvate carboxylase deficiency on metabolism and lysine production in *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **40:**857–863.
8. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
9. **Jetten, M. S. M., M. E. Gubler, M. M. McCormick, G. E. Colon, M. T. Follettie, and A. J. Sinskey.** 1993. Molecular organization and regulation of the biosynthetic pathway for aspartate-derived amino acids in *Corynebacterium glutamicum*, p. 97–104. *In* R. H. Baltz, G. D. Hegeman, and P. L. Skatrud (ed.), Industrial microorganisms: basic and applied molecular genetics. American Society for Microbiology, Washington, D.C.
10. **Jetten, M. S. M., and A. J. Sinskey.** 1993. Characterization of phosphoenol pyruvate carboxykinase from *Corynebacterium glutamicum*. FEMS Microbiol. Lett. **111:**183–188.
11. **Llanos, R. M., C. J. Harris, A. J. Hillier, and B. E. Davidson.** 1993. Identification of a novel operon in *Lactococcus lactis* encoding three enzymes for lactic acid synthesis: phosphofructokinase, pyruvate kinase, and lactate dehydrogenase. J. Bacteriol. **175:** 2541–2551.
12. **Miyajima, R., and I. Shiio.** 1970. Regulation of aspartate family amino acid biosynthesis in *Brevibacterium flavum*. III. Properties of homoserine dehydrogenase. J. Biochem. **68:**311–319.
13. **Mori, M., and I. Shiio.** 1987. Pyruvate formation and sugar metabolism in an amino acid-producing bacterium, *Brevibacterium flavum*. Agric. Biol. Chem. **51:**129–138.
14. **Mori, M., and I. Shiio.** 1987. Phosphoenol pyruvate:sugar phosphotransferase systems and sugar metabolism in *Brevibacterium flavum*. Agric. Biol. Chem. **51:**2671–2678.
15. **Ozaki, H., and I. Shiio.** 1969. Regulation of the TCA and glyoxylate cycles in *Brevibacterium flavum*. II. Regulation of phosphoenol pyruvate carboxylase and pyruvate kinase. J. Biochem. **66:**297–311.
16. **Sakai, H., and T. Ohta.** 1993. Molecular cloning and nucleotide sequence of the gene for pyruvate kinase of *Bacillus stearothermophilus* and the production of the enzyme in *Escherichia coli*. Eur. J. Biochem. **211:**851–859.
17. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning, a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. **Sano, K., and I. Shiio.** 1970. Microbial production of L-lysine. III. Production by mutants resistant to *S*-(2-aminoethyl)-L-cysteine. J. Gen. Microbiol. **16:**373–391.
19. **Schäfer, A., J. Kalinowski, R. Simon, A.-H. Seep-Feldhaus, and A. Pühler.** 1990. High-frequency conjugal plasmid transfer from gram-negative *Escherichia coli* to various gram-positive coryneform bacteria. J. Bacteriol. **172:**1663–1666.
20. **Schwarzer, A., and A. Pühler.** 1991. Manipulation of *Corynebacterium glutamicum* by gene disruption and replacement. Bio/Technology **9:**84–87.
21. **Stephanopoulos, G., and J. J. Vallino.** 1991. Network rigidity and metabolic engineering in metabolite overproduction. Science **252:** 1675–1681.
22. **Tani, K., M. C. Yoshida, H. Satoh, K. Mitamura, T. Noguchi, T. Tanaka, H. Fujii, and S. Miwa.** 1988. Human M_2-type pyruvate kinase: cDNA cloning, chromosomal assignment and expression in hepatoma. Gene **73:**509–516.
23. **Tosaka, O., and K. Takinami.** 1978. Pathway and regulation of lysine biosynthesis in *Brevibacterium lactofermentum*. Agric. Biol. Chem. **42:**95–100.
24. **Tosaka, O., K. Takinami, and Y. Hirose.** 1978. L-lysine production by *S*-(2-aminoethyl)-L-cysteine and α-amino-β-hydroxyvaleric acid resistant mutants of *Brevibacterium lactofermentum*. Agric. Biol. Chem. **42:**745–752.
25. **Yoshihama, M., K. Higashiro, E. A. Rao, M. Akedo, W. G. Shanabruch, M. T. Follettie, G. C. Walker, and A. J. Sinskey.** 1985. Cloning vector system for *Corynebacterium glutamicum*. J. Bacteriol. **162:**591–597.