JOURNAL OF BACTERIOLOGY, Oct. 1995, p. 5719–5722
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology
Vol. 177, No. 19

Cloning of the Two Pyruvate Kinase Isoenzyme Structural Genes from *Escherichia coli*: the Relative Roles of These Enzymes in Pyruvate Biosynthesis

ELIZABETH PONCE, NOEMÍ FLORES, ALFREDO MARTINEZ, FERNANDO VALLE, AND FRANCISCO BOLÍVAR*

Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México (UNAM), Cuernavaca, Morelos 62271, Mexico

Received 12 May 1995/Accepted 20 July 1995

We report the cloning of the *pykA* and *pykF* genes from *Escherichia coli*, which code for the two pyruvate kinase isoenzymes (ATP:pyruvate 2-*O*-phosphotransferases; EC 2.7.1.40) in this microorganism. These genes were insertionally inactivated with antibiotic resistance markers and utilized to interrupt one or both *pyk* genes in the *E. coli* chromosome. With these constructions, we were able to study the role of these isoenzymes in pyruvate biosynthesis.

Pyruvate is a key intermediate in catabolic and biosynthetic reactions, and this is the reason why there are several metabolic routes that can deliver this compound (Fig. 1). *Escherichia coli*, when growing on glucose as the only carbon source, synthesizes most of its pyruvate through the coupled mechanism of glucose transport by the phosphotransferase transport system (PTS or PT system) (5). During this process, the phosphate group from phosphoenolpyruvate (PEP) is transferred to glucose, generating pyruvate and glucose-6-phosphate (9, 10). Pyruvate can also be synthesized from gluconate through the Entner-Doudoroff pathway (3). Another mechanism to synthesize pyruvate is through the action of pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase; EC 2.7.1.40), which catalyzes the conversion of PEP and ADP into pyruvate and ATP, at the final stage of the glycolytic pathway. In *E. coli*, there are two pyruvate kinase isoenzymes, PykF and PykA, encoded by the *pykF* and *pykA* genes, respectively.

Garrido-Pertierra and Cooper have reported that in *E. coli*, under aerobic conditions and in the absence of pyruvate kinase activities, a functioning PT system can provide enough pyruvate to sustain wild-type normal growth rates. In the same report, it was mentioned that the absence of PykA did not seem to affect growth on any of many carbon sources; therefore, in that study, the role of PykA was not clearly defined (4).

We report here the cloning of both *pykA* and *pykF* structural genes from *E. coli* and the construction and analysis of strain derivatives in which the chromosomal copy of one or both genes was interrupted by antibiotic resistance markers. We present data on cell growth effects of these mutations, in the wild-type background and in strains that lack the PT system but are capable of glucose transport by using the galactose permease (GalP) (2). Results obtained strongly suggest that, at least under conditions where glucose is the only carbon source, both pyruvate kinase isoenzymes have an active role in pyruvate biosynthesis, but it appears that the PykF isoenzyme contributes to a greater extent. As expected, a double *pykA pykF* mutant is capable of growing on glucose and on gluconate but incapable of growing on ribose as the only carbon source, and the triple *pts pykA pykF* mutant is capable of growing on gluconate but incapable of growing on glucose or ribose.

Cloning and sequencing of the *E. coli pykA* and *pykF* structural genes. Using PCR standard techniques and primers specific for both of the two pyruvate kinase genes, we cloned these two structural genes into plasmids. The nucleotide sequences of both genes were obtained and were found to be almost identical to the deposited sequences (GenBank release 86.0) (data not shown).

Generation of *pykA* and *pykF* insertional inactivation mutants. *E. coli* mutants altered in one or both *pyk* genes were isolated after insertional mutagenesis of the *pykA* or *pykF* gene. This was performed by using antibiotic resistance cassettes (Table 1). These insertional mutations were separately integrated into the chromosome of *E. coli* ATCC 47002 (F^- *recB21 recC22 sbc-15 leu-6 ara-14 his-4* λ^-) (8) and subsequently transduced into strain JM101 [*supE thi* Δ(*lac-proAB*) (F' *traD36 proAB lacI*^{q}*Z*ΔM15)] or PB12 [same as JM101 but Δ(*ptsH-I-crr*)::*kan*; Glc^+]. Chromosomal gene interruptions were confirmed, in all cases, by Southern hybridization (data not shown).

Effect of *pyk* and *pts* mutations on cell growth on glucose as the only carbon source. To study the relative roles of the two *pyk* genes and their products in cell metabolism, one or both genes were interrupted in the bacterial chormosome. Also, because the PT system is the major source of pyruvate in *E. coli* (5), we isolated a mutant capable of glucose transportation through the GalP permease (2), in a background strain that carries a deletion of the PT system (a deletion mutant lacking *ptsH*, *ptsI*, and *crr* genes) (6). Using this *pts* Glc^+ mutant PB12, we separately incorporated each or both of the *pyk* mutations in this background. When these strains were grown in a 6-liter fermentor with M9 medium supplemented with glucose and Casamino Acids (11), no differences in growth patterns were observed for any of them; all grew as well as the parental strain JM101 (data not shown). However, if the M9 medium used did not contain Casamino Acids, interesting differences were observed. In this type of medium, all cultures of mutants and parental strains reached the same final optical density (data not shown). However, strains carrying *pyk* or *pts* mutations presented different growth rates, reported here as the generation time (t_D) obtained during the exponential growth phase

* Corresponding author. Mailing address: Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México (UNAM), Apdo. Postal 510-3, Cuernavaca, Morelos 62271, México. Phone and fax: (52) (73) 17-23-99. Electronic mail address: valle@pbr322.ceingebi.unam.mx.

Downloaded from http://jb.asm.org/ on October 31, 2020 by guest

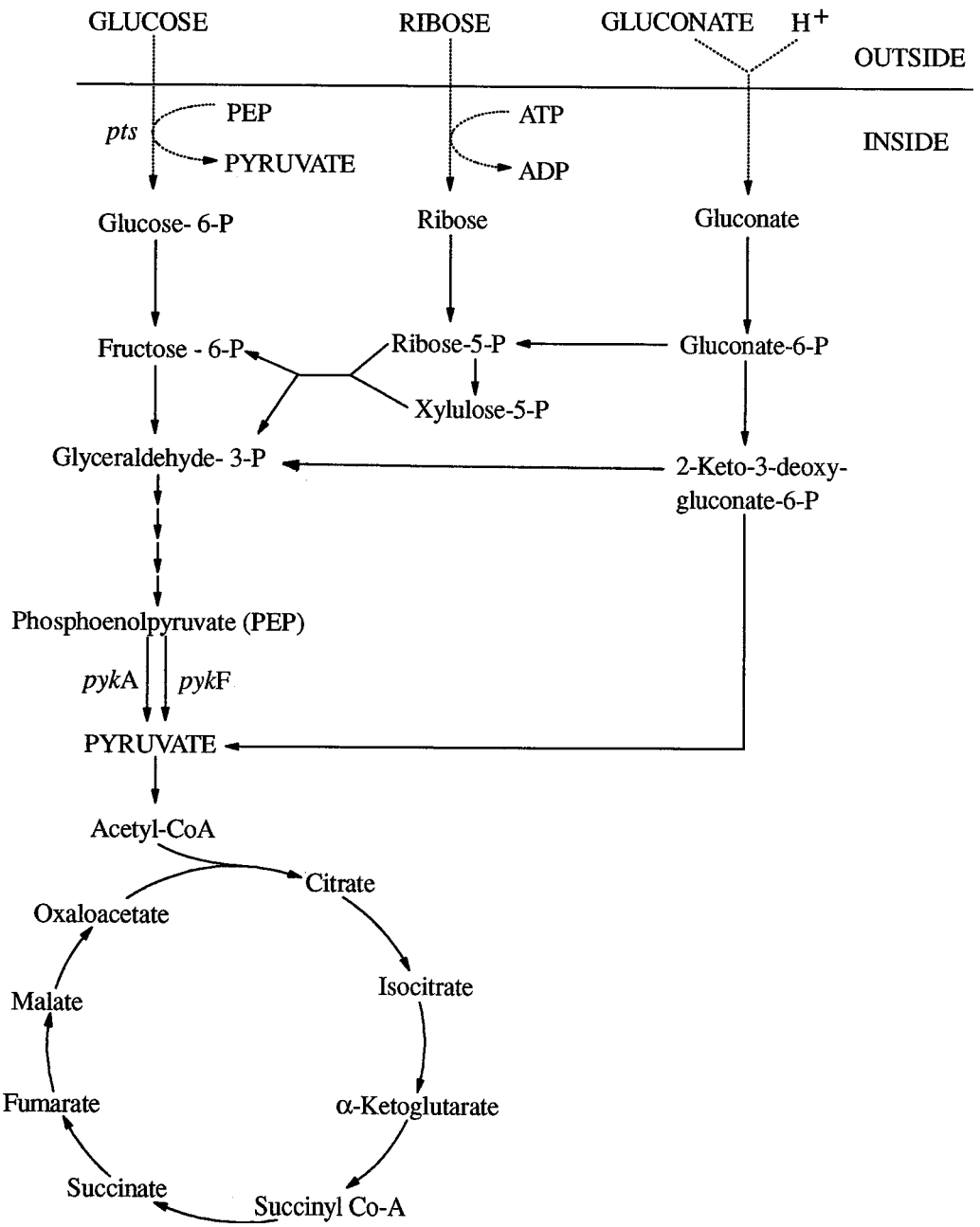

FIG. 1. Metabolic routes in *E. coli* that allow the synthesis of pyruvate from different carbon sources. CoA, coenzyme A; Glucose-6-P, glucose 6-phosphate.

(Table 1). Strains carrying the single *pykA* or *pykF* mutations, in a wild-type (Pts^+) background, showed a slight increase in their t_D in comparison with the parental strain. The strain carrying the double *pykA pykF* mutation showed a t_D 40% higher than that observed for JM101 (Table 1). The wild-type strain and the single *pyk* mutants needed 7 and 8 h, respectively, to reach maximum cell density (data not shown). PB12 (*pts* Glc^+) showed a t_D of 106.4 min (80% higher than the t_D obtained for the wild-type strain and 40% higher than that for the strain carrying the *pykA pykF* double mutation). The t_D obtained for the *pts pykA* mutant (98.5 min) was similar to the t_D of the strain carrying only the *pts* deletion, and this double mutant also took the same time to reach stationary phase. However, the introduction of the *pykF* mutation into the *pts* (Glc^+) background had the strongest effect in slowing cell growth. This strain showed a t_D of 141 min (an increase of almost 140% over the t_D shown by the wild-type strain). Finally, as expected, the triple *pts pykA pykF* mutant was incapable of growing on glucose as the only carbon source (Table 1).

Measurements of the two pyruvate kinase specific activities in the *pyk* and *pts* mutants on glucose as the only carbon source. The specific activities of both pyruvate isoenzymes were measured for the wild type and the *pyk* and *pts* mutants in the same set of experiments as those described above, by the method reported by Malcovati and Valentini (7). The results presented in Table 1 show that under these conditions, in the wild-type strain the specific activity of the PykF enzyme was

Downloaded from http://jb.asm.org/ on October 31, 2020 by guest

TABLE 1. t_Ds, pyruvate kinase specific activities, and comparative growth on different carbon sources for the wild type and strains carrying the *pyk* and *pts* mutations

Strain (description)	t_D (min)[a]	Sp act (IU/mg of protein)[a]		Comparative growth on carbon source[b]:		
		PykF	PykA	Glucose	Ribose	Gluconate
JM101 (wild type)	58.8 ± 2.10	0.42 ± 0.03	0.026 ± 0.002	+++++	+++++	+++++
PB22 (same as JM101 but *pykA*::*cat*[c])	69.3 ± 3.95	0.50 ± 0.04	ND	++++	++++	++++
PB24 (same as JM101 but *pykF*::*cat*)	69.4 ± 1.72	ND	0.029 ± 0.003	++++	+++	+++
PB25 (same as JM101 but *pykA*::*kan*[d] *pykF*::*cat*)	82.2 ± 0.60	ND	ND	+++	−	++
PB12[e] [same as JM101 but Δ(*ptsH-I-crr*)::*kan*; Glc$^+$]	106.4 ± 2.79	0.25 ± 0.02	0.037 ± 0.003	+++	++	++++
PB26 (same as PB12 but *pykA*::*cat*)	98.5 ± 1.62	0.25 ± 0.03	ND	+++	++	+++
PB27 (same as PB12 but *pykF*::*cat*)	141.0 ± 0.06	ND	0.042 ± 0.004	++	+	++
PB28 (same as PB12 but *pykA*::*cat pykF*::*gen*[f])		ND	ND	−	−	+

[a] Data are presented as the average of two to four independent measurements. Cultures were grown aerobically in a 6-liter fermentor in M9 medium supplemented with glucose as the only carbon source. ND, not detected.
[b] The growth response was observed after 48 h of cultivation at 37°C on minimal medium plates supplemented with either glucose (10 mM), ribose (15 mM), or gluconate (10 mM) as the only carbon source.
[c] The chloramphenicol resistance gene was obtained, as a *Sma*I fragment, from plasmid PCAT19 (1).
[d] The kanamycin resistance gene was obtained, as a *Bam*HI fragment, from plasmid pNK862 (13).
[e] An article giving a description and analysis of this strain is in preparation (2).
[f] The gentamycin resistance gene was obtained, as a *Sma*I fragment, from plasmid pGMΩ1 (12).

more than 15 times higher than that of the PykA isoenzyme. When one of the two *pyk* genes was interrupted, the enzymatic level of the remaining pyruvate kinase was slightly increased. Interestingly, strains with the *pts* background showed differences in both pyruvate kinase specific activities in comparison with those in the parental and single *pyk* backgrounds. In the *pts* background, the PykF specific activity decreased from 0.42 IU/mg of protein (in the parental strain) to 0.25 IU/mg of protein. On the other hand, the PykA specific activity in the *pts* background increased slightly over the level in the wild-type strain (Table 1).

The results presented thus far demonstrate that under the growth conditions tested, with glucose as the only carbon source, mutations in one or both *pyk* genes did affect cell growth kinetics in strains with a functional PT system. These effects were most obvious in the *pykF pykA* double mutant.

These results are in agreement with the proposition that *E. coli* in fact needs both pyruvate kinases to reach maximal growth rates (Table 1). However, cultures of the *pyk* mutants finally reached the same final optical density as did those of the parental strain JM101 (data not shown). One explanation for these results could be that the PT system and the two pyruvate kinase enzymes are coordinated in order to maintain a very low level of PEP, an allosteric regulator of several glycolytic enzymes and therefore a key intermediate in the biosynthesis of pyruvate and glucose 6-phosphate.

A deletion of the PT system, on the other hand, caused a strong increase in the generation time in comparison with that of the parental strain. Interestingly, the PykF specific activity decreased 40% in the *pts* background compared with the activity in the wild-type strain, while in the same background the PykA specific activity increased approximately by the same percentage. Nevertheless, in the *pts* mutant, under these conditions, the PykF specific activity was still eight times superior to that of PykA.

In addition, it was observed that both pyruvate kinase isoenzymes maintained the same specific activities throughout the exponential and stationary phases in each particular strain (data not shown). These results suggest that both Pyk enzymes are important throughout the life cycle of the organism and not only in a particular phase.

Effects of *pyk* and *pts* mutations on cell growth on other carbon sources. When ribose, a non-PT system sugar, was used as the only carbon source (Table 1), single *pykA* or *pykF* mutants were capable of growing on this sugar. However, the strain carrying the double *pykA pykF* mutation was unable to grow on ribose but capable of growing on gluconate, which is catabolized through the Entner-Doudoroff pathway to yield pyruvate (3). The double *pykA pykF* mutant did not grow on ribose because it lacks the ability to produce pyruvate. However, if the *E. coli* strain carries only one of the *pyk* mutations, the strain is capable of growing on ribose because pyruvate is synthesized via the remaining pyruvate kinase (Fig. 1). Finally, it is important to notice that a *pykA* strain grew better than a *pykF* strain with ribose as the only carbon source (Table 1).

Taken together, these results suggest that both pyruvate kinase isoenzymes have active roles in pyruvate biosynthesis, but it appears that the PykF enzyme contributes to a greater extent. Since pyruvate is a key intermediate in cell metabolism, it is important for the bacterial cell to have several alternatives to allow its synthesis. These different mechanisms are probably differentially regulated in order to have adequate responses to environmental changes.

We thank Paul Gaytán and Eugenio López for oligonucleotide synthesis and Alan Berry for a critical review of the manuscript.

This work was supported by grants 2031-N9302 from Consejo Nacional de Ciencia y Tecnología, México, PNUD/MEX/93/019 from Programa de las Naciones Unidas para el Desarrollo, and Genencor International.

REFERENCES

1. **Clairborne, W. F.** 1992. An improved chloramphenicol resistance gene cassette for site-directed marker replacement mutagenesis. BioTechniques **12:** 223–225.
2. **Flores, N., et al.** Unpublished data.
3. **Fraenkel, D. G.** 1987. Glycolysis, pentose phosphate pathway, and Entner-Doudoroff pathway, p. 142–149. *In* F. C. Neidhardt, J. L. Ingraham, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
4. **Garrido-Pertierra, A., and R. A. Cooper.** 1977. Pyruvate formation during the catabolism of simple hexose sugars by *Escherichia coli*: studies with pyruvate kinase-negative mutants. J. Bacteriol. **129:**1208–1214.
5. **Holms, W. H.** 1986. The central metabolic pathway of *Escherichia coli*: relationship between flux and control at a branch point, efficiency of conversion to biomass, and excretion of acetate, p. 69–105. *In* B. L. Horecker and E. R. Stadtman (ed.), Current topics in cell regulation. Academic Press, Inc., New York.
6. **Lévy, S., G.-Q. Zeng, and A. Danchin.** 1990. Cyclic AMP synthesis in *Esch-*

Downloaded from http://jb.asm.org/ on October 31, 2020 by guest

erichia coli strains bearing known deletions of the *pts* phosphotransferase operon. Gene **86:**27–33.
7. **Malcovati, M., and G. Valentini.** 1982. AMP- and fructose 1,6-bisphosphate-activated pyruvate kinases from *Escherichia coli*. Methods Enzymol. **90:**170–179.
8. **Oden, K. L., L. C. DeVeaux, C. R. T. Vibat, J. E. Cronan, Jr., and R. B. Gennis.** 1990. Genomic replacement in *Escherichia coli* K-12 using covalently closed circular plasmid DNA. Gene **96:**29–36.
9. **Postma, P. W., J. W. Lengeler, and G. R. Jacobson.** 1993. Phosphoenolpyruvate: carbohydrate phosphotransferase system of bacteria. Microbiol. Rev. **57:**543–594.
10. **Saier, M. H., Jr., and A. M. Chin.** 1990. Energetics of bacterial phosphotransferase system in sugar transport and the regulation of carbon metabolism, p. 273–299. *In* T. A. Krulwich (ed.), The bacteria: a treatise on structure and function, vol. XII. Bacterial energetics. Academic Press, Inc., New York.
11. **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.
12. **Schweizer, H. P.** 1993. Small broad-host-range gentamycin resistance gene cassettes for site-specific insertion and deletion mutagenesis. BioTechniques **15:**831–833.
13. **Way, J. C., M. A. Davies, D. Morisato, D. E. Roberts, and N. Klecner.** 1984. New Tn10 derivatives for transposon mutagenesis and for construction of *lac*Z operon fusions by transposition. Gene **32:**369–379.

Downloaded from http://jb.asm.org/ on October 31, 2020 by guest