APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1996, p. 1808–1810 Vol. 62, No. 5
0099-2240/96/$04.00+0

Enhanced Production of Succinic Acid by Overexpression of Phosphoenolpyruvate Carboxylase in *Escherichia coli*

CYNTHIA SANVILLE MILLARD,[1] YUN-PENG CHAO,[2] JAMES C. LIAO,[2] AND MARK I. DONNELLY[1]*

Department of Chemical Engineering, Texas A&M University, College Station, Texas 77843,[2] and Environmental Research Division, Argonne National Laboratory, Argonne, Illinois 60439[1]

Received 20 July 1995/Accepted 22 February 1996

Fermentative production of succinic acid from glucose by *Escherichia coli* was significantly increased by overexpression of phosphoenolpyruvate carboxylase. In contrast, overexpression of phosphoenolpyruvate carboxykinase had no effect. Under optimized conditions, induction of the carboxylase resulted in a 3.5-fold increase in the concentration of succinic acid, making succinic acid the major fermentation product by weight.

Dicarboxylic acids produced through fermentation represent a potential route to the production of commodity chemicals from renewable feedstocks (12). Formation of succinic acid from glucose through the reductive reactions of the tricarboxylic acid cycle involves incorporation of carbon dioxide and, theoretically, can result in a net increase in mass relative to the carbohydrate substrate. Succinic acid itself can subsequently serve as a feedstock for chemical conversion to commodity chemicals such as 1,4-butanediol and tetrahydrofuran (12) or to new "green" solvents or polymers through esterification reactions. The best current technology for the biological production of succinic acid employs the strict anaerobe *Anaerobiospirillum succiniciproducens*. Under optimized conditions, this organism ferments glucose to a mixture of succinic and acetic acids in a two-to-one molar ratio at an 87% yield of succinic acid, based on weight (7). In order to expand the range of organisms that could be used in the production of succinic acid or other dicarboxylic acids, we have initiated efforts to increase the production of succinic acid in *Escherichia coli* cells through metabolic engineering.

Phosphoenolpyruvate (PEP) occupies a critical position in the metabolism of *E. coli*. It is the final intermediate of glycolysis and is the substrate which is carboxylated in the first step of succinic acid formation. Its high-energy enol-phosphate ester bond represents the net energetic gain of glycolysis. PEP is also a required cosubstrate for uptake of glucose, the first intermediate in gluconeogenesis from many compounds, and a substrate for the first committed step in the biosynthesis of aromatic amino acids (4). Accordingly, the partitioning of PEP is highly regulated. *E. coli* elaborates two enzymes capable of carboxylating PEP to produce oxaloacetate (OAA). PEP carboxylase functions aerobically to replenish oxaloacetate consumed in biosynthetic reactions (1). Under fermentative conditions, PEP carboxylase also has a catabolic function; it directs a portion of the PEP to succinic acid (11). The second enzyme, PEP carboxykinase, functions physiologically in gluconeogenesis, catalyzing the nucleotide triphosphate-dependent decarboxylation and phosphorylation of oxaloacetate to yield PEP (5, 21). The pattern of induction of these two enzymes reflects these roles; PEP carboxylase is produced during growth on glycolytic substrates, and PEP carboxykinase is produced during growth on succinate and other four-carbon compounds (10, 20).

In the mixed-acid fermentation of *E. coli*, succinic acid is normally a minor product (3, 6). We have evaluated the effect of overexpression of PEP carboxylase and PEP carboxykinase on the distribution of fermentation products, in particular, on the amount of succinic acid produced. Cloning of the genes for the two *E. coli* enzymes, *ppc* for PEP carboxylase and *pck* for PEP carboxykinase, has been reported previously (4). Plasmids pPC201 and pCK601 contain the genes *ppc* and *pck*, respectively, cloned into the expression vector pJF118EH (9). In this vector, expression is under the control of the *tac* promoter, which allows induction by isopropyl-β-D-thiogalactopyranoside (IPTG) in the presence of glucose. The host strain, JCL1208, lacks the *lac* operon but contains a chromosomally inserted *lacI*^{q} gene.

Cultures were grown anaerobically on 10 ml of Luria-Bertani medium supplemented with glucose in sealed serum tubes that also contained 0.15 g of solid $MgCO_3$ to stabilize the pH. At the end of fermentation, pH values were approximately 6.0. The headspace gas was exchanged for sterile, anoxic CO_2 by use of a gassing manifold (2) and pressurized to 1 atm (101.29 kPa). As appropriate, the medium also contained ampicillin or carbenicillin (100 μg/ml) and 0.1 mM IPTG (4). To evaluate overexpression of the genes, cells were grown in serum tubes without $MgCO_3$, and cell density was monitored with a Spectronics 20 spectrometer. At an optical density at 600 nm of 0.5 to 1.0, the cultures were centrifuged. The medium was then expelled by use of a Vacutainer needle and replaced with fresh anoxic medium containing 0.1 mM IPTG, and the tubes were repressurized. The cultures were harvested after 6 h, and extracts were prepared by osmotic lysis following treatment of cells with lysozyme (1 mg/ml) on ice for 1 h and a single freeze-thaw cycle. Assays were performed according to published procedures (10, 19). Endogenous malate and lactate dehydrogenases were also measured.

Induction by 100 μM IPTG under fermentative conditions with glucose (11 g/liter) as the carbon source resulted in expression of the carboxykinase and carboxylase to levels 50- to 100-fold higher than those observed with the host strain (Table 1). The PEP carboxykinase activity was slightly higher than that of the carboxylase under the assay conditions employed. Denaturing polyacrylamide gel electrophoresis showed that, in each case, a protein of the expected molecular weight, approximately 95,000 for PEP carboxylase or 51,000 for PEP carboxykinase (8, 15), was very highly overexpressed (data not shown). The presence of IPTG had no significant effect on

* Corresponding author. Mailing address: Argonne National Laboratory, Bldg. 202, Rm. BE111, 9700 So. Cass Ave., Argonne, IL 60439. Phone: (708) 252-7432. Fax: (708) 252-7709. Electronic mail address: donnelly@anl.gov.

TABLE 1. Induction of enzymatic activities

Strain	IPTG	Sp act (μmol/min/mg of protein)			
		PEP carboxylase	PEP carboxykinase	Malate dehydrogenase	Lactate dehydrogenase
JCL1208	−	0.21	0.13	0.52	2.10
	+	0.25	0.23	0.63	2.27
JCL1208(pPC201)	−	0.28	0.12	0.75	1.42
	+	12.30	0.19	0.49	0.90
JCL1208(pCK601)	−	0.10	0.86	0.80	2.00
	+	0.14	15.10	0.59	0.90

expression of chromosomal *E. coli* genes, as reflected by the activities observed for JCL1208.

To determine the potential of the two enzymes to divert PEP to succinic acid, we cultured the strains as described above, but with 23 g of glucose per liter, and analyzed the distribution of products formed after 18 h, at which time all the glucose had been consumed (Table 2). Six replicate cultures were analyzed. Products were separated by high-pressure liquid chromatography on a Shimadzu LC-10A chromatographic system, detected by UV absorbance and refractive index, and quantified by comparison to standards of known concentration. A Bio-Rad Aminex HPX-87H column (7.8 by 300 mm) was eluted isocratically with 5 mM sulfuric acid, and data were collected and analyzed with an EZChrom chromatographic data system (Scientific Software, Inc.).

Overexpression of PEP carboxylase caused a significant and reproducible increase in the amount of succinic acid formed, whereas overexpression of PEP carboxykinase did not (Table 2). The average amount of succinic acid formed increased from 3.27 to 4.44 g/liter when the carboxylase was induced. When PEP carboxykinase was induced, on the other hand, succinic acid formation was not affected; the distribution of the fermentation products was the same within experimental error as that observed with the host strain (Table 2).

Because the fermentations required 18 h to complete and lowered the pH of the medium, the ampicillin initially present most likely was destroyed and selective pressure for maintaining the plasmid was lost. Therefore, we optimized the production of succinic acid by using the antibiotic carbenicillin, which is stabler at lower pH values, and periodically added more carbenicillin to the medium to replace that destroyed enzymatically. Glucose was increased to 37 g/liter, and $MgCO_3$ was increased to 0.5 g in 10 ml of Luria-Bertani medium in sealed serum tubes. A control culture of JCL1208 lacked carbenicillin and IPTG. Under these conditions, induction of PEP carboxylase increased the production of succinic acid 3.5-fold, from 3.0 g/liter in the control culture to 10.7 g/liter, making succinic acid the major fermentation product by weight (Table 3). The amounts of all other products were significantly smaller than in the control culture. Lactic acid, which was present at higher levels in the control culture under these conditions, was the most affected.

These results demonstrate the potential of overexpression of a critical branchpoint enzyme, PEP carboxylase, to alter the distribution of fermentation products in *E. coli* cells. In principle, it should be possible to increase succinic acid production further by inactivation of the lactate dehydrogenase gene. The results also demonstrate constraints on metabolic manipulations; overexpression of PEP carboxykinase had no effect on product distribution. This result is significant because *A. succiniciproducens* employs a PEP carboxykinase in its succinate-acetate fermentation, not a PEP carboxylase (18). A carboxykinase may be more suitable for a pathway that makes predominately succinic acid because it generates ATP, conserving the energy gained in glycolysis, whereas a PEP carboxylase would dissipate that energy. A possible explanation of the results is that the PEP carboxykinase of *A. succiniciproducens* has kinetic properties which make it better suited for its catabolic role. However, K_m values reported for the *E. coli* and *A. succiniciproducens* PEP carboxykinases—70 and 54 μM, respectively, for PEP, 50 and 420 μM for ADP, and 13 and 17 mM for bicarbonate (14, 17)—if anything suggest that the *E. coli* enzyme would perform better under physiological conditions. Allosteric inhibition of the *E. coli* PEP carboxykinase could also explain its lack of effect, but the enzyme is a monomer, and previous reports of allosteric inhibition by NADH have been refuted (13, 22). In contrast, allosteric regulation of PEP carboxylase is well documented (16).

The most notable difference between the two *E. coli* enzymes is their affinity for bicarbonate. The PEP carboxylase has a K_m toward bicarbonate of 0.15 mM, compared with the value of 13 mM for the carboxykinase. However, the PEP carboxykinase from *A. succiniciproducens* also has a high K_m value for bicarbonate, 17 mM, yet functions effectively in the succinate-acetate fermentation. Possibly, differences in the internal concentration of bicarbonate in the two organisms result in the different physiological consequences. Alternatively, unknown regulatory controls at the enzyme level may prevent the *E. coli* PEP carboxykinase from functioning in the reverse direction in vivo.

TABLE 2. Effect of *ppc* and *pck* genes on product distribution

Strain	Amt (g/liter) of product ± SD[a]				
	Succinic acid	Lactic acid	Formic acid	Acetic acid	Ethanol
JCL1208	3.27 ± 0.25	1.24 ± 0.10	2.67 ± 0.26	4.66 ± 0.12	3.92 ± 0.09
JCL1208(pPC201)	4.44 ± 0.14	1.01 ± 0.11	2.38 ± 0.12	4.64 ± 0.16	3.80 ∓ 0.13
JCL1208(pCK601)	3.26 ± 0.37	1.14 ± 0.18	2.80 ± 0.21	4.65 ± 0.11	3.87 ± 0.14

[a] Values are means of three replicates.

TABLE 3. Optimization of diversion of PEP to succinic acid

Strain	Amt (g/liter) and yield (mol%) of product											
	Succinic acid		Lactic acid		Pyruvic acid		Formic acid		Acetic acid		Ethanol	
	Amt	Yield	Amt	Yield	Amt	Yield	Amt	Yield	Amt	Yield	Amt	Yield
JCL1208	3.0	12	4.5	24	0.2	1	2.5	26	6.5	51	7.8	80
JCL1208(pPC201)	10.7	45	2.4	13	0.1	1	1.5	16	5.5	45	7.0	74

This work was supported by the Alternative Feedstock Program of the U.S. Department of Energy (DOE), Office of Industrial Technology, and by DOE's Assistant Secretary for Energy Efficiency and Renewable Energy, under contract W-31-109-Eng-38.

REFERENCES

1. **Ashworth, J. M., and H. L. Kornberg.** 1966. The anaplerotic fixation of carbon dioxide by *Escherichia coli*. Proc. R. Soc. Ser. B **165:**179–188.
2. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. **32:**781–791.
3. **Blackwood, A. C., A. C. Neish, and G. A. Ledingham.** 1956. Dissimilation of glucose at controlled pH values by pigmented and non-pigmented strains of *Escherichia coli*. J. Bacteriol. **72:**497–499.
4. **Chao, Y.-P., and J. C. Liao.** 1993. Alteration of growth yield by overexpression of phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase in *Escherichia coli*. Appl. Environ. Microbiol. **59:**4261–4265.
5. **Chao, Y.-P., R. Patnaik, W. D. Roof, R. F. Young, and J. C. Liao.** 1993. Control of gluconeogenesis by *pps* and *pck* in *Escherichia coli*. J. Bacteriol. **175:**6939–6944.
6. **Clark, D. P.** 1989. The fermentation pathways of *Escherichia coli*. FEMS Microbiol. Rev. **63:**223–234.
7. **Datta, R., D. A. Glassner, M. K. Jain, and J. R. Vick Roy.** 1991. European patent application EP 405707.
8. **Fujita, N., T. Miwa, S. Ishijima, K. Izui, and H. Katsuki.** 1984. The primary structure of phosphoenolpyruvate carboxylase of *Escherichia coli*. Nucleotide sequence of the *ppc* gene and deduced amino acid sequence. J. Biochem. **95:**909–916.
9. **Furste, J. P., W. Pansegrau, R. Frank, H. Blocker, P. Scholz, M. Bagdasarian, and E. Lanka.** 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene **48:**119–131.
10. **Goldie, A. H., and B. D. Sanwal.** 1980. Allosteric control by calcium and mechanism of desensitization of phosphoenolpyruvate carboxykinase of *Escherichia coli*. J. Biol. Chem. **255:**1399–1405.
11. **Gottschalk, G.** 1985. Bacterial metabolism, 2nd ed. Springer-Verlag, New York.
12. **Jain, M. K., R. Datta, and J. G. Zeikus.** 1989. High-value organic acids fermentation—emerging processes and products, p. 366–389. *In* T. K. Ghose (ed.), Bioprocess engineering: the first generation. Ellis Horwood, Chichester, United Kingdom.
13. **Krebs, A., and W. A. Bridger.** 1976. On the monomeric structure and proposed regulatory properties of phosphoenolpyruvate carboxykinase of *Escherichia coli*. Can. J. Biochem. **54:**22–26.
14. **Krebs, A., and W. A. Bridger.** 1980. The kinetic properties of phosphoenolpyruvate carboxykinase of *Escherichia coli*. Can. J. Biochem. **58:**309–318.
15. **Medina, V., R. Pontarollo, D. Glaeske, H. Tabel, and H. Goldie.** 1990. Sequence of the *pckA* gene of *Escherichia coli* K-12: relevance to genetic and allosteric regulation and homology of *E. coli* phosphoenolpyruvate carboxykinase with the enzymes from *Trypanosoma brucei* and *Saccharomyces cerevisiae*. J. Bacteriol. **172:**7151–7156.
16. **Morikawa, M., K. Izui, M. Taguchi, and H. Katsuki.** 1980. Regulation of *Escherichia coli* phosphoenolpyruvate carboxylase by multiple effectors *in vivo*. J. Biochem. **87:**441–449.
17. **Podkovyrov, S. M., and J. G. Zeikus.** 1993. Purification and characterization of phosphoenolpyruvate carboxykinase, a catabolic CO_2-fixing enzyme, from *Anaerobiospirillum succiniciproducens*. J. Gen. Microbiol. **139:**223–228.
18. **Samuelov, N. S., R. Lamed, S. Lowe, and J. G. Zeikus.** 1991. Influence of CO_2-HCO_3^- levels and pH on growth, succinate production, and enzyme activities of *Anaerobiospirillum succiniciproducens*. Appl. Environ. Microbiol. **57:**3013–3019.
19. **Terada, K., T. Murata, and K. Izui.** 1991. Site-directed mutagenesis of phosphoenolpyruvate carboxylase from *E. coli*: the role of his-579 in the catalytic and regulatory functions. J. Biochem. **109:**49–54.
20. **Teraoka, H., T. Nishikido, K. Izui, and H. Katsuki.** 1970. Control of the synthesis of phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase in *Escherichia coli*. J. Biochem. **67:**567–575.
21. **Utter, M. F., and H. M. Kolenbrander.** 1972. Formation of oxaloacetate by CO_2 fixation on P-enolpyruvate, p. 117–165. *In* P. D. Boyer (ed.), The enzymes, vol. 7. Academic Press, New York.
22. **Wright, J. A., and B. D. Sanwal.** 1969. Regulatory mechanisms involving nicotinamide adenine nucleotides as allosteric effectors. II. Control of phosphoenolpyruvate carboxykinase. J. Biol. Chem. **244:**1838–1845.