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Effect of Overexpression of *Actinobacillus succinogenes* Phosphoenolpyruvate Carboxykinase on Succinate Production in *Escherichia coli*

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Succinate fermentation was investigated in *Escherichia coli* strains overexpressing *Actinobacillus succinogenes* phosphoenolpyruvate carboxykinase (PEPCK). In *E. coli* K-12, PEPCK overexpression had no effect on succinate fermentation. In contrast, in the phosphoenolpyruvate carboxylase mutant *E. coli* strain K-12 *ppc*::*kan*, PEPCK overexpression increased succinate production 6.5-fold.

Succinate is a four-carbon dicarboxylic acid, which has many applications in agriculture, food, and pharmaceutical industries. It is also a potential intermediary commodity chemical feedstock derived from biomass. Succinate production from glucose involves reductive CO_2 fixation. Several enzymes involved in CO_2 fixation have been overexpressed in *Escherichia coli* to enhance succinate production. Succinate production was increased by overexpressing pyruvate carboxylase, phosphoenolpyruvate (PEP) carboxylase (PPC), and malic enzyme, whereas overexpression of *E. coli* PEP carboxykinase (PEPCK) had no effect (3, 4, 8, 12, 18). In this paper, we call PPC the enzyme that catalyzes the formation of oxaloacetate plus P_i from PEP plus CO_2 (i.e., EC 4.1.1.38), and we call PEPCK the enzyme that catalyzes the formation of oxaloacetate plus ATP from PEP, ADP, and CO_2 (i.e., EC 4.1.1.49) (5). *Actinobacillus succinogenes* is a natural succinate producer (6, 7). In contrast to *E. coli*, PEPCK is the main CO_2-fixing enzyme in the *A. succinogenes* succinate production pathway (17). We have cloned and sequenced the *A. succinogenes pckA* gene (P. Kim, M. Laivenieks, J. McKinlay, C. Vieille, and J. G. Zeikus, submitted for publication). In this report, we describe the effect of *A. succinogenes* PEPCK overexpression on succinate production in wild-type and *ppc* mutant *E. coli* strains.

The characteristics of the *E. coli* strains used in this study are described in Table 1. The molecular biology techniques used in this study were performed as described previously (15). To construct a knockout K-12 *ppc* mutant, a *ppc-5*::*kan* gene was introduced into *E. coli* K-12 by P1 transduction with a P1 lysate of strain JCL1242 (Table 1) as described previously (13). *E. coli* DH5α was used as the host for the subcloning experiments. To clone the *A. succinogenes pckA* gene into an expression system, the *A. succinogenes pckA* gene (Kim et al., submitted) was amplified by using *A. succinogenes* chromosomal DNA as the template and oligonucleotides 5′-GCGAGAG TACTGACTTAAACAAACTCG (where the underlined sequence creates a *Sca*I site) and 5′-ACGCGTCGACCTCAGC CTTATTTTTCAG (where the underlined sequence creates a *Sal*I site) as the forward and reverse primers, respectively. The 1.6-kb PCR product was cloned into pCRII (Invitrogen, Carlsbad, Calif.) and sequenced. The *pckA* gene was then subcloned into the *Ehe*I and *Sal*I sites of expression vector pProEx-1 (Invitrogen), yielding plasmid pAsPCK. In this construct, PEPCK is expressed with an N-terminal His tag followed by a TEV protease cleavage site. Plasmid pAsPCK was used to overexpress *A. succinogenes* PEPCK in *E. coli*.

Single colonies of *E. coli* cells (parental strains and transformants) were transferred into 5 ml of Luria-Bertani broth and incubated at 37°C for 12 h with shaking. Five hundred microliters of these precultures was used to inoculate 50 ml of glucose-based medium (per liter, 9 g [50 mM] of glucose, 5 g of yeast extract, 10 g of $NaHCO_3$, 8.5 g of $NaH_2PO_4 \cdot H_2O$, and 15.5 g of K_2HPO_4 [pH 7.0]) in 125-ml butyl rubber-stoppered serum vials under a CO_2 atmosphere. Ampicillin was supplemented at 50 μg/ml for the strains harboring a plasmid; kanamycin was added at 20 μg/ml for the *ppc*::*kan* K-12 derivatives. IPTG (isopropyl-β-D-thiogalactopyranoside [0.6 mM]) was added to induce *A. succinogenes* PEPCK expression. Fermentations were performed without shaking at 37°C. Samples were withdrawn every 2 h during the fermentation to determine the biomass, glucose, and metabolites. Biomass was estimated by measuring optical density at 600 nm (OD_{600}). Glucose and metabolites were analyzed on a Breeze high-performance liquid chromatograph (Waters, Milford, Mass.) equipped with an Aminex-87H ion-exchange column (Bio-Rad, Hercules, Calif.) and a refractive index (RI) detector. A UV detector was also used to confirm the organic acid RI data. The mobile phase was 4 mM H_2SO_4 (isocratic flow, 0.6 ml $\cdot$ min^{-1}), and the column was maintained at 40°C. To measure PEPCK activity, bacteria were harvested in the exponential phase and disrupted in a French press as described previously (17). Protein content in the extracts was determined with the Bio-Rad protein assay kit with bovine serum albumin as the standard. PEPCK activity was calculated by measuring ATP formation at 37°C with the Sigma Diagnostics ATP kit (Sigma, St. Louis, Mo.). The reaction mixture contained 100 mM Tris-HCl (pH 6.6), 35 mM $NaHCO_3$, 16 mM $MgCl_2$, 0.3 mM NADH, 2 U of phosphoglycerate phosphokinase/glyceraldehyde phosphate dehydro-

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TABLE 1. Strains used in this study

Strain	Characteristics	Source
DH5α	F$^-$ ϕ80 d*lac*Z ΔM15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17* ($r_K^-m_K^+$) *deoR thi-1 phoA supE44* λ$^-$ *gyrA96 relA1*	Invitrogen Carlsbad, Calif.
K-12	Wild-type *E. coli* strain	Laboratory collection
K-12 *ppc*	*E. coli* K-12 *ppc-5*::*kan*	This study
JCL1242	*ppc-5*::*kan DE(argF-lac)169* λ$^-$	CGSC 7728
342-167	*thr-1 leuB6(Am) fhuA2 lacY1 glnV44(AS) gal-6 hisG1(Fs) rfbD1 galP63 rpsL9 malT1(LamR) xylA7 mtlA2 ppc-1 argC32 thi-1* λ$^-$	CGSC 3594
DL10	Δ*(aroP-aceF)73 galK2(Oc)* λ$^-$ IN*(rrnD-rrnE)1 rpsL200 pck-11, ppc-1 maeA1 maeB3 maeR2*	CGSC 7061
NJ27	[*araD139*] Δ*(argF-lac)169* λ$^-$ *manZ26* Δ*(his-gnd)296 fruA25 relA1 rpsL150* (*strR*) Δ*argH1* Δ*(xylE-malB)262 deoC1 ppc-3*	CGSC 6812

genase, 1 mM dithiothreitol, 10 mM ADP, 1.8 mM 3-phosphoglycerate, 5 mM PEP, and the cell extract. The extinction coefficient for NADH was 6.22 cm^{-1} mM^{-1} at 340 nm.

The growth properties and fermentation balances of the *E. coli* K-12 and K-12 *ppc* strains were compared in the presence and absence of *A. succinogenes* PEPCK overexpression. Fermentation balances were determined at the 18-h time point [K-12, K-12(pAsPCK), and K-12 *ppc*(pAsPCK)] and at the 22-h time point (K-12 *ppc*) (Table 2), when all of the glucose was just depleted and before any fermentation product could be recycled. The *E. coli* K-12 *ppc* strain grew slower and more poorly than strain K12 (as indicated by a 40% reduction in biomass). This result is not surprising, since *E. coli ppc* strains do not grow on glucose-minimal medium (11). In our fermentation, the K-12 *ppc* strain derives its tricarboxylic acid cycle intermediates from the yeast extract present in the medium. Succinate production by the K-12 *ppc* strain decreased by more than 70%, while its lactate production increased. The two strains harboring plasmid pAsPCK showed at least 60-fold-higher PEPCK activity than the strains containing no plasmid, indicating that *A. succinogenes* PEPCK (with an N-terminal His tag and TEV protease site) is expressed as a functional enzyme in *E. coli*. With the same amount of biomass produced by K-12 *ppc*(pAsPCK) as that by K-12, *A. succinogenes* PEPCK overexpression restored full growth to K-12 *ppc*. In *E. coli* K-12, *A. succinogenes* PEPCK overexpression did not significantly affect succinate production, nor did it affect the overall fermentation balance. In contrast, PEPCK overexpression in K-12 *ppc* increased succinate production more than sixfold. The amount of succinate produced by K-12 *ppc*(pAsPCK) reached twice the amount produced by K-12 (Table 2). Concomitantly, lactate production decreased by almost 30%, while levels of acetate, formate, and ethanol production remained unchanged. In all cases, the theoretical redox balance based on the flux estimations was between 0.68 and 0.89 (data not shown). Because the carbon recoveries were all about 100%, these redox balance values significantly below 1 suggest that some of the electrons are directed toward the production of hydrogen (not determined) or that they are consumed in uncounted reactions involving components of the yeast extract.

Since no PPC activity is detectable in *A. succinogenes*, PEPCK is the only enzyme that mediates PEP carboxylation (17). In this organism, the ratio of pyruvate kinase and PEPCK activities probably determines the balance between the C3 and C4 metabolic branches, as reported in other rumen bacteria (1). In *E. coli*, however, PPC is the enzyme that carboxylates PEP during growth on glucose. PEPCK seems better suited than PPC for succinate production, though, because it generates ATP-conserving energy during glycolysis, whereas PPC dissipates that energy. Still, PPC overexpression in *E. coli* JCL1208 (a Δ*lac* strain containing a chromosomally inserted *lacI*^{q} gene (12) increased succinate production, whereas overexpression of *E. coli* PEPCK had no effect (12). As expected, the same result was obtained when we overexpressed *A. succinogenes* PEPCK in *E. coli* K-12. A possible explanation for these results is the difference in kinetics between PPC and PEPCK, as extensively discussed in reference 12. *E. coli* PPC has a K_m toward bicarbonate of 0.15 mM (14), whereas the K_ms toward bicarbonate of *E. coli* and *Anaerobiospirillum succiniciproducens* PEPCKs are 13 mM (9) and 30 mM (10), respectively. With 74% sequence identity and 84% similarity between *A. succinogenes* and *E. coli* PEPCKs (Kim et al., submitted), it is likely that *A. succinogenes* PEPCK has a K_m toward bicarbonate in the same range as *E. coli* PEPCK does. This hypothesis could be verified in the future by kinetic studies of *A. succinogenes* PEPCK. Millard et al. (12) expressed the alternative

TABLE 2. Effect of *A. succinogenes* PEPCK overexpression on the fermentation balance of *E. coli* K-12 and K-12 *ppc*

Strain	Concn of biomass and metabolites (mM)[a]						PEPCK activity (nmol/min · mg of protein^{-1})	Succinate ratio[c]	C recovery[d]
	Biomass[b]	Succinate	Lactate	Acetate	Formate	Ethanol			
K-12	33.8 ± 0.5	11.4 ± 0.4	37.4 ± 1.2	31.1 ± 0.6	44.4 ± 1.8	6.5 ± 0.2	3.1 ± 1.1	0.132	1.00 ± 0.04
K-12(pAsPCK)	34.7 ± 0.4	10.6 ± 0.5	35.9 ± 2.2	29.9 ± 1.6	43.9 ± 2.0	6.5 ± 0.2	194.4 ± 14.8	0.128	0.97 ± 0.03
K-12 *ppc*	20.0 ± 1.2	3.1 ± 0.1	48.8 ± 1.6	27.4 ± 0.4	45.2 ± 1.4	6.7 ± 0.7	1.3 ± 0.3	0.074	0.96 ± 0.07
K-12 *ppc*(pAsPCK)	35.2 ± 0.6	20.2 ± 0.9	35.0 ± 0.9	28.0 ± 0.8	45.9 ± 2.1	6.0 ± 0.1	200.9 ± 18.2	0.226	1.04 ± 0.02

[a] Biomass and metabolite concentrations were determined at the 18-h time point [K-12, K-12(pAsPCK), and K-12 *ppc*(pAsPCK)] and at the 22-h time point (K12 *ppc*), when glucose was just completely depleted.
[b] Biomass carbon was calculated by using the ratio 13.001 mM biomass/OD_{660} based on the formula for $CH_2O_{0.5}N_{0.2}$ (16).
[c] Calculated as succinate/(lactate + acetate + ethanol + succinate).
[d] The balance was corrected for CO_2 fixation to succinate (17).

TABLE 3. Effect of *A. succinogenes* PEPCK overexpression on succinate production by *E. coli ppc* strains 342-167, DL10, and NJ27

Strain	Growth (OD_{660})[a]	Succinate concn (mM)[a]
342-167	1.27 ± 0.06	8.2 ± 5.7
342-167(pAsPCK)	3.12 ± 0.04	26.4 ± 2.8
DL10	2.43 ± 0.11	4.7 ± 4.7
DL10(pAsPCK)	2.69 ± 0.24	12.1 ± 1.9
NJ27	1.73 ± 0.17	3.0 ± 0.5
NJ27(pAsPCK)	1.97 ± 0.16	12.5 ± 1.2

[a] Data were collected at the 24-h time point.

hypothesis that "unknown regulatory controls at the enzyme level may prevent *E. coli* PEPCK from functioning in the reverse direction in vivo." In the K-12 *ppc* strain, though, *A. succinogenes* PEPCK can replace PPC as the PEP-carboxylating enzyme in a glucose-based medium. Since *A. succinogenes* and *E. coli* PEPCKs are highly similar, it is likely that *E. coli* PEPCK would be able to replace PPC as the PEP-carboxylating enzyme in a *ppc* mutant *E. coli* strain. Our results suggest that in strains JCL1208(pCK601) (12) and K-12(pAsPCK) (this study), PEPCK does not function as the PEP-carboxylating enzyme due to PPC's and PEPCK's respective kinetic properties in relation to intracellular metabolite concentrations. This hypothesis concurs with the recent findings of Yang et al. (19). Contrary to the common belief that *E. coli* PEPCK is only expressed during gluconeogenesis, Yang et al. showed that PEPCK shows significant activity in glucose-grown cultures, in which it decarboxylates oxaloacetate back to PEP. A detailed metabolic flux analysis suggested that the opposing fluxes generated by PPC and PEPCK activities are controlled at the kinetic level by PEP and oxaloacetate concentrations (19).

Chao and Liao (2) tested the growth of *E. coli* strain JCL4212(pCK601) (a Δ[*argF-lac*]169 *ppc* mutant strain overexpressing *E. coli* PEPCK) on minimal medium containing glucose plus succinate. This strain grew poorly, and Chao and Liao concluded that PEPCK overexpression was inhibitory for growth. This observation probably led to the comment by Gokarn et al. (3) that PEPCK "was unable to complement the absence of PPC in *E. coli*." Our results with K-12 *ppc* (pAsPCK) (i.e., normal growth rate and enhanced succinate production) contradict this statement and Chao and Liao's results. To confirm our results, we performed similar experiments with three other *ppc* mutant *E. coli* strains (i.e., strains 342-167, DL10, and NJ27) (Table 1). PEPCK activity levels increased between 68- and 189-fold in the three strains overexpressing *A. succinogenes* PEPCK (data not shown). As observed with strain K-12 *ppc*, PEPCK overexpression improved growth of the poorly growing strain 342-167 by 2.5-fold (Table 3). Finally, overexpression of *A. succinogenes* PEPCK in 342-167, DL10, and NJ27 increased succinate production 3.0-, 4.2-, and 2.6-fold, respectively. The results with these last three strains show that our results are not strain specific. In addition to other mutations, strain NJ27 contains the same Δ[*argF-lac*] 169 deletion as that in strain JCL4212. Under our fermentation conditions, overexpression of PEPCK in NJ27 did not inhibit growth (Table 3), but it did increase succinate production significantly. What differentiates our experiment with NJ27 (pAsPCK) from Chao and Liao's experiment with JCL4212 (pCK601) is the presence in our fermentation medium of 0.5% yeast extract. In the K-12–MG1655 genomic sequence (at www.tigr.org), the *argF-lac* region mutation encompasses approximately 70 genes and putative genes. The poor growth of JCL4212(pCK601) on minimal medium-glucose-succinate (2) could have been due to the absence of any or several of these genes rather than to growth inhibition by PEPCK.

The main results of this work are that PEPCK can replace PPC as the PEP-carboxylating enzyme in *E. coli* and that PEPCK overexpression results in a significant increase in succinate production. K-12 *ppc*(pAsPCK) produces almost twice as much succinate as K-12 does. Upon PPC overexpression in *E. coli* JCL1208, Millard et al. (12) observed increases in succinate production in nonoptimized and optimized fermentation media of 1.36- and 3.5-fold, respectively. Because the fermentation medium we used is closer in composition to the nonoptimized medium used by Millard et al., one would expect PEPCK overexpression to increase succinate production more than it already does, under optimized conditions. In this respect, since PEPCK's K_m for HCO_3^- is 2 orders of magnitude higher than that of PPC, optimizing the HCO_3^- concentration might be a critical step toward increasing succinate production through PEPCK overexpression. Because the increase in succinate production observed in this study is coupled to the substitution of PPC by PEPCK (an enzyme that couples ATP production to PEP carboxylation), this engineering step represents an attractive option for the design of a succinate-producing *E. coli* strain.

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