Increasing the Acetyl-CoA Pool in the Presence of Overexpressed Phosphoenolpyruvate Carboxylase or Pyruvate Carboxylase Enhances Succinate Production in *Escherichia coli*

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An in vivo strategy to apply the activation effect of acetyl-CoA on phosphoenolpyruvate carboxylase (PEPC) and pyruvate carboxylase (PYC) to increase succinate production in *Escherichia coli* was studied. This approach relies on the increased intracellular acetyl-CoA and CoA levels by overexpressing *E. coli* pantothenate kinase (PANK). The results showed that coexpression of PANK and PEPC, and PANK and PYC, did improve succinate production compared to the individual expression of PEPC and PYC, respectively. The intracellular acetyl-CoA and CoA levels were also measured, and each showed a significant increase when the PANK was overexpressed. Another effect observed was a decrease in lactate production. The least amount of lactate was produced when PANK and PEPC, and PANK and PYC, were coexpressed. This result showed increased competitiveness of the succinate pathway at the phosphoenolpyruvate and pyruvate nodes for the carbon flux, as a result reducing the carbon flux toward the lactate pathway. The study also demonstrates a feasible method for metabolic engineering to modulate enzyme activity in vivo through specific activators and inhibitors.

Introduction

One common limitation in overexpressing enzymes for metabolic engineering purposes is the inability to modulate their in vivo activities. One method of manipulating enzymatic activities is by utilizing the enzymes' specific activators or inhibitors. Activators can significantly enhance the activities of the corresponding enzymes. Acetyl-CoA has been shown to be a powerful allosteric activator of phosphoenolpyruvate carboxylase (PEPC) from a range of species of organisms. PEPC from *Escherichia coli* has been shown to be strongly activated by acetyl-CoA (*1*). PEPC from *Corynebacterium glutamicum* exhibited 6-fold higher activity in the presence of acetyl-CoA when expressed in the transgenic plant *Solanum tuberosum* (*2*). Acetyl-CoA, in general, is known to be a better activator of bacterial PEPCs than plant PEPCs (*3*). Despite this, individual cases still need to be examined for PEPCs from different species. For example, acetyl-CoA showed no effect on the activity of the PEPC from the thermophilic cyanobacterium *Synechococcus vulcanus* (*3*). PEPC branches from glycolysis by converting phosphoenolypruvate (PEP) to oxaloacetate (OAA) through a carboxylation reaction. Pyruvate carboxylase (PYC) activity has also been shown to be largely dependent on acetyl-CoA. For example, PYC from the photosynthetic bacterium *Rhodobacter capsulatus* was shown to be absolutely dependent on acetyl-CoA (*4*). Acetyl-CoA also effectively protected the *R. capsulatus* PYC from thermal denaturation (*4*). PYC converts pyruvate to OAA also through fixation of CO_2. In glycolysis, PEP is converted to pyruvate in a one-step reaction by pyruvate kinase or during uptake of sugars by the phosphotransferase system (PTS).

Studies of activation of PEPC or PYC by acetyl-CoA or other activators have involved in vitro methods through enzyme extraction and activity measurement (*3*, *5*). The activation effect of acetyl-CoA on PEPC and PYC has never been accessed and applied in vivo by any means. Manipulation of intracellular enzymatic activity has been an ongoing interest and effort in metabolic engineering. The ability to modulate a particular enzyme's activity in vivo means the possibility to successfully control the cellular system for achieving a desired purpose, such as enhancing the production of a certain product.

In this study, we seek to provide and corroborate a new means of exploiting the acetyl-CoA activation effect on PEPC and PYC entirely in vivo. In the context of metabolic engineering, this approach will be applied to enhance the production of succinate. PEPC and PYC are the first enzymatic steps in the succinate pathway. Overexpression of *E. coli* PEPC or *Rhizobium etli* PYC in *E. coli* has been shown to enhance succinate production (*6*, *7*). Interest in succinate production has been fueled by its practicality as a specialty industrial chemical and by an ever-increasing effort to replace chemical processes with biocatalysts for production. To increase the intracellular acetyl-CoA pool, *E. coli* pantothenate kinase (PANK) is overexpressed in *E. coli*. This strategy has been shown to significantly enhance both the acetyl-CoA and CoA pools (*8*). Strains bearing the PANK plasmid will be used to coexpress a malate feedback inhibition resistant *Sorghum* PEPC and also, separately, a PYC from *Lactococcus lactis*. PYC is not indigenous in *E. coli*

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(*7*), and therefore the *L. lactis* PYC is used as the model PYC to be studied in the *E. coli* host system. Both the *L. lactis* PYC and *Sorghum* PEPC have been shown in our lab to increase succinate production in *E. coli*. The increased intracellular acetyl-CoA pool will be used to study its effect on the native *E. coli* PEPC, *Sorghum* PEPC, and *Lactococcus lactis* PYC through the subsequent effect on succinate production. By simultaneously overexpressing PANK and PEPC, or PANK and PYC, succinate production was found to increase as compared to individual overexpression of PEPC or PYC.

Materials and Methods

Plasmids. The laboratory wild-type *E. coli* is GJT001, a spontaneous *cadR* mutant of MC4100 (*9*). All plasmid transformation and gene expression studies were performed in GJT001. A malate feedback inhibition resistant *Sorghum pepc* was cloned from pKK313 (*10*) into the high-copy-number plasmid pDHK29 (*11*) to yield pHL333. The *trc* promoter and transcription terminators of the *Sorghum pepc* on pKK313 were also included in the PCR product. The *rTth* DNA polymerase was used (Applied Biosystems), and pKK313 served as the DNA template. The forward primer is 5′-CAGCTCGAGCGGTAAATCACTGCATAATTCG-3′, and the reverse primer is 5′-CACCATGGGGTTATCAGGGTTATTGTCTCATGAGC-3′. The PCR product of *Sorghum pepc* was first ligated into the intermediate plasmid pCR2.1-TOPO (Invitrogen). The 3.8-kb *Sorghum pepc* fragment was then digested from the pCR2.1-TOPO vector using *SpeI* and *EcoRI* and then ligated into pDHK29. The *Sorghum pepc* cloned in pDHK29 is designated pHL333, which confers kanamycin (Km) resistance.

The *L. lactis pyc* was digested from pCPYC1 (*12*) with *SmaI* and *PstI* and then ligated into pTrc99A (Pharmacia). The final vector is designated pHL413 and confers ampicillin (Ap) resistance.

The *E. coli panK* was cloned from pSJ380 (*13*) into both pDHK29 and pUC19 (*14*) to yield pRV480 (*15*) and pRV380 (*8*), respectively. The origin of replication of pDHK29 is compatible with the ColE1 origin of pTrc99A and pUC19. This feature allows concurrent overexpression of two genes in the same system. The antibiotic markers are also compatible since pDHK29 has kanamycin resistance and pTrc99A and pUC19 express ampicillin resistance.

Medium and Cultivation. The medium used throughout the study was Luria-Bertani broth (LB) adjusted to pH of 7.5. A 20 g/L concentration of glucose was supplemented. Kanamycin concentration used was 100 mg/L, and ampicillin concentration used was 1 g/L to maintain plasmid stability. $MgCO_3$ concentration of 20 g/L was also added to the LB. $MgCO_3$ serves to buffer the pH during fermentation and provides an indirect means of supplying CO_2 required in succinate synthesis. For induction of gene expression, 1 mM of isopropyl-β-D-thiogalactoside (IPTG) was used in the medium. Pantothenic acid was added to the culture medium at a concentration of 5 mM. This is a required substrate for the pantothenate kinase.

The experiments were performed in 250-mL flasks sealed with rubber plugs containing 15 mL of media. The results from this experimental setup were demonstrated to be reproducible through repetition of the experiments. All experiments were done in triplicates. The fermentation runs were for 24 h. The flask cultures were grown at 37 °C at 250 rpm in an orbital shaker.

Analytical Techniques. For analyzing the extracellular metabolites such as succinate and lactate, 1 mL of culture was centrifuged and the supernatant was then filtered through a 0.45-μm syringe filter for HPLC analysis. The HPLC system (Shimadzu-10A Systems, Shimadzu, Columbia, MD) used was equipped with a cation-exchange column (HPX-87H, BioRad Labs, Hercules, CA), a UV detector (Shimadzu SPD-10A), and a differential refractive index detector (Waters 2410, Waters, Milford, MA). A 0.6 mL/min mobile phase using 2.5 mM H_2SO_4 solution was applied to the column. The column was operated at 55 °C.

To quantify intracellular acetyl-CoA and CoA compounds, 40 optical density (OD) units of cell culture was taken ($OD_{660\ nm} \times$ vol (mL) = 40) and centrifuged at 5000*g* at 4 °C for 10 min. The pellet was then resuspended in 1 mL of 6% perchloric acid to lyse the cells. Then 0.3 mL of 3 M potassium carbonate was added while vortexing to neutralize the acid. The solution is then centrifuged, and the supernatant was filtered through a 0.2-μm PVDF membrane syringe filter.

Acetyl-CoA and CoA were analyzed on HPLC (Thermofinnigan, San Jose, CA) using a UV detector and a 5-μm octyldecyl silane column (Cell Technologies, Inc., Houston, TX). Two mobile phases of buffer were used at a flow rate of 1 mL/min. One buffer is 0.2 M sodium phosphate (pH 5.0), and the other buffer is 800 mL of 0.25 M sodium phosphate (pH 5.0) mixed with 200 mL of 100% acetonitrile).

Enzyme Assays. To prepare crude extracts for enzyme assays, mid-exponential phase cultures were taken and washed twice with cold 100 mM Tris-HCl buffer at pH 8.0. Centrifugation was done at 4000*g* and 4 °C for 20 min for these washing steps. The cells were then resuspended in the same solution and subjected to sonication for 6 min in an ice bath. The sonicated cells were centrifuged at 1500*g* and 4 °C for 60 min to remove cell debris. PEPC and PYC activities were measured on the basis of the citrate synthase coupled assay by the method of Payne and Morris (*16*). The oxaloacetate produced by PYC or PEPC is reacted with acetyl-CoA by citrate synthase to yield CoA. The CoA then reacts with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) to produce a yellow colored product that can be measured spectrophotometrically at 412 nm. All reactions were performed in duplicates and carried out at 37 °C. Reactions were initiated by adding the crude extract. The PYC assay differed from the PEPC assay in that pyruvate and ATP were added for the PYC assay. For PEPC, phosphoenolpyruvate was added instead of pyruvate and ATP. The specific activity of the enzymes was measured in μmol product/mg protein/h (DTNB extinction coefficient used is 1.36×10^4 M^{-1} cm^{-1}). Total protein concentration of crude extract was measured by Lowry's method (Sigma Lowry Reagent, Modified) using bovine serum albumin as standard.

Results and Discussion

The intracellular acetyl-CoA level is increased by overexpressing *E. coli* PANK and adding pantothenic acid to the medium, a necessary substrate for PANK (*8*). The effect of modulating intracellular PEPC or PYC activity is therefore studied with coexpression of PEPC and PANK and coexpression of PYC and PANK. The subsequent effect on succinate production and other metabolites is then examined.

To perform the coexpression effectively with two plasmids without the difficulty of imbalanced plasmid replication, two origin-compatible plasmids were used. For PEPC and PANK coexpression, PEPC was cloned into pDHK29 to form pHL333, and PANK was cloned into

Table 1. Strains and Plasmids Used

strain	genotype	reference
GJT001	laboratory wildtype	9
GJT(pDHK29,pUC19)	control for PEPC and PANK study	this study
GJT(pDHK29,pRV380)	overexpression of PANK only	this study
GJT(pHL333,pUC19)	overexpression of PEPC only	this study
GJT(pHL333,pRV380)	overexpression of PEPC and PANK	this study
GJT(pTrc99A,pDHK29)	control for PYC and PANK study	this study
GJT(pTrc99A,pRV480)	overexpression of PANK only	this study
GJT(pHL413,pDHK29)	overexpression of PYC only	this study
GJT(pHL413,pRV480)	overexpression of PYC and PANK	this study

plasmid	genotype	reference
pUC19	ColE1 origin control vector Ap^R	14
pTrc99A	ColE1 origin control vector Ap^R	Pharmacia
pDHK29	ColE1 compatible control vector Km^R	11
pHL333	*Sorghum pepc* in pDHK29 Km^R	this study
pHL413	*L. lactis pyc* in pTrc99A Ap^R	this study
pRV380	*E. coli panK* in pUC19 Ap^R	8
pRV480	*E. coli panK* in pDHK29 Km^R	15
pKK313	S8D mutant *Sorghum pepc* Ap^R	10
pCPYC1	*L. lactis pyc* Cm^R	12

pUC19 to form pRV380. For PYC and PANK coexpression, PYC was cloned into pTrc99A to form pHL413, and PANK was cloned into pDHK29 to form pRV480. The vector, pTrc99A, is also compatible with pDHK29. Plasmids pRV380 and pRV480 have both been shown to be similar in significantly increasing the acetyl-CoA and CoA pools (*8*, *15*).

Coexpression of PEPC and PANK. To systematically study the effect of the individual enzymes and then the combined effect, four strains cotransformed with two plasmids were created. The strains are described in Table 1 for PEPC and PANK coexpression. The strain GJT-(pDHK29,pUC19) is the control strain without PEPC or PANK overexpression. The strain GJT(pDHK29,pRV380) overexpresses PANK only, the strain GJT(pHL333,-pUC19) overexpresses *Sorghum* PEPC only, and the strain GJT(pHL333,pRV380) overexpresses *Sorghum* PEPC and PANK simultaneously. Experiments with these four strains were performed simultaneously, and succinate, lactate, and intracellular acetyl-CoA and CoA levels were measured. All four strains consumed the same amount of glucose at the end of the experiment, and no pyruvate accumulation was detected. The acetate production was similar for all four strains.

To show that the increase in succinate was due to the increased acetyl-CoA pool enhancing the activation of PEPC rather than differences in enzyme activity levels, enzyme assays were performed on the four strains GJT-(pDHK29,pUC19), GJT(pDHK29,pRV380), GJT(pHL333,-pUC19), and GJT(pHL333,pRV380). For the enzyme assays, pantothenic acid was not added during cultivation, since it could be subsequently converted to acetyl-CoA by PANK. This could prevent differences in acetyl-CoA levels among the strains during the assay, which could have affected the activity of PEPC. For comparison, the basal chromosomal PEPC activities of GJT(pDHK29,-pUC19) and GJT(pDHK29,pRV380) were observed to be the same (these two strains have no PEPC overexpressed) (data not shown). The basal PEPC activities of GJT-(pHL333,pUC19) and GJT(pHL333,pRV380) were also observed to be the same (both strains overexpress the *Sorghum* PEPC) (data not shown). Therefore, the increase in succinate production by the two strains (GJT-(pDHK29,pRV380) and GJT(pHL333,pRV380)) overexpressing PANK could only be attributed to the effect of increased acetyl-CoA pool on the native and *Sorghum* PEPC.

The results showed that the strain overexpressing PEPC and PANK concurrently resulted in the highest succinate final concentration at the 24 h endpoint (Figure 1a). This strain had an intracellular acetyl-CoA concentration of 8.05 μM as compared to its control (PEPC and no PANK), which had only 0.24 μM, at the 24 h endpoint (statistically significantly different at the 95% confidence level). Previous studies have shown that overexpressing PANK consistently yields higher acetyl-CoA levels throughout the exponential and stationary phases (*8*). This significantly increased acetyl-CoA pool may have enhanced the activation of the abundant intracellular *Sorghum* PEPC throughout the cell culture, causing an increase in succinate production. Individual overexpression of PANK or PEPC also increased succinate production compared to the control with no PEPC or PANK overexpression (GJT(pDHK29,pUC19)). The increase in succinate production caused by overexpression of PANK alone was likely a result of the effect of elevated acetyl-CoA level on the native *E. coli* PEPC. Acetyl-CoA has been shown to be an effective activator of *E. coli* PEPC (*1*). This increased activation of the native PEPC, therefore, would cause a subsequent increase in succinate production.

Lactate production decreased significantly when both PANK and PEPC were overexpressed compared to the control without PANK or PEPC overexpression (statistically significantly different at the 95% confidence level) (Figure 1b). When the individual genes were overexpressed, lactate also decreased substantially as compared to the control with no PEPC and PANK overexpression, but not as substantially as when both genes were coexpressed. Lactate is formed from pyruvate by lactate dehydrogenase (LDH). As a result, succinate increased and lactate decreased. The decline in lactate was caused by the PEPC being more competitive than the LDH for the carbon flux at the PEP-to-pyruvate node. The elevated in vivo pool of acetyl-CoA likely enhanced the activity of PEPC, resulting in more succinate production and less lactate production. This effect became more prominent when both PEPC and PANK were overexpressed simultaneously. No pyruvate accumulation was detected at the end of fermentation. Acetate production was similar in all four strains studied (data not shown). Apparently, overexpression of PEPC and PANK did not have substantial effect on the acetate level. In addition, formate and ethanol production were observed to be

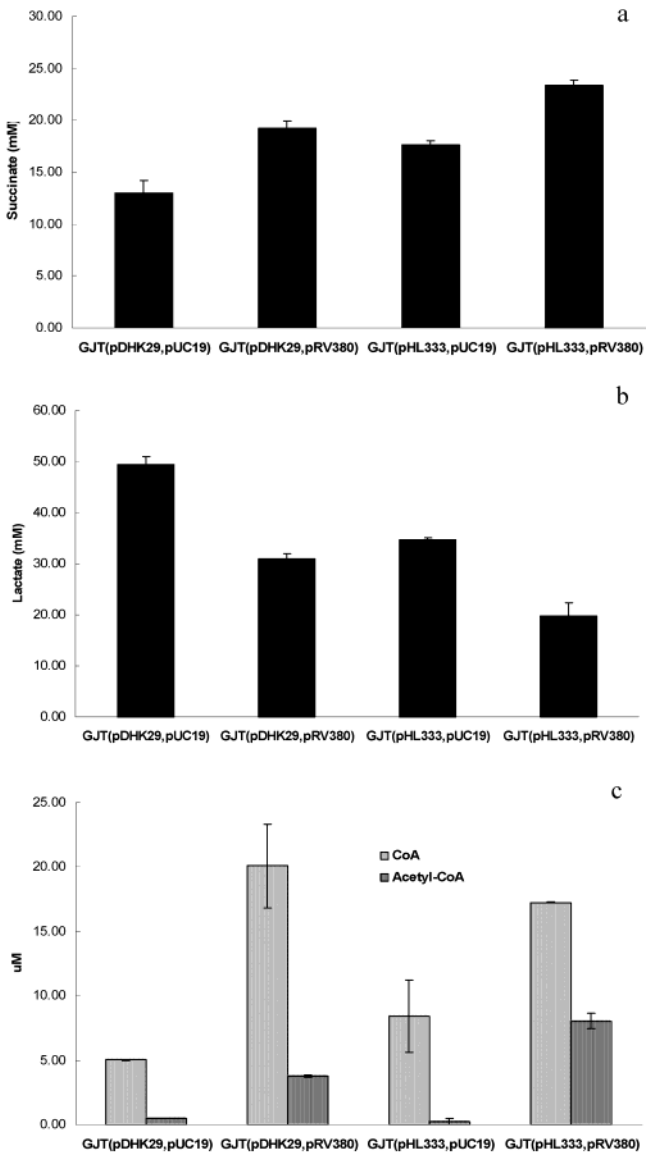

Figure 1. (a) Effect on succinate production by PEPC and PANK coexpression. (b) Effect on lactate production by PEPC and PANK coexpression. (c) Effect of PANK on acetyl-CoA and CoA levels. GJTpDHK29pUC19 is control, GJTpDHK29pRV380 overexpresses PANK only, GJTpHL333pUC19 overexpresses PEPC only, and GJTpHL333pRV380 overexpresses both PEPC and PANK. Total glucose consumed is the same for all four strains. No pyruvate accumulation was detected.

higher in the strains overexpressing PANK compared to the same strains not overexpressing PANK (data not shown).

Figure 1c shows the intracellular acetyl-CoA and CoA levels of the four strains at the end of the experiment. The two strains carrying pRV380 overexpressing PANK showed significantly higher acetyl-CoA and CoA levels than their respective controls carrying pUC19 (statistically significantly different at the 95% confidence level). Especially the acetyl-CoA levels in the two strains with pRV380 were manyfold higher than their respective controls. The acetyl-CoA and CoA levels have also been previously shown to be consistently higher throughout the growth phase when overexpressing the PANK in a batch bioreactor setting (*8*).

Coexpression of PYC and PANK. Coexpression of PYC and PANK was studied in the same manner as coexpression of PEPC and PANK. Four strains were created, each carrying different combinations of two plasmids. These strains are described in Table 1 for PYC and PANK coexpression. The first strain, GJT(pTrc99A,-pDHK29), is the control without overexpression of both PYC and PANK. The strain GJT(pTrc99A,pRV480) overexpresses only PANK, and the strain GJT(pHL413,-pDHK29) overexpresses only PYC. The fourth strain, GJT(pHL413,pRV480), overexpresses both PYC and PANK simultaneously. All four strains consumed the same amount of glucose at the end of the experiment, and no pyruvate accumulation was detected. The acetate production was the same for all four strains.

PYC assays were performed on the four strains GJT-(pTrc99A,pDHK29), GJT(pTrc99A,pRV480), GJT(pHL413,-pDHK29), and GJT(pHL413,pRV480) to show that the increase in succinate was due to the increased acetyl-CoA pool enhancing the activation of PYC rather than differences in PYC level. Again, no pantothenic acid was added during cultivation to prevent any differences in acetyl-CoA levels in the extract affecting the measured activity. There was very minimal activity detected in the strains GJT(pTrc99A,pDHK29) and GJT(pTrc99A,pRV480) when the PYC assay was performed (data not shown). This is expected, since *E. coli* does not possess a native PYC. The increase in succinate concentration produced by GJT(pTrc99A,pRV480), therefore, could only be the effect of increased acetyl-CoA on the native chromosomal PEPC. The basal PYC activities of GJT(pHL413,pDHK29) and GJT(pHL413,pRV480) were observed to be the same (data not shown). Therefore, succinate increase by GJT-(pHL413,pRV480) overexpressing PANK during the experiment could only be attributed to the effect of an increased acetyl-CoA pool on the *L. lactis* PYC.

The results of PYC and PANK coexpression showed similar trends as coexpression of PEPC and PANK for succinate, lactate, acetyl-CoA, and CoA. These similar trends are expected since acetyl-CoA is a potential allosteric activator for these two types of carboxylases. The enhanced PYC activity would increase conversion of pyruvate to OAA, resulting in subsequent increase of succinate. Therefore, when PYC and PANK were both overexpressed, the succinate production was the highest after 24 h compared to the other three strains (Figure 2a). The succinate production was also higher in the strain GJT(pTrc99A,pRV480) overexpressing only PANK than in the control, GJT(pTrc99A,pDHK29), with no overexpression of PYC or PANK. This increase in succinate production was caused by the enhanced activation of the native *E. coli* PEPC, since PYC is absent in *E. coli*. Although the strain GJT(pHL413,pRV480), overexpressing both PYC and PANK, showed the highest succinate production, it was not substantially higher than the strain GJT(pHL413,pDHK29), overexpressing PYC alone (Figure 2a). This may be because this particular PYC from *L. lactis* is not too sensitive to acetyl-CoA activation.

Lactate decreased significantly when both PYC and PANK were overexpressed compared to the control without PYC or PANK overexpressed (statistically significantly different at the 95% confidence level) (Figure 2b). Overexpression of PYC or PANK separately exhibited a lesser decrease in lactate. The formation of lactate for the strain overexpressing both PYC and PANK was not substantially less, though, than the strain overexpressing PYC alone. This may also be attributed to the low sensitivity of *L. lactis* PYC to acetyl-CoA. Nonetheless, the decrease in lactate can be similarly explained for PYC as with PEPC. At the pyruvate junction, the enhanced PYC activity by the increased acetyl-CoA level became more competitive for the carbon flux. As a result, the carbon flux toward the lactate pathway was reduced

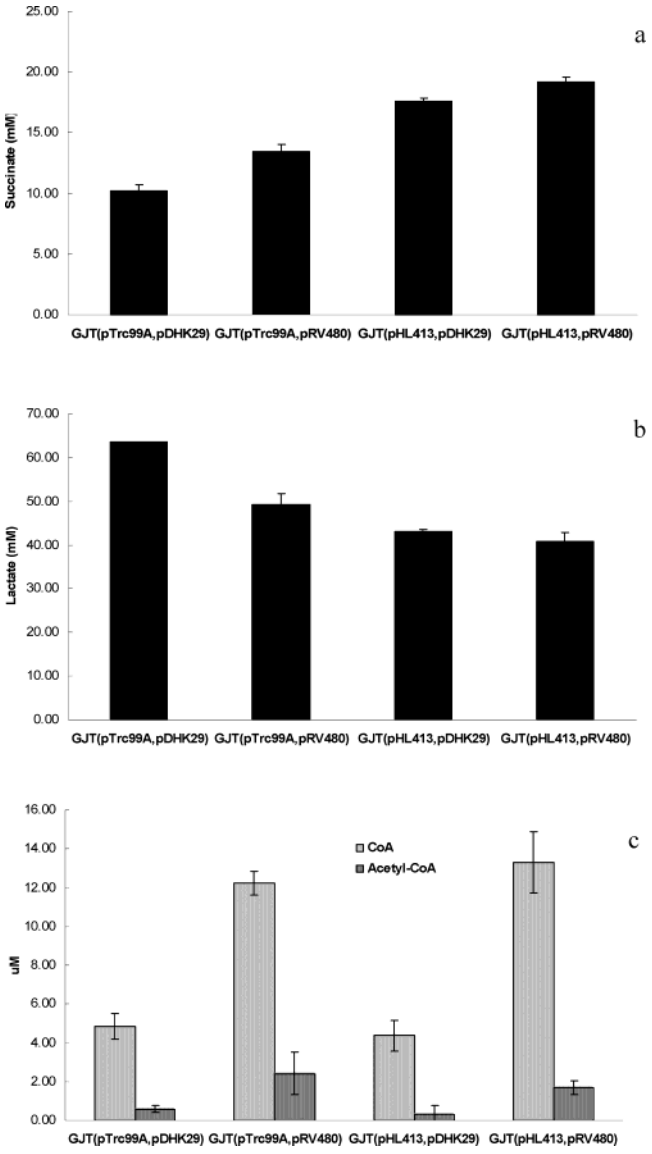

Figure 2. (a) Effect on succinate production by PYC and PANK coexpression. (b) Effect on lactate production by PYC and PANK coexpression. (c) Effect of PANK on acetyl-CoA and CoA levels. GJTpTrc99ApDHK29 is control, GJTpTrc99ApRV480 overexpresses PANK only, GJTpHL413pDHK29 overexpresses PYC only, and GJTpHL413pRV480 overexpresses both PYC and PANK. Total glucose consumed is the same for all four strains. No pyruvate accumulation was detected.

and channeled more toward the succinate pathway. No pyruvate accumulation was detected in all four strains. Acetate production was similar in all four strains studied (data not shown). Overexpressing PYC and PANK did not have substantial effect on the acetate level. Formate and ethanol production were also observed to increase in the strains overexpressing PANK compared to the same strains not overexpressing PANK (data not shown). Figure 2c shows that the intracellular acetyl-CoA and CoA levels were significantly higher at the 24 h endpoint for the strains carrying pRV480 than the strains carrying the control plasmid pDHK29 (statistically significantly different at the 95% confidence level.

Conclusion

The capability of regulating in vivo enzymatic activity in cellular systems still stands a tremendous challenge in metabolic engineering. This study shows an example of a method to modulate the activity of two specific enzymes, PEPC and PYC, through one of their activators, acetyl-CoA. The increased intracellular acetyl-CoA pool does effectively increase succinate production even without the coexpression of *Sorghum* PEPC or *L. lactis* PYC, possibly by enhancing the activity of the native *E. coli* PEPC. Coexpression of *Sorghum* PEPC with *E. coli* PANK and *L. lactis* PYC with *E. coli* PANK further enhances succinate production. This acetyl-CoA and CoA manipulation strategy, therefore, demonstrates the feasibility of modulating in vivo enzymatic activity. PEPC and PYC from other species can also be tested in the future using this technique to understand the effect of acetyl-CoA on their in vivo activities and the subsequent effect on the flux to the succinate pathway.

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