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Alteration of Growth Yield by Overexpression of Phosphoenolpyruvate Carboxylase and Phosphoenolpyruvate Carboxykinase in *Escherichia coli*

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Phosphoenolpyruvate and oxaloacetate are key intermediates at the junction between catabolism and biosynthesis. Alteration of carbon flow at these branch points will affect the growth yield and the formation of products. We attempted to modulate the metabolic flow between phosphoenolpyruvate and oxaloacetate by overexpressing phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase from a multicopy plasmid under the control of the *tac* promoter. It was found that overexpression of phosphoenolpyruvate carboxylase decreased the rates of glucose consumption and organic acid excretion, but the growth and respiration rates remained unchanged. Consequently, the growth yield on glucose was improved. This result indicates that the wild-type level of phosphoenolpyruvate carboxylase is not optimal for the most efficient glucose utilization in batch cultures. On the other hand, overexpression of phosphoenolpyruvate carboxykinase increased glucose consumption and decreased oxygen consumption relative to those levels required for growth. Therefore, the growth yield on glucose was reduced because of a higher rate of fermentation product excretion. These data provide useful insights into the regulation of central metabolism and facilitate further manipulation of pathways for metabolite production.

One of the common issues in metabolic engineering is the redistribution of metabolic flux at branch points. The split of carbon flow affects the yield, which often is the most significant parameter determining the manufacturing cost. To achieve rational manipulation of metabolic pathways, it is essential to understand how the flux distribution can be altered and how the cell responds to metabolic perturbations introduced by genetic manipulations.

In *Escherichia coli* and many other microorganisms, phosphoenolpyruvate (PEP) and oxaloacetate (OAA) are metabolites at the junction between biosynthesis and catabolism (Fig. 1). PEP is a precursor of aromatic amino acids and a phosphate donor in the phosphotransferase system. OAA is the precursor of amino acids derived from aspartate and an intermediate in the tricarboxylic acid cycle. When cells grow in glucose minimal medium, OAA is derived from PEP via PEP carboxylase (Ppc) reaction. When cells grow in succinate or other four-carbon metabolites, OAA is converted to PEP to supply carbon source for gluconeogenesis. Flux between PEP and OAA can therefore be modulated by Ppc or phosphoenolpyruvate carboxykinase (Pck).

The regulation of Ppc and Pck activities at the enzyme level has been well studied. Ppc is inhibited by aspartate and malate and activated by acetyl coenzyme A, fructose 1,6-diphosphate, GTP, guanosine-5′-diphosphate-3′-diphosphate, and fatty acids (11–13, 20, 24). Pck is allosterically activated by calcium (8) and inhibited by ATP and PEP (16). The regulation at the gene expression level is not completely understood. It is known that Pck is under the control of catabolite repression and that it is induced at the onset of stationary phase (10). Under normal physiological conditions, the regulations at both the activity level and the gene expression level control the carbon flow between PEP and OAA, which in part determines the flux distribution between catabolism and biosynthesis.

In this study, we attempted to alter the flux between PEP and OAA by overexpressing either Ppc or Pck from a multicopy plasmid under glycolytic conditions. To eliminate regulations at the gene expression level, both genes were cloned and expressed under an artificial promoter. Physiological responses such as the growth rate and the rates of glucose and oxygen consumption were measured under balanced growth at different expression levels of Ppc or Pck. It would be interesting to see whether metabolic regulation at the enzyme activity level can stabilize the flux distributions in the presence of overexpressed Ppc or Pck.

MATERIALS AND METHODS

Materials. D-Glucose, amino acids, nucleotides, and all salts were purchased from Sigma Chemical Co. (St. Louis, Mo.). Protein assay reagents and chemicals for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad (Richmond, Calif.). Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from U.S. Biochemicals (Cleveland, Ohio). Restriction enzymes, *Taq* DNA polymerase, and T4 DNA ligase were from Promega (Madison, Wis.), and the protein molecular weight standard was from GIBCO BRL (Gaithersburg, Md.).

Cloning of *ppc* and *pck*. To eliminate the natural promoter and other control sequences, the structural gene of *ppc* was cloned by use of polymerase chain reaction (PCR). The primers were designed on the basis of the published sequence (6) and contained *Eco*RI sites on both 5′- and 3′-end primers. The resulting PCR product of the *ppc* gene is without its natural promoter but contains a putative ribosome binding site. The PCR product of the *ppc* gene, a 2.7-kb fragment, was inserted into pJF118EH (7) to yield pPC201, which expresses the *ppc* gene from the *tac* promoter. PCR cloning of the *pck* structural gene was described

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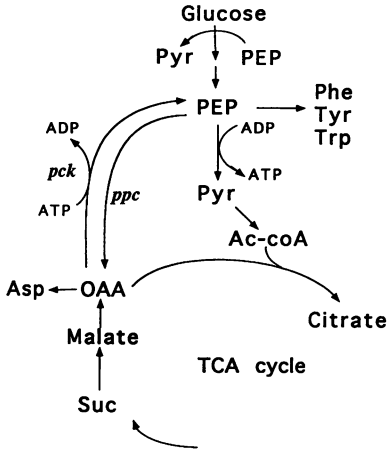

FIG. 1. Schematic diagram of PEP and OAA metabolism. Abbreviations: Ac-coA, acetyl coenzyme A; Asp, aspartate; Phe, phenylalanine; Pyr, pyruvate; Trp, tryptophan; Tyr, tyrosine; Suc, succinate; TCA, tricarboxylic acid.

previously (3). Plasmid pCK601 was constructed by ligating the *Eco*RI-*Bam*HI fragment of pCK401 (3) to the corresponding sites downstream of the *tac* promoter in pJF118EH. The *pck* gene on this plasmid is also without its natural promoter but contains its own ribosome binding site. All DNA manipulations essentially followed the procedures published earlier (23).

Bacterial strains. The *E. coli* strains used are listed in Table 1. The wild-type F^- λ^- *E. coli* K-12 strain (VJS632) and its derivative (VJS676) were generous gifts from Valley Stewart, Cornell University. VJS632 was made *lacI*^{q} and *recA* by P1 transduction with lysates of appropriate strains. The resulting strain was designated JCL1208. To construct a *ppc* strain, the *ppc* gene on plasmid pPC201 was first inactivated by inserting a kanamycin cassette (4) at the *Stu*I site. To integrate the *ppc*::Km marker into the chromosome, this plasmid was linearized and transformed into a *recD* strain, DPB271 (1). Km^r Ap^s transformants were isolated, and the Ppc$^-$ phenotype was confirmed by enzyme assay. The *ppc*::Km^r marker on the chromosome was then transduced into VJS676 by P1 transduction, and the resulting strain was designated JCL1242.

Media and growth conditions. For strain construction, the cells were grown on Luria-Bertani medium (19). For physiological characterizations, all strains were cultured on M9 minimal medium (19) with 0.2% glucose unless stated otherwise. Various amounts of IPTG (see Fig. 2 to 6) were added after about one to two doubling times postinoculation. The specific growth rate, the glucose consumption rate, and the oxygen consumption rate were measured at least six generations after inoculation and when the cell densities were between ca. 2×10^7 and 6×10^7 cells per ml. During this phase, the physiological parameters measured remained approximately constant. All cultures were grown at 37°C on a rotary shaker, and ampicillin (50 μg/ml) was added as a selection pressure for plasmid-bearing strains.

TABLE 1. *E. coli* strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
DPB271	λ^- *recD1903*::mini-Tet	S. N. Cohen
VJS632	Wild-type *E. coli* K-12 but F^- λ^-	V. J. Stewart
VJS676	As VJS632 but Δ(*argF-lac*) U169	V. J. Stewart
JCL1225	As VJS632 but *lacI*^{q} *lacZ*::Tn*5*	This study
JCL1208	As JCL1225 but Δ*recA zfi*::Tn*10*	This study
JCL1242	As VJS676 but *ppc*::Km	This study
Plasmids		
pJF118EH	*bla lacI*^{q} *tacP*	M. Bagdasarian
pCK601	As pJF118EH but *pck*$^+$	This study
pPC201	As pJF118EH but *ppc*$^+$	This study

Calculation of physiological variables. The specific growth rate (k) was calculated as ln2 divided by the doubling time. It can be interpreted as the amount of cell mass produced per unit of cell mass per hour. The specific consumption rate for a substrate (Q_{Glc} and Q_{O_2} for glucose and oxygen, respectively) is the substrate consumed per hour per unit of cell mass. The growth yield on glucose (Y_{Glc}) is the cell mass produced per unit of glucose consumed. Similarly, the growth yield for oxygen (Y_{O_2}) is the cell mass produced per unit of oxygen consumed. In balanced growth, Y_{Glc} is equal to k/Q_{Glc}, and Y_{O_2} is equal to k/Q_{O_2}. Y_{Glc} was calculated by plotting residual glucose levels against cell weight (dry weight). This curve becomes a straight line when a cell reaches balanced growth (ca. 2×10^7 to 6×10^7 cells per ml), and the negative slope of this straight line is Y_{Glc}. The yield coefficients can be interpreted as the stoichiometry of growth with respect to glucose or oxygen in balanced growth.

Enzyme assays. The preparation of cell-free extracts was described elsewhere (9, 21). The Pck activity was measured spectrophotometrically by monitoring the appearance of OAA (8, 21). The Ppc activity was measured in a similar way except that the reaction buffer contained 0.1 M Tris (pH 8.5), 20 mM magnesium acetate, and 20 mM $NaHCO_3$ and the initial substrate was 10 mM PEP. Total protein content was determined by use of the Bio-Rad dye reagent (Bradford assay) with bovine serum albumin as the protein standard.

Measurement of metabolites. The glucose concentration in the medium was measured by use of the dinitrosalicylic acid assay for total reducing sugars as described previously (18, 21). Excreted fermentation products were measured by high-pressure liquid chromatography (HPLC) over an organic acid column (Aminex HPX-87H; Bio-Rad) with 0.01 N sulfuric acid as the eluent and at a flow rate of 0.6 ml/min. The column temperature was maintained at 60°C, and the peaks were detected by measuring UV A_{210}. To measure the rate of oxygen consumption, a Clark-type polarographic oxygen probe was used to monitor the disappearance of oxygen from a sealed chamber. The experimental procedure was described previously (21).

RESULTS

Overexpression of Ppc and Pck. The structural genes *ppc* and *pck* were cloned into vector pJF118EH as described above. The resulting plasmids, pPC201 and pCK601, were transformed into JCL1208, and the activities of Ppc and Pck were measured at different IPTG concentrations. Figure 2 shows that the expression of these genes was well controlled by IPTG. SDS-PAGE of the cell extract showed that the molecular weights of these two proteins agreed with the previously reported values (approximately 95,000 [6] and 51,000 [17] for Ppc and Pck, respectively).

To determine whether Ppc and Pck overexpression reduces the cell growth on glucose, the specific growth rates

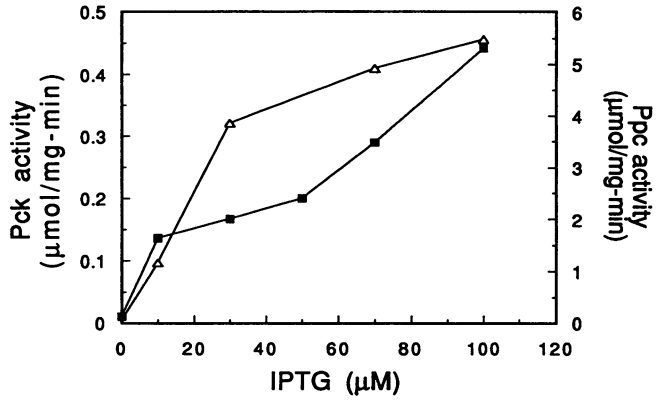

FIG. 2. Induction of Pck and Ppc activities by IPTG. JCL1208 containing pCK601 or pPC201 was cultured in M9 glucose medium with 50 μg of ampicillin per ml and with the indicated amounts of IPTG. Cells were harvested when the cell density reached about 1.5 × 10^8 cells per ml. Enzyme activities were measured as described in the text. Symbols: △, Pck activity from JCL1208/pCK601; ■, Ppc activity from JCL1208/pPC201.

were measured at different IPTG concentrations (Fig. 3). We found that overexpression of Ppc had little effect on the rate of growth in glucose minimal medium. However, the overexpression of Pck reduced the growth rate by more than 40% in glucose minimal medium at a moderate IPTG concentration (100 μM). The growth retardation by Pck overproduction was not due to the formation of inclusion bodies, since none was observed in these cells. It is unclear whether it is solely due to the ribosome load caused by protein overexpression.

The growth inhibition by overexpressed Pck under glycolytic conditions may be due to futile cycling between Pck and Ppc reactions, since chromosomal Ppc is also expressed under these conditions. The specific activity of Ppc from a chromosome was measured to be 0.049 μmol/mg/min in JCL1208. To test this hypothesis, we constructed a *ppc* strain which has the same genetic background as JCL1208.

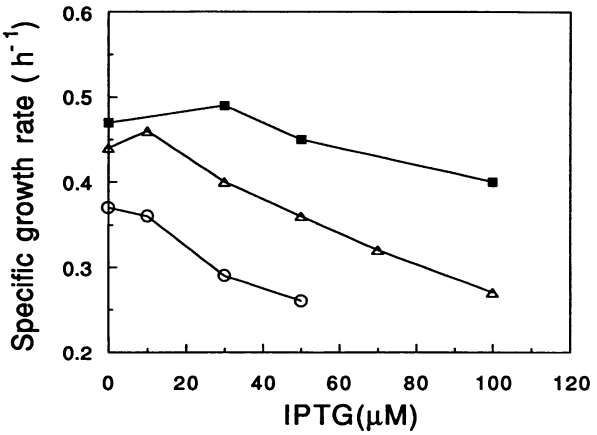

FIG. 3. Specific growth rates of cells overexpressing Ppc or Pck. Cells were cultured as described in the text. The strains used were a wild-type host containing a Ppc-overexpressing plasmid (JCL1208/pPC201 [■]), a wild-type host containing a Pck-overexpressing plasmid (JCL1208/pCK601 [△]), and a *ppc* strain containing a Pck-overexpressing plasmid (JCL1242/pCK601 [○]).

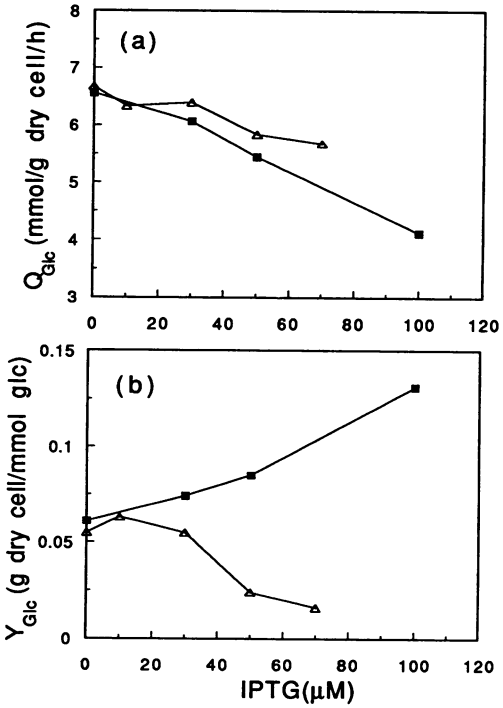

FIG. 4. Specific glucose consumption rate (Q_{Glc}) (a) and growth yield on glucose (Y_{Glc}) (b). The strains used were a Ppc-overexpressing strain, JCL1208/pPC201 (■), and a Pck-overexpressing strain, JCL1208/pCK601 (△).

Since *ppc* strains cannot grow on glucose as the sole carbon source, succinate (0.05%) was added to the glucose M9 medium. Upon overexpression of Pck, the *ppc* strain still showed growth inhibition (Fig. 3), suggesting that the futile cycling between PEP and OAA is not the reason for the reduced growth rate.

Specific glucose consumption rate and the growth yield on glucose. Since PEP is the phosphate donor in the phosphotransferase system, changing the metabolism of PEP may affect the rate of glucose transport. We therefore measured the glucose consumption rate in cultures of JCL1208 containing pPC201 or pCK601 at different IPTG concentrations (Fig. 4a). We observed that overproduction of either Ppc or Pck decreased the specific glucose consumption rate. Because Pck overexpression caused growth inhibition, the glucose consumption rate may be reduced because of a lower growth rate. To control for the growth rate difference, the growth yield on glucose was calculated (Fig. 4b); the result showed that overexpression of Ppc increased the growth yield on glucose, whereas the Pck overexpression decreased it. These data suggest that Ppc overexpression led to a lower glucose consumption rate relative to the growth rate, whereas Pck overexpression showed the opposite trend.

Specific oxygen consumption rate and growth yield for oxygen. Overexpression of Ppc is likely to channel carbon flow to biosynthesis at the expense of catabolism. On the other hand, overexpression of Pck may decrease the metabolic flow in the tricarboxylic acid cycle because of a potential drain in the intracellular OAA pool. To test these possibilities, we measured the oxygen consumption rate of strains overproducing these enzymes (Fig. 5a). We found that the overexpression of Ppc did not change the respiration

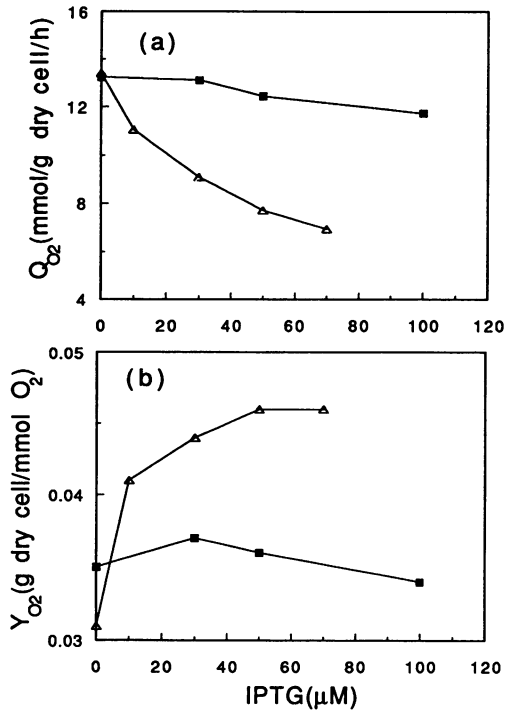

FIG. 5. Specific oxygen consumption rate (Q_{O_2}) (a) and growth yield for oxygen (Y_{O_2}) (b). The strains used were a Ppc-overexpressing strain, JCL1208/pPC201 (■), and a Pck-overexpressing strain, JCL1208/pCK601 (△).

rate, whereas Pck overexpression reduced the respiration rate by approximately 50%. Since high levels of Pck retarded growth on glucose, Y_{O_2} (k/Q_{O_2}) would be a better index for the effect on respiration. These yield values (Fig. 5b) show that the overproduction of Pck indeed decreased the respiration relative to the growth rate, whereas Ppc overproduction has an insignificant effect.

Fermentation products. Since Ppc overexpression reduced the specific glucose consumption rate but did not change the growth rate or respiration rate significantly, the decreased glucose uptake must result in decreased product formation. Overproduction of Pck increased glucose consumption relative to growth, but the excess carbon flow did not go through the tricarboxylic acid cycle, since the respiration rate was decreased. The excess carbon flow in a Pck-overproducing strain must be excreted as fermentation products. To test these hypotheses, we measured the fermentation products of strains overproducing Ppc or Pck. As predicted, Ppc overexpression reduced product formation, whereas Pck overexpression increased it. Among the major peaks identified by HPLC, acetate was quantified as shown in Fig. 6. Overexpression of Ppc also decreased two other fermentation products, which were not identified from the chromatogram. These results suggest that reduced fermentation product formation explains the improved growth yield on glucose for strains overexpressing Ppc. Similarly, the decreased growth yield on glucose for Pck-overproducing strains can be attributed to increased product formation. The Pck-overexpressing strain also excreted pyruvate, which was absent in the cultures of the Ppc-overproducing strain. Interestingly, pyruvate excretion was also observed for strains overexpressing PEP synthase under glycolytic conditions (21).

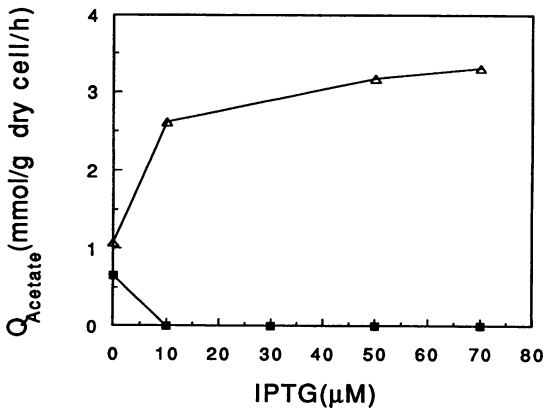

FIG. 6. The specific acetate production rate ($Q_{Acetate}$) for strains overexpressing Ppc (JCL1208/pPC201 [■]) and overexpressing Pck (JCL1208/pCK601 [△]).

DISCUSSION

To explain the above results, we follow Saier and Chin (22) in proposing that the PEP/pyruvate ratio is one of the factors determining the glucose uptake rate through the phosphotransferase system. Overexpression of Ppc is expected to decrease this ratio and thus decrease the glucose consumption rate. Overexpression of Pck tends to increase this ratio and thus increase the glucose consumption rate relative to the growth rate. Therefore, the expression levels of Ppc and Pck also control the glucose uptake rate indirectly. Unfortunately, the intracellular PEP level is difficult to detect under physiological conditions because of its low quantity and fast turnover. The results presented here provide indirect evidence consistent with the model. It is unclear whether any known phosphotransferase system regulators such as cyclic AMP and enzyme II^{Glc} in the phosphotransferase system (5) are involved in this regulation.

Decreased glucose consumption due to Ppc overexpression does not reduce the growth rate. Instead, it results in reduced fermentation product formation, which means more effective utilization of the carbon source. On the other hand, the increased glucose consumption relative to growth (shown by Y_{Glc}) in the Pck-overproducing strain leads to increased fermentation product formation. These results indicate that the growth is not limited by the glucose uptake rate under the experimental conditions used here and that the wild-type Ppc level is not optimized for maximal yield on glucose in batch cultures. As commonly assumed, wild-type *E. coli* consumes glucose as fast as possible, even though the growth is limited by other factors, and utilizes fermentation products when glucose is depleted.

The reduced oxygen consumption relative to growth in the Pck-overproducing strain (shown by Y_{O_2}) is perhaps due to the limitation of OAA or other four-carbon metabolites. This limitation may in turn cause the Pck-induced growth inhibition. However, the addition of four-carbon metabolites such as succinate or malate did not rescue the growth inhibition (unpublished data). The intracellular OAA concentration was too low to measure accurately. Therefore, the cause of these phenomena remains unclear. As indicated by the yield value for oxygen, more biomass is produced per unit of oxygen consumed because of additional ATP formation through increased acetate production.

In general, metabolic responses to perturbations in central metabolism are still incompletely understood. With some

exceptions (2, 14, 15, 25), most previous investigations of metabolic regulation focused on normal physiological states. Relatively little is known about metabolic regulation under extreme conditions such as enzyme overexpression. These conditions are important for the production of metabolites, which often requires moderately high levels of enzymes in the desired pathway. The change in enzyme activity in central metabolism will perturb the metabolite pools, which in turn may cause secondary responses important for practical reasons. Overexpression of Ppc is expected to favor the production of amino acids in the OAA family. One might therefore attempt to cause overexpression of Ppc for lysine production. The present study shows that such a practice may limit the glucose uptake rate but reduce fermentation product formation. The overall benefit of Ppc overexpression in lysine biosynthesis remains to be seen. Overexpression of Pck may favor the production of aromatic amino acids, since it tends to increase the PEP concentration. However, it reduces the growth rate of *E. coli* for unknown reasons. The underlying mechanisms for these metabolic responses call for further investigation.

ACKNOWLEDGMENTS

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