

Engineering of *Escherichia coli* Central Metabolism for Aromatic Metabolite Production with Near Theoretical Yield

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Received 4 April 1994/Accepted 15 August 1994

***Escherichia coli* and many other microorganisms synthesize aromatic amino acids through the condensation reaction between phosphoenolpyruvate (PEP) and erythrose 4-phosphate to form 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP). It has been shown that overexpression of transketolase increases the production of DAHP in an *aroB* mutant strain (unable to further metabolize DAHP) with elevated DAHP synthase. However, the yield (percent conversion) of DAHP from glucose is still low. Stoichiometric analysis shows that many enzymes compete for intracellular PEP. In particular, the phosphotransferase system, responsible for glucose transport in *E. coli*, uses PEP as a phosphate donor and converts it to pyruvate, which is less likely to recycle back to PEP. This stoichiometric limitation greatly reduces the yield of aromatic metabolites. To relieve this limitation, we overexpressed PEP synthase in the presence of glucose and showed that it increased the final concentration and the yield of DAHP by almost twofold, to a near theoretical maximum. The PEP synthase effect is not observed without overproduced transketolase, suggesting that erythrose 4-phosphate is the first limiting metabolite. This result demonstrates the utility of pathway analysis and the limitation of central metabolites in the high-level overproduction of desired metabolites.**

Production of biochemicals from microorganisms has long been an important application of biotechnology. Typically, the steps involved in developing a production strain include (i) selection of a proper host organism, (ii) elimination of pathways leading to by-products, (iii) deregulation of pathways at both the enzyme activity level and the transcriptional level, and (iv) overexpression of enzymes in the desired pathways. The last three steps can now be achieved by a variety of in vivo and in vitro methods. These processes are particularly user-friendly in well-studied microorganisms such as *Escherichia coli*. Therefore, many articles on the engineering of microorganisms for physiological characterization (6, 18, 25) and metabolite production (2, 3, 12) have been published. In most cases, the first target for engineering is the terminal pathway leading to the desired product, and the results are usually successful. However, further improvement of productivity (product formation rate) and yield (percent conversion) calls for the alteration of central metabolic pathways which supply necessary precursors and energy for biosyntheses.

Aromatic metabolites such as tryptophan, phenylalanine, and tyrosine are essential amino acids for humans and animals. In addition, phenylalanine is used in aspartame production (15, 19), and the tryptophan pathway can be modified to produce indigo (8). In *E. coli*, aromatic metabolites are generated from the condensation reaction between phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) to form 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP). This step is the first committed step toward synthesis of aromatic metabolites and is mediated by three DAHP synthases. These isoenzymes are encoded by the genes *aroF*, *aroG*, and *aroH*, whose products are feedback inhibited by tyrosine, phenylalanine, and tryptophan, respectively. The production of tryptophan and phenylalanine by *E. coli* has been well documented

(1, 9, 11, 17). As expected, the productivity and yield have increased as a result of deleting branched pathways, deregulating enzymes, and overexpressing terminal pathways. For example, Aiba et al. (1) have reported a tryptophan overproducer that contains overexpressed genes in the tryptophan operon in a host strain that is *trpR* and *trnA* (encoding tryptophanase) negative. Moreover, various enzymes, such as the *trpE* gene product (1), have been mutated to resist feedback inhibition. Similar work has been reported for phenylalanine production. In addition to engineering the terminal pathways, Miller et al. (17) attempted to direct more carbon flux into the amino acid pathway by use of a PEP carboxylase (coded by *ppc*)-deficient mutant. Draths et al. (7) reported that overexpression of transketolase (coded by *tktA*) and a feedback-resistant (Fbr) DAHP synthase [coded by *aroG*(Fbr)] improves the production of DAHP from glucose. However, the yields of these processes are still far below the theoretical values.

To channel more carbon flux into the aromatic pathway, one has to overcome pathways competing for PEP. PEP is also used as a phosphate donor in the phosphotransferase system, which is responsible for glucose uptake in *E. coli*. Moreover, PEP can be converted to pyruvate by pyruvate kinases and to oxaloacetate (OAA) by PEP carboxylase. Once PEP is converted to pyruvate by either the phosphotransferase system or pyruvate kinases, it is less likely to recycle back to PEP because of the high energy cost. As a result, a large amount of carbon flux is channelled through pyruvate and eventually to organic acids, carbon dioxide, or cell mass. To overcome this stoichiometric limitation, we overexpressed PEP synthase (Pps) in the presence of glucose and attempted to direct more carbon flux into the aromatic pathway to produce DAHP.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* AB2847 *aroB mal T6*^r (20), obtained from the *E. coli* Genetic Stock Center, Yale University, was used as the host strain for DAHP production. BJ502 *ikt-2 fhuA22 garB10 ompF627 fadL701 relA1 pit-10 spoT1*

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype	Source or reference
Strains		
AB2847	<i>aroB mal T6^r</i>	<i>E. coli</i> Genetic Stock Center
BJ502	<i>tkl-2</i>	<i>E. coli</i> Genetic Stock Center
JC7623	<i>recB21 recC22 sbcB15</i>	26
HG4	<i>pck-2 pps-3</i>	Hughes Goldie
POII1734	<i>araD139 ara::(Mu cts)3 Δ(lac)X74 galU galK rpsL</i> with Mu dIII1734 <i>lac⁺</i> (Km ^r)	4
CAG12151	<i>zdh-925::Tn10</i>	22
JCL1242	<i>Δ(argF-lac)U169</i> but <i>ppc::Km</i>	5
JCL1283	As for AB2847 but <i>ppc::Km</i>	This study
JCL1362	As for AB2847 but <i>pps::Mu dIII1734</i>	This study
Plasmids		
pUHE23-2	Ap ^r ; IPTG-inducible T7(A1) early promoter	H. Bujard
pPS341	As for pUHE23-2 but <i>pps⁺</i>	18
pPS341X1	As for pPS341 but <i>pps-50</i> (2-codon insertion)	18
pPS1734	pPS341::Mu dIII1734 <i>lac⁺</i> (Km ^r)	This study
pRW5	pACYC184 derivative, Cm ^r but tandem <i>lac</i> promoters <i>aroG</i> (Fbr) ⁺	Alan Berry
pAT1	As for pRW5 but <i>tklA⁺</i>	This study

mcrB1 phoM510, also from the *E. coli* Genetic Stock Center, was used in the identification of the *tkl* clone. JCL1242 *ppc::Km* was constructed as described previously (5) by inserting a kanamycin cassette into a cloned *ppc* gene, which is then integrated into the chromosome by homologous recombination.

Plasmid pPS341 was constructed as described previously (18) by cloning a fragment of *E. coli* chromosomal DNA containing the *pps* gene into an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression vector, pUHE23-2 (a pBR322 derivative). Plasmid pPS341X1 containing the inactive gene product of *pps* was constructed by codon insertion mutagenesis, the details of which are also described by Patnaik et al. (18). The *pps* gene on pPS341 was inserted with a Mu dIII1734 *lac⁺* Km^r (MudK) according to published protocol (4). Briefly, a Mu lysate was made from donor strain POII1734/pPS341, which was lysogenized by the mini-Mu element and a *Mu cts*. The lysate was used to infect a Mu lysogen of HG4 *pps pck*, and colonies were selected for Ap^r and Km^r simultaneously to ensure that the mini-Mu element hopped to the plasmid. Colonies were further screened for the Pps⁻ phenotype (inability to grow on pyruvate). Restriction analysis of plasmid DNA further confirmed the insertion of the MudK element into the *pps* gene on plasmid pPS341. Twenty percent of these selected colonies showed IPTG-dependent expression of β-galactosidase, indicating an in-frame insertion. The plasmid from one such colony is named pPS1734; it was linearized at the *ScaI* site and then transformed into strain JC7623 *recB21 recC22 sbcB15* (26). Transformants were selected for Km^r and scored for Ap sensitivity. Such colonies presumably contained *pps::MudK* on the chromosome. By means of P1 transduction, this locus was moved to AB2847, and Km^r transductants were further screened for the inability to grow on pyruvate. One such colony was designated JCL1362 and used for later studies. The MudK insertion into chromosomal *pps* was further confirmed by cotransduction frequency (89%) with Tet^r markers from strain CAG12151 *zdh-925::Tn10* (22).

Plasmid pRW5, a kind gift of A. Berry of Genencor International, South San Francisco, Calif., is a pACYC derivative and contains *aroG*(Fbr). This plasmid also contains a *lacI* gene, and *aroG*(Fbr) is expressed from a *lac* promoter. To construct plasmid pAT1 containing both *aroG*(Fbr) and *tklA*, a 5-kb *Bam*HI fragment of *E. coli* DNA was cut from phage 473 of the Kohara miniset (thanks to Yuji Kohara of the National

Institute of Genetics, Japan) and was inserted into the *Bam*HI site of pRW5. This fragment was reported to contain the *tklA* gene (23), which was confirmed by its ability to complement a *tkl* strain (BJ502) for growth on ribose and also by the migration distance of the gene product on a sodium dodecyl sulfate–12% polyacrylamide gel (molecular weight, ca. 72,500 [7]). The strains and plasmids used are summarized in Table 1.

Media and growth conditions. All cloning procedures were carried out in Luria-Bertani medium. Yeast extract (YE) medium (7) contained (per liter) K₂HPO₄ (14 g), KH₂PO₄ (16 g), (NH₄)₂SO₄ (5 g), MgSO₄ (1 g), yeast extract (15 g), and D-glucose (15 g). Minimal medium used for high-cell-density resuspension cultures contained, per liter, K₂HPO₄ (14 g), KH₂PO₄ (16 g), (NH₄)₂SO₄ (5 g), MgSO₄ (1 g), and D-glucose (15 g) and was also supplemented with thiamine (1 mg), shikimic acid (50 mg), L-tyrosine (8 mg), L-phenylalanine (8 mg), and L-tryptophan (4 mg). The minimal medium was supplemented with succinate (0.1 g/liter) when the *ppc* mutant and its control were grown. For the stable maintenance of plasmids, ampicillin (100 mg/ml) and chloramphenicol (50 mg/ml) were added to the culture medium. The concentrations of the antibiotics were reduced by half when minimal medium was used.

Overnight cultures in YE medium were grown at 37°C in a roller drum and then were subcultured in the same medium with appropriate drugs. Cultures were grown in 250-ml shake flasks at 37°C in a gyratory water bath shaken at 200 rpm. After 4 h of incubation (optical density at 550 nm, 2 to 3), cultures were induced with IPTG (1 mM). Cells were harvested from late stationary phase by centrifugation at 6,000 × g and were washed twice with minimal medium before being resuspended in the same minimal medium supplemented with appropriate drugs and IPTG (1 mM). The initial optical densities at 550 nm of all high-density resuspension cultures were about 4.0. Samples from the resuspension cultures were withdrawn periodically for assaying the DAH(P) and glucose concentrations in the medium.

Determination of glucose and DAH(P). Cells were removed from samples by centrifugation and the supernatants were stored at 4°C until all samples had been collected. The level of residual glucose in the culture supernatant was determined by the dinitrosalicylic acid assay for total reducing sugars (16, 18). The concentration of DAH(P) in the supernatant was deter-

mined by the thiobarbiturate assay (7, 10). This assay does not distinguish between DAH and DAHP.

Enzyme assays. Cells were harvested by centrifugation at $6,000 \times g$ and were washed and resuspended in 50 mM potassium phosphate buffer (pH 7) or 5 mM Tris-Cl-1 mM $MgCl_2$ (pH 7.4) for a DAHP synthase or Pps assay, respectively. Cell extracts were prepared by rupturing cells with a French pressure cell (SLM Aminco, Urbana, Ill.) at 16,000 lb/in². DAHP synthase activity was assayed by the procedure of Schoner and Herrmann (21). Pps activity was assayed as described previously (18). Total protein in the extracts was determined with the Bio-Rad dye reagent (Bradford assay) and with bovine serum albumin as the standard.

RESULTS

Overexpression of Pps increases the production of DAHP. AB2847 is unable to utilize DAHP and accumulates DAHP in the medium if DAHP synthase is overexpressed. This strain was used as a host for detecting the flux committed to the aromatic pathways. Since Draths et al. (7) have shown a possible limitation of E4P in the production of DAHP, we transformed pAT1 [containing both *aroG*(Fbr) and *tklA*] into AB2847 to eliminate the limitation of E4P. To test whether PEP supply is also limiting in the production of DAHP, we overexpressed Pps in AB2847/pAT1 by transforming plasmid pPS341 into this strain. As a control, we replaced pPS341 with pPS341X1 (18), which encodes an inactive but stable *pps* gene product. The use of the inactive Pps control allows one to distinguish between the effect of Pps activity and that of protein overexpression. We also used AB2847/pAT1/pUHE23-2 and AB2847/pAT1 without any other plasmid as additional controls to identify the effect of the cloning vector, pUHE23-2, on DAHP production.

As described above, the strains were grown in a rich medium (YE) with IPTG and resuspended in a minimal medium. Since the overexpression of Pps under glycolytic conditions may cause growth inhibition (18), resuspension cultures were used to minimize the effect of cell growth on the biocatalytic conversion. After resuspension, the excreted DAHP and residual glucose were measured periodically. At 27 h after resuspension, samples were taken for Pps and AroG assays. Fig. 1A shows that the strain overexpressing active Pps increased DAHP production almost twofold. The strains containing pPS341X1 or pUHE23-2 produced the same amount of DAHP as the one containing only pAT1. Figure 1B shows that, as expected, Pps activity in the strain containing pPS341 is overexpressed 10-fold, whereas the AroG activity in all strains remains almost constant. These data strongly suggest that the activity of Pps is responsible for the increase in DAHP production, whereas the inactive Pps or the cloning vector has no observable effect on DAHP production.

The specific glucose consumption rates of these strains were not influenced by the presence of active or inactive Pps or by the cloning vector (data not shown). Therefore, the strain overexpressing Pps showed an almost twofold increase in overall DAHP yield (ca. 90% molar yield) compared with those of the controls (ca. 52% molar yield), suggesting that Pps improves both the productivity and the yield of DAHP production. The maximum theoretical yield from glucose to DAHP is 86% (discussed below), which is slightly lower than the measured yield from the strain overexpressing Pps. Because both glucose and DAHP measurements were reasonably reproducible, the discrepancy may be attributed to the inaccuracy of the extinction coefficient (24) used to calculate DAHP concentration. However, we have calibrated the extinction

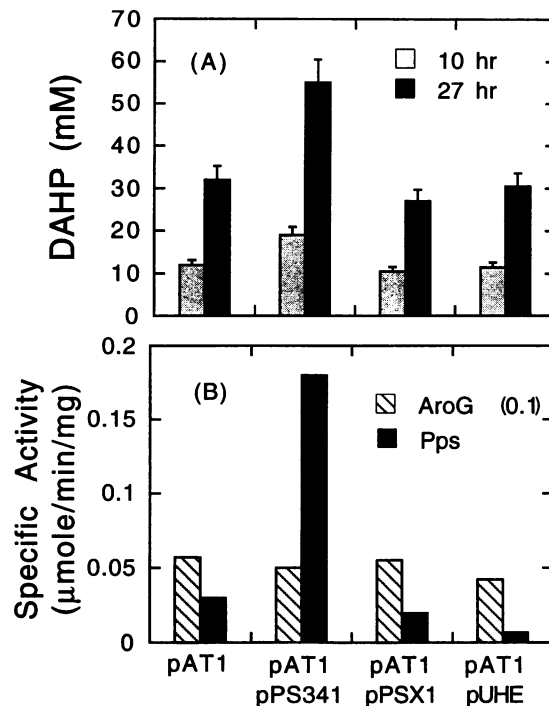


FIG. 1. Overexpression of Pps increased the production of DAHP. The host strain was AB2847, and the plasmids were as labeled in the figure. Note that pPSX1 and pUHE stand for pPS341X1 and pUHE23-2, respectively. These strains were cultured first in YE medium (a rich medium) to late stationary phase and then washed and resuspended in a minimal medium (see the text). (A) DAHP concentrations measured at 10 and 27 h after resuspension; (B) activities of DAHP synthase (AroG, 10^{-1}) and PEP synthase (Pps) measured at 27 h after resuspension.

coefficient by using biosynthesized DAHP from cell extract and known amounts of E4P and PEP. Results show that the extinction coefficient is roughly within 30% accuracy. Therefore, the yield of DAHP is reasonably close to the theoretical maximum, even though it may be lower than the theoretical value.

To determine whether the Pps effect requires overexpressed Tkt, we used plasmid pRW5, which contains only *aroG*(Fbr), to replace pAT1 in the experiments described above. We found that overproduction of Pps did not increase DAHP production (Fig. 2A) without the elevated Tkt activity. Therefore, so far as limitation of small molecules in the biosynthesis of DAHP is concerned, the first limitation arises from the supply of E4P. This bottleneck shifts to the supply of PEP when Tkt is overexpressed, which is believed to increase the supply of E4P.

Chromosomal null mutations of *pps* and *ppc*. As shown above, Pps overexpression improved DAHP production from glucose. It was interesting to know whether the basal level of Pps expression in glucose medium contributed to the production of DAHP. We therefore knocked out the chromosomal *pps* gene in strain AB2847. The resulting strain (JCL1362) was used as the host to repeat the above-described experiments. Results show that inactivation of chromosomal *pps* did not significantly affect DAHP production in strains containing pRW5 or pAT1 (Fig. 2B). Therefore, the basal level of *pps* expression in glucose medium did not contribute to DAHP production.

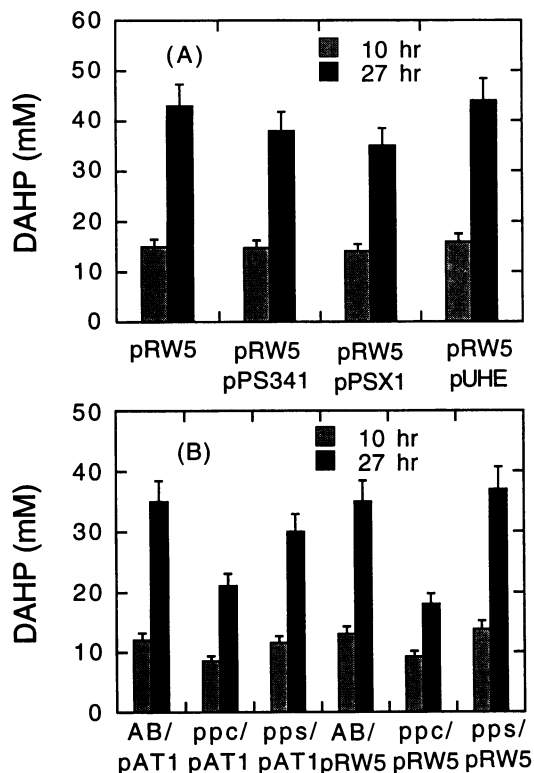


FIG. 2. DAHP production at 10 and 27 h after resuspension. (A) Strain AB2847 with the plasmids shown below the x axis. pPSX1 and pUHE stand for pPS341X1 and pUHE23-2, respectively. (B) Strains AB2847 (AB), JCL1283 *ppc::Km* (*ppc*), and JCL1362 *pps::MudK* (*pps*) with the plasmids shown below the x axis.

Since PEP is also converted to OAA by Ppc, the deletion of this enzyme may increase the supply of PEP. We therefore inactivated the *ppc* gene on the chromosome of AB2847 in an attempt to increase DAHP production without Pps overexpression. This was done by transducing AB2847 with a P1 lysate grown on JCL1242 *ppc::Km*. The resulting transductant, JCL1283 *aroB ppc::Km*, was then transformed with pAT1 or pRW5 and tested for DAHP production in the resuspension culture as described above. To avoid limitation of OAA in the *ppc* strain, the culture medium was supplemented with succinate, which was shown to have no effect on DAHP production (data not shown). Contrary to the expectation, the *ppc* mutation did not increase the production of DAHP (Fig. 2B), suggesting that the metabolic flux from PEP to OAA was not significant under the experimental conditions tested here. In fact, the *ppc* mutation actually decreased DAHP production for unknown reasons.

DISCUSSION

According to theoretical analyses (9, 13), the maximum yield of aromatic amino acids from glucose can be increased twofold if pyruvate is recycled back to PEP. The maximum yield is calculated by assuming that the branching pathways are blocked and that the carbon flow is directed by the most efficient pathways with minimum loss to carbon dioxide and other metabolites. Under these conditions, the relative flux through each step at the steady state can be calculated by balancing the input and output fluxes from each metabolite

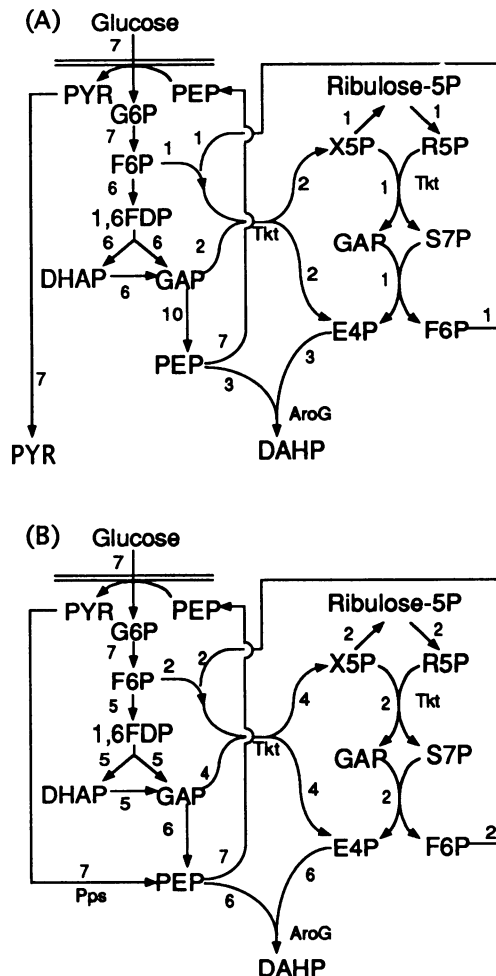


FIG. 3. Reaction pathways for maximal conversion of glucose to DAHP for strains without Pps (A) and strains overexpressing Pps (B). The numbers are the relative fluxes needed to convert 7 mol of glucose to DAHP. G6P, glucose 6-P; F6P, fructose 6-P; 1,6FDP, 1,6-fructose diphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-P; R5P, ribose 5-P; X5P, xylulose 5-P; S7P, sedoheptulose 7-P; PYR, pyruvate.

pool. As shown in Fig. 3A, for maximum yield of DAHP production by strains without Pps overproduction, 7 mol of glucose is needed to produce 3 mol of DAHP (43% molar yield) and 7 mol of pyruvate, which is further metabolized. The relative flux through each intermediate step is shown in Fig. 3A. The formation of pyruvate is necessary because of the stoichiometry of the phosphotransferase system for glucose uptake. In the presence of glucose, pyruvate is not recycled back to PEP efficiently because the enzyme Pps is not induced. However, if pyruvate is effectively recycled to PEP via overexpressed Pps in the presence of glucose, the maximum yield will increase twofold, reaching the theoretical level. As shown in Fig. 3B, 6 mol of DAHP can now be produced from 7 mol of glucose (86% molar yield). The nonoxidative part of the pentose pathway provides E4P, and the Pps reaction recycles pyruvate to PEP.

The data shown above are in agreement with this flux distribution model. Although it is difficult to measure flux mediated by Pps directly, the controls with inactive Pps and

with no Pps demonstrate that the activity of Pps is required to achieve this high yield. Unfortunately, measurements of intracellular PEP are difficult because of its small quantity and high turnover rate. According to Lowry et al. (14), the PEP concentration is below 0.5 $\mu\text{mol/g}$ (dry weight) with about 50% standard deviations. In our hands, the measurement was not sensitive enough to detect the change in PEP level due to Pps overproduction, particularly because PEP is further converted to DAHP by overexpressed DAHP synthase. The observation that the Pps effect on DAHP production requires the overexpressed Tkt suggests that E4P is the first limiting substrate for DAHP synthase. Therefore, without a sufficient supply of E4P, increasing the flux to PEP with Pps does not lead to increased DAHP production. However, supplying E4P alone by Tkt overexpression cannot increase the yield to near the theoretical level (Fig. 2A) because of the stoichiometric limitation in pyruvate recycling to PEP (Fig. 3A).

In our previous work (18), we demonstrated that overexpression of Pps in the presence of glucose led to growth inhibition, increased glucose consumption, and excretion of pyruvate and acetate. To overcome the problem of growth impairment, we had to use high-density resuspension cultures which have high levels of metabolic activity but very low growth rates. In actively growing cultures, the effects of Pps on DAHP production are not as significant and the impairment of growth caused by Pps overexpression negates the positive effects on DAHP production. The stimulation of glucose consumption in the previous work was attributed to the altered PEP/pyruvate ratio. It was hypothesized that an increased PEP/pyruvate ratio stimulates the phosphotransferase system for increased glucose consumption, which in turn results in the excretion of pyruvate. In the present study, we redirected PEP to the aromatic pathway, and thus the PEP/pyruvate ratio is likely to decrease. This flux redirection explains the insensitivity of the specific glucose consumption rate to Pps overexpression in the experimental system used in this study. The increased DAHP production from glucose caused by Pps overexpression also suggests that Pps actually functions in its physiological direction (from pyruvate to PEP) *in vivo*, even under glycolytic conditions.

It has been reported that the deletion of *ppc* increased the production of phenylalanine and acetate (17). Moreover, our group has shown that the overexpression of Ppc in a wild-type host reduces acetate production (5). Both results suggest that the flux through Ppc (from PEP to OAA) is reasonably significant under those conditions, and thus, the modulation of Ppc expression level affects the utilization of PEP. In the present work, however, we did not observe any positive effect on DAHP production by deleting the chromosomal *ppc* gene, suggesting that the flux through Ppc is not important under the experimental conditions used here. The discrepancy may be attributed to the nongrowth condition used in our experiment and to the phenotypical variation in the host strains. Note that the reaction scheme for theoretical yield (Fig. 3B) does not require pyruvate kinases or the oxidative part of the pentose pathway. Therefore, it is possible that the inactivation of pyruvate kinases or the glucose 6-P dehydrogenase during the production phase may ensure the high yield of DAHP.

This work demonstrates that engineering the central metabolism according to the stoichiometric analysis can lead to improvements in both the productivity and yield. However, such analysis is predicated on the assumptions that all the reaction steps are known and that the physiological role of each step *in vivo* is also known under the conditions of interest. Despite the extensive research on *E. coli* metabolism, it is still possible that unknown physiological roles of existing enzymes

or new reactions exist, which may invalidate the stoichiometric analysis.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation (grant BCS-9257351), the Welch Foundation (grant A-1251), and the Texas Higher Education Coordinating Board (grant 999903-084).

REFERENCES

1. Aiba, S., H. Tsunekawa, and T. Imanaka. 1982. New approach to tryptophan production by *Escherichia coli*: genetic manipulation of composite plasmids *in vitro*. *Appl. Environ. Microbiol.* **43**:289-297.
2. Bailey, J. E. 1991. Toward a science of metabolic engineering. *Science* **252**:1668-1675.
3. Cameron, D. C., and I.-T. Tong. 1993. Cellular and metabolic engineering: an overview. *Appl. Biochem. Biotechnol.* **38**:105-140.
4. Castilho, B. A., P. Olfson, and M. J. Casadaban. 1984. Plasmid insertion mutagenesis and *lac* gene fusion with mini-Mu bacteriophage transposons. *J. Bacteriol.* **158**:488-495.
5. 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.
6. Chao, Y.-P., and J. C. Liao. 1994. Metabolic responses to substrate futile cycling in *Escherichia coli*. *J. Biol. Chem.* **269**:5122-5126.
7. Draths, K. M., D. L. Pompliano, D. L. Conley, J. W. Frost, A. Berry, G. L. Disbrow, R. J. Staversky, and J. C. Lievens. 1992. Biocatalytic synthesis of aromatics from D-glucose: the role of transketolase. *J. Am. Chem. Soc.* **114**:3956-3962.
8. Ensley, B. D., B. J. Ratzkin, T. D. Osslund, M. J. Simon, L. P. Wackett, and D. T. Gibson. 1983. Expression of naphthalene oxidation genes in *Escherichia coli* results in the biosynthesis of indigo. *Science* **222**:167-169.
9. Forberg, C., T. Eliaeson, and L. Haggstrom. 1988. Correlation of theoretical and experimental yields of phenylalanine from non-growing cells of a *rec Escherichia coli* strain. *J. Biotechnol.* **7**:319-332.
10. Gollub, E., H. Zalkin, and D. B. Sprinson. 1971. Assay for 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthase. *Methods Enzymol.* **17A**:349-350.
11. Imanaka, T., H. Tsunekawa, and S. Aiba. 1980. Phenotypic stability of *trp* operon recombinant plasmids in *Escherichia coli*. *J. Gen. Microbiol.* **118**:253-261.
12. Ingram, L. O., T. Conway, D. P. Clark, G. W. Sewell, and J. F. Preston. 1987. Genetic engineering of ethanol production in *Escherichia coli*. *Appl. Environ. Microbiol.* **53**:2420-2425.
13. Liao, J. C. 1989. Some practical issues in metabolic pathway engineering, paper 29b. *Abstr. Annu. Meet. Am. Inst. Chem. Eng.*
14. Lowry, O. H., J. Carter, J. B. Wood, and L. Glaser. 1971. The effect of carbon and nitrogen sources on the level of metabolic intermediates in *Escherichia coli*. *J. Biol. Chem.* **246**:6511-6521.
15. Mazur, R. H., J. M. Schlatter, and A. H. Goldkamp. 1969. Structure-taste relationships of some dipeptides. *J. Am. Chem. Soc.* **91**:2684-2691.
16. Miller, G. L. 1958. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.* **31**:426-428.
17. Miller, J. E., K. C. Backman, J. M. O'Connor, and T. R. Hatch. 1987. Production of phenylalanine and organic acids by phosphoenolpyruvate carboxylase-deficient mutants of *Escherichia coli*. *J. Ind. Microbiol.* **2**:143-149.
18. Patnaik, R., W. D. Roof, R. F. Young, and J. C. Liao. 1992. Stimulation of glucose catabolism in *Escherichia coli* by a potential futile cycle. *J. Bacteriol.* **174**:7527-7532.
19. Pietsch, H. 1976. Synthese von S-Aspartyl-S-Phenylalaninmethyl-ester (ASPARTAM) aus S-4-Vinylazetid-2-On. *Tetrahedron Lett.* **45**:4053-4056.
20. Pittard, J., and B. J. Wallace. 1966. Distribution and function of genes concerned with aromatic biosynthesis in *Escherichia coli*. *J. Bacteriol.* **91**:1494-1508.
21. Schoner, R., and K. M. Herrmann. 1976. 3-Deoxy-D-arabino-

- heptulosonate 7-phosphate synthase. *J. Biol. Chem.* **251**:5440–5447.
22. **Singer, M., T. A. Baker, G. Schnitzler, S. M. Deischel, M. Goel, W. Dove, K. J. Jaacks, A. D. Grossman, J. W. Erickson, and C. A. Gross.** 1989. A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. *Microbiol. Rev.* **53**:1–24.
 23. **Sprenger, G. A.** 1992. Location of the transketolase (*tkt*) gene on the *Escherichia coli* physical map. *J. Bacteriol.* **174**:1707–1708.
 24. **Srinivasan, P. R., and D. B. Sprinson.** 1958. 2-Keto-3-deoxy-D-arabo-heptonic acid 7-phosphate synthetase. *J. Biol. Chem.* **234**:716–722.
 25. **Walsh, K., and D. E. Koshland, Jr.** 1985. Characterization of rate-controlling steps *in vivo* by use of an adjustable expression vector. *Proc. Natl. Acad. Sci. USA* **82**:3577–3581.
 26. **Winans, S. C., S. J. Elledge, J. H. Krueger, and G. C. Walker.** 1985. Site-directed insertion and deletion mutagenesis with cloned fragments in *Escherichia coli*. *J. Bacteriol.* **161**:1219–1221.