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A direct comparison of approaches for increasing carbon flow to aromatic biosynthesis in *Escherichia coli*

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Different approaches to increasing carbon commitment to aromatic amino acid biosynthesis were compared in isogenic strains of *Escherichia coli*. In a strain having a wild-type PEP:glucose phosphotransferase (PTS) system, inactivation of the genes encoding pyruvate kinase (*pykA* and *pykF*) resulted in a 3.4-fold increase in carbon flow to aromatic biosynthesis. In a strain already having increased carbon flow to aromatics by virtue of overexpression of the *tktA* gene (encoding transketolase), the *pykA* and/or *pykF* mutations had no effect. A PTS$^-$ glucose$^+$ mutant showed a 1.6-fold increase in carbon flow to aromatics compared to the PTS$^+$ control strain. In the PTS$^-$ glucose$^+$ host background, overexpression of *tktA* caused a further 3.7-fold increase in carbon flow, while inactivation of *pykA* and *pykF* caused a 5.8-fold increase. When all of the variables tested (PTS$^-$ glucose$^+$, *pykA*, *pykF*, and overexpressed *tktA*) were combined in a single strain, a 19.9-fold increase in carbon commitment to aromatic biosynthesis was achieved.

Keywords: amino acids; aromatics; *E. coli*; DAHP; PEP

Introduction

The aromatic amino acid biosynthetic pathway is now widely recognized as a potential source of a variety of aromatic compounds of commercial interest [10]. The advantages of microbial production of aromatic compounds via fermentation (versus traditional chemical syntheses) are synthesis from renewable resources (eg glucose, biomass) and more environmentally friendly manufacturing processes.

Until recently, the spectrum of products derived from the aromatic pathway was limited to those compounds that fell within the native genetic/physiological potential of the production organism. However, with the advent of heterologous gene expression, the range of aromatic compounds produced by a given microorganism was greatly expanded. For example, expression in *Escherichia coli* of the *qad* gene from *Klebsiella pneumoniae*, the *nahA* gene cluster from *Pseudomonas putida* and the *mel* genes from *Streptomyces antibioticus*, results in production of quinic acid [7], indigo [8], and melanin [5], respectively. *E. coli* lacks the native potential for synthesis of any of these compounds.

The commercial viability of producing aromatic compounds using microorganisms depends on achieving the maximum conversion of raw material (glucose) to the desired product. Frost and Lievense [10] and Patnaik *et al* [16,18] described the significant gains to be made in the yield of aromatic products from glucose by increasing the intracellular supply of phosphoenolpyruvate (PEP), a direct precursor of aromatic biosynthesis. Figure 1 shows the central metabolic pathways in *E. coli* that compete for PEP (only those steps relevant to the present work are shown).

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The PEP:glucose phosphotransferase system (PTS) and the pyruvate kinase isozymes (encoded by the *pykA* and *pykF* genes) are major consumers of PEP in *E. coli*, and as such are the focus of the present investigation. In addition, amplification of the *pps* gene (encodes PEP synthase) increases the yield of aromatics from glucose [16,18], and is therefore also included in the present work. These different approaches to increasing PEP availability were tested with and without simultaneous overproduction of the pentose phosphate pathway enzyme transketolase, encoded by the *tktA* gene. Amplifiation of *tktA* increases carbon flow to aromatics, presumably by increasing the intracellular level of erythrose 4-phosphate (E4P; Figure 1) [6].

In this report, we use carbon commitment to the aromatic amino acid pathway as a means of comparing different approaches to increasing the intracellular supply of PEP in *E. coli*. Previous reports [14,16,18] described the effects of the individual approaches to increasing PEP levels on aromatic biosynthesis. However, this is the first report where the various approaches have been combined and examined in isogenic *E. coli* strains.

Materials and methods

Strains and plasmids

The relevant genotypes for the *E. coli* strains and the plasmids used in this study are listed in Table 1. NF9 is a PTS$^-$ glucose$^+$ derivative of PB103 [9]. The *pykA* and *pykF* derivatives of PB103 and NF9 were obtained by P1 transduction according to Miller [15], using lysates prepared on *E. coli* strains PB22 (*pykA::cat*) [19], PB24 (*pykF::cat*) [19] and ATCC-FGm and ATCC-FKm (*pykF::gen* and *pykF::kan*, respectively, Table 1). Transductants were selected for resistance to the appropriate antibiotic, and the insertions of the antibiotic resistance markers in the *pyk* genes were confirmed by PCR using primers specific for

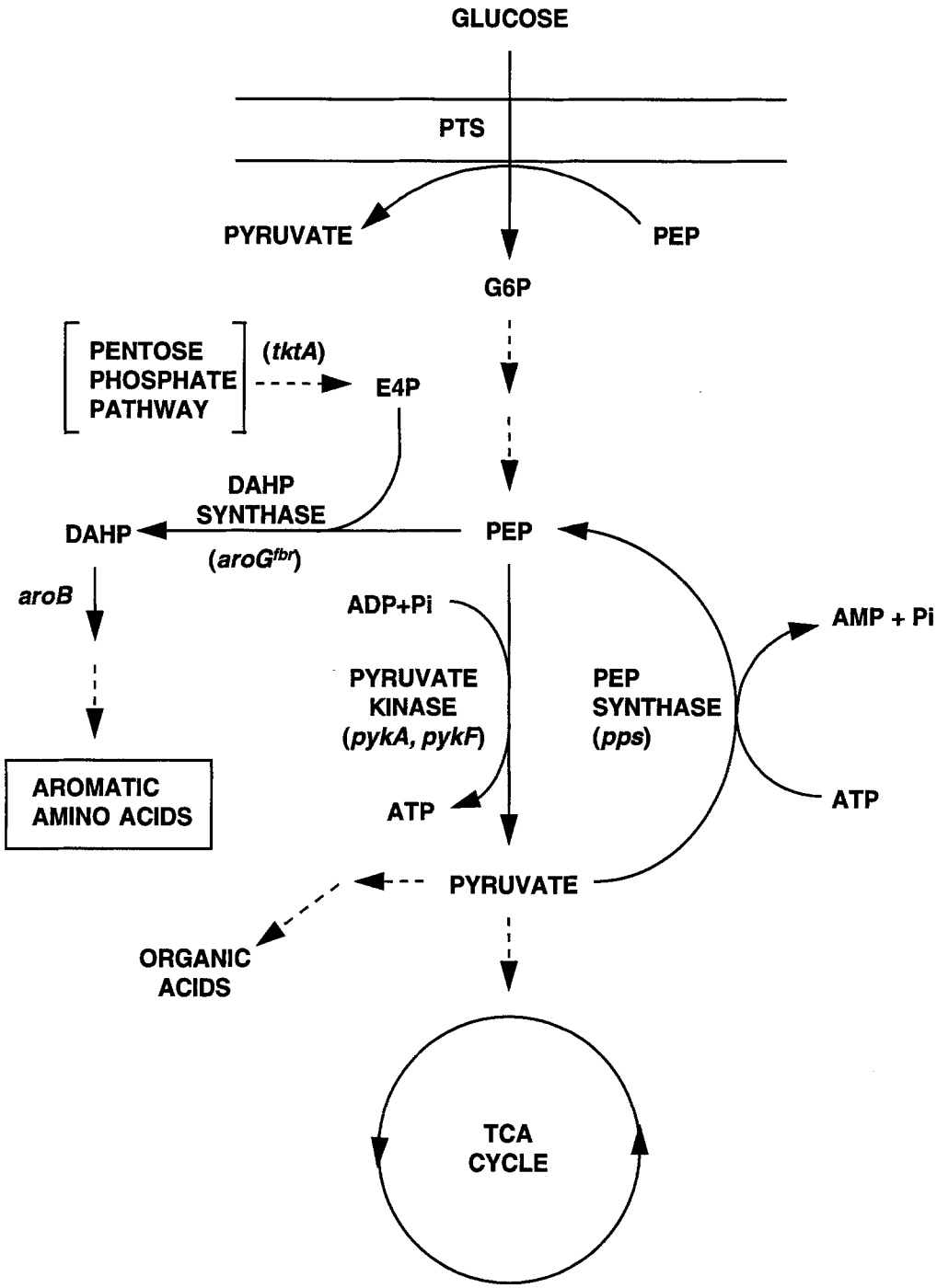

Figure 1 Central metabolic pathways related to the formation and consumption of PEP, and the proximity of PEP to aromatic amino acid biosynthesis. Dashed lines represent multiple enzymatic steps. Reproduced and modified with permission from [17].

pykA and *pykF*. Plasmids pRW300 and pRW5 were constructed in the pBR322 [3] and pACYC184 [4] vector backbones, respectively, and contain the same P_{lacUV5}-*aroG*^{fbr} gene cassette. This cassette contains tandem *lacUV5* promoters [1] controlling expression of the *E. coli aroG*^{fbr} gene [13]. The *aroG*^{fbr} gene encodes the first enzyme of the aromatic amino acid pathway, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase (Figure 1). The 'fbr' designation indicates that the *aroG* gene used here contains a mutation that renders its product (DAHP synthase) resistant to feedback inhibition by phenylalanine (the normal allosteric effector) [13]. Both pRW300 and pRW5 also contain a cloned *E. coli lacI* gene to regulate expression of *aroG*^{fbr}. Plasmid pCLtkt was constructed by cloning a 5-kb *Bam*HI fragment containing the *E. coli tktA* gene [6] into vector pCL1920 [12]. Plasmid pPS341 contains the *E. coli pps* gene cloned under control of the *tac* promoter [17].

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or cloned gene(s)	Source or reference
Strains		
ATCC 47002	*F⁻ lacZ⁺ leu-6 his-4 ara-14 recB21 recC22 sbc15 λ⁻*	[21]
ATCC-FGm	same as ATCC 47002, but *pykF::gen*	This study
ATCC-FKm	same as ATCC 47002, but *pykF::kan*	This study
JM101	F′ *traD36 lacI^q Δ (lacZ)M15 proA⁺B⁺/supE thi Δ (lac-proAB)*	[22]
PB22	same as JM101, but *pykA::cat*	[19]
PB24	same as JM101, but *pykF::cat*	[19]
PB103	W3110 F⁻ *Δ (argF-lac)U169 trpR tnaA2*	This study
PB103A	same as PB103, but *pykA::cat*	This study
PB103F	same as PB103, but *pykF::cat*	This study
PB103AF	same as PB103A, but *pykF::kan*	This study
NF9	same as PB103, but PTS⁻ glucose⁺	[9]
NF9A	same as NF9, but *pykA::cat*	This study
NF9F	same as NF9, but *pykF::gen*	This study
NF9AF	same as NF9A, but *pykF::gen*	This study
Plasmids		
pRW5	P_{lacUV5}-*aroG*^{fbr} (pACYC184 replicon, chloramphenicol-resistant)	[6]
pRW5tkt	same as pRW5, but also contains *tktA*	[6]
pRW300	P_{lacUV5}-*aroG*^{fbr} (pBR322 replicon, tetracycline-resistant)	This study
pCLtkt	*tktA* (pCL1920 replicon, spectinomycin-resistant)	This study
pPS341	P_{tac}-*pps* (pBR322 replicon, ampicillin-resistant)	[17]

Medium and growth conditions

The medium used was a modified version of YE [6], containing per liter: 15 g yeast extract, 14 g K_2HPO_4, 16 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 22.5 g glucose, 1 g $MgSO_4 \cdot 7H_2O$ and one drop of P-2000 antifoam. The antibiotics spectinomycin, tetracycline, chloramphenicol and carbenicillin were used (each at a final concentration of 50 $\mu g\ ml^{-1}$) as needed for plasmid selection and maintenance. Cultures (30-ml) were grown at 35°C in 250-ml baffled flasks. The cultures were aerated by shaking at 250 rpm. Overnight seed cultures were centrifuged, and the cells resuspended to an $OD_{660\,nm}$ of 10 in 200 mM potassium phosphate buffer (pH 7.0). Aliquots of these suspensions were then used to inoculate the experimental flasks such that the initial $OD_{660\,nm}$ was 0.2. When the $OD_{660\,nm}$ of the cultures reached 2.0, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce expression of the *aroG*^{fbr} gene (and the *pps* gene in strains carrying plasmid pPS341 [17]). The pH of the cultures was monitored manually at 1-h intervals, adding 45% KOH as needed to maintain the pH at 6.5. The $OD_{660\,nm}$ of the cultures was monitored throughout the experiment, and used to calculate the dry cell weight. Starting 2 h after IPTG induction, 0.6-ml samples were taken, the cells were removed by centrifugation and the supernatant phase was stored at 4°C to await assay for DAH(P).

Measurement of metabolites

Levels of DAH(P) in sample supernatant fluids were determined using the thiobarbituric assay [20]. The levels are expressed as DAH(P) because the thiobarbituric acid assay does not distinguish between DAH and DAHP. The glucose concentration in the supernatant fluids was measured using a Monarch 2000 glucose analyzer (Instrumentation Laboratory, Lexington, MA, USA).

Results and discussion

The first enzymatic step in aromatic amino acid biosynthesis is the condensation of E4P and PEP to form DAHP (Figure 1). This step (catalyzed by DAHP synthase) is irreversible, and since DAHP has no other known function in the cell, accumulation of DAHP in the extracellular medium is a good indicator of carbon commitment to aromatic biosynthesis. To follow DAHP production, workers in our laboratory and others have used an *aroB* mutant of *E. coli* that is blocked at the second step of aromatic biosynthesis, 3-dehydroquinate synthase (Figure 1). Since this mutant cannot consume DAHP, overexpression of DAHP synthase causes excretion of high levels of DAH(P) into the culture medium.

We have found [9] that even in wild-type *E. coli* strains that have a functional *aroB* gene, overproduction of DAHP synthase (via introduction of a plasmid containing the P_{lacUV5}-*aroG*^{fbr} cassette) causes excretion of DAH(P) due to a natural rate limitation at the *aroB* step. We have exploited this finding in the present work.

The parental *E. coli* host strain used was PB103, a host which, when carrying the cloned genes necessary for tryptophan biosynthesis, is capable of producing high levels of tryptophan (unpublished results). Our goal was to begin with this strain that has the demonstrated capacity for high level production of aromatic amino acids, and in this host background determine the effect of different genetic approaches to increasing the intracellular supply of PEP on carbon commitment to aromatic biosynthesis. Carbon flux to aromatics was measured as DAH(P) accumulation in the culture medium of the different strains.

Strains PB103 (control), NF9 (PTS⁻ glucose⁺) and their *pykA* and *pykF* derivatives were transformed with a plasmid containing the P_{lacUV5}-*aroG*^{fbr} gene cassette (pRW300 or pRW5) to establish a high level of DAHP synthase activity. In some strains, the level of transketolase (encoded by *tktA*) was also increased, either by substituting plasmid pRW5tkt for pRW5, or by transforming with plasmid pCLtkt. In addition, in some strains the level of PEP synthase (encoded by the *pps* gene) was increased by transforming them with plasmid pPS341 [17].

DAH(P) production

Table 2 shows DAH(P) production and doubling times for all of the strains studied. As stated above, all strains carried a plasmid containing the P_{lacUV5}-*aroG*^{fbr} gene cassette, and therefore had high DAHP synthase activity. Because of different plasmids present in certain groups of strains, it is important to compare strains to the most appropriate control strain. Thus in Table 2, strain 1 is the control for strains 2–8; strain 9 is the control for strains 10–12; and strain 13 is the control for strains 14–20.

The baseline control strain PB103(pRW300) produced 0.37 mmol DAH(P) g^{-1} DCW. Inactivation of either the *pykA* gene or the *pykF* gene had no significant effect on

Table 2 DAH(P) production and doubling times

	Strain	Relevant property	mmol DAH(P) g^{-1} dry cell weight		Doubling time (h)	
			Mean[a]	s.d.[b]	Mean	s.d.
1.	PB103(pRW300)	control	0.37	0.08	1.91	0.07
2.	PB103A(pRW300)	*pykA*$^-$	0.54	0.16	1.85	0.11
3.	PB103F(pRW300)	*pykF*$^-$	0.44	0.07	1.83	0.12
4.	PB103AF(pRW300)	*pykA*$^-$ *pykF*$^-$	1.24	0.08	1.91	0.11
5.	PB103(pRW300, pCLtkt)	*tktA*$^{++}$	1.66	0.26	1.78	0.24
6.	PB103A(pRW300, pCLtkt)	*pykA*$^-$ *tktA*$^{++}$	1.81	0.20	1.75	0.21
7.	PB103F(pRW300, pCLtkt)	*pykF*$^-$ *tktA*$^{++}$	1.63	0.05	1.76	0.22
8.	PB103AF(pRW300, pCLtkt)	*pykA*$^-$ *pykF*$^-$ *tktA*$^{++}$	1.94	0.12	1.85	0.07
9.	PB103(pRW5)	control	0.77	0.07	2.07	0.37
10.	PB103(pRW5tkt)	*tktA*$^{++}$	1.65	0.23	2.06	0.46
11.	PB103(pRW5, pPS341)	*pps*$^{++}$	1.37	0.30	2.05	0.40
12.	PB103(pRW5tkt, pPS341)	*pps*$^{++}$ *tktA*$^{++}$	2.93	0.63	2.15	0.59
13.	NF9(pRW300)	PTS$^-$	0.58	0.24	1.88	0.12
14.	NF9A(pRW300)	PTS$^-$ *pykA*$^-$	1.61	0.68	2.19	0.52
15.	NF9F(pRW300)	PTS$^-$ *pykF*$^-$	0.26	0.12	1.95	0.33
16.	NF9AF(pRW300)	PTS$^-$ *pykA*$^-$ *pykF*$^-$	3.39	0.68	2.26	0.35
17.	NF9(pRW300, pCLtkt)	PTS$^-$ *tktA*$^{++}$	2.15	0.94	2.00	0.14
18.	NF9A(pRW300, pCLtkt)	PTS$^-$ *pykA*$^-$ *tktA*$^{++}$	3.29	0.59	1.85	0.26
19.	NF9F(pRW300, pCLtkt)	PTS$^-$ *pykF*$^-$ *tktA*$^{++}$	1.18	0.60	1.87	0.13
20.	NF9AF(pRW300, pCLtkt)	PTS$^-$ *pykA*$^-$ *pykF*$^-$ *tktA*$^{++}$	7.37	0.37	3.24	0.37

[a]Results shown are averages of three independent experiments. Within each experiment, each strain was tested in duplicate or triplicate.
[b]Standard deviation.

DAH(P) production. However, simultaneous inactivation of *pykA* and *pykF* caused a 3.4-fold increase in DAH(P) production. These results indicate that under the culture conditions used, a reduction in pyruvate kinase activity can cause an increase in the intracellular supply of PEP, which in our system is manifested as higher DAH(P) production. Overexpression of the *tktA* gene caused the DAH(P) level to increase 4.5-fold over the control, as has been reported previously [6,18]. In the strain having overexpressed *tktA*, the *pykA* and *pykF* mutations, singly or in combination, afforded no significant additional increase in DAH(P) production. Essentially identical results to those described above were obtained with *E. coli* strains derived from a different lineage than PB103 (G Gosset, unpublished results).

Compared to the appropriate control strain, a strain overexpressing the *pps* gene produced 1.8-fold more DAH(P). This increase was similar to the increase caused by overexpression of *tktA* in the same strain background. When both the *tktA* and *pps* genes were simultaneously overexpressed in the same strain, DAH(P) production increased to 3.8-fold over the appropriate control strain. Similar results have been reported using an *aroB* strain of *E. coli* that overexpresses the same complement of cloned genes [18].

The results shown in Table 2 for all of the PB103-based strains were highly reproducible, both in terms of replicate samples within an experiment as well as from one independent experiment to the next. On the other hand, while the NF9-based strains gave reproducible replicate samples within a given experiment, there was considerable variation in DAH(P) production in independent experiments, hence the higher standard deviations for some strains (Table 2). While this raises some doubt about the statistical significance of the differences between some strains, the positive effect of certain genetic modifications on DAH(P) production cannot be disputed.

The PTS$^-$ glucose$^+$ strain NF9(pRW300) produced 0.58 mmol DAH(P) g^{-1} DCW. This represents a 1.6-fold increase in DAH(P) production compared to the isogenic PTS$^+$ control strain PB103(pRW300). This increase has been observed previously [9] and illustrates the extent to which elimination of PEP-dependent glucose transport can affect the availability of PEP for other metabolic processes (eg aromatic biosynthesis). When *pykA* was inactivated in the PTS$^-$ glucose$^+$ host background, a further 2.8-fold increase in DAH(P) production was observed. Inactivation of *pykF*, on the other hand, caused a decrease in DAH(P) production to 45% of that observed for the appropriate control strain. Inactivation of both *pyk* isozymes in the PTS$^-$ glucose$^+$ backgound caused a 5.8-fold increase in DAH(P) production over the appropriate control. These results indicate that it is possible to increase further the already high carbon commitment to aromatics in the PTS$^-$ glucose$^+$ strains by inactivating *pykA* alone or in combination with *pykF*. In the PTS$^-$ glucose$^+$ strain containing both *pyk* mutations, two of the three major metabolic activities that consume PEP have been inactivated (the remaining one is PEP carboxylase).

When the *tktA* gene was overexpressed in the PTS$^-$ glucose$^+$ host background, the level of DAH(P) produced was 2.15 mmol DAH(P) g^{-1} dry cell weight. This represents a 3.7-fold increase in DAH(P) production over the PTS$^-$ glucose$^+$ control strain lacking amplified *tktA*. Further inactivation of *pykA* in this background led to an additional 1.5-fold increase in DAH(P) production, while inactivation of *pykF* caused a 45% decrease. Finally, the PTS$^-$ glucose$^+$ strain containing inactivated *pykA* and *pykF*, and amplified

tktA produced 7.37 mmol DAH(P) g^{-1} dry cell weight. This increase in DAH(P) production is undoubtedly statistically significant, and is extraordinary in that it represents a 12.7-fold increase over the PTS^- $glucose^+$ control strain and a 19.9-fold increase over the baseline control strain in which the central metabolic pathways related to E4P and PEP have not been manipulated.

The results with the strains containing interruptions in only the *pykF* gene must be interpreted with some caution. In the PB103 host background, inactivation of *pykF* caused no significant change in DAH(P) production relative to the appropriate control strain (Table 2). However, in the NF9 host background, the *pykF* mutation caused a significant decrease in DAH(P) production. To avoid duplication of antibiotic markers, the *pykF* gene in the NF9 strains was interrupted with a gentamycin resistance gene, while in the PB103 strains *pykF* was interrupted with either chloramphenicol or kanamycin resistance genes. Therefore, the decrease in DAH(P) production in the NF9 strains carrying the *pykF::gen* mutation could be due to the expression of the gentamycin resistance gene. However, if this is true, the problems associated with gentamycin resistance must be overcome in the NF9AF double mutant (*pykA::cat, pykF::gen*) since in the NF9AF-based strains the DAH(P) production was the highest of all the strains tested (Table 2).

Table 2 shows only the total amount of DAH(P) accumulated by the cultures in the 24 h after IPTG induction. While the data are normalized to account for differences in total dry cell weight, they do not reflect the differences in the rates of DAH(P) production. Figure 2a shows the complete DAH(P) production profile for six of the strains represented in Table 2. The data in Figure 2a show that there is a direct correlation between the final level of DAH(P) produced g^{-1} dry cell weight and the rate of production in the first 7 h after IPTG induction. The results observed for the highest DAH(P) producing strain [NF9AF(pRW300, pCLtkt)] were even more striking when one considers that the amount of glucose consumed by this strain in the first 7 h after IPTG induction was about half of that consumed by the other strains during the same time interval (see below).

Growth properties

Figure 2b and c shows the actual growth curves and the glucose consumption for the six strains represented in Figure 2a. The doubling times during exponential growth for all of the strains used are presented in Table 2. Rich yeast extract medium was used in this work to minimize subtle differences in the growth of the different strain/plasmid combinations, thereby allowing a clearer correlation between glucose consumption and product formation. All of the strains showed similar doubling times with the exception of strain NF9AF(pRW300, pCLtkt), which had a significantly increased doubling time. This is reflected in the growth and glucose consumption curves (Figure 2b and c). The slower growing strain did reach a final density similar to the other strains (Figure 2b). In all strains, the growth rate during exponential growth was directly related to the rate of glucose consumption (Figure 2b and c).

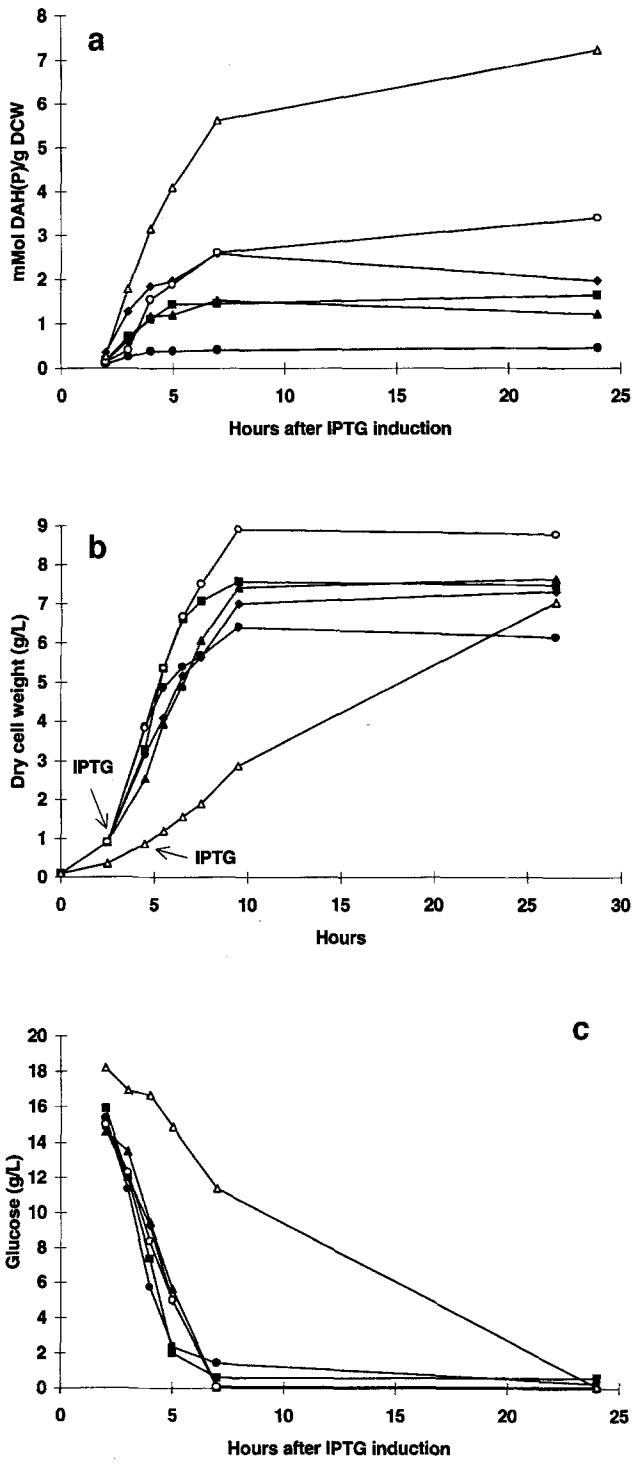

Figure 2 DAH(P) production (panel a), growth (panel b) and glucose consumption (panel c) for selected strains. In panel b, the arrows show the time of IPTG addition, which was the same for all strains except NF9AF(pRW300, pCLtkt), which was induced 2 h later because of the slower growth of the culture. The strains are represented by the same symbols in all three panels. PB103(pRW300) [●]; PB103AF (pRW300) [▲]; PB103(pRW300, pCLtkt) [■]; PB103AF(pRW300, pCLtkt) [◆]; PB103(pRW5tkt, pPPS341) [○]; and NF9AF(pRW300, pCLtkt) [△].

Practical significance

The results presented here illustrate the potential of engineering the central metabolic pathways of microorganisms to increase the yield of metabolites produced by fermentation. With respect to production of aromatic compounds by fermentation, these approaches to increasing product yield will be required, since even without directed genetic manipulation of the intracellular PEP supply, we have been able to produce tryptophan at the theoretical maximum yield from glucose [2]. The use of a combination of genetic approaches to increasing PEP availability should have general applicability in the production of any fermentation product that derives its carbon skeleton in part from PEP (eg lysine and threonine). However, the best approach may vary, depending on the desired product and the production organism. For example, elimination of pyruvate kinase activity decreased lysine production in *Corynebacterium lactofermentum*, contrary to expectation [11].

From an industrial point of view, the results presented here are most encouraging. However, in a fundamental sense our results are difficult to explain. For example, increased carbon commitment to DAH(P) via amplification of transketolase (*tktA*) has been well documented [6,18]. The main effect of amplified transketolase on DAH(P) production is believed to be to increase the availability of E4P, a product of the pentose phosphate pathway (Figure 1) [6]. Since amplification of *tktA* does in fact increase carbon commitment to aromatics, one would assume that PEP levels are not limiting. However, inactivation of *pykA* and *pykF*, or elimination of the PEP-dependent glucose PTS, in the absence of amplified (cloned) *tktA* also caused increased DAH(P) production. This suggests that PEP is in fact limiting under the experimental conditions used here. These results illustrate our lack of a complete understanding of the relationships between the central metabolic pathways related to PEP (and E4P). Nevertheless, our results suggest directions for future strain improvements for increased production of aromatic compounds from glucose.

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