Identification and Removal of Impediments to Biocatalytic Synthesis of Aromatics from D-Glucose: Rate-Limiting Enzymes in the Common Pathway of Aromatic Amino Acid Biosynthesis

K. A. Dell and J. W. Frost*

Contribution from the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907

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Abstract: Increasing the flow of D-glucose equivalents into the common pathway of aromatic amino acid biosynthesis creates a metabolic situation where individual common pathway enzymes become rate-limiting. Such enzymes are unable to convert substrate to product at a rate sufficient to avoid intracellular accumulation of substrate. Export of the accumulating substrate by the host microbe into its culture supernatant results in lowered percent conversions and decreased purity of desired aromatic products. Methodology has now been developed which facilitates rapid identification and removal of impediments to biocatalytic synthesis of aromatics from D-glucose which are caused by rate-limiting, common pathway enzymes. An *Escherichia coli* mutant, D2704, incapable of L-tryptophan and L-tyrosine synthesis was transformed with a plasmid which increased the in vivo catalytic activity of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) synthase and transketolase. Analysis by ^{1}H NMR of the culture supernatant of this construct revealed the accumulation of L-phenylalanine and phenyllactate along with common pathway enzyme substrates and related metabolites. Plasmid-borne genes encoding common pathway enzymes were then introduced into D2704 individually and in various combinations followed by ^{1}H NMR analysis of these constructs' culture supernatants. Progress toward increasing the catalytic activity of rate-limiting enzymes was indicated by a decrease in the number of accumulated enzyme substrates and increased L-phenylalanine synthesis and phenyllactate synthesis. In this fashion, 3-dehydroquinate (DHQ) synthase, shikimate kinase, 5-enolpyruvoylshikimate 3-phosphate (EPSP) synthase, and chorismate synthase were identified as rate-limiting enzymes. A feedback loop in *E. coli* was also identified involving inhibition of shikimate dehydrogenase by shikimic acid.

Although microbial aromatic amino acid biosynthesis[1] can be manipulated to produce an impressive array of aromatic structures from nontoxic, inexpensive D-glucose, several different challenges confront the use of intact microbes as catalysts in the synthesis of industrial and medicinal aromatics.[2] Those factors which affect yield, conversion rate, or product purity are of particular importance. For instance, the highest possible percentage of D-glucose consumed by the microbe must be directed into synthesis of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP), the first committed intermediate of aromatic amino acid biosynthesis (Scheme I). This surge in the synthesis of DAHP has to then be delivered by way of the enzymes of the common pathway of aromatic amino acid biosynthesis (Scheme I) into increased synthesis of chorismic acid. Finally, carbon flow at the end of the common pathway needs to be directed into the various pathways which convert chorismate into L-phenylalanine, L-tyrosine, and L-tryptophan along with a host of related secondary metabolites.

Prior efforts have successfully identified methods for increasing the percentage of D-glucose consumed by *Escherichia coli* which is channeled into the common pathway. Such methods include increasing the in vivo catalytic activity of DAHP synthase[3,4] and, more recently, amplified expression of transketolase.[5] Delivery of the surge in DAHP synthesis into increased synthesis of chorismic acid, which is the focus of this study, has received much less attention.[6] Increases in the in vivo catalytic activity of DAHP synthase and transketolase create a metabolic situation where individual common pathway enzymes become rate-limiting. Such enzymes are unable to catalyze conversion of substrate to product at a rate sufficient to avoid intracellular accumulation of substrate. Because of the rapid export by *E. coli* of intracellularly accumulating metabolites into the culture supernatant,[6a,7] substrates of rate-limiting enzymes are effectively lost to aromatic biosynthesis. Resulting reduction in percent yield, conversion rate, and decreased purity of aromatic product are major problems.

The ability of microbes to export accumulating intermediate metabolites has now been employed to biocatalytic advantage as the basis for a method of identifying rate-limiting enzymes in the

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(6) (a) Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1990**, *112*, 9630. (b) Matsui, K.; Miwa, K.; Sano, K. U.S. Patent 5 017 481, 1991. (c) Tribe, D. E. U.S. Patent 4 681 852, 1987.

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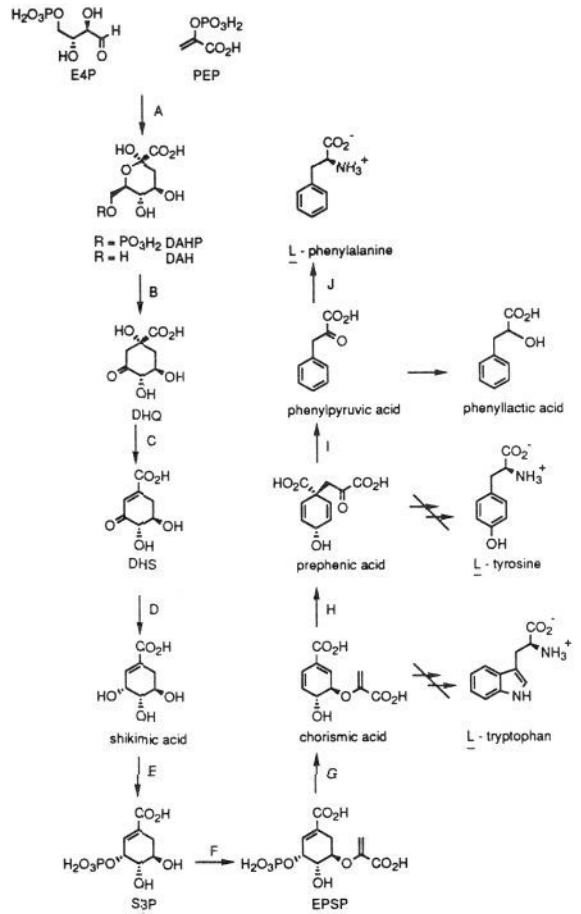
Scheme I[a]



[a](A) DAHP synthase (*aroF aroG aroH*); (B) DHQ synthase (*aroB*); (C) DHQ dehydratase (*aroD*); (D) shikimate dehydrogenase (*aroE*); (E) shikimate kinase (*aroL aroK*); (F) EPSP synthase (*aroA*); (G) chorismate synthase (*aroC*); (H) chorismate mutase (*pheA tyrA*); (I) prephenate dehydratase (*pheA*); (J) aromatic aminotransferase (*tyrB*).

common pathway of aromatic amino acid biosynthesis. ^{1}H NMR is used to identify common pathway substrates and related metabolites which accumulate in the culture supernatant of an *E. coli* auxotroph constructed to express amplified levels of DAHP synthase and transketolase. Genes encoding common pathway enzymes whose substrates or related metabolites accumulate are then introduced into the *E. coli* auxotroph. The disappearance of a previously accumulating metabolite is interpreted as evidence for an increase in the catalytic activity of an enzyme to a point where it is no longer rate-limiting. In this fashion, constructs have been assembled in which increases in carbon flow can be delivered from the beginning to the end of the common pathway of aromatic amino acid biosynthesis.

Results

Analysis for Rate-Limiting Enzymes. Identification and removal of impediments to the flow of carbon through the common pathway followed from the analysis by ^{1}H NMR of the culture supernatant of *E. coli* D2704,[8] a *pheA*, *tyrA*, Δ*trpE-C* strain requiring L-tyrosine and L-tryptophan supplementation for growth. The various mutations in D2704 ensure that the chorismate produced by the common pathway is converted into phenylpyruvate, which subsequently partitions between L-phenylalanine and phenyllactic acid biosynthesis. A surge of carbon flow through the common pathway of D2704 was created by transforming this

(8) Mascarenhas, D.; Ashworth, D. J.; Chen, C. S. *Appl. Environ. Microbiol.* **1991**, *57*, 2995.

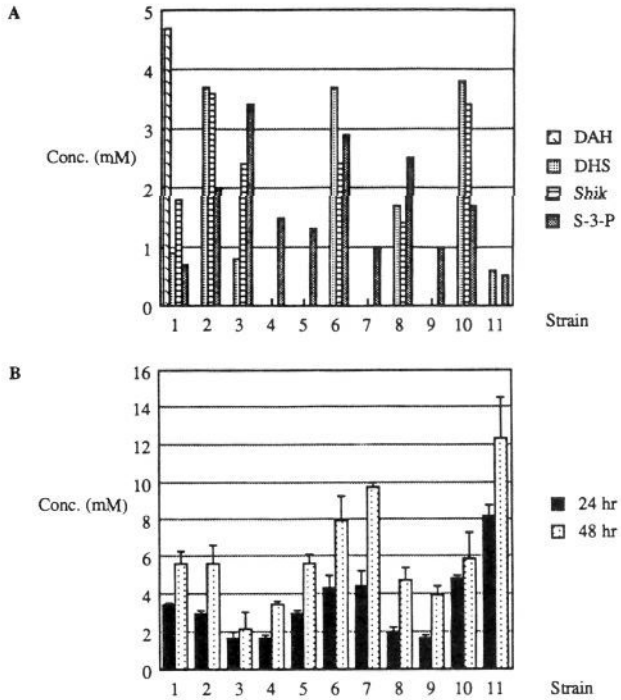


Figure 1. (A) Common pathway intermediates extracellularly accumulated by D2704 strains after 24 h of culturing in minimal medium which initially contained 56 mM D-glucose. (B) Average, combined accumulations of L-phenylalanine and phenyllactate after 24 h and 48 h. Strains studied include (1) D2704/pKD130A; (2) D2704/pKD136; (3) D2704/pKD136/pKD28; (4) D2704/pKD136/pKAD34; (5) D2704/pKD136/pKAD31; (6) D2704/pKD136/pKAD38; (7) D2704/pKD136/pKAD43; (8) D2704/pKD136/pKAD39; (9) D2704/pKD136/pKAD51; (10) D2704/pKD136/pKAD44; (11) D2704/pKD136/pKAD50.

auxotroph with pKD130A,[5a] a plasmid which carried the *tkt* gene encoding transketolase and the *aroF* gene encoding the tyrosine-sensitive isozyme of DAHP synthase. As with all the constructs examined, D2704/pKD130A was initially grown in rich medium (LB), harvested, and then cultured for 48 h in minimal medium (M9) containing 56 mM D-glucose. A portion of the culture supernatant was withdrawn from the minimal medium at 24 and 48 h and analyzed by ^{1}H NMR. Accumulation of common pathway metabolites was best observed at 24 h of culturing in minimal medium while end-product L-phenylalanine and phenyllactate concentrations reached their maximum values by 48 h of culturing in minimal medium. 3-Deoxy-D-*arabino*-heptulosonic acid (DAH), 3-dehydroshikimate (DHS), shikimate, and shikimate 3-phosphate (S3P) were detected after 24 h of culturing D2704/pKD130A in minimal medium (Figure 1A/entry 1 and Figure 2A). On the basis of the enzymes for which these or closely related metabolites are substrates, DHQ synthase, shikimate dehydrogenase, shikimate kinase, and EPSP synthase were tentatively identified as rate-limiting enzymes. Phenyllactate and L-phenylalanine were detected in the culture supernatant of D2704/pKD130A after 24 h (Figure 1B/entry 1, Figure 2A) and increased to 5.6 ± 0.7 mM after 48 h of culturing in minimal medium (Figure 1B/entry 1). Prephenate was present in the culture supernatant of D2704/pKD130A at 24 h but had largely disappeared by 48 h of culturing in minimal medium.

Preventing Accumulation of Pathway Intermediates. Previous efforts[6a] have shown DHQ synthase to be a metabolic block in the strain AB2834, an *aroE* auxotroph, which can be removed when in vivo activities of the enzyme are increased. Transformation of D2704 with pKD136, a plasmid[6a] which carries *aroB* (encoding DHQ synthase), *tkt* (encoding transketolase), and *aroF* (encoding the tyrosine-sensitive isozyme of DAHP synthase), resulted (Figure 1A, 1B/entry 2) in the disappearance of DAH from the culture supernatant and increased accumulation of DHS,

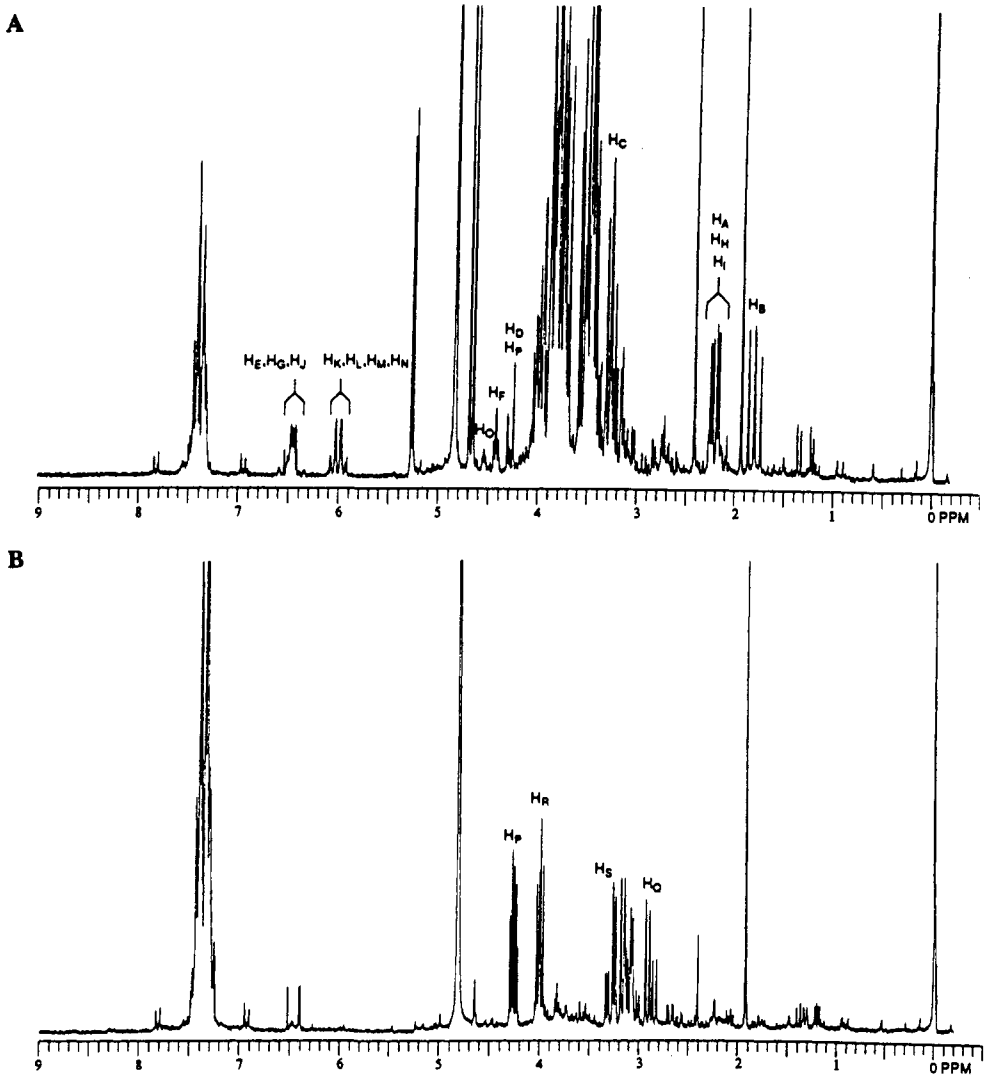


Figure 2. (A) Before removal of impediments to carbon flow: ^{1}H NMR of the supernatant of D2704/pKD130A after 24 h of culturing in minimal medium initially containing 56 mM D-glucose. Observable resonances for accumulated molecules are DAH (H_A, H_B, H_C), DHS (H_D, H_E), shikimate (H_F, H_G, H_H), shikimate 3-phosphate (H_I, H_J), prephenic acid (H_K, H_L, H_M, H_N, H_O), phenyllactic acid (H_P and aromatic resonances at 7.4 ppm), and phenylalanine (aromatic resonances at 7.4 ppm). (B) After removal of impediments to carbon flow: ^{1}H NMR of the culture supernatant of D2704/pKD136/pKAD50 after 48 h. Observable resonances for accumulated molecules are phenyllactic acid (H_P, H_Q, and aromatic resonances at 7.4 ppm) and phenylalanine (H_R, H_S, and aromatic resonances at 7.4 ppm).

shikimate, and shikimate 3-phosphate. Increased accumulation of L-phenylalanine and phenyllactate was not observed. Thus the removal of the impediment to carbon flow at DHQ synthase allowed unrestricted carbon flow through the initial portion of the common pathway but failed to increase end-product synthesis due to the continued presence of other rate-limiting enzymes.

The lack of a suitably situated polylinker for the insertion of additional genetic fragments into pKD136 led to the use of a two-plasmid system. Plasmid pKD136 was retained as the plasmid which directed the surge of carbon flow into the common pathway and removed the impediment to this flow of carbon at DHQ synthase. Genes encoding other rate-limiting enzymes were inserted into the polylinker region of either pSU18 or pSU19 to form the second plasmid (Table I). Accumulation of 3.1 ± 0.1 mM phenyllactate and L-phenylalanine by D2704/pKD136/pSU18 indicated that this second plasmid (pSU18) was a significant burden on the microbe's (D2704/pKD136) metabolism.

Plasmid pKD28, derived from insertion of *aroE* (encoding shikimate dehydrogenase) into pSU18 (Table I), was transformed into D2704/pKD136 in an effort to remove accumulation of DHS. While a decrease in the levels of DHS in the culture supernatant of D2704/pKD136/pKD28 was observed, introduction of the second plasmid did not completely eliminate DHS accumulation (Figure 1A/entry 3). Shikimate and shikimate 3-phosphate were still present in the culture supernatant, and the total production of L-phenylalanine and phenyllactate declined to 2.1 ± 0.9 mM after 48 h. Additional insights into prevention of DHS accumulation were afforded by efforts to compensate for the rate-limiting character of shikimate kinase. Plasmid pKAD34 (Table I) was constructed which carried *aroL* (encoding shikimate kinase) and *aroE* (encoding shikimate dehydrogenase) inserts. No DHS or shikimate could be detected in the culture supernatant of D2704/pKD136/pKAD34, leaving shikimate 3-phosphate as the only accumulated common pathway intermediate (Figure 1A/entry 4). No improvement was achieved in the levels of L-phenylalanine and phenyllactate synthesized by D2704/pKD136/pKAD34 (Figure 1B/entry 4).

DHS and shikimate were also absent in the culture supernatant (Figure 1A/entry 5) of D2704/pKD136/pKAD31. Plasmid pKAD31 carried only an *aroL* (encoding shikimate kinase) insert (Table I). The accumulation of shikimate 3-phosphate was still observed, and the total production of L-phenylalanine and phenyllactate (Figure 1B/entry 5) of 5.6 ± 0.5 mM was essentially unchanged relative to the case of D2704/pKD136. Removal of both DHS and shikimate accumulation with a second plasmid

Table I. Restriction Enzyme Maps of Plasmids Used to Increase the in Vivo Catalytic Activity of Rate-Limiting, Common Pathway Enzymes[a]

Plasmid	Vector	Plasmid Map
pKD28 (3.9 kb)	pSU18	EB H — Plac *aroE* Cm
pKAD31 (3.3 kb)	pSU19	HPSXB K — Plac *aroL* Cm
pKAD34 (4.9 kb)	pSU19	X B K — Plac *aroE* *aroL* Cm
pKAD38 (4.7 kb)	pSU18	EK P — Plac *aroA* Cm
pKAD39 (4 kb)	pSU19	HPS B K — Plac *aroC* Cm
pKAD43 (5.7 kb)	pSU19	PSB K — Plac *aroA* *aroL* Cm
pKAD44 (6.4 kb)	pSU19	PS B KE — Plac *aroA* *aroC* Cm
pKAD50 (7.4 kb)	pSU19	PS B KE — Plac *aroA* *aroC* *aroL* Cm
pKAD51 (5.0 kb)	pSU19	S B KE — Plac *aroC* *aroL* Cm

[a] Restriction enzyme sites are abbreviated as follows: E = *Eco*RI, B = *Bam*HI, H = *Hin*dIII, X = *Xba*I, K = *Kpn*I, P = *Pst*I, S = *Sal*I.

possessing only an *aroL* insert suggested that the apparent rate-limiting character of shikimate dehydrogenase might not be due to inadequate expression levels. Rather, shikimate accumulating due to rate-limiting shikimate kinase activity might be feedback inhibiting shikimate dehydrogenase. To test this hypothesis, shikimate dehydrogenase was purified to homogeneity and the inhibition of the enzyme was measured in the presence of increasing concentrations of shikimate. Shikimate was found to be a product inhibitor exhibiting linear mixed-type inhibition[9] (Figure 3) with an inhibition constant (K_i) of 0.16 mM.

Improving End Product Accumulation. Removing those impediments to carbon flow responsible for DAH, DHS, and shikimate accumulation failed to provide an increase in the accumulation of L-phenylalanine and phenyllactate. This suggestion of a bottleneck later in the common pathway led to consideration of EPSP synthase, since shikimate 3-phosphate, the only accumulating common pathway metabolite which remained, was the substrate for this enzyme. Plasmid pKAD38 containing *aroA* (encoding EPSP synthase) was constructed (Table I) to increase the levels of EPSP synthase. Evaluation of D2704/pKD136/pKAD38 revealed total L-phenylalanine and phenyllactate production of 7.9 ± 1.3 mM after 48 h (Figure 1B/entry 6), which was a significant increase relative to end-product accumulations in strains lacking plasmid-borne *aroA*. It is noteworthy that this improved end-product accumulation was achieved in D2704/pKD136/pKAD38 despite the continued accumulation (Figure 1A/entry 6) of DHS, shikimate, and shikimate 3-phosphate. Given the earlier success in removing both DHS and shikimate accumulation with amplified shikimate

(9) Segel, I. H. *Biochemical Calculations*; Wiley: New York, 1976; p 261.

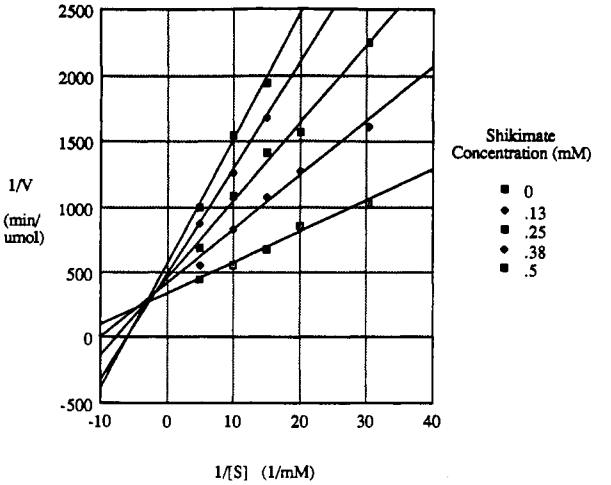


Figure 3. Lineweaver-Burk plot of shikimate dehydrogenase inhibition by product shikimate.

kinase activity, plasmid pKAD43 which carried both *aroA* (encoding EPSP synthase) and *aroL* (encoding shikimate kinase) was constructed (Table I). The strain D2704/pKD136/pKAD43 produced 9.7 ± 0.3 mM of L-phenylalanine and phenyllactate (Figure 1B/entry 7) with the accumulation of only one common pathway intermediate, shikimate 3-phosphate (Figure 1A/entry 7).

The possibility now arose that rate-limiting chorismate synthase activity might contribute to the continued presence (Figure 1A/entry 7) of shikimate 3-phosphate in the culture supernatants of constructs such as D2704/pKD136/pKAD43 which expressed

Table II. Ratios of the Specific Activities of Amplified Common Pathway Enzymes Relative to the Specific Activities of Unamplified Common Pathway Enzymes in *E. coli* D2704 Constructs

strain	DHQ synthase	shikimate kinase	EPSP synthase	chorismate synthase
D2704[a]	1.0	1.0	1.0	1.0
D2704/pKD136	3.1			
D2704/pKD136/pKAD31	1.2	120		
D2704/pKD136/pKAD38	0.7		19	
D2704/pKD136/pKAD43	1.1	24	1.6	
D2704/pKD136/pKAD50	2.0	41	8.3	4.2

[a] D2704 enzyme activity values (units/mg) are as follows: DHQ synthase, 0.023; shikimate kinase, 0.0023; EPSP synthase, 0.0099; chorismate synthase, 0.0017. One unit is defined as one μmol of product formed per min.

amplified EPSP synthase activity. As a result, plasmid pKAD50 was constructed (Table I) which carried *aroA* (encoding EPSP synthase), *aroL* (encoding shikimate kinase), and *aroC* (encoding chorismate synthase). The strain D2704/pKD136/pKAD50 accumulated small quantities of DHS and shikimate 3-phosphate (Figure 1A/entry 11) at 24 h and 12.3 ± 2.2 mM L-phenylalanine and phenyllactate at 48 h (Figure 1B/entry 11 and Figure 2B). The observed increase in end-product levels and reduction in shikimate 3-phosphate accumulation suggested that chorismate synthase was an impediment to the flow of carbon through the common pathway. To further evaluate the role of chorismate synthase, plasmids pKAD39, pKAD51, and pKAD44 respectively carrying *aroC*, *aroC aroL*, and *aroA aroC* were constructed (Table I) and inserted into D2704/pKD136. None of these constructs synthesized the (Figure 1B/entries 8–10) levels of end products synthesized by D2704/pKD136/pKAD50 (Figure 1B/entry 11).

Enzyme Activities. The ability of D2704, a *pheA* and *tyrA* mutant, to synthesize L-phenylalanine could be consistent with nonenzymatic conversion of chorismate into end product or might indicate the presence of residual chorismate mutase or prephenate dehydratase activity. Specific activities were therefore measured in D2704 for chorismate mutase (8.5×10^{-5} units/mg) and prephenate dehydratase (2.8×10^{-5} units/mg). Comparison with the specific activities of chorismate mutase (1.1×10^{-2} units/mg) and prephenate dehydratase (9.2×10^{-3} units/mg) in the wild-type strain *E. coli* RB791 indicates that enzymatic conversion of chorismate into phenylpyruvate is essentially absent in D2704. Enzyme activities were also measured (Table II) for DHQ synthase, shikimate kinase, EPSP synthase, and chorismate synthase in those strains which either gave a significant increase in end-product accumulation or a significant improvement in product purity. The levels of overexpression (Table II) of DHQ synthase, EPSP synthase, and chorismate synthase were generally modest relative to the overexpression of shikimate kinase.

Measured Improvement in Carbon Flow. The 12.3 ± 2.2 mM concentration (74% of theoretical maximum yield)[2a] of L-phenylalanine and phenyllactate synthesized (Figure 1B/entry 11) by D2704/pKD136/pKAD50 relative to the 5.6 ± 0.7 mM concentration (34% of theoretical maximum yield)[2a] of L-phenylalanine and phenyllactate synthesized by D2704/pKD130A (Figure 1B/entry 1) clearly attests to the improvement in carbon flow delivered to the end of the common pathway attendant with increasing the in vivo catalytic activities of rate-limiting enzymes. A complementary way to measure progress toward removal of impediments to carbon flow is to determine whether incremental improvements in carbon flow directed into the common pathway translate into incremental improvements in carbon flow exiting the common pathway. As has already been mentioned, amplified in vivo activity of DAHP synthase and amplified in vivo activity of transketolase are two strategies for increasing the flow of carbon directed into aromatic amino acid biosynthesis. Therefore, it is informative to determine the impact (Figure 4) of amplified expression levels of rate-limiting, common pathway enzymes on the levels of synthesized end products when the catalytic activity

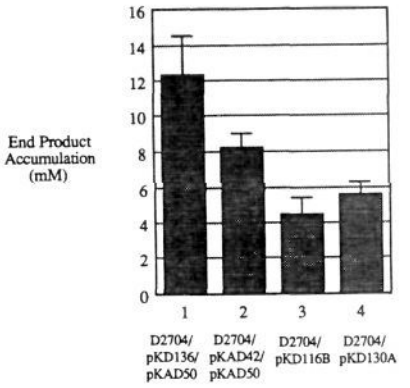


Figure 4. Averaged, combined accumulations (48 h) of L-phenylalanine, phenyllactate, and prephenate by various *E. coli* D2704 constructs after 48 h of culturing in minimal medium which initially contained 56 mM D-glucose.

of DAHP synthase is varied and, in separate experiments, when the catalytic activity of transketolase is varied.

An important construct employed in these analyses was D2704/pKAD42/pKAD50. Plasmid pKAD42 carries *aroB* and *aroF* inserts but lacks a *tkt* insert. Culturing of D2704/pKAD42/pKAD50 produced large amounts of acetate and lactate. Resulting acidification of the culture medium resulted in premature death of the construct. To alleviate this problem, the pH of the accumulation medium was monitored and neutralized when necessary. Maintaining a neutral pH resulted in a pronounced accumulation of prephenic acid. This may reflect prephenate's greater stability at neutral versus acidic pH.[10] As a result, comparisons of end-product accumulation (Figure 4) with D2704/pKAD42/pKAD50 required quantification of prephenate concentrations in addition to L-phenylalanine and phenyllactate concentrations. D2704/pKAD42/pKAD50 was the only strain which had to be neutralized during the 48 h culturing in minimal medium.

D2704/pKD116B, which expresses amplified levels of DAHP synthase activity, synthesizes 4.5 ± 0.9 mM L-phenylalanine, phenyllactate, and prephenate (Figure 4/entry 3). D2704/pKAD42/pKAD50, which expresses amplified levels of DAHP synthase activity like D2704/pKD116B, synthesizes 8.2 ± 0.8 mM L-phenylalanine, phenyllactate, and prephenate (Figure 4/entry 2). The difference between these two DAHP synthase overexpressing strains is that the in vivo catalytic activities of rate-limiting, common pathway enzymes are increased in D2704/pKAD42/pKAD50 while the activities of these same enzymes are unchanged in D2704/pKD116B. On the basis of end product accumulations, more of the incremental improvement in carbon flow directed into the common pathway by amplified DAHP synthase activity is being delivered to the end of the pathway in D2704/pKAD42/pKAD50.

Amplified transketolase activity expressed by D2704/pKD130A distinguishes this construct from D2704/pKD116B. Both of these constructs overexpress DAHP synthase. Accumulation (Figure 4/entry 4) of L-phenylalanine, phenyllactate, and prephenate in D2704/pKD130A is 5.7 ± 0.6 mM while D2704/pKD116B accumulates (Figure 4/entry 3) a 4.5 ± 0.9 mM concentration of end products. This percentage increase in L-phenylalanine, phenyllactate, and prephenate accumulation is significantly less than that observed when the activities of the rate-limiting, common pathway enzymes are simultaneously increased with transketolase activity. As an example, D2704/pKAD42/pKAD50 expresses amplified levels of DAHP synthase and rate-limiting, common pathway enzymes while D2704/pKD136/pKAD50 overexpresses these same enzyme activities and, in addition, overexpresses

(10) Weiss, U.; Gilvarg, C.; Mingioli, E. S.; Davis, B. D. *Science* **1954**, *119*, 774.

transketolase, D2704/pKD136/pKAD50 synthesizes 12.3 ± 2.2 mM L-phenylalanine, phenyllactate, and prephenate (Figure 4/entry 1) while only an 8.2 ± 0.8 mM concentration of end products (Figure 4/entry 2) is synthesized by D2704/pKAD42/pKAD50.

Discussion

Removal of the impediments to the flow of carbon through the common pathway of aromatic amino acid biosynthesis required (a) direction of a surge of carbon flow into the common pathway and (b) development of analysis methodology suitable for identification of rate-limiting enzymes. For the purposes of this study, the wild-type *aroF* locus encoding the tyrosine-sensitive isozyme of DAHP synthase was localized on a plasmid. The native, strong promoter and the copy number of the plasmid combined to afford ample overexpression of DAHP synthase. Although not pursued in this study, additional improvements in DAHP synthase in vivo catalytic activity could likely be achieved if an *aroF* or *aroG* locus containing a point mutation rendering the gene product insensitive to feedback inhibition were employed.[4]

In vivo catalytic activity of DAHP synthase can be increased to a point beyond which additional carbon flow is not directed into the common pathway. One possible explanation for this observation is that the availability of phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P), the two substrates of DAHP synthase, ultimately becomes a limiting factor. Approaches to increase the in vivo concentration of both PEP[11] and E4P[5] have been explored. Increasing E4P availability by increasing the in vivo catalytic activity of transketolase has proven to be particularly successful.[5] At levels of DAHP synthase catalytic activity where additional increases in catalytic activity do not lead to increased DAHP synthesis, amplification of transketolase expression with plasmid-encoded *tkt* provides an approximately twofold increase in synthesis of DAHP.[5b] Consequently, efforts to identify and remove impediments in the common pathway have utilized pBR325 derivatives such as pKD130A and pKD136 which contain transketolase-encoding *tkt* and DAHP synthase-encoding *aroF* loci.

Identification of rate-limiting enzymes entailed the use of auxotrophic *E. coli* mutants lacking individual common pathway enzymes. For instance, *E. coli aroB*/pKD130A, which lacks catalytically active DHQ synthase, accumulated 20 mM 3-deoxy-D-*arabino*-heptulosonic acid (DAH) in its culture supernatant when cultured in medium containing 56 mM D-glucose. DAH is the dephosphorylated substrate of DHQ synthase. Subsequent introduction of transketolase and DAHP synthase-encoding pKD130A into *E. coli aroD*, which lacks catalytically active DHQ dehydratase, would be expected to accumulate 20 mM 3-dehydroquinate, the substrate of DHQ dehydratase. Instead, *E. coli aroD*/pKD130A accumulated only 14 mM DHQ when cultured in medium containing 56 mM D-glucose. In addition to DHQ, 5 mM DAH was discovered in the culture supernatant. The reduction in accumulated substrate (or closely related metabolite) is consistent with an intervening, rate-limiting enzyme. Introduction of pKD136 which encodes DHQ synthase along with transketolase and DAHP synthase into *E. coli aroD* resulted in disappearance of DAH from the culture supernatant and accumulation of 19 mM DHQ. These data could be interpreted as indicative of DHQ synthase being a rate-limiting enzyme whose partial blockage of the common pathway was removed upon amplification of the enzyme. *E. coli aroE*/pKD136, which lacked catalytically active shikimate dehydrogenase, accumulated approximately 30 mM 3-dehydroshikimate.[6a] This level of DHS accumulation and lack of DHQ accumulation indicated that DHQ dehydratase was not a rate-limiting enzyme.[6a]

Because *E. coli* possesses two isozymes of shikimate kinase (encoded by *aroK* and *aroL*)[12,13] *E. coli aroL*/pKD136 failed to accumulate shikimate. In addition, *E. coli aroA*/pKD136 (lacking EPSP synthase) and *E. coli aroC*/pKD136 (lacking chorismate synthase) failed to accumulate significant concentrations of substrates of the missing enzymes.[7c] This led to examination of *E. coli* D2704 (lacking chorismate mutase, prephenate dehydratase, prephenate dehydrogenase, and anthranilate synthase) which is unable to process chorismic acid due to mutations in the *pheA* and *tyrA* loci and a deletion mutation spanning *trpE–C*.[8] D2704 was initially chosen for its expected ability to accumulate chorismate. However, D2704 was found to accumulate primarily L-phenylalanine along with smaller amounts of phenyllactic acid. This reflects the precedented ability of chorismate mutase to spontaneously undergo nonenzymatic[14] rearrangement to prephenic acid, whose transient formation by D2704 can be detected. Nonenzymatic[10] decarboxylation and dehydration of prephenate affords phenylpyruvate, which undergoes predominant transamination to form L-phenylalanine while the remaining portion is reduced to phenyllactate.

Although *tyrB*-encoded aromatic aminotransferase, *aspC*-encoded aspartate transaminase, and *ilvE*-encoded branched-chain aminotransferase can catalyze the transamination leading to L-phenylalanine, the aromatic aminotransferase has the greatest affinity for the phenylpyruvate.[15,1d] That not all of the phenylpyruvate is transaminated reflects insufficient in vivo activity of the aromatic aminotransferase. This may indicate that the flow of carbon directed into phenylpyruvate synthesis is in excess of what the aromatic aminotransferase can transaminate. Alternatively, the initial culturing of the D2704 variants in rich medium may result in the microbe's consumption of enough L-tyrosine that expression of *tyrR* regulon genes such as the *tyrB* locus which encodes the aromatic transaminase may be transcriptionally repressed[16] even during subsequent culturing in medium lacking L-tyrosine supplementation. Lower aromatic aminotransferase activity may also reflect disruption of the putative complexes which this transferase forms in vivo with chorismate mutase–prephenate dehydrogenase and chorismate mutase–prephenate dehydratase.[17] This could be due to the mutations which have been introduced into the *tyrA* and *pheA* loci, which respectively encode the aforementioned bifunctional enzymes. Reduction of accumulating phenylpyruvate to phenyllactate may be exploited by the microbe as a mechanism for cycling NADH back to NAD^+ when the culture medium becomes partially limiting in oxygen availability. Reduction of another α-ketocarboxylic acid, pyruvic acid, by lactate dehydrogenase is a route for regenerating NAD^+ under anaerobic conditions.[18]

Measurement of carbon flow through the common pathway of aromatic amino acid biosynthesis is simplified in *E. coli* D2704, since two of the three major routes by which chorismic acid is processed have been eliminated. The combined concentration of L-phenylalanine and phenyllactate determined by ^1H NMR was then a convenient way to quantitate the carbon flow delivered to the end of the common pathway. This follows from the one to one mole relationship between chorismate processed and L-phenylalanine synthesized as well as chorismate processed and phenyllactate synthesized. Determination of which enzymes in the common pathway are rate-limiting was then based on the identification of metabolites other than L-phenylalanine and

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phenyllactate, which accumulated in the culture supernatant of *E. coli* D2704/pKD130A. In this fashion, a single ^{1}H NMR was able to quantitate carbon flow through the common pathway *and* identify rate-limiting enzymes in the common pathway.

In addition to L-phenylalanine and phenyllactate, DAH, DHS, shikimate, and shikimate 3-phosphate were observed in the culture supernatant of *E. coli* D2704/pKD130A. The indication that DHQ synthase is rate-limiting and DHQ dehydratase is not rate-limiting corresponds nicely with the ^{1}H NMR analyses of *E. coli aroD*/pKD130A and *E. coli aroE*/pKD130A and demonstrates that those enzymes identified to be rate-limiting or not rate-limiting are not an artifact of the host *E. coli* strain being scrutinized. The generality of the overall strategy to remove rate-limiting enzymes in the common pathway is further verified with the elimination of DAH accumulation upon amplification of DHQ synthase in *E. coli* D2704/pKD136. Observation of DHS, shikimate, and shikimate 3-phosphate in the culture supernatant of *E. coli* D2704/pKD136 indicated other potentially rate-limiting, common pathway enzymes. In systematic fashion, amplified expression of *aroE*-encoded shikimate dehydrogenase and *aroL*-encoded shikimate kinase eliminated both DHS and shikimate accumulation.

The discovery that the rate-limiting nature of both shikimate dehydrogenase and shikimate kinase could be removed upon amplified expression of *aroL* came as a surprise. Feedback inhibition of shikimate dehydrogenase by shikimate had been previously reported in plants,[19] but the existence of this same feedback loop in *E. coli* had not previously been reported. The impact of amplified expression of *aroL* also indicated that an enzyme's apparent rate-limiting character may not result from inadequate expression levels or catalytic sluggishness but rather could be a consequence of feedback inhibition by the substrate of a distal, rate-limiting enzyme in the pathway. Shikimate kinase, like the aromatic aminotransferase, is part of the *tyrR* regulon.[14d,13] Residual L-tyrosine from the initial growth in rich medium might contribute to transcriptional repression of shikimate kinase expression levels. Such transcriptional repression is likely to always be problematic, since even the low L-tyrosine levels resulting from de novo biosynthesis in completely unsupplemented medium are sufficient to result in accumulation of shikimate in culture supernatants of *E. coli* constructs.[20] One approach to removing the transcriptional repression is to introduce a mutation in the *tyrR* locus which encodes the regulatory protein controlling expression of the various *tyrR* regulon enzymes. This approach was not pursued due to the adverse metabolic burden likely placed on the microbe upon derepression of all of the various *tyrR* regulon enzymes. In addition, stability of *aroF*-containing plasmids has been reported[21] to be problematic in hosts possessing genomic mutations in *tyrR*.

Even with the removal of DAH, DHS, and shikimate from the culture supernatant, significant improvements in the levels of L-phenylalanine and phenyllactate synthesis had still not been achieved. Attention then turned to unraveling the basis for shikimate 3-phosphate accumulation. Rate-limiting EPSP synthase (encoded by *aroA*) catalytic activity was an obvious possibility. However, rate-limiting chorismate synthase could also contribute, since EPSP accumulation could be converted back to shikimate 3-phosphate by EPSP synthase. Conversion of EPSP and inorganic phosphate into shikimate 3-phosphate and PEP is one commonly employed method for assay of EPSP synthase.[22] Introducing plasmid-encoded *aroA* had a pronounced impact on accumulation of L-phenylalanine and phenyllactate even in lieu of amplified shikimate kinase activity. Increasing the catalytic activities of both EPSP synthase and chorismate synthase was ultimately required to significantly reduce shikimate 3-phosphate levels. Plasmid-encoded *aroA* and *aroC* along with plasmid-encoded *aroB* and *aroL* ultimately led to the highest levels of carbon flow through the common pathway as evidenced by the levels of L-phenylalanine and phenyllactate synthesized by D2704/pKD136/pKAD50. Almost twice as much carbon flow is being delivered (Figure 1B) to the end of the common pathway in this construct relative to D2704/pKD130A, which expresses comparable levels of transketolase and DAHP synthase but lacks plasmid-localized genes encoding the rate-limiting pathway enzymes.

Analysis of D2704 demonstrates that the combined use of simple ^{1}H NMR spectroscopy with the proper auxotrophic mutant can result in the straightforward identification of rate-limiting enzymes in the common pathway of a biosynthetic cascade. For the common pathway of aromatic amino acid biosynthesis, DHQ synthase, shikimate kinase, EPSP synthase, and chorismate synthase are rate-limiting enzymes. DHQ dehydratase and shikimate dehydrogenase are not rate-limiting. It is particularly important in identifying the rate-limiting enzymes to point out (Table II) that massive overexpression of the relevant loci is not necessarily required to remove impediments to carbon flow. For instance, less than twofold increases (Table II) in the specific activity of DHQ synthase completely compensated for the rate-limiting character of DHQ synthase. The minimum level of overexpresssion required to compensate for the rate-limiting character of DHQ synthase, shikimate kinase, EPSP synthase, and chorismate synthase remains to be established. This is an important consideration for the future, since achieving the highest possible percent conversions of D-glucose into industrial and medicinal aromatics will require that metabolic currency not be wasted on unneeded enzyme overexpression. In route to creation of such optimal microbial catalysts, the analysis methodology developed and rate-limiting enzymes identified in this study are important steps.

Experimental Section

General Methods. Chorismic acid, prephenic acid, phosphoenolpyruvate, and porcine heart diaphorase were purchased from Sigma. The sodium salt of 3-(trimethylsilyl)propionic-*2, 2, 3, 3-d*$_4$ acid was purchased from Aldrich. ^{14}C-Shikimate was obtained from New England Nuclear. ((Diethylamino)ethyl)cellulose (DE-52) was obtained from Whatman. Protein assay solution was purchased from Bio-Rad. ^{1}H NMR spectra were recorded on a Varian Gemini-200 spectrometer at 200 MHz.

Bacterial Strains. Construction of BJ502*aroB* has been described.[5b] RB791[23a] (W3110 *lacL8I*^{q}) was obtained previously by this laboratory. D2704[8] [W3110 F$^-$ (*argF-lac*)del U169 (*gal-bio*)del (*trp* del61-*intc*226 *trp-lac* W205) *trpR* Gal$^+$ *tna* Sal$^+$ *tyrA4* (*pheA*)del], AB2834/pIA321,[23b] JP1672/pMU371,[23c] AB2829/pKD501,[23d] AB2349/pGM602,[23e] and AB2829/pRW5*tkt*/pMU377*aroB* were obtained from Genencor International, Rochester, NY. AB2834[23f] [*tsx-352 supE42* λ$^-$ *aroE353 malA352* (λ$^-$)] and AB2849[23f] (*tsx-357 supE42* λ$^-$ *aroC355*) were obtained from the *E. coli* Genetic Stock Center at Yale University.

Culture Medium. All solutions were prepared in distilled, deionized water. LB medium[24] contained (per L) tryptone (10 g), yeast extract (5 g), and sodium chloride (10 g). M9 salts contained (per L) Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NaCl (0.5 g), and NH_4Cl (1 g). M9 growth medium contained glucose (10 g), $MgSO_4$ (0.12 g), and thiamine (1 mg) in 1 L of M9 salts. Isopropyl β-D-thiogalactopyranoside (IPTG) (0.2 mM) was added to all cultures of strains possessing plasmids derived from pSU18 and pSU19. Chloramphenicol (20 mg/L) and ampicillin (50 mg/L)

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were added to all appropriate cultures. Solutions of inorganic salts, magnesium salts, and carbon sources were autoclaved separately and then mixed. Antibiotics, thiamine, and IPTG were sterilized through 0.2-μm membranes prior to addition to the culture medium.

Culture Conditions. LB medium (1 L in a 4-L Erlenmeyer flask) containing IPTG and antibiotics was inoculated with 5 mL of an overnight culture and incubated at 37 °C for 12 h with agitation (250 rpm). Cells were collected by centrifugation, washed three times with 300-mL portions of M9 salts, and resuspended in M9 medium (1 L) containing IPTG and antibiotics. Cultures were returned to 37 °C and 250 rpm agitation.

NMR Analysis of Culture Supernatant. An aliquot (25 mL) of culture supernatant was taken at the indicated time interval and cells were removed by centrifugation. The supernatant was neutralized by the addition of 2 N NaOH, concentrated to dryness several times from D_2O, and redissolved in D_2O containing a known concentration of the sodium salt of 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid (TSP). Concentrations of cellular metabolites in the supernatant were determined by comparison of the integrals of known metabolite resonances and the resonance corresponding to TSP in the ^{1}H NMR. Cultures were grown in triplicate to establish mean values and standard deviations.

Genetic Manipulations. Recombinant DNA manipulations were performed as outlined in Sambrook *et al.*[25] T4 DNA ligase was used for all ligations, and blunt ends were formed by removing protruding ends of DNA with mung bean nuclease. Construction of pKD130A[5a] and pKD136[6a] have been described elsewhere. The vectors pSU18 and pSU19 are medium copy number vectors derived from the plasmids pSU2718/pSU2719.[26] Plasmids pSU18 and pSU19 possess chloramphenicol resistance, a *lac* promoter, and a p15A origin of replication and differ in the orientation of the pUC18 multiple cloning site with respect to the *lac* promoter. Plasmids carrying loci encoding rate-limiting, common pathway enzymes were constructed using *E. coli* DNA in the vectors pSU18 and pSU19 and are shown in Table I.

pKD28. A 1.6-kb *Bam*HI/*Hin*dIII fragment containing the *aroE* gene expressed from a *tac* promoter was isolated from pIA321[23b] and ligated into the vector pSU18.

pKAD31. A 1.0-kb *Bam*HI/*Kpn*I *aroL* fragment from pMU371[23c] was isolated and ligated into pSU19.

pKAD34. This 4.9-kb *aroE aroL* plasmid was constructed by manipulation of the flanking restriction sites of *aroE* from pKD28 followed by ligation into pKAD31. pKD28 was linearized with *Bam*HI, and the 5′ protruding ends of DNA were removed. *Xba*I linkers were ligated to the resulting blunt ends, and the plasmid was recircularized forming pKAD32. pKAD32 was digested with *Hin*dIII and blunt ended, *Bam*HI linkers were attached, and the plasmid was recircularized forming pKAD33. The *aroE* gene, now localized on a 1.6-kb *Xba*I/*Bam*HI fragment, was isolated from pKAD33 and ligated into pKAD31 to form pKAD34.

pKAD38. This 4.7-kb plasmid was created by ligation of a 2.4-kb *Kpn*I/*Pst*I fragment encoding *aroA* derived from pKD501[23d] behind the *lac* promoter of pSU18. The absence of a native promoter on the *aroA* fragment necessitates its placement behind a promoter for expression.

pKAD43. This 5.7-kb *aroA aroL* plasmid was created by isolation of an *aroA* fragment from pKAD38 followed by its ligation into pKAD31. pKAD38 was linearized with *Kpn*I, and the resulting 3′ ends of DNA were removed. Subsequent digestion with *Pst*I yielded a 2.4-kb blunt end/*Pst*I *aroA* fragment. pKAD31 was digested with *Hin*dIII and treated with mung bean nuclease. Digestion with *Pst*I afforded a 3.3-kb blunt end/*Pst*I linearized vector suitable for ligation with the prepared *aroA* fragment.

pKAD39. This 4.0-kb *aroC* plasmid was constructed by isolating an *aroC* fragment from pGM602[23e] followed by its insertion into pSU19. pGM602 was digested with *Cla*I and treated with mung bean nuclease. Digestion with *Sal*I yielded a 1.7-kb *Sal*I/blunt end fragment. pSU19 was digested simultaneously with *Sal*I and *Sma*I and ligated to the prepared *aroC* fragment.

pKAD50. The 7.4-kb *aroA aroC aroL* plasmid was constructed by inserting the *aroC* fragment from pKAD39 into pKAD43. pKAD39 was linearized with *Kpn*I, and the 3′ protruding ends of DNA were removed. A 1.7-kb *Sal*I/blunt end *aroC* fragment was isolated upon digestion of the DNA with *Sal*I. pKAD43 was digested with *Bam*HI and blunt ended, and the resulting DNA was digested with *Sal*I. The 5.7-kb *Sal*I/blunt end fragment from pKAD43 was ligated to the prepared 1.7-kb *aroC* fragment, yielding pKAD50.

pKAD44. The 6.4-kb *aroC aroA* plasmid was created by isolation of an *aroA* fragment from pKAD38 followed by insertion into pKAD39. pKAD38 was digested with *Kpn*I forming a linear DNA fragment. Subsequent mung bean nuclease treatment followed by *Pst*I digestion yielded a 2.4-kb *aroA* fragment. Plasmid pKAD39 was digested with *Hin*dIII, the cohesive ends were removed, and the resulting DNA was digested with *Pst*I. The resulting blunt end/*Pst*I pKAD39 fragment was ligated to the *aroA* fragment affording pKAD44.

pKAD51. The 5-kb *aroC aroL* plasmid was constructed by isolation of *aroC* from pKAD39 followed by its ligation into pKAD31. pKAD39 was digested with *Kpn*I, blunt ended, and digested with *Sal*I to liberate the *aroC* gene. pKAD31 was linearized with *Bam*HI, treated with mung bean nuclease, and digested with *Sal*I. The resulting blunt ended/*Sal*I pKAD31 fragment was ligated to *aroC*.

pKAD42. The 10-kb *aroF aroB* plasmid was constructed from pKD136 by removal of the 5-kb *tkt* insert as a *Bam*HI fragment followed by recircularization.

pKAD46A. The 7-kb *serC aroA* plasmid was constructed by isolating a 4.7-kb *Pst*I fragment containing the *serC aroA* operon from pKD501[23d] and inserting it in the *Pst*I site of pSU19.

pKAD52. Construction of the 4.2-kb *pheA* plasmid was achieved by isolating *pheA* from pKB45[27] as an *Eco*RI/*Bsp*1286I fragment[28] followed by insertion into pSU18.

Preparation of DAHP, DHS, Shikimate 3-Phosphate, and EPSP. Strains for metabolite accumulations were grown in LB medium and resuspended in M9 medium as described under culture conditions. Metabolites were isolated from the culture supernatant upon removal of cells.

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate was synthesized from methyl (methyl 3-deoxy-D-*arabino*-heptulopyranosid)onate according to the procedure of Frost and Knowles.[7a] DAH was synthesized as described previously using *E. coli* BJ502*aroB*/pKD130A.[5a] DAH was purified and subsequently converted to methyl (methyl 3-deoxy-D-*arabino*-heptulopyranosid)onate as described by Reimer.[29]

3-Dehydroshikimate was isolated from the culture supernatant (1 L) of *E. coli* AB2834*aroE*/pKD136.[6a] Following removal of the cells, the supernatant (24 mmol of DHS) was adjusted to pH 2.5 by addition of 6 N HCl. After continuous extraction with ethyl acetate, the organic fraction was dried over $MgSO_4$ and concentrated. Subsequent recrystallization of the brown oil from ethyl acetate afforded 1.6 g of DHS (40% yield).

Shikimate 3-phosphate was isolated from the culture supernatant of AB2829/pRW5*tkt*/pMU377*aroB*. Host strain AB2829 accumulates shikimate 3-phosphate due to a mutation in the gene which encodes EPSP synthase. Plasmid pRW5*tkt*[5b] contains a feedback-resistant *aroG* gene and *tkt*. Plasmid pMU377*aroB*, a pBR322[30] derivative, encodes *aroL* and *aroB*. Two liters of cell-free culture supernatant (3.6 mmol of shikimate 3-phosphate) were passed through a column (500 mL) of Dowex 50 (H^+ form) at 4 °C. The column was washed with 1.5 L of water, and the resulting eluent was adjusted to pH 8.0 by addition of 5 M LiOH. Water was removed from the sample in vacuo. Methanol (500 mL) was added to the resulting solid, and the mixture was stirred at 4 °C for 1.5 h. After removal of the precipitate by filtration, the sample was concentrated to dryness. The residue was dissolved in water (50 mL), the pH of the resulting solution was adjusted to 7.0 by addition of 1 M LiOH, and a white precipitate was removed by filtration and discarded. The solution was loaded onto a column of DE-52 (5 cm × 19.5 cm) at 4 °C. The column was washed with 1 column volume of water and subsequently eluted with a linear gradient (1.5 L + 1.5 L, 0–400 mM) of triethylammonium bicarbonate, pH 7.2. Column fractions (25 mL) were analyzed for total phosphorous and for inorganic phosphate.[31] Fractions that contained shikimate 3-phosphate but were free of inorganic phosphate were pooled and concentrated under reduced pressure to 100 mL. Buffer was removed by azeotropic distillation with 2-propanol (three times). After concentrating the sample, the resulting white powder was dissolved in water and passed down a column (100 mL) of Dowex 50

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(Na^+ form). The sample was eluted with three column volumes of water and concentrated to dryness to afford 0.21 g of the trisodium shikimate 3-phosphate (18% yield). Spectroscopic characterization of the purified shikimate 3-phosphate correctly correlated with literature data.[32]

5-Enolpyruvoylshikimate 3-phosphate was synthesized enzymatically as previously described[33] from phosphoenolpyruvate and sodium shikimate 3-phosphate. EPSP synthase was partially purified from DH5α/pKAD46A using the procedure of Levin and Sprinson.[33b]

Enzyme Assays. Cells were grown in LB media for 12 h as described under culture conditions. Cells were isolated by centrifugation and disrupted by two passages through a French press at 16 000 psi. Cellular debris was removed by centrifugation at 48 000*g* for 20 min. Protein was quantified using the Bradford dye-binding procedure.[34] A standard curve was prepared with bovine serum albumin.

Chorismate mutase was assayed[35] using the method of Cotton *et al.* Prephenic dehydratase was assayed[35] by following the formation of phenylpyruvic acid from prephenic acid at 320 nm. DHQ synthase activity was measured by monitoring the disappearance of DAHP over time[23a] using the thiobarbituric acid assay[36] to quantify DAHP. Shikimate kinase was assayed by monitoring the formation of ^{14}C-shikimate 3-phosphate from ^{14}C-shikimate using the method of De Feyter.[37] EPSP synthase was assayed in the reverse direction as described by Boocock and Coggins.[22] Chorismate synthase was assayed[38] by monitoring the formation of chorismate through its subsequent conversion to phenylpyruvate at 320 nm using chorismate mutase and prephenate dehydratase as coupling enzymes. $FADH_2$ required for activity was generated in situ from FAD, NADH, and porcine heart diaphorase as described.[38] A crude extract of the coupling enzymes was prepared from AB2849/pKAD52.

Inhibition of Shikimate Dehydrogenase. Shikimate dehydrogenase was purified[39a] from AB2834/pIA321 and stored at −70 °C at a final concentration of 1600 units/mL in 50% glycerol, 50 mM Tris-HCl, pH 7.5, 1 mM benzamidine, 0.4 mM dithiothreitol, and 50 mM KCl. For the inhibition studies, the conversion of dehydroshikimate to shikimate with the subsequent oxidation of NADPH was monitored[39b] at 340 nm and 24 °C. The assay contained (final volume 1 mL) 0.1 M KH_2PO_4, pH 7.4, 0.21 mM NADPH, DHS, shikimate, and 0.01 mL of diluted enzyme. The K_i for shikimate was determined under the following conditions: NADPH (K_m 0.03 mM) was held constant at 0.21 mM; DHS concentrations (K_m 0.072 mM) were 0.033, 0.050, 0.067, 0.10, and 0.20 mM; shikimate concentrations were 0, 0.13, 0.25, 0.38, and 0.50 mM. Enzyme samples were diluted 1:4000 directly from the frozen stock in 0.05 M Na_2HPO_4, pH 7.4, 0.1 mM dithiothreitol, and 0.1 mg/mL acetylated bovine serum albumin. The K_i for shikimate was obtained from a replot of the slope of a Lineweaver–Burk[40] plot (Figure 3) versus inhibitor concentration. Points at high inhibitor concentrations and low substrate concentrations in the Lineweaver–Burk plot were found to deviate from linearity and were removed.

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