Synthetic Modification of the *Escherichia coli* Chromosome: Enhancing the Biocatalytic Conversion of Glucose into Aromatic Chemicals

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Abstract: The yield of L-phenylalanine, racemic phenyllactic acid, and prephenic acid synthesized from glucose has been doubled and contamination of these aromatic end products by biosynthetic intermediates drastically reduced. These improvements resulted from increasing the in vivo catalytic activity of specific enzymes in the common pathway of aromatic amino acid biosynthesis by chromosomal modification of *Escherichia coli*. The centerpiece of these changes was the synthesis of a multigene cassette carrying *aroA* (encoding EPSP synthase), *aroC* (encoding chorismate synthase), and *aroB* (encoding DHQ synthase). Chromosomal insertion of the synthesized multigene cassette into *E. coli* KAD29B, a strain having a mutation in the *tyrR* locus which relieves transcriptional repression of *aroL* (encoding shikimate kinase), resulted in biocatalysts KAD1D and KAD11D. Improved catalytic activities of individual common pathway enzymes have previously been accomplished with extrachromosomal plasmids encoding the appropriate loci. By contrast, the chromosomal alterations possessed by KAD1D and KAD11D circumvent potential problems associated with plasmid instability and unnecessary overexpression of plasmid-encoded, common pathway enzymes. The described modifications of the *E. coli* chromosome and the methods utilized to achieve these changes will also simplify construction of future generations of aromatic-synthesizing biocatalysts.

The diversity of biosynthetic enzymes possessed by microorganisms creates a wide range of opportunities for synthesis of aromatic chemicals from abundant and inexpensive D-glucose.[1] However, the industrial viability of microbe-catalyzed aromatic syntheses hinges on percent yield, rates of conversion, and product purity. A number of innovative approaches have been developed that increase the percentage of carbon consumed by a microbe that is channeled into the common pathway of aromatic amino acid biosynthesis (Scheme 1).[2−5] By contrast, comparatively little attention has been focused on the intact delivery of carbon flow[6−8] from 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP), the first intermediate of the common pathway, to chorismic acid, the last intermediate of the common pathway (Scheme 1).[1b,9] Not all of the intervening common pathway enzymes situated between DAHP and chorismic acid are capable of catalyzing the conversion of their substrates to products at a sufficiently rapid rate to avoid substrate accumulation.[6,7] Substrates that accumulate as a consequence of these rate-limiting common pathway enzymes are exported by the microbial biocatalyst from the cytosol to the culture supernatant. The result is a reduction in yield and compromised purity of aromatic end products.

Previous attempts to improve carbon flow through the common pathway (Scheme 1) in *Escherichia coli* relied on transformation of the microbial host with plasmids carrying genes that encoded rate-limiting pathway enzymes.[6] The final *E. coli* construct carried two multicopy plasmids. While effective in reducing enzymatic impediments to carbon flow, the design of the biocatalyst clearly required modification. A gene localized on a multicopy plasmid may lead to enzyme expression levels in excess of what is required to ameliorate an enzyme's rate-limiting character. This metabolic burden is often reflected in slower growth rates and lower rates and yields of biosynthesized products. Metabolic burden coupled with the metabolic demands imposed by stable maintenance of two plasmids was anticipated to cause problems under the large-volume, high-density growth conditions typically employed in industrial biocatalysis. Diminished biocatalyst growth rates, end product yields, and plasmid stability were of particular concern. In addition, genetic manipulation of a biocatalyst to alter the aromatic or metabolically related chemical synthesized from glucose is complicated by the presence of excessive plasmid-

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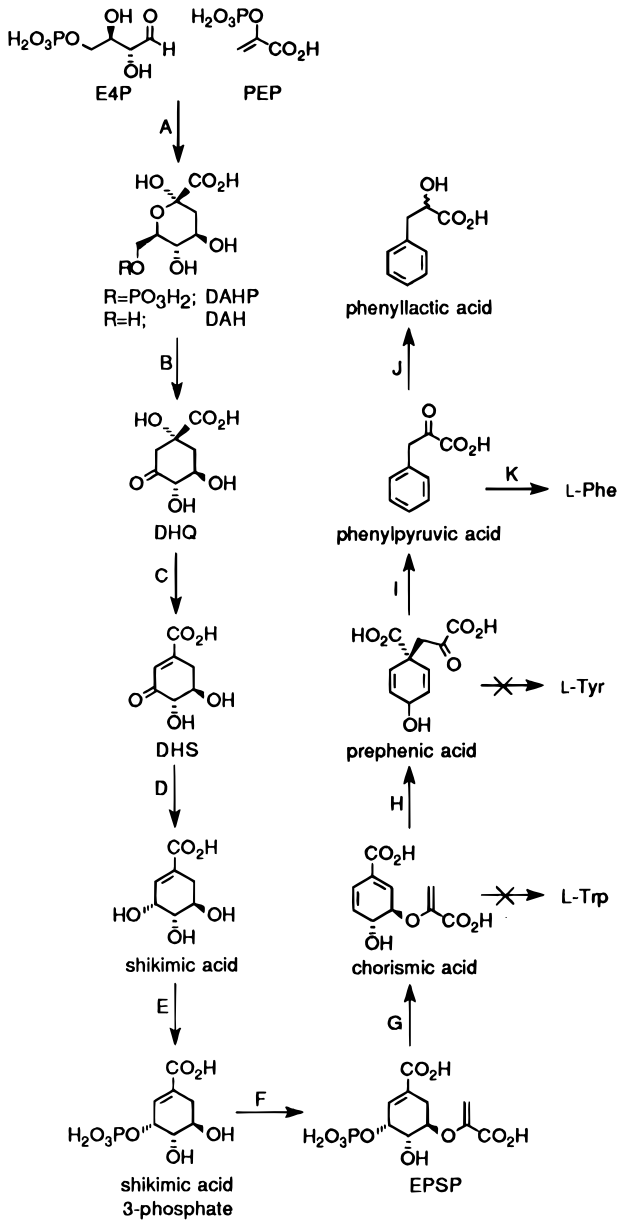
Scheme 1[a]



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

encoded DNA. There is thus a need to minimize the amount of extrachromosomal DNA. Along these lines, impediments to the flow of carbon from glucose to aromatic end products caused by rate-limiting enzymes in the common pathway (Scheme 1) have now been rectified with a strategy which relies on redesigning the *E. coli* chromosome.

Results

Biocatalyst Selection and Percent Yield Consideration. In wild-type *E. coli*, chorismic acid is channeled into biosynthesis of L-phenylalanine, L-tyrosine, L-tryptophan, ubiquinones, menaquinones, enterobactin, and folic acid.[1b,9] *E. coli* D2704[10] (Table 1) was selected as the host biocatalyst due to mutations that eliminate L-tyrosine and L-tryptophan biosynthesis (Scheme 1). As a result, carbon flow exiting the common pathway of aromatic amino acid biosynthesis can be conveniently measured in *E. coli* D2704 by use of ^{1}H NMR to quantitate prephenic acid, L-phenylalanine, and racemic phenyllactic acid concentrations which accumulate in the microbe's culture supernatant.[6]

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Table 1. Bacterial Strains and Plasmids Used in This Study

strain/ plasmid	relevant characteristics	reference or source
strain		
DH5α	*lacZ* ΔM15 *hsdR recA*	BRL
D2704	(Δ*trpC*−*E*)*trpR tyrA4* Δ(*pheA*)	ref 10, GCI
JB5	*tyrR*	ref 44
AB2829	*aroA354*	ref 26, B. Bachmann
AB2849	*aroC355*	ref 26, B. Bachmann
AB2847	*aroB351*	ref 26, B. Bachmann
KAD27C	D2704 *tyrR*	this study
KAD29B	D2704 *tyrR*	this study
KAD1D	KAD29B *serA*::(P_{tac}*aroAaroCaroBkan*^{R})	this study
KAD11D	KAD29B *serA*::(P_{tac}*aroAaroCaroBkan*^{R})	this study
plasmid		
pBR325	Ap^R Cm^R Tc^R, pMB1 replicon	ref 49, BRL
pSU18	Cm^R, P_{lac} *lacZ*′, p15A replicon, pUC18 MCS	ref 24
pSU19	Cm^R, P_{lac} *lacZ*′, p15A replicon, pUC19 MCS	ref 24
pCL1920	Sp^R, P_{lac} *lacZ*′, pSC101 replicon	ref 27, GCI
pCL1920-lac	Sp^R, pCL1920 without P_{lac} *lacZ*′	this study
pBLSCR SK+	Ap^R, P_{lac} *lacZ*′	ref 25, Stratagene
pDR540	Ap^R, P_{tac}	ref 47, Pharmacia
p*Trc*99A	Ap^R, P_{trc}	ref 46, Pharmacia
p*Trc*99A−E	Ap^R, P_{trc}, *Eco*RI removed from MCS	this study
p34E	Ap^R, inverted repeat of MCS	ref 48
pKD501	*aroA* plasmid	ref 18, GCI
pGM602	*aroC* plasmid	ref 43, GCI
pJB14	Ap^R, *aroB* fragment in pKK223−3	ref 44
pMB2190	Ap^R, *kan*^{R} fragment	GCI
pD2625	*serA* plasmid	GCI
pMAK705	Cm^R, *lacZ*′, *ts*-pSC101 replicon	ref 29, Kushner
pKD116A	Ap^R Cm^R, *aroF* fragment in pBR325	ref 3a
pKD130A	Ap^R, *tktA* and *aroF* fragments in pBR325	ref 3a
pKD136	Ap^R, *tktA*, *aroF*,and *aroB* fragments in pBR325	ref 7
pKAD31	Cm^R, *aroL* fragment in pSU19	ref 6
pKAD46A	Cm^R, *aroA* fragment in pSU19	ref 6
pKAD49	Cm^R, P_{tac} fragment in pSU18	this study
pKAD50	Cm^R, *aroA*, *aroC*, and *aroL* fragments in pSU19	ref 6
pKAD52	Cm^R, *pheA* fragment in pSU18	ref 6
pKAD61	Ap^R, *Hin*dIII site of p*Trc*99A-E MCS replaced by *Eco*RI	this study
pKAD62A	Ap^R, *kan*^{R} fragment in pKAD61	this study
pKAD63	Ap^R, *serA* fragment in p34E	this study
pKAD64	Cm^R, *aroL* fragment in pSU18	this study
pKAD66	Ap^R, *tktA*, *aroF*, *aroL* fragments in pBLSCR SK+	this study
pKAD68	Ap^R, *aroCaroB* fragment in pBLSCR SK+	this study
pKAD69	Cm^R, *aroA* fragment in pSU19	this study
pKAD70	Ap^R, *aroC* fragment in pBLSCR SK+	this study
pKAD72A	Cm^R, P_{tac}*aroAaroCaroBkan*^{R} cassette in pSU18	this study
pKAD73	Cm^R, P_{tac}*aroA* in pSU18	this study
pKAD74	Cm^R, *aroCaroBkan*^{R} fragment in pSU18	this study
pKAD76A	Cm^R, *serA* fragment in pMAK705	this study
pKAD77A	Sp^R, P_{tac}*aroAaroCaroBkan*^{R} cassette in pCL1920-lac	this study
pKAD80A	Cm^R, P_{tac}*aroAaroCaroBkan*^{R} cassette in pKAD76A	this study

D2704 is still capable of biosynthesizing ubiquinone, menaquinone, enterobactin and folic acid, although the amount of chorismic acid committed to these terminal pathways is small.

The percentage of carbon directed into the common pathway in *E. coli* D2704 increases upon transformation with plasmid pKD130A.[3] This pBR325-based plasmid carries *aroF*, which encodes the DAHP synthase isozyme sensitive to feedback inhibition by L-tyrosine,[11] and *tktA*, which encodes the predominant transketolase activity in *E. coli*.[3,12] Along with L-phenylalanine and racemic phenyllactic acid, 3-deoxy-D-*arabino*-heptulosonic acid (DAH), 3-dehydroshikimic acid (DHS), shikimic acid, and shikimic acid 3-phosphate were detected (Scheme 1) in the culture supernatant of *E. coli* D2704/pKD130A.[6] Accumulation of these metabolites, based on earlier precedent, indicated that 3-dehydroquinate (DHQ) synthase, shikimate kinase, 5-enolpyruvylshikimic acid 3-phosphate (EPSP) synthase, and chorismate synthase were rate-limiting enzymes impeding carbon flow through the common pathway. While attention focused on increasing the in vivo catalytic activity of these enzymes with modifications of the D2704 chromosome, expression of plasmid pKD130A remained the method used throughout this study for directing carbon flow into the common pathway.

Introduction of a *tyrR* Mutation into D2704. Two shikimate kinase isozymes are expressed in *E. coli*.[13] Shikimate kinase II encoded by the *aroL* locus[14] accounts for the majority of enzyme activity due to a 100-fold lower K_m for shikimic acid[9a,14a] than that of shikimate kinase I, encoded by *aroK*.[15] Efforts to adjust shikimate kinase specific activities therefore focused on *aroL*. The *aroL* gene is a component of the *tyrR* regulon.[16] In combination with tyrosine or tryptophan, TyrR inhibits transcription of *aroL*. Starvation of cells for tyrosine and tryptophan or mutation of *tyrR* result in a 10-fold increase in shikimate kinase II specific activity.[9a]

A mutant allele of *tyrR* was introduced into D2704 via phage transduction. Selection for *tyrR* mutations typically relies on screening colonies for the ability to synthesize tyrosine in the presence of *m*-fluorotyrosine.[2b] Unfortunately, a mutation in the *tyrA* locus of D2704 encoding chorismate mutase-prephenate dehydrogenase precludes de novo tyrosine biosynthesis. Colonies were instead screened on M9 plates containing *m*-fluorotyrosine and supplemented with *p*-hydroxyphenylpyruvic acid, the immediate precursor of tyrosine. Although *p*-hydroxyphenylpyruvic acid relieves tyrosine auxotrophy in D2704, addition of *m*-fluorotyrosine inhibits this growth due to TyrR-mediated repression of *tyrB*, the locus that encodes aromatic aminotransferase.[17] While D2704 grew slowly on M9 plates lacking L-phenylalanine, supplementation of the medium with L-phenylalanine in addition to L-tryptophan improved growth characteristics.

Following transduction of D2704 with P1 lysate propagated from *E. coli* JB5, KAD27C and KAD29B were isolated that acquired the ability to grow on M9 containing L-phenylalanine, L-tryptophan, *p*-hydroxyphenylpyruvic acid, and *m*-fluorotyrosine. KAD27C and KAD29B were incapable of growth in the absence of either L-tryptophan or *p*-hydroxyphenylpyruvic acid, indicating that the *tyrA* Δ*trpE*−*C* genotype of the originating D2704 was still intact. Growth of KAD27C and KAD29B on M9 medium containing L-tyrosine and L-tryptophan in the absence of L-phenylalanine indicated that derepression of *tyrB* increased L-phenylalanine biosynthesis.

Comparison of shikimate kinase specific activities for D2704, KAD27C, and KAD29B revealed the impact of the *tyrR* mutation on *aroL* expression. Each strain was grown in 1 L of LB medium (see Experimental Section), and enzyme specific activities were measured in crude cellular lysates. Shikimate kinase specific activities of 0.0023, 0.09, and 0.12 unit/mg were obtained for D2704, KAD27C, and KAD29B, respectively. The observed 40−50-fold increase in shikimate kinase specific activity for KAD27C and KAD29B relative to D2704 was indicative of derepression of *aroL* expression.

Impact of *aroL* Derepression on Metabolite Accumulation. To determine whether derepression of *aroL* relieved the rate-limiting character of shikimate kinase, intermediate metabolites that accumulated in culture supernatants of D2704/pKD136, KAD27C/pKD136, and KAD29B/pKD136 were compared. Plasmid pKD136[7] (Table 1) is a derivative of pKD130A which contains the *aroB* locus in addition to *tktA* and *aroF*. Inclusion of plasmid-encoded *tktA* and *aroF* served to increase carbon flow into the common pathway while plasmid-encoded *aroB* relieved the rate-limiting character of DHQ synthase, preventing extracellular DAH accumulation.[7] As with all constructs examined in this study, D2704/pKD136, KAD27C/pKD136, and KAD29B/pKD136 were initially grown in LB medium, harvested, and then cultured for 48 h in M9 medium that contained 56 mM glucose. Portions of the culture were removed after 24 h and 48 h in M9, and the culture supernatants were analyzed by ^{1}H NMR. Accumulation of common pathway metabolites was best observed after 24 h of culturing in M9 medium while end products L-phenylalanine and phenyllactic acid reached maximum values after 48 h of culturing in M9 medium.

Detection of 3.7 mM DHS and 3.6 mM shikimic acid in the supernatant of D2704/pKD136 (Figure 1A, entry 1) reflects the rate-limiting character of shikimate kinase. Shikimic acid is exported to the supernatant due to insufficient shikimate kinase levels whereas accumulation of DHS is likely due to feedback inhibition of shikimate dehydrogenase by shikimic acid.[6] The DHS and shikimate concentrations that were detected in cultures of KAD27C/pKD136 (Figure 1A, entry 2) were reduced to 0.4 and 0.6 mM, respectively, while neither intermediate was detected in cultures of KAD29B/pKD136 (Figure 1A, entry 3). Removal of the rate limitation of shikimate kinase in KAD29B/pKD136 also resulted in higher concentrations of end products L-phenylalanine and phenyllactic acid. After 48 h in M9 medium, KAD29B/pKD136 synthesized 6.9 mM combined concentration of L-phenylalanine and phenyllactic acid (Figure 1B, entry 3) as compared to the 5.6 mM concentration of end products synthesized by D2704/pKD136 (Figure 1B, entry 1). The increase in shikimate kinase specific activity in KAD27C/pKD136 (Figure 1B, entry 2) resulted in no significant increase in L-phenylalanine and phenyllactic acid synthesis relative to D2704/pKD136.

Preparation of P_{tac}*aroA*B*aroC*k*an^R Cassette. Removal of common pathway rate limitations resulting from modest increases in enzyme specific activities afforded by plasmid-encoded *aroB*, *aroA*, and *aroC*[6] suggested that insertion of a single additional copy of each gene into the chromosome of D2704 might be sufficient to achieve this same goal. Integration of additional copies of *aroB*, *aroA*, and *aroC* was facilitated by preparation of a single cassette that contained each gene

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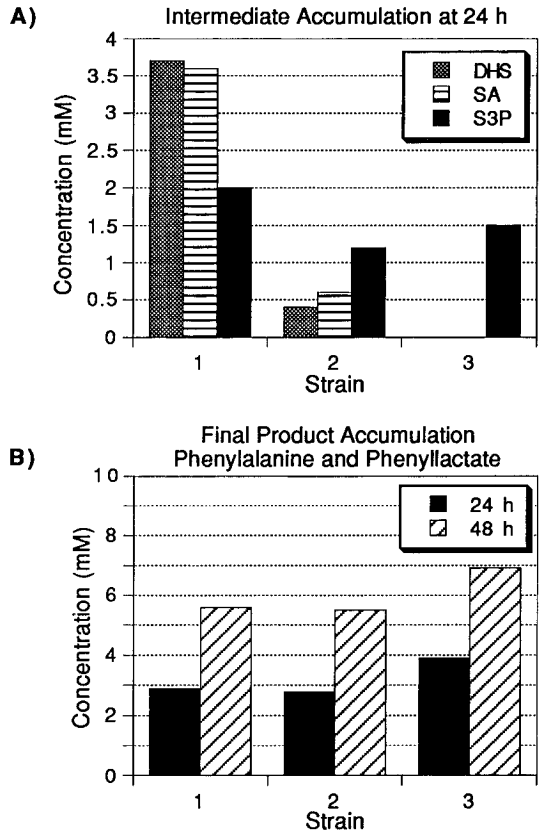
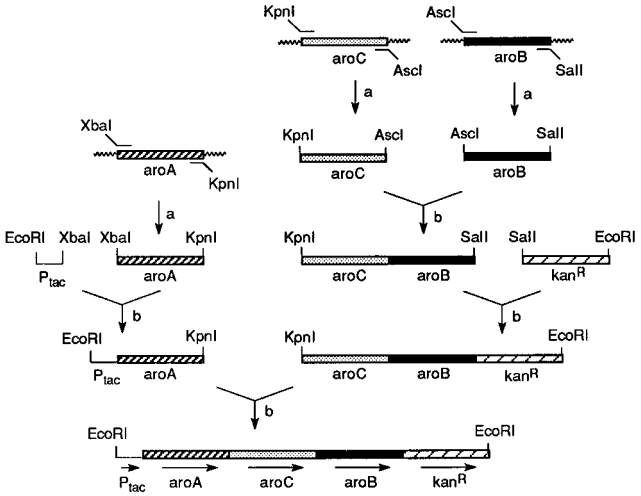


Figure 1. (A) Common pathway intermediates which accumulated extracellularly after 24 h of culturing in minimal medium which initially contained 56 mM D-glucose. (B) Combined concentrations of L-phenylalanine and phenyllactic acid which accumulated after 24 h and 48 h of culturing. Strains include (1) D2704/pKD136; (2) KAD27C/pKD136; and (3) KAD29B/pKD136.

Scheme 2[a]



[a] (a) Vent polymerase, dNTP's; (b) T4 DNA ligase, ATP.

(Scheme 2). Although no effort was made to determine the minimum increase in EPSP synthase specific activity needed to remove its rate-limitation, the 8-fold increase in specific activity resulting from plasmid-localized *aroA* was the largest increase of the three genes to be included in the cassette. Furthermore, there is no transcriptional promoter sequence immediately upstream from the *E. coli aroA* gene, which is located in an operon behind the *serC* locus.[18] It was concluded that optimal expression of EPSP synthase might best be achieved by placement of *aroA* behind the *tac* promoter sequence (P_{tac}) (Scheme 2).[19] The *aroC* and *aroB* loci, each of which included their native promoters, were positioned behind *aroA* such that transcription of each gene would proceed in the same direction as *aroA* transcription. Finally the kanamycin resistance gene (kan^R) encoding aminoglycoside 3′-phosphotransferase was included in the cassette to facilitate eventual screening for integration of the cassette into the D2704 genome.

Using the polymerase chain reaction (PCR),[20] *aroA*, *aroC*, and *aroB* were each amplified from individual plasmids. Inclusion of restriction recognition sequences at the 5′-end of each PCR primer simplified the necessary cloning operations for final preparation of the cassette. Complete nucleotide sequences for *aroA*,[21] *aroC*,[22] and *aroB*[23] were critical for selection of compatible restriction recognition sequences. After PCR amplification, each DNA fragment was localized in a plasmid. Localization of the 1.3 kb *aroA* fragment in pSU19[24] resulted in pKAD69 while the 1.3 kb *aroC* and 1.3 kb *aroB* genes were localized in pBLSCR SK+[25] to afford pKAD68. Complementation of *E. coli* aromatic auxotrophs[26] AB2829 with pKAD69 and both AB2849 and AB2847 with pKAD68 confirmed that PCR-amplified gene products yielded active proteins.

Assembly of the cassette was simplified using a stepwise approach, the details of which can be found in the Experimental Section. Final ligation of P_{tac}*aroA* and *aroCaroBkan*^{R} fragments into the *Eco*RI site of pSU18 afforded pKAD72A (Table 2). The cassette was localized in pSU18 immediately downstream and in the same orientation as a vector-encoded *E. coli lac* promoter (P_{lac}). Plasmid pSU18 is a low-copy (approximately 10−15 copies/cell) vector that contains the p15A replicon.[24] The completed cassette was also inserted into a second low-copy vector, pCL1920, which utilizes the pSC101 replicon and is usually present in approximately 5 copies per cell.[27] While pCL1920 also contains the *lac* promoter immediately upstream from the multiple cloning site, digestion of the plasmid with *Eco*RI and religation afforded pCL1920-lac in which the *lac* promoter was removed from the vector. Localization of the cassette into the *Eco*RI site of pCL1920-lac resulted in pKAD77A (Table 2).

Impact of the Plasmid-Localized Cassette on Metabolite Accumulation. Prior to integration into the genome of KAD29B, the impact of the plasmid-localized P_{tac}*aroAaroC-aroBkan*^{R} cassette on intermediate and end product accumulation was assessed. Plasmids pKAD72A and pKAD77A were transformed separately into KAD29B/pKD130A. In the presence of the plasmid-localized cassette, KAD29B was expected to possess increased specific activities of all four common pathway rate-limiting enzymes while *tktA*- and *aroF*-encoding pKD130A was expected to increase carbon flow into the common pathway. Metabolite accumulation was compared with D2704/pKD130A in which none of the rate-limiting enzymes

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Table 2. Restriction Enzyme Maps of Plasmids Containing the Multigene Cassette

Plasmid	Vector	Plasmid Map[a]
pKAD72A (7.8 kb)	pSU18	E EX K A S E Cm^R P_{lac} P_{tac} aroA aroC aroB kan^R
pKAD77A (9.8 kb)	pCL1920	E EX K A S E Sp^R P_{tac} aroA aroC aroB kan^R
pKAD80A (12.9 kb)	pMAK705	Sp Sp EX K A S E Sp Cm^R rep(ts) P_{lac} serA P_{tac} aroA aroC aroB kan^R serA

[a] Restriction enzymes sites are abbreviated as follows: E = EcoRI, X = XbaI, K = KpnI, A = AscI, S = SalI, Sp = SphI.
— vector DNA — insert DNA

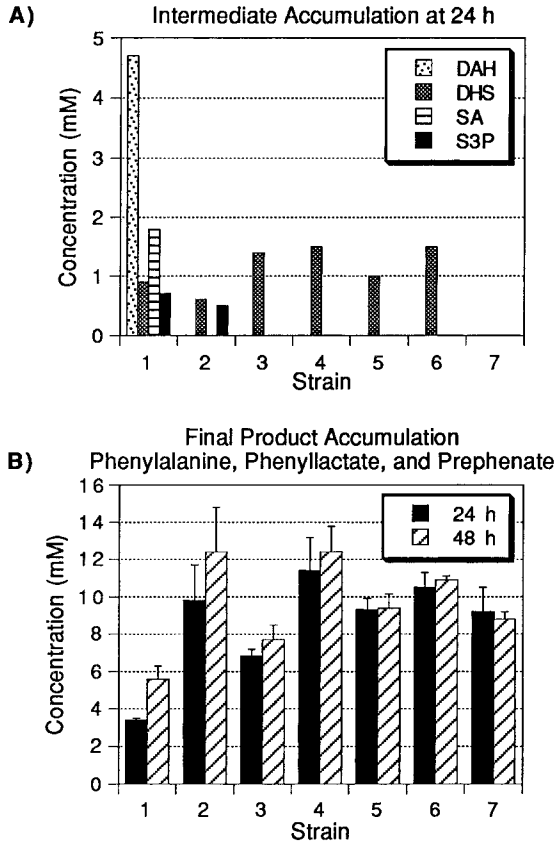

Figure 2. (A) Common pathway intermediates which accumulated extracellularly after 24 h of culturing in minimal medium which initially contained 56 mM D-glucose. (B) Average, combined concentrations of prephenic acid, L-phenylalanine, and phenyllactic acid which accumulated after 24 h and 48 h of culturing. Strains include (1) D2704/pKD130A; (2) D2704/pKD136/pKAD50; (3) KAD29B/pKD130A/pKAD72A; (4) KAD29B/pKD130A/pKAD77A; (5) KAD1D/pKD130A; (6) KAD11D/pKD130A; and (7) KAD1D/pKAD66.

were amplified and with D2704/pKD136/pKAD50 in which the four rate-limiting enzymes were localized on plasmids.[6] After 24 h in M9 medium, the culture supernatant of KAD29B/pKD130A/pKAD72A (Figure 2A, entry 3) contained 1.4 mM DHS. DAH, shikimic acid, and shikimate 3-phosphate were not detected in the medium. In contrast, significant concentrations of DAH, DHS, shikimic acid, and shikimate 3-phosphate were detected in the culture medium of D2704/pKD130A (Figure 2A, entry 1) after 24 h of culturing. Metabolite accumulation for KAD29B/pKD130A/pKAD72A (Figure 2A, entry 3) was more similar to D2704/pKD136/pKAD50 (Figure 2A, entry 2) which contained 0.6 mM DHS and 0.5 mM shikimate 3-phosphate after 24 h in M9 medium.

After 48 h of culturing in M9, KAD29B/pKD130A/pKAD72A (Figure 2B, entry 3) synthesized 7.7 ± 0.8 mM combined concentration of L-phenylalanine and phenyllactic acid. This represents a slight improvement relative to the 5.6 ± 0.7 mM end products synthesized by D2704/pKD130A (Figure 2B, entry 1). Surprisingly, while KAD29B/pKD130A/pKAD72A (Figure 2A, entry 3) and D2704/pKD136/pKAD50 (Figure 2A, entry 2) accumulated comparable concentrations of common pathway intermediates, D2704/pKD136/pKAD50 (Figure 2B, entry 2) synthesized 12.4 ± 2.4 mM L-phenylalanine and phenyllactic acid, considerably higher end product output than KAD29B/pKD130A/pKAD72A (Figure 2B, entry 3).

The same experiment was performed with KAD29B/pKD130A/pKAD77A to determine if changing the number of copies of the cassette in the organism would significantly impact intermediate accumulation and end product synthesis. Using identical culture conditions, the pH of the medium for KAD29B/pKD130A/pKAD77A fell to approximately 5.0 during the initial 24 h in M9 medium and resulted in a significant decrease in the concentration of common pathway intermediates and end products that were synthesized. The presence of significant glucose concentrations remaining in the supernatants after 24 h in M9 suggested that a rapid decrease in pH resulted in reduced cell viability. To overcome this problem, the pH of the culture was carefully monitored and maintained in the range of pH 6.8−7.0 by periodic addition of sodium hydroxide.

Under pH-controlled conditions, KAD29B/pKD130A/pKAD77A (Figure 2A, entry 4) accumulated 1.5 mM DHS. DAH, shikimic acid, and shikimate 3-phosphate were not detected in the supernatant. The major product synthesized by KAD29B/pKD130A/pKAD77A was prephenic acid rather than L-phenylalanine and phenyllactic acid. This was likely due to the chemical stability of prephenic acid in neutral solutions.[28] After 48 h in M9, KAD29B/pKD130A/pKAD77A (Figure 2B, entry 4) synthesized 12.4 ± 1.4 mM combined concentration of prephenic acid, L-phenylalanine, and phenyllactic acid. Localization of the cassette onto low-copy vector pCL1920 thus resulted in concentrations of end product comparable to those produced by D2704/pKD136/pKAD50 (Figure 2B, entry 2).

Chromosomal Integration of the *P_{tac}aroAaroCaroBkan^R* Cassette. Recombination of the *P_{tac}aroAaroCaroBkan^R* cassette into the KAD29B chromosome utilized integration vector pMAK705 which contains a temperature-sensitive pSC101 replicon.[29] To improve the chance for recombination into a specific chromosomal locus, the cassette was flanked by a nucleotide sequence corresponding to *serA* which encodes 3-phosphoglycerate dehydrogenase, an enzyme necessary for

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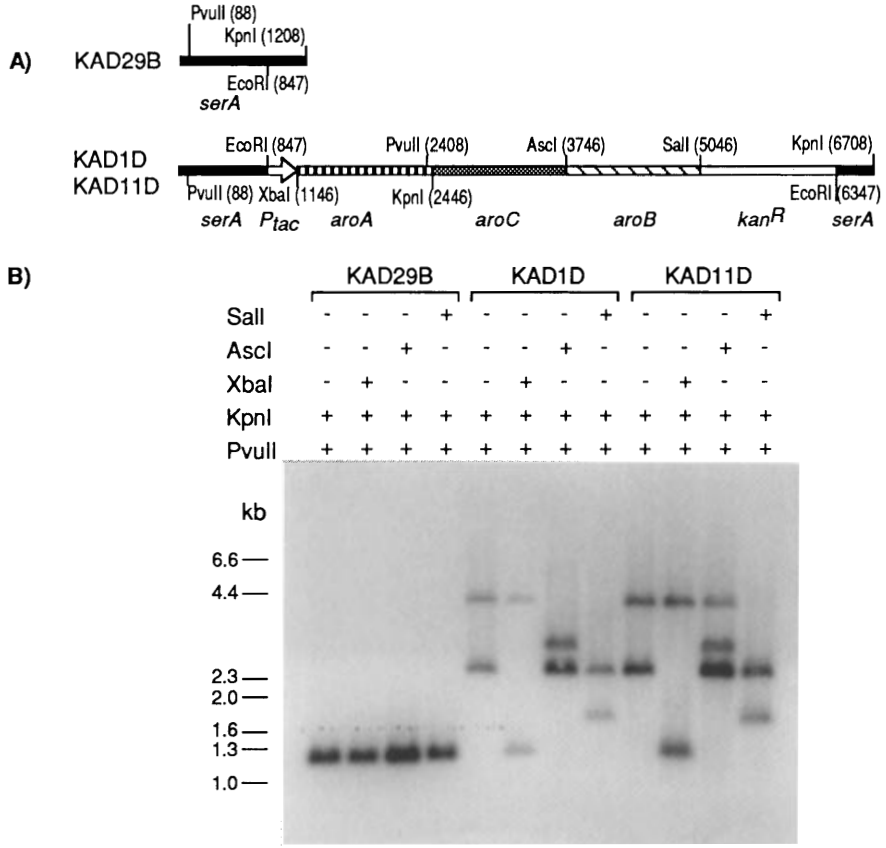

Figure 3. (A) Schematic representation and (B) Southern analysis of the *serA* locus of strains KAD29B, KAD1D, and KAD11D. Genomic DNA isolated from KAD29B, KAD1D, and KAD11D was digested with the indicated restriction enzymes. Reactions were electrophoresed (0.7% agarose), transferred to a nylon membrane, and hybridized to ^{32}P-labeled probe prepared by random primer extension of the 1.1 kb *Pvu*II−*Kpn*I *serA* fragment.

serine biosynthesis.[30] Excision of the *serA* gene from pKAD63 as a 1.9 kb *Sph*I fragment was followed by its insertion into the *Sph*I site of pMAK705. Subsequent insertion of the P_{tac}*aroAaroCaroBkan*^{R} cassette into an *Eco*RI site located within *serA* afforded pKAD80A (Table 2).

Transformation of KAD29B with pKAD80A was followed by several rounds of temperature-dependent selection for two recombinant events.[29,31] KAD1D and KAD11D were isolated as a consequence of their resistance to kanamycin and sensitivity to chloramphenicol. Neither KAD1D nor KAD11D was capable of growth on M9 plates containing L-phenylalanine, L-tryptophan, and *p*-hydroxyphenylpyruvic acid although addition of serine to plates containing the aforementioned aromatic supplementation resulted in growth of both isolates. Confirmation that the full cassette was inserted into the *serA* locus of KAD29B also relied on Southern blot analysis using radiolabeled *serA* as probe (Figure 3). Bands obtained on the Southern blot correlated to the bands expected from insertion of the entire P_{tac}*aroAaroCaroBkan*^{R} cassette into the *serA* locus. An additional band detected in the lane corresponding to digestion of KAD11D DNA with *Pvu*II, *Asc*I, and *Kpn*I was indicative of incomplete digestion of the genomic DNA.

Impact of Genome-Localized Cassette on Metabolite Accumulation. KAD1D and KAD11D were each transformed with pKD130A. As described for KAD29B/pKD136/pKAD77A, both KAD1D/pKD130A and KAD11D/pKD130A were cultured in M9 medium under pH-controlled conditions. KAD1D/pKD130A (Figure 2A, entry 5) accumulated 1.0 mM DHS while KAD11D/pKD130A (Figure 2A, entry 6) accumulated 1.5 mM DHS. No other common pathway intermediates were detected in the supernatants of either organism. After 48 h in M9 medium, KAD1D/pKD130A (Figure 2B, entry 5) synthesized 9.4 ± 0.8 mM combined concentration of prephenic acid, L-phenylalanine, and phenyllactic acid, whereas KAD11D/pKD130A (Figure 2B, entry 6) synthesized 10.9 ± 0.2 mM of these same products. The concentrations of end products synthesized by both KAD1D/pKD130A and KAD11D/pKD130A are statistically equivalent to the concentrations of end products synthesized by D2704/pKD136/pKAD50 (Figure 2B, entry 2) in which plasmid-based genes were utilized to increase rate-limiting enzyme specific activities.

Table 3. Ratios of the Specific Activities of Amplified Common Pathway Enzymes Relative to the Specific Activities of Unamplified Common Pathway Enzymes in D2704

strain	DHQ synthase	shikimate kinase	EPSP synthase	chorismate synthase
D2704[a]	1.0	1.0	1.0	1.0
D2704/pKD136/pKAD50	2.0	41	8.3	4.2
KAD29B/pKD130A/pKAD77A	12	4.0	12	25
KAD1D/pKD130A	1.7	4.1	2.4	3.1
KAD1D	3.6	6.5	11	6.5

[a] D2704 enzyme specific activities (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 1 μmol of product formed/min.

Rate-Limiting Enzyme Specific Activities. Specific activities for rate-limiting enzymes DHQ synthase, shikimate kinase, EPSP synthase, and chorismate synthase were measured (Table 3) for strains that accumulated decreased concentrations of common pathway intermediates and increased synthesis of end products. Enzyme specific activities were also measured for

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KAD1D, and activities are reported relative to D2704. Specific activities obtained for D2704/pKD136/pKAD50 and KAD1D/pKD130A provided an interesting comparison of the plasmid-based and genome-based approach for modification of rate-limiting specific activities. The most significant difference in enzyme specific activity between these two microbes was a 10-fold decrease in shikimate kinase in KAD1D/pKD130A relative to D2704/pKD136/pKAD50. Although introduction of the *tyrR* mutation into D2704 was earlier found to increase shikimate kinase specific activities by 50-fold in KAD29B, KAD1D/pKD130A expressed only 4.1-fold higher shikimate kinase activity relative to D2704. Similarly, KAD29B/pKD130A/pKAD77A which also utilizes the *tyrR* mutation to derepress *aroL* transcription contained only 4.0-fold higher shikimate kinase specific activity relative to D2704. Apparently the metabolic burden resulting from expression of the P_{tac}*aroA-aroCaroBkan*^{R} cassette significantly reduces transcription of *aroL* from the bacterial chromosome.

Impact of Plasmid-Localized *aroL* on Metabolite Accumulation. In order to increase shikimate kinase specific activity above the level obtained in KAD1D/pKD130A, an additional copy of the *aroL* gene was introduced onto pKD130A. Plasmid pKAD66 was prepared which contained DNA fragments encoding *tktA*, *aroF*, and *aroL*. After 24 h of culturing KAD1D/pKAD66 in M9 medium, no common pathway intermediates were detected in the supernatant (Figure 2A, entry 7). This provided the first instance of complete removal of all four rate-limiting common pathway enzymes. After 48 h in M9 medium, KAD1D/pKAD66 (Figure 2B, entry 7) synthesized 8.8 $\pm$ 0.4 mM combined concentration of prephenic acid, L-phenylalanine, and phenyllactic acid.

Discussion

Metabolite Accumulation. Metabolites observed to accumulate in culture supernatants were once again used to identify and gauge progress toward removal of impediments to carbon flow caused by rate-limiting enzymes in the common pathway.[6,7] The relationship between an accumulating metabolite and a rate-limiting enzyme can be a direct one as in the case of DAH, the dephosphorylated substrate of DHQ synthase, and shikimic acid, the substrate of shikimate kinase. Increasing the expression levels of DHQ synthase completely eliminates DAH accumulation while amplified expression of shikimate kinase leads to sizable reductions in shikimic acid concentrations in the culture supernatant.

The relationship between an accumulating metabolite and a rate-limiting enzyme can also be less straightforward as demonstrated by DHS and shikimate 3-phosphate accumulation. DHS accumulation correctly implicates shikimate dehydrogenase as a rate-limiting enzyme. However, the best way of removing this impediment to carbon flow is by amplified expression of shikimate kinase, which is situated immediately after shikimate dehydrogenase in the common pathway (Scheme 1). This curious solution to a rate-limiting enzyme's in vivo activity reflects the sensitivity of shikimate dehydrogenase to feedback inhibition by shikimic acid.[6] Reductions in shikimic acid concentrations realized with increased shikimate kinase expression levels eliminate feedback inhibition of shikimate dehydrogenase, which in turn, reduces DHS accumulation.

Shikimate 3-phosphate accumulation provides an example where only a single metabolite increases in concentration as a result of two rate-limiting enzyme activities. A portion of shikimate 3-phosphate accumulation is caused by the rate-limiting character of EPSP synthase. However, rate-limiting chorismate synthase also contributes to the concentration of shikimate 3-phosphate found in culture supernatants. This follows from EPSP synthase-catalyzed reaction of EPSP with inorganic phosphate.[32] As a result, EPSP accumulation caused by rate-limiting chorismate synthase activity is converted by EPSP synthase back into shikimate 3-phosphate.

End Product Accumulation. Increased concentrations of common pathway end products was the final criterion used for successful removal of impediments to carbon flow. Synthesis of prephenic acid, in addition to L-phenylalanine and racemic phenyllactic acid, was observed during expression of either the plasmid-localized or genome-localized synthetic cassette. However, L-phenylalanine and racemic phenyllactic acid were the only aromatic end products that required quantitation during previous experiments employing plasmid-encoded genes to amplify expression of rate-limiting common pathway enzymes. This difference in end product accumulation reflects an experimental modification. Culture supernatants were not maintained at pH 7 during previous experiments, while the culture supernatants of D2704/pKD130A carrying either the plasmid-localized or genome-localized synthetic cassette required maintenance of neutral solution pH. In lieu of base addition to maintain pH 7, acidification of culture supernatants resulted in cessation of aromatic biosynthesis.

The end products synthesized by D2704 and the dependence of product matrices on solution pH reflect the unique combination of mutations which this microbial host carries.[10] A deletion mutation (Δ*trpC*$-$*E*) eliminates conversion of chorismic acid to anthranilic acid while mutations in the *pheA* and *tyrA* loci remove both chorismate mutase isozymes that catalyze the conversion of chorismic acid to prephenic acid (Scheme 1). The *pheA* and *tyrA* mutations also eliminate, respectively, enzymatic decarboxylation/dehydration of prephenic acid to phenylpyruvic acid and enzymatic decarboxylation/dehydrogenation of prephenic acid to *p*-hydroxyphenylpyruvic acid (Scheme 1). Although D2704 might be anticipated to synthesize chorismic acid, this final metabolite of the common pathway undergoes a rapid nonenzymatic rearrangement to prephenic acid.[33] Prephenic acid is observed to accumulate along with phenyllactic acid and L-phenylalanine approximately 24 h after resuspension of the biocatalyst in glucose-containing, M9 minimal salts culture medium.

When the pH of the minimal salts solution is not maintained near neutrality, the culture becomes acidic and prephenic acid is not detected in the culture medium by 48 h. Nonenzymatic decarboxylation/dehydration of synthesized prephenic acid results in formation of phenylpyruvic acid,[34] which subsequently partitions between transaminase-catalyzed conversion to L-phenylalanine and reduction to phenyllactic acid. This reduction is likely catalyzed by D- and L-lactate dehydrogenase.[35] Presumably phenylpyruvic acid formed in the culture supernatant is transported back into the D2704 cytosol, where enzyme-catalyzed reduction or transamination occurs. Alternatively, maintenance of the culture medium at pH 7 by periodic addition of base results in significant concentrations of prephenic acid remaining in the culture medium at 48 h. When the culture supernatant is maintained at neutral pH, decarboxylation/dehydration is retarded. This results in the need to include the concentration of prephenic acid along with L-phenylalanine and phenyllactic acid concentrations when quantitating end product accumulation.

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Redesign of the Chromosome. Two approaches were explored for amplifying expression of the four rate-limiting enzymes from the chromosome of D2704. Homologous recombination of a single cassette containing the *aroA*, *aroC*, and *aroB* loci increased the copy number of each gene in the chromosome to 2. Inclusion of the strong *tac* promoter sequence[19] upstream from the genes was intended to promote adequate transcription from the cassette. Enzyme specific activities determined for KAD1D containing no plasmids revealed that localization of the cassette in the D2704 genome afforded from 3.6- to 11-fold higher activity for the three enzymes. Following transformation of KAD1D with pKD130A, all three enzyme activities were reduced at least 2-fold. This reduction in activity was likely a reflection of the metabolic burden resulting from overexpression of plasmid-encoded *tktA* and *aroF*.

Examination of KAD1D/pKD130A and D2704/pKD136/pKAD50 provides a direct comparison of genome-encoded and plasmid-encoded *aroA*, *aroC*, and *aroB*. Similar specific activities for DHQ synthase and chorismate synthase were obtained in both organisms, although KAD1D/pKD130A expressed only approximately one-third the EPSP synthase as was found in D2704/pKD136/pKAD50. Since shikimate 3-phosphate was not detected in the culture medium of KAD1D/pKD130A, the 2.4-fold increase in EPSP synthase activity in this microbe relative to D2704 was apparently sufficient to eliminate the rate limitation at this step of the pathway.

The second approach explored for amplifying expression of a rate-limiting enzyme relied on introduction of a *tyrR* mutation in the D2704 genome. Derepression of *aroL* transcription mediated by *tyrR* resulted in 40−50-fold increases in shikimate kinase specific activities in KAD27C and KAD29B relative to D2704. Shikimate kinase overexpression in KAD29B/pKD136 eliminated accumulation of both DHS and shikimic acid which was characteristic of D2704/pKD136. Unfortunately, metabolic burden resulting from overexpression of the *aroA*-, *aroC*-, and *aroB*-encoding cassette severely limited shikimate kinase amplification. KAD1D/pKD130A, in which the cassette was genome-localized, and KAD29B/pKD136/pKAD77A, in which the cassette was plasmid-localized, possessed only 4-fold higher shikimate kinase specific activity relative to D2704. KAD1D/pKD130A, KAD11D/pKD130A, and KAD29B/pKD130A/pKAD77A accumulated from 1.0 to 1.5 mM DHS in their respective cultures. While none of these strains exported shikimic acid to the medium, it is likely that increased intracellular shikimic acid concentrations resulting from rate-limiting shikimate kinase activity caused DHS to be exported to the medium. This is due to feedback inhibition of the *E. coli* shikimate dehydrogenase by shikimic acid. Localization of an additional copy of *aroL* on *tktA*- and *aroF*-encoding pKD130A afforded pKAD66. No common pathway intermediates were detected in the culture medium of KAD1D/pKAD66.

While shikimate kinase amplification requires additional modification, the rate-limiting character of DHQ synthase, EPSP synthase, and chorismate synthase was alleviated by integration of the cassette into the D2704 chromosome. KAD1D/pKD130A and KAD11D/pKD130A achieved combined concentrations of end products prephenic acid, L-phenylalanine, and phenyllactic acid that were comparable to those achieved with D2704/pKD136/pKAD50. Significantly reduced standard deviations for KAD1D/pKD130A and KAD11D/pKD130A may be indicative of improved biocatalyst stability.

It is useful to compare the percent conversion of glucose into aromatic end products catalyzed by KAD11D/pKD130A with the theoretical maximum yield that is possible for such a bioconversion. Since each molecule of prephenic acid and phenyllactic acid can conceivably be converted into a molecule of L-phenylalanine given appropriate modification of the biocatalytic organism, the individual yields of these molecules can be summed to provide an aromatic end product yield. The theoretical maximum percent conversion of glucose into L-phenylalanine is 30%.[36] KAD11D/pKD130A achieved a 20% conversion of glucose into aromatic end products. For comparison, only a 10% conversion of glucose into aromatic end products is realized with D2704/pKD130A which has not been genomically modified to remove impediments to carbon flow through the common pathway.

In future experiments, it will be intriguing to examine yields in constructs optimized for synthesis of L-phenylalanine which express adequate chorismate mutase, prephenate dehydratase, and aromatic transaminase activities along with feedback-resistant DAHP synthase activity.[2d,e] Carbon flow into the common pathway has also recently been increased beyond that achievable with amplified expression of DAHP synthase and transketolase by simultaneous amplified expression of PEP synthase.[4] The methods employed to introduce the genomic modifications made in KAD1D and KAD11D can be readily applied to these constructs to remove impediments to carbon flow through the common pathway. From this perspective, the value of KAD1D and KAD11D is not just the demonstration that genomic modifications can be used to remove impediments to carbon flow. These constructs are of equal importance as a consequence of the ease with which the methods used to introduce the genomic changes can be applied to other biocatalysts.

Experimental Section

General Methods. Porcine heart diaphorase, pyruvate kinase, and lactate dehydrogenase were purchased from Sigma. The sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid was purchased from Lancaster. [^{14}C]Shikimic acid was obtained from New England Nuclear. Protein assay solution was purchased from Bio-Rad. DEAE cellulose disks (DE-81) were obtained from Whatman. Phosphoenolpyruvate was prepared as described by Hirschbein.[37] DAHP was synthesized from methyl (methyl 3-deoxy-D-*arabino*-heptulopyranosid)-onate according to the procedure of Frost and Knowles.[38] DAH was synthesized using *E. coli* BJ502*aroB*/pKD130A[3a] and was subsequently purified and converted to methyl (methyl 3-deoxy-D-*arabino*-heptulopyranosid)onate as described by Reimer.[39] EPSP was synthesized enzymatically from PEP and sodium shikimate 3-phosphate.[40] EPSP synthase was partially purified from DH5α/pKAD46A[40b] as previously described. Sodium shikimate 3-phosphate was isolated from a bacterial culture supernatant as described by Dell.[6a]

Bacterial Strains and Media. Bacterial strains and plasmids used in this study are listed in Table 1. All solutions were prepared in distilled, deionized water. LB medium[41] contained tryptone (10 g), yeast extract (5 g), and NaCl (10 g) in 1 L of H_2O. M9 salts contained Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NaCl (0.5 g), and NH_4Cl (1 g) in 1 L of H_2O. M9 medium[41] contained glucose (10 g), $MgSO_4$ (0.12 g), and thiamine (1 mg) in 1 L of M9 salts. Antibiotics were added where appropriate to the following final concentrations: chloramphenicol (Cm), 20 μg/mL; ampicillin (Ap), 50 μg/mL; kanamycin (Kan), 50 μg/mL; and spectinomycin (Sp), 50 μg/mL. Isopropyl β-D-thiogalac-

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topyranoside (IPTG) was added to appropriate cultures to a final concentration of 0.2 mM. L-Phenylalanine, L-tryptophan, L-tyrosine, *p*-hydroxyphenylpyruvic acid, and serine were added to M9 medium as indicated to a final concentration of 40 mg/L. Solid medium was prepared by addition of 1.5% (w/v) Difco agar to LB and 1.5% (w/v) agarose (Sigma, Type II, EEO) to M9 medium.

Genetic Manipulations. Standard procedures were used for construction, purification, and analysis of plasmid DNA.[42] *E. coli* DH5α served as the host strain for all plasmid constructions.

Transduction of the *tyrR* locus via P1 phage from JB5[44] into D2704[10] proceeded as described by Miller.[41] Following the transduction, cells were washed twice with 10 mM Tris-Cl, pH 7.4, 1 mM $MgSO_4$ before resuspension in 10 mM Tris-Cl, pH 7.4, 1 mM $MgSO_4$, 100 mM sodium citrate. Cells were spread onto M9 medium containing L-phenylalanine, L-tryptophan, *p*-hydroxyphenylpyruvic acid and 80 μM *m*-fluorotyrosine. After incubation for 48 h at 37 °C, eight colonies that came up were streaked onto LB to afford single colonies and subsequently plated onto M9 medium containing L-phenylalanine, L-tryptophan, *p*-hydroxyphenylpyruvic acid, and 120 μM *m*-fluorotyrosine. KAD27C and KAD29B were identified which possessed the following growth characteristics: growth on M9 containing L-phenylalanine, L-tryptophan, and *p*-hydroxyphenylpyruvic acid; growth on M9 containing L-phenylalanine, L-tryptophan, *p*-hydroxyphenylpyruvic acid, and *m*-fluorotyrosine; growth on M9 containing L-tyrosine and L-tryptophan; no growth on M9 containing L-phenylalanine and L-tryptophan; and no growth on M9 containing L-phenylalanine and L-tyrosine.

PCR amplifications were carried out as described by Sambrook.[42] Each reaction (0.1 mL) contained 10 mM KCl, 20 mM Tris-Cl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, dATP (0.2 mM), dCTP (0.2 mM), dGTP (0.2 mM), dTTP (0.2 mM), template DNA, 0.5 μM of each primer, and 2 units of Vent polymerase. Initial template concentrations varied from 0.01 μg to 0.7 μg.

The *aroA* gene was amplified by PCR from pKD501[18] as a 1329 bp fragment. The resulting *aroA* fragment contained no promoter sequence upstream from the gene and required placement behind a promoter for expression of EPSP synthase. Inclusion of *Xba*I and *Kpn*I recognition sequences at the 5′- and 3′-ends, respectively, of the gene fragment facilitated further cloning. Localization of the resulting 1.3 kb *Xba*I−*Kpn*I fragment into pSU19 afforded pKAD69. Vectors pSU18 and pSU19 encode resistance to chloramphenicol, the *lac* promoter sequence (P_{lac}), and a p15A origin of replication.[24] pSU18 and pSU19 differ in the orientation of the pUC18 multiple cloning site with respect to P_{lac}.

PCR primers for *aroC* amplification were designed to include two putative promoter regions located upstream of the gene. Amplification of *aroC* from a 1.7 kb *Eco*RI−*Sal*I fragment isolated from pGM602[43] yielded a 1341 bp fragment. *Kpn*I and *Asc*I recognition sequences were included at the 5′- and 3′-ends, respectively, of the amplified *aroC* fragment. Localization of the amplified *aroC* fragment as a *Kpn*I−blunt ended fragment in *Kpn*I and *Sma*I-digested pBLSCR SK+[25] afforded pKAD70.

The *aroB* gene was amplified with its native promoter by PCR from pJB14[44] to yield a 1343 bp product. *Asc*I and *Sal*I recognition sequences were included at the 5′- and 3′-ends, respectively, of the *aroB* fragment. Inability to digest the *Sal*I site at the end of the amplified fragment necessitated the following cloning strategy. The 1.3 kb *aroB* PCR fragment was digested with *Asc*I and subsequently ligated to the *Kpn*I−*Asc*I *aroC* fragment isolated from pKAD70. Ligation of the resulting 2.6 kb *Kpn*I−blunt ended *aroCaroB* fragment into *Kpn*I and *Sma*I-digested pBLSCR SK+ afforded pKAD68. Transformation of aromatic auxotrophs AB2849 and AB2847[26] with pKAD68 restored their ability to grow on M9 agarose in the absence of L-phenylalanine, L-tyrosine, and L-tryptophan supplementation. Similarly, transformation of AB2829[26] with pKAD69 restored the ability to grow on M9 in the absence of aromatic supplementation.

The kan^R gene[45] was obtained as a 1.3 kb fragment following digestion of pMB2190 with *Pst*I. The *Pst*I ends of the kan^R fragment were changed to *Sal*I and *Eco*RI recognition sequences at the 5′- and 3′-ends, respectively, via the following sequence. The *Eco*RI site of p*Trc*99A[46] was eliminated by digestion of the plasmid with *Eco*RI, treatment with mung bean nuclease, and subsequent religation of the blunt ends to afford p*Trc*99A-E. Reintroduction of an *Eco*RI site at the opposite end of the multiple cloning site resulted from digestion of p*Trc*99A-E with *Hin*dIII, treatment with mung bean nuclease, and attachment of *Eco*RI synthetic linkers to afford pKAD61. Subsequent localization of the kan^R *Pst*I fragment in pKAD61 resulted in pKAD62A. The kan^R fragment was now ready for excision from pKAD62A as a *Sal*I−*Eco*RI fragment.

Assembly of the P_{tac}*aroAaroCaroBkan*^{R} cassette utilized a stepwise approach. A 0.3 kb *Eco*RI−*Bam*HI fragment containing the *tac* promoter sequence (P_{tac}) was isolated from pDR540[47] and ligated into pSU18 to afford pKAD49. Subsequent digestion of pKAD49 with *Eco*RI and *Xba*I liberated the 0.3 kb P_{tac} sequence while digestion of pKAD69 with *Xba*I and *Kpn*I liberated the 1.3 kb *aroA* fragment. Ligation of these two fragments into pSU18 that was previously digested with *Eco*RI and *Kpn*I resulted in pKAD73 in which *aroA* was expressed from P_{tac}. Digestion of pKAD68 with *Kpn*I and *Sal*I liberated a 2.6 kb *aroCaroB* fragment while digestion of pKAD62A with *Sal*I and *Eco*RI liberated the kan^R fragment. Ligation of these two fragments into pSU18 that was previously digested with *Kpn*I and *Eco*RI resulted in pKAD74. Finally, digestion of pKAD73 with *Eco*RI and *Kpn*I liberated the P_{tac}*aroA* fragment while digestion of pKAD74 with the same enzymes afforded the *aroCaroBkan*^{R} fragment. Ligation of these two fragments into the *Eco*RI site of pSU18 afforded pKAD72A (Table 2). The orientation of the cassette localized in pKAD72A was such that transcription from P_{tac} and both the native promoters of *aroC* and *aroB* were in the same direction as the vector-encoded P_{lac}.

Plasmid pCL1920 is a low-copy vector that contains the pSC101 replicon, P_{lac}, and the gene responsible for spectinomycin resistance.[27] Digestion of pCL1920 with *Eco*RI followed by religation afforded pCL1920-lac in which the P_{lac} sequence and the multiple cloning site were removed from the vector. Localization of the 5.5 kb *Eco*RI fragment containing the P_{tac}*aroAaroCaroBkan*^{R} cassette in the *Eco*RI site of pCL1920-lac resulted in pKAD77A (Table 2).

Plasmid pMAK705[29] containing a temperature-sensitive pSC101 replicon facilitated recombination of the P_{tac}*aroAaroCaroBkan*^{R} cassette into the genome of KAD29B. Since plasmids derived from pMAK705 replicate at 30 °C but are unstable at 44 °C, isolation of all pMAK705 derivatives required culturing of cells at 30 °C. Localization of the *serA*[30] gene in pMAK705 followed by insertion of the cassette into an *Eco*RI site internal to the *serA* gene directed recombination of the cassette into the *serA* locus of the genome. Digestion of pD2625 with *Eco*RV and *Dra*I liberated a 1.9 kb *serA* fragment that was subsequently ligated into the *Sma*I site of p34E[48] to afford pKAD63. Digestion of pKAD63 with *Sph*I released the 1.9 kb *serA* fragment, which was then inserted into the *Sph*I site of pMAK705 to yield pKAD76A. Insertion of the P_{tac}*aroAaroCaroBkan*^{R} cassette into the *Eco*RI site of *serA* was complicated by two additional *Eco*RI sites in pKAD76A. Following partial digestion of pKAD76A with *Eco*RI, the resulting DNA fragments were resolved on an agarose gel and the 7.4 kb fragment corresponding to the linearized plasmid was isolated. Ligation of the linearized plasmid to the 5.5 kb *Eco*RI fragment containing the cassette afforded pKAD80A (Table 2).

Conditions for homologous recombination of the cassette were based on those previously described.[29,31] Competent KAD29B was transformed with pKAD80A. Following heat-shock treatment, cells were incubated in LB at 44 °C for 1 h and subsequently plated onto LB containing Cm and Kan. Plates were incubated at 44 °C for approximately 24 h before colonies appeared. The resulting cointegrates were inoculated into 5 mL of LB containing no antibiotics, and the cells were grown at 30 °C for 12 h to allow excision of the plasmid from the genome. Cultures were diluted (1:20000) in LB without antibiotics, and two more cycles of growth at 30 °C for 12 h were carried out to enrich cultures for more rapidly growing cells that had lost the temperature-sensitive replicon. Cultures were then diluted (1:

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20000) into LB and grown at 44 °C for 12 h to promote plasmid loss from the cells. Serial dilutions of each culture were spread onto LB plates containing Kan and incubated at 44 °C overnight to select for cells which had retained the *kan*^{R} locus in the genome. The resulting colonies were further screened on LB containing Cm at 44 °C to identify colonies which had lost Cm resistance. Both KAD1D and KAD11D were *Kan*^{R} and *Cm*^{S} and displayed the following growth characteristics: growth on M9 containing L-phenylalanine, L-tryptophan, *p*-hydroxyphenylpyruvic acid, and serine; and no growth on M9 containing L-phenylalanine, L-tryptophan, and *p*-hydroxyphenylpyruvic acid.

Preparation of pKAD66A began with linearization of pKAD31 with *Kpn*I. Subsequent treatment of the product with mung bean nuclease eliminated the 5′-overhangs and further digestion with *Bam*HI liberated the 1.0 kb *Bam*HI−blunt end *aroL* fragment. Digestion of pSU18 with *Xba*I followed by treatment with mung bean nuclease and digestion with *Bam*HI resulted in pSU18 possessing *Bam*HI and blunt ends. Ligation of the *Bam*HI−blunt end *aroL* fragment into the resulting vector afforded pKAD64. Digestion of pKAD64 with *Bam*HI and *Sph*I liberated the 1.0 kb *aroL* fragment, which was subsequently ligated into *Bam*HI- and *Sph*I-digested pKD116B to yield pKAD65. pKD116B[3a] is a pBR325-derived plasmid[49] that contains an *aroF*-encoding fragment. Ligation of a 5.0 kb *tktA Bam*HI fragment isolated from pKD136[7] into the *Bam*HI site of pKAD65 afforded pKAD66A.

Genomic DNA was isolated as described by Silhavy.[50] DNA probes were labeled with [α-^{32}P]dCTP using the random-primer method of Feinberg and Vogelstein.[51] Southern blot hydridizations were performed as described by Sambrook.[42]

Culture Conditions. Bacterial strains were grown as follows for analysis of common pathway intermediate accumulation and product synthesis. One liter of LB (4 L Erlenmeyer flask) containing the appropriate antibiotics and IPTG was inoculated with 5 mL of a culture grown overnight in LB. Cultures were grown at 37 °C in a gyratory shaker at 250 rpm for 12 h. Cells were collected by centrifugation at 4000*g* and were washed three times with 300 mL portions of M9 salts before being resuspended in 1 L of M9 medium (4 L Erlenmeyer flask) containing the appropriate antibiotics and IPTG. Cultures were returned to 37 °C in the gyratory shaker at 250 rpm. Cultures that required pH adjustment were monitored periodically during the initial 24 h in M9 medium. Sodium hydroxide (5 N) was added to readjust the pH to 7.0.

Analysis of Culture Supernatants by ^{1}H NMR. Samples (25 mL) of culture supernatant were taken at the indicated intervals, and cells were removed by centrifugation. Following neutralization of an aliquot (10 mL) of the culture supernatant with 2 N NaOH, the solution was concentrated to dryness using a rotary evaporator. The residue was redissolved in D_2O and concentrated to dryness (two times). The residue was then redissolved in D_2O containing a known concentration of the sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (TSP). The ^{1}H NMR spectrum was recorded on a Varian Gemini-200 spectrometer at 200 MHz. Concentrations of cellular metabolites in the supernatant were determined by comparison of the integrals of known metabolite resonances to the resonance corresponding to TSP in the ^{1}H NMR. Cultures were grown in triplicate to establish mean values and standard deviations.

Enzyme Assays. Enzymes were assayed following growth of cells in LB for 12 h as described under culture conditions. Cells were collected by centrifugation and disrupted by two passages through a French press (16 000 psi). Cellular debris was removed by centrifugation at 48000*g* for 20 min. Protein concentrations were determined using the Bradford dye-binding procedure.[52] Protein concentrations were determined by comparison to a standard curve prepared using bovine serum albumin.

DHQ synthase activity was measured by following the disappearance of DAHP over time.[44] DAHP concentrations were determined using the thiobarbituric acid assay.[53] Shikimate kinase activity was determined by monitoring formation of [^{14}C]shikimate 3-phosphate from [^{14}C]shikimic acid as described by De Feyter.[54] EPSP synthase was measured as described by Boocock[32] by coupling the release of PEP from the reverse reaction to the pyruvate kinase and lactate dehydrogenase reactions. Oxidation of NADH was monitored at 340 nm. Chorismate synthase was assayed as described by Morell[55] by monitoring formation of phenylpyruvic acid at 320 nm using chorismate mutase and prephenate dehydratase as coupling enzymes. A crude cellular lysate of AB2849/pKAD52 was used for chorismate mutase and prephenate dehydratase activity. AB2849/pKAD52 cells were grown in LB, harvested by centrifugation, and washed in 0.9% NaCl. The cells were then resuspended in 100 mM KH_2PO_4, pH 7.0, and disrupted by two passages through a French pressure cell (16 000 psi). Cellular debris was removed by centrifugation at 48000*g* for 20 min. The chorismate mutase and prephenate dehydratase extract was stored at −20 °C and maintained sufficient activity for at least 1 month.

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