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Published on Web 04/24/2007

Directed Evolution of 2-Keto-3-deoxy-6-phosphogalactonate Aldolase To Replace 3-Deoxy-D-*arabino*-heptulosonic Acid 7-Phosphate Synthase

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Abstract: Directed evolution of 2-keto-3-deoxy-6-phosphogalactonate (KDPGal) aldolase for microbial synthesis of shikimate pathway products provides an alternate strategy to circumvent the competition for phosphoenolpyruvate between 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) synthase and the phosphoenolpyruvate:carbohydrate phosphotransferase system in *Escherichia coli*. *E. coli* KDPGal aldolase was evolved using a combination of error-prone polymerase chain reaction, DNA shuffling, and multiple-site-directed mutagenesis to afford KDPGal aldolase variant NR8.276-2, which exhibits a 60-fold improvement in the ratio k_{cat}/K_M relative to that of wild-type *E. coli* KDPGal aldolase in catalyzing the addition of pyruvate to D-erythrose 4-phosphate to form DAHP. On the basis of its nucleotide sequence, NR8.276-2 contains seven amino acid changes from the wild-type *E. coli* KDPGal aldolase. Amplified expression of NR8.276-2 in the DAHP synthase and shikimate dehydrogenase-deficient *E. coli* strain NR7 under fed-batch fermentor-controlled cultivation conditions resulted in synthesis of 13 g/L 3-dehydroshikimic acid in 6.5% molar yield from glucose. Increased coexpression of the irreversible downstream enzyme 3-dehydroquinate synthase increased production of 3-dehydroshikimic acid to 19 g/L in 9.7% molar yield from glucose. Coamplification with transketolase, which increases D-erythrose 4-phosphate availability, afforded 16 g/L 3-dehydroshikimic acid in 8.5% molar yield.

Introduction

The shikimate pathway starts with the stereospecific condensation of phosphoenolpyruvate and D-erythrose 4-phosphate to form 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) catalyzed by DAHP synthase (Scheme 1). Competition between DAHP synthase and the sugar-transporting phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) for cytoplasmic supplies of phosphoenolpyruvate limits the concentrations and yields of chemicals microbially synthesized via the shikimate pathway.[1] Strategies to alleviate this limitation examined previously focused on increasing phosphoenolpyruvate availability in *Escherichia coli*. Two methods that have been elaborated to increase phosphoenolpyruvate availability include overexpression of *ppsA*-encoded phosphoenolpyruvate synthase to recycle pyruvate back to phosphoenolpyruvate[1] and avoidance of the expenditure of phosphoenolpyruvate during glucose transport. The latter strategy was achieved by replacing the native *E. coli* PTS with *Zymomonas mobilis glf*-encoded glucose facilitator[2] or *E. coli galP*-encoded galactose permease.[3]

A new strategy to synthesize DAHP in which an enzyme is evolved to condense pyruvate with D-erythrose 4-phosphate, thereby avoiding consumption of phosphoenolpyruvate, was recently reported.[4] *E. coli dgoA*-encoded 2-keto-3-deoxy-6-phosphogalactonate (KDPGal) aldolase, which normally catalyzes the reversible cleavage of KDPGal to pyruvate and D-glyceraldehyde 3-phosphate, was evolved by directed evolution to catalyze a reversible condensation of pyruvate and D-erythrose 4-phosphate to form DAHP (Scheme 1). During *E. coli* PTS-mediated glucose transport, one molecule of pyruvate is generated from phosphoenolpyruvate for each molecule of glucose transported into the cytoplasm and phosphorylated to form glucose 6-phosphate.[5] Utilization of the PTS byproduct pyruvate as a substrate for KDPGal aldolase-catalyzed DAHP synthesis avoids competition for phosphoenolpyruvate with the PTS (Scheme 1).

In an extension of our preliminary studies, in this paper we describe the use of multiple-site-directed mutagenesis to further improve DAHP synthesizing activity of KDPGal aldolase.

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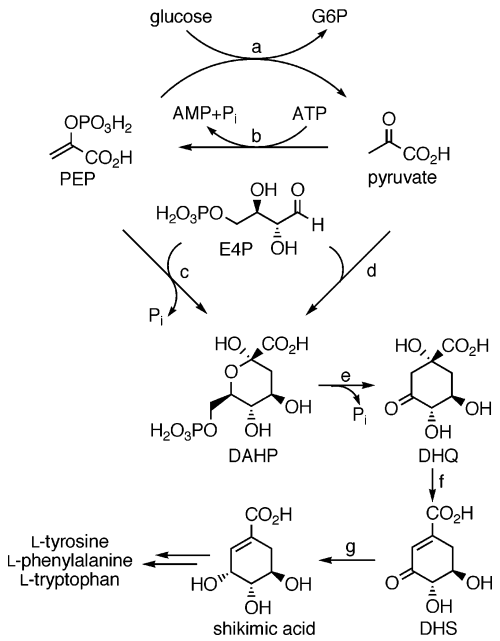
10.1021/ja067330p CCC: $37.00

Scheme 1. Shikimate Pathway Variants[a,b]



[a] Abbreviations: G6P, glucose 6-phosphate; E4P, D-erythrose 4-phosphate; PEP, phosphoenolpyruvate; DAHP, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate; DHQ, 3-dehydroquinic acid; DHS, 3-dehydroshikimic acid. [b]Enzymes and encoding genes: (a) PEP:carbohydrate phosphotransferase system (*ptsH*, *ptsI*, *crr*, *ptsG*); (b) phosphoenolpyruvate synthase (*ppsA*); (c) DAHP synthase (*aroF*, *aroG*, *aroH*); (d) KDPGal aldolase (*dgoA*); (e) 3-dehydroquinate synthase (*aroB*); (f) 3-dehydroquinate dehydratase (*aroD*); (g) shikimate dehydrogenase (*aroE*).

Extension of the research to include the application of evolved KDPGal aldolase for the synthesis of shikimate pathway intermediate 3-dehydroshikimic acid is also described. In addition, amplified expression of both 3-dehydroquinate synthase, an irreversible enzyme downstream from the evolved aldolase, and transketolase, a D-erythrose 4-phosphate-generating enzyme, was investigated for increasing both the titer and yield of 3-dehydroshikimic acid. The strategy described in this study to avoid the competition between the PTS and DAHP synthase for cellular phosphoenolpyruvic acid supplies may be applicable to synthesis of shikimic acid,[6] a starting material for the manufacture of the oral antiinfluenza agent Tamiflu,[7] as well as to the syntheses of a plethora of commodity, pseudocommodity, and fine chemicals derived from the shikimate pathway. These chemicals include adipic acid,[8] phenol,[9] hydroquinone,[10] catechol,[11] *p*-hydroxybenzoic acid,[12] vanillin,[13] indigo,[14] and gallic acid and pyrogallol.[15]

Table 1. Kinetic Parameters of Wild-Type KDPGal Aldolases and Evolved Mutants

entry	enzyme	K_M(E4P) (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}\ s^{-1}$)
1	wild-type *E. coli* DgoA	570	0.94	1.6×10^{-3}
2	wild-type *K. pneumoniae* DgoA	1500	1.4	9.3×10^{-4}
3	wild-type *S. typhimurium* DgoA	690	0.60	8.7×10^{-4}
4	EC03-1	120	2.5	2.1×10^{-2}
5	NR8.165-2	160	2.5	1.6×10^{-2}
6	NR8.165-4	120	4.8	4.0×10^{-2}
7	NR8.276-2	49	4.9	1.0×10^{-1}

Results

Directed Evolution of 2-Keto-3-deoxy-6-phosphogalactonate Aldolase. In a previous paper, subjection of wild-type *E. coli* KDPGal aldolase to two rounds of error-prone polymerase chain reaction (PCR) and one round of DNA shuffling was described.[4] A subset of the resulting 2-keto-3-deoxy-6-phosphogalactonate aldolase variants were capable of supporting growth of *E. coli* CB734,[4] a DAHP synthase-deficient strain, in minimal salt medium in the absence of L-tyrosine, L-phenylalanine, and L-tryptophan supplementation. The KDPGal aldolase mutant with the highest specific activity, designated EC03-1, carried six amino acid mutations (F33I, D58N, Q72H, A75V, V85A, V154F). The DAHP synthesizing specific activity of EC03-1 in crude lysate showed an 8-fold improvement relative to that of wild-type *E. coli* KDPGal aldolase.[4]

In the present study, the wild-type *E. coli* KDPGal aldolase and the evolved EC03-1 were expressed as N-terminal fusion proteins with glutathione *S*-transferase by cloning each gene into pGEX-4T-1. The resulting soluble fusion proteins were purified to homogeneity in one step on glutathione−Sepharose under nondenaturing conditions. The K_M for D-erythrose 4-phosphate and k_{cat} of the glutathione *S*-transferase-tagged KDPGal aldolases were determined by measuring DAHP synthesizing activity at various concentrations of D-erythrose 4-phosphate and a pyruvate concentration of 2 mM. The wild-type *E. coli* KDPGal aldolase showed a k_{cat} of 0.94 s^{-1} and a K_M of 570 μM for D-erythrose 4-phosphate (Table 1, entry 1). The KDPGal aldolase mutant EC03-1 showed a 2.5-fold increase in k_{cat} and a 5-fold decrease in K_M relative to those of the wild-type *E. coli* enzyme, leading to an improvement in k_{cat}/K_M of 13-fold (Table 1, entry 4 vs entry 1).

In addition to the KDPGal aldolase from *E. coli*, putative KDPGal aldolase orthologues have also been identified on the basis of sequence similarity searches using BLAST through the National Center for Biotechnology Information[16] and ERGO (Integrated Genomics, Chicago, IL)[17] databases. Among the aldolases identified, KDPGal aldolase activity of the putative KDPGal aldolases from *Klebsiella pneumoniae* has been determined.[4] The wild-type *K. pneumoniae* KDPGal aldolase and the putative *Salmonella typhimurium* KDPGal aldolase each have 80% DNA sequence identity with the wild-type *E. coli*

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enzyme, which is adequate sequence similarity for efficient DNA shuffling between the sequences.[18] The KDPGal aldolases from *K. pneumoniae* and *S. typhimurium* were expressed and purified as N-terminal glutathione *S*-transferase-tagged proteins. The wild-type *K. pneumoniae* KDPGal aldolase exhibits a higher k_{cat} of 1.4 s^{-1} and a larger K_M of 1500 μM for D-erythrose 4-phosphate compared with the wild-type *E. coli* KDPGal aldolase (Table 1, entry 2 vs entry 1). The DAHP synthesis activity of the putative *S. typhimurium* enzyme was confirmed and shows a lower k_{cat} of 0.60 s^{-1} and a larger K_M of 690 μM relative to those of the *E. coli* KDPGal aldolase (Table 1, entry 3 vs entry 1).

Wild-type *S. typhimurium dgoA* was subjected to two rounds of error-prone PCR followed by two rounds of DNA shuffling using a method described in the previous paper.[4] The *dgoA* mutants were then expressed in *E. coli* CB734 and selected for growth on glucose-containing minimal salt medium. Due to the inactivation of all three isozymes of DAHP synthases, *E. coli* CB734 is unable to biosynthesize its own L-tyrosine, L-phenylalanine, and L-tryptophan. The evolved *S. typhimurium dgoA* plasmid insert in *E. coli* CB734/pST04-5 allowed growth in the absence of aromatic amino acid supplementation after 72 h of incubation at 37 °C. To further increase KDPGal aldolase activity toward D-erythrose 4-phosphate, three KDPGal aldolase mutants, EC03-1 (F33I, D58N, Q72H, A75V, V85A, V154F),[4] KP03-3 (I10V, E71G, V85A, P106S, V154F, E187D, Q191H, F196I),[4] and ST04-5 (D20E, V28M, S42T, I89T, P150L, D178G), evolved from wild-type *E. coli*, *K. pneumoniae*, and *S. typhimurium dgoA*, respectively, were shuffled using a method of single-stranded DNA family shuffling.[19] Sequencing a library of 76 random mutants obtained from the DNA shuffling revealed a crossover rate of approximately 1.4 per *dgoA* gene (0.6 kb). The resulting chimeric *dgoA* hybrids were transformed into *E. coli* CB734 and selected for a faster growth rate on the selective medium plates. From a library of 3×10^5 transformants, no colonies were found which grew significantly faster than the control, *E. coli* CB734 expressing the KDPGal aldolase mutant EC03-1. Plasmids from two of the selected mutants, NR8.165-2 and NR8.165-4, were purified and sequenced. Mutant NR8.165-2 is a hybrid of *S. typhimurium* and *E. coli* KDPGal aldolase with one crossover in the first 60−80 base pair region. The sequence of NR8.165-4 contains segments from *E. coli*, *K. pneumoniae*, and *S. typhimurium* sequences with two crossovers located in the first 110−130 base pair region and 470−490 base pair region. The NR8.165-2 and NR8.165-4 were subsequently expressed as N-terminal fusion proteins with glutathione *S*-transferase and purified. Neither the k_{cat} nor K_M of NR8.165-2 and NR8.165-4 showed any significant improvement relative to those of the previously obtained *E. coli* KDPGal aldolase mutant EC03-1 (Table 1, entries 5 and 6 vs entry 4).

With the observation that error-prone PCR and DNA family shuffling did not provide KDPGal aldolase with an improved k_{cat}/K_M, a new strategy was employed using multiple-site-directed mutagenesis to further increase the DAHP synthesizing activity. In the absence of structural information from crystallographic studies, a homology model of wild-type *E. coli* KDPGal aldolase was constructed using Swiss-Model.[20] The

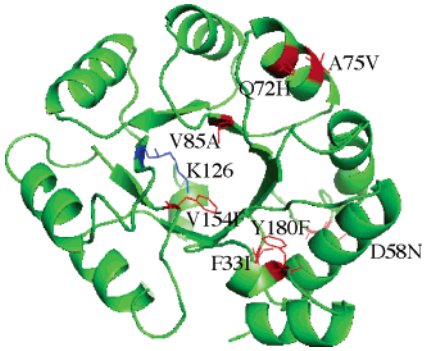

Figure 1. Structure of modeled *E. coli* wild-type KDPGal aldolase with amino acid mutations identified in the KDPGal aldolase mutant NR8.276-2.

protein structure was rendered with PyMOL.[21] Like other type I aldolases, the *E. coli* KDPGal aldolase appears to have an $(\alpha/\beta)_8$ barrel structure.[22] The *E. coli* KDPGal aldolase shows 25% amino acid sequence identity and 43% sequence similarity with *E. coli* 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase. A putative active site residue, Lys-126, in the *E. coli* KDPGal aldolase was identified on the basis of sequence alignment with the *E. coli* 2-keto-3-deoxy-6-phosphogluconate aldolase,[23] in which Lys-133 has been identified as the Schiff base-forming lysine residue.[22a,24] In KDPGal aldolase, Lys-126 is situated at the bottom of a funnel-shaped pocket created by the eight loops that connect the β-strains with the α-helices (Figure 1). Multiple-site-directed mutagenesis was employed to mutate 12 amino acid residues in the proximity of the putative active site of the *E. coli* KDPGal aldolase. Amino acid residues L61(*V*), I62(*V*), L68(*T*,M,P), T103(*V*,I,A), V104(*T*,I,A), C105(*S*,T,G), P106(*I*,T,L), C108(*F*), A173(*F*,V,S), L175(*V*), S177(*T*), and Y180(*F*,N,I) in the KDPGal aldolase mutant EC03-1 were mutated using an overlap extension method.[25] These amino acid residues were mutated to incorporate naturally occurring variants (shown in italics) found in other putative KDPGal aldolase orthologues identified using BLAST. Several nonnaturally occurring variants were also generated from the codon changes. Resulting mutants from the multiple-site-directed mutagenesis were cloned into vector pTrc99A, transformed into *E. coli* CB734, and spread onto selective medium plates. Mutant NR8.276-2 was identified from a library of 2×10^5 transformants that enabled *E. coli* CB734 to grow on the selective medium plate after 48 h of incubation at 37 °C, as compared to the 72 h incubation required for *E. coli* CB734 expressing the parental aldolase EC03-1. The mutant NR8.276-2 contains one amino acid mutation (Y180F) and four silent mutations (g519t, a529t, g530c, and c531g) relative to EC03-1. The distance between the mutated residue Y180 and the active site residue K126 is 11 Å on the basis of the homology model of the wild-type *E. coli* KDPGal aldolase. Compared with the wild-type *E. coli*

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Table 2. Synthesis of 3-Dehydroshikimic Acid by Various *E. coli* Constructs

entry	strain/plasmid	vector	genes in plasmid	DHS[a] (g/L)	DHS yield[b] (%)
1	NR7/pNR8.075	pTrc99A	P_{trc}*dgoA*$_{EC}$, *serA*	2.0	0.9
2	NR7/pNR8.288	pTrc99A	P_{trc}*NR8.276-2*, *serA*	13	6.5
3	NR7/pNR8.294	pTrc99A	P_{trc}*NR8.276-2*, *serA*, *aroB*	19	9.7
4	NR7/pNR9.056	pTrc99A	P_{trc}*NR8.276-2*, *serA*, P_{T5}*aroB*$_{orf}$	13	8.1
5	NR7/pNR8.286	pTrc99A	P_{trc}*NR8.276-2*, *serA*, *tktA*	16	8.5
6	NR7/pNR9.088	pTrc99A	P_{trc}*NR8.276-2*, *serA*, *aroB*, *tktA*	17	8.7
7	NR7/pNR9.127	pJF118HE	P_{tac}*NR8.276-2*, *serA*, *aroB*	16	7.4
8	NR7/pKL4.79	pJF118EH	P_{tac}*aroF*^{FBR}, *serA*	40	18

[a] Abbreviations: 3-dehydroshikimic acid (DHS), wild-type *E. coli* KDPGal aldolase (*dgoA*$_{EC}$). [b] Given as (moles of DHS)/(mole of glucose consumed) × 100.

KDPGal aldolase, the evolved mutant NR8.276-2 showed a 5-fold increase in k_{cat} and a 12-fold decrease in K_M, resulting in a 60-fold improvement in k_{cat}/K_M relative to that of the wild-type *E. coli* enzyme (Table 1, entry 7 vs entry 1). An additional round of error-prone PCR was carried out on the aldolase mutant NR8.276-2. No significant improvement was obtained from the variants produced by error-prone PCR.

Synthesis of 3-Dehydroshikimic Acid in *E. coli* Using the Evolved KDPGal Aldolase. *E. coli* NR7 was previously constructed from *E. coli* KL3 using site-specific chromosomal insertions to inactivate all three chromosomal DAHP synthase isozymes (Table 4).[4] *E. coli* KL3 synthesized 3-dehydroshikimic acid as a result of a mutation in the *aroE* locus encoding shikimate dehydrogenase. Synthesis of 3-dehydroshikimic acid by *E. coli* KL3 has been extensively examined and used as a measure of the amount of carbon flow directed into the shikimate pathway.[1b,3b]

Due to inactivation of the DAHP synthases, *E. coli* NR7/pNR8.288 (Table 5) relied exclusively on the expression of plasmid-localized KDPGal aldolase mutant NR8.276-2 for the synthesis of 3-dehydroshikimic acid. *E. coli* NR7 also contains a second copy of the *aroB* gene in its *serA* locus, which encodes 3-phosphoglycerate dehydrogenase. Inactivation of the NR7 *serA* gene eliminates de novo synthesis of L-serine. Therefore, plasmid pNR8.288 also contains the *serA* gene for plasmid maintenance during cultivation in minimal salt medium lacking L-serine supplementation. This strategy for plasmid maintenance has been successfully applied in previously reported microbial syntheses of 3-dehydroshikimic acid.[1b,3b,26] *E. coli* NR7/pNR8.288 was cultured on a 1 L scale in a 2 L working capacity fermentor at pH 7.0 and 36 °C in minimal salt medium with glucose as the carbon source and aromatic amino acid and aromatic vitamin supplementations.[1b] Glucose was maintained at a concentration range of 55−110 mM, and dissolved oxygen was maintained at 20% air saturation. Cultivation of *E. coli* NR7/pNR8.288 under these fermentor-controlled conditions resulted in synthesis of 13 g/L 3-dehydroshikimic acid in 6.5% (mol/mol) yield from glucose (Table 2, entry 2). When cultured under identical conditions, *E. coli* NR7/pNR8.075 expressing the wild-type *E. coli* KDPGal aldolase synthesized only 2 g/L 3-dehydroshikimic acid in 0.9% (mol/mol) yield (Table 2, entry 1).

Table 3. Specific Activities (U/mg) of KDPGal Aldolase (DAHP Synthesis), 3-Dehydroquinate Synthase, and DAHP Synthase in Various *E. coli* Constructs

		KDPGal aldolase measured for DAHP synthesis[a]			DHQ synthase[b]		
entry	strain/plasmid	24 h	36 h	48 h	24 h	36 h	48 h
1	NR7/pNR8.075	ND[c]	ND	ND	0.015	0.016	0.016
2	NR7/pNR8.288	0.18	0.24	0.21	0.017	0.017	0.016
3	NR7/pNR8.294	0.11	0.12	0.10	0.043	0.039	0.023
4	NR7/pNR9.056	0.18	0.29	0.41	0.53	0.92	1.17
5	NR7/pNR9.127	0.17	0.22	0.18	0.027	0.022	0.024

		DAHP synthase[d]			DHQ synthase[b]		
entry	strain/plasmid	24 h	36 h	48 h	24 h	36 h	48 h
6	NR7/pKL4.79	0.21	0.70	0.28	0.009	0.010	0.011

[a] One unit (U) of KDPGal aldolase (DAHP synthesis) corresponds to formation of 1 μmol of DAHP per minute at 25 °C when incubated with pyruvate and D-erythrose 4-phosphate. [b] One unit (U) of 3-dehydroquinate synthase corresponds to consumption of 1 μmol of DAHP per minute at 25 °C. [c] ND = not detectable. [d] One unit (U) of DAHP synthase corresponds to the formation of 1 μmol of DAHP from phosphoenolpyruvate and D-erythrose 4-phosphate per minute at 37 °C.

DAHP synthesizing specific activity and 3-dehydroquinate synthase specific activity were measured for cells removed from the fermentor 24, 36, and 48 h after inoculation of the culture medium (Table 3). The DAHP synthesizing specific activity of the evolved mutant NR8.276-2 remained stable at about 0.20 U/mg throughout the fermentation (Table 3, entry 2). In comparison, the DAHP synthesizing specific activity of the wild-type *E. coli* KDPGal aldolase was undetectable (Table 3, entry 1). 3-Dehydroquinate synthase specific activities remained constant at approximately 0.016 U/mg (Table 3, entries 1 and 2) for both *E. coli* NR7/pNR8.075 and NR7/pNR8.288 over the course of the fermentations.

DAHP synthase catalyzes an irreversible condensation of phosphoenolpyruvate and D-erythrose 4-phosphate to form DAHP and inorganic phosphate. In contrast, KDPGal aldolase catalyzes a reversible condensation between pyruvate and D-erythrose 4-phosphate to produce DAHP. The second enzyme in the shikimate pathway, 3-dehydroquinate synthase, also catalyzes an irreversible conversion of DAHP to 3-dehydroquinate and inorganic phosphate. Therefore, increasing the activity of 3-dehydroquinate synthase was expected to drive KDPGal aldolase-catalyzed reversible aldol condensation toward the formation of DAHP. To evaluate the impact of increased 3-dehydroquinate synthase activities on 3-dehydroshikimic acid synthesis, plasmids pNR8.294 and pNR9.056 were constructed with the evolved KDPGal aldolase *NR8.276-2*, *serA*, and *aroB* encoding 3-dehydroquinate synthase. Plasmid pNR9.056 differed from plasmid pNR8.294 in that the *aroB* carried on plasmid pNR9.056 is expressed under the control of a *T5* promoter in contrast to the native *aroB* promoter used in plasmid pNR8.294. Using culture conditions identical to those used for NR7/pNR8.288, *E. coli* NR7/pNR8.294 synthesized 19 g/L 3-dehydroshikimic acid in 9.7% (mol/mol) yield from glucose (Table 2, entry 3) and exhibited an approximate 2-fold increase in 3-dehydroquinate synthase specific activities relative to those of *E. coli* NR7/pNR8.288 (Table 3, entry 3 vs entry 2). *E. coli* NR7/pNR9.056 synthesized only 13 g/L 3-dehydroshikimic acid in 8.1% (mol/mol) yield (Table 2, entry 4) under the same

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cultivation conditions. The 3-dehydroquinate synthase specific activity in *E. coli* NR7/pNR9.056 showed a significant increase ranging from 31-fold at 24 h to 73-fold at 48 h compared to that of *E. coli* NR7/pNR8.288 (Table 3, entry 4 vs entry 2).

D-Erythrose 4-phosphate is the common substrate shared by DAHP synthase and KDPGal aldolase. Previous studies revealed that amplified coexpression of *tktA*-encoded transketolase and feedback-insensitive DAHP synthase isozyme AroF^{FBR} in *E. coli* KL3 increased the yield of 3-dehydroshikimic acid synthesized from glucose by approximately 30%.[26] To evaluate the impact of amplified expression of transketolase on the synthesis of 3-dehydroshikimic acid in an *E. coli* construct expressing the evolved KDPGal aldolase, plasmid pNR8.286 carrying *NR8.276-2*, *serA*, and *tktA* was constructed. Cultivation of *E. coli* NR7/pNR8.286 under fermentor-controlled conditions led to synthesis of 16 g/L 3-dehydroshikimic acid in 8.5% (mol/mol) yield, representing a 30% increase in yield relative to that of *E. coli* NR7/pNR8.288 (Table 2, entry 5 vs entry 2). To examine the impact of amplified expression of both transketolase and 3-dehydroquinate synthase on the synthesis of 3-dehydroshikimic acid, plasmid pNR9.088, which carried *NR8.276-2*, *serA*, *tktA*, and *aroB* under the control of its own promoter, was constructed. Under the same cultivation conditions, *E. coli* NR7/pNR9.088 produced 17 g/L 3-dehydroshikimic acid in 8.7% (mol/mol) yield (Table 2, entry 6).

Microbial synthesis of 3-dehydroshikimic acid by *E. coli* KL3/pKL4.79, which carried an *aroF*^{FBR} under the control of a *tac* promoter and a *serA* gene in vector pJF118EH, has been previously examined.[26] Under the fermentor-controlled conditions, *E. coli* NR7/pKL4.79 synthesized 40 g/L 3-dehydroshikimic acid in 18% (mol/mol) yield from glucose (Table 2, entry 8). The DAHP synthase specific activity in *E. coli* NR7/pKL4.79 increased from 0.21 U/mg at 24 h to 0.70 U/mg at 36 h and then dropped to 0.28 U/mg at 48 h (Table 3, entry 6). In this study, the wild-type KDPGal aldolase and evolved mutants examined were expressed under the control of a *trc* promoter in vector pTrc99A. To compare the synthesis of 3-dehydroshikimic acid using the KDPGal aldolase variant NR8.276-2 with AroF^{FBR} under the same conditions, *E. coli* NR7/pNR9.127 was constructed which contained *NR8.276-2* under the control of a *tac* promoter and *aroB* and *serA* in vector pJF118HE. The vector pJF118HE differs from pJF118EH only in the orientation of the multiple-cloning site. When cultured under identical fermentor-controlled conditions, *E. coli* NR7/pNR9.127 synthesized 16 g/L 3-dehydroshikimic acid in 7.4% (mol/mol) yield from glucose (Table 2, entry 7). The DAHP synthesizing specific activity in *E. coli* NR7/pNR9.127 remained stable between 0.17 and 0.22 U/mg over the course of the fermentations, which was slightly higher relative to that of *E. coli* NR7/pNR8.294 (Table 3, entry 5 vs entry 3).

Discussion

Glucose transport mediated by the PTS in *E. coli* competes with the shikimate pathway for cellular supply of phosphoenolpyruvate. One molecule of phosphoenolpyruvate is consumed and one molecule of pyruvate is generated per molecule of glucose transported into the cytoplasm and phosphorylated. In wild-type *E. coli*, pyruvate is apparently oxidized via the TCA cycle to carbon dioxide. The competition between the shikimate pathway and the PTS for cellular phosphoenolpyruvate and the subsequent generation of pyruvate limit the theoretical maximal yield to 43% (mol/mol) for biosynthesis of 3-dehydroshikimic acid and shikimic acid from glucose.[6,26]

3-Dehydroshikimic acid is the precursor to shikimic acid and the key hydroaromatic intermediate in the biocatalytic conversion of glucose into a variety of industrial chemicals such as adipic acid,[8] catechol,[11] and vanillin.[13] Strategies elaborated to increase the availability of phosphoenolpyruvate in *E. coli* have been examined using synthesis of 3-dehydroshikimic acid as a common measure under identical fermentor-controlled cultivation conditions.[1b,3b] *E. coli* constructs examined in these studies all carried a plasmid-localized *aroF*^{FBR}, a *tktA* insert, and a *serA* locus encoding, respectively, a feedback-insensitive DAHP synthase isozyme, transketolase, and 3-phosphoglycerate dehydrogenase. Fermentor-controlled fed-batch cultivation conditions were used to maintain the dissolved oxygen concentration, pH, and glucose concentration throughout the cultivation. The benchmark *E. coli* KL3/pKL5.17A with its native PTS-mediated glucose transport synthesized 49 g/L 3-dehydroshikimic acid in 26% yield (mol/mol) from glucose after 42 h of cultivation.[3b] Amplified expression of *ppsA*-encoded phosphoenolpyruvate synthase to increase phosphoenolpyruvate availability was first reported by Liao and co-workers.[1a] Phosphoenolpyruvate synthase catalyzes the reaction of pyruvate with ATP to form phosphoenolpyruvate along with byproducts AMP and inorganic phosphate. *E. coli* KL3/pJY1.216A, with optimal expression of phosphoenolpyruvate synthase, synthesized 69 g/L 3-dehydroshikimic acid in 35% (mol/mol) yield from glucose under fermentor-controlled conditions.[1b]

In addition to amplified expression of phosphoenolpyruvate synthase, a promising strategy entails the use of PTS-independent glucose transport systems. The alternative glucose uptake systems examined include facilitated diffusion mediated by *Z. mobilis glf*-encoded glucose facilitator[2] and galactose-proton symport mediated by *E. coli galP*-encoded galactose permease.[3] Ingram and co-workers were the first to construct an *E. coli* mutant with its native PTS-mediated glucose transport inactivated and replaced by the *Z. mobilis glf*-encoded glucose facilitator and *glk*-encoded glucose kinase.[2] Utilization of heterologously expressed *Z. mobilis* glucose facilitator and glucose kinase in *E. coli* JY1/pJY2.183A, a derivative of *E. coli* KL3 lacking PTS-mediated glucose transport, resulted in production of 31 g/L 3-dehydroshikimic acid in 19% (mol/mol) yield after 42 h.[3b] *E. coli* JY1/pJY2.183A also produced 9.5 g/L acetate under the glucose-rich fermentation conditions. Cultivation of *E. coli* JY1/pJY2.183A under glucose-limited conditions eliminated acetate accumulation and resulted in 60 g/L 3-dehydroshikimic acid in 34% (mol/mol) yield.[3b] The impact on 3-dehydroshikimic acid yield in *E. coli* when PTS-mediated glucose transport is replaced by the *galP*-encoded galactose permease was examined with JY1.3/pKL5.17A. The GalP-utilizing *E. coli* JY1.3 was isolated by deletion of PTS-mediated glucose transport in *E. coli* KL3 followed by selection for fast growth on glucose. Recruitment of up-regulated *E. coli galP*-encoded galactose permease for glucose transport in *E. coli* JY1.3/pKL5.17A resulted in synthesis of 60 g/L 3-dehydroshikimic acid in 36% (mol/mol) yield from glucose after an optimal cultivation time of 60 h under the same set of fermentor-controlled conditions.[3b] All those results indicate that the cellular

phosphoenolpyruvate availability indeed limited the microbial synthesis of shikimate pathway products.

Evolving KDPGal aldolase to replace DAHP synthase in *E. coli* provided an alternative strategy to circumvent the competition for phosphoenolpyruvate. The created shikimate pathway variant uses pyruvate as a substrate in place of phosphoenolpyruvate. The intracellular concentration of pyruvate in *E. coli* at the exponential phase grown in glucose-containing minimal salt medium under aerobic conditions is approximately 1.6 mM, while the phosphoenolpyruvate concentration is about 0.1 mM.[27] Utilization of the more abundant pyruvate as a substrate for the shikimate pathway could lead to more carbon flow into the shikimate pathway. In addition, pyruvate is the byproduct of PTS-mediated glucose transport in *E. coli*. The theoretical maximal molar yield of 3-dehydroshikimic acid synthesized from glucose by this approach is 86%. Furthermore, it might be advantageous to utilize this shikimate pathway variant for microbial synthesis of L-tyrosine, L-phenylalanine, and L-tryptophan, in which an additional molecule of phosphoenolpyruvate is required to form an intermediate common to the synthesis of all three aromatic amino acids, 5-enolpyruvylshikimate 3-phosphate (EPSP).

Among the three wild-type KDPGal aldolases examined, the wild-type *E. coli* KDPGal aldolase is the most efficient at condensing pyruvate and D-erythrose 4-phosphate to form DAHP. However, amplified expression of wild-type *E. coli* KDPGal aldolase in *E. coli* NR7, in which all three DAHP synthase isozymes have been inactivated, only synthesized 2 g/L 3-dehydroshikimic acid from glucose (Table 2, entry 1). Evolving KDPGal aldolase to efficiently synthesize DAHP was critical to the creation of a pyruvate-utilizing shikimate pathway. The KDPGal aldolase mutant NR8.276-2 was evolved from the wild-type *E. coli* enzyme via two rounds of error-prone PCR and one round of DNA shuffling of the best mutants, followed by one round of multiple-site-directed mutagenesis on the obtained mutant EC03-1. NR8.276-2 contained seven amino acid changes from the wild type. Three mutated amino acid residues (V85A, V154F, Y180F) were found in the proximity of the putative active site on the basis of the protein model (Figure 1). With a k_{cat} of 4.9 s^{-1} and a K_M of 49 μM for D-erythrose 4-phosphate, NR8.276-2 exhibited a 60-fold improvement in the ratio k_{cat}/K_M relative to that of the *E. coli* wild-type KDPGal aldolase (Table 1, entry 7 vs entry 1). Of the three *E. coli* DAHP synthase isozymes, the *aroF*-encoded L-tyrosine-sensitive DAHP synthase has a k_{cat} of 30 s^{-1} and K_M of 81 μM for D-erythrose 4-phosphate.[28] The *aroG*-encoded L-phenylalanine-sensitive DAHP synthase shows a k_{cat} of 32 s^{-1} and a K_M of 86 μM for D-erythrose 4-phosphate,[29] and the *aroH*-encoded L-tryptophan-sensitive DAHP synthase exhibits a k_{cat} of 21 s^{-1} and a K_M of 35 μM.[30] In comparison with the DAHP synthases, the K_M value of the evolved KDPGal aldolase NR8.276-2 is approximately the same but k_{cat} is still 4-fold to 6-fold lower.

With an evolved KDPGal aldolase to efficiently catalyze the condensation of pyruvate and D-erythrose 4-phosphate, an alternative shikimate pathway was assembled. Amplified expression of the KDPGal aldolase variant NR8.276-2 in DAHP synthase-deficient *E. coli* NR7/pNR8.288 under fed-batch fermentor-controlled conditions produced 13 g/L 3-dehydroshikimic acid in 6.5% (mol/mol) yield from glucose. Synthesis of 3-dehydroshikimic acid by way of the created shikimate pathway variant demonstrated the viability of directed evolution in creating a novel enzyme and pathway for biosynthesis. The concentration and yield of 3-dehydroshikimic acid were further improved by amplified expression of the downstream *aroB*-encoding 3-dehydroquinate synthase. The 3-dehydroquinate synthase catalyzes an irreversible cyclization of DAHP to form 3-dehydroquinate.[31] *E. coli* NR7 already expressed increased specific activity of 3-dehydroquinate synthase due to insertion of a second *aroB* gene into its genomic *serA* locus.[32] Thus, expression of a plasmid-localized *aroB* was examined in *E. coli* NR7/pNR8.294 to further increase the 3-dehydroquinate synthase activity. *E. coli* NR7/pNR8.294 exhibited an additional 2-fold increase in 3-dehydroquinate synthase specific activity and synthesized 19 g/L 3-dehydroshikimic acid in 9.7% (mol/mol) yield from glucose (Table 2, entry 3 vs entry 2).

Overexpression of *tktA*-encoded transketolase in *E. coli* strains expressing *aroF*^{FBR}- or *aroG*^{FBR}-encoded DAHP synthase[26,33] was reported to result in an increase in yield of shikimate pathway products. Transketolase catalyzes the reversible transfer of a ketol group from D-xylulose 5-phosphate and D-erythrose 4-phosphate to D-fructose 6-phosphate and D-glyceraldehyde 3-phosphate in the nonoxidative pentose phosphate pathway. Increasing the expression of transketolase presumably increases the availability of D-erythrose 4-phosphate for aromatic amino acid biosynthesis. However, D-erythrose 4-phosphate is prone to dimerization and has never been detected in cell cytoplasm.[34] Considering the K_M of DAHP synthases for D-erythrose 4-phosphate, it was hypothesized that D-erythrose 4-phosphate was channeled from the transketolase to DAHP synthase.[34] Hence, it is important to note that amplified expression of a plasmid-localized *tktA* in *E. coli* NR7/pNR8.286 expressing the evolved KDPGal aldolase, NR8.276-2, also afforded a 30% increase in the concentration and yield of 3-dehydroshikimic acid synthesized from glucose (Table 2, entry 5 vs entry 2).

Amplified expression of the KDPGal aldolase mutant NR8.276-2 and *aroB*-encoding 3-dehydroquinate synthase from plasmid vector pTrc99A in *E. coli* NR7/pNR8.294 and from plasmid vector pJF118HE in *E. coli* NR7/pNR9.127 synthesized 19 and 16 g/L 3-dehydroshikimic acid, respectively (Table 2, entries 3 and 7). For comparison, *E. coli* NR7/pKL4.79 expressing a feedback-insensitive DAHP synthase isozyme, AroF^{FBR}, from plasmid vector pJF118EH synthesized 40 g/L 3-dehydroshikimic acid (Table 2, entry 8). The lower production of 3-dehydroshikimic acid by *E. coli* expressing the KDPGal aldolase NR8.276-2 might be attributed to its lower DAHP synthesizing activity compared with that of the *aroF*^{FBR}-encoded DAHP synthase. The specific activity of the KDPGal aldolase variant NR8.276-2 remained stable in *E. coli* NR7/pNR9.127, but was lower than the DAHP synthase activities in *E. coli* NR7/

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Table 4. Bacterial Strains and Plasmids Used in This Study

strain/plasmid	characteristics	source
DH5α	*lacZ*ΔM15 *hsdR recA*	Gibco BRL
CB734	C600 Δ(*gal-aroG-nadA*)*50 aroF::Cm*^{R} Δ*aroH::Kan*^{R} *recA*	Bauerle
AB2834	*aroE*353	Pittard et al.[36]
NR7	AB2834 *serA::aroB aroF::Cm*^{R} *aroH::Kan*^{R} *aroG::Tc*^{R}	Ran et al.[4]
pTrc99A	Ap^R, *lacI*^{Q} in pKK233-2, pMB1 replicon	Amann et al.[37]
pJF118EH	Ap^R, *lacI*^{Q} in pKK223-3, pMB1 replicon	Fürste et al.[38]
p34E	Ap^R	Tsang et al.[39]
pRC1.55B	Cm^R, *serA* in pSU18	Yi et al.[3b]
pMF51A	Ap^R, *tktA* in pBR325	Li et al.[26]
pQE30	Ap^R	Qiagen
pNR6.252	Ap^R, P_{trc}*dgoA*$_{KP}$ in pJF118EH	Ran et al.[4]
pNR7.088	Ap^R, P_{trc}*dgoA*$_{EC}$ in pTrc99A	Ran et al.[4]
pNR7.120	Ap^R, P_{trc}*dgoA*$_{ST}$ in pJF118EH	this study
pNR8.075	Ap^R, P_{trc}*dgoA*$_{EC}$, *serA* in pTrc99A	Ran et al.[4]
pNR8.276-2	Ap^R, P_{trc}*NR8.276-2* in pTrc99A	this study
pNR8.123	Ap^R, *serA* in p34E	this study
pNR8.146	Ap^R, *serA*, *tktA* in p34E	this study
pNR8.286	Ap^R, P_{trc}*NR8.276-2*, *serA*, *tktA* in pTrc99A	this study
pNR8.288	Ap^R, P_{trc}*NR8.276-2*, *serA* in pTrc99A	this study
pNR8.294	Ap^R, P_{trc}*NR8.276-2*, *serA*, *aroB* in pTrc99A	this study
pNR9.043	Ap^R, P_{trc}*dgoA*$_{EC}$, *serA*, *tktA* in pTrc99A	this study
pNR9.045	Ap^R, P_{trc}*dgoA*$_{EC}$, *serA*, *aroB* in pTrc99A	this study
pNR9.056	Ap^R, P_{trc}*NR8.276-2*, *serA*, P_{T5}*aroBorf* in pTrc99A	this study
pNR9.088	Ap^R, P_{trc}*NR8.276-2*, *serA*, *aroB*, *tktA* in pTrc99A	this study
pNR9.127	Ap^R, P_{tac}*NR8.276-2*, *serA*, *aroB* in pJF118HE	this study
pKL4.79	Ap^R, P_{tac}*aroF*^{FBR}, *serA* in pJF118EH	Li et al.[26]
pGEX-4T-1	Ap^R, *lacI*^{Q}, P_{tac}*GST*	Amersham Biosciences
pGEX-dgoA$_{EC}$	Ap^R, *dgoA*$_{EC}$ in pGEX-4-1	this study
pGEX-dgoA$_{KP}$	Ap^R, *dgoA*$_{KP}$ in pGEX-4-1	this study
pGEX-dgoA$_{ST}$	Ap^R, *dgoA*$_{ST}$ in pGEX-4-1	this study
pGEX-EC03-1	Ap^R, *EC03-1* in pGEX-4-1	this study
pGEX-NR8.165-2	Ap^R, *NR8.165-2* in pGEX-4-1	this study
pGEX-NR8.165-4	Ap^R, *NR8.165-4* in pGEX-4-1	this study
pGEX-NR8.276-2	Ap^R, *NR8.276-2* in pGEX-4-1	this study

pKL4.79 throughout the course of fermentation (Table 3, entry 5 vs entry 6). The hypothesized channeling of D-erythrose 4-phosphate between transketolase and DAHP synthase might also contribute to the higher production of 3-dehydroshikimic acid by *E. coli* NR7/pKL4.79. Further improvement of the DAHP synthesizing activity of the KDPGal aldolase mutant is clearly required for practical production of shikimate pathway products via the created shikimate pathway variant. The existence of this D-erythrose 4-phosphate channeling and the extent of its contribution to DAHP synthesis have yet to be investigated.

Experimental Section

Culture Media. All solutions were prepared with distilled, deionized water. LB medium (1 L) contained Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g). M9 salts (1 L) contained Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NaCl (0.5 g), and NH_4Cl (1 g). The 2× YT medium (1 L) contained Bacto tryptone (16 g), Bacto yeast extract (10 g), and NaCl (5 g). M9 minimal medium contained D-glucose (10 g), $MgSO_4$ (0.12 g), and thiamine hydrochloride (0.001 g) in 1 L of M9 salts. M9 medium (1 L) was supplemented with L-phenylalanine (0.040 g), L-tyrosine (0.040 g), L-tryptophan (0.040 g), *p*-hydroxybenzoic acid (0.010 g), potassium *p*-aminobenzoate (0.010 g), and 2,3-dihydroxybenzoic acid (0.010 g). The selective medium for directed evolution of KDPGal aldolase contained D-glucose (10 g), $MgSO_4$ (0.12 g), thiamine hydrochloride (0.001 g), L-leucine (0.025 g), nicotinic acid (0.006 g), and isopropyl β-D-thioglucopyranoside (IPTG; 0.05 mM) in 1 L of M9 salts. Antibiotics were appropriately added to the following final concentrations: ampicillin (Ap), 50 μg/mL; chloramphenicol (Cm), 20 μg/mL; kanamycin (Kan), 50 μg/mL. Antibiotics, IPTG, thiamine, nicotinic acid, L-phenylalanine, L-tyrosine, L-tryptophan, and L-leucine solutions were sterilized through 0.22 μm membranes prior to addition. Solid medium was prepared by addition of Difco agar to a final concentration of 1.5% (w/v) to the liquid medium.

DNA Manipulations. *E. coli* DH5α served as the host strain for all plasmid constructions. *E. coli* CB734 was generously provided by Professor Ronald Bauerle (University of Virginia). *E. coli* NR7 was constructed as previously reported.[4] Unless otherwise indicated, cultures of *E. coli* strains were grown at 37 °C with agitation at 250 rpm. Standard protocols were used for construction and analysis of plasmid DNA.[35] Plasmid DNA was isolated using the Plasmid Maxi Kit (Qiagen, Valencia, CA), and DNA fragments were isolated from agarose gels using the DNA Clean & Concentrator Kit (Zymo Research, Orange, CA). DNA ligation was performed using the Fast-Link DNA Ligation Kit (Epicentre, Madison, WI). PCR amplifications were carried out in a Mastercycler gradient thermal cycler (Eppendorf AG, Hamburg, Germany). *Taq* polymerase, a large fragment of DNA polymerase I (Klenow fragment), and calf intestinal alkaline phosphatase were purchased from Invitrogen. *Pfu* polymerase was purchased from Stratagene. λ exonuclease was purchased from New England Biolabs, and DNase I was purchased from Roche Diagnostics. *E. coli* strains were electroporated using a Bio-Rad GenePulser/Pulse Controller at 12.5 kV/cm, 25 μF, and 200 Ω. All strains and plasmids used in this study are listed in Table 4.

pNR8.286. A *serAtktA* cassette liberated from plasmid pNR8.146 by digestion with *Xba*I was inserted into the *Xba*I site of plasmid pNR8.276-2 to produce the 8.6 kb plasmid pNR8.286. Plasmid pNR8.146 was constructed by ligation of a *serA* gene excised from plasmid pRC1.55B[3b] into the *Sma*I site of plasmid p34E[39] to afford

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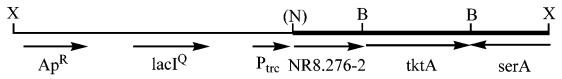
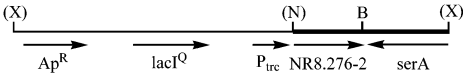
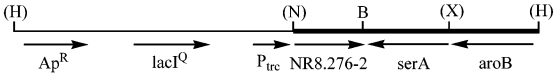
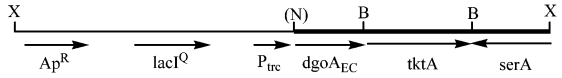
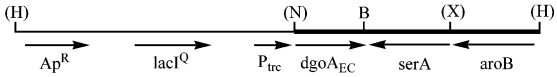
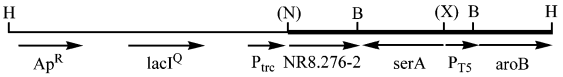
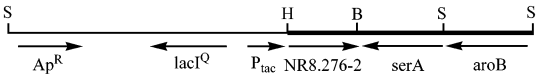
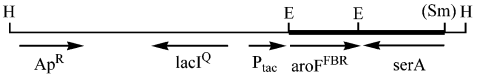
Table 5. Restriction Enzyme Maps of Plasmids

plasmid (size)	plasmid map[a]
pNR8.286 (8.6 kb)	
pNR8.288 (6.4 kb)	
pNR8.294 (8.1 kb)	
pNR9.043 (8.6 kb)	
pNR9.045 (8.1 kb)	
pNR9.056 (7.8 kb)	
pNR9.127 (9.2 kb)	
pKL4.79 (8.3 kb)	

[a] Restriction enzyme sites are abbreviated as follows: X = *Xba*I, B = *Bam*HI, E = *Eco*RI, H = *Hin*dIII, N = *Nco*I, S = *Sph*I, Sm = *Sma*I. Parentheses indicate that the designated restriction site has been eliminated. Lightface lines indicate vector DNA, and boldface lines indicate insert DNA.

plasmid pNR8.123, followed by insertion of the *tktA* locus excised from plasmid pMF51A[26] into the *Bam*HI site of the plasmid pNR8.123. Plasmid pNR8.276-2 contains the evolved *dgoA* mutant *NR8.276-2* located downstream of the *trc* promoter of vector pTrc99A. Transcription of *serA* is in the opposite direction compared with that of the *trc* promoter.

pNR8.288. A 1.6 kb DNA fragment encoding *serA* was excised from plasmid pRC1.55B by digestion with *Sma*I. Plasmid pNR8.276-2 was digested with *Xba*I, and the overhanging ends were blunted by treatment with the Klenow fragment. Subsequent ligation of the 1.6 kb *serA* fragment into pNR8.276-2 previously digested with *Xba*I afforded the 6.4 kb plasmid pNR8.288. Transcription of *serA* is in the opposite direction compared with that of the *trc* promoter.

pNR8.294. A 1.7 kb DNA fragment encoding the *aroB* gene was excised from plasmid pKL3.82[26] by digestion with *Eco*RI and treated with the Klenow fragment. Ligation of the *aroB* locus to pNR8.288, which was previously digested with *Hin*dIII and treated with the Klenow fragment, afforded the 8.1 kb plasmid pNR8.294. The *aroB* gene is transcribed in the opposite direction compared with *NR8.276-2*.

pNR9.043. A 0.6 kb DNA fragment encoding the wild-type *E. coli dgoA* gene was amplified from *E. coli* W3110 genomic DNA using *Pfu* polymerase and the following primers: 5′-GTCACT*CGTCTC*ACATGCAGTGGCAAACTAA and 5′-CTCAGT*CGTCTC*AGATCCTCATTGCACTGCCTCTCG. The *dgoA* fragment was digested with *Esp*3I and ligated into vector pTrc99A previously digested with *Nco*I and *Bam*HI to afford the 4.8 kb plasmid pNR9.042. The *dgoA* locus is oriented in the same direction as the *trc* promoter. A 3.8 kb *serAtktA* cassette was liberated by digestion of plasmid pNR8.146 with *Xba*I. This fragment was ligated to plasmid pNR9.042 previously digested with *Xba*I to afford pNR9.043.

pNR9.045. A 1.6 kb *serA* gene was excised from plasmid pNR8.123 by digestion with *Xba*I. Ligation of the *serA* locus to plasmid pNR9.042 that had been digested with *Xba*I afforded the 6.4 kb plasmid pNR9.044. The *serA* gene is transcribed in the opposite direction compared with *E. coli dgoA*. An *aroB* fragment was liberated from plasmid pKL3.82[26] by digestion with *Eco*RI and subsequently treated with the Klenow fragment. The *aroB* gene was ligated into plasmid pNR9.044 previously digested with *Hin*dIII and treated with the Klenow fragment to afford the plasmid pNR9.045.

pNR9.056. A 1.1 kb DNA fragment encoding the *aroB* open reading frame was amplified from *E. coli* W3110 genomic DNA using *Pfu* polymerase and the following primers: 5′-C*GGATCC*ATGGAGAGGATTGTCGTTACTCT and 5′-AGT*CTGCAG*TTACGCTGATTGACAATCGG. The amplified *aroB* fragment was digested with *Bam*HI and *Pst*I and subsequently ligated into vector pQE30 previously digested with *Bam*HI and *Pst*I to afford plasmid pNR9.040. The *aroB* locus is transcribed in the same orientation as the *T5* promoter. The P_{T5}*aroB* fragment was excised from pNR9.040 by digestion with *Xho*I and *Hin*dIII. Subsequent ligation of P_{T5}*aroB* into the *Sal*I−*Hin*dIII site of pNR8.288 yielded the 7.8 kb plasmid pNR9.056 in which the P_{T5}*aroB* fragment is transcribed in the same direction as the *dgoA* variant *NR8.276-2*.

pNR9.127. A 3.9 kb DNA fragment containing the *NR8.276-2serAaroB* cassette was amplified from plasmid pNR8.294 using a mixture of *Pfu* and *Taq* polymerase (1:1) and the following primers: 5′-TGAAGG*AAGCTT*ATGCAGTGGCAAACTAAACTC and 5′-TTGAAC*GTCGAC*TTCTCTCATCCGCCAAAACA. The PCR product was digested with *Hin*dIII and *Sal*I, and the liberated 2.2 kb fragment containing the *NR8.276-2serA* cassette was ligated into the *Hin*dIII−*Sal*I site of vector pJF118HE to yield plasmid pNR9.126. Subsequent

ligation of the liberated 1.7 kb *aroB* locus into the *Sal*I site of pNR9.126 afforded the 9.2 kb plasmid pNR9.127. The *aroB* gene is transcribed in the opposite direction compared with the *dgoA* variant *NR8.276-2*.

Multiple-Site-Directed Mutagenesis. Multiple-site-directed mutagenesis of the *E. coli* KDPGal aldolase mutant EC03-1 was performed by an overlap extension method that included two rounds of PCR.[25] A total of 12 amino acid residues, L61, I62, L68, T103, V104, C105, P106, C108, A173, L175, S177, and Y180, in the KDPGal aldolase mutant EC03-1 were mutated. In the first round of PCR amplification, four DNA fragments encompassing the entire protein coding regions of the *EC03-1* sequence were amplified. The oligonucleotide primers for amplifying the first region were 5′-GTCACT*CGTCTC*ACATG-CAGTGGCAAACTAA and 5′-CGCCTTGTTGCCGTACG. The primer pairs used for amplifying the second, the third, and the fourth fragments each contained one flanking primer that hybridized at one end of the *EC03-1* sequence and one mutagenetic primer. The primers for amplifying the second region were 5′-CGTACGGCAACAAGGCGKTGRT-TGGCGCAGGTACGGTAMYGAAACCTGAACATGTCGA and 5′-CATGCCGTAGCCTACCG. Oligonucleotide primers 5′-CGGTAG-GCTACGGCATGRYCRYCDSCMYCGGCTKCGCGACAG-CGACCGAAGC and 5′-CCCTGCACAACCTGCGT were used to amplify the third fragment, and the last fragment was amplified using primers 5′-ACGCAGGTTGTGCAGGGKYTGGCKTAGGCWCGG-ATCTCWWTCGCGCCGGGCAATCCGT and 5′-CTCAGT*CGTCTC*AGATCCTCATTGCACTGCCTCTCG. The four DNA fragments generated in the first PCR were purified, pooled, and used as templates as well as primers in the subsequent amplification reaction to recover the entire *dgoA* sequence. The desired 0.6 kb PCR fragment was digested with *Esp*3I and ligated into the pTrc99A vector that was previously digested with *Eco*RI and *Bam*HI. The resulting plasmids were transformed by electroporation into *E. coli* CB734 electrocompetent cells. The library size was estimated as 2×10^5 transformants. Approximately 100 colonies grew up on the selective medium after incubation at 37 °C for a period of approximately 48−60 h. From these colonies, the fastest-growing colony was selected and the encoding *dgoA* mutant *NR8.276-2* was characterized.

Enzyme Assays and Enzyme Kinetics. 2-Keto-3-deoxy-6-phosphogalactonate aldolase,[4] DAHP synthase,[40] 3-dehydroquinate synthase,[41] and transketolase[42] were assayed according to procedures described previously. Cells were collected by centrifugation at 4000*g* and 4 °C. Harvested cells were resuspended in buffer containing potassium phosphate (20 mM, pH 7.5) and phenylmethylsulfonyl fluoride (PMSF; 1 mM) for assay of 2-keto-3-deoxy-6-phosphogalactonate aldolase and 3-dehydroquinate synthase. Harvested cells were resuspended in buffer containing potassium phosphate (50 mM, pH 7.0), $CoCl_2$ (0.05 mM), and potassium phosphoenolpyruvate (10 mM) for assay of DAHP synthase and in buffer containing potassium phosphate (50 mM, pH 7.4), $MgCl_2$ (1 mM), and dithiothreitol (DTT; 0.2 mM) for assay of transketolase. The harvested cells in appropriate buffers were disrupted by two passages through a French pressure cell (16000 psi). Cellular debris was removed by centrifugation at 48000*g* for 20 min. Protein concentrations were determined using the Bradford dye-binding method. Protein assay solution was purchased from Bio-Rad. Protein concentrations were determined by comparison to a standard curve prepared using bovine serum albumin. UV kinetic assays were performed on a Hewlett-Packard 8452A UV/vis spectrophotometer.

The kinetics of wild-type and evolved KDPGal aldolases were measured as follows. The native *E. coli dgoA* and the evolved *EC03-1* and *NR8.276-2* were amplified from plasmids pNR7.088,[4] pEC03-1,[4] and pNR8.276-2, respectively, under standard PCR conditions using the following primers: 5′-CGC*GGATCC*ATGCAGTGGCAAACTAAA and 5′-TCT*CCCGGG*TCATTGCACTGCCTCTCG. The native *K. pneumoniae dgoA* gene was amplified from pNR6.252[4] with the following primers: 5′-CGC*GGATCC*ATGCAGTGGCAAACTAAC and 5′- TCT*CCCGGG*TCATTGCACTGCCTCTCG. The native *S. typhimurium dgoA* was amplified from plasmid pNR7.120 with the following primers: 5′-CGC*GGATCC*ATGCAGTGGCAAACTAAT and 5′- TCT*CCCGGG*TCATTGCACTGCCTCTCG. The *dgoA* mutant *NR8.165-2* was amplified from plasmid pNR8.165-2 with the following primers: 5′-CGC*GGATCC*ATGCAGTGGCAAACTAAT and 5′-TCT*CCCGGG*TCATTGCACTGCCTCTCG. The evolved *dgoA* variant *NR8.165-4* was amplified from plasmid pNR8.165-4 using the following primers: 5′-CGC*GGATCC*CAGTGGCAAACTAAACTC and 5′-TCT*CCCGGG*TCATTGCACTGCCTCTCG. The PCR products were digested with *Bam*HI and *Sma*I and ligated into vector pGEX-4T-1 (Amersham Biosciences) previously digested with *Bam*HI and *Sma*I to afford the corresponding plasmids pGEX-dgoA_{EC}, pGEX-EC03-1, pGEX-NR8.276-2, pGEX-dgoA_{KP}, pGEX-dgoA_{ST}, pGEX-NR8.165-2, and pGEX-NR8.165-4, respectively.

The plasmids containing wild-type and evolved *dgoA* were transformed into *E. coli* BL21 competent cells and were cultured in 2 L of 2× YT medium containing ampicillin (50 μg/mL) at 37 °C. When the culture reached an OD_{600} of 0.6, gene expression was induced by addition of IPTG to a final concentration of 1 mM. The culture was shaken for an additional 4 h at 37 °C. *E. coli* cells were recovered by centrifugation at 4000*g* for 20 min, and the pelleted cells were washed once with 0.9% NaCl and resuspended in 40 mL of 1× PBS buffer (1.4 mM NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.3) containing 1 mM PMSF. The cells were lysed by two passages through a French pressure cell at 16000 psi, and the cell debris was removed by centrifugation at 48000*g* for 20 min at 4 °C. The glutathione *S*-transferase-tagged KDPGal aldolases were purified using a glutathione−Sepharose 4B resin column provided in bulk GST purification modules (Amersham Biosciences).

Kinetic parameters for the glutathione *S*-transferase-tagged KDPGal aldolases were determined by measuring DAHP formation using the KDPGal aldolase assay described previously.[4] The reaction (1 mL) contained morpholinepropanesulfonic acid (MOPS) buffer (50 mM, pH 7.5), 50 μM $CoCl_2$, 10 μM NAD, D-erythrose 4-phosphate, 2 mM pyruvate, 1 U of 3-dehydroquinate synthase, 1 U of 3-dehydroquinate dehydratase, and wild-type or evolved KDPGal aldolase. The concentration of D-erythrose 4-phosphate ranged from 0.02 to 2 mM. The reaction was initiated by addition of purified aldolase to the assay solution, and the absorbance at 234 nm was monitored continuously for 5 min. One unit of aldolase catalyzes the formation of 1 μmol of 3-dehydroshikimate ($\epsilon = 1.19 \times 10^4$ M^{-1} cm^{-1}) per minute at 25 °C. The K_M and k_{cat} were derived using the nonlinear regression program of Prism 4 (GraphPad Software, San Diego, CA) based on the Michaelis−Menton equation. The resulting curves represent best-fit values for the data. The proteins were assayed side by side under identical conditions.

Fed-Batch Fermentation. Glucose-rich fed-batch fermentation was performed in a 2.0 L working capacity B. Braun M2 culture vessel. The fermentation medium was prepared as previously reported.[1b] The initial glucose concentration in the fermentation medium was 20 g/L. The temperature, pH, and dissolved O_2 were controlled with proportional-integral-derivative (PID) control loops. The temperature was maintained at 36 °C, and the pH was maintained at 7.0 by addition of concentrated NH_4OH or 2 N H_2SO_4. The pO_2 was monitored using a Mettler-Toledo 12 mm sterilizable O_2 sensor fitted with an Ingold A-type O_2-permeable membrane and maintained at 20% air saturation. Antifoam 204 (Sigma) was added as needed.

An inoculant was prepared by introducing a single colony of an *E. coli* strain into a sterile test tube containing 5 mL of M9 medium. The culture was grown at 37 °C with agitation at 250 rpm for 18−24 h and then transferred to a 500 mL shake flask containing 100 mL of M9

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medium. The culture was grown at 37 °C with agitation for 10−12 h until an OD_{600} in the range of 1.5−2.5 was reached. The culture was then transferred to the fermentor to initiate the fermentation. Three-staged methods were used to maintain the pO_2 at 20% air saturation during the course of the fermentation. With the airflow at an initial setting of 0.06 L/min, the pO_2 was maintained at 20% air saturation by increasing the impeller speed from its initial set value of 50 rpm to a preset maximum of 750 rpm. With the impeller speed constant at 750 rpm, the mass flow controller then maintained the pO_2 by increasing the airflow from 0.06 L/min to a preset maximum of 1.0 L/min. After the preset maxima of 750 rpm and 1.0 L/min were reached, the third stage of the fermentation was initiated in which glucose (65%, w/v) was added to the vessel at a rate sufficient to maintain a glucose concentration in the range of 10−20 g/L for the remainder of the run. Airflow was maintained at 1.0 L/min, and the impeller speed was allowed to vary to maintain the pO_2 at 20% air saturation. The impeller speed typically varied from 750 to 2000 rpm during the remainder of the run. When 12 h of fermentation had passed since inoculation, IPTG (23.8 mg) was added every 6 h until the end of the run. The only exception was when *E. coli* NR7/pKL4.79 was cultured. In that case, 4.8 mg of IPTG was added using the same condition regimen described above.[26] The *E. coli* cell density was determined by dilution of the fermentation broth with water followed by measurement of absorption at 600 nm (OD_{600}). The dry cell weight (g/L) was calculated using a conversion coefficient of 0.43 (g/L)/OD_{600}.

The concentration of 3-dehydroshikimic acid was determined by ^{1}H NMR as follows. *E. coli* cells were removed by centrifugation, and the supernatant containing 3-dehydroshikimic acid was concentrated to dryness under reduced pressure, concentrated to dryness one additional time from D_2O, and finally dissolved in D_2O containing 10 mM sodium salt of 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid (TSP; Lancaster Synthetic Inc.). The concentration of 3-dehydroshikimic acid was determined by comparison of the integral corresponding to 3-dehydroshikimic acid (δ 4.28, d, 1H) with the integral corresponding to TSP (δ 0.0, s, 9H) in ^{1}H NMR. A response factor of 0.95 was determined by using an authentic 3-dehydroshikimic acid standard for ^{1}H NMR analysis. All ^{1}H NMR spectra were recorded on a Varian VXR-300 FT-NMR spectrometer (300 MHz).

Acknowledgment. This research was supported by a grant from the National Institutes of Health.

JA067330P