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Creation of a Shikimate Pathway Variant

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Phosphoenolpyruvate is a substrate for the first committed step in the shikimate pathway (Scheme 1) and is also used by the carbohydrate phosphotransferase (PTS) system for microbial transport and phosphorylation of glucose.[1] The resulting competition between the shikimate pathway and PTS-mediated glucose transport for cytoplasmic supplies of phosphoenolpyruvate limits the concentrations and yields of natural products microbially synthesized by way of the shikimate pathway. This account explores whether pyruvate can replace phosphoenolpyruvate in an enzyme-catalyzed condensation with D-erythrose 4-phosphate to form 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP, Scheme 1). The centerpiece of the successful creation of this shikimate pathway variant is the directed evolution of 2-keto-3-deoxy-6-phosphogalactonate (KDPGal, Scheme 1) aldolase.

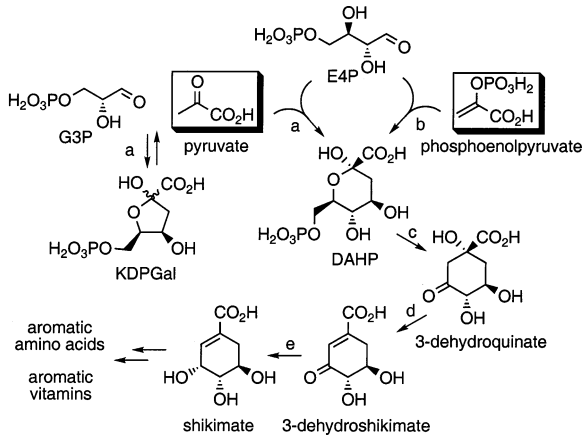
By catalyzing the reversible cleavage of KDPGal to pyruvate and D-glyceraldehyde 3-phosphate (G3P, Scheme 1), KDPGal aldolase enables *Escherichia coli* to use D-galactonate as a sole carbon source.[2] KDPGal aldolase from *Pseudomonas cepacia* has been reported to catalyze the condensation of pyruvate with various aldehydes including D-erythrose.[3] To explore the catalytic activity of KDPGal aldolase toward phosphorylated D-erythrose 4-phosphate (E4P, Scheme 1), *E. coli dgoA*-encoded KDPGal aldolase was overexpresssed, partially purified, and incubated with pyruvate, D-erythrose 4-phosphate, 3-dehydroquinate synthase, and 3-dehydroquinate dehydratase. Formation of 3-dehydroshikimate in 90% yield established the ability of KDPGal aldolase to catalyze the reaction of pyruvate with D-erythrose 4-phosphate as well as the ability of 3-dehydroquinate synthase to drive this reaction nearly to completion. Dehydratase-catalyzed dehydration of 3-dehydroquinate provides in product 3-dehydroshikimate a chromophore suitable for continuous spectrophotometric assay.

With KDPGal aldolase-catalyzed condensation of pyruvate and D-erythrose 4-phosphate established in vitro, attention turned to gauging the impact of this activity in *E. coli* CB734, which lacks all isozymes of DAHP synthase.[4] Growth of *E. coli* CB734 on glucose-containing minimal salts medium required supplementation with L-phenylalanine, L-tyrosine, L-tryptophan, and aromatic vitamins (entry 1, Table 1). *E. coli* CB734/pNR7.088 with its plasmid-encoded *E. coli dgoA* was able to biosynthesize its own aromatic vitamins (entry 2, Table 1). Plasmid-encoded *Klebsiella pneumoniae dgoA* afforded a 4-fold higher KDPGal aldolase specific activity in *E. coli* CB734/pNR6.252 relative to plasmid-encoded *E. coli dgoA* in *E. coli* CB734/pNR7.088 (Table 2). *E. coli* CB734/pNR6.252 was able to provide for its own aromatic vitamin and L-tryptophan requirements (entry 3, Table 1).

K. pneumoniae dgoA and *E. coli dgoA* were subjected to two rounds of error-prone PCR mutagenesis[5] followed by one round of DNA shuffling.[6] CB734/pKP01 (entry 4, Table 1), which carried a mutant *dgoA* plasmid insert resulting from the first round of PCR mutagenesis performed on the *K. pneumoniae dgoA* plasmid insert in *E. coli* CB734/pNR6.252, required only L-phenylalanine supplementation for growth. The second round of PCR mutagenesis led to the mutant *dgoA* plasmid insert in CB734/pKP02 (entry 5, Table 1) that enabled this construct to grow in the absence of aromatic amino acid supplements. Subsequent gene shuffling gave the evolved *dgoA* plasmid insert in CB734/pKP03 (entry 6, Table 1) that facilitated growth in the absence of aromatic amino acid supplements when evolved KDPGal aldolase expression was reduced by lowering IPTG concentrations.

The seven most active *K. pneumoniae* mutants and the seven most active *E. coli* mutants were selected for characterization. Each mutant contained 4−9 amino acid substitutions. No insertion or deletion mutants were found. Two amino acid substitutions (V85A, V154F) were observed in all of the examined *K. pneumoniae dgoA* and *E. coli dgoA* mutants. KP03-3, the most active evolved *K. pneumoniae* KDPGal aldolase, showed a 4-fold higher DAHP formation specific activity and a 30-fold lower KDPGal cleavage

Scheme 1 [a,b]



[a] Metabolites: G3P, D-glyceraldehyde 3-phosphate; E4P, D-erythrose 4-phosphate; KDPGal, 2-keto-3-deoxy-6-phosphogalactonate; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate. [b] Enzymes (genes): (a) KDPGal aldolase (*dgoA*); (b) DAHP synthase (*aroF, aroG, aroH*); (c) 3-dehydroquinate synthase (*aroB*); (d) 3-dehydroquinate dehydratase (*aroD*); (e) shikimate dehydrogenase (*aroE*).

Table 1. Directed Evolution of KDPGal Aldolase (DgoA)

entry	construct[f]	M9[a]	M9[b]	F[c]	FY[c]	FYW[c]	FYWvit[c]
1	*E. coli* CB734	−[d]	−	−	−	−	+[e]
2	CB734/pNR7.088	−	−	−	−	+	+
3	CB734/pNR6.252	−	−	−	+	+	+
4	CB734/pKP01	−	−	+	+	+	+
5	CB734/pKP02	−	+	+	+	+	+
6	CB734/pKP03	+	+	+	+	+	+

[a] Contained 0.05 mM IPTG. [b] Contained 0.2 mM IPTG. [c] Supplements added to M9 medium containing 0.2 mM IPTG included: F, L-phenylalanine; Y, L-tyrosine; W, L-tryptophan; vit, *p*-aminobenzoate, *p*-hydroxybenzoate, 2,3-dihydroxybenzoate. [d] No growth (−). [e] Growth (+). [f] All native and evolved *K. pneumoniae dgoA* genes were inserted into plasmid pJF118EH with transcription controlled by a P_{tac} promoter. All native and evolved *E. coli dgoA* genes were inserted into plasmid pTrc99A with transcription controlled by a P_{trc} promoter.

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Table 2. Specific Activities of Native and Evolved DgoA Isozymes

enzyme	description	DAHP assay[a]	KDPGal assay[a]
K. pneumoniae DgoA	native enzyme	0.29[b]	77[b]
KP03-3	I10V, E71G, V85A, P106S, V154F, E187D, Q191H, F196I	1.30[c]	2.6[c]
E. coli DgoA	native enzyme	0.068[d]	6.7[d]
EC03-1	F33I, D58N, Q72E, A75V, V85A, V154F	0.56[e]	1.0[e]

[a] Specific activity is defined as units of enzyme activity per mg of protein in crude cell lysate. One unit of activity = one μmol of DAHP formed or KDPGal cleaved per minute. See Suporting Information for assay protocols. Crude cell lysates were prepared from: [b] *E. coli* CB734/pNR6.252; [c] *E. coli* CB734/pKP03-3; [d] *E. coli* CB734/pNR7.088; [e] *E. coli* CB734/pEC03-1. See Supporting Information for full descriptions of these plasmids.

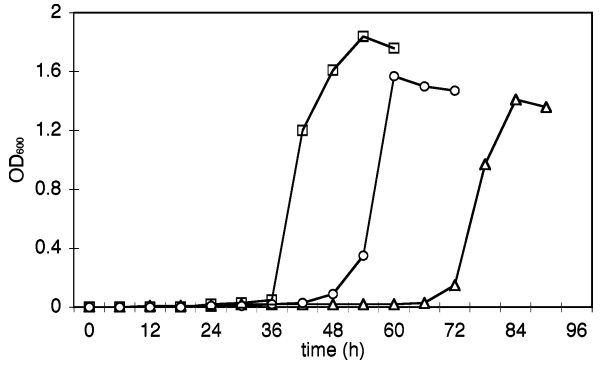

Figure 1. Growth in the absence of aromatic supplements in glucose-containing minimal salts medium under shake-flask conditions. *E. coli* CB734/pNR7.126 expressing native AroF^{FBR} (squares); *E. coli* CB734/pEC03-1 expressing evolved *E. coli* DgoA (circles); *E. coli* CB734/pKP03-3 expressing evolved *K. pneumoniae* DgoA (triangles).

specific activity relative to native *K. pneumoniae* KDPGal aldolase (Table 2). EC03-1, the most active evolved *E. coli* KDPGal aldolase, exhibited an 8-fold higher DAHP formation specific activity and a 7-fold reduced KDPGal cleavage specific activity relative to the native *E. coli* KDPGal aldolase (Table 2).

Constructs expressing evolved *dgoA* were examined for growth rates and synthesis of 3-dehydroshikimate. *E. coli* CB734/pEC03-1 and *E. coli* CB734/pKP03-3 were completely dependent on plasmid-encoded, evolved DgoA isozymes EC03-1 and KP03-3, respectively, for the formation of DAHP. *E. coli* CB734/pNR7.126 relied on plasmid-encoded, feedback-insensitive AroF^{FBR} for DAHP synthase activity. When cultured under identical conditions where growth was dependent on de novo synthesis of aromatic amino acids and aromatic vitamins, *E. coli* CB734/pEC03-1 and *E. coli* CB734/pKP03-3 entered the logarithmic phases of their growths 12 and 36 h, respectively, later than *E. coli* CB734/pNR7.126 (Figure 1).

Synthesis of 3-dehydroshikimate employed *E. coli* NR7, which was constructed from *E. coli* KL3 using site-specific chromosomal insertions to inactivate all DAHP synthase isozymes. *E. coli* KL3 has been extensively used in studies[7] examining the impact of phosphoenolpyruvate availability on the synthesis of 3-dehydroshikimate. Constructs were cultured under identical fermentor-controlled conditions. *E. coli* NR7/pKP03-3serA synthesized 8.3 g/L of 3-dehydroshikimate in 48 h in 5% yield from glucose. Only a trace amount of 3-dehydroshikimate was synthesized by NR7/pNR8.074, which expressed plasmid-encoded, native *K. pneumoniae dgoA*. *E. coli* NR7/pEC03-1serA synthesized 12 g/L of 3-dehydroshikimate in 5% yield from glucose. For comparison, 2.0 g/L of 3-dehydroshikimate was synthesized in 0.9% yield by *E. coli* NR7/pNR8.075, which expressed plasmid-encoded, native *E. coli dgoA*.

With evolved KDPGal aldolase, the first reaction in the shikimate pathway can consume the pyruvate byproduct instead of competing for the phosphoenolpyruvate substrate required by PTS-mediated glucose transport.[8] This constitutes a fundamental departure from all previous strategies employed to increase phosphoenolpyruvate availability in microbes.[9] Beyond increasing the maximum theoretical yield for 3-dehydroshikimate synthesis from 43 to 86% (mol/mol),[7a] a shikimate pathway variant based on condensation of pyruvate with D-erythrose 4-phosphate may be important as a theoretical construct. Growth environments can be envisioned where minimizing expenditure of phosphoenolpyruvate by the shikimate pathway might be a metabolic advantage. The shikimate pathway variant outlined in this account may thus serve as a model of a naturally occurring aromatic biosynthetic pathway that remains to be discovered.

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Supporting Information Available: Construction of plasmids and *E. coli* NR7; enzyme assays; in vitro synthesis of 3-dehydroshikimate; directed evolution of KDPGal aldolase (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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