

Hydroaromatic Equilibration During Biosynthesis of Shikimic Acid

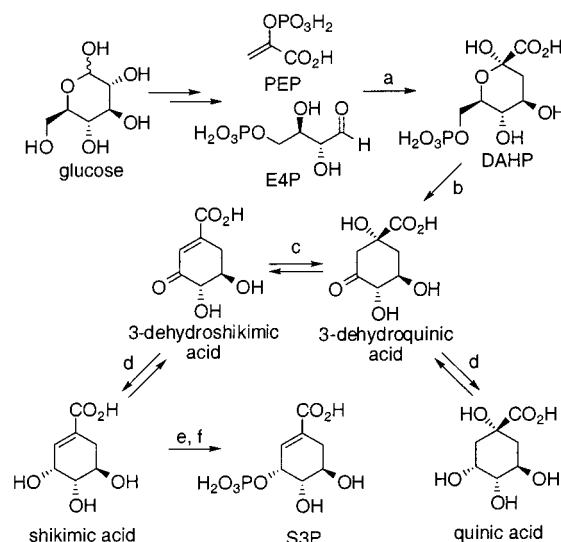
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Received April 12, 2001

Abstract: The expense and limited availability of shikimic acid isolated from plants has impeded utilization of this hydroaromatic as a synthetic starting material. Although recombinant *Escherichia coli* catalysts have been constructed that synthesize shikimic acid from glucose, the yield, titer, and purity of shikimic acid are reduced by the sizable concentrations of quinic acid and 3-dehydroshikimic acid that are formed as byproducts. The 28.0 g/L of shikimic acid synthesized in 14% yield by *E. coli* SP1.1/pKD12.138 in 48 h as a 1.6:1.0:0.65 (mol/mol/mol) shikimate/quinic/dehydroshikimate mixture is typical of synthesized product mixtures. Quinic acid formation results from the reduction of 3-dehydroquinic acid catalyzed by *aroE*-encoded shikimate dehydrogenase. Is quinic acid derived from reduction of 3-dehydroquinic acid prior to synthesis of shikimic acid? Alternatively, does quinic acid result from a microbe-catalyzed equilibration involving transport of initially synthesized shikimic acid back into the cytoplasm and operation of the common pathway of aromatic amino acid biosynthesis in the reverse of its normal biosynthetic direction? *E. coli* SP1.1/pSC5.214A, a construct incapable of de novo synthesis of shikimic acid, catalyzed the conversion of shikimic acid added to its culture medium into a 1.1:1.0:0.70 molar ratio of shikimate/quinic/dehydroshikimate within 36 h. Further mechanistic insights were afforded by elaborating the relationship between transport of shikimic acid and formation of quinic acid. These experiments indicate that formation of quinic acid during biosynthesis of shikimic acid results from a microbe-catalyzed equilibration of initially synthesized shikimic acid. By apparently repressing shikimate transport, the aforementioned *E. coli* SP1.1/pKD12.138 synthesized 52 g/L of shikimic acid in 18% yield from glucose as a 14:1.0:3.0 shikimate/quinic/dehydroshikimate mixture.

Shikimic acid is a hydroaromatic intermediate (Scheme 1) in the common pathway of aromatic amino acid biosynthesis.¹ Although the common pathway has been observed in plants,² microbes,³ and parasites,⁴ this wide distribution does not translate into abundant availability of the pathway's biosynthetic intermediates such as shikimic acid. A tedious, multistep process is used for isolation of shikimic acid from *Illicium spp.*,⁵ a plant whose cultivation has never benefited from large-scale monoculture. The carbocyclic array of chirality displayed by shikimic acid has attracted attention as the basis for synthesis of a large combinatorial library.⁶ However, synthetic utilization of shikimic acid has been much more restricted in comparison to the more abundantly available and less expensive quinic acid (Scheme 1).⁷ Shikimic acid's expense and limited availability has also

Scheme 1^a

^a Biosynthetic intermediates (abbreviations): phosphoenolpyruvic acid (PEP), D-erythrose 4-phosphate (E4P), 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP), shikimate 3-phosphate (S3P). Enzymes (encoding genes): (a) DAHP synthase (*aroF^{FBR}*), (b) 3-dehydroquinase (*aroB*), (c) 3-dehydroquinase dehydratase (*aroD*), (d) shikimate dehydrogenase (*aroE*), (e,f) shikimate kinase (*aroK*, *aroL*).

complicated its use as the starting material for the synthesis of neuraminidase inhibitors such as the antiinfluenza drug marketed as Tamiflu.⁸

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In this account, the mechanism underlying the formation of quinic acid and 3-dehydroshikimic acid during biosynthesis of shikimic acid under glucose-limited conditions is further delineated. All experiments rely on cultivation of *E. coli* in fermentors in order to control pH, temperature, dissolved O₂ concentrations, and the concentration of glucose in cultures. An intriguing picture emerges of the common pathway operating in the reverse of its normal biosynthetic direction with transport of initially synthesized shikimic acid back into the cytoplasm of *E. coli* playing a significant role in the formation of quinic

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pKD12.112

pKD12.138

pSC5.214A

pJB5.291

^a Restriction enzyme sites are abbreviated as follows: B = *Bam*HI, Bg = *Bgl*III, E = *Eco*RI, H = *Hind*III, K = *Kpn*I, N = *Nco*I, P = *Pst*I, S = *Sac*I, Sl = *Sal*I, Sm = *Sma*I, Sp = *Sph*I, X = *Xba*I. Parentheses indicate that the designated enzyme site has been eliminated. Lightface line indicates vector DNA; boldface line indicates insert DNA.

acid. This mechanistic insight has, in turn, resulted in the identification of methodologies that can be conveniently used to minimize formation of quinic acid during microbial synthesis of shikimic acid from glucose even when expression of *tktA*-encoded transketolase is amplified. The result is a significant improvement in the yield and titer of shikimic acid synthesized from glucose.

Results

Shared Genetic Elements. Both shikimate kinase isozymes (Scheme 1) encoded by *aroK* and *aroL* were inactivated in all *E. coli* constructs used in this study by successive P1 phage-mediated transductions of *aroL478::Tn10* and *aroK17::Cm^R* into the appropriate *E. coli* host.¹¹ While ensuring that carbon flow directed into the common pathway did not proceed beyond synthesis of shikimic acid, inactivation of the shikimate kinases also precluded de novo biosynthesis of aromatic amino acids and aromatic vitamins. Growth of all constructs therefore required supplementation with L-phenylalanine, L-tyrosine, L-tryptophan, *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, and 2,3-dihydroxybenzoic acid. These supplements could potentially create a problem in that feedback inhibition of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) synthase by aromatic amino acids plays a prominent role in controlling carbon flow directed into the common pathway.¹² Accordingly, an isozyme of DAHP synthase encoded by plasmid-localized *aroF^{FBR}* that was insensitive to feedback inhibition by aromatic amino acids was carried as a plasmid-localized insert (Table 1: pKD12.112, pKD12.138, and pJB5.291; details of strains and plasmids can be found in Table 2).¹³

With increased carbon flow resulting from amplified expression of *aroF*^{FBR}, DAHP is not converted into 3-dehydroquinic acid by *aroB*-encoded 3-dehydroquinate synthase at a rate sufficiently rapid to avoid dephosphorylation of DAHP and export of 3-deoxy-D-*arabino*-heptulosonic acid (DAH) into the

Table 2. Bacterial Strains and Plasmids Used in This Study

strain/ plasmid	relevant characteristics	source/ reference
strain		
DH5 α	<i>lacZ</i> Δ M15 <i>recA</i>	Gibco BRL
RB791	<i>lacI</i> ^h	ATCC 53622
AL0807	F ⁻ <i>aroL478::Tn10 aroK17::Cm</i> ^R	Marinus
JP11123	<i>aroD shiA354::Kan</i> ^R	Pittard
CL451	<i>aroD25::Tn10</i>	Stockner
KL3	AB2834 <i>aroE353 serA::aroB</i>	lab strain
SP1.1	RB791 <i>serA::aroB aroL478::Tn10 aroK17::Cm</i> ^R	this study
SP1.1shiA	SP1.1 <i>shiA354::Kan</i> ^R	this study
JB4	KL3 <i>aroL- aroK17::Cm</i> ^R <i>aroD25::Tn10</i>	this study
plasmid		
pSU18	Cm ^R , <i>P_{lac}lacZ'</i> , p15A replicon	Bartolome
pMAK705	Cm ^R , <i>lacZ'</i> , ts-pSC101 replicon	Kushner
pKK223-3	Ap ^R , <i>P_{tac}</i> , pBR322 replicon	Pharmacia
pKL3.82A	Cm ^R , <i>serA::aroB</i> in pMAK705	lab plasmid
pIA321	<i>P_{tac}aroE</i> source	Coggins
pD2625	<i>serA</i> source	Genencor Intl.
SP3	<i>N. tabacum aroD-E</i> source	Bonner
pKD12.112	Ap ^R , <i>aroF</i> ^{FBR} , <i>P_{tac}aroE</i> , <i>serA</i> in pSU18	this study
pKD12.138	<i>tkiA</i> in pKD12.112	this study
pSC5.214A	Ap ^R , <i>P_{tac}aroE</i> , <i>serA</i> in pSU18	this study
pJB5.291	Cm ^R , <i>aroF</i> ^{FBR} , <i>tkiA</i> , <i>serA</i> , <i>N. tabacum aroD-E</i> in pSU18	this study

culture medium (Scheme 1). By inserting a cassette consisting of *aroB* flanked with *serA* nucleotide sequences into the genomic *serA* locus, 3-dehydroquinase synthase activity was increased and DAH accumulation avoided because of the expression of a second genomic copy of *aroB*.¹⁴ Complementation of the disrupted genomic *serA* locus with plasmid-localized *serA* provided the basis for plasmid maintenance (Table 1: pKD12.112, pKD12.138, pSC5.214A, and pJB5.291) in all *E. coli* constructs used in this study. The *serA* locus encodes 3-phosphoglycerate dehydrogenase, an enzyme necessary for biosynthesis of L-serine. Growth in culture medium lacking L-serine supplementation was only possible by expression of plasmid-localized *serA*.

In addition to *aroB*-encoded 3-dehydroquinase synthase, feedback inhibition of shikimate dehydrogenase by shikimic acid has previously been demonstrated to be a significant impediment to the flow of carbon through the common pathway and is reflected by accumulation of 3-dehydroshikimic acid in the culture medium.¹⁵ Shikimate dehydrogenase activity was therefore increased either by expression of plasmid-localized *E. coli aroE* (Table 1: plasmids pKD12.112, pKD12.138, and pSC5.214A) or *N. tabacum aroD-E* (Table 1: plasmid pJB5.291) from a strong *P_{tac}* promoter. A potential complication of shikimate dehydrogenase overexpression was this enzyme's ability to reduce both 3-dehydroquinic acid and 3-dehydroshikimic acid.¹⁰ Increasing shikimate dehydrogenase activity, while compensating for the feedback inhibition of this enzyme by shikimic acid, could also interfere with synthesis of shikimic acid by reducing intermediate 3-dehydroquinic acid to quinic acid.

In addition to feedback-inhibition by aromatic amino acids, the in vivo activity of overexpressed DAHP synthase is limited by the availability of its substrate phosphoenolpyruvic acid and D-erythrose 4-phosphate. A significant body of research has demonstrated that expression levels of DAHP synthase are reached at which point further increases in specific activity do not result in increases in the flow of carbon directed into the common pathway until the availability of D-erythrose 4-phosphate is first increased.¹⁶ This requires increasing expression levels of either *tkiA*-encoded transketolase^{16a-c} or *talA*-encoded transaldolase^{16d} to increase the rate that the nonoxidative pentose

Table 3. Hydroaromatics Biosynthesized under Glucose-Limited Culture Conditions

<i>K_c</i> ^a	<i>E. coli</i> :			
	SP1.1/pKD12.112		SP1.1/pKD12.138	
	0.1	0.8	0.1	0.8
SA ^b	26	14	28	12
QA ^b	8.1	1.2	19	9.4
DHS ^b	6.5	4.6	11	5.7
SA/QA ^c	3.1	12	1.6	1.4
SA yield ^d	13%	10%	14%	8%
total yield ^e	20%	14%	29%	18%

^a Proportional gain of glucose PID control loop. ^b g/L of shikimic acid (SA), quinic acid (QA), 3-dehydroshikimic acid (DHS). ^c (mol SA)/(mol QA). ^d (mol SA)/(mol glucose). ^e (mol SA + QA + DHS)/(mol glucose).

phosphate pathway catalyzes interchange of C-6 and C-4 carbohydrates. Plasmid pKD12.112, which lacked a *tkiA* insert (Table 1), and pKD12.138, which carried a *tkiA* insert (Table 1), were constructed for the purpose of evaluating the impact of transketolase overexpression on the biosynthesis of shikimic acid. Plasmids pSC5.214A and pJB5.291 also carried a *tkiA* insert (Table 1). Strategies for increasing in vivo phosphoenolpyruvate availability were not pursued in this study.

Amplified Expression of Transketolase. The glucose addition rate to microbe-catalyzed syntheses is a critical consideration. Excessive rates of glucose addition result in accumulation of byproducts such as acetic acid, which can lead to inhibition of microbial growth and reduction in the yields of the desired product.¹⁷ For this reason, *E. coli* SP1.1/pKD12.112 and *E. coli* SP1.1/pKD12.138 were cultured under glucose-limited conditions at 33 °C, pH 7.0, with dissolved oxygen maintained at a set point of 10% air saturation. Glucose addition was controlled by dissolved O₂ concentration and was a critical control parameter during syntheses of shikimic and quinic acids by *E. coli* SP1.1/pKD12.112 and *E. coli* SP1.1/pKD12.138. When dissolved oxygen levels exceeded the set point value indicating decreased microbial metabolism, the rate of glucose addition was increased. When dissolved oxygen levels declined below the set point value indicating increased microbial metabolism, the rate of glucose addition was decreased.

For *E. coli* SP1.1/pKD12.112 and *E. coli* SP1.1/pKD12.138, the rate of glucose addition was governed by a proportional-integral-derivative (PID) control loop. When the proportional gain (*K_c*) on the glucose PID control loop was set to 0.1 for culturing *E. coli* SP1.1/pKD12.112, substantial concentrations of quinic acid were synthesized (Table 3). Increasing *K_c* to 0.8, and thus increasing the average glucose concentration during cultivation of *E. coli* SP1.1/pKD12.112, significantly reduced the formation of quinic acid (Table 3).¹⁰ Higher levels of quinic acid were synthesized by *E. coli* SP1.1/pKD12.138 relative to *E. coli* SP1.1/pKD12.112 when cultured with a *K_c* of 0.1 (Table 3). Unlike *E. coli* SP1.1/pKD12.112, a substantial concentration of quinic acid was still formed as a byproduct as evidenced by the 1.4:1.0:0.68 molar ratio of shikimate/quinic/dehydroshikimate synthesized after 42 h by *E. coli* SP1.1/pKD12.138 when a *K_c* of 0.8 was employed during cultivation (Table 3).

A *K_c* of 0.1 led to comparatively constant glucose addition rates during cultivation of *E. coli* SP1.1/pKD12.112 and *E. coli* SP1.1/pKD12.138. By contrast, fermentor runs for these same constructs that utilized a *K_c* of 0.8 were difficult to control beyond 42 h because of oscillations in glucose addition rates and glucose concentrations in the culture medium. Yields and titers of synthesized hydroaromatics were also lower (Table 3) for both *E. coli* SP1.1/pKD12.112 and *E. coli* SP1.1/pKD12.138

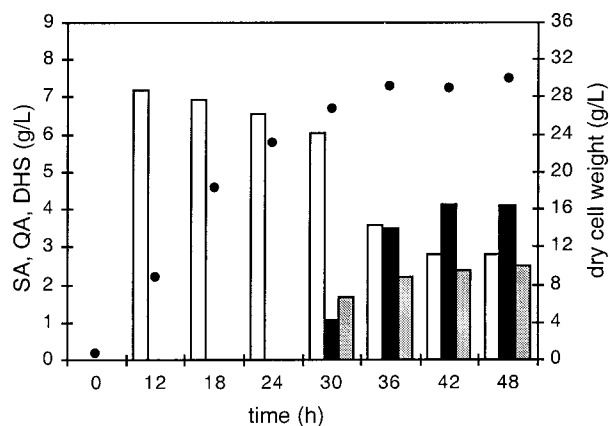


Figure 1. Equilibration of shikimic acid added to the culture medium of *E. coli* SP1.1/pSC5.214A. Legend: shikimic acid (white bar), quinic acid (black bar), 3-dehydroshikimic acid (gray bar), dry cell weight (●).

when cultured at a K_c of 0.8 relative to a K_c of 0.1. This deleterious impact of higher K_c values on hydroaromatic biosynthesis and the failure to control quinic acid formation during cultivation of *E. coli* SP1.1/pKD12.138 clearly indicated that a different strategy was needed to control hydroaromatic equilibration in constructs expressing amplified levels of transketolase activity. As a first step, a more detailed understanding of the mechanism underlying the formation of quinic acid during shikimic acid biosynthesis was needed when a K_c of 0.1 for glucose addition was employed. These culture conditions maintain a steady-state concentration of glucose of approximately 0.20 mM and are referred to as glucose-limited conditions.

Hydroaromatic Equilibration under Glucose-Limited Conditions. Microbe-catalyzed conversion of shikimic acid into 3-dehydroshikimic and quinic acids had previously been examined by harvesting fermentor-grown *E. coli* SP1.1/pKD12.112 and resuspending these cells in minimal salts medium.¹⁰ Equilibration of shikimic acid added to the culture medium was then examined under shake-flask conditions.¹⁰ The goal in this study was to examine hydroaromatic equilibration under fermentor conditions similar to those used for de novo synthesis of shikimic acid from glucose. To ensure that quinic acid formation was due to equilibration of shikimic acid and not due to reduction of 3-dehydroquinic acid synthesized from glucose, *E. coli* SP1.1/pSC5.214A was constructed (Table 1). Plasmid pKD12.138A was linearized by digestion with *Bgl*III restriction enzyme, treated with Klenow fragment, and the blunt ends subsequently ligated to form pSC5.214A. After transformation, the resulting *E. coli* SP1.1/pSC5.214A expressed DAHP synthase activity only from its chromosomal *aroF*, *aroG*, and *aroH* loci. All of the DAHP synthase activity expressed by *E. coli* SP1.1/pSC5.214A was thus sensitive to feedback inhibition by L-phenylalanine, L-tyrosine, or L-tryptophan.³

E. coli SP1.1/pSC5.214A was first cultured under glucose-limited conditions. L-Phenylalanine (0.7 g), L-tyrosine (0.7 g), and L-tryptophan (0.35 g) were added to the culture medium (1 L) at the beginning of the fermentor run as required for growth and at 18 and 30 h to inhibit DAHP synthase activity. The absence of quinic acid, 3-dehydroshikimic acid, and shikimic acid in the culture medium after 48 h indicated an absence of DAHP synthase activity. Shikimic acid (10 g) was subsequently added at 12 h (Figure 1) to *E. coli* SP1.1/pSC5.214A cultured again with aromatic amino acid supplements to negate DAHP synthase activity and de novo hydroaromatic biosynthesis. Formation of quinic acid and 3-dehydroshikimic acid (Figure

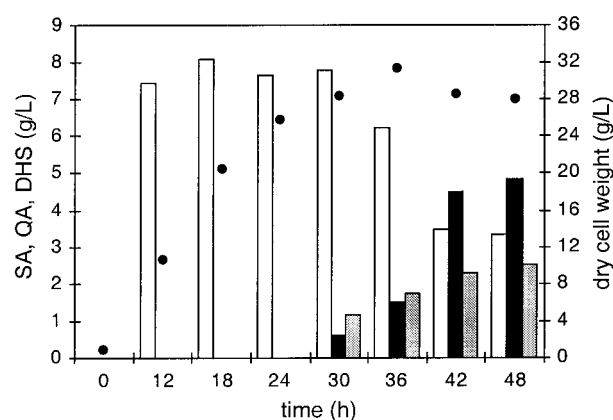


Figure 2. Equilibration of shikimic acid added to the culture medium of *E. coli* SP1.1.shiA/pSC5.214A. Legend: shikimic acid (white bar), quinic acid (black bar), 3-dehydroshikimic acid (gray bar), dry cell weight (●).

1) was observed. The 1.1:1.0:0.70 molar ratio of shikimate/quinic/dehydroshikimate formed by 36 h (Figure 1) clearly illustrated transport of shikimic acid from the culture medium into the microbial cytoplasm and subsequent operation of the common pathway in the reverse of its normal biosynthetic direction.

Inactivation of ShiA-Encoded Shikimate Transport. Mutagenic inactivation of shikimic acid transport provided an approach to differentiate between de novo biosynthesis of quinic acid from glucose and formation of quinic acid resulting from transport and equilibration of shikimic acid. Pittard and co-workers have characterized a system encoded by a locus designated as *shiA* that is apparently responsible for transport of shikimic acid in *E. coli*.¹⁸ This group has also constructed *E. coli* JP11123, which carries catalytically inactive ShiA linked to a transposable element.^{18c} P1 phage-mediated transduction of *shiA*::Kan^R from *E. coli* JP11123 into *E. coli* SP1.1 produced *E. coli* SP1.1.shiA.

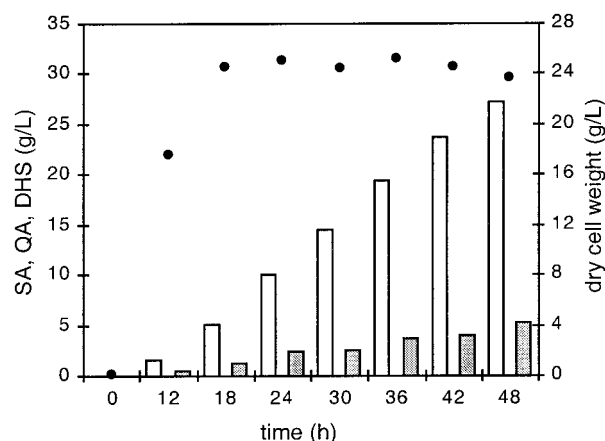
E. coli SP1.1.shiA was then transformed with plasmid pKD12.138 and the resulting *E. coli* SP1.1.shiA/pKD12.138 cultured under glucose-limited conditions. Synthesis of quinic acid and 3-dehydroshikimic acid along with shikimic acid was observed throughout the fermentor run. *E. coli* SP1.1.shiA/pKD12.138 synthesized 23 g/L of shikimic acid, 22 g/L of quinic acid, and 9.6 g/L of 3-dehydroshikimic acid over a 48 h period. To determine if hydroaromatic equilibration was occurring, *E. coli* SP1.1.shiA was transformed with pSC5.214A to generate a construct lacking *shiA*-encoded shikimate transport, DAHP synthase activity, and the attendant capacity for de novo biosynthesis of hydroaromatics when cultured with aromatic amino acid supplements. Shikimic acid was added at 12 h to *E. coli* SP1.1.shiA/pSC5.214A cultured under conditions identical to those employed to study hydroaromatic equilibration in *E. coli* SP1.1/pSC5.214A. Formation of quinic acid and 3-dehydroshikimic acid (Figure 2) was observed. Although hydroaromatic equilibration was slower than for SP1.1/pSC5.214A, the 0.85:1.0:0.57 molar ratio of shikimate/quinic/dehydroshikimate formed by 42 h (Figure 2) clearly indicated that transport and equilibration of shikimic acid were occurring in *E. coli* SP1.1.shiA/pSC5.214A.

Glucose-Limited Cultures and Methyl- α -D-glucopyranoside. As the average glucose concentration increased with increasing K_c value, a concentration of glucose may have been realized that resulted in catabolic repression¹⁹ of the transcription of genes involved in shikimic acid transport. This may explain the success of utilizing increased K_c values to control quinic

Table 4. Hydroaromatics Biosynthesized under Glucose-Limited Culture Conditions with Added Methyl- α -D-glucopyranoside and under Glucose-Rich Culture Conditions

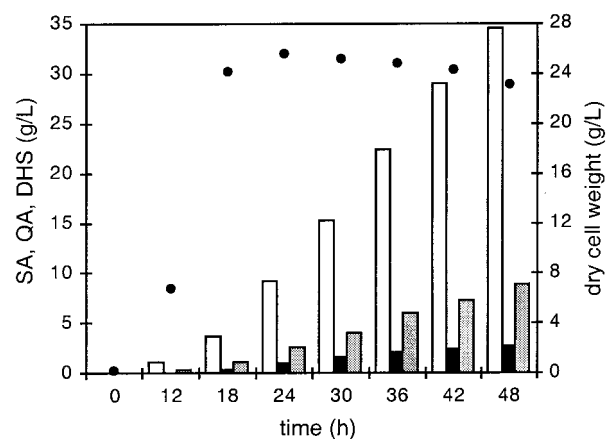
	<i>E. coli</i>			
	SP1.1/pKD12.112		SP1.1/pKD12.138	
	glucose-limited ^a	glucose-rich ^b	glucose-limited ^a	glucose-rich ^a
SA ^c	27	38	35	52
QA ^c	0.0	2.0	2.8	4.1
DHS ^c	5.3	6.4	8.8	11
SA/QA ^d	—	20	14	14
SA yield ^e	15%	12%	19%	18%
total yield ^f	18%	15%	25%	24%

^a K_c of 0.1 with 1 mM methyl- α -D-glucopyranoside. ^b 25 g/L glucose. ^c g/L of shikimic acid (SA), quinic acid (QA), 3-dehydroshikimic acid (DHS). ^d (mol SA)/(mol QA). ^e (mol SA)/(mol glucose). ^f (mol SA + QA + DHS)/(mol glucose).

**Figure 3.** Biosynthesis of shikimic acid under glucose-limited conditions by *E. coli* SP1.1/pKD12.112 in the presence of 1 mM methyl- α -D-glucopyranoside. Legend: shikimic acid (white bar), quinic acid (black bar), 3-dehydroshikimic acid (gray bar), dry cell weight (●).

acid formation during biosynthesis of shikimic acid in the absence of overexpressed transketolase. An alternate approach might be to add some concentration of a glucose mimic to the culture medium of a shikimate-synthesizing *E. coli*. Methyl- α -D-glucopyranoside was chosen as the glucose mimic, because it is hydrolytically stable under the solution conditions used to culture shikimate-synthesizing *E. coli* constructs. Although methyl- α -D-glucopyranoside is transported and phosphorylated by *E. coli*, it is not otherwise metabolized.²⁰

Cultivation of SP1.1/pKD12.112 with 1 mM methyl- α -D-glucopyranoside under glucose-limited conditions with a K_c of 0.1 resulted in the synthesis of 27 g/L of shikimic acid in 15% yield after 48 h (Table 4, Figure 3). Although 3-dehydroshikimic acid was also synthesized (Table 4, Figure 3), there was no detectable formation of quinic acid. The 1 mM concentration of methyl- α -D-glucopyranoside that inhibited quinic acid formation was apparently sufficiently low to avoid the reduction in yield of shikimic acid associated with increasing the K_c to 0.8. Cultivation of SP1.1/pKD12.138 with 1 mM methyl- α -D-glucopyranoside under glucose-limited conditions using a K_c of 0.1 led to the synthesis of 35 g/L of shikimic acid in 19% yield after 48 h (Table 4, Figure 4). Although quinic acid formation was not completely eliminated, less quinic acid was formed than 3-dehydroshikimic acid (Table 4, Figure 4). The molar ratio of shikimate/quinic/dehydroshikimate was 14:1.0:3.5 at 48 h. No control loss of glucose addition was encountered with methyl- α -D-glucopyranoside present. This allowed fermentations to be controlled for 48 h during cultivation of both

**Figure 4.** Biosynthesis of shikimic acid under glucose-limited conditions by *E. coli* SP1.1/pKD12.138 in the presence of 1 mM methyl- α -D-glucopyranoside. Legend: shikimic acid (white bar), quinic acid (black bar), 3-dehydroshikimic acid (gray bar), dry cell weight (●).

SP1.1/pKD12.112 and SP1.1/pKD12.138 and contrasts with the inability to maintain control of glucose addition typically encountered after 42 h for cultivation of these shikimate-synthesizing *E. coli* at a K_c of 0.8. Most importantly, use of methyl- α -D-glucopyranoside to inhibit formation of quinic acid established (Table 4) that amplified expression of transketolase by *E. coli* SP1.1/pKD12.138 increased the yield of shikimic acid, the titer of shikimic acid, and the total yield of hydroaromatics synthesized from glucose.

Glucose-Rich Cultures. Perhaps the simplest strategy for catabolite repression¹⁹ of shikimate transport is to culture the microbe under glucose-rich conditions. Such culture conditions were initially avoided because of precedent indicating that abundant glucose availability can increase formation of byproducts such as acetic acid and lower yields in *E. coli*-catalyzed conversions.¹⁷ To determine how glucose-rich culture conditions compared with addition of methyl- α -D-glucopyranoside in conjunction with glucose-limited conditions, *E. coli* constructs SP1.1/pKD12.112A and SP1.1/pKD12.138A were cultured at 33 °C and pH 7.0 with glucose concentrations maintained at 25 g/L (140 mM). Dissolved oxygen levels were controlled at a set point of 10% air saturation by allowing the impeller speed to vary over the range 750–1600 rpm. Baffles were also introduced into the fermentor vessel to enhance mixing and attendant aeration of cultures.

Under glucose-rich conditions, constructs synthesized shikimic acid for a longer period of time (60 h) relative to cultivation under glucose-limited conditions in the absence (42 h) or in the presence (48 h) of methyl- α -D-glucopyranoside. *E. coli* SP1.1/pKD12.112 synthesized 38 g/L of shikimic acid in 12% yield after 60 h of cultivation (Table 4, Figure 5). The molar ratio of shikimate/quinic/dehydroshikimate at 60 h was 21:1.0:3.6. Although its formation was not completely eliminated under the glucose-rich conditions, less quinic acid was formed relative to 3-dehydroshikimic acid (Table 4, Figure 5). The yield of shikimic acid and total yield of hydroaromatics synthesized by *E. coli* SP1.1/pKD12.112 under glucose-rich conditions was only marginally reduced relative to cultivation of this same strain under glucose-limited conditions in the presence of methyl- α -D-glucopyranoside (Table 4).

Hydroaromatic equilibration was also inhibited when transketolase-overexpressing *E. coli* SP1.1/pKD12.138 was cultured under glucose-rich conditions (Table 4, Figure 6). *E. coli* SP1.1/pKD12.138 synthesized 52 g/L of shikimic acid in 18% yield after cultivation for 60 h (Table 4, Figure 6). The molar ratio

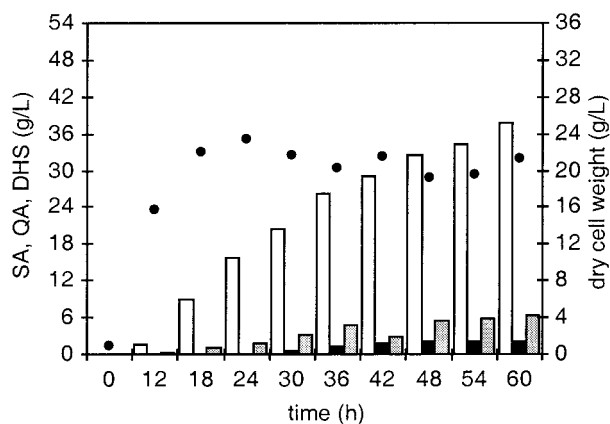


Figure 5. Biosynthesis of shikimic acid under glucose-rich conditions by *E. coli* SP1.1/pKD12.112. Legend: shikimic acid (white bar), quinic acid (black bar), 3-dehydroshikimic acid (gray bar), dry cell weight (●).

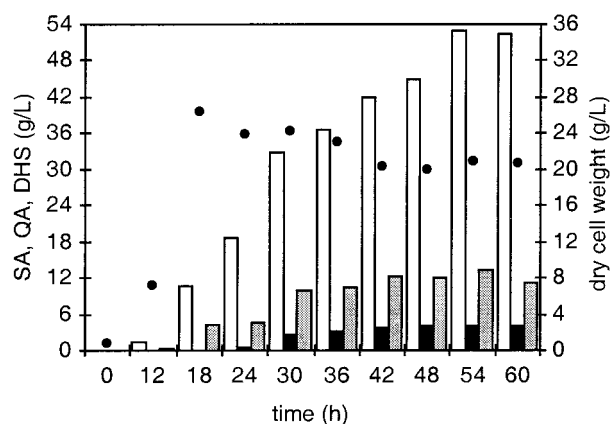


Figure 6. Biosynthesis of shikimic acid under glucose-rich conditions by *E. coli* SP1.1/pKD12.138. Legend: shikimic acid (white bar), quinic acid (black bar), 3-dehydroshikimic acid (gray bar), dry cell weight (●).

of shikimate/quinic/dehydroshikimate synthesized by *E. coli* SP1.1/pKD12.138 at 60 h under glucose-rich conditions was 14:1.0:3.0. Once again, the yield of shikimic acid and total yield of hydroaromatics synthesized by *E. coli* SP1.1/pKD12.138 under glucose-rich conditions was not significantly reduced relative to cultivation of this construct under glucose-limited conditions in the presence of methyl- α -D-glucopyranoside (Table 4).

Nicotiana tabacum aroD•E. With the success in controlling hydroaromatic equilibration under glucose-limited culture conditions by adding methyl- α -D-glucopyranoside or by use of glucose-rich culture conditions, 3-dehydroshikimic acid became the major contaminant in *E. coli*-catalyzed syntheses of shikimic acid from glucose. In plants, 3-dehydroquinic acid dehydratase and shikimate dehydrogenase activities are associated with a single bifunctional enzyme.² The gene encoding this bifunctional enzyme has been isolated from a *Nicotiana tabacum* cDNA library and designated as *aroD•E*.²¹ To test the possibility that metabolite channeling²² in AroD•E might reduce accumulation of 3-dehydroshikimic acid, a shikimate-synthesizing *E. coli* construct expressing *N. tabacum aroD•E* was needed that was deficient in native *aroD*-encoded 3-dehydroquinic acid dehydratase and native *aroE*-encoded shikimate dehydrogenase.

E. coli KL3 was selected as the starting point for construction of the host strain for expression of *N. tabacum aroD•E*. Synthesis of 3-dehydroshikimic acid by this mutant has been

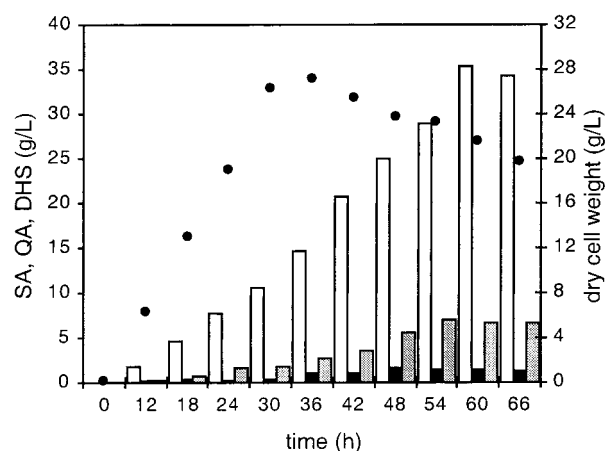


Figure 7. Biosynthesis of shikimic acid under glucose-limited conditions by *E. coli* JB4/pJB5.291. Legend: shikimic acid (white bar), quinic acid (black bar), 3-dehydroshikimic acid (gray bar), dry cell weight (●).

extensively studied.²³ In addition to a mutation in its *aroE* locus inactivating shikimate dehydrogenase activity, *E. coli* KL3 contains a second copy of *aroB* inserted into its *serA* locus. Conversion of KL3 from a 3-dehydroshikimate-synthesizing strain to a shikimate-synthesizing strain began with successive P1 phage-mediated transductions of *aroK17::Cm^R* and *aroL478::Tn10*.¹¹ The resulting KL3 *aroK::Cm^R aroL::Tn10* was then cultured in the presence of D-cycloserine to select for strains that had lost transposon Tn10 inserts to become sensitive to tetracycline resistance (*Tc^S*)²⁴ while retaining the *aroK aroL* phenotype. P1 phage-mediated transduction of an *aroD25::Tn10* allele²⁵ into KL3 *aroK::Cm^R aroL::Tc^S* afforded *E. coli* JB4, which possessed the desired *aroD aroE aroK aroL* phenotype. *E. coli* JB4 was then transformed with plasmid pJB5.291, which carried *N. tabacum aroD•E* expressed from a *P_{tac}* promoter along with *aroF^{FBR}*, *tktA*, and *serA* inserts (Table 1).

Although no reduction in the amount of 3-dehydroshikimic acid formed during synthesis of shikimic acid was observed for *E. coli* JB4/pJB5.291, quinic acid formation was surprisingly reduced (Figure 7). *E. coli* JB4/pJB5.291 was cultivated in the absence of methyl- α -D-glucopyranoside under glucose-limited conditions at 36 °C, pH 7.0 and with dissolved O₂ maintained at 10% of air saturation. This resulted in the synthesis of 34 g/L of shikimic acid in 15% yield after 66 h with a 29:1.0:5.7 molar ratio of shikimate/quinic/dehydroshikimate. *E. coli* JB4/pJB5.291 was cultured at a higher temperature because of the sluggish growth characteristics of this construct at 33 °C. ¹H NMR analysis of *N. tabacum aroD•E* incubated with NADPH (25 mM) and 3-dehydroquinic acid (25 mM) in phosphate buffer (100 mM, pH 7.5) at 30 °C for 6 h led to a 54:1.0:5.7 molar ratio of shikimate/quinic/dehydroshikimate. Inhibition by shikimic acid of the binding of 3-dehydroshikimic acid to the shikimate dehydrogenase portion of *N. tabacum aroD•E* was also measured. Shikimic acid was a competitive inhibitor with *K_i* = 1 mM.

Discussion

Hydroaromatic Equilibration. Formation of quinic acid results from the reduction of 3-dehydroquinic acid. Delineating the source of the 3-dehydroquinic acid that is reduced thus becomes central to understanding the mechanism responsible

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for byproduct formation during shikimic acid biosynthesis. Two mechanisms are possible. 3-Dehydroquinic acid synthesized from glucose may be reduced by *aroE*-encoded shikimate dehydrogenase prior to its conversion to 3-dehydroshikimic acid catalyzed by *aroD*-encoded 3-dehydroquinic acid dehydratase (Scheme 1). Alternatively, microbe-synthesized shikimic acid that accumulates in the culture supernatant may be transported back into the microbial cytoplasm followed by microbe-catalyzed equilibration. Conversion of transported shikimic acid into 3-dehydroquinic acid would require shikimate dehydrogenase-catalyzed oxidation of shikimic acid to 3-dehydroshikimic acid followed by hydration of this hydroaromatic catalyzed by 3-dehydroquinic acid dehydratase.

Demonstrating (Figure 1) the ability of *E. coli* to catalyze the formation of 3-dehydroshikimic acid and quinic acid from shikimic acid under fermentor-controlled, glucose-limited conditions was essential to establishing that hydroaromatic equilibration was a possible mechanism explaining the formation of quinic acid during shikimic acid biosynthesis. However, demonstration of hydroaromatic equilibration does not necessarily establish that this is the mechanism involved in formation of quinic acid during microbial synthesis of shikimic acid from glucose. Delineation of the role of shikimic acid transport in quinic acid formation provided an essential second line of mechanistic information. Continued formation of quinic acid in a shikimate-synthesizing *E. coli* construct deficient in shikimic acid transport would be consistent with a mechanism involving shikimate dehydrogenase-catalyzed reduction of intermediate 3-dehydroquinic acid prior to synthesis of shikimic acid. A pronounced decline or the absence of quinic acid generated by this same *E. coli* construct lacking the ability to transport shikimic acid from its growth medium to its cytoplasm would be indicative of equilibration of initially synthesized shikimic acid.

Transport of shikimic acid in *E. coli* has been traced to the *shiA* locus.¹⁸ The availability^{18c} of a transposon-linked mutation in *shiA* made construction of a shikimate-synthesizing *E. coli shiA* strain a relatively straightforward exercise. One such construct deficient in *shiA*-encoded transport of shikimic acid, *E. coli* SP1.1*shiA*/pKD12.138, produced quinic acid and 3-dehydroshikimic acid during de novo biosynthesis of shikimic acid from glucose. This result, upon first inspection, appeared to be consistent with a mechanism for quinic acid formation involving reduction of intermediate 3-dehydroquinic acid prior to formation of shikimic acid. However, subsequent evaluation of the *ShiA*-deficient *E. coli* SP1.1*shiA*/pSC5.214A revealed that quinic acid and 3-dehydroshikimic acid were formed when shikimic acid was added to this construct's culture medium (Figure 2). This observation indicated that *E. coli* employed at least one system for shikimic acid transport that was not encoded by *shiA*.

Additional insight into the role of shikimic acid transport and formation of quinic acid came from consideration of shikimic acid's role as a source of carbon for microbial growth and metabolism. Numerous microbes can use hydroaromatics such as shikimic acid as a sole source of carbon during growth.²⁶ One of these species, *Klebsiella spp.*, is evolutionarily related to *E. coli*.²⁷ Although unable to utilize shikimic acid as a sole source of carbon for growth, shikimic acid transport in *E. coli* may be an evolutionary remnant of a former capacity to catabolize shikimic acid and may also reflect the multiple transport systems microbes often possess for transport of carbon

sources. Consideration of shikimic acid as a source of carbon for growth and metabolism also leads to a possible strategy for repression of shikimate transport. When another carbon source in addition to glucose is available at any one time for growth of microbes such as *E. coli*, glucose is frequently consumed first.^{19,28} Expression of genes required for transport and metabolism of non-glucose carbon sources can be prevented by catabolite repression.¹⁹

Both small (1 mM) concentrations of methyl- α -D-glucopyranoside under glucose-limited culture conditions (0.20 mM glucose) and glucose-rich (140 mM glucose) culture conditions successfully inhibited quinic acid formation (Table 4, Figures 3–6) during shikimic acid biosynthesis. These observations are consistent with catabolic repression of all systems employed by *E. coli* to transport shikimic acid. Inhibition of quinic acid formation attendant with repression of shikimic acid transport, in turn, indicates that quinic acid formation during shikimic acid biosynthesis is best viewed as an equilibration of initially synthesized shikimic acid as opposed to reduction of intermediate 3-dehydroquinic acid prior to formation of shikimic acid. In essence, the common pathway is operating in an intact organism in the reverse of its normal biosynthetic direction.

3-Dehydroshikimic Acid and Shikimate Dehydrogenase Activity. Formation of quinic acid and 3-dehydroshikimic acid when shikimic acid was added to the culture medium of *E. coli* SP1.1/pSC5.214A (Figure 1) also provided insights into formation of 3-dehydroshikimic acid during shikimic acid biosynthesis. Accumulation of 3-dehydroshikimic acid has typically been attributed to the inability of shikimate dehydrogenase to catalyze the turnover of its substrate into shikimic acid at a rate sufficiently rapid to avoid accumulation of 3-dehydroshikimic acid when the flow of carbon into the common pathway is increased.^{14,15} However, it is also possible that accumulation of 3-dehydroshikimic acid may reflect rate-limiting hydration of 3-dehydroshikimic acid by 3-dehydroquinic acid dehydratase when the common pathway is operating in the reverse of its normal biosynthetic direction. Such a mechanism is consistent with the accumulation of 3-dehydroshikimic acid formed during equilibration of shikimic acid catalyzed by *E. coli* SP1.1/pSC5.214A (Figure 1) and *E. coli* SP1.1*shiA*/pSC5.214A (Figure 2).

With the successful inhibition of quinic acid formation (Table 4, Figures 3–6), most of the accumulated 3-dehydroshikimic acid can be reasonably attributed to feedback inhibition of shikimate dehydrogenase by shikimic acid.¹⁵ The standard approach to reducing accumulation of 3-dehydroshikimic acid entails overexpression of shikimate dehydrogenase by plasmid-localization of the *aroE* gene. However, 3-dehydroshikimic acid accumulated as the major byproduct during shikimic acid biosynthesis when hydroaromatic equilibration was inhibited. This prompted the evaluation of the *aroD•E* gene from *N. tabacum*.²¹ The proximity of 3-dehydroquinic acid dehydratase and shikimate dehydrogenase activities in AroD•E might allow for metabolic channeling of enzyme-bound 3-dehydroquinic acid directly into shikimic acid formation and avoid accumulation of 3-dehydroshikimic acid in the cytoplasm.² Minimizing or completely avoiding accumulation of 3-dehydroshikimic acid may be important for plants, given the precedented enzymatic^{9c} and nonenzymatic²⁹ conversion of this hydroaromatic into gallic acid. Heterologous expression of *N. tabacum aroD•E* had

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previously been demonstrated with the successful complementation of *E. coli aroD* and *E. coli aroE* auxotrophs.²¹

E. coli JB4/pJB5.291, which lacked wild-type *E. coli aroD* and *aroE* activities and expressed *N. tabacum aroD•E*, still accumulated substantial quantities of 3-dehydroshikimic acid. *N. tabacum aroD•E*-encoded shikimate dehydrogenase activity was also just as sensitive to feedback inhibition as wild-type *E. coli aroE*-encoded shikimate dehydrogenase. An unanticipated surprise when culturing *E. coli* JB4/pJB5.291 under glucose-limited conditions was the low quantities of quinic acid that were formed during shikimic acid biosynthesis under glucose-limited culture conditions. Reduced formation of quinic acid was ultimately traced to the selectivity of *N. tabacum AroD•E* favoring reduction of 3-dehydroshikimic acid to shikimic acid over reduction of 3-dehydroquinic acid to quinic acid. This selectivity adds a new twist to the list of enzymes displaying dehydrogenase activity toward intermediates in the common pathway of aromatic amino acid biosynthesis.

The first reported microbial synthesis of quinic acid relied on heterologous expression in a 3-dehydroquinic-acid-synthesizing *E. coli* construct of the *Klebsiella pneumoniae qad* gene encoding quinate dehydrogenase.³⁰ Qad catalyzed the reduction of 3-dehydroquinic acid to quinic acid. In *K. pneumoniae*, Qad oxidizes quinic acid to 3-dehydroquinic acid, which is the first step in the catabolism of quinic acid.³¹ Synthesis of quinic acid by *E. coli* strains such as SP1.1/pKD12.112 and SP1.1/pKD12.138 (Table 3, Figure 1) is thus noteworthy given that *E. coli* has not previously been reported to biosynthesize quinic acid or to exploit this hydroaromatic as a sole source of carbon for growth. The ability of *aroE*-encoded shikimate dehydrogenase to catalyze the reduction of 3-dehydroquinic acid in addition to its reduction of 3-dehydroshikimic acid (Scheme 1) to shikimic acid had apparently evaded the intense mechanistic scrutiny afforded enzymes in the common pathway. With *N. tabacum AroD•E*, a true shikimate dehydrogenase has been identified given this enzyme's selective reduction of 3-dehydroshikimic acid over 3-dehydroquinic acid.

Purity, Titer, and Yield. Three methods are now available for controlling hydroaromatic equilibration during shikimic acid biosynthesis. Under glucose-limited culture conditions, shikimate-synthesizing *E. coli* constructs can be cultivated in the presence of low concentrations (1 mM) of methyl- α -D-glucopyranoside. Hydroaromatic equilibration can also be avoided under glucose-limited culture conditions by expressing *N. tabacum aroD•E* in an *E. coli* host lacking native *aroE*-encoded shikimate dehydrogenase activity. Finally, cultivating shikimate-synthesizing *E. coli* constructs under glucose-rich conditions inhibits hydroaromatic equilibration. These methods can be individually evaluated and compared with one another on the basis of the purity, titer, and yield of shikimic acid synthesized by *E. coli* from glucose.

3-Dehydroshikimic acid is separated from shikimic acid and quinic acid in culture supernatants by acidification, heating, and absorption of the resulting protocatechuic acid on activated carbon. Removal of quinic acid requires a fractional recrystallization of crude shikimic acid product after removal of 3-dehydroshikimic acid. Contamination levels of quinic acid thus become important, because the presence of quinic acid in excess of 10% (mol/mol) relative to shikimic acid is difficult to remove by crystallization. Although the concentration of quinic acid

relative to the shikimic acid synthesized by *E. coli* SP1.1/pKD12.138 was 2-fold higher than that realized with *E. coli* JB4/pJB5.291, this higher level of quinic acid contamination was still readily removed from product shikimic acid by crystallization.

Peak shikimic acid concentrations using *E. coli* JB4/pJB5.291 took significantly longer to achieve (60 h) relative to use of *E. coli* SP1.1/pKD12.138 under glucose-rich (54 h) or glucose-limited (48 h) culture conditions. This increased length of time for synthesis of shikimic acid reflects the slower growth characteristics of *E. coli* JB4/pJB5.291, which had to be cultured at 36 °C relative to the 33 °C culture conditions where vigorous growth was observed for *E. coli* SP1.1/pKD12.138. The 18–19% (Table 4) yield of shikimic acid and the 24–25% (Table 4) combined yield of shikimic acid, quinic acid, and 3-dehydroshikimic acid were essentially unaffected (Table 4) by the use of glucose-rich versus glucose-limited conditions for cultivation of *E. coli* SP1.1/pKD12.138. However, the 52 g/L of shikimic acid in fermentation broths synthesized by *E. coli* SP1.1/pKD12.138 when cultured under glucose-rich conditions was substantially higher relative to the 35 g/L synthesized by this same construct under glucose-limited conditions in the presence of methyl- α -D-glucopyranoside.

Another outcome of controlling hydroaromatic equilibration during shikimic acid biosynthesis is the ability to take full advantage of the improvement in carbon flow directed into the common pathway (Scheme 1) by overexpression of transketolase and the attendant increase in the availability of D-erythrose 4-phosphate for aromatic amino acid biosynthesis.¹⁶ *E. coli* SP1.1/pKD12.112, which expresses wild-type expression levels of transketolase, synthesized 38 g/L of shikimic acid in 12% yield. This titer and yield contrast sharply with the 52 g/L of shikimic acid synthesized in 18% yield under identical culture conditions by *E. coli* SP1.1/pKD12.138, which overexpresses transketolase. Prior to delineating the mechanism associated with hydroaromatic equilibration and developing methodologies appropriate for controlling this phenomenon, it was not possible to evaluate the impact of transketolase overexpression on the yields and titers of shikimic acid synthesized by *E. coli* from glucose. With the ability to control hydroaromatic equilibration when D-erythrose 4-phosphate availability is increased, microbe-catalyzed synthesis of shikimic acid from glucose can now be used as a paradigm to study the impact on product yields and titers when the in vivo availability of phosphoenolpyruvic acid is increased.

Experimental Section

Culture Medium. All solutions were prepared in distilled, deionized water. LB (Luria–Bertani) medium³² contained (1 L) Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g). M9 salts (1 L) contained Na₂HPO₄ (6 g), KH₂PO₄ (3 g), NH₄Cl (1 g), and NaCl (0.5 g). M9 minimal medium contained D-glucose (10 g), MgSO₄ (0.12 g), and thiamine hydrochloride (0.001 g) in 1 L of M9 salts. M9 medium (1 L) was supplemented where appropriate 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). L-Serine and shikimic acid were added to a final concentration of 40 mg/L where indicated. Antibiotics were added where appropriate to the following final concentrations: ampicillin (Ap), 50 μ g/mL; chloramphenicol (Cm), 20 μ g/mL; kanamycin (Kan), 50 μ g/mL; and tetracycline (Tc), 25 μ g/mL. Solutions of M9 salts, MgSO₄, and glucose were autoclaved individually and then mixed. Solutions of amino acids, aromatic vitamins, shikimic acid, thiamine hydrochloride

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ride, and antibiotics were sterilized through 0.22- μ m membranes. Solid medium was prepared by addition of Difco agar to a final concentration of 1.5% (w/v) to the liquid medium.

The standard fermentation medium (1 L) contained K_2HPO_4 (7.5 g), ammonium iron (III) citrate (0.3 g), citric acid monohydrate (2.1 g), L-phenylalanine (0.7 g), L-tyrosine (0.7 g), L-tryptophan (0.35 g), and concentrated H_2SO_4 (1.2 mL). Fermentation medium was adjusted to pH 7.0 by addition of concentrated NH_4OH before autoclaving. The following supplements were added immediately prior to initiation of the fermentation: glucose, $MgSO_4$ (0.24 g), *p*-hydroxybenzoic acid (0.010 g), potassium *p*-aminobenzoate (0.010 g), 2,3-dihydroxybenzoic acid (0.010 g), and trace minerals, including $(NH_4)_6(Mo_7O_{24}) \cdot 4H_2O$ (0.0037 g), $ZnSO_4 \cdot 7H_2O$ (0.0029 g), H_3BO_3 (0.0247 g), $CuSO_4 \cdot 5H_2O$ (0.0025 g), and $MnCl_2 \cdot 4H_2O$ (0.0158 g). Methyl- α -D-glucopyranoside was added to a final concentration of 1 mM where indicated. Glucose, $MgSO_4$ (1 M), and methyl- α -D-glucopyranoside (100 mM) solutions were autoclaved separately, while solutions of aromatic vitamins and trace minerals were sterilized through 0.22- μ m membranes. Shikimic acid was added to the fermentation medium where indicated. A solution of shikimic acid (10 g) in water (10 mL) was adjusted to pH 7 by addition of KOH and sterilized through a 0.22- μ m membrane prior to addition to the fermentation medium. In cases where additional aromatic amino acids were added while the batch was in progress, L-phenylalanine (0.7 g), L-tyrosine (0.7 g), and L-tryptophan (0.35 g) were added as dry powders. Antifoam (Sigma 204) was added as needed.

Bacterial Strains and Plasmids. *E. coli* K-12 strain RB791 was obtained from the American Type Culture Collection (ATCC strain 53622). *E. coli* AL0807¹¹ was provided by Professor M. G. Marinus (University of Massachusetts), *E. coli* JP11123^{18c} was provided by Professor A. J. Pittard (University of Melbourne), and *E. coli* CL451²⁵ was provided by Professor B. A. D. Stocker (Stanford University). Plasmid constructions were carried out in *E. coli* DH5 α , which is available from Gibco BRL Products. Homologous recombinations utilized plasmid pMAK705,³³ which was obtained from Professor S. R. Kushner (University of Georgia).

Genetic Manipulations. Standard protocols were used for construction, purification, and analysis of plasmid DNA.³⁴ T4 DNA ligase and Large Fragment of DNA polymerase I (Klenow fragment) were purchased from Gibco BRL Products. Calf intestinal alkaline phosphatase was purchased from Roche Molecular Biochemicals. PCR amplifications were carried out as previously described.³⁴ Each amplification reaction (0.1 mL) contained 10 mM KCl, 20 mM Tris-HCl (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 (0.02 μ g-1 μ g), 0.5 μ M of each primer, and 2 units of Vent polymerase. Primers were synthesized by the Macromolecular Structure Facility at Michigan State University.

SP1.1. Construction of SP1.1 began with the homologous recombination of the *aroB* gene into the *serA* locus of *E. coli* RB791 resulting in RB791 *serA::aroB*. Plasmid pKL3.82A, which contained the *serA::aroB* locus, was described previously.^{23a} The procedure for homologous recombination has also been described.^{33,35} Competent RB791 was transformed with pKL3.82A and plated out onto LB plates containing Cm. Colonies were observed after incubation of the plates at 44 °C for 12 h. The resulting cointegrates were inoculated into 5 mL LB and grown at 30 °C for 12 h to allow excision of the plasmid from the genome. Cultures were diluted (1:20 000) in LB, and two more growth cycles were performed at 30 °C for 12 h to enrich the cultures for more rapidly growing cells that had lost the temperature-sensitive replicon. Cultures were then diluted (1:20 000) 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 and incubated at 30 °C for 12 h. The resulting colonies were screened on multiple plates to select the desired recombination product. RB791 *serA::aroB* was isolated on the basis of the following growth characteristics: no growth on M9,

growth on M9 containing serine, growth on LB, and no growth on LB containing Cm.

RB791 *serA::aroB* was subjected to two successive P1 phage-mediated transductions to transfer the *aroL478::Tn10* and *aroK::Cm^R* loci of ALO807¹¹ into the genome. P1 phage was propagated from *E. coli* ALO807, and transductions were carried out using protocols described by Miller.³² RB791 *serA::aroB aroL::Tn10 aroK::Cm^R* was selected on the basis of the following growth characteristics: no growth on M9 containing aromatic amino acids and aromatic vitamins; no growth on M9 containing serine; growth on M9 containing aromatic amino acids, aromatic vitamins, and serine; and growth on LB containing Tc and Cm. RB791 *serA::aroB aroL::Tn10 aroK::Cm^R* was renamed SP1.1.

SP1.1sh*iA*. *E. coli* SP1.1sh*iA* was prepared by transduction of the *shiA354::Kan^R* locus from JP11123^{18c} into SP1.1. SP1.1sh*iA* was selected on the basis of its ability to grow on LB containing Cm, Tc, and Kan. The inability of SP1.1sh*iA* to grow in the absence of aromatic amino acid and aromatic vitamin supplementation and in the absence of serine supplementation was confirmed separately.

JB4. Construction of *E. coli* JB4 began with KL3,^{23a} which already contains the *serA::aroB* locus as well as a mutation in the *aroE* locus. Two successive P1 phage transductions using phage propagated from ALO807¹¹ were carried out on KL3 to afford KL3 *aroK::Cm^R aroL::Tn10*. To make the strain sensitive to tetracycline without losing the mutation in *aroL*, colonies were selected for resistance to D-cycloserine.²⁴ A single colony of KL3 *aroK::Cm^R aroL::Tn10* was introduced into 5 mL of LB and grown overnight at 37 °C with agitation. The overnight culture was diluted (1:500) into LB, and the cells were grown for an additional 5 h. The cells were then collected by centrifugation, resuspended in LB containing Tc, and cultured for 1 h at 37 °C with agitation. D-Cycloserine was added to the culture to a final concentration of 6 mM, and cell growth was continued for 3.5 h. Cells were collected from 1 mL of the culture, washed with 1 mL of LB, and finally resuspended in 1 mL of LB. A portion (10 μ L) of the suspension was used to inoculate 5 mL of fresh LB, and the cells were grown overnight. After a second identical treatment with D-cycloserine, the surviving cells were plated onto LB. Colonies which grew were then replicate-plated onto LB and LB/Tc plates. Approximately 10% of the colonies that grew on LB were sensitive to Tc. One of these colonies was named KL3 *aroK::Cm^R aroL::Tn^S*. Subsequent transduction of KL3 *aroK::Cm^R aroL::Tn^S* with P1 phage propagated from CL451²⁵ was followed by selection of colonies for resistance to Tc. KL3 *aroK::Cm^R aroL::Tn^S aroD::Tn10* colonies were selected on the basis of the following growth characteristics: no growth on M9 containing serine; no growth on M9 containing shikimic acid and serine; growth on M9 containing aromatic amino acids, aromatic vitamins, and serine; and growth on LB containing Cm and Tc. KL3 *aroK::Cm^R aroL::Tn^S aroD::Tn10* was renamed JB4.

pKD12.112A. Plasmid pKD12.112 is a pSU18-derived plasmid³⁶ that encodes *aroF^{FBR}*, *aroE*, *serA*, and Ap^R. Construction of pKD12.112 began with pKL4.20B, a 3.6-kb plasmid that was described elsewhere.^{23a} Plasmid pKL4.20B contains a 1.2-kb fragment that encodes *aroF^{FBR}*.¹³ PCR amplification of the *P_{tac} aroE* sequence from pIA321³⁷ afforded a 1.2-kb fragment that was subsequently localized in pKL4.20B to afford pKD12.036. A 1.9-kb *serA*-encoding fragment was liberated from pD2625 by digestion with *EcoRV* and *DraI*. Insertion of the *serA* fragment into pKD12.036 yielded pKD12.047. Resistance to chloramphenicol conferred by pKD12.047 was eliminated by inserting the gene that confers ampicillin resistance into the *NcoI* site of pKD12.047. PCR amplification of a 1.0-kb Ap^R-encoding fragment from pUC18³⁸ followed by insertion of the fragment into the *NcoI* site of pKD12.047 afforded the 7.7-kb plasmid pKD12.112.

pKD12.138. Insertion of a 2.2-kb *tktA*-encoding fragment^{16b,39} into pKD12.112 afforded the 9.9-kb plasmid pKD12.138.

pSC5.214A. Plasmid pSC5.214A is identical to pKD12.138 except that pSC5.214A does not contain a functional *aroF^{FBR}* gene. To

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inactivate the *aroF^{FBR}* gene of pKD12.138, advantage was taken of a unique *Bgl*III recognition site located internal to the gene.¹³ *Bgl*III digestion of pKD12.138 was followed by treatment of the linearized plasmid with the Klenow fragment and a cocktail of dNTPs. Subsequent religation of the plasmid afforded pSC5.214A.

pJB5.291. Plasmid pJB5.291 is a pSU18-derived plasmid that encodes *aroF^{FBR}*, *tktA*, *serA*, and the *N. tabacum aroDE* locus. Construction of pJB5.291 began with pJB3.141, a 7.6-kb plasmid that encoded *aroF^{FBR}*, *tktA*, *serA*. The *N. tabacum aroDE* locus²¹ was cloned as a 1.9-kb fragment behind the *P_{lac}* promoter of pKK223-3⁴⁰ to afford pJB5.272A. Liberation of a 2.2-kb *P_{lac} aroDE* fragment from pJB5.272A followed by insertion of the fragment into pJB3.141 afforded pJB5.291.

Fed-Batch Fermentations (General). Fermentations employed a 2.0 L working capacity B. Braun M2 culture vessel. Utilities were supplied by a B. Braun Biostat MD controlled by a DCU-1. Data acquisition utilized a Dell Optiplex Gs⁺ 5166M personal computer (PC) equipped with B. Braun MFCS/Win software (v1.1). Temperature and pH were controlled with PID control loops. Glucose feeding was controlled with a PID control loop unless otherwise indicated. Temperature was maintained at 33 °C for all fermentations with the exception of the JB4/pJB5.291, which was cultured at 36 °C. pH was maintained at 7.0 by addition of concentrated NH₄OH or 2 N H₂SO₄. Dissolved oxygen (D.O.) was measured using a Mettler-Toledo 12 mm sterilizable O₂ sensor fitted with an Ingold A-type O₂ permeable membrane. D.O. was maintained at 10% air saturation. The initial glucose concentration in the fermentation medium ranged from 20 to 30 g/L.

Inoculants were started by introduction of a single colony picked from an agar plate into 5 mL of M9 medium. For strains possessing a plasmid that confers resistance to ampicillin, ampicillin was added to the medium at each step of the inoculant preparation. Cultures were grown at 37 °C with agitation at 250 rpm until they were turbid (12–36 h) and subsequently transferred to 100 mL of M9 medium. Cultures were grown at 37 °C and 250 rpm for an additional 8–12 h. After the culture reached an appropriate OD₆₀₀ (1.0–3.0), the inoculant was transferred into the fermentation vessel and the batch fermentation was initiated (*t* = 0 h).

Fed-Batch Fermentations (Glucose-Limited Conditions). Three staged methods were used to maintain D.O. concentrations at 10% air saturation during the fermentations. With the airflow at an initial setting of 0.06 L/L/min, the D.O. concentration was maintained by increasing the impeller speed from its initial set point of 50 rpm to its preset maximum of 940 rpm. With the impeller rate constant at 940 rpm, the mass flow controller then maintained the D.O. concentration by increasing the airflow rate from 0.06 L/L/min to a preset maximum of 1.0 L/L/min. At constant impeller speed and constant airflow rate, the D.O. concentration was finally maintained at 10% air saturation for the remainder of the fermentation by oxygen sensor-controlled glucose feeding. At the beginning of this stage, the D.O. concentration fell below

10% air saturation because of residual initial glucose in the medium. This lasted for approximately 0.5–1 h before glucose (65% w/v) feeding commenced. The glucose feed PID control parameters were set to 0.0 s (off) for the derivative control (τ_D) and 999.9 s (minimum control action) for the integral control (τ_I). X_p was set to 950% to achieve a K_c of 0.1 and 125% to achieve a K_c of 0.8 as required.

Fed-Batch Fermentations (Glucose-Rich Conditions). For fermentations that employed glucose-rich conditions, a stainless steel baffle cage consisting of four 1/2" × 5" baffles was placed in the fermentation vessel. Three staged methods were used to maintain the D.O. concentration at 10% air. With the airflow at an initial setting of 0.06 L/L/min, the D.O. concentration was maintained by increasing the impeller speed from its initial set point of 50 rpm to a preset maximum of 750 rpm. With the impeller rate constant at 750 rpm, the mass flow controller then maintained the D.O. concentration by increasing the airflow rate from 0.06 L/L/min to a preset maximum of 1.0 L/L/min. After the preset maxima of 750 rpm and 1.0 L/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 of 20–30 g/L for the remainder of the run. Airflow was maintained at 1.0 L/L/min, and the impeller was allowed to vary in order to maintain the D.O. concentration at 10% air saturation. The impeller speed typically varied from 750 to 1600 rpm during the remainder of the run.

Analysis of Fermentation Broth. Samples (5–10 mL) of fermentation broths were taken at the indicated timed intervals. Cell densities were determined by dilution of fermentation broth with water (1:100) followed by measurement of absorbance at 600 nm (OD₆₀₀). Dry cell weight (g/L) was calculated using a conversion coefficient of 0.43 g/L/OD₆₀₀. The remaining fermentation broth was centrifuged to obtain cell-free broth.

Glucose concentrations in cell-free broth were measured using the Glucose Diagnostic Kit obtained from Sigma. Solute concentrations in the cell-free broth were quantified by ¹H NMR. Solutions were concentrated to dryness under reduced pressure, concentrated to dryness one additional time from D₂O, and then redissolved in D₂O containing a known concentration of the sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-*d*₄ acid (TSP, Lancaster Synthesis Inc.). All ¹H NMR spectra were recorded on a Varian VXR-300 FT-NMR Spectrometer (300 MHz). Concentrations were determined by comparison of integrals corresponding to each compound with the integral corresponding to TSP (δ = 0.00 ppm). A standard concentration curve was determined for each metabolite using solutions of authentic, purified metabolites. Compounds were quantified using the following resonances: shikimic acid (δ 4.57, d, 1 H); quinic acid (δ 4.16, m, 1 H); and 3-dehydroshikimic acid (δ 4.28, d, 1 H).

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

JA0109444

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