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Biocatalytic Production of Aromatics from D-Glucose

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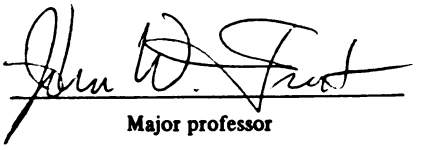

Major professor

Date 4/3/96

BIOCATALYTIC PRODUCTION OF AROMATICS FROM D-GLUCOSE

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Chemistry

1996

ABSTRACT

BIOCATALYTIC PRODUCTION OF AROMATICS FROM D-GLUCOSE

By

Michael Anthony Farabaugh

Transaldolase catalysis had a notable impact on the yield of aromatics produced from D-glucose. This effect is attributed to the fact that transaldolase catalyzes the formation of D-erythrose 4-phosphate and can increase the in vivo availability of this metabolite. The carbon flow entering the common pathway of aromatic amino acid biosynthesis was quantitated by measuring the concentration of 3-dehydroshikimate (DHS) in the culture supernatant of *Escherichia coli aroE* strains. Under certain conditions, overexpression of transaldolase raised the yield of DHS to near theoretical maximum.

The *Citrobacter freundii* gene encoding tyrosine phenol-lyase (*tpl*) was introduced into an *E. coli* construct that synthesizes catechol in order to achieve a biocatalytic synthesis of L-3,4-dihydroxyphenylalanine (L-DOPA) from D-glucose. The synthesis was impeded by an unfavorable reaction equilibrium. In the presence of catechol, cells expressing tyrosine phenol-lyase synthesized L-DOPA in 12% yield.

To my wife Karen, for her constant love and support.

ACKNOWLEDGMENTS

I would like to thank Professor John Frost for his enthusiasm and encouragement. I would also like to acknowledge all the members of the Frost group who have given me their advice, support, and friendship. I wish them the very best in their endeavors.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

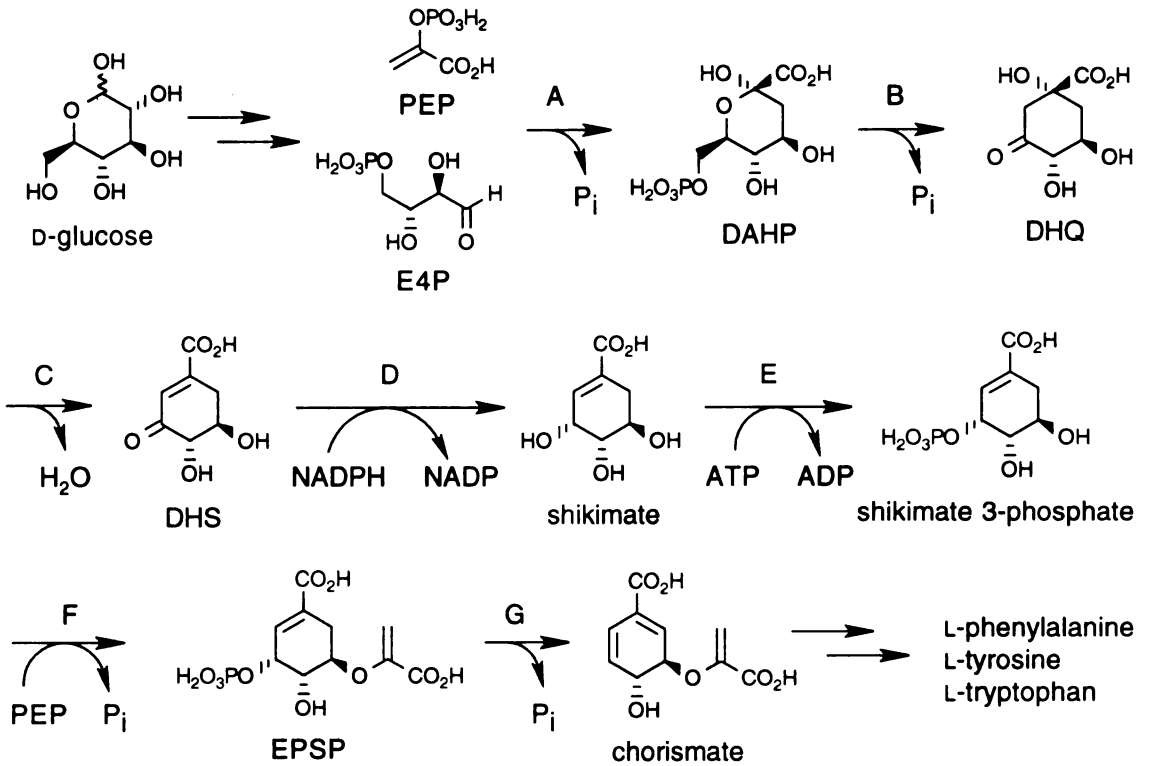
Ap	ampicillin
bp	base pair
Cm	chloramphenicol
DAH	3-deoxy-D-*arabino*-heptulosonic acid
DAHP	3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate
DHQ	3-dehydroquinate
DHS	3-dehydroshikimate
E4P	D-erythrose 4-phosphate
EPSP	5-enolpyruvoylshikimate 3-phosphate
h	hour
HPLC	high pressure liquid chromatography
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase
min	minute
NMR	nuclear magnetic resonance spectroscopy
PEP	phosphoenolpyruvate
PCR	polymerase chain reaction
phe	L-phenylalanine
Tc	tetracycline
trp	L-tryptophan
TSP	sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$
tyr	L-tyrosine

CHAPTER 1

INTRODUCTION

The Common Pathway

The common pathway of aromatic amino acid biosynthesis is found in plants, bacteria, and fungi and is named for the fact that chorismic acid is the common branch point from which the aromatic amino acids and related secondary metabolites are synthesized.[1] The intermediates of the seven enzymatic reactions that convert phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P) into chorismic acid (Figure 1) were completely identified by the early 1960s from studies of bacterial auxotrophs of *Escherichia coli* and *Klebsiella aerogenes*. The first committed step of aromatic amino acid biosynthesis involves the condensation of PEP and E4P to form 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP), catalyzed by DAHP synthase.[2] Three isozymes of DAHP synthase exist in *E. coli*, each of which is sensitive to feedback inhibition by one of the three aromatic amino acids. The genes *aroF*, *aroG*, or *aroH* encode for DAHP synthase (tyr), DAHP synthase (phe), and DAHP synthase (trp), respectively.[1] DAHP is converted into 3-dehydroquinate (DHQ) via DHQ synthase in a reaction that requires NAD. DHQ dehydratase catalyzes the formation of 3-dehydroshikimate (DHS) which contains the first double bond of the aromatic ring. The reduction of DHS by shikimate dehydrogenase in the presence of NADPH affords shikimate. Shikimate kinase produces shikimate 3-phosphate from shikimate and ATP. The reversible condensation of shikimate 3-phosphate and PEP to form 5-enolpyruvoylshikimate 3-phosphate (EPSP) is catalyzed by EPSP synthase. Chorismate synthase catalyzes the elimination of inorganic phosphate from



(A) DAHP synthase (*aroF aroG aroH*); (B) DHQ synthase (*aroB*); (C) DHQ dehydratase (*aroD*); (D) shikimate dehydrogenase (*aroE*); (E) shikimate kinase (*aroK aroL*); (F) EPSP synthase (*aroA*); (G) chorismate synthase (*aroC*).

Figure 1 - The Common Pathway of Aromatic Amino Acid Biosynthesis

EPSP to give chorismate, which contains the second double bond of the aromatic ring. Three terminal pathways lead from chorismate to L-phenylalanine, L-tryptophan, and L-tyrosine. Chorismate also serves as the precursor for other essential aromatic metabolites such as ubiquinone, enterochelin, and folic acid, which are involved in electron transport, iron uptake, and coenzyme biosynthesis, respectively.[1]

Synthesis of Aromatics with *E. coli*

Microorganisms possess a variety of biosynthetic enzymes, in addition to those of the common pathway, which can be utilized for the synthesis of aromatics from D-glucose. The advantage of using D-glucose is that it is inexpensive, nontoxic, and derived from

renewable resources such as corn starch.[3] This contrasts with traditional chemical synthesis of aromatics, which relies on petroleum-derived resources and typically involves carcinogenic starting materials such as benzene. Using *Escherichia coli* as the microbial host for aromatic production is derived from the fact that a tremendous amount of information has been accumulated on the biochemistry and molecular biology of this organism.[1] It has become the archetype for extensive studies on protein expression, genetic engineering, and biocatalysis.

One of the essential goals in the development of a microbial catalyst for aromatic synthesis is to direct a high percentage of the carbon consumed by the microbe into the common pathway of aromatic amino acid biosynthesis. Typically this has been accomplished by increasing the specific activity of the first enzyme of the pathway, DAHP synthase. This can be done by localizing a gene that encodes for one of the three isozymes of DAHP synthase on an extrachromosomal plasmid[4] or by eliminating transcriptional control of one of the DAHP synthase genes.[5] The result is an increased intracellular concentration of DAHP synthase verified by elevated enzyme activity. A complementary strategy is to introduce a mutation in one of the DAHP synthase genes resulting in a protein that is resistant to feedback inhibition by the aromatic amino acids.[6]

Increasing DAHP synthase activity does increase the percentage of carbon (D-glucose equivalents) committed to aromatic amino acid biosynthesis. This has been measured using *E. coli aroB* strains that possess an inactive DHQ synthase.[7] Because these strains are incapable of converting DAHP into DHQ, the DAHP that accumulates intracellularly is exported into the culture medium, along with the dephosphorylated product 3-deoxy-D-arabino-heptulosonic acid (DAH).[4] The amount of DAH(P) present in the culture supernatant can be directly quantified[8] to provide a measure of the carbon flow directed into the common pathway. Experiments with *E. coli aroB* strains that contain a feedback-resistant copy of *aroG*-encoded DAHP synthase localized on an extrachromosomal plasmid demonstrated that increased DAHP synthase activity and

improved DAHP production are observed.[7b] However, a point was reached where further increases in DAHP synthase activity had no impact on the delivery of carbon flow into the common pathway.

Increasing In Vivo Availability of D-Erythrose 4-Phosphate

At this point it becomes necessary to focus on the in vivo availability of the substrates of DAHP synthase: D-erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP). If the intracellular concentration of either E4P or PEP is limiting, DAHP formation will be impeded, even in the presence of overexpressed DAHP synthase. Initial attention centered on E4P as the limiting substrate, for several reasons. D-Erythrose 4-phosphate has never been convincingly detected in a living system.[9] The absence of intracellular E4P accumulation could be attributed to its rapid utilization subsequent to its formation inside the cell.[9b,c] Considering that this aldose phosphate is rather unstable and can easily undergo dimerization,[10] it may be that biological systems have evolved to maintain very low steady-state concentrations of E4P. Nevertheless, when the demand for aromatic amino acid biosynthesis is high, limiting E4P levels may hinder the rate of formation of DAHP. Investigation into increasing the in vivo levels of E4P led to the enzymes of the pentose phosphate pathway.

The pentose phosphate pathway forms a bridge between glycolysis and several biosynthetic pathways. It consists of an oxidative and a non-oxidative branch. The oxidative branch converts D-glucose 6-phosphate into D-ribose 5-phosphate and carbon dioxide, with concomitant production of NADPH. The non-oxidative branch converts D-fructose 6-phosphate (a D-glucose equivalent) into a variety of C-3 through C-7 monophosphate sugars. Two enzymes of the nonoxidative branch,[11] transketolase and transaldolase, catalyze reactions that lead to the formation of D-ribose 5-phosphate, D-sedoheptulose 7-phosphate, and D-erythrose 4-phosphate (Figure 2). These sugars are required for the biosynthesis of nucleotides, lipopolysaccharides, and aromatic amino

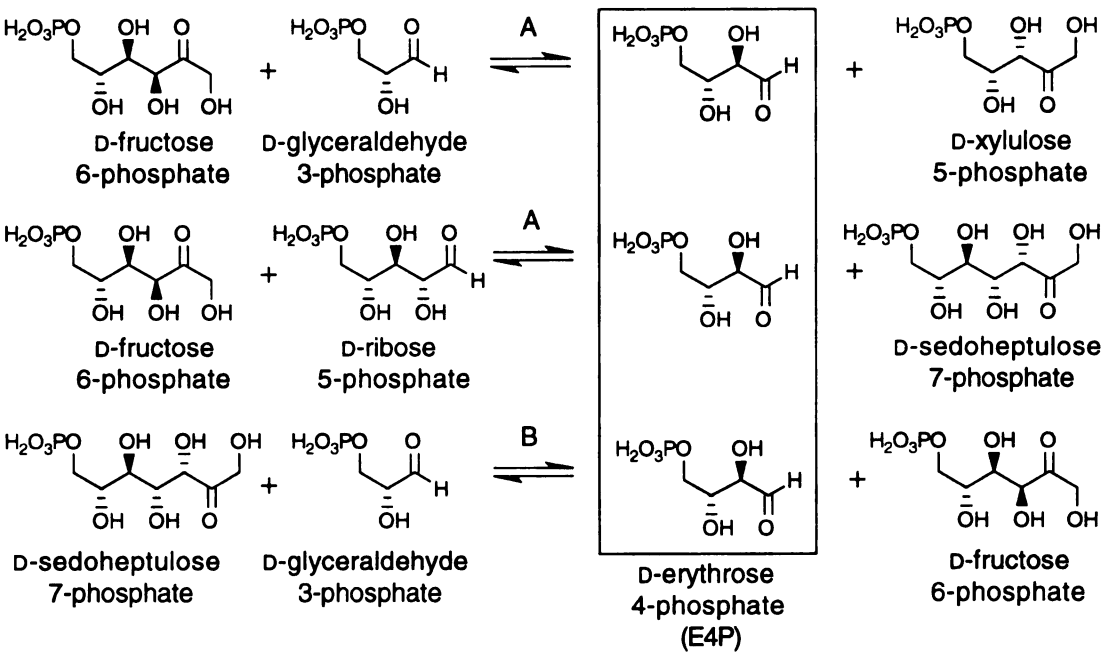


Figure 2 - Reactions Catalyzed by Transketolase (A) and Transaldolase (B)

acids, respectively. The only source of D-erythrose 4-phosphate in bacterial biosynthesis is derived from reactions catalyzed by transketolase and transaldolase.

Although both transketolase and transaldolase are responsible for E4P formation, efforts were focused on increasing transketolase activity for the following reasons. Transketolase catalyzes the reversible transfer of a two carbon ketol group between various aldose acceptors. The equilibrium constant for transketolase-catalyzed conversion of D-fructose 6-phosphate into E4P favors D-fructose 6-phosphate.[12] The unwieldy chemical characteristics of free E4P might be avoided by maintaining an intracellular supply of D-fructose 6-phosphate that serves as a precursor to E4P.[10a] Furthermore, *E. coli* mutants with extremely low levels of transketolase are unable to grow in the absence of aromatic amino acid supplementation, presumably due to limitations in E4P availability.[13]

A gene encoding transketolase was isolated from an *E. coli* genomic library by complementation of a mutant possessing reduced transketolase levels.[7b] The insert was

subcloned to give a 5 kb fragment that contained the *tkt* gene. Transketolase was purified to homogeneity from both wild-type *E. coli* and an overexpressing strain containing the 5 kb insert localized on an extrachromosomal plasmid. The protein is a homodimer with a subunit size of 72 kDa,[7b] which agrees closely with nucleotide sequence information.[14] Recently a second gene for transketolase (designated *tktB*) was identified and sequenced.[15] However this isozyme of transketolase accounts for a small percentage of wild-type transketolase activity.[15]

The isolation of the transketolase gene (now designated *tktA*) and successful overexpression in *E. coli* led to experiments that measured the impact of transketolase on the delivery of D-glucose equivalents into the common pathway of aromatic amino acid biosynthesis. This was illustrated by comparison of DAHP formation in two strains that possessed comparable levels of DAHP synthase, AB2847*aroB*/pRW5 and AB2847*aroB*/pRW5tkt.[7b] The only difference between these two strains is that AB2847*aroB*/pRW5tkt carries an extrachromosomal copy of *tktA* resulting in elevated levels of transketolase activity. Under the same culturing conditions AB2847*aroB*/pRW5tkt synthesized twice as much DAHP relative to AB2847*aroB*/pRW5, which confirmed that increasing transketolase activity leads to higher in vivo availability of E4P and increased carbon flow into the common pathway.

Although it has been demonstrated that amplification of transketolase can increase the yield of DAHP, the effect of increasing the specific activity of transaldolase has not been explored. One of the goals of this thesis is to determine the impact of transaldolase on directing carbon flow into the common pathway.

Increasing In Vivo Availability of Phosphoenolpyruvate

In addition to D-erythrose 4-phosphate, phosphoenolpyruvate (PEP) has been proposed to limit the percentage of D-glucose committed to the common pathway.[16] Stoichiometric analysis of the pathways for DAHP production in *E. coli* suggests that the

theoretical yield of DAHP production from D-glucose is limited by PEP due to the phosphotransferase system (PTS) for sugar uptake. During glucose transport PEP serves as a phosphate donor and is converted to pyruvate. In wild-type *E. coli*, this pyruvate is not likely to be recycled back to PEP because of the high energy cost. As a result, carbon flow directed through pyruvate goes on to produce organic acids, carbon dioxide, or cell mass. The problem of limited PEP availability has been addressed with several strategies.

One plan to alleviate the burden of limited PEP supply is to increase the specific activity of PEP synthase, which catalyzes the conversion of pyruvate into PEP.[16] Theoretical analyses predict that the maximum yield of DAHP produced from D-glucose can be increased twofold (from 43 mol % to 86 mol %) if pyruvate is recycled back to PEP.[16] The overexpression of PEP synthase was accomplished by localization of the *pps* gene on an extrachromosomal plasmid. When DAHP synthase, transketolase, and PEP synthase were overexpressed in an *E. coli aroB* strain in the presence of D-glucose, the final concentration and yield of DAHP approached the theoretical maximum.[16] Notably, the effect of PEP synthase was not observed without the overproduction of transketolase, suggesting that E4P is the first limiting metabolite. However, transketolase alone cannot increase the yield of DAHP from glucose to the theoretical maximum value because of the stoichiometric limitation of PEP.

Other strategies to compensate for the limitations in PEP availability include elimination of enzymatic pathways that compete for intracellular PEP. Studies have been done with mutants lacking PEP carboxylase activity[16a,17] (converting PEP to oxaloacetate) and with mutants lacking pyruvate kinase activity[18] (converting PEP to pyruvate). These approaches have been met with limited success. Changing the carbon source from D-glucose to D-xylose, a sugar whose uptake does not involve the phosphotransferase system, has been explored.[16b] The theoretical yield of DAHP from D-xylose is not limited by PEP and can reach 71 mol % without pyruvate recycling.[16b]

Rate-Limiting Enzymes of the Common Pathway

An increased surge of carbon flow into the common pathway creates a metabolic situation where individual common pathway enzymes become rate-limiting. These rate-limiting enzymes cannot convert substrate to product at a sufficient rate to avoid accumulation of substrate inside the cell. Export of the accumulating substrate into the culture supernatant occurs, resulting in lower percent conversions and reduced purity of desired aromatic products. Using ^{1}H NMR analysis, common pathway substrates and related metabolites were identified from the culture supernatant of an *E. coli* auxotroph that had been designed to overexpress DAHP synthase and transketolase.[19] The genes for common pathway enzymes whose substrates were present in the culture supernatant were introduced on extrachromosomal plasmids into the *E. coli* auxotroph. When a metabolite no longer accumulated as a result of elevated levels of a common pathway enzyme, the rate-limiting character of that enzyme was said to be removed. Progress toward decreasing the number of accumulating common pathway enzyme substrates and increased synthesis of aromatics has been achieved. In this fashion, 3-dehydroquinate (DHQ) synthase, shikimate kinase, 5-enolpyruvoylshikimate 3-phosphate (EPSP) synthase, and chorismate synthase were identified as rate-limiting enzymes.[19]

Other methods that have been used to remove the rate-limiting character of common pathway enzymes include inactivation of the TyrR protein in *E. coli*. The TyrR protein, in combination with tyrosine or tryptophan, inhibits the transcription of *aroL*, which encodes shikimate kinase.[1a,20] The introduction of a mutant allele of *tyrR* into an *E. coli* strain that produces phenylalanine resulted in an increased specific activity of shikimate kinase, a decrease in the accumulation of shikimate, and a higher yield of aromatic end products.[21] Recently, chromosomal insertion of a synthetic multi-gene cassette carrying *aroB* (encoding DHQ synthase), *aroA* (encoding EPSP synthase), and *aroC* (encoding chorismate synthase) into an aromatic-synthesizing *E. coli* strain was accomplished in an effort to

avoid potential problems associated with plasmid instability and unnecessary overexpression of plasmid-encoded, common pathway enzymes.[21]

Assembly of a Heterologous Microbial Biocatalyst

Intermediates of the common pathway of aromatic amino acid biosynthesis can be transformed into a variety of aromatic-derived chemicals.[22] Often this requires incorporation of enzyme activities not possessed by *E. coli*. Examples of this process are illustrated in the biocatalytic production of quinic acid,[23] catechol,[24] and adipic acid[25] from D-glucose. Quinic acid is an important chiral starting material used widely in multistep chemical synthesis whose isolation from plant sources is expensive. The biocatalytic synthesis of quinic acid (Figure 3) has been reported using inexpensive, abundant D-glucose as starting material.[23]

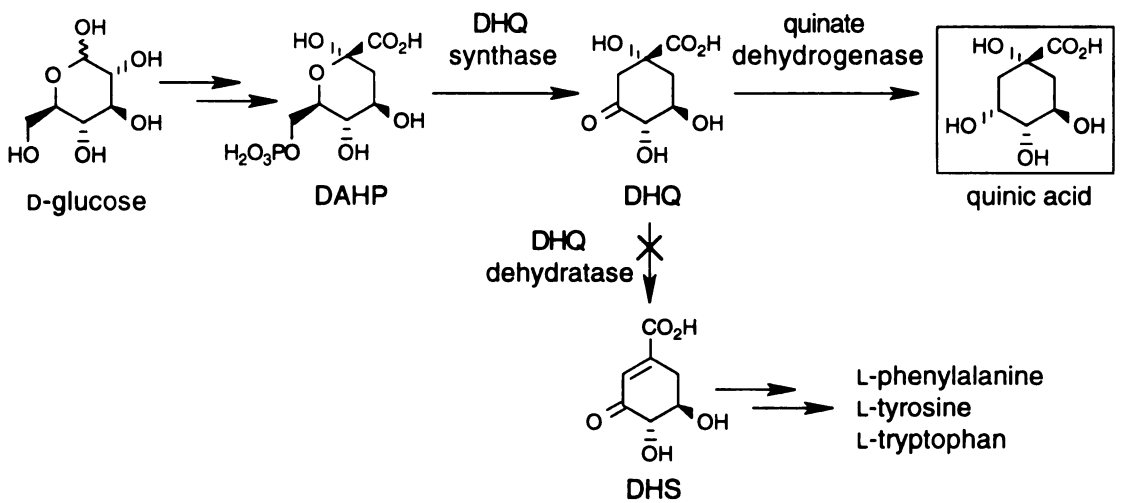


Figure 3 - Biocatalytic Synthesis of Quinic Acid from D-Glucose

Quinic acid synthesis in *E. coli* requires the conversion of D-glucose into 3-dehydroquinate (DHQ) via the common pathway. DHQ can be converted into quinic acid by quinic acid dehydrogenase,[26] an enzyme not found in *E. coli*. The quinic acid

dehydrogenase gene (*qad*) was isolated from the microorganism *Klebsiella pneumoniae*, which shares a close evolutionary relationship with *E. coli*.[27] This relationship is evidenced by the fact that *K. pneumoniae* genes have been successfully expressed from their native promoters in *E. coli*.[28] Although *K. pneumoniae* utilizes quinic acid dehydrogenase for the catabolism of quinic acid to DHQ, the reduction of DHQ to quinic acid is the thermodynamically preferred direction of reaction.[26] The overexpression of quinate dehydrogenase, DAHP synthase, DHQ synthase, and transketolase on extrachromosomal plasmids was achieved in an *E. coli aroD* strain incapable of converting DHQ into DHS. The result was the creation of a heterologous microbe that converts D-glucose into quinic acid in 31 mol % yield.[23]

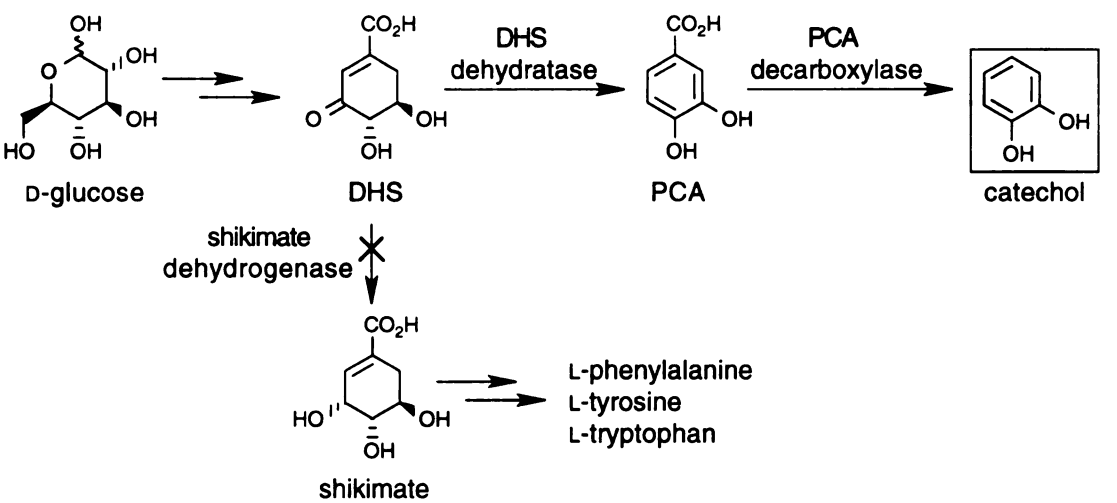
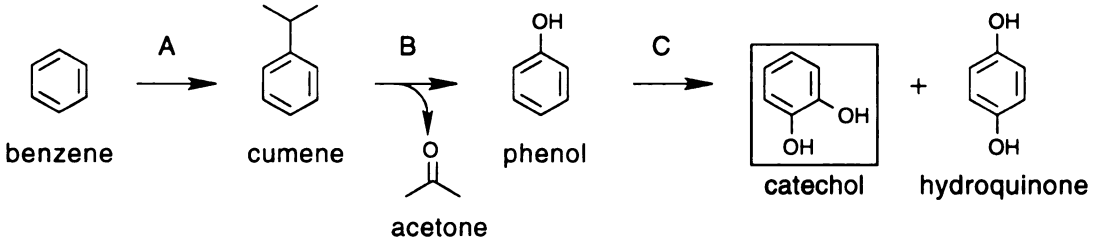


Figure 4 - Biocatalytic Synthesis of Catechol from D-Glucose

The biocatalytic synthesis of catechol from D-glucose also involved the recruitment of genes from *K. pneumoniae*.[24] The microbial biocatalyst was created by utilizing enzymes from the common pathway of aromatic amino acid biosynthesis and from hydroaromatic catabolism[29] (Figure 4). D-Glucose is converted to 3-dehydroshikimate

(DHS) through the common pathway. Catechol is formed from DHS by the action of two enzymes found in *K. pneumoniae*. DHS dehydratase[30] (encoded by *aroZ*) catalyzes the formation of protocatechuic acid (PCA) from DHS, and PCA decarboxylase[31] (encoded by *aroY*) converts PCA into catechol. Plasmid-based overexpression of DAHP synthase, DHQ synthase, and transketolase in an *E. coli aroE* strain that cannot convert DHS into shikimic acid results in the production of DHS from D-glucose.[32] Isolation of the *K. pneumoniae* genes *aroZ* and *aroY* was followed by amplification of DHS dehydratase and PCA decarboxylase in the DHS-producing construct. This heterologous biocatalyst synthesized catechol in 33 mol % yield from D-glucose.[24]



(A) propylene, solid H_3PO_4 catalyst, 200-260°C, 400-600 psi. (B) O_2, 80-130°C then SO_2, 60-100°C. (C) 70% H_2O_2, EDTA, Fe^{+2} or Co^{+2}, 70-80°C.

Figure 5 - Industrial Catechol Manufacture

This environmentally compatible synthesis contrasts sharply with the dominant method[33] of industrial catechol manufacture, which starts from petroleum-derived benzene (Figure 5). Benzene undergoes Friedel-Crafts alkylation to give cumene, which is subsequently converted into acetone and phenol in a Hock-type, air oxidation. Phenol is then oxidized with 70% hydrogen peroxide either in the presence of transition metal catalysts or in formic acid solution where the actual oxidant is performic acid. The resulting mixture of catechol and hydroquinone is separated into its pure components through successive distillations. The biocatalytic synthesis of catechol from D-glucose

simultaneously avoids the use of carcinogenic, petroleum-based starting materials and highly corrosive hydrogen peroxide.

Catechol can be transformed into *cis,cis*-muconic acid with catechol 1,2-dioxygenase encoded by *catA*.[29a] This gene is not possessed by *E. coli*, but has been isolated from *Acinetobacter calcoaceticus*.[34] Expression of *catA* in a microbe designed to produce catechol resulted in a new heterologous biocatalyst that converted D-glucose into *cis,cis*-muconic acid (Figure 6) in 30 mol % yield.[25] Catalytic hydrogenation of the *cis,cis*-muconic acid from the culture supernatant at 50 psi for 3 h at room temperature afforded adipic acid in 90% yield.[25]

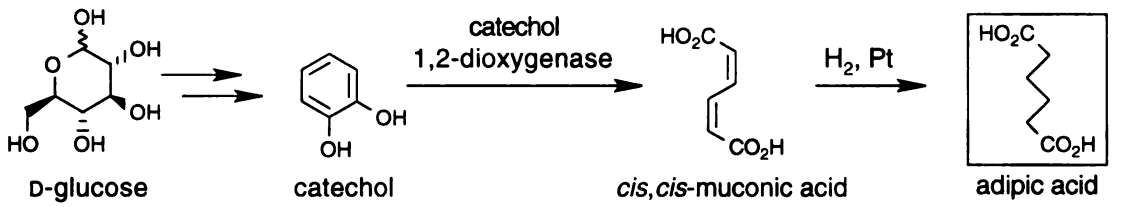
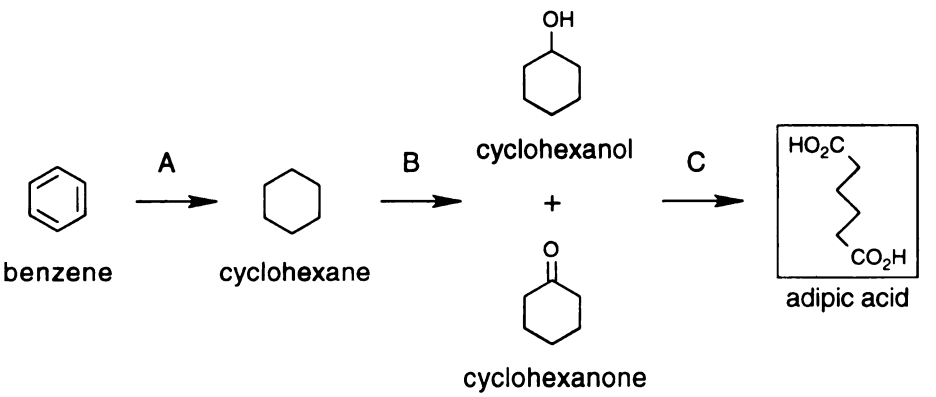


Figure 6 - Biocatalytic Synthesis of Adipic Acid from D-Glucose

As with the synthesis of catechol, industrial synthesis of adipic acid[33a-b,35] (Figure 7) poses several threats to the environment. Benzene is hydrogenated to produce cyclohexane, which is then oxidized in air to give a mixture of cyclohexanol and cyclohexanone. Nitric acid oxidation affords adipic acid. The byproduct of this last reaction, nitrous oxide,[36] is involved in depletion of the ozone layer and the greenhouse effect.[37] Some 10% of the annual increase in atmospheric nitrous oxide levels is related to adipic acid manufacture.[36] The industrial process requires forcing reaction conditions, with temperatures as high as 250°C and pressures reaching 800 psi.[33a-b,35] However, biocatalytic synthesis of adipic acid involves mild temperatures and pressures, avoids



(A) Ni-Al_2O_3, 370-800 psi, 150-250°C. (B) Co, O_2, 120-140 psi, 150-160°C. (C) Cu, NH_4VO_3, 60% HNO_3, 60-80°C.

Figure 7 - Industrial Adipic Acid Manufacture

generation of nitrous oxide, and starts with nontoxic D-glucose derived from abundant, renewable resources.

Design of microbial catalysts often involves recruitment of enzyme activities not found in *E. coli*. This has been illustrated in the biocatalytic syntheses of quinic acid, catechol, and adipic acid. A similar strategy was proposed for creating a biocatalyst capable of converting D-glucose into L-3,4-dihydroxyphenylalanine (L-DOPA), a compound that is primarily used for treatment of Parkinson's disease.[38] This required the activity of tyrosine phenol-lyase,[39] an enzyme not found in *E. coli*, which converts catechol, pyruvate, and ammonia into L-DOPA. In addition to the transaldolase study mentioned previously, this thesis will describe the isolation of the *tpl* gene encoding tyrosine phenol-lyase from *Citrobacter freundii* and the expression of *tpl* in a catechol-producing biocatalyst.

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CHAPTER 2

IMPACT OF TRANSALDOLASE ON THE BIOCATALYTIC PRODUCTION OF AROMATICS

Background

Biocatalytic synthesis of aromatics requires that a large percentage of the carbon source consumed by the microbe be committed to the common pathway of aromatic amino acid biosynthesis. This can be accomplished by increasing the specific activity of DAHP synthase, the first enzyme of the common pathway.[1] However, a point has been reached where the flow of carbon into the common pathway is not limited by the catalytic activity of DAHP synthase, but rather by the in vivo availability of the substrates D-erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP). Transketolase and transaldolase are the two enzymes of the pentose phosphate pathway that catalyze reactions leading to E4P (Figure 2, Chapter 1). Transketolase has been successfully overexpressed in *E. coli* and has been used to increase the delivery of carbon (D-glucose equivalents) into the common pathway.[2] However, the effect of transaldolase on the biosynthesis of aromatics has not been explored.

Transaldolase was first described by Horecker and Smyrniotis[3] in 1953. Present in animal, plant, and microbial cells, transaldolase is named for the fact that it catalyzes the transfer of aldol linkages from one sugar to another. Specifically, transaldolase transfers a three carbon dihydroxyacetone unit from a phosphorylated ketose donor to an aldose acceptor[4] (Figure 8). The mechanism of the reaction[4a] involves the initial formation of a Schiff base intermediate between the carbonyl group of the ketose substrate and the ε-amino group of a lysine residue at the active site of transaldolase (Figure 9). The proton of

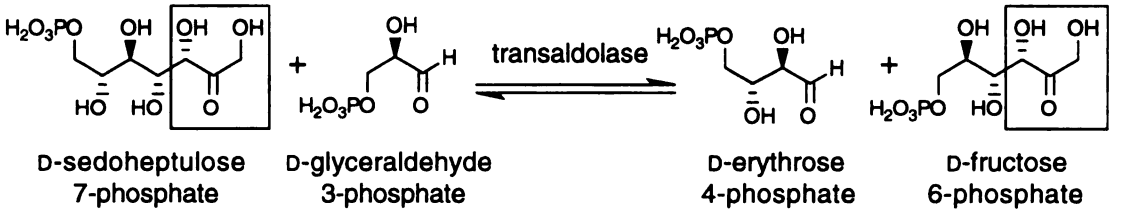


Figure 8 - Transaldolase Involves the Transfer of a Dihydroxyacetone Unit

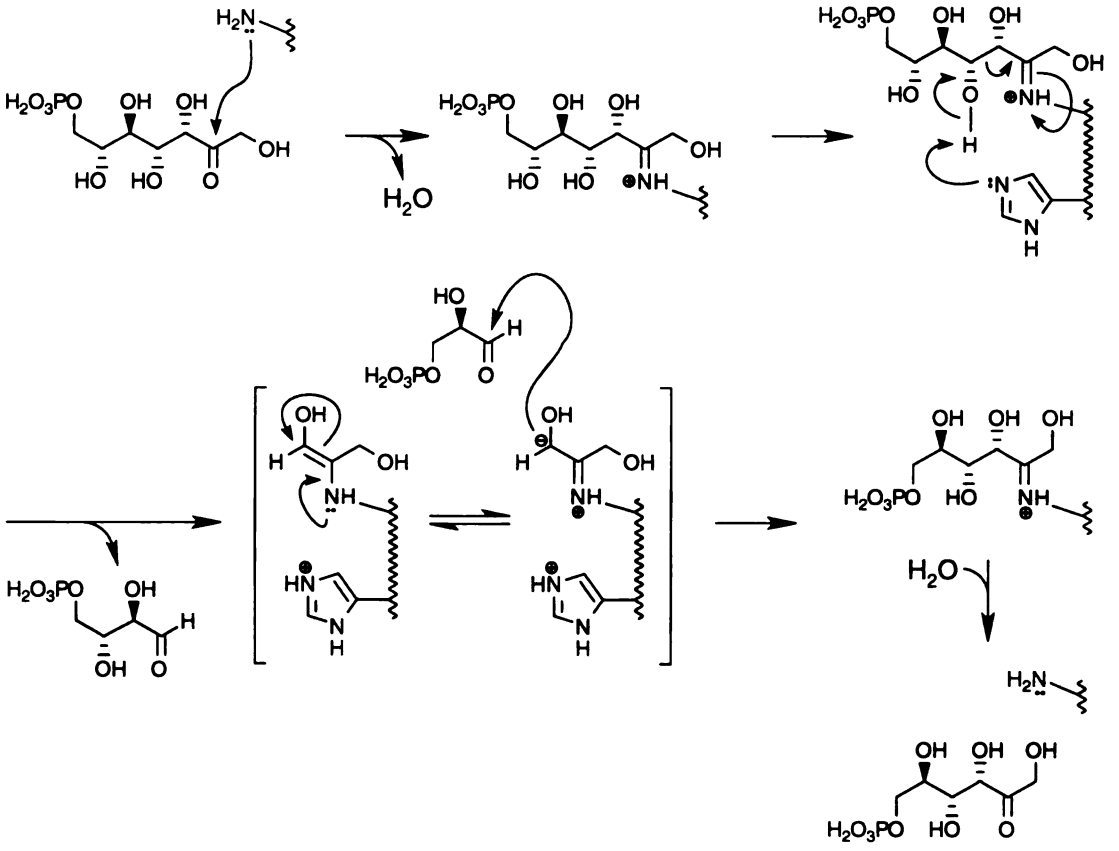


Figure 9 - Mechanism of Transaldolase

the C-4 hydroxyl group is abstracted by the imidazole of a histidine residue, promoting release of the aldose product. The Schiff base carbanion can then react with a suitable aldose acceptor that produces a ketose product upon hydrolysis.

The first step toward overexpression of transaldolase (or any enzyme) in *E. coli* is to isolate the gene that encodes for it. Once this has been achieved, the gene can be localized on an extrachromosomal plasmid vector.[5] Because genes that are plasmid-encoded are present in multiple copies, they are often expressed at higher levels than chromosomal genes. This leads to increased enzyme activity for the corresponding gene products. Technical advances in molecular biology have greatly facilitated the use of plasmids. It is generally easier and faster to increase gene expression by introducing plasmids into the cell rather than performing manipulations directly on the bacterial chromosome.

Overexpression of Transaldolase in *E. coli*

Isolation of the gene encoding transketolase was facilitated with an *E. coli* auxotrophic mutant.[5b] This strain possessed extremely low levels of transketolase and therefore was unable to grow on pentoses such as D-ribose, D-xylose, and D-arabinose. Complementation of this mutant with an *E. coli* genomic library led to the isolation of the *tktA* gene. Unfortunately, this strategy could not be applied to isolation of the transaldolase gene, because no *E. coli* transaldolase mutants are known. Perhaps *E. coli* possesses more than one enzyme (or a transaldolase isozyme) that can catalyze the reactions of transaldolase. In this case, deletion of transaldolase activity in *E. coli* might not result in an easily identifiable phenotype.

Two different regions of the *E. coli* chromosome have been identified which share homology with the transaldolase protein[6] from *Saccharomyces cerevisiae*. One is located at 53 min and was identified as an open reading frame upstream of *tktB*, a gene encoding the minor isozyme of transketolase.[7] The size of the open reading frame, designated as *talA*,

was not specified, and only 200 bp of the 3' end of the gene sequence was reported.[7] The second putative *tal* locus is at 0 min, and was discovered as a pair of open reading frames during the sequencing of the 0-2.4 min region of the *E. coli* genome.[8] Since the entire sequence of this region was available, it was amplified using the polymerase chain reaction (PCR).

PCR is an in vitro method for amplifying DNA fragments.[9] From the published sequence information, two oligonucleotide primers were designed to flank the locus at 0 min. The DNA that was amplified by PCR included the sequence that exhibited homology with yeast transaldolase[6] (954 bp) plus an additional 322 bp outside this region. This was done to ensure that the entire gene and its native promoter would be amplified. Genomic DNA from wild-type *E. coli* strain RB791[10] served as the template for PCR. The size of the major product from PCR was 1.3 kb, as expected.

The next step was to determine if this 1.3 kb DNA fragment (designated *talB*) encoded for transaldolase. This required localization of the fragment onto a plasmid vector. For cloning purposes, the 5'-ends of each PCR primer had been designed to contain the recognition site for endonuclease *Nco*I. The PCR-amplified DNA fragment was digested with *Nco*I and cloned into the *Nco*I site of pBR325[11] (Figure 10). The resulting plasmid, pMF52A, was transformed into wild-type *E. coli* strain DH5α.[12]

Transaldolase activity was quantified from crude extracts of DH5α and DH5α/pMF52A. This is a coupled enzyme assay[13] that measures the change in absorbance at 340 nm resulting from the disappearance of NADH. The specific activity of transaldolase from DH5α/pMF52A (8.6 units mg^{-1}) was approximately 6-fold higher than that of DH5α (1.5 units mg^{-1}), which verified that this 1.3 kb DNA fragment (*talB*) encodes for transaldolase.

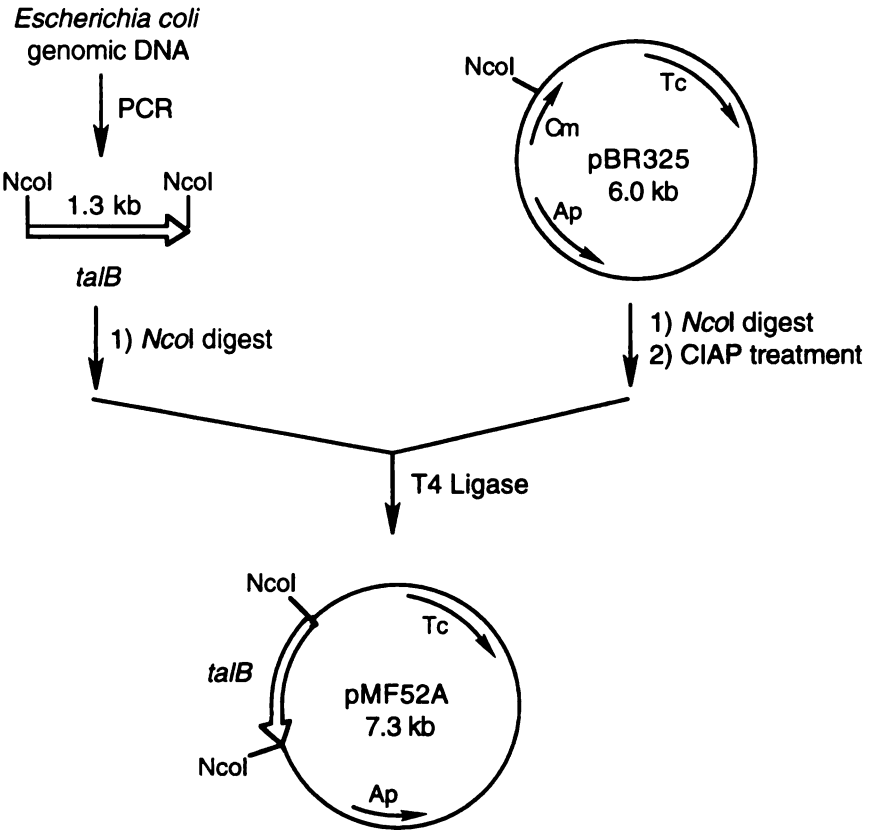


Figure 10 - Preparation of Plasmid pMF52A

Table 1 - Plasmids Constructed for DHS Accumulation Experiments

Plasmid	Genes Encoded
pMF61A	*aroF aroB*
pMF60A	*aroF aroB talB*
pMF63A	*aroF aroB tktA*
pMF65A	*aroF aroB talB tktA*

Impact of Transaldolase on Aromatic Production

Successful overexpression of transaldolase in *E. coli* led to experiments that measured the impact of transaldolase on delivery of carbon flow into the common pathway. Previous experiments with transketolase had been performed with *E. coli aroB* strains that possess an inactive DHQ synthase.[2] The DAHP that accumulates intracellularly is exported into the culture supernatant and can be quantified using a colorimetric assay.[14] The concentration of DAHP produced by the strain is a direct measure of the percentage of D-glucose committed to aromatic amino acid biosynthesis. However, results with transaldolase were attained using an *E. coli aroE* mutant for the following reasons. An *aroE* mutant blocked at shikimate dehydrogenase results in the accumulation of 3-dehydroshikimate (DHS) in the culture supernatant. Quantitation of DHS can be done with ^{1}H NMR analysis by comparison of the integrated resonances corresponding to DHS to the integrated resonance of an internal standard. This has advantages over the colorimetric assay, in that it is more reliable and reproducible. In addition, ^{1}H NMR facilitates detection and identification of any metabolite that may be accumulating in the culture medium.

For this study four plasmids were constructed (Table 1). Each plasmid contains *aroF* (encoding DAHP synthase) and *aroB* (encoding DHQ synthase). Amplified expression of DAHP synthase increases the amount of carbon flow directed into the

common pathway.[1] DHQ synthase is overexpressed to overcome the rate-limiting character of this enzyme.[15] The genes for transketolase (*tktA*) and transaldolase (*talB*) were then added in all possible combinations.

The *aroF* gene had previously been localized on a 2.4 kb *Eco*RI fragment.[2a,c] However, the *aroF* open reading frame is only 1068 bp.[16] In order to eliminate the extraneous DNA sequence, the *aroF* gene including its native promoter was amplified by PCR to give a 1.25 kb *Eco*RI fragment (Figure 11). This shortened *aroF* gene was then cloned into pBR325 to form plasmid pMF58A (Figure 11). Furthermore, the gene for transketolase had been originally isolated on a 5 kb *Bam*HI fragment,[2] but the size of the open reading frame is only 1992 bp.[17] The *tktA* gene was also amplified by PCR to eliminate nonessential DNA sequence, giving a 2.2 kb *Bam*HI fragment containing *tktA* with its native promoter (Figure 12). This 2.2 kb *tktA* fragment was cloned into pBR325 to give pMF51A (Figure 12). The *aroB* gene had been previously cloned as a 1.65 kb *Sph*I fragment into plasmid pKD136.[15a] This 1.65 kb DNA fragment was not shortened further by PCR. Instead, the *aroB* gene was simply isolated from *Sph*I digestion of pKD136.

The assembly of the four plasmids listed in Table 1 was carried out as follows. Plasmid pMF61A encodes *aroF* and *aroB* and was prepared by cloning the 1.65 kb *aroB* fragment into plasmid pMF58A (Figure 13). Plasmid pMF60A containing *aroF*, *aroB*, and *talB* was constructed in two steps. The *aroB* fragment was first cloned into pMF52A to give pMF57A (Figure 14). Then the 1.25 kb *aroF* fragment was ligated into pMF57A, forming pMF60A (Figure 14). Plasmid pMF63A containing *aroF*, *aroB*, and *tktA* was constructed by cloning *tktA* into pMF61A. (Figure 15). Finally, *talB* was cloned into pMF63A to produce pMF65A (Figure 15).

Each of these four plasmids (pMF60A, pMF61A, pMF63A, and pMF65A) was transformed separately into *E. coli* AB2834 *aroE*.[18] Because all plasmids were derived from pBR325,[11] they contain the gene that confers resistance to the antibiotic ampicillin.

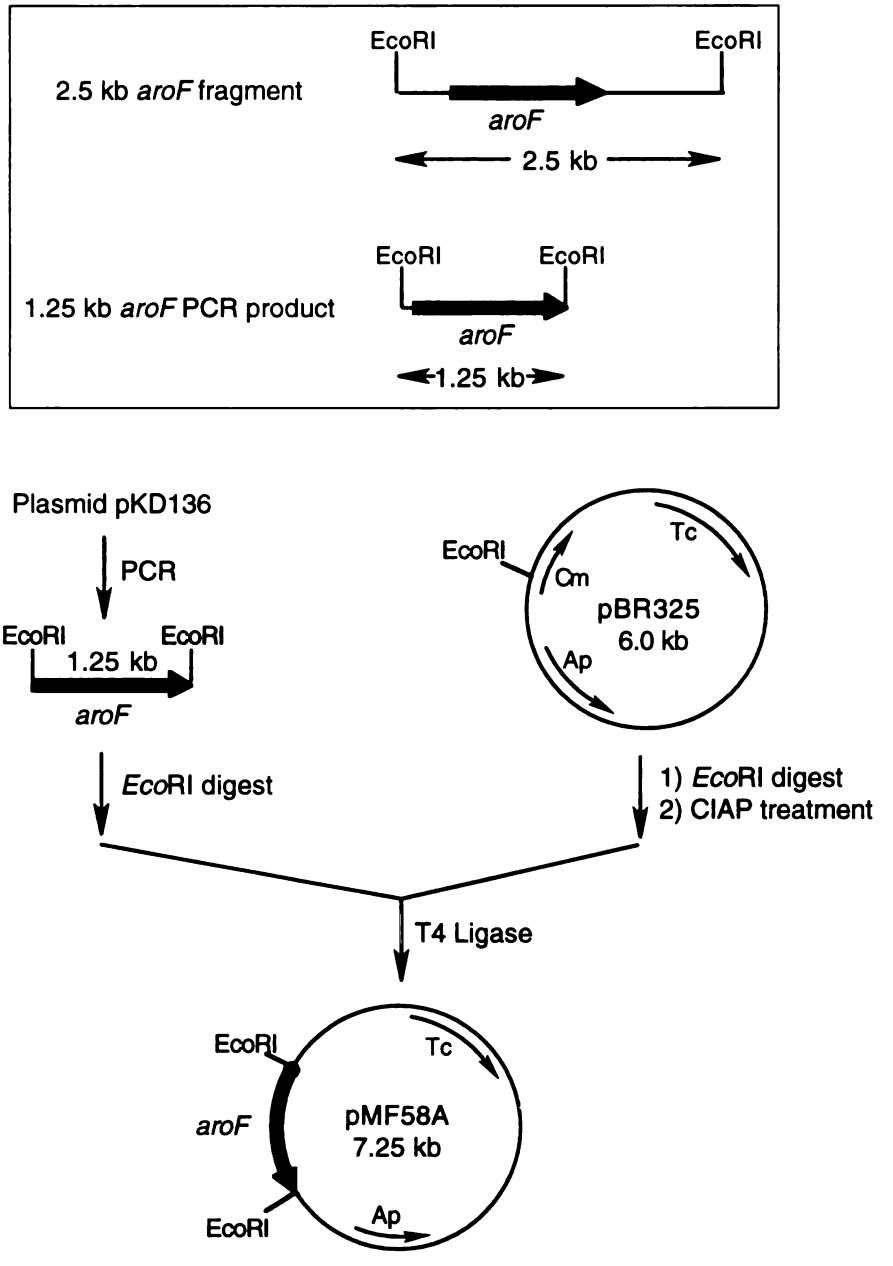


Figure 11 - Cloning the 1.25 kb *aroF* Fragment: Preparation of pMF58A

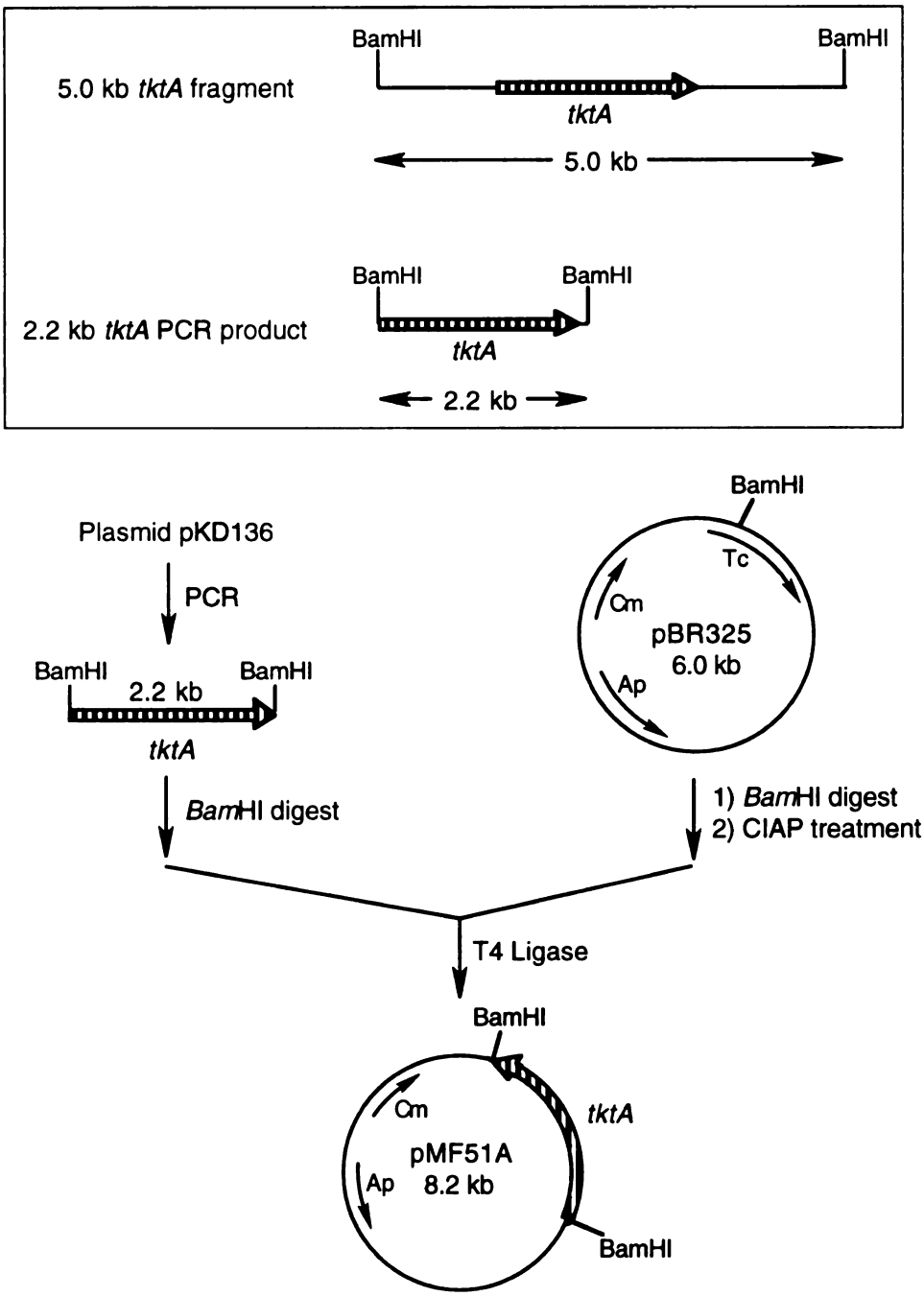


Figure 12 - Cloning the 2.2 kb *tktA* Fragment: Preparation of pMF51A

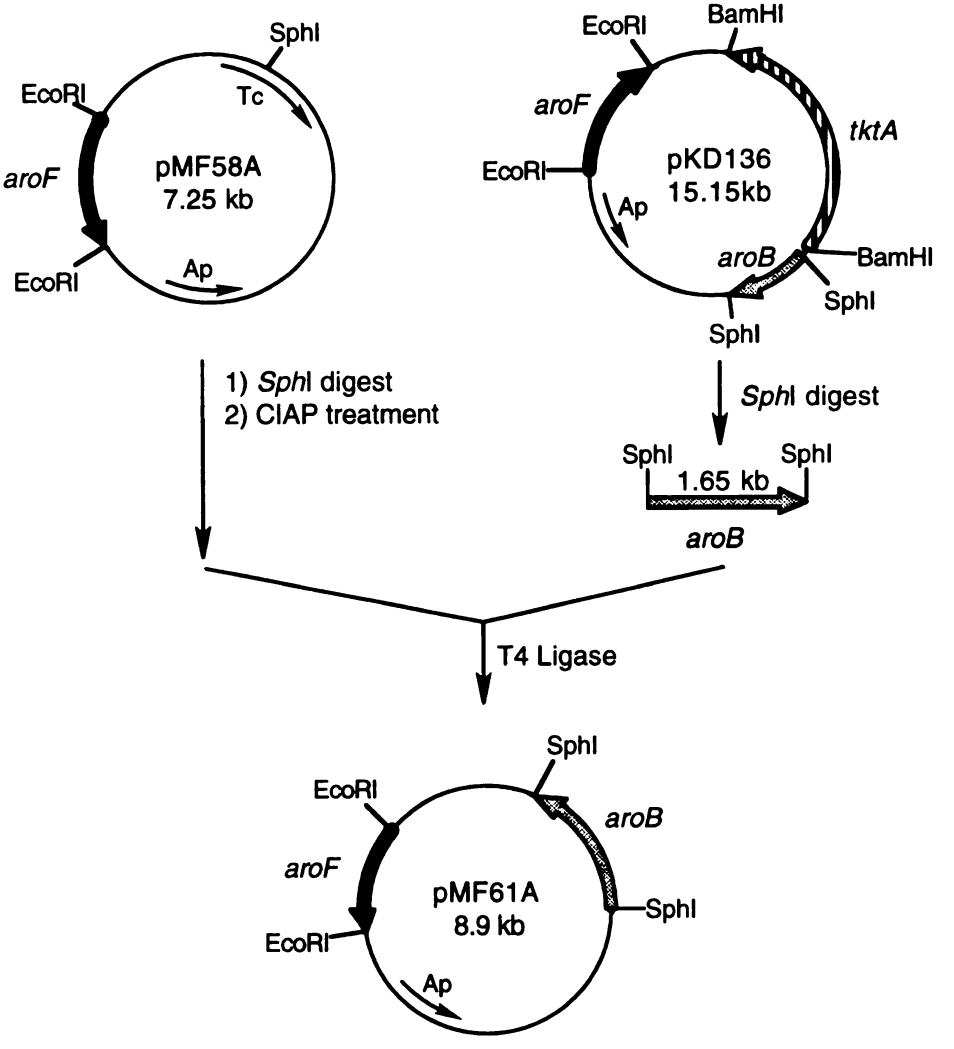


Figure 13 - Preparation of Plasmid pMF61A

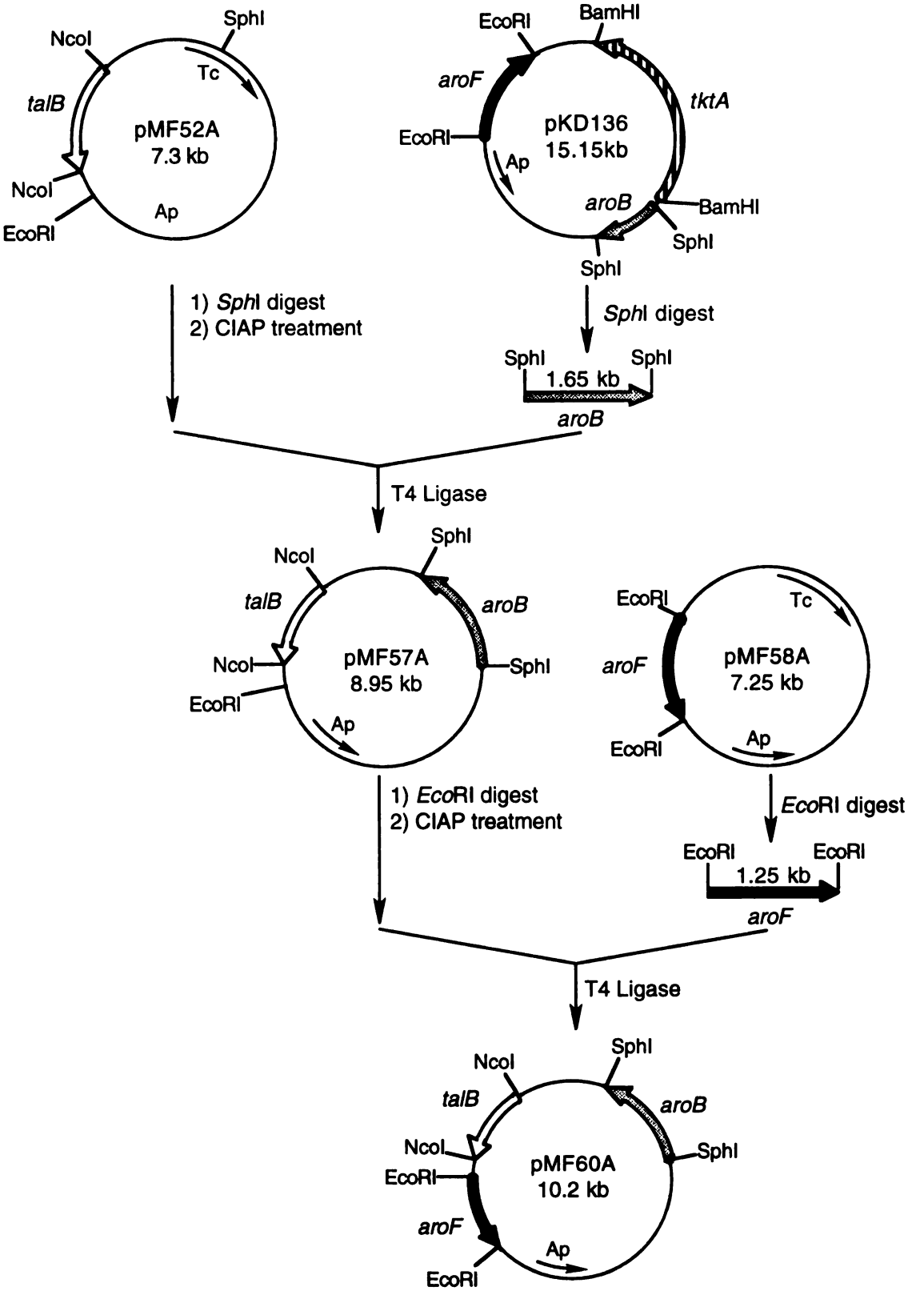


Figure 14 - Preparation of Plasmids pMF57A and pMF60A

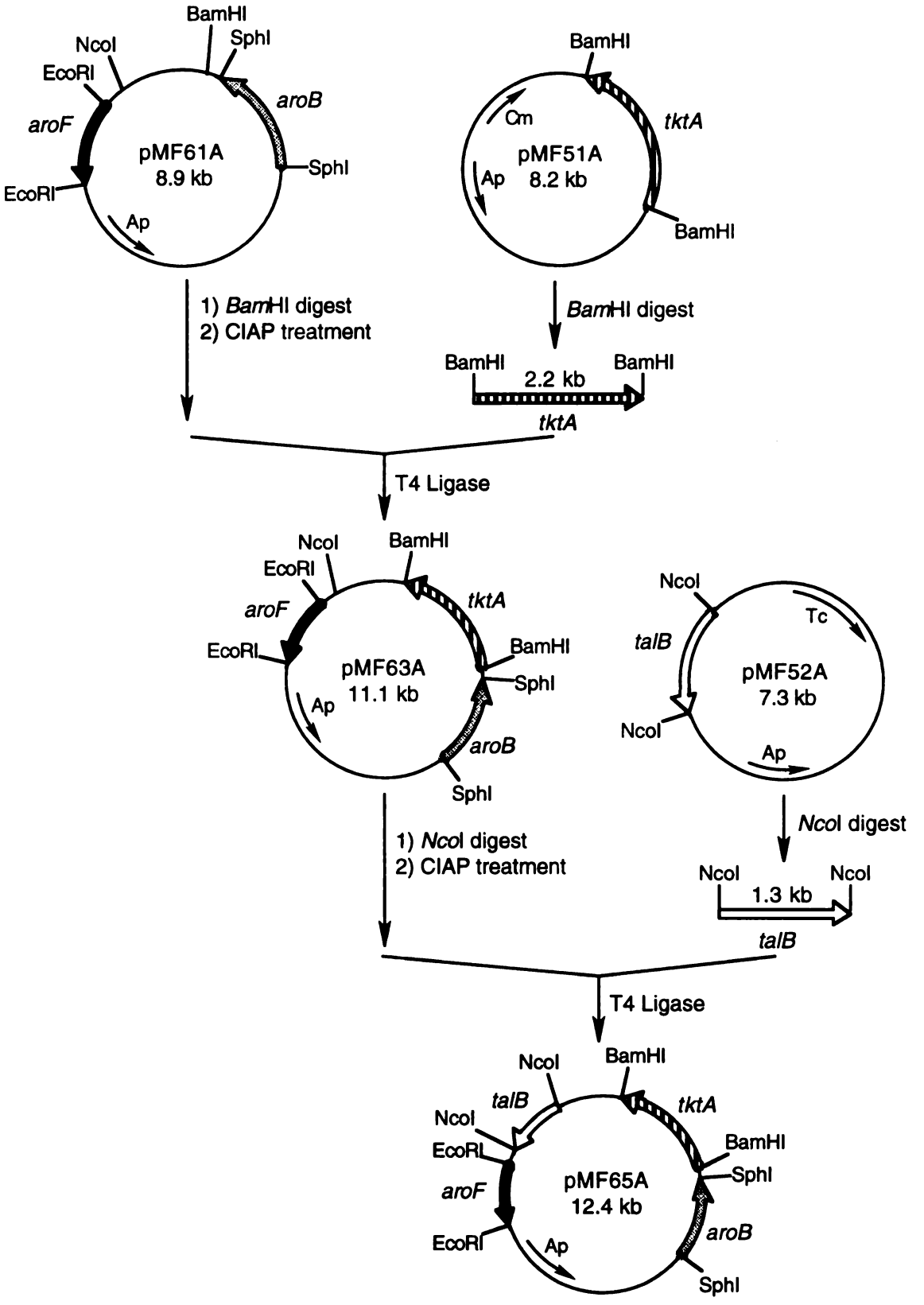


Figure 15 - Preparation of Plasmids pMF63A and pMF65A

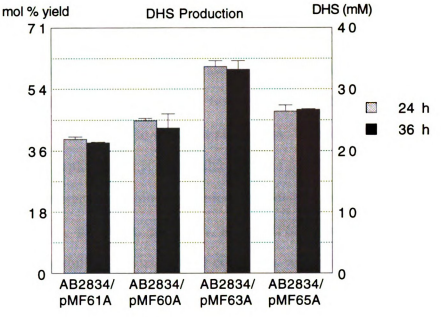


Figure 16 - DHS Production in Different *E. coli aroE* Strains

Addition of ampicillin to the culture medium provides selective pressure for the strain to maintain the plasmid. These strains were initially grown in rich medium, harvested, and then cultured in minimal medium that contained D-glucose. The culture supernatants were analyzed by ^{1}H NMR. The results from each strain are summarized in Figure 16.

^{1}H NMR analysis indicated that overexpression of transaldolase (AB2834/pMF60A compared to AB2834/pMF61A) improved the yield of DHS slightly, from 39 mol % to 44 mol %. Transketolase overexpression (AB2834/pMF63A) was more effective, producing DHS in 60 mol % yield. The strain that overexpressed both transketolase and transaldolase (AB2834/pMF65A) did not lead to further improvements in DHS production. The yield of DHS produced by AB2834/pMF65A was actually lower compared to AB2834/pMF63A, in which only transketolase was overexpressed. At this point it became necessary to determine if a metabolic burden was being imposed on AB2834/pMF65A resulting from

the overexpression of four proteins. The specific activity of DAHP synthase might be reduced to a point where the flow of carbon into the common pathway is limited.

In order to compensate for the potential limitations of DAHP synthase activity, *aroF* was localized on a second plasmid to increase expression levels further. To ensure plasmid compatibility and plasmid maintenance, two vectors must have compatible replication origins and encode resistance to different antibiotics. Plasmid pSU18[19] was chosen by virtue of its p15A replicon that is compatible with the pMB1 replicon of pBR325-derived plasmids. The resistance to chloramphenicol encoded by pSU18 distinguishes it from plasmids pMF60A, pMF61A, pMF63A, and pMF65A, which encode resistance to ampicillin. The stability of two-plasmid constructs was maintained in culture medium containing both antibiotics. The *aroF* gene was cloned into pSU18, generating pMF66A (Figure 17). Transformation of each of the strains listed in Figure 16 with pMF66A was followed by ^{1}H NMR analysis to determine the impact on DHS production. The specific activity of DAHP synthase was also measured[20] from crude extracts of all eight strains. The results are summarized in Figure 18.

The addition of *aroF*-encoding pMF66A increased the specific activity of DAHP synthase at least tenfold. However, this increase in enzyme activity did not significantly improve the DHS production, since the maximum yield of DHS was still 60 mol %. Furthermore, transaldolase and transketolase seemed to have little or no effect on improving the carbon flow into the common pathway in the presence of high levels of DAHP synthase. This suggested that the in vivo availability of D-erythrose 4-phosphate (E4P) was no longer rate-limiting under these conditions. Thus it became necessary to focus on increasing the intracellular supply of phosphoenolpyruvate (PEP).

Techniques have been developed that increase the PEP available for aromatic biosynthesis. It has been demonstrated that overexpression of PEP synthase increases the production of DAHP from D-glucose.[21] The PEP synthase effect is not observed without amplified expression of transketolase, which suggests that E4P is the first limiting

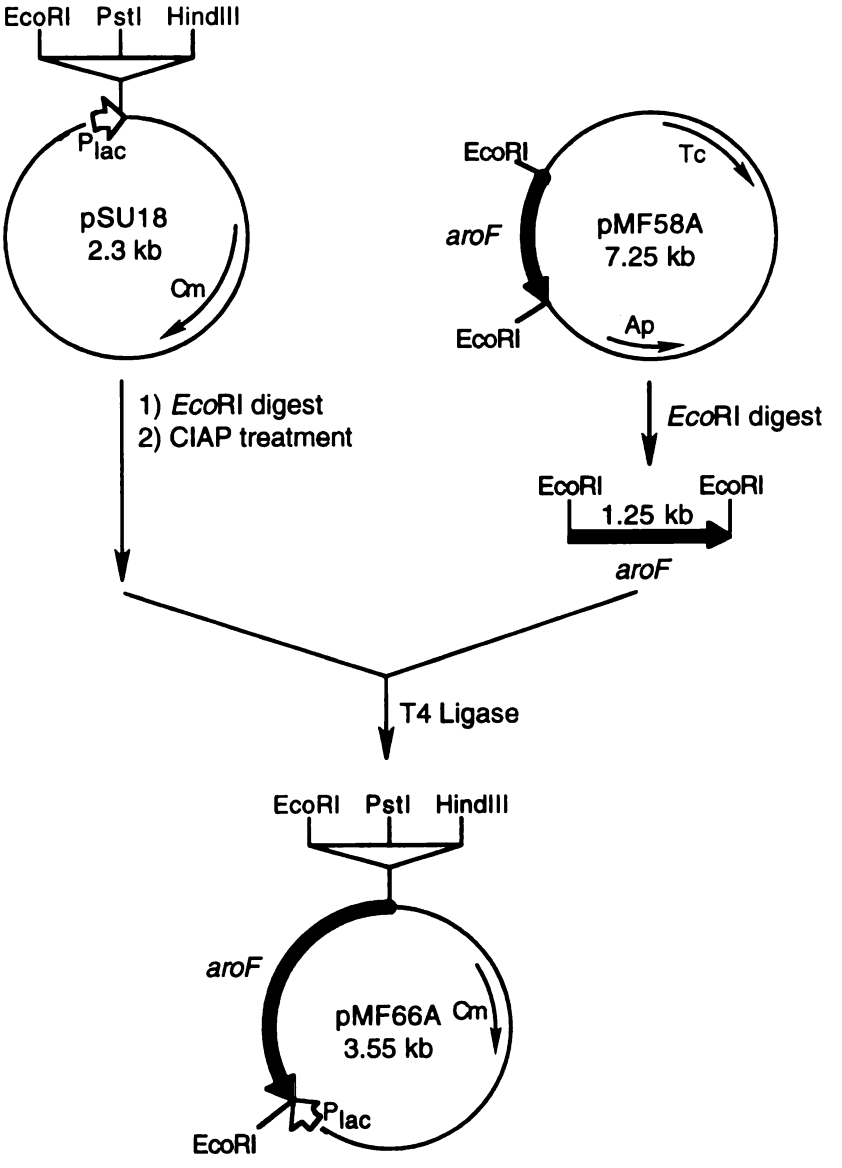


Figure 17 - Preparation of Plasmid pMF66A

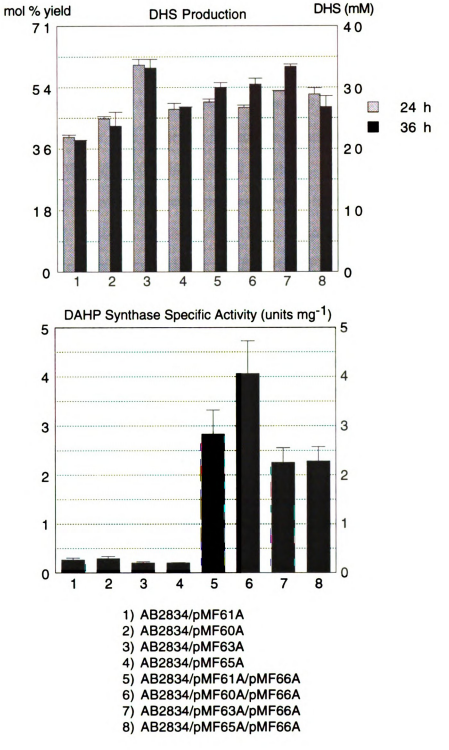


Figure 18 - Effect of Plasmid pMF66A on DHS Production

metabolite.[21a] Overexpression of PEP synthase (encoded by *pps*) was carried out in this laboratory by Kai Li. A 3.1 kb PstI fragment containing the *pps* gene was amplified by PCR and cloned into pSU19 to create pKL1.87A. The only difference between pSU19 and pSU18 is the orientation of its multiple cloning site relative to a *lac* promoter. The *aroF* gene was cloned into pKL1.87A to give pMF67 (Figure 19).

Transformation of each of the strains listed in Figure 16 with pMF67 was followed by analysis of the DHS production in each new construct. The results are listed in Figure 20. AB2834/pMF61A/pMF67, which expresses elevated levels of DAHP synthase, DHQ synthase, and PEP synthase, synthesized DHS in 55 mol % yield from D-glucose. Additional overexpression of transaldolase in AB2834/pMF60A/pMF67 improved the yield to 66 mol %. Transketolase overexpression in AB2834/pMF63A/pMF67 resulted in a 79 mol % yield of DHS. There was not a significant increase in the yield of DHS resulting from overexpression of both transketolase and transaldolase under these conditions, as AB2834/pMF65A/pMF67 synthesized DHS in 81 mol % yield.

At this point the effect of PEP synthase had only been observed under conditions where DAHP synthase activity was quite high. Maximizing the yield of DHS might not necessarily require such elevated DAHP synthase levels in the presence of overexpressed PEP synthase. Consequently each of the strains listed in Figure 16 were transformed with *pps*-encoding pKL1.87A. The DHS production from each new construct is reported in Figure 21. The results from these experiments illustrate the role of transaldolase in directing carbon flow into the common pathway. Comparing AB2834/pMF60A/pKL1.87A to AB2834/pMF61A/pKL1.87A, it appears that the overexpression of transaldolase had no impact on the production of DHS. However, comparison of AB2834/pMF65A/pKL1.87A to AB2834/pMF63A/pKL1.87A is quite different. An increase in the yield of DHS from 69 mol % to 80 mol % was obtained by increasing the catalytic activity of transaldolase.

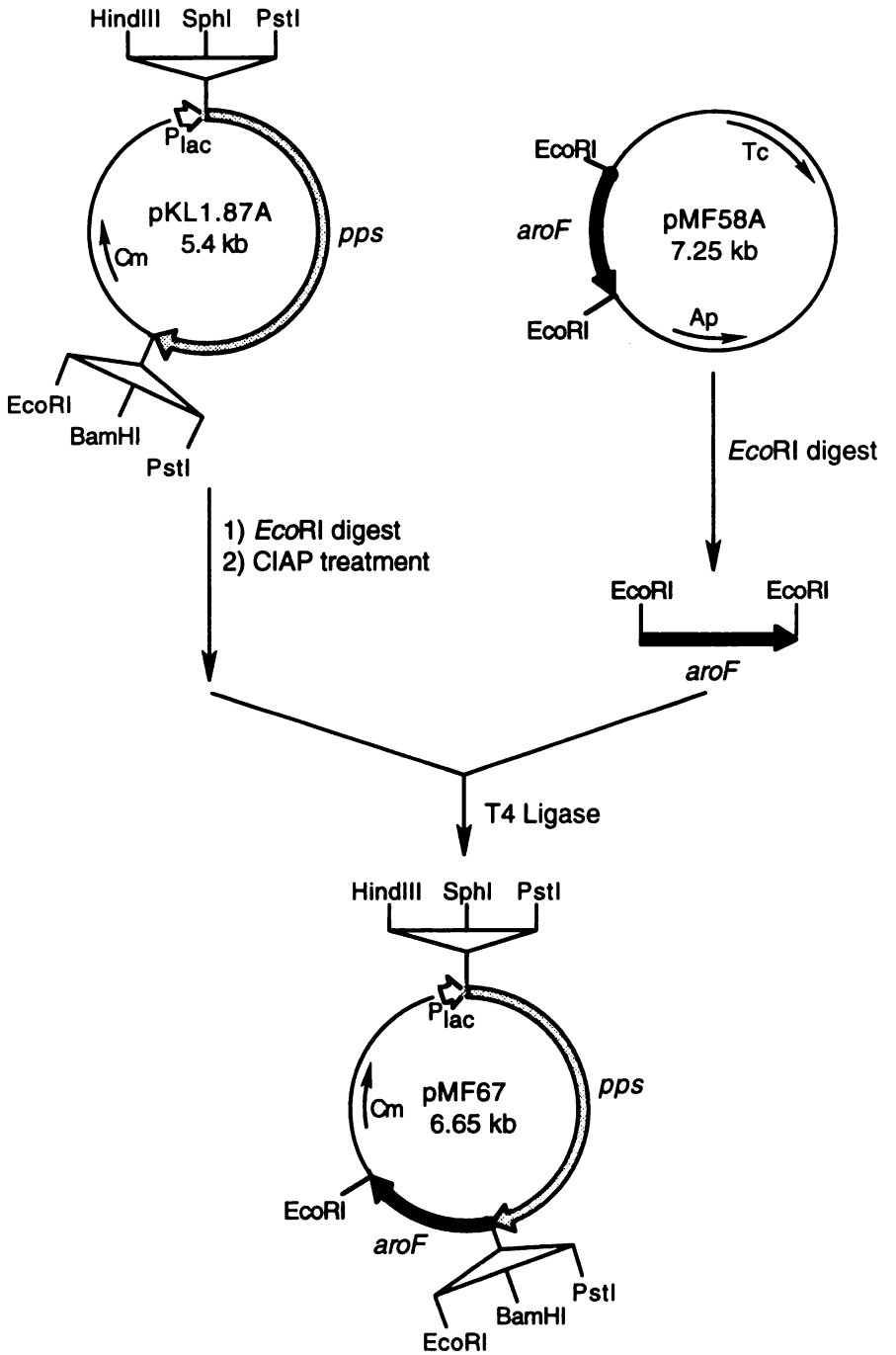
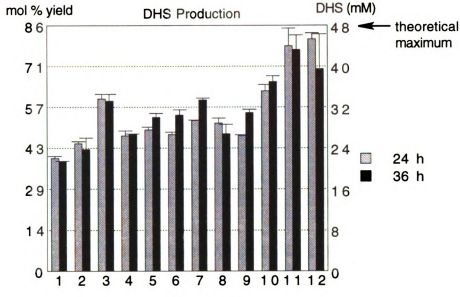
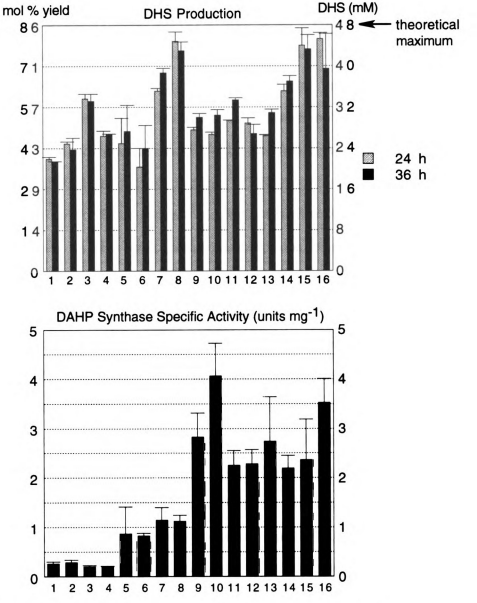


Figure 19 - Preparation of Plasmid pMF67



1) AB2834/pMF61A
2) AB2834/pMF60A
3) AB2834/pMF63A
4) AB2834/pMF65A
5) AB2834/pMF61A/pMF66A
6) AB2834/pMF60A/pMF66A
7) AB2834/pMF63A/pMF66A
8) AB2834/pMF65A/pMF66A
9) AB2834/pMF61A/pMF67
10) AB2834/pMF60A/pMF67
11) AB2834/pMF63A/pMF67
12) AB2834/pMF65A/pMF67

Figure 20 - Effect of Plasmid pMF67 on DHS Production



1) AB2834/pMF61A
2) AB2834/pMF60A
3) AB2834/pMF63A
4) AB2834/pMF65A
5) AB2834/pMF61A/pKL1.87A
6) AB2834/pMF60A/pKL1.87A
7) AB2834/pMF63A/pKL1.87A
8) AB2834/pMF65A/pKL1.87A
9) AB2834/pMF61A/pMF66A
10) AB2834/pMF60A/pMF66A
11) AB2834/pMF63A/pMF66A
12) AB2834/pMF65A/pMF66A
13) AB2834/pMF61A/pMF67
14) AB2834/pMF60A/pMF67
15) AB2834/pMF63A/pMF67
16) AB2834/pMF65A/pMF67

Figure 21 - Effect of Plasmid pKL1.87A on DHS Production

Discussion

Engineering *E. coli* to synthesize the highest possible yield of aromatics requires consideration of DAHP synthase activity as well as the availability of substrates E4P and PEP. It has been demonstrated that once DAHP synthase has been overexpressed to a certain level, E4P availability becomes limiting.[2] This limitation can be relieved by overexpressing transketolase (AB2834/pMF63A relative to AB2834/pMF61A). After in vivo E4P levels have been increased with transketolase, results suggest that the concentration of PEP becomes limiting. This problem can be addressed with overexpression of PEP synthase (AB2834/pMF63A/pKL1.87A relative to AB2834/pMF63A). At this point there are two options for augmenting the carbon flow into the common pathway. One option is to increase further the catalytic activity of DAHP synthase (AB2834/pMF63A/pMF67 relative to AB2834/pMF63A/pKL1.87A). Alternatively, the specific activity of transaldolase can be increased (AB2834/pMF65A/pKL1.87A relative to AB2834/pMF63A/pKL1.87A). Both strategies increase the yield of DHS to a level approaching the theoretical maximum[21a] (86 mol %). Considering that unnecessary overexpression of common pathway enzymes is a waste of metabolic currency, achieving a high yield of DHS with only modest increases in DAHP synthase activity (AB2834/pMF65A/pKL1.87A) is an important result.

The plasmids used in this study contained a wild-type *aroF* gene that encodes for the isozyme of DAHP synthase that is sensitive to feedback inhibition by tyrosine. Further improvements in the yield of aromatics might be achieved with a DAHP synthase protein that is feedback-resistant to the aromatic amino acids. Although a feedback-resistant *aroF* isozyme was not available in this laboratory, DHS production in AB2834*aroE* strains was examined with plasmids in which the wild-type *aroF* gene was replaced with a feedback resistant *aroG* locus.[2b, 21] However, no improvement in the yield of DHS relative to wild-type *aroF* was observed. This result might be explained by the fact that the cultures were initially grown in rich medium and then transferred to minimal medium containing glucose.

A feedback insensitive DAHP synthase would be essential for large scale fermentation processes in which growth and production occur simultaneously in minimal medium. The growth of auxotrophs such as AB2834*aroE* in minimal medium requires supplementation with aromatic amino acids that could inhibit wild-type DAHP synthase.

Although transaldolase was used to increase the flow of carbon into the common pathway, other advantages of transaldolase overexpression may be realized in the future. For example, amplification of transaldolase could benefit the health of the microbial construct by lowering intracellular levels of D-sedoheptulose-7-phosphate (S7P). A perturbation in S7P levels resulting from elevated transketolase activity might have a general toxic effect, since this heptose phosphate is required for the biosynthesis of lipopolysaccharide, a cellular wall component in Gram-negative bacteria. While the full impact of transaldolase on microbial biocatalysis has not been completely defined, the results described in this chapter suggest that further studies of primary metabolic pathways in *E. coli* are essential for improving and optimizing the biocatalytic synthesis of aromatics.

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CHAPTER 3

PRODUCTION OF L-DOPA WITH TYROSINE PHENOL-LYASE

Background

Creating a surge of carbon flow into the common pathway of aromatic amino acid biosynthesis is essential for the development of microbial biocatalysts that synthesize aromatics. However, this is only part of the process. The desired biosynthetic pathway often requires recruitment of enzyme activities not found in *E. coli*. This has been described in the biocatalytic synthesis of catechol.[1] *Klebsiella pneumoniae* genes encoding 3-dehydroshikimate (DHS) dehydratase[2] and protocatechuic acid (PCA) decarboxylase[3] were introduced into an *E. coli* strain that synthesizes elevated levels of DHS. The resulting biocatalyst AB2834/pKD136/pKD9.069A synthesized catechol from D-glucose in 33 mol % yield[1] (Chapter 1, Figure 4).

Chemical products derived from catechol include flavors, agrochemicals, polymerization inhibitors, and antioxidants.[4] Catechol is also used to synthesize pharmaceuticals, such as L-3,4-dihydroxyphenylalanine (L-DOPA), which is primarily used in the treatment of Parkinson's disease.[5] A strategy was proposed for the biocatalytic conversion of D-glucose into L-DOPA (Figure 22). This required the activity of tyrosine phenol-lyase,[6] an enzyme not present in *E. coli*, which catalyzes the formation of L-DOPA from catechol, pyruvate, and ammonia.

Tyrosine phenol-lyase was identified in 1953 as the enzyme responsible for converting L-tyrosine to phenol in bacterial cultures.[7] A few years later it was demonstrated that this L-tyrosine-inducible enzyme catalyzes the stoichiometric conversion

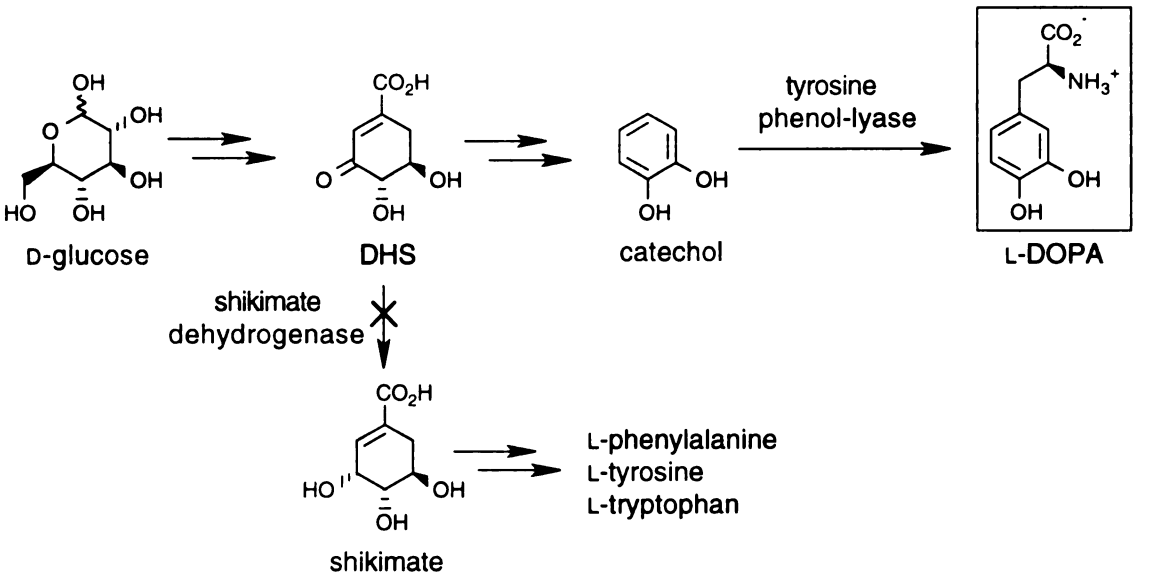


Figure 22 - Proposed Synthesis of L-DOPA from D-Glucose

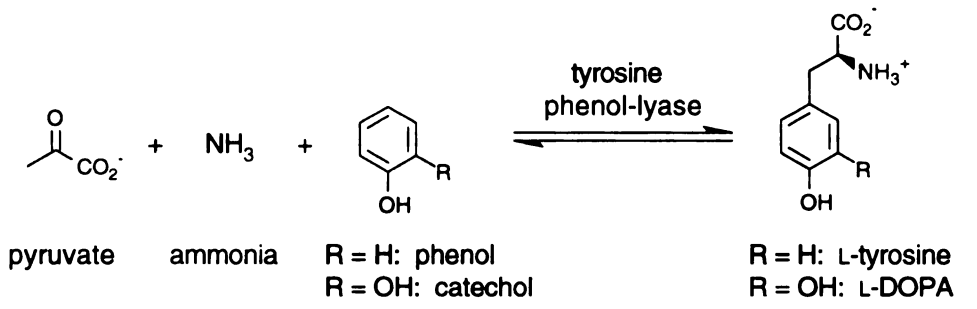


Figure 23 - Reactions Catalyzed by Tyrosine Phenol-Lyase

of L-tyrosine to phenol, pyruvate, and ammonia (Figure 23) and requires pyridoxal 5'-phosphate as a cofactor.[8] This α,β-elimination reaction is readily reversible at high concentrations of pyruvate and ammonia, leading to the formation of L-tyrosine from phenol.[9,10] When catechol is substituted for phenol, L-DOPA is formed[9,11] (Figure 23).

Spectrophotometric studies of tyrosine phenol-lyase indicated that ammonia is the first substrate that interacts with bound pyridoxal 5'-phosphate.[10b] Kinetic results showed that pyruvate is the second substrate bound, thus phenol (or catechol) is the third.[10b] The proposed mechanism[10b] for this reversible reaction is represented in Figure 24. Enzyme-bound pyridoxal 5'-phosphate interacts with ammonia, then pyruvate, resulting in the formation of an enzyme-bound α-aminoacrylate intermediate. Phenol is then added, which leads to the release of the L-tyrosine product. The reverse of this reaction scheme would most likely occur in the degradation of L-tyrosine.

Overexpression of Tyrosine Phenol-Lyase in *E. coli*

Tyrosine phenol-lyase has been studied extensively in *Escherichia intermedia*,[6a-c] *Erwinia herbicola*,[6d] and *Citrobacter freundii*.[6e] The gene encoding this enzyme (*tpl*) has been cloned and expressed in *E. coli*, and the DNA sequence has been published.[12] PCR amplification of the *tpl* gene was performed using genomic DNA isolated from *C. freundii* (ATCC 29063). Initially, a 2.1 kb *Kpn*I fragment of DNA was obtained from PCR that included the complete *tpl* gene. This DNA fragment was cloned into pSU18 to give pMF38A (Figure 25). Transformation of wild-type *E.coli* strain DH5α with pMF38A was followed by an enzyme assay[6e] for tyrosine phenol-lyase. The specific activity of tyrosine phenol-lyase from the crude extract of DH5α/pMF38A was 0.008 units mg^{-1}. Tyrosine phenol-lyase activity in DH5α/pMF38A was increased to a maximum value (0.033 units mg^{-1}) by addition of 0.1% L-tyrosine to the culture medium. This reflects the fact that expression of tyrosine phenol-lyase is normally induced by L-tyrosine.[9,13]

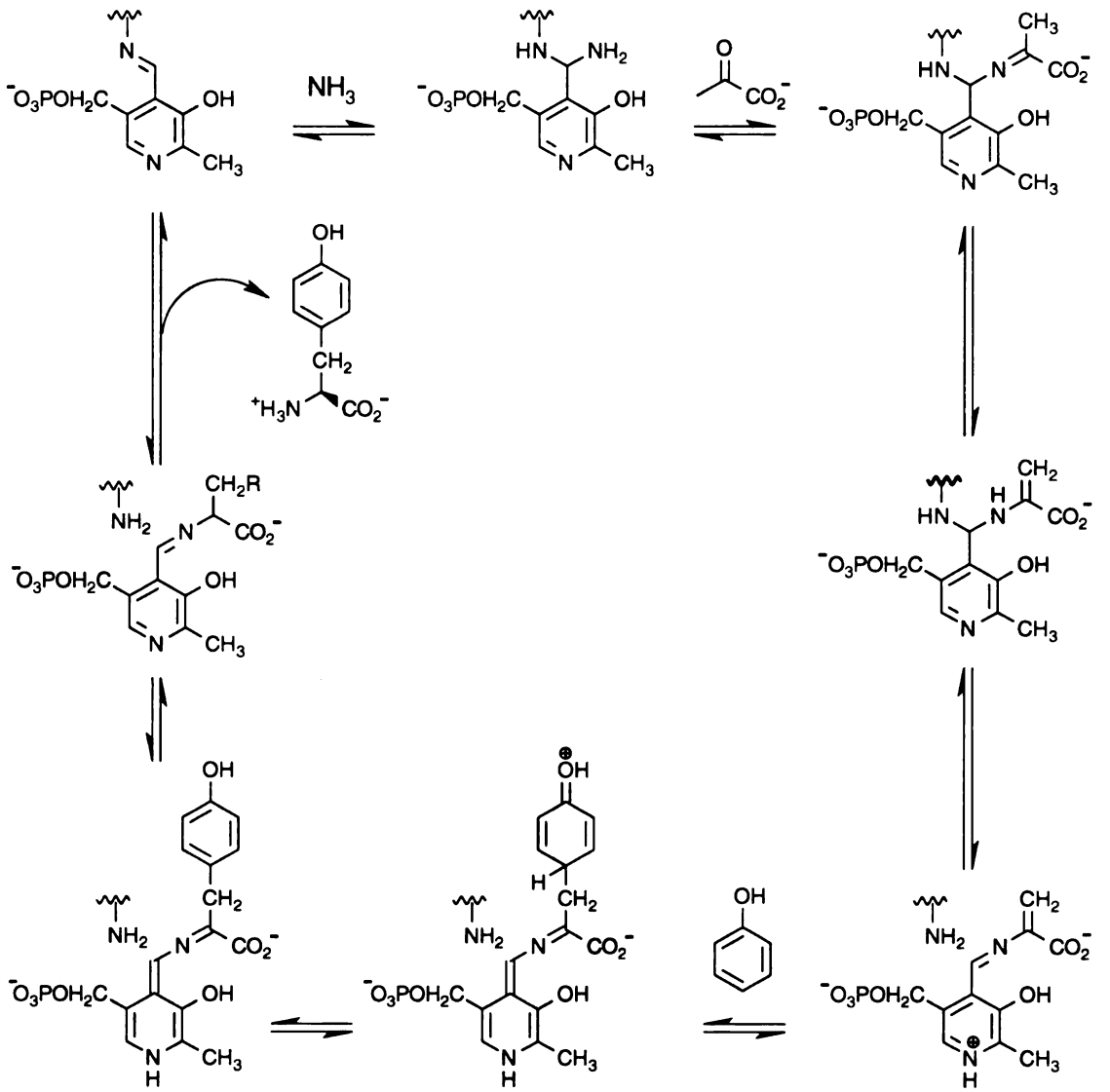


Figure 24 - Proposed Mechanism of Tyrosine Phenol-Lyase

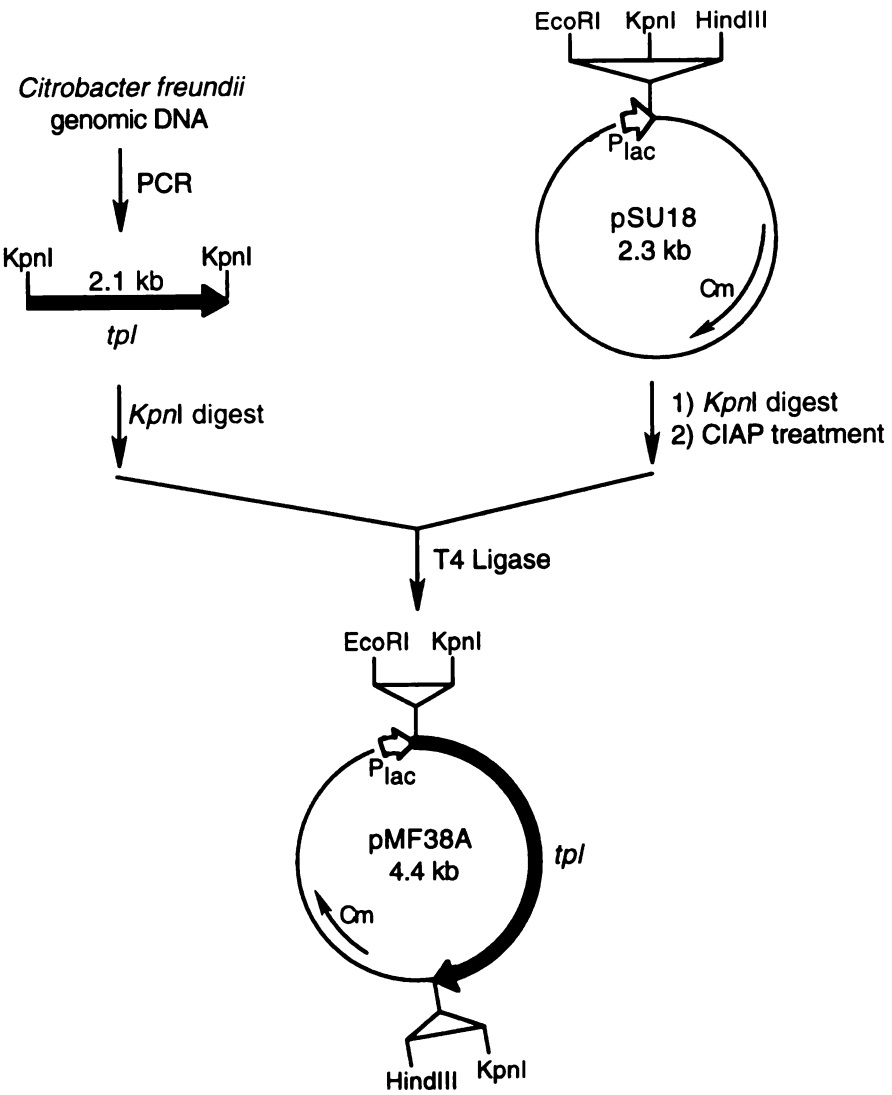
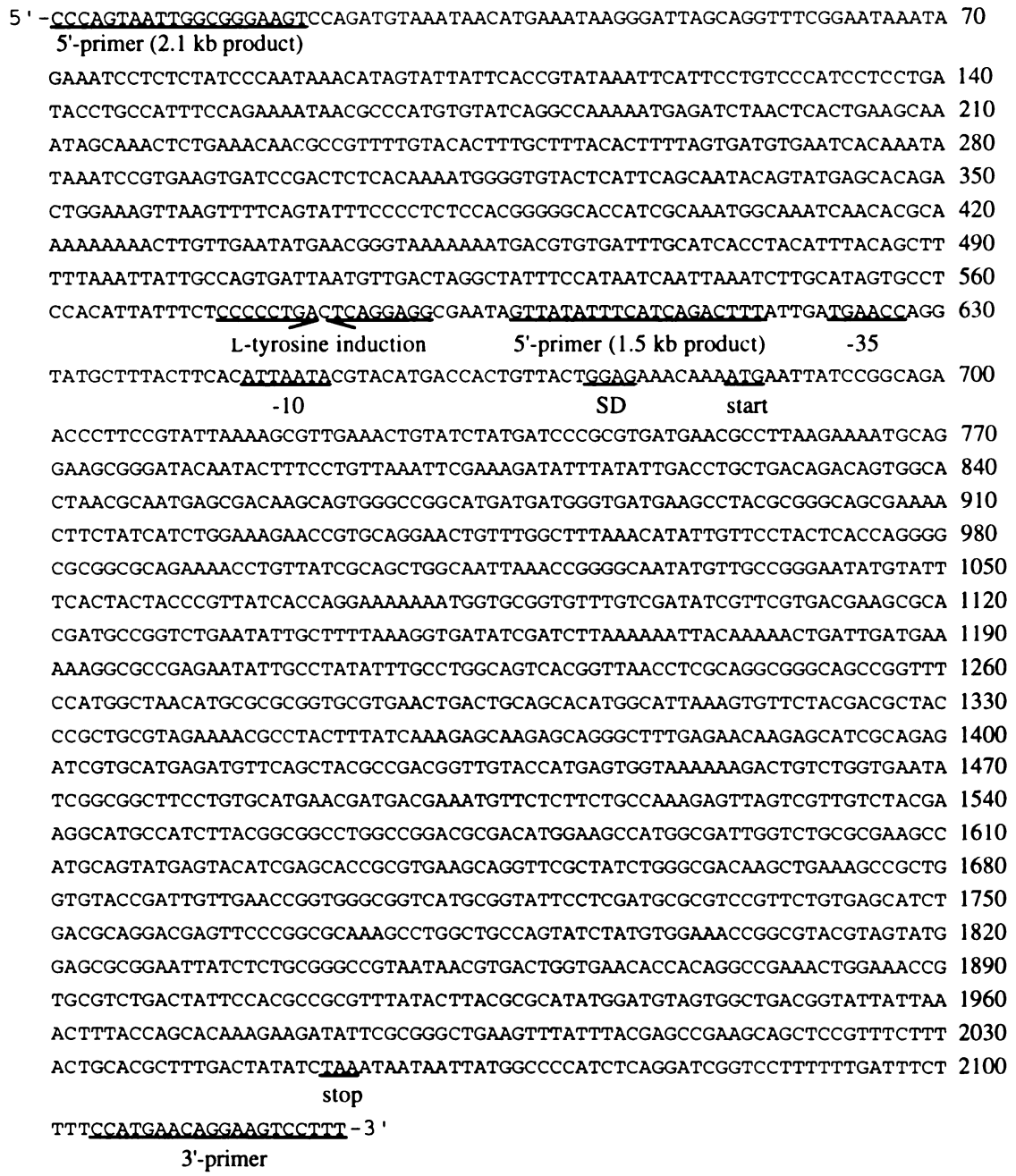


Figure 25 - Preparation of Plasmid pMF38A

Because the presence of L-tyrosine in the culture medium would be problematic, PCR of the *tpl* gene was repeated with a new set of oligonucleotide primers. This time the DNA sequence that was amplified by PCR did not include the region of DNA presumed to control induction by L-tyrosine[12a] (Figure 26). The product of this PCR reaction, a 1.5 kb *Kpn*I fragment, was cloned into pSU18 to form pMF42A (Figure 27). The specific activity of tyrosine phenol-lyase from crude extracts of DH5α/pMF42A was 0.065 units mg^{-1}. When 0.1% L-tyrosine was added to the culture medium, the tyrosine phenol-lyase activity was unchanged (0.068 units mg^{-1}), which verified that the induction by L-tyrosine had been eliminated.

Exploiting Tyrosine Phenol-Lyase for the Synthesis of L-DOPA

Successful overexpression of tyrosine phenol-lyase in *E. coli* was followed by incorporation of *tpl* into the catechol-producing construct AB2834/pKD136/pKD9.069A.[1] AB2834 is an *E. coli aroE* mutant that cannot convert DHS into shikimate. Plasmid pKD136[14] carries the genes *aroF*, *tktA*, and *aroB* encoding for DAHP synthase, transketolase, and DHQ synthase respectively. Amplified expression of DAHP synthase and transketolase increases the carbon flow directed into DAHP synthesis. Elevated levels of DHQ synthase ensure that DAHP is completely converted into DHS. Plasmid pKD9.069A contains the genes for DHS dehydratase[2] (*aroZ*) and PCA decarboxylase[3] (*aroY*). AB2834/pKD136/pKD9.069A synthesizes catechol in 33 mol % yield from D-glucose.[1] In order to create an L-DOPA-producing construct, the 1.5 kb *tpl* fragment from pMF42A was cloned into pKD9.069A to give pMF43A (Figure 28). AB2834/pKD136 was transformed with pMF43A. Despite the fact that AB2834/pKD136/pMF43A possesses all the genes required for the synthesis of L-DOPA from D-glucose, only catechol was observed as an end product. This required closer examination of the literature regarding the use of tyrosine phenol-lyase to synthesize L-DOPA from catechol.



The putative ribosomal binding site (SD), promoter sequences (-35, -10), start codon, and stop codon are underlined. The region that is responsible for induction by L-tyrosine is indicated by arrows. Two different 5'-primers were used for PCR. The size of the PCR product associated with each 5'-primer is indicated in parentheses.

Figure 26 - Nucleotide Sequence of the *Citrobacter freundii tpl* Gene

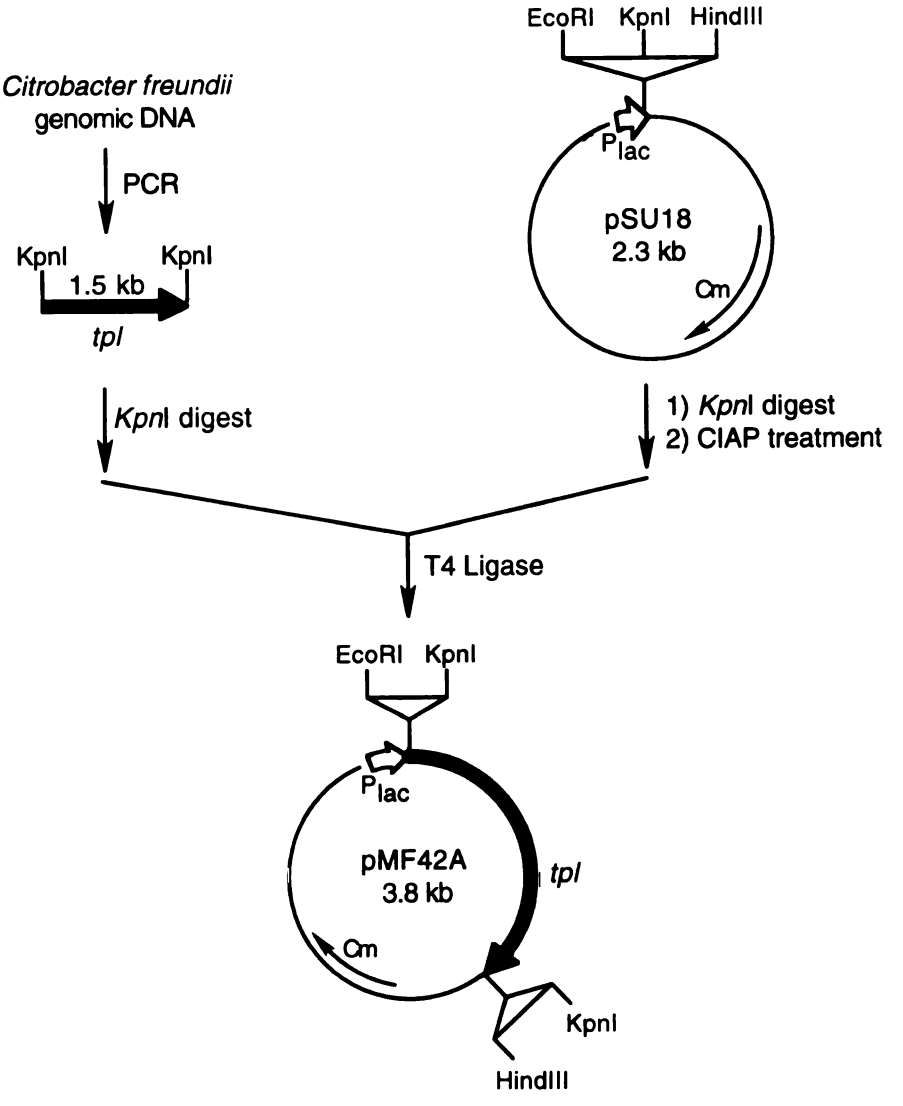


Figure 27 - Preparation of Plasmid pMF42A

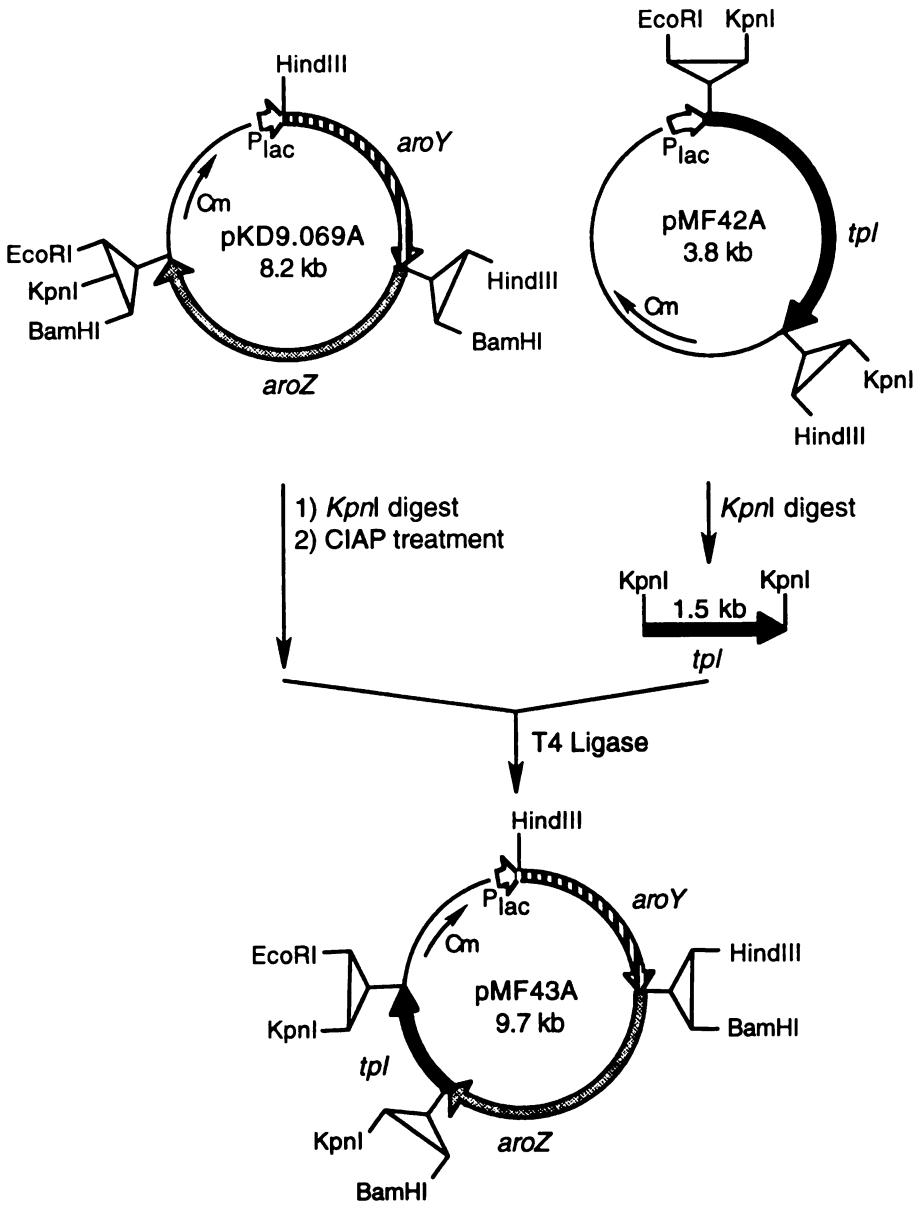


Figure 28 - Preparation of Plasmid pMF43A

When whole cells expressing elevated levels of tyrosine phenol-lyase are used to synthesize L-DOPA from catechol, the temperature of the reaction is typically 15-25 °C, and the pH is maintained at 8.0-8.5.[9,11b] The concentration of catechol is very high[11b] (230 mM) or maintained at a steady concentration[9] (about 65 mM) by frequent additions to the reaction mixture. These conditions contrast with the method in this laboratory for producing catechol from D-glucose. Biocatalysts such as AB2834/pKD136/pKD9.069A are incubated at 37 °C in a medium containing glucose buffered to near pH 7.0. In order to achieve the conversion of D-glucose to L-DOPA, variations in culture conditions were explored.

Biocatalytic conversion of D-glucose into catechol was extremely sensitive to the temperature at which the cells were incubated. As the temperature was lowered from 37 °C to room temperature, the purity of catechol produced by AB2834/pKD136/pMF43A was compromised by increasing levels of protocatechuic acid (PCA) in the culture supernatant. Decreased temperature also led to slower rates of D-glucose consumption, reducing the combined yield of end products. The effect of pH adjustment was also explored. As the pH of the culture medium was increased from 7.0 to 8.0, the amount of PCA synthesized by AB2834/pKD136/pMF43A increased at the expense of catechol. In an effort to drive the reaction equilibrium toward L-DOPA formation, pyruvate and ammonia were added to the culture medium. This decreased the consumption of D-glucose dramatically, which may have been the result of catabolic inhibition by pyruvate. Whole cells of AB2834/pKD136/pMF43A and DH5α/pMF42A, which both overexpress tyrosine phenol-lyase, were used to synthesize L-DOPA from catechol (230 mM), pyruvate (330 mM), and ammonia (330 mM) using a modified procedure described by Foor.[11b] Formation of L-DOPA was monitored by HPLC. The yield of L-DOPA produced from both strains was 12 mol % based on catechol. However, no experimental conditions were identified for converting D-glucose into L-DOPA with AB2834/pKD136/pMF43A.

Further information about the characteristics of tyrosine phenol-lyase was attained by determining the K_m values for catechol and L-tyrosine using Lineweaver-Burk analysis.[15] In the synthetic direction from catechol to L-DOPA, the K_m for catechol was 8 mM. In the degradation of L-tyrosine to phenol, however, the K_m for L-tyrosine was 0.4 mM. This suggests that the affinity of tyrosine phenol-lyase for L-tyrosine is roughly 20 times greater than for catechol. Therefore it is likely that the conversion of D-glucose to L-DOPA with AB2834/pKD136/pMF43A was hampered by an unfavorable reaction equilibrium. The kinetic data is also consistent with the fact that L-DOPA synthesis with tyrosine phenol-lyase requires high concentrations of catechol, pyruvate, and ammonia, presumably to drive the reaction forward.

Discussion

At this point it was suggested that the addition of tyrosine phenol-lyase activity to an L-tyrosine-producing construct might lead to the synthesis of phenol. This would require that carbon flow be directed through the common pathway and through the terminal pathway leading from chorismate to L-tyrosine. The most critical consideration for such a process is percent conversion. The theoretical maximum percent conversion of glucose into L-tyrosine is reduced (from 43 mol %) to 30 mol % because of the additional PEP required for EPSP synthesis.[16] Impediments to the biocatalytic synthesis of L-tyrosine that are caused by rate-limiting, common pathway enzymes would need to be removed. Selection of an appropriate host organism and elimination of pathways leading to undesirable by-products would also be essential. Although construction of a microbial biocatalyst that can convert D-glucose into phenol seems plausible, development of a biocatalytic route to phenol that can compete with traditional synthetic methods represents a more challenging task.

An alternate approach which might be developed for producing L-DOPA from D-glucose involves the catalytic action of tyrosine hydroxylase. In mammals, tyrosine

hydroxylase catalyzes the conversion of L-tyrosine into L-DOPA. The gene encoding this enzyme has been isolated from rat and mouse cDNA libraries and successfully expressed in *E. coli*.[17] The addition of tyrosine hydroxylase activity to an L-tyrosine-producing construct might lead to the synthesis of L-DOPA. However, while in vitro catalytic activity of tyrosine hydroxylase expressed in *E. coli* has been clearly demonstrated, achieving in vivo catalytic activity is problematic. Tyrosine hydroxylase requires 5,6,7,8-tetrahydrobiopterin as a cofactor. Dihydropteridine reductase is needed to regenerate the cofactor during the course of the reaction. *E. coli* does not biosynthesize biopterin nor does it possess a reductase system for regenerating it. The reductase could possibly be cloned and expressed in *E. coli*, or a different microbial host might be used which does synthesize and catalytically recycle biopterin. The production of L-DOPA from D-glucose might eventually be realized, provided that solutions are found for all of the impediments to tyrosine hydroxylase-catalyzed conversion of L-tyrosine to L-DOPA.

A number of techniques have been developed that increase the percentage of carbon flow directed into the common pathway of aromatic amino acid biosynthesis,[18-21] as well as deliver the carbon flow from DAHP to chorismate.[22] Recruitment of foreign genes into *E. coli* has been used to create microbial constructs that can synthesize aromatic chemicals from abundant, inexpensive D-glucose. The results of this chapter illustrate that the construction of *E. coli* strains for the production of aromatics is not as straightforward as it appears. Nevertheless, the experiments described in this thesis provide information that can be applied toward the development of future biocatalysts. Optimization of current processes and the engineering of new biosynthetic pathways in microorganisms represent fundamental challenges in biocatalysis. However, the effort required to surmount these challenges is justified by the benefits of environmentally compatible synthesis.

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CHAPTER 4

EXPERIMENTAL

General Methods

Chromatography

HPLC analysis utilized a Rainin HPLC system with a Rheodyne injector, HPXL pumps, and a model UV-1 detector. A Rainin C18 Microsorb-MV analytical column (4.6 mm x 25 cm) was used for separation and identification of components of reaction mixtures.

Dowex 50 (H^+ form, 100-200 mesh) was purchased from Sigma. Dowex 50 was cleaned before use as follows. An aqueous suspension of resin was adjusted to pH 14 with solid potassium hydroxide and allowed to cool to room temperature. Bromine (approximately 1 mL per 100 mL of resin) was added until the supernatant was clear yellow, and the suspension was left at room temperature for at least 3 h with occasional agitation. Dowex 50 resin was collected by filtration and washed extensively with water followed by 6 N HCl. The resin was poured into a glass column and washed with 6 N HCl (5 column volumes) followed by exhaustive rinsing with water (at least 5 column volumes).

Spectroscopic Measurements

^1H NMR spectra were recorded on a Varian Gemini-300 spectrometer at 300 MHz. Chemical shifts were reported in parts per million (ppm) downfield from internal sodium 3-

(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (TSP, δ = 0.00) with D_2O as solvent. TSP was purchased from Lancaster. UV and visible measurements were recorded on a Perkin-Elmer Lambda 3b UV-vis spectrophotometer connected to an R100A chart recorder. Additional UV spectra were measured on a Hewlett Packard 8452A Diode Array Spectrophotometer equipped with HP 89532A UV-Visible Operating Software.

Bacterial Strains

E. coli DH5α[1] [*F' endA1 hsdR17*($r^-_K m^+_K$) *supE44 thi-1 recA1 gyrA relA1 ϕ80lacZΔM15 Δ(lacZYA-argF)$_{U169}$*] and RB791[2] (W3110 *lacL8I^q*) were obtained previously by this laboratory. AB2834[3] [*tsx-352 supE42 λ^- aroE353 malA352 (λ^-)*]was obtained from the *E. coli* Genetic Stock Center at Yale University. *Citrobacter freundii* (ATCC 29063)[4] was obtained from the American Type Culture Collection.

Storage of Bacterial Strains and Plasmids

All bacterial strains were stored at -78 °C in glycerol. Plasmids were transformed into DH5α for permanent storage. Glycerol samples were prepared by adding 0.75 mL of an overnight culture to a sterile vial containing 0.25 mL of 80% (v/v) glycerol. The solution was mixed, left at room temperature for 2 h, and then stored at -70 °C.

Culture Medium

All solutions were prepared in distilled, deionized water. LB medium[5] contained (per liter) tryptone (10 g), yeast extract (5 g), and NaCl (10 g). M9 medium[5] contained (per liter) Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NH_4Cl (1 g), NaCl (0.5 g), $MgSO_4$ (0.12 g), thiamine (1 mg), and D-glucose (10 g). TB medium contained (per liter) tryptone (10 g), NaCl (5 g), and $MgSO_4$ (1.2 g). Isopropyl β-D-thiogalactopyranoside (IPTG) (0.2 mM)was added to the culture medium for strains possessing plasmids derived from pSU18 and pSU19. Ampicillin (50 μg mL^{-1}) and chloramphenicol (20 μg mL^{-1}) were added to

appropriate cultures. Stock solutions of antibiotics were prepared in water with the exception of chloramphenicol (100% ethanol). Solutions of inorganic salts, magnesium salts, and carbon sources were autoclaved separately and then mixed. Antibiotics, thiamine, and IPTG were sterilized through 0.2 μm membranes prior to addition to the culture medium. Solid medium was prepared by addition of 1.5 % (w/v) Difco agar to LB and 1.5 % (w/v) agarose (Sigma, type II: medium EEO) to M9 medium.

Culture Conditions

For analysis of product accumulation, bacterial strains were cultured as follows. One liter of LB (4 L Erlenmeyer flask) containing the appropriate antibiotics and IPTG was inoculated with 10 mL of an overnight culture. Cultures were grown at 37 °C in a gyratory shaker at 250 rpm for 10 h. Cells were collected by centrifugation (4000 x *g*, 5 min) and resuspended in 1 L of M9 medium (4 L Erlenmeyer flask) containing the appropriate antibiotics and IPTG. Cultures were then returned to the shaker (37 °C, 250 rpm).

^{1}H NMR Analysis of Culture Supernatant

Samples (25 mL) of the culture were taken at timed intervals, and the cells were removed by centrifugation (4000 x *g*, 5 min). A portion (2 mL) of the culture supernatant was concentrated to dryness under reduced pressure. The residue was redissolved in D_2O and concentrated to dryness (2 times). The residue was then redissolved in D_2O containing a known concentration of sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (TSP, $\delta = 0.00$). Concentrations of cellular metabolites in the supernatant were determined by comparison of the integrals of known metabolite resonances to the resonance corresponding to TSP in the ^{1}H NMR. Cultures were grown in triplicate to establish mean values and standard deviations.

General Genetic Manipulations

Recombinant DNA manipulations generally followed methods described in Sambrook *et al.*[6] All restriction enzymes were purchased from Gibco BRL or New England Biolabs. T4 DNA ligase was obtained from Gibco BRL. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. Agarose (electrophoresis grade) was obtained from Gibco BRL. Phenol was prepared by addition of 0.1 % (w/v) 8-hydroxyquinoline to distilled, liquefied phenol. Extraction with an equal volume of 1 M Tris-HCl pH 8.0 (two times) was followed by extraction with 0.1 M Tris-HCl pH 8.0 until the pH of the aqueous layer was greater than 7.6. Phenol was stored at 4 °C under an equal volume of 0.1 M Tris-HCl pH 8.0. SEVAG was a mixture of chloroform and isoamyl alcohol (24:1 v/v). TE buffer contained 10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0. Endostop solution (10X concentration) contained 50% glycerol (v/v), 0.1 M Na_2EDTA, pH 7.5, 1% sodium dodecyl sulfate (SDS) (w/v), 0.1% bromophenol blue (w/v), and 0.1% xylene cyanole FF (w/v). DNAse-free RNAse (10 mg mL^{-1}) was prepared according to Sambrook et al.,[6] and 0.12 mL RNAse was added to 1 mL of 10X endostop.

Large Scale Purification of Plasmid DNA

Plasmid DNA was purified on a large scale (0.5 mg) using a modified alkaline lysis method described by Sambrook *et al.*[6] LB (500 mL in a 2 L Erlenmeyer flask) was inoculated with a strain that contained the plasmid. The culture was incubated in a gyratory shaker (250 rpm) for 16 h at 37 °C. Cells were harvested by centrifugation (4000 x *g*, 5 min, 4 °C) and then resuspended in 10 mL of cold GETL solution [50 mM glucose, 20 mM Tris-HCl (pH 8.0), 10 mM Na_2EDTA (pH 8.0) into which lysozyme (5 mg mL^{-1}, Sigma) had been added immediately before use]. The suspension was stored on ice for 5 min. Addition of 20 mL of 1% (w/v) sodium dodecyl sulfate in 0.2 N NaOH was followed by gentle mixing and storage on ice for 15 min. To the sample was added 15 mL

of cold KOAc solution (3 M K^+, 5 M acetate, prepared by combining 60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid, and 28.5 mL of H_2O). Vigorous shaking resulted in formation of a white precipitate. After the suspension was stored on ice for 10 min, the cellular debris was removed by centrifugation (50000 x *g*, 20 min, 4 °C). The supernatant was transferred to two centrifuge bottles and isopropanol (0.6 volumes) was added to precipitate the DNA. After the samples were left at room temperature for 15 min, the DNA was recovered by centrifugation (20000 x *g*, 20 min, 4 °C). The DNA pellet was then rinsed with 70% ethanol and dried under vacuum (10 min).

Further purification of the DNA sample involved precipitation with polyethylene glycol (PEG). The DNA was dissolved in TE (3 mL) and transferred to a Corex tube. Cold 5 M LiCl (3 mL) was added, and the solution was gently mixed. The sample was then centrifuged (12000 x *g*, 10 min, 4 °C) to remove high molecular weight RNA. The supernatant was transferred to another Corex tube and isopropanol (6 mL) was added, followed by gentle mixing. The precipitated DNA was collected by centrifugation (12000 x *g*, 10 min, 4 °C). The DNA was then rinsed with 70% ethanol and dried. After redissolving the DNA in 1 mL of TE containing 20 µg of RNAse, the solution was transferred to a 1.5 mL microcentrifuge tube and stored at room temperature for 30 min. To this sample was added 500 µL of 1.6 M NaCl containing 13% PEG-8000 (w/v) (Sigma). The solution was mixed and centrifuged (microcentrifuge, 5 min, 4 °C) to recover the precipitated DNA. The supernatant was completely removed, and the DNA pellet was then redissolved in 400 µL of TE. The sample was extracted sequentially with phenol (400 µL), phenol and SEVAG (400 µL each), and finally SEVAG (400 µL). Ammonium acetate (10 M, 100 µL) was added to the aqueous DNA solution. After thorough mixing, 95% ethanol (1 mL) was added to precipitate the DNA. The sample was left at room temperature for 5 min and then centrifuged (microcentrifuge, 5 min, 4 °C). The DNA was rinsed with 70% ethanol, dried, and then redissolved in 500µL of TE.

The concentration of DNA in the sample was determined as follows. An aliquot (20 μL) of the DNA was diluted to 1 mL in TE, and the absorbance at 260 nm was measured relative to the absorbance of TE. The concentration of DNA was calculated based on the fact that the absorbance at 260 nm of a 50 μg mL^{-1} sample of plasmid DNA is 1.0. The purity of the DNA sample was estimated by calculating the ratio of the absorbance at 260 nm to the absorbance at 280 nm. If this ratio was greater than 1.8, the purity of the DNA was sufficient for cloning purposes.

Small Scale Purification of Plasmid DNA

Plasmid DNA was purified on a small scale (25 μg) using a modified alkaline lysis method described by Sambrook *et al.*[6] An aliquot (1.5 mL) from an overnight culture (5 mL) of the strain containing the plasmid was transferred to a microcentrifuge tube. The cells were collected by centrifugation (1 min, room temperature) and the culture supernatant discarded. The cell pellet was liquefied by vortexing (30 sec) and then resuspended in 0.1 mL of cold GETL solution. The solution was stored on ice for 10 min. Addition of 0.2 mL of 1% (w/v) sodium dodecyl sulfate in 0.2 N NaOH was followed by gentle mixing and storage on ice for 10 min. To the sample was added 0.15 mL of cold KOAc solution (3 M K^+, 5 M acetate). The solution was shaken vigorously and stored on ice for 5 min before centrifugation (15 min, 4 °C). The supernatant was transferred to another microcentrifuge tube and extracted with phenol (0.2 mL) and SEVAG (0.2 mL). DNA was precipitated by the addition of 95% ethanol (1 mL). The sample was left at room temperature for 5 min before centrifugation (15 min, room temperature) to collect the DNA. The DNA pellet was rinsed with 70% ethanol, dried, and redissolved in 100 μL TE. The DNA isolated from this method was used for routine cloning and restriction enzyme analysis, and the concentration was not quantified.

Restriction Enzyme Digestion of DNA

Restriction enzyme digests were performed using buffer solutions supplied by BRL or New England Biolabs. A typical digest contained approximately 1 μg of DNA in 8 μL TE, 1 μL of restriction enzyme buffer (10X concentration), and 1 μL of restriction enzyme (10 units). The reaction was incubated at 37 °C for 2 h. The digest was completed by addition of 1.1 μL of endostop solution (10X concentration) followed by agarose gel electrophoresis. When gel electrophoresis was not required, the reaction was stopped by addition of 1 μL of 0.5 M EDTA (pH 8.0) followed by precipitation of the DNA. DNA was precipitated by addition of 0.1 volume of 3 M NaOAc (pH 5.2), thorough mixing, and addition of 3 volumes of 95% ethanol. Samples were stored for at least 2 h at -78 °C. Precipitated DNA was recovered by centrifugation (15 min, 4 °C). To the DNA pellet was added 70% ethanol (100 μL), and the sample was centrifuged again (15 min, 4 °C). The DNA was dried and redissolved in TE.

Agarose Gel Electrophoresis

Agarose gels were run in TAE buffer containing 40 mM Tris-acetate and 2 mM EDTA (pH 8.0). The concentration of the gels was typically 0.7% agarose (w/v) in TAE buffer. Higher concentrations of agarose (1%) were used to resolve DNA fragments less than 1 kb. Ethidium bromide (0.5 μg mL^{-1}) was included in the agarose to visualize the DNA fragments with a UV lamp. The size of the DNA was determined by using two sets of DNA standards: λ DNA digested with *Hin*dIII or λ DNA digested with *Eco*RI and *Hin*dIII.

Isolation of DNA from Agarose

The band of agarose containing the DNA of interest was excised from the gel and chopped into smaller pieces with a spatula in a 1.5 mL microcentrifuge tube. Phenol (400 μL) was added to the agarose, and the sample was vortexed for several minutes. The

sample was placed in a dry ice/ethanol bath for 15 min and then centrifuged (15 min, 4 °C). The aqueous layer was transferred to another 1.5 mL microcentrifuge tube and then extracted sequentially with phenol (400 μL), phenol and SEVAG (400 μL each), and finally SEVAG (400 μL). The DNA was precipitated with 3 M NaOAc and 95% ethanol as previously described and redissolved in TE.

Treatment of Vector DNA with Calf Intestinal Alkaline Phosphatase

Plasmid vectors digested with a single restriction enzyme were dephosphorylated to prevent self-ligation. Digested vector DNA was dissolved in TE (88 μL). To this sample was added 10 μL of dephosphorylation buffer (10X concentration, Boehringer Mannheim) and 2 μL of calf intestinal alkaline phosphatase (2 units). The reaction was incubated at 37 °C for 1 h. The phosphatase was inactivated by addition of 1 μL of 0.5 M EDTA (pH 8.0) followed by heat treatment (65 °C, 20 min). The sample was extracted with phenol and SEVAG (100 μL each) to remove the phosphatase protein, and the DNA was precipitated as previously described and redissolved in TE.

Ligation of DNA

DNA ligations were designed so that the molar ratio of insert to vector was at least 3 to 1. A typical ligation reaction contained 0.1 to 0.5 μg of vector and 0.5 to 1 μg of insert in a total volume of 14 μL. To this sample was added 4 μL of ligation buffer (5X concentration, BRL) and 2 μL of T4 DNA ligase (2 units). The reaction was incubated at 16 °C for at least 4 h and then used to transform competent cells.

Preparation and Transformation of Competent Cells

Competent cells were prepared using a procedure modified from Sambrook *et al.*[6] An aliquot (1 mL) from an overnight culture (5 mL) was used to inoculate 100 mL of LB (500 mL Erlenmeyer flask) containing the appropriate antibiotics. The cells were cultured

in a gyratory shaker (37 °C, 250 rpm) until they reached the mid-log phase of growth (judged from the absorbance at 600 nm reaching 0.6). The culture was poured into a large centrifuge bottle that had been previously sterilized with bleach and rinsed with autoclaved water. The cells were collected by centrifugation (4000 x *g*, 5 min, 4 °C) and the culture medium discarded. At this point all further manipulations were carried out on ice. The cell pellet was washed with 100 mL of cold 0.9% (w/v) NaCl and then resuspended in 50 mL of cold 100 mM $CaCl_2$. The suspension was stored on ice for at least 30 min and then centrifuged (4000 x *g*, 5 min, 4 °C). The cell pellet was resuspended in 4 mL of cold 100 mM $CaCl_2$ containing 15% glycerol (v/v). Aliquots (0.25 mL) were dispensed into 1.5 mL microcentrifuge tubes and immediately frozen in liquid nitrogen. The competent cells were stored at -78 °C with no significant decrease in transformation efficiency over a period of several months.

Frozen competent cells were thawed on ice for 5 min before transformation. A small aliquot (1 to 10 µL) of plasmid DNA or a ligation reaction was added to the thawed competent cells (0.25 mL). The solution was gently mixed and stored on ice for 30 min. The cells were then heat shocked at 42 °C for 2 min and placed on ice briefly (30 s). LB (1 mL, no antibiotics) was added to the cells, and the sample was incubated at 37 °C (no agitation) for 1 h. Cells were collected in a microcentrifuge (30 s), resuspended in a small volume of LB (0.1 mL), and then spread onto LB plates that contained the appropriate antibiotics. A sample of competent cells with no DNA added was also carried through the transformation procedure as a control. These cells were used to check the viability of the competent cells and to verify the absence of growth on selective medium.

Purification of Genomic DNA

Genomic DNA was purified using a modified method described by Silhavy.[7] A single colony of the strain was inoculated into 100 mL of TB medium (500 mL Erlenmeyer flask). The cells were cultured in a gyratory shaker (37 °C, 250 rpm) for 12 h.

Centrifugation (4000 x *g*, 5 min, 4 °C) of the culture was followed by resuspension of the cell pellet in 5 mL of buffer [50 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0)] and storage at -20 °C for 20 min to freeze the suspension. To the frozen cells was added 0.5 mL of 0.25 M Tris-HCl (pH 8.0) that contained 5 mg of lysozyme. The suspension was thawed at room temperature in a water bath with gentle mixing and then stored on ice for 45 min. The sample was then transferred to a Corex tube. After addition of 1 mL of STEP solution [25 mM Tris-HCl (pH 7.4), 200 mM EDTA (pH 8.0), 0.5% SDS (w/v), and proteinase K (1 mg mL^{-1}, Sigma), prepared just before use], the mixture was incubated at 50 °C for at least 1 h with gentle, periodic mixing. The solution was then divided into two Corex tubes, and the contents of each tube were extracted with phenol (4 mL). The organic and aqueous layers were separated by centrifugation (1000 x *g*, 15 min, room temperature), and the aqueous layer was transferred to another Corex tube. All transfers of the aqueous layer were carried out using wide bore pipette tips to minimize shearing of the genomic DNA. The contents of each tube were extracted again with a mixture of phenol (3 mL) and SEVAG (3 mL). Extractions with phenol/SEVAG were repeated (6 to 7 times) until the aqueous layer was clear.

Genomic DNA was precipitated by addition of 0.1 volume of 3 M NaOAc (pH 5.2), gentle mixing, and addition of 2 volumes of 95% ethanol. Threads of DNA were spooled onto a sealed Pasteur pipette and transferred to a Corex tube that contained 5 mL of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), and 1 mg of RNAse. The mixture was stored at 4 °C overnight to allow the DNA to dissolve completely. The solution was then extracted with SEVAG (5 mL) and centrifuged (1000 x *g*, 15 min, room temperature). The aqueous layer was transferred to another Corex tube and the genomic DNA was precipitated as before with 3 M NaOAc and 95% ethanol. The threads of DNA were spooled onto a Pasteur pipette and redissolved in 2 mL of 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA (pH 8.0). Genomic DNA was stored at 4 °C.

Enzyme Assays

Cells were grown in LB medium as described under Culture Conditions. Centrifugation of the culture (4000 x *g*, 5 min, 4 °C) was followed by resuspension of the cell pellet in a buffer appropriate for the enzyme assay. The volume of resuspension buffer (mL) was usually twice the wet weight (g) of the cells. The cells were disrupted by two passages through a French pressure cell (SLM Aminco) at 18000 psi. Cellular debris was removed from the lysate by centrifugation (48000 x *g*, 20 min, 4 °C). Protein was quantified using the Bradford dye-binding procedure.[8] A standard curve was prepared using bovine serum albumin. The protein assay solution (5X concentration) was purchased from Bio-Rad.

Chapter 2

D-Erythrose 4-Phosphate

D-Erythrose 4-Phosphate (E4P) was synthesized by oxidation of D-glucose 6-phosphate with lead tetraacetate.[9] D-Glucose 6-phosphate (0.56 g, 2 mmol) was dissolved in water (4 mL) and added to 500 mL of glacial acetic acid in a 1 L three-necked round bottom flask. A solution of lead tetraacetate (1.51 g, 3.4 mmol) in 80 mL of acetic acid containing 1.2 mL of 6 N H_2SO_4 was added dropwise over a 2 h period under a nitrogen atmosphere with stirring. A fine white precipitate formed over the course of the reaction, which was removed upon filtration of the reaction mixture through Celite. The filtrate was concentrated by rotary evaporation to 80 mL and excess acetic acid was removed by azeotropic distillation with water (at least 5 times). The solution (80 mL) was loaded onto a column of Dowex 50 (H^+ form, 90 mL) and eluted with 300 mL of water. The concentration of E4P in the eluent was determined by using an aliquot of the solution as a substrate in the transaldolase assay,[10] which measures the change in absorbance at 340 nm resulting from a decrease in NADH (ε = 6220 L mol^{-1} cm^{-1}). Dilute E4P solutions (less

than 4 mM) were stored in a dark bottle at room temperature. The yield of E4P was 75% based on D-glucose 6-phosphate.

Assays

DAH(P)

The combined concentration of DAH and DAHP was determined by thiobarbituric acid visualization of the periodate cleavage products.[11] An aliquot (0.1 mL) of solution containing DAH/DAHP was added to 0.2 M $NaIO_4$ in 8.2 M H_3PO_4 (0.1 mL) and incubated at 37 °C for 5 min. The periodate oxidation was quenched by adding 0.5 mL of a solution containing 0.8 M $NaAsO_2$ and 0.5 M Na_2SO_4 in 0.1 M H_2SO_4. Thorough mixing of this sample was followed by addition of 3 mL of 0.04 M thiobarbituric acid in 0.5 M Na_2SO_4 (pH 7.0). The mixture was incubated at 100 °C for 15 min and the pink chromophore was extracted into 4 mL of distilled cyclohexanone. The organic and aqueous layers were separated by centrifugation (2000 x *g*, 15 min, room temperature). The absorbance of the organic layer was measured at 549 nm (ε = 68000 L mol^{-1} cm^{-1}).

DAHP synthase

DAHP synthase activity was measured according to the procedure described by Schoner.[12] Harvested cells were resuspended in 50 mM potassium phosphate (pH 6.5) that contained 10 mM PEP and 0.05 mM $CoCl_2$. The cells were then disrupted as previously described. The lysate was diluted in potassium phosphate (50 mM), PEP (0.5 mM), and 1,3-propanediol (250 mM), pH 7.0. A dilute solution of E4P was concentrated to 12 mM by rotary evaporation and neutralized with 5 N KOH. A solution was then prepared that contained E4P (6 mM), PEP (12 mM), ovalbumin (1 mg mL^{-1}), and potassium phosphate (25 mM), pH 7.0. An aliquot (0.5 mL) of the diluted lysate was combined with 1 mL of the E4P/PEP solution. After these solutions were mixed (time = 0), the reaction was incubated at 37 °C for 3 min. Aliquots (0.15 mL) were removed at

timed intervals and quenched with 0.1 mL of 10% trichloroacetic acid (w/v). Precipitated protein was removed by microcentrifugation, and the DAH(P) in each sample was quantified by the thiobarbituric acid assay. One unit of DAHP synthase activity was defined as the formation of 1 μmol of DAHP per min.

Transaldolase

Transaldolase was assayed according to the method described by Tsolas.[10] The transaldolase-catalyzed conversion of D-fructose 6-phosphate and E4P into D-sedoheptulose 7-phosphate and D-glyceraldehyde 3-phosphate (GAP) was monitored. For the determination of transaldolase activity the GAP formed per unit time was related to the loss of NADH by coupling enzymes triosephosphate isomerase (TIM) and glycerophosphate dehydrogenase (GDH). Cells were resuspended in 50 mM potassium phosphate (pH 7.6) and disrupted in a French pressure cell as previously described. The assay solution contained D-fructose 6-phosphate (2.7 mM), E4P (0.2 mM), NADH (0.1 mM), triethanolamine (91 mM), pH 7.6, EDTA (91 mM), TIM (14 units), and GDH (2 units) in a total volume of 2.7 mL. The absorbance at 340 nm was monitored at room temperature until a stable baseline was obtained. An aliquot (0.05 mL) of diluted lysate was added and the absorbance at 340 nm monitored for 10 min. One unit of transaldolase activity was defined as the loss of one μmol of NADH ($\varepsilon = 6220$ L mol^{-1} cm^{-1}) per min.

Transketolase

Transketolase activity was quantified using a procedure described by Paoletti.[13] The transketolase-catalyzed conversion of E4P and β-hydroxypyruvate into D-fructose 6-phosphate and CO_2 was assayed. Formation of D-fructose 6-phosphate was related to NADPH formation through coupling enzymes phosphoglucoisomerase (PGI) and glucose 6-phosphate dehydrogenase (G6PDH). Cells were resuspended in buffer containing potassium phosphate (50 mM), pH 7.4, $MgCl_2$ (1 mM), and dithiothreitol (0.2 mM).

Cells were disrupted as previously described. The assay solution (0.98 mL) contained triethanolamine (125 mM), pH 7.6, $MgCl_2$ (5 mM), thiamine pyrophosphate (0.1 mM), β-hydroxypyruvate (0.4 mM), NADP (0.4 mM), E4P (0.1 mM), G6PDH (3 units), and PGI (10 units). The solution was incubated at room temperature for several minutes until the absorbance at 340 nm was constant. An aliquot (0.02 mL) of diluted lysate was then added and the reaction was monitored at 340 nm for 20 min. One unit of transketolase activity was defined as the formation of one μmol of NADPH (ε = 6220 L mol^{-1} cm^{-1}) per min.

PCR Amplification of *talB*, *tktA*, and *aroF*

PCR reactions were performed in a single-block thermocycler purchased from Ericomp. $Vent_R$® DNA polymerase was purchased from New England Biolabs. Oligonucleotide primers were synthesized by the Macromolecular Structure Facility in the Department of Biochemistry at Michigan State University. Stock solutions of 2'-deoxynucleoside 5'-triphosphates were purchased from Pharmacia. Plasmids used as templates for PCR were purified by PEG precipitation. Genomic DNA used as templates for PCR was isolated as previously described. The concentration of DNA from solutions of oligonucleotide primers was determined by measuring the absorbance at 260 nm. The extinction coefficient for each primer was calculated from the sum of the extinction coefficients for each dNTP[14] (A = 15400 L mol^{-1} cm^{-1}; C = 7400 L mol^{-1} cm^{-1}; G = 11700 L mol^{-1} cm^{-1}; T = 8700 L mol^{-1} cm^{-1}). Primers were dissolved in water or TE and diluted to 10 μM. A typical PCR reaction contained the following: 10 μL of 2 mM dNTPs, 20 μL of each primer (10 μM), 10 μL of $Vent_R$® polymerase reaction buffer (10X concentration), $Vent_R$® polymerase (2 units) and template DNA in a total volume of 100 μL. The amount of template DNA was usually 1 μg for genomic DNA and 0.05 μg for plasmid DNA. PCR reactions were carried out in 0.6 mL microcentrifuge tubes. Mineral oil (100 μL) was added to each reaction mixture immediately before starting PCR to minimize evaporation of the sample. After completion of the temperature cycling program, the PCR reaction

mixture was removed from the mineral oil. A small aliquot of the sample was analyzed by agarose gel electrophoresis. The remainder of the sample was extracted with a mixture of phenol and SEVAG (0.1 mL each). DNA was precipitated by addition of 0.1 volume of 3 M NaOAc (pH 5.2), thorough mixing, and addition of 3 volumes of 95% ethanol.

A 1.3 kb fragment containing the *talB* gene was amplified from *E. coli* RB791 genomic DNA. Oligonucleotide primers used were as follows:

JWF46 (5'-GTACGTACCCATGGTTTAAGAAGTATATACGCTA-3')

JWF47 (5'-CATGCATGCCATGGTTAAACAGTCTCGTTAAACA-3')

The following temperature cycling program was used: 1 cycle of 94 °C (4 min); 30 cycles of 94 °C (1 min), 60 °C (1 min), 72 °C (1.3 min). The size of the product was confirmed on a 0.7% agarose gel. The PCR product was digested with *Nco*I and purified by agarose gel electrophoresis. Vector pBR325[15] was digested with *Nco*I and treated with calf intestinal alkaline phosphatase (CIAP). Ligation of the *talB* fragment into pBR325 resulted in the formation of plasmid pMF52A (7.3 kb). The orientation of the insert with respect to the host vector was determined by analysis of the DNA fragments that resulted from digestion with *Bam*HI.

A 2.2 kb *tktA* fragment was amplified from plasmid pKD130A.[16] The template DNA was prepared by linearizing pKD130A with *Sph*I before use. The following primers were used:

JWF38 (5'-CGGGATCCTGGTCCGCAAACGGACATTA-3')

JWF39 (5'-CGGGATCCAGAGATTTCTGAAGC-3')

The program for PCR was as follows: 1 cycle of 94 °C (4 min); 25 cycles of 55 °C (1 min), 72 °C (2.2 min), 94 °C (1 min). The size of the fragment was verified on a 0.7% agarose gel. The *tktA* fragment was digested with *Bam*HI and gel purified. The insert was then ligated to pBR325 that had been previously digested with *Bam*HI and treated with CIAP, resulting in the formation of pMF51A (8.2 kb). The orientation of the insert with

respect to the host vector was determined by analysis of the DNA fragments that resulted from digestion with *Ava*I.

The *aroF* gene was amplified as a 1.25 kb fragment from plasmid pKD130A that had been previously linearized by *Sph*I. The primers used for PCR were as follows:

JWF19 (5'-GGAATTCTTAAGCCACGCGAGCCGT-3')

JWF22 (5'-GGAATTCAAAGGGAGTGTAAATTTAC-3')

The following program was used for PCR: 1 cycle of 94 °C (4 min); 30 cycles of 94 °C (1 min), 55 °C (1 min), 72 °C (1.3 min). The size of the PCR product was confirmed by running a 0.7% agarose gel. The DNA fragment was digested with *Eco*RI and isolated from an agarose gel. After digestion of pBR325 and treatment with CIAP, the vector was ligated to the *aroF* fragment to give pMF58A (7.25 kb). The orientation of the insert with respect to the host vector was determined by analysis of the DNA fragments that resulted from digestion with *Pvu*II.

Plasmid Constructions

Plasmid pMF61A

Plasmid pMF58A was digested with *Sph*I and treated with CIAP. The 1.65 kb *aroB* fragment was isolated from an agarose gel following *Sph*I digestion of plasmid pKD136. Ligation of these two fragments gave plasmid pMF61A (8.9 kb). The orientation of the insert with respect to the host vector was determined by analysis of the DNA fragments that resulted from digestion with *Aat*II.

Plasmid pMF60A

Plasmid pMF52A was digested with *Sph*I and treated with CIAP. The 1.65 *aroB* fragment was ligated to pMF52A to give pMF57A (8.95 kb). Digestion of pMF57A with *Eco*RI was followed by CIAP treatment. The 1.25 kb *aroF* fragment was purified by

agarose gel electrophoresis from an *Eco*RI digest of pMF58A. Ligation of the *aroF* fragment to pMF57A yielded pMF60A (10.2 kb).

Plasmid pMF63A

Plasmid pMF61A was digested with *Bam*HI and treated with CIAP. The 2.2 kb *tktA* fragment was isolated from an agarose gel following *Bam*HI digestion of pMF51A. Ligation of *tktA* into pMF61A resulted in plasmid pMF63A (11.1 kb).

Plasmid pMF65A

Plasmid pMF63A was digested with *Nco*I followed by CIAP treatment. Isolation of the 1.3 kb *talB* fragment from an *Nco*I digest of pMF52A was followed by ligation to pMF63A to give pMF65A (12.4 kb).

Plasmid pMF66A

Vector pSU18[17] was digested with *Eco*RI and treated with CIAP. Ligation of the 1.25 kb *aroF* fragment to pSU18 yielded pMF66A (3.55 kb).

Plasmid pMF67

Plasmid pKL1.87A[18] contains a 3.1 kb *pps* fragment in pSU19. Digestion of pKL1.87A with *Eco*RI and CIAP treatment was followed by ligation to the 1.25 kb *aroF* fragment. This resulted in the formation of plasmid pMF67 (6.65 kb).

Chapter 3

Tyrosine Phenol-Lyase Assay

Tyrosine phenol-lyase activity was assayed according to the method described by Kumagai, which measures the amount of pyruvate formed from L-tyrosine.[19] Cells were

resuspended in 50 mM potassium phosphate (pH 8.0) and disrupted with a French pressure cell as previously described. The assay solution (3.9 mL) contained L-tyrosine (1.25 mM) and pyridoxal 5'-phosphate (0.025 mM) in 50 mM potassium phosphate buffer (pH 8.0). Lysate (0.1 mL) was added (time = 0), and the mixture was incubated at 30 °C in a water bath with gentle shaking for 20 min. Aliquots (0.5 mL) were removed at timed intervals and quenched with 10% trichloroacetic acid (3 mL). Precipitated protein was removed by centrifugation (2000 x *g*, 15 min, room temperature).

The pyruvate in each sample was quantified as the 2,4-dinitrophenylhydrazone derivative by the method of Friedemann.[20] A 2,4-dinitrophenylhydrazine (2,4-DNP) reagent solution was prepared as follows. Solid 2,4-DNP was ground in a mortar with addition of small amounts of 2 N HCl until 100 mL was added, followed by gravity filtration of the solution. The 2,4-DNP reagent was stored at 4 °C and was prepared freshly each week. An aliquot (3 mL) of the deproteinized supernatant from the tyrosine phenol-lyase assay was added to 1 mL of 2,4-DNP reagent in a large test tube. The mixture was vortexed (15 s), and the hydrazone derivative was extracted into benzene (3 mL). The aqueous layer was discarded, and to the organic layer was added 10% Na_2CO_3 (6 mL). After this solution was vortexed (30 s), an aliquot (5 mL) of the aqueous layer was withdrawn and added to 1.5 M NaOH (5 mL) in another test tube. This solution was then mixed briefly and left at room temperature for 10 min. The absorbance of this solution at 520 nm was measured. The concentration of pyruvate in the sample was determined from a standard curve that had been prepared with pyruvate. One unit of tyrosine phenol-lyase activity was defined as the formation of 1 μmol of pyruvate per min.

PCR Amplification of *tpl*

The PCR conditions were identical to those previously described under Chapter 2 with the exception that oligonucleotide primers were synthesized by National Biosciences.

Initially a 2.1 kb fragment containing the *tpl* gene was amplified from *Citrobacter freundii* (ATCC 29063) genomic DNA. Oligonucleotide primers used were as follows:

Primer A (5'-TTGGTACCCCCAGTAATTGGCGGGAAGT-3')

Primer B (5'-ACGGTACCAAAGGACTTCCTGTTCATGG-3')

The following temperature cycling program was used: 1 cycle of 94 °C (5 min); 25 cycles of 94 °C (1 min), 55 °C (1 min), 72 °C (2.1 min). The size of the product was confirmed on a 0.7% agarose gel. The PCR product was digested with *Kpn*I and purified by agarose gel electrophoresis. Vector pSU18 was digested with *Kpn*I and treated with CIAP. Ligation of the 2.1 kb *tpl* fragment into pSU18 gave plasmid pMF38A (4.4 kb). The orientation of the insert with respect to the host vector was determined by analysis of the DNA fragments that resulted from digestion with *Sph*I.

Iwamori identified a palindromic region of DNA located approximately 100 bp upstream from the translational start site for *tpl*.[21] It was presumed that this region was concerned with the induction of L-tyrosine.[21] In order to generate a *tpl* fragment that did *not* require L-tyrosine induction, a new PCR primer was designed to anneal to the 5'-end of the *tpl* gene immediately downstream from the aforementioned palindromic sequence.

New Primer A (5'-GGGGTACCGTTATATTTCATCAGACTTT-3')

With New Primer A and Primer B, a 1.5 kb tpl fragment was amplified from *C. freundii* genomic DNA. The PCR program was as follows: 1 cycle of 94 °C (5 min); 25 cycles of 94 °C (1 min), 55 °C (1 min), 72 °C (1.5 min). The size of the PCR product was verified on a 0.7% agarose gel. The 1.5 kb *tpl* fragment was digested with *Kpn*I, gel purified, and ligated to pSU18 that had been digested with *Kpn*I and treated with CIAP. This resulted in the formation of plasmid pMF42A (3.8 kb).

Construction of Plasmid pMF43A

Plasmid pKD9.069A[22] contains a 3.5 kb *aroZ* fragment and a 2.4 kb *aroY* fragment localized in pSU19. Digestion of pKD9.069A with *Kpn*I was followed by CIAP

treatment. Isolation of the 1.5 kb *tpl* fragment from a *Kpn*I digest of pMF42A was followed by ligation of this insert to pKD9.069A to give pMF43A (9.7 kb)

Culture Conditions for Converting D-Glucose into L-DOPA

The culture conditions described previously under General Methods were tried initially with AB2834/pKD136/pMF43A, but no production of L-DOPA was observed. The effect of pH on the reaction was explored as follows. Six samples of LB (50 mL in a 250 mL Erlenmeyer flask) were each inoculated with 0.25 mL of an overnight culture of AB2834/pKD136/pMF43A. The cells were grown in a gyratory shaker (37 °C, 250 rpm) for 12 h. The cells were collected by centrifugation (4000 x *g*, 5 min) in bleach-sterilized bottles and washed with 10 mL of 50 mM potassium phosphate (pH 8.0). Each of the six cell pellets was resuspended in M9 medium (25 mL in a 125 mL Erlenmeyer flask) that had been adjusted to a different pH value (7.0, 7.2, 7.4, 7.6, 7.8, and 8.0). The cultures were then returned to the shaker (37 °C, 250 rpm). An aliquot (3 mL) of each culture was removed at 24h and 48h and the culture supernatant was analyzed by ^{1}H NMR.

The effect of temperature on the reaction was explored as follows. The procedure for growing the cells was the same as for the pH study, except that only three cultures were used. Each culture was incubated in M9 (pH 7.0) at a different temperature (37 °C, 30 °C, and 25 °C). An aliquot (3 mL) of each culture was removed at 24h and 48h and the culture supernatant was analyzed by ^{1}H NMR.

Addition of excess pyruvate and ammonia to the M9 culture was performed as follows. AB2834/pKD136/pMF43A was grown exactly as for the pH study. Six cultures were used. The M9 media was adjusted in the following manner. Pyruvate (330 mM) was added to two of the M9 cultures and the pH adjusted to 7.5 and 8.0. Ammonium chloride (330 mM) was added to two of the M9 cultures and the pH adjusted to 7.5 and 8.0. Both pyruvate (330 mM) and NH_4Cl (330 mM) were added to two of the M9 cultures and the pH adjusted to 7.5 and 8.0. The six cell pellets were each resuspended in one of the M9

cultures and returned to the shaker (37 °C, 250 rpm). An aliquot (3 mL) of each culture was removed at 24h and 48h and the culture supernatant was analyzed by ^{1}H NMR.

Culture Conditions for Converting Catechol into L-DOPA

The synthesis of L-DOPA from catechol was carried out using a modified procedure described by Foor.[23] LB (50 mL) was inoculated with 0.25 mL of an overnight culture from a strain that overexpressed tyrosine phenol-lyase. The cells were harvested (4000 x *g*, 5 min) and washed with 10 mL of 50 mM potassium phosphate (pH 8.0). The cells were then resuspended in a 25 mL of a solution that contained catechol (230 mM), pyruvate (330 mM), NH_4Cl (330 mM), $B(OH)_3$ (240 mM), Na_2SO_3 (30 mM), and EDTA (7 mM) (adjusted to pH 8.5 with 5 N NH_4OH). The cell suspension was incubated at room temperature for 24 h. An aliquot of the culture was removed and the culture supernatant analyzed by HPLC. The yield of L-DOPA was 12 mol % based on catechol.

HPLC Analysis of Reaction Mixtures

For the HPLC analysis of catechol and L-DOPA, an aliquot of the supernatant from the reaction was diluted in 25 mM potassium phosphate (pH 3.0). Diluted samples (20 to 50 μL) were analyzed on a Rainin HPLC using a C18 analytical column (4.6 mm x 25 cm). The HPLC program was as follows. For the first 5 min, the mobile phase was 5% (v/v) methanol in 25 mM potassium phosphate (pH 3.0). Then from 5 min to 20 min, methanol was increased over a linear gradient to 60 %. The absorbance at 280 nm was monitored and the flow rate was 1 mL min^{-1}. The retention time for L-DOPA and catechol under these conditions was about 5.6 min and about 15.6 min, respectively.

Determination of K_M Values for Tyrosine Phenol-Lyase

For the determination of the K_M for catechol (8 mM), the assay solution (4 mL) contained 50 mM potassium phosphate (pH 8.0), 200 mM NH_4Cl, 120 mM pyruvate,

0.025 mM pyridoxal 5'-phosphate, 1 unit of tyrosine phenol-lyase, and catechol concentrations of 5, 10, 15, 20, 25, and 30 mM. The samples were incubated in a water bath at 30 °C with gentle shaking. Aliquots (0.5 mL) were removed at timed intervals and quenched with concentrated H_2SO_4 (5 µL). The precipitated protein was removed by microcentrifugation and the concentration of L-DOPA in the sample determined by HPLC analysis. A Lineweaver-Burk plot[24] of V^{-1} versus $[catechol]^{-1}$ was generated, where V is the velocity of the reaction (µmol L-DOPA formed per min) and [catechol] is the concentration of catechol (M). The x-intercept of this plot is equal to $-K_M^{-1}$.

The K_M for L-tyrosine (0.4 mM) was determined as follows. The normal tyrosine phenol-lyase assay (as previously described) was performed. The reaction contained 0.2 units of enzyme and varying concentrations of L-tyrosine (0.05, 0.1, 0.25, 0.5, 1, and 2 mM). A plot of V^{-1} versus $[L\text{-tyrosine}]^{-1}$ was generated, where V is the velocity of the reaction (µmol pyruvate formed per min) and [L-tyrosine] is the concentration of L-tyrosine (M). The x-intercept of this plot is equal to $-K_M^{-1}$.

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