Environmentally Compatible Synthesis of Catechol from D-Glucose

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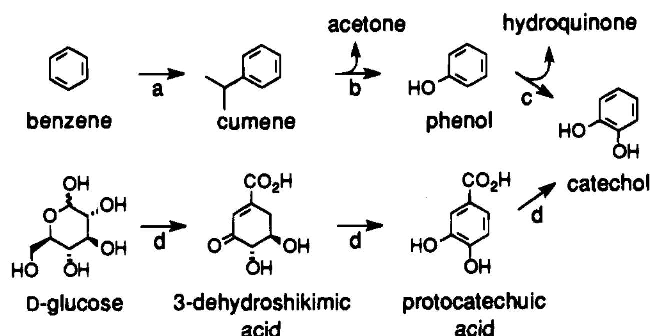
Abstract: A biocatalytic alternative to currently employed industrial synthesis of catechol has been developed. *Klebsiella pneumoniae* genes encoding 3-dehydroshikimate dehydratase (*aroZ*) and protocatechuic acid decarboxylase (*aroY*) were introduced into an *Escherichia coli* construct that synthesizes elevated levels of 3-dehydroshikimic acid. One of the resulting biocatalysts, *E. coli* AB2834/pKD136/pKD9.069A, synthesizes 18.5 ± 2.0 mM catechol from 56 mM D-glucose on 1 L scale.

Of the chemicals produced in the U.S. in excess of 2.2×10^7 kg per year, 98% are derived from petroleum and natural gas.[1] Catechol manufacture provides a useful paradigm of the fossil fuel bias that has gripped the chemical industry throughout the 20th century. While some catechol is distilled from coal tar, the dominant method[2] (Scheme 1) of production begins with Friedel–Crafts alkylation of petroleum-derived benzene to afford cumene. Subsequent Hock-type, air oxidation of the cumene leads to formation of acetone and phenol. The phenol is then oxidized using 70% hydrogen peroxide either in the presence of transition metal catalysts or in formic acid solution where performic acid is the actual oxidant. The catechol and hydroquinone mixture that is generated is separated into its pure components by successive distillations. Chemical products derived from the purified catechol include pharmaceuticals (L-DOPA, adrenaline, papaverine), flavors (vanillin, eugenol, isoeugenol), agrochemicals (carbofuran, propoxur), and polymerization inhibitors and antioxidants (4-*tert*-butylcatechol, veratrol).[2]

Several aspects of contemporary catechol manufacture are environmentally problematic. Petroleum is a nonrenewable resource that has been historically plagued by inadvertent releases into the environment. The benzene starting material used in catechol synthesis is carcinogenic,[3] and intermediate phenol is toxic.[3] Benzene is also included by the Environmental Protection Agency on the list of chemicals covered by the Chemical Manufacturing Rule that requires drastic reductions in emissions of hazardous organic air pollutants.[4] Hydrogen peroxide used as the oxidant in catechol synthesis is a highly energetic, corrosive material which requires special safety precautions to ensure safe storage and handling.[5] Strict regulations regarding transport of solutions that exceed 52% hydrogen peroxide concentration attest to the risk associated with hydrogen peroxide transport.[5]

Scheme 1[a]



[a] (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. (d) *E. coli* AB2834/pKD136/pKD9.069A, 37 °C.

A synthesis of catechol has now been elaborated (Scheme 1) that utilizes D-glucose as the starting material and a genetically modified microbe, *Escherichia coli* AB2834/pKD136/pKD9.069A, as a catalyst. D-glucose is currently derived primarily from corn starch.[6] Future sources of D-glucose will likely include plants such as switchgrass that require minimal chemical inputs during cultivation and can be harvested multiple times during a single growing season.[6] Catechol is readily isolated from the bacterial culture supernatant where it accumulates as essentially the only aromatic product. Benzene starting material, phenol intermediacy, and use of hydrogen peroxide oxidant are completely avoided. As opposed to other reported biocatalytic syntheses that convert benzene,[7] benzoate,[8] or phenol[9] to catechol, the bioconversion that has now been developed completely avoids petroleum-derived synthetic inputs. The amount of catechol synthesized from D-glucose by *E. coli* AB2834/pKD136/pKD9.069A corresponds to approximately 77% of the calculated theoretical maximum mol % yield for this bioconversion.

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(1) Szmant, H. H. *Organic Building Blocks of the Chemical Industry*; Wiley: New York, 1989; p 4.
(2) (a) Franck, H.-G.; Stadelhofer, J. W. *Industrial Aromatic Chemistry*; Springer-Verlag: New York, 1988; pp 183–190. (b) Varagnat, J. In *Kirk-Othmer Encyclopedia of Chemical Technology*, 3rd ed.; Grayson, M., Ed.; Wiley: New York, 1981; Vol. 13, pp 39–69. (c) Szmant, H. H. *Organic Building Blocks of the Chemical Industry*; Wiley: New York, 1989; pp 512–519.
(3) Lewis, R. J. *Hazardous Chemicals Desk Reference*, 2nd ed.; Van Nostrand Reinhold: New York, 1991.
(4) (a) Ember, L. *Chem. Eng. News* **1994**, *72*, 4 (March 7). (b) Noah, T. *Wall Street J.* **1994**, 132 (March 2). (c) *Washington Post* **1994**, A3 (March 2).
(5) Kirchner, J. R. In *Kirk-Othmer Encyclopedia of Chemical Technology*, 3rd ed; Grayson, M., Ed; Wiley: New York, 1981; Vol. 13, pp 12–38.
(6) Lee, H. In *Industrial Uses of Agricultural Materials*; Commodity Economics Division, Economic Research Service, U.S. Department of Agriculture: 1993 (June); IUS-1.
(7) (a) Shirai, K. *Agric. Biol. Chem.* **1986**, *50*, 2875. (b) Shirai, K. *Agric. Biol. Chem.* **1987**, *51*, 121. (c) Nishimura, T.; Ishikawa, T. *Yuki Gosei Kagaku Kyokai Shi* **1976**, *34*, 652.
(8) Kuwahara, M.; Tsuji, M. *J. Ferment. Technol.* **1976**, *54*, 782.
(9) (a) Nei, N.; Tanaka, Y.; Takata, N. *J. Ferment. Technol.* **1974**, *52*, 28. (b) Gurujeyalakshmi, G.; Oriel, P. *Biotechnol. Lett.* **1989**, *2*, 10. (c) Subramanian, R.; Worden, R. M. *Appl. Biochem. Biotech.* **1993**, *39/40*, 509.

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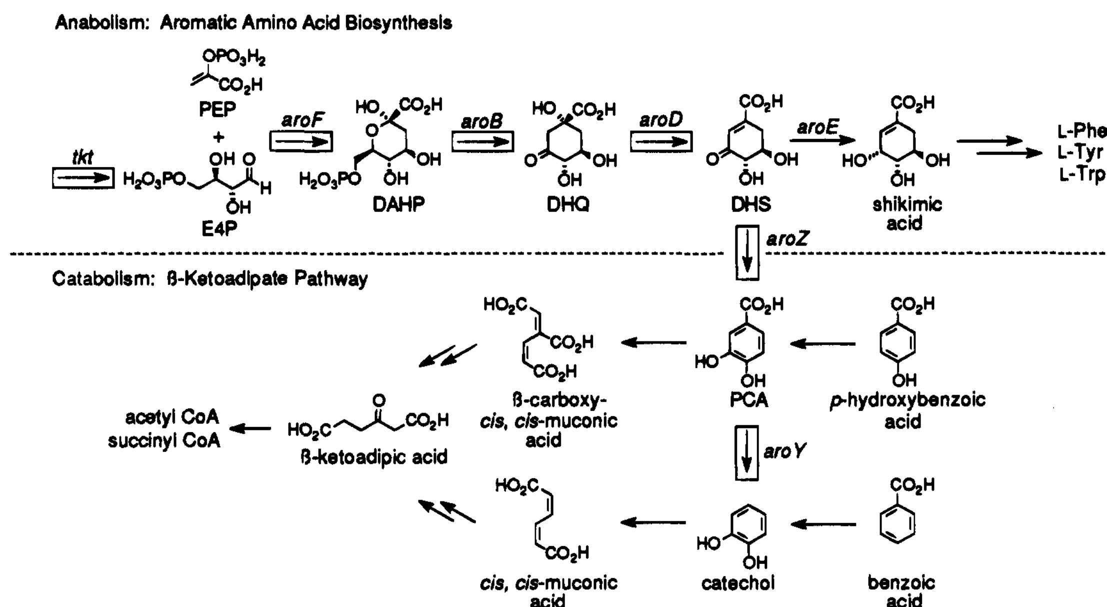
Scheme 2



Results and Discussion

Creation of a microbial biocatalyst capable of synthesizing catechol from D-glucose required recruitment of enzyme activities from both aromatic anabolism[10] and hydroaromatic catabolism[11] (Scheme 2). Aromatic anabolic enzymes were needed for initial conversion of D-glucose into 3-dehydroshikimic acid (DHS) while hydroaromatic catabolic enzymes were essential for subsequent conversion of DHS into catechol. The auxotroph *Escherichia coli* AB2834 was chosen as the microbial host due to a mutation in this strain's *aroE* genomic locus that renders shikimate dehydrogenase catalytically inactive. Since DHS is not converted into shikimic acid by shikimate dehydrogenase, DHS is made available for catechol synthesis instead of aromatic amino acid biosynthesis. Synthesis of DHS (Scheme 2) by *E. coli* AB2834 was substantially enhanced upon transformation with plasmid pKD136.[12] The *tkt*, *aroF*, and *aroB* genes localized on pKD136, respectively, encode the enzymes transketolase, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) synthase, and 3-dehydroquinate (DHQ) synthase. Resulting amplified expression in *E. coli* AB2834/pKD136 of transketolase and DAHP synthase increases the amount of carbon flow directed into DAHP synthesis.[13] Amplified expression of DHQ synthase ensures that accumulation of DHQ does not occur during conversion of DAHP into DHS.[12]

Detection of low concentrations of catechol has been reported for certain genetically modified *E. coli* constructs cultured in medium where D-glucose is the carbon source.[14] However, a high-yielding conversion of D-glucose into catechol (Scheme 2) requires amplified expression of genes encoding DHS dehydratase and protocatechuic acid (PCA) decarboxylase in DHS-synthesizing *E. coli* AB2834/pKD136. DHS dehydratase[15] is one of the critical enzyme activities that enables microbes to use hydroaromatics such as shikimic and quinic acids as sole sources of carbon for growth. The PCA product of DHS dehydratase catalysis is an intermediate in the *p*-hydroxybenzoate branch (Scheme 2) of the catabolic β-ketoadipate pathway.[11a] While *E. coli* is incapable of exploiting hydroaromatics as a sole source of carbon, other microbes that are capable of such catabolism were targeted as the source of the structural gene and associated promoter that would lead to amplified expression of DHS dehydratase. Genes encoding DHS dehydratase have been isolated from both *Aspergillus nidulans*[16] and *Neurospora crassa*[11b,17] although neither of these genes has been expressed in *E. coli*. Concerns about codon usage and whether another microbe's gene would be expressed from its native promoter upon introduction into *E. coli* resulted in the choice of *Klebsiella pneumoniae* as the source of the locus (designated *aroZ*) encoding DHS dehydratase. This strategy reflects the successful expression[18] of *K. pneumoniae* genes from their native promoters in *E. coli* and the close evolutionary relationship[19] between *Klebsiella pneumoniae* and *Escherichia coli*.

The role of PCA decarboxylase in microbial metabolism has never been clearly elaborated. The *p*-hydroxybenzoate and benzoate branches of the β-ketoadipate pathway (Scheme 2) were originally formulated to be linked by PCA decarboxylase-catalyzed decarboxylation of PCA to form catechol.[20] This

(10) (a) Pittard, A. J. In *Escherichia coli and Salmonella typhimurium*; Neidhardt, F. C., Ed.; American Society for Microbiology: Washington, 1987; Vol. 1, pp 368−394. (b) Haslam, E. *Shikimic Acid Metabolism and Metabolites*; Wiley: New York, 1993.

(11) (a) Ornston, L. N.; Niedle, E. L. In *The Biology of Acinetobacter*; Towner, K. J., Bergogne-Bérézin, E., Fewson, C. A., Eds.; Plenum: New York, 1991; pp 201−237. (b) Giles, N. H.; Case, M. E.; Baum, J.; Geever, R.; Huiet, L.; Patel, V.; Tyler, B. *Microbiol. Rev.* **1985**, *49*, 338.

(12) Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1990**, *112*, 9630.

(13) (a) Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1990**, *112*, 1657. (b) Draths, K. M.; Pompliano, D. L.; Conley, D. L.; Frost, J. W.; Berry, A.; Disbrow, G. L.; Staversky, R. J.; Lievense, J. *J. Am. Chem. Soc.* **1992**, *114*, 3956. (c) Frost, J. W. U.S. Patent 5 168 056, 1992.

(14) (a) Pittard, A. J. *Biochim. Biophys. Acta* **1961**, *49*, 485. (b) Pittard, A. J.; Gibson, F.; Doy, C. H. *Biochim. Biophys. Acta* **1962**, *57*, 290. (c) Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1991**, *113*, 9361. (d) Frost, J. W.; Draths, K. M. U.S. Patent 5 272 073, 1993.

(15) (a) Gross, S. R. *J. Biol. Chem.* **1958**, *233*, 1146. (b) Tatum, E. L.; Gross, S. R. *J. Biol. Chem.* **1956**, *219*, 797. (c) Strøman, P.; Reinert, W. R.; Giles, N. H. *J. Biol. Chem.* **1978**, *253*, 4593.

(16) (a) Hawkins, A.; DaSilva, A. J. F.; Roberts, C. F. *Current Genetics* **1985**, *9*, 305. (b) Lamb, H. K.; Hawkins, A. R.; Smith, M.; Harvey, I. J.; Brown, J.; Turner, G.; Roberts, C. F. *Mol. Gen. Genet.* **1990**, *223*, 17.

(17) (a) Schweizer, M.; Case, M. E.; Dykstra, C. C.; Giles, N. H.; Kushner, S. R. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 5086. (b) Schweizer, M.; Case, M. E.; Dykstra, C. C.; Giles, N. H.; Kushner, S. R. *Gene* **1981**, *14*, 23. (c) Rutledge, B. J. *Gene* **1984**, *32*, 275.

(18) (a) Draths, K. M.; Ward, T. L.; Frost, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 9725. (b) Peng, H.-L.; Wang, P.-Y.; Wu, C.-M.; Hwang, D.-C.; Chang, H.-Y. *Gene* **1992**, *117*, 125. (c) Feldmann, S. D.; Sahm, H.; Sprenger, G. A. *Mol. Gen. Genet.* **1992**, *234*, 201. (d) Hama, H.; Wilson, T. H. *J. Biol. Chem.* **1992**, *267*, 18371.

(19) Jensen, R. A. *Mol. Biol. Evol.* **1985**, *2*, 92.

(20) (a) Patel, J. C.; Grant, D. J. W. *Antonie van Leeuwenhoek* **1969**, *35*, 53. (b) Grant, D. J. W. *Antonie van Leeuwenhoek* **1970**, *36*, 161.

proposal is inconsistent with more recent literature.[21] Nonetheless, the observed ability of intact *K. pneumoniae* cells to convert PCA into catechol is suggestive that PCA decarboxylase activity does exist even though its role in hydroaromatic and aromatic catabolism is nebulous. Efforts to isolate the locus (designated *aroY*) thus focused on genomic libraries constructed from *K. pneumoniae*.

Isolation and Expression of *aroZ*-Encoded DHS Dehydratase. Identification of the *aroZ* gene in a *K. pneumoniae* genomic library was based on the expectation that expression of DHS dehydratase in a host strain that normally synthesizes DHS would result in PCA formation. Chromogenic[22] agarose plates containing *p*-toluidine and ferric citrate, which in the presence of PCA develop a brown color, were used to identify PCA-synthesizing colonies. Although screening a library in AB2834/pKD136 was contemplated because of the high concentration of DHS synthesized by the organism, AB2834 could not be infected with λ phage because of an additional mutation AB2834 carries[23] in its *malA* locus. This problem was circumvented by screening a genomic library constructed from *K. pneumoniae* in *E. coli* DH5α/pKD136. Due to the rate-limiting character of genome-encoded shikimate dehydrogenase,[24] DH5α/pKD136 synthesizes low concentrations of DHS.

Genomic DNA was purified from *K. pneumoniae* A170−40 and partially digested with *Bam*H I. The resulting genomic DNA fragments were ligated into cosmid pLAFR3 to yield concatamers that were then packaged in λ phage. Infection of DH5α/pKD136 was followed by selection for resistance to ampicillin and tetracycline to obtain colonies possessing both pKD136 and pLAFR3. Approximately 1.0×10^3 colonies resistant to both antibiotics were then screened on chromogenic agarose plates. Following 48 h of incubation, the agarose surrounding two colonies had turned brown. These colonies were separately cultured in rich medium (LB) to the early stationary phase of growth, harvested, and then resuspended in minimal medium (M9) containing 56 mM D-glucose. An aliquot was withdrawn after 48 h of culturing in minimal medium, cells were removed, and the supernatant was analyzed by ^{1}H NMR. PCA (8.8 mM) in one colony's culture supernatant confirmed the presence of the *K. pneumoniae aroZ* gene. No PCA was observed in the culture supernatant derived from the other colony. Purification of the cosmid (p5−87) from the PCA-synthesizing colony followed by analysis of the fragments generated by restriction enzyme digestion indicated that 14 kb of *K. pneumoniae* DNA had been localized in pLAFR3.

Though ideal for identifying the *aroZ* gene, the lack of a complete sequence for pLAFR3 and its limited multiple cloning site made this vector[25] undesirable for localization of multiple genes in the final biocatalyst. A lack of convenient cloning sites also precluded localization of the *aroZ* gene in *tkt*, *aroF*, and *aroB*-encoding pKD136 which had to be retained in the final construct in order to ensure high-yielding conversion of D-glucose into DHS. As a result, a second plasmid was recruited for expression of DHS dehydratase and, ultimately, PCA decarboxylase. Low copy plasmids pSU18 and pSU19 were chosen by virtue of their p15A replicon that is compatible with the pMB1 replicon of pKD136. The resistance to chloramphenicol (Cm^R) encoded by both pSU18 and pSU19 and resistance to ampicillin (Ap^R) encoded by pKD136 were expected to facilitate stable plasmid maintenance in a construct

(21) Doten, R. C.; Ornston, L. N. *J. Bacteriol.* **1987**, *169*, 5827.
(22) Parke, D. *Appl. Environ. Microbiol.* **1992**, *58*, 2694.
(23) Pittard, J.; Wallace, B. J. *J. Bacteriol.* **1966**, *91*, 1494.
(24) Dell, K. A.; Frost, J. W. *J. Am. Chem. Soc.* **1993**, *115*, 11581.
(25) Staskawicz, B.; Dahlbeck, D.; Keen, N.; Napoli, C. *J. Bacteriol.* **1987**, *169*, 5789.

Table 1. Restriction Enzyme Maps of Plasmids Containing *aroZ* and *aroY*[a]

Plasmid	Vector	Plasmid Map
pSU1-28 (5.8 kb)	pSU19	B E — H B — S — B; Cm^R, P$_{lac}$
pSU1-31 (5.8 kb)	pSU19	B E — H B — S — B; Cm^R, P$_{lac}$
pSU204R (4.3 kb)	pSU18	S H — E B — S; Cm^R, P$_{lac}$
pSU204 (4.3 kb)	pSU19	B E — H S — B; Cm^R, P$_{lac}$
pKD9.069A (8.2 kb)	pSU19	B E — H — BB — H B — S — B; Cm^R, P$_{lac}$
pKD9.080A (8.2 kb)	pSU18	H — E B — S — B H — BB — H; Cm^R, P$_{lac}$

— vector DNA ▬ *aroZ*-encoding DNA ┈ *aroY*-encoding DNA

[a] Restriction enzyme sites: B = *Bam*H I, E = *Eco*R I, H = *Hind* III, and S = *Sph* I.

cultured in medium containing both antibiotics. A difference between pSU18 and pSU19 in the orientation of the multiple cloning site relative to a *lac* promoter provided additional flexibility.

Since both *aroZ* and *aroY* needed to be inserted into the second plasmid and to avoid expression of proteins not associated with DHS dehydratase activity, the 14 kb genetic fragment containing the *aroZ* locus had to be shortened. Plasmid p5−87 was digested with *Bam*H I, and the resulting DNA fragments were ligated into pSU19. Transformation into DH5α/pKD136 followed by screening the resulting colonies on chromogenic agarose plates resulted in plasmids pSU1−28 and pSU1−31 which expressed DHS dehydratase catalytic activity. Analysis of restriction enzyme digestions indicated that while both plasmids contained a 3.5 kb *Bam*H I insert, they differed in the orientation of the fragment relative to the vector-encoded *lac* promoter (Table 1). Further efforts to subclone the *aroZ* fragment were facilitated by a restriction map which was generated of the 3.5 kb *Bam*H I fragment (Table 1). Plasmids pSU204 and pSU204R (Table 1) were prepared which localized the *aroZ* gene to a 2.0 kb *Bam*H I−*Sph* I region. However, measurement of DHS dehydratase specific activities indicated that significantly less activity was obtained from the 2.0 kb fragment than from the 3.5 kb fragment.

The four plasmids expressing DHS dehydratase were transformed into AB2834/pKD136, and the resulting strains were evaluated for their ability to convert D-glucose into PCA. The specific activity of DHS dehydratase was determined for each strain at the point of transfer from rich medium into minimal medium. AB2834/pKD136/pSU1−28 (Figure 1, strain 3) and AB2834/pKD136/pSU1−31 (Figure 1, strain 4), which expressed DHS dehydratase from the 3.5 kb *Bam*H I fragment, accumulated 28.0 ± 2.4 mM and 22.4 ± 3.3 mM PCA, respectively, in their culture supernatants. DHS was not detected in either of the culture supernatants. However, AB2834/pKD136/pSU204R (Figure 1, strain 5) and AB2834/pKD136/pSU204 (Figure 1, strain 6), which expressed DHS dehydratase from the 2.0 kb *Bam*H I-*Sph* I *aroZ* insert, synthesized a mixture of DHS and PCA. Specific activities of DHS dehydratase in AB2834/pKD136/pSU204R (0.01 units/mg) and AB2834/pKD136/pSU204 (0.02 units/mg) apparently were insufficient to catalyze conversion of DHS to PCA at a rate sufficient to avoid intracellular accumulation of DHS and its subsequent export into the culture supernatant. Higher DHS dehydratase specific activities resulting from the 3.5 kb *aroZ* insert of AB2834/pKD136/pSU1−28 (0.1 units/mg) and AB2834/pKD136/pSU1−31 (0.08 units/mg) eliminated DHS accumulation in culture supernatants.

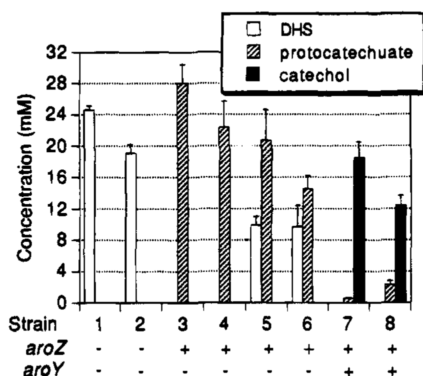


Figure 1. Concentration of DHS, PCA, and catechol which accumulated extracellularly after 48 h of culturing in minimal medium which initially contained 56 mM D-glucose. Strains studied include (1) AB2834/pKD136; (2) AB2834/pKD136/pSU19; (3) AB2834/pKD136/pSU1−28; (4) AB2834/pKD136/pSU1−31; (5) AB2834/pKD136/pSU204R; (6) AB2834/pKD136/pSU204; (7) AB2834/pKD136/pKD9.069A; and (8) AB2834/pKD136/pKD9.080A.

In going from DHS-synthesizing AB2834/pKD136 to the PCA-synthesizing constructs, it was possible that a decrease in yield of the desired end product might be observed due to the metabolic burden imposed on the microbe by expression of the *aroZ* locus. However, the 28.0 ± 2.4 mM of PCA synthesized by AB2834/pKD136/pSU1−28 (Figure 1, strain 3) was roughly comparable to the 24.6 ± 0.5 mM DHS synthesized by AB2834/pKD136 (Figure 1, strain 1). A different assessment of DHS synthesis and PCA synthesis relied on comparison of percent conversions achieved with two strains carrying the same number of plasmids. Surprisingly, AB2834/pKD136/pSU19 (Figure 1, strain 2) synthesized 19.1 ± 1.1 mM DHS, which represents a 30% reduction in the final concentration of end product DHS relative to end product PCA accumulated by AB2834/pKD136/pSU1−28 (Figure 1, strain 3). Expression of the *aroZ* locus clearly did not adversely affect the percent conversion of D-glucose to desired end product.

Isolation and Expression of *aroY*-Encoded PCA Decarboxylase. Isolation of the *aroY* gene utilized a derivative of pLAFR3 that contained an *aroZ* gene. A second library of *K. pneumoniae* was constructed in this *aroZ*-encoding cosmid. Packaging in λ phage and infection of DH5α/pKD136 primarily yielded PCA-synthesizing colonies. Colonies synthesizing catechol due to expression of PCA decarboxylase were distinguished from PCA-synthesizing colonies by use of the same chromogenic agarose plates described for isolation of the *aroZ* gene. While the chromogenic[22] agarose forms a brown color in the presence of catechol similar to that observed for PCA, the rate of color formation is noticeably faster for catechol relative to PCA.

Cosmid p4−20 was prepared by inserting the 3.5 kb *Bam*H I *aroZ* fragment into pLAFR3. *K. pneumoniae* A170−40 genomic DNA was partially digested with *Eco*R I, ligated into the *Eco*R I site of p4−20, and packaged in λ phage heads. Subsequent infection of DH5α/pKD136 yielded colonies which were selected for resistance to ampicillin and tetracycline. Approximately 1.0 × 10^3 colonies were then screened on chromogenic agarose plates. Following incubation for 24 h, a single colony was selected that was producing pigmentation significantly darker than background. Purification of the cosmid (p2−47) followed by analysis after restriction enzyme digestion indicated that an approximately 20 kb fragment of *K. pneumoniae* DNA was localized in the *Eco*R I site. Confirmation that p2−47 expressed both DHS dehydratase and PCA decarboxylase relied on ^{1}H NMR analysis of the culture supernatant of AB2834/pKD136/p2−47. After 48 h in minimal medium, the culture supernatant contained 12.8 ± 2.3 mM catechol, thereby confirming the presence of both the *aroY* and *aroZ* genes on p2−47.

Shortening the genetic fragment containing the *aroY* locus encoding PCA decarboxylase proceeded in stages. Digestion of p2−47 with *Eco*R I yielded an 11.9 kb fragment which was subsequently localized in *aroZ*-encoding pSU1−31 to afford pSU157−27. Further digestion of pSU157−27 with *Hin*d III resulted in a 2.4 kb fragment from which assayable PCA decarboxylase activity was expressed.

Biocatalyst Appraisal. Both the mol % conversion of D-glucose into catechol and the purity of product catechol hinge on the relative ratio of DHS dehydratase and PCA decarboxylase catalytic activities. Microbial synthesis of PCA at a rate in excess of the rate of conversion of PCA to catechol catalyzed by PCA decarboxylase would lead to PCA accumulation. This PCA accumulation would reduce the yield and purity of product catechol. To explore the optimal ratio of DHS dehydratase and PCA decarboxylase catalytic activities, plasmids pKD9.069A and pKD9.080A (Table 1), respectively, were constructed from pSU19 and pSU18 using the 3.5 kb *aroZ* and the 2.4 kb *aroY* inserts. Both DHS dehydratase and PCA decarboxylase were expressed from their native promoters. However, the orientation of these genes relative to the vector-encoded *lac* promoter was expected to have an effect on enzyme expression levels.

Plasmids pKD9.069A and pKD9.080A were transformed into AB2834/pKD136, and the resulting strains were evaluated both for the concentration and purity of catechol synthesized. DHS dehydratase and PCA decarboxylase specific activities were determined for each strain at the point of transfer from rich medium into minimal medium. The 18.5 ± 2.0 mM catechol synthesized by AB2834/pKD136/pKD9.069A (Figure 1, strain 7) was significantly higher than 12.4 ± 1.3 mM achieved by AB2834/pKD136/pKD9.080A (Figure 1, strain 8). Extracellular accumulation of PCA was observed for both constructs. Although the specific activity of PCA decarboxylase for AB2834/pKD136/pKD9.080A (Figure 1, strain 8) of 0.14 units/mg was nearly twice the specific activity for AB2834/pKD136/pKD9.069A (Figure 1, strain 7) of 0.08 units/mg, a higher concentration of PCA was detected in the supernatant of AB2834/pKD136/pKD9.080A. The specific activity of DHS dehydratase in AB2834/pKD136/pKD9.080A (Figure 1, strain 8) was 0.06 units/mg, while the specific activity of this same enzyme in AB2834/pKD136/pKD9.069A (Figure 1, strain 7) was 0.04 units/mg.

PCA and Catechol Toxicity. The combined mol % yield of catechol and PCA synthesized by AB2834/pKD136/pKD9.069A represented a roughly 30% decrease relative to the mol % of PCA synthesized by AB2834/pKD136/pSU1−28. These observations suggested that the accumulating catechol might be toxic to the biocatalyst, while PCA accumulation did not interfere with the biocatalyst's metabolism. To determine the nature of this potential toxicity, growth of AB2834/pKD136 in the presence of catechol versus PCA was examined. A different measure of toxicity was also employed where AB2834/pKD136 was grown in the absence of catechol and PCA and then resuspended in medium containing varying concentrations of catechol or PCA. The effects of catechol and PCA on the biocatalyst's metabolism were measured by their respective impact on DHS accumulation in the culture supernatant of AB2834/pKD136.

Catechol's impact on microbial growth was determined by spreading liquid suspensions of AB2834/pKD136 onto rich (LB) agarose plates containing varying concentrations of catechol or

Table 2. Number of *E. coli* AB2834/pKD136 Colonies that Grew on LB Plates in the Presence of Varying Concentrations of Catechol and PCA

metabolite added	concentration (mM)	colonies
catechol	0	71 ± 9
	0.1	70 ± 3
	1.0	75 ± 5
	2.5	0
PCA	0	89 ± 18
	5	88 ± 13
	10	90 ± 7
	25	93 ± 15

Table 3. Concentration of DHS Synthesized by *E. coli* AB2834/pKD136 in the Presence of Varying Concentrations of Catechol and PCA

metabolite added	concentration (mM)	DHS concentration (mM)
catechol	0	29.3
	5	28.8
	10	24.9
	25	6.3
PCA	0	29.3
	5	28.3
	10	28.7
	25	26.2

PCA. The number of colonies on each plate was determined after incubating the plates for 24 h. While no effect on the number of colonies was observed in the presence of 1 mM catechol, addition of 2.5 mM catechol completely prevented cell growth (Table 2). On the other hand, 25 mM PCA had no impact on the number of colonies which grew, although a decreased rate of growth was observed even at 5 mM PCA concentrations.

AB2834/pKD136 was then grown in rich (LB) medium, harvested, and resuspended in minimal medium containing 56 mM D-glucose and varying concentrations of catechol or PCA. An aliquot of each resuspended culture was withdrawn after 24 h, and the concentration of DHS synthesized was determined by ^{1}H NMR. A 15% reduction in the concentration of synthesized DHS was observed following addition of 10 mM catechol, while an 80% reduction resulted from addition of 25 mM catechol to the medium (Table 3). In contrast, PCA had a modest impact on the concentration of DHS synthesized by AB2834/pKD136 initially grown in rich medium lacking PCA. Addition of 25 mM PCA to the medium of resuspended AB2834/pKD136 resulted in only a 10% reduction in DHS production (Table 3).

Catechol clearly interferes with the biocatalyst's metabolism particularly during microbial growth. However, this metabolic disruption does not prevent synthesis and decarboxylation of PCA in microbes initially grown in the absence of catechol. This has important implications relative to future optimization of catechol-synthesizing biocatalysts.

Catechol Purification and Yield Considerations. The medium of AB2834/pKD136/pKD9.069A and all other catechol-synthesizing organisms in this study were brown-colored after 48 h of culturing. However, analysis of the crude culture supernatant of AB2834/pKD136/pKD9.069A by ^{1}H NMR (Figure 2) indicated that except for acetate (4.1 mM) and trace quantities of PCA, catechol was the only detectable product in

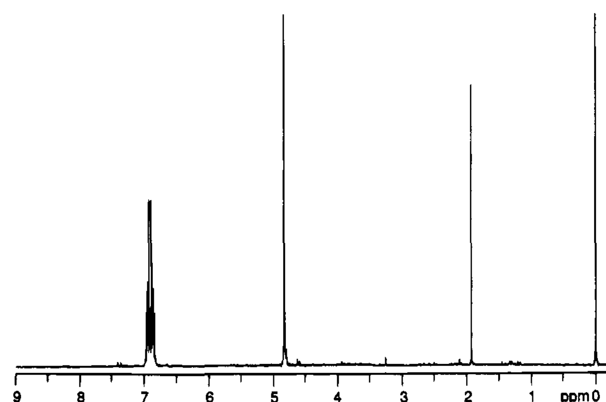
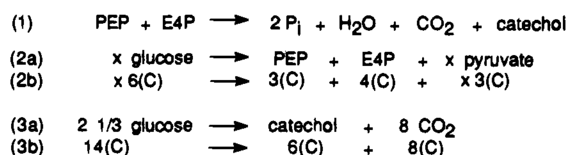


Figure 2. ^{1}H NMR of the culture supernatant of AB2834/pKD136/pKD9.069A after 48 h of culturing in minimal medium which initially contained 56 mM D-glucose. Observable resonances for accumulated molecules are catechol at δ 6.8−7.0 (m, 4H) and acetate δ 1.9 (s, 3H).

the culture medium. Extraction of catechol from the medium (pH 6.7) with ethyl acetate afforded a quantitative recovery of catechol that was free of PCA and acetate impurities.



Determining the theoretical yield[26] for biocatalytic synthesis of catechol begins with balancing (eq 1) the substrate and cosubstrate inputs with the products and byproducts formed by the pathway constructed in AB2834/pKD136/pKD9.069A. The phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P) input required (Scheme 2) for catechol synthesis is then equated to the amount of D-glucose that is required to form these substrates (eq 2a). A pyruvate term is added (eq 2a) to take into consideration the operation of the PEP phosphotransferase system[27] in *E. coli*. This system converts one molecule of PEP into one molecule of pyruvate with the uptake of each molecule of D-glucose by *E. coli*. Coefficients (eq 2b) are determined to balance the number of carbon atoms in the glucose input with the total number of carbon atoms formed in PEP, E4P, and pyruvate. The coefficient for the D-glucose term (eq 2b) is then used in the carbon balance (eq 3b) for the overall conversion (eq 3a). Total carbon dioxide formation (eq 3a) reflects the one molecule of carbon dioxide resulting from PCA decarboxylase activity and the three molecules of carbon dioxide produced from each molecule of pyruvate. A requirement of 2.33 molecules of D-glucose for synthesis of each molecule of catechol indicates (eq 3a) that the theoretical maximum mol % yield of catechol from D-glucose is 43%.

Catechol levels synthesized by AB2834/pKD136/pKD9.069A corresponds to a 33 mol % yield. This yield does not take into consideration the amount of D-glucose required to grow the biocatalyst. Biocatalytic synthesis of catechol in fermentors with controlled nutrient feed rates, dissolved oxygen levels, and agitation conditions will also be needed for obtaining yields scalable to larger volume conversions. Nonetheless, the yield obtained with AB2834/pKD136/pKD9.069A is a useful first approximation of the achievable yield for conversion of D-glucose into catechol. *E. coli* AB2834/pKD136/pKD9.069A thus provides an invaluable starting point for further biocatalyst improvement and optimization.

Experimental Section

General Methods. Shikimic acid was obtained from Aldrich. The sodium salt of 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid (TSP) was

(26) Föberg, C.; Eliaeson, T.; Häggström, L. *J. Biotechnol.* **1988**, *7*, 319.

(27) Postma, P. W. In *Escherichia coli and Salmonella typhimurium*; Neidhardt, F. C., Ed.; American Society for Microbiology: Washington, 1987; Vol. 1, pp 127−141.

purchased from Lancaster Synthesis Inc. Protein assay solution was purchased from Bio-Rad. Packagene Lambda DNA Packaging System was obtained from Promega. ^{1}H NMR spectra were recorded on a Varian Gemini-200 spectrophotometer at 200 MHz. Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim.

Bacterial Strains. *E. coli* DH5α is *F′ endA1 hsdR17($r^-_K m^+_K$) supE44 thi-1 recA1 gyrA relA1* Φ80lacZΔ*M15* Δ*(lacZYA-argF)*$_{U169}$.[28] *Klebsiella pneumoniae* A170−40 was obtained from the American Type Culture Collection (strain 25597).[29] *E. coli* AB2834 [*tsx-352 supE42* λ^- aroE353 malA352 (λ^-)][30] was obtained from the *E. coli* Genetic Stock Center at Yale University.

Culture Medium. All solutions were prepared in distilled, deionized water. LB medium[31] contained Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g) in 1 L of water. M9 minimal medium[31] contained Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NaCl (0.5 g), NH_4Cl (1 g), D-glucose (10 g), $MgSO_4$ (0.12 g), and thiamine (1 mg) in 1 L of water. Shikimic acid (40 mg) supplementation was added to M9 minimal medium, although identical experiments carried out in M9 lacking shikimic acid yielded comparable results (data not shown). Isopropyl β-D-thiogalactopyranoside (IPTG) (0.2 mM) was added to all cultures of strains possessing plasmids derived from pSU18 and pSU19. Ampicillin (50 mg L^{-1}), chloramphenicol (20 mg L^{-1}), and tetracycline (12.5 mg L^{-1}) were added to all appropriate cultures. Solutions of inorganic salts, magnesium salts, and glucose were autoclaved separately and then mixed. Antibiotics, thiamine, shikimic acid, 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.

Chromogenic[22] agarose plates were prepared in M9 medium and contained (per L) D-glucose (4 g), ferric citrate (70 mg), *p*-toluidine (1.9 g), and shikimic acid (40 mg). Antibiotics were added as appropriate. *p*-Toluidine was added to the autoclaved medium as a concentrated solution in ethanol (0.6 g/mL).

Culture Conditions. Inoculants were grown in LB medium (50 mL in a 250-mL Erlenmeyer flask) containing the appropriate antibiotics for 12 h at 37 °C with agitation. A 10 mL aliquot of inoculant was then added to LB medium (1 L in a 4-L Erlenmeyer flask) containing antibiotics and IPTG. Cells were grown at 37 °C for 10 h with agitation (250 rpm). Cells were collected by centrifugation at 4000*g* for 5 min and then resuspended immediately in sterile M9 medium (1 L) containing antibiotics and IPTG. Cultures were returned to 37 °C and 250 rpm agitation.

NMR Analysis of Culture Supernatants. An aliquot (10−25 mL) of each culture was removed at the indicated time interval, and cells were removed by centrifugation. A portion (2−5 mL) of the culture supernatant was concentrated to dryness under reduced pressure, concentrated to dryness two additional times from D_2O, and then redissolved in D_2O containing a known concentration of TSP (δ = 0.00 ppm). Concentrations of metabolites in the supernatant were determined by comparison of integrals corresponding to each metabolite with the integral corresponding to TSP in the ^{1}H NMR. Mean values and standard deviations were determined from cultures which were grown in triplicate.

General Genetic Manipulations. Standard techniques[28,32] were used for restriction digests, preparation of plasmid DNA, transformation of bacterial cells, and cloning *aroZ* and *aroY*. Construction of pKD136 has been described elsewhere.[12] Vectors pSU18 and pSU19 are medium copy number vectors derived from pSU2718 and pSU2719.[33] Plasmids pSU18 and pSU19 encode resistance to chloramphenicol, a *lac* promoter, and a p15A origin of replication and differ in the orientation of the pUC18 multiple cloning site with respect to the *lac* promoter.

Cosmid libraries of *K. pneumoniae* were prepared in pLAFR3[25] or a pLAFR3 derivative using the following general procedure. Genomic DNA was purified[34] from *K. pneumoniae* A170−40 as described previously. Genomic DNA was partially digested with a single restriction enzyme to afford fragments in the range of 20−30 kb. The resulting DNA fragments were ligated to pLAFR3 which had been previously digested with the same restriction enzyme and treated with alkaline phosphatase. Ligated DNA was packaged in phage λ using Packagene extract as recommended by the manufacturer. The phage library was transfected into DH5α/pKD136 and colonies were selected on LB plates for resistance to ampicillin and tetracycline. Antibiotic-resistant colonies were then screened on chromogenic agarose plates. Colonies producing PCA darkened the surrounding medium after 48 h at 37 °C, while those producing catechol darkened the surrounding medium after 24 h at 37 °C.

Enzyme Assays. Cells were grown in LB medium containing the appropriate antibiotics for 10 h as described under culture conditions. Cells were isolated by centrifugation and disrupted by two passes through a French press at 16 000 psi. Cellular debris was removed by centrifugation at 40 000*g* for 20 min. Protein concentrations were determined using the Bradford dye-binding procedure.[35] A standard curve was prepared with bovine serum albumin.

DHS dehydratase was assayed as described by Strøman[15c] by monitoring formation of PCA at 290 nm. The reaction mixture contained 1 mM DHS, 25 mM $MgCl_2$, and 0.1 M Tris-HCl at a pH of 7.5 in a total volume of 1 mL. Specific activity was expressed as μmol of PCA generated per min per mg of protein at 24 °C. A molar extinction coefficient of 3.89×10^3 M^{-1} cm^{-1} (290 nm) was used for PCA.

PCA decarboxylase was assayed by monitoring the disappearance of PCA. The assay solution contained 0.3 mM PCA and 75 mM NaH_2PO_4 at a pH of 6.0 in a total volume of 1 mL. The absorbance of the reaction was monitored at 290 nm. Specific activity was expressed as μmol of PCA lost per min per mg of protein at 24 °C.

Toxicity of Catechol versus PCA: Microbial Growth. A 1 L culture of AB2834/pKD136 was grown in LB medium for 10 h as described under culture conditions. An aliquot was removed after 10 h, and serial dilutions (final volume 1.5 mL) were prepared in LB. Aliquots (0.1 mL) of the 10^{-6} dilution were spread onto LB plates containing ampicillin and either 0, 0.1, 1.0, or 2.5 mM catechol or 0, 5, 10, or 25 mM PCA. Agarose plates were incubated for 24 h at 37 °C. Samples were run in triplicate to establish mean values and standard deviations.

Toxicity of Catechol versus PCA: DHS Synthesis. A 1 L culture of AB2834/pKD136 was grown in LB medium for 10 h as described under culture conditions. Cells were harvested by centrifugation from 100 mL aliquots of the culture and immediately resuspended in 100 mL of sterile M9 medium (500-mL flask) containing 0, 5, 10, or 25 mM catechol or 0, 5, 10, or 25 mM PCA. The resuspended cells were cultured at 37 °C with 250 rpm agitation. After 24 h in minimal medium, each culture supernatant was evaluated by ^{1}H NMR as previously described. Synthesized DHS was reported as the mean value of duplicate cultures.

Isolation of Catechol from the Culture Supernatant. AB2834/pKD136/pKD9.069A was grown as described under culture conditions. After resuspension in minimal medium (24 h), the cells were removed by centrifugation. ^{1}H NMR analysis of the culture supernatant indicated a catechol concentration of 20.4 mM. The culture supernatant (900 mL) was extracted two times with 900 mL portions of ethyl acetate. The organic fractions were combined, dried over $MgSO_4$, and concentrated under reduced pressure to afford a quantitative recovery of the synthesized catechol.

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(28) *Current Protocols in Molecular Biology*; Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., Struhl, K., Eds.; Wiley: New York, 1987.

(29) Weiss, U.; Mingioli, E. S. *J. Am. Chem. Soc.* **1956**, *78*, 2894.

(30) Pittard, J.; Wallace, B. J. *J. Bacteriol.* **1966**, *91*, 1494.

(31) Miller, J. H. *Experiments in Molecular Genetics*; Cold Spring Harbor Laboratory: Plainview, 1972.

(32) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory: Plainview, 1990.

(33) Martinez, E.; Bartolome, B.; de la Cruz, F. *Gene* **1988**, *68*, 159.

(34) Silhavy, T. J.; Berman, M. L.; Enquist, L. W. *Experiments with Gene Fusions*; Cold Spring Harbor Laboratory: Plainview, 1984.

(35) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.