

MIT Open Access Articles

Engineering Escherichia coli coculture systems for the production of biochemical products

The MIT Faculty has made this article openly available. **Please share**
how this access benefits you. Your story matters.

Citation: Zhang, Haoran, Brian Pereira, Zhengjun Li, and Gregory Stephanopoulos. "Engineering Escherichia Coli Coculture Systems for the Production of Biochemical Products." Proc Natl Acad Sci USA 112, no. 27 (June 25, 2015): 8266-8271.

As Published: <http://dx.doi.org/10.1073/pnas.1506781112>

Publisher: National Academy of Sciences (U.S.)

Persistent URL: <http://hdl.handle.net/1721.1/101117>

Version: Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

Terms of Use: Article is made available in accordance with the publisher's policy and may be subject to US copyright law. Please refer to the publisher's site for terms of use.



Massachusetts Institute of Technology

Engineering *Escherichia coli* coculture systems for the production of biochemical products

Haoran Zhang, Brian Pereira, Zhengjun Li¹, and Gregory Stephanopoulos²

Chemical Engineering Department, Massachusetts Institute of Technology, Cambridge, MA 02139

Edited by Arnold L. Demain, Drew University, Madison, NJ, and approved June 5, 2015 (received for review April 6, 2015)

Engineering microbial consortia to express complex biosynthetic pathways efficiently for the production of valuable compounds is a promising approach for metabolic engineering and synthetic biology. Here, we report the design, optimization, and scale-up of an *Escherichia coli*-*E. coli* coculture that successfully overcomes fundamental microbial production limitations, such as high-level intermediate secretion and low-efficiency sugar mixture utilization. For the production of the important chemical *cis,cis*-muconic acid, we show that the coculture approach achieves a production yield of 0.35 g/g from a glucose/xylose mixture, which is significantly higher than reported in previous reports. By efficiently producing another compound, 4-hydroxybenzoic acid, we also demonstrate that the approach is generally applicable for biosynthesis of other important industrial products.

metabolic engineering | microbial coculture | muconic acid | 4-hydroxybenzoic acid

Metabolic engineering and synthetic biology have made great strides in constructing and optimizing metabolic pathways for biochemical product synthesis in a pure culture (1, 2). There are, however, situations where this approach may be limited, as in the cases where (*i*) a single host cell cannot provide an optimal environment for the functioning of all pathway enzymes, (*ii*) biosynthetic efficiency is reduced due to overwhelming metabolic stress from the overexpression of long and complex pathways (3, 4), or (*iii*) pathway intermediates are secreted yielding undesired byproducts and reducing substrate utilization (5, 6). The above limitations can potentially be overcome through the use of rationally designed microbial coculture systems. Different from previous modular engineering approaches, such coculture-based systems completely modularize and segregate a biosynthetic pathway into two separate cells, each of which carries a portion of the pathway, and thus can be engineered independently to achieve optimal functioning of the combined pathway.

The concept of microbial cocultures is not new. However, previous research on microbial consortia was primarily concerned with the study of mixed population stability and dynamic interactions (7–11), although a few recent studies have reported the engineering of microbial consortia for utilization of simple sugars to make small molecules of central carbon metabolism, such as ethanol and lactate (12, 13). Progress was made recently, when a full n-butanol pathway was expressed in two separate *E. coli* cells to achieve higher production (14) and when a bacterium-yeast coculture was used to address the difficulties of functional reconstitution of a pathway involving prokaryotic and eukaryotic enzymes in a consortium, and thus improved production of complex pharmaceutical molecules (15). Here, we expand the generality of the coculture engineering by demonstrating that microbial cocultures can also be engineered to overcome more universal challenges in metabolic engineering, including high-level intermediate secretion and low-efficiency sugar mixture utilization. In particular, we show that without the employment of additional microbial species, two cells of the same species can be engineered to form an integrated system for accomplishing biosynthesis that is difficult to achieve by a single cell. The use of one microbial species may increase culture

stability compared with multispecies consortia, and represents a promising and relatively unexplored approach in the context of metabolic engineering and synthetic biology. As a proof of concept, we show that a stable coculture system can be established to accommodate the requirements of the complex pathway of the synthesis of commodity chemical *cis,cis*-muconic acid (MA) optimally and result in high-level production from sugar mixtures that can be derived from renewable lignocellulose at an industrially relevant yield.

The *cis,cis*-MA is an important compound for making a variety of high-demand bulk chemicals, including nylon- and polyurethane-precursor adipic acid and terephthalic acid, a monomer of the plastic polymer polyethylene terephthalate (16). Current industrial production of adipic acid and terephthalic acid relies exclusively on petroleum and coal feedstocks. Production methods using renewable resources, such as lignocellulose, are potentially attractive, provided that they can be cost-effective. However, despite progress in microbial MA production, previous studies have suffered from low yields and titers and high accumulation of intermediate metabolites (17–22).

Results and Discussion

MA is synthesized via the shikimate pathway, whereby precursor metabolites of central carbon metabolism erythrose-4-phosphate (E4P) and phosphoenolpyruvate condense to synthesize 3-dehydroshikimic acid (DHS), a key MA precursor. DHS is then converted to MA by three enzymes: DHS dehydratase, protocatechualic acid (PCA) decarboxylase, and catechol (CA) 1,2-dioxygenase

Significance

Production of industrial compounds by using engineered microorganisms is a robust method to reduce our reliance on nonrenewable petroleum resources and increase the utility of renewable resources, such as lignocellulose. Because there are major limitations for engineering a single microbial cell to achieve high-yield production, we developed microbial co-culture systems consisting of two different microbial cell types to use sugar mixtures that can be derived from lignocellulose efficiently. We demonstrate that this approach is successful for achieving high-level production of two important value-added molecules, *cis,cis*-muconic acid and 4-hydroxybenzoic acid. This accomplishment establishes a previously unidentified technology for advancing future research in metabolic engineering and synthetic biology.

Author contributions: H.Z. and G.S. designed research; H.Z., B.P., and Z.L. performed research; H.Z., Z.L., and G.S. analyzed data; and H.Z., B.P., and G.S. wrote the paper.

The authors declare no conflict of interest.

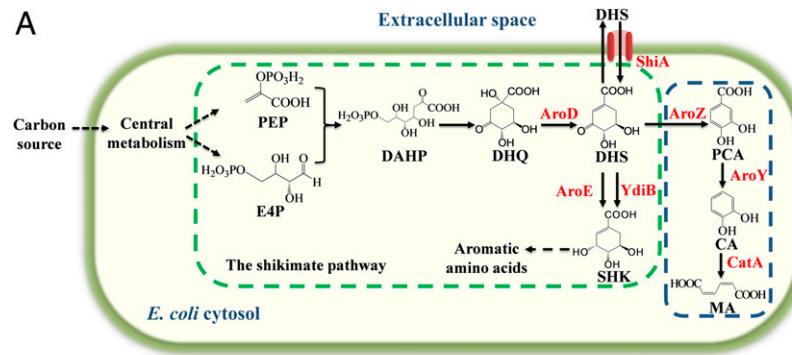
This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

¹Present address: Beijing Key Laboratory of Bioprocess, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, People's Republic of China.

²To whom correspondence should be addressed. Email: gregstep@mit.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1506781112/-DCSupplemental.



B

	Wild type	<i>aroD</i> mutant	<i>aroD shiA</i> mutant
Minimal Medium (MM)	+++	-	-
MM + DHS	+++	+	-
MM + DHS + plasmid-borne <i>ShiA</i>	+++	++	++

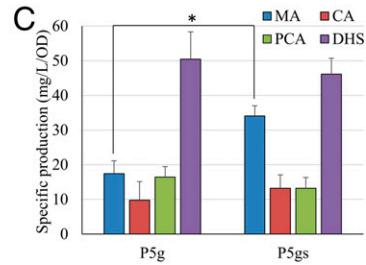


Fig. 1. MA production using a single-cell strategy. (A) Schematic of the *cis,cis*-MA biosynthetic pathway and intermediate DHS mass transfer. Compound abbreviations are listed and described in *SI Appendix*, Table S2. (B) Phenotypic confirmation of *ShiA* activity for cross-membrane DHS transport. *aroD* mutant, *E. coli* BW25113 with disrupted shikimate pathway by removal of the *aroD* gene; *aroD shiA* mutant, *aroD* mutant with the further removal of the *shiA* transporter gene; WT, *E. coli* BW25113. +++, strong growth; ++, moderate growth; +, poor growth; -, no growth. (C) MA production improvement by overexpression of *ShiA* transporter. P5g, *E. coli* with the engineered shikimate pathway and MA biosynthesis pathway; P5gs, P5g with overexpression of *ShiA*. *P < 0.005 (Student's t test).

(Fig. 1A). We selected the DHS dehydratase AroZ and the PCA decarboxylase AroY from *Klebsiella pneumoniae* and the CA 1,2-dioxygenase CatA from *Acinetobacter calcoaceticus* to assemble the heterologous DHS-to-MA pathway based on previous studies and an *in vivo* activity assay of the enzymes from different microorganisms (16, 17) (*SI Appendix*, Fig. S1). To eliminate the undesired metabolic flux to aromatic amino acid biosynthesis, we deleted genes encoding the competing enzymes YdiB and AroE from a previously constructed *Escherichia coli* tyrosine overproducer's chromosome (23). The resulting *E. coli* strain P5g was then used to produce MA from a glucose/xylose sugar mixture. However, only 68 mg/L MA and 187 mg/L DHS were produced from 3.3 g/L xylose and 6.6 g/L glucose (typical of the ratio of these sugars in lignocellulose). Further analysis showed that the majority of DHS accumulated outside the cell, suggesting that the intracellular availability of DHS for downstream conversion to MA could be a limiting step. DHS accumulation was also observed in several previous studies (18–20, 24, 25). Thus, secretion of DHS and accumulation in the medium have plagued prior work aiming at yield improvement despite many attempts to reduce DHS secretion through overexpression of enzymes downstream of DHS.

To improve MA production further, we engineered a membrane-bound transporter, *ShiA*, specifically to assimilate DHS back into the cell. *ShiA* is a 12-helix transmembrane permease and has been characterized as a symporter for protons and the metabolite shikimate, which is structurally similar to DHS (26). However, there was no prior report confirming *ShiA*'s transport activity for DHS. To ascertain *ShiA*'s functionality, as well as to explore its potential use for DHS importation, a phenotype assay was designed and performed (Fig. 1B). We found that disruption of the shikimate pathway by removal of the *aroD* gene resulted in no *E. coli* growth on a minimal medium. However, the *aroD* mutant was able to assimilate exogenous DHS substrate to complete the shikimate pathway if the *ShiA* transporter was active. The *aroD* and *shiA* double-mutant strain was not able to grow even in the presence of exogenous DHS, indicating that

transporter *ShiA* is actively responsible for DHS importation. Supplementation of the *shiA* gene enabled the *aroD* and *shiA* double mutant to restore the DHS importation ability, and thus to grow on minimal medium with exogenous DHS. Our results showed, for the first time to our knowledge, that *ShiA* is indeed a functional transporter for DHS.

Although chromosomal expression of native *shiA* gene is active, it was also reported to be repressed under normal growth conditions (26, 27). In an effort to improve DHS transport and intracellular availability, the *E. coli* *shiA* gene was overexpressed in strain P5gs. The specific MA production (the production concentration normalized by the cell density) of P5gs was higher than the specific MA production of P5g (Fig. 1C), indicating that *shiA* overexpression did help import DHS back to the cytosol for MA formation as expected. Further screening of other *E. coli* permeases with high amino acid sequence similarity confirmed that *ShiA* was the best DHS transporter (*SI Appendix*, Fig. S2). Notably, 3 g/L xylose remained unconsumed at the end of P5gs cultivation, partially due to the glucose catabolite repression that delayed effective assimilation of xylose. In fact, glucose catabolite repression and the resultant ineffective simultaneous assimilation of glucose and xylose have presented a generic challenge in the engineering of single microbial cells for treating sugar mixtures (28).

Given the persisting high extracellular DHS levels and low sugar utilization efficiency (despite overexpression of the DHS-consuming genes), we sought to improve MA synthesis through the use of an *E. coli*-*E. coli* coculture system. Compared with two microbes of different species, two *E. coli* cells of the same species have a similar growth rate. As such, the use of an *E. coli*-*E. coli* coculture would help minimize problems of dominance of one species and culture instability. To this end, the entire MA biosynthetic pathway was split into two independent modules, each of which was contained in a separate *E. coli* cell. The first cell comprised the engineered shikimate pathway solely dedicated to the production of the intermediate DHS, whereas the second cell harbored the heterologous DHS-to-MA pathway, as well as the

engineered DHS transporter (Fig. 2A). Because the DHS-to-MA conversion relies on functional expression of three heterologous enzymes, we introduced the DHS-to-MA biosynthetic pathway into an *E. coli* expression strain BL21(DE3) that is commonly used for expression of foreign enzymes. Indeed, it was found that the DHS-to-MA heterologous pathway showed higher efficiency in *E. coli* BL21(DE3) than K-12(DE3), which is the precursor of *E. coli* P5g and P5gs used previously (*SI Appendix*, Fig. S3). Therefore, *E. coli* BL21(DE3) was engineered to overexpress the complete DHS-to-MA pathway and the DHS transporter, and the resulting strains were used for the coculture production of MA.

The use of a coculture system also provides a powerful method to overcome glucose catabolite repression. Specifically, the phosphotransferase system in the first cell type was removed, such that the resulting strain, P6.2, prefers to use xylose for growth, and the *xylA* gene was deleted from the chromosome of the second cell type [(BL21(DE3) derivatives], yielding strains BXS and BXC (for inducible and constitutive expression, respectively) that cannot consume xylose (*SI Appendix*, Fig. S4). The resulting consortium of P6.2 and BXS/BXC strains simultaneously consumed xylose and glucose (Fig. 2A). Moreover, this design ensured that

the two cells do not compete for carbon sources, making each cell's growth more compatible with the other cell's growth.

This coculture strategy indeed improved MA production. Compared with single-cell P5gs-based monoculture, the P6.2/BXS consortium with a 1:1 inoculation ratio was able to convert 3.3 g/L xylose and 6.6 g/L glucose to a fourfold higher titer of MA, accompanied by significantly increased CA and PCA concentrations (Fig. 2B). The MA production did not vary with the inoculation ratio of P6.2/BXS (*SI Appendix*, Fig. S5). We then switched the regulation of expression of the three heterologous enzymes and the transporter ShiA from the inducible T7 promoter to the constitutive *Zymomonas mobilis* pyruvate decarboxylase promoter, generating a new downstream cell, BXC. This strategy eliminated the need for the costly isopropyl- β -D-thiogalactopyranoside (IPTG) inducer and further reduced the unnecessary metabolic burden caused by the overly strong T7 promoter. The new coculture system (i.e., P6.2/BXC) yielded different MA and intermediate production profiles by fine-tuning the initial inoculation ratio between the two cells instead of modulating the expression strength of individual modules in one cell, as used by traditional metabolic engineering. At the optimal inoculation ratio of 1:1, 850 mg/L MA and low levels of intermediates were produced by complete consumption of xylose and glucose (Fig. 2B and *SI Appendix*, Fig. S6).

The time course of the mixed population constituents (Fig. 2C) shows that the percentage of BXC in the whole population gradually increased with time, so that the evolved coculture system exhibited a dynamic growth balance between the two constituent strains. The high final BXC percentage was due to its faster consumption of glucose compared with P6.2's consumption of xylose (Fig. 2D). As a result, the large BXC population exhibited a strong and steady capacity for DHS-to-MA conversion and reduced the accumulation of other intermediates over time, as shown in Fig. 2E.

The coculture system also exhibited a remarkable capacity for handling variable sugar mixtures and converting them to the MA product. For example, with a sugar mixture of 6.6 g/L xylose and 3.3 g/L glucose (high xylose content), the P6.2/BXC coculture produced 682 mg/L MA at the best inoculation ratio of 1:1, compared with 36 mg/L MA produced by a monoculture based on the single-cell P5gs. The time profiles of the BXC population, intermediate accumulation, and sugar concentration changes showed altered patterns compared with the case of the low xylose medium (*SI Appendix*, Fig. S7).

The very low accumulation of DHS in the coculture system (Fig. 2B) indicated that total MA synthesis was limited by DHS supply, and therefore that MA production could be further improved by engineering the upstream cell so as to increase the supply of DHS to the downstream cell. Three key upstream enzymes, PpsA, TktA, and AroG, were targeted because they play a material role in increasing the flux to the first committed step of the shikimate pathway (29) (Fig. 3A). When the feedback-resistant *aroG* gene (30) and the *pssA* gene were overexpressed in strains P6.5 and P6.6, respectively, these engineered strains cocultured with BXC on the low xylose medium yielded an increased MA titer of 1,185 mg/L and 1,209 mg/L, respectively (Fig. 3B). Notably, such production improvement was not achieved in the monoculture systems comprising a single-cell type, because the supply of DHS was not the limiting step in the overall MA pathway (*SI Appendix*, Table S1). Further overexpression of the *tktA* gene resulted in reduced production, indicating that it decreased the availability of the shikimate pathway precursor E4P.

Next, the best strain combination, P6.6 and BXC, was cultivated in a 1.3-L batch-mode bioreactor to scale up the production (*SI Appendix*, Fig. S8A). MA (4 g/L) was produced from 6.6 g/L xylose and 13.2 g/L glucose, and the intermediate accumulation was maintained at low levels throughout the whole production process (Fig. 3C). Because BXC assimilated glucose and grew

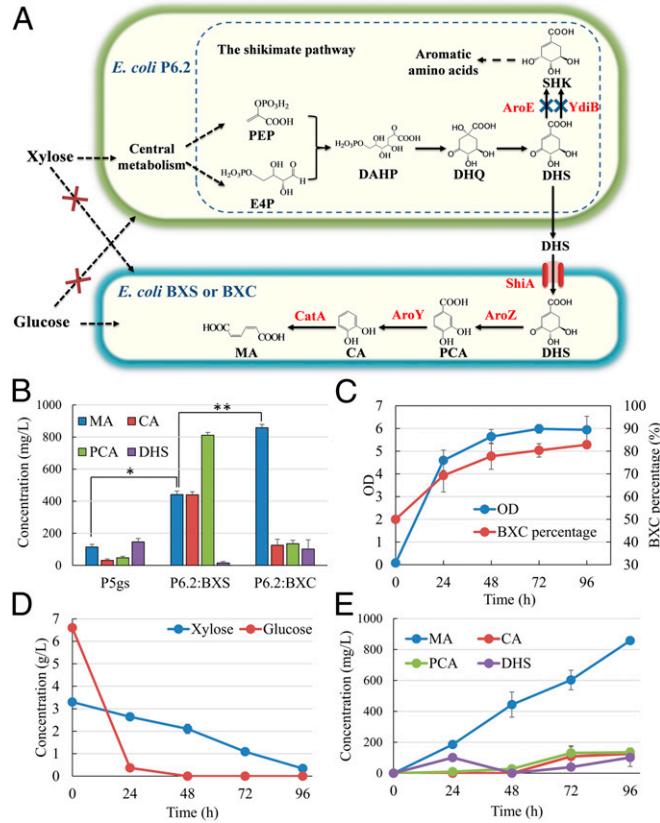


Fig. 2. Conversion of a sugar mixture to the value-added MA product using an *E. coli*-*E. coli* coculture strategy. (A) Schematic of the *E. coli*-*E. coli* coculture approach to accommodate the complex MA biosynthetic pathway and convert a xylose and glucose mixture to MA. (B) Production comparison between the monoculture approach (P5gs) and coculture approach with inducible (P6.2:BXS) and constitutive (P6.2:BXC) expression strategies. The medium contained 3.3 g/L xylose and 6.6 g/L glucose. * $P < 0.001$; ** $P < 0.001$ (Student's *t* test). (C) Overall cell density and BXC percentage profiles of the P6.2:BXC coculture with the inoculation ratio of 1:1. (D) Xylose and glucose uptake during the course of P6.2 and BXC cocultivation with the inoculation ratio of 1:1. (E) Time profile of MA and the pathway intermediate production using P6.2:BXC coculture with the inoculation ratio of 1:1. Error bars represent the SEMs from four independent experiments.

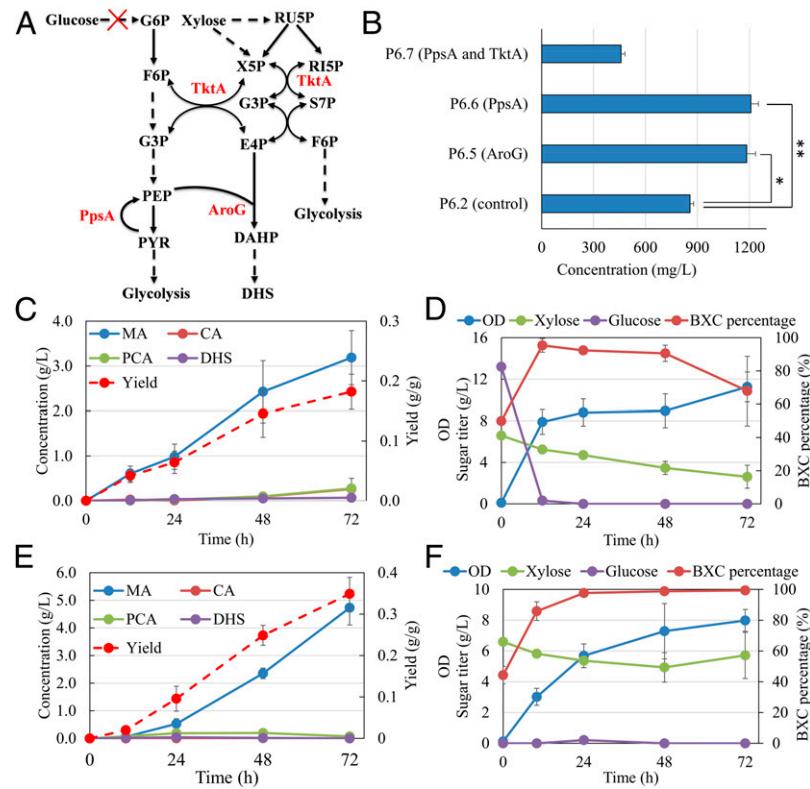


Fig. 3. High-level MA production by modular engineering and scale-up of the coculture systems. (A) Schematic of the metabolic network leading from xylose to the shikimate pathway. AroG, feedback-resistant 2-dehydro-3-deoxyphosphoheptonate aldolase (30); PpsA, phosphoenolpyruvate synthetase; TktA, transketolase. (B) MA production by the coculture systems using the engineered upstream cells P6.5, P6.6, and P6.7. The specified strains were cocultivated with BXC with the inoculation ratio of 1:1. P6.2, control strain; P6.5m overexpressed feedback-resistant AroG; P6.6, overexpressed PpsA; P6.7, overexpressed PpsA and TktA. Error bars represent the SEMs from three independent experiments. *P < 0.001; **P < 0.001 (Student's t test). (C and D) Batch bioreactor cocultivation of P6.6 and BXC on 6.6 g/L xylose and 13.2 g/L glucose. The P6.6 and BXC were cocultivated with the inoculation ratio of 1:1. (C) Time profile of MA and intermediate production and overall production yield. (D) Time profile of the P6.6/BXC coculture overall cell density (OD), BXC percentage, and sugar titers. Error bars represent SEMs from three independent experiments. (E and F) Fed-batch bioreactor cocultivation of P6.6 and BXC. The P6.6 and BXC were cocultivated with the inoculation ratio of 1:1. (E) Time profile of MA and intermediate production and overall production yield. (F) Time profile of the P6.6/BXC coculture overall cell density, BXC percentage, and sugar titers. Error bars represent the SEMs from three independent experiments.

much faster than P6.6 grown on xylose, the majority of the coculture population was dominated by BXC (Fig. 3D). However, the majority of glucose was consumed by BXC at the initial phase of the coculture cultivation when only a small amount of DHS was produced by the upstream P6.6 cell. In an effort to avoid unnecessary glucose uptake, a fed-batch bioreactor was used to optimize the production further. Under this new condition, the MA production titer and yield were improved to 4.7 g/L and 0.35 g/g, respectively (Fig. 3E and *SI Appendix*, Fig. S8B). Glucose was depleted throughout the whole cocultivation process in the fed-batch bioreactor, whereas the xylose concentration fluctuated as a combined result of new xylose addition and xylose uptake by P6.6. Despite reduced glucose consumption, BXC was still the dominant cell type in the coculture (Fig. 3F). These results confirmed that our *E. coli*-*E. coli* coculture system can be stably scaled up to use sugar mixtures efficiently. Moreover, the achieved yield is significantly higher than reported in any previous reports, all of which used a single glucose sugar, instead of more challenging sugar mixtures, for MA production (18–22). The yield of 0.35 g/g corresponds to 51% of the theoretical maximum that a chemical conversion of sugar to MA via DHS can achieve, even without consideration of the carbon loss for cell growth (*SI Appendix*, Eq. S1).

The coculture approach was also used for the conversion of sugar mixtures to the industrially important aromatic compound 4-hydroxybenzoic acid (4HB) (31–33). The modular nature of

our coculture system facilitated a plug-and-play design whereby the upstream DHS precursor provider cell (P6.2) was combined with the downstream 4HB producer cell (BH2.2) (Fig. 4A). The production of 4HB from 3.3 g/L xylose and 6.6 g/L glucose was improved by 8.6-fold when biosynthesis was switched from the monoculture strategy to the coculture strategy. To reduce the accumulated shikimate and DHS intermediates, extra copies of downstream enzymes and the ShiA transporter were cloned in a new strain, BH2.9. Here, the independence of the constituent cells of the coculture system allowed for dedicated engineering of the downstream cell without any negative metabolic impact on the upstream cell. As a result, the consortium of P6.2 and BH2.9 further improved the 4HB titer by another 1.9-fold (Fig. 4B). Finally, the fed-batch bioreactor technique was employed to scale up the 4HB production. Despite the intermediate chorismic acid accumulation, the coculture system achieved a production of 2.3 g/L 4HB. The production yield was also increased to 0.11 g/g, which equals the highest yield reported previously (31) (Fig. 4C and *SI Appendix*, Fig. S9).

Materials and Methods

Bacterial Cultivation Conditions. All strains used in this study were cultivated in 14-mL culture tubes containing 2 mL of medium at 250 rpm and 37 °C (Thermo Scientific MaxQ 4000 orbital shaker with an orbit of 1.9 cm). The media used in this work are as follows. One liter of sugar medium contained 2.0 g of NH₄Cl, 5.0 g of (NH₄)₂SO₄, 3.0 g of KH₂PO₄, 7.3 g of K₂HPO₄, 8.4 g of 3-(*N*-morpholino)propanesulfonic acid, 0.5 g of NaCl, 0.24 g of MgSO₄, 0.5 g

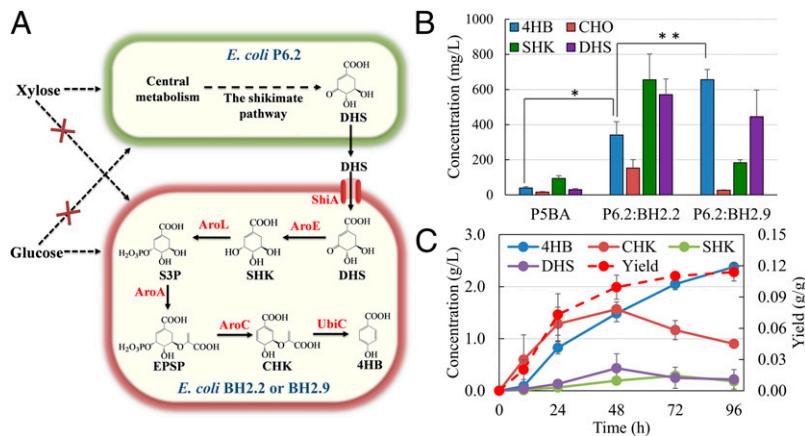


Fig. 4. Production of 4HB using the *E. coli* coculture engineering. (A) Schematic of the *E. coli*-*E. coli* coculture approach to accommodate the 4HB biosynthetic pathway and convert a xylose and glucose mixture to 4HB. BH2.2 overexpressed the enzymes converting DHS to 4HB, as well as the ShiA transporter. BH2.9 carried extra copies of enzymes for DHS-to-4HB conversion and an extra copy of the ShiA transporter. (B) Comparison between the single-cell (P5BA) and coculture (P6.2:BH2.2 and P6.2:BH2.9) strategy for 4HB production from the 3.3-g/L xylose and 6.6-g/L glucose mixture. Error bars represent the SEMs from three independent experiments. * $P < 0.001$; ** $P < 0.01$ (Student's *t* test). (C) Time profile of 4HB and intermediate production and overall production yield achieved by fed-batch bioreactor production. Error bars represent the SEMs from three independent experiments.

of yeast extract, 40 mg of tyrosine, 40 mg of phenylalanine, 40 mg of tryptophan, 10 mg of 4-HB, and desired amounts of xylose and glucose. The low xylose medium contained 3.3 g/L xylose and 6.6 g/L glucose, whereas the high xylose medium contained 6.6 g/L xylose and 3.3 g/L glucose. Trace elements were also supplemented to the media, giving final concentrations of 0.4 mg/L Na₂EDTA, 0.03 mg/L H₃BO₃, 1 mg/L thiamine, 0.94 mg/L ZnCl₂, 0.5 mg/L CoCl₂, 0.38 mg/L CuCl₂, 1.6 mg/L MnCl₂, 3.77 mg/L CaCl₂, and 3.6 mg/L FeCl₂.

For the ShiA phenotype screen study, a minimal medium was used to cultivate the constructed strains. One liter of minimal medium contained 15 g of glucose, 2 g of (NH₄)₂SO₄, 0.2 g of MgSO₄, 3.82 g of Na₂HPO₄, 1.5 g of KH₂PO₄, 0.05 g of Fe(III)-NH₄-citrate, 0.02 g of CaCl₂, 0.06 mg of ZnSO₄, 0.02 mg of MnCl₂, 0.3 mg of H₃BO₃, 0.11 mg of CoCl₂, 0.006 mg of CuSO₄, 0.0011 mg of NiCl₂, 0.028 mg of NaMoO₄, and 0.5 mmol of HCl. The working concentrations of antibiotics were 100 mg/L ampicillin, 50 mg/L kanamycin, and 34 mg/L chloramphenicol.

For *E. coli* P5g, P5gs, and P5BA monoculture cultivation, 40 μ L of overnight LB culture was inoculated into 2 mL of the sugar medium containing appropriate antibiotics and grown for 96 h. At the time of inoculation, 0.1 mM IPTG was added. For *E. coli*-*E. coli* coculture cultivation, 40 μ L of overnight LB culture of the first cell type (P6.2) and the desired amount of overnight LB culture of the second cell type (BXS, BXC, BH2.2, and BH2.9) were inoculated into the sugar medium at the specified inoculation ratio simultaneously. The mixed culture was then cultivated at 250 rpm at 37 °C for 96 h (Thermo Scientific MaxQ 4000 orbital shaker with an orbit of 1.9 cm). For P6.2/BXS, P6.2/BH2.2, and P6.2/BH2.9 cocultures, 0.1 mM IPTG was added at the time of inoculation.

For batch bioreactor cultivation for MA production, 14 mL of overnight LB culture of *E. coli* P6.6 and 14 mL of overnight LB culture of *E. coli* BXC were inoculated into a 1.3-L BioFlo110 modular fermenter system (New Brunswick Scientific) with 0.7 L of sugar medium containing 6.6 g/L xylose and 13.2 g/L glucose. Culture conditions were maintained as follows: airflow rate, 0.5 L/min; pH value, 6.8; and temperature, 37 °C. The agitation was set up to maintain a dissolved oxygen (DO) of 12% air saturation until glucose was depleted, after which the agitation rate was maintained at 100 rpm. For fed-batch bioreactor cultivation for MA production, 14 mL of an overnight LB culture of *E. coli* P6.6 and 14 mL of an overnight LB culture of *E. coli* BXC were inoculated into a 1.3-L BioFlo110 modular fermenter system with 0.7 L of sugar medium containing only 6.6 g/L xylose. A stock solution of 120 g/L xylose and 240 g/L glucose was fed into the bioreactor at a feeding rate of 0.45 mL·h⁻¹. Culture conditions were maintained as follows: airflow rate, 0.5 L/min; pH value, 7.1; and temperature, 37 °C. The agitation was set up to maintain a DO of 25%. The bioreactor volume increase due to addition of the sugar mixture and acid and base solutions was considered when calculating the production yield.

For fed-batch bioreactor cultivation for 4HB production, 14 mL of overnight LB culture of *E. coli* P6.2 and 14 mL of overnight LB culture of *E. coli* BH2.9 were inoculated into a 1.3-L BioFlo110 modular fermenter system with 0.7 L of sugar medium containing 6.6 g/L xylose and 6.6 g/L glucose. A stock solution of 120 g/L xylose and 240 g/L glucose was fed into the

bioreactor at a feeding rate of 0.27 mL·h⁻¹ throughout the whole cultivation process. Culture conditions were maintained as follows: airflow rate, 0.4 L·min⁻¹; pH value, 7.1; and temperature, 37 °C. The agitation was set up to maintain a DO of 12%. The bioreactor volume increase due to addition of the sugar mixture and acid and base solutions was considered when calculating the production yield.

For comparison of the heterologous pathway efficiency in *E. coli* WT and BL21M, 40 μ L of overnight LB culture of each strain was inoculated into the sugar medium supplemented with 5 g/L glucose and 2 g/L DHS, followed by a 24-h growth period at 37 °C before the liquid chromatography (LC)/MS analysis. For the sugar consumption preference test, overnight LB cultures of *E. coli* P5, P6.2, and BXC were inoculated (2% vol/vol) into a sugar medium containing 4.5 g/L xylose and 5 g/L glucose. Sugar concentration changes over time were monitored and compared.

Phenotype Assay. For the phenotypic confirmation of ShiA activity, 10 μ L of overnight LB cultures of WT and mutant strains were centrifuged at 9,800 \times *g*, and the cell pellets were washed with sterile water two times before being inoculated into 1 mL of above-mentioned minimal medium. When needed, 50 mg/L DHS was supplemented into the medium. The inoculated cultures were then incubated at 250 rpm at 37 °C (Thermo Scientific MaxQ 4000 orbital shaker with an orbit of 1.9 cm) for 24 h.

Determination of Ratios Between the Constituent Cells of the Coculture System. The real-time cell-to-cell ratio of the two components in the coculture was analyzed by either fluorescence microscopy analysis or blue-white screening, because the first module cell (P6.2) contained a plasmid-borne GFP and a disrupted lacZ gene, whereas the second module cells (BL21 derivatives) contained an intact lacZ gene. For this study, blue-white screening was used as the primary method for distinguishing the two cells because the expression of GFP in P6.2 was found to be low and unstable, which caused inaccurate results by the fluorescence-based analysis. To conduct blue-white screening, 20 μ L of the coculture sample was diluted 10⁵- to 10⁶-fold before being spread onto an LB agar plate containing X-Gal. After 24 h of incubation, the phenotypic screening was conducted by identification and counting of the blue and white colonies. It should be noted that the results obtained by this method indicated the ratio of the viable upstream and downstream cells rather than the ratio of these cells' overall biomass, including viable and nonviable cells.

Metabolite Analysis. One milliliter of cell-free culture supernatants was filtered through 0.2- μ m pore-sized polytetrafluoroethylene membrane syringe filters (VWR International) and mixed with 10 μ L of 1 g/L *p*-coumaric acid internal standard before being subjected to LC/tandem MS analysis using an Applied Biosystems API2000 system equipped with a Waters symmetry 5- μ m C8 column. Target molecules were eluted using a linear gradient of 5% acetonitrile and 95% 10 mM acetic acid (0 min) to 75% acetonitrile and 25% 10 mM acetic acid (10 min) at a flow rate of 0.3 mL·min⁻¹.

To determine xylose concentration, an aliquot of coculture samples was centrifuged at 12,000 $\times g$ for 2 min, followed by filtration of the supernatant using 0.2- μm pore-sized polytetrafluoroethylene membrane syringe filters. Ten microliters of this treated supernatant was injected into an HPLC system (Waters 2695 separation module coupled to a Waters 410 differential refractometer) for xylose quantification. Isocratic elution through a Bio-Rad HPX-87H column was conducted using 14 mM sulfuric acid as a mobile phase at a flow rate of 0.7 mL·min⁻¹. To measure the concentration of glucose, the

filtered culture supernatant was also injected into a Yellow Springs Instruments YSI 7100 Multiparameter Bioanalytical System equipped with immobilized enzyme electrodes.

ACKNOWLEDGMENTS. We thank Dr. Ellen L. Neidle (University of Georgia) for the generous gift of the CA 1,2-dioxygenase gene. We are grateful for insightful suggestions made by Kang Zhou, Kangjian Qiao, and Thomas Wasylenko. This work was supported by US Department of Energy Grant DE-SC0008744.

1. Woolston BM, Edgar S, Stephanopoulos G (2013) Metabolic engineering: Past and future. *Annu Rev Chem Biomol Eng* 4:259–288.
2. Khalil AS, Collins JJ (2010) Synthetic biology: Applications come of age. *Nat Rev Genet* 11(5):367–379.
3. Ro DK, et al. (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440(7086):940–943.
4. Ajikumar PK, et al. (2010) Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli*. *Science* 330(6000):70–74.
5. Lee JW, et al. (2012) Systems metabolic engineering of microorganisms for natural and non-natural chemicals. *Nat Chem Biol* 8(6):536–546.
6. Zhou K, Zou R, Stephanopoulos G, Too HP (2012) Metabolite profiling identified methylerythritol cyclodiphosphate efflux as a limiting step in microbial isoprenoid production. *PLoS ONE* 7(11):e47513.
7. Keller L, Surette MG (2006) Communication in bacteria: An ecological and evolutionary perspective. *Nat Rev Microbiol* 4(4):249–258.
8. Agapakis CM, Boyle PM, Silver PA (2012) Natural strategies for the spatial optimization of metabolism in synthetic biology. *Nat Chem Biol* 8(6):527–535.
9. Fredrickson AG, Stephanopoulos G (1981) Microbial competition. *Science* 213(4511): 972–979.
10. Bayer TS, et al. (2009) Synthesis of methyl halides from biomass using engineered microbes. *J Am Chem Soc* 131(18):6508–6515.
11. Minty JJ, et al. (2013) Design and characterization of synthetic fungal-bacterial consortia for direct production of isobutanol from cellulosic biomass. *Proc Natl Acad Sci USA* 110(36):14592–14597.
12. Xia T, Eiteman MA, Altman E (2012) Simultaneous utilization of glucose, xylose and arabinose in the presence of acetate by a consortium of *Escherichia coli* strains. *Microb Cell Fact* 11(11):77.
13. Eiteman MA, Lee SA, Altman R, Altman E (2009) A substrate-selective co-fermentation strategy with *Escherichia coli* produces lactate by simultaneously consuming xylose and glucose. *Biotechnol Bioeng* 102(3):822–827.
14. Saini M, Hong Chen M, Chiang CJ, Chao YP (2015) Potential production platform of n-butanol in *Escherichia coli*. *Metab Eng* 27:76–82.
15. Zhou K, Qiao K, Edgar S, Stephanopoulos G (2015) Distributing a metabolic pathway among a microbial consortium enhances production of natural products. *Nat Biotechnol* 33(4):377–383.
16. Xie NZ, Liang H, Huang RB, Xu P (2014) Biotechnological production of muconic acid: Current status and future prospects. *Biotechnol Adv* 32(3):615–622.
17. Draths KM, Frost JW (1994) Environmentally compatible synthesis of adipic acid from D-glucose. *J Am Chem Soc* 116(1):399–400.
18. Niu W, Draths KM, Frost JW (2002) Benzene-free synthesis of adipic acid. *Biotechnol Prog* 18(2):201–211.
19. Curran KA, Leavitt JM, Karim AS, Alper HS (2013) Metabolic engineering of muconic acid production in *Saccharomyces cerevisiae*. *Metab Eng* 15:55–66.
20. Weber C, et al. (2012) Biosynthesis of *cis,cis*-muconic acid and its aromatic precursors, catechol and protocatechuic acid, from renewable feedstocks by *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 78(23):8421–8430.
21. Lin Y, Sun X, Yuan Q, Yan Y (2014) Extending shikimate pathway for the production of muconic acid and its precursor salicylic acid in *Escherichia coli*. *Metab Eng* 23:62–69.
22. Sun X, Lin Y, Huang Q, Yuan Q, Yan Y (2013) A novel muconic acid biosynthesis approach by shunting tryptophan biosynthesis via anthranilate. *Appl Environ Microbiol* 79(13):4024–4030.
23. Santos CNS, Xiao W, Stephanopoulos G (2012) Rational, combinatorial, and genomic approaches for engineering L-tyrosine production in *Escherichia coli*. *Proc Natl Acad Sci USA* 109(34):13538–13543.
24. Li W, Xie D, Frost JW (2005) Benzene-free synthesis of catechol: Interfacing microbial and chemical catalysis. *J Am Chem Soc* 127(9):2874–2882.
25. Juminaga D, et al. (2012) Modular engineering of L-tyrosine production in *Escherichia coli*. *Appl Environ Microbiol* 78(1):89–98.
26. Whipp MJ, Camakaris H, Pittard AJ (1998) Cloning and analysis of the *shiA* gene, which encodes the shikimate transport system of *Escherichia coli* K-12. *Gene* 209(1–2): 185–192.
27. Prévost K, et al. (2007) The small RNA RyhB activates the translation of *shiA* mRNA encoding a permease of shikimate, a compound involved in siderophore synthesis. *Mol Microbiol* 64(5):1260–1273.
28. Kim JH, Block DE, Mills DA (2010) Simultaneous consumption of pentose and hexose sugars: An optimal microbial phenotype for efficient fermentation of lignocellulosic biomass. *Appl Microbiol Biotechnol* 88(5):1077–1085.
29. Herrmann KM, Weaver LM (1999) The shikimate pathway. *Annu Rev Plant Physiol Plant Mol Biol* 50:473–503.
30. Lütke-Eversloh T, Stephanopoulos G (2007) L-tyrosine production by deregulated strains of *Escherichia coli*. *Appl Microbiol Biotechnol* 75(1):103–110.
31. Barker JL, Frost JW (2001) Microbial synthesis of p-hydroxybenzoic acid from glucose. *Biotechnol Bioeng* 76(4):376–390.
32. Verhoef S, Ruijsenaars HJ, de Bont JA, Wery J (2007) Bioproduction of p-hydroxybenzoate from renewable feedstock by solvent-tolerant *Pseudomonas putida* S12. *J Biotechnol* 132(1):49–56.
33. McQualter RB, et al. (2005) Initial evaluation of sugarcane as a production platform for p-hydroxybenzoic acid. *Plant Biotechnol J* 3(1):29–41.