ACS Catalysis

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Transforming Flask Reaction into Cell-Based Synthesis: Production of Polyhydroxylated Molecules via Engineered *Escherichia coli*

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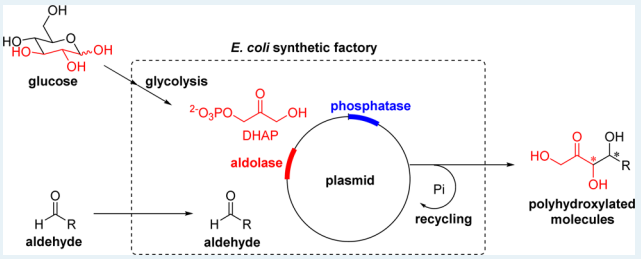
Supporting Information

ABSTRACT: Dihydroxyacetone phosphate (DHAP)-dependent aldolases have been intensively studied and widely used in the synthesis of carbohydrates and complex polyhydroxylated molecules. However, strict specificity toward donor substrate DHAP greatly hampers their synthetic utility. Here, we transformed DHAP-dependent aldolases-mediated by in vitro reactions into bioengineered *Escherichia coli* (*E. coli*). Such flask-to-cell transformation addressed several key issues plaguing in vitro enzymatic synthesis: (1) it solves the problem of DHAP availability by in vivo-hijacking DHAP from the glycolysis pathway of the bacterial system, (2) it circumvents purification of recombinant aldolases and phosphatase, and (3) it dephosphorylates the resultant aldol adducts in vivo, thus eliminating the additional step for phosphate removal and achieving in vivo phosphate recycling. The engineered *E. coli* strains tolerate a wide variety of aldehydes as acceptor and provide a set of biologically relevant polyhydroxylated molecules in gram scale.



KEYWORDS: *DHAP-dependent aldolase, synthetic biology, metabolic engineering, E. coli synthetic factory, polyhydroxylated molecules*

Aldolases exhibit unrivaled efficiency in the synthesis of polyhydroxylated molecules and β-hydroxy-α-amino acids,[1−4] which are difficult to prepare and handle by conventional chemical synthesis. Among known aldolases, DHAP-dependent aldolases have been intensively studied and widely used in the synthesis of carbohydrates and complex polyhydroxylated molecules,[5−18] because configurations of two newly generated stereogenic centers can be chosen and controlled by an appropriate choice of four known DHAP-dependent aldolases.[19] Fructose-1,6-bisphosphate aldolase (FruA) provides a 3*S*,4*R* configuration product; fuculose-1-phosphate aldolase (FucA) provides a 3*R*,4*R* configuration product; tagatose-1,6-bisphosphate aldolase (TagA) provides a 3*S*,4*S* configuration product; rhamnulose-1-phosphate aldolase (RhuA) provides a 3*R*,4*S* configuration product. Therefore, a complete set of four aldol products could be provided (Figure 1).

However, the strict specificity toward donor substrate DHAP greatly hampers their synthetic utility because of the high cost and lability of DHAP.[3,20] Therefore, effective production of DHAP is instrumental, and several chemical and enzymatic approaches have been developed for its synthesis. Chemical approaches focus on producing storable precursors that can be easily converted to DHAP immediately before its use;[5,21−28]

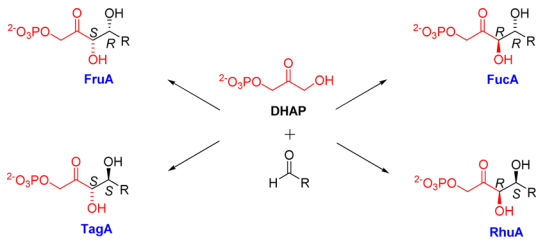


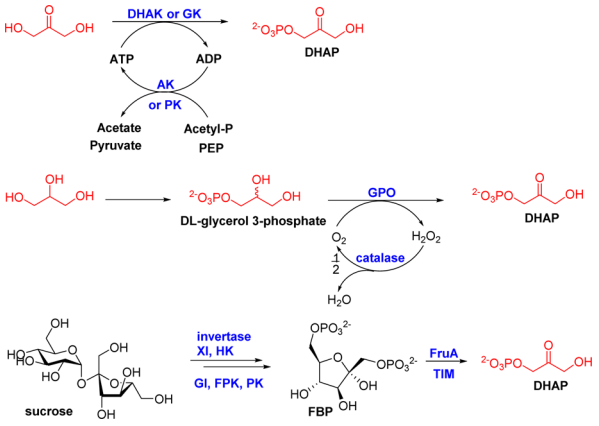
Figure 1. DHAP-dependent aldolase-mediated aldol reaction.

however, they suffer from low yields, complicated workup, or toxic reagents or catalysts.[29] Enzymatic approaches generate DHAP in situ and follow three general routes: phosphorylation of dihydroxyacetone (DHA),[14,30−34] oxidation of glycerol 3-phosphate,[35−39] and mimicking glycolysis.[5,40] Although enzymatic approaches start from cheap nonphosphorylated precursors (DHA, glycerol, sucrose), they employ multiple costly isolated enzymes (Scheme 1). Both types of approaches require further improvement to serve as a basis for scalable and

Received: May 6, 2015
Revised: May 27, 2015
Published: May 29, 2015

DOI: 10.1021/acscatal.5b00953
ACS Catal. 2015, 5, 4060−4065

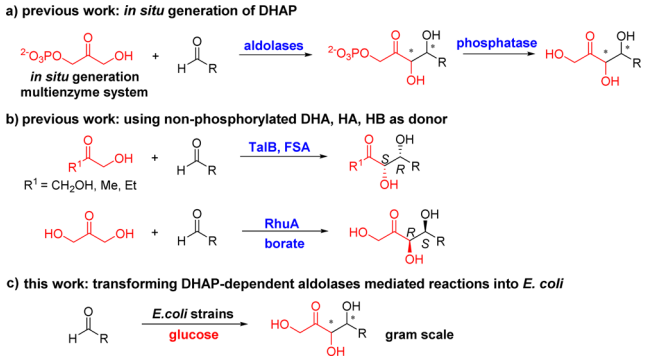
Scheme 1. Principal Enzymatic Routes to DHAP[a]



[a]DHAK, dihydroxyacetone kinase; GK, glycerol kinase; AK, acetate kinase; PK, pyruvate kinase; GPO, L-glycerol 3-phosphate oxidase; XI, xylose isomerase; HK, hexokinase; GI, glucose 6-phosphate isomerase; FPK, fructose 6-phosphate kinase; TIM, triose phosphate isomerase; PEP, phosphoenolpyruvate; and FBP, fructose 1,6-biphosphate.

cost-effective production of DHAP. Another issue associated with synthetic utility of DHAP-dependent aldolases is that an additional step is required to remove phosphate group of aldol adducts (Scheme 2a).

Scheme 2. DHAP-Dependent Aldolase-Mediated Aldol Reaction

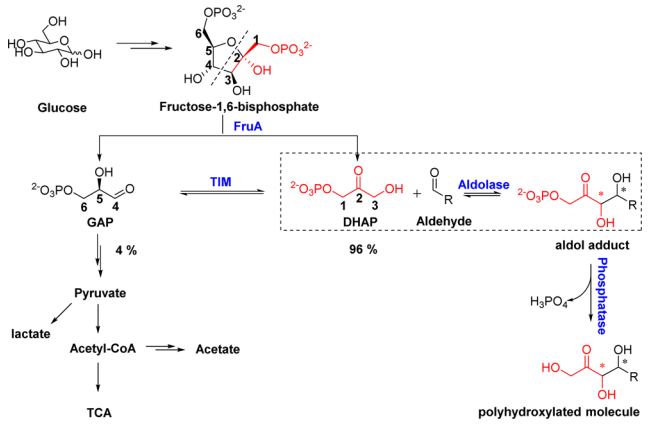


A desirable solution is to eliminate the requirement of DHAP and use readily available, inexpensive DHA. Recent efforts in overcoming DHAP-dependence by directed evolution of aldolases, reaction/substrate engineering, and exploitation of newly discovered enzymes have achieved great success. Fructose 6-phosphate aldolase (FSA)[20,41−44] and engineered transaldolase B[45−47] can accept DHA, hydroxyacetone (HA), and hydroxylbutone (HB) as donor substrate. FSA even can accept glycolaldehyde as a donor substrate.[43] In addition, engineered RhuA[48] or wild type RhuA with the presence of borate buffer[49,50] also can accept DHA as a donor substrate; however, these DHAP-independent methods can provide only 3*S*,4*R* and 3*R*,4*S* configuration products (Scheme 2b). Access to 3*R*,4*R* and 3*S*,4*S* products still requires DHAP as the donor, and to the best of our knowledge, none of these methods has ever been realized on more than lab scale. Therefore, developing a general method to solve the above-mentioned issues is greatly desired.

Synthetic biology, a recently emerging discipline that utilizes elaborate bioengineered organisms as "programmable synthetic machinery" to execute transformations inside cells has completely revolutionized conventional enzymatic synthesis.[51−53] Advances in DNA technologies and bioinformatics enable the reconstruction and perfection of such genetic devices. Thus, synthetic biology would provide appealing opportunities and solutions to the aforementioned issues by technically manipulating microbial hosts to execute the aldolase-catalyzed reactions inside cells and afford the desired products.

Here, we report the transformation of DHAP-dependent aldolase-mediated in vitro reactions into engineered *E. coli* for facile and effective production of polyhydroxylated molecules (Scheme 2c). In glycolysis, glucose is metabolized into fructose 1,6-bisphosphate via three enzymatic steps and is then split by FruA into two interconvertible triose phosphates, DHAP and D-glyceraldehyde-3-phosphate (GAP).[54] The concentration ratio of DHAP to GAP is 96% to 4% because of the favored formation of DHAP by TIM.[55] We envision that by introducing and overexpressing an aldolase gene and a phosphatase gene in *E. coli* cells, overexpressed aldolase can hijack DHAP from the glycolytic pathway and couple it with exogenous aldehyde to provide phosphorylated aldol adduct, which is dephosphorylated by overexpressed phosphatase and released from the host cell to give the desired product (Scheme 3).

Scheme 3. Hijacking DHAP from the Glycolytic Pathway



To fulfill such a transformation, three major issues need to be addressed. First, a suitable aldolase gene must be introduced and overexpressed in the host cell. The expressed aldolase would couple the glycolysis pathway with the aldol reaction to afford phosphorylated aldol adducts. Second, an appropriate phosphatase gene needs to be introduced and overexpressed. High intracellular accumulation of phosphorylated aldol adducts is expected to be toxic or, at least, a burden to cells. The phosphatase should be able to selectively dephosphorylate the resultant aldol adducts under physiological conditions but without any interference to other phosphorylated metabolic intermediates, which may have a negative influence on the whole engineered system. After dephosphorylation, the final product would be secreted out of host cells. This will shift glycolysis and the aldol reaction toward product formation and make purification of the final product much easier. In addition, the removed phosphate could be recycled inside cells. Third, bacteria growth and product synthesis must be well balanced. Hijacking DHAP from glycolysis will greatly reduce energy production, disrupt the redox balance, and reduce growth in

DOI: 10.1021/acscatal.5b00953
ACS Catal. 2015, 5, 4060−4065

host cells. A suitable condition must be explored to maximize production while maintaining bacteria growth.

We are very interested in FruA, one class of DHAP-dependent aldolase, owing to its high stereoselectivity and relaxed aldehyde substrate specificity. FruA from *Staphylococcus carnosus* ($FruA_{S.car}$) encoded by gene *fda* displays unusual stability across a wide range of temperature and pH conditions while retaining quite relaxed acceptor specificity.[56,57] At the same time, $FruA_{S.car}$ has been demonstrated to exhibit high enantioselectivity in aldol reactions, selectively furnishing aldol products with a 3*S*,4*R*-threo configuration.[57] Thus, we chose $FruA_{S.car}$ as the aldolase. YqaB from *E. coli* ($YqaB_{E. coli}$) encoded by gene *yqaB* could dephosphorylate D-fructose-1-phosphate but shows no activity toward aldose phosphates, ketose-terminal phosphates, or other phosphorylated metabolic intermediates.[58] Our in vitro study also showed that $YqaB_{E. coli}$ could dephosphorylate D-sorbose-1-phosphate, D-psicose-1-phosphate, L-tagatose-1-phosphate, and L-fructose-1-phosphate but showed almost no activity toward DHAP under neutral conditions. Therefore, we chose $YqaB_{E. coli}$ as the phosphatase. Meanwhile, we chose 3-trifluoroacetamido propanal **1** as the model aldehyde acceptor owing to its excellent solubility in water.

To construct an recombinant *E. coli* strain, *fda* and *yqaB* genes were cloned into pCDFDuet-1 (Novagen), a vector that allows high-level expression of two proteins under induction of isopropyl *β*-D-1-thiogalactopyranoside (IPTG). The resulting plasmid pCDF-fda-Y was transformed into *E. coli* BL21Star (DE3) to provide recombinant *E. coli* strain FruA-Y, which was grown aerobically at 37 °C, 220 rpm in LB broth medium until its OD_{600} value reached 1.0. Then the temperature was switched to 30 °C, and IPTG was added to induce coexpression of FruA and YqaB for 12 h. Subsequently, **1** and glucose were fed. To our delight, after purification, 234 mg of **2** was provided with 11.3% yield.

After validation of such transformation, we set out to optimize the fermentation conditions by using **1** as a model acceptor. Several key parameters critical to fermentation yield were investigated (Table 1). Glucose is the energy source and carbon source of living cell systems, and a high concentration of glucose will lead to catabolite repression. To determine the optimal initial glucose concentration in a medium, a variety of glucose concentrations, ranging from 2 to 12 g/L, were examined (entries 1−6). After a glucose concentration was over the 4 g/L threshold, the yield of **2** would not increase further and remained almost constant until it reached 12 g/L, at which point the yield of **2** began to decrease. Therefore, the initial glucose concentration was chosen to be 4 g/L. Notably, if glucose was fed during the cell proliferation phase or induction stage, the yield of **2** would decrease dramatically.

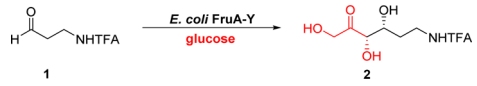
Addition of IPTG would affect the growth of cells and expression of the target genes. Therefore, IPTG was added at different levels of cell density, determined by corresponding OD_{600} values, to investigate the effect of IPTG addition time point on fermentation yield. After 12 h of induction, the OD_{600} value of all cell cultures reached around 2.3. As illustrated in entries 2 and 7−11, the yield of **2** increased in an almost linear relationship with the increase of the cell OD_{600} values until the OD_{600} equaled 1.8, at which point the yield of **2** reached a plateau at 41.7%. Thus, the optimum time point of IPTG addition is the time at which the OD_{600} value is ≥1.8.

LB Broth medium is a nutritionally rich medium and supplies essential growth factors that *E. coli* would otherwise have to synthesize, although ECAM (see recipe in Supporting Information) is a mineral salt medium and provides all kinds of metals and essential trace elements for *E. coli*. Thus, to probe the effect of the culture medium on the output of the *E. coli* strain, LB Broth and ECAM media were examined, as well as their combinations. As demonstrated in Table 1, entries 11−15, LB Broth turned out to be far superior to ECAM with respect to yield of **2** (entry 11 vs 15); however, an appropriate combination of these two media can increase the yield of **2**. When these two media were mixed in a 3:1 volumetric ratio, the yield of **2** was increased from 41.7 to 49.8% (entry 11 vs entry 12). The yield of **2** was further increased to 50.0% by mixing LB Broth and ECAM in a 1:1 volumetric ratio (entry 13).

A further increase in the ECAM ratio led to a decrease in the production yield (entry 14). Therefore, the optimal medium is a combination of LB Broth and ECAM in a 1:1 volumetric ratio. In addition, when *E. coli* strain BL21Star (DE3) was used to perform fermentation under optimized conditions, no product was detected (entry 16), which indicated that the background reactions were negligible.

After we identified the optimal fermentation conditions, we set out to investigate the scope and limitations of such a FruA-Y *E. coli* synthetic factory by using a variety of cell-membrane-permeable aldehydes as acceptors. The FruA-Y strain demonstrated superb promiscuity toward different aldehyde acceptors. As indicated in Table 2, a set of small aldehydes were taken up by FruA-Y *E. coli* cells and subjected to the aldol reaction with glycolytic intermediate DHAP, followed by in situ

Table 1. Condition Optimization for Maximum Production



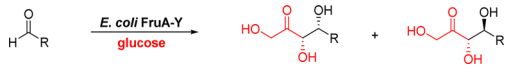
1 2

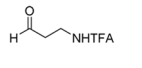
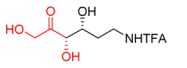
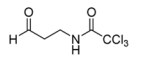
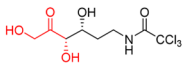
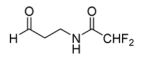
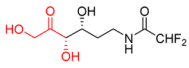
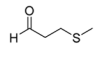
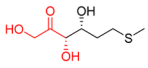
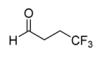
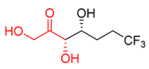
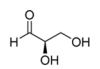
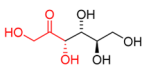
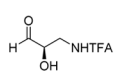
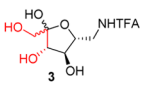
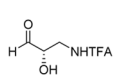
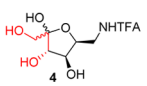
entry[a]	medium	glucose (g/L)[b]	cell density[c] OD_{600}	incubation time (h)	yield (%)[d]
1	LB Broth	2	1.0	12	19.7
2	LB Broth	4	1.0	12	27.6
3	LB Broth	6	1.0	12	27.5
4	LB Broth	8	1.0	12	27.6
5	LB Broth	10	1.0	12	27.4
6	LB Broth	12	1.0	12	15.4
7	LB Broth	4	1.2	11	30.1
8	LB Broth	4	1.4	10	33.5
9	LB Broth	4	1.6	9	36.7
10	LB Broth	4	1.8	8	41.7
11	LB Broth	4	1.95	8	41.7
12	LB Broth/ECAM (3/1, v/v)	4	>1.8	8	49.8
13	LB Broth/ECAM (1/1, v/v)	4	>1.8	8	50.0
14	LB Broth/ECAM (1/3, v/v)	4	>1.8	8	40.4
15	ECAM	4	>1.8	11	28.5
16[e]	LB Broth/ECAM (1/1, v/v)	4	>1.8	8	0

[a]Fermentation conditions: strain FruA-Y was grown aerobically at 37 °C, 220 rpm in 200 mL medium until OD_{600} reached certain value, then the temperature was switched to 30 °C, and IPTG was added to induce coexpression of FruA and YqaB for 12 h. Subsequently, 8 mmol **1** and glucose were fed. [b]Initial glucose concentration in medium. [c]At this cell density, IPTG was added. [d]HPLC yield based on acceptor **1**. [e]*E. coli* strain BL21Star (DE3) was used.

DOI: 10.1021/acscatal.5b00953
ACS Catal. 2015, 5, 4060−4065

Table 2. Production of Polyhydroxylated Molecules via *E. coli* Strain FruA-Y



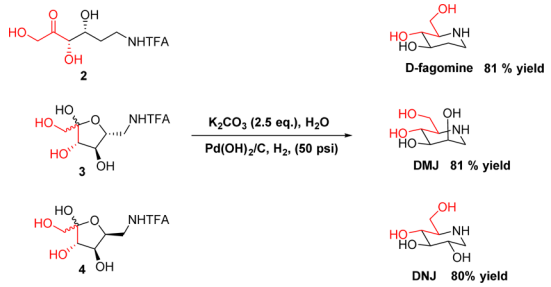
entry[a]	acceptor	product	incubation time (h)	isolated yield	dr[b]
1			8	2.47 g / 23.8 % (5.18g / 50.0 %)[c]	> 95:5
2			10	4.41 g / 35.7 %	92:8
3			10	2.26 g / 23.4 %	92:8
4[d]			24	327 mg / 2.8 %	92:8
5[e]			20	716 mg / 4.7 %	87:13
6[f]			20	947 mg / 26.3 % (2.36 g / 65.6 %)[c]	> 95:5[g]
7[h]			10	827 mg / 11.0 %	ND
8[h]			10	513 mg / 6.8 %	ND

[a]Fermentation conditions: strain FruA-Y was grown aerobically at 37 °C, 220 rpm in 1 L LB Broth/ECAM (1/1, v/v) medium until the OD_{600} reached 1.8−2.0, then the temperature was switched to 30 °C, and IPTG was added to induce coexpression of FruA and YqaB for 12 h. Subsequently, the aldehyde acceptor and glucose were fed. [b]Determined by ^{1}H NMR. [c]HPLC yield based on aldehyde acceptor. [d]60 mmol aldehyde was used. [e]70 mmol aldehyde was used. [f]1 L of ECAM was used as the medium and 20 mmol of aldehyde was used. [g]Determined by HPLC. [h]27.3 mmol aldehyde was used. ND, not determined.

dephosphorylation, to yield the desired aldol products. When **1** was used as acceptor, 2.47 g of **2** was provided with 23.8% yield as a single stereoisomer (dr > 95:5). However, concentration of **2** in the fermentation medium was 5.18 g/L (determined by HPLC), and the corresponding yield was 50.0% (entry 1). The low isolated yield is mainly attributed to loss of product during purification. Along the same line, 3-trichloroacetamido propanal gave 4.41 g of (3*S*,4*R*)-6-trichloroacetamido-1,3,4-trihydroxyhexan-2-one with 35.7% yield and 92:8 dr (entry 2); 3-difluoroacetamido propanal gave 2.26 g of (3*S*,4*R*)-6-difluoroacetamido-1,3,4-trihydroxyhexan-2-one with 23.4% yield and 92:8 dr (entry 3); 3-(methylthio)propanal gave 327 mg of (3*S*,4*R*)-1,3,4-trihydroxy-6-(methylthio)hexan-2-one with 2.8% yield and 92:8 dr (entry 4); and 4,4,4-trifluorobutanal gave 716 mg of (3*S*,4*R*)-7,7,7-trifluoro-1,3,4-trihydroxyheptan-2-one with 4.7% yield and 87:13 dr (entry 5). Low fermentation yields of 3-(methylthio)propanal and 4,4,4-trifluorobutanal are mainly due to vaporization of aldehyde acceptors during incubation and their poor cell membrane permeability. When D-glyceraldehyde was used as the acceptor, 947 mg of D-fructose was afforded with 26.3% yield; however, the HPLC yield before purification was 65.6% (entry 6). In addition, when (*R*)-3-trifluoroacetamido-2-hydroxypropanal and (*S*)-3-trifluoroacetamido-2-hydroxypropanal were used as acceptors, 827 mg of **3** and 513 mg of **4** were afforded (entries 7−8).

D-Fagomine, 1-deoxymannojirimycin (DMJ), 1-deoxynojirimycin (DNJ), and their derivatives are effective inhibitors of glycosidases and glycosyltransferases and exert a profound effect on *N*-linked glycoprotein processing and maturation, as well as cell−cell and cell−virus recognition.[59] Therefore, they have gained considerable clinical importance in the treatment of cancer, type II diabetes, viral diseases such as HIV, hepatitis B and C, Gaucher's disease, and other glycosphingolipid storage disorders.[60−63] For example, D-fagomine can effectively reduce the blood glucose peak when taken together with sucrose or starch, without stimulating insulin release and can help to eliminate the excess of enterobacteria and lower weight gain by selectively agglutinating fimbriated enterobacteria and inhibiting their adhesion to the intestinal mucosa.[64,65] Miglitol, *N*-hydroxyethyl DNJ, is currently used as a potent second-generation digestive α-glucosidase inhibitor for treatment of type II diabetes; Miglustat, *N*-butyl DNJ, is currently used for treatment of type 1 Gaucher disease (GD1) and Niemann−Pick type C (NPC) disease.[66−68] By simple deprotection and reductive amination, product **2**, **3**, and **4** were transformed to D-fagomine, DMJ and DNJ respectively with high yields, and no other diastereomers were detected by ^{1}H NMR analysis (Scheme 4).

Scheme 4. Synthesis of D-Fagomine, DMJ, and DNJ

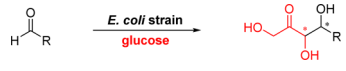


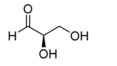
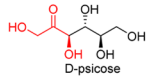
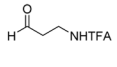
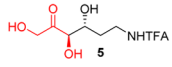
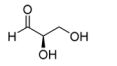
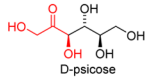
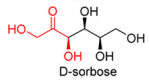
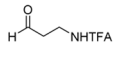
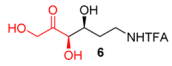
To further expand applications of such transformation, recombinant *E. coli* strains FucA-Y and RhuA-Y were constructed following the same procotol of FruA-Y with the *fucA* gene encoding L-fuculose-1-phosphate aldolase (FucA) from *Thermus thermophilus* HB8 and *rhuA* gene encoding L-rhamnulose-1-phosphate aldolase (RhuA) from *E. coli*. For the FucA-Y strain, D-glyceraldehyde gave 960 mg of D-psicose with 35.6% yield and 92:8 dr (Table 3, entry 1), and **1** gave 665 mg of (3*R*,4*R*)-6-trifluoroacetamido-1,3,4-trihydroxyhexan-2-one **5** with 12.8% yield and 87:13 dr (Table 3, entry 2). For the RhuA-Y strain, D-glyceraldehyde provided 281 mg of D-piscose and 286 mg of D-sorbose with 10.4% and 10.6% yields, respectively (Table 3, entry 3); **1** yielded 1.16 g of (3*R*,4*S*)-6-trifluoroacetamido-1,3,4-trihydroxyhexan-2-one **6** with 22.4% yield and 89:11 dr (Table 3, entry 4). The successful application of D-glyceraldehyde and **1** in strains FucA-Y and RhuA-Y indicated that many other aldehydes also could be applied in these two *E. coli* strains.

FruA, FucA, and RhuA also were used to synthesize **2**, **5**, and **6** in vitro via a one-pot, four-enzyme system (Table 4). GPO catalyzed the in situ generation of DHAP from DL-glycerol 3-phosphate, then aldolases coupled DHAP with aldehyde **1** to give the phosphorylated aldol adducts, which were dephosphorylated by acid phosphatase (AP) to give the aldol products **2**, **5**, and **6**. FruA gave **2** exclusively (dr > 95:5, entry 1), FucA provided **5** with 87:13 dr (entry 2), and RhuA yielded **6** with

DOI: 10.1021/acscatal.5b00953
ACS Catal. 2015, 5, 4060−4065

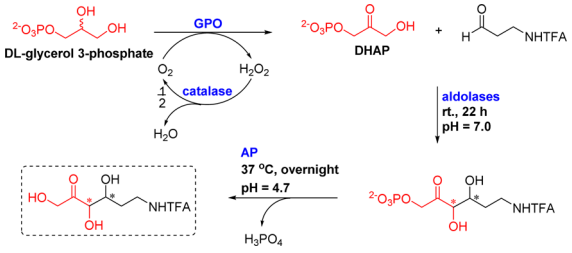
Table 3. Production of Polyhydroxylated Molecules via *E. coli* Strains FucA-Y and RhuA-Y

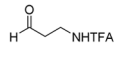
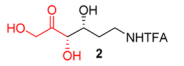
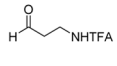
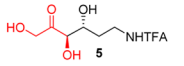
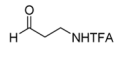
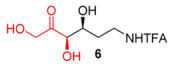


entry[a]	strain	acceptor	product	incubation time (h)	isolated yield	dr
1[b]	FucA-Y		 D-psicose	20	960 mg 35.6 %	92:8[d]
2[c]	FucA-Y		 5	12	665 mg 12.8 %	87:13[e]
3[b]	RhuA-Y		 D-psicose  D-sorbose	20	281 mg 10.4 % 286 mg 10.6 %	49:51[d]
4[c]	RhuA-Y		 6	6	1.16g 22.4 %	89:11[e]

[a]Fermentation conditions: same as Table 2. [b]1 L of ECAM was used as the medium and 15 mmol of aldehyde was used. [c]1 L of LB Broth/ECAM (1/1, v/v) was used as the medium, and 20 mmol of aldehyde was used. [d]Determined by HPLC. [e]Determined by ^{1}H NMR.

Table 4. One-Pot Four-Enzyme Synthesis of 2, 5, and 6



entry	aldolase	acceptor	product	isolated yield	dr[a]
1	FruA		 2	53 %	> 95:5
2	FucA		 5	48 %	87:13
3	RhuA		 6	42 %	89:11

[a]Determined by ^{1}H NMR.

89:11 dr (entry 3). The stereoselectivities of FruA, FucA, and RhuA are consistent with our in vivo results (Table 2, entry 1; Table 3, entry 2 and 4); therefore, these engineered *E. coli* strains can serve as a general and effective method for the practical production of polyhydroxylated molecules.

To confirm that the C1, C2, C3 carbons of products were derived from supplemented glucose via DHAP of the glycolytic pathway, FruA-Y was fed with [U-$^{13}C_6$] glucose as the sole carbon source and **1** as the aldehyde acceptor. Fermentation was carried out using the same protocol as described previously by using citric acid-free ECAM as the culture medium. After purification, the product was characterized by ^{1}H and ^{13}C NMR, which indicated that the C1, C2, and C3 carbons truly came from [U-$^{13}C_6$] glucose as integrals of the three carbons are much stronger than other carbons as a result of 100% ^{13}C abundance (see Supporting Information).

In conclusion, we have successfully transformed DHAP-dependent aldolases mediated in vitro reactions into engineered *E. coli* for practical and effective production of polyhydroxylated molecules. Such a transformation solves the problem of DHAP availability by hijacking the DHAP from the glycolytic pathway, circumvents purification of recombinant aldolases and phosphatase, dephosphorylates the aldol adducts in vivo, and recycles phosphate inside *E. coli* cells. Operational simplicity, low cost, and easy scalability of fermentation shows such a transformation holds enormous value in the synthesis of biologically relevant polyhydroxylated molecules on an industrial scale. Efforts on enhancing production efficiency by optimizing *E. coli* strains via bioengineering and simplifying purification procedures are ongoing.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.5b00953.

Experimental procedure, characterization data for all new compounds, selected NMR spectra (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interests.

■ ACKNOWLEDGMENTS

This work was supported by the NIH (GM085267, AI083754). Dr. Z. Li is grateful for support from the National Natural Science Foundation of China (21302069).

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DOI: 10.1021/acscatal.5b00953
ACS Catal. 2015, 5, 4060−4065