ACS SyntheticBiology

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Systematically Engineering *Escherichia coli* for Enhanced Production of 1,2-Propanediol and 1-Propanol

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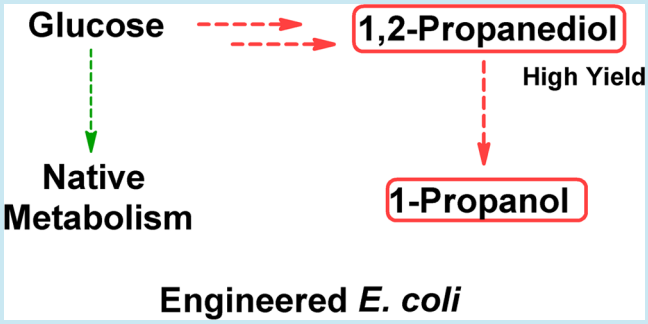
Supporting Information

ABSTRACT: The biological production of high value commodity 1,2-propanediol has been established by engineering the glycolysis pathway. However, the simultaneous achievement of high titer and high yield has not been reported yet, as all efforts in increasing the titer have resulted in low yields. In this work, we overcome this limitation by employing an optimal minimal set of enzymes, channeling the carbon flux into the 1,2-propanediol pathway, increasing NADH availability, and improving the anaerobic growth of the engineered *Escherichia coli* strain by developing a cell adaptation method. These efforts lead to 1,2-propanediol production at a titer of 5.13 g/L with a yield of 0.48 g/g glucose in 20 mL shake flask studies. On this basis, we pursue the enhancement of 1-propanol production from the 1,2-propanediol platform. By constructing a fusion diol dehydratase and developing a dual strain process, we achieve a 1-propanol titer of 2.91 g/L in 20 mL shake flask studies. To summarize, we report the production of 1,2-propanediol at enhanced titer and enhanced yield simultaneously in *E. coli* for the first time. Furthermore, we establish an efficient system for the production of biofuel 1-propanol biologically.



KEYWORDS: *1,2-propanediol, 1-propanol, metabolic engineering, Escherichia coli, biofuels*

A vast number of high value chemicals, fine chemicals, and other commodity products rely on the petrochemical industry for large-scale manufacture. It is reported that currently about 3 billion barrels of oil are consumed annually for the production of industrial chemicals.[1] These processes are responsible for releasing about 30 billion tons of carbon dioxide into the atmosphere, as reported for the year 2008.[1] The ongoing depletion of fossil fuels will lead to the increase in the cost of manufacturing these chemicals in the near future and also pose a threat to the environment by releasing vast quantities of carbon dioxide. An alternative to this is the biological manufacture through the development of synthetic metabolic routes via metabolic engineering[2] and fermentation, which reduces the dependence on oil by using renewable sources as raw materials and decreases the release of greenhouse gases into the atmosphere.[1,3]

Metabolic engineering efforts via fermentation optimization, utilization of different carbon sources, strain engineering and growth improvement strategies have led to the establishment of biological platforms for the manufacturing of ethanol,[4,5] succinic acid,[6] lactic acid,[7,8] etc. These approaches have proven to be efficient for the production of indigenous metabolites. However, the manufacturing of non-native high-value chemicals or commodities requires the construction of novel synthetic pathways by the expansion of native metabolism via combinatorial biosynthesis and manipulation of cellular metabolism. These strategies have led to the establishment of novel biosynthetic pathways for the production of advanced biofuels such as longer chain alcohols,[9,10] various C2−C6 platform chemicals,[3,11,12] polymers,[13,14] antioxidants,[15,16] and pharmaceuticals.[17,18]

Although the biological manufacture of 1,2-propanediol (propylene glycol) and 1-propanol has been achieved, at present, they are manufactured from the petrochemical industry at a large scale.[19,20] 1,2-Propanediol has an annual market of over 1 billion pounds in U.S.A. alone and is primarily used in the manufacturing of antifreeze and deicers, and also in the manufacture of pharmaceuticals, polyester resin, laundry detergent, cosmetics, and so on.[21] 1-Propanol has been proposed as a potential biofuel and is currently used in the following industries: pharmaceuticals, textiles, printing ink, cosmetics, etc.[20,22]

The biosynthesis of 1,2-propanediol has been achieved in *E. coli* and *Saccharomyces cerevisiae*, by engineering the glycolysis pathway.[19,21,23−28] Metabolic engineering efforts such as selection of optimal carbon source,[21] optimization of fermentation media and conditions,[23] scale up to 400 or 2000 mL fed batch fermentation[23,27] and strain engineering have resulted in 1,2-propanediol production at low titers and

Received: October 28, 2014
Published: December 9, 2014

yields (Supporting Information Table S1). To achieve both high titer and high yield is challenging and has not been reported yet. For instance, the highest reported titer of 1,2-propanediol of 5.6 g/L was achieved at a low yield of 21.3% (w/w) from engineered *E. coli* in a fermenter with 400 mL media using glycerol as the carbon source.[27] Whereas, the highest reported yield of 0.20 g/g using glucose as the carbon source was achieved at a low titer of 1.4 g/L in 150 mL shake flask studies.[23] A major limitation in these established methods is the dominance of the native metabolism in dragging the carbon flux toward byproduct formation, thereby undermining the synthetic pathway. In order to promote industrial interest, it is necessary to achieve high titer without sacrificing yield, utilize inexpensive media and prevent the loss of carbon to harmful byproduct accumulation.

The biosynthesis of 1-propanol has been well established from the keto-acid pathway[29] and the citramalate pathway.[22] By engineering the amino acid biosynthetic pathway, 1-propanol was produced from a key keto-acid intermediate- 2-ketobutyrate at about 1 g/L in an engineered *E. coli* strain.[20] Further enhancement of 1-propanol production in *E. coli* was achieved (3.5 g/L) by constructing a citramalate pathway, utilizing an evolved citramalate synthase capable of preventing feedback inhibition by isoleucine.[22] By engineering an L-threonine overproducing strain, 1-propanol production was achieved at 10.8 g/L using glucose and at 10.3 g/L using glycerol in bioreactors with 2 L medium.[30] Recently, it was demonstrated that the synergy between the threonine pathway and citramalate pathway can be modulated, proving to be more efficient than the individual pathways. This resulted in a 1-propanol yield of 0.15 g/g glucose at a titer of 8 g/L.[29]

In our previous work, we expanded the 1,2-propanediol pathway to establish a novel 1-propanol biosynthesis approach anaerobically in wild type *E. coli* (Figure 1). In doing so, the 1,2-propanediol (about 0.8 g/L) was dehydrated by the action of *Klebsiella oxytoca* diol dehydratase to 1-propanal, following which native reduction resulted in 1-propanol production at 0.25 g/L using wild type *E. coli*.[26] Our previous results suggested two major limitations: (a) dominance of native metabolism toward byproduct formation, thereby limiting 1,2-propanediol titer (b) low conversion efficiency of 1,2-propanediol to 1-propanol.

In this work, we overcome both of the above-mentioned limitations, resulting in boosting 1,2-propanediol titer without sacrificing yield and also enhancing 1,2-propanediol conversion efficiency to 1-propanol. We first pursue the enhancement of 1,2-propanediol production by systematically engineering *E. coli*. By overexpressing the optimal set of enzymes, redirecting the carbon flux, utilizing a system for intracellular NADH regeneration and adapting the cells to improve growth, we accomplish 1,2-propanediol production at the highest reported titer and yield simultaneously in shake flask studies. We achieve 1,2-propanediol production at 5.13 g/L in 20 mL shake flask studies, which is comparable to the highest reported titer (5.6 g/L) using a fermenter with 400 mL media.[27] Our process also achieved a yield of 0.48 g/g glucose, which is more than double the yields reported until date. Furthermore, we demonstrate the improvement in 1-propanol production from the established 1,2-propanediol platform. The combined effect of creating a fusion diol dehydratase and a dual strain strategy leads to1-propanol production at 2.91 g/L in 20 mL shake flask studies. This work represents a step forward in making the biological manufacture of 1,2-propanediol and 1-propanol more economically viable.

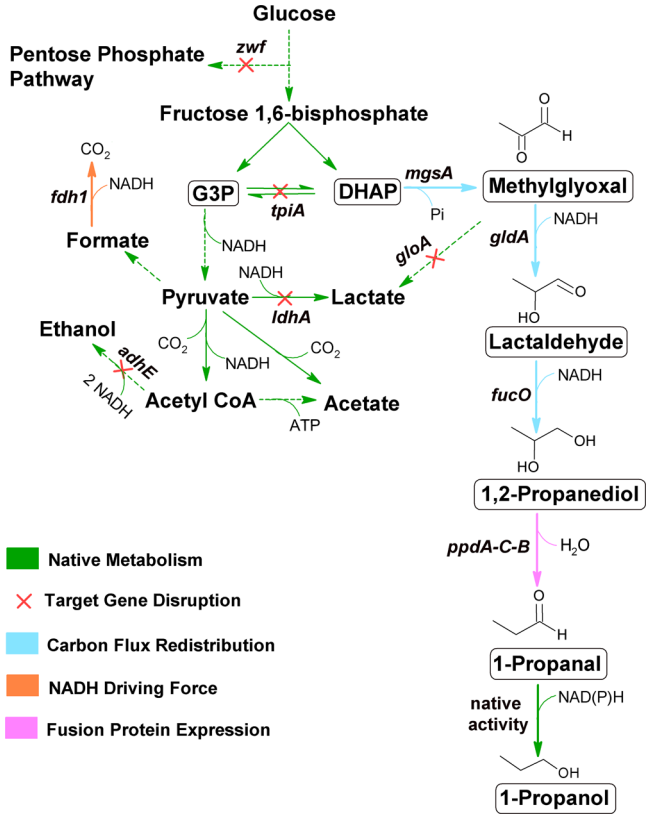


Figure 1. Schematic representation of 1,2-propanediol pathway, 1-propanol pathway and major native competing pathways in *E. coli*. G3P: glyceraldehyde 3-phosphate. DHAP: dihydroxyacetone phosphate.

RESULTS AND DISCUSSION

Identification of the Optimal Minimal Set of Enzymes for 1,2-Propanediol Production. The production of 1,2-propanediol from glycolytic intermediate dihydroxyacetone phosphate has been well established.[19,21,23] The expression of either methylglyoxal synthase or secondary alcohol dehydrogenase has been shown to be sufficient for 1,2-propanediol production.[19] It has also been demonstrated that the coexpression of methylglyoxal synthase with a secondary alcohol dehydrogenase[19] or the expression of the complete pathway (methylglyoxal synthase, secondary alcohol dehydrogenase, 1,2-propanediol oxidoreductase)[23] leads to the production of 1,2-propanediol at various levels. The overexpression of too many enzymes in *E. coli* may lead to metabolic burdens,[31−33] which may affect the cell growth and product yield. Hence, our objective was to identify the optimal minimal set of enzymes required for efficient production of 1,2-propanediol.

As several candidate enzymes are available for each step of the pathway and different combinations of their expression is possible, we performed a systematic study by overexpressing candidate enzymes in a stepwise manner. We had previously characterized methylglyoxal synthase from eight different bacterial sources and identified the methylglyoxal synthase from *Clostridium acetobutylicum* and *Bacillus subtilis* as the most proficient. We first overexpressed the two synthases using plasmids pRJ1 and pRJ2[26] in wild type *E. coli* individually.

dx.doi.org/10.1021/sb500345t | *ACS Synth. Biol.* 2015, 4, 746−756

Fermentations were carried out according to the method described in the Methods as "shake flask fermentations".

After 48 h of fermentation, it was found that 1,2-propanediol was produced at a low amount. As shown in Figure 2, the overexpression of methylglyoxal synthase from *B. subtilis* resulted in 1,2-propanediol production at 0.46 g/L. Whereas, the overexpression of methylglyoxal synthase from *C. acetobutylicum* resulted in 1,2-propanediol production at 0.29 g/L.

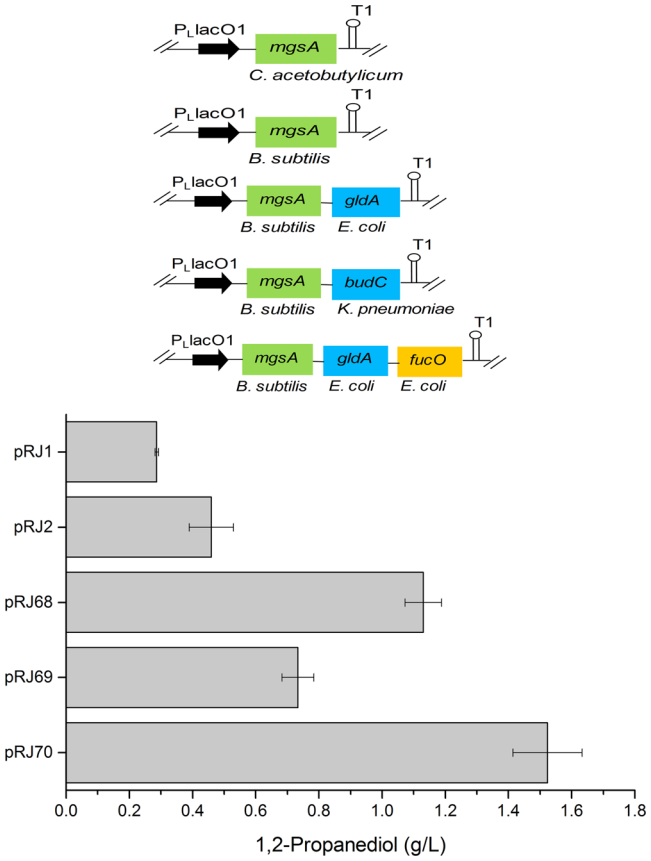


Figure 2. Identification of optimal minimal set of enzymes for 1,2-propanediol production in 20 mL anaerobic shake flask studies using wild type *E. coli* BW25113 transformed with different plasmids. The data were generated from three independent experiments.

Next, we overexpressed secondary alcohol dehydrogenases in addition to the *B. subtilis* methylglyoxal synthase to evaluate if their overexpression is necessary to enhance the production of 1,2-propanediol. The plasmids pRJ68 carrying *B. subtilis mgsA*, *E. coli gldA* and pRJ69 carrying *B. subtilis mgsA*, *Klebsiella pneumoniae budC* were transferred into wild type *E. coli* independently for fermentation studies. After 48 h of fermentation, the *E. coli* strain transformed with pRJ68 increased the 1,2-propanediol titer to 1.13 g/L (Figure 2). We had previously determined that the diol dehydrogenase (*budC*) from *K. pneumoniae* had a higher *in vitro* catalytic efficiency than *E. coli gldA* toward methylglyoxal.[26] Contrary to our expectation, the coexpression of *budC* from *K. pneumoniae* with *B. subtilis mgsA* (pRJ69) led to lower levels of 1,2-propanediol production (0.73 g/L) as compared to when *E. coli gldA* was coexpressed. This result demonstrated the beneficial effect of overexpressing *E. coli* native dehydrogenase toward 1,2-propanediol production over the heterologous dehydrogenase despite having a lower catalytic efficiency *in vitro*.

Finally, we proceeded to evaluate if the overexpression of 1,2-propanediol oxidoreductase (*fucO*) can further facilitate the increase of 1,2-propanediol production. To test this, plasmid pRJ70 carrying *mgsA* from *B. subtilis*, *gldA*, and *fucO* from *E. coli* was transferred into wild type *E. coli* for fermentation studies. After 48 h of fermentation, the *E. coli* strain carrying pRJ70 further increased the 1,2-propanediol titer to 1.52 g/L (yield = 0.13 g/g glucose). Thus, we identified the optimal minimal set of enzymes to be overexpressed for 1,2-propanediol production. This stepwise approach resulted in increasing the titer from 0.29 g/L to 1.52 g/L (Figure 2). The plasmid pRJ70 was selected for all further studies as it resulted in the highest titer. However, the carbon flux toward the glycolytic pathway remained dominant in the wild type strain overexpressing the 1,2-propanediol pathway. The major competing pathway was found to be lactate, which was produced at 6.21 g/L (Supporting Information Table S2). No pyruvate accumulation was observed, suggesting that most of it was channeled toward lactate generation due to the possible increase in lactate dehydrogenase activity (*ldhA*).[34] Ethanol, acetate, formate and succinate were also produced at 0.53 g/L, 2.03 g/L, 0.89 g/L, and 0.26 g/L, respectively (Supporting Information Table S2).

Disruption of Lactate Producing Pathways. As seen from the above results, the dominant byproduct lactate was accumulated as a result of the carbon flux being dominant toward glycolysis. We speculated that the elimination of lactate generation would result in making more carbon available for utilization into the 1,2-propanediol pathway. In order to study the effect of disrupting both the lactate generating pathways (through glyoxalase system and lactate dehydrogenase), we constructed an *E. coli* strain RJ31 with *gloA* and *ldhA* gene disruptions. We then tested this strain transformed with pRJ70 for 1,2-propanediol production via 48 h fermentation studies. As expected, lactate production decreased from 6.21 g/L to 0.54 g/L (Supporting Information Table S2). Surprisingly, this strain resulted in reducing 1,2-propanediol titer by nearly 41% of the wild type to 0.90 g/L (yield = 0.07 g/g glucose). Interestingly, pyruvate was accumulated at 7.18 g/L and ethanol generation increased from 0.53 g/L to 2.77 g/L. Succinate generation increased from 0.26 g/L to 1.41 g/L and formate from 0.89 g/L to 2.45 g/L. Acetate production decreased from 2.03 g/L to 0.99 g/L.

Although lactate production was drastically lowered, a significant amount of pyruvate was accumulated along with an increase in other fermentative byproducts. This result indicated the over-riding capability of glycolysis to redirect the carbon flux toward production of indigenous metabolites. This suggested that merely the deletion of the major fermentative byproduct pathway was not an efficient strategy to tap into the carbon flux for the production of 1,2-propanediol.

Carbon Flux Redistribution. In glycolysis, the three-carbon intermediate dihydroxyacetone phosphate is converted into glyceraldehyde 3-phosphate by the action of triose phosphate isomerase (*tpiA*).[26] This native activity results in dragging the carbon flux away from the 1,2-propanediol pathway's starter molecule—dihydroxyacetone phosphate as indicated by the above results. So, we discerned that disrupting this major competing pathway for carbon flux is essential in order to conserve the carbon for the 1,2-propanediol pathway. We also speculated that the action of glucose 6-phosphate dehydrogenase (*zwf*) leading to pentose phosphate pathway

dx.doi.org/10.1021/sb500345t | *ACS Synth. Biol.* 2015, 4, 746−756

Table 1. 1,2-Propanediol Titer and Yield Achieved after 48 h of IPTG Induction from 20 mL Anaerobic Shake Flask Studies[a]

strain/plasmid	optical density (OD_{600})	glucose consumed (g/L)	1,2-PD titer (g/L)	yield (1,2-PD titer/S) (g/g)
BW25113/pRJ70	1.89 ± 0.06	11.75 ± 0.40	1.52 ± 0.11	0.13 ± 0.01
RJ31/pRJ70	2.41 ± 0.13	12.91 ± 0.60	0.90 ± 0.04	0.07 ± 0.01
RJ57/pRJ70	0.38 ± 0.03	1.64 ± 0.29	0.35 ± 0.00	0.22 ± 0.04
BW25113/pRJ70 + pRJ58	1.07 ± 0.08	14.94 ± 0.30	2.14 ± 0.07	0.14 ± 0.01
RJ57/pRJ70 + pRJ58 (A-48 h)	0.41 ± 0.04	1.75 ± 0.44	0.59 ± 0.02	0.34 ± 0.07
RJ57/pRJ70 + pRJ58 (B-48 h)	0.51 ± 0.18	5.35 ± 0.20	2.12 ± 0.20	0.40 ± 0.02
RJ57/pRJ70 + pRJ58 (C-48 h)	0.57 ± 0.02	5.52 ± 0.88	2.39 ± 0.27	0.44 ± 0.02
RJ57/pRJ70 + pRJ58 (C-120 h)	0.80 ± 0.02	10.60 ± 0.33	5.13 ± 0.14	0.48 ± 0.01

[a](A) regular strategy; (B) cell adaptation strategy; (C) optimized cell adaptation strategy; OD_{600}: optical density measured at 600 nm. S: grams of substrate consumed (glucose). 1,2-PD: 1,2-propanediol. The data were generated from three independent experiments.

may further result in the loss of carbon. With this cognition, we also hypothesized that in addition to *zwf* and *tpiA* disruptions, the disruption of other major competing pathways for the carbon flux under anaerobic conditions would also reduce byproduct formation. To test these hypotheses, we constructed *E. coli* strain RJ57 with the following gene disruptions: *zwf* encoding glucose 6-phosphate dehydrogenase, *tpiA* encoding triose phosphate isomerase, *ldhA* encoding lactate dehydrogenase, *gloA* encoding glyoxalase I, and *adhE* encoding alcohol dehydrogenase (ethanol generating pathway).

E. coli strain RJ57 was transformed with pRJ70 and fermentation was carried out for 48 h. As expected, this strain generated the least amount of byproducts, with lactate at 0.14 g/L, succinate at 0.22 g/L, formate at 0.33 g/L, acetate at 0.65 g/L, and ethanol at 0.05 g/L. Interestingly, this strain did not accumulate any pyruvate. However, 1,2-propanediol was produced at merely 0.35 g/L with a yield of 0.22 g/g glucose. As seen from Table 1, the amount of glucose consumed was drastically lower in the engineered strain (1.64 g/L) as compared to the wild type strain (11.75 g/L) after the fermentation study. We reasoned that this led to a decrease in the rate of glycolysis thereby diminishing the cell's capacity to generate carbon flux and NADH. It was also observed that at the end of the fermentation study that the optical density of the engineered strain was dramatically lower (OD_{600} = 0.38) than the wild type strain after fermentation (OD_{600} = 1.89) (Supporting Information Table S2). We then pursued to surmount these limitations by increasing NADH availability and enhancing cell growth.

Increasing NADH Availability as a Driving Force. We analyze that with the disruption of *zwf* and *tpiA* genes (strain RJ57), the carbon flux will be split equally among glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. As a consequence, with every cycle of glycolysis only one molecule of NADH can be generated in the engineered strain. The production of acetate via acetyl CoA results in the production of an additional NADH molecule per molecule of glucose (Figure 1). Furthermore, with the disruption of *ldhA* and *adhE*, major NADH competing pathways were disrupted. Since two molecules of NADH are consumed by the 1,2-propanediol pathway, this system can be regarded as NADH balanced at steady state conditions. Although NADH balance is achieved, this system may not favor the production of 1,2-propanediol as NADH may also be utilized by the cell for other anabolic reactions in the form of NADPH.

We then hypothesized that increasing the NADH availability would serve as a driving force for 1,2-propanediol production. We aimed at increasing the cellular NADH levels by overexpressing *Candida boidinii* formate dehydrogenase (*fdh1*), which has previously been shown to regenerate intracellular NADH.[35] An *in vitro* enzyme assay confirmed that the enzyme had sufficient catalytic efficiency to generate NADH from formate (k_m = 12.69 mM, k_{cat} = 26.33 min^{-1}).

To test this hypothesis, strain RJ57 was transformed with plasmids pRJ70 and pRJ58 (plasmid carrying *Candida boidinii fdh1*), and fermentations were carried out for 48 h. It was seen that the titer of 1,2-propanediol was increased to 0.59 g/L as compared to 0.35 g/L from the engineered strain without NADH regeneration system (Table 1). This result indicates that the NADH regeneration system facilitates in driving 1,2-propanediol production. As expected the yield showed a dramatic increase, from 0.22 g/g to 0.34 g/g glucose. The optical density (OD_{600}), however, remained low at 0.41 at the end of the study.

Additionally, we tested this NADH regeneration system in wild type *E. coli*. As expected, after 48 h of fermentation, 1,2-propanediol production was increased to 2.14 g/L as compared to 1.52 g/L from the wild type strain without NADH regeneration system. This strain accomplished a yield of 0.14 g/g glucose and accumulated the maximum amount of byproducts, with lactate at 8.22 g/L (Supporting Information Table S2). This positioned our process wherein high titer (2.14 g/L) was achieved in the wild type strain at a low yield (0.14 g/g glucose) or a high yield (0.34 g/g glucose) was achieved in an engineered strain at a low titer (0.59 g/L) (Figure 3A).

Cell Adaptation Studies. As seen from the above results, when the optical density of the engineered strain remained low (OD_{600} = 0.41), the 1,2-propanediol titer was also low (0.59 g/L) even with the NADH regeneration system. Since this strain achieved higher yield (0.34 g/g glucose), we speculated that improving the cell growth may result in increasing the 1,2-propanediol titer without sacrificing yield. We then channeled our efforts toward increasing the cell growth of the engineered strain (RJ57 transformed with plasmids pRJ70 and pRJ58) with the objective of achieving both high titer and high yield simultaneously. Preliminary experiments were conducted to test the effect of increasing the inoculum volume by various amounts, changing IPTG induction time, and changing fermentation temperature. However, these experiments failed to recover the cell growth (data not provided). We then hypothesized that by adapting the engineered strain we could emulate the fermentation environment prior to commencement of study, which would recover cell growth. To test this hypothesis, this strain was then subjected to "cell adaptation", by growing the overnight culture anaerobically in the low phosphate formate media followed by fermentation studies as described in Methods.

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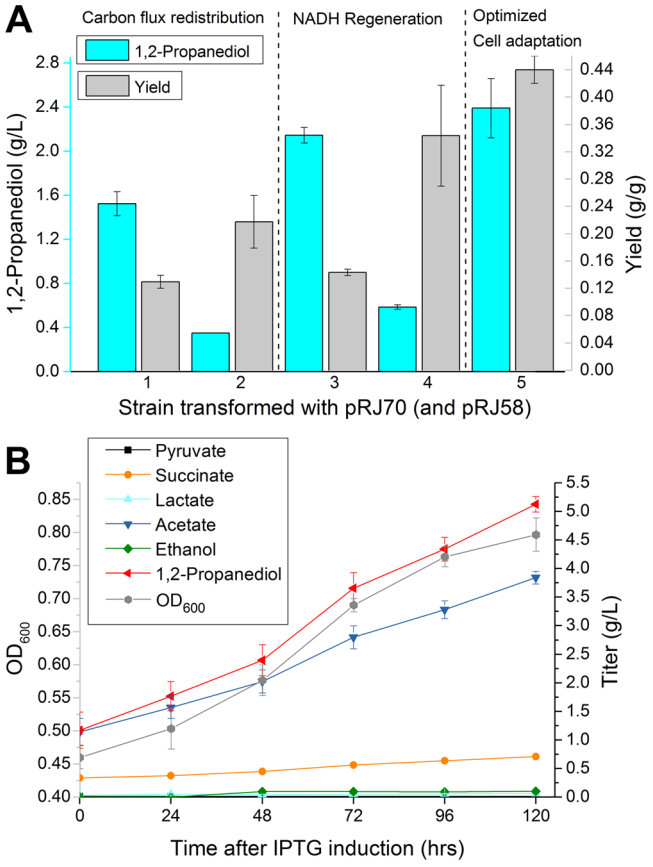

Figure 3. Comparison of 1,2-propanediol titers and yields and time course of 1,2-propanediol production. (A) Comparison of 1,2-propanediol titers (g/L) and yields (g/g glucose consumed) using different strategies after 48 h of study. 1: BW25113/pRJ70. 2: RJ57/pRJ70. 3: BW25113/pRJ70 + pRJ58. 4: RJ57/pRJ70 + pRJ58. 5: RJ57/pRJ70 + pRJ58. (B) Time course of 1,2-propanediol production, major cellular byproducts and cell growth from a 120 h shake flask study using strain RJ57/pRJ70 + pRJ58 grown with optimized cell adaptation strategy. The data were generated from three independent experiments.

As a result, cell growth (OD_{600}) was increased to 0.51 after 48 h. Furthermore, the amount of glucose consumed was also increased to 5.35 g/L after 48 h as compared to 1.75 g/L from this strain without cell adaptation (Table 1). Due to this effect, the titer of 1,2-propanediol was dramatically increased to 2.12 g/L after 48 h with a yield of 0.40 g/g glucose. Thus, the cell adaptation strategy successfully increased the cell growth of this strain and resulted in achieving both high titer and high yield simultaneously.

Furthermore, by adjusting the IPTG induction time even higher titers and yield of 1,2-propanediol was achieved as described below. Using this "optimized cell adaptation strategy" a 120 h fermentation study was carried out where IPTG was added to the cultures when the optical density reached 0.46. As seen from Table 1, this strain consumed 5.52 g/L of glucose, achieved an optical density of 0.57 and produced 2.39 g/L of 1,2-propanediol after 48 h (after IPTG induction) with a yield of 0.44 g/g glucose. This represents a more than 3 fold increase in glucose consumption as compared to this strain without cell adaptation. Also, this result indicated a higher titer of 2.39 g/L and a higher yield (0.44 g/g glucose) as compared to the wild type strain carrying plasmids pRJ70 and pRJ58 (2.14 g/L with a yield of 0.14 g/g glucose) after 48 h of fermentation (Table 1).

At the end of the fermentation study (120 h after IPTG induction), 1,2-propanediol was produced at 5.13 g/L with a yield of 0.48 g/g glucose (Figure 3B). This strain achieved a final optical density of 0.8 and consumed a total of 10.6 g/L of glucose. The major byproducts (lactate, ethanol, and pyruvate) were nearly eliminated (<0.1 g/L) and the minor byproduct (succinate) was accumulated at 0.71 g/L. The dominant byproduct for this case was acetate, which was produced at 3.84 g/L. Thus, the optimized cell adaptation strategy achieved both high titer and high yield of 1,2-propanediol simultaneously. Interestingly, this strain produced 1.16 g/L of 1,2-propanediol prior to IPTG induction. This result indicated that the leaky expression of the pathway enzymes stimulated the production of 1,2-propanediol. This conclusion is also supported by various studies confirming that the transcription of operons may occur even with the absence of a derepressor (IPTG/lactose) in the environment of pLac promoter.[36]

1-Propanol Production. In our previous work, we had established a novel metabolic route for the production of 1-propanol in wild type *E. coli* by expanding the 1,2-propanediol pathway (Figure 1). A whole cell conversion study showed 100% conversion of 1,2-propanediol (5 g/L) to 1-propanol. However, during fermentation studies, most of the 1,2-propanediol remained unconverted (0.46 g/L), producing only 0.25 g/L of 1-propanol.[26] In this work, we pursued the enhancement of 1-propanol production building on our efficient 1,2-propanediol platform. In order to identify any potential hurdles, we first tested 1-propanol production in a wild type strain instead of directly utilizing the enhanced 1,2-propanediol production system. We first transferred plasmids pRJ70 and pYY93 (a medium copy number plasmid carrying *ppdABC* operon) in wild type *E. coli*, following which fermentations were carried out for 48 h. As a result, 0.11 g/L of 1-propanol was produced with 0.95 g/L of 1,2-propanediol remaining unconverted. Similar to our previous work, this result depicted lower catalytic efficiency of the diol dehydratase during fermentation. We speculated that this could be due to pathway intermediate accumulation, resulting in inhibition of diol dehydratase activity. However, after thorough HPLC analysis, no pathway intermediates were detected from the shake flask fermentations. Hence, we concluded that the effect of pathway intermediates accumulation on dehydratase activity was not of concern.

We then speculated that the use of a high copy number plasmid (pZE12-luc) to express the 1,2-propanediol pathway may have led to plasmid incompatibility with pYY93, thereby affecting the diol dehydratase activity. To test this hypothesis, a 24 h study was performed as described in Methods as "whole cell conversion studies". Wild type *E. coli* transformed with pYY93 served as the control for diol dehydratase activity resulting in 100% conversion of 1,2-propanediol to 1-propanol, which was consistent with our previous study.[26] As seen from Figure 4A, the *E. coli* strain transformed with plasmids pYY93 and pZE12-luc (without 1,2-propanediol pathway operon) also achieved 100% conversion to 1-propanol. Hence, we concluded that there was no incompatibility between plasmids pYY93 and pZE12-luc.

Since there were no pathway intermediates detected and plasmid incompatibility was not of concern, we speculated that the overexpression of too many enzymes (1,2-propanediol pathway) may affect the diol dehydratase activity by interfering with its native enzyme complex formation. The *K. oxytoca* diol dehydratase enzyme consists of a complex of three subunits

dx.doi.org/10.1021/sb500345t | *ACS Synth. Biol.* 2015, 4, 746−756

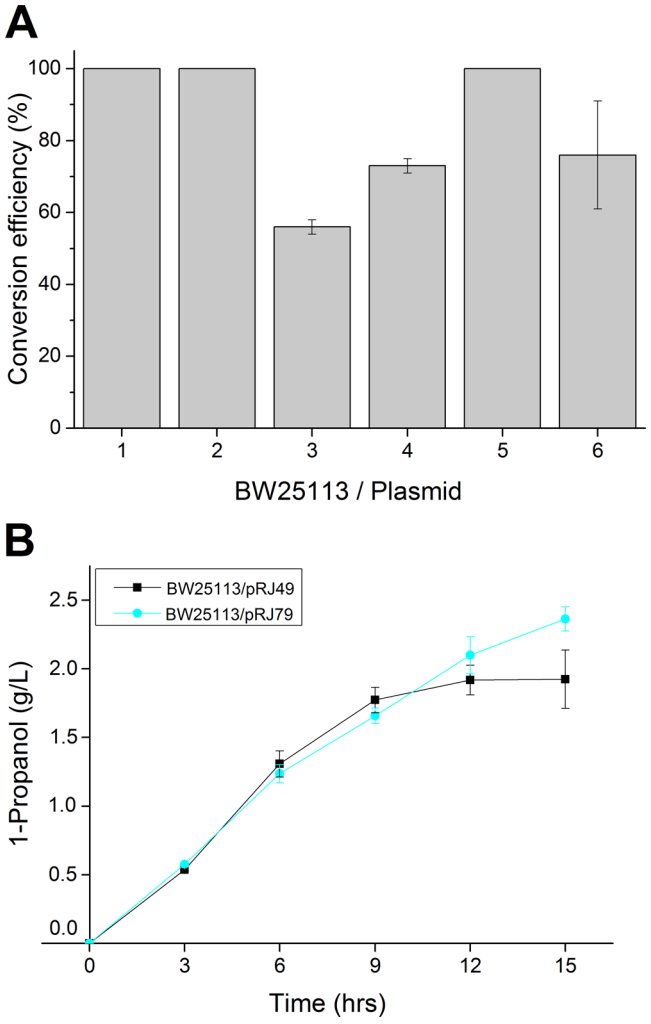

Figure 4. Comparison of the conversion efficiency of fusion diol dehydratases and natural diol dehydratase. (A) Whole cell conversion studies for 1-propanol production with 1,2-propanediol feeding. 1: BW25113/pYY93. 2: BW25113/pYY93 + pZE12-luc. 3: BW25113/pYY93 + pRJ70. 4: BW25113/pRJ49. 5: BW25113/pRJ79. 6: BW25113/pRJ81. (B) Comparison of 1-propanol production using superior fusion diol dehydratase (pRJ79) as compared to natural diol dehydratase (pRJ49) using wild type *E. coli* BW25113 via whole cell conversion studies. The data were generated from three independent experiments.

(*ppdA*, *ppdB*, *ppdC*). The crystal structure had determined that the enzyme complex exists as a dimer of the three subunits.[37] Therefore, any perturbation to this complex formation may hinder its catalytic efficiency. To test this hypothesis, plasmids pRJ70 and pYY93 were transferred into *E. coli* for another whole cell conversion study. It was seen that the *E. coli* strain transformed with pYY93 and pRJ70 resulted in a 44% decrease in conversion efficiency to 1-propanol as compared to the *E. coli* strain transformed with only pYY93 (Figure 4A). Hence, we inferred that the overexpression of the 1,2-propanediol pathway operon reduced the diol dehydratase catalytic efficiency by possibly interfering with the enzyme complex formation.

Interestingly, when the *ppdABC* operon was inserted into high copy number plasmid pZE12-luc (pRJ49) and was transferred into *E. coli* for a whole cell conversion study, it resulted in a conversion efficiency of 73% (Figure 4A), even without the overexpression of the 1,2-propanediol pathway operon. This result depicts a decrease in conversion efficiency by 27% compared to its expression via a medium copy number plasmid (pYY93) as shown above. Hence, it indicated that too much expression of the diol dehydratase subunits itself may reduce the catalytic efficiency possibly due to interference with the enzyme subunits' recognition and assembly.

To address this concern, we developed an approach which would facilitate the enzyme complex formation as well as achieve an equimolar level of the three subunits even with higher levels of expression. To achieve this, all three subunits were expressed as a fusion protein under the control of a common RBS as described below. By carefully examining the crystal structure of the diol dehydratase in both substrate free (PDBid: 1IWB)[37] and substrate bound (PDBid: 1UC5)[38] forms, we deduced the distances between the C and N terminuses of each subunit using Pymol software. By evaluating different subunit alignment possibilities we inferred that in order to facilitate the native enzyme complex formation a suitable approach was to link the C terminus of *ppdA* with N terminus of *ppdC* and the C terminus of *ppdC* with N terminus of *ppdB* (*ppdA-C-B*). To achieve this, a 13 amino acid linker between C terminus of *ppdA* and the N terminus of *ppdC* and a 9 amino acid linker between C terminus of *ppdC* and the N terminus of *ppdB* were inserted as described in Methods ("DNA manipulations"). The length of the amino acid linkers were chosen so as to ensure flexibility among the subunits. Similarly, another possible approach to facilitate the native enzyme complex formation was also tested (*ppdC-B-A*).

The sequence of *ppdA-C-B* and the *ppdC-B-A* reading frames have been provided in Supporting Information.

Plasmids pRJ79 and pRJ81carrying the *ppdA-C-B* and the *ppdC-B-A* reading frames respectively were transferred into *E. coli* individually for a whole cell conversion study. From Figure 4A it was seen that the *E. coli* strain transformed with pRJ79 showed 100% conversion of 1,2-propanediol to 1-propanol, whereas the *E. coli* strain transformed with pRJ81 showed a lower conversion efficiency of 76%. Thus, the optimal fusion diol dehydratase (*ppdA-C-B*) restored the conversion efficiency to 100% even with a high level expression of the subunits using a high copy number plasmid (pZE12-luc). Furthermore, the catalytic activity of this fusion diol dehydratase was found to be superior to the native one in a whole cell conversion study (Figure 4B). As seen from Figure 4B, the native and fusion diol dehydratases showed similar activity up to 9 h, however, after 9 h the activity of the native diol dehydratase became lower than that of the fusion diol dehydratase. We then hypothesized that the coexpression of this fusion diol dehydratase with the 1,2-propanediol pathway operon would enhance the production of 1-propanol.

To test this hypothesis, a high copy number plasmid pRJ93 carrying the *ppdA-C-B* reading frame and the 1,2-propanediol pathway genes was transferred into wild type *E. coli* for a 48 h fermentation study. Similarly, 1-propanol production was also tested using medium (pRJ99) and low copy number (pRJ100) plasmids. It was seen that when the high copy number plasmid was used to express the complete pathway, 1-propanol was produced at 0.62 g/L with 1.51 g/L of 1,2-propanediol remaining unconverted at the end of the study. Whereas, when the medium and low copy number plasmids were used to express the complete pathway, 0.29 g/L and 0.30 g/L of 1-propanol were produced with 1.14 g/L and 0.31 g/L of 1,2-propanediol remaining unconverted, respectively. Thus, even

dx.doi.org/10.1021/sb500345t | *ACS Synth. Biol.* 2015, 4, 746−756

with the utilization of a fusion diol dehydratase, the conversion efficiency to 1-propanol could not be enhanced during fermentation. We reasoned that since a large portion of 1,2-propanediol is generated during the later stages of fermentation (Figure 3B) where the catalytic activity of diol dehydratase might be lower, the conversion of 1,2-propanediol to 1-propanol might be hampered. This inference is also supported by the observation from the whole cell conversion study (Figure 4B), where the diol dehydratases showed higher catalytic efficiency during the initial phase of the study with a gradual decrease in the efficiency with time.

Dual Strain Strategies for Improvement of 1-Propanol Production. To address the above-mentioned concern, we hypothesized that using the "optimized cell adaptation strategy", 1,2-propanediol production can be achieved prior to the addition of the strain expressing the downstream 1,2-propanediol conversion pathway. This would make a large portion of 1,2-propanediol available when the diol dehydratase activity would be at its highest, thus resulting in enhanced 1-propanol production.

To test this hypothesis, the strain expressing the 1,2-propanediol pathway (strain RJ57 transformed with plasmids pRJ70 and pRJ58) was grown utilizing "optimized cell adaptation strategy" for 72 h. Following this, the strain expressing the fusion diol dehydratase (wild type *E. coli* strain transformed with plasmids pRJ79 and pRJ58) was added into the cultures via resuspension strategy or direct addition (as described in Methods as "dual strain strategies for 1-propanol production"). It was seen that after 72 h, 3.66 g/L of 1,2-propanediol was produced (prior to the addition of the strain expressing the downstream pathway). After the addition of the strain expressing the downstream pathway to the culture via the resuspension strategy, 0.89 g/L of 1-propanol was produced with 2.61 g/L of 1,2-propanediol remaining unconverted at the end of the study. Whereas, when the strain expressing the downstream pathway was added to the culture via the direct addition strategy, 1.5 g/L of 1-propanol was produced with 2.1 g/L of 1,2-propanediol remaining unconverted. Thus, by expressing the two portions of the pathway in different strains, 1-propanol production was enhanced to 1.5 g/L. The addition of the downstream strain directly proved to be more efficient than the resuspension strategy. However, most of the 1,2-propanediol still remained unconverted.

Alternatively, we utilized another approach for 1-propanol production by availing the leaky expression of the 1,2-propanediol pathway and coupling it with the dual strain strategy. In this approach, 1,2-propanediol production was carried out via "optimized cell adaptation strategy", however, without IPTG induction. It was seen that even without IPTG induction, 3.36 g/L of 1,2-propanediol was produced after 120 h (prior to the addition of the strain expressing the downstream pathway) due to leaky expression. After 120 h of fermentation, 1 mL of the strain expressing the downstream pathway was added to the cultures directly and the study was then carried out as described in Methods ("dual strain strategies for 1-propanol production"). As seen from Figure 5, 2.91 g/L of 1-propanol was produced with 0.20 g/L of 1,2-propanediol remaining unconverted at the end of the study. This result showed that nearly all of the 1,2-propanediol was converted to 1-propanol using this method. Thus, the utilization of an optimized cell adaptation strategy with leaky operon expression for 1,2-propanediol production coupled with a dual strain strategy resulted in enhancing 1-propanol production to 2.91 g/L. This result represents over 11-fold increase in 1-propanol production compared with our previous work (0.25 g/L).[26]

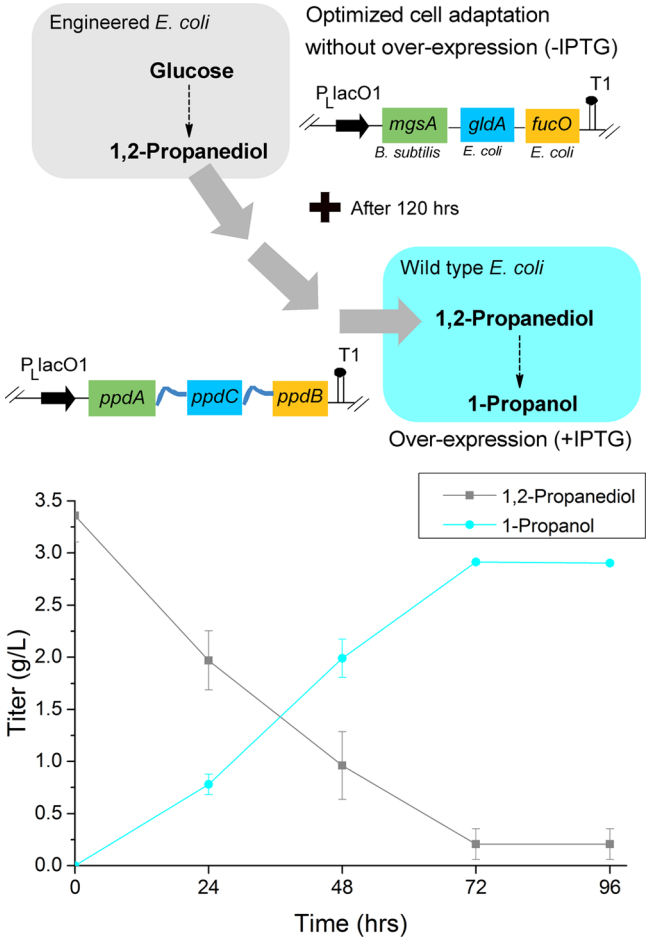

Figure 5. Schematic representation of a dual strain strategy and time course of 1-propanol production. The 1,2-propanediol producing strain lacked IPTG induction; the 1-propanol producing strain was added to the cultures after 120 h. The data were generated from three independent experiments.

Discussion. In order to conserve more carbon and NADH for 1,2-propanediol production, we first disrupted the major byproduct pathway (lactate). Interestingly, the expression of 1,2-propanediol pathway in this strain (RJ31) resulted in lowering 1,2-propanediol production by nearly 40% of the wild type strain and accumulated pyruvate at 7.18 g/L (Supporting Information Table S2). This led to the understanding that since the carbon flux was dominant toward the glycolytic pathway, the disruption of competing pathways for carbon is vital. In order to direct the carbon effectively into the 1,2-propanediol pathway, the major carbon competing pathways were disrupted (strain RJ57). However, the expression of the 1,2-propanediol pathway in this strain resulted in lowering the titer to just 0.35 g/L. We then hypothesized that the development of a NADH regeneration system would serve as a driving force for 1,2-propanediol production. It is seen that with the introduction of this NADH regeneration system, 1,2-propanediol production was increased in both the engineered (RJ57) and wild type strains (Table 1). With the development of cell adaptation strategy, the growth of the engineered strain was improved and 1,2-propanediol production was achieved at a high titer (5.13 g/L) with a high yield of 0.48 g/g glucose.

dx.doi.org/10.1021/sb500345t | *ACS Synth. Biol.* 2015, 4, 746−756

The theoretical maximum yield for anaerobic production of 1,2-propanediol was previously calculated to be 1.2 mol/mol glucose (0.51 g/g glucose).[39] It should be noted that we used yeast extract in the culture media to facilitate cell growth, which has been extensively used in metabolic engineering. The contribution of yeast extract toward target product yield was evaluated using yeast extract as the sole carbon source, following which it was determined that the contribution of yeast extract toward product yield was minor or even negligible.[40] Therefore, we investigated the contribution of yeast extract toward 1,2-propanediol yield using the same approach. Control experiments were then carried out using yeast extract as the sole carbon source. At the end of fermentation study, it was determined that no 1,2-propanediol was produced in both the wild type and the engineered strains. Hence, the contribution of yeast extract toward product yield was considered to be negligible. Thus, we concluded that strain RJ57 expressing the 1,2-propanediol pathway grown with cell adaptation strategy achieved 94% of theoretical maximum yield (0.48 g/g glucose).

With a highly efficient system established for 1,2-propanediol, we then channeled our efforts toward resolving its low conversion efficiency to 1-propanol. As seen from Figure 4A, with the creation of a fusion diol dehydratase, the conversion efficiency was recovered to 100% in whole cell conversion studies. However, during fermentation, most of the 1,2-propanediol remained unconverted. We reasoned that since the catalytic activity of the diol dehydratases showed gradual decrease with time, the production of 1-propanol was hampered when 1,2-propanediol is generated gradually. To circumvent this predicament, a dual strain strategy was utilized where 1,2-propanediol production was first achieved, following which the strain expressing the fusion diol dehydratase was added to the cultures. By relying on the leaky expression of the upstream pathway for 1,2-propanediol production, this dual strategy resulted in converting nearly all of the 1,2-propanediol to 1-propanol, boosting the titer to 2.91 g/L (Figure 5).

Conclusion. Majority of the world's demand for high value/bulk chemicals and energy is met by utilization of fossil fuels. With the emergence of metabolic engineering, better solutions are emerging to meet the global demand. Here, we provide effective steps toward the future scale up and industrial scale biological manufacture of current petrochemical based compounds 1,2-propanediol and 1-propanol. In this work, the production of 1,2-propanediol is pursued followed by the production of 1-propanol by adapting different metabolic engineering strategies to surpass the hurdles in enhancing their production.

METHODS

Chemicals and Reagents. 1,2-Propanediol and methylglyoxal standards were procured from Sigma-Aldrich (St. Louis, MO); acetol (hydroxyacetone) was procured from Acros Organics (New Jersey, U.S.A.) and 1-propanol was purchased from Fisher Scientific (Atlanta, GA). For amplifying DNA in order for cloning, phusion high fidelity polymerase (New England Biolabs, Beverly, MA) and KOD DNA polymerase (EMD Chemicals Inc., NJ) were used. In order to verify knockout strains, GoTaq DNA polymerase (Promega, Madison, WI) was utilized. Restriction enzymes were procured from New England Biolabs (Beverly, MA). For ligation reactions either Rapid DNA ligase (Roche Applied Science, Indianapolis, IN) or Quick DNA ligase (New England Biolabs, Beverly, MA) were used.

Bacterial Strains and Plasmids. *E. coli* strain BW25113 (*E. coli* Genetic Resource Center) as wild type and its knockout derivatives were used for shake flask experiments.[41] Using P1 transduction, target genes were disrupted to construct engineered strains with more than one gene disruption.[42] Plasmid pCP20 was used to remove kanamycin resistance gene from target strains.[43] Verification of gene disruption/antibiotic resistance loss was done via colony PCR. For DNA manipulations, *E. coli* strain XL1-Blue (Stratagene, CA) was used. For enzyme assays, *E. coli* BL21 Star (DE3) was used. Plasmids pZE12-luc,[44] pCS27,[20] pSA74,[2] and pETDuet-1 (EMD Chemicals Inc., NJ) were used for DNA cloning. The characteristics of strains and plasmids are described in Table 2.

DNA Manipulations. The procedures used for DNA manipulations were performed according to the methods described previously.[45] Plasmids pRJ1, pRJ2, and pYY93 were constructed previously.[26] To create pRJ68, the *E. coli gldA* gene was inserted downstream to the *mgsA* gene in pRJ2 using SphI and XbaI. Similarly to create pRJ69, the *budC* gene from *K. pneumoniae* was inserted downstream to the *mgsA* gene in pRJ2 using SphI and XbaI. To create pRJ70, the genes *gldA* and *fucO* were inserted simultaneously downstream to the *mgsA* gene in pRJ2 using SphI, *Bam*HI, and XbaI. The *Candida boidinii fdh1* gene encoding formate dehydrogenase was codon optimized for expression in *E. coli*. The sequence is provided in the Supporting Information. To create pRJ58, the *fdh1* gene was synthesized via PCR based oligonucleotide assembly and inserted into pCS27 using Acc65I and *Bam*HI. In order to express his-tagged FDH protein, pRJ98 was constructed with the *fdh1* gene inserted into pET-Duet-1 using *Bam*HI and *Sal*I.

To construct pRJ49, the *ppdABC* operon was digested with BsrGI and XbaI and inserted into plasmid pZE12-luc digested with Acc65I and XbaI. In order to create fusion diol dehydratase proteins, we first linked the *ppdA*, *ppdB*, and *ppdC* genes into single reading frames (*ppdA-C-B* or *ppdC-B-A*). Overlap PCR was used achieve these, with the removal of corresponding start and stop codons using the designed primers. Plasmid pRJ79 was constructed, with the *ppdA-C-B* reading frame inserted into pZE12-luc using *Eco*RI and XbaI. Similarly, pRJ81 was constructed by inserting *ppdC-B-A* reading frame into pZE12-luc using *Eco*RI and XbaI. To create pRJ93, the *ppdA-C-B* reading frame and the *mgsA-gldA-fucO* operon were cloned simultaneously intopZE12-luc using *Eco*RI, BsrGI, and XbaI. To construct plasmid pRJ99, the *ppdA-C-B* and the *mgsA-gldA-fucO* operon (obtained using pRJ93 as the template) as a single DNA fragment were digested with BsiWI and XhoI. Following digestion, the fragment was inserted into the plasmid pCS27 digested with Acc65I and *Sal*I. Similarly pRJ100 was constructed by cloning this fragment into plasmid pSA74.

Formate Dehydrogenase Enzyme Assay. *E. coli* BL21 Star (DE3) harboring plasmid pRJ98 was used to evaluate the activity of formate dehydrogenase. 500 μL of the overnight cultures was added to 50 mL fresh LB and was grown for 3 h at 37 °C. IPTG was added to a final concentration of 1 mM to induce protein expression, and the cultures were then grown for another 3 h at 30 °C. Next, the cultures were centrifuged and the cells were lysed using Mini Bead Beater (Biospec). Following another round of centrifugation, the resulting supernatant was collected. His-Spin Protein Miniprep kit (Zymo Research, Irvine, CA) was used to obtain the purified protein from the supernatant. Protein concentration estimation

Table 2. List of Strains and Plasmids Used in This Study

strain	genotype	ref
E. coli BW25113	*F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ⁻, rph-1, Δ(rhaD-rhaB)568, hsdR514*	Yale CGSC
E. coli XL-1 Blue	*recA1 endA1gyrA96thi-1hsdR17supE44relA1lac [F′ proAB lacIqZDM15Tn10 (TetR)]*	Stratagene
E. coli BL21 Star (DE3)	*FompT hsdS_B ($r_B^-m_B^-$) gal dcm (DE3)*	Invitrogen
JW1375-1	BW25113 Δ*ldhA::kan*	Yale CGSC
JW1643-2	BW25113 Δ*gloA::kan*	Yale CGSC
JW3890-2	BW25113 Δ*tpiA::kan*	Yale CGSC
JW1841-1	BW25113 Δ*zwf::kan*	Yale CGSC
JW1228-1	BW25113 Δ*adhE::kan*	Yale CGSC
RJ31	BW25113 Δ*gloA* Δ*ldhA*	this study
RJ57	BW25113 Δ*zwf* Δ*tpiA* Δ*ldhA* Δ*gloA* Δ*adhE*	this study
plasmid	**description**	**ref**
pZE12-luc	pLlacO1; luc; *ColE1 ori*; Amp^R	44
pCS27	pLlacO1; *p15A ori*; Kan^R	20
pSA74	pLlacO1; *pSC101ori*; Cm^R	2
pETDuet-1	two T7 promoters; two MCS; *pBR322 ori*; Amp^R	Novagen
pYY93	*ppdABC* from *K. oxytoca* cloned into pCS27	26
pRJ1	*mgsA* from *C. acetobutylicum* cloned into pZE12-luc	26
pRJ2	*mgsA* from *B. subtilis* cloned into pZE12-luc	26
pRJ49	*ppdABC* from *K. oxytoca* cloned into pZE12-luc	this study
pRJ58	*fdh1* from *C. boidinii* cloned into pCS27	this study
pRJ68	*mgsA* from *B. subtilis* and *gldA* from *E. coli* cloned into pZE12-luc	this study
pRJ69	*mgsA* from *B. subtilis* and *budC* from *K. pneumoniae* cloned into pZE12-luc	this study
pRJ70	*mgsA* from *B. subtilis*, *gldA* from *E. coli* and *fucO* from *E. coli* cloned into pZE12-luc	this study
pRJ79	Fusion *ppdA-C-B* cloned into pZE12-luc	this study
pRJ81	Fusion *ppdC-B-A* cloned into pZE12-luc	this study
pRJ93	Fusion *ppdA-C-B*, *mgsA* from *B. subtilis*, *gldA* from *E. coli* and *fucO* from *E. coli* cloned into pZE12-luc	this study
pRJ98	*fdh1* from *C. boidinii* cloned into pET-Duet-1	this study
pRJ99	Fusion *ppdA-C-B*, *mgsA* from *B. subtilis*, *gldA* from *E. coli* and *fucO* from *E. coli* cloned into pCS27	this study
pRJ100	Fusion *ppdA-C-B*, *mgsA* from *B. subtilis*, *gldA* from *E. coli* and *fucO* from *E. coli* cloned into pSA74	this study

was done using the Pierce BCA protein assay kit (Thermo Scientific, Atlanta, GA). The formate dehydrogenase enzyme assay using the purified protein was done with modifications to the method described previously.[35] The reaction system (1 mL) consisted of 50 mM sodium phosphate buffer (pH 7.0), 0.25 mM NAD^+, 50 μL of purified enzyme and substrate sodium formate. The concentration of substrate sodium formate was varied from 0 mM to 75 mM. The molecular weight of the protein along with his-tag was estimated to be 41748 Da. The reaction rates were monitored for 30 s at 37 °C by measuring the change in absorbance at 340 nm.

Culture Medium. Modified M9 medium was used for the whole cell conversion study of propanediol dehydratase, which consisted of (per liter): 20 g glucose, 5 g yeast extract, 12.8 g $Na_2HPO_4{\cdot}7H_2O$, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 0.5 mM $MgSO_4$, and 0.05 mM $CaCl_2$. The modified low phosphate medium was used for shake flask fermentations and consisted of (per liter): 40 g glucose, 5 g NaCl, 5 g yeast extract, 1 g NH_4Cl, 1.5 g KCl, 0.2 g $MgCl_2$, 0.07 g Na_2SO_4, and 0.005 g $FeCl_3$. The media was then buffered to pH 6.8 with 13.3 g of $NaHCO_3$ and 10 g of 3-[N-morpholino] propanesulfonic acid (MOPS).[23,26] For experiments with the expression of formate dehydrogenase, in order to regenerate cellular NADH, 50 mM sodium formate was added to the modified low phosphate media to make low phosphate formate media.[35]

Whole Cell Conversion Studies. For whole cell conversion studies, 2 mL of seed cultures were prepared in LB containing necessary antibiotics and grown overnight at 37 °C in a shaker set at 250 rpm. After overnight incubation, 100 μL of the cultures were inoculated into 10 mL of modified M9 media containing appropriate antibiotics in 125 mL screw cap bottles and grown at 37 °C for 4 h. After this, 0.1 mM IPTG, 10 μM coenzyme B12, and 5 g/L 1,2-propanediol were added to the media and grown at 30 °C for 24 h. Samples (1 mL) were collected at the end of the study for HPLC analysis.

Shake Flask Fermentations. For shake flask fermentation studies, 200 μL of the overnight cultures (grown in LB) were inoculated into 20 mL modified low phosphate media or low phosphate formate media in 150 mL serum bottles. The cultures were made anaerobic by sparging with a mixture of nitrogen and carbon dioxide gas and grown in a shaker at 37 °C, 250 rpm. IPTG was added into the cultures to a final concentration of 0.1 mM, after 3 h (unless mentioned otherwise) to induce protein expression. For experiments involving 1-propanol production, 10 μM coenzyme B12 was added to the cultures along with IPTG. Fermentations were then carried out at 30 °C in a shaker set at 250 rpm. Samples were collected and analyzed for cell growth and products.

Cell Adaptation. The overnight cultures of strain RJ57 harboring plasmids pRJ70 and pRJ58 were initially made in 3 mL LB and grown until exponential growth phase (until OD_{600} = 0.7). Following this, 1 mL of the cultures were added to 10 mL of fresh low phosphate formate media in vacutainer glass tubes (BD) and made anaerobic immediately. The cultures were then grown until OD_{600} reached about 0.2. Next, 1 mL of these subcultures were added into 20 mL of fresh low phosphate formate media for shake flask fermentation studies as described above.

Dual Strain Strategies for 1-Propanol Production. We used a dual strain strategy to express the upstream 1,2-

dx.doi.org/10.1021/sb500345t | ACS Synth. Biol. 2015, 4, 746−756

propanediol pathway and the downstream 1-propanol pathway in different strains. The strain RJ57 harboring plasmids pRJ70 and pRJ58 was used to express the upstream 1,2-propanediol pathway and the wild type strain harboring plasmids pRJ79 and pRJ58 was used to express the downstream 1-propanol pathway. First, 1,2-propanediol production was achieved, following which the strain expressing 1-propanol pathway was prepared and added to the cultures as described below. The strain RJ57 harboring plasmids pRJ70 and pRJ58 was grown using the methods described in "cell adaptation", following which, fermentations were carried out for 72 or 120 h as described in "shake flask fermentations", with the addition of IPTG when the optical density reached 0.46. Meanwhile, the wild type strain harboring plasmids pRJ79 and pRJ58 was prepared for sequential inoculation. The overnight cultures (60 μL of 2 mL) of the strain expressing 1-propanol pathway was used to inoculate 6 mL of fresh LB, which was grown at 37 °C for 3 h. Next, IPTG (0.1 mM) and 10 μM coenzyme B12 were added in order to facilitate protein expression and the cultures were grown at 30 °C for another 5 h (OD_{600} was approximately 2.0). These cultures were then added to the 72 or 120 h cultures (OD_{600} = 0.69 or OD_{600} = 0.79 after 72 or 120 h, respectively) of the strain expressing the upstream 1,2-propanediol pathway in two different strategies. In the first strategy, the cultures (6 mL) of the strain expressing the downstream 1-propanol pathway was centrifuged and resuspended in 1 mL of fresh low phosphate formate media. This 1 mL resuspended cultures were added to the 72 h cultures along with 0.1 mM IPTG and 10 μM Coenzyme B12. In the second strategy, 1 mL cultures of the strain expressing the downstream pathway was directly added to the 72 or 120 h cultures along with 0.1 mM IPTG and 10 μM Coenzyme B12. Fermentations were then carried out at 30 °C and samples were collected for analysis.

Analytical Procedures. Cell growth was analyzed by measuring the optical density at 600 nm using a UV-650 spectrophotometer (Beckman Instruments, San Jose, CA, U.S.A.). The samples from fermentation studies and whole cell conversion studies were analyzed via HPLC-RID. First, the samples were prepared via centrifugation at 15 000 rpm for 15 min. Following this, the resulting supernatants were filtered and used for analysis in a HPLC (Shimadzu) equipped with a Coregel-64H column (Transgenomic) with 4 mN H_2SO_4 as the mobile phase. The flow rate of mobile phase was 0.6 mL/min and the oven temperature was set at 60 °C.[46] For the separation of 1,2-propanediol and acetol and also for the separation of ethanol and MOPS, the oven temperature was set at 40 °C.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Y.Y. and R.J. conceived the study. Y.Y. and Q.Y. supervised the project. R.J. and X.S. designed the experiments. R.J. performed the experiments. R.J. analyzed the data and wrote the manuscript. Y.Y. revised the manuscript.

Notes

The authors declare the following competing financial interest(s): The University of Georgia has filed a patent application on this technology.

■ ACKNOWLEDGMENTS

This work has been supported by the start-up funds from the College of Engineering, University of Georgia, Athens, and a National Science Foundation grant (1335856).

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