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Directing enzyme devolution for biosynthesis of alkanols and 1,n-alkanediols from natural polyhydroxy compounds

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ABSTRACT

Primordial enzymes are proposed to possess broad specificities. Through divergence and evolution, enzymes have been refined to exhibit specificity towards one reaction or substrate, and are thus commonly assumed as "specialists". However, some enzymes are "generalists" that catalyze a range of substrates and reactions. This property has been defined as enzyme promiscuity and is of great importance for the evolution of new functions. The promiscuities of two enzymes, namely glycerol dehydratase and diol dehydratase, were herein exploited for catalyzing long-chain polyols, including 1,2-butanediol, 1,2,4-butanetriol, erythritol, 1,2-pentanediol, 1,2,5-pentanetriol, and 1,2,6-hexanetriol. The specific activities required for catalyzing these six long-chain polyols were studied via *in vitro* enzyme assays, and the catalytic efficiencies were increased through protein engineering. The promiscuous functions were subsequently applied *in vivo* to establish 1,4-butanediol pathways from lignocellulose derived compounds, including xylose and erythritol. In addition, a pathway for 1-pentanol production from 1,2-pentanediol was also constructed. The results suggest that exploiting enzyme promiscuity is promising for exploring new catalysts, which would expand the repertoire of genetic elements available to synthetic biology and may provide a starting point for designing and engineering novel pathways for valuable chemicals.

1. Introduction

Alkanols and 1,n-alkanediols (C4-C6) are important chemical compounds, with a wide range of applications in modern industry (Lin et al., 2015; Yim et al., 2011). Aliphatic alcohols (C4-C6) have recently emerged as alternative biofuels because of their higher energy density (Buijs et al., 2013; Serrano-Ruiz et al., 2012) and lower hygroscopicity (Atsumi et al., 2008; Zhang et al., 2011). 1,n-Alkanediols are important commodity chemicals, which have gained increasing interest in modern manufacturing for the production of pharmaceuticals, polyesters and polyurethane resins (Burt et al., 2017; Koso et al., 2009). However, most of these alcohols cannot be naturally synthesized in cells. By contrast, polyhydroxy compounds, with similar structures to the alkanols and 1,n-alkanediols, are ubiquitous in nature such as all kinds of sugars and sugar alcohols. Of these compounds, glucose and xylose are the most abundant monosaccharides present in lignocellulosic biomass (An et al., 2011; Farwick et al., 2014). Erythritol (ERY) is a four-carbon sugar alcohol produced on the industrial scale using mainly sucrose, glucose or fructose syrup (Moon et al., 2010; Oh et al., 2001). Given that these chemicals are cheaply available and their structures are somewhat similar to aliphatic polyols, it is attractive to explore an enzyme/enzymes capable of reducing the number of hydroxyl groups, so as to produce the desired alcohols.

Enzyme promiscuity has become popular in the fields of enzyme engineering. Some researchers regard it as a starting point for divergent evolution (Hult and Berglund, 2007). Low promiscuous activities towards non-native substrates, by accumulation of one or more beneficial mutations, might turn the enzyme into a much more proficient catalyst (Gupta, 2016). The promiscuous functions can be further reinforced by rational design or directed laboratory evolution in an effort to create a de-evolved new enzyme (Humble and Berglund, 2011; Renata et al., 2015). Recently, improvements in promiscuous activity and changes in substrate specificity have been obtained through protein engineering (Bigley et al., 2013; Colin et al., 2015; Dorr et al., 2014). It is notable that glycerol dehydratase (GDHt, EC 4.2.1.30) and diol dehydratase (DDHt, EC 4.2.1.28) are isofunctional enzymes that catalyze the coenzyme B_{12}-dependent conversion of C2-C3 polyols to the corresponding aldehydes (Kinoshita et al., 2008; Wang et al., 2017b; Yamanishi et al., 2002). For example, GDHt is an essential enzyme in glycerol fermentation, leading to the production of 1,3-propanediol (Knietsch et al.,

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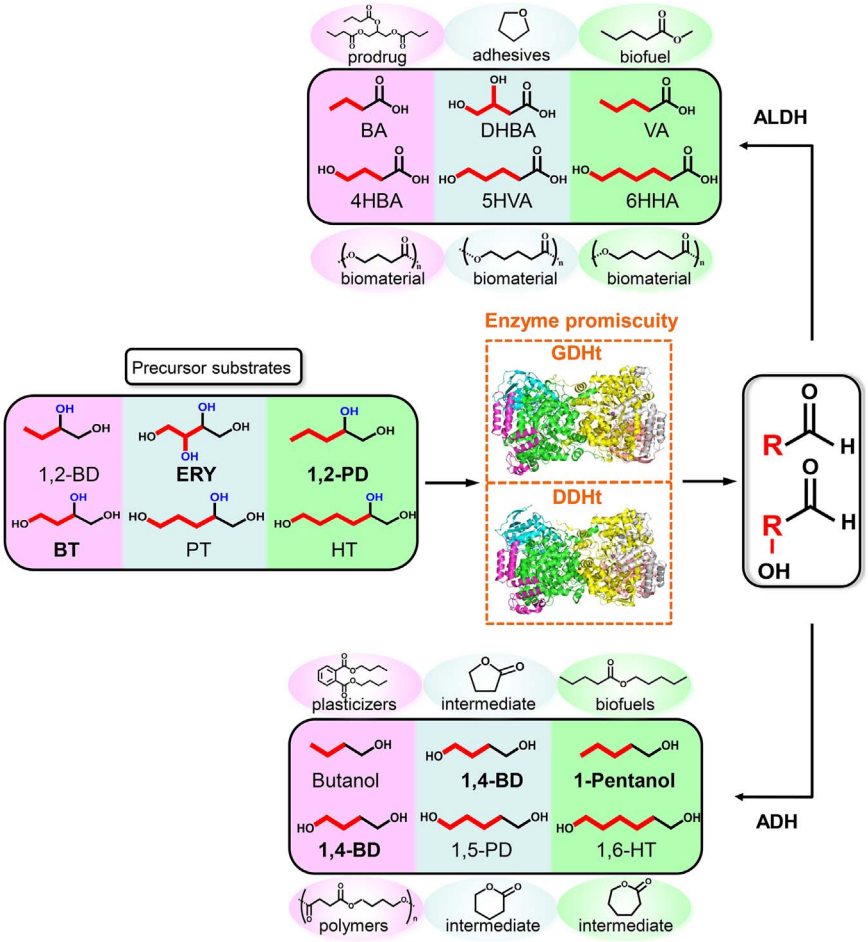

Fig. 1. Schematic pathways of the aliphatic alcohols and acids production (C4-C6). The precursor substrates used in the system are depicted enclosed by a rectangular box. Corresponding aliphatic alcohols and acids are color coded accordingly with precursor substrates. Substrates applied to *in vivo* productions were labeled in bold. Enzyme symbols: GDHt, glycerol dehydratase; DDHt, diol dehydratase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase. The crystal structures shown in the figure belong to GDHt (PDB ID: 1MMF) and DDHt (PDB ID: 1DIO).

2003), while DDHt is a key enzyme in 1,2-propanediol degradation, resulting in the synthesis of 1-propanol (Jain et al., 2015). The property of GDHt and DDHt to catalyze various substrates might enable the production of higher-order alcohols. This possibility prompts the redesign of promiscuous GDHt/DDHt to enable the conversion of other long-chain polyols, thereby leading to the production of alkanols and 1,n-alkanediols.

In this study, both GDHt and DDHt were explored for the potential of long-chain polyols catalysis. The activities towards six polyols were determined via *in vitro* enzyme assays, and products formed from coupled enzyme reactions were detected via GC-MS. Site-directed mutagenesis of GDHt and DDHt was performed to increase catalytic efficiencies towards these long-chain polyols. The mutants were subsequently applied *in vivo* to produce important chemicals including 1,4-butanediol (1,4-BD) and 1-pentanol by extending heterologous pathways in *Escherichia coli*. This demonstrated a platform for biological synthesis of various alcohols and acids (Fig. 1).

2. Materials and methods

2.1. Chemicals and strains

1,2-Butanediol (1,2-BD, 98%) and 1-butanol (99.7%) were purchased from Sigma-Aldrich (St. Louis, USA). 1,2,4-Butanetriol (BT, 98%), 1,4-butanediol (1,4-BD, 99%), erythritol (ERY, 99%), 1,2-pentanediol (1,2-PD, 98%), 1-pentanol (99.5%), 1,5-pentanediol (1,5-PD, 97%), 1,2,6-hexanetriol (HT, 97%), and 1,6-hexanediol (1,6-HD, GC standard) were purchased from Aladdin (Shanghai, China). 1,2,5-Pentanetriol (PT, 97%) was purchased from TCI (Tokyo, Japan). Isopropyl β-D-thiogalactoside (IPTG) and antibiotics (ampicillin, kanamycin and chloramphenicol) were obtained from Sangon Biotech (Shanghai, China). Other chemicals were of analytical grade. Strains, plasmids and primers are listed in Table S1 and Table S2. *E. coli* DH5α was used for plasmid construction. *E. coli* BL21 (DE3) was used for expression of dehydratases and various pathway genes.

2.2. DNA manipulations

Isolation of vectors, restriction enzyme digestion, agarose gel electrophoresis, and other DNA manipulations were carried out using standard protocols (Joseph and David, 2001). Plasmids used in this study were constructed using pEASY-Uni Seamless Cloning and Assembly Kit (TransGen, Beijing, China) according to the manufacturer's protocol. The *gldABC* gene encoding glycerol dehydratase (GDHt) of *Klebsiella pneumoniae* ATCC 25955 was inserted into pET28a between *Eco*RI and *Hin*dIII sites to create pT-*gldABC*. The *pddABC* gene encoding diol dehydratase (DDHt) of *Klebsiella oxytoca* ATCC 8724 was cloned into pET28a using *Bam*HI and *Xho*I to create pT-*pddABC*. pT-DM-*gldABC* was obtained through two rounds of site-directed mutagenesis. GDHt-1F/GDHt-S302 and GDHt-S302R/GDHt-2R pairs were first used for a Ser-to-Ala substitution at position 302 (S302A) of GDHt. The second amplification with primer pairs GDHt-1F/GDHt-Q337 and GDHt-Q337R/GDHt-2R was performed to introduce another mutation into *gldABC* such that the encoded protein carried alanine residues at both position 302 and 337 instead of serine and glutamine. Similarly to create pT-DM-*pddABC*, DDHt-1F/DDHt-S301 and DDHt-S301R/DDHt-2R pairs were used for the first round of mutagenesis. The amplification using DDHt-1F/DDHt-Q336 and DDHt-Q336R/DDHt-2R was then performed to introduce another mutation into DDHt. The *yqhD* gene encoding alcohol dehydrogenase (ADH) of *E. coli* BL21 (DE3) was inserted