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Use of modular, synthetic scaffolds for improved production of glucaric acid in engineered *E. coli*

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ABSTRACT

The field of metabolic engineering has the potential to produce a wide variety of chemicals in both an inexpensive and ecologically-friendly manner. Heterologous expression of novel combinations of enzymes promises to provide new or improved synthetic routes towards a substantially increased diversity of small molecules. Recently, we constructed a synthetic pathway to produce D-glucaric acid, a molecule that has been deemed a "top-value added chemical" from biomass, starting from glucose. Limiting flux through the pathway is the second recombinant step, catalyzed by *myo*-inositol oxygenase (MIOX), whose activity is strongly influenced by the concentration of the *myo*-inositol substrate. To synthetically increase the effective concentration of *myo*-inositol, polypeptide scaffolds were built from protein–protein interaction domains to co-localize all three pathway enzymes in a designable complex as previously described (Dueber et al., 2009). Glucaric acid titer was found to be strongly affected by the number of scaffold interaction domains targeting upstream Ino1 enzymes, whereas the effect of increased numbers of MIOX-targeted domains was much less significant. We determined that the scaffolds directly increased the specific MIOX activity and that glucaric acid titers were strongly correlated with MIOX activity. Overall, we observed an approximately 5-fold improvement in product titers over the non-scaffolded control, and a 50% improvement over the previously reported highest titers. These results further validate the utility of these synthetic scaffolds as a tool for metabolic engineering.

1. Introduction

Synthetic biology is an evolving field involving the creation of new biological components and systems, such as enzymes, signaling molecules, and metabolic pathways (Benner and Sismour, 2005; Keasling, 2008; Leonard et al., 2008). Synthetic biologists seek to design and characterize interchangeable parts from which one can build devices and systems that can both help to understand natural biological systems and facilitate the creation of new biological "machines." Achievements in the field include rewiring signaling pathways (Park et al., 2003) as well as the development of microbes that can synthesize bulk chemicals (Nakamura and Whited, 2003), fuels (Atsumi et al., 2008), and drugs (Martin et al., 2003; Ro et al., 2006). In the latter examples, synthetic biology intersects directly with metabolic engineering in using enzymes as interchangeable parts for the construction or re-constitution of metabolic pathways. These pathways can be naturally existing, recruited from a heterologous organism, or they may be formed from novel combinations of enzymes to produce both natural compounds and products not yet observed in nature (Prather and Martin, 2008). Metabolic engineering has traditionally focused on the improvement of metabolic pathways for increased productivity. To this end, one focus of synthetic biology is to provide additional tools for producing high-value compounds cheaply, efficiently, and cleanly (Arkin and Fletcher, 2006; Keasling, 2008; Tyo et al., 2007).

We recently constructed a synthetic pathway for the production of D-glucaric acid from D-glucose in *Escherichia coli* (Moon et al., 2009a). D-glucaric acid has been identified as a "top value-added chemical from biomass" (Werpy and Petersen, 2004), and has been studied for therapeutic purposes including cholesterol reduction (Walaszek et al., 1996) and cancer chemotherapy (Singh and Gupta, 2003, 2007). Its primary use is as a starting material for hydroxylated nylons (Werpy and Petersen, 2004). D-Glucaric acid is currently produced by chemical oxidation of glucose, a non-selective and expensive process using nitric

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acid as the oxidant. There is a known route for the production of D-glucaric acid from D-glucose in mammals; however, this is a lengthy pathway, consisting of more than ten conversion steps. Our synthetic pathway was assembled by recruiting enzyme activities from disparate sources into *E. coli* (Moon et al., 2009a). Co-expression of the genes encoding *myo*-inositol-1-phosphate synthase (Ino1) from *Saccharomyces cerevisiae*, *myo*-inositol oxygenase (MIOX) from *Mus musculus* (mouse), and uronate dehydrogenase (Udh) from *Pseudomonas syringae* led to production of D-glucaric acid at titers of ~1 g/L. We next aimed to improve this level of productivity.

Other examples of the design and construction of synthetic pathways from the combination of heterologous enzymes have been recently reported (Atsumi et al., 2008; Martin et al., 2003; Nakamura and Whited, 2003; Niu et al., 2003); however, the main focus of metabolic engineering has been global optimization of metabolic flux (Stephanopoulos and Jensen, 2005). To this end, various approaches have been successfully implemented, including modulation of enzyme expression by varying the strengths of promoters and ribosome binding sites, control of mRNA processing by introducing tunable intergenic regions, and improvement of rate-limiting enzymes by directed evolution (Alper et al., 2005; Bloom et al., 2005; Pfleger et al., 2006; Pitera et al., 2007; Stephanopoulos, 1999). Recently, an orthogonal, but compatible method for improving pathway efficiency was described (Dueber et al., 2009). In this method, pathway enzymes were colocalized using synthetic scaffolds built from protein–protein interaction domains that specifically bound corresponding ligands fused to the metabolic enzymes. By taking advantage of the modularity of these interaction domains, scaffold architectures were optimized to achieve a 77-fold improvement of mevalonate production at low expression levels of pathway enzymes (Dueber et al., 2009). In this same report, we were able to demonstrate a three-fold improvement in D-glucaric acid titer by co-localizing Ino1 and MIOX in a 1:1 ratio, although the baseline of 0.6 g/L in the absence of scaffolding was somewhat lower than the titers previously achieved with different expression machinery (Moon et al., 2009a). In particular, the original system utilized the very strong T7 promoter, while the second-generation system employed a P_{lac} promoter. Here we take advantage of the modular scaffold design to control enzyme stoichiometry at the synthetic complex in a targeted manner for further titer improvements.

Our interest in scaffolding the glucaric acid pathway is based on two prior observations. First, we observed that the activity of MIOX was lowest of the three enzymes in the recombinant system, more than two orders of magnitude lower than that of the most active enzyme (Udh) (Moon et al., 2009a). Second, we confirmed previous reports that high MIOX activity in *E. coli* is strongly influenced by exposure to high concentrations of *myo*-inositol, its substrate (Arner et al., 2004; Moon et al., 2009a). Based on these observations, we hypothesized that beyond merely reducing diffusion distance and transit time, recruitment of the pathway enzymes, particularly Ino1 and MIOX, to the synthetic scaffold could result in increased effective concentrations of the *myo*-inositol substrate. This, in turn, could lead to increased MIOX activity and improved D-glucaric acid production. In the current work, we report on further investigation of the effectiveness of these modular scaffolds to improve D-glucaric acid titers. We first examined, more fully, the effects of recruiting only Ino1 and MIOX to the scaffold that were previously reported (Dueber et al., 2009) in order to determine whether any impact on MIOX activity was observed. We next created synthetic scaffolds to co-localize all three enzymes on constructs that allowed the independent manipulation of scaffold and enzyme concentration. Finally, we varied the number of interaction domains targeting Ino1 and MIOX to modulate the effective concentration of *myo*-inositol at the synthetic complex and to improve glucaric acid titers.

2. Materials and methods

2.1. E. coli strains, plasmids and scaffold construction

E. coli strains and plasmids used in this study are listed in Table 1. All molecular biology manipulations were carried out according to standard practices (Sambrook and Russell, 2001).

Scaffold devices consisting of GBD, SH3 and PDZ protein interaction domains (Dueber et al., 2009) were assembled using the BglBrick strategy with BamHI and BglII cohesive ends compatible for ligation (doi: 1721.1/46747). Basic parts were made such that they would be flanked on the 5′ end by a BglII site and on the 3′ end by BamHI and XhoI sites. Composite parts were then constructed by digesting the backbone vector with BamHI and XhoI, and a 3′ part was added as a BglII/XhoI-digested insert. The resultant parts could then be sub-cloned into the pWW306 and pWW308 expression plasmids carrying either a tetracycline-inducible (P_{tet}) or a lactose-inducible promoter (P_{lac}), respectively, upstream of a BglII/XhoI multi-cloning site. Both expression plasmids were constructed as a modification of pSB1A2, a plasmid obtained from the MIT Registry of Standard Biological Parts (http://partsregistry.org/Main_Page).

2.2. Synthesis of degenerate versions of SH3 domain

To build robust constructs with more than four repeats of the interaction domains within the scaffold sequences, it was necessary to make degenerate versions of the SH3 domain. The sequence of the SH3 domain of mouse protein Crk (residues 134–191) was jumbled, optimized for *E. coli*, and diagnostic restriction enzyme sites incorporated using the online tool *Gene Design* at http://baderlab.bme.jhu.edu/gd/. This was done iteratively with some codon changes made by eye with alignment analysis in an attempt to maximize degeneracy of the five

Table 1
E. coli strains and plasmids. All production strains were made by transforming BL21 Star™ (DE3) (F⁻ *ompT* *hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm rne*131 (DE3), Invitrogen Corporation, Carlsbad, CA) with pJD727 (Ino1, MIOX, and Udh under *lac* promoter control; p15A origin) and the scaffold plasmid as indicated.

Strains	Scaffold plasmid[a]	No. of GBD domain (for Udh)	No. of SH3 domain (for Ino1)	No. of PDZ domain (for MIOX)
JT1	pJD757	1	1	1
JT2	pJD758	1	1	2
JT3	pJD759	1	1	4
JT4	pJD760	1	2	1
JT5	pJD761	1	2	2
JT6	pJD762	1	2	4
JT7	pJD763	1	4	1
JT8	pJD764	1	4	2
JT9	pJD765	1	4	4
JT10	pWW306[b]	0	0	0
JTK1	pJD788[c]	1	6	2
JTK2	pJD789[c]	1	8	2
JTK3	pJD790[c]	1	3	2
JTK4	pJD791[c]	1	2	2
JTK5	pJD824	1	0	2
JTK6	pJD825[c]	1	4	2

[a] Scaffold plasmids are under P_{tet} control and contain ColE1 origin.
[b] pWW306 is a control plasmid containing no scaffold.
[c] These plasmids contain degenerate sequences for SH3 domains.