Use of Modular, Synthetic Scaffolds for Improved Production of Glucaric Acid in Engineered *E. coli*

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Improved Production of Glucaric Acid with Synthetic Scaffolds

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.

Keywords: glucaric acid; synthetic biology; metabolic pathway engineering; scaffold; modularity; colocalization.

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; Singh and Gupta, 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 nonselective and expensive process using nitric 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 *Escherichia 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. Escherichia coli strains, plasmids and scaffold construction

Escherichia 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 additional coding versions of the Crk SH3 domain. *Gene Design* returned oligonucleotide sequences that could be PCR assembled to produce the desired SH3-encoding product. These were made into basic BglBrick parts as described above.

2.3. Culture and analysis conditions for D-glucaric acid production

Cultures were grown in LB medium supplemented with 10 g/L D-glucose (or without D-glucose) and induced at the exponential phase as indicated in the Results

(IPTG = 0.025 to 0.2 mM; aTc = 27 to 215 nM). An inoculum was prepared in LB medium, and 1 % (v/v) was used to inoculate 250-mL baffled flasks containing 50 mL of medium. The cultures were incubated at 30°C and 250 rpm for 2 days, and then D-glucaric acid titer was analyzed using HPLC as described previously (Moon et al., 2009a).

2.4. Assay for activity of MIOX, Ino1, and Udh

Assays for MIOX activity were performed using lysates as described previously (Moon et al., 2009b). Briefly, lysates were prepared by suspending cell pellets in sodium phosphate buffer (50 mM, pH 8.0) with 1 mg/mL lysozyme and EDTA-free protease inhibitor cocktail tablets (Roche Applied Science, Indianapolis, IN) and then by sonication, followed by centrifugation to remove insolubles. The total protein concentration of lysates was determined using the Bradford method (Bradford, 1976). The MIOX activity was measured by monitoring D-glucuronic acid produced when excess (60 mM) *myo*-inositol was incubated with lysates. For the determination of D-glucuronic acid produced, the reaction mixture also contained excess NAD^+ and purified Udh (Yoon et al., 2009), stoichiometrically generating NADH which can be determined by absorbance at 340 nm. Control reactions were established without *myo*-inositol to account for background. Assays for Ino1 and Udh activity were also performed using lysates as described previously (Moon et al., 2009a).

2.5. Western Blots

Lysates samples were separated by SDS-PAGE and transferred onto a nitrocellulose blotting membrane (Pall Life Sciences, Port Washington, NY) according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Anti-MIOX antibody was used as described by the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA), and immuno-detection was performed using anti-goat IgG-HRP and Western Blotting Luminal Reagent (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's instructions.

3. Results

3.1. The effect of Ino1 and MIOX co-localization on D-glucaric acid titer and MIOX activity

Previous studies on the production of D-glucaric acid showed that the MIOX-catalyzed step is limiting and that MIOX activity is strongly influenced by the concentration of *myo*-inositol, its substrate (Moon et al., 2009a). We previously designed a simple scaffold that co-recruits Ino1 and MIOX in a 1:1 ratio and showed that D-glucaric acid titers were improved by 200% (Dueber et al., 2009). To determine whether this titer improvement was accompanied by activity changes in the most limiting step, we measured MIOX activity in lysate samples. Activity in the scaffolded system was 19.0±0.9 nmol/min/mg, more than 25% higher than the scaffold-free system activity of 15.0±1.3 nmol/min/mg ($p=0.013$). Given this result, we inquired whether increased MIOX activities could be observed by changing the scaffold architecture.

3.2. Constructing various synthetic scaffold devices

The previous results suggested that MIOX activity was indeed enhanced by co-localization, but that system was limited by the inability to independently control scaffold and enzyme expression levels. Additionally, only one scaffold architecture of a 1:1 recruiting domain ratio was tested, which may not be optimal for maximizing the local *myo*-inositol concentration. Here, we took advantage of the modular design of the synthetic scaffolds to vary the number of interaction domains of Ino1 and MIOX for control over relative enzyme stoichiometry. In particular, our hypothesis predicts improved titers to be achievable for architectures with higher numbers of Ino1-recruiting Src homology 3 (SH3) domains on the scaffold that would produce higher concentrations of *myo*-inositol at the complex. The increased numbers of SH3 domains should result in increased MIOX activation with a concomitant increase in D-glucaric acid production. However, if increasing the number of MIOX-recruiting PSD95/DlgA/Zo-1 (PDZ) domains results in substantial titer improvement, the scaffold effect is more likely due to shorter transit time of substrates and higher effective concentrations of MIOX, balancing the overall flux. Of course, these two mechanisms are not mutually exclusive.

For independent expression control of the glucaric acid synthetic pathway and the scaffolds, we used orthogonal IPTG-inducible (P_{lac}) and tetracycline-inducible (P_{tet}) promoters, respectively, to drive expression. We used a two plasmid expression platform: the enzymes for biosynthesis of glucaric acid were cloned into a p15A-based medium copy plasmid, and the scaffolds were cloned into a ColE1-based high copy plasmid (Table 1). Scaffolds were constructed by assembling three protein-protein

interaction domains linked together by nine-residue glycine-serine linkers predicted to be flexible and unstructured. The GTPase binding domain (GBD), SH3, and PDZ domains were used to target Udh, Ino1, and MIOX, respectively. As the first test, a matrix of nine constructs was designed: $GBD_aSH3_cPDZ_b$ where a=1 and b and c are 1, 2, or 4 (Table 1). Because Udh activity was more than 2 orders of magnitude higher than that of Ino1 and MIOX (Moon et al., 2009a), all the scaffolds were designed to contain only one GBD domain (a=1).

To determine the most favorable expression levels, D-glucaric acid production by $GBD_1SH3_4PDZ_4$ was tested at different aTc and IPTG concentrations (Fig. 1). The highest titers were observed with IPTG and aTc concentrations of 0.05 mM and 108 nM, respectively, which led to ~5-fold titer improvement (compared to scaffold-free control). It is well-known that over-expression of recombinant enzymes can have a deleterious effect on cell growth in an unpredictable manner and that, ultimately, expression levels that are too high actually lead to decreases in productivity (Bentley et al., 1990; Birnbaum and Bailey, 1991; Jones et al., 2000; Kane and Hartley, 1988; Moon et al., 2009a; Tyo et al., 2009). This metabolic burden effect may be the reason that productivity decreases with increasing IPTG concentration. However, an expression level that is too low might reduce the encounter frequency between scaffolds and enzymes, diluting the pathway enzymes on the scaffolds and sequestering enzymes from their substrates. The sequestering effect (Burack and Shaw, 2000; Levchenko et al., 2000) is especially probable when the scaffold expression level is high (215 nM aTc).

3.3. Testing various synthetic scaffold devices and demonstrating that increased MIOX activity plays an important role in titer improvement

Using the previously identified IPTG concentration of 0.05 mM, we tested the matrix of scaffold constructs JT1 to JT9 at several scaffold inducer concentrations (215, 108, and 54 nM aTc) (Fig. 2). The results indicate that D-glucaric acid titer primarily depends on the number of Ino1-recruiting SH3 domains, not on the number of MIOX-recruiting PDZ domains. This observation supports the hypothesis that enhancement of MIOX activation by the *myo*-inositol substrate is responsible for the majority of the scaffolding's beneficial effect, since recruiting additional Ino1 enzymes should increase the local *myo*-inositol concentration while leaving the number of localized MIOX enzymes unchanged. The highest titers were achieved at moderate scaffold induction levels, consistent with the expectation that too high of a scaffold to enzyme ratio would result in a small number of recruited enzymes per scaffold molecule, resulting in small titer improvement with increasing number of SH3 domains targeting Ino1.

Given that the number of PDZ domains targeting MIOX does not affect the titer considerably, we constructed additional scaffolds where the number of PDZ domains was held constant at two and the number of SH3 domains was varied up to 8 (Fig. 3). Because the probability of recombination increases as the number of identical repeat sequences is increased, we built this new class of scaffolds with degenerate coding sequences for the SH3 domain with consideration of *E. coli* preferred codon usage. To verify that it is the interaction activities of the domains and not the codon usage that determines the scaffolding effect, two different constructs for $GBD_1SH3_2PDZ_2$ and

$GBD_1SH3_4PDZ_2$ were compared: one with the same SH3 sequences (JT5 and JT8, respectively), and the other with the degenerate sequences (JTK4 and JTK6, respectively). There were no statistically significant differences in the titers observed between constructs with identical and degenerate nucleotide sequences (Table 2). Increasing the number of SH3 domains past four repeats was not accompanied by an increase in titer (Fig. 3). In fact, there was a decrease in titers at the higher concentrations of scaffold inducer (81 and 108 nM aTc). There are several potential explanations for this decrease: 1. Sequestering effects, where the enzymes bind separate scaffold molecules and leave many unoccupied sites, are expected to become more prevalent with an increasing number of SH3 domains. 2. The recruited enzymes (e.g., Ino1 and MIOX) may become oriented in less efficient ways with increasing domain number. 3. Longer scaffolds with higher domain numbers may exhibit reduced stability and/or solubility. Any sequestering effect could potentially be overcome by an increase in enzyme levels via the concentration of the inducer IPTG. To investigate this possibility, we tested D-glucaric acid production by $GBD_1SH3_cPDZ_2$ where c is 3, 6, and 8 at four different IPTG concentrations (Fig. 4). Higher IPTG concentrations did not lead to titer enhancement, suggesting that metabolic burden effects are affecting productivity. We should also note that in this system, the synthetic pathway is consuming glucose-6-phosphate, the entry molecule for carbon flux into endogenous metabolism. Thus, in addition to burden specifically associated with recombinant protein expression, negative effects on productivity may result from variations in the flux distribution at this node that result from competition between the glycolysis and pentose phosphate pathways and glucaric acid production. No clear trends were evident from an examination of the cell densities

(represented as optical density at 600 nm) from the various scaffold architectures and induction levels (Supplementary Tables S1-S3). However, in several cases, the highest titers did correspond to the highest specific productivities.

For direct evidence that the scaffolds affect D-glucaric acid titer by improving MIOX activation, we measured MIOX activity from multiple systems with various scaffold architectures and compared the specific activities with the corresponding titers (Fig. 5). Given that (1) the D-glucaric acid titer is proportional to MIOX activity and (2) MIOX activity depends on the effective number of Ino1 molecules colocalized, we hypothesized that titer improvement by scaffolds in our systems is strongly affected by MIOX activation, most likely as the result of increased local *myo*-inositol concentration. However, an alternative explanation is that the scaffolds inherently alter the stability and/or activity of the MIOX enzyme and this effect is independent of substrate concentration. To test this hypothesis, we first examined relative MIOX protein levels using Western blots with anti-MIOX antibodies, with protein levels normalized to a sample with non-scaffolded proteins (scaffold architecture 0:0:0) (Fig. 6). The results indicate that there is a difference observed in the relative concentrations of MIOX present in scaffolded systems; however, this difference is not consistent with MIOX recruitment. For example, in the top panel, MIOX levels are generally decreased relative to the non-scaffolded control, while in the bottom panel, the protein levels are generally increased. Recall, however, that the high titers correlated with MIOX activities were observed when Ino1 was recruited to the scaffold via the SH3 interaction domain. A comparison of MIOX protein levels between samples with and without Ino1 recruitment (i.e., with and

without SH3 domains) indicates a general *decrease* in enzyme levels with Ino1. Therefore, the increase in MIOX activity is not the result of higher protein levels in this system as the result of increased stability. Next, we investigated the dependence on substrate. The *myo*-inositol in this system is produced from glucose. Thus, if the scaffold effect is indeed independent of metabolite concentration, then the MIOX activity should be independent of glucose presence. In all scaffold architectures examined, the MIOX activity was low in the absence of glucose, and there are no significant differences in activity with non-scaffolded and scaffolded MIOX (Fig. 7 – compare, for example, scaffolds 0:0:0 and 1:6:2 in the absence of glucose). Likewise, in all cases, activity was improved with the addition of glucose. However, this improvement was moderate when Ino1 was not recruited to the scaffold (architectures 0:0:0 and 1:0:2), but increased by 18-fold to 49-fold when the enzyme was recruited. No similar effects on activity were observed for Ino1 and Udh (Supplementary Figures S1 and S2). These results indicate that the improvement in MIOX activity is dependent on *both* the scaffold *and* substrate being present, supporting our hypothesis that MIOX is activated in a substrate-dependent manner, improved by co-localization of Ino1 to the scaffold.

4. Discussion

Enzymatic activities of engineered metabolic pathways must be balanced to achieve high titers, especially when these enzymes are heterologous to the production host. As a result, this has been an area of intense focus where enzyme expression levels are controlled or enzyme activities are improved via directed evolution (Alper et al.,

2005; Bloom et al., 2005; Pfleger et al., 2006; Pitera et al., 2007; Stephanopoulos, 1999). Impressive successes have been achieved for the *in vivo* production of multiple targets (Atsumi et al., 2008; Menzella et al., 2005; Nakamura and Whited, 2003; Ro et al., 2006); however, a great need exists for additional strategies that are orthogonal, but additive, to achieve further gains in production yields if metabolic engineering is to prove viable for a wider spectrum of compounds, especially bulk compounds. One such strategy may be the use of synthetic scaffolds to co-target metabolic enzymes to the same complex to increase the effective concentration of each pathway component. Recently, scaffolding was shown to be highly effective for improving titers of the mevalonate pathway while simultaneously lowering the enzyme expression levels required to achieve these titers (Dueber et al., 2009). As a result, the production cells grew considerably faster than the non-scaffolded pathway expressed to levels required to achieve comparable titers. In the present study, the modular architecture was utilized for the glucaric acid pathway to optimize the effective concentration of the intermediate *myo*-inositol since the bottleneck enzyme MIOX had been observed to be more active when produced in the presence of *myo*-inositol (Moon et al., 2009a).

It was reported that MIOX harbors a coupled dinuclear iron cluster which is perturbed by *myo*-inositol binding (Brown et al., 2006; Xing et al., 2006c). Interestingly, MIOX with the mixed-valent (II/III) diiron cluster is the catalytically active form, instead of MIOX (II/II) or MIOX (III/III) (Xing et al., 2006a). It was suggested that *myo*-inositol binding conditions the diiron cluster for activation of oxygen, the other substrate of MIOX (Xing et al., 2006a; Xing et al., 2006b; Xing et al., 2006c). The authors showed

that in the presence of saturating *myo*-inositol and limiting oxygen, MIOX (II/II) with *myo*-inositol bound (MIOX (II/II)•MI) was converted into MIOX (II/III)•MI as a stable product with a low yield of D-glucuronic acid; in contrast, MIOX (II/III) with *myo*-inositol bound (MIOX (II/III)•MI) reacted with limiting oxygen to stoichiometrically generate D-glucuronic acid with regeneration of MIOX (II/III)•MI. Given those findings and our results, we speculate that the increased local *myo*-inositol concentration near the scaffolded enzymes leads to an increase in the fraction of MIOX in the active (II/III) state, followed by activation of oxygen by MIOX (II/III)•MI and conversion of *myo*-inositol to D-glucuronic acid, with concomitant titer improvement. We also previously observed a dependence on oxidation state in non-scaffolded cultures, where D-glucaric acid titers were sensitive to aeration in shake flasks (Moon et al., 2009a).

Although our synthetic pathway only requires three heterologous enzymes, an endogenous phosphatase is required to de-phosphorylate *myo*-inositol-1-phosphate, the product of the Ino1 enzyme (Moon et al., 2009a). The exact enzyme that performs this function *in vivo* is unknown, although the protein product of the *suh*B gene has been shown to possess activity against *myo*-inositol-1-phosphate (Matsuhisa et al., 1995). We have over-expressed the *suh*B gene in an attempt to improve glucaric acid productivity; however, the titers have always *decreased* in response to *suh*B (data not shown). For this reason, we decided to focus on the heterologous enzymes for targeting to the scaffold. The resulting improvements in MIOX activity could mean that phosphatase activity remains sufficiently high and proximal to the scaffolds to produce *myo*-inositol. Alternatively, the activation effect that we propose may also be mediated by *myo*-

inositol-1-phosphate. As indicated in Fig. 7 and Supplementary Figure S2, MIOX activity still remains significantly below the most active enzyme, Udh. Understanding the proper role of the phosphatase and altering its activity might provide additional enhancements in MIOX activity and glucaric acid titer.

We were unable to achieve as large an improvement in glucaric acid titers as previously achieved for mevalonate biosynthesis (77-fold). Multiple reasons for this difference, as well as potential challenges of generalizing this scaffold strategy towards other systems, were well reviewed by DeLisa and Conrado (DeLisa and Conrado, 2009). Understanding the limitations of the scaffold device is indeed important for maximizing its utility; therefore, we point out several significant differences between the mevalonate system and the synthetic glucaric acid pathway. Firstly, the bottleneck enzymatic step in the mevalonate biosynthetic pathway results in an accumulation of a toxic intermediate (HMG-CoA). Thus, testing the matrix of scaffolds for improved titers was equivalent to screening for improved flux of this bottleneck step and reduction of toxicity to the *E. coli* production host. Unfortunately, these simultaneous effects cannot be easily deconvoluted. For the glucaric acid production pathway, the bottleneck is MIOX activity that is improved with increased substrate concentrations, but there is no indication of intermediate toxicity. Consequently, the scaffolds that worked best were the ones predicted to increase the effective concentration of *myo*-inositol at the resultant complexes and not necessarily those that formed complexes with structures resulting in improved, balanced fluxes of each enzymatic step. Secondly, in the glucaric acid system, the increase in activity of the rate-limiting MIOX enzyme as the result of co-localization

with Ino1 affects both the characteristic diffusion and characteristic reaction times, whereas co-recruitment of the enzymes in the mevalonate pathway should not affect the underlying kinetics. Again, because of the nature of the enhancement, we cannot deconvolute these two effects. Lastly, it should also be emphasized that we do not have the ability to predict the structural properties of the scaffolded complexes. These are likely to be complicated by the oligomerization state of the pathway enzymes. For example, a tetrameric enzyme will contain four scaffold-recruiting peptide ligands. Thus, the final scaffolded complex could be quite large and the optimal mevalonate biosynthetic complex is likely to differ considerably from the optimal glucaric acid biosynthetic complex. Given these factors, it is difficult to predict the performance of the scaffolds when applied to a new pathway. However, in the absence of such factors, we would expect the scaffolds to be most useful in diffusion-limited systems. Indeed, the mevalonate pathway intermediates are significantly larger than those of the glucaric acid pathway and should thus be expected to suffer from relativity lower diffusivity. Additionally, the baseline titers achieved for glucaric acid production were significantly higher, leaving much less room for improvement.

In this study, we were able to treat metabolic enzymes as modular parts that can be combined in novel combinations to produce the high-value product glucaric acid. Similarly, we were able to take advantage of the functional and physical modularity of the metazoan protein-protein interaction domains to build synthetic scaffolds for the directed purpose of forming a synthetic complex with increased local concentrations of intermediate *myo*-inositol. Further, we were able to change the relative product titers

depending on the number of enzymes producing *myo*-inositol expected to be recruited to the complex, consistent with a titration in this local metabolite concentration. In this manner, we used modularity as an engineering strategy similar to how it has facilitated evolution of new signaling connections in living cells (Pawson and Nash, 2003). We observed maximum titers of ~2.5 g/L glucaric acid. This value represents a 5-fold improvement over the non-scaffolded enzymes expressed from the same vectors, and an approximately 50% improvement of our previously highest reported titers (Dueber et al., 2009). While reasonable titers for production scale are currently unknown, a recent review indicates that production titers for organic acids range from 29 g/L to 771 g/L (Sauer et al., 2008). Thus, additional work is necessary to improve productivity of glucaric acid, including scale-up and process engineering. Nevertheless, these modular strategies should prove to be generalizable towards other engineered pathways for improvement of production yields in addition to the gains made by conventional strategies.

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Table 1. *E. coli* strains and plasmids. All production strains were made by transforming BL21 Star™ (DE3) (F^- *ompT* *hsd*S_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.

Table 2. Comparison of titers achieved using scaffolds with the same SH3 sequences (JT5 and JT8) versus scaffolds with degenerate coding sequences (JTK4 and JTK6), with 0.05 mM IPTG. Numbers are the averages ± standard deviations, in g/L.

aTc (nM)	**JT5 ($GBD_1SH3_2PDZ_2$)**	**JTK4 ($GBD_1SH3_2PDZ_2$)**	**JT8 ($GBD_1SH3_4PDZ_2$)**	**JTK6 ($GBD_1SH3_4PDZ_2$)**
108	1.43±0.01	1.22±0.31	2.27±0.23	2.28±0.35
81	1.21±0.28	1.20±0.02	Not measured	Not measured
54	1.31±0.12	1.21±0.12	2.20±0.08	2.37±0.30
27	0.89±0.10	1.02±0.13	Not measured	Not measured

Figure Captions

Fig. 1. Effect of induction levels (IPTG for pathway enzyme expression; aTc for scaffold expression) on the production of D-glucaric acid using the scaffold $GBD_1SH3_4PDZ_4$ (JT9). For the 0.025 mM IPTG/215 nM aTc condition, no experiment was performed. Data are the averages of three replicates and the standard deviations are not higher than 23% of the averages.

Fig. 2. Effect of various scaffold architectures (constructs JT1 to JT9) on the production of D-glucaric acid at 0.05 mM IPTG and three different aTc concentrations as indicated. Δ = 54 nM aTc; ■ = 108 nM aTc; ◊ = 215 nM aTc.

Fig. 3. Effect of the number of Ino1-recruiting SH3 domains on D-glucaric acid titer with 0.05 mM IPTG in $GBD_1SH3_cPDZ_2$, where c is 0, 1, 2, 3, 4, 6, and 8. Experiments were performed at four different aTc concentrations as indicated. The scaffolds with degenerate coding sequences were used for c = 2, 3, 4, 6, and 8. ● = 27 nM aTc; Δ = 54 nM aTc; ■ = 81 nM aTc; ◊ = 108 nM aTc.

Fig. 4. Effect of IPTG concentration on D-glucaric acid titer at 54 nM aTc in $GBD_1SH3_cPDZ_2$, where c is 0, 3, 6, and 8. Experiments were performed at four different inducer concentrations: from left to right, 0.05, 0.075, 0.1, and 0.2 mM IPTG. Data are the averages and standard deviations of two replicates.

Fig. 5. Correlation between D-glucaric acid titer and MIOX activity across various scaffold architectures. Data are from JT1-10 at 0.05 mM IPTG, and 54 or 215 nM aTc; and JTK1-4, JT5, and JT10 at 0.05 mM IPTG and 108, 81, 54, or 27 nM aTc. Data points represent the average values of MIOX activity and D-glucaric acid titer. The standard deviations are not higher than 57% (MIOX activity) and 37% (D-glucaric acid titer) of the averages.

Fig. 6. Determination of relative MIOX enzyme levels for various scaffold architectures and induction levels as indicated. The effect of glucose addition is also examined. Relative protein levels, as determined by relative intensities, are normalized to MIOX levels in the non-scaffolded control (far right, scaffold architecture 0:0:0).

Fig. 7. MIOX activity as a function of scaffold architecture and glucose addition. Glucose presence ("Yes") or absence ("No") is indicated for each sample. Data are from JT5, JT9, and JT10 (at 108 nM aTc and 0.05 mM IPTG) and JTK1 and JTK5 (at 54 nM aTc and 0.05 mM IPTG).