Project Title: Synthetic metabolic channels for improving microbial production of 1,2,4-butanetriol

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A. Scientific and Technical Objectives

The long-term objective of our proposed studies is the development of a broad-spectrum platform for engineering complexes of metabolic enzymes in bacteria that is expected to dramatically improve the yield of virtually any biosynthetic target produced in microorganisms by accounting for enzyme organization and minimizing metabolic cross-talk. Related studies are currently funded under ONR YIP grant # N000140610565 and focus on the production of 1,2-propylene glycol (1,2-PG). The objective of this particular application is to improve microbial synthesis of D-1,2,4-butanetriol (D-BT), a precursor to the energetic material D-butanetriol trinitrate (D-BTTN), using our technology for engineering metabolic complexes in living cells. We have proposed the following specific aims:

Specific Aim 1: Co-localization of D-BT enzymes into functional metabolic complexes. Under this aim, we will assemble the D-BT pathway enzymes into functional complexes using covalent and non-covalent methods of assembly/crosslinking developed here and in conjunction with studies under N000140610565. We will also explore the use of computational tools as a means to design protein-protein interactions *de novo*.

Specific Aim 2: Enable combinatorial engineering of metabolic complexes via metabolite sensors. We will engineer a collection of protein-based switches that are capable of dynamically responding to our desired end-product D-BT over a broad concentration range. Such sensors will enable fine-tuning (e.g., laboratory evolution) of our engineered D-BT channels.

B. Approach

Our approach to improving D-BT biosynthesis is to sequentially tether enzymes together to form synthetic metabolic complexes inside living bacterial cells. The rationale for building synthetic complexes is that D-BT titers can be increased by accounting for pathway enzyme organization in a manner that (1) eliminates intracellular enzyme/substrate diffusion barriers and thus increases catalytic efficiency by co-localizing enzymes to discrete locations in the cells; (2) enables control over the molecular ratios of pathway enzymes; and (3) minimizes metabolic cross-talk and side reactions by focusing metabolic flux towards the production of D-BT and away from known side products. To accomplish our objective, sequential tethering of D-BT pathway enzymes into either static or dynamic channels will be performed by: (1) construction of fusion proteins (static); (2) post-translational protein assembly using covalent (static) or non-covalent bonds (dynamic); and (3) enzyme co-immobilization to an intracellular scaffold (dynamic). Once we have assembled the D-BT pathway in a complex inside living cells, we will quantify the formation of D-BT as well as all unwanted side products to determine the net effect of different enzyme assembly strategies. While our approach is largely experimental in nature, we will also explore the use of computational studies to design alternative assembly strategies whereby interactions between metabolic enzymes is designed *de novo*. Finally, in parallel to the assembly of the D-BT metabolic enzymes, we will use develop a set of D-BT sensors based on a chemical genetic reporter of protein stability. These reporters will be useful for direct monitoring of intracellular D-BT titers in living cells and are expected to open the door to laboratory evolution of our engineered metabolic complexes.

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C. Concise Accomplishments

The major accomplishments to date include:

- (1) We have assembled a functional D-BT pathway in *E. coli* cells and quantified D-BT accumulation in these cells.
- (2) We have co-localized D-BT pathway enzymes via the creation of enzyme fusions and found that these constructs increase D-BT titers without the need for genetic knockout of competing metabolic reactions.
- (3) We have used a directed evolution strategy to create a fluorescent D-BT biosensor that will be used for optimizing our current and future enzyme assemblies.

D. Expanded Accomplishments

Summary. Engineering microbes is an attractive approach for the biosynthesis of ONR-relevant chemicals including D-1,2,4-butanetriol (D-BT), a precursor to the energetic material D-butanetriol trinitrate (D-BTTN). One significant bottleneck in the biosynthesis of D-BT in *Escherichia coli* results from competing side reactions that divert carbon flux and limit the overall yield. To overcome this, we have applied our expertise in creating synthetic metabolic complexes in the cytoplasm of living *E. coli* cells to control the diffusion of the diverted intermediates. To date, we constructed a recombinant pathway in *E. coli* for D-BT production (section D1) and have subsequently co-localized the D-BT pathway enzymes via protein fusions (section D2). We are now extending our studies to include the creation of non-covalent enzyme assemblies using modular protein and DNA scaffolds for docking individual D-BT pathway enzymes. We have also employed a directed evolution strategy to create a fluorescent D-BT biosensor that can be used to optimize our current and future enzyme assemblies (section D3). We anticipate that engineered enzyme organization will provide a general strategy for optimizing numerous metabolic pathways where metabolic bottlenecks such as diverted carbon flux or toxic intermediates are present.

D1. Creation of a metabolic pathway for D-BT production. Due to difficulties in obtaining the genes for D-BT biosynthesis from Drafts Corporation, the construction of a D-BT pathway was significantly delayed. However, during the past year, we were able to assemble a recombinant D-BT pathway in *E. coli* comprised of the following four enzymes - *C. crescentus* D-xylose dehydrogenase (Xdh), *E. coli* D-xylonate dehydratase (YjhG), *P. putida* benzoylformate decarboxylase (MdlC) and *E. coli* alcohol dehydrogenase (AdhP) (Fig. 1a). Co-expression of these 4 enzymes in wildtype (wt) *E. coli* cells resulted in no measurable D-BT activity (Fig. 1b). However, when we deleted the *xylA* gene, encoding D-xylose isomerase that diverts carbon flux away from D-BT, the D-BT levels reached 0.1 g/L (Fig. 1b). Inactivation of a second competing reaction was accomplished by deletion of the *yjhH* gene, which encodes 2-keto acid aldolase. This resulted in a further increase in D-BT levels to ~0.5 g/L.

D2. Synthetic assemblies for D-BT production enabled by protein fusions. To determine if directed enzyme assembly could be used to increase D-BT titers without genetic deletion of competing pathways, we created a variety of enzyme fusions between the 4 D-BT pathway enzymes and tested these in different *E. coli* host strains. None of the fusion constructs were able to increase D-BT levels when expressed in wt *E. coli* cells (data not shown), indicating that elimination of D-xylose isomerase was essential for making D-BT in *E. coli*. However, fusions between the second and third or the third and fourth D-BT pathway enzymes was able to

increase D-BT titers in $\Delta xyIA$ *E. coli* without deleting *yjhH* (Fig. 1b), suggesting that the increase in metabolic efficiency conferred by the fusion constructs was sufficient to overcome the diverted flux created by the 2-keto acid aldolase. This result has important consequences when it comes to optimizing metabolic efficiency in engineered *E. coli* cells especially in cases where the competing reactions are not known and thus genetic deletion is not a viable strategy.

Figure 1. (a) Recombinant pathway for converting D-xylose to D-BT in *E. coli*. (b) D-BT titers produced by wildtype (wt) *E. coli*, $\Delta xyIA$ *E. coli* or $\Delta xyIA \Delta yjhH$ *E. coli*. First three lanes depict cells co-expressing unfused D-BT pathway enzymes. Last four lanes depict D-BT titers from cells expressing fusions between the second and third enzymes in different orientations (2-3 or 3-2) or the third and fourth enzymes in different orientations (3-4 or 4-3). Error bars represent the standard error of the mean from three replicate experiments.

D3. Directed evolution of a fluorescent D-BT biosensor. Since there are currently no generic reporters for intracellular metabolites, we sought to develop a tool for sensing such compounds. Previously, we developed a protein conformational switch comprised of an unstable domain and a reporter protein (Fig. 2). The unstable domain was selected such that introduction of a small molecule ligand that stabilizes the domain would restore stability of the entire fusion and thus lead to measurable activity of the reporter protein. The reporter for our switch was chosen as the green fluorescent protein (GFP) so that upon introduction of small molecules that stabilize the unstable domain, a large increase in cell fluorescence would result (Fig. 3a). For the unstable domain, we chose the TraR transcriptional activator from *Agrobacterium tumefaciens* (Fig. 3a). In the absence of its natural ligand, the freely diffusible quorum signaling molecule 3-oxooctanoyl-L-homoserine lactone (OHHL), the TraR protein is a monomer that is highly unstable in the cytoplasm of *Agrobacterium tumefaciens* and *E. coli* (1). However, upon binding of OHHL, TraR forms an extremely stable dimer (2). We have observed this same OHHL-dependent stability with an engineered TraR-GFP fusion protein. That is, in the absence of OHHL, TraR-GFP is highly unstable and cells expressing the fusion are relatively non-fluorescent (Fig. 3b). However, upon addition of OHHL, the TraR-GFP protein is stabilized (presumably in a dimeric conformation) and the cells become highly fluorescent in an OHHL dose-dependent fashion (Fig. 3b).

Figure 2. Chemical genetic control of protein stability.

Figure 3. (a) TraR-GFP fusion as a reporter of small molecules in the cytoplasm of *E. coli* cells. (b) Dose-dependent response of the TraR-GFP-expressing *E. coli* to various concentrations of OHHL added exogenously to the growth medium. Cells were grown in 96-well plates and assayed using a fluorescent plate reader.

During the last year, we have begun engineering the GFP-TraR conformational switch for sensing molecules other than OHHL. Our targets of interest are propanediol (1,2-PD) and D-BT. To evolve the substrate specificity of TraR, we created a combinatorial gene library where mutations were generated randomly to active site residues (Fig. 4a) via an NNK degenerate primer strategy. DNA libraries containing $\sim 10^8$ members were transformed in *E. coli* carrying enzymes for 1,2-PD or D-BT biosynthesis resulting in cell libraries of $\sim 10^6$ members (Fig. 4b). Cells were screened via FACS to identify variants that could bind to either 1,2-PD or D-BT and become fluorescent. In the case of D-BT biosynthesis, 15 clones were identified as putative hits and are now undergoing further characterization. We expect that this will be a general strategy for creating a collection of small molecule switches based on (1) the GFP-TraR backbone and (2) the application of protein design and/or laboratory evolution to change the substrate specificity of TraR from OHHL to other compounds of interest.

Figure 4. (a) The TraR OHHL binding pocket. (b) Directed evolution strategy for engineering TraR substrate specificity.

E. Work Plan

The workplan for the short remainder of this grant will focus on the following key issues:

(1) Exploration of alternative experimental strategies for enzyme assembly to the D-BT enzymes in a manner identical to our metabolic engineering work on 1,2-propanediol (see ONR YIP grant # N000140610565). We will explore several different methods for the assembly of these enzymes. This will include the use of “protein interacting domains” whereby dimerization domains from eukaryotic proteins (e.g., jun/fos) will be fused onto each pathway enzyme, thereby creating artificial interaction domains that will catalyze non-covalent enzyme assembly. This will allow us to explore the extent to which the nature of the assembly (i.e., covalent bonds vs. non-covalent bonds) affects the efficiency of the metabolic conversion (i.e., D-BT production). We will initially focus on the assembly of just two enzymes, namely Pp-MdIC and Ec-AdhP, because these two enzymes are at the crucial branch point where side reactions occur and because these enzymes gave the highest titers when directly fused to each other. If successful, we will then explore the assembly of three- and four-enzyme assemblies. Time permitting, this will potentially expand to include testing of protein and/or plasmid DNA scaffolds (Fig. 5) (3).

Figure 5. Synthetic protein scaffolds for co-localization of D-BT pathway enzymes. Binding domains from eukaryotic signaling proteins (SH3, PDZ and GBD) along with their corresponding peptide ligands can be used for assembling pathway enzymes. Variables such as orientation, spacing, stoichiometry and affinity for the scaffold can all be addressed using this design strategy.

(2) Our results to date have clearly demonstrated that the TraR-GFP fusion has an exquisite ability to be stabilized by the binding of a small molecule ligand and thus “sense” the presence of extremely small compounds. We have also demonstrated a plasticity of the binding pocket to recognize different substrates including D-BT. Moving forward, we will focus on detailed biochemical characterization of the 15 TraR variants isolated thus far. Studies will seek to prove D-BT binding and whether this binding induces a conformational change in the protein. In parallel, we have also begun to develop metabolite sensors based on another flexible scaffold, namely the AraC response regulator proteins (Fig. 6). Studies using the AraC scaffold for sensing D-BT and 1,2-PD in collaboration with Dr. Pat Cirino (Penn State) are underway.

Figure 6. Engineered metabolite sensors based on TraR (left) or AraC (right). In the latter case, AraC undergoes a conformational change following ligand binding that leads to promoter (PBAD) binding. Insertion of the PBAD promoter in front of the *gfp* gene results in strong cell fluorescence in response to AraC binding.

F. Major Problems/Issues. We do not see any major impediments to completing the work plan as outlined above.

G. Technology Transfer. No new technology transfer.

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