Metabolic Engineering 16 (2013) 130–139

Contents lists available at SciVerse ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Regular Article

Expression of the sub-pathways of the *Chloroflexus aurantiacus* 3-hydroxypropionate carbon fixation bicycle in *E. coli*: Toward horizontal transfer of autotrophic growth

Matthew d. Mattozzi [a,b,*], Marika Ziesack [a,b,c], Mathias J. Voges [a,b,d], Pamela A. Silver [a,b], Jeffrey C. Way [a,b]

[a] *Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA 02115, USA*
[b] *Department of Systems Biology, Harvard Medical School, Boston, MA 02115, USA*
[c] *Institute of Pharmacy and Molecular Biotechnology, Ruprecht–Karls-Universität, Heidelberg, Germany*
[d] *Department of Biotechnology, Delft University of Technology, Delft, Netherlands*

ARTICLE INFO

Article history:
Received 24 September 2012
Received in revised form
8 January 2013
Accepted 17 January 2013
Available online 29 January 2013

Keywords:
Metabolic engineering
Carbon fixation
3-hydroxypropionate
Chloroflexus aurantiacus
E. coli
Diaminopimelic acid
Propionate biosensor
Propionate toxicity

ABSTRACT

The 3-hydroxypropionate (3-HPA) bicycle is unique among CO_2-fixing systems in that none of its enzymes appear to be affected by oxygen. Moreover, the bicycle includes a number of enzymes that produce novel intermediates of biotechnological interest, and the CO_2-fixing steps in this pathway are relatively rapid. We expressed portions of the 3-HPA bicycle in a heterologous organism, *E. coli* K12. We subdivided the 3-HPA bicycle into four sub-pathways: (1) synthesis of propionyl-CoA from acetyl-CoA, (2) synthesis of succinate from propionyl-CoA, (3) glyoxylate production and regeneration of acetyl-CoA, and (4) assimilation of glyoxylate and propionyl-CoA to form pyruvate and regenerate acetyl-CoA. We expressed the novel enzymes of the 3-HPA bicycle in operon form and used phenotypic tests for activity. Sub-pathway 1 activated a propionate-specific biosensor. Sub-pathway 2, found in non-CO_2-fixing bacteria, was reassembled in *E. coli* using genes from diverse sources. Sub-pathway 3, operating in reverse, generated succinyl-CoA sufficient to rescue a *sucAD*$^-$ double mutant of its diaminopimelic acid (DAP) auxotrophy. Sub-pathway 4 was able to reduce the toxicity of propionate and allow propionate to contribute to cell biomass in a *prpC*$^-$(2 methylcitrate synthase) mutant strain. These results indicate that all of the sub-pathways of the 3-HPA bicycle can function to some extent *in vivo* in a heterologous organism, as indicated by growth tests. Overexpression of certain enzymes was deleterious to cell growth, and, in particular, expression of MMC-CoA lyase caused a mucoid phenotype. These results have implications for metabolic engineering and for bacterial evolution through horizontal gene transfer.

1. Introduction

There are six different mechanisms for CO_2 assimilation known today (Bar-Even et al., 2011). In the most common, the ubiquitous Calvin–Benson–Bassham cycle, CO_2 reacts with a five-carbon sugar, yielding two carboxylic acids from which sugar is regenerated (Bassham and Calvin, 1962). This cycle operates in plants, algae and cyanobacteria; its key enzyme is ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). Other pathways are the reductive citric acid or Arnon–Buchanan cycle (Buchanan and Arnon, 1990), the reductive acetyl-CoA or Wood–Ljungdahl pathway (Ljungdahl et al., 1965), the dicarboxylate/4-hydroxybutyrate cycle (Huber et al., 2008) and the 3-hydroxypropionate/4-hydroxybutyrate cycle (Berg et al., 2007). All of these alternative pathways include oxygen-inhibited enzymes, whereas in the Calvin cycle, Rubisco catalyzes a wasteful reaction with oxygen, but is not actually inhibited by oxygen. Another alternative CO_2 fixation pathway is the 3-hydroxypropionate (3-HPA) bicycle, which was fully described in 2009 by Fuchs, Zarzycki and colleagues (Holo and Sirevåg 1986; Zarzycki et al., 2009). This bicycle appears not to have reactions that are affected by oxygen. The carboxylases of the 3-HPA bicycle are relatively rapid ($k_{cat} \approx 30$–80/s, Hügler et al., 2003; Menendez et al., 1999; Chuakrut et al., 2003; Ishii et al., 2004) compared to Rubisco ($k_{cat} \approx 1$–10/s, as reviewed in Sage (2001)). They are the acetyl- and propionyl-CoA carboxylases retooled from fatty acid synthesis, and may in fact work faster in this context (Ishii et al., 2004; Zhang et al., 2010). None of the 3-HPA enzymes require exotic cofactors except for methylmalonyl-CoA mutase, which requires Vitamin B_{12} (which is

* Corresponding author at: Wyss Institute for Biologically Inspired Engineering, Harvard University Center for Life Sciences Boston, 3 Blackfan Circle, Room 528-13C, Boston, MA 02115, United States. Fax: +1 617 432 7828.
E-mail addresses: matt.mattozzi@wyss.harvard.edu, matt.mattozzi@gmail.com (M.d. Mattozzi).

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http://dx.doi.org/10.1016/j.ymben.2013.01.005

made by many bacteria but not *E. coli* (Foster et al., 1964; Guest et al., 1964; Roy and Leadlay, 1992; Froese et al., 2009)).

The 3-HPA bicycle is used by the green nonsulfur bacterium *Chloroflexus aurantiacus*. This organism lives commensally with cyanobacteria in dense mats in hot springs (Psencík et al., 2009; Tang et al., 2011). The bicycle is likely optimized for 55 °C, the optimal growth temperature of *Chloroflexus* (Rathnasingh et al., 2011). Inspection of the bicycle (Fig. 1) suggests that it may function both as a self-sufficient CO_2 fixation pathway and a salvage pathway for glycolate and glyoxylate, allowing *C. aurantiacus* to use these products of photorespiration that may be excreted by its cyanobacterial neighbors (Zarzycki and Fuchs, 2011). The oxygenase reaction of Rubisco is favored at high temperatures (e.g. in *Chloroflexus*' native hot spring or on a warming planet), so the 3-HPA bicycle may confer a particular selective advantage due to its oxygen-insensitivity and its ability to absorb glyoxylate from cyanobacteria that are suffering from high levels of photorespiration.

The 3-HPA bicycle is an attractive target for metabolic engineering because of its insensitivity to oxygen, high rate of CO_2 fixation, and production of novel intermediates. The eponymous compound 3-HPA has been explored recently as a bioplastic precursor (Meng et al., 2012; Zhou et al., 2011; Wang et al., 2012a, 2012b), showing promise for engineering the bicycle in *E. coli*. Rational expression of multiple enzymes constituting large *de novo* metabolic pathways is still a significant challenge (Zhang et al., 2011; Keasling, 2010; Dunlop, 2011). We therefore expressed the novel enzymes of the 3-HPA bicycle in *E. coli* as sub-pathways, and determined functional expression through genetic and phenotypic assays. Here, we demonstrate that all the sub-pathways of the *Chloroflexus* carbon fixation bicycle do indeed express heterologously, sufficient to complement host mutations. These then can be engineered further for the synthesis of industrial bioproducts, biofuels, and biopharmaceuticals, perhaps with an artificially autotrophic host cell.

2. Materials and methods

2.1. Media

Cells were grown in Luria–Bertani or M9 minimal medium (Miller, 1972; Sigma-Aldrich) with appropriate antibiotics. Noble agar (1.5–2%) was used for selected nutritional studies to minimize growth on trace carbon sources in the agar. In experiments that assay methylmalonyl-CoA mutase (also termed 'sleeping beauty mutase'), 1 μM Vitamin B_{12} (hydroxocobalamin, Sigma-Aldrich) was added to the medium.

2.2. Strains and plasmids

E. coli K strains were used primarily in this work; the B strain JSB (a BL21 derivative, Lee and Keasling, 2005) was used for the GFP biosensor assay (Fig. 2). Strains and plasmids are listed in Table 1. *E. coli* integrations were performed via P1 transduction (Miller, 1972; Murphy, 1998) or lambda-red integration (Datsenko and Wanner, 2000; Court et al., 2002; Thomason et al., 2007). Plasmids were constructed with traditional cloning

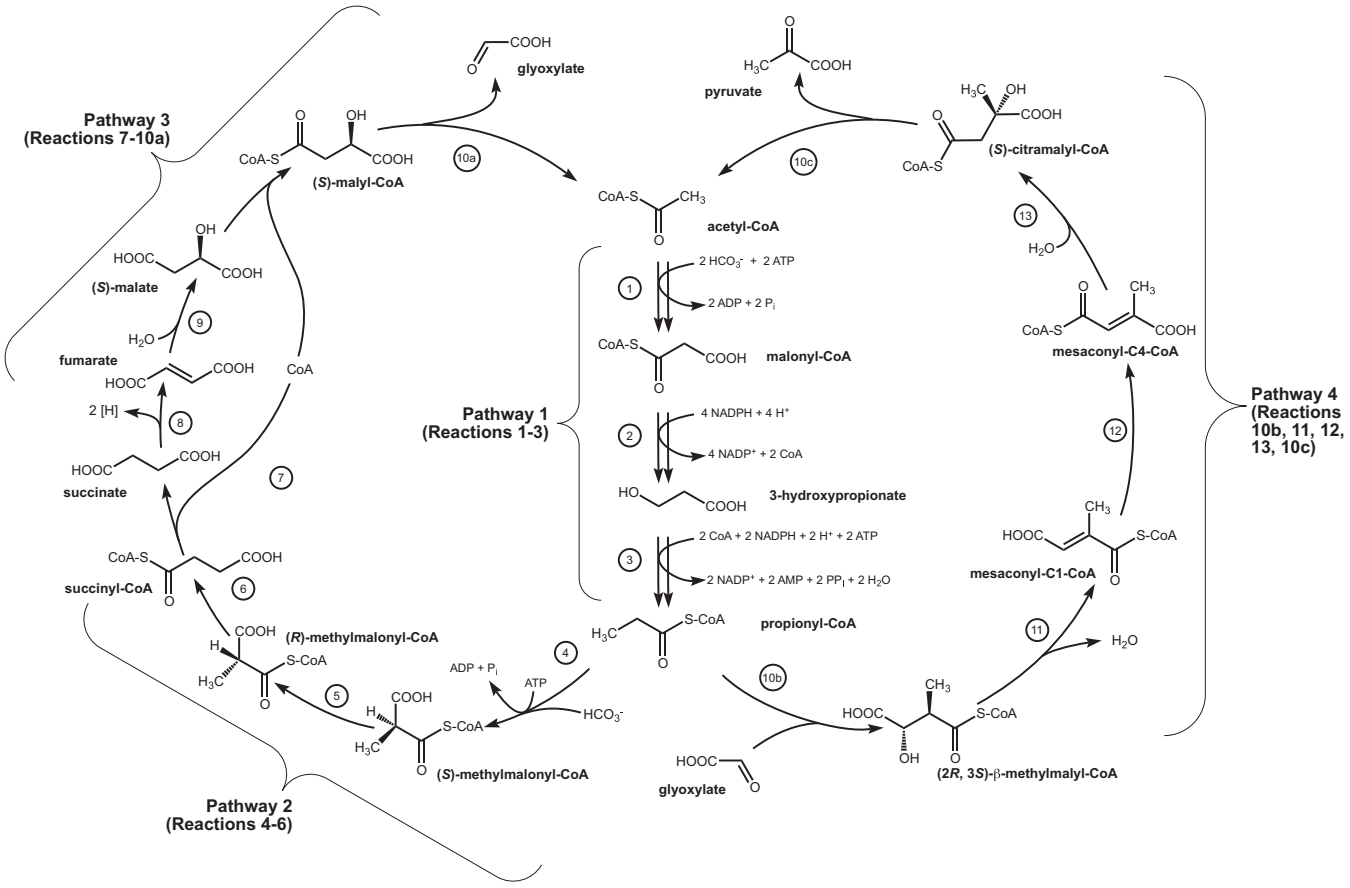

Fig. 1. *Chloroflexus aurantiacus* 3-hydroxypropionate bicycle (redrawn from (Zarzycki et al., 2009)), with the bicycle divided into four functional sub-pathways. Individual enzymatic steps are numbered within circles and described in Section 3.1. In sub-pathway 1, the double arrows indicate that this pathway operates twice per bicycle. Glyoxylate produced from sub-pathway 3 (Step 10a) is consumed at the beginning of sub-pathway 4 (Step 10b).

methods (Sambrook and Russell, 2001), BglBricks (Anderson et al., 2010), or Gibson assembly (Gibson, 2011) using genes codon-optimized for *E. coli* expression (Genscript, Piscataway, NJ).

For the genetic tests done in this study, several *E. coli* multiple knockouts needed to be constructed (Table 1). The vast majority of them were obtained directly from the Keio collection (Baba et al., 2006), with the kanamycin resistance marker removed with pCP20 (Datsenko and Wanner, 2000). Bacteriophage P1 was used successively to transduce multiple kanamycin resistance-marked Keio deletions into the same background. Experiments were generally performed in kanamycin-sensitive backgrounds with plasmids expressing the transgenes.

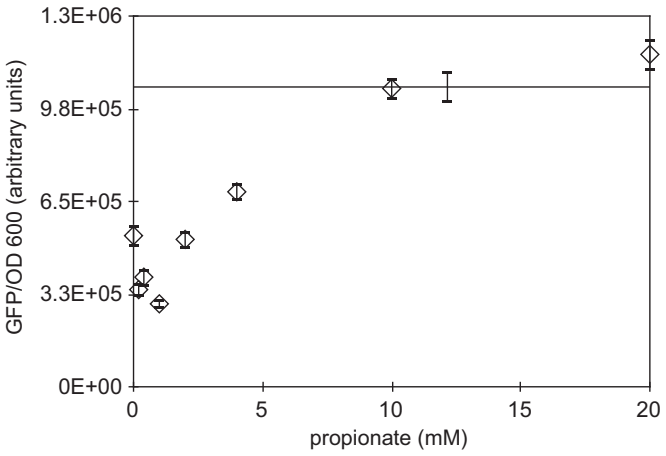

Fig. 2. *E. coli* produces propionyl-CoA from *C. aurantiacus* sub-pathway 1. Diamonds: a standard curve of GFP expression levels versus external propionate concentration for *E. coli* BW25113 containing pPro33-GFP and pCM62-MP. Measurements were performed in triplicate (Section 2). GFP fluorescence units are arbitrary. The fluorescence of cells in which sub-pathway 1 from pCM62-MP is induced (horizontal line with error bar) corresponds to cells without sub-pathway 1 but with a concentration of about 10 ± 0.6 mM propionate in the medium.

2.3. GFP propionate biosensor assay

To demonstrate functional activity of *mcr-pcs*, we employed the propionate-responsive inducible promoter constructed by Lee and Keasling (2005) as a GFP biosensor. In strain *E. coli* JSB (BL21(DE3) Δ*sbm-ygfDGHI*), the competing propionate catabolic pathway, which carboxylates it to methylmalonyl-CoA and succinate, has been knocked out, ensuring all propionate is metabolized via 2-methylcitrate. Plasmids containing the *mcr-pcs* and empty vector were co-transformed via electroporation into *E. coli* JSB with pPro33gfp. The end product of the *mcr-pcs* sub-pathway is propionyl-CoA, which is converted to 2-methylcitrate by host metabolism. 2-Methylcitrate binds to the PrpR regulator encoded by pPro33gfp, activating the promoter P_{prpC} driving GFP expression. Previous work has shown that GFP expression increases with propionate concentration, so we constructed a standard curve using varying propionate concentrations with our empty vector. We measured GFP fluorescence (excitation 395 nm, emission 509 nm, gain automatically determined by the instrument) and optical density at 600 nm using an Infinite plate reader (TECAN, Männedorf, Switzerland) and optically clear 96 well plates (Greiner Bio-One catalog number 655087, Monroe, NC). Three 100 μL cultures grown in M9 0.4% glycerol were measured for

Table 1
Bacterial strains and plasmids.

E. coli strain	Genotype	Source
BW25113	*lacI*^{q}*rrnB*$_{T14}$Δ*lacZ*$_{WJ16}$*hsdR514*Δ*araBAD*$_{AH33}$Δ*rhaBAD*$_{LD78}$	Datsenko and Wanner (2000); Baba et al. (2006)
prpC$^-$	BW25113 Δ*prpC*::*FRT*	Baba et al. (2006)
prpE$^-$	BW25113 Δ*prpE*::*FRT*	Baba et al. (2006)
sucAD$^-$	BW25113 Δ*sucA*::*kanR* Δ*sucD*::*FRT*	This work, Yu et al. (2006)
JSB	BL21(DE3)Δ*sbm-ygfDGHI*	Lee and Keasling (2005)
sbm$^-$	BW25113 Δ*sbm*::*FRT*	Baba et al. (2006)
sbm$^+$	BW25113 CmR-LacP→*sbm-ygfDGHJ*	This work
prpC$^-$*sbm*$^-$	BW25113 Δ*prpC*::*FRT*Δ*sbm*::*FRT*	This work
prpC$^-$*sbm*$^+$	BW25113 Δ*prpC* CmR-LacP→*sbm-ygfDGHJ*	This work
MVB8	BW25113 Δ*aceBAK*::*FRT*, Δ*prpC*::*FRT*, Δ*gcl*::*FRT*	This work
MVB9	BW25113 Δ*aceBAK*, Δ*prpC*, Δ*gcl* CmR-LacP-*sbm-ygfDGHJ*	This work
MVC5	BW25113 Δ*sucA*Δ*sucD*, Δ*prpC*, CmR-LacP-*sbm-ygfDGHJ*	This work
Plasmid	**Relevant features**	
pPro33gfp	*prpR*P$_{prpB}$→GFP	Lee and Keasling (2005)
pYW1200	pET21c with *S. coelicolor pcc-acc*	Zhang et al. (2010)
pUC19	Empty high copy *E. coli* vector	New England Biolabs
pUC57	Empty high copy *E. coli* vector	New England Biolabs
pCM62xt	Empty broad host vector	Marx and Lidstrom (2001); Mattozzi and Keasling (2010)
pCM62-*mcr*	Expresses malonyl-CoA reductase	This work
pCM62-*pcs*	Expresses propionyl-CoA synthase	This work
pCM62-MP	pCM62 with middle sub-pathway (sub-pathway 1)	This work
pUC57-2034	Expresses putative *C. aurantiacus* PCC (Caur2034)	This work
pUC57-3435	Expresses putative *C. aurantiacus* PCC (Caur3435)	This work
pUC19–10–12–13–11	Expresses synthetic operon constituting sub-pathway 4	This work, Weiss et al. (2008)
pUC19–11–12–13	Expresses synthetic operon constituting sub-pathway 4 without Enzyme 10	This work
pUC19–10–12–13	Expresses synthetic operon constituting sub-pathway 4 without Enzyme 11	This work
pUC19–10–11–13	Expresses synthetic operon constituting sub-pathway 4 without Enzyme 12	This work
pUC19–10–11–12	Expresses synthetic operon constituting sub-pathway 4 without Enzyme 13	This work
p19–10only	Expresses only enzyme 10 for assays of sub-pathways 3 or 4	This work
pUC57-7ab	Expresses both genes of Enzyme 7 heterodimer of sub-pathway 3	This work
pUC57-7ab10	Expresses sub-pathway 3	This work
pET21c-PCCACC-MMCE	pYW1200 (*pcc-acc*) also with methylmalonyl-CoA epimerase from *C. aurantiacus*	This work
pUC57-MMCE	Expresses methylmalonyl-CoA epimerase from *C. aurantiacus*	This work

every treatment. Plates were shaken in the reader at 900 rpm at 37 °C, and OD 600 and GFP fluorescence measurements were taken every 15 min. An endpoint at 8 h was used to calculate the standard curve using pCM62xt and propionate production using pCM62-MP driving the JSB:pPro33gfp biosensor. The expression of GFP per unit OD 600 increased with the propionate concentration.

2.4. Propionate detoxification assay

Propionate is a potent growth inhibitor of *E. coli* K12 strains (Supplemental Fig. 3). Assays for pathways 2 and 4 are based on their ability to shuttle flux away from propionate and propionyl-CoA. The inhibitory concentration of propionate varied as a function of the plasmid burden of the cell, whether or not the cells carried a *prpC*$^-$(2-methylcitrate synthase) mutation, and whether or not the other carbon source could mediate catabolite repression of the endogenous *prp* operon. Cells were grown in M9 minimal medium at an inhibitory concentration of propionate with supplemental glucose or glycerol. Cultures were initiated with a 100-fold dilution of an overnight culture in M9 glucose medium with appropriate antibiotics, for a starting calculated optical density of 0.005. Cells were grown at 37 °C in 96-well plates at 750 rpm. Optical densities of fast-growing 100 μL cultures were measured every 15 min with a Synergy H1 plate reader (Biotek, Winooski, VT); slower-growing 1 mL cultures in deep-well plates were manually sampled daily. Improved growth in a high propionate regimen indicated functional expression of the pathways.

2.5. DAP titration assay

E. coli requires succinyl-CoA to make diaminopimelic acid (DAP), a precursor of cell wall synthesis (Yu et al., 2006). Other succinyl-CoA downstream products (e.g. heme) are not essential for growth in rich medium (Haddock and Schairer, 1973). We constructed a *sucAD*$^-$ double mutant by transducing the KEIO Δ*sucA*::*kanR* strain into a Δ*sucD*::*FRT* strain and providing the resultant cells with 120 μM DAP (Sigma-Aldrich). During the isolation of the doubly mutated transductant, it appeared that the strain may have acquired a secondary mutation(s) that improved its growth, so it was subjected to whole-genome sequencing (Supplemental Table 1). The *sucAD*$^-$ cells require DAP for growth, but the amount can be reduced by expressing enzymes that produce succinyl-CoA from an alternative pathway. Cell optical densities (600 nm) were measured as described above.

3. Results

3.1. Engineering strategy

The *Chloroflexus* bicycle contains 19 reactions catalyzed by 13 enzymes, 10 of which are not characterized in *E. coli* (Fig. 1) (Zarzycki et al., 2009; Zarzycki and Fuchs, 2011), and likely optimized for expression at 55 °C (Rathnasingh et al., 2011). The bicycle can be considered to start when acetyl-CoA carboxylase (ACC) fixes bicarbonate to acetyl-CoA at the expense of an ATP (reaction 1), releasing malonyl-CoA as an intermediate; *E. coli* already performs this reaction as the first step of fatty acid synthesis. Malonyl-CoA is converted to 3-hydroxypropionate and then to propionyl-CoA at the cost of 3 NADPH reducing equivalents and an ATP (reactions 2 and 3). Here, the bicycle branches. In the left cycle, propionyl-CoA carboxylase (PCC) fixes another bicarbonate, resulting in (S)-methylmalonyl-CoA. Based on analogy with other systems (Strauss and Fuchs, 1993), it is thought that in *Chloroflexus* an epimerase (reaction 5) converts this intermediate to the R enantiomer, which is converted to succinyl-CoA by the methylmalonyl-CoA mutase (reaction 6). Coenzyme A is removed, and the resulting succinate is converted to malate by TCA cycle reactions, and then to malyl-CoA (reactions 7–9); in reaction 7 CoA is transferred from succinate to malate. Malyl-CoA is split to regenerate acetyl-CoA and a molecule of glyoxylate (reaction 10a). From this acetyl-CoA, the central pathway of the bicycle is repeated, and the glyoxylate is combined with a propionyl-CoA to form β-methylmalyl-CoA (reaction 10b), initiating a second cycle. Here, β-methylmalyl-CoA is converted through a series of novel intermediates to regenerate acetyl-CoA and pyruvate (10c–13).

To heterologously express the entire bicycle, we divided the overall bicycle into four sub-pathways as diagrammed in Fig. 1. Our experimental plan was to separately express the few enzymes that define each sub-pathway and demonstrate their coordinated activity, using functional readouts such as complementation of host mutations or induction of reporter genes in live cells. All of the enzymes have been characterized *in vitro* in literature (Roy and Leadlay, 1992; Strauss and Fuchs, 1993; Evans et al., 1993; Haller et al., 2000; Alber and Fuchs, 2002; Herter et al., 2002a, 2002b; Hügler et al., 2002; Friedmann et al., 2006, 2007; Zarzycki et al., 2009; Froese et al., 2009; Zhang et al., 2010; Boghigian et al., 2011; Zarzycki and Fuchs, 2011). However, expression of the enzymes themselves in the form of reconstituted metabolic pathways has not been demonstrated, except for a portion of the central sub-pathway. MCR and PCS have been used to generate 3-hydroxypropionate and 3-hydroxypropionyl-CoA (Fukui et al., 2009; Andreessen and Steinbüchel, 2010; Meng et al., 2012; Wang et al., 2012a, 2012b; Zhou et al., 2011).

3.2. Detection of propionyl-CoA from sub-pathway 1 with a GFP biosensor

The first enzymatic reaction of the *Chloroflexus* 3-hydroxypropionate bicycle is also the universal first reaction in fatty-acid synthesis: addition of one bicarbonate ion to acetyl-CoA. This fixed carbon is then released as CO_2 in the next step in fatty acid synthesis.

Since the first step of sub-pathway 1 is catalyzed natively by *E. coli*, we constructed an operon containing two genes: *mcr* and *pcs*, whose products catalyze reactions 2 and 3 (Fig. 1). *C. aurantiacus* has exactly one malonyl-CoA reductase gene (*mcr*, 3.7 kb (Hügler et al., 2002)) and one propionyl-CoA synthase (*pcs*, 5.4 kb (Alber and Fuchs, 2002)), corresponding to the genes Caur2614 and Caur0613 in the *C. aurantiacus* J-10-fl genome (Tang et al., 2011). We codon-optimized and cloned *mcr* and *pcs* into the broad-host range BioBrick compatible vector pCM62xt (Marx and Lidstrom, 2001; Mattozzi and Keasling, 2010) for essentially constitutive expression. As the two genes encode rather large multidomain bacterial proteins (133 and 203 kD, respectively) we identified them on an SDS-PAGE gel of a total cell protein lysate (Supplemental Fig. 1).

Propionyl-CoA is not required for essential reactions in *E. coli*, so we designed a biosensor for propionyl-CoA. The Keasling lab developed a propionate-inducible expression system as a low-cost alternative to IPTG, using the propionate-inducible *prpB* promoter and *prpR* regulatory gene (Lee and Keasling, 2005, 2006a, 2006b, 2008). *E. coli*'s primary propionate detoxification pathway first assimilates propionate to propionyl-CoA (by the product of *prpE*), and then to 2-methylcitrate (by the product of *prpC*). 2-Methylcitrate is the activator of the *prpR*/*prpB* regulator/promoter system. We used a derivative of this propionate-inducible system to function as a biosensor for intracellular production of propionyl-CoA. Plasmid pPro33gfp contains a medium-copy broad-host-range p15A origin, a chloramphenicol marker, a constitutively expressed *prpR* gene, and the *prpB*

promoter transcribing GFP gene as a reporter (Supplemental Fig. 2). Control treatments indicated that small amounts of propionate in the medium (<2 mM) repressed constitutive expression, and higher concentrations (up to 20 mM) induced GFP expression (Fig. 2), as consistent with previous observations (Lee and Keasling, 2005).

Expression of the *C. aurantiacus mcr* and *pcs* genes induced GFP expression from the propionate-inducible reporter system (Fig. 2). When *mcr* and *pcs* were expressed from an IPTG-inducible promoter on a compatible multi-copy plasmid, the GFP expression from pPro33gfp corresponded to that seen with about 10 mM propionate in the medium. (Since the system responds to 2-methylcitrate metabolized from propionate via propionyl-CoA, a precise level of MCR-PCS activity cannot be inferred.) Induction of *mcr* and *pcs* with IPTG had only a slight effect on propionyl-CoA levels, while the induction of the corresponding proteins was significant (Supplemental Fig. 1), suggesting that substrate, rather than *mcr* and *pcs* expression, may be limiting 2-methylcitrate synthesis. Induced cells produced propionate at a concentration corresponding to that at 10.0 ± 0.6 mM in the medium.

3.3. Selection of an appropriate propionyl-CoA carboxylase enzyme complex for sub-pathway 2

The steps in the 3-HPA bicycle that lead from propionyl-CoA to succinyl-CoA (sub-pathway 2 in Fig. 1) also occur in other bacteria, and were reconstructed in various configurations by the Pfeifer lab for metabolic engineering purposes (Pfeifer et al., 2001; Zhang et al., 2009, 2010; Boghigian et al., 2011). Propionyl-CoA carboxylase (PCC, Enzyme 4) is the first step in this sub-pathway, and represents another carbon-fixing step.

We compared the *Streptomyces coelicolor* propionyl-CoA carboxylase (Pfeifer et al., 2001; Zhang et al., 2009, 2010; Boghigian et al., 2011) with two candidate PCCs annotated in the *C. aurantiacus* genome (Tang et al., 2011). Both PCC and acetyl-CoA carboxylase (ACC) are three-subunit enzymes that may be encoded by three genes or fusions thereof. We obtained the *C. aurantiacus* genes Caur3435 and Caur2034, and codon-optimized them for expression in *E. coli* (Genscript). None of the *C. aurantiacus* propionyl-CoA carboxylase and acetyl-CoA carboxylase genes were linked to other genes encoding 3-HPA bicycle enzymes, so no functional grouping was inferred.

To determine which of the three propionyl-CoA carboxylases (Enzyme 4 in Fig. 1) would be most active in *E. coli*, we functionally tested their ability to confer resistance to propionate, which is toxic to many bacteria and is often used as a food preservative. While the exact method of propionate toxicity is not known, it is believed that propionyl-CoA selectively inhibits citrate synthase (Horswill et al., 2001; Man et al., 1995). The toxicity of propionate is enhanced in *prpC*$^-$ mutants (2-methylcitrate synthase) and reduced in *prpE*$^-$ mutants (propionyl-CoA synthetase; Supplemental Fig. 3). *E. coli* K cells (BW25113 *prpC*$^-$) were grown in M9 minimal medium at an inhibitory concentration of propionate (0.26% w/v) with supplemental glucose (0.14% w/v; Fig. 3). *E. coli* expressing the PCC/ACC from *Streptomyces coelicolor* grew faster and reached a higher OD 600 than all the other constructs tested; we have observed this behavior in all other concentrations of propionate and glucose we have tested (data not shown). We therefore designed subsequent constructs utilizing the *S. coelicolor* enzymes.

3.4. Propionate detoxification with a hybrid propionyl-CoA carboxylase pathway (sub-pathway 2)

To further assay function of sub-pathway 2 downstream of the propionyl-CoA carboxylase (Enzyme 4 in Fig. 1), we tested *E. coli* for propionate detoxification while overexpressing enzymes from various organisms annotated to perform the relevant reactions. Methylmalonyl-CoA epimerase (MMCE) from *C. aurantiacus* (Tang et al., 2011; Zarzycki et al., 2009) is a 139 aa protein that is annotated to interconvert (*R*)- and (*S*)-enantiomers of methylmalonyl-CoA (Enzyme 5). We obtained the *C. aurantiacus* gene Caur3037 and codon-optimized it for expression in *E. coli* (Genscript). The next reaction, catalyzed by methylmalonyl-CoA mutase (SBM), converts methylmalonyl-CoA into succinyl-CoA for assimilation into the TCA cycle (Enzyme 6). This enzyme, also known as "sleeping beauty mutase", was one of the earliest reactions to be identified bioinformatically in the *E. coli* genome (Roy and Leadlay, 1992), in a "promoterless operon". Though its reactions have been described, some of its interactions with other proteins in the host cell have only been recently elucidated (Froese et al., 2009).

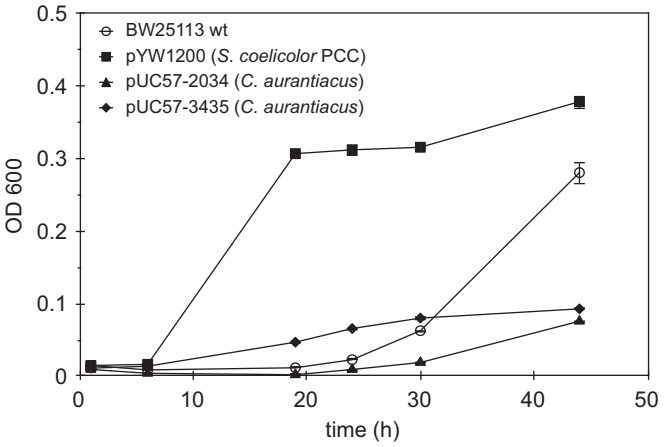

Fig. 3. Identification of an optimal propionyl-CoA carboxylase for function in *E. coli*. BW25113 strains harboring either an empty vector pUC19, plasmid pYW1200 expressing *S. coelicolor* PCC/ACC, pUC57-2034, or pUC57-3435 were compared. The latter two plasmids express the two genes annotated in the *C. aurantiacus* genome as propionyl-CoA carboxylases. Cells were grown in M9 minimal medium supplemented with inhibitory propionate (0.26%) and supplemental glucose (0.14%). Cells were inoculated at the same OD and error bars represent standard deviations from three independent cultures.

To complete sub-pathway 2, we expressed MMCE (Enzyme 5) in the same operon as PCC/ACC, and we upregulated SBM by inserting a Lac promoter 5′ of its native operon (*sbm-ygfDGH*) via lambda-red recombination (Datsenko and Wanner, 2000). Propionyl-CoA and its metabolite 2-methylcitrate are expected to be toxic intermediates (Evans et al., 1993; Haller et al., 2000; Horswill et al., 2001; Brock et al., 2002, Man et al., 1995) and the *prpC*$^-$ mutant is more sensitive to propionate than wild-type *prpC*$^+$. Growth in the presence of propionate is thus expected to reflect elimination of propionate toxicity and possibly, the use of propionate as a carbon source. Cells were also engineered to contain a *prpC*$^-$ (2-methylcitrate synthase) mutation to enhance propionate sensitivity. *E. coli* BW25113 *prpC*$^-$ cells were grown with a high amount of propionate (0.32% w/v), and a small amount of supplemental glucose (0.08% w/v). Cells expressing all three sub-pathway 2 enzymes (PCC/ACC, MMCE, and SBM/methylmalonyl-CoA mutase) showed an increase in growth relative to strains without these genes (Fig. 4) at 0.32% propionate 0.08% glucose, as well as at a 0.26% propionate 0.14% glucose concentration (Supplemental Fig. 4). Strains expressing sub-pathway 2 genes grew at an average growth rate of $\mu \approx 0.3$–0.4/d; empty vector constructs grew at about $\mu \approx 0.2$/d. However, strains with the full pathway did not show simple exponential growth; the growth rate was higher in the later part of the experiment, presumably due to a period of propionate detoxification that resulted in an extended lag phase (Supplemental Fig. 4). No differences were observed at lower propionate/glucose ratios, and no cells grew at 0.4% propionate 0% glucose (data not shown).

3.5. Growth enhancement of a sucAD⁻ mutant with sub-pathway 3: A succinyl-CoA:(S)-malate-CoA transferase operon

Sub-pathway 3 in Fig. 1 consists of two heterologous reactions, reaction 7, succinyl-CoA:(S)-malate-CoA transferase, and one of the reactions catalyzed by enzyme complex 10: (S)-malyl-CoA lyase, both unique to *Chloroflexus*. In addition, two steps of the TCA cycle are required: succinate dehydrogenase and fumarate hydratase; the CoA group from succinyl-CoA is transferred to malate by reaction 7, and malate is converted to glyoxylate and acetyl-CoA by reaction 10a. We hypothesized that these reactions should also run in reverse using the cells' native pools of acetyl-CoA and glyoxylate, and thus providing the cells with succinyl-CoA: the steps in the TCA cycle are reversible, and reaction 7 is also reversible (Friedmann et al., 2006). Reaction 10a is also expected to be reversible based on thermodynamic principles and by analogy with reaction 10b. The product of sub-pathway 3, operating in reverse, is succinyl-CoA.

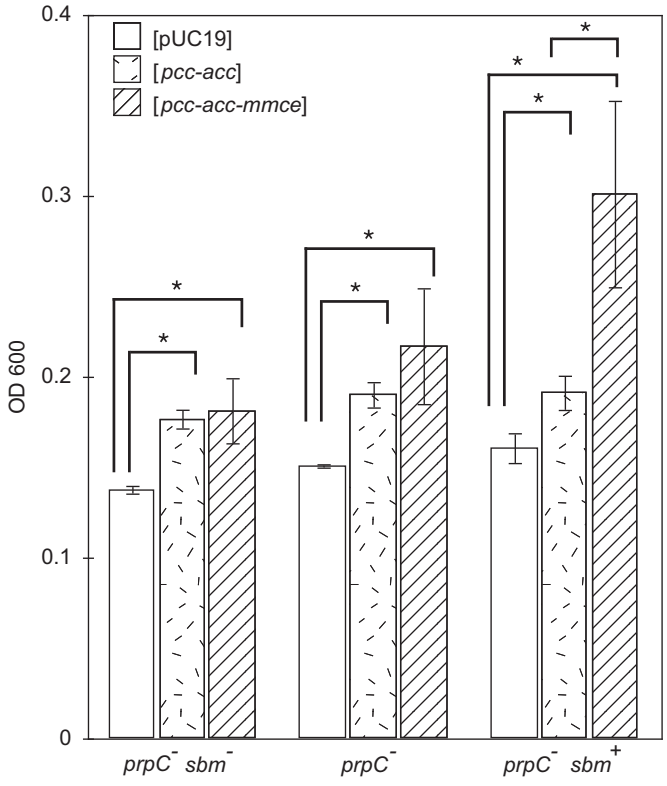

Fig. 4. Increased growth in propionate due to expression of a hybrid sub-pathway 2. *E. coli prpC*⁻ cells were grown in 0.32% propionate and 0.08% glucose at 37 °C for five days. The *E. coli sbm* gene encodes a putative methylmalonyl-CoA mutase corresponding to Reaction 6 of the 3-HPA bicycle. *sbm*⁻ is a knockout and *sbm*⁺ is upregulated with a lac promoter 5′ from *sbm*. Cells also contained either an empty vector, *S. coelicolor* PCC-ACC as expressed on pYW1200, or pET21c-PCCACC-MMCE. Cells were inoculated at the calculated OD 600 of 0.05. Error bars represent standard deviations from three independent cultures, and * represents significance at a level of $p=0.05$ using a Student's *t*-test.

To create a genetic assay for succinyl-CoA synthesis, we constructed an *E. coli* strain in which genes *sucA* and *sucD*, encoding subunits of the 2-oxoglutarate dehydrogenase and succinyl-CoA ligase, are deleted. An earlier study (Yu et al., 2006) reported that *sucAB* and *sucCD* in *E. coli* are synthetic lethal. However, inspection of the biochemical pathways of *E. coli* suggested that succinyl-CoA might be important for only two compounds that might not be supplemented by rich medium: 5-aminolevulinic acid for heme synthesis, and diaminopimelic acid (DAP) for cell wall synthesis. We constructed a *sucA*⁻ *sucD*⁻ double mutant by standard techniques culminating in a P1 transduction of *sucA*::*KanR* into a *sucD*::*FRT* strain (Materials and Methods). The *sucAD*⁻ strain grows, albeit slowly, on LB supplemented with DAP and succinate.

Expression of Reactions 7 and 10 of the 3-HPA bicycle rescued the DAP auxotrophy of the *sucAD*⁻ strain, such that supplementation with very small amounts of DAP was sufficient to allow cell growth (Fig. 5). We compared cells with the constructs pUC57 (empty vector), pUC57-7ab (expressing both subunits of succinyl-CoA:(S)-malate-CoA transferase encoded by Caur0178 and Caur0179), and pUC57-7ab10 (additionally expressing the trifunctional (S)-malyl-CoA/β-methylmalyl-CoA/(S)-citramalyl-CoA (MMC) lyase encoded by Caur0174). We transformed these plasmids into the *sucAD*⁻ mutant and grew them in LB liquid culture with decreasing DAP concentrations. Growth curves of bacteria incubated in LB with ampicillin, succinate and 120, 24, 12, or 6 μM DAP indicated that all strains grew at 120 μM DAP; 7ab and 7ab10 transformants grew after an extended lag-phase compared to the empty vector transformant. When reducing the DAP concentration to 24 μM, the *sucAD*⁻ mutants with pUC57-7ab10 and empty vector pUC57 grew with an extended lag phase compared to growth at 120 μM DAP. At a concentration of 12 μM DAP, the *sucAD*⁻ mutant started growing after 12 h only when transformed with pUC57-7ab10. At 6 μM DAP, none of the strains grew within the time period of 16 h. Plasmid pUC57-7ab10 enables growth of the *sucAD*⁻ mutant in lower DAP concentrations compared to 7ab alone and empty vector. Expression

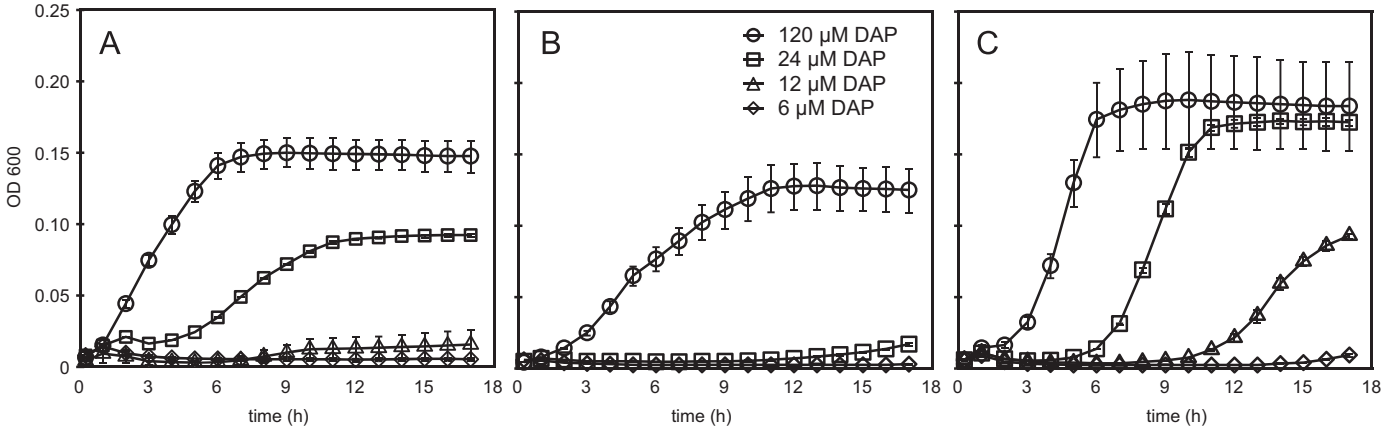

Fig. 5. Enzymes 7ab and 10 enable growth of BW25113 *sucAD*⁻ at low diaminopimelic acid (DAP) concentrations. BW25113 *sucAD*⁻ transformed with an empty vector (a), pUC57-7ab (b), and pUC57-7ab10 (c) were incubated at 37 °C in LB with 100 ng/μL ampicillin, 6.5 mM succinate and either 120 μM, 24 μM, 12 μM and 6 μM DAP. Error bars indicate standard deviation of three independent cultures.

of enzymes 7a and 7b alone appears to impair growth, possibly due to protein expression burden and/or wasteful generation of malyl-CoA.

3.6. Growth in the presence of propionate mediated by sub-pathway 4

Sub-pathway 4 consists of five enzymatic reactions unique to Chloroflexi, converting propionyl-CoA and glyoxylate to acetyl-CoA and pyruvate. In brief, propionyl-CoA and glyoxylate are condensed to form β-methylmalonyl-CoA by the second reaction of the enzyme 10 complex (*mcl*); β-methylmalonyl-CoA is dehydrated to form mesaconyl-C1-CoA by β-methylmalyl-CoA dehydratase (*mch*); the CoA is transferred to the other side of the molecule by mesaconyl-C1-C4-CoA transferase (*mct*); this mesaconyl-C4-CoA is rehydrated to form citramalyl-CoA by mesaconyl-C4-CoA hydratase (*meh*); which is converted to pyruvate by the lyase activity of the enzyme 10. This also regenerates acetyl-CoA, allowing the cycle to repeat.

We constructed an artificial operon encoding sub-pathway enzymes 10–13 using Gibson assembly (Gibson, 2011) from codon-optimized *C. aurantiacus* genes (Genscript, Tang et al., 2011). The gene order and ribosome binding sites (Weiss et al., 2008) were chosen based on activities of the enzymes in the operon, with the slow Enzyme 10 encoded first (Zarzycki et al., 2009). The order of the genes in the synthetic operon is *mcl* Caur174, *mct* Caur175, *meh* Caur180, and *mch* Caur173, catalyzing reactions 10–12–13–11 (Fig. 1). Additionally we made constructs of each gene separately, as well as operons encoding all but one of each enzyme (Table 1).

To test the function of these gene sets, *prpC*$^{-}$ *E. coli* were grown in the presence of propionate and glycerol. In this strain, propionate is metabolized to propionyl-CoA by the *prpE* product, but further metabolism by the endogenous pathway is blocked. Propionyl-CoA and its byproduct 2-methylcitrate are expected to be toxic intermediates (Evans et al., 1993; Haller et al., 2000; Horswill et al., 2001; Brock et al., 2002, Man et al., 1995) and the *prpC*$^{-}$ mutant is more sensitive to propionate than wild-type *prpC*$^{+}$. Growth in the presence of propionate is thus expected to reflect elimination of propionate toxicity and possibly, the use of propionate as a carbon source.

Typical results are seen in Fig. 6. In the presence of 0.36% propionate and 0.04% glycerol, strains with a complete operon or an operon expressing reactions 10, 12 and 13 grew the fastest (average growth rate $\mu \approx 1.0$/d), but growth overall is still heavily suppressed by the high propionate concentration. Strains with a control vector or operons encoding reactions 11, 12, and 13 grew at an intermediate rate ($\mu \approx 0.8$/d), while strains expressing operons with reactions 10, 11 and 12 or 10, 11 and 13 grew relatively slowly ($\mu \approx 0.7$/d). One interpretation of these results is that strains expressing all of enzymes 10–13 were able to metabolize propionyl-CoA to pyruvate, such that the propionate is both detoxified and contributes to biomass. Enzyme 11 catalyzes a dehydration reaction that may occur spontaneously, such that sub-pathway 4 can still function in its absence when the other enzymes are overproduced. Strains expressing Enzyme 10 without a complete sub-pathway grew particularly slowly ($\mu \approx 0.7$/d) because of deleterious effects of this enzyme.

To test the behavior of the entire 3-HPA bicycle, we performed preliminary experiments using M9 minimal petri dishes solidified with 1.5% noble agar and supplemented with 1 μg/mL Vitamin B_{12}. We lambda-red integrated (Datsenko and Wanner, 2000) formate dehydrogenases from *Pseudomonas sp.* 101 and *Bulkholderia sp.* (Lamzin et al., 1992; Hatrongjit and Packdibamrung, 2010), and expressed the 3-HPA subpathways on plasmids or integrated via lambda-red. Cells expressing sub-pathways 1–4 were able to grow using 0.8% propionate + 0.8% glycolate + 50 mM sodium formate as carbon sources, but not on propionate or formate alone (data not shown). Wild-type *E. coli* BW25113 did not grow under these concentrations of propionate + glycolate + formate. Expression of the bicycle thus allowed for some reduction in propionate toxicity in solid media, but not autotrophic growth. Presumably additional engineering is required to achieve balanced functioning of this system (see Section 4).

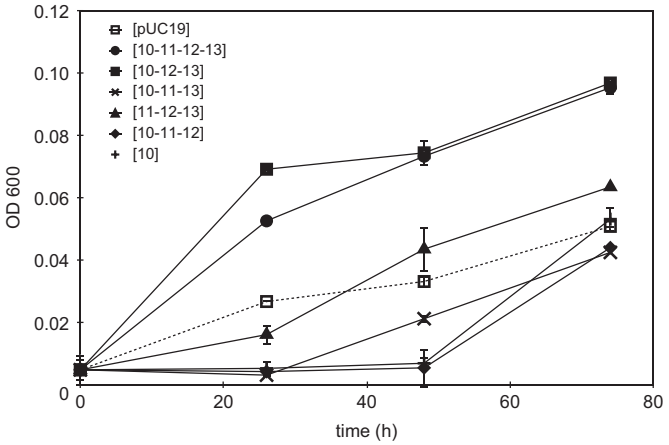

Fig. 6. Functional expression of sub-pathway 4 as observed by propionate detoxification. BW25113 *prpC*$^{-}$ was transformed with pUC19 expressing enzymes encoding either Reactions 10–12–13–11 (full sub-pathway 4), Reactions 10–12–13, no sub-pathway genes (pUC19 empty vector), Reactions 11–12–13, Reactions 10–11–13, Reactions 10–11–12, and Reaction 10 only. Cells were cultured in M9 minimal medium supplemented with 0.36% propionate and 0.04% glycerol at 37 °C. Error bars indicate standard deviation of three independent cultures.

3.7. Secondary phenotypes of 3-HPA enzyme expression

In the course of performing these experiments, we noted that expression of Enzyme 10 inhibited growth of *E. coli* in rich and minimal media, and on plates caused a 'whitish' colony color phenotype that becomes mucoid as the colony reaches about 3 mm in diameter. These phenotypes may result from the action of Enzyme 10 on other substrates in the cell.

4. Discussion

The 3-hydroxypropionate bicycle for carbon fixation includes seven enzymes that are not found in most bacteria, and much of the bicycle functions independently of central metabolism. Of the 19 distinct enzymatic reactions in this bicycle, only three are clearly extant in *E. coli* (Reactions 1, 8 and 9). This contrasts with the Calvin cycle, which includes only three distinctive enzymes and relies on nine other enzymes from central metabolism. Transfer of carbon fixation pathways to heterotrophic organisms is of interest both for metabolic engineering, and to understand natural mechanisms of horizontal gene transfer and functional incorporation of foreign metabolic pathways into microbes.

We have expressed all of the novel enzymes of the 3-HPA bicycle in *E. coli* and demonstrated that they can be integrated into *E. coli* metabolism. These enzymes were grouped into functional units, expressed in operons, and tested under conditions that demanded some level of *in vivo* enzymatic activity without requiring regulation. The overall bicycle was divided into four sub-pathways that were independently assayed (Fig. 1): (1) the 'middle sub-pathway' that uses acetyl-CoA carboxylase, malonyl-CoA reductase and propionyl-CoA synthase; (2) the lower left-side sub-pathway that uses propionyl-CoA carboxylase, methylmalonyl-CoA epimerase, and methylmalonyl-CoA mutase; (3) the upper left-side sub-pathway that includes a novel trifunctional lyase and succinyl-CoA:malyl-CoA transferase as well as succinate dehydrogenase and fumarase from the TCA cycle; and (4) the right-side sub-pathway that consists of four novel enzymes.

Sub-pathway 1 was reconstructed by expression of malonyl-CoA reductase and propionyl-CoA synthase. Endogenous acetyl-CoA carboxylase, which also catalyzes the first step in fatty acid synthesis, was sufficient to initiate this sub-pathway. Production of propionyl-CoA was observed using a propionate-inducible construct (Lee and Keasling, 2005) modified to serve as a biosensor (Fig. 2). Previously, Fukui et al. demonstrated that MCR and a portion of PCS lacking the reductase activity could be co-expressed to generate intracellular hydroxypropionyl-CoA, an attractive substrate for bioplastic synthesis (Fukui et al., 2009).

Portions of sub-pathway 2, which converts propionyl-CoA to succinyl-CoA, was previously reconstructed in *E. coli* by Pfeifer and colleagues (Pfeifer et al., 2001; Zhang et al., 2009, 2010; Boghigian et al., 2011). This sub-pathway includes a second carbon fixation step by propionyl-CoA carboxylase. We found that the *S. coelicolor* PCC works more efficiently than either of those from *C. aurantiacus*, and that sub-pathway 2 can be reconstructed from heterologous expression of the *S. coelicolor* PCC and the *C. aurantiacus* methylmalonyl-CoA epimerase, along with activation of the endogenous *E. coli* methylmalonyl-CoA mutase (Froese et al., 2009).

Function of enzymes in the upper left sub-pathway was measured by testing for formation of succinyl-CoA. This involved running the sub-pathway in reverse, producing succinyl-CoA from endogenous glyoxylate and acetyl-CoA. Fumarase, succinate dehydrogenase, and succinyl-CoA:malyl-CoA transferase (Friedmann et al., 2006) are known to be reversible, and the multifunctional MMC lyase (Zarzycki et al., 2009) should catalyze its reaction in both directions based on fundamental thermodynamic principles and by analogy with reaction 10b, also catalyzed by this enzyme. To test for synthesis of succinyl-CoA *in vivo*, we constructed a *sucA*$^-$ *sucD*$^-$ double mutant of *E. coli*, which is blocked in both 2-oxoglutarate dehydrogenase and succinyl-CoA ligase; this strain is unable to grow in the absence of diaminopimelic acid (DAP). The DAP requirement of this strain was significantly reduced when succinyl-CoA:malyl-CoA transferase and MMC lyase were co-expressed in the *sucAD*$^-$ double mutant strain (Fig. 5).

We tested the function of sub-pathway 4 (Reactions 10b-11-12-13-10c) by coexpressing these enzymes in *prpC*$^-$ *E. coli*. Cells could not tolerate propionate as a sole carbon source, so a small amount of glycerol was added. Strains expressing all of the enzymes or all but methylmalyl-CoA dehydratase (Enzyme 11) showed faster initial growth rates in propionate/glycerol medium (Fig. 6). Cells with an empty vector or a sub-pathway 4 operon lacking Enzyme 10 showed intermediate growth, while cells with an incomplete sub-pathway 4 operon still expressing Enzyme 10 grew very poorly, presumably due to deleterious effects of this enzyme. The lack of a strict requirement for the enzyme catalyzing Reaction 11, the dehydration of methylmalyl-CoA, suggests that this reaction may occur spontaneously at a sufficient rate to allow sub-pathway function.

These results indicate that the novel enzymes of the 3-HPA bicycle can function to some degree in *E. coli* as part of simple, linear pathways with some level of flux. Co-expression of all of the genes in the bicycle, either on the plasmids as presented in this study or integrated by lambda-red (Datsenko and Wanner, 2000) did not yield autotrophic growth (Section 3).

Reconstruction of the entire bicycle or larger sub-pathways will require regulation of carbon flux that is normally achieved through transcriptional, allosteric and other post-transcriptional mechanisms that are not in place. These mechanisms are more difficult to predict and design rationally. More sophisticated analyses and metabolic flux models are being constructed for this purpose (Boyle and Morgan, 2011). For example, the reaction set 10b–11–12–13–10c could be sufficient to allow growth on propionate as the sole carbon source in a *prpC*$^-$ strain, but this was not observed. For such growth to occur, for each acetyl-CoA and pyruvate generated by Reaction 10c, at least one glyoxylate would need to be generated via the glyoxylate shunt and not consumed by malate synthase. As isocitrate lyase and malate synthase are coordinately regulated such that most or all glyoxylate produced by the first enzyme is consumed by the second, failure to grow on propionate is not surprising. Directed evolution is a clear next step, starting with propionate as a carbon source.

Conversion of *E. coli* metabolism to fully autotrophic growth would require a number of host modifications and would also require correct regulation of flux at certain branchpoints. For example, aerobic *E. coli* metabolism normally involves significant flux through the TCA cycle, which involves conversion of fixed carbon into CO_2 to generate reducing equivalents, while anaerobic metabolism involves a branched TCA cycle in which succinate dehydrogenase operates in the reverse direction; operation of the 3-HPA bicycle would involve a distinct flux pattern and would most likely require an absence of 2-oxoglutarate dehydrogenase, which could be achieved by mutation of *sucA* (Yu et al., 2006). Carbon flux would need to be managed at several branch points, such as propionyl-CoA between Enzyme 10 and Enzyme 4 within the 3-HPA bicycle (Fig. 1), malonyl-CoA going into the 3-HPA bicycle or into fatty acid synthesis, malate going to malyl-CoA in the 3-HPA bicycle or into oxaloacetate and glyoxylate remaining in the 3HPA bicycle or entering the glyoxylate shunt. Based on its genome sequence, *C. aurantiacus* appears to encode a full complement of the enzymes in central metabolism but must somehow regulate these branch points (Tang et al., 2011), but the underlying mechanisms are unknown. Finally, to achieve autotrophic growth, a source of reducing equivalents is needed. It would be difficult to introduce light-cycle reactions into *E. coli*, but introduction of an NAD(P)-dependent formate dehydrogenase (Lamzin et al., 1992; Hatrongjit and Packdibamrung, 2010) could be used to generate reducing equivalents (and CO_2) without contributing reduced carbon to metabolism. It may also be useful to express a bicarbonate transporter to increase intracellular levels.

In sum, we have demonstrated the heterologous intracellular function in *E. coli* of all of the novel enzymes in the 3-HPA bicycle. These enzymes, when appropriately grouped, can use substrates and generate products that move in and out of *E. coli*'s endogenous metabolism. These results indicate that this unusual set of enzymes can be used to generate a variety of novel metabolites. In addition, it may be possible to reconstruct the entire autotrophic bicycle in heterologous organisms, although this may require extensive modification of host metabolism.

Acknowledgments

This work was supported by a cooperative agreement with the Department of Energy ARPA-E agency (Award# DE-0000079). We thank Jay Keasling for his gracious gift of plasmid pPro33gfp and Blaine Pfeifer for his gift of plasmid pYW1200. We would also like to thank Colby Stoddard and Anna Chen for helpful discussions, and Wade Hicks for technical assistance with whole genome sequencing and strains containing an NAD(P)H-dependent formate dehydrogenases.

Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2013.01.005.

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