Mitochondrial targeting increases specific activity of a heterologous valine assimilation pathway in Saccharomyces cerevisiae

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Bio-based isobutanol is a sustainable ‘drop in’ substitute for petroleum-based fuels. However, well-studied production routes, such as the Ehrlich pathway, have yet to be commercialized despite more than a century of research. The more versatile bacterial valine catabolism may be a competitive alternate route producing not only an isobutanol precursor but several carboxylic acids with applications as biomonomers, and building blocks for other advanced biofuels. Here, we transfer the first two committed steps of the pathway from pathogenic Pseudomonas aeruginosa PAO1 to yeast to evaluate their activity in a safer model organism. Genes encoding the heterologeric branched chain keto-acid dehydrogenase (BCKAD; bkdA1, bkdA2, bkdB, lpdV), and the homooligomeric acyl-CoA dehydrogenase (ACD; acd1) were tagged with fluorescence epitopes and targeted for expression in either the mitochondria or cytoplasm of S. cerevisiae. We verified the localization of our constructs with confocal fluorescence microscopy before measuring the activity of tag-free constructs. Despite reduced heterologous expression of mitochondria-targeted enzymes, their specific activities were significantly improved with total enzyme activities up to 138% greater than those of enzymes expressed in the cytoplasm. In total, our results demonstrate that the choice of protein localization in yeast has significant impact on heterologous activity, and suggests a new path forward for isobutanol production.

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1. Introduction

Yeast microbial factories for biochemical production are competitive alternatives to bacterial platforms. As simple eukaryotes, yeast offer wider product portfolios (Adrio and Demain, 2010; Demain and Vaishnav, 2009) and improved tolerance to toxic value-added products (Ingram, 1986), while remaining relatively simple to engineer. These improved capabilities arise from their ability to compartmentalize their metabolic functions. By spatially separating reactions in this manner, eukaryotes locally concentrate precursors and enzymes where they are needed (Fukuda et al., 2005; Kumar et al., 2002; Paltauf et al., 1992), enhancing the efficiency of these processes. Compartmentalization also establishes fixed environmental conditions (e.g. pH (Orij et al., 2009) and redox potential (Hu et al., 2008)), which affect the enzyme kinetics and potential chemistries within them. For instance, disulfide bond formation is thermodynamically favored in the oxidizing environment of the endoplasmic reticulum (Woycechowsky and Raines, 2000), while the substantial pH gradient across the mitochondrial membrane drives ATP biosynthesis (Mitchell, 1961). The thermodynamic and kinetic advantages offered by these environments, however, have historically been ignored in engineering applications due to the increased complexity of eukaryote biology and the poorly defined nature of many localization signal sequences (Kim and Hwang, 2013).

The resurgent interest in the Ehrlich pathway for the production of advanced ‘drop-in’ biofuels such as isobutanol (Avalos et al., 2013; Brando et al., 2013; Smith and Lio, 2011) has led to innovative strategies for pathway design. One of the most promising leveraging the improved tolerance of yeast, and their ability to localize key heterologous enzymes to the mitochondria where they have ready access to needed precursors (Avalos et al., 2013). In comparison to traditional cytosolic expression, yields of isobutanol were improved by more than 2-fold due to the increased local concentration of precursors and enzymes in the compartment’s reduced volume (Avalos et al., 2013). Despite

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these successes, the titers were insufficient for commercialization and future efforts may benefit from alternate metabolic routes to product. More importantly, however, the impact of the compartment’s environment on heterologous activity remains unexamined.

A promising alternate route to isobutanol is through the bacterial valine assimilation pathway (Fig. 1). Bacterial catabolism oxidatively decarboxylates α-keto acids to acyl-CoAs that may be cleaved with a thioesterase and reduced to release fusel alcohols (Sheppard et al., 2014). Extensions of the native pathway also produce a number of other valuable products such as biomonomers, and fatty alcohols (Massey et al., 1976). To evaluate the commercial potential of this pathway, we first examined the feasibility of heterologous expression of these enzymes in more fuel-tolerant non-pathogenic yeast. We cloned and expressed the oxidative decarboxylase, branched chain keto-acid dehydrogenase (BCKAD), and the subsequent oxidase of the native pathway, acyl-CoA dehydrogenase (ACD), from Pseudomonas aeruginosa PAO1. These enzymes were targeted for expression in either the mitochondria or cytoplasm of S. cerevisiae in a multi-plasmid/multigene background to recreate the metabolic burden of a complete production pathway. Here, we quantify their recombinant activity and examine the effects of enzyme localization on specific activity.

2. Materials and methods

2.1. Strains and plasmids

S. cerevisiae and E. coli strains and plasmids used in this study are listed in Table 1. Sub-cloning was carried out according to standard practices (Sambrook and Russell, 2001) and plasmids were maintained in E. coli XL1B. acd1 (PA0746), bkdA1 (PA2247), bkdA2 (PA2248), bkdB (PA2249), and IpDV (PA2250), encoding the acyl-CoA dehydrogenase and branched chain keto acid dehydrogenase complex, respectively, from Pseudomonas aeruginosa PAO1, were PCR amplified from plasmid template (pMMA202) provided by Mitsubishi Rayon. These amplicons were cloned into the BglII sites of the galactose-inducible pYES-mtGFP vector, graciously provided by Prof. Dr. Westermann, Universität Bayreuth, Germany, with the primers listed in Table 2. These constructs target expression of a C-terminal gene-Green Fluorescent Protein (GFP) fusion to the mitochondrion via the included mitochondrial (mt) leader peptide.
| Name | Relevant genotype | Vector backbone | Plasmid type | Source |
|------|-------------------|----------------|--------------|--------|
| **E. coli strains** | | | | |
| XL1B | endA1 gyrA96(nalR) thi-1 recA1 relA1 lacIq Δ(lacZ)M15 hsdR17(rK- mK+) | pYES | 2μ | Stratagene (Santa Clara, CA) |
| CKY263 | MATa leu2-3, 112 ura3-52 GAL | pYES | 2μ | (Frand and Kaiser, 1999) |
| BJS464 | MATα ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL | pYES | 2μ | (Jones, 1991) |
| **Plasmids** | | | | |
| pYESSmGFP | YEp bla pBR322 URA3 GAL1-mtGFP-CYCT | pRS316 | 2μ | (Westermann and Neupert, 2000) |
| pRS316-P-Pfu_bglA | YEp bla pBR322 URA3 GAL1 PrpGfbgl1-His10 CYCT | pRS316 | 2μ | This study |
| pYES-acd-GFP | YEp bla pBR322 URA3 GAL1-acd1-GFP-CYCT | pRS316 | 2μ | This study |
| pYES-ntacd-GFP | YEp bla pBR322 URA3 GAL1-ntacd1-GFP-CYCT | pRS316 | 2μ | This study |
| pYES-acd | YEp bla pBR322 URA3 GAL1-acd1-CYCT | pRS316 | 2μ | This study |
| pYES-ntacd | YEp bla pBR322 URA3 GAL1-ntacd1-CYCT | pRS316 | 2μ | This study |
| pYES-bkdA1-GFP | YEp bla pBR322 URA3 GAL1-bkdA1-GFP-CYCT | pRS316 | 2μ | This study |
| pYES-bkdA2-GFP | YEp bla pBR322 URA3 GAL1-bkdA2-GFP-CYCT | pRS316 | 2μ | This study |
| pYES-bkdB-GFP | YEp bla pBR322 URA3 GAL1-bkdB-GFP-CYCT | pRS316 | 2μ | This study |
| pYES-mtbkdA1-GFP | YEp bla pBR322 URA3 GAL1-mtbkdA1-GFP-CYCT | pRS316 | 2μ | This study |
| pYES-bkdA2-GFP | YEp bla pBR322 URA3 GAL1-bkdA2-GFP-CYCT | pRS316 | 2μ | This study |
| pYES-mtbkdA2-GFP | YEp bla pBR322 URA3 GAL1-mtbkdA2-GFP-CYCT | pRS316 | 2μ | This study |
| pYES-bkdB-GFP | YEp bla pBR322 URA3 GAL1-bkdB-GFP-CYCT | pRS316 | 2μ | This study |
| pYES-mtbkdB-GFP | YEp bla pBR322 URA3 GAL1-mtbkdB-GFP-CYCT | pRS316 | 2μ | This study |
| pYES-lpdV-GFP | YEp bla pBR322 URA3 GAL1-lpdV-GFP-CYCT | pRS316 | 2μ | This study |
| pYES-mtlpdV-GFP | YEp bla pBR322 URA3 GAL1-mtlpdV-GFP-CYCT | pRS316 | 2μ | This study |
| pACD1 | YEp bla pBR322 URA3 GAL1-acd1-CYCT | pRS316 | 2μ | This study |
| pmtACD1 | YEp bla pBR322 URA3 GAL1-mtacd1-CYCT | pRS316 | 2μ | This study |
| pBCKAD4 | YEp bla pBR322 LEU2 GAL1-bkdA1-CYCT GAL1-bkdA2-CYCT GAL1-bkdB-CYCT GAL1-lpdV-CYCT | pRS315 | 2μ | This study |
| pmtBCKAD4 | YEp bla pBR322 LEU2 GAL1-mtbkdA1-CYCT GAL1-mtbkdA2-CYCT GAL1-mtbkdB-CYCT GAL1-mtlpdV-CYCT | pRS315 | 2μ | This study |
| pCoA2 | YEp bla pBR322 LEU2 GAL1-hchA-CYCT GAL1-echA3-CYCT | pRS314 | 2μ | This study |
| pmtCoA2 | YEp bla pBR322 LEU2 GAL1-mtchA-CYCT GAL1-mtechA3-CYCT | pRS314 | 2μ | This study |
To simulate the metabolic burden of carboxylic acid production, echA3 (PA0745) and hchA (PA0744) encoding enoyl-CoA hydratases were cloned between the BglII and EcoRI sites of pYES-mtGFP with the primers in Table 2 to target expression to the mitochondria via the included mt leader peptide sequence and fluorescence epitope (Westermann and Neupert, 2000). Cytoplasmic expression was achieved by removing the mt leader sequence in these plasmids by cleaving with HindIII and circularizing the resulting product. As a fluorescent negative control, a beta-glucosidase construct from Pyrococcus furiosus (prepro-BglA-His10) was cloned into pRS316 from pITY—PP—Pfu_bglA (O’Malley et al., 2012) via the flanking KpnI and SacI sites.

To reconstitute functional enzyme complexes and test activity, genes encoding heterologous enzymes were cloned together into a single plasmid using an idempotent cloning strategy inspired by the BioBrick standard (Fig. 2) (Knight, 2003). First, gene cassettes were amplified from the pYES series of vectors using universal primers 5pYES (5′-GATGATCCACTAGTACGGATTAG-3′) and 3Cyct (5′-CTATCTAACGGAGGGCCGC-3′) containing a native SpeI site (underlined) and an introduced AvrII site (bold). The amplicons generated with these primers lack the C-terminal GFP tag to preclude any issues with oligomerization and activity. These sites produce compatible cohesive ends (CTAG), which allow for the sequential addition of gene cassettes using the same restriction enzymes without the loss of previously integrated cassettes. (mt) acd1 was cloned into pRS316 to make p(mt)ACD1, (mt) bkdA1, (mt) bkdA2, (mt) bkdB, and (mt) lpdV were cloned into pRS315 to make p(mt)BCKAD4, while (mt) echA3 and (mt) hchA were cloned into pRS314 to make p(mt)CoA2. This cloning strategy generated 3 enzyme ‘modules’ (oxidative decarboxylation – p(mt)BCKAD4, subsequent reduction – p(mt)ACD1, and thioester cleavage to carboxylic acids via the hydratases – p(mt)CoA2) targeted to either the mitochondria or cytoplasm for 6 plasmids in total (Fig. 2).

Unless otherwise noted, PCR amplifications were performed with Phusion High-Fidelity DNA Polymerase (NEB, Ipswich, MA) and oligonucleotides synthesized by Eurofins Genomics (Huntsville, AL).

### 2.2. Culture conditions

Constructed plasmids were expressed in haploid S. cerevisiae strains CKY263 and BJ5464 after transformation using standard LiAc chemical transformation methods (Daniel Gietz and Woods, 2002). Bacterial strains were propagated on Luria-Bertani (LB) medium at 37 °C supplemented with 100 µg/ml ampicillin.

**Table 2**

| Gene | Forward (5′ → 3′) | Reverse (5′ → 3′) |
|------|-------------------|-------------------|
| acd1 | CTATAGATCTAGCCTATGGATTTGACCTACCC | CTATAGATCTAGGAGGGCATCGAG |
| bkdA1 | CTATAGATCTAGCCTATGGATTTGACCTACCC | CTATAGATCTAGGAGGGCATCGAG |
| bkdA2 | CTATAGATCTAGCCTATGGATTTGACCTACCC | CTATAGATCTAGGAGGGCATCGAG |
| bkdB | CTATAGATCTAGCCTATGGATTTGACCTACCC | CTATAGATCTAGGAGGGCATCGAG |
| lpdV | CTATAGATCTAGCCTATGGATTTGACCTACCC | CTATAGATCTAGGAGGGCATCGAG |
| hchA | CTATAGATCTAGCCTATGGATTTGACCTACCC | CTATAGATCTAGGAGGGCATCGAG |
| echA3 | CTATAGATCTAGCCTATGGATTTGACCTACCC | CTATAGATCTAGGAGGGCATCGAG |

Bolded sequence indicates introduced BglII restriction sites. Underlined sequence indicates introduced HindIII sites. Bold italicized sequences indicate an introduced EcoRI restriction site.

**Fig. 2.** Modular enzyme expression constructs. Gene subunits of each enzyme module are expressed together from a single galactose inducible plasmid. These plasmids target expression to either the cytoplasm or mitochondria (as indicated above) and contain compatible auxotrophic markers for co-expression as shown.
Initially, yeast strains were revived on glucose-rich YPD media before being transferred to either synthetic complete medium (SD-CAA) with the appropriate auxotrophic markers dropped out (Sherman, 2002), and supplemented with either 2% glucose or galactose. Cultures were induced on galactose at an OD of ~0.1 and expressed for at least 7 h before assaying their activity.

2.3. Protein localization

*S. cerevisiae* CKY263 cultures were propagated overnight in 5 ml of synthetic complete media (SD-SCAA) lacking uracil, and supplemented with 2% galactose to induce expression of the GFP-tagged protein constructs. ∼10⁶ cells were pelleted and the mitochondrial stained with Mito-ID Red detection reagent diluted 1:2500 (Mito-ID Red detection kit - Cat# ENZ-51007-500, Enzo Life Sciences, Ann Arbor, MI) according to manufacturer instructions before being affixed to polylysine-coated slides for visualization (Mazia et al., 1975). Mitochondria visualization and protein localization were determined by confocal microscopy with an Olympus Fluoview 1000 Spectral Confocal with a 60x objective (Olympus America, Center Valley, PA). Protein expression was detected via green fluorescence (excitation=488 nm, emission=510 nm) while mitochondria were stained to fluoresce red (excitation=559 nm, emission=598 nm). All samples were visualized at similar gain, PMT voltage, and magnification to directly compare fluorescence levels.

2.4. Enzyme activity assays

All enzyme activity assays were performed on crude lysates generated by disrupting cell pellets resuspended in 500 μl of 50 mM Tris–HCl (pH=7.5) with ~100 μl of 0.5mm diameter zirconia/silica beads (BioSpec Products, Bartlesville, OK) in 5 × 5 s pulses at max speed on a vortexer with the samples maintained on ice for 30 s between pulses. The resulting lysates were clarified by centrifugation before being assayed for either ACD or BCKAD activity. ACD activity was measured in a continuous spectrophotometric assay at 25 °C in a redox reaction described by Izai and coworkers (Izai et al., 1992). ACD oxidizes its substrate isobutyryl-CoA to methacryloyl-CoA, transferring electrons to the intermediate electron carrier phenazine methosulfate (PMS). These electrons are then transferred to the electron acceptor 2, 6-dichlorophenolindophenol (DCPIP) with a simultaneous decrease in absorbance at 600 nm (ε=21 mol-1 cm-1 at pH=8 (McD. Armstrong, 1964). 1 U of activity corresponds to the oxidation of 1 mmol of isobutyryl-CoA/min in the presence of 1 mM N-ethylmaleimide, 0.03 mM isobutyryl-CoA, 1.6 mM PMS, 0.035 mM DCPIP, 0.4 mM FAD and 100 mM potassium phosphate buffer (pH = 8). BCKAD activity was measured spectrophotometrically at 37 °C in an assay first described by Reed and Mukherjee (Reed and Mukherjee, 1969). Reaction progress is monitored by the reduction of NAD⁺ at 340 nm. 1 U of activity corresponds to the decarboxylation and oxidation of 1 μmol of 2-oxoisovalerate/min in the presence of 1 mM MgCl₂, 0.2 mM thiamine pyrophosphate, 0.2 mM coenzyme A/2 mM diithiothreitol (DTT; Fisher Scientific, Pittsburgh, PA), 2 mM NAD⁺, 2 mM L-valine, 4 mM 2-oxoisovalerate and 100 mM potassium phosphate buffer (pH=7). Enzyme activities reported are normalized by total protein levels as measured with a BCA total protein assay (ThermoFisher Scientific, formerly Pierce Biotechnology, Waltham, MA). All reagents from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise.

3. Results and discussion

3.1. Production and mitochondrial targeting of heterologous valine degradation pathway genes

To evaluate the feasibility of isobutanol production via bacterial valine catabolism in yeast, we first needed to sub-clone constructs that could be expressed and localized within yeast. We constructed gene fusions of the first two enzymes (BCKAD and ACD) incorporating an N-terminal mt leader peptide and a C-terminal GFP tag for each enzyme to achieve mitochondrial expression and observe its localization. This mt leader sequence consisted of the 69 amino acid presequence of the S9 mitochondrial F₀-ATPase found in *N. crassa* that was previously shown to direct mitochondrial expression in *S. cerevisiae* (Westermann and Neupert, 2000). These constructs were tagged with a 56ST mutant of GFP, allowing for rapid and bright visualization of protein expression (Heim et al., 1995). Each gene fusion construct was expressed independently both with and without the mt leader peptide to test the ability of yeast to successfully target expression of proteins derived from the prokaryote *Pseudomonas aeruginosa*. We verified the localized expression of these genes by monitoring the green fluorescence of the C-terminal GFP epitope and by mitochondrial staining.

All of the valine degradation gene constructs tested, both with and without the mt leader sequence, were readily expressed in *S. cerevisiae* following ~24 h induction with galactose as evidenced by cellular green fluorescence vs. control strains (Fig. 3). In all cases, the presence of the mt presequence had a direct impact on the intensity and distribution of green fluorescence throughout the cell. As anticipated, constructs lacking the mt leader showed diffuse fluorescence consistent with non-localized expression of these genes within the cytoplasm. This distribution of fluorescence was distinct from the Mito-ID Red mitochondrial staining, which revealed discrete structures that crowd the periphery of the cell. In contrast, the mt leader sequence dramatically reduced the amount of fluorescence to localized punctate regions along the cellular margins that generally aligned with mitochondrial staining. That is, the mt leader peptide appeared to direct expression towards the mitochondria of the cell. However, this targeted expression dramatically reduced the amount of functional protein as visualized by the reduced green fluorescence, which may be due to the high energy cost involved in translocating proteins across the mitochondrial membranes and their folding within the matrix (Mokranjac and Neupert, 2008; Neupert and Herrmann, 2007). The effects of this energy penalty do not appear to be exclusively correlated with protein size. For instance acd1, encoding a 42.6 kDa protein, expresses very strongly in the cytoplasm but weakly in the mitochondria (Fig. 3A) while the slightly larger lpdV (48.6 kDa), expresses at comparable levels in both the cytoplasm and mitochondria (Fig. 3E). Despite this penalty, however, mitochondrial expression well above background (Fig. 3F) was observed for each construct with fluorescence approaching roughly half that of the positive mitochondrial fluorescence control (Fig. 3G). These nonlinear effects hint at the complex relationship between compartment environment (e.g. pH, redox potential) and protein expression, and suggests that the mitochondria presents an optimal environment for the proper folding and activity of the valine assimilation pathway.

3.2. Compartimentalization results in higher specific activities of Acd and bckad

Following the successful targeting of this isobutanol producing candidate pathway to either the mitochondria or the cytoplasm, we measured the total protein activity and assessed the impact of compartmentalization. acd1 encodes an acyl-CoA dehydrogenase, which belongs to the family of flavin adenine dinucleotide-containing enzymes involved in the β-oxidation of fatty acids and
catabolism of many amino acids in many domains of life (Battaile et al., 2002). While the crystal structure for the \textit{P. aeruginosa} ACD is unknown, homologous \textit{acd}1 gene products typically form homotetramers or homodimers with the C-terminus forming an \( \alpha \)-helix that mediates FAD\(^+\) binding and participates in oligomerization (Djordjevic et al., 1995). Thus, the C-terminal GFP epitope of the cloned \textit{acd}1 constructs was anticipated to sterically hinder substrate binding and enzyme assembly, and was removed to evaluate its activity. Similarly, the BCKAD enzyme complex, encoded by \textit{bkdA1}, \textit{bkdA2}, \textit{bkdB}, and \textit{lpdV} forms a complex heteroligomer with octahedral symmetry with as many as 60 subunits (Ævarsson et al., 2000). This large enzyme oxidatively decarboxylates branched short-chain \( \alpha \)-ketoacids in a 3-stage reaction and is the first committed step of branched chain amino acid degradation. Given the intricacy of the enzyme complex, we removed the GFP epitope to preserve activity.

We amplified the (mt)\textit{acd}1 gene cassettes without the GFP epitope and created constructs that were anticipated to oligomerize to form active ACD (pYES-mtacd/pYES-acd). After 7 h and 24 h of induction and expression in \textit{S. cerevisiae} CKY263 with 2% galactose, cells were harvested, lysed and assayed for activity. Both cytoplasmic and mitochondrial constructs displayed activity above background suggesting routes to carboxylic acid biomonomers from bacterial valine catabolism are potentially feasible. The presence of the mt leader peptide was shown to delay expression of functional ACD, possibly due to the energy intensive and dynamic nature of mitochondrial translocation (Fig. 4A). Unlike proteins which are readily translated and folded in the cytoplasm, mitochondrial proteins must be maintained in an unfolded state, actively pumped across the mitochondrial membranes, and then folded correctly and oligomerized (if necessary) before the enzyme is active against substrate (Neupert and Herrmann, 2007). Thus, the nascent energy reserves of early exponential phase may create a bottleneck in translocation, reducing mitochondrial protein activity relative to the cytoplasmic variant at early times as seen in Fig. 4A. However, at 24 h and beyond, activity of mitochondrial ACD surpasses that of its cytoplasmic variant by up to 138\% (Fig. 4A). This observation is reproduced in multiple strains (CKY263 and the vacuolar proteases deficient BJ5464), in the presence of multiple plasmids, across vector backbones (pYES vs pRS316), and across induction times (Fig. 4). Experiments performed in triplicate in BJ5464 [p(mt)\textit{ACD1} p(mt)\textit{BCKAD4} p(mt)]

![Image](https://via.placeholder.com/150)

**Fig. 3.** Valine degradation pathway genes are directed to the yeast mitochondria with the mt leader peptide. \textit{S. cerevisiae} CKY263 cells were induced for 24 h with galactose before being stained with the Mito-ID Red kit and visualized by confocal microscopy (Materials and Methods). A) \textit{acd}1; B) \textit{bkdA1}; C) \textit{bkdA2}; D) \textit{bkdB}; E) \textit{lpdV}. F) fluorescence (-) control – bgA G) Mitochondrial fluorescence (+) control - mtGFP. All images were taken under comparable gain and PMT voltage settings to allow for direct comparison of pixel intensities.
CoA2] at 24 h replicate this result with cytoplasmic cultures showing activity indistinguishable from background while mitochondrial cultures display 1.14 ± 0.86 U ACD activity per 10⁸ cells. When coupled with the fluorescence data of Fig. 3A, this observation is even more remarkable. Despite the reduced fluorescence and lower expression levels of mitochondrial protein, mitochondrially targeted ACD is more active than cytoplasmic ACD. That is, the specific activity of mitochondrially expressed ACD is higher than cytoplasmic expression variants. This observation is even more striking given the diffuse cytoplasmic expression of this enzyme in its native prokaryotic host. One hypothesis for this behavior is the increased local concentrations of acd1 monomer and/or substrate (Avalos et al., 2013) stabilize the protein and favor ACD oligomerization leading to a higher specific activity. Moreover, the reduced number of protein degradation pathways within the mitochondria (Käser and Langer, 2000), and abundance of chaperonins, may confer additional protein stability that further improves activity.

Similar experiments with the first committed step of valine catabolism from P. aeruginosa, (mt)BCKAD [(mt)bkdA1, (mt)bkdA2, (mt)bkdB, (mt)lpdV] show heterologous activity in yeast despite the presence of native homologs (Richard Dickinson, 2000) (Fig. 5). That is, bacterial valine catabolism may be used in yeast to produce precursors for isobutanol and biomonomer production. After 48 h induction in S. cerevisiae BJ5464, cytoplasmic and mitochondrial expression of the heterooligomeric BCKAD enzyme complex yield comparable activity. Like ACD, mitochondrial expression of these subunits is dramatically reduced relative to the cytoplasm (Figs. 3B–3E). However, proper folding and assembly of this large oligomeric complex appears to be favored in the mitochondrial matrix leading to comparable total activities, or a higher mitochondrial specific activity. As seen here, heterologous expression in a compartment different from the native host may be more optimal for enzyme specific activity of an engineered pathway. Future engineering efforts with this pathway are, thus, more likely to be successful by pursuing a mitochondrial-expression approach.

4. Conclusions

In these studies, we evaluated the feasibility of bacterial valine assimilation in non-pathogenic yeast as an alternate route to monomers and alcohols such as isobutanol, an advanced biofuel. We expressed the first two enzymes of the pathway, BCKAD and ACD, and confirmed that they could be heterologously expressed in yeast and targeted to either the cytoplasm or mitochondria for expression. Although heterologous mitochondrial protein expression was lower than cytoplasmic expression, the total enzyme activity was the same if not greater in the mitochondria. Countertuitively, the choice of heterologous expression compartment need not be limited by the ones used by the native host as our results demonstrate that non-native environments can improve specific activity. More specifically, the data suggests that the mitochondria is a more optimal environment than the yeast cytosol for folding and assembly of cytoplasmic bacterial valine catabolism. This benefit is likely multiplicative with the increased local enzyme and precursor concentration (Avalos et al., 2013) for future isobutanol and biomonomer production efforts. More broadly, however, our results underscore the importance of the cellular environment in heterologous protein expression and highlights a key parameter to be optimized in recombinant protein expression.
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