A synthetic biosensor to detect peroxisomal acetyl-CoA concentration for compartmentalized metabolic engineering

Herbert M Huttanus [1], **Ryan S Senger** [Corresp. 1, 2]

[1] Department of Biological Systems Engineering, Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, Virginia, United States

[2] Department of Chemical Engineering, Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, Virginia, United States

Corresponding Author: Ryan S Senger
Email address: senger@vt.edu

Background. Sub-cellular compartmentalization is used by cells to create favorable microenvironments for various metabolic reactions. These compartments concentrate enzymes, separate competing metabolic reactions, and isolate toxic intermediates. Such advantages have been recently harnessed by metabolic engineers to improve the production of various high-value chemicals via compartmentalized metabolic engineering. However, measuring sub-cellular concentrations of key metabolites represents a grand challenge for compartmentalized metabolic engineering. **Methods.** To this end, we developed a synthetic biosensor to measure a key metabolite, acetyl-CoA, in a representative compartment of yeast, the peroxisome. This synthetic biosensor uses highly efficient enzyme re-localization via PTS1 signal peptides to construct a metabolic pathway in the peroxisome, which converts acetyl-CoA to polyhydroxybutyrate (PHB) via three enzymes, phaA phaB and phaC. The PHB is then quantified by HPLC. **Results.** The biosensor demonstrated the difference in relative peroxisomal acetyl-CoA availability under various culture conditions and was also applied to screening a library of single knockout yeast mutants. The screening identified several mutants with drastically reduced peroxisomal acetyl-CoA and one with potentially increased levels. We expect our synthetic biosensors can be widely used to investigate sub-cellular metabolism and facilitate the "design-build-test" cycle of compartmentalized metabolic engineering.

1 # A Synthetic Biosensor to Detect Peroxisomal Acetyl-CoA
2 # Concentration for Compartmentalized Metabolic
3 # Engineering

5 Herbert M. Huttanus[1], Ryan S. Senger[1,2]
6
7 [1]Department of Biological Systems Engineering, Virginia Polytechnic Institute and State
8 University, Blacksburg, VA, USA
9 [2]Department of Chemical Engineering, Virginia Polytechnic Institute and State University,
10 Blacksburg, VA, USA
11
12 Corresponding Author:
13 Ryan S. Senger[1,2]
14 Department of Biological Systems Engineering, Virginia Tech, 1230 Washington St., 301C
15 HABB1, Blacksburg, VA, 24061, USA
16 Email address: senger@vt.edu

17

18 **Keywords:** acetyl-CoA, biosensor, compartmentalization, peroxisome, quantification
19
20 **Abbreviations: CoA,** coenzyme A; **DCW,** dry cell weight; **HPLC,** high performance liquid
21 chromatography; **PHB,** polyhydroxybutyrate; **PTS,** peroxisomal targeting sequence

22

Abstract

Background. Sub-cellular compartmentalization is used by cells to create favorable microenvironments for various metabolic reactions. These compartments concentrate enzymes, separate competing metabolic reactions, and isolate toxic intermediates. Such advantages have been recently harnessed by metabolic engineers to improve the production of various high-value chemicals via compartmentalized metabolic engineering. However, measuring sub-cellular concentrations of key metabolites represents a grand challenge for compartmentalized metabolic engineering.

Methods. To this end, we developed a synthetic biosensor to measure a key metabolite, acetyl-CoA, in a representative compartment of yeast, the peroxisome. This synthetic biosensor uses highly efficient enzyme re-localization via PTS1 signal peptides to construct a metabolic pathway in the peroxisome, which converts acetyl-CoA to polyhydroxybutyrate (PHB) via three enzymes, phaA phaB and phaC. The PHB is then quantified by HPLC.

Results. The biosensor demonstrated the difference in relative peroxisomal acetyl-CoA availability under various culture conditions and was also applied to screening a library of single knockout yeast mutants. The screening identified several mutants with drastically reduced peroxisomal acetyl-CoA and one with potentially increased levels. We expect our synthetic biosensors can be widely used to investigate sub-cellular metabolism and facilitate the "design-build-test" cycle of compartmentalized metabolic engineering.

Introduction

Sub-cellular compartmentalization is used by all eukaryotes and some prokaryotes as a means to create favorable micro-environments for various metabolic reactions. These compartments concentrate enzymes and substrates(Choi & Montemagno, 2006), increasing efficiency. Compartmentalization also helps to keep incompatible pathways separate(Zecchin et al., 2015) and can provide a safe area for toxic intermediates. Recently, metabolic engineers have explored the concept of pathway compartmentalization to enhance the performance of metabolic pathways(Avalos, Fink & Stephanopoulos, 2013; Zecchin et al., 2015; Zhou et al., 2016).

One challenge to the continued progress of compartmentalized metabolic engineering is the lack of tools for measuring local metabolite concentrations at a sub-cellular scale. There are many proprietary colorimetric and fluorescent assays (such as glucose peroxidase assays(Ngo & Lenhoff, 1980) or enzymatic acetyl-CoA assays(Fritz et al., 2013)) but these *in vitro* methods often require cell lysis. Cell fractionation involves separating cell compartments of interest beforehand, but contamination with similarly sized organelles is possible following differential or density gradient centrifugation(Kikuchi et al., 2004). *In vivo* biosensors are another popular method for screening the response of metabolites to various perturbations. This technique often involves the use of transcription factors that recognize the analyte and activate bioluminescence or fluorescence reporter genes(Michener et al., 2012; Mustafi et al., 2012; Brognaux et al., 2013; Li & Yu, 2015; Mahr et al., 2016; Skjoedt et al., 2016). This, generally, means that the detection of the analyte largely occurs in the cytoplasm or nucleus.

To provide a tool for measuring metabolite availability in sub-cellular compartments, we propose using a localized synthetic biosensor as a form of metabolic assay. In this method, heterologous enzymes are expressed with localization tags to produce some reporter compound only in the compartment of interest. With high levels of enzyme expression, we hypothesize that the production of the reporter compound will be rate-limited by the substrate concentration, allowing for relative quantification of the substrate. The reporter compound can then be extracted from the cell and quantified. Here, we showcase the use of one such synthetic biosensor to measure acetyl-CoA in the peroxisomes of the yeast *Saccharomyces cerevisiae*. Acetyl-CoA is a core metabolite whose availability has implications in many important metabolic pathways. Measuring total, cellular acetyl-CoA levels is a simple matter using commercially available enzymatic assay kits(Jose & Suraishkumar, 2016), but measuring acetyl-CoA in organelles is more challenging since conventional assays would require the organelles to first be isolated by cell fractionation. Polyhydroxybutyrate (PHB) production can also be used as an indirect marker of acetyl-CoA availability. Only three enzymes are needed for the conversion of acetyl-CoA into PHB(Wang & Yu, 2007) and they can be efficiently localized to the peroxisome by inclusion of C-terminal PTS1 peptide tags(Kim & Hettema, 2015). Acetyl-CoA does not readily cross membranes(Chen, Siewers & Nielsen, 2012), so the peroxisome-localized enzymes should only

83 have access to the peroxisomal pool of acetyl-CoA (*Fig. 1*). In this paper, a localized enzymatic
84 assay and HPLC analysis of the cellular PHB content were used to qualify the response of acetyl-
85 CoA in the peroxisome to several culturing conditions and gene knockout perturbations.
86

Materials and methods

Media and strains

91 Minimal media was prepared as previously reported(Blank & Sauer, 2004; Guo et al., 2016) with
92 a glucose concentration of 0.5% w/v. Yeast nitrogenous media YN contains 0.67% w/v yeast
93 nitro base, 0.1% yeast extract and 0.5% glucose. Synthetic complete media contained 0.17%
94 yeast nitro base, 0.5% ammonium sulfate, 0.5% glucose and 1x CSM Ura supplement from MP
95 biomedicals. All cultures were incubated at 30°C in a shaking incubator. The experiment testing
96 for the effect of media composition used 10 mL cultures each and the mutant screening cultures
97 were 3 mL cultures each from which only 2 mL was harvested at the end of culturing. The
98 growth/production curve experiments used 20 mL cultures from which 2 mL were drawn at each
99 time point. All of the homologous recombinations were performed in *Saccharomyces cerevisiae*
100 Invitrogen strain INVSc1. Plasmids were transferred to *E. coli* strain Top10 for verification and
101 long-term storage. All cultures used to collect and measure PHB were performed in
102 *Saccharomyces cerevisiae* strain BY4741 or BY4741-derived single gene knockouts.
103

Cloning

106 All plasmids, strains, and primers are shown in Supplementary Methods *Table S1*. The three *pha*
107 genes were codon-optimized for yeast and kindly provided by Jens Nielsen(Kocharin et al.,
108 2012). Construction of the plasmids pPHBc and pPHBp was accomplished by an established,
109 yeast homologous recombination-based method, DNA assembler(Shao & Zhao, 2009). Briefly,
110 DNA fragments were amplified by PCR using primers that add homologous overlap between
111 adjacent fragments. The fragments were then co-transformed into *S. cerevisiae* along with a
112 linearized backbone to assemble elements in a single step(Shao, Luo & Zhao, 2011). Gene
113 fragments used to construct plasmid pPHBp used modified primers for the addition of PTS1
114 localization tags(Liu & Naismith, 2008). Additional details are provided in the Supporting
115 Information.
116

HPLC analysis

119 Culture samples of 10 mL were centrifuged at 4000 rpm for five minutes, and rinsed twice with
120 10 mL of ddH$_2$0. Pellets were then dried for 48 hours at 70°C. PHB is insoluble, so it is regularly
121 converted to crotonic acid monomers by sulfuric acid digestion(Karr, Waters & Emerich, 1983).
122 The pulverized dry pellets (or thin films for smaller samples) were digested with 100 μL of

123   concentrated H_2SO_4 per mL of the pre-dried sample. Acid digests were carried out at 95°C for 1
124   hour. The digest was diluted with 400 μL of ddH_20 for every 100 μL of acid used. The
125   carbonified cell debris was removed by centrifugation at 13,000 rpm for 10 min. These samples
126   were analyzed by HPLC with an Aminex HPX-87H ion exclusion column with a temperature of
127   60°C and a flow rate of 0.6 mL min-1 and with 5 mM H_2SO_4 as the mobile phase.
128

129   **Results**

130

131   **Dependence on fatty acids**

132

133   To test the viability of the localized synthetic biosensor, a proof-of-concept study was performed
134   in which yeast cells with PHB-producing genes (with or without peroxisomal localization) were
135   analyzed to compare acetyl-CoA levels in the cytosol and peroxisome. PHB production was
136   achieved by expressing three enzymes from *Ralstonia eutropha* H16. The first enzyme (*phaA*)
137   consumed two acetyl-CoA to produce acetoacetyl-CoA. The next enzyme (*phaB*) reduced the
138   acetoacetyl-CoA in order to generate 3-hydroxybutyric-CoA monomers. The monomers were
139   then polymerized by the final enzyme (*phaC*)(Wang & Yu, 2007). These genes were previously
140   codon-optimized for use in yeast cells(Kocharin et al., 2012). All three genes were combined in
141   each of two plasmids. Plasmid pPHBc contained the codon-optimized genes without peroxisomal
142   localization and thus the enzymes end up in the cytoplasm. Plasmid pPHBp contained the same
143   three enzymes with high efficiency peroxisomal localization tags(DeLoache, Russ & Dueber,
144   2016) added by homologous recombination(Joska et al., 2014). We chose PHB as our reporter
145   compound because of the following two considerations. First, PHB is detected relatively easily
146   and is non-native to yeast cells. Therefore, the PHB signal is representative of peroxisomal
147   acetyl-CoA concentration. Second, the PHB pathway is relatively short, with only three
148   enzymes, which reduces the risk of metabolic burden.

149

150   To validate our hypothesis that peroxisomal acetyl-CoA could lead to PHB production, we
151   cultured the peroxisomal and cytosolic variants of the PHB producing strains (phbP and phbC
152   respectively) in various media with various concentrations of oleic acid for 48 hours. Three
153   media were used: synthetic complete yeast media (SC), minimal media, and yeast nitro base
154   (YN) media. Oleic acid content ranging from 0%, 0.2% and 0.5% w/v was added into each of the
155   media. The cytosolic *pha* genes were able to produce similar levels of PHB with any of the
156   media and at all of the oleic acid concentrations present (*Fig. 2A*). No PHB was produced by the
157   peroxisomal strain phbP when oleic acid was absent from the media (*Fig. 2B*). Also, we found
158   that with the increase of oleic acid concentration in the YN media, the PHB production also
159   increased.

160

161   **Growth and production curves**

162

163 Both strains were then cultured in YN media with 0.5% oleic acid and sampled periodically until
164 PHB production plateaued (*Fig. 3*). Strain phbC achieved a maximal PHB level of around 30
165 mg/g DCW within only 24 hours, which was the same amount of time it took to achieve a
166 maximum cell density (~5 O.D.). Strain phbP reached the same cell density in those 24 hours,
167 but only started producing PHB after that point. PHB production in the phbP strain reached a
168 maximum of about 7 mg/g DCW after five days.
169
170 **Knockout screening**
171
172 We demonstrated the application of our compartmentalized biosensor by screening several
173 mutants for altered levels of peroxisomal acetyl-CoA. In brief, thirteen yeast single knockout
174 mutants were selected and transformed with pPHBp. Three of the genes, *adr1, aat2* and *ino1* are
175 transcription factors that promote fatty acid synthesis or fatty acid utilization. Three of the genes,
176 *rpd3*, *opi1* and *sin3* are transcription factors that repress fatty acid synthesis. The genes *pot1* and
177 *pox1* were selected as integral enzymes required for beta-oxidation. Genes *pex5* and *pex7* are
178 peroxisome transport proteins responsible for import of peroxisome-specific proteins. The gene
179 *inp2* was selected for its role in peroxisomal inheritance and the two genes *slt2* and *atg36* were
180 selected for their role in pexophagy. The peroxisomal *pha* enzymes were expressed in all thirteen
181 knockout mutants and the wild-type yeast for five days in YN media at varied concentrations of
182 oleic acid (*Fig. 4*). Beta oxidation gene knockouts Δ*pot1*, and Δ*pox1*, as well as peroxisomal
183 transport protein knockouts Δ*pex5* and Δ*pex7* exhibited dramatic decreases in PHB production.
184 Knockout strain Δ*aat2* showed relatively little change relative to the wild type while Δ*adr1*
185 exhibited decreased PHB production(*Fig. 4a*). None of the three fatty acid synthesis repressor
186 knockouts (*Δrpd3*, *Δsin3* and *Δopi1*) had a significant effect on PHB levels at 0.2% or 0% oleate
187 concentrations but *Δsin3* and *Δopi1* exhibited decreased production of PHB at 0.5% oleate
188 relative to the wild type.
189
190
191 **Discussion**
192
193 The compartmentalized peroxisomal biosensor for acetyl-CoA was constructed and
194 demonstrated (*Fig. 2*). The cytosolic variant of the PHB pathway was not significantly affected
195 by oleate concentrations because acetyl-CoA in the cytosol was mostly derived from glucose.
196 The peroxisomal variant of the PHB pathway, however, demonstrated a strong correlation
197 between oleic acid content and PHB production, because acetyl-CoA in the peroxisome is mostly
198 derived from beta-oxidation of fatty acids such as oleic acid(Chen, Siewers & Nielsen, 2012).
199 There was also no detectable PHB produced from the peroxisomal pathway in *Fig. 2* when oleic
200 acid was absent, suggesting minimal leakage of cytosolic acetyl-CoA into the peroxisome.
201 Therefore, it is appropriate to use PHB production from the compartmentalized biosensor to
202 indicate the peroxisomal acetyl-CoA level.

203

204 Even with similar levels of glucose and oleic acid, the peroxisomal variant strain, phbP did not

205 produce as much PHB as the cytosolic variant, phbC, within 48 hours *(Fig. 2)*, so it was

206 hypothesized that two days of incubation was not long enough for the yeast cells to fully switch

207 from glucose utilization to fatty acid utilization(Gurvitz & Rottensteiner, 2006). Growth and

208 production curves, shown in *Fig. 3* demonstrate that PHB production in the phbP strain started

209 only after 24 hours, which is the same amount of time required to reach stationary phase. This

210 suggests that the yeast cells used glucose for cellular growth until it was depleted, then switched

211 to beta-oxidation of oleic acid. However, the strain phbP was never able to generate as much

212 PHB as the cytosolic control, phbC, even when given similar quantities of oleic acid and glucose

213 and ample time to utilize oleic acid.

214

215 The compartmentalized biosensor was then applied to screening several single-gene knockouts

216 for altered acetyl-CoA availability in the peroxisome. As expected, Δ*pot1*, Δ*pox1*, Δ*pex5* and

217 Δ*pex7* all exhibited dramatic decreases in peroxisomal PHB production as those genes are

218 necessary for beta oxidation. Similarly, Δ*adr1* reduced PHB production because of its role in

219 activating fatty acid utilization. It is puzzling, however, that the Δ*pex5* strain did not exhibit high

220 levels of cytosolic PHB production. Since pex5 is a transport protein needed for peroxisomal

221 localization of PTS1-tagged proteins, it was expected that the biosensor enzymes would

222 accumulate in the cytosol as has been observed for PTS1 tagged yellow fluorescent

223 protein(DeLoache, Russ & Dueber, 2016). However, the *pex5* knockouts in this study did not

224 perform similarly to the strains using enzymes without PTS1 tags. The reason for this is still

225 unclear.

226

227 Other knockouts were predicted to increase peroxisomal acetyl-CoA. Fatty acids generated by

228 the cell could be used to supplement the exogenous source of oleic acid for beta-oxidation. As

229 repressors of fatty acid synthesis, Δ*rpd3*, Δ*sin3* and Δ*opi1* had potential to increase PHB levels

230 by providing additional fatty acids from the cytosol to be digested in the peroxisome. However,

231 no dramatic increase in PHB was observed for these knockouts.

232

233 Unlike the two-day cultures used for *Fig. 2*, the five-day cultures in the knockout experiments

234 showed low levels of PHB production even in the absence of oleate. This could be caused by

235 acetate being produced and converted to acetyl-CoA in the peroxisome by the pyruvate

236 dehydrogenase bypass after the glucose had been depleted(Nielsen, 2014). PHB might also be

237 produced by low, native availability of acetoacetyl-CoA and 3-hydroxybutyric-CoA

238 intermediates. In a study by Leaf et al., yeast cells with only the exogenous phaC enzyme were

239 able to produce PHB, albeit at only 1 mg/g DCW(Leaf et al., 1996). Intermediates produced in

240 this manner would still be positively correlated to acetyl-CoA availability in the peroxisome, and

241 thus, do not interfere with the intended function of the biosensor.

242

243   Overall, this study illustrates only one specific application of a compartmentalized biosensor, but
244   the strategy could be applied to other metabolites in other compartments. For example, the
245   concentrations of acetyl-CoA in the mitochondria have been estimated to be 20-30 times higher
246   than in the cytosol(Weinert et al., 2017) and a recent study involved re-localizing heterologous
247   enzymes for the production of valencene to the mitochondria of *S. cerevisiae* via targeting signal
248   peptides resulting in increased production compared to cytosolic expression of those same
249   enzymes(Farhi et al., 2011). Further optimization of this localized pathway could benefit from a
250   biosensor similar to ours to detect the critical precursor, farnesyl diphosphate (FDP). FDP can be
251   detected using a fluorescent enzymatic assay(Dozier & Distefano, 2012) and
252   compartmentalization of this assay could be accomplished by introducing N-terminal
253   mitochondrial localization signal (MLS) tags such as from subunit IV of the yeast cytochrome C
254   oxidase (CoxIV) to the enzymes of the fluorescent assay(Avalos, Fink & Stephanopoulos, 2013).
255

Conclusions

257
258   We developed an assay that used three compartmentalized enzymes to convert peroxisomal
259   acetyl-CoA to a reporter compound, PHB. By quantifying PHB levels, we successfully applied
260   this compartmentalized biosensor to screen a library of yeast mutants and identified knockouts
261   with drastically impaired PHB synthesis, which suggest the possibility that overexpression of
262   some those genes might result in increased PHB production. Metabolic engineering of
263   compartmentalized pathways requires measurement tools for pathway design and evaluation.
264   Traditional methods for measuring metabolite concentrations are generally limited to cytosolic or
265   whole-cell sources of the metabolite. In contrast, as shown in this study, compartmentalized
266   biosensors could be a valuable addition to current synthetic toolkits on analyzing intracellular
267   metabolites. We anticipate that the compartmentalized biosensors can be used to better
268   understand the compartmentalized metabolism in various organelles and facilitate "design-build-
269   test" cycle of compartmentalized metabolic engineering.
270

References

Avalos JL, Fink GR, Stephanopoulos G. 2013. Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols. *Nature Biotechnology* 31:335–341. DOI: 10.1038/nbt.2509.

Blank LM, Sauer U. 2004. TCA cycle activity in Saccharomyces cerevisiae is a function of the environmentally determined specific growth and glucose uptake rates. *Microbiology* 150:1085–1093. DOI: 10.1099/mic.0.26845-0.

Brognaux A, Thonart P, Delvigne F, Neubauer P, Twizere J, Francis F, Gorret N. 2013. Direct and indirect use of GFP whole cell biosensors for the assessment of bioprocess performances: Design of milliliter scale-down bioreactors. *Biotechnology Progress* 29:48–59. DOI: 10.1002/btpr.1660.

Chen Y, Siewers V, Nielsen J. 2012. Profiling of cytosolic and peroxisomal acetyl-CoA metabolism in Saccharomyces cerevisiae. *PLoS One* 7:e42475. DOI: 10.1371/journal.pone.0042475.

Choi H-J, Montemagno CD. 2006. Biosynthesis within a bubble architecture. *Nanotechnology* 17. DOI: 10.1088/0957-4484/17/9/020.

DeLoache WC, Russ ZN, Dueber JE. 2016. Towards repurposing the yeast peroxisome for compartmentalizing heterologous metabolic pathways. *Nat Commun* 7:11152. DOI: 10.1038/ncomms11152.

Dozier JK, Distefano MD. 2012. An enzyme-coupled continuous fluorescence assay for farnesyl diphosphate synthases. *Anal Biochem* 421:158–63. DOI: 10.1016/j.ab.2011.10.038.

Farhi M, Marhevka E, Masci T, Marcos E, Eyal Y, Ovadis M, Abeliovich H, Vainstein A. 2011. Harnessing yeast subcellular compartments for the production of plant terpenoids. *Metabolic Engineering* 13:474–481. DOI: 10.1016/j.ymben.2011.05.001.

Fritz V, Benfodda Z, Henriquet C, Hure S, Cristol JP, Michel F, Carbonneau MA, Casas F, Fajas L. 2013. Metabolic intervention on lipid synthesis converging pathways abrogates prostate cancer growth. *Oncogene* 32:5101–10. DOI: 10.1038/onc.2012.523.

Guo W, Chen Y, Wei N, Feng X. 2016. Investigate the Metabolic Reprogramming of Saccharomyces cerevisiae for Enhanced Resistance to Mixed Fermentation Inhibitors via 13C Metabolic Flux Analysis. *PLoS One* 11:e0161448. DOI: 10.1371/journal.pone.0161448.

Gurvitz A, Rottensteiner H. 2006. The biochemistry of oleate induction: transcriptional upregulation and peroxisome proliferation. *Biochim Biophys Acta* 1763:1392–402. DOI: 10.1016/j.bbamcr.2006.07.011.

Jose S, Suraishkumar GK. 2016. High carbon (CO_2) supply leads to elevated intracellular acetyl CoA levels and increased lipid accumulation in Chlorella vulgaris. *Algal Research* 19:307–315. DOI: 10.1016/j.algal.2016.08.011.

309   Joska TM, Mashruwala A, Boyd JM, Belden WJ. 2014. A universal cloning method based on
310   yeast homologous recombination that is simple, efficient, and versatile. *J Microbiol*
311   *Methods* 100:46–51. DOI: 10.1016/j.mimet.2013.11.013.
312   Karr DB, Waters JK, Emerich DW. 1983. Analysis of Poly-β-Hydroxybutyrate in Rhizobium
313   japonicum Bacteroids by Ion-Exclusion High-Pressure Liquid Chromatography and UV
314   Detection †. *Appl Environ Microbiol* 46:1339–44.
315   Kikuchi M, Hatano N, Yokota S, Shimozawa N, Imanaka T, Taniguchi H. 2004. Proteomic
316   analysis of rat liver peroxisome: presence of peroxisome-specific isozyme of Lon
317   protease. *J Biol Chem* 279:421–8. DOI: 10.1074/jbc.M305623200.
318   Kim PK, Hettema EH. 2015. Multiple pathways for protein transport to peroxisomes. *J Mol Biol*
319   427:1176–90. DOI: 10.1016/j.jmb.2015.02.005.
320   Kocharin K, Chen Y, Siewers V, Nielsen J. 2012. Engineering of acetyl-CoA metabolism for the
321   improved production of polyhydroxybutyrate in Saccharomyces cerevisiae. *AMB Express*
322   2:52. DOI: 10.1186/2191-0855-2-52.
323   Leaf TA, Peterson MS, Stoup SK, Somers D, Srienc F. 1996. Saccharomyces cerevisiae
324   expressing bacterial polyhydroxybutyrate synthase produces poly-3-hydroxybutyrate.
325   *Microbiology* 142 (Pt 5):1169–80. DOI: 10.1099/13500872-142-5-1169.
326   Li Y, Yu Z. 2015. Construction and evaluation of a genetic construct for specific detection and
327   measurement of propionate by whole-cell bacteria. *Biotechnology and Bioengineering*
328   112:280–287. DOI: 10.1002/bit.25358.
329   Liu H, Naismith JH. 2008. An efficient one-step site-directed deletion, insertion, single and
330   multiple-site plasmid mutagenesis protocol. In: *BMC Biotechnol*. 91. DOI: 10.1186/1472-
331   6750-8-91.
332   Mahr R, von Boeselager RF, Wiechert J, Frunzke J. 2016. Screening of an Escherichia coli
333   promoter library for a phenylalanine biosensor. *Applied Microbiology and Biotechnology*
334   100:6739–6753. DOI: 10.1007/s00253-016-7575-8.
335   Michener JK, Thodey K, Liang JC, Smolke CD. 2012. Applications of genetically-encoded
336   biosensors for the construction and control of biosynthetic pathways. *Metabolic*
337   *Engineering* 14:212–222. DOI: 10.1016/j.ymben.2011.09.004.
338   Mustafi N, Grünberger A, Kohlheyer D, Bott M, Frunzke J. 2012. The development and
339   application of a single-cell biosensor for the detection of l-methionine and branched-
340   chain amino acids. *Metabolic engineering* 14:449–457. DOI:
341   10.1016/j.ymben.2012.02.002.
342   Ngo TT, Lenhoff HM. 1980. A sensitive and versatile chromogenic assay for peroxidase and
343   peroxidase-coupled reactions. *Anal Biochem* 105:389–97.
344   Nielsen J. 2014. Synthetic Biology for Engineering Acetyl Coenzyme A Metabolism in Yeast.
345   In: *mBio*. DOI: 10.1128/mBio.02153-14.
346   Shao Z, Luo Y, Zhao H. 2011. Rapid characterization and engineering of natural product
347   biosynthetic pathways via DNA assembler. *Mol Biosyst* 7:1056–9. DOI:
348   10.1039/c0mb00338g.

349   Shao Z, Zhao H. 2009. DNA assembler, an in vivo genetic method for rapid construction of
350   biochemical pathways. *Nucleic Acids Res* 37:e16. DOI: 10.1093/nar/gkn991.
351   Skjoedt ML, Snoek T, Kildegaard KR, Arsovska D, Eichenberger M, Goedecke TJ, Rajkumar
352   AS, Zhang J, Kristensen M, Lehka BJ, Siedler S, Borodina I, Jensen MK, Keasling JD.
353   2016. Engineering prokaryotic transcriptional activators as metabolite biosensors in
354   yeast. *Nature Chemical Biology* 12:951–958. DOI: 10.1038/nchembio.2177.
355   Wang J, Yu HQ. 2007. Biosynthesis of polyhydroxybutyrate (PHB) and extracellular polymeric
356   substances (EPS) by Ralstonia eutropha ATCC 17699 in batch cultures. *Appl Microbiol*
357   *Biotechnol* 75:871–8. DOI: 10.1007/s00253-007-0870-7.
358   Weinert BT, Denmark TNC for PRF of HSU of CC, Iesmantavicius V, Denmark TNC for PRF
359   of HSU of CC, Moustafa T, Austria I of MBU of GG, Schölz C, Denmark TNC for PRF
360   of HSU of CC, Wagner SA, Denmark TNC for PRF of HSU of CC, Magnes C, Austria
361   H– I for B and HSJRG, Zechner R, Austria I of MBU of GG, Choudhary C, Denmark
362   TNC for PRF of HSU of CC. 2017. Acetylation dynamics and stoichiometry in
363   Saccharomyces cerevisiae. *Molecular Systems Biology* 10. DOI: 10.1002/msb.134766.
364   Zecchin A, Stapor PC, Goveia J, Carmeleit P. 2015. Metabolic pathway compartmentalization:
365   an underappreciated opportunity? *Current Opinion in Biotechnology* 34:73–81. DOI:
366   10.1016/j.copbio.2014.11.022.
367   Zhou YJ, Buijs NA, Zhu Z, Gomez DO, Boonsombuti A, Siewers V, Nielsen J. 2016.
368   Harnessing Yeast Peroxisomes for Biosynthesis of Fatty-Acid-Derived Biofuels and
369   Chemicals with Relieved Side-Pathway Competition. *J Am Chem Soc* 138:15368–15377.
370   DOI: 10.1021/jacs.6b07394.
371
372
373

Figure 1

Figure 1 . Mechanism of the localized biosensor.

The three enzymes phaA, phaB and phaC were expressed from a plasmid and contained enhanced PTS1 localization tags. The tags were rapidly recognized by the pex5 protein that shuttled the enzymes into the peroxisome. There, the enzymes converted peroxisomal acetyl-CoA into PHB which was subsequently extracted and detected by HPLC.
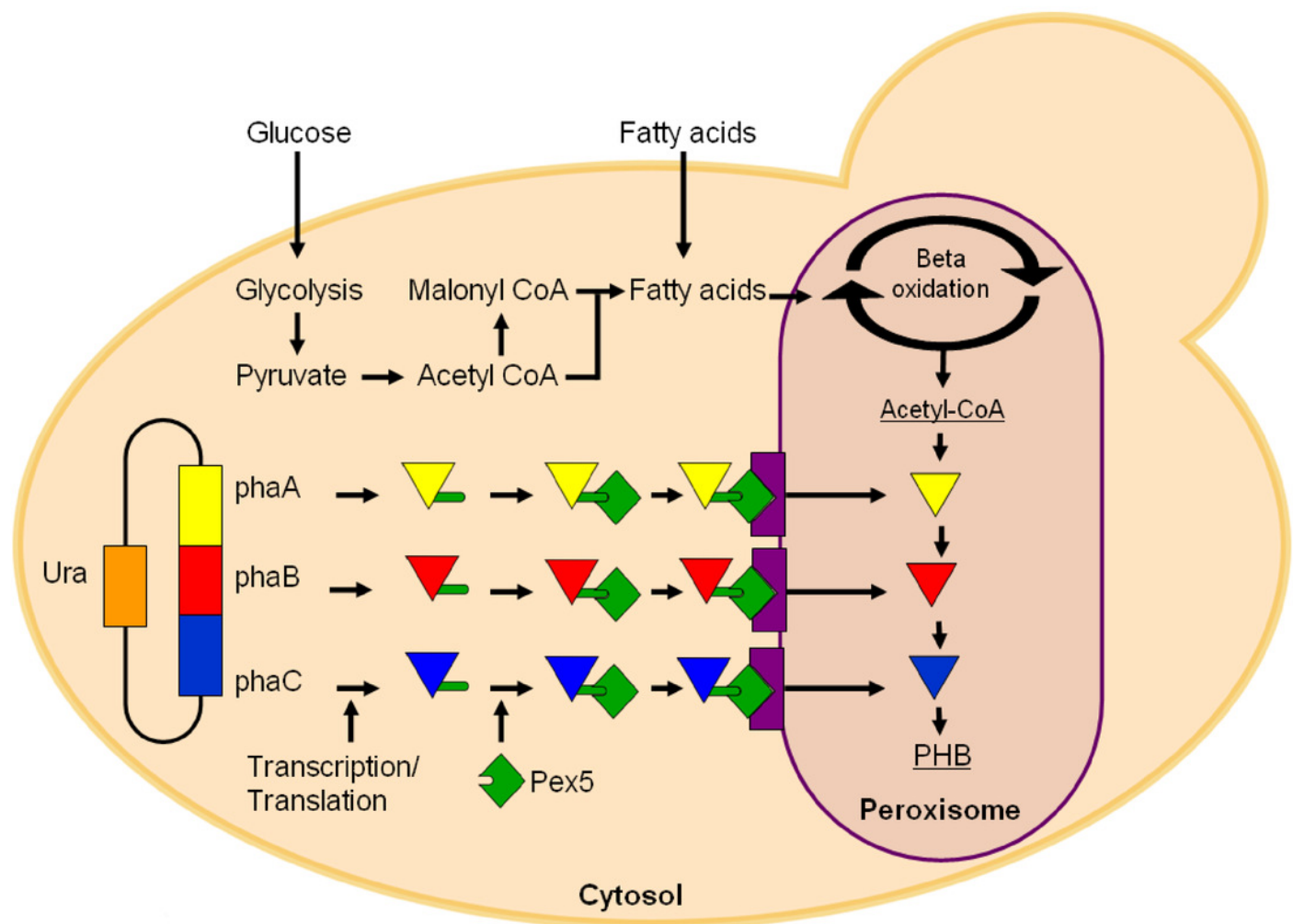
Figure 2

Figure 2. Oleic acid dependency of peroxisomal PHB.

Yeast strains with PHB producing enzymes were incubated for 48 hours in several media and with varied concentrations of oleic acid. A) Yeast strain phbC containing the three pha enzymes without enzyme localization. B) Yeast strain phbP with the three pha enzymes with localization tags. ND: Not Detectable. Error bars show estimated instrument error.
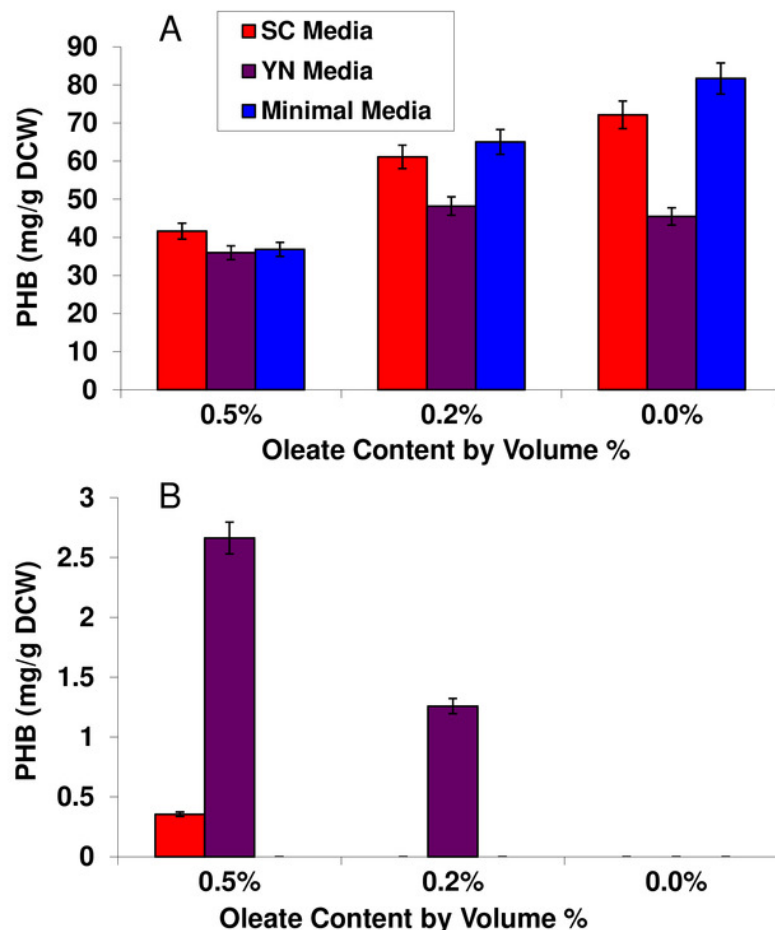
Figure 3

Figure 3. PHB production and growth curves for cytosolic and peroxisomal strains.

Both strains were cultured for five days in YN media with 0.5% oleic acid. A) PHB content vs. time for the cytosolic strain phbC. B) PHB content vs. time for phbP. C) Growth curve for the phbC. D) Growth curve for phbP. Error bars show standard deviation among triplicate cultures.
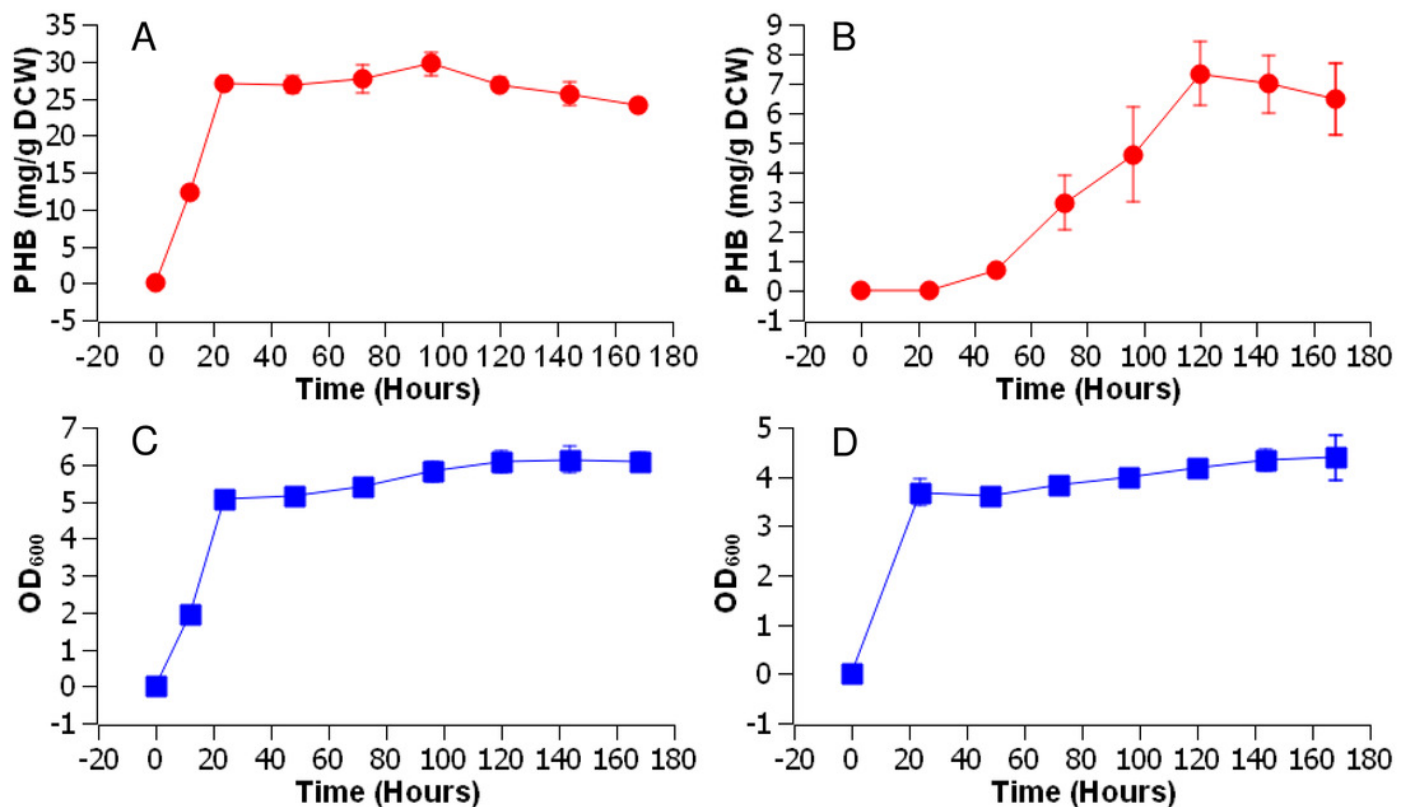
Figure 4

Figure 4. Screening of mutants using the peroxisomal acetyl-CoA assay.

Yeast knockout strains and the wild type (WT) were incubated for five days in YN media with various oleic acid concentrations. A) PHB concentration. B) Cell density (in OD_{600}) of the mutant screening cultures. ND: Not Detectable. Error bars show estimated instrument error.