Production of fatty acids in *Ralstonia eutropha* H16 by engineering β-oxidation and carbon storage

Janice S Chen, Brendan Colón, Brendon Dusel, Marika Ziesack, Jeffrey C Way, Joseph P Torella

Ralstonia eutropha H16 is a facultatively autotrophic hydrogen-oxidizing bacterium capable of producing polyhydroxybutyrate (PHB)-based bioplastics. As PHB's physical properties may be improved by incorporation of medium-chain-length fatty acids (MCFAs), and MCFAs are valuable on their own as fuel and chemical intermediates, we engineered *R. eutropha* for MCFA production. Expression of *UcFatB2*, a medium-chain-length-specific acyl-ACP thioesterase, resulted in production of 14 mg/L laurate in wild-type *R. eutropha*. Total fatty acid production (22 mg/L) could be increased up to 2.5-fold by knocking out PHB synthesis, a major sink for acetyl-CoA, or by knocking out the acyl-CoA ligase *fadD3,* an entry point for fatty acids into β-oxidation. As Δ*fadD3* mutants still consumed laurate, and because the *R. eutropha* genome is predicted to encode over 50 acyl-CoA ligases, we employed RNA-Seq to identify acyl-CoA ligases upregulated during growth on laurate. Knockouts of the three most highly upregulated acyl-CoA ligases increased fatty acid yield significantly, with one strain (Δ*A2794*) producing up to 62 mg/L free fatty acid. This study demonstrates that homologous β-oxidation systems can be rationally engineered to enhance fatty acid production, a strategy that may be employed to increase yield for a range of fuels, chemicals, and PHB derivatives in *R. eutropha.*

1 **Production of fatty acids in *Ralstonia eutropha* H16 by engineering β-oxidation and carbon**

2 **storage**

4 Janice S. Chen[1], Brendan Colón[2], Brendon Dusel[1], Marika Ziesack[2], Jeffrey C. Way[1], and Joseph

5 P. Torella[2]

7 [1]Wyss Institute for Biologically Inspired Engineering, Harvard Medical School, Boston, MA,

8 USA

9 [2]Department of Systems Biology, Harvard Medical School, Boston, MA, USA

11 Corresponding Author:

12 Jeffrey C. Way

13 Wyss Institute for Biologically Inspired Engineering, Harvard Medical School, Boston, MA,

14 02115, USA

15 Email address: Jeff.Way@wyss.harvard.edu

Abstract

25   *Ralstonia eutropha* H16 is a facultatively autotrophic hydrogen-oxidizing bacterium capable of

26   producing polyhydroxybutyrate (PHB)-based bioplastics. As PHB's physical properties may be

27   improved by incorporation of medium-chain-length fatty acids (MCFAs), and MCFAs are

28   valuable on their own as fuel and chemical intermediates, we engineered *R. eutropha* for MCFA

29   production. Expression of *UcFatB2*, a medium-chain-length-specific acyl-ACP thioesterase,

30   resulted in production of 14 mg/L laurate in wild-type *R. eutropha*. Total fatty acid production

31   (22 mg/L) could be increased up to 2.5-fold by knocking out PHB synthesis, a major sink for

32   acetyl-CoA, or by knocking out the acyl-CoA ligase *fadD3,* an entry point for fatty acids into β-

33   oxidation. As Δ*fadD3* mutants still consumed laurate, and because the *R. eutropha* genome is

34   predicted to encode over 50 acyl-CoA ligases, we employed RNA-Seq to identify acyl-CoA

35   ligases upregulated during growth on laurate. Knockouts of the three most highly upregulated

36   acyl-CoA ligases increased fatty acid yield significantly, with one strain (Δ*A2794*) producing up

37   to 62 mg/L free fatty acid. This study demonstrates that homologous β-oxidation systems can be

38   rationally engineered to enhance fatty acid production, a strategy that may be employed to

39   increase yield for a range of fuels, chemicals, and PHB derivatives in *R. eutropha.*

40

Introduction

42   *Ralstonia eutropha* H16 is a facultatively chemolithoautotrophic bacterium capable of using H_2

43   and CO_2 as sole sources of energy and carbon, respectively (1). Under conditions of carbon

44   abundance and nutrient deprivation, *R. eutropha* accumulates polyhydroxybutyrate (PHB), an

45   intracellular carbon and energy storage polymer (2). The naturally high production rate and yield

46   of PHB and PHB-derived bioplastics, coupled with the industrial scalability and facile genetics

47   of *R. eutropha*, have made it an attractive host for biofuel and industrial chemical production (3).

48   Recently, it has been shown that through the expression of heterologous enzymes, precursors in

49   PHB synthesis can be redirected to the synthesis of short-chain fusel alcohols such as

50   isopropanol and isobutanol (4-6). This work has demonstrated the plausibility of using *R.*

51   *eutropha* as a chassis for fuel and chemical production beyond PHB-based bioplastics.

52

53   The capacity of *R. eutropha* to grow autotrophically using CO_2 and H_2/formate has also made it

54   an attractive chassis organism for "electrosynthesis": the use of electricity to drive biological

55   carbon fixation for renewable chemical production (7, 8), an alternative to non-renewable oil-

56   based methods. Owing to the difficulty of electrochemically reducing CO_2 to infrastructure-

57   compatible carbon-based fuels (9), and the forty-year-old knowledge that *R. eutropha* is capable

58   of robust growth in actively electrolyzed media (10), electrosynthesis using *R. eutropha* has

59   emerged as an attractive future technology for renewable fuel and chemical production. Efforts

60   by Li and colleagues demonstrated the ability to integrate precious metal-based electrochemical

61   formate production with engineered *R. eutropha* to convert electricity and CO_2 to isobutanol and

62   3-methyl-1-butanol (5). In recent work from our lab, we coupled *R. eutropha* with scalable,

63   earth-abundant electrodes to produce isopropanol at high efficiency, and achieved biomass yields

64   comparable to those of terrestrial plants (11). These studies provide a proof of principle

65   demonstration that *R. eutropha* may be useful for the renewable production of fuels and

66   chemicals from electricity and CO_2.

67

68   While many efforts to engineer *R. eutropha* have focused on the production of bioplastics or

69   short-chain alcohols by redirecting the PHB synthesis pathway, few have focused on engineering

70   more chemically diverse products for fuel and chemical applications. Fatty acids represent a

71   broad category of important chemicals that are facile to produce from precursors in bacterial

72   metabolism (Fig. 1), and may be converted biologically or chemically into a range of biofuels

73   and valuable industrial chemicals including alcohols, alkanes, and wax esters (12-14). Moreover,

74   medium-chain fatty acids (MCFAs) may be incorporated into the PHB production pathway to

75   make copolymers with superior physical properties compared to pure PHB (15).

76

77   Recent efforts to produce fatty acids and fatty acid-related chemicals in *R. eutropha* include the

78   successful production of long-chain fatty acids and methyl ketones (16), as well as alcohols,

79   aldehydes and alkanes (17). While significant yields of methyl ketones have been achieved, the

80   fatty acid precursors to ketones have only been produced at a maximum yield of 0.017 mg/L in

81   2% fructose minimal medium and 10 mg/L in LB medium (16). This limitation is likely due to

82   extensive fatty acid degradation, or β-oxidation. *R. eutropha* expresses over 50 homologues of

83   some of its β-oxidation enzymes, far exceeding that of other model organisms such as

84   *Escherichia coli*, which contains only one or two homologues for each enzyme in its β-oxidation

85   pathway (18) (Fig. 1). This apparent redundancy may pose a significant challenge to engineering

86   the production of fatty acids and related chemicals in *R. eutropha*.

87

88   In this work, we engineered *R. eutropha* to produce the medium-chain fatty acid laurate by

89   expressing *UcFatB2*, a medium-chain-length-specific acyl-ACP thioesterase, and obtained

90   laurate yields of up to 14 mg/L. Fatty acid yields were increased by deleting one of several acyl-

91   CoA ligases (*fadD3, A0285, A2794*) that catalyze the first step in fatty acid β-oxidation. These

92   were identified by RNA-Seq as genes strongly upregulated in *R. eutropha* during growth on

93 lauric acid. Fatty acid yield could also be increased by knocking out PHB synthesis (*phaCAB*). In

94 all, we achieved maximum production of 62 ± 2 mg/L free fatty acids, the highest yield of fatty

95 acids reported in this organism to date. This work provides a starting point for the production of

96 fatty acid-derived fuels and chemicals at high yield, including biofuels and PHB-derived

97 bioplastics, in *R. eutropha*.

98

99 **Materials and Methods**

100 **Bacterial growth and induction.** Strains and plasmids used in this study are listed in

101 Supplementary Table 1. *E. coli* strains were grown at 37˚C in lysogeny broth (LB) supplemented

102 with 50 µg/ml kanamycin as appropriate. *R. eutropha* strains were grown at 30˚C in rich medium

103 (16 g/L nutrient broth, 10 g/L yeast extract, 5 g/L $(NH_4)_2SO_4$) + 10 µg/ml gentamicin, and

104 supplemented with 200 µg/ml kanamycin as appropriate. Rich medium plates were prepared

105 from the same media plus 1.5% agar (BD). Lauric acid minimal media plates were produced

106 with 1.5% agar and the following salts: 4.0 g/L NaH_2PO_4, 4.6 g/L Na_2HPO_4, 0.45 g/L

107 K_2SO_4, 0.39 g/L $MgSO_4$, 0.062 g/L $CaCl_2$, 0.05 % (*w/v*) NH_4Cl, 0.1% lauric acid, 1% NP40 and

108 1 ml/L of a trace metal solution (15 g/L $FeSO_4 \cdot 7H_2O$, 2.4 g/L $MnSO_4 \cdot H_2O$, 2.4 g/L

109 $ZnSO_4 \cdot 7H_2O$, and 0.48 g/L $CuSO_4 \cdot 5H_2O$ in 0.1 M HCl). For lauric acid minimal media plate

110 growth experiments, *R. eutropha* strains were streaked out onto rich medium plates for 2 days at

111 30˚C, and then a single colony was transferred onto minimal media plates containing 0.1%

112 laurate and grown at 30˚C for 5 days before imaging with a Canon EOS Rebel T3i Digital SLR

113 camera.

114

115   To generate liquid cultures of *E. coli* or *R. eutropha*, a single freshly-grown colony was

116   inoculated into 10 mL of culture medium in a 14 mL BD Falcon disposable polypropylene

117   culture tube and shaken at 220 rpm overnight. *R. eutropha* free fatty acid (FFA) production

118   experiments were conducted in 2 mL 96-well plates (Thermo Scientific) or 30 mL glass culture

119   tubes. In both cases, individual *R. eutropha* colonies were first inoculated in 10 mL rich medium

120   with appropriate antibiotics and grown at 220 rpm at 30˚C for 20-22 h. For FFA production in

121   96-well plates, *R. eutropha* overnight cultures were diluted 1:20 into 1 mL fresh rich medium

122   plus necessary antibiotics and shaken at 30°C, 1200 rpm on a Titramax 1000 platform shaker

123   (Heidolph) for 4-5h before induction with 0.5% L-arabinose. For FFA production in glass culture

124   tubes, *R. eutropha* overnight cultures were diluted 1:20 into 1 ml fresh rich medium

125   supplemented with required antibiotics and shaken at 180 RPM at 30˚C. After 20h, these cultures

126   were induced with 0.5% L-arabinose and a 20% (v/v) dodecane overlay added. OD$_{600}$ was

127   measured using an Ultrospec 10 spectrophotometer (Amersham).

128

129   **Construction of plasmids.** All L-arabinose-inducible plasmids were generated by conventional

130   restriction cloning. L-arabinose-inducible mCherry was digested from pJT390 (constructed by

131   Joseph P. Torella) with SpeI and NaeI and ligated into SpeI and EcoRV sites in pBBR1MCS2 to

132   generate pJC007. C-terminally his-tagged *UcFatB2* (codon-optimized for expression in

133   *Synechococcus elongatus*) was PCR amplified from pJT204 (constructed by Joseph P. Torella)

134   using forward primers JC016-JC019 and 5'-phosphorylated reverse primer JC023 to generate

135   four RBS variants driving UcFatB2. The resulting PCR products were digested with XbaI and

136   inserted into XbaI and AleI sites in pJC007 to construct plasmids pJC022-pJC025. All

137   markerless deletion plasmids were generated by conventional restriction cloning or Gibson

138 assembly. A gene deletion fragment consisting of 240 bp stretches directly upstream and

139 downstream the *A0285* open reading frame was linked by a PacI restriction site, and the 498 bp

140 product with flanking BamHI sites was synthesized directly by Integrated DNA Technologies

141 (IDT). The synthesized insert and backbone vector pCB96 was digested with the double-cutter

142 BamHI, and ligated to generate the *A0285* markerless deletion plasmid, pJC043. The *B1148*

143 markerless deletion plasmid was generated as described by Lu *et al.* (5), using primer pairs

144 pJC078/JC079 and pJC080/JC081 for overlap extension PCR. The resulting PCR product and

145 parent plasmid pCB96 were digested with XbaI and SacI and ligated to generate pJC046. The

146 gene deletion fragment for *A2794* was synthesized by IDT as described above, except the

147 fragment was flanked upstream by XbaI and downstream by SacI, with ~30 bp overlap regions to

148 pCB96 on both ends. The fragment was assembled to the pCB96 backbone (linearlized by

149 XbaI/SacI) via Gibson Assembly to generate the *A2794* markerless deletion plasmid, pJC045.

150 All newly-ligated plasmids and Gibson assembly reactions were transformed into TSS-

151 competent *E. coli* TURBO or Mach1 cells, isolated and re-transformed into *E. coli* S17-1 cells

152 for conjugative transfer of plasmid DNA into *R. eutropha*. Following conjugation, *UcFatB2*

153 thioesterase plasmids (pJC022-pJC025) were extracted from *R. eutropha* and frozen for

154 subsequent electroporation. Primers used in plasmid construction are listed in Supplementary

155 Table 2.

156

157 **Genomic modifications.** Genetic knockouts were constructed as described previously in

158 Brigham *et al.* (23). *R. eutropha* was conjugated with a donor *E. coli* strain S17-1 harboring a

159 markerless gene deletion plasmid containing homology to the 250 bp regions upstream and

160 downstream of the gene of interest, and containing a kanamycin resistance cassette and *sacB*

161 gene for sucrose-based counter-selection. To perform the mating procedure, 10 ml of *R. eutropha*

162 and *E. coli* were grown separately in rich medium at 30˚C and LB at 37˚C, respectively. Each

163 culture was harvested after 20 h of shaking, washed twice and resuspended in 1 ml 0.85% NaCl.

164 Next, 30 µl of resuspended *R. eutropha* was mixed with 20 µl of resuspended *E. coli* and the 50

165 µl mixture plated onto an LB plate without selection for 24h at 30˚C. Following conjugation, a

166 cell scraper was used to remove half the cells from the plate, which were then transferred to an

167 eppendorf tube and resuspended in 300 µl 0.85% NaCl. To select for *R. eutropha* with successful

168 plasmid integration, either 100 µL or 10 µL of the cell suspension was plated onto rich medium

169 agar plates with 10 µg/ml gentamicin and 200 µg/ml kanamycin and grown for 48h at 30˚C. *R.*

170 *eutropha* colonies were picked and restreaked onto rich medium plates supplemented with

171 gentamicin and kanamycin and grown for 24h at 30˚C. To allow the second recombination event

172 to occur, the restreaked colonies were grown in 10 mL rich medium with 10 µg/ml gentamicin at

173 30˚C. After 20h, 1 ml from the overnight culture was harvested, washed twice with 0.85% NaCl

174 and resuspended in 1 ml 0.85% NaCl. 10 µl of resuspended cells were then plated onto rich

175 medium plates containing 10 µg/ml gentamicin and 5% sucrose. The plates were incubated at

176 30˚C for 36-48h, and colonies screened with colony PCR for successful recombination-based

177 loss of the gene of interest. Primers used in strain verification are listed in Supplementary Table

178 2. Note that although the *B1148* markerless deletion plasmid was constructed, we were unable to

179 knock out the *B1148* gene in parallel with other *fadD* homologues identified by RNA-Seq. The

180 lack of viable colonies from multiple attempts to knock out *B1148* in WT and Δ*fadD3*

181 background strains may reflect that the gene is essential, or be a consequence of the locally high

182 GC content of the genome.

183

184   **Generation of *R. eutropha* MCFA production strains.** Freshly-prepared electrocompetent *R.*

185   *eutropha* strains were generated for all FFA production experiments using *UcFatB2* thioesterase

186   plasmids (pJC022-pJC025). Single *R. eutropha* colonies were grown overnight in rich medium,

187   diluted 1:10 in 10 ml rich medium in 15 ml falcon tubes, and harvested at $OD_{600} = 0.8$. Cultures

188   were submerged in ice for 10 min, pelleted and washed twice with 1 ml ice-cold 10% sucrose.

189   The cell pellet was resuspended in 1 ml 10% sucrose, and 50 ul electrocompetent cells were

190   combined with 1-2 μg of plasmid DNA. The cell suspension was transferred to a pre-chilled 0.1

191   cm gap cuvette and electroporated using the Biorad electroporator manually set at 1.15 kV.

192   Immediately following electroporation, 250 μl rich medium was added and the cells were

193   transferred to an eppendorf tube. Electroporated cells were recovered at 30˚C for 2 h before

194   being plated on selective medium and grown for 2-3 days at 30˚C.

195

196   **Enzymatic assays for FFA measurements.** Fatty acids were measured using the enzymatic

197   Free Fatty Acid Half Micro Test (Roche) as described by Torella *et al.* (28). For FFA

198   measurements with 20% dodecane, 5 μl of the overlay was diluted into 20 ul dodecane before

199   assaying with the same sample volume as previously discussed. Because acyl-CoAs are not cell

200   permeable, detection of these compounds by the enzymatic assay is not possible in the cell

201   supernatant or dodecane overlay.

202

203   **GCMS identification of lauric acid.** Fatty acids were extracted from 400 μl of acidified culture

204   with ethyl acetate and esterified with ethanol. Fatty ethyl esters were extracted with hexane and

205   run on an Agilent GC-MS 5975/7890 (Agilent Technologies) using an HP-5MS (length: 30 m;

206   diameter: either 0.25 or 0.50 mm; film: 0.25 μm) column, as described by Torella *et al.* (28).

207   This method is unable to detect acyl-CoAs, because they cannot be extracted into the solvent

208   layer due to their hydrophilic properties.

209

210   **Isolation of RNA and RNAseq analysis.** For RNA-Seq experiments, individual colonies of *R.*

211   *eutropha* strain Re2061 ($\Delta phaCAB$) were inoculated in triplicate into rich medium, grown

212   overnight, then diluted 1:100 into 30 mL minimal medium (23) in a 125 mL flask, supplemented

213   with 2.0% Nondiet-P40 (v/v), 0.54% NH_4Cl, 10 µg/ml gentamicin, and either 0.4% fructose

214   (w/v) or 0.1% lauric acid (w/v). Cells were grown to stationary phase, diluted 1:100 again into

215   the same medium, and each culture monitored at 2 h intervals for cell density by OD_{600}. Once a

216   given culture entered mid-log phase (OD_{600} = 0.5-0.6), 1 mL was immediately taken and

217   stabilized with RNA Protect (Qiagen), followed by purification with an RNEasy Mini Kit

218   (Qiagen). The purified RNA was then analyzed by Genewiz on an Illumina HiSeq-2500, and the

219   resulting data analyzed with Rockhopper (26) for alignment to the *R. eutropha* H16 genome and

220   differential gene expression analysis.

221

222   ## Results

223   **Expression of the medium-chain thioesterase UcFatB2 enables MCFA production in *R.***

224   ***eutropha***

225   To produce the MCFA lauric acid, we cloned *UcFatB2*, a thioesterase selective for 12-carbon

226   acyl-ACP substrates from the plant *U. californica* (19), into an arabinose-inducible, *R. eutropha*-

227   compatible replicative vector (pJC007; Supplementary Table 1). Ribosome Binding Sites (RBS)

228   with a range of predicted expression strengths (Translational Initiation Region (TIR) values from

229   14,000 to 84,000 in *R. eutropha*) were designed using the RBS Calculator (20) and cloned

230   upstream of *UcFatB2* (pJC022-pJC025; Supplementary Table 1). One variant (pJC024, TIR =

231   66,000) resulted in 22 mg/L total free fatty acid (FFA) production within 48 h (Fig. 2A). The

232   absence of detectable fatty acid production in the variant with the highest predicted TIR (84,000,

233   pJC025) may reflect toxicity or inclusion body formation resulting from high expression, or

234   insufficient protein production (e.g. in the case that the actual TIR is much lower than predicted).

235

236   GCMS analysis of the ReH16-pJC024 culture confirmed production of lauric acid at 14.3 ± 2.6

237   mg/L after 24 h of induction; laurate production did not occur in wild-type ReH16 (Fig. 2B).

238   Compared to lauric acid, significantly smaller amounts of myristic ($C_{14:0}$) and palmitic ($C_{16:0}$)

239   acids were detected from both wild-type and *UcFatB2*-expressing strains (Fig. 2B, ~2 mg/L),

240   while the concentration of shorter and unsaturated products was negligible.

241

242   Although FFA production was significant within the first ~24 h following arabinose induction,

243   by 50h post-induction FFAs were undetectable in the culture (Fig. 3A). This behavior is

244   consistent with the expectation that fatty acids should be re-consumed by *R. eutropha* through β-

245   oxidation, something previously observed in other microbes engineered to produce fatty acids

246   (21, 22).

247

248   **Fatty acid production in *R. eutropha* is limited by β-oxidation and PHB synthesis**

249   To evaluate the role of β-oxidation in FFA reconsumption, we expressed *UcFatB2* in *R. eutropha*

250   strain Re2303, which contains knockouts in two putative β-oxidation operons and is incapable of

251   growth on oleic acid (23). In contrast to the wild-type, Re2303 (Δβ-oxidation) produced FFA

252   concentrations up to 20 mg/L without reconsumption over a 150 h time course, indicating that β-

253   oxidation in the wild-type contributes to the decline in FFA yields (Fig. 3A).

254

255   Although Re2303 lacks two β-oxidation operons, the acyl-CoA ligases that catalyze entry of

256   fatty acids into the β-oxidation pathway are not located in these operons. We therefore

257   hypothesized that FFA yield might be limited by uptake of fatty acids and conversion to acyl-

258   CoAs and other β-oxidation intermediates, which are not detected in either of our fatty acid

259   assays (Methods). We assayed FFA production in Re2312, which contains a deletion in a known

260   acyl-CoA ligase in *R. eutropha*, *fadD3* (23), and observed a burst of 30 mg/L FFAs within 4h

261   post-induction along with a steady increase up to 54 mg/L FFA over the following week. By

262   150h post-induction, Re2312 (Δ*fadD3*) led to a 2.8-fold improvement over the Re2303 (Δβ-

263   oxidation) mutant strain and an 8.5-fold increase over wild-type parent strain (Fig. 3 A,B),

264   indicating that disruption of the first step in β-oxidation enhanced the production of FFAs

265   beyond what is achieved by blocking downstream steps in β-oxidation (e.g. in Re2303).

266

267   As PHB synthesis is a sink both for acetyl-CoA (24) and β-oxidation intermediates (18) in

268   *R. eutropha*, we hypothesized that it may also limit FFA yield. Expression of *UcFatB2* in a

269   *phaCAB* deletion strain (Re2061) resulted in a considerable 5-fold increase in FFA yield over

270   wild-type by 150 h. Unlike the initial burst of FFAs observed in Re2312 (Δ*fadD3*), the increase

271   in FFA production by Re2061 (Δ*phaCAB*) began 40h after arabinose induction, suggesting a

272   different mechanism for increasing FFA yield. Indeed, PHB synthesis is induced in response to

273   nutrient limitation (2), which may explain the increase in FFA synthesis at a late time-point in

274   this strain (Fig. 3A,B). It is worth noting that simultaneously knocking out PHB synthesis and

275   beta-oxidation genes was unable to enhance FFA production over that achieved by *ΔfadD3* alone

276   (Supplementary Figure 1), suggesting that beta oxidation is the limiting factor for FFA yield. Our

277   study therefore focused on manipulating the acyl-CoA ligases responsible for lauric acid

278   consumption to enhance fatty acid yields in *R. eutropha*.

279

280   Consistent with the hypothesis that Re2312 (Δ*fadD3*) had increased FFA production due to a

281   lower rate of FFA consumption, we found that it had a slower rate of exogenous FFA uptake

282   (Fig. 3C). Wild-type *R. eutropha*, Re2303, Re2061 and Re2312 (none expressing *UcFatB2*) were

283   incubated with 120 mg/L laurate and the levels of extracellular FFA monitored over a 24 hour

284   time period. The wild-type, Re2303 (Δβ-oxidation) and Re2061 (Δ*phaCAB*) strains had similar

285   consumption rates with laurate no longer detectable in the media by 12 h. Re2312 (Δ*fadD3*) cells

286   exhibited a slower rate of consumption relative to the other strains, with laurate becoming

287   undetectable by 24 h (Fig. 3C). Laurate consumption was not blocked entirely, suggesting that

288   other acyl-CoA ligases may still be active in Re2312. Indeed, we found that Re2312 was still

289   capable of growth on laurate minimal medium (Supplementary Figure 2).

290

291   **Identification of three additional acyl-CoA ligases that limit FFA yield through RNA-Seq**

292   Deletion of the primary acyl-CoA ligase in *R. eutropha*, *fadD3*, results in slower uptake of fatty

293   acids (Fig. 3C) but does not block their utilization as a sole carbon source (23). This is consistent

294   with computational predictions that the *R. eutropha* genome harbors an apparently redundant β-

295   oxidation system (1), estimated to contain 51 homologues of the acyl-CoA ligase alone (25) (Fig.

296   4A).

297

298   To narrow down the list of acyl-CoA ligases likely to limit the production of fatty acids, a

299   preliminary RNA-Seq experiment was performed to identify acyl-CoA ligases that are up-

300   regulated during growth on lauric acid. Three independent cultures of Re2061 were grown from

301   an OD_{600} of 0.02 in minimal medium containing fructose or lauric acid as a sole carbon source

302   (Fig. 4B). During mid-exponential phase (OD 0.3-0.6), a sample of each culture was taken for

303   analysis by RNA-Seq (Methods). Successfully aligned transcript frequency was low (~40-50%

304   of reads), likely due to poor sample quality, and we therefore elected not to analyze the resulting

305   data in full here (a future manuscript is forthcoming). Nevertheless, it provided enough

306   information on acyl-CoA ligase expression patterns to engineer improved FFA production in

307   *R. eutropha*.

308

309   Read counts normalized to upper-quartile gene expression (26, 27) were used to identify four

310   genes annotated as acyl-CoA ligases in the *R. eutropha* genome that were upregulated strongly

311   ($\geq$ 5-fold) and significantly (q-value < 0.001) during growth on laurate as compared to fructose:

312   *fadD3*, *A2794*, *A0285*, and *B1148* (Fig. 4C). We generated mutants of each gene alone and in

313   combination with *fadD3* with the exception of *B1148*, which we were unable to knock out after

314   several attempts (Methods). FFA production and OD_{600} were measured for each strain 96 h after

315   induction; a large increase in FFA production was associated with knocking out *fadD3* (Re2312),

316   *A0285* (JC357) and *A2794* (JT338), with *A2794* providing the greatest FFA yield at 62 ± 2 mg/L

317   (Fig. 4D). The $\Delta A0285\Delta fadD3$ double knockout (JC358) failed to increase FFA yield

318   significantly above what was achieved for either knockout alone, while the $\Delta A2794\Delta fadD3$

319   double knockout (JT339) actually decreased FFA yield. As the latter was the only strain tested

320   with impaired biomass yield (Fig. 4D, bottom panel), this reduced mean yield may reflect a

321   synthetic loss of fitness associated with the $\Delta A2794\Delta fadD3$ double knockout.

322

Discussion

324   *R. eutropha* is a versatile industrial organism capable of synthesizing a range of biofuels and

325   bioplastics either heterotrophically from organic substances or autotrophically from H_2 and CO_2.

326   In this study, we engineered medium-chain fatty acid (MCFA) synthesis in *R. eutropha* by

327   optimizing the expression of a heterologously expressed thioesterase, *UcFatB2*. Deletion of the

328   acyl-CoA ligase *fadD3* increased fatty acid yields without completely abolishing fatty acid

329   uptake, implying the action of additional acyl-CoA ligases. Because 51 such enzymes have been

330   annotated in the *R. eutropha* genome, we employed RNA-Seq to identify which were

331   upregulated during growth on the MCFA lauric acid. Of the four candidate genes identified,

332   knockouts in three (*fadD3, A0285, A2794*) led to higher fatty acid yields, with one (*ΔA2794)*

333   providing the highest yield of fatty acids yet reported in *R. eutropha* (62 mg/L).

334

335   Expression of *UcFatB2* in *R. eutropha* resulted in relatively selective production of lauric acid,

336   as laurate production increased by 15 mg/L, while all other detected fatty acids increased by less

337   than 1 mg/L (Fig. 2B). This level of selectivity appears higher than in *E. coli*, where expression

338   of *UcFatB2* has previously been shown to liberate a significant amount of decanoate, myristate

339   and palmitate (28). Likewise, although *UcFatB2* has previously been shown to produce methyl

340   ketones in *E. coli* (presumably by catalyzing hydrolysis of β-ketoacyl-ACPs (19)), we failed to

341   detect methyl ketones in *R. eutropha* expressing *UcFatB2* by GCMS. These results may suggest

342   different rates of product degradation, a difference in interaction between *UcFatB2* and *R.*

343   *eutropha* ACP, or a distinct distribution of acyl- and β-ketoacyl-ACPs in *R. eutropha* that result

344   in altered product profiles.

345

346   During our fatty acid production timecourse, the *ΔfadD3* strain produced an initial burst of

347   30 mg/ml free fatty acids in the 4h following thioesterase induction that was not observed in the

348   wild-type (Fig. 3A). These kinetics suggest that the rate of acyl-ACP hydrolysis by *UcFatB2* is

349   faster than the rate at which free fatty acids leave the cell, and that the intracellular pool of free

350   fatty acids are rapidly acted on by FadD3 in the wild-type to generate acyl-CoAs. This

351   hypothesis is consistent with a previous observation by Muller *et al.*, in which *R. eutropha*

352   expressing a thioesterase in a Δβ-oxidation background produced free fatty acids that accounted

353   for only ~0.00016% of the fructose carbon consumed (16). However, addition of a heterologous

354   methyl ketone pathway that acts on β-oxidation intermediates produced greater than 50 mg/L

355   methyl ketones, or about 2% of the fructose carbon consumed (16).

356

357   Unlike in the *ΔfadD3* strain, in the *ΔphaCAB* strain the largest increase in fatty acid production

358   began at a ~30h delay post-induction (Fig 3A). This difference in timing may reflect a difference

359   in mechanism for increasing fatty acid yields. Under conditions of nutrient deprivation, WT

360   *R. eutropha* normally induce PHB synthesis (29); *phaCAB* strains, however, accumulate acetyl-

361   CoA, which is converted to pyruvate or can be diverted by engineered pathways to other

362   products (e.g. isopropanol or isobutanol (4-6)). High acetyl-CoA levels stimulate fatty acid

363   synthesis and inhibit beta-oxidation (28, 30), and could explain the increase in fatty acid

364   production observed in the *ΔphaCAB* mutant. Moreover, as acetyl-CoA would only accumulate

365   after nutrient depletion, the observed ~30 h delay may reflect the time required to deplete an

366   essential nutrient in the growth medium.

367

368   Our identification of differential expression of *fadD* homologs (*fadD3*, *A0285*, *A2794* and

369   *B1148*) in *R. eutropha* during growth on lauric acid and fructose using RNA-Seq (Fig. 4C) was

370   consistent with a previously published microarray study (23), and hints at the possibility of

371   differential regulation of β-oxidation enzymes in response to fatty acids of different chain

372   lengths. Previous work by Brigham *et al.* (23) identified significant upregulation of *fadD3*

373   (*A3288*) and *A2794* during growth on trioleate relative to fructose, similar to our observation that

374   these genes are upregulated during growth on laurate. However, they did not observe increased

375   expression of *A0285* or *B1148*, though these were strongly upregulated in response to laurate in

376   our system. These results may indicate that different β-oxidation enzymes in *R. eutropha* are

377   induced by structurally distinct fatty acid substrates, discriminating for example on the basis of

378   saturation and chain length.

379

380   Knockouts of *fadD3*, *A0285* and *A2794* expressing *UcFatB2* increased FFA production by 8.9,

381   10.2 and 12.5-fold (respectively) over WT at 96h post-induction, without altering total biomass

382   yield (Fig. 4D). Surprisingly, knocking out candidate acyl-CoA ligases in conjunction with

383   *fadD3* failed to further improve yield: the Δ*A0285*Δ*fadD3* double mutant did not yield any added

384   FFA increase over the individual mutations, and the Δ*A2794*Δ*fadD3* double mutant had lower

385   yields than either of the single mutants alone. The failure to increase yield in these double

386   mutants may be due to compensatory regulation of acyl-CoA ligases in response to an increase in

387   laurate levels, to kinetic features of *R. eutropha*'s extensive β-oxidation system, or both. In the

388   specific case of *ΔA2794ΔfadD3*, the drop in productivity may also be due to a fitness defect,

389   indicated by the relative decrease in total biomass yield at 96 h compared to its parent strains

390   (Fig 4D). As FadD is essential for stationary phase survival in *E. coli* (31), it is possible that

391   acyl-CoA ligases in *R. eutropha* perform compensatory roles in the maintenance of stationary

392   phase fitness, and that the deletion of some combinations of ligases carry a stationary phase

393   fitness cost. In all, these results indicate a potentially complex metabolic and regulatory

394   relationship among the acyl-CoA ligases in *R. eutropha* that may be addressed through more

395   systematic engineering, as well as transcriptomic and metabolic investigation.

396

397   Although the roles of many of the unannotated β-oxidation homologs remain elusive, identifying

398   transcriptomic changes in response to a range of fatty acid chain lengths may help elucidate these

399   functions, while providing the information necessary to engineer *R. eutropha* for industrially

400   relevant FFA yields. It will also be important to better understand the relationship between PHB

401   synthesis and fatty acid metabolism, both because their co-regulation complicates efforts to

402   produce FFAs at high yields, and because of the importance of *R. eutropha* as a chassis for

403   industrial polyhydroxyalkanoate (PHA) production. As PHA co-polymers containing medium-

404   chain-length side chains have superior physical properties compared to PHB (32) and these side

405   chains are derived from β-oxidation intermediates (33, 34), a desirable application of the present

406   research will be to engineer *R. eutropha* for the production, partial β-oxidation, and incorporation

407   of MCFAs into PHA co-polymers. In particular, the link between β-oxidation and PHA synthesis

408   has been demonstrated in two related PHA-producing species, *Pseudomonas putida* and

409   *Pseudomonas entomophila*: knocking out key enzymes in β-oxidation was sufficient to increase

410   metabolic flux through the PHA synthesis pathway, and this enabled chain-length-specific

411   production of PHA consisting of 3-hydroxydodecanoate when grown on lauric acid (37, 38). We

412   also note that while our study lays a foundation for improved FFA production by *R. eutropha*

413   during growth in rich medium, it will be important to demonstrate high-yield production of FFAs

414   in minimal carbon media and on H_2 and CO_2 in order to exploit the full capacity of this

415   metabolically versatile, chemolithotrophic bacterium. In particular, H_2 and CO_2-based FFA

416   production would enable integration with water splitting catalysts for efficient solar-to-fuel

417   transformation (5, 11), offering new, sustainable routes for the production of chemicals,

418   bioplastics, and fuels.

419

Acknowledgements

421   We thank P.A. Silver for project guidance, J. Lu and A.J. Sinskey for plasmids and reagents; J.

422   Lu, C.J. Brigham, T.J. Ford, D.C. MacKellar and O. Mavrothalassitis for helpful discussion.

423

References

425   1.   **Pohlmann A, Fricke WF, Reinecke F, Kusian B, Liesegang H, Cramm R, Eitinger T,**

426   **Ewering C, Potter M, Schwartz E, Strittmatter A, Voss I, Gottschalk G, Steinbuchel**

427   **A, Friedrich B, Bowien B.** 2006. Genome sequence of the bioplastic-producing

428   "Knallgas" bacterium Ralstonia eutropha H16. Nat Biotechnol **24:**1257-1262.

429   2.   **Verlinden RA, Hill DJ, Kenward MA, Williams CD, Radecka I.** 2007. Bacterial

430   synthesis of biodegradable polyhydroxyalkanoates. J Appl Microbiol **102:**1437-1449.

431   3.   **Henderson RA, Jones CW.** 1997. Physiology of poly-3-hydroxybutyrate (PHB)

432   production by Alcaligenes eutrophus growing in continuous culture. Microbiology

433   **143:**2361-2371.

434 4. **Grousseau E, Lu J, Gorret N, Guillouet SE, Sinskey AJ.** 2014. Isopropanol production

435   with engineered Cupriavidus necator as bioproduction platform. Appl Microbiol

436   Biotechnol **98:**4277-4290.

437 5. **Li H, Opgenorth PH, Wernick DG, Rogers S, Wu TY, Higashide W, Malati P, Huo**

438   **YX, Cho KM, Liao JC.** 2012. Integrated electromicrobial conversion of CO2 to higher

439   alcohols. Science **335:**1596.

440 6. **Lu J, Brigham CJ, Gai CS, Sinskey AJ.** 2012. Studies on the production of branched-

441   chain alcohols in engineered Ralstonia eutropha. Appl Microbiol Biotechnol **96:**283-297.

442 7. **Nevin KP, Woodard TL, Franks AE, Summers ZM, Lovley DR.** 2010. Microbial

443   electrosynthesis: feeding microbes electricity to convert carbon dioxide and water to

444   multicarbon extracellular organic compounds. MBio **1**.

445 8. **Rabaey K, Butzer S, Brown S, Keller J, Rozendal RA.** 2010. High current generation

446   coupled to caustic production using a lamellar bioelectrochemical system. Environ Sci

447   Technol **44:**4315-4321.

448 9. **Kuhl KP, Cave ER, Abram DN, Jaramillo TF.** 2012. New insights into the

449   electrochemical reduction of carbon dioxide on metallic copper surfaces. Energy &

450   Environmental Science **5:**7050-7059.

451 10. **Schlegel HG, Lafferty R.** 1965. Growth of /`Knallgas/'' Bacteria (Hydrogenomonas)

452   using Direct Electrolysis of the Culture Medium. Nature **205:**308-309.

453 11. **Torella JP, Gagliardi CJ, Chen JS, Bediako DK, Colon B, Way JC, Silver PA,**

454   **Nocera DG.** 2015. Efficient solar-to-fuels production from a hybrid microbial-water-

455   splitting catalyst system. Proc Natl Acad Sci U S A **112:**2337-2342.

456   12.   **Steen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A, del Cardayre SB,**

457   **Keasling JD.** 2010. Microbial production of fatty-acid-derived fuels and chemicals from

458   plant biomass. Nature **463:**559-562.

459   13.   **Lennen RM, Pfleger BF.** 2013. Microbial production of fatty acid-derived fuels and

460   chemicals. Curr Opin Biotechnol **24:**1044-1053.

461   14.   **Akhtar MK, Turner NJ, Jones PR.** 2013. Carboxylic acid reductase is a versatile

462   enzyme for the conversion of fatty acids into fuels and chemical commodities. Proc Natl

463   Acad Sci U S A **110:**87-92.

464   15.   **Lageveen RG, Huisman GW, Preusting H, Ketelaar P, Eggink G, Witholt B.** 1988.

465   Formation of Polyesters by Pseudomonas oleovorans: Effect of Substrates on Formation

466   and Composition of Poly-(R)-3-Hydroxyalkanoates and Poly-(R)-3-Hydroxyalkenoates.

467   Appl Environ Microbiol **54:**2924-2932.

468   16.   **Muller J, MacEachran D, Burd H, Sathitsuksanoh N, Bi C, Yeh YC, Lee TS, Hillson**

469   **NJ, Chhabra SR, Singer SW, Beller HR.** 2013. Engineering of Ralstonia eutropha H16

470   for autotrophic and heterotrophic production of methyl ketones. Appl Environ Microbiol

471   **79:**4433-4439.

472   17.   **Bi C, Su P, Muller J, Yeh Y-C, Chhabra S, Beller H, Singer S, Hillson N.** 2013.

473   Development of a broad-host synthetic biology toolbox for ralstonia eutropha and its

474   application to engineering hydrocarbon biofuel production. Microbial Cell Factories

475   **12:**107.

476   18.   **Shimizu R, Chou K, Orita I, Suzuki Y, Nakamura S, Fukui T.** 2013. Detection of

477   phase-dependent transcriptomic changes and Rubisco-mediated CO_2 fixation into poly

478   (3-hydroxybutyrate) under heterotrophic condition in Ralstonia eutropha H16 based on

479   RNA-seq and gene deletion analyses. BMC Microbiol **13:**169.

480 19. **Voelker TA, Davies HM.** 1994. Alteration of the specificity and regulation of fatty acid

481   synthesis of Escherichia coli by expression of a plant medium-chain acyl-acyl carrier

482   protein thioesterase. Journal of Bacteriology **176:**7320-7327.

483 20. **Salis HM, Mirsky EA, Voigt CA.** 2009. Automated design of synthetic ribosome

484   binding sites to control protein expression. Nat Biotechnol **27:**946-950.

485 21. **Lu X, Vora H, Khosla C.** 2008. Overproduction of free fatty acids in E. coli:

486   Implications for biodiesel production. Metabolic Engineering **10:**333-339.

487 22. **Runguphan W, Keasling JD.** 2014. Metabolic engineering of Saccharomyces cerevisiae

488   for production of fatty acid-derived biofuels and chemicals. Metabolic Engineering

489   **21:**103-113.

490 23. **Brigham CJ, Budde CF, Holder JW, Zeng Q, Mahan AE, Rha C, Sinskey AJ.** 2010.

491   Elucidation of beta-oxidation pathways in Ralstonia eutropha H16 by examination of

492   global gene expression. J Bacteriol **192:**5454-5464.

493 24. **Cook A, Schlegel H.** 1978. Metabolite concentrations in Alcaligenes eutrophus H 16 and

494   a mutant defective in poly-β-hydroxybutyrate synthesis. Archives of Microbiology

495   **119:**231-235.

496 25. **Shimizu R, Chou K, Orita I, Suzuki Y, Nakamura S, Fukui T.** 2013. Detection of

497   phase-dependent transcriptomic changes and Rubisco-mediated CO_2 fixation into poly

498   (3-hydroxybutyrate) under heterotrophic condition in Ralstonia eutropha H16 based on

499   RNA-seq and gene deletion analyses. BMC Microbiology **13:**169.

500   26.   **McClure R, Balasubramanian D, Sun Y, Bobrovskyy M, Sumby P, Genco CA,**

501   **Vanderpool CK, Tjaden B.** 2013. Computational analysis of bacterial RNA-Seq data.

502   Nucleic Acids Res **41:**e140.

503   27.   **Bullard JH, Purdom E, Hansen KD, Dudoit S.** 2010. Evaluation of statistical methods

504   for normalization and differential expression in mRNA-Seq experiments. BMC

505   Bioinformatics **11:**94.

506   28.   **Torella JP, Ford TJ, Kim SN, Chen AM, Way JC, Silver PA.** 2013. Tailored fatty

507   acid synthesis via dynamic control of fatty acid elongation. Proc Natl Acad Sci U S A

508   **110:**11290-11295.

509   29.   **Wang J, Yu HQ.** 2007. Biosynthesis of polyhydroxybutyrate (PHB) and extracellular

510   polymeric substances (EPS) by Ralstonia eutropha ATCC 17699 in batch cultures. Appl

511   Microbiol Biotechnol **75:**871-878.

512   30.   **Dellomonaco C, Clomburg JM, Miller EN, Gonzalez R.** 2011. Engineered reversal of

513   the beta-oxidation cycle for the synthesis of fuels and chemicals. Nature **476:**355-359.

514   31.   **Farewell A, Diez AA, DiRusso CC, Nystrom T.** 1996. Role of the Escherichia coli

515   FadR regulator in stasis survival and growth phase-dependent expression of the uspA,

516   fad, and fab genes. J Bacteriol **178:**6443-6450.

517   32.   **Nomura CT, Tanaka T, Gan Z, Kuwabara K, Abe H, Takase K, Taguchi K, Doi Y.**

518   2004. Effective enhancement of short-chain-length-medium-chain-length

519   polyhydroxyalkanoate copolymer production by coexpression of genetically engineered

520   3-ketoacyl-acyl-carrier-protein synthase III (fabH) and polyhydroxyalkanoate synthesis

521   genes. Biomacromolecules **5:**1457-1464.

522 33. **Tsuge T, Yano K, Imazu S-i, Numata K, Kikkawa Y, Abe H, Taguchi S, Doi Y.**

523   2005. Biosynthesis of Polyhydroxyalkanoate (PHA) Copolymer from Fructose Using

524   Wild-Type and Laboratory-Evolved PHA Synthases. Macromolecular Bioscience **5:**112-

525   117.

526 34. **Insomphun C, Mifune J, Orita I, Numata K, Nakamura S, Fukui T.** 2014.

527   Modification of beta-oxidation pathway in Ralstonia eutropha for production of poly(3-

528   hydroxybutyrate-co-3-hydroxyhexanoate) from soybean oil. J Biosci Bioeng **117:**184-

529   190.

530 35. **Simon R, Priefer U, Puhler A.** 1983. A Broad Host Range Mobilization System for In

531   Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. Nat

532   Biotech **1:**784-791.

533 36. **Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, 2nd, Peterson**

534   **KM.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS,

535   carrying different antibiotic-resistance cassettes. Gene **166:**175-176.

536 37. **Liu Q, Luo G, Zhou XR, Chen GQ.** 2011. Biosynthesis of Poly(3-hydroxydecanoate)

537   and 3-Hydroxydodecanoate Dominating Polyhydroxyalkanoates by β-Oxidation Pathway

538   Inhibited Pseudomonas putida. Metabolic Engineering **13**:11-17

539 38. **Chung AL, Jin HL, Huang LJ, Ye HM, Chen JC, Wu Q, Chen GQ.** 2011.

540   Biosynthesis and Characterization of Poly(3-hydroxydodecanoate) by β-Oxidation

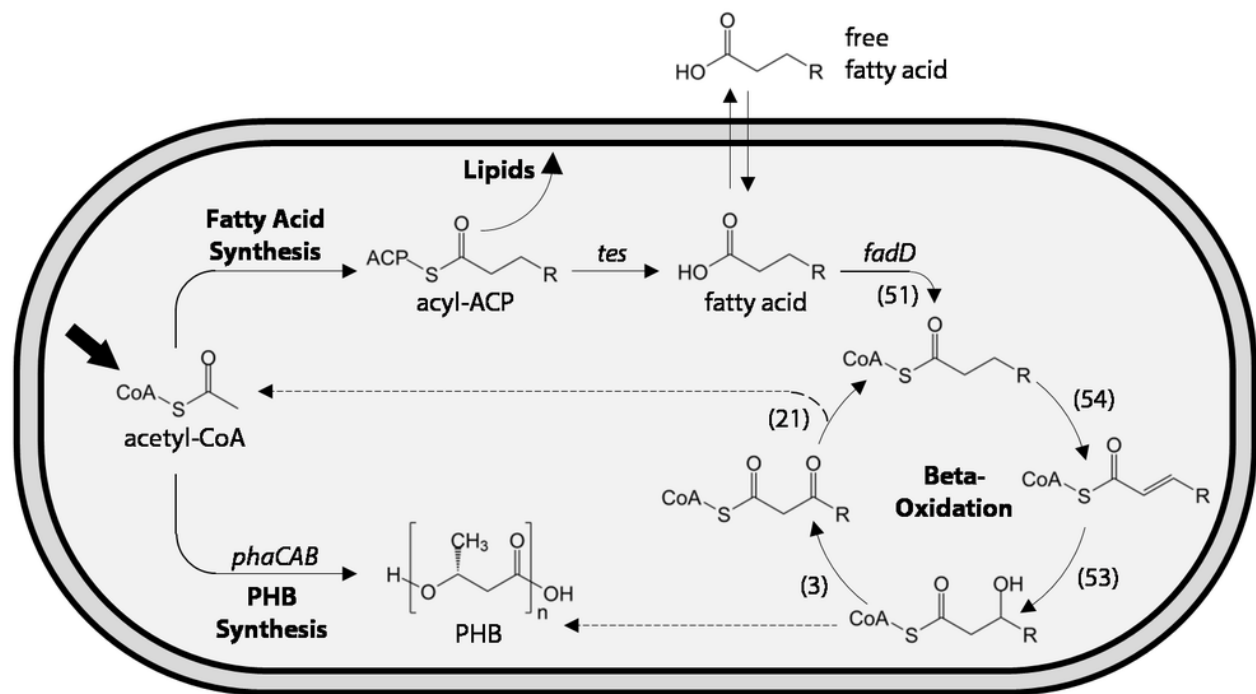
541   Inhibited Mutant of Pseudomonas entomophila L48. Biomacromolecules **12**:3559–3566

542

543

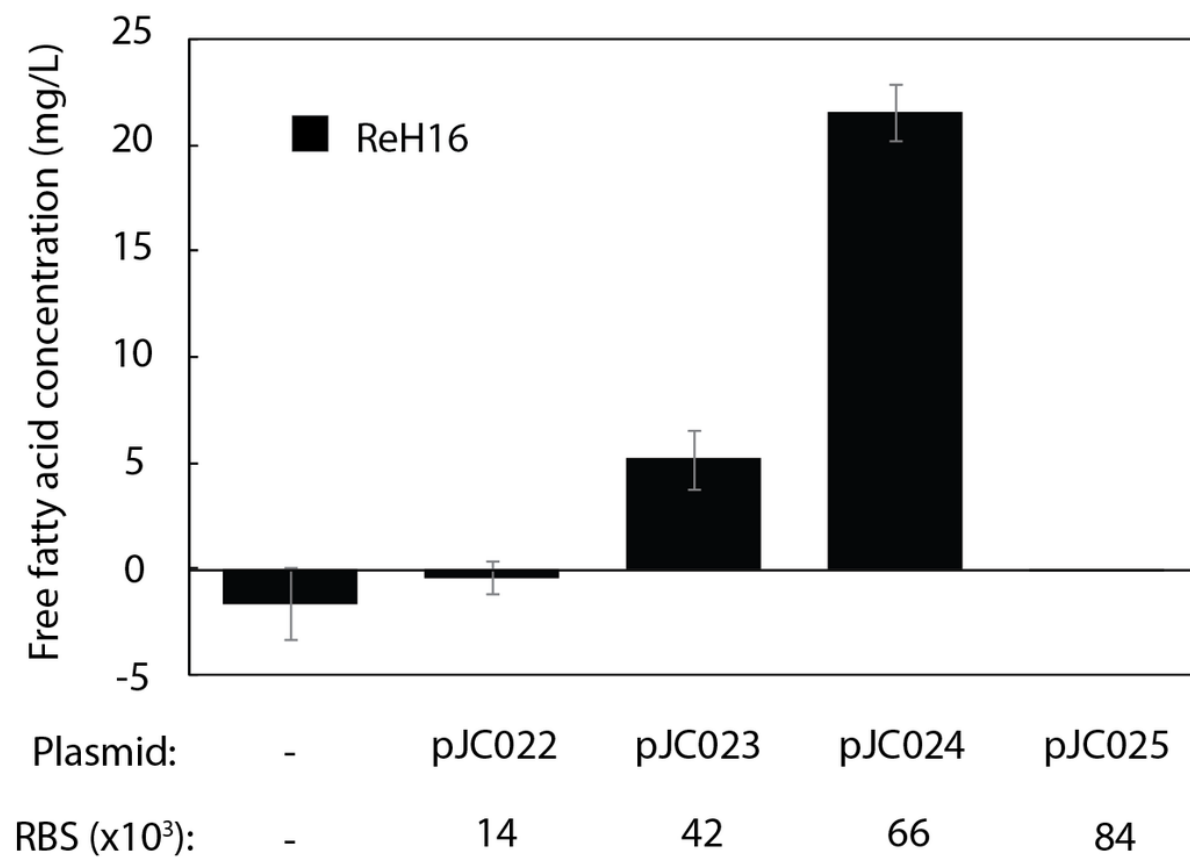
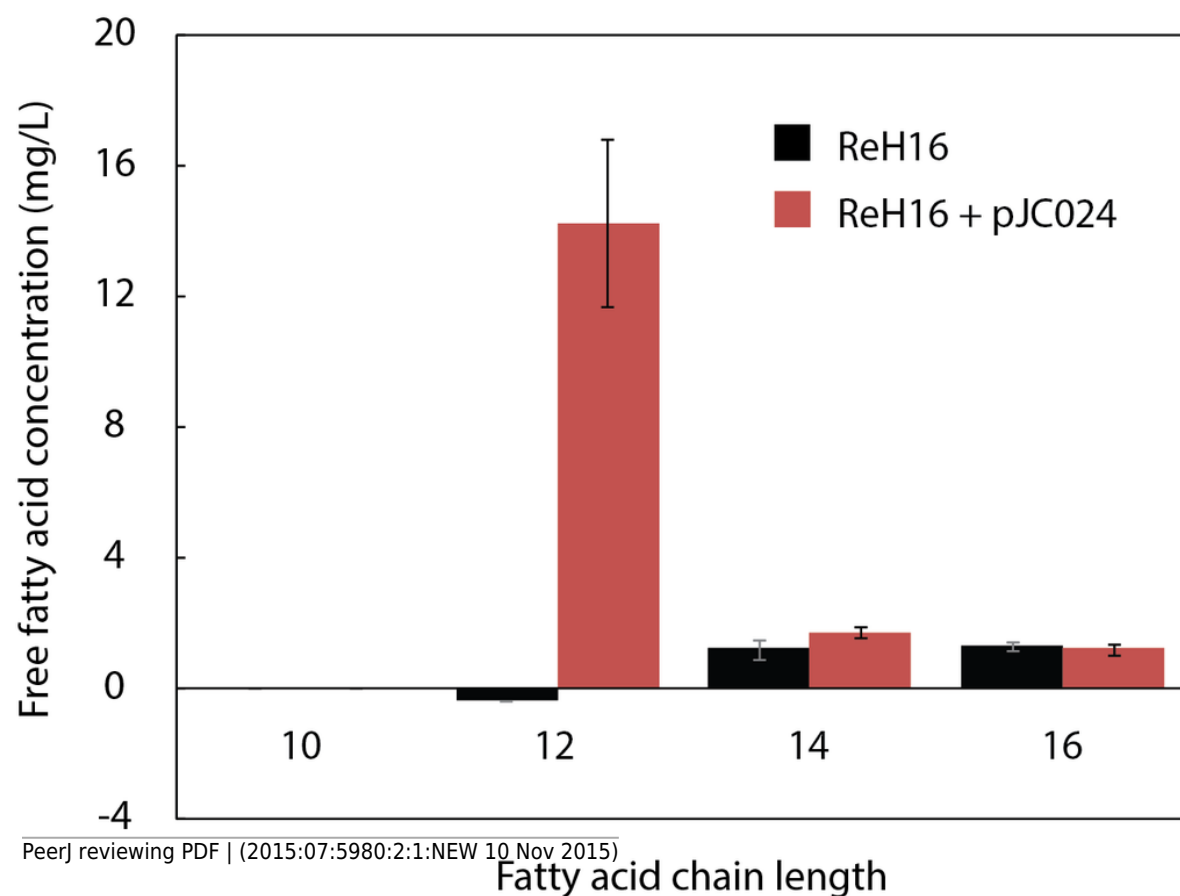
1

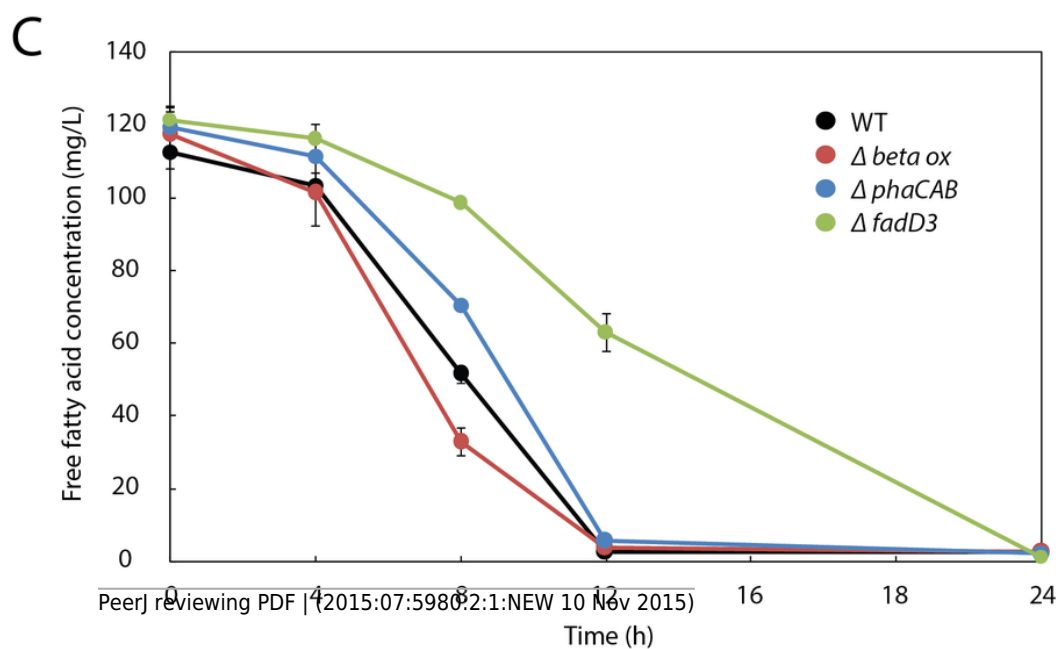
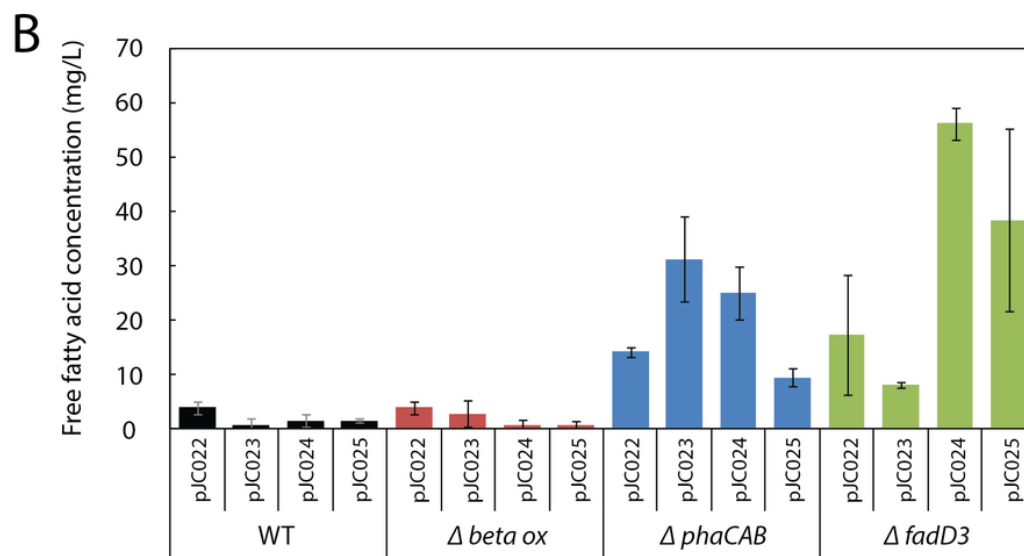
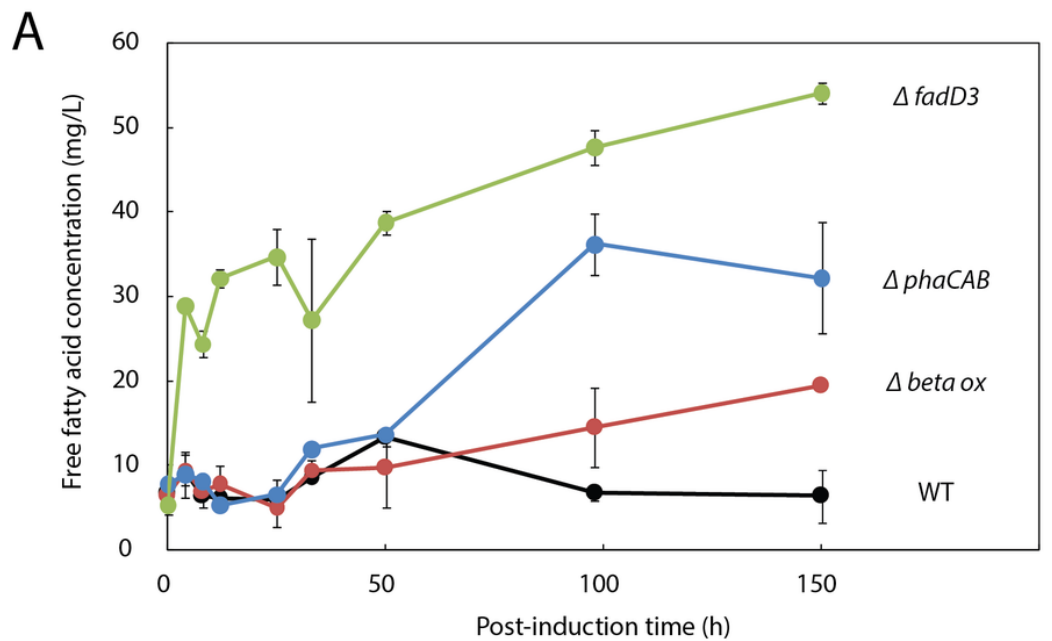
Schematic of fatty acid production and uptake by *Ralstonia eutropha* H16.

Heterotrophic and autotrophic growth of *R. eutropha* H16 both result in production of the central metabolite acetyl-CoA (bold arrow). During fatty acid synthesis, derivatives of acetyl-CoA are iteratively condensed on an acyl carrier protein (ACP) scaffold to yield a series of acyl-ACPs of increasing length for lipid synthesis. Heterologous expression of chain-length-specific acyl-ACP thioesterases (*tes*) enables the hydrolysis of acyl-ACPs and release of fatty acids, some of which are capable of crossing the lipid membrane. Fatty acids can be re-consumed through the β-oxidation pathway, which iteratively removes two carbons from the fatty acid to yield acetyl-CoA (top dashed line). Entry of fatty acids into β-oxidation is catalyzed by one or more acyl-CoA ligases (*fadD* homologues). Multiple homologues of each step of the β-oxidation pathway have been identified in the genome of *R. eutropha* H16, with the number of homologues indicated in parentheses. Acetyl-CoA may also be converted to polyhydroxybutyrate (PHB) through the action of three enzymes (*phaCAB*) under periods of carbon excess but limitation of nutrients such as nitrogen or phosphate. β-oxidation and PHB synthesis share common metabolic intermediates and may be co-regulated. The dashed line indicates the potential for 3-hydroxyl-acyl-CoAs to enter PHB synthesis pathway and be incorporated into the polymer.

2

Production of lauric acid by engineered *R. eutropha*.

(A) Total free fatty acids (FFA) produced by ReH16 grown on rich broth (RB) and expressing UcFatB2 from plasmids (top row) of different predicted RBS strengths (RBS Calculator; bottom row). FFAs were detected in culture supernatant 48 h after arabinose induction by the fatty acid half-micro assay (Methods). Negligible amounts of free fatty acid (0.04 ± 0.06 mg/L) were detected for ReH16 expressing pJC025. (B). Chain length distribution of saturated fatty acids extracted from total culture of ReH16-pJC024 at 48 h post-induction via GCMS. For all experiments in this figure, error bars represent the standard error of the mean (S.E.M.) from N ≥ 3 independent experiments.

3

β-oxidation limits fatty acid yield in engineered *R. eutropha*.

(A) Time course of free fatty acid (FFA) production by different R. eutropha mutants in RB following arabinose induction (0 h time point). (B) Total FFA production at 24 h as a function of genetic background and UcFatB2 expression plasmid; each plasmid has a different RBS and predicted expression strength. (C) Time course of fatty acid uptake by R. eutropha of different genetic backgrounds incubated with 120 mg/L lauric acid in RB. For all experiments in this figure, FFA concentration was measured using the fatty acid half-micro assay (Methods), and error bars represent S.E.M. for N ≥ 3 independent experiments.

4

Three additional acyl-CoA ligases limit FFA yield.

(A) Schematic diagram of β-oxidation in *R. eutropha*. The first enzymatic step of β-oxidation is carried out by an acyl-CoA ligase (ACL) followed by an acyl-CoA dehydrogenase (ACD), enoyl-CoA hydratase (ECH), 3-hydroxyacyl-CoA dehydrogenase (HCD) and β-ketoacyl-CoA thiolase (BKT). Each β-oxidation gene has multiple annotated homologues in the R. eutropha genome (number indicated in parentheses). (B) Time course of Re2061 growth on fructose or laurate minimal medium. Error bars represent S.E.M. from N = 3 independent experiments. The grey arrow indicates the point at which cultures were sampled for RNA-Seq analysis. (C) Expression levels of four acyl-CoA ligases upregulated during growth on laurate, measured by RNA-Seq and normalized by the upper quartile method (Methods). Numbers above bars indicate fold-increase in laurate media as compared to fructose media. Error bars represent S.E.M. of the normalized expression levels for three independent experiments. Differences in expression between fructose and laurate conditions are significant for all four genes after correcting for multiple hypothesis testing (q-value < 0.001). (D) Total FFA production 96 h after arabinose induction by R. eutropha knockout mutants, as measured by the fatty acid half-micro assay (top). OD600 96 h after induction for each mutant. Error bars represent S.E.M. from N = 4 independent experiments in both panels. * indicates a significant difference in mean FFA production with p < 0.05, evaluated using the one-tailed student's t-test.