The essential role of 2,4-dienoyl-CoA reductase for degradation of complex fatty acid mixtures

Veronica Schiaffi*, Viola Pavoncello*, Frédéric Barras, Emmanuelle Bouveret[#]

SAMe Unit, Department of Microbiology, UMR CNRS 6047, Université Paris-Cité, Institut Pasteur, Paris, France

* contributed equally to this work

to whom correspondence should be sent: emmanuelle.bouveret@pasteur.fr

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ABSTRACT

Fatty acids (FAs) can be used as carbon and energy source by most bacteria. FAs are very diverse and show variations in aliphatic chain length, degree and kind of branching, and number of double bonds. After their activation by a thioester link to Coenzyme A, FAs are degraded by the β-oxidation machinery. The core enzymes of the β-oxidation machinery can degrade most FAs, except for those that bear an unsaturation at even-numbered carbons. Such FAs include arachidonic acid or linoleic acid, which are essential FAs of the mammalian diet. We studied the role of the 2,4-dienoyl-CoA reductase FadH in *E. coli* FA metabolism. We showed that *fadH* is essential for growth on linoleic acid and that Cys residues connecting FadH-bound [Fe-S] cluster are essential for activity *in vivo*. Moreover, we showed that when mixed with other FAs, linoleic acid prevents growth of the *fadH* mutant. These results underline the key role of FadH in complex environments like the gut containing diverse FAs. Eukaryotes also use 2,4-dienoyl-CoA reductases for β-oxidation in mitochondria, but these enzymes belong to a different family than FadH, with different co-factors equipment and mechanism. Yet, we showed that eukaryotic 2,4-dienoyl-CoA reductases DECR can complement the *E. coli fadH* mutant for growth on linoleic acid and for relief of linoleate mediated jamming of the β-oxidation, paving the way to search for chemicals targeting DECR activity. Altogether these studies demonstrate the key role of prokaryotic and eukaryotic 2,4-dienoyl-CoA reductases in complex environments containing mixtures of saturated and unsaturated FAs.

40 **IMPORTANCE**

41 Bacteria and eukaryotes can harness energy from fatty acids (FAs) through the process
42 of β-oxidation. However, information on the β-oxidation in bacteria stems from studies in
43 which degradation of only a limited set of saturated or monounsaturated FAs were
44 investigated, far from reflecting the wide chemical diversity of FAs found in Nature. Here we
45 evidenced the physiological importance of dienoyl-CoA reductase enzymes required for the
46 degradation of specific unsaturated fatty acids in complex mixtures of fatty acids, and how
47 their absence leads to the congestion of the β-oxidation machinery. These results will permit
48 to better understand the impact of FA degradation in enterobacteria, living in the complex
49 gut environment where FAs are available from the diet or from host lipids. Furthermore, we
50 showed that eukaryotic enzymes can replace the prokaryotic ones, opening the possibility of
51 biomedical application in structure/function studies of the eukaryotic dienoyl-CoA
52 reductases.

53

54 **INTRODUCTION**

55 Fatty acids (FAs) are present in the membranes of all organisms as building blocks of lipids,
56 but they can also be used as a source of energy. In bacteria, free FAs can arise either from
57 the recycling of the lipids of the envelope or are imported from the environment.
58 Degradation of FAs through β-oxidation leads to the production of acetyl-Coenzyme A that
59 can enter the TCA cycle, and to the production of reduced NADH and $FADH_2$ cofactors (1–3)
60 (Figure 1A).

61 To be used by the β-oxidation cycle, free FAs are first linked as thioesters to CoA by
62 the acyl-CoA synthetase FadD. Then, each round of β-oxidation consists of sequential
63 activities of FadE, an acyl-CoA dehydrogenase, FadB, an enoyl-CoA hydratase and
64 hydroxyacyl-CoA dehydrogenase, and FadA, a 3-ketoacyl-CoA thiolase (Figure 1A). FadA and
65 FadB form a so-called core-tri-functional multienzyme complex, catalyzing the last 3 steps
66 listed above. Noticeably, FadB also possesses 3,2-enoyl-CoA isomerase and 3-hydroxyacyl-
67 CoA epimerase activities, which are both required for the degradation of unsaturated FAs
68 (UFA) with double bonds at odd-numbered positions. In contrast, degradation of UFA with
69 double bond*s* at even-numbered positions requires an additional enzyme. Indeed,
70 degradation of such UFA leads to a 2-*trans*,4-*cis*-dienoyl-CoA intermediate that cannot be
71 further catabolized by the core trifunctional enzymatic complex (Figure 1A) (4). Biochemical
72 investigations showed that *E. coli* contains a 2,4-dienoyl-CoA reductase, FadH, which can act
73 on such UFAs, while a *fadH* mutant was found to preclude growth on petroselinic acid that
74 contains a double bond at carbon 6 (5–7).

75 *In vit*ro, FadH catalyzes the reduction of 2,4-dienoyl-CoA to 2-*trans*-enoyl-CoA. FadH
76 is a 72 kDa monomeric protein (7) that contains several cofactors: NADPH, a [4Fe-4S] cluster,
77 a flavin adenine dinucleotide (FAD), and a flavin mononucleotide (FMN) (8, 9). FadH uses the

78 reducing power from NADPH to remove the C_4-C_5 double bond and produce an enoyl-CoA
79 that can re-enter the β-oxidation cycle (Figure 1A) (6, 10). In the reduction process, two
80 reducing equivalents are transferred from NADPH to FAD, then 2 electrons are further
81 transferred to FMN via the [4Fe-4S] cluster (9, 11). The fully reduced FMN together with the
82 Tyr-166 and His-252 residues proposed to form a catalytic dyad at the active center of the
83 protein perform the final reduction of the dienoyl-CoA substrate (9). Interestingly, FadH
84 lacks stereospecificity and can catalyze the reduction of both *cis* and *trans* double bonds
85 (12).

86 Eukaryotes synthesize two 2,4-dienoyl-CoA reductases (euDECR), a mitochondrial and
87 a peroxisomal one (13–15). The mitochondrial and peroxisomal euDECR carry out similar
88 reactions, and they share 35% sequence identity (15). In contrast, they widely differ from the
89 prokaryotic FadH. The eukaryotic DECR are homotetramers with a total molecular mass of
90 124 kDa. They are strictly NADPH-dependent but differ from FadH by lacking FAD and
91 [4Fe-4S] cofactors. In euDECR, the reducing equivalents are directly supplied from NADPH to
92 the substrate to form 3-*trans*-enoyl-CoA (10). Then, an additional isomerase is required to
93 convert the 3-*trans*-enoyl-CoA to 2-*trans*-enoyl-CoA which can reenter in the β-oxidation
94 cycle. The eukaryotic DECR can also metabolize FAs with double bonds at odd-numbered
95 positions (7, 16), whereas there is no evidence for such activity in the *E. coli* FadH.

96 In the present study, we show that *fadH* is essential for *E. coli* to use linoleic acid
97 (9-*cis,* 12-*cis*-octadecadienoic acid). Moreover, we demonstrate that FadH activity is
98 essential to prevent linoleic acid degradation products from jamming the β-oxidation and
99 thereby inhibiting degradation of other types of FA. An extension of our study to eukaryotic
100 2,4-dienoyl-CoA reductases show that they can substitute for FadH in *E. coli*, both for growth
101 on linoleate and preventing inhibition of other FA degradation. Our results highlight the

102 crucial role that enoyl-CoA reductases may play in the degradation of FAs in natural
103 environments, either for the use of FAs as energy, or for detoxification of host-derived lipids
104 with antimicrobial effects (17).
105

106 **RESULTS**

107 ***fadH* is essential for growth of *E. coli* on linoleic acid.**

108 We compared the growth of a *fadH* mutant with the wild-type strain on minimal medium
109 containing oleate or linoleate as unique carbon and energy source (Figure 1B). Growth of the
110 wild-type strain appeared as robust on linoleate as on oleate. In contrast, the *fadH* mutant
111 was unable to grow on linoleate, whereas it was able to grow on oleate. Growth on linoleate
112 of the *fadH* mutant was restored when the mutant strain was complemented with a pTrc-
113 *fadH* plasmid (Figure 1B). Note that this complementation was observed in the absence of
114 induction with IPTG, showing that low amounts of *fadH* expression from the leaky Plac
115 promoter was sufficient for complementation.

116

117 **The [Fe-S] cluster of FadH is required for FadH activity *in vivo*.**

118 FadH binds a [4Fe-4S] cluster, predicted to be liganded by 4 cysteine residues (Cys335,
119 Cys338, Cys342, and Cys354) as shown by structural analysis (9). Purified FadH variants with
120 a Cys338Ala or a double Cys335Ala-Cys338Ala mutation have drastically reduced activity *in*
121 *vitro* (11). Therefore, we tested the importance of the two remaining Cys342 and Cys354
122 residues. We mutated each of the 4 Cysteine residues in Alanine. We first verified that the
123 mutant proteins were produced similarly as the wild-type FadH protein (Figure S1) and then
124 tested the ability of these mutants to complement the growth phenotype of Δ*fadH* on
125 linoleate plates (Figure 2). When expressed from plasmids, none of the mutants enabled
126 complementation. Our results show that Cys335, Cys338, Cys342, and Cys354 are all
127 essential for FadH function *in vivo*, and by inference that the [4Fe-4S] cluster is essential for
128 FadH activity *in vivo*.

129

130 **The unsaturated linoleic acid inhibits growth of the *ΔfadH* mutant on other fatty acids**

131 During the course of this project, we tested growth of *E. coli* on several commercial sources
132 for oleate. Surprisingly, the *fadH* mutant showed clear growth defect when using powders
133 rated less than 90% pure, whereas it exhibited wild-type like growth on pure oleate
134 preparations (Figure 3A). Changing the type of neutralization salt (sodium or potassium) did
135 not modify the growth defect phenotype (Figure 3A). Given the known biochemical
136 specificity of FadH for UFA with double bonds at even-numbered carbon position, we
137 surmised that such type of UFA may be contaminating the oleate preparations. We tested
138 this hypothesis by mixing linoleate with high quality oleate powder. Strikingly, the presence
139 of linoleate prevented the use of pure oleate by the Δ*fadH* mutant (Figure 3B). A possibility
140 was that contaminating UFA somehow inhibited FA degradation as a whole by blocking β-
141 oxidation. If so, the prediction was that the presence of linoleate should prevent the use of
142 any kind of FAs by the *fadH* mutant, but not the use of other types of carbon source.
143 Consistent with this prediction, Δ*fadH* failed to grow on a mix of linoleate and myristate (a
144 14-carbon saturated FA) (Figure 3B). Then, a Δ*fadR*Δ*fadH* double mutant was used to test
145 linoleate inhibitory effect on medium chain fatty acids (MCFA), such as decanoate (C10) and
146 dodecanoate (C12). While the Δ*fadR*Δ*fadH* mutant grew well on decanoate and
147 dodecanoate, it was unable to use MCFA when linoleate was added (Figure 3C). Note that a
148 Δ*fadR* mutation was used because MCFA are unable otherwise to alleviate FadR-mediated
149 repression of the FA degradation *fad* regulon as shown by the lack of growth of the wild-type
150 strain on decanoate or dodecanoate alone (Figure 3C).

151 Last, we tested whether the linoleate was inhibiting use of FAs specifically. This
152 proved to be the case as growth of the Δ*fadH* strain was not affected in linoleate containing
153 media added with succinate or glycerol as carbon sources (Figure 3D).

154

155 **FAs accumulate inside *ΔfadH* mutant cells exposed to linoleate.**

156 A possible explanation to account for the linoleate mediated inhibition of FA utilization in a
157 *ΔfadH* mutant was that the Fad machinery got jammed by unprocessed products of linoleic
158 acid degradation. This was tested three ways.

159 Firstly, we reasoned that preventing the entrance of linoleic acid inside the cell might
160 prevent the jamming of the machinery. FAs are imported via the outer membrane located
161 FadL transporter. Therefore, we tested whether a Δ*fadL* mutation would suppress growth
162 defect of Δ*fadH*Δ*fadR* strain on linoleate added with decanoate (a 10 carbon FA), a condition
163 for which growth inhibition was the strongest. The *ΔfadL* mutation was transduced in the
164 Δ*fadH*Δ*fadR* strain and the resulting transductants were able to grow on decanoate even in
165 the presence of linoleate (Figure 4A), demonstrating that the linoleate inhibitory effect
166 required linoleic acid to be imported inside the cells.

167 Secondly, we searched for suppression mutations in the Δ*fadH*Δ*fadR* strain grown on
168 linoleate added to decanoate. We obtained suppressors that acquired the capability to grow
169 in this condition (Figure 4A). Most of the suppressors had an inactivated *fadL* gene, either by
170 the insertion of an IS1 element, or by the presence of nucleotide deletions inside the *fadL*
171 ORF (Table S2). Systematic nucleotide genome sequencing of other $fadL^+$ suppressors
172 uncovered enrichment of a 100 kb large genomic region, which includes genes potentially

173 related to FA metabolism (ubiquinone synthesis, lipases, transcription regulators). The
174 molecular basis of their suppressor effect will be investigated in a separate study.

175 Thirdly, total lipid content from wild-type and Δ*fadH* strains was analyzed. Both
176 strains were grown in minimal medium with oleate as the sole carbon source for two days,
177 then these cultures were diluted in minimal medium containing oleate with or without
178 linoleate and incubated for 2 more days. Note that the growth of the Δ*fadH* strain was
179 strongly affected with linoleate+oleate as described above, but we could obtain enough cell
180 material in these growth conditions with a preincubation with oleate. Thin Layer
181 Chromatography analysis of the neutral lipids revealed accumulation of free FAs inside the
182 cells in the Δ*fadH* mutant exposed to linoleate (Figure 4B). When the Δ*fadH* strain was
183 transformed with the pTrc-*fadH* plasmid, liquid growth in linoleate+oleate was restored and
184 similar to the growth of the wild-type strain. This was concomitant with a strong reduction in
185 the amount of accumulating free FAs (Figure 4B).

186 Taken together, these results strongly suggest that linoleate exerts its inhibitory
187 effect in the Δ*fadH* mutant by blocking the β-oxidation machinery.

188

189 **Linoleic acid jams the β-oxidation in a Δ*fadH* mutant.**

190 In order to investigate the basis of the linoleate mediated inhibitory effect on oleate
191 degradation, we tested whether there could be actual competition between the two FAs.
192 Supporting this view, we observed that the extent of growth inhibition of the Δ*fadH* mutant
193 was proportional to the concentration of linoleate present in the linoleate/oleate mix (Figure
194 S2). A logical follow up was that linoleate inhibited oleate degradation by competing out FA
195 degradation machinery components. As a matter of fact, linoleate inhibitory effect on oleate

196 degradation was not as strong in the Δ*fadR*Δ*fadH* mutant as in the Δ*fadH* mutant (Figure 3B).
197 Because Δ*fadR* mutation turns on constitutive synthesis of the Fad degradation machinery,
198 this suggested that the jamming of the machinery could be suppressed by increasing the
199 levels of the Fad proteins for which oleic acid and linoleic acid were competing.

200 We then tested whether overexpression of *fad* genes could act as multicopy
201 suppressors of the linoleate inhibitory effect in the Δ*fadH* mutant. We found that the
202 overproduction of the acyl-CoA synthetase FadD improved the growth of the Δ*fadH* mutant
203 on the oleate/linoleate mix (Figure 5A and S3A) or on the decanoate/linoleate mix (Figure
204 5B). None of the other Fad enzymes improved growth on linoleate + oleate (Figure S3A). To
205 test if suppression by increased *fadD* gene dosage was due to acyl-CoA synthase activity of
206 FadD enzyme, we constructed the Y213A or E361A variants. These residues are located in
207 the ATP/AMP signature motif, and the corresponding FadD mutated enzymes retain only
208 10% or less than wild-type activity *in vitro* (18). The cognate alleles were introduced in the
209 pTrc-*fadD* plasmid. *In vivo*, we observed that only the FadD(E361A) mutant was severely
210 impaired in FadD activity (Figure S3B), therefore we selected this mutant for further
211 experiments. Compared to wild-type FadD, the FadD(E361A) mutant was not able to restore
212 growth on linoleate + oleate or on linoleate + decanoate in the Δ*fadH* mutant (Figure 5A and
213 5B), showing that the enzymatic activity of FadD was required to relieve the jamming of the
214 FA degradation machinery by linoleate.

215 All together, these experiments demonstrated that the *fadH* gene is essential for *E.*
216 *coli* to use FA optimally in a complex environment containing mixtures of FA and UFA, a
217 situation likely to reflect one found in the gut.

218

219 **Eukaryotic 2,4 dienoyl-CoA reductases (euDECR) can complement an *E. coli* Δ*fadH* mutant.**

220 Like bacteria, eukaryotic cells use FA as source of energy and carbon through their β-
221 oxidation in organelles. Several recent studies highlighted the role of FA β-oxidation in the
222 survival and proliferation of some types of cancer cells. In particular, euDECR was found to
223 be induced in breast cancer and in the treatment-resistance prostate cancer, correlating the
224 gene to a higher malignancy and proliferation of the tumor (19, 20). Given that β-oxidation is
225 highly conserved between prokaryotes and eukaryotes, it was intriguing that eukaryotes
226 make use of a 2,4-dienoyl-CoA reductase that exhibits structural and functional differences
227 with FadH (especially the absence of an [Fe-S] cluster in euDECR). In addition, the different
228 nature of the end-products of the reaction, i.e. bacterial-like FadH produces 2-*trans*-enoyl-
229 CoA, while euDECR produces 3-*trans*-enoyl-CoA, calls for an additional as yet
230 uncharacterized isomerization step to obtain 2-*trans*-enoyl-CoA in the *E. coli* system (10).

231 Since euDECR might represent a new therapeutical target for cancer treatment (20),
232 we tested whether it could complement an *E. coli fadH* mutant. We chose to test both
233 mitochondrial DECR and peroxisomal DECR (called thereafter DECR1 and DECR2
234 respectively). We also tested both the human and the rat homologs (14, 21). DECRs
235 sequences (deleted of the transit peptide sequence in the case of mitochondrial DECR1
236 genes) were synthesized and codon-optimized for production in *E. coli*. For human DECR2,
237 we used the original cDNA sequence that has been cloned before (14). Then, the sequences
238 were cloned in the pTrc vector, under the control of the Plac promoter. The Δ*fadH* mutant
239 was transformed by the DECR expressing plasmids and growth on linoleate was assayed
240 using different concentrations of IPTG inducer (Figure 6A). Interestingly, different behaviors
241 were observed depending on the DECR constructs. Human DECR1 and rat DECR2 were able

242 to complement for the absence of FadH without induction, hence with basal expression from
243 the Plac promoter. Strikingly, this complementation was lost when induced with IPTG,
244 suggesting a toxic effect of the over expression. This is similar to the expression of *fadH* itself
245 that complemented the Δ*fadH* mutant without induction, but which was toxic when induced
246 at 0.5mM IPTG (Figure 6A). In contrast, the human DECR2 was only able to complement
247 upon induction with IPTG. Finally, we did not observe complementation with the rat DECR1
248 construct. To test if these differences in complementation were due to functional
249 specificities or rather from a difference in protein production levels, we tested the
250 overproduction of the different DECR proteins (Figure 6B). Interestingly, it was not possible
251 to detect the human DECR2 proteins that require induction for complementation. In
252 contrast, human DECR1 and rat DECR2 proteins were easily identified on a Coomassie blue
253 stained gel, similarly to FadH. These proteins correspond to the ones that complemented
254 without the need of induction, like FadH. Finally, it was also possible to detect rat DECR1
255 overproduction. In conclusion, it seems that the differences in complementation of the
256 Δ*fadH* mutant were rather due to different efficiencies of production of the proteins. Only
257 rat DECR1 seemed not to be able to complement in any conditions even though we could
258 detect it on gel.

259 Next, we tested whether use of *E. coli* could help to investigate further functional
260 aspects of eukaryotic DECR. As a proof of concept, we mutagenized residues of human
261 DECR2 that have been proposed to be involved in its enzymatic activity (15) and tested their
262 ability to complement the *E. coli* Δ*fadH* mutant for growth on linoleate. Mutations of acidic
263 (D86, D137, D186, D268) and conserved K182 residues around the active site abolished the
264 complementation (Figure S4). Mutation of the conserved N117 residue retained some
265 complementation upon overproduction.

266 Finally, we asked if the activity conferred by euDECR enzymes was able to relieve the
267 jamming of the β-oxidation machinery by linoleate in the Δ*fadH* mutant. We chose the
268 euDECR1h and euDECR2r plasmids that complemented the Δ*fadH* mutant for growth on
269 linoleate alone without the need of adding IPTG, like the pTrc-*fadH* plasmid (Figure 6A).
270 These two enzymes were also able to relieve the inhibition of oleate degradation by
271 linoleate in the Δ*fadH* mutant (Figure 6C).

272 These results show that 2,4-dienoyl-CoA reductases from bacteria and eukaryotes are
273 indeed able to perform the same reaction *in vivo* in *E. coli*. Interestingly, it suggests that in
274 *E. coli*, an isomerase activity distinct from FadH is present to enable conversion from
275 2-*trans*-enoyl-CoA produced by euDECR to 3-*trans*-enoyl-CoA. Finally, these results of *in vivo*
276 complementation of an *E. coli fadH* mutant may provide a practical and powerful tool to
277 study the structure/function of the eukaryotic enzymes important for human health.

278

279 **DISCUSSION**

280 In the gut, enterobacteria can encounter a mix of diverse FAs, which they can use as a source
281 of energy for growth. Noticeably, recent evidence showed that cancer cells also rely heavily
282 on FA metabolism for their growth and proliferation (22). In contrast, host-derived FAs can
283 also display antimicrobial activities for which bacterial β-oxidation might play a
284 detoxification role (17). Here we demonstrated that the 2,4-dienoyl-CoA reductase FadH is
285 essential for *E. coli* to use linoleic acid as the sole carbon and energy source. Moreover, we
286 showed that FadH is essential for preventing jamming of the FA degradation machinery in a
287 situation wherein *E. coli* is exposed to mixed heterogenous environments, including
288 saturated and (poly)unsaturated FAs, a situation likely to reflect natural conditions.
289 Strikingly, we showed that eukaryotic DECR enzymes that show wide structural and
290 biochemical differences with FadH, can complement a Δ*fadH* mutant. Given the implication
291 of euDECR enzymes in human health, especially some types of cancer (19, 23), this result
292 opens the way to study euDECR structure/function in *E. coli* and eventually offering an easier
293 cellular model for screening of anti-euDECR inhibitors.

294 In this work, we observed that FadH is essential for *E. coli* to use linoleic acid as an
295 energy source but also to use other types of FAs in the presence of linoleic acid. Quite
296 strikingly, we showed here that lack of FadH not only prevents linoleic acid degradation but
297 precludes degradation of any type of FA tested in the presence of linoleic acid. We showed
298 that this inhibitory effect is due to accumulation of intermediates of linoleic acid degradation
299 that are likely to set an inhibitory competition situation. This hypothesis was supported by
300 the fact that increasing amounts of the machinery by deleting the *fadR* repressor relieved
301 the jam. Moreover, this result indicated that the *fad* genes were not completely derepressed
302 in the presence of oleate and linoleate.

303 Interestingly, while increasing the expression of the *fad* genes in the Δ*fadH*Δ*fadR*
304 mutant was sufficient to allow degradation of oleate in the presence of linoleate, it was not
305 enough to allow degradation of decanoate (compare figure 3B and 3C). This suggests that
306 the inhibitory effect might be due to a conjunction of titration of the machinery and of a
307 stronger specificity of some enzymes of the machinery for long chain FAs. In addition, we
308 observed that increasing the levels of acyl-CoA inside the cell by overproducing FadD was
309 also able to relieve the inhibition of the FA degradation machinery by linoleate in the Δ*fadH*
310 mutant (Figure 5). It was reported that overproduction of FadD increased the expression of
311 *fadE* gene, and that it even permitted the cell to grow on MCFAs (24). So, in a manner similar
312 to the deletion of *fadR*, the increase in acyl-CoA level might further derepress the expression
313 of the FA degradation machinery genes. However, as underlined above, increasing the
314 expression of the *fad* genes in the Δ*fadH*Δ*fadR* mutant was not enough to allow the
315 consumption of decanoate in the presence of linoleate, while it was allowing the
316 consumption of oleate. Hence, the positive effect of FadD overproduction might be
317 explained rather by an increased activity of the β-oxidation enzymes due to the higher acyl-
318 CoA substrate concentration.

319 Linoleic acid is an 18-carbon long UFA with two double bonds at carbons 9 and 12
320 (Figure 1). Thus, in theory, four cycles of partial degradation of linoleic acid should be
321 possible before the degradation process reaches the 10-carbon long intermediate step
322 requiring the FadH activity. Therefore, it could have been expected that a Δ*fadH* mutant
323 might still be able to grow on the energy given by this partial degradation. However, the
324 jamming that we describe here explains why it is not the case, and the machinery is blocked
325 at the 10-carbon long intermediate. Any FA containing an unsaturation at an even-numbered
326 carbon is expected to show the same behavior. For instance, in addition to linoleic acid that

327 is essential and can be obtained only from the diet, most polyunsaturated fatty acids found
328 in vegetal oils will contain unsaturations at an even position. The results we obtained with
329 low quality oleate powders showed that even small amounts of polyunsaturated FAs can
330 block β-oxidation in a Δ*fadH* mutant, suggesting an essential role of *fadH* in the complex and
331 heterogenous environment of the gut.

332 FAs are used as energy source both in bacteria and in eukaryotes by a similar
333 β-oxidation mechanism, including the 2,4-dienoyl-CoA reductase activity required for the
334 complete degradation of FAs containing an unsaturation at an even position. Interestingly,
335 this reaction is performed by two distinct protein families, the bacterial FadH-like containing
336 a [4Fe-4S] cluster and the eukaryote protein family lacking such a cofactor. We showed here
337 that all four Cysteine residues binding the cluster in FadH are required for *in vivo* activity and
338 strongly support the notion of the essential role of the cluster for the reductase activity. The
339 question of why eukaryotes have selected a reductase that functions without [Fe-S] cluster is
340 open. One possibility is that [Fe-S] independent 2,4-dienoyl-CoA reductases are more
341 resistant to oxidative stress in the mitochondrial environment. Such argument has been
342 used for other types of enzymes, such as fumarases, to strengthen the idea that eukaryote
343 genomes have steadily evolved to get rid of enzymes requiring [4Fe-4S] clusters that are
344 sensitive to oxidation (25). However, such a view is somehow disputed by the fact that we
345 were unable to observe any decrease in FadH activity in *E. coli* strains exposed to oxidative
346 stress (our unpublished work). The fact that the cluster is deeply buried in FadH structure
347 and strongly liganded by 4 Cysteine residues is consistent with the notion that it is shielded
348 from solvent and highly stable. Moreover, a bacterial-like enzyme homologous to FadH has
349 been found in eukaryotes such as Leishmania, indicative of a lateral transfer, and confirming
350 the equivalence of the reactions performed by the enzymes of the two families (26). Further

351 phylogenomic analysis might shed lights on the evolutive pattern of both types of enzyme
352 families and possibly provide a rationale for the emergence of [Fe-S]-less euDECR. Another
353 intriguing potential twist in the evolution of this protein family is provided by an enzyme of
354 *Helicobacter pylori*, FabX, which possesses FMN and [4Fe-4S] cofactors like FadH, yet is
355 required for the synthesis of unsaturated FAs and not for their degradation (27). Here too, it
356 would be interesting to delineate the phylogenetic relationships between FadH and FabX
357 enzymes.

358 In conclusion, our results show that it is crucial to understand better the degradation
359 of various types of FAs, how these FAs impose different constraints on cell metabolism,
360 whether prokaryotes or eukaryotes, which in turn will be differentially impacted by
361 starvation or other types of stresses in the natural environment. In particular, we showed
362 that enzymes of distinct protein families in bacteria or eukaryotes are able to perform the
363 same 2,4-dienoyl-CoA reductase reaction *in vivo* in *E. coli*. This opens towards new lines of
364 research as it suggests that in *E. coli*, an isomerase activity distinct from FadH is present to
365 enable conversion from 3-*trans*-enoyl-CoA produced by euDECR to 2-*trans*-enoyl-CoA.
366 Moreover, *E. coli fadH* mutant may provide a practical and powerful tool to study the
367 structure/function of the eukaryotic enzymes important for human health.

368

369

370 **MATERIALS AND METHODS**

371

372 **Media and growth conditions**

373 Bacterial strains were routinely grown in aeration at 37°C in Luria-Bertani broth (LB;
374 bactotryptone [10 g/liter], yeast extract [5 g/liter], NaCl [10 g/liter], pH 7.5) or M9 minimal
375 medium (Na_2HPO4-$7H_2O$ [6 g/liter], KH_2PO_4 [3 g/liter], NaCl [0.5g/liter], NH_4Cl [1 g/liter],
376 $MgSO_4$ [2 mM]), complemented with 1.5% agar for solid media. When required, ampicillin
377 was added at the concentrations of 100 µg/ml. Growth on fatty acids was tested on
378 synthetic M9 medium plates containing 1 g/L of potassium oleate or sodium linoleate.
379 Potassium Oleate 98% and Sodium Linoleate 95% were purchased from TCI. Medium chain
380 fatty acids and other oleate stocks were purchased from Sigma, as indicated in the figure
381 legends. All fatty acids were prepared at 100 mg/ml in 10% NP-40, and then diluted to a final
382 concentration of 1 mg/ml in the culture media.

383

384 **Strain and plasmid constructions**

385 The *E. coli* strains, plasmids, and oligonucleotides used in this study are listed respectively in
386 tables 1, 2, and table S1. *E. coli* deletion mutants were constructed by P1 transduction from
387 strains of the Keio collection (28) into our laboratory MG1655 reference strain. The coding
388 sequences of DECR1 deleted of the N-terminal targeting peptide and of DECR2 from human
389 and rat have been codon-optimized for expression in *E. coli* and genes have been ordered
390 from Twist Bioscience (TWB). Additionally, the natural coding sequence of human DECR2
391 have been amplified from the pBAD-DECR2 plasmid (14). Amplified DNA fragments were
392 cloned in pTrc99a plasmid using conventional cloning with restriction enzymes and T4 DNA

393 ligase, as indicated in table 2. Mutations were introduced by PCR mutagenesis using the
394 oligonucleotides indicated (Tables 2 and S1).
395
396 **SDS-PAGE and protein expression**
397 Wild type *E. coli* transformed by the different pTrc plasmids were grown in LB supplemented
398 with ampicillin at 37°C. At OD_{600nm}=0.8, expression was induced with 1mM IPTG for 2 hours.
399 After centrifugation of 1 ml culture, total extracts were prepared by resuspending cell pellets
400 in 1X Laemmli Loading buffer and heating 10 minutes at 95°C. Sodium dodecyl sulfate-
401 polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (29)
402 and proteins revealed by Coomassie Blue staining.
403
404 **Lipid extraction and analysis by thin layer chromatography**
405 Wild-type and mutant strains were grown in 100 ml M9 minimal medium containing oleate
406 (O) or oleate plus linoleate (O+L) as the sole carbon source. At OD=1, cells were pelleted and
407 washed twice with phosphate-buffered saline. Total lipid extraction was performed through
408 a neutral Bligh Dyer method (30), following the protocol detailed in (31). Lipids were
409 analyzed by thin layer chromatography using a solvent for separation of neutral lipids
410 (Heptane, Ether, Formic acid 55:45:1). After drying the plate, lipids were visualized by
411 spraying with primuline, and the picture was taken under UV exposure.
412

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Table 1 : Strains

Lab code	name	description	Reference
FBE353	MG1655	Wild-type reference strain	Lab collection
FBE765	Δ*fadH*	MG1655 Δ*fadH*::kana^{R}	This work
FBE1197	Δ*fadH*Δ*fadR*	MG1655 Δ*fadH* Δ*fadR* cured	This work
FBE434	Δ*fadL*	MG1655 Δ*fadL*::kana^{R}	This work
FBE425	Δ*fadD*	MG1655 Δ*fadD*::kana^{R}	This work
VSS50	Δ*fadH*Δ*fadR*Δ*fadL*	MG1655 Δ*fadH* Δ*fadR* Δ*fadL*::kana^{R}	This work

Table 2 : Plasmids

Lab code	name	description	Reference
pVP114	pTrc99a	amp^R, pTac promoter, *lacI*^{q}	(32)
pVP234	pTrc-*fadH*	PCR Ebm1742/1743 on *E. coli* genomic DNA EcoRI/XhoI in pTrc99a (EcoRI/SalI)	This work
pVP241	pTrc-*fadH*(C335A)	Mutagenesis PCR Ebp335/336 on pVP234	This work
pVP265	pTrc-*fadH*(C338A)	Mutagenesis PCR Ebp383/384 on pVP234	This work
pVP243	pTrc-*fadH*(C342A)	Mutagenesis PCR Ebp339/340 on pVP234	This work
pVP244	pTrc-*fadH*(C354A)	Mutagenesis PCR Ebp341/342 on pVP234	This work
pVP240	pBAD-6His-DECR2_human_cDNA		(14)
pVP247	pTrc-DECR2_human_cDNA	PCR Ebp348/349 on pVP240 EcoRI/XhoI in pTrc99a (EcoRI/SalI)	This work
pVP332	pTrc-DECR1ΔTP_human	PCR Ebp449/424 on synthetic fragment EcoRI/XhoI in pTrc99a (EcoRI/SalI)	This work
pVP333	pTrc-DECR1ΔTP_rat	Ebp450/428 on synthetic fragment EcoRI/XhoI in pTrc99a (EcoRI/SalI)	This work
pVP318	pTrc-DECR2_rat	Ebp429/430 on synthetic fragment EcoRI/XhoI in pTrc99a (EcoRI/SalI)	This work
pVP394	pTrc-*fadD*	PCR Ebm302/303 on *E. coli* genomic DNA EcoRI/XhoI in pTrc99a (EcoRI/SalI)	This work
pVP400	pTrc-*fadD*(Y213A)	Mutagenesis PCR ebp516/517 on pVP394	This work
pVP401	pTrc-*fadD*(E361A)	Mutagenesis PCR ebp518/519 on pVP394	This work
pVP395	pTrc-*fadB*	PCR Ebm1731/1732 on *E. coli* genomic DNA EcoRI/XhoI in pTrc99a (EcoRI/SalI)	This work
pVP396	pTrc-*fadE*	PCR Ebm1740/1741 on *E. coli* genomic DNA EcoRI/XhoI in pTrc99a (EcoRI/SalI)	This work
pVP397	pTrc-*fadBA*	PCR Ebm1731/1725 on *E. coli* genomic DNA EcoRI/XhoI in pTrc99a (EcoRI/SalI)	This work
pVP398	pTrc-*fadIJ*	PCR Ebm1733/305 on *E. coli* genomic DNA EcoRI/HindIII in pTrc99a (EcoRI/HindIII)	This work

519 **Figures**

520

521 **Figure 1 : A. Degradation of fatty acids in *E. coli*.** After their import in the cell, fatty acids
522 must be linked by a thioester to Coenzyme A to enter the β-oxidation cycle, an activity
523 performed by the acyl-CoA synthase FadD. Each round of β-oxidation consists of the
524 successive reactions of acyl-CoA dehydrogenase (FadE), enoyl-CoA hydratase (FadB),
525 hydroxyacyl-CoA dehydrogenase (FadB), and finally 3-ketoacyl-CoA thiolase (FadA).
526 Degradation of FA with unsaturations at even-numbered carbon (indicated in red in the
527 linoleic acid formula) leads to the production of a 2-*trans*,4-*cis* dienoyl-CoA intermediate. In
528 the case of linoleic acid, this happens after 4 cycles of degradation, producing a 10 carbon
529 long 2,4-decadienoyl-CoA molecule. The 2,4-dienoyl-CoA reductase FadH takes reducing
530 power from NADPH to catalyze the formation of an enoyl-CoA that can then re-enter the β-
531 oxidation cycle at the enoyl-CoA hydratase step performed by FadB. **B.** Wild-type and *fadH*
532 mutant (FBE765) strains were transformed by pTrc99a or pTrc-*fadH* plasmids. After
533 overnight growth in LB, cells were washed in minimal medium, serially diluted, and spotted
534 on M9 minimal medium with oleate or linoleate as sole carbon source. Plates were
535 incubated 3 days at 37°C.

536

537 **Figure 2** : **A.** The *fadH* deletion strain (FBE765) was transformed by pTrc99a empty vector,
538 *fadH* vector, or plasmids encoding *fadH* containing point mutations replacing the indicated
539 cysteine residues by alanines. After overnight growth in LB, cells were washed in minimal
540 medium, serially diluted, and spotted on M9 minimal medium with oleate or linoleate as
541 sole carbon source. Plates were incubated 3 days at 37°C.

542

543

544 **Figure 3: A.** Wild-type and *fadH* mutant (FBE765) strains were grown overnight in LB, cells
545 were washed in minimal medium, serially diluted, and spotted on M9 minimal medium
546 containing the indicated commercial sources of oleate. **B.** Wild-type, *fadH* (FBE765), and
547 Δ*fadH*Δ*fadR* (FBE1197) mutant strains were grown overnight in LB, cells were washed in
548 minimal medium, serially diluted, and spotted on M9 minimal medium containing FAs. Plates
549 were prepared with 0.1% oleate or myristate, with or without the addition of 0.1% linoleate
550 as indicated. **C.** Wild type and Δ*fadH*Δ*fadR* mutant (FBE1197) strains were grown overnight
551 in LB, cells were washed and serially diluted in minimal medium, and spotted on M9 minimal
552 plates containing 0.1% decanoate or dodecanoate, with or without the addition of 0.1%
553 linoleate, as indicated. Plates shown were incubated at 37°C for 3 days. **D.** Wild-type and
554 Δ*fadH* mutant (FBE765) strains were grown overnight in LB, cells were washed and serially
555 diluted in minimal medium, and spotted on M9 minimal plates containing 0.2% glycerol or
556 0.4% succinate, with or without 0.1% linoleate. Plates shown were incubated at 37°C for 3
557 days. Each panel shows a picture taken from one plate; the white lines indicate a cut to show
558 different parts of the same plate.
559
560 **Figure 4 : A.** The wild-type, Δ*fadH*, Δ*fadH*Δ*fadR*, Δ*fadH*Δ*fadR*Δ*fadL*, and 3 suppressor strains
561 (SUP1, SUP2, SUP3) were isolated on M9 minimal medium plates containing 0.1% decanoate
562 and 0.1% linoleate and incubated 3 days at 37°C. **B.** Total lipids were extracted from cultures
563 of wild-type and Δ*fadH* (FBE765) strains transformed with empty vector or pTrc-*fadH*, grown
564 in M9 minimum medium containing oleate (O) or containing oleate plus linoleate (O+L) (see
565 text for growth conditions). Neutral lipids were analyzed by thin layer chromatography and
566 detected with primuline staining as described in the Materials and Methods.
567

568 **Figure 5 :** The *fadH* deletion strain (FBE765) was transformed by pTrc99a empty vector, and
569 plasmids expressing *fadH*, *fadD*, or *fadD*(E361A) inactive mutant. After overnight growth in
570 LB, cells were washed in minimal medium, serially diluted, and spotted on M9 minimal
571 medium containing oleate or containing oleate plus linoleate (**A**) or containing decanoate +
572 linoleate (**B**). Plates were incubated 3 days (A) or 7 days (B) at 37°C.
573
574 **Figure 6** : **A.** The *fadH* deletion strain (FBE765) was transformed by pTrc99a empty vector
575 (e.v.), *fadH* vector, or plasmids encoding the indicated eukaryote DECR constructions. After
576 overnight growth in LB, cells were washed in minimal medium, serially diluted, and spotted
577 on M9 minimal medium containing linoleate as sole carbon source, with or without 0.5 mM
578 IPTG. Plates were incubated 2 days at 37°C. Each panel shows a picture taken from one
579 plate; the white lines indicate a cut in the image to show different parts of the same plate. **B.**
580 Wild-type *E. coli* strain was transformed by the indicated plasmids. Production of the
581 proteins was then assayed as described in Materials and Methods. Reds dots indicate the
582 position of the apparent overproduced proteins. The white separation indicates a cut in the
583 image to show different parts of the same gel. **C.** The wild-type and Δ*fadH* mutant (FBE765)
584 strains were transformed by the pTrc empty vector (e.v.) or by the pTrc plasmids expressing
585 *fadH*, DECR1h, or DECR2r genes as indicated. After overnight growth in LB, cells were
586 washed in minimal medium, serially diluted, and spotted on M9 minimal plates containing
587 0.1% oleate as the sole carbon source, with or without 0.1% linoleate. Plates shown were
588 incubated at 37°C for 3 days. Each panel show a picture taken from one plate; the white lines
589 indicate a cut to show different parts of the same plate.
590

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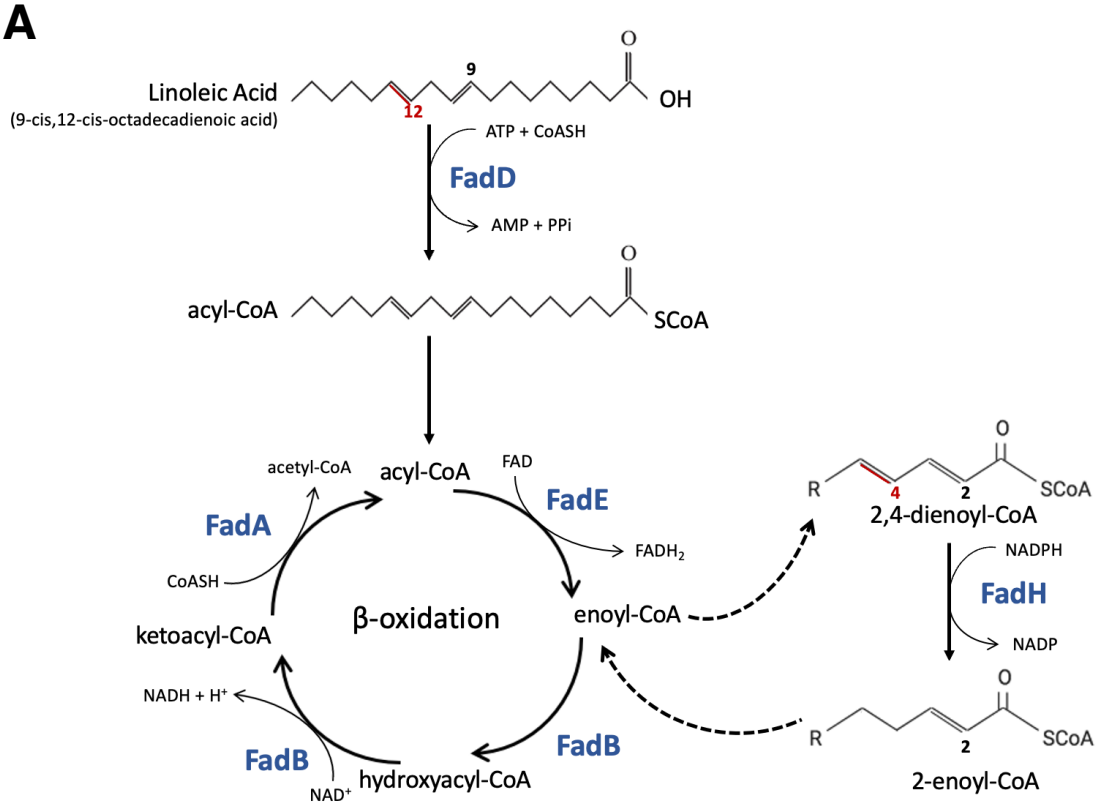
Figure 1

A


Linoleic Acid
(9-cis,12-cis-octadecadienoic acid)
9
12
OH
ATP + CoASH
FadD
AMP + PPi
acyl-CoA
SCoA
acyl-CoA
acetyl-CoA
FAD
FadE
FadA
FADH2
CoASH
β-oxidation
enoyl-CoA
ketoacyl-CoA
NADH + H+
FadB
FadB
NAD+
hydroxyacyl-CoA
R
4
2
SCoA
2,4-dienoyl-CoA
NADPH
FadH
NADP
R
2
SCoA
2-enoyl-CoA

B

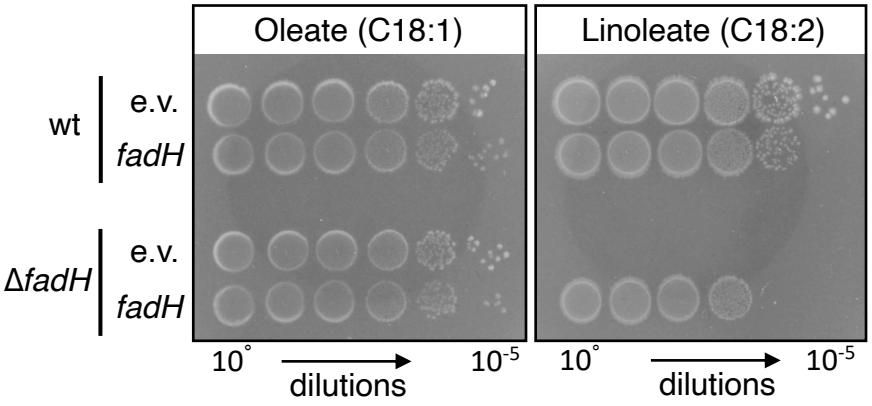

Oleate (C18:1)
Linoleate (C18:2)
wt
e.v.
fadH
ΔfadH
e.v.
fadH
10°
dilutions
10-5
10°
dilutions
10-5

Figure 2

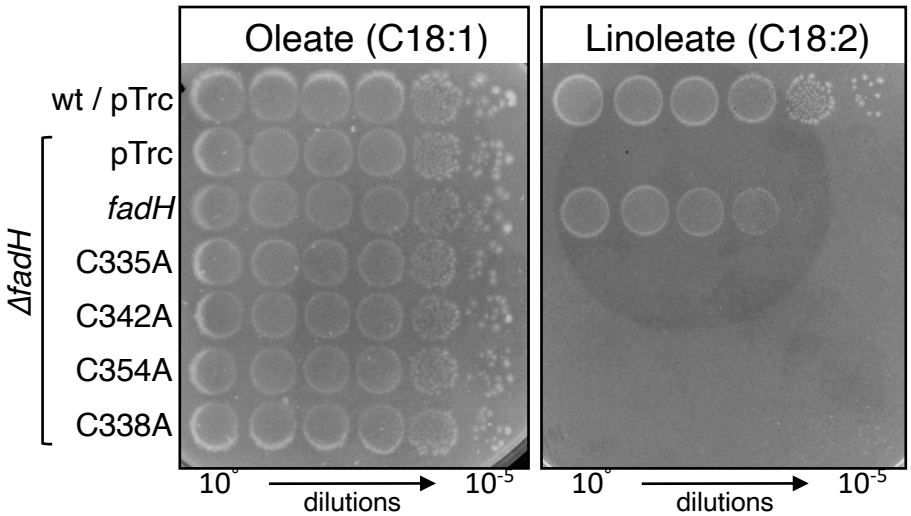
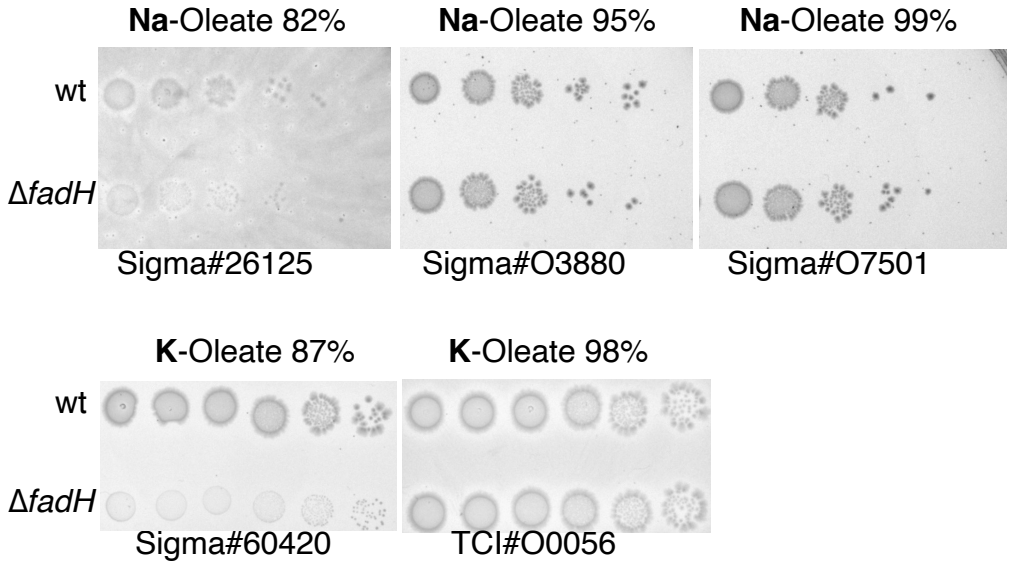

Oleate (C18:1)
Linoleate (C18:2)
wt / pTrc
pTrc
fadH
C335A
C342A
C354A
C338A
ΔfadH
10°
dilutions
10-5
10°
dilutions
10-5

Figure 3

A


Na-Oleate 82%
Na-Oleate 95%
Na-Oleate 99%
wt
Δ*fadH*
Sigma#26125
Sigma#O3880
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K-Oleate 87%
K-Oleate 98%
wt
Δ*fadH*
Sigma#60420
TCI#O0056

B

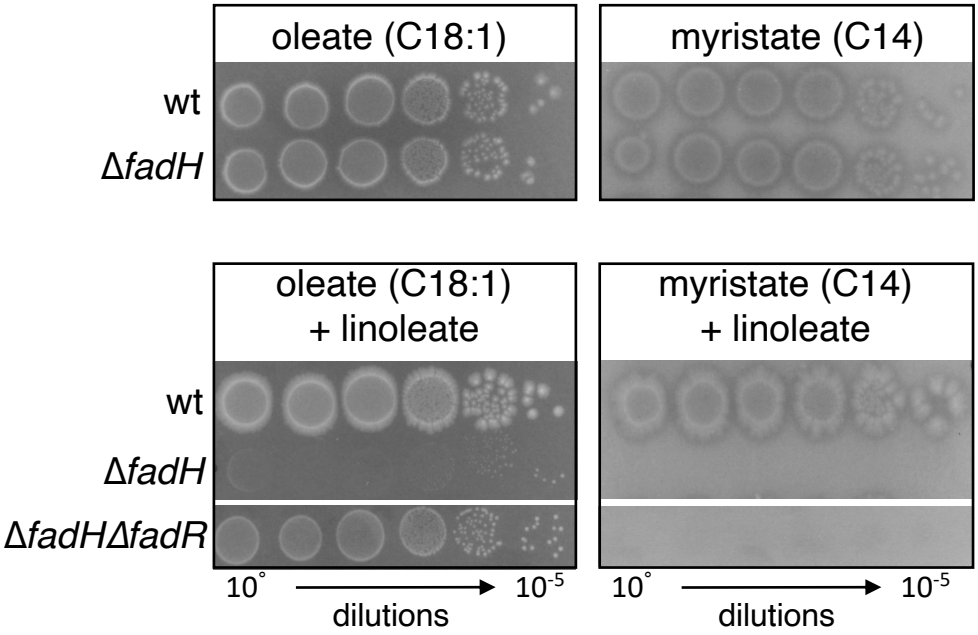
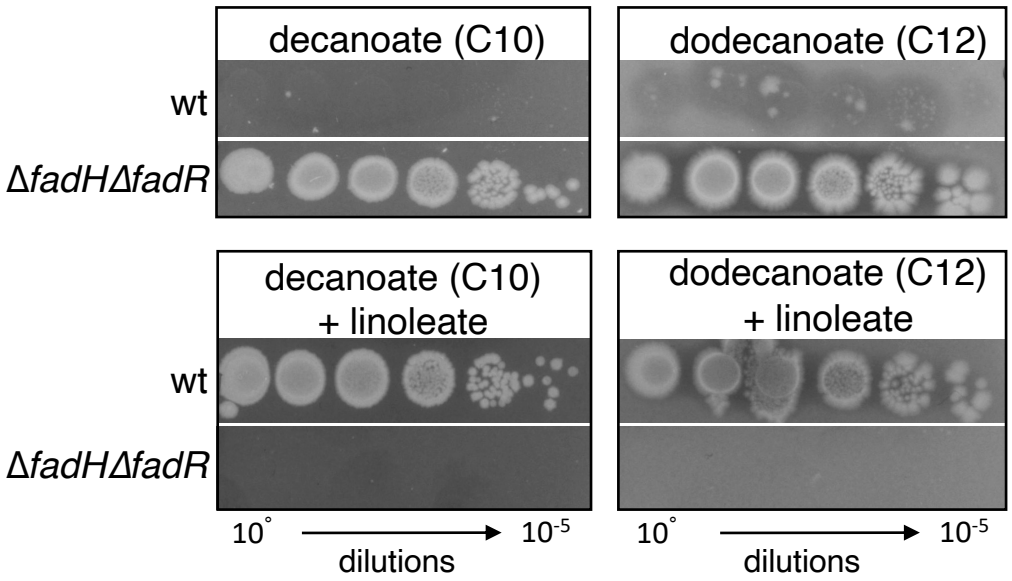

oleate (C18:1)
myristate (C14)
wt
Δ*fadH*
oleate (C18:1)
+ linoleate
myristate (C14)
+ linoleate
wt
Δ*fadH*
Δ*fadH*Δ*fadR*
10°
10-5
dilutions
10°
10-5
dilutions

Figure 3

C


decanoate (C10)
dodecanoate (C12)
wt
ΔfadHΔfadR
decanoate (C10)
+ linoleate
dodecanoate (C12)
+ linoleate
wt
ΔfadHΔfadR
10°
10-5
dilutions
10°
10-5
dilutions

D

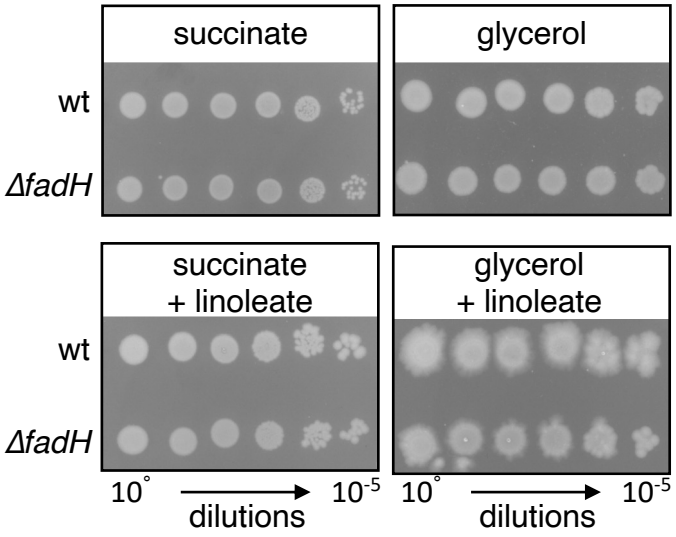
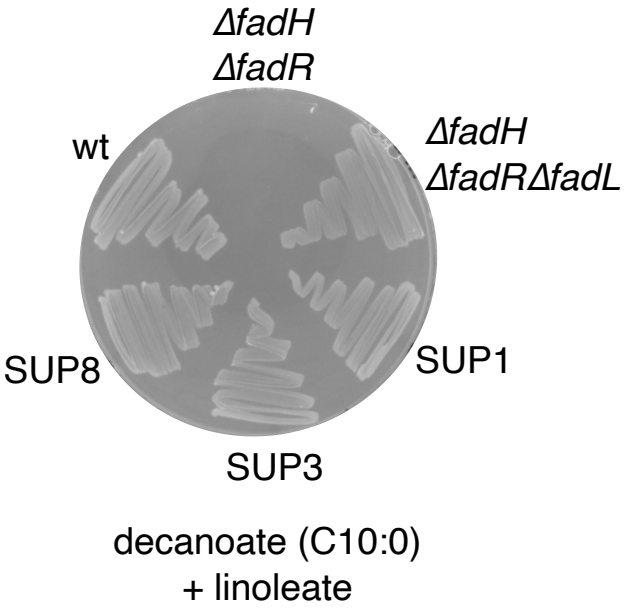

succinate
glycerol
wt
ΔfadH
succinate
+ linoleate
glycerol
+ linoleate
wt
ΔfadH
10°
10-5
dilutions
10°
10-5
dilutions

Figure 4

A


ΔfadH
ΔfadR
wt
ΔfadH
ΔfadRΔfadL
SUP8
SUP1
SUP3
decanoate (C10:0)
+ linoleate

B

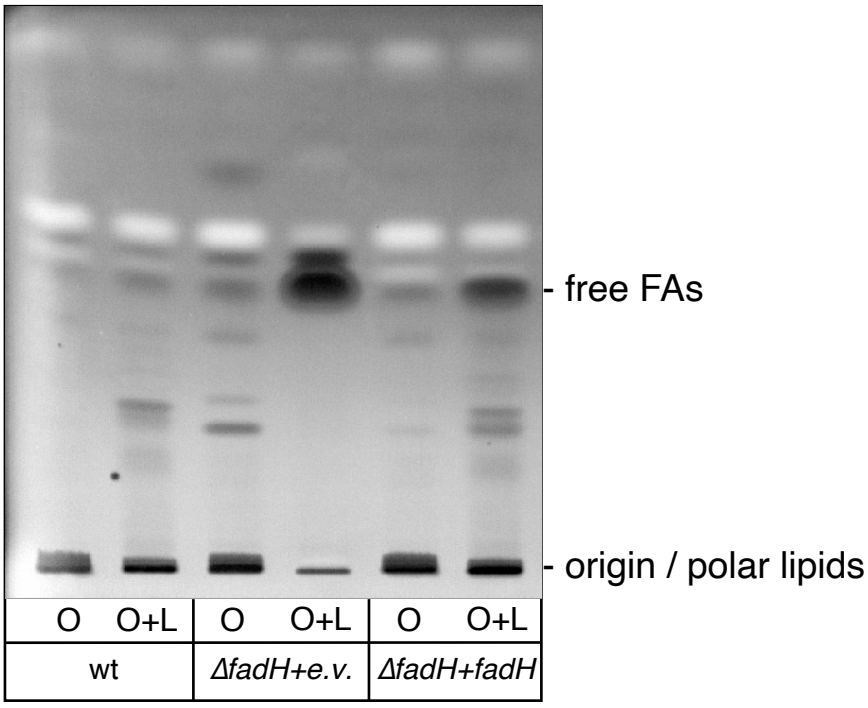
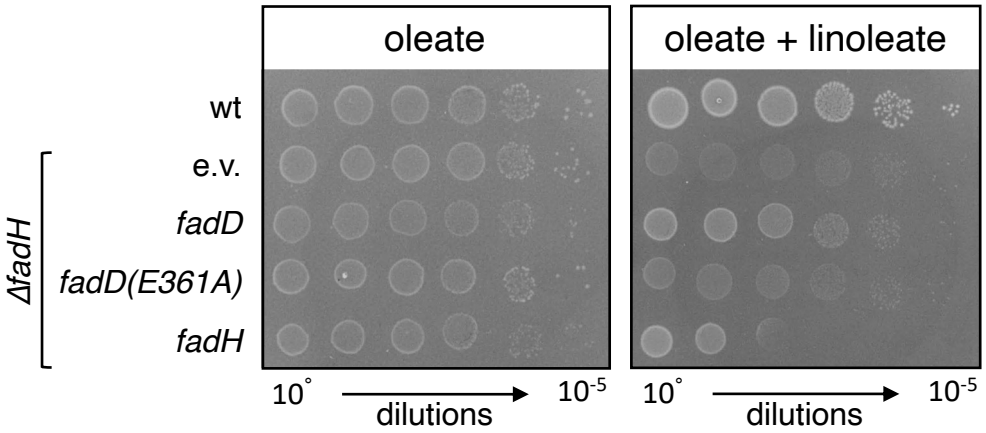

- free FAs
- origin / polar lipids
O
O+L
O
O+L
O
O+L
wt
ΔfadH+e.v.
ΔfadH+fadH

Figure 5

A


oleate
oleate + linoleate
wt
e.v.
fadD
fadD(E361A)
fadH
ΔfadH
10°
dilutions
10-5
10°
dilutions
10-5

B

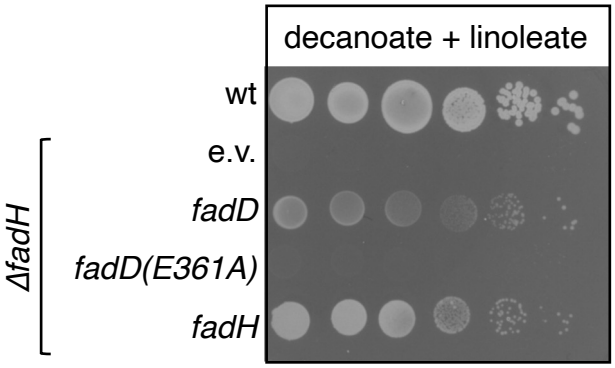
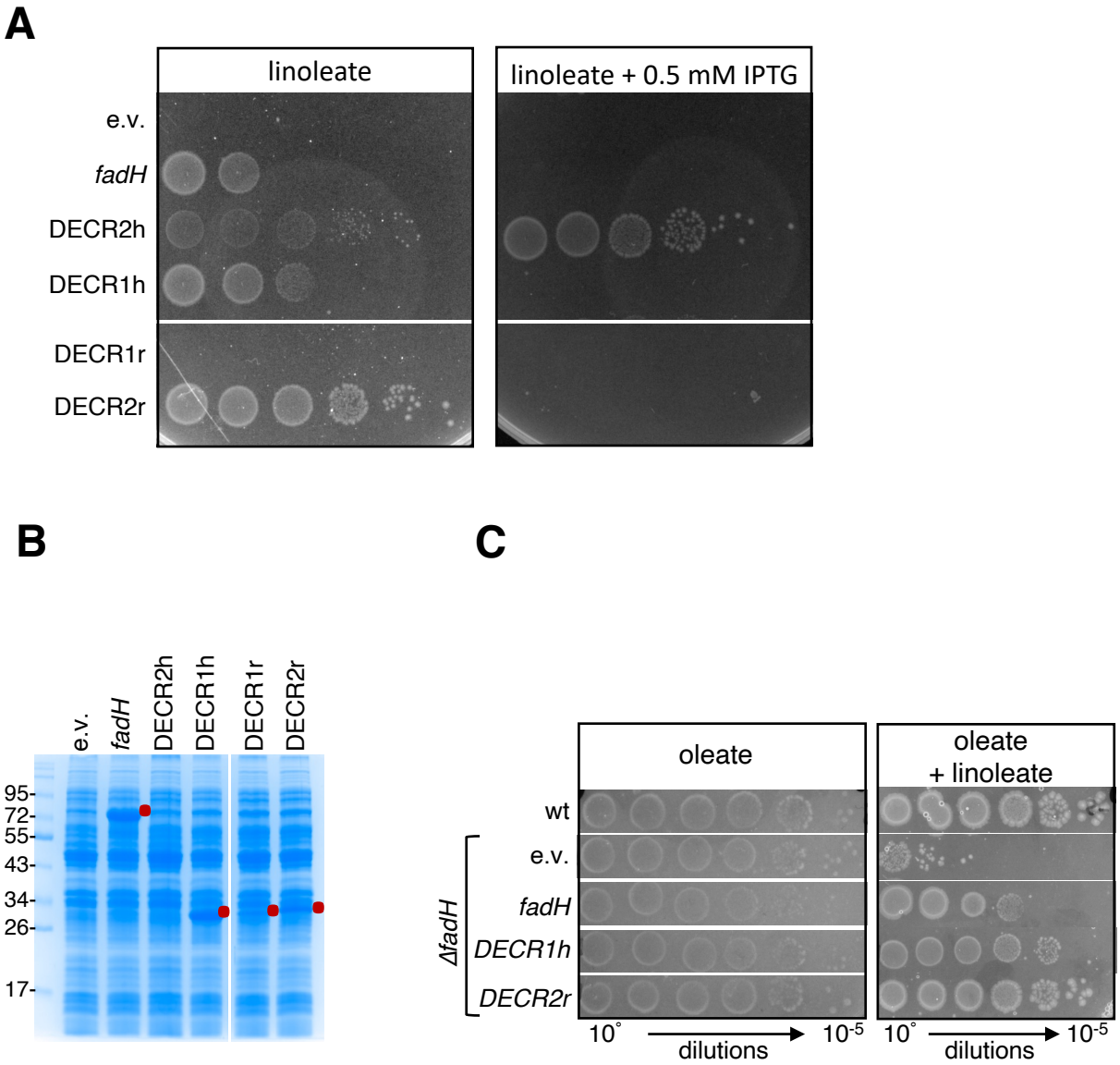

decanoate + linoleate
wt
e.v.
fadD
fadD(E361A)
fadH
ΔfadH

Figure 6


A
linoleate
linoleate + 0.5 mM IPTG
e.v.
fadH
DECR2h
DECR1h
DECR1r
DECR2r
B
e.v.
fadH
DECR2h
DECR1h
DECR1r
DECR2r
95-
72-
55-
43-
34-
26-
17-
C
oleate
oleate
+ linoleate
wt
e.v.
fadH
DECR1h
DECR2r
ΔfadH
10˚
dilutions
10-5
10˚
dilutions
10-5