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## Recent Work

### Title

A metabolic pathway for catabolizing levulinic acid in bacteria.

### Permalink

<https://escholarship.org/uc/item/7wj2z10w>

### Journal

Nature microbiology, 2(12)

### ISSN

2058-5276

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### Publication Date

2017-12-01

### DOI

10.1038/s41564-017-0028-z

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Peer reviewed

1Identification and characterization of the levulinic acid catabolic pathway in *Pseudomonas*  
2*putida*

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19

## 20Abstract

21Microorganisms have the ability to catabolize a wide range of organic compounds and therefore  
22the potential to perform many industrially relevant bioconversions. One barrier to realizing the  
23full potential of bio-refining strategies lies in the incomplete knowledge of metabolic pathways,  
24including those that can be used assimilate naturally abundant or easily generated feedstocks. For  
25instance, levulinic acid (LA) is a carbon source that is readily obtainable as a dehydration  
26product of lignocellulosic biomass molecules and can serve as the sole source of carbon for some  
27bacteria. Despite the importance of LA catabolism for growth on certain biomass hydrolysates,  
28both the genetics and intermediates of this pathway have remained unknown. Here, we report the  
29identification and characterization of an operon responsible for LA catabolism in *Pseudomonas*  
30*putida* KT2440. The seven-gene operon, designated *lva*, is under the control of a *prpR*-like  
31regulator, and encodes five enzymatic proteins alongside two transporters. *In vitro* reconstitution  
32of the pathway using purified enzymes demonstrated that LA is converted to 3-hydroxyvaleryl-  
33CoA through the intermediates 4-hydroxyvaleryl-CoA and 4-phosphovaleryl-CoA. This  
34discovery will allow for more efficient utilization of carbon from biomass hydrolysates and  
35engineering of bacteria to perform novel bioconversions using LA as a feedstock.

### 36Introduction

37Levulinic acid (LA) is a five carbon  $\gamma$ -keto acid that can be readily obtained from biomass  
38through non-enzymatic, acid hydrolysis of a wide range of feedstocks<sup>1,2</sup>. LA was named one of  
39the US Department of Energy's "Top 12 value-added chemicals from biomass"<sup>3</sup> because it can be  
40used as a renewable feedstock for generating a variety of molecules, such as fuel additives<sup>4-10</sup>,  
41flavors, fragrances<sup>11,12</sup> and polymers<sup>13,14</sup>, through chemical catalysis. In addition, microbes can  
42use LA as a sole carbon source and have been shown to convert LA into  
43polyhydroxyalkanoates<sup>15-18</sup>, short chain organic acids<sup>19-21</sup>, and trehalose<sup>19</sup>. All of these  
44bioconversion studies were conducted with natural bacterial isolates because the enzymes  
45comprising a LA assimilation pathway were unknown<sup>19</sup>. This knowledge gap limits metabolic  
46engineering and the potential of creating novel LA bioconversions.

47While the enzymes responsible for LA assimilation were unknown at the time of these  
48bioconversion demonstrations, other studies identified putative intermediates and suggested  
49pathways for LA catabolism. In a study where crude cell lysates of *Cupriavidus necator* were fed  
50LA, the concentration of LA and free CoA decreased over time while acetyl-CoA and propionyl-  
51CoA concentrations increased, suggesting that LA is catabolized via CoA thioesters like other  
52short-chain organic acids<sup>22</sup>. In a second study, cultures of *Pseudomonas putida* KT2440  
53expressing a heterologous TesB thioesterase were fed LA. Here, 4-hydroxyvalerate (4HV) and 3-  
54hydroxyvalerate (3HV) transiently accumulated extracellularly before ultimately disappearing<sup>23</sup>.  
55This observation strongly suggested that 4HV and 3HV (or their CoA thioesters) were pathway  
56intermediates. Lastly, a metabolomic study of rat livers suggested that LA is catabolized to  
57acetyl-CoA and propionyl-CoA via a unique phosphorylated acyl-CoA<sup>24,25</sup>. In sum, these

58 observations suggest a relatively direct route from LA to beta-oxidation intermediates, but the  
59 enzymes comprising such a pathway remain unknown.

60 In order to better utilize LA as a substrate for microbial growth or bioconversion, a detailed  
61 understanding of the metabolic pathway and enzymes involved is necessary. In this work, we  
62 investigated the genetic and biochemical factors that allow *P. putida* KT2440 to catabolize LA.  
63 Using a loss of function screen of a transposon library, we identified a putative LA utilization  
64 operon. The operon consists of seven genes, two homologs for membrane transporters and five  
65 enzymatic proteins. We reconstituted the pathway *in vitro* and determined that all five enzymatic  
66 proteins are required for complete conversion of LA into 3HV-CoA, an intermediate in the  $\beta$ -  
67 oxidation of odd-chain fatty acids. A closer inspection of the CoA ligase encoded in the operon  
68 revealed a broad substrate promiscuity including C<sub>4</sub> to C<sub>6</sub> organic acids. A putative regulator  
69 proximal to the operon activated transcription of the LA catabolic genes in the presence of LA or  
70 4HV. The induction tests revealed that while the CoA ligase might have nonspecific activity  
71 towards similar chain length acids, the promoter is only responsive when cells were provided LA  
72 or 4HV. Altogether, the catabolism of LA to acetyl-CoA and propionyl-CoA requires at least 2  
73 ATP that likely come from respiration and the TCA cycle.

## 74 Results

### 75 Identification of Genes Involved in Levulinic Acid Metabolism

76 *P. putida* KT2440 is known to metabolize LA as a sole carbon source and demonstrates diauxic  
77 growth in the presence of glucose and LA (**Supplementary Figure 1**). Therefore, we initiated a  
78 genetic study to identify genes involved in LA catabolism. We constructed a mutant library with  
79 a Tn5 mini transposase and screened for *P. putida* mutants lacking the ability to grow on LA as

80the sole carbon source<sup>26</sup>. Thirteen out of 7,000 colonies screened demonstrated LA growth  
81deficiencies. The location of each transposon insertion was determined by sequencing PCR  
82products created with a primer nested in the transposon paired with a degenerate random primer.  
83**Table 1** shows the ten unique isolates from these thirteen hits and the putative function of the  
84disrupted genes. Two mutants had disruptions in genes involved in propionate metabolism,  
85supporting the hypothesis that LA is catabolized to the central metabolites, acetyl-CoA and  
86propionyl-CoA. Three transposon mutants had disruptions in a putative operon that had not been  
87previously characterized (disrupting genes PP\_2791, PP\_2793, and PP\_2794). Other mutants had  
88disruptions in genes with no obvious connection to LA catabolism (*bioH*, *gcvP*, a hypothetical  
89zinc protease, *mrda*, and *fpvA*). To confirm that we had screened a sufficient number of clones,  
90we performed random bar code transposon-site sequencing (RB-TnSeq) for cultures enriched by  
91growth on LA and 4HV relative to growth on glucose. RB-TnSeq is an efficient method for  
92determining gene essentiality under different conditions with high genomic coverage<sup>27</sup>. This  
93analysis identified additional genes involved in LA metabolism including an acetoacetyl-CoA  
94transferase important for growth on LA, genes functioning in  $\beta$ -oxidation and propionyl-CoA  
95metabolism, and 14 transcriptional regulators potentially involved in LA metabolism. The RB-  
96TnSeq dataset also revealed that 3-hydroxybutyryl-CoA dehydrogenase and  $\beta$ -ketothiolase are  
97also necessary for growth on LA and 4HV, supporting our hypothesis that LA metabolism  
98terminates through  $\beta$ -oxidation. For a more complete summary and analysis of the fitness data,  
99please see **Supplementary Table 1** and **Supplementary Note:RB-TnSeq**.

101**Table 1.** *P. putida* Levulinic Acid Transposon Insertion Sites

Locus	Insertion Point*	Gene Name	Description/Homology
PP_0364	442685	<i>bioH</i>	pimeloyl-ACP methyl ester esterase
PP_0988	1128706	<i>gcvP-1</i>	glycine dehydrogenase
PP_2332	2660666	N/A	ATP-dependent zinc protease family
PP_2336	2666405	<i>acnA-II</i>	aconitate hydratase
PP_2337	2666944	<i>prpF</i>	aconitate isomerase
PP_2791	3181098	N/A	Phosphotransferase family
PP_2793	3182533	N/A	acyl-CoA dehydrogenase family protein
PP_2794	3183601	N/A	short chain dehydrogenase/reductase family
PP_3741	4271628	<i>mrdA-I</i>	transpeptidase
PP_4217	4765953	<i>fpvA</i>	TonB-dependent outer membrane ferripyoverdine receptor

102\*Insertion point based on location from *P. putida* KT2440 origin

### 103Operon Characterization and Induction

104Given the propensity of bacteria to cluster related genes into operons, we examined the putative  
105seven-gene operon, PP\_2791-PP\_2797, which contained three of our transposon hits (PP\_2791,  
106PP\_2793 and PP\_2794). We analyzed the sequence homology of the seven genes in the operon  
107using the basic local alignment search tool (BLAST<sup>28</sup>) and assigned predicted functions, listed in  
108**Table 2**. We were unable to find any published studies about these genes beyond the automated  
109sequence annotations. Therefore, we investigated the expression and function of these genes  
110involved in LA catabolism. First, we isolated RNA from wild type *P. putida* grown in minimal  
111media with LA as the carbon source and demonstrated that we could locate all seven genes by  
112PCR amplification of cDNA created with a reverse primer specific to PP\_2797 (**Figure 1**  
113**A,B,C**). The transcription start site (TSS) of the operon was isolated by 5' RACE<sup>29</sup> (**Figure 1D**)  
114and implicated a different start codon for PP\_2791, 72 bp downstream of the one originally  
115reported<sup>30,31</sup>. A  $\sigma^{54}$  promoter sequence located upstream of PP\_2791 was identified by comparing  
116upstream of the new TSS with published  $\sigma^{54}$  promoter consensus sequences<sup>32</sup> (**Supplementary**

117**Figure 1**). The data presented below suggests the proteins encoded by this operon are important  
118in LA catabolism and we propose that the polycistronic genes be designated as *lvaABCDEFG*.

119Upstream of *lvaABCDEFG*, we identified a gene oriented divergently from the operon  
120(PP\_2790) and predicted to encode a transcription factor with a  $\sigma^{54}$  interaction domain and  
121homology to the propionate metabolism activator, *prpR*. The genomic organization strongly  
122suggested that the gene encoded a regulator for the *lva* operon. Consequently, we deleted  
123PP\_2790 and evaluated growth of *P. putida* strains on both LA and a likely intermediate, 4HV.  
124The  $\Delta$ PP\_2790 mutant was unable to grow on LA and 4HV suggesting that it acts as an activator  
125for the operon. Expression of PP\_2790 on a plasmid restored growth of the deletion strain on LA  
126and 4HV. To identify compounds that activate *lvaABCDEFG* expression, we built a  
127transcriptional reporter system that linked sfGFP to the  $\sigma^{54}$  promoter sequence located upstream  
128of *lvaA*. The reporter cassette was cloned onto a broad host range vector (**Figure 2A**) and the  
129resulting construct was transformed into wild type *P. putida*. We tested a variety of short and  
130medium chain length acids by adding them to rich media and evaluating the corresponding  
131sfGFP expression levels. We observed strong sfGFP fluorescence only when LA or 4HV were  
132added to the system (**Figure 2B**). For these reasons, we suggest that PP\_2790 encodes  
133transcriptional regulator responsive to the LA pathway and should be designated *lvaR*.

#### 134**Genetic and Biochemical Studies of *lvaABCDEFG* Operon**

135To confirm the involvement of the *lva* operon in LA catabolism, we created a deletion mutant of  
136each *lva* gene predicted to encode an enzymatic protein and a corresponding complementation  
137plasmid using the  $P_{araBAD}$  promoter. We tested the ability of the resulting strains to grow on LA  
138and 4HV (**Table 2, Supplementary Figure 2**). In addition, we purified the five enzymes from



139cultures of *E. coli* BL21 (DE3), reconstituted the enzymatic reactions *in vitro*, and used liquid  
140chromatography/mass spectrometry (LC/MS) to identify reaction products. We used selective ion  
141scanning to monitor the masses for likely intermediates based on prior studies<sup>22,23,25</sup> and the  
142following hypothesized pathway (**Figure 3, Supplementary Figure 3**). First, LA is activated as  
143a coenzyme A-thioester, levulinyl-CoA (LA-CoA). Second, LA-CoA is reduced to 4-  
144hydroxyvaleryl-CoA (4HV-CoA). Third, 4HV-CoA is phosphorylated at the  $\gamma$ -position to yield 4-  
145phosphovaleryl-CoA (4PV-CoA). Fourth, 4PV-CoA is dephosphorylated to yield a pentenoyl-  
146CoA species (likely 3-pentenoyl-CoA). Last, pentenoyl-CoA is hydrated to yield 3-  
147hydroxyvaleryl-CoA (3HV-CoA) which can be further oxidized via  $\beta$ -oxidation to yield acetyl-  
148CoA and propionyl-CoA or incorporate 3HV-CoA into PHA polymers. The remainder of this  
149manuscript will provide evidence supporting our hypothesized metabolic pathway for converting  
150LA to 3-hydroxyvaleryl-CoA (3HV-CoA) and assignment of enzymes to each reaction.

152**Table 2.** *P. putida* LA Operon Knockout and Complementation

Genotype	Predicted Function	Growth on LA		Growth on 4HV	
		EV	Complement	EV	Complement
WT		++	N/A	++	N/A
$\Delta lvaR$	$\sigma^{54}$ dependent sensory box protein	-	++	-	++
$\Delta lvaA$	Phosphotransferase family	-	++	-	++
$\Delta lvaB$	Hypothetical protein	-	++	-	++
$\Delta lvaC$	acyl-CoA dehydrogenase family protein	-	++	+	++
$\Delta lvaD$	short chain dehydrogenase/reductase family	-	++	++	++
$\Delta lvaE$	Acyl-CoA synthetase	++	++	-	+

153(EV) empty vector plasmid; (N/A) not applicable; (-) No growth; (+) Visible growth; (++) Robust growth

154

### 155*lvaE*

156The presence of an enzyme (encoded by *lvaE*) with homology to an acyl-CoA synthetase  
157(including a putative CoA binding region and an AMP binding site) suggested that the  
158degradation pathway acts on CoA thioesters and begins with the activation of acids to acyl-  
159CoA's. The  $\Delta lvaE$  strain grew on LA but not on 4HV, indicating that LA may also be activated  
160by other CoA-synthetases in *P. putida*. We quantified the activity of purified LvaE (6x-His N-  
161terminal fusion) on a variety of organic acid substrates using the EnzChek<sup>®</sup> Pyrophosphate Assay  
162Kit which detects pyrophosphate released in the first half reaction to creating the acyl-AMP  
163intermediate (**Figure 4A**). LvaE demonstrated activity on C<sub>4</sub>-C<sub>6</sub> carboxylic acids, including LA  
164and 4HV (**Figure 4B**), but showed minimal activity on other organic acids (lactate, pyruvate,

165acetate, propionate, octanoate). Using LC/MS to detect reaction products (**Figure 5**), we  
166demonstrated that LvaE was necessary and sufficient to catalyze the ligation of CoA to LA,  
167generating levulinyl-CoA (LA-CoA). None of the other enzymes from the operon catalyzed this  
168or any other reaction using LA as a substrate (**Supplementary Figure 4**), confirming that the  
169pathway proceeds via acyl-CoA intermediates.

### 170*lvaD*

171The second step in our proposed pathway is the reduction of LA-CoA to 4HV-CoA which we  
172predicted to be catalyzed by *lvaD*. *lvaD* is annotated as an oxidoreductase containing an NADH  
173binding domain and was found to be required for growth on LA but not necessary for growth on  
1744HV (**Table 2**). We purified LvaD in a similar manner to LvaE but used an N-terminal maltose  
175binding protein (MBP) tag to increase the solubility of the enzyme<sup>33</sup>. The *in vitro* reaction  
176containing LvaD and LvaE verified that LvaD is involved in the production of 4HV-CoA (**Figure**  
177**5A**). Furthermore, LvaDE was the only enzyme combination capable of generating 4HV-CoA *in*  
178*vitro* (**Figure S2**). LvaD can catalyze the reduction of LA-CoA with either NADH or NADPH  
179(**Supplementary Figure 4**).

### 180*lvaAB*

181We hypothesized that the third intermediate would be 4-phospho-valeryl-CoA (4PV-CoA) based  
182off its observation in LA degradation in rat livers<sup>24,25</sup>. The first gene in the operon, *lvaA*, has  
183putative homology regions, including an ATP binding site, that associated it with the kinase  
184superfamily and phosphotransferase family of enzymes. The second protein in the operon (LvaB)  
185has no listed function and is predicted to be only 12 kDa in size. Orthologous sequence  
186alignments of *lvaB* reveal that in all other organisms this hypothetical protein is located

187immediately downstream of an *lvaA* ortholog. Therefore, a pull down experiment was used to  
188determine if the two proteins interact<sup>34,35</sup>. LvaA was N-terminally tagged with MBP and cloned  
189into a pET expression vector. LvaB was cloned directly downstream of LvaA as it is found in *P.*  
190*putida*'s native genome sequence. The recombinant proteins were expressed in *E. coli* BL21  
191(DE3) and purified using the MBP tag. An SDS-page gel of the eluent contained two bands at 85  
192kDa and 12 kDa, closely matching the predicted sizes of MBP-LvaA and untagged LvaB  
193respectively (**Supplementary Figure 5**). We performed MALDI-TOF-TOF mass spectrometry  
194on a trypsin digest of the 12 kDa band and identified the protein sequence to be LvaB  
195(**Supplementary Figure 5**).

196Growth studies of deletion mutants revealed that *lvaA* and *lvaB* are both required for growth on  
197either LA or 4-HV. This supports the hypothesis that they are involved in a reaction after the  
198conversion of LA-CoA to 4HV-CoA. To confirm that the association between LvaA and LvaB is  
199important for enzymatic activity, we tested the following enzymatic combinations: i) LvaA,  
200LvaD and LvaE, ii) LvaB, LvaD, and LvaE, iii) LvaAB, LvaD and LvaE. We observed a  
201decrease of 4HV-CoA and an increase of the predicted 4PV-CoA intermediate only when all four  
202of the enzymes were present (**Figure 5A, Supplementary Figure 4**).

203To verify the identity of 4PV-CoA, we performed tandem mass spectrometry (**Figure 6,**  
204**Supplementary Figure 6, Supplementary Table 2**). We compared the MS/MS spectra of 4HV-  
205CoA and 4PV-CoA and detected major ion fragments at  $m/z$  786.191, 537.106 and 519.095  
206(4HV-CoA) and 866.158, 617.072 and 599.061 (4PV-CoA). For each compound, these fragments  
207can be assigned to the cleavage of a P-O bond, an O-C bond and the dehydration of O-C cleaved  
208product, respectively. Both compounds are fragmenting at the same bonds, but the resulting  $m/z$

209values for the daughter ions differ by 79.967. This mass corresponds to the m/z of PO<sub>3</sub>H<sup>-</sup>,  
210supporting the existence of the phosphorylated 4HV-CoA species, 4PV-CoA.

### 211*lvaC*

212The final step in the hypothesized pathway is the formation of 3HV-CoA. Given that the  
213combination of LvaABDE was responsible for generating 4PV-CoA and no 3HV-CoA was  
214detected in these reactions, we postulated that LvaC was responsible for the final conversion  
215steps. LvaC has homology to the dehydrogenase family of enzymes and 30% amino acid  
216sequence identity to the *E. coli* acyl-CoA dehydrogenase protein. The  $\Delta$ *lvaC* strain was unable to  
217grow on LA, but grew weakly on 4HV. LvaC was purified as an MBP fusion and the resulting  
218protein pellet displayed a yellow hue. This is often indicative of a co-purified flavoprotein and an  
219absorbance scan of the protein revealed absorbance maxima that are consistent with a flavin co-  
220factor (**Supplementary Figure 5**). When the LvaC sample was treated with trichloroacetic acid  
221and centrifuged<sup>36</sup>, a white protein pellet and a yellow hued supernatant were observed (data not  
222shown). This indicates that the co-factor was not covalently bound to LvaC.

223When LvaC was added to the *in vitro* reaction mixture, the concentrations of reaction  
224intermediates (LA-CoA, 4HV-CoA, 4PV-CoA) were reduced while the abundance of 3HV-CoA  
225and a pentenoyl-CoA species increased (**Figure 5A**). This species is likely either 2-pentenoyl-  
226CoA and/or 3-pentenoyl-CoA, which could not be resolved with our methods. Both compounds  
227eluted at the same retention time with the same molecular mass. To test if LvaC is solely  
228responsible for the conversion of 4PV-CoA to 3HV-CoA, we ran a two-step reaction. First, we  
229performed the LvaABDE reaction with LA, CoA, ATP, NAD(P)H and separated the CoA  
230products from the enzymes. To the enzyme-free mixture, we added LvaC without additional co-

231factors. After 30 min, we observed signals for both pentenoyl-CoA and 3HV-CoA. This indicated  
232that the putative oxidoreductase, LvaC, is responsible for both the removal of the phosphate  
233group to produce the enoyl-CoA and the hydration of the enoyl to the 3-hydroxyl compound. To  
234reconstitute the whole pathway, we set-up a time course reaction with all five Lva enzymes and  
235LA as the starting substrate. Over time, we observed a rapid increase in pentenoyl-CoA followed  
236by a slow disappearance that mirrored the increase in the 3HV-CoA signal (**Figure 5B**). This  
237suggests that the hydration reaction may be the limiting step in the overall pathway.

### 238*lvaFG*

239Based on homology alignments, *lvaG* is predicted to encode a protein with 95% amino acid  
240sequence identity to a *Pseudomonas aeruginosa* cation acetate symporter and *lvaF* shares 33%  
241amino acid sequence identity with the *E. coli* inner membrane protein Yhjb<sup>28</sup>. Sequence  
242alignments of *lvaF* orthologs indicate that *lvaF* and *lvaG* are found with the same spatial  
243relationship to each other in many organisms (data not shown). These proteins are likely  
244involved in organic acid transport but are unlikely to be involved in the catabolism of LA given  
245that they were not necessary for the enzymatic conversion of LA to 3HV-CoA *in vitro*.

### 246Discussion

247The work described herein identified an operon responsible for assimilating LA into the  $\beta$ -  
248oxidation pathway of *P. putida*. Through an integrated genetic and *in vitro* biochemistry study,  
249we demonstrated that the genes *lvaABCDE* are upregulated in the presence of LA and are  
250sufficient for the conversion of LA to 3HV-CoA, an intermediate of native  $\beta$ -oxidation.  
251Removing any enzyme from the reaction mixture abolished 3HV-CoA production, indicating all  
2525 genes are necessary for this pathway. The biochemical assays confirmed the presence of 4PV-

253CoA, an intermediate previously observed in the metabolism of LA in rat livers. In sum, the  
254pathway consumes at least 2 ATP and one reducing equivalent to produce 3HV-CoA (**Figure 3**).  
255 $\beta$ -oxidation of 3HV-CoA to acetyl-CoA and propionyl-CoA would recover the reducing  
256equivalent. Given the energy demands of the pathway, growth on LA must be performed  
257aerobically or in the presence of an alternative electron acceptor to enable ATP synthesis via  
258respiration.

259Like many catabolic pathways, expression of the *lva* operon is regulated by the presence of the  
260pathway substrates. Using a transcriptional reporter assay, we demonstrated that the *lva* operon is  
261upregulated by a transcriptional activator encoded by the divergent *lvaR* gene. Additionally, we  
262suspect that the *lva* operon is also regulated by Crc, a global carbon catabolite repressor. Crc is  
263an mRNA binding protein that prevents protein translation when bound to a specific mRNA  
264sequence in *P. putida*, AAnAAnAA<sup>37-40</sup>. This sequence pattern is found immediately upstream of  
265*lvaE* (**Supplementary Figure 1**), which encodes an acyl-CoA synthetase that initiates the  
266pathway. The presence of the Crc target sequence suggests that the operon is also subject to *P.*  
267*putida*'s carbon catabolite repression system which may explain the diauxic growth curves  
268observed for mixtures of glucose and LA.

269While LvaB was shown to be essential for LA catabolism, its exact role remains unclear. LvaB is  
270a small protein (~100 amino acids) that is unlikely to contain enzymatic activity by-itself.  
271Furthermore, LvaB co-purifies with LvaA, is essential for the phosphorylation of 4HV-CoA, and  
272its orthologs are consistently found adjacent to orthologs of *lvaA* in the genomes of other  
273organisms. Similar examples where small proteins provide critical support to a variety of  
274biological functions, including metabolism and enzymatic function, have been indentified<sup>41,42</sup>.  
275For example, nonribosomal peptide synthetase gene clusters often contain a small protein that

276 belongs to the MbtH-like protein family, a family of proteins that are known to bind adenylation  
277 domains and enable catalytic activity. MbtH-like proteins form the necessary complexes required  
278 for domain activation but are not predicted to interact directly with the catalytic site<sup>43-45</sup>.  
279 Although *lvaB* does not share significant sequence homology with known MbtH-like proteins,  
280 we speculate that it could be playing a similar role with LvaA, where the presence of LvaB is  
281 required to form an active LvaAB complex. Without a crystal structure, the specific interaction  
282 between LvaA and LvaB and its role in catalysis will be difficult to unlock.

283 Interestingly, the isomerization of 4HV-CoA to 3HV-CoA in *P. putida* proceeds through a  
284 phosphorylated intermediate, 4PV-CoA, a compound also observed in a study of LA metabolism  
285 in rat livers<sup>25</sup>. This study suggested the 3HV-CoA was generated via a pathway comprised of  
286 complex phosphorylated intermediates. We did not detect MS peaks corresponding to any of  
287 these compounds in our *in vitro* reaction mixtures. Instead, based on changes we observed in  
288 total ion abundance over time, we propose that 4PV-CoA is dephosphorylated to an enoyl-CoA  
289 and subsequently rehydrated to 3HV-CoA. We suspect that the phosphorylation of 4HV-CoA by  
290 LvaAB generates a better leaving group and makes the subsequent dehydration more  
291 thermodynamically favorable. However, the mechanism for these last steps remains unclear.

292 Previous groups studying the nonmevalonate pathway have identified phosphate elimination  
293 steps for the formation of a double bond that is reminiscent of the intermediates we observed<sup>46,47</sup>,  
294 but these reactions do not include a rehydration step. The time course measurements that we  
295 collected for the full reaction indicate that the formation of the pentenoyl-CoA happens fairly  
296 quickly, but the transition from the pentenoyl-CoA to the 3HV-CoA is a much slower reaction  
297 (**Figure 5B**). Our tests indicate that LvaC is capable of converting 4PV-CoA to 3HV-CoA, but



298those reactions still contain a higher abundance of pentenoyl-CoA compared to 3HV-CoA. A  
299more detailed mechanistic study of the final steps may clarify the specific role of *IvaC*.

300Understanding how LA metabolism works is important because LA is a common byproduct of  
301biomass hydrolysis and is often present in the final feedstock. High concentrations of LA in the  
302feedstock can lead to microbial inhibition and represents an underused source of carbon in  
303traditional sugar fermentations. By discovering the catabolic pathway, microbes can be  
304engineered to detoxify the media and/or utilize LA as a source of carbon, maximizing the overall  
305carbon conversion from biomass into high value products. Additionally, identifying the structure  
306of LA metabolism will improve metabolic models and enable pathway design for novel LA-  
307based bioconversions.

### 308**Acknowledgements**

309Work in the Pflieger lab was funded by the National Science Foundation (CBET-114678) and the  
310William F. Vilas Trust. Work in the Deutschbauer lab was funded by ENIGMA, a Scientific  
311Focus Area Program, supported by the U. S. Department of Energy, Office of Science, Office of  
312Biological and Environmental Research and Genomics:GTL Foundational Science through  
313contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U. S.  
314Department of Energy.. Work in the Amador-Noguez lab was funded by the HHMI International  
315Student Research Fellowship. R.L.C. was supported by the NIH NHGRI Genomic Sciences  
316Training Program (T32 HG002760). A.L.M. was supported by NSF SEES fellowship (GEO-  
3171215871).

318We want to thank J. Escalante for providing the plasmid pK18*mobsacB* and J. Altenbuchner for  
319providing the strain *P. putida* KTU and the plasmid pJOE6261.2. We would also like to thank the  
320Mass Spectrometry/ Proteomics Facility at the UW-Madison Biotechnology Center for  
321performing the in-gel digest and providing the MALDI-TOF-TOF results.

### 322**Author Contribution**

323J.M.R. D.E.A. and B.F.P. conceived the study. J.M.R. designed and performed the experiments  
324and analyzed the data with the following exceptions. T.P. and D.A.M. designed the LC/MS/MS  
325experiments and T.P. performed the LC/MS and LC/MS/MS experiments. D.E.A. and J.M.T.  
326performed the transposon library screen. C.E.C. assisted with the promoter and CoA ligase assay.

327A.L.M. proposed, and helped design and perform, the pull-down experiment. Y.S. and J.R.  
328prepared the RB-TnSeq mutant library of *P. putida* KT2440 (Putida\_ML5). K.M.W., R.L.C, and  
329A.M.D. performed the fitness assays with the Putida\_ML5 library. M.N.P. performed the data  
330analysis to determine fitness values. R.L.C. prepared the supplementary analysis of the  
331Putida\_ML5 fitness experiments. A.M.D. and A.P.A. managed the Bar-Seq experiments. J.M.R.  
332and B.F.P wrote the manuscript.

### 333**Methods**

334Please see the **Supplementary Methods** for additional details.

### 335**Chemicals, Strains, and Media**

336All chemicals were obtained from Sigma-Aldrich or Fisher Scientific. 4-hydroxyvalerate was  
337made through the saponification of  $\gamma$ -valerolactone (GVL)<sup>23</sup>. Bacterial strains and plasmids used  
338in this study are summarized in **Table 3**. Plasmid sequences are listed in the **Supplementary**  
339**Material**. *E. coli* strains were grown at 37°C and *P. putida* strains were grown at 30°C unless  
340otherwise noted. Kanamycin was used at final concentration of 50  $\mu\text{g/ml}$ . 5-Fluorouracil was  
341used at a final concentration of 20  $\mu\text{g/mL}$ .

342

343 **Table 3. Strains and Plasmid List**

Strain/Plasmid	Relevant genotype/property	Source or Reference
<i>Strains</i>		
<i>Pseudomonas putida</i>		
KT2440	Wild Type	ATCC 47054
KTU	$\Delta upp$	Altenbuchner et al <sup>51</sup>
$\Delta lvaR$	$\Delta upp \Delta PP_{2970}$	This work
$\Delta lvaA$	$\Delta upp \Delta PP_{2971}$	This work
$\Delta lvaB$	$\Delta upp \Delta PP_{2972}$	This work
$\Delta lvaC$	$\Delta upp \Delta PP_{2973}$	This work
$\Delta lvaD$	$\Delta PP_{2974}$	This work
$\Delta lvaE$	$\Delta upp \Delta PP_{2975}$	This work
<i>Escherichia coli</i>		
CC118 $\lambda$ pir	$\Delta(ara-leu), araD, \Delta lacX174, galE, galK, phoA, thi1, rpsE, rpoB, argE (Am), recA1, lysogenic \lambda pir$	de Lorenzo et al <sup>26</sup>
DH5 $\alpha$	F <sup>-</sup> $\Phi 80 lacZ \Delta M15 \Delta(lacZYA-argF) U169 recA1 endA1 hsdR17 (r_k^-, m_k^+) phoA supE44 thi-1 gyrA96 relA1 \lambda^-$	Invitrogen
<i>Plasmids</i>		
pBAM1	<i>tnpA</i> , Amp <sup>R</sup> , Kan <sup>R</sup> , <i>oriR6K</i>	de Lorenzo et al <sup>26</sup>
pJOE6261.2	<i>upp</i> (from <i>P. putida</i> ), Kan <sup>R</sup> , ColE1 origin	Altenbuchner et al <sup>51</sup>
pJOE-lvaR	pJOE6261.2 with up- and downstream regions of <i>lvaR</i>	This work
pJOE-lvaA	pJOE6261.2 with up- and downstream regions of <i>lvaA</i>	This work
pJOE-lvaB	pJOE6261.2 with up- and downstream regions of <i>lvaB</i>	This work
pJOE-lvaC	pJOE6261.2 with up- and downstream regions of <i>lvaC</i>	This work
pJOE-lvaE	pJOE6261.2 with up- and downstream regions of <i>lvaE</i>	This work
pBAD35	P <sub>BAD</sub> promoter, Kan <sup>R</sup> , pBBR1 origin	Lennen et al <sup>52</sup>
pBAD-lvaA	pBAD35 carrying <i>lvaA</i>	This work
pBAD-lvaB	pBAD35 carrying <i>lvaB</i>	This work
pBAD-lvaC	pBAD35 carrying <i>lvaC</i>	This work
pBAD-lvaD	pBAD35 carrying <i>lvaD</i>	This work
pBAD-lvaE	pBAD35 carrying <i>lvaE</i>	This work
pK18 <i>mobsacB</i>	<i>sacB</i> , Kan <sup>R</sup> , pMB1 origin	Schafer et al <sup>53</sup>
pK18-lvaD	pK18 <i>mobsacB</i> containing up- and downstream regions of <i>lvaD</i>	This work
pJMR74	pBAD35 with P <sub>BAD</sub> promoter and <i>araC</i> replaced with <i>lvaA</i> promoter and <i>lvaR</i> ( <i>P. putida</i> ) carrying sfGFP	This work

### 345 **Transposon Library and Screening**

346 The *P. putida* transposon library was created following a protocol adapted from Martinez-Garcia  
347 et al<sup>26</sup>. Suicide vector delivery was achieved through bi-parental mating. The transposon library  
348 was screened by replica plating colonies from the M9 citrate plates onto LB, M9 glucose and M9  
349 LA plates supplemented with kanamycin. Positive hits were identified as colonies that exhibited  
350 growth on LB and glucose plates but not on LA plates.

### 351 **RNA Experiments**

352 RNA was extracted from *P. putida* using a protocol adapted from Pinto *et al*<sup>54</sup>. The transcription  
353 start site for genes *lvaR* and *lvaA* were isolated using an adapted 5' Race protocol from Schramm  
354 et al<sup>29</sup>. Reverse transcription PCR was performed using primers listed in **Supplementary Table**  
3553.

### 356 ***P. putida* Knockouts**

357 The genetic knockout of *lvaD* was performed following the protocol from Schafer et al<sup>53</sup>.  
358 Knockouts of the remaining genes in *P. putida* were performed following the protocol from Graf  
359 et al<sup>51</sup>. Knockout cassettes were designed with 500 bp of homology up and down stream of the  
360 deletion site flanking kanR and 5-FU markers. Cassettes were introduced to *P. putida* via a  
361 suicide vector. Disruption mutants were selected on kanamycin and clean deletions were selected  
362 on 5-FU. Colonies were then screened by colony PCR to confirm deletion strains.

### 363 **Transcriptional Reporter Assay**

364 *P. putida* KT2440 was transformed with a broad host range plasmid (pJMR74) containing a kan<sup>R</sup>  
365 marker, *lvaR*, and sfGFP cloned under the native promoter for *lvaA*. *P. putida* KT2440 harboring  
366 an empty vector (no sfGFP) was used as a negative control. The fluorescence of cultures exposed  
367 to 20 mM of the appropriate carboxylic acid (acetate, propionate, butyrate, valerate, LA, 4HV, or  
368 hexanoate) was measured in a Tecan infinite m1000 (ex 485 nm/em 510 nm).

### 369 **CoA Ligase Assay**

370 A CoA ligase activity assay was performed with the EnzChek<sup>®</sup> Pyrophosphate Assay Kit. The  
371 final reaction volume of 100  $\mu$ L contained 0.1 mM ATP, 0.1 mM CoA, 0.2  $\mu$ M LvaE, 0.2 mM  
372 MESG, 1 U purine nucleoside phosphorylase, 0.01 U pyrophosphatase, 50 mM Tris-HCL, 1 mM  
373 MgCl<sub>2</sub> and 0.1 mM substrate (sodium acetate, sodium propionate, butyric acid, valeric acid,  
374 levulinic acid, hexanoic acid, octanoic acid, 4HV, 2-pentenoic acid, 3-pentenoic acid, 3HV,  $\gamma$ -  
375 valerolactone, pyruvate, lactic acid, L-carnitine). All substrate stocks were adjusted to pH 7  
376 before use. Reactions were incubated at 25°C for 30 minutes before measuring the absorbance at  
377 360 nm in a Tecan infinite m1000.

### 378 **Protein Production and Purification**

379 Expression vectors for each *lva* gene were constructed using the pET28b backbone and  
380 expressed in *E. coli* BL21 (DE3). After induction with 1 mM IPTG at OD600 0.8, cultures were  
381 chilled on ice for 10 minutes and incubated at 16°C for 18 hours. Proteins were purified using a  
382a GE Äkta Start System with a 1 mL HisTrap HP column (LvaE) or 1 mL MBPTrap HP column  
383 (LvaABCD). Eluted proteins were buffer exchanged using a GE PD-10 column and concentrated  
384 with an Amicon® Ultra 4 mL Centrifugal Filter with a 10 kDa cut-off size. Proteins were stored at  
385-80°C until use.

### 386 Enzyme Assays and Metabolite Purification

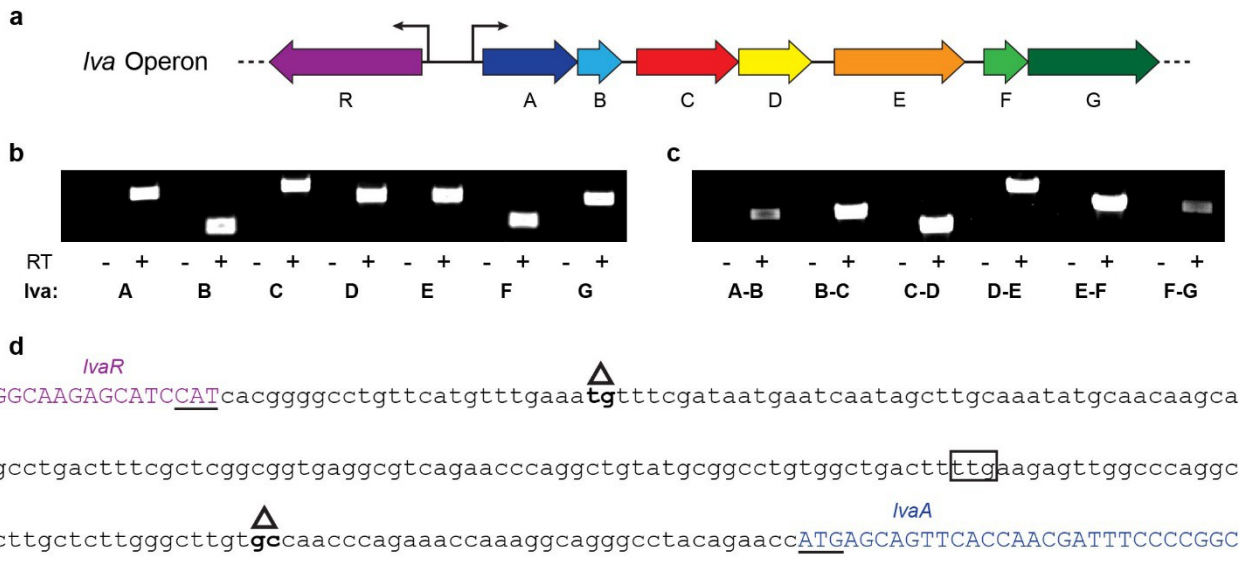
387 All *in vitro* enzyme assays were performed in a 30° water bath at a pH of 6.5 and contained 50  
388 mM Tris-HCL, 1 mM MgCl<sub>2</sub>, and 2 mM DTT. Final reaction concentrations included the  
389 following components, depending on enzymes added: 0.5 mM LA, 0.55 mM CoA, 0.55 mM ATP  
390 (1.05 mM ATP when *lvaAB* were present), 0 mM NAD(P)H (0.55 mM NAD(P)H when *lvaD*  
391 was present). Final protein concentrations were: LvaA (0.2 μM), LvaB (0.8 μM), LvaAB (0.4  
392 μM), LvaC (0.4 μM), LvaD (0.2 μM), and LvaE (0.2 μM) (**Supplementary Figure 5**). The *in*  
393 *vitro* enzyme assays were incubated for 30 minutes, excluding the timecourse which was  
394 incubated for various intervals up to 60 minutes. Reaction metabolites were purified following a  
395 modified protocol from Zhang *et al*<sup>24</sup> and stored at -80°C until LC/MS analysis.

### 396 Liquid Chromatography Mass spectrometry (LC/MS, LC/MS/MS)

397 Samples were analyzed using an HPLC-MS/MS system consisting of a Vanquish™ UHPLC  
398 system (Thermo Scientific) coupled by electrospray ionization (ESI; negative polarity) to a  
399 hybrid quadrupole - high-resolution mass spectrometer (Q Exactive orbitrap, Thermo Scientific)  
400 operated in full scan mode for detection of targeted compounds based on their accurate masses.  
401 Fragmentation of CoA, 4HV-CoA, and phosphorylated 4HV-CoA was achieved using parameters  
402 indicated in the Supplementary Methods.

404**Figures**

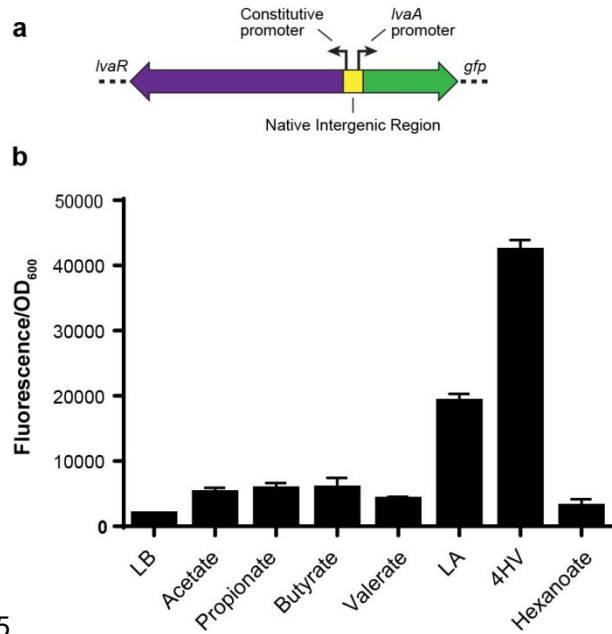
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406

407**Figure 1.** *P. putida lva* operon genetic characterization A. Organization of the *lvaRABCDEFG* (9,323 bp) operon. B.  
408Reverse Transcriptase (RT) PCR demonstrates that each gene is expressed in cells grown on LA. Samples were  
409compared with the negative control (-RT) where reverse transcriptase was omitted from the reaction. C. RT-PCR of  
410cDNA created with primer JMR237 demonstrates that operon is polycistronic. Note that a product spanning each  
411intergenic region was observed. D. The transcription start sites (TSS) of regulator *IvaR* and *IvaA* were identified by  
4125'-RACE. Underlined sequence indicates ATG start codon. Triangle highlights experimentally determined TSS.  
413Boxed sequence indicates previously annotated translation start site for *IvaA*.

414

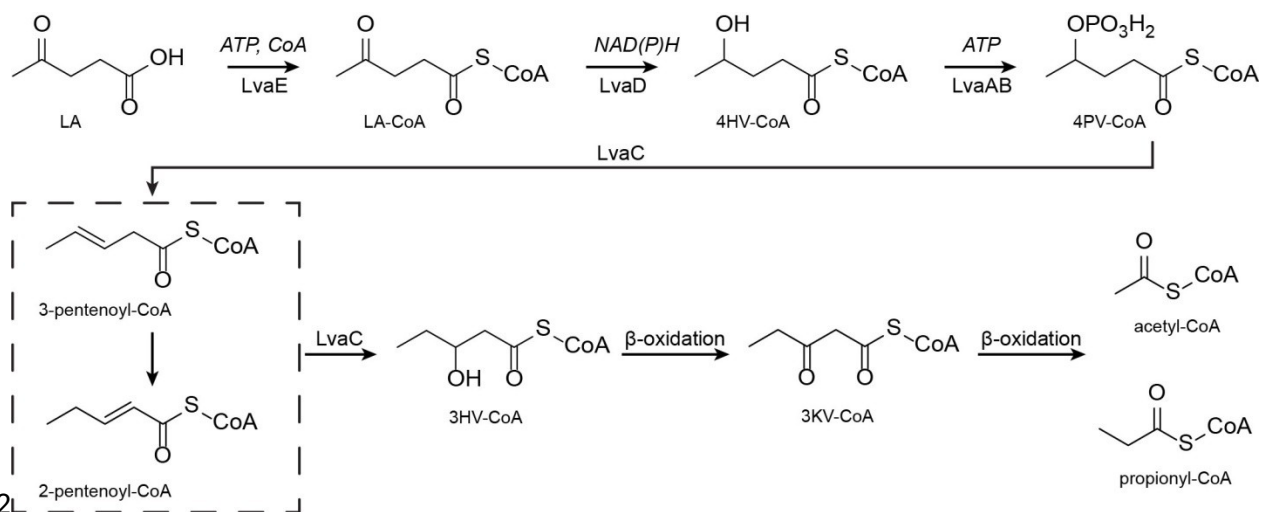


415

416 **Figure 2.** *Iva* operon induction assay. A. Schematic of transcriptional GFP fusion used to test induction of the *Iva*  
 417 operon. *IvaR* was cloned onto a plasmid containing its native constitutive promoter and the native promoter region  
 418 for *IvaA*. The fluorescent protein sfGFP was cloned in place of *IvaA*. B. GFP fluorescence was measured from LB-  
 419 cultures supplemented with various organic acids (20 mM).

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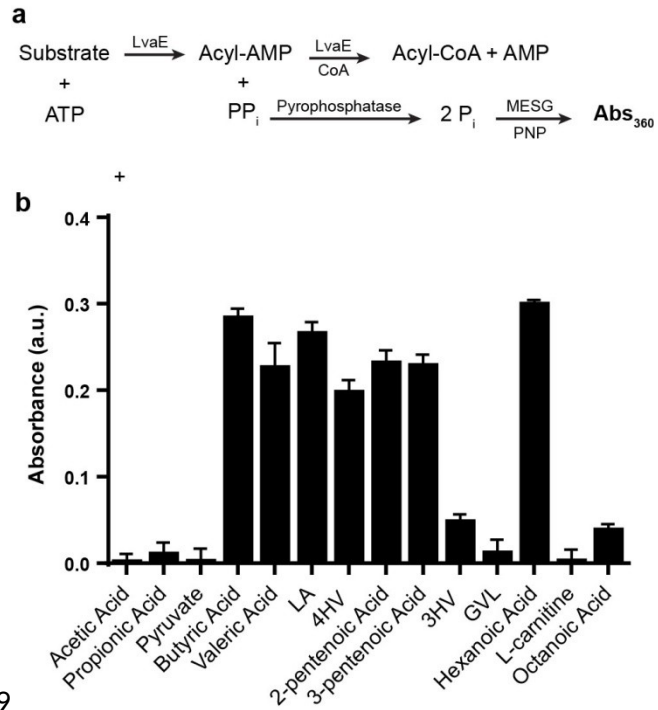


422L

423 **Figure 3.** Proposed pathway for LA metabolism. LA, levulinic acid; 4HV, 4-hydroxyvalerate; 3HV, 3-  
 424 hydroxyvalerate; LA-CoA, levulinyl-CoA; 4HV-CoA, 4-hydroxyvaleryl-CoA; CoA, coenzyme-A; ATP, adenosine  
 425 triphosphate; 4PV-CoA, 4-phosphovaleryl-CoA; 3KV-CoA, 3-ketovaleryl-CoA; NAD(P)H, Nicotinamide adenine  
 426 dinucleotide (phosphate) reduced.

427

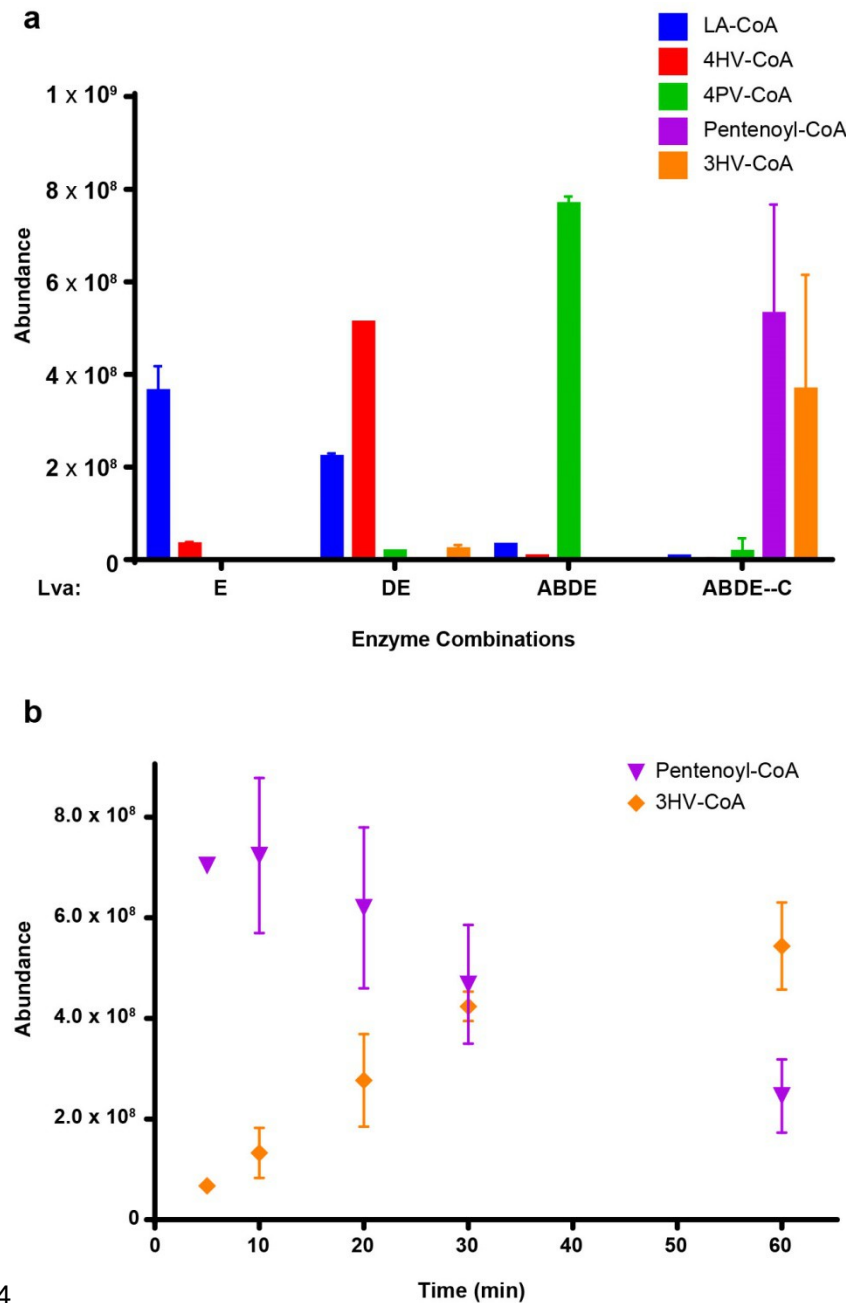




429

**430**Figure 4. LvaE CoA-Ligase Activity A. Schematic of CoA-ligase activity assay. Using the Enzchek®  
**431**Pyrophosphatase Assay kit, the amount of pyrophosphate released during the CoA ligase reaction was measured as  
**432**an increase of absorbance at 360 nm. B. Activity of LvaE towards a variety of short and medium chain acids.

433

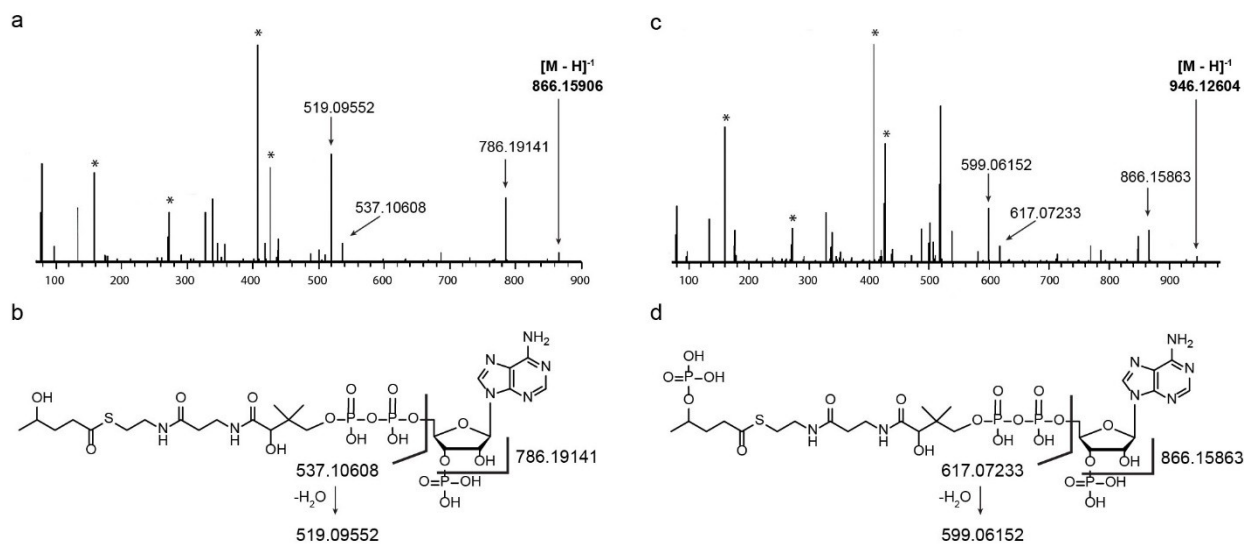


434

435 **Figure 5.** CoA species abundance in LC/MS analysis of *in vitro* enzyme combinations. A. Abundance of CoA  
 436 species created after 30 min of incubating LA, ATP, NAD(P)H with varying enzyme combinations. ABDE—C  
 437 indicates that the LvaABDE reaction was performed first, metabolites were separated from LvaABDE, and the  
 438 resulting solutions was supplemented with LvaC solely. The reaction confirms that LvaC is capable of converting  
 439 4PV-CoA to 3HV-CoA. B. Abundance of CoA species over a 60 minute timecourse for a mixture of LvaABCDE,  
 440 LA, ATP, and NAD(P)H.

441

442



444

445 **Figure 6.** Comparison of 4HV-CoA and 4PV-CoA MS/MS spectra. A. MS/MS spectra for 4HV-CoA. B.  
 446 Assignment of selected fragments from 4HV-CoA. C. MS/MS spectra for 4PV-CoA. D. Assignments of selected  
 447 fragments from 4PV-CoA. The masses between the selected fragments of 4PV-CoA and 4HV-CoA differ by the  
 448 mass of  $\text{PO}_3\text{H}^-$  (79.967), indicating 4PV-CoA contains a phosphate group not found in 4HV-CoA. **Bold** values  
 449 indicate the mass of the parent ion. Peaks identified with the symbol (\*) are fragments resulting from coenzyme A.  
 450 See **Supplementary Figure 6** and **Supplementary Table 2** for additional fragmentation information.

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