Supplemental Data

A metabolic pathway for catabolizing levulinic acid in bacteria

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Supplementary Note

To further investigate genes involved in LA metabolism, random bar code transposon-site sequencing (RB-TnSeq) was performed for the growth of *P. putida* on LA and 4HV. RB-TnSeq is an efficient method for determining gene importance under different conditions with high genomic coverage[1]. A summary of genes identified as particularly interesting to the authors is shown in **Supplementary Table 1** including fitness scores for growth on minimal media with LA or 4HV relative to minimal media with glucose or the initial inoculum grown in LB. All data from these experiments is available through the fitness browser at http://fit.genomics.lbl.gov/cgi-bin/exps.cgi?orgId=Putida&expGroup=carbon%20source[2].

RB-TnSeq Results are Consistent with Evidence Provided in the Main Text

All genes mentioned in the main text are shown with their fitness scores for growth on LA and 4HV in **Supplementary Table 1**. Genes that were identified as transposon library hits have their gene loci highlighted in red italics.

RB-TnSeq analysis suggests the genes identified as constituting the LA metabolism operon *lvaABCDEFG* as well as the proposed regulator *lvaR* were important for growth on both LA and 4HV with a few exceptions described as follows. *lvaB* was excluded from the data summary for growth on LA and 4HV due to insufficient barcode insertions in this small gene and *lvaE* (shown to not be essential for growth on LA in the main text) shows no phenotype on LA.

RB-TnSeq analysis suggests *lvaF* and *lvaG* are not important for growth on LA or 4HV, suggesting they are not required for transport of these metabolites at the concentrations used in the experiments. The positive fitness scores of these genes for growth on 4HV suggest that the 4HV concentrations used in this experiment had negative effects on fitness, an effect that would be alleviated by elimination of import system (See section below: *Potential Induction of Quorum-Sensing Systems by γ-Valerolactone*). None of the remaining transposon library hits from the main text exhibited interesting phenotypes in the RB-TnSeq experiment, suggesting they may have been dependent upon the transposon library experiment.

In addition to genes identified in the main text, genes of interest shown in **Supplementary Table 1** were identified using the following criteria:

Important for Fitness in LA and 4HV: Fitness scores lower than -2 for both LA and 4HV.

Important for Fitness in LA but not 4HV: Fitness score for LA lower than -2 and fitness score for 4HV greater than -2.

Important for Fitness in 4HV but not LA: Fitness score for 4HV lower than -2 and fitness score for LA greater than -2.

Enhanced Fitness in 4HV: Fitness score greater than 2 for 4HV.

This list of genes of interest was further refined by eliminating genes that shared a phenotype with growth on acetate as these results were considered not relevant to the scope of this work.

β-Oxidation of 3-Hydroxyvaleryl-CoA to Propionyl-CoA and Acetyl-CoA by Genes Important for Growth on LA and 4HV

As proposed in the main text, the 3-hydroxyvaleryl-CoA metabolite produced in LA metabolism could be utilized through β-Oxidation to form Propionyl-CoA and Acetyl-CoA. RB-TnSeq analysis helped to identify potential candidate genes for this pathway:

PP_3755 is annotated as a 3-hydroxybutyryl-CoA dehydrogenase, suggesting that this enzyme catalyzes the conversion of 3-hydroxyvaleryl-CoA to 3-ketovaleryl-CoA.

PP_3754 is annotated as a β-ketothiolase, suggesting that this enzyme catalyzes the conversion of 3-ketovaleryl-CoA to propionyl-CoA and Acetyl-CoA.

PP_3753 is annotated as a transcriptional regulator and its location directly upstream of the two previous genes suggests a role in the regulation of these two β-oxidation genes.

Propionyl-CoA Metabolism by Genes Important for Growth on LA and 4HV

After propionyl-CoA is formed through the mechanism proposed in the previous section, it could be further metabolized to form succinate and pyruvate through the 2-methylcitrate cycle. PP_2337 is annotated as a methylaconitate isomerase (prpF), suggesting that the pathway utilized is the 2-methylcitrate cycle II that passes through a *trans*-2-methyl-aconitate intermediate. RB-TnSeq analysis helped to identify potential candidate genes for this pathway:

PP_2335 is annotated as a methylcitrate synthase, suggesting that this enzyme catalyzes the reaction of propionyl-CoA with oxaloacetate to form 2-methylcitrate.

PP_2336 is annotated as an aconitate hydratase. PP_2339, an additional gene in close chromosomal proximity but with insufficient BarSeq data for analysis is also annotated as an aconitate hydratase. These results suggest that some combination of these two enzymes catalyze both the conversion of 2-methylcitrate to *trans*-2-methylaconitate and the downstream conversion of *cis*-2-methylaconitate to 2-methylisocitrate.

PP_2337 is annotated as a methylaconitate isomerase, suggesting that this enzyme catalyzes the conversion of *trans*-2-methylaconitate to *cis*-2-methylaconitate.

PP_2334 is annotated as a 2-methylisocitrate lyase, suggesting that this enzyme catalyzes the conversion of 2-methylisocitrate to succinate and pyruvate.

PP_2333 is annotated as a transcriptional regulator and its location directly upstream of the PP_2334-2339 genes suggests a role in the regulation of these propionyl-CoA metabolism genes.

Potential LA CoA Transferase

In the main text, *lvaE* was shown to catalyze the conversion of LA to levulinyl-CoA as well as the conversion of 4HV to 4-hydroxyvalerly-CoA. *lvaE* is essential for growth on 4HV but not essential for growth on LA,

suggesting that there is another enzyme capable of catalyzing the conversion of LA to levulinyl-CoA. PP_3122 and PP_3123 are annotated as acetoacetyl CoA-transferase subunits A and B respectively and are both important for growth on LA but not 4HV, suggesting they could fill the role of the additional catalyst for levulinyl-CoA formation. PP_3121 is also important for growth on LA but not 4HV and is annotated as a transcriptional regulator. Its genomic context suggests it regulates the expression of PP_3122 and PP_3123. This set of genes is analogous to the *dhcAB* operon involved in catabolism of carnitine in *Pseudomonas aeruginosa*. PP_3121 shares 72% sequence identity across 95% of its sequence with *dhcR* (PA1998) and PP_3122 and PP_3123 share 86% and 90% identity across their entire sequences with *dhcA* (PA1999) and *dhcB* (PA2000), respectively. *dhcR* regulates expression of the *dhcAB* operon encoding a predicted 3-ketoacid CoA-transferase with evidence of activity on 3-dehydrocarnitine[3]. PP_3121-PP_3123 could serve a similar role in catabolism of LA.

Transcriptional Regulators Control Both Beneficial and Detrimental Systems for Fitness Under LA and 4HV Metabolism

PP_3286 and PP_3753 are annotated as transcriptional regulators and RB-TnSeq analysis suggests they are important for growth on LA and 4HV. The annotation for PP_3286 suggests involvement in the regulation of phenylacetic acid metabolism. As previously stated, genomic context suggests the involvement of PP_3753 in the regulation of the probable β-oxidation genes PP_3754-3755.

PP_3121 and PP_4515 are annotated as transcriptional regulators and RB-TnSeq analysis suggests they are important for growth on LA but not important for growth on 4HV. As previously stated, genomic context suggests PP_3121 regulates expression of the potential acetoacetyl-CoA transferase subunits PP_3122-3123. The regulatory role of PP_4515 is unclear.

Conversely, PP_0995, PP_1328, PP_1968, PP_2333, and PP_2436 are annotated as transcriptional regulators and RB-TnSeq analysis suggests they are important for growth on 4HV, but not important for growth on LA. PP_0995 shares 41% homology across its entire sequence with a gene in *Caulobacter crescentus* (CC3252) thought to be involved in sigma factor regulation for heavy metal stress, although its regulatory role in *Pseudomonas putida* is unclear[4]. As previously stated, genomic context suggests the involvement of PP_2333 in the regulation of the probable propionyl-CoA metabolism genes PP_2333-2339. The regulatory functions of PP_1328, PP_1968, and PP_2436 are unclear.

PP_0191, PP_1236, PP_2144, PP_3603, and PP_4734 are annotated as transcriptional regulators and RB-TnSeq analysis suggests their deletions are beneficial for growth on 4HV. PP_0191 is annotated as a regulator of alginate bioaccumulation, suggesting a role in biofilm formation. PP_1236 is annotated as a regulator of a glycine cleavage system and a close homolog in *Pseudomonas aeruginosa* (PA1009) is involved in the regulation of host colonization[5]. PP_2144 has a close homolog in *Pseudomonas syringae* (*psrA*) that is involved in the regulation of epiphytic fitness, quorum-sensing, and plant host interactions[6].

PP_3603 and PP_4734 are annotated as fatty acid responsive transcriptional regulators with unknown regulatory roles.

Potential Induction of Quorum-Sensing Systems by γ-Valerolactone

4HV used in the RB-TnSeq experiments was synthesized from γ-valerolactone as described in the methods section of the main text. As a result, residual γ-valerolactone was likely present in the experiments for growth on 4HV. Several molecules in the lactone family are known to be used as quorum sensing signals in *Pseudomonads*[7]. Quorum sensing responses would likely cause physiological responses towards the formation of a biofilm in the culture vessel. Cells with disruptions in these regulatory systems would replicate themselves to a higher degree resulting in a perceived increase in fitness as is the case with the transcriptional regulators PP_0191, PP_1236, and PP_2144 discussed above. As γ-Valerolactone is being investigated as a promising solvent for nonenzymatic sugar production from biomass[8], its effect on the quorum sensing systems of potential platform host organisms for bioprocessing should be further investigated.

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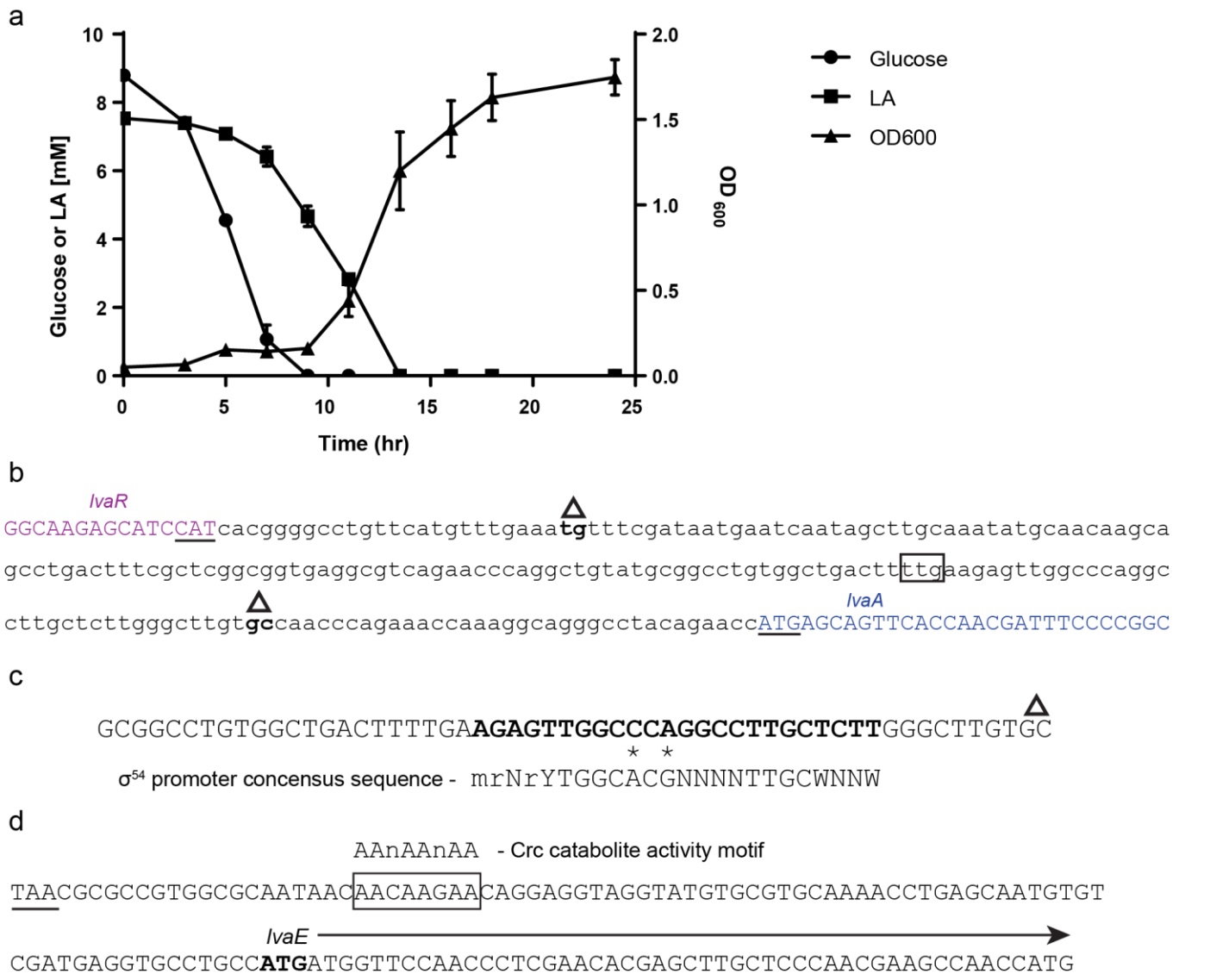
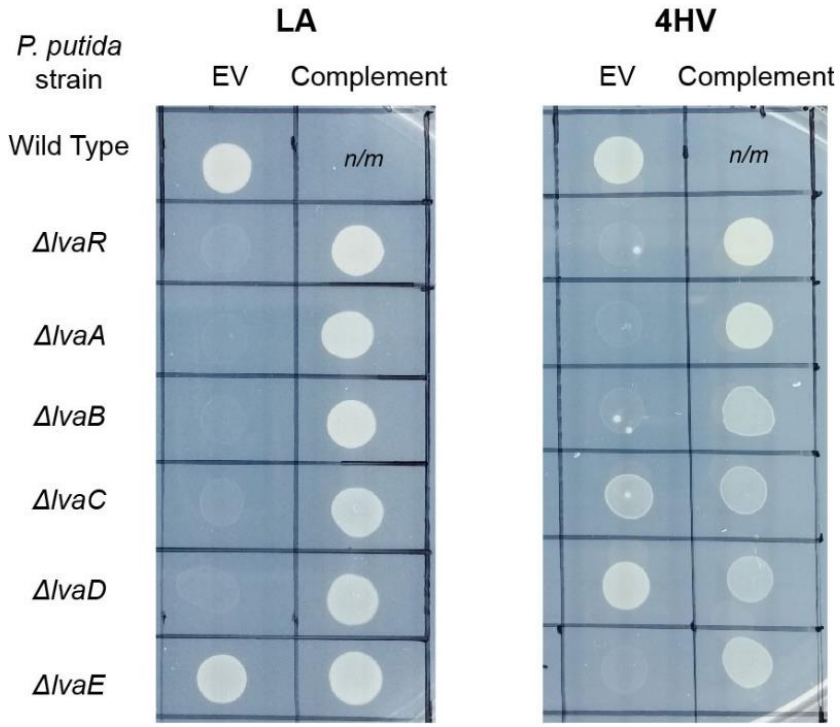
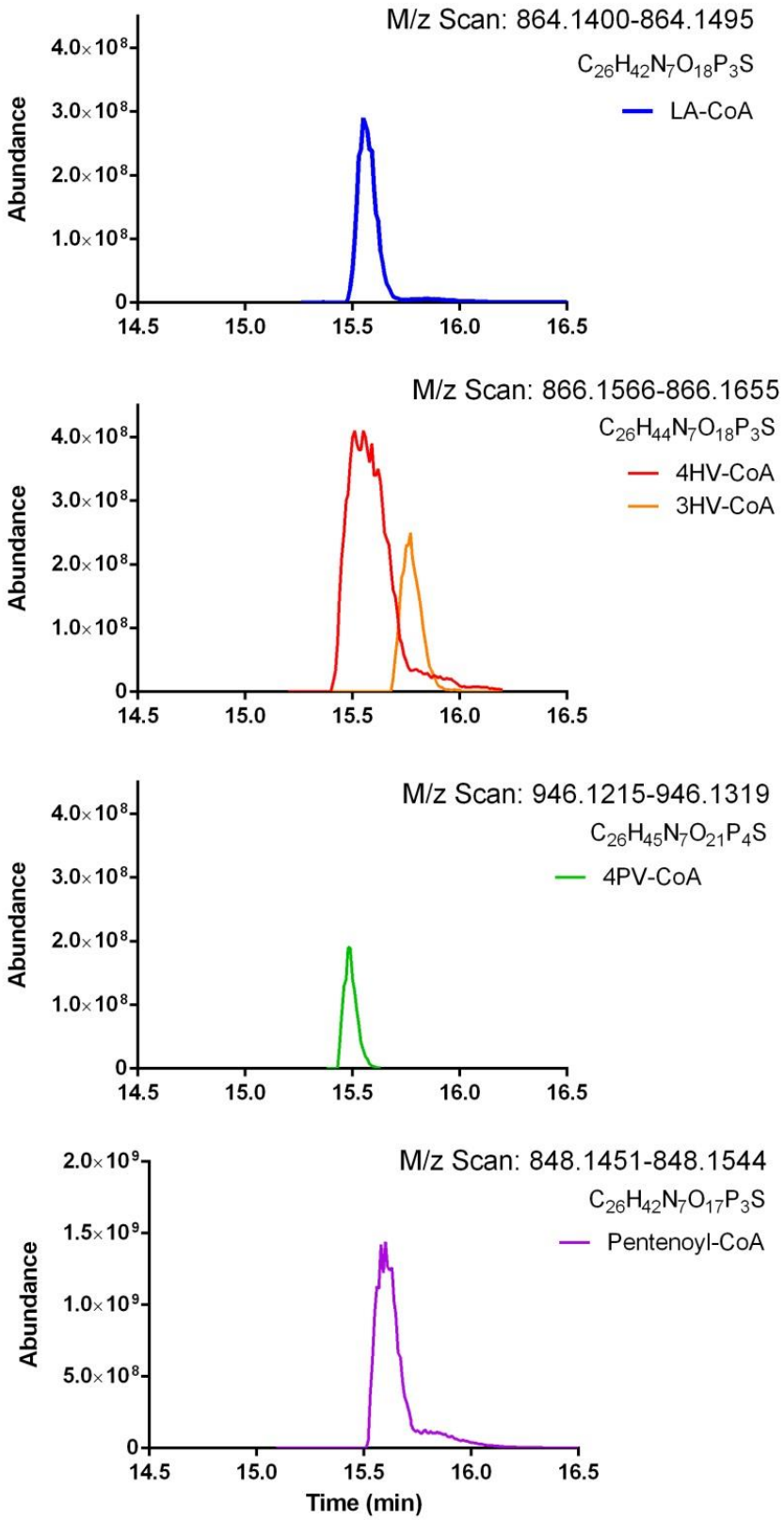
Supplementary Figures



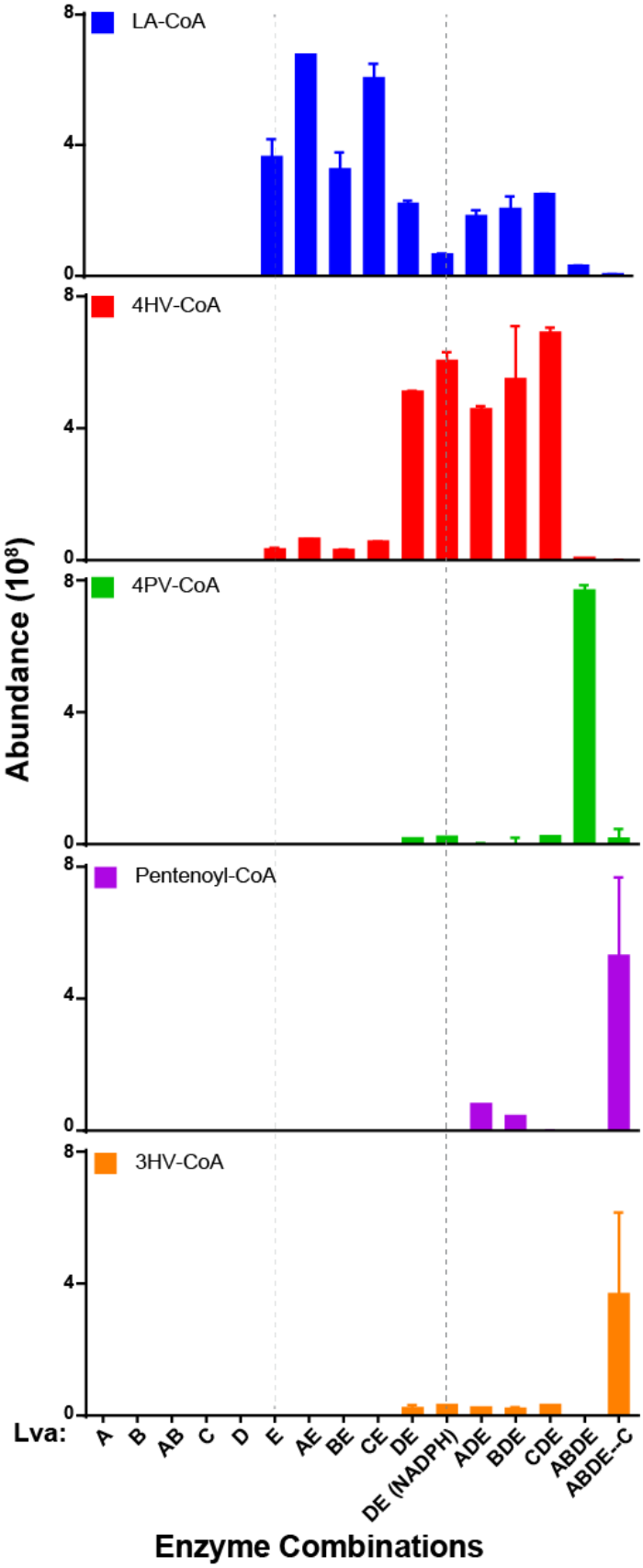
Supplementary Figure 1. a, Growth curve of *P. putida* KT2440 in the presence of both glucose and LA. Glucose is metabolized before LA, supporting the theory that the *lva* operon is under catabolite repression (*n*=3, biological). Circles indicate glucose concentration [mM], squares indicate LA concentration [mM] and triangles indicate culture optical density (OD) at 600 nm. **b,** The transcription start sites (TSS) of regulator *lvaR* and *lvaA* were identified by 5'-RACE. Underlined sequence indicates ATG start codon. Triangle highlights experimentally determined TSS. Boxed sequence indicates previously annotated translation start site for *lvaA*. **c,** σ^{54} promoter sequence for *lvaA*. Bold triangle indicates *lvaA* TSS, bold letters indicate *lvaA* promoter sequence, below is the σ^{54} consensus sequence and (*) indicate bases that differ from the consensus. **d,** Identification of Crc protein binding motif upstream of *lvaE* gene. Bold letters indicate start codon for *lvaE*, underlined sequence is stop codon for the preceding gene, *lvaD*, and boxed region indicates the found Crc catabolite activity motif. Above is the consensus sequence for Crc binding motifs. Error bars represent s.d.



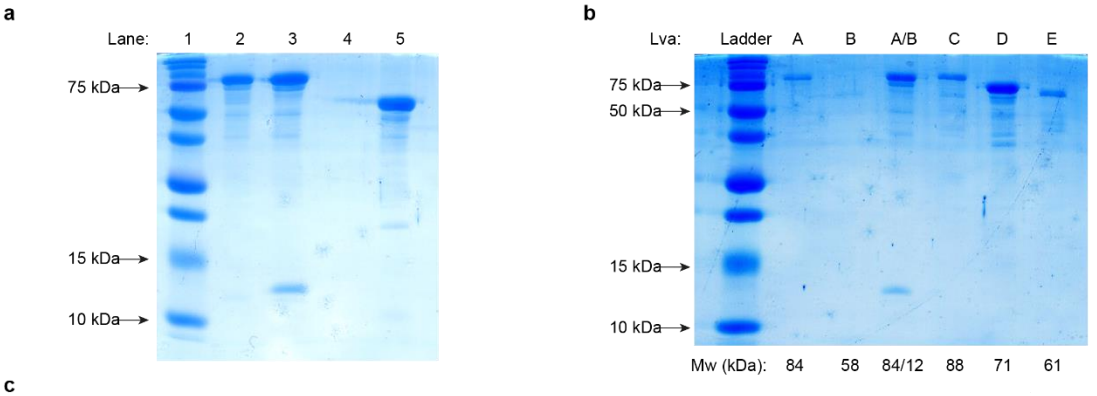
Supplementary Figure 2. Growth of *P. putida lva* mutants and complementation strains on minimal plates. KO strains contain empty vector pBAD35 plasmid. Complementation strains contain gene of interest on arabinose inducible pBAD35 vector. Plates are 20 mM LA or 4HV, 0.2% arabinose and MOPS minimal media. EV, empty vector; n/m, not made; KO, knockout.



Supplementary Figure 3. Retention times for compounds of interest identified by selective ion scanning modes. The graphs show the different m/z ratios used for scanning and the molecular formula of each compound. Since 4HV-CoA and 3HV-CoA have the same molecular formula, the method used was optimized to separate the individual peaks.



Supplementary Figure 4. CoA metabolites observed after 30 min incubation with LA, ATP, CoA, NAD(P)H and various enzyme combinations (*n*=3, technical). For reactions involving LvaD, NADH was the primary co-factor added excluding the sample DE (NADPH), where NADPH was used to test for activity of the LA-CoA reductase. ABDE—C indicates that the LvaABDE reaction was performed first, metabolites were separated from LvaABDE, and the resulting solution was supplemented with LvaC solely. Error bars represent s.d.



c

Matched peptides shown in **Bold Red**

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  1 MTQPNAHELL EIARATLLEQ LLPALPGELR YPALMIANAM AIAARENRLG
 51 AQAEDQEQAR LAALVDDAPS TLPDLRRQLA RAIRQGSHDA PQTRRTLVET
101 LRQITVARLA ISNPKALP
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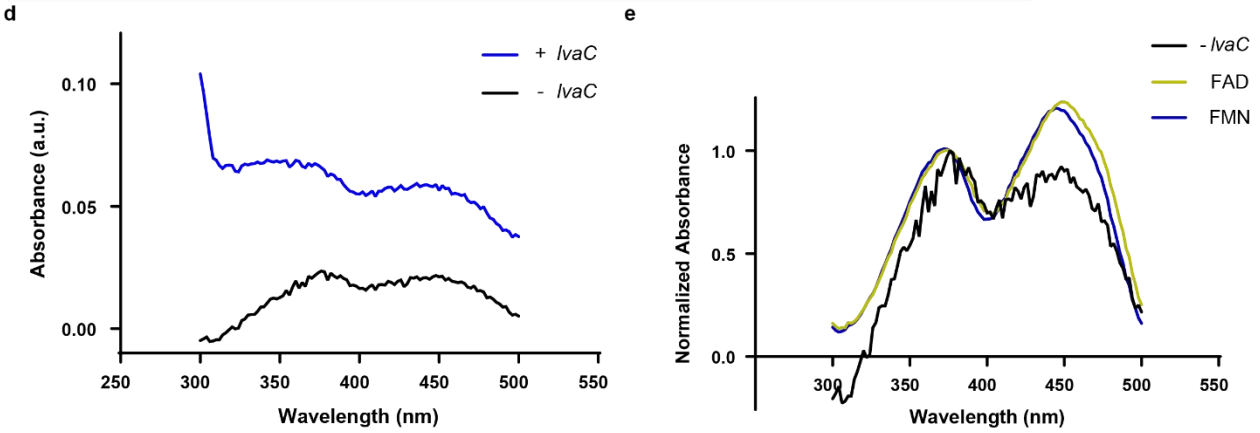
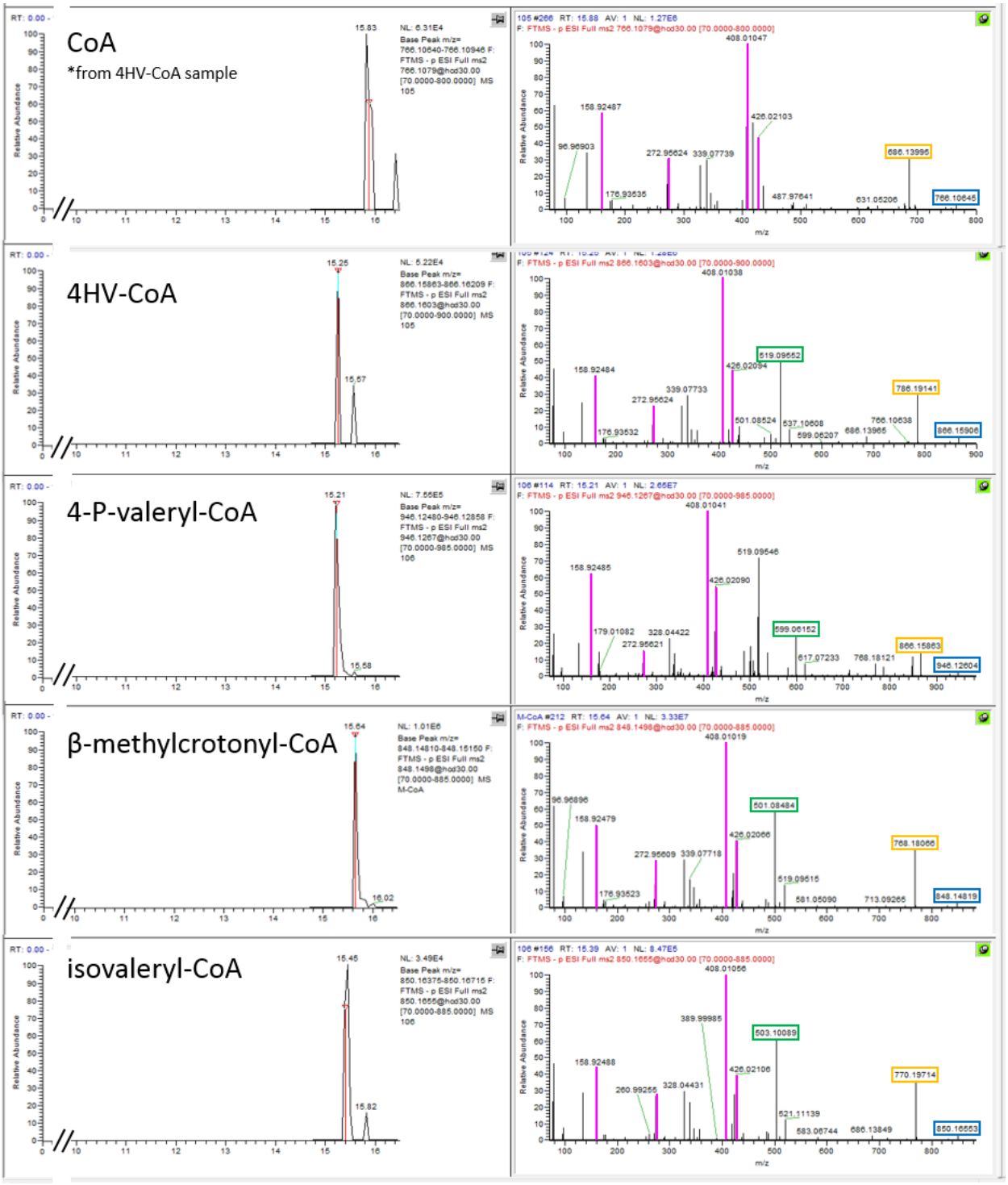


Supplementary Figure 5. a, LvaAB pull-down. 15% SDS-page protein gel of proteins purified by Dextrin Sepharose™ affinity chromatography. Lane 1: Ladder. Lane 2: N-terminal MBP tagged LvaA. Lane 3: N-terminal MBP tagged LvaA with native LvaB. Lane 4: N-terminal His tagged LvaA with native LvaB. Lane 5: N-terminal MBP tagged LvaA containing frameshift stop codon with native LvaB. MBP tagged LvaA has a full length molecular weight of 85 kDa. Untagged LvaB has a molecular weight of 12 kDa. **b,** 15% SDS-page protein gel of all purified proteins used in experiments. Because of the faint band seen for the original dilution of LvaB, the actual concentration of LvaB in the reactions was increased to correct for this over dilution. **c,** The results from an in gel digest and LC/MS/MS analysis. The 12 kDa band isolated from the pull-down experiment was submitted for LC/MS/MS analysis and the experimentally determined sequence was compared with the known amino acid sequence for LvaB and the peptides in agreement are shown in red. **d,** The absorbance spectra of purified LvaC. The blue indicates the absorption of dilute LvaC flavoprotein and the black is the absorbance of trichloroactetic acid treated LvaC supernatant where the protein was removed by centrifugation. **e,** LvaC flavoprotein absorbance directly compared with known flavins. Black is the protein flavin supernatant from A, yellow is pure FAD and blue is pure FMN. FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; MBP, maltose binding protein.



Supplementary Figure 6. MS/MS fragmentation of C_5 CoA compounds. Peaks highlighted in pink are fragments common to CoA. The blue box indicates parent mass, the green box highlights where the parent compound has lost an adenylated phosphoribose and yellow is the parent compound loses a phosphate group. **Supplementary Table 2** has a more descriptive list of the various fragments.

Supplementary Tables

Supplementary Table 1. Genes of Interest Identified from RB-TnSeq Experiments

Genes Mentioned in the Main Text				
Locus	Name	Annotation	LA/Gluc	4HV/Gluc
PP_0364	*bioH*	pimeloyl-ACP methyl ester esterase	0.3	0.02
PP_0988	*gcvP-1*	glycine dehydrogenase	-0.02	-0.003
PP_2332	-	ATP-dependent zinc protease family	-0.1	0.2
PP_2336	*acnA-II*	aconitate hydratase	-4.5	-3.5
PP_2337	*prpF*	aconitate isomerase	-4.4	-3.8
PP_2790	*lvaR*	Sigma-54 dependent sensory box protein	-3.9	-5.0
PP_2791	*lvaA*	Aminoglycoside phosphotransferase	-5.2	-4.2
PP_2792	*lvaB*	Hypothetical protein	NA	NA
PP_2793	*lvaC*	acyl-CoA dehydrogenase/reductase family	-5.2	-4.1
PP_2794	*lvaD*	Oxidoreductase, short chain dehydrogenase/reductase family	-6.5	-5.3
PP_2795	*lvaE*	Acyl-CoA synthetase	0.4	-4.6
PP_2796	*lvaF*	conserved protein of unknown function	0.2	0.7
PP_2797	*lvaG*	acetate permease	0.1	1.7
PP_3741	*mrdA-I*	transpeptidase	0.0	-0.06
PP_4217	*fpvA*	TonB-dependent outer membrane ferripyoverdine receptor	0.3	0.02
Important for Fitness in LA and 4HV				
PP_2217		enoyl-CoA hydratase	-2.0	-2.2
PP_2334		2-methylisocitrate lyase	-4.9	-3.3
PP_2335		methylcitrate synthase	-5.1	-4.7
PP_3286		DNA-binding transcriptional repressor PaaX(phenylacetyl-CoA)	-4.3	-4.1
PP_3753		Transcriptional regulator, AraC family	-4.8	-2.6
PP_3754		Beta-ketothiolase BktB	-5.8	-3.2
PP_3755		3-hydroxybutyryl-CoA dehydrogenase	-2.9	-3.1
Important for Fitness in LA but not 4HV				
PP_1291		PhoH family protein	-2.5	0.3
PP_2333		GntR family transcriptional regulator	-4.5	-0.7
PP_3121		transcriptional regulator, LysR family	-4.1	-0.3
PP_3122		acetoacetyl CoA-transferase (subunit A)	-2.3	-0.1
PP_3123		acetoacetyl CoA-transferase (subunit B)	-3.1	-0.02
PP_3925		conserved protein of unknown function	-2.1	-0.9
PP_4515		Transcriptional regulator, MarR family	-2.2	0.03
PP_4628		conserved protein of unknown function	-3.6	-1.3

Supplementary Table 1 (*continued*)

Important for Fitness in 4HV but not LA				
PP_0951		Ribosome hibernation promoting factor	0.2	-2.4
PP_0995		Putative sigma factor regulator	-0.5	-2.6
PP_1328		Protein MraZ	-0.5	-4.2
PP_1764		Phosphoglycolate phosphatase 2	-1.3	-2.7
PP_1778		Lipopolysaccharide ABC export system, permease protein	0.2	-4.8
PP_1779		Lipopolysaccharide ABC export system, ATP-binding protein	0.003	-4.0
PP_1968		TetR family transcriptional regulator	-0.8	-2.1
PP_2082		phosphoenolpyruvate synthetase	-0.2	-2.7
PP_2436		Transcriptional regulator, LysR family	-0.3	-2.4
PP_4342		flagellar synthesis regulator, putative ATPase	-1.4	-2.0
PP_4571		cysteine synthase A	-0.1	-3.5
PP_4762		Acyl-CoA thioesterase II	0.3	-4.3
PP_4824		Sensor histidine kinase/response regulator	-0.7	-2.2
PP_4895		tRNA dimethylallyltransferase	-1.7	-2.0
PP_5203		5-formyltetrahydrofolate cyclo-ligase	-0.01	-3.0
PP_5210		Alcohol dehydrogenase, zinc-containing	-0.2	-2.5
PP_5502		ribosome modulation factor	-0.2	-3.5
Enhanced Fitness in 4HV				
PP_0191		Transcriptional regulatory protein AlgQ	0.6	2.7
PP_0395		putative type IV piliation protein	-0.3	3.2
PP_0396		conserved protein of unknown function	-0.5	3.1
PP_0397		protein kinase	-0.2	3.0
PP_0674		ADP/ATP ratio sensor and inhibitor of translation	-0.2	2.1
PP_1236		putative Glycine cleavage system transcriptional repressor	1.1	2.3
PP_2144		Transcriptional regulator, TetR family	1.3	5.6
PP_3603		Transcriptional regulator, GntR family	-0.5	2.3
PP_4734		Transcriptional regulator, GntR family	0.3	3.2
PP_4874		50S ribosomal protein L9	-0.5	2.6
PP_5145		phosphoenolpyruvate-dependent regulator	-1.2	4.6
PP_5146		RNA pyrophosphohydrolase	-1.0	2.9
PP_5232		conserved protein of unknown function	-1.4	3.4

Supplementary Table 2. Target MS/MS parameters for confirmation of phosphorylation of 4HV-CoA

Metabolites	Parent mass (m/z)	Parent molecular formula	Fragment mass (m/z) of interest*	Fragment molecular formula*	Names of fragments	Notes
CoA	767.11576	C21H36N7O16P3S	426.02103	C10H15N5O10P2	ADP	common CoA fragments
			408.01047	C10H13N5O9P2	ADP minus H2O	
			272.95624	C5H8O9P2	deoxyribose diphosphate	
			158.92487	(P2H2O6)n	Oligophosphate	
			686.13995	C21H35N7O13P2S		parent loses a phosphate group
4HV-CoA	***866.16036***	***C26H44N7O18P3S***	426.02094	C10H15N5O10P2	ADP	common CoA fragments
			408.01038	C10H13N5O9P2	ADP minus H2O	
			272.95624	C5H8O9P2	deoxyribose diphosphate	
			158.92484	(P2H2O6)n	Oligophosphate	
			786.19141	***C26H43N7O15P2S***		parent loses a phosphate group
			519.09552	***C16H30N2O11P2S***		parent loses a adenylylated phospho ribose
			537.10608	***C16H32N2O12P2S***		
4PV-CoA	***946.12669***	***C26H45N7O21P4S***	426.02090	C10H15N5O10P2	ADP	common CoA fragments
			408.01041	C10H13N5O9P2	ADP minus H2O	
			272.95621	C5H8O9P2	deoxyribose diphosphate	
			158.92485	(P2H2O6)n	Oligophosphate	
			866.15863	***C26H44N7O18P3S***	4HV-CoA	parent loses a phosphate group
			599.06152	***C16H31N2O14P3S***		parent loses a adenylylated phospho ribose
			617.07233	***C16H33N2O15P3S***		
β-methylcrotonyl CoA	848.14980	C26H42N7O17P3S	426.02066	C10H15N5O10P2	ADP	common CoA fragments
			408.01019	C10H13N5O9P2	ADP minus H2O	
			272.95609	C5H8O9P2	deoxyribose diphosphate	
			158.92479	(P2H2O6)n	Oligophosphate	
			768.18066	C26H41N7O14P2S		parent loses a phosphate group
			501.08484	C16H28N2O10P2S		parent loses a adenylylated phospho ribose
isovaleryl CoA	850.16545	C26H44N7O17P3S	426.02106	C10H15N5O10P2	ADP	common CoA fragments
			408.01056	C10H13N5O9P2	ADP minus H2O	
			272.95620	C5H8O9P2	deoxyribose diphosphate	
			158.92488	(P2H2O6)n	Oligophosphate	
			770.19714	C26H43N7O14P2S		parent loses a phosphate group
			503.10089	C16H30N2O10P2S		parent loses a adenylylated phospho ribose

mass difference by a single phosphate group (m/z = 79.967)

Supplementary Table 3. List of mutations from evolved strains M141 and M142

Position	Gene	Mutation	Change
Genomic mutations			
Common			
243014	fadE	C →T	Trp → stop codon
2323064	atoC (M141)	Transposable element insertion	Early stop codon
2322858	atoC (M142)	Transposable element insertion	Early stop codon
M141			
205559	dnaE	G →A	Arg →His
261153	proB	C →T	His →Tyr
3390059	aaeR	A →C	Lys →Asn
M142			
2395921	nuoI	C →T	Ser →Asn
4161154	fabR	A →C	Thr →Pro
Plasmid mutations			
pJMR5	RBS	G →T	Increased RBS strength

Supplementary Table 4. *E. coli* LS5218 Growth on LA

LS5218 Strains[a]	Growth on LA
Wild Type	--
Δ*fadE*	++
Δ*atoC*	--
Δ*fadE* Δ*atoC*	++
M142	++

[a] All strains carrying pJMR32; --, no growth; ++, robust growth

Supplementary Table 5. Species with LvaABCD homologs.
Included as an excel document.

Supplementary Table 6. Species with LvaACD homologs.
Included as an excel document.

Supplementary Table 7. Strains and Plasmid List

Strain/Plasmid	Relevant genotype/property	Source or Reference
Strains		
Pseudomonas putida		
KT2440	Wild Type	ATCC 47054
KTU	Δ*upp*	Altenbuchner et al[9]
Δ*lvaR*	Δ*upp* ΔPP_2970	This work
Δ*lvaA*	Δ*upp* ΔPP_2971	This work
Δ*lvaB*	Δ*upp* ΔPP_2972	This work
Δ*lvaC*	Δ*upp* ΔPP_2973	This work
Δ*lvaD*	ΔPP_2974	This work
Δ*lvaE*	Δ*upp* ΔPP_2975	This work
Escherichia coli		
CC118λpir	Δ*(ara-leu), araD,* Δ*lacX174, galE, galK, phoA, thi1, rpsE, rpoB, argE (Am), recA1,* lysogenic λ*pir*	de Lorenzo et al[10]
DH5α	F^- Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (r_k^-,m_k^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-	Invitrogen
MG1655	F^- λ^- *ilvG*$^-$ *rfb*-50 *rph*-1	ECGSC
LS5218	F^+ λ^+ *fadR601 atoC512*(Const)	ECGSC
M141	LS5218 mutant evolved on LA	This work
M142	LS5218 mutant evolved on LA	This work
Δ*fadE*	LS5218 Δ*fadE*	This work
Δ*fadE* Δ*atoC*	LS5218 Δ*fadE* Δ*atoC*	This work
Plasmids		
pBAM1	*tnpA*, Amp^R, Kan^R, *ori*R6K	de Lorenzo et al[10]
pJOE6261.2	*upp* (from *P. putida*), Kan^R, ColE1 origin	Altenbuchner et al[9]
pJOE-lvaR	pJOE6261.2 with up- and downstream regions of *lvaR*	This work
pJOE-lvaA	pJOE6261.2 with up- and downstream regions of *lvaA*	This work
pJOE-lvaB	pJOE6261.2 with up- and downstream regions of *lvaB*	This work
pJOE-lvaC	pJOE6261.2 with up- and downstream regions of *lvaC*	This work
pJOE-lvaE	pJOE6261.2 with up- and downstream regions of *lvaE*	This work
pBAD35	P_{BAD} promoter, Kan^R, pBBR1 origin	Lennen et al[11]
pBAD-*lvaA*	pBAD35 carrying *lvaA*	This work
pBAD-*lvaB*	pBAD35 carrying *lvaB*	This work
pBAD-*lvaC*	pBAD35 carrying *lvaC*	This work
pBAD-*lvaD*	pBAD35 carrying *lvaD*	This work
pBAD-*lvaE*	pBAD35 carrying *lvaE*	This work
pK18*mobsacB*	*sacB*, Kan^R, pMB1 origin	Schafer et al[12]
pK18-*lvaD*	pK18*mobsacB* containing up- and downstream regions of *lvaD*	This work
pJMR74	pBAD35 with P_{BAD} promoter and *araC* replaced with *lvaA* promoter and *lvaR* (*P. putida*) carrying sfGFP	This work
pBbS2k-mCherry	Kan^R, SC101 ori, P_{Tet} promoter, mCherry	Addgene[13]
pJMR5	pBbS2k carrying *lva* operon in front of mCherry	This work
p2	pJMR5 mutant evolved on LA	This work
pJMR32	pJMR5 with increased RBS for *lva* operon, mCherry removed	This work

Supplementary Table 8. Primer List

Primer Name	Sequence	Function
5' Race primers		
JMR2	AACCTGGACGGTGAAGAGCG	Reverse primer for *lvaR* cDNA
JMR287	GAACGGACAGGAAGCACAG	Reverse primer for *lvaA* cDNA
GG318	GGCCACGCGTCGACTAGTACCCCCCCCCCC CC	Amplification primer for dGTP tailing reactions
ALM244	GGCCACGCGTCGACTAGTACGGGIIGGGIIG GGIIG	Amplification primer for dCTP tailing reactions
JMR150	CCAATGCCCGTAGCAGGTCGC	Reverse primer for *lvaR*
JMR296	GAACTCCTGTTCACGGTCAAG	Reverse primer for *lvaA*
Operon cDNA Reverse Transcription Primer		
JMR237	TCAATGATCGACGGCACCG	Reverse primer for operon cDNA
Operon – individual genes		
JMR3	ACGCTGTGCTTCCTGTCCGTT	*lvaA* Forward
JMR325	GTTCTTCACCGGACAGATGG	*lvaA* Reverse
JMR576	CCCACGAATTGCTCGAGATC	*lvaB* Forward
JMR577	GCAGGTCGGGCAATGTCG	*lvaB* Reverse
JMR290	CATGCCCGTTCGTGCTTC	*lvaC* Forward
JMR572	CAGGTCCATCATGTTGTCGGC	*lvaC* Reverse
JMR330	ACGAGCCGTGAGGACATCT	*lvaD* Forward
JMR293	CGAGCGCAACTTGTCACC	*lvaD* Reverse
JMR294	GCTGGTGTGCATCAACATCC	*lvaE* Forward
JMR571	GCAGTGGAACATCGGCAAGG	*lvaE* Reverse
JMR573	TGTTATACGCGCGTGTTCG	*lvaF* Forward
JMR574	GGTACACGTAGAACGCCGAC	*lvaF* Reverse
JMR575	CATGGTGTTCGTGCTGTTCACC	*lvaG* Forward
JMR579	GCCGAACAGCAACCTGATCA	*lvaG* Reverse
Operon – individual genes		
JMR3	ACGCTGTGCTTCCTGTCCGTT	*lvaA* Forward
JMR289	CAGGTCGGGCAATGTCG	*lvaB* Reverse
JMR576	CCCACGAATTGCTCGAGATC	*lvaB* Forward
JMR578	GAAGCACGAACGGGCATGG	*lvaC* Reverse
JMR301	GCCGACAACATGATGGACCTG	*lvaC* Forward
JMR299	CGTGGTCCCAGGTTTGATCATC	*lvaD* Reverse
JMR292	GCTCGACACCAACCTCAAGG	*lvaD* Forward
JMR333	GCCAAGAACGCTTCGTAGTC	*lvaE* Reverse
JMR11	CAC GGT GCT GGA TAC CGA CA	*lvaE* Forward
JMR574	GGTACACGTAGAACGCCGAC	*lvaF* Reverse
JMR573	TGTTATACGCGCGTGTTCG	*lvaF* Forward
JMR579	GCCGAACAGCAACCTGATCA	*lvaG* Reverse

Supplementary Table 9. Other parameters for targeted MS/MS

Resolution	70,000
AGC target	1E6
Maximum IT	40 ms
Isolation width	1.4 (m/z)
Fixed first mass	70 m/z
(N)CE/stepped (N)CE	15, 30, 45
Default charge	1
Polarity	negative

Supplementary Table 10. Modified Oligonucleotides used for BarSeq

Oligo Name	Sequence
Barseq_P1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTCGACCTGCAGCGTACG
Barseq_P1_4N	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNGTCGACCTGCAGCGTACG
Barseq_P1_3N	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNGTCGACCTGCAGCGTACG
Barseq_P1_2N	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNGTCGACCTGCAGCGTACG