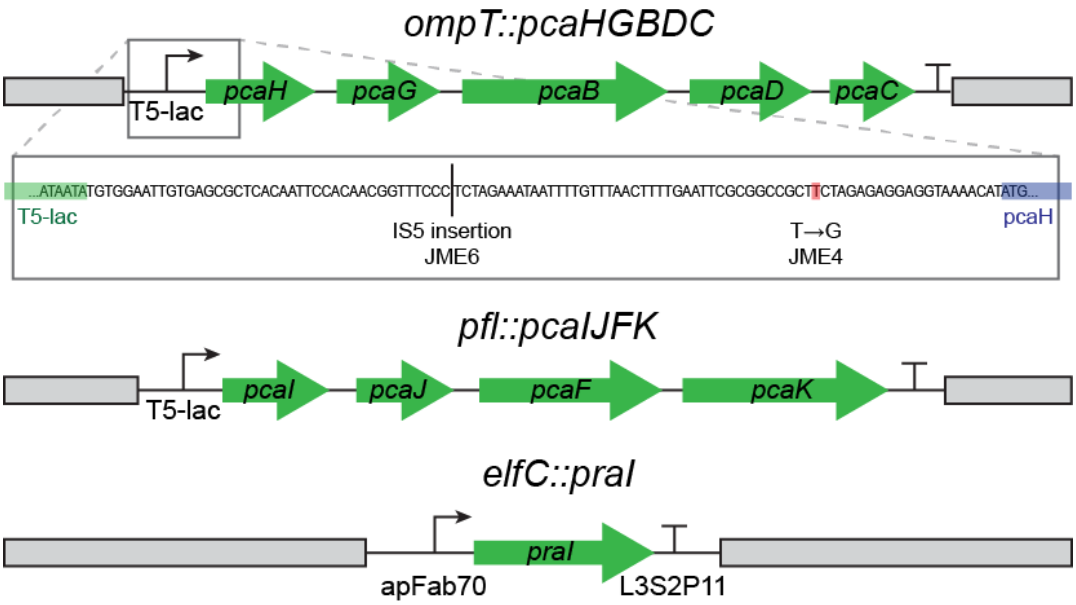
Supplementary Table 1: Mutations observed in resequenced isolates. All nucleotide references are relative to AG978 (BW25113 *ompT::pcaHGBDC pflB::pcaIJFK*)

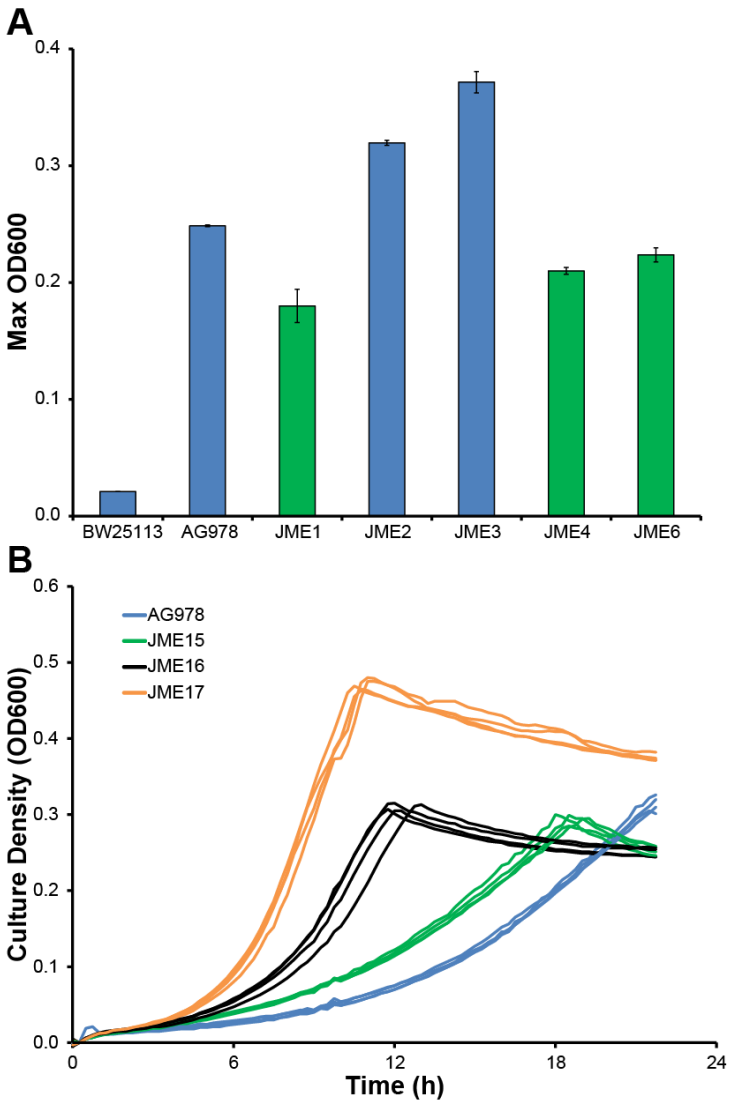
Strain	**Nucleotide Mutation**	**Notes**
JME1	Deletion, 266,713-365,993	Contains *lacI*, *mhpABC*
	Duplication, 570,874-689,163	Contains *pcaHBGDC* IS5 flanks each side of duplication
	Duplication, 2,065,704-2,105,550	Contains *flu* IS5 flanks each side of duplication
	G2,076,174T	Ag43 G684C, in both copies of *flu*
	C2,853,953T	Intergenic
	Deletion, 2,971,431-2,971,442	In-frame deletion of 12 bp in *ptsP*
	Duplication, 3,624,082-3,767,566	Spans *rhsB* to *rhsA*, frequently duplicated
	Deletion, 4,409,630-4,409,639	10 bp frameshift mutation in *rnr*
JME2	Deletion, 266,713-365,993	Contains *lacI*, *mhpABC*
	Duplication, 570,874-689,163	Contains *pcaHBGDC* IS5 flanks each side of duplication
	Deletion, 1,198,056-1,213,105	Prophage excision
	T1,311,771G	TopA M533R
	Deletion, 2,967,196-2,967,197	2 bp frameshift mutation in *ptsP*
	Deletion, 4,409,630-4,409,639	10 bp frameshift mutation in *rnr*
JME3	Duplication, 570,874-689,163	Contains *pcaHBGDC* IS5 flanks each side of duplication
	Duplication, 3,624,082-3,767,566	Spans *rhsB* to *rhsA*, frequently duplicated
	Duplication, 3,940,660-4,034,652	
	T3,991,518A	CyaA W1072R
	C4,174,713A	SecE A29E
JME4	T580,465G	*pcaH* RBS See Supplementary Figure 1
	Deletion, 1,198,056-1,213,105	Prophage excision
	C2,072,530T	Silent mutation in Ag43
	Deletion, 2,072,288-2,072,443	In-frame 156 bp deletion in *flu*
	Duplication, 3,624,082-3,767,566	*rhsB* to *rhsA*
JME6	IS5 insertion	Between promoter and RBS of *pcaH* See Supplementary Figure 1
	Duplication, 2,065,704-2,105,550	*flu* at 2,071,645-2,074,769 IS5 flanks each side of duplication
	Deletion, 2,072,681-2,072,941	In-frame 261 bp deletion in both copies of *flu*
	Duplication, 3,624,082-3,767,566	*rhsB* to *rhsA*
	Deletion, 3,816,442	Intergenic

Supplementary Table 2: Expression of Ag43 in evolved isolates.

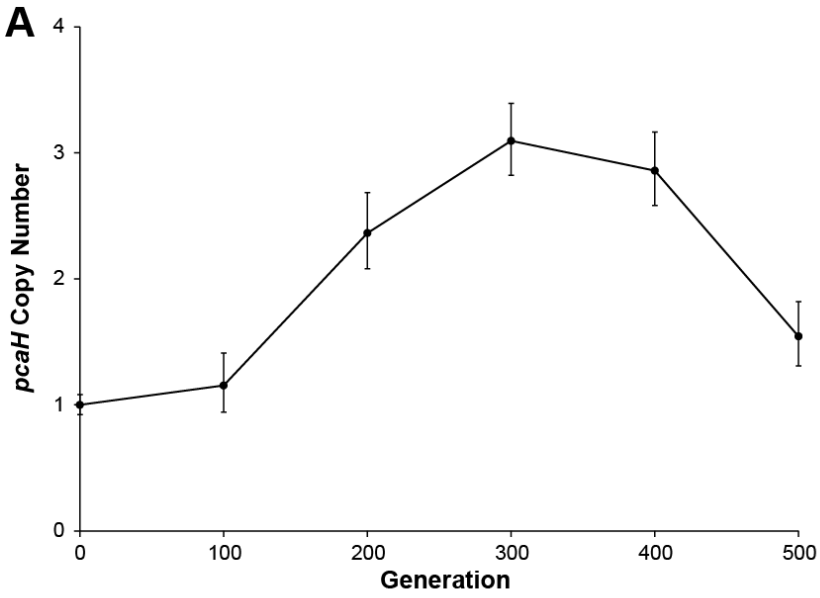
Strain	Relative expression
AG978	1.0 ± 0.1
JME1	110. ± 10.
JME4	65 ± 9
JME6	190. ± 3
JME17	2.4 ± 1.1

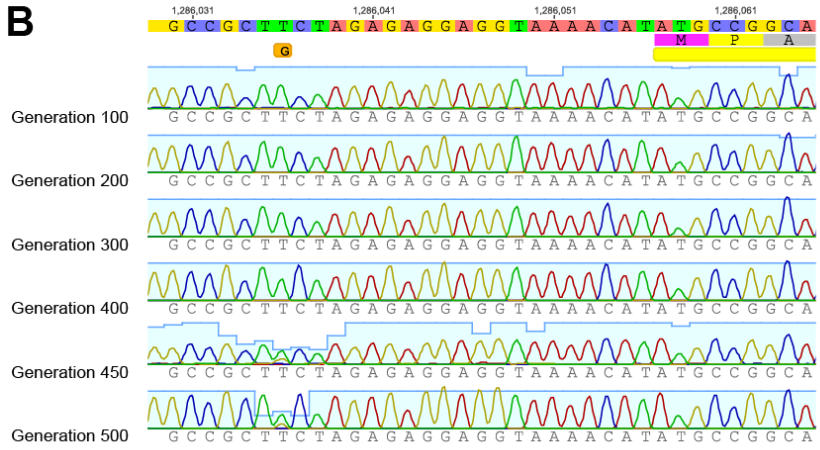


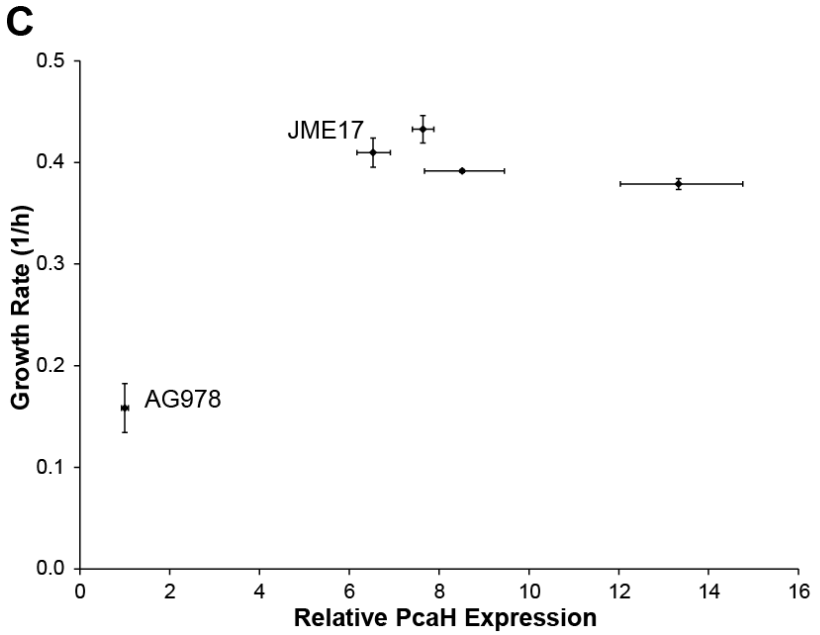
Supplementary Figure 1: Construct design. The eight-gene pathway for ortho PCA degradation, along with the PCA transporter *pcaK* was designed as two operons, each expressed from a T5-lac promoter. These operons were integrated into the indicated chromosomal loci in AG978. The mutations to the *ompT::pcaHGBDC* operon found in JME4 and JME6 are indicated. A separate expression construct for the 4-hydroxybenzoate 3-monooxygenase *praI* was inserted into the *elfC* locus in strains JME7 and JME50.



Supplementary Figure 2: Mutations to *flu* decrease maximum optical density. (A) During growth with PCA, the maximum OD600 varies between strains with wildtype *flu* (in blue) and mutant forms of *flu* (in green). Cultures containing the parental strain AG978 turn a darker red color, indicating that more of the substrate has oxidized, which likely accounts for the intermediate yield of this strain. Error bars show one standard deviation, calculated from three biological replicates. (B) A 261-bp deletion in *flu* was introduced into AG978 and JME17, producing strains JME15 and JME16, respectively. Strains were grown in minimal medium containing 1 g/L PCA as the sole source of carbon and energy.


A
pcaH Copy Number
Generation
0
1
2
3
4
0
100
200
300
400
500


B
1,286,031
1,286,041
1,286,051
1,286,061
G C C G C T T C T A G A G A G G A G G T A A A A C A T A T G C C G G C A
M P A
G
Generation 100
Generation 200
Generation 300
Generation 400
Generation 450
Generation 500


C
Growth Rate (1/h)
Relative PcaH Expression
JME17
AG978
0.0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
8
10
12
14
16

Supplementary Figure 3: A two-fold increase in RBS strength is the dominant strategy for PcaH overexpression. (A) As measured by qPCR, the population averaged *pcaH* copy number increases in replicate population B. Error bars show one standard deviation, calculated from three biological replicates. The curve is a guide for the eye. (B) The RBS mutation, a T->G substitution, is first detected as a mixed base in generation 450. (C) The RBS mutation, reconstructed in JME17, increases PcaH expression by 7-fold relative to the parent, AG978. Engineered mutants with further increases in predicted RBS strength do not lead to a corresponding increase in growth rate. Error bars show one standard deviation, calculated from three biological replicates. Note that, in some cases, the error bars are difficult to visually distinguish from the data points.