

Table S1. Strains used in this study.

Name	Strain	Genotype	Source
NEB5a	<i>E. coli</i> NEB5a	<i>fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	NEB (Ipswich, MA)
BW25113	<i>E. coli</i> BW25113	<i>Δ(araD-araB)567 ΔlacZ4787(::rrnB-3) λ- rph-1 Δ(rhaD-rhaB)568 hsdR514</i>	Baba et al., 2006
<i>ΔfrmA</i>	<i>E. coli</i> BW25113 <i>ΔfrmA</i>	BW25113 <i>ΔfrmA::kan</i>	Whitaker et al., 2017
<i>ΔfrmA ΔihfA</i>	<i>E. coli</i> BW25113 <i>ΔfrmA ΔihfA</i>	BW25113 <i>ΔfrmA::FRT ΔihfA::kan</i>	Bennett et al., 2020a

Table S2. Plasmids used in this study.

Name	Plasmid	Characteristics	Source
pKD13	pKD13	<i>R6K ori, bla, kan, FRT</i>	Datsenko and Wanner, 2000
pKD46	pKD46	<i>oriR101, repA101(ts), bla, araB, 1 red bet, 1 red gam, 1 red exo</i>	Datsenko and Wanner, 2000
pCP20	pCP20	<i>repA101(ts), cI857(ts), bla, cat, S. cerevisiae flp</i>	Datsenko and Wanner, 2000
pETM6	pETM6	<i>ColE1 ori, bla, fl ori, lacI, T7 MCS</i>	Xu et al., 2012
pUD9	pETM6_P _{trc} _mdh_hps_phi	pETM6, P _{trc} , <i>B. stearothermophilus mdh</i> , <i>B. methanolicus hps</i> , <i>B. methanolicus phi</i>	Bennett et al., 2018a
pACM4	pACM4	<i>P15A ori, cat, fl ori, lacI, T7 MCS</i>	Xu et al., 2012
pC _{rp}	pACM4_P _{tac} _crp	pACM4, P _{tac} , <i>E. coli</i> BW25113 <i>crp</i>	Bennett et al., 2020a

Table S3. Primers used in this study.

Primer	Sequence (5' to 3')	Purpose
ihfA KO F	AGAGGCATTAAAAGAGCGATTCCAGGCATCATTGAGGGATTGAACCTATGATTCCGGGGATCCGTCGACC	Gene knockout
ihfA KO R	CGCAGAGCGGCCCTTTTAGTTAGATCAGATTACTCGTCTTTGGGCGAAGCTGTAGGCTGGAGCTGCTTCG	
ihfA F	GGATGCCAAAGAACTGGTTG	Gene-specific confirmation
ihfA R	CACGCAGATCGAAGTTACCA	
ihfA up F	TTGGTTTGTGTGGGGTTGTT	Locus-specific confirmation
ihfA down R	TGAAGTGTCATGGCGTTGAT	
kan F	TGAATGAACGTCAGGACGAG	Gene-specific confirmation
kan R	AATATCACGGGTAGCCAACG	
pACM4 F Gib	TCGAGTCTGGTAAAGAAAC	PCR for Gibson assembly
pACM4 R Gib	TGTATATCTCCTTCTTAAAGTTAAAC	
pACM4 up F	CGACTCCTGCATTAGGAAGC	Sequencing confirmation
pACM4 down R	GGCAGTTATTGGTGCCCTTA	
crp F pAC Gib	TTTAACTTTAAGAAGGAGATATACAATGGTGCTTGGCAAACCG	PCR for Gibson assembly
crp R pAC Gib	GCAGCGGTTTCTTACCAGACTCGATTAAACGAGTGCCGTAAACG	

Table S4. Maximum growth rates of parent and evolved *E. coli* strains in LB medium supplemented with methanol or formaldehyde at the specified concentrations. Data represent average $\pm$ standard error (n=2).

	+ Plasmid		- Plasmid	
	Parent	Evolved	Parent	Evolved
Methanol (M)	Growth Rate (h⁻¹)			
0	1.2 $\pm$ 0.02	1.2 $\pm$ 0.02	1.4 $\pm$ 0.02	1.2 $\pm$ 0.00
1	1.0 $\pm$ 0.01	1.1 $\pm$ 0.00	1.3 $\pm$ 0.00	1.2 $\pm$ 0.01
2	0.47 $\pm$ 0.02	0.65 $\pm$ 0.01	0.66 $\pm$ 0.00	0.66 $\pm$ 0.01
3	0.07 $\pm$ 0.00	0.11 $\pm$ 0.01	0.18 $\pm$ 0.00	0.15 $\pm$ 0.00
Formaldehyde (mM)	Growth Rate (h⁻¹)			
0.25	-	-	1.0 $\pm$ 0.03	1.1 $\pm$ 0.01
0.5	-	-	0.75 $\pm$ 0.01	0.76 $\pm$ 0.00
1	-	-	0.33 $\pm$ 0.01	0.34 $\pm$ 0.00
1.5	-	-	0.16 $\pm$ 0.01	0.18 $\pm$ 0.00

Table S5. Unique sequence variants of evolved clones determined via whole genome sequence with *E. coli* BW25113 as the reference genome. I=insertion, D=deletion, nucleotide changes are indicated by '→'. Cells highlighted in blue indicate common mutations in tRNAs. Cells highlighted in yellow indicate common mutations in 30S ribosomal subunit proteins.

Strain	Position in BW25113 genome	Nucleotide change (+ strand)	Amino acid change	Gene
KB201	1011663	C→G	Trp93Cys	substitution in <i>fabA</i> , beta dehydroxydecanoyl thioester dehydrase
	3033130	T→A	n/a	substitution in <i>xerD</i> promoter
	3441364	G→A	Arg49Cys	substitution in <i>rplN</i> , 50S ribosomal subunit
	3441836	T→G	His31Pro	substitution in <i>rpsQ</i> , 30S ribosomal subunit
	4210706	large insertion	n/a	large insertion in <i>arpA</i> , ankyrin repeat protein
Evolved $\Delta ihfA$ Clone	86049	DT	n/a	deletion in <i>mraZ</i> promoter, methyltransferase inhibitor
	219445	DA	frameshift	deletion in <i>gmhB</i> , D,D-heptose 1,7-bisphosphate phosphatase
	2434696	C→G	Gly130Ala	substitution in <i>fabB</i> , 3-oxoacyl-synthase I
	3441836	T→G	His31Pro	substitution in <i>rpsQ</i> , 30S ribosomal subunit
Original Evolved Strain ($\Delta frmA$ +pUD9)	557147	DG	frameshift	deletion in <i>sfmD</i> , putative outer membrane protein
	1415901	DC	frameshift	deletion in cryptic prophage Rac conserved protein <i>ydaU</i>
	1584807	G→A	n/a	substitution in <i>yneL</i> pseudogene promoter
	1632320	G→A	Ala16Val	substitution in <i>ynfN</i> , cold-shock protein in Qin prophage
	1763729	DG	frameshift	deletion in <i>ydiK</i> , inner membrane protein
	2196843	DG	frameshift	deletion in <i>yehI</i> , putative regulator
	2398891	DC	n/a	deletion upstream of <i>nuoA</i> , oxidoreductase, membrane subunitA
	3456961	C→T	Pro227Ser	substitution in <i>gspK</i> , transport protein
	3461340	C→T	Ala625Thr	substitution in <i>chiA</i> , periplasmic endochitinase
	3467638	C→T	Gly92Ser	substitution in <i>rpsL</i> , 30S ribosomal subunit
	3498571	C→T	Ser270Asn	substitution in <i>yhfS</i> , aminotransferase family protein
	3528705	C→T	Ala175Thr	substitution in <i>envZ</i> , sensor histidine kinase for <i>ompC</i> and <i>ompF</i>
	3721471	DC	frameshift	deletion in <i>xylB</i> , xylulokinase
	4328920	G→A	silent	substitution in <i>adiA</i> , arginine decarboxylase
	4596140	DG	n/a	deletion in <i>leuQ</i> , tRNA leucine