Supplementary Information

Mining RNA-seq data reveals the massive regulon of GcvB small RNA and its physiological significance in maintaining amino acid homeostasis in *Escherichia coli*

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This supplement contains:

Supplementary Figures S1 to S2

Supplementary Tables S1 to S8

Supplementary Figure S1

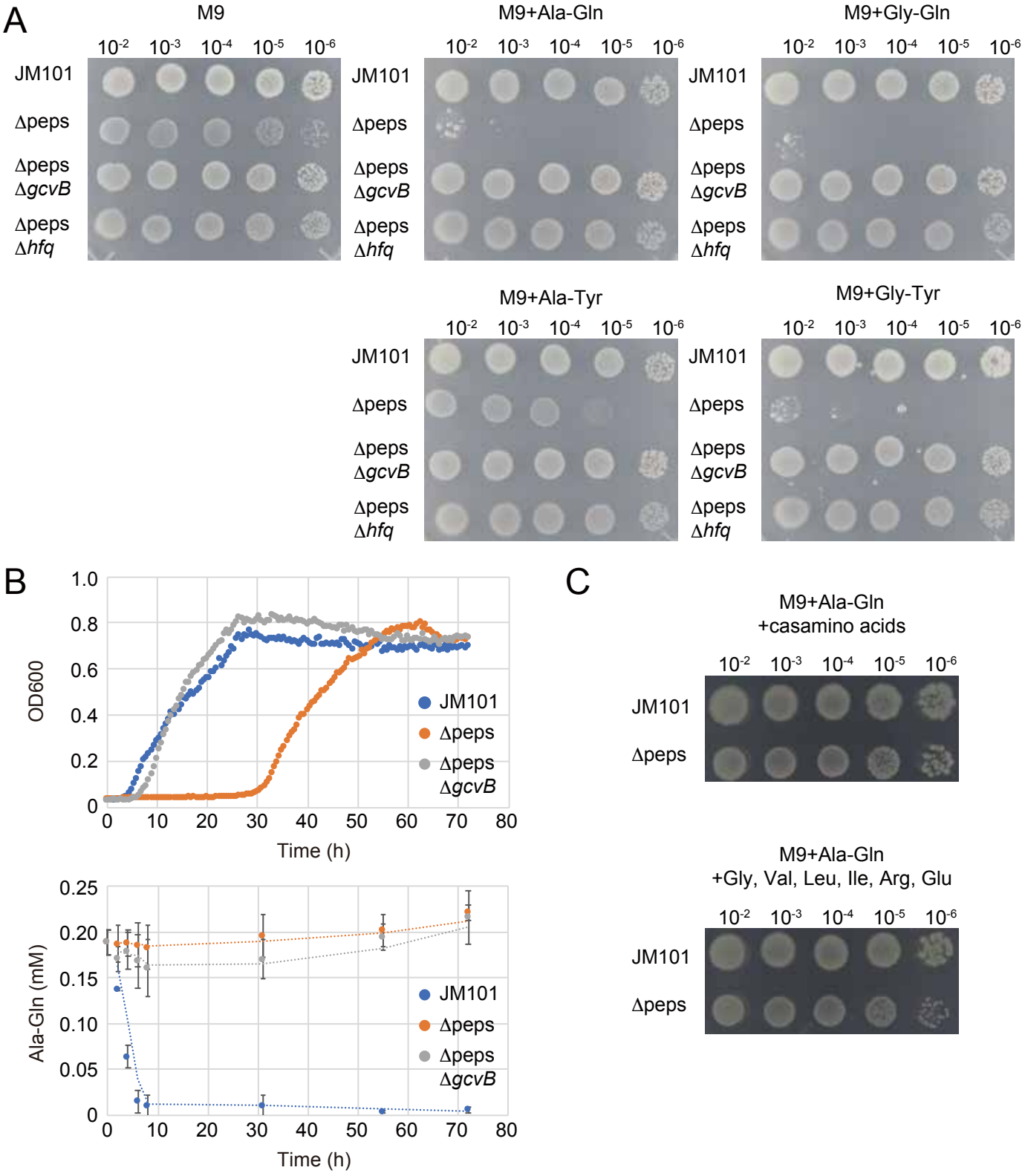

Figure S1. Growth inhibition by dipeptides. (A) Growth on M9 plates was compared among the wild-type JM101 strain, Δpeps strain, ΔpepsΔ*gcvB* and ΔpepsΔ*hfq*. M9 plates were aupplemented with 0.2 mM dipeptides as indicated. (B) Growth curve of JM101, Δpeps, and ΔpepsΔ*gcvB* strains in M9 liquid medium and Ala-Gln concentration in the supernatant. (C) Growth of JM101 and Δpeps strains on M9 plates supplemented with 1 mg/mL casamino acids (upper panel) and six amino acids (100 μg/mL each, lower panel) in addition to 0.2 mM Ala-Gln. The plates were incubated at 30°C for two days.

Supplementary Figure S2

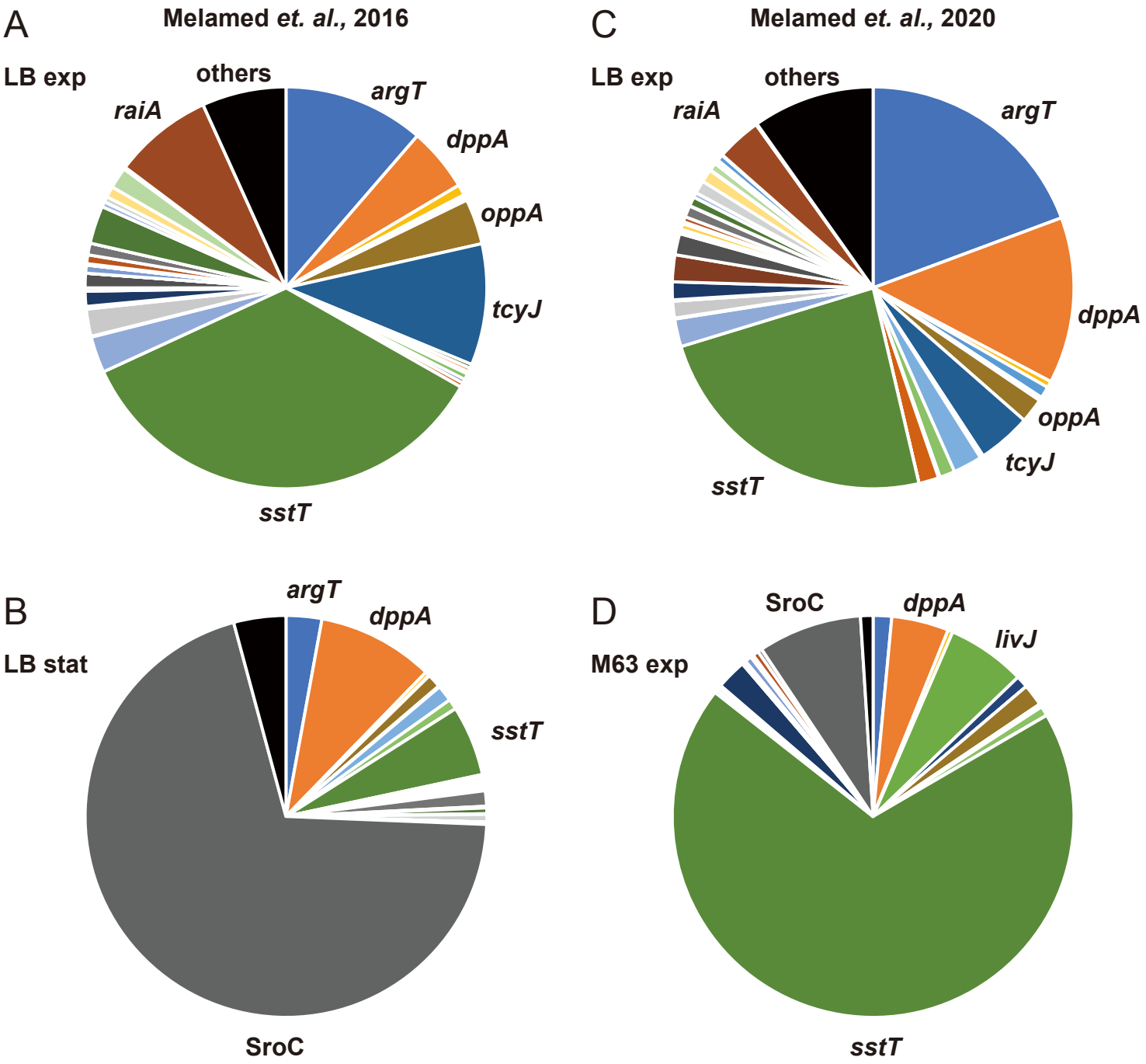

Figure S2. Transition of GcvB regulon depending on growth conditions. Pie chart representation of the proportion of each GcvB interactants to the total GcvB chimeric reads calculated from the RIL-seq datasets shown in Table 1. A and B were obtained from the Hfq RIL-seq datasets of E. coli grown in LB medium to early exponential (OD ~0.5) and stationary phase, respectively (Melamed et al., 2016). C and D were obtained from Hfq RIL-seq datasets of E. coli grown to late exponential phase (OD ~1.0) in LB medium and M63 minimal medium, respectively (Melamed et al., 2020).

Table S1. Genes repeatedly detected in the datasets of RIL-seq, CLASH, and MAPS.
All genes overlapping among the interactome datasets are listed. Previously identified and newly validated GcvB targets are indicated in bold black and red fonts, respectively.

Gene	Description	MAPS
acs	**acetyl-CoA synthetase**	**34.7**
argT	**lysine/arginine/ornithine transporter subunit**	**64.2**
aroC	**chorismate synthase : N5-glutamine methyltransferase**	**20.2**
asd	**asparagine synthetase B**	**15.8**
asnB	**aspartate-semialdehyde dehydrogenase, NAD(P)-binding**	**37.8**
aspV	*aspV.yafT*.IGR	16.3
bax	putative glucosaminidase	51.0
cfa	**cyclopropane fatty acyl phospholipid synthase**	**10.5**
cstA	**carbon starvation protein**	**12.6**
cycA	**D-alanine/D-serine/glycine transporter**	**279.3**
dppA	**dipeptide/heme ABC transporter periplasmic binding protein**	**119.6**
fecA	KpLE2 phage-like element; ferric citrate outer membrane transporter	8.0
ftsB	cell division protein	29.8
gatY	**D-tagatose 1,6-bisphosphate aldolase 2, catalytic subunit**	**21.1**
gdhA	**glutamate dehydrogenase, NADP-specific**	**147.0**
gltI	**glutamate and aspartate transporter subunit**	**236.3**
gltP	**glutamate/aspartate:proton symporter**	**19.4**
icd	**isocitrate dehydrogenase, specific for NADP+, e14 prophage;**	**16.6**
ilvC	**ketol-acid reductoisomerase, NAD(P)-binding**	**2.1**
kgtP	**alpha-ketoglutarate transporter**	**34.5**
lipA	lipoate synthase	16.3
livK	**leucine transporter subunit**	**10.2**
lrp	**DNA-binding transcriptional dual regulator, leucine-binding**	**547.4**
maeB	fused malic enzyme predicted oxidoreductase	8.4
mgrR	sRNA	12.2
mltC	**membrane-bound lytic murein transglycosylase C**	**41.3**
mtfA	anti-repressor for DgsA(Mlc)	30.2
ompF	outer membrane porin 1a (Ia;b;F) : asparaginyl tRNA synthetase	14.6
oppA	**oligopeptide transporter subunit**	**52.3**
panD	**aspartate 1-decarboxylase**	**207.9**
raiA	cold shock protein associated with 30S ribosomal subunit	96.9
rbsK	**ribokinase**	**29.0**
rmf	ribosome modulation factor	91.2
rodZ	cytoskeletal protein required for MreB assembly	19.6
serA	**D-3-phosphoglycerate dehydrogenase**	**90.9**
sstT	**sodium:serine/threonine symporter**	**97.2**
tcyJ (fliY)	**cystine ABC transporter periplasmic binding protein**	**95.2**
thrL	***thr* operon leader peptide**	**124.7**
thrU	coaA.thrU.IGR	7.1
wzzB	regulator of length of O-antigen component of lipopolysaccharide chains	18.2
yeeX	UPF0265 family protein	10.4
yfhM	alpha-2-macroglobulin	7.0
ygeR	putative peptidase lipoprotein	13.3
ygfF	putative NAD(P)-dependent oxidoreductase	51.0
yghU	putative S-transferase	47.9
yifK	**putative APC family amino acid transporter**	**35.2**
ysgA	**putative carboxymethylenebutenolidase**	**105.0**

Table S2. Comparative genome analysis of Δ*peps* suppressor mutants.

Breakpoints of deletion were detected by sprites v0.3.0 based on the K12 reference genome (NC_000913.3). Deletions found in all the Δ*peps* strains are indicated in red fonts. Large deletions acquired in the 10 suppressor mutants are highlighted in yellow. + and - represent presence and absence of a mutation respectively.

start	end	JM101	Δ*peps*	1-1	1-2	2-1	2-2	3-1	3-2	4-1	4-2	5-1	5-2	total	annotation
254,246	255,735	–	+	+	+	+	+	+	+	+	+	+	+	11	*pepD*
256,125	371,374	+	+	+	+	+	+	+	+	+	+	+	+	12	
990,600	993,256	–	+	+	+	+	+	+	+	+	+	+	+	11	*pepN*
1,299,498	1,300,694	+	+	+	+	+	+	+	+	+	+	+	+	12	
1,978,502	1,979,271	+	+	+	+	+	+	+	+	+	+	+	+	12	
2,558,709	2,565,481	+	+	+	+	+	+	+	+	+	+	+	+	12	
2,655,110	2,656,323	–	+	+	+	+	+	+	+	+	+	+	+	11	*pepB*
3,625,588	3,720,629	–	–	+	+	–	–	–	+	–	–	–	–	3	95kb deletion
3,625,590	3,720,631	–	–	–	–	+	–	+	–	+	–	+	+	5	95kb deletion
4,484,475	4,485,916	–	+	+	+	+	+	+	+	+	+	+	+	11	*pepA*
4,606,166	4,606,314	–	+	+	+	+	+	+	+	+	+	+	+	11	tRNA-Leu(CAG)

SNP calling was performed by GATK-4.0.5.2 based on the K12 reference genome (NC_000913.3). SNPs found in all the Δ*peps* strains are indicated in red fonts. SNPs acquired in the 10 suppressor mutants are highlighted in yellow. + and - represent presence and absence of a mutation respectively.

position	SNP	WT	Δpeps	1-1	1-2	2-1	2-2	3-1	3-2	4-1	4-2	5-1	5-2	annotation
1276421	C->A	–	+	+	+	+	+	+	+	+	+	+	+	*narX* (D->Y)
2942810	G->A	–	–	–	–	–	–	–	–	–	+	–	–	*gcvB*
2971188	T->A	–	–	–	–	–	–	–	–	–	+	–	–	
3334738	C->A	–	+	+	+	+	+	+	+	+	+	+	+	
3702998	C->A	–	–	–	–	–	+	–	–	–	–	–	–	*dppD* (C->F)
3704067	A->C	–	–	–	–	–	–	–	–	–	+	–	–	*dppC* (L->R)

Table S3. Bacterial strains used in this study.

Strain	Relevant markers/ genotype	Reference/ source
E. coli		
BW25113	F^- λ^- *rrnB3* Δ*lacZ*4787 *hsdR*514 Δ(*araBAD*)567 Δ(*rhaBAD*)568 *rph*-1	NBPR strain
Δ*gcvB*	BW25113 Δ*gcvB:: kan*	This study
Δ*sroC*	BW25113 Δ*sroC::*FRT	This study
Δ*gcvB*Δ*sroC*	BW25113 Δ*gcvB:: kan* Δ*sroC::*FRT	This study
gdhA::3xFLAG	BW25113 *gdhA*::3xFLAG *kan*	This study
Δ*gcvB gdhA*::3xFLAG	BW25113 Δ*gcvB::* FRT *gdhA*::3xFLAG *kan*	This study
JM101 F^-	*supE*, *thi*-1, Δ(*lac-proAB*), F^- (*pepD*)	Wachi laboratory stock
Δ*peps*	JM101 F^- (*pepD*), *pepN*, *pepB*, *pepA*	Hayashi et al. 2010
Δ*peps*Δ*dpp*	Δ*peps* Δ*dppABCDF:: kan*	This study
Δ*peps*Δ*gcvB*	Δ*peps gcvB*::*kan*	This study
Δ*peps*Δ*hfq*	Δ*peps hfq*::*kan*	This study
Δ*peps*Δ*ydeE*	Δ*peps* Δ*ydeE:: kan*	This study

Table S4. DNA oligonucleotides used in this study.

Name	Sequence (5´ -> 3´ direction)	Used for
Northern blot		
JVO-0322	CTACGGCGTTTCACTTCTGAGTTC	Probe for 5S rRNA
JVO-0749	TTCGTTCCGGCTCAGGA	Probe for GcvB 5´ region
GcvB cloning and mutagenesis		
JVO-0237	ACTTCCTGAGCCGGAAC	GcvB cloning
MMO-0086	GTTTTTTCTAGATAACGATACCGGTATGATTTC	GcvB cloning
MMO-0184 (JVO-0744)	ATTGGTCTGCGATTCAGA	GcvB R1 deletion
MMO-0185	ACCGTAAGCCAAAAGTTCA	GcvB R1 deletion
MMO-0196 (JVO-0895)	ACATTTACCCTGTCTGTCC	GcvB R2 deletion
MMO-0197	GAAAAAAGGTAGCTTTGCTAC	GcvB R2 deletion
MMO-0768	ATTAATGTAGCACCGCCTAA	GcvB R3 deletion
MMO-0769	TAAATGTACAGGAAGTGAAAAAAG	GcvB R3 deletion
JVO-9214	CCTGTCTCTCCATAGTGATTAATGTAGCAC	GcvB G160C mutation
JVO-9215	CTATGGAGAGACAGGGTAAATGTACAGG	GcvB G160C mutation
MMO-0342	TACCCTCtctgtccATAGTGATTAATG	GcvB G156C mutation
MMO-0343	GACAGAGAGGGTAAATGTACAGGAAG	GcvB G156C mutation
MMO-0391	TACCGACAGtgtccATAGTGATTAATG	GcvB mutR3 mutation
MMO-0392	GACACTGTCGGTAAATGTACAGGAAG	GcvB mutR3 mutation
MMO-0776	GTCTGTGCATAGTGATTAATGTAGCAC	GcvB C162G mutation
MMO-0777	ACTATGCACAGACAGGGTAAATGTA	GcvB C162G mutation
GcvB target cloning		
MMO-0199	GTTTTATGCATGCAAAAGCACATGACATA	*gdhA* GFP fusion cloning
MMO-0201	GTTTTGCTAGCGAGGAATGACTCCAGAGA	*gdhA* GFP fusion cloning
MMO-0327	GTTTTATGCATATCAAAGAGTTGCTGGAAG	*sucB-sucC* GFP fusion cloning
MMO-0328	GTTTTGCTAGCTTGTTTTGCCTGATATTCA	*sucB-sucC* GFP fusion cloning
MMO-0459	GTTTTATGCATACTTACATATATATTGTCGGTATC	*map* GFP fusion cloning
MMO-0461	GTTTTTGCTAGCGACGCGCATTTTTTCGAT	*map* GFP fusion cloning
MMO-0462	GTTTTATGCATAATGTAACATCTCTATGGACAC	*cstA* GFP fusion cloning
MMO-0464	GTTTTTGCTAGCGATCTGTTCCCCACGAT	*cstA* GFP fusion cloning
MMO-0465	GTTTTATGCATATTTTTGCCATGCTATTTCTTTA	*ydeE* GFP fusion cloning
MMO-0466	GTTTTTGCTAGCGGTTAATAACAACGACGAGG	*ydeE* GFP fusion cloning
MMO-0469	GTTTTATGCATCACAAATATGACAGTGGCG	*rmf* GFP fusion cloning
MMO-0470	GTTTTTGCTAGCGGCCATTACTACCCTGTC	*rmf* GFP fusion cloning
MMO-0477	GTTTTATGCATTACTTCGATTTTGATGTTTATGG	*hisJ-hisQ* GFP fusion cloning
MMO-0478	GTTTTTGCTAGCTAAAATAACACCTGAAAACCC	*hisJ-hisQ* GFP fusion cloning
MMO-0790	GTTTTTATGCATCTTTACAAAAAAAATAAACAAAAGC	*kgtP* GFP fusion cloning
MMO-0791	GTTTTTGCTAGCGACATAGAAATCGAACCAC	*kgtP* GFP fusion cloning
MMO-0792	GTTTTTATGCATATAACGCGCATCTTTCATGA	*icd* GFP fusion cloning
MMO-0793	GTTTTTGCTAGCTTGTGCCGGAACAACTAC	*icd* GFP fusion cloning
MMO-0794	GTTTTTATGCATAAAAAAATCTACAAACCAACGC	*gltP* GFP fusion cloning
MMO-0795	GTTTTTGCTAGCGCTATGGTAGTGCAGGTAG	*gltP* GFP fusion cloning
MMO-0798	GTTTTTATGCATAAGAAACTCAACATGATGA	*yggX-mltC* GFP fusion cloning
MMO-0799	GTTTTTGCTAGCCAGAATATCAAAACCGTTG	*yggX-mltC* GFP fusion cloning
MMO-0800	GTTTTTATGCATAAAGGCGAGAAAGTTCAG	*rbsB-rbsK* GFP fusion cloning
MMO-0801	GTTTTTGCTAGCATTAAGAATGTGGTCAGCA	*rbsB-rbsK* GFP fusion cloning
MMO-0803	GTTTTTATGCATCAATATCCGGATGTTCCGTTC	*prmB-aroC* GFP fusion cloning
MMO-0804	GTTTTTGCTAGCAACACCATCGACGATGCA	*prmB-aroC* GFP fusion cloning
MMO-0805	GTTTTTATGCATAACTTCTTTGATGTAAACAAAT	*aroP* GFP fusion cloning
MMO-0806	GTTTTTGCTAGCAATATGGCGGTTTTTAAGG	*aroP* GFP fusion cloning
MMO-0817	GTTTTTATGCATAGCGGGCTTTTTTTTGAAC	*trpE* GFP fusion cloning
MMO-0818	GTTTTTGCTAGCCCCACACAACTGGTGAAA	*trpE* GFP fusion cloning
MMO-0819	GTTTTTATGCATCCTACAAGGAGAACAAAAGC	*acs* GFP fusion cloning
MMO-0820	GTTTTTGCTAGCTTGTTGATACATCGCCTCG	*acs* GFP fusion cloning
MMO-0821	GTTTTTATGCATATGTGCCAAGAGGAGACC	*asd* GFP fusion cloning
MMO-0822	GTTTTTGCTAGCGCGTTGCATGAGAACGGA	*asd* GFP fusion cloning
MMO-0925	GTTTTTATGCATACACGTCCGCAGTTTGGT	*purU* GFP fusion cloning

MMO-0926	GTTTTTGCTAGCGATCAGACCTTTTTGGTCCGG	*purU* GFP fusion cloning
MMO-0861	GTTTTTATGCATAAATATTGTTAAACACAAAACCAACAAG	*ivbL* GFP fusion cloning
MMO-0862	GTTTTTGCTAGCTAGTTTTGCGTTGAGCATGG	*ivbL* GFP fusion cloning
MMO-0863	GTTTTTATGCATTTTTTCGTTTAAGCACCTCCC	*ilvB* GFP fusion cloning
MMO-0864	GTTTTTGCTAGCAACGATAAATTCTGCGCCG	*ilvB* GFP fusion cloning
MMO-0865	GTTTTTATGCATATTAAACGCATCATAAAAATCGGCC	*ilvL* GFP fusion cloning
MMO-0889	GTTTTTGCTAGCGGCTGTCATTTTGTCTTTTCTTGC	*ilvL* GFP fusion cloning
MMO-0867	GTTTTTATGCATACCTTAAAAACATAACCGAGGAGC	*ilvX* GFP fusion cloning
MMO-0868	GTTTTTGCTAGCGTTCCCCGTCCTGAATCTTGAG	*ilvX* GFP fusion cloning
MMO-0890	GTTTTTATGCATGAAACCGAAGATAAATGGGGCTGG	*ilvE-ilvD* GFP fusion cloning
MMO-0870	GTTTTTGCTAGCACCCGCCATATTACGACCATGAG	*ilvE-ilvD* GFP fusion cloning
MMO-0871	GTTTTTATGCATTACCTGCTTCATGTCTCAATCGACG	*ilvG-ilvM* GFP fusion cloning
MMO-0872	GTTTTTGCTAGCCACGCGTAAAACACGTTCTAAGGTTTC	*ilvG-ilvM* GFP fusion cloning
GcvB target mutagenesis		
MMO-0335	GATGGAGAGAACACATGAACTTACATGA	*sucC* C-8G
MMO-0454	TGTTCTCTCCATCCTTCAGTAATCGA	*sucC* C-8G
MMO-0492	GATGGAGAGAATTAATGGCTATCTCAATCAAGAC	*map* C-8G
MMO-0493	AATTCTCTCCATCAGCGTCGGTGA	*map* C-8G
MMO-0494	GCCGGAGAGACAAATGAACTTATCCCTACGACG	*ydeE* C-7G
MMO-0495	TTGTCTCTCCGGCAGTGCGTTTCG	*ydeE* C-7G
MMO-0784	TCTATGCACACGCACACGGATAA	*cstA* G-22C
MMO-0785	GCGTGTGCATAGAGATGTTACATTATGCATG	*cstA* G-22C
MMO-0736	GGATGCTGTCAACACATGAACTTACATGAA	*sucC* mutR3
MMO-0737	GTGTTGACAGCATCCTTCAGGTAATCGT	*sucC* mutR3
MMO-0774	TCTATGCATCAGACATATTCTCTGG	*gdhA* G4C
MMO-0775	TCTGATGCATAGATATAAAACCCTTATATATTAA	*gdhA* G4C
MMO-0784	TCTATGCACACGCACACGGATAA	*cstA* G-22C
MMO-0785	GCGTGTGCATAGAGATGTTACATTATGCATG	*cstA* G-22C
MMO-0930	GACAGAGACTGGGAGTAAATAAAGTATGC	*ilvD* C-19G
MMO-0931	CCCAGTCTCTGTCTCGTAAATGGGAC	*ilvD* C-19G
Lambda Red recombination		
JVO-0131	TTCTACCAGCAAATACCTATAGTGGCGGCACTTCCTGAGCCGGAA**GTGTAGGCTGGAGCTGCTTC**	*gcvB* deletion with pKD4
JVO-0132	TCGCGATCGCAAGGTAAAAAAAAGCACCGCAATTAGGCGGTGCTA**GGTCCATATGAATATCCTCCTTAG**	*gcvB* deletion with pKD4
JVO-7614	CGAAATGAAAGCACTGTTCAAAGAACCGAATGACAAGGCACTGAACTAA**GTGTAGGCTGGAGCTGCTTC**	*sroC* deletion with pKD4
JVO-7615	ACATAAATCTACTCCAGAAAAAAGAGGGTAGCAGCGTTAACTGCTACCC**GGTCCATATGAATATCCTCCTTAG**	*sroC* deletion with pKD4
MMO-0206	TGTGAAGGTTGCCGATGCGATGCTGGCGCAGGGTGTGATT**GACTACAAAGACCATGACGG**	*gdhA*::3xFLAG insertion with pSUB13
MMO-0207	TGTGCCCATTTGTAGGCCTGATAAGCGTAGCGCCATCAGGT**CCATATGAATATCCTCCTTAG**	*gdhA*::3xFLAG insertion with pSUB13
dppA-P1-R	GCATCCCCACCTCATAACGTTGACCCGACCGGGCAA**GTGTAGGCTGGAGCTGCTTC**	*dppABCDF* deletion with pKD13
dppF-P4-F	CTGTACGGCATTTTGCTATGCTTGTCGCCACTGTTG**ATTCCGGGGATCCGTCGACC**	*dppABCDF* deletion with pKD13
ydeE-P1-R	AATTAAATCTTTATTGTGTTCGACTTAAATCAACAAAGCGCGGGCTGCCC**GTGTAGGCTGGAGCTGCTTC**	*ydeE* deletion with pKD13
ydeE-P4-F	TACATCTCTAAAACAAAACATAACGAAACGCACTGCCGGACAGACAAATG**CAAACATGAGAATTAATTCC**	*ydeE* deletion with pKD13
***ydeE* cloning**		
pSydeE-5´	ATAGAATTCTTAACAGTTGATTCGTTAGTC	*ydeE* cloning
pSydeE-3´	ATAGGATCCTCAACAAAGCGCGGGCTGCCC	*ydeE* cloning

Table S5. Plasmids used in this study.

Name	Relevant fragment	Comment	Origin / marker	Reference
pTP11	control plasmid	Control plasmid based on pJV300, ColE1 origin replaced by p15A origin	p15A / Amp^R	Sharma et al., 2011
pP_L-*gcvB*	$P_{Llac\text{-}o}$ *gcvB*	*E. coli gcvB* mid-copy expression plasmid, *gcvB* is controlled by the constitutive P_{Llac0} promoter	p15A / Amp^R	this study
pP_L-*gcvB*ΔR1	$P_{L\,lac\text{-}o}$-*gcvB*ΔR1	*E. coli gcvB* deletion of position 66 – 91	p15A / Amp^R	this study
pP_L-*gcvB*ΔR2	$P_{L\,lac\text{-}o}$-*gcvB*ΔR2	*E. coli gcvB* deletion of position 136 – 144	p15A / Amp^R	this study
pP_L-*gcvB*ΔR3	$P_{L\,lac\text{-}o}$-*gcvB*ΔR3	*E. coli gcvB* deletion of position 152 – 169	p15A / Amp^R	this study
pP_L-*gcvB*ΔR12	$P_{L\,lac\text{-}o}$-*gcvB*ΔR12	*E. coli gcvB* deletion of position 66 – 91 and 136 – 169	p15A / Amp^R	this study
pP_L-*gcvB*ΔR13	$P_{L\,lac\text{-}o}$-*gcvB*ΔR13	*E. coli gcvB* deletion of position 66 – 91 and 152 – 169	p15A / Amp^R	this study
pP_L-*gcvB*ΔR123	$P_{L\,lac\text{-}o}$-*gcvB*ΔR123	*E. coli gcvB* deletion of position 66 - 169	p15A / Amp^R	this study
pP_L-*gcvB*ΔR1mutR3	$P_{L\,lac\text{-}o}$-*gcvB*ΔR1mutR3	*E. coli gcvB*ΔR1 mutant in position 154 – 158 (CTGTC->GACAG)	p15A / Amp^R	this study
pP_L-*gcvB*ΔR1G156C	$P_{L\,lac\text{-}o}$-*gcvB*ΔR1G156C	*E. coli gcvB*ΔR1 mutant in position 156 (G->C)	p15A / Amp^R	this study
pP_L-*gcvB*ΔR1C160G	$P_{L\,lac\text{-}o}$-*gcvB*ΔR1C160G	*E. coli gcvB*ΔR1 mutant in position 160 (C->G)	p15A / Amp^R	this study
pP_L-*gcvB*ΔR1C162G	$P_{L\,lac\text{-}o}$-*gcvB*ΔR1C162G	*E. coli gcvB*ΔR1 mutant in position 162 (C->G)	p15A / Amp^R	this study
pXG-10sf	P_{Ltet0}- *lacZ::gfp*	Plasmid for construction of translational sfGFP fusion	pSC101* / Cm^R	Corcoran et al., 2012
pXG-30sf	P_{Ltet0}-FLAG::*glmU-glmS::gfp*	Plasmid for construction of translational sfGFP fusions of dicistronic targets	pSC101* / Cm^R	Corcoran et al., 2012
pXG-10sf-*gdhA*	P_{Ltet0}-*gdhA::gfp*	*E. coli gdhA* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-10sf-*map*	P_{Ltet0}- *map::gfp*	*E. coli map* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-10sf-*cstA*	P_{Ltet0}- *cstA::gfp*	*E. coli cstA* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-10sf-*ydeE*	P_{Ltet0}- *ydeE::gfp*	*E. coli ydeE* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-10sf-*rmf*	P_{Ltet0}- *rmf::gfp*	*E. coli rmf* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-10sf-*kgtP*	P_{Ltet0}- *kgtP::gfp*	*E. coli kgtP* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-10sf-*icd*	P_{Ltet0}- *icd::gfp*	*E. coli icd* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-10sf-*gltP*	P_{Ltet0}- *gltP::gfp*	*E. coli gltP* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-10sf-*aroP*	P_{Ltet0}- *aroP::gfp*	*E. coli aroP* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-10sf-*trpE*	P_{Ltet0}- *trpE::gfp*	*E. coli trpE* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-10sf-*acs*	P_{Ltet0}- *acs::gfp*	*E. coli acs* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-10sf-*asd*	P_{Ltet0}- *asd::gfp*	*E. coli asd* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-10sf-*purU*	P_{Ltet0}- *purU::gfp*	*E. coli purU* translational GFP fusion plasmid	pSC101*/ Cm^R	this study

pXG-10sf-*ivbL*	P_{LtetO}- *ivbL::gfp*	*E. coli ivbL* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-10sf-*ilvB*	P_{LtetO}- *ilvB::gfp*	*E. coli ilvB* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-10sf-*ilvL*	P_{LtetO}- *ilvL::gfp*	*E. coli ilvL* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-10sf-*ilvX*	P_{LtetO}- *ilvX::gfp*	*E. coli ilvX* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-30sf-*ilvED*	P_{LtetO}-FLAG::*ilvE-ilvD::gfp*	*E. coli ilvED* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-30sf-*ilvGM*	P_{LtetO}-FLAG::*ilvG-ilvM::gfp*	*E. coli ilvGM* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-30sf-*sucBC*	P_{LtetO}-FLAG::*sucB-sucC::gfp*	*E. coli sucBC* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-30sf-*hisJQ*	P_{LtetO}-FLAG::*hisJ-hisQ::gfp*	*E. coli hisJQ* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-30sf-*yggX-mltC*	P_{LtetO}-FLAG::*yggX-mltC::gfp*	*E. coli yggX-mltC* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-30sf-*rbsBK*	P_{LtetO}-FLAG::*rbsB-rbsK::gfp*	*E. coli rbsBK* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pXG-30sf-*prmB-aroC*	P_{LtetO}-FLAG::*prmB-aroC::gfp*	*E. coli prmB-aroC* translational GFP fusion plasmid	pSC101*/ Cm^R	this study
pSydeE	P_{lac}-*ydeE*	*E. coli ydeE* expression plasmid	pACYC184/ Cm^R	this study

Table S6. Inserts of GcvB mutant plasmids.
Black letters indicate the *gcvB* wild-type sequence, R1, R2, and R3 seed sequences are highlighted in yellow, green and cyan, respectively. The modified nucleotides are highlighted in magenta.

Plasmid	**Insert from +1 to end of *gcvB* terminator**	**Positions deleted or mutated**
pP_L-*gcvB*	ACTTCCTGAGCCGGAACGAAAAGTTTTATCGGAATGCGTGTTCTGGTGAACTT TTGGCTTACGGTTGTGATGTTGTGTTGTTGTGTTTGCAATTGGTCTGCGATTC AGACCATGGTAGCAAAGCTACCTTTTTTCACTTCCTGTACATTTACCCTGTCT GTCCATAGTGATTAATGTAGCACCGCCTAATTGCGGTGCTTTTTTTT	none
pP_L-*gcvB*ΔR1	ACTTCCTGAGCCGGAACGAAAAGTTTTATCGGAATGCGTGTTCTGGTGAACTT TTGGCTTACGGTATTGGTCTGCGATTCAGACCATGGTAGCAAAGCTACCTTTT TTCACTTCCTGTACATTTACCCTGTCTGTCCATAGTGATTAATGTAGCACCGC CTAATTGCGGTGCTTTTTTTT	66 - 91
pP_L-*gcvB*ΔR2	ACTTCCTGAGCCGGAACGAAAAGTTTTATCGGAATGCGTGTTCTGGTGAACTT TTGGCTTACGGTTGTGATGTTGTGTTGTTGTGTTTGCAATTGGTCTGCGATTC AGACCATGGTAGCAAAGCTACCTTTTTTCCATTTACCCTGTCTGTCCATAGTG ATTAATGTAGCACCGCCTAATTGCGGTGCTTTTTTTT	136 - 144
pP_L-*gcvB*ΔR3	ACTTCCTGAGCCGGAACGAAAAGTTTTATCGGAATGCGTGTTCTGGTGAACTT TTGGCTTACGGTTGTGATGTTGTGTTGTTGTGTTTGCAATTGGTCTGCGATTC AGACCATGGTAGCAAAGCTACCTTTTTTCACTTCCTGTACATTTAATTAATGT AGCACCGCCTAATTGCGGTGCTTTTTTTT	152 - 169
pP_L-*gcvB*ΔR12	ACTTCCTGAGCCGGAACGAAAAGTTTTATCGGAATGCGTGTTCTGGTGAACTT TTGGCTTACGGTATTGGTCTGCGATTCAGACCATGGTAGCAAAGCTACCTTTT TTCCATTTACCCTGTCTGTCCATAGTGATTAATGTAGCACCGCCTAATTGCGG TGCTTTTTTTT	66 – 91 136 - 144
pP_L-*gcvB*ΔR13	ACTTCCTGAGCCGGAACGAAAAGTTTTATCGGAATGCGTGTTCTGGTGAACTT TTGGCTTACGGTATTGGTCTGCGATTCAGACCATGGTAGCAAAGCTACCTTTT TTCACTTCCTGTACATTTAATTAATGTAGCACCGCCTAATTGCGGTGCTTTTT TTT	66 - 89 152 – 169
pP_L-*gcvB*ΔR123	ACTTCCTGAGCCGGAACGAAAAGTTTTATCGGAATGCGTGTTCTGGTGAACTT TTGGCTTACGGTATTAATGTAGCACCGCCTAATTGCGGTGCTTTTTTTT	66 - 169
pP_L-*gcvB*ΔR1mutR3	ACTTCCTGAGCCGGAACGAAAAGTTTTATCGGAATGCGTGTTCTGGTGAACTT TTGGCTTACGGTATTGGTCTGCGATTCAGACCATGGTAGCAAAGCTACCTTTT TTCACTTCCTGTACATTTACCGACAGTGTCCATAGTGATTAATGTAGCACCGC CTAATTGCGGTGCTTTTTTTT	66 - 89 154 – 158 (CTGTC->GACAG)
pP_L-*gcvB*ΔR1G156C	ACTTCCTGAGCCGGAACGAAAAGTTTTATCGGAATGCGTGTTCTGGTGAACTT TTGGCTTACGGTATTGGTCTGCGATTCAGACCATGGTAGCAAAGCTACCTTTT TTCACTTCCTGTACATTTACCCTCTCTGTCCATAGTGATTAATGTAGCACCGC CTAATTGCGGTGCTTTTTTTT	66 - 89 156 (G->C)
pP_L-*gcvB*ΔR1C160G	ACTTCCTGAGCCGGAACGAAAAGTTTTATCGGAATGCGTGTTCTGGTGAACTT TTGGCTTACGGTATTGGTCTGCGATTCAGACCATGGTAGCAAAGCTACCTTTT TTCACTTCCTGTACATTTACCCTGTCTGTCCATAGTGATTAATGTAGCACCGC CTAATTGCGGTGCTTTTTTTT	66 - 89 160 (C->G)
pP_L-*gcvB*ΔR1C162G	ACTTCCTGAGCCGGAACGAAAAGTTTTATCGGAATGCGTGTTCTGGTGAACTT TTGGCTTACGGTATTGGTCTGCGATTCAGACCATGGTAGCAAAGCTACCTTTT TTCACTTCCTGTACATTTACCCTGTCTGTGCATAGTGATTAATGTAGCACCGC CTAATTGCGGTGCTTTTTTTT	66 - 89 162 (C->G)

Table S7. Details of GFP fusion plasmids.

Target gene	Backbone	Oligos used to amplify insert	Insert digested with	Upstream ORF [bp]	Intergenic region [bp]	Downstream ORF [bp]	Insert length [bp]	Translational fusion to N-terminal FLAG [aa]	Translational fusion to C-terminal GFP [aa]
gdhA	pXG-10sf	MMO-0199 x MMO-0201	NsiI/NheI	-	63	33	96	-	11
map	pXG-10sf	MMO-0459 x MMO-0461	NsiI/NheI	-	47	48	95	-	16
cstA	pXG-10sf	MMO-0462 x MMO-0464	NsiI/NheI	-	39	96	135	-	32
ydeE	pXG-10sf	MMO-0465 x MMO-0466	NsiI/NheI	-	69	60	129	-	20
rmf	pXG-10sf	MMO-0469 x MMO-0470	NsiI/NheI	-	132	165	297	-	55
kgtP	pXG-10sf	MMO-0790 x MMO-0791	NsiI/NheI	-	66	120	186	-	40
icd	pXG-10sf	MMO-0792 x MMO-0793	NsiI/NheI	-	162	30	192	-	10
gltP	pXG-10sf	MMO-0794 x MMO-0795	NsiI/NheI	-	103	90	193	-	30
aroP	pXG-10sf	MMO-0805 x MMO-0806	NsiI/NheI	-	99	60	159	-	20
trpE	pXG-10sf	MMO-0817 x MMO-0818	NsiI/NheI	-	36	90	126	-	30
acs	pXG-10sf	MMO-0819 x MMO-0820	NsiI/NheI	-	20	90	110	-	30
asd	pXG-10sf	MMO-0821 x MMO-0822	NsiI/NheI	-	61	60	121	-	20
purU	pXG-10sf	MMO-0925 x MMO-0926	NsiI/NheI	-	127	60	187	-	20
ivbL	pXG-10sf	MMO-0861 x MMO-0862	NsiI/NheI	-	35	30	65	-	10
ilvB	pXG-10sf	MMO-0863 x MMO-0864	NsiI/NheI	-	53	60	113	-	20
ilvL	pXG-10sf	MMO-0865 x MMO-0889	NsiI/NheI	-	104	9	113	-	3
ilvX	pXG-10sf	MMO-0867 x MMO-0868	NsiI/NheI	-	29	48	77	-	16
ilvED	pXG-30sf	MMO-0890 x MMO-0870	NsiI/NheI	45	64	51	160	15	17
ilvGM	pXG-30sf	MMO-0871 x MMO-0872	NsiI/NheI	96	-4	69	161	32	23
sucBC	pXG-30sf	MMO-0327 x MMO-0328	NsiI/NheI	48	274	30	352	16	10
hisJQ	pXG-30sf	MMO-0477 x MMO-0478	NsiI/NheI	30	89	30	149	10	10
yggX-mltC	pXG-30sf	MMO-0798 x MMO-0799	NsiI/NheI	120	64	120	304	40	40
rbsBK	pXG-30sf	MMO-0800 x MMO-0801	NsiI/NheI	60	125	60	245	20	20
prmB-aroC	pXG-30sf	MMO-0803 x MMO-0804	NsiI/NheI	120	34	90	244	40	30

Table S8. Inserts of GFP fusion plasmids.

E. coli gene sequences are indicated in which black letters correspond to 5´UTR parts and red letters to ORF parts for pXG-10sf derivatives. For the intraoperonic fusions in pXG30-sf, upstream ORF fused with FLAG, intergenic region, downstream ORF fused with GFP are indicated in blue, black, and red, respectively. The overlapping region between ORFs is highlighted in magenta. NsiI and NheI sites used for cloning are highlighted in bold in cyan and green, respectively.

GFP fusion	Insert
gdhA::gfp	ATGCATgcaaaagcacatgacataaacaacataagcacaatcgtattaatatataaggttttatatctatggatcagac atattctctggagtcattcctcGCTAGC
map::gfp	ATGCATacttacatatatattgtcggtatcaccgacgctgatggacagaattaatggctatctcaatcaagacccagaa gatatcgaaaaaatgcgcgtcGCTAGC
cstA::gfp	ATGCATaatgtaacatctctatggacacgcacacggataacaactatgaacaaatcagggaaatacctcgtctggacagt gctctctgtaatgggagcatttgctctgggatacattgctttaaatcgtggggaacagatcGCTAGC
ydeE::gfp	ATGCATatttttgccatgctatttctttacatctctaaaacaaaacataacgaaacgcactgccggacagacaaatgaac ttatccctacgacgctctaccagcgcccttcttgcctcgtcgttgttattaaccGCTAGC
rmf::gfp	ATGCATcacaaatatgacagtggcgtgaattttgcgcattgacggcagttatgattcgcggtattgcttaactgtgattg cacatttagtaatcactgttttcttttccaccagaaaccagtatgagggaaacgaggcatgaagagacaaaaacgagatc gcctggaacgggcacatcaacgtggttatcaggccggcatcgccggacgctcaaaagaaatgtgtccctatcagacgctg aatcaaaggtcacaatggctgggaggctggcgagaagccatggcggacagggtagtaatggccGCTAGC
kgtP::gfp	ATGCATctttacaaaaaaaatcaaacaaaagcgaccgacaaaagcatcggattacggcaggagacataatggcatggctga aagtactgtaacggcagacagcaaactgacaagtagtgatactcgtcgccgcatttgggcgattgtgggggcctcttcag gtaatctggtcgagtggttcgatttctatgtcGCTAGC
icd::gfp	ATGCATataacgcgcatctttcatgacggcaaacaatagggtagtattgacaagccaattacaaatcattaacaaaaaat tgctctaaagcatccgtatcgcaggacgcaaacgcatatgcaacgtggtggcagacgagcaaaccagtagcgctcgaagg agaggtgaatggaaagtaaagtagttgttccggcacaaGCTAGC
gltP::gfp	ATGCATaaaaaaatctacaaaccaacgcaacacaattcatgccctggcagtatgtcacgttctcgcgtttctgaacgggg aacggcgctccattgaggaagtcattcatatgaaaaatataaaattcagcctggcctggcagattctgtttgctatggtg ctgggcattctcctgggaagctacctgcactaccatagcGCTAGC
aroP::gfp	ATGCATaacttctttgatgtaaacaaattaatacaacaaacggaattgcaaacttacacacgcatcactgcgtagatcaa aaaaacaaccaccgcacgaggtttcatgatggaaggtcaacagcacggcgagcagctaaagcgcggccttaaaaaccgcc atattGCTAGC
trpE::gfp	ATGCATagcgggctttttttttgaacaaaattagagaataacaatgcaaacacaaaaaccgactctcgaactgctaacctg cgaaggcgcttatcgcgacaatcccaccgcgcttttttcaccagttgtgtgggGCTAGC
acs::gfp	ATGCATcctacaaggagaacaaaagcatgagccaaattcacaaacacaccattcctgccaacatcgcagaccgttgcctg ataaaccctcagcagtacgaggcgatgtatcaacaaGCTAGC
asd::gfp	ATGCATatgtgccaagaggagaccggcacatttatacagcacacatctttgcaggaaaaaaacgcttatgaaaaatgttg gttttatcggctggcgcggtatggtcggctccgttctcatgcaacgcGCTAGC
aspC::gfp	ATGCATgacttcccttctgtaaccataatggaacctcgtcatgtttgagaacattaccgccgctcctgccgacccgattc tgggcctggccgatctgtttcgtgccgatgaacgtcccggcaaaattaacGCTAGC
purU::gfp	ATGCATacacgtccgcagtttggtcgcaacgatccctgcccttgtggttcaggtaaaaaatttaaaaagtgctgcggcca ataatggttgacggtacggtttagcaaacactctcaacaaggttttccagcaatgcattcactccaacgtaaagttctg cgtactatttgtccggaccaaaaaggtctgatcGCTAGC
ivbL::gfp	ATGCATaaatattgttaaacacaaaaccaacaaggtcccca atgactacttccatgctcaacgcaaaacta
ilvB::gfp	ATGCATttttt cgtttaagcacctcccggaaagtcggcccagaagaaaaggactggagcatggcaagttcgggcacaaca tcgacgcgtaagcgctttaccggcgcagaatttatcgttGCTAGC
ilvL::gfp	ATGCATattaaacgcatcataaaaatcggccaaaaaatatcttgtactatttacaaaacctatggtaactctttaggcat tccttcgaacaagatgcaagaaaagacaaaatgacagccGCTAGC
ilvX::gfp	ATGCATaccttaaaaacataaccgaggagcagacaatgaataacagcacaaaattctgtttctcaagattcaggacgggg aacGCTAGC
ilvD::gfp	ATGCATgaaaccgaagataaatggggctggttagatcaagttaatcaataaatacaaaaaatgggacggcacgcaccgtc ccatttacgagacagacactgggagtaaataaagtatgcctaagtaccgttccgccaccaccactcatggtcgtaatatg gcgggtGCTAGC
ilvM::gfp	ATGCATtacctgcttcatgtctcaatcgacgaacttgagaacgtctggccgctggtgccgcctggcgccagtaattcaga aatgttggagaaattatcatgtgcaacatcaggtcaatgtatcggctcgcttcaatccagaaaccttagaacgtgtttt acgcgtgGCTAGC
P$_{Ltet0}$-FLAG::*sucB-sucC::gfp*	ATGCATatcaaagagttgctggaagatccgacgcgtctgctgctggacgtgtagtagtttaagtttcacctgcactgtag accggataaggcattatcgccttctccggcaattgaagcctgatgcgacgctgacgcgtcttatcaggcctacgggacca ccaatgtaggtcggataaggcgcaagcgccgcatccgacaagcgatgcctgatgtgacgtttaacgtgtcttatcaggcc tacgggtgaccgacaatgcccggaagcgatacgaaatattcggtctacggtttaaaagataacgattactgaaggatgga cagaacacatgaacttacatgaatatcaggcaaaacaaGCTAGC
P$_{Ltet0}$-FLAG::*hisJ-hisQ::gfp*	ATGCATtacttcgattttgatgtttatggtggctaatcgccatcggtgaaagaaagcccgctcccttcgggtaaccggag ggagacaagaggcggtaattcaccacacacgacaggacaggcagcatgttgtatgggttttcaggtgttattttaGCTAG C
P$_{Ltet0}$-FLAG::*yggX-mltC::gfp*	ATGCATaagaaactcaacatgatgaatgccgagcaccgcaagctgcttgagcaggagatggtcaacttcctgttcgaggg taaagaggtgcatatcgagggctatacgccggaagataaaaaataaaaacagtgccggagcacgcctccggcaacttgca taaaaacaaacacaacacgcacccggaatgatgaaaaaatatctcgcgctggctttgattgcgccgttgctcatctcctg ttcgacgaccaaaaaaggcgatacctataacgaagcctgggtcaaagatacccaacggttttgatattctgGCTAGC
P$_{Ltet0}$-FLAG::*rbsB-rbsK::gfp*	ATGCATaaaggcgagaaagttcaggctaagtatccggttgatctgaaactggttgttaagcagtagttttaatcaggttg tatgacctgatggtgacataaatacgtcatcgacagatgaacgtgtaatataaagaaaagcagggcacgcgccaccctaa cacggtggcgcattttatggacatcccgaatatgcaaaacgcaggcagcctcgttgttcttggcagcattaatgctgacc acattcttaatGCTAGC
P$_{Ltet0}$-FLAG::prmB-*aroC::gfp*	ATGCATcaatatccggatgttccgttcacctggctggagtttgataacggcggcgatggtgtgtttatgctcaccaaaga gcagcttattgccgcacgagaacatttcgcgatttataaagattaagtaaacacgcaaacacaacaataacggagccgtg atggctggaaacacaattggacaactctttcgcgtaaccaccttcggcgaatcgcacgggctggcgctcggctgcatcgt cgatggtgttGCTAGC