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*

Masatoshi Miyakoshi, Haruna Okayama, Maxence Lejars, Takeshi Kanda, Yuki Tanaka, Kaori Itaya, Miki Okuno, Takehiko Itoh, Noritaka Iwai, Masaaki Wachi

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Figure S1. Growth inhibition by dipeptides. (A) Growth on M9 plates was compared among the wild-type JM101 strain, Δpeps strain, ΔpepsΔgcvB and ΔpepsΔhfq. M9 plates were supplemented with 0.2 mM dipeptides as indicated. (B) Growth curve of JM101, Δpeps, and ΔpepsΔgcvB 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.
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   | aspartase-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 |
| 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   | aspartase 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  |
| tcyf (flIV) | 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   |
| yggR   | putative peptidase lipoprotein                                              | 13.3  |
| ygfF   | putative NAD(P)-dependent oxidoreductase                                    | 51.0  |
| yghI   | putative S-transferase                                                      | 47.9  |
| yifK   | putative APC family amino acid transporter                                  | 35.2  |
| ysgA   | putative carboxymethylenebutenolidasan                                     | 103.0 |
Table S2. Comparative genome analysis of Δpeps 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 Δpeps 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 | Δpeps | 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 Δpeps 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 | -  | -     | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | gcvcB       |
| 2971188  | T>A | -  | -     | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |            |
| 334738   | 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 ΔlacZ4787 hsdR514 Δ(araBAD)Δ(rhaBAD)567 Δ(rhaBAD)568 rph-1                     | NBPR strain                     |
| ΔgcvB           | BW25113 ΔgcvB:: kan                                                                       | This study                      |
| ΔsroC           | BW25113 ΔsroC::FRT                                                                        | This study                      |
| ΔgcvBΔsroC      | BW25113 ΔgcvB:: kan ΔsroC::FRT                                                            | This study                      |
| gdhA::3xFLAG    | BW25113 gdhA::3xFLAG kan                                                                  | This study                      |
| ΔgcvB gdhA::3xFLAG | BW25113 ΔgcvB:: FRT gdhA::3xFLAG kan                                                      | This study                      |
| JM101 F-        | supE, thi-1, Δ(lac-proAB), F- (pepD)                                                      | Wachi laboratory stock          |
| Δpeps           | JM101 F- (pepD), pepN, pepB, pepA                                                         | Hayashi et al. 2010             |
| ΔpepsΔdpp       | Δpeps ΔdppABCDF:: kan                                                                     | This study                      |
| ΔpepsΔgcvB      | Δpeps gcvB::kan                                                                           | This study                      |
| ΔpepsΔhfq       | Δpeps hfq::kan                                                                            | This study                      |
| ΔpepsΔydeE      | Δpeps ΔydeE:: kan                                                                         | This study                      |
| Name          | Sequence (5' - 3' direction)                                                                 | Used for                                    |
|--------------|-----------------------------------------------------------------------------------------------|---------------------------------------------|
| **Northern blot** |                                                                                               |                                             |
| JVO-0322     | CTACGGCGTTCTACATCTCTGAGTTC                                                                    | Probe for SS rRNA                           |
| JVO-0749     | TCTGTTTCGGCGCTAGGA                                                                           | Probe for GevB 5' region                    |
| **GevB cloning and mutagenesis** |                                                                                               |                                             |
| JVO-0237     | ACTCTCATACACCGAAC                                                                            | GevB cloning                               |
| MMO-0086     | GCTTTTCTCACATAACGACTGCATCTGATGTTT                                                         | GevB cloning                               |
| MMO-0184     | ATGCTTCTGGCTACAGA                                                                           | GevB R1 deletion                            |
| MMO-0185     | ACCGTAGCAGAAGACAGTCC                                                                       | GevB R1 deletion                            |
| MMO-0196     | ACATTATACCTGTCTGCC                                                                         | GevB R2 deletion                            |
| MMO-0197     | GAAAAAGTTAGCTTGGTCT                                                                       | GevB R3 deletion                            |
| MMO-0768     | ATATAGTACAGCGGCTAA                                                                         | GevB R3 deletion                            |
| MMO-0769     | TAAATGACAGAGAGTGAAA                                                                         | GevB R3 deletion                            |
| JVO-9214     | CCTGCTTCTCACATAGTTAAGTTGAGG                                                                | GevB G160C mutation                         |
| JVO-9215     | CTATGGACAGACGAGTTAAAGTGGTACGG                                                             | GevB G160C mutation                         |
| MMO-0342     | TACCTCTCTGCTATTTGCGGG                                                                       | GevB G156C mutation                         |
| MMO-0343     | GACAGAGGCGATGAAGCTAGGGA                                                                    | GevB G156C mutation                         |
| MMO-0391     | TACCTCAGATCTAGTTAAGGGA                                                                     | GevB mutR3 mutation                         |
| MMO-0392     | GACAGAGGCGATGAAGCTAGGGA                                                                    | GevB mutR3 mutation                         |
| MMO-0776     | GTCTGCTTCTACATAGTTAAGGGA                                                                  | GevB C162G mutation                         |
| MMO-0777     | ATATGACAGAGAGTGGTCTAGG                                                                      | GevB C162G mutation                         |
| **GevB target cloning** |                                                                                               |                                             |
| MMO-0199     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | gdhA GFP fusion cloning                     |
| MMO-0201     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | gdhA GFP fusion cloning                     |
| MMO-0327     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | sucB-sucC GFP fusion cloning               |
| MMO-0328     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | sucB-sucC GFP fusion cloning               |
| MMO-0459     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | map GFP fusion cloning                       |
| MMO-0461     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | map GFP fusion cloning                       |
| MMO-0462     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | catA GFP fusion cloning                      |
| MMO-0464     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | catA GFP fusion cloning                      |
| MMO-0465     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | ydeE GFP fusion cloning                     |
| MMO-0466     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | ydeE GFP fusion cloning                     |
| MMO-0469     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | rrf GFP fusion cloning                       |
| MMO-0470     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | rrf GFP fusion cloning                       |
| MMO-0477     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | hisf-hisQ GFP fusion cloning               |
| MMO-0478     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | hisf-hisQ GFP fusion cloning               |
| MMO-0790     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | kgtP GFP fusion cloning                     |
| MMO-0791     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | kgtP GFP fusion cloning                     |
| MMO-0792     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | ildC GFP fusion cloning                     |
| MMO-0793     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | ildC GFP fusion cloning                     |
| MMO-0794     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | gphB GFP fusion cloning                     |
| MMO-0795     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | gphB GFP fusion cloning                     |
| MMO-0798     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | yggX-mtxC GFP fusion cloning               |
| MMO-0799     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | yggX-mtxC GFP fusion cloning               |
| MMO-0800     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | rbsB-rbsK GFP fusion cloning               |
| MMO-0801     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | rbsB-rbsK GFP fusion cloning               |
| MMO-0803     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | prmb-aroC GFP fusion cloning               |
| MMO-0804     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | prmb-aroC GFP fusion cloning               |
| MMO-0805     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | aroF GFP fusion cloning                     |
| MMO-0806     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | aroF GFP fusion cloning                     |
| MMO-0817     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | trpE GFP fusion cloning                     |
| MMO-0818     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | trpE GFP fusion cloning                     |
| MMO-0819     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | acaC GFP fusion cloning                     |
| MMO-0820     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | acaC GFP fusion cloning                     |
| MMO-0821     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | acaC GFP fusion cloning                     |
| MMO-0822     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | acaC GFP fusion cloning                     |
| MMO-0925     | GCTTTTCTACATCGCAAAACACACATGACATTA                                                          | purUGFP fusion cloning                     |
| MM0-0926 | GATTCCTGCGCGATACAGCCCTTTTTTGCCCGG | puriGFP fusion cloning |
| MM0-0861 | GGTTAAAATGATCTTTATGTTAAAAACAAAACACAAG | ivblGFP fusion cloning |
| MM0-0862 | GTTTGGTCTAGTTTTCATTGCGGACATGG | ivblGFP fusion cloning |
| MM0-0863 | GTTTGTATGCATTTTCTGTTTTAGGACCTTCC | ivblGFP fusion cloning |
| MM0-0864 | GTTTTGGTCTAGCTATTTCCATTGCCAATCTCGCCCGG | ivblGFP fusion cloning |
| MM0-0865 | GTTTTGGTCTAGCTATTTCCATTGCCAATCTCGCCCGG | ivblGFP fusion cloning |
| MM0-0866 | GTTTTGGTCTAGCTATTTCCATTGCCAATCTCGCCCGG | ivblGFP fusion cloning |
| MM0-0867 | GTTTTGGTCTAGCTATTTCCATTGCCAATCTCGCCCGG | ivblGFP fusion cloning |
| MM0-0868 | GTTTTGGTCTAGCTATTTCCATTGCCAATCTCGCCCGG | ivblGFP fusion cloning |
| MM0-0869 | GTTTTGGTCTAGCTATTTCCATTGCCAATCTCGCCCGG | ivblGFP fusion cloning |
| MM0-0871 | GTTTTGGTCTAGCTATTTCCATTGCCAATCTCGCCCGG | ivblGFP fusion cloning |
| MM0-0872 | GTTTTGGTCTAGCTATTTCCATTGCCAATCTCGCCCGG | ivblGFP fusion cloning |

**GeVβ target mutagenesis**

| MM0-0355 | GATCCAAAACACACACTGAATTACATGA | sucCC-8G |
| MM0-0454 | TGCTGGTATACCGTGATACGAGATACGA | sucCC-8G |
| MM0-0492 | GATGACGATAGATTGACTGACGTACTCTAATACGAC | map C-8G |
| MM0-0493 | AGATTCAATCATCTACGACGTACTCTAATACGAC | map C-8G |
| MM0-0494 | GATGACGATAGATTGACTGACGTACTCTAATACGAC | ydeE-7G |
| MM0-0495 | TGCTGGTATACCGTGATACGAGATACGA | ydeE-7G |
| MM0-0775 | TGCTGGTATACCGTGATACGAGATACGA | ydeE-7G |
| MM0-0784 | TGCTGGTATACCGTGATACGAGATACGA | ydeE-7G |
| MM0-0785 | TGCTGGTATACCGTGATACGAGATACGA | ydeE-7G |
| MM0-0736 | GATGACGATAGATTGACTGACGTACTCTAATACGAC | sucCmutR3 |
| MM0-0737 | GATGACGATAGATTGACTGACGTACTCTAATACGAC | sucCmutR3 |
| MM0-0774 | GATGACGATAGATTGACTGACGTACTCTAATACGAC | gdhA 4AC |
| MM0-0775 | GATGACGATAGATTGACTGACGTACTCTAATACGAC | gdhA 4AC |
| MM0-0784 | GATGACGATAGATTGACTGACGTACTCTAATACGAC | gdhA 4AC |
| MM0-0785 | GATGACGATAGATTGACTGACGTACTCTAATACGAC | gdhA 4AC |
| MM0-0930 | GATGACGATAGATTGACTGACGTACTCTAATACGAC | gdhA 4AC |
| MM0-0931 | GATGACGATAGATTGACTGACGTACTCTAATACGAC | gdhA 4AC |

**Lambda Red recombination**

| JVO-0131 | TTCTAGACAACTACTACATATTAGTGGGCGGATAGGTTAGCGGGGCACTTTCTCGGACGCGGGAATTTGAGGCCTGAGCTGTC | gcvB deletion with pKD4 |
| JVO-0132 | TTCTAGACAACTACTACATATTAGTGGGCGGATAGGTTAGCGGGGCACTTTCTCGGACGCGGGAATTTGAGGCCTGAGCTGTC | gcvB deletion with pKD4 |
| JVO-7614 | TTCTAGACAACTACTACATATTAGTGGGCGGATAGGTTAGCGGGGCACTTTCTCGGACGCGGGAATTTGAGGCCTGAGCTGTC | sroC deletion with pKD4 |
| JVO-7615 | TTCTAGACAACTACTACATATTAGTGGGCGGATAGGTTAGCGGGGCACTTTCTCGGACGCGGGAATTTGAGGCCTGAGCTGTC | sroC deletion with pKD4 |
| MM0-0206 | TTCTAGACAACTACTACATATTAGTGGGCGGATAGGTTAGCGGGGCACTTTCTCGGACGCGGGAATTTGAGGCCTGAGCTGTC | gdhB:3xFLAG insertion with pSUB13 |
| MM0-0207 | TTCTAGACAACTACTACATATTAGTGGGCGGATAGGTTAGCGGGGCACTTTCTCGGACGCGGGAATTTGAGGCCTGAGCTGTC | gdhB:3xFLAG insertion with pSUB13 |
| dppA-P1-R | GCTGACGGGTACGATAGGTTAGCGGGGCACTTTCTCGGACGCGGGAATTTGAGGCCTGAGCTGTC | dppABCDF deletion with pKD13 |
| dppF-P4-F | GCTGACGGGTACGATAGGTTAGCGGGGCACTTTCTCGGACGCGGGAATTTGAGGCCTGAGCTGTC | dppABCDF deletion with pKD13 |
| ydeE-P1-R | GCTGACGGGTACGATAGGTTAGCGGGGCACTTTCTCGGACGCGGGAATTTGAGGCCTGAGCTGTC | ydeE deletion with pKD13 |
| ydeE-P4-F | GCTGACGGGTACGATAGGTTAGCGGGGCACTTTCTCGGACGCGGGAATTTGAGGCCTGAGCTGTC | ydeE deletion with pKD13 |

**ydeE cloning**

| psydeE-5 | ATACGACAACTACTACATATTAGTGGGCGGATAGGTTAGCGGGGCACTTTCTCGGACGCGGGAATTTGAGGCCTGAGCTGTC | ydeE cloning |
| psydeE-3' | ATACGACAACTACTACATATTAGTGGGCGGATAGGTTAGCGGGGCACTTTCTCGGACGCGGGAATTTGAGGCCTGAGCTGTC | 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<sup>a</sup> | Sharma et al, 2011 |
| pPlc-gcvB        | P<sub>lac</sub>-gcvB | E. coli gcvB mid-copy expression plasmid, gcvB is controlled by the constitutive P<sub>lac</sub> promoter | p15A / Amp<sup>a</sup> | this study         |
| pPlc-gcvBΔR1     | P<sub>lac</sub>-gcvBΔR1 | E. coli gcvB deletion of position 66 – 91                              | p15A / Amp<sup>a</sup> | this study         |
| pPlc-gcvBΔR2     | P<sub>lac</sub>-gcvBΔR2 | E. coli gcvB deletion of position 136 – 144                            | p15A / Amp<sup>a</sup> | this study         |
| pPlc-gcvBΔR3     | P<sub>lac</sub>-gcvBΔR3 | E. coli gcvB deletion of position 152 – 169                            | p15A / Amp<sup>a</sup> | this study         |
| pPlc-gcvBΔR12    | P<sub>lac</sub>-gcvBΔR12 | E. coli gcvB deletion of position 66 – 91 and 136 – 169              | p15A / Amp<sup>a</sup> | this study         |
| pPlc-gcvBΔR13    | P<sub>lac</sub>-gcvBΔR13 | E. coli gcvB deletion of position 66 – 91 and 152 – 169               | p15A / Amp<sup>a</sup> | this study         |
| pPlc-gcvBΔR123   | P<sub>lac</sub>-gcvBΔR123 | E. coli gcvB deletion of position 66 - 169                           | p15A / Amp<sup>a</sup> | this study         |
| pPlc-gcvBΔR1mutR | P<sub>lac</sub>-gcvBΔR1mutR | E. coli gcvBΔR1 mutant in position 154 – 158 (CTGTC->GACAG)            | p15A / Amp<sup>a</sup> | this study         |
| pPlc-gcvBΔR1G156C| P<sub>lac</sub>-gcvBΔR1G156C | E. coli gcvBΔR1 mutant in position 156 (G->C)              | p15A / Amp<sup>a</sup> | this study         |
| pPlc-gcvBΔR1C160G| P<sub>lac</sub>-gcvBΔR1C160G | E. coli gcvBΔR1 mutant in position 160 (G->C)              | p15A / Amp<sup>a</sup> | this study         |
| pPlc-gcvBΔR1C162G| P<sub>lac</sub>-gcvBΔR1C162G | E. coli gcvBΔR1 mutant in position 162 (G->C)              | p15A / Amp<sup>a</sup> | this study         |
| pXG-10sf         | P<sub>lac</sub>-lacZ::gfp | Plasmid for construction of translational sfGFP fusion                  | pSC101* / Cm<sup>a</sup> | Corcoran et al, 2012 |
| pXG-30sf         | P<sub>lac</sub>-FLAG::glmU::gfp | Plasmid for construction of translational sfGFP fusions of dicistronic targets | pSC101* / Cm<sup>a</sup> | Corcoran et al, 2012 |
| pXG-10sf-gdhA     | P<sub>lac</sub>-gdhA::gfp | E. coli gdhA translational GFP fusion plasmid                          | pSC101* / Cm<sup>a</sup> | this study         |
| pXG-10sf-map      | P<sub>lac</sub>-map::gfp | E. coli map translational GFP fusion plasmid                           | pSC101* / Cm<sup>a</sup> | this study         |
| pXG-10sf-cstA     | P<sub>lac</sub>-cstA::gfp | E. coli cstA translational GFP fusion plasmid                          | pSC101* / Cm<sup>a</sup> | this study         |
| pXG-10sf-ydeE     | P<sub>lac</sub>-ydeE::gfp | E. coli ydeE translational GFP fusion plasmid                          | pSC101* / Cm<sup>a</sup> | this study         |
| pXG-10sf-rmf      | P<sub>lac</sub>-rmf::gfp | E. coli rmf translational GFP fusion plasmid                           | pSC101* / Cm<sup>a</sup> | this study         |
| pXG-10sf-kgtP     | P<sub>lac</sub>-kgtP::gfp | E. coli kgtP translational GFP fusion plasmid                         | pSC101* / Cm<sup>a</sup> | this study         |
| pXG-10sf-icd      | P<sub>lac</sub>-icd::gfp | E. coli icd translational GFP fusion plasmid                          | pSC101* / Cm<sup>a</sup> | this study         |
| pXG-10sf-glpP     | P<sub>lac</sub>-glpP::gfp | E. coli glpP translational GFP fusion plasmid                         | pSC101* / Cm<sup>a</sup> | this study         |
| pXG-10sf-aroP     | P<sub>lac</sub>-aroP::gfp | E. coli aroP translational GFP fusion plasmid                         | pSC101* / Cm<sup>a</sup> | this study         |
| pXG-10sf-trpE     | P<sub>lac</sub>-trpE::gfp | E. coli trpE translational GFP fusion plasmid                         | pSC101* / Cm<sup>a</sup> | this study         |
| pXG-10sf-acs      | P<sub>lac</sub>-acs::gfp | E. coli acs translational GFP fusion plasmid                         | pSC101* / Cm<sup>a</sup> | this study         |
| pXG-10sf-asd      | P<sub>lac</sub>-asd::gfp | E. coli asd translational GFP fusion plasmid                         | pSC101* / Cm<sup>a</sup> | this study         |
| pXG-10sf-purU     | P<sub>lac</sub>-purU::gfp | E. coli purU translational GFP fusion plasmid                         | pSC101* / Cm<sup>a</sup> | this study         |
| constructs          | promoters | transgene             | hosts                        | plasmids       | references     |
|---------------------|-----------|-----------------------|------------------------------|----------------|---------------|
| pXG-10sf-ilvB       | LTtetO    | ilvB::gfp             | E. coli ilvB translational GFP fusion plasmid | pSC101*/Cm® | this study    |
| pXG-10sf-ilvL       | LTtetO    | ilvL::gfp             | E. coli ilvL translational GFP fusion plasmid | pSC101*/Cm® | this study    |
| pXG-10sf-ilvX       | LTtetO    | ilvX::gfp             | E. coli ilvX translational GFP fusion plasmid | pSC101*/Cm® | this study    |
| pXG-30sf-ilvED      | LTtetO    | FLAG::ilvE-ilvD::gfp | E. coli ilvED translational GFP fusion plasmid | pSC101*/Cm® | this study    |
| pXG-30sf-ilvGM      | LTtetO    | FLAG::ilvG-ilvM::gfp | E. coli ilvGM translational GFP fusion plasmid | pSC101*/Cm® | this study    |
| pXG-30sf-sucBC      | LTtetO    | FLAG::sucB-sucC::gfp | E. coli sucBC translational GFP fusion plasmid | pSC101*/Cm® | this study    |
| pXG-30sf-hisJQ      | LTtetO    | FLAG::hisJ-hisQ::gfp | E. coli hisJQ translational GFP fusion plasmid | pSC101*/Cm® | this study    |
| pXG-30sf-yggX-mltC   | LTtetO    | FLAG::yggX-mltC::gfp | E. coli yggX-mltC translational GFP fusion plasmid | pSC101*/Cm® | this study    |
| pXG-30sf-rbsBK      | LTtetO    | FLAG::rbsB-rbsK::gfp | E. coli rbsBK translational GFP fusion plasmid | pSC101*/Cm® | this study    |
| pXG-30sf-prmB-aroC   | LTtetO    | FLAG::prmB-aroC::gfp | E. coli prmB-aroC translational GFP fusion plasmid | pSC101*/Cm® | this study    |
| pSydeE              | lacP       | ydeE                 | E. coli ydeE expression plasmid | pACYC184/Cm® | 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*:gcvB      | ACTTCCTGAGCGGAGAAGAAAGTTTTTATCGGAAATGCCGCTTCTGGTAGAACTTTTGCGCTCCCAGTAATTTATTGGAATTCGGGATATGTTCTGCTGACCGACCGCTAAATTTCGGGGCTCTTTTTTTTT | none                         |
| pP*:gcvcBΔR1  | ACTTCCTGAGCGGAGAAGAAAGTTTTTATCGGAAATGCCGCTTCTGGTAGAACTTTTGCGCTCCCAGTAATTTATTGGAATTCGGGATATGTTCTGCTGACCGACCGCTAAATTTCGGGGCTCTTTTTTTTT | 66 - 91                      |
| pP*:gcvcBΔR2  | ACTTCCTGAGCGGAGAAGAAAGTTTTTATCGGAAATGCCGCTTCTGGTAGAACTTTTGCGCTCCCAGTAATTTATTGGAATTCGGGATATGTTCTGCTGACCGACCGCTAAATTTCGGGGCTCTTTTTTTTT | 136 - 144                    |
| pP*:gcvcBΔR3  | ACTTCCTGAGCGGAGAAGAAAGTTTTTATCGGAAATGCCGCTTCTGGTAGAACTTTTGCGCTCCCAGTAATTTATTGGAATTCGGGATATGTTCTGCTGACCGACCGCTAAATTTCGGGGCTCTTTTTTTTT | 152 - 169                    |
| pP*:gcvcBΔR12 | ACTTCCTGAGCGGAGAAGAAAGTTTTTATCGGAAATGCCGCTTCTGGTAGAACTTTTGCGCTCCCAGTAATTTATTGGAATTCGGGATATGTTCTGCTGACCGACCGCTAAATTTCGGGGCTCTTTTTTTTT | 66 - 91                      |
| pP*:gcvcBΔR13 | ACTTCCTGAGCGGAGAAGAAAGTTTTTATCGGAAATGCCGCTTCTGGTAGAACTTTTGCGCTCCCAGTAATTTATTGGAATTCGGGATATGTTCTGCTGACCGACCGCTAAATTTCGGGGCTCTTTTTTTTT | 66 - 91 152 - 169             |
| pP*:gcvcBΔR123| ACTTCCTGAGCGGAGAAGAAAGTTTTTATCGGAAATGCCGCTTCTGGTAGAACTTTTGCGCTCCCAGTAATTTATTGGAATTCGGGATATGTTCTGCTGACCGACCGCTAAATTTCGGGGCTCTTTTTTTTT | 66 - 69                      |
| pP*:gcvcBΔR1 mutR3 | ACTTCCTGAGCGGAGAAGAAAGTTTTTATCGGAAATGCCGCTTCTGGTAGAACTTTTGCGCTCCCAGTAATTTATTGGAATTCGGGATATGTTCTGCTGACCGACCGCTAAATTTCGGGGCTCTTTTTTTTT | 66 - 91 154 - 158 (CTGTC→GACAG) |
| pP*:gcvcBΔR1G156C | ACTTCCTGAGCGGAGAAGAAAGTTTTTATCGGAAATGCCGCTTCTGGTAGAACTTTTGCGCTCCCAGTAATTTATTGGAATTCGGGATATGTTCTGCTGACCGACCGCTAAATTTCGGGGCTCTTTTTTTTT | 66 - 69 156 (G→C)             |
| pP*:gcvcBΔR1C160G | ACTTCCTGAGCGGAGAAGAAAGTTTTTATCGGAAATGCCGCTTCTGGTAGAACTTTTGCGCTCCCAGTAATTTATTGGAATTCGGGATATGTTCTGCTGACCGACCGCTAAATTTCGGGGCTCTTTTTTTTT | 66 - 99 160 (L→G)             |
| pP*:gcvcBΔR1C162G | ACTTCCTGAGCGGAGAAGAAAGTTTTTATCGGAAATGCCGCTTCTGGTAGAACTTTTGCGCTCCCAGTAATTTATTGGAATTCGGGATATGTTCTGCTGACCGACCGCTAAATTTCGGGGCTCTTTTTTTTT | 66 - 99 162 (L→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                                        |
| ctaA        | 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                                        |
| rnf         | pXG-10sf | MMO-0469 x MMO-0470           | NsiI/NheI           | -                 | 132                   | 165                | 297               | -                                        | 55                                        |
| hqfP        | 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                                        |
| gfpP        | pXG-10sf | MMO-0794 x MMO-0795           | NsiI/NheI           | -                 | 103                   | 90                 | 193               | -                                        | 30                                        |
| aroP        | pXG-10sf | MMO-0805 x MMO-0818           | NsiI/NheI           | -                 | 99                    | 60                 | 159               | -                                        | 20                                        |
| trpE        | pXG-10sf | MMO-0817 x MMO-0818           | NsiI/NheI           | -                 | 36                    | 90                 | 126               | -                                        | 30                                        |
| aca         | 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                                        |
| ibdL        | pXG-10sf | MMO-0861 x MMO-0862           | NsiI/NheI           | -                 | 35                    | 30                 | 65                | -                                        | 10                                        |
| ivbB        | pXG-10sf | MMO-0863 x MMO-0864           | NsiI/NheI           | -                 | 53                    | 60                 | 113               | -                                        | 20                                        |
| ilvL        | pXG-10sf | MMO-0865 x MMO-0869           | 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-0897           | 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                                        |
| hisIQ       | pXG-30sf | MMO-0477 x MMO-0478           | NsiI/NheI           | 30                | 89                    | 30                 | 149               | 10                                       | 10                                        |
| yggX         | pXG-30sf | MMO-0798 x MMO-0799           | NsiI/NheI           | 120               | 64                    | 120                | 304               | 40                                       | 40                                        |
| ribS        | pXG-30sf | MMO-0800 x MMO-0801           | NsiI/NheI           | 60                | 125                   | 60                 | 245               | 20                                       | 20                                        |
| prnB-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  | ATGACAT gcacacaagcatgacataaaacacacacacagattatataagggattatatctatagatcagacatatctctggagatcctgctgATGCAT |
| map::gfp   | ATGACAT actaaacatatatcctggatgcagacgtcattgaccagaattaattggtctctcatctcaagacccgaaaatagcataaaacagctgctgATGCAT |
| ctsA::gfp  | ATGACAT aatgtaatactcatatagcacgcacaggtataacgataacacaggaattaacactctctgaccactgtctgctgATGCAT |
| ydeE::gfp  | ATGACAT atttttgcaatgtatttctcaatctctccaacaaaaactacgaaacaggacacacactggccagagagatgtggaggatcctgctgATGCAT |
| rmf::gfp   | ATGACAT caacaaatagttgctggtaatcctttgagcattactactagttactacggtttacagtctgacctgtgctgctgATGCAT |
| kgtP::gfp  | ATGACAT taaaactgcatatattcagctggactcatctcagagatagtggctgtatttcagctgctgATGCAT |
| icd::gfp   | ATGACAT taaaactgcatatattcagctggactcatctcagagatagtggctgtatttcagctgctgATGCAT |
| gfp::gfp   | ATGACAT taaaactgcatatattcagctggactcatctcagagatagtggctgtatttcagctgctgATGCAT |
| gdi::gfp   | ATGACAT taaaactgcatatattcagctggactcatctcagagatagtggctgtatttcagctgctgATGCAT |
| aspC::gfp  | ATGACAT gactctctctgttaatgaaagctgcaatattataaggctgggtcaaatgatcatggatcagagacagcagctgctgATGCAT |
| trpE::gfp  | ATGACAT gactctctctgttaatgaaagctgcaatattataaggctgggtcaaatgatcatggatcagagacagcagctgctgATGCAT |
| acc::gfp   | ATGACAT cttaaaccgagacaaaggatgatgcacaaaaacacatctctccagcagacggctgctgATGCAT |
| aspC::gfp  | ATGACAT gactctctctgttaatgaaagctgcaatattataaggctgggtcaaatgatcatggatcagagacagcagctgctgATGCAT |
| purU::gfp  | ATGACAT atgcccacctggctttgattgcgccgttgctcatctcctggaatagagccatgcagcccaccccaataatctggactggtgctgATGCAT |
| nhb::gfp   | ATGACAT atttttgtaattttctgtagtaaatctgtaataatctgaaattctctattcattgtggctgctgATGCAT |
| nhn::gfp   | ATGACAT atgggttatattctcttctcctattataattcgtttctctctttcttatgctgctgATGCAT |
| hcl::gfp   | ATGACAT atgggttatattctcttctcctattataattcgtttctctctttcttatgctgctgATGCAT |
| nhm::gfp   | ATGACAT atgggttatattctcttctcctattataattcgtttctctctttcttatgctgctgATGCAT |
| Psu::FLAG::sucB::sucC::gfp | ATGACAT atgggttatattctcttctcctattataattcgtttctctctttcttatgctgctgATGCAT |
| Psu::FLAG::sucB::sucC::gfp | ATGACAT atgggttatattctcttctcctattataattcgtttctctctttcttatgctgctgATGCAT |
| Psu::FLAG::sucB::sucC::gfp | ATGACAT atgggttatattctcttctcctattataattcgtttctctctttcttatgctgctgATGCAT |
| Psu::FLAG::sucB::sucC::gfp | ATGACAT atgggttatattctcttctcctattataattcgtttctctctttcttatgctgctgATGCAT |