

1 Translational profiling of stress-induced small proteins uncovers an 2 unexpected connection among distinct signaling systems

3
4 Sangeevan Vellappan^{1,2,3}, Junhong Sun¹, John Favate^{2,3}, Pranavi Jagadeesan¹, Debbie
5 Cerda^{1,2}, Premal Shah^{2,3}, Srujana S. Yadavalli^{1,2*}

6 ¹Waksman Institute of Microbiology, Rutgers University, Piscataway, NJ USA

7 ²Department of Genetics, School of Arts and Sciences, Rutgers University, Piscataway, NJ USA

8 ³Human Genetics Institute of New Jersey, Rutgers University, Piscataway, New Jersey, USA

9
10
11 *Corresponding author

12 Waksman Institute of Microbiology

13 Rutgers University

14 190 Frelinghuysen Rd

15 Piscataway NJ 08854

16 Phone: 848-445-0251

17 Email: sam.yadavalli@rutgers.edu

18 19 20 Highlights

- 21 • Ribo-RET identifies 17 small proteins induced under low Mg²⁺ stress in *E. coli*
- 22 • Many of these proteins are transcriptionally activated by PhoQP signaling system
- 23 • Half of the stress-induced small proteins localize to the membrane
- 24 • Deletion or overexpression of specific small proteins affects growth under stress
- 25 • Small protein Yoal connects PhoR-PhoB and EnvZ-OmpR signaling networks

26 27 28 Summary

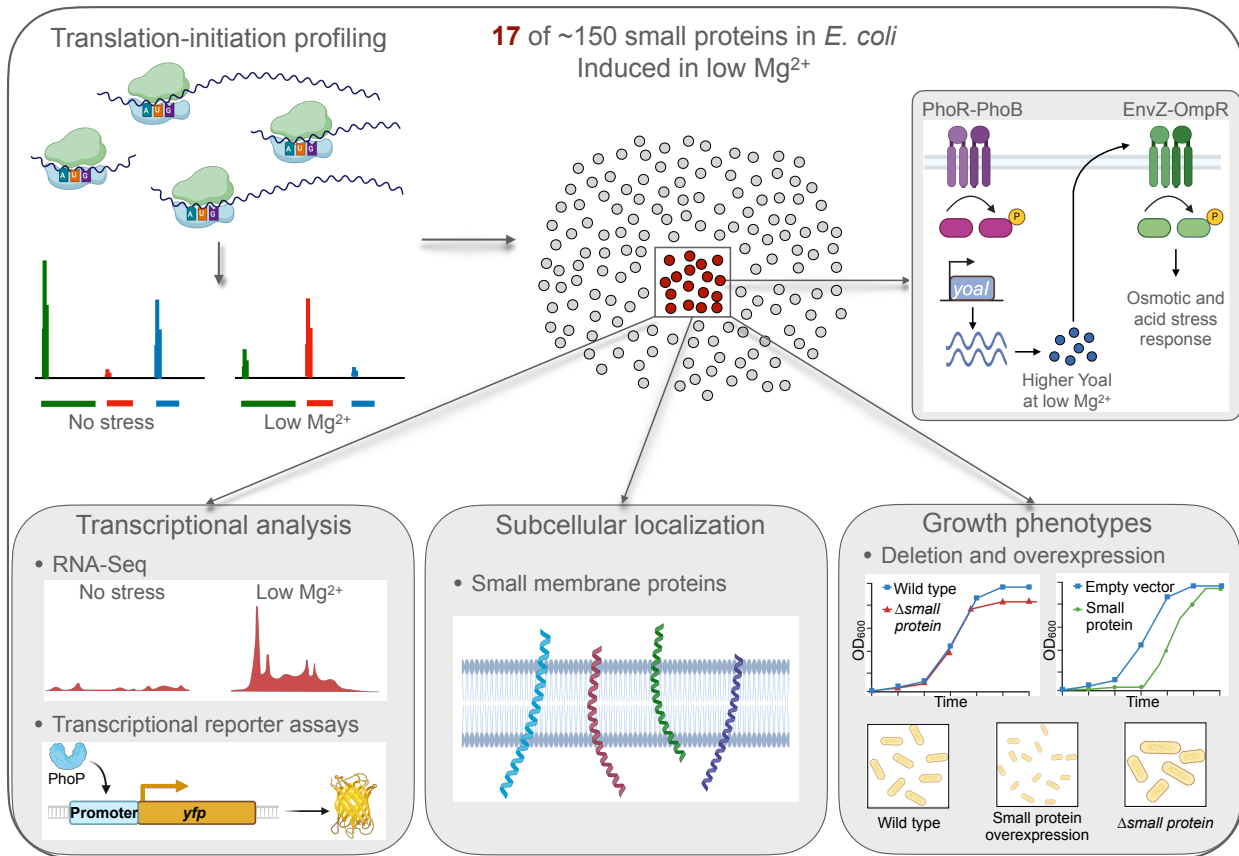
29 Signaling networks in bacteria enable sensing and adaptation to challenging environments by
30 activating specific genes that help counteract stressors. Small proteins (≤ 50 amino acids long)
31 are a rising class of bacterial stress response regulators. *Escherichia coli* encodes over 150
32 small proteins, most of which lack known phenotypes and their biological roles remain elusive.
33 Using magnesium limitation as a stressor, we investigate small proteins induced in response to
34 stress using ribosome profiling, RNA sequencing, and transcriptional reporter assays. We
35 uncover 17 small proteins with increased translation initiation, a majority of which are
36 transcriptionally upregulated by the PhoQ-PhoP two-component signaling system, crucial for
37 magnesium homeostasis. Next, we describe small protein-specific deletion and overexpression
38 phenotypes, which underscore the physiological significance of their expression in low
39 magnesium stress. Most remarkably, our study reveals that a small membrane protein Yoal is
40 an unusual connector of the major signaling networks – PhoR-PhoB and EnvZ-OmpR in *E. coli*,
41 advancing our understanding of small protein regulators of cellular signaling.

42

43

44 Graphical abstract

45



46

47

48

49 Keywords

50 Bacterial stress response, translation-profiling, small proteins, magnesium starvation, two-
51 component signaling, PhoQ-PhoP, fluorescent reporters

52

53

54 Introduction

55 Living organisms sense and respond to environmental stressors through a wide variety of gene
56 regulatory mechanisms¹. In bacteria, signal transduction is primarily carried out by two-
57 component signaling systems²⁻⁴. Gene expression regulation can occur at various stages
58 including transcription, post-transcription, translation, and post-translation⁵⁻⁸. While some of
59 these mechanisms involving regulators such as transcription factors and small RNAs have been
60 extensively studied⁹⁻¹¹, other regulatory pathways essential for stress adaptation remain less
61 well understood. Small proteins (≤ 50 amino acids in prokaryotes and ≤ 100 amino acids in
62 eukaryotes), encoded by authentic small open reading frames, are emerging as key regulators
63 of stress response¹²⁻¹⁴, highlighting a need for further investigation into their roles and
64 mechanisms of action.

65

66 Advances in bioinformatics, gene expression studies, and ribosome profiling have identified
67 hundreds of previously unannotated small proteins in all kingdoms of life including humans^{13,15–}
68 ²⁰. In *Escherichia coli*, more than 150 small proteins have been documented^{13,15}. While there
69 has been significant progress in identifying small proteins, a major gap in our knowledge is the
70 lack of understanding of the functions of most of these proteins^{12,15}. Small proteins are typically
71 non-essential, at least under standard growth conditions used in the labs, and their requirement
72 is likely conditional when the activity of a larger target needs fine-tuning. In support of this idea,
73 it was previously observed that some small proteins accumulate at higher levels in a specific
74 growth phase, medium, or stress condition^{21,22}. Magnesium (Mg²⁺) is an important cofactor
75 affecting the catalysis and stability of numerous proteins and RNAs involved in vital cellular
76 processes, including translation²³. Given the central role of Mg²⁺, its deprivation poses a major
77 stress to the cells. The signal transduction and physiological response to low Mg²⁺ is fairly well
78 understood, which involves the activation of the master regulator, PhoQP two-component
79 signaling system in gammaproteobacteria^{23–25}.

80
81 So far, three small proteins are known to mediate stress response to magnesium limitation in
82 bacteria. Firstly, MgrB, a 48-aa transmembrane protein negatively regulates the sensor kinase
83 PhoQ of the PhoQP signaling system^{26,27}. During magnesium starvation, the absence of MgrB
84 leads to hyperactivation of the PhoQP pathway, resulting in cell division inhibition and
85 filamentation in *E. coli*²⁸. In addition to its role in Mg²⁺ stress, loss of MgrB in *E. coli* increases
86 tolerance to trimethoprim, an antibiotic commonly used to treat bacterial infections²⁹. In clinical
87 isolates of *Klebsiella pneumoniae*, disruption of MgrB-mediated inhibition of PhoQ also leads to
88 acquired colistin resistance³⁰. A second small membrane protein, MgtS is also induced under
89 low Mg²⁺ stress and is important for magnesium homeostasis^{31,32}. MgtS protects the
90 magnesium transporter, MgtA, from degradation when Mg²⁺ is limited³¹. PmrR is the third small
91 membrane protein shown to be expressed under low Mg²⁺ stress in *Salmonella enterica*^{33,34}.
92 PmrR is activated by the PmrAB two-component system^{33,35,36} in a PhoP-dependent manner
93 and inhibits the lipopolysaccharide modification enzyme, LpxT.

94
95 Here we ask, what subset of the ~150 small proteins in *E. coli* are induced under magnesium
96 limitation? We hypothesized that, like MgrB, MgtS, and PmrR, many other small proteins with
97 potential regulatory roles are yet to be identified as part of this stress response. We leveraged
98 the translation-initiation profiling method, Ribo-RET^{37,38} to identify small proteins induced by low
99 magnesium stress, revealing a set of 17 proteins representing a substantial proportion (~11%)
100 of the documented small proteins in *E. coli*. To investigate the transcriptional regulation of the
101 stress-induced small proteins, we utilized RNA-Seq and transcriptional reporter assays, we
102 investigated the transcriptional regulation of these stress-induced small proteins, shedding light
103 on the genomic regions responsible for their expression and discerning any regulatory influence
104 from the PhoQP system. Additionally, using epitope tagging, microscopy, and biochemical
105 analysis, we meticulously examined the localization, overexpression, and loss-of-function
106 phenotypes of these small proteins. Through these experiments, we unexpectedly uncovered
107 how the small membrane protein Yoal, transcriptionally controlled by the PhoRB signaling
108 pathway³⁹, displays increased protein levels under magnesium stress and activates another
109 well-studied two-component system EnvZ-OmpR⁴⁰ in *E. coli*.

110 Results

111 **Identification of low magnesium stress-induced small proteins in *E. coli* using** 112 **translation-initiation profiling.**

113 To identify small proteins that accumulate under low magnesium stress, we adapted the
114 translation initiation profiling method called Ribo-RET (Figure 1A) – a modified ribosome
115 profiling approach utilizing translation initiation inhibitors for the identification of small proteins in
116 bacteria⁴¹. Retapamulin (RET) is an antimicrobial compound that can stall ribosomes at the
117 translation start sites to help identify previously unannotated open reading frames^{37,38}. In this
118 study, we prepared Ribo-RET libraries of wild-type *E. coli* cells grown in media with or without
119 magnesium to examine small proteins expressed in response to magnesium starvation.

120
121 In our Ribo-RET data, we observe an increase in reads mapping to a specific distance of the
122 ribosomal P-site codon from the 3' end of the reads in our libraries, ~6-10 nucleotides (nt)
123 downstream of the expected start codon (Figure S1), similar to the pattern observed in previous
124 translation initiation profiling studies³⁸. We calculated ribosome density at annotated translation
125 initiation sites using reads mapped from 4 to 20 nt downstream of the first nucleotide in the start
126 codon, using a broad window of 16 nt to capture all relevant footprint sizes. In our analysis, the
127 ribosome footprint sizes range from 16-24 nt, as illustrated in Figure S1, as anticipated for the
128 bacterial systems⁴².

129
130 The PhoQP signaling system is upregulated under Mg²⁺ limitation⁴³ (Figures S2A-B). As
131 expected, the read counts at the translation start site for PhoP and PhoQ were more than 4-fold
132 and 10-fold higher, respectively, under stress conditions compared to no stress. Three small
133 proteins known to be induced under low Mg²⁺ stress – MgrB, MgtS, and PmrR, show a 9-fold, 5-
134 fold, and 5-fold increase in the reads mapping to the translation start sites, respectively, under
135 stress vs. no stress (Figures 1B-C, S2C). Overall, in our Ribo-RET data, we see a moderate
136 correlation between the reads mapping to the start sites of all proteins expressed under these
137 two conditions (Figure 1C), which is indicative of the anticipated changes in the translation
138 initiation of proteins during stress response.

139
140 We detected 379 proteins with ≥3-fold increase in read counts under stress, several among
141 these are small proteins <50 aa in length (Table S4). We narrowed down a set of 17 small
142 proteins namely MgtS, MgrB, PmrR, MgtT, YmiC, YmiA, Yoal, YobF, YddY, YriB, YadX, YkgS,
143 YriA, YdgU, Yqhl, YadW and DinQ for further analysis (Table 1). Ribosome footprints at the
144 start site of an open reading frame are a good proxy for the expression of the corresponding
145 protein. However, it is critical to establish whether a given small open reading frame (sORF)
146 results in a protein product of that specific size. All but one protein Yoal were previously
147 validated for expression via genomic epitope-tagging (Table S5). In the case of Yoal, the protein
148 could not be detected in rich media or specific growth conditions, including envelope stress, acid
149 stress, and heat shock^{21,22}. Given the strong signal for expression of Yoal in our Ribo-RET data
150 (~12-fold increase in RPM for stress vs. no stress) (Figure S4A), we wondered if Yoal is
151 conditionally expressed during magnesium limitation. To examine the expression of Yoal, we
152 utilized a strain carrying chromosomally-encoded fusion protein Yoal-SPA^{21,22}. We grew cells in

153 media containing different levels of Mg^{2+} , prepared membrane and cytoplasmic fractions, and
154 performed western blot analysis. Consistent with our hypothesis, we detected expression of
155 Yoal in a magnesium-dependent manner, where the protein level is highest at the lowest
156 concentration of magnesium (Figure S4B). In addition, this result also confirms that Yoal is
157 predominantly associated with the membranes, consistent with the bioinformatic prediction that
158 it is a membrane protein (Table S6).

159
160 14 of the 17 shortlisted small proteins were not previously associated with expression under low
161 magnesium stress. Small proteins YobF^{22,44} and DinQ⁴⁵ have been linked to other stress
162 responses in *E. coli*, here we find the expression of these two proteins in low magnesium stress
163 as well. Many of these small proteins have not been characterized biochemically and have no
164 documented function yet. In the following sections, we systematically study these low- Mg^{2+} -
165 induced small proteins by investigating their transcriptional regulation and cellular localization
166 and characterizing their overexpression and deletion phenotypes.

167
168 **Transcriptional regulation of low magnesium stress-induced small proteins.**
169 To determine changes in the levels of transcripts encoding stress-induced small proteins, we
170 performed RNA-Seq using cells grown to early exponential phase in high and low Mg^{2+}
171 conditions. It is well-established that in cells depleted for Mg^{2+} , the PhoQP signaling system is
172 stimulated, leading to PhoP-mediated transcriptional activation of hundreds of genes necessary
173 for the stress response²⁴. Not surprisingly, we see an increase in the mRNA abundances for
174 *phoQ* and *phoP* (Figure S3). Differential expression analysis of our RNA-seq data revealed
175 significant upregulation (fold change threshold of >2 and a p-value threshold of <0.05) in
176 transcription under low Mg^{2+} stress for at least 10 of the 17 small proteins shortlisted, including
177 MgtS, MgtT, and MgrB (Figure 2). It is worth noting that YadX also showed a small increase
178 (1.7-fold) although it falls below the >2-fold threshold we applied in our data analysis.
179 Intriguingly, YriA, whose open reading frame overlaps with YriB by ~60% did not show an
180 increase in the transcript levels. In addition, we did not see a significant change in the mRNA
181 levels corresponding to Yoal, YobF, YkgS, and DinQ. In these cases, it is possible that the
182 change in transcript abundances is generally low and therefore fell below the significance
183 threshold. Alternatively, gene expression might be regulated at a post-transcriptional or
184 translational level under low magnesium stress. Indeed, *yobF* expression is induced post-
185 transcriptionally during heat shock response²². The expression of *yobF* under magnesium
186 limitation may be controlled via a similar post-transcriptional regulatory mechanism.

187 To delve deeper into the transcriptional control mechanism of the small proteins we sought to:
188 1) identify the regulatory regions upstream of each sORF, and 2) determine if the expression is
189 PhoQ-dependent. To identify the regions containing the putative promoters of low Mg^{2+} -induced
190 small proteins, we carefully selected ~200 to 500 base pairs upstream of each sORF (Table 2).
191 In general, we did not include any annotated full-length ORFs or non-coding RNAs occurring
192 within the regulatory region being tested, to avoid interference from the ectopic expression of
193 the corresponding product. In cases where a sORF appeared to be in the middle of an operon,
194 we selected two regions to test: one upstream of the entire operon and another immediately
195 upstream of the sORF of interest. We designed transcriptional reporter constructs to measure

196 the activity of each putative regulatory region, as shown in the schematic (Figure 3A). To
197 analyze which of these sORFs are regulated by the PhoQP two-component system, we
198 measured the transcriptional reporter activities in a *phoQ* deletion strain and compared them to
199 that of the wild type (Figure 3B).

200

201 Consistent with our RNA-seq data, we saw an increase in the transcriptional activity for
202 regulatory regions corresponding to 11 sORFs with this upregulation under low Mg²⁺ condition
203 being PhoQ-dependent (Figure 3B). Two sets of overlapping (out-of-frame) sORFs including
204 *mgtS-mgtT* and *yriA-yriB*, strongly suggest their association within the same operon. Three
205 other genes, *yadW*, *ymiC*, and *ydgU* are likely regulated as part of the operons, *yadX-clcA-*
206 *yadW*, *ymiA-yciX-ymiC*, and *asr-ydgU*, respectively. For all the sORFs occurring within an
207 established or putative operon, the region upstream of the operon showed a higher
208 transcriptional reporter activity in response to stress, while a second region immediately
209 upstream of the given sORF had negligible activity (Table 2, Figure 3B). Based on the reporter
210 assay, the transcriptional activity for the 11 sORFs (*mgrB*, *mgtS*, *mgtT*, *ymiA*, *ymiC*, *yddY*, *yriA*,
211 *yriB*, *yadX*, *yadW*, and *ydgU*) peaks at ~6-10 hours. The RNA-Seq data represent
212 transcriptomic abundances at a given time point of growth (early exponential phase). This
213 transcriptional reporter assay captures temporal changes in the reporter activity and serves as a
214 complementary approach to RNA-Seq, where changes in low-abundance transcripts would be
215 missed. For instance, we did not observe a significant upregulation of *yadX* and *yadW* in the
216 RNA-Seq data, but we see a PhoQ-dependent increase in the reporter activity for the putative
217 regulatory region P_{*yadX-clcA-yadW*} region under low magnesium stress. Conversely, for *pmrR* and
218 *yqhl*, while the RNA-Seq data suggested upregulation, we did not detect a significant increase
219 in the transcriptional reporter activity under stress suggesting that there may be distal or
220 additional regulatory factors, outside of our reporter construct, involved in controlling their
221 transcription. Indeed, *pmrR* is shown to be transcriptionally activated by the PmrA-PmrB
222 signaling system during magnesium limitation through the PhoQP-regulated connector protein
223 PmrD in *Salmonella enterica*³³. This connection between the PhoQ-PhoP and PmrA-PmrB
224 signaling pathways is also active in *E. coli* under specific conditions including high stimulation of
225 the PhoQ-PhoP system^{35,46}. We did not observe a reporter activity for *dinQ*, *ykgS*, *yobF*, and
226 *yoal* in either the wild-type or Δ *phoQ* cells, consistent with the results from the RNA-Seq.

227

228 In summary, our RNA-Seq and transcriptional reporter assays reveal transcriptional
229 upregulation of 13 of the 17 small proteins, of which PhoQ regulates at least 11 under
230 magnesium starvation.

231

232 **Regulation of small membrane protein Yoal under Mg²⁺ stress.**

233 Yoal is known to be regulated by the PhoRB two-component system⁴⁷⁻⁴⁹, which responds to
234 phosphate starvation³⁹. PhoRB signaling is also activated under low magnesium stress due to
235 the slowdown of protein synthesis and reduction of cytoplasmic phosphate levels⁵⁰.

236 Consistently, we noticed a 10-fold increase in reads mapping to the PhoB translation start site
237 under magnesium stress (Figure S2D). We then tested our transcriptional reporter for *yoal*
238 (P_{*yoal*}-YFP) in a low-phosphate medium and saw increased transcriptional activity for P_{*yoal*} when
239 compared to no-stress condition, in a PhoB-dependent manner (Figure 4A). This transcriptional

240 activation is PhoQ-independent as we see an amplification of P_{yoal} reporter in Δ phoQ cells as
241 well (Figure 4B). Taken together, these results show that yoal transcription is controlled by
242 PhoB, and there is no significant effect of low magnesium stress on yoal transcription based on
243 RNA-Seq and transcriptional reporter data (Figures 2, 3B). Yet Yoal protein levels increase
244 under low magnesium conditions, and we wondered if PhoQ controls Yoal abundance. To test
245 this possibility, we examined Yoal protein expression in wild-type and Δ phoQ cells in high and
246 low magnesium conditions by western blotting. Our results demonstrate that Yoal's expression
247 remains magnesium-dependent in a Δ phoQ strain, mirroring the pattern seen in the wild type
248 (Figure 4C), suggesting that PhoQ does not affect Yoal levels. Therefore, Yoal expression is
249 induced in a PhoQ-independent manner under magnesium limitation potentially via a post-
250 transcriptional regulatory mechanism that enhances translation initiation.

251

252 **About half of the low Mg²⁺ stress-induced small proteins localize to the membrane.**

253 As a first step to classifying the 17 stress-induced small proteins, we checked if a given small
254 protein is likely associated with the membrane or cytoplasm. Most of these proteins have limited
255 or no documented information, so their cellular localization can provide insights into their
256 potential mechanisms of action and interactions within the cell. Using bioinformatic tools
257 TMHMM⁵¹, TMPred⁵², and Phobius⁵³ to predict membrane helices, we find that at least 9 of
258 our candidates are putative membrane proteins. Three of them, MgrB, MgtS, and DinQ, were
259 previously shown to localize to the membrane in *E. coli*^{21,45}. Additionally, PmrR from *S. enterica*
260 was shown to be membrane-bound³³. Our bioinformatic analyses predict that small proteins
261 YdgU, YmiA, YmiC, Yoal, and YobF also localize to the membrane (Table S6).

262

263 Next, to confirm the membrane association and visualize the small protein localization we used
264 epitope-tagging with GFP. Taking into account the predicted orientation of the putative
265 transmembrane protein, we created either an N- or C-terminal fusion of GFP by tagging the end
266 that is expected to face the cytoplasm (Figure S5A, Table S6) to ensure proper folding of GFP
267⁵⁴. Accordingly, YdgU, YmiA, and YmiC were tagged at the N-terminus, and Yoal was tagged at
268 the C-terminus. Upon observation by fluorescence microscopy, cells expressing GFP-tagged
269 YdgU, YmiA, YmiC, and Yoal revealed bright fluorescence at the cell periphery indicative of
270 membrane localization, similar to the known membrane protein, MgrB (Figure S5B). As a
271 control, cells expressing GFP alone showed uniform fluorescence indicative of cytoplasmic
272 localization.

273

274 For PmrR, we constructed an N-terminal fusion with GFP, where the tag was expected to
275 localize to the cytoplasm. However, we did not observe membrane localization (Figure S5B).
276 Considering the predicted membrane helices, this suggests that the tag interferes with PmrR
277 localization. Alternately, we tagged the C-terminus of *E. coli* PmrR with a hexahistidine (6XHis)
278 tag (Figure S5C) and performed western blotting using membrane and cytoplasmic fractions.
279 PmrR-6XHis strongly associated with the membrane fraction (Figure S5D), consistent with the
280 bioinformatic prediction and data from *S. enterica* PmrR. As in the case of PmrR, YobF-GFP
281 localized to the cytoplasm, which was inconsistent with its predicted membrane localization
282 (Table S6). Therefore, we used a 6XHis-tagged version of YobF, which showed a greater

283 association with the membranes than the cytoplasmic fraction (Figure S5D). Overall, among the
284 17 small proteins induced under low Mg²⁺, we find that 9 of them are membrane proteins.
285

286 **Three small proteins exhibit stress-specific growth defects upon gene deletion.**

287 To identify potential defects in growth and cellular morphology caused by the absence of each
288 small protein in the cell, we utilized single gene deletions of *mgtS*, *mgtT*, *pmrR*, *yadW*, *yadX*,
289 *yddY*, *ykgS*, *ymiC*, *yqhl*, *yobF*, *dinQ*, *ydgU*, *ymiA*, and *yoal* in the *E. coli* genome. *mgtT* lies in an
290 operon with *mgtS*³⁸, with an out-of-frame overlap between the stop codon of MgtS and the start
291 codon of MgtT. Therefore, we carefully generated an *mgtS* deletion without affecting the start
292 codon of MgtT, and similarly, an *mgtT* deletion while keeping the stop codon for MgtS intact (for
293 details see materials and methods, Table SI). In the case of *yriAB*, the two small proteins YriA
294 and YriB have overlapping open reading frames, where ~80% of the *yriB* gene overlaps with that
295 of *yriA* (Figure 6C, schematic), so, we first deleted the combined *yriAB* ORF to see if there is a
296 phenotype. 12 out of the 17 small proteins displayed no discernible growth defects upon deletion
297 relative to the wild-type strain when grown under magnesium limitation. However, four mutants –
298 namely $\Delta pmrR$, $\Delta yobF$, $\Delta yqhl$, and $\Delta yriAB$ – exhibit reduced growth yields when grown over 24
299 hours (Figure 5). The cells carrying these deletions entered the stationary phase earlier than the
300 wild type. Notably, PmrR and YobF are membrane-bound proteins, while Yqhl, YriA, and YriB are
301 putative soluble proteins (Figure S5D, Table S6).
302

303 To determine whether these growth defects are specifically related to the loss of these small
304 proteins, we complemented the deletion strains with plasmids encoding the corresponding small
305 proteins and induced with IPTG. Successful rescue of growth is observed for $\Delta pmrR$, and $\Delta yobF$
306 (Figure 6A, B; top panel). To confirm that this complementation is specific to the small protein and
307 not due to a cryptic ORF or regulatory element present within the small protein coding region, we
308 prepared plasmids carrying *pmrR* and *yobF* where their start codons are mutated to stop codons.
309 When tested for complementation in $\Delta pmrR$ and $\Delta yobF$, respectively, the start codon mutants
310 retained the growth defect similar to that observed for the empty vector (Figure 6A, B; top panel),
311 reinforcing that the growth phenotype is specific to the small proteins themselves. In addition, we
312 investigated whether the growth defect observed with *pmrR* and *yobF* deletions is specific to low
313 magnesium stress. Our results indicate that deletions of *pmrR* and *yobF* do not cause growth
314 defects in the absence of stress (Figure 6A, B; bottom panel), and the phenotype is specific to
315 magnesium-limited conditions. Intriguingly, expression of plasmid-encoded PmrR in the absence
316 of stress resulted in a growth defect (Figure 6A, bottom panel), suggesting that increased
317 expression of PmrR under no-stress conditions may be detrimental.
318

319 Given the growth phenotype observed for $\Delta yriAB$, we performed complementation with either
320 plasmid-encoded YriA and/or YriB to establish if the loss of either protein is responsible for the
321 growth defect. Intriguingly, complementation with either YriA or YriB alone failed to restore growth
322 but a plasmid expressing the region from *yriB* to *yriA*, mimicking the native genomic arrangement
323 (*yriAB*), partially rescued the growth defect (Figure 6C, top panel). In our plasmid construct
324 encoding YriB and YriAB, there is a possibility of a spurious small protein produced due to the
325 intact, out-of-frame YriA start codon within the *yriB* ORF (Figure 6C, schematic) that potentially
326 interferes with the complementation. To address this issue, we created a modified *yriB* construct

327 where YriA start codon ATG is mutated to TAA, this substitution changes codons 4 (Asp) and 5
328 (Val) in YriB to 4 (Ile) and 5 (Ile). This variant of YriB confers full complementation (Figure 6C, top
329 panel), indicating that loss of YriB contributes to the growth phenotype, at least in part. In addition,
330 we see that the growth defect associated with *yriAB* deletion is specific to low magnesium stress
331 condition (Figure 6C, bottom panel).

332
333 Finally, the growth defect observed in $\Delta yqhl$ is not complemented with a plasmid encoding Yqhl
334 (Figure 6D), which suggests that this phenotype is not directly related to the Yqhl protein itself.
335 Intriguingly, we did not see a growth defect in $\Delta mgtS$ strain when grown in a low magnesium
336 medium (Figure 5), contrasting with the previous report³¹. This discrepancy might be due to
337 differences in strain construction and/or growth media. Together, our results underscore the
338 complexities in studying overlapping small ORFs and their phenotypes.

339

340 **Seven small proteins affect growth and cell morphology upon overexpression.**

341 As a parallel approach to analyzing loss-of-function phenotypes, we explored the phenotypes
342 associated with the overexpression of small proteins. We cloned the genes corresponding to the
343 untagged small proteins into either an IPTG-inducible (all except DinQ as we were unable to
344 generate a DinQ clone in the IPTG-inducible pEB52 background) or arabinose-inducible (DinQ)
345 vector. Upon induction, slow growth or a prolonged lag phase is observed for 7 out of the 17 small
346 proteins tested: MgtS, MgrB, PmrR, YmiA, Yoal, YkgS, and DinQ (Figure 7). Notably, six of these
347 proteins are membrane-bound (MgtS, MgrB, PmrR, YmiA, Yoal, and DinQ). In the case of MgtS,
348 MgrB, PmrR, YmiA, and Yoal, induction of expression with IPTG leads to an extended lag phase
349 lasting for ~8-10 hours, after which the cells grow exponentially at growth rates similar to that of
350 control cells carrying empty vector, eventually reaching saturation at comparable levels.
351 Interestingly, cells expressing small proteins YkgS and DinQ did not display a long lag phase but
352 grew at a slower rate, eventually catching up to the same growth yield as the control cells. The
353 growth defect due to DinQ overexpression aligns with previous studies indicating that its
354 overexpression disrupts membrane potential and depletes intracellular ATP levels⁴⁵. It is worth
355 noting that overexpression of the other 10 small proteins, including 3 membrane proteins YdgU,
356 YmiC, and YobF had a negligible impact on cell growth under the same condition, suggesting that
357 cells may tolerate high amounts of these proteins (Figure 7). More importantly, the lack of a
358 phenotype for most of these small proteins indicates that the growth defects observed here are
359 not due to a generic burden on the cell from plasmid-driven protein overexpression.

360

361 To be cautious in interpreting the overexpression data, we considered the possibility that the
362 observed growth defects might be attributed to a cryptic small open reading frame or regulatory
363 element within the small ORF being overexpressed. To check if the growth defects are indeed
364 small protein-dependent, we prepared plasmid constructs containing variants of the small protein
365 where the start codon (ATG) is substituted with a stop codon (TAA). For all 7 small proteins
366 displaying overexpression phenotypes, we repeated the growth assay with the start codon
367 mutants of each small protein. Expression of the mutant small proteins rescued the growth defect
368 with the cells exhibiting similar growth patterns to those carrying the empty vector (Figure 8A),
369 reinforcing that the observed growth defects are specific to the overexpression of these small
370 proteins.

371
372 In addition to the impact on growth, we explored whether overexpression of these 7 small proteins
373 (MgtS, MgrB, PmrR, YmiA, Yoal, YkgS, and DinQ) influences cell size and morphology, especially
374 given the delayed growth (Figures 7, 8A). We hypothesized that cells overexpressing these small
375 proteins may undergo morphological changes as part of adaptation during the extended lag and
376 log phases of growth. Using phase contrast microscopy, we imaged cells induced for expression
377 of the small proteins during the lag and exponential growth phases. As controls, we used wild-
378 type cells carrying either an empty vector or a plasmid containing a variant of the small ORF
379 where its start codon (ATG) is mutated to a stop (TAA). The control cells exhibited a roughly
380 uniform cell size with volumes around 1.1-1.3 fL and 1.2-1.4 fL in lag and log phases, respectively.
381 Intriguingly, overexpression of small proteins MgtS, MgrB, PmrR, YkgS, Yoal, and YmiA causes
382 distinct changes in cell size (Figures 8B, S6). Specifically, overexpression of MgtS results in
383 smaller cell size (Figure 8B), with a reduction in cellular volume to ~30-36% of the control cells
384 carrying either empty vector or start codon mutants in both lag and log phases (Figure S6).
385 Similarly, overexpression of PmrR and Yoal leads to a ~29% decrease in cell size and volume
386 during the lag phase, however, the cell size reverts to that of control cells during the exponential
387 phase (Figures 8B, S6). In contrast, overexpression of MgrB leads to an increase in cell size, with
388 an average volume of 2.2 fL, ~1.6-fold larger than the controls in the lag phase (Figures 8B, S6),
389 akin to the $\Delta phoQ$ cells grown in low magnesium conditions (Figure S7). This finding is remarkable
390 because MgrB is known to inhibit PhoQ^{26,27}, consistently, plasmid overexpression of MgrB likely
391 leads to strong inhibition of PhoQ, resulting in a cellular morphology similar to that of $\Delta phoQ$.
392 These cells eventually return to normal size (cell volume of 1.2 fL) in the exponential phase.
393 Overexpression of YkgS and YmiA causes varying extents of cell elongation affecting cell size
394 and volume (Figures 8B, S6). Cells overexpressing DinQ did not show a significant change in cell
395 size.

396
397 Overall, the slow or delayed growth observed here along with the diverse morphological
398 phenotypes at distinct growth stages reflects how cells adapt in response to the overexpression
399 of small proteins mentioned above.

400 401 **Small protein Yoal connects distinct signaling systems – PhoQ-PhoP, PhoR-PhoB, and** 402 **EnvZ-OmpR.**

403 We wondered whether the growth delay observed upon overexpression of the 7 small proteins
404 described above is PhoQ-dependent, given the pivotal role of PhoQ in low magnesium stress. To
405 explore this, we evaluated the effects of overexpressing these proteins in a $\Delta phoQ$ strain under
406 magnesium limitation. Notably, the growth phenotype associated with protein overexpression is
407 retained in the $\Delta phoQ$ cells, similar to those observed in the wild-type strain for 6 out of 7
408 candidates – MgrB, MgtS, PmrR, YmiA, YkgS, and DinQ (Figures 8C), indicating that these
409 effects are PhoQ-independent. Interestingly, in the case of Yoal overexpression, the extended
410 lag phase observed in wild-type cells is completely abolished in the $\Delta phoQ$ cells, suggesting a
411 direct or indirect link between PhoQ and Yoal where PhoQ functions upstream of Yoal in this
412 regulatory pathway.

413

414 To check if Yoal and PhoQ physically interact with each other, we employed a bacterial two-hybrid
415 (BACTH) assay based on reconstituting split adenylyl cyclase (CyaA)^{55,56}. In this system, CyaA
416 activity is restored when the two fragments T18 and T25 come close leading to an increase in
417 cyclic AMP (cAMP) levels and subsequent expression of β -galactosidase (β -gal) from the *lac*
418 promoter. We fused the T18 fragment to Yoal and tested it against T25-PhoQ. We included the
419 T18-MgrB and T25-PhoQ pair as a positive control and two other histidine kinases, PhoR and
420 EnvZ, each fused to the T25 fragment as additional controls. In this experiment, Yoal did not show
421 an interaction with either PhoQ or PhoR, however, to our surprise there was a strong signal
422 between Yoal and EnvZ that is comparable to the positive control (Figure 9A). EnvZ is primarily
423 known to be an osmosensor, integral to the EnvZ-OmpR two-component system, which regulates
424 porins in response to changes in osmolarity, pH, temperature, and growth phase⁴⁰. Several small
425 membrane proteins are known to modulate the activities of sensor kinases¹⁴. A 127-amino acid
426 protein, MzrA regulates EnvZ resulting in higher levels of phosphorylated OmpR⁵⁷. Based on the
427 BACTH interaction between Yoal and EnvZ, we suspected Yoal might influence the activity of the
428 EnvZ-OmpR signaling system. To address this possibility, we utilized an EnvZ-OmpR-regulated
429 transcriptional reporter strain with chromosomal fusion of mScarlet to P_{omrB} ⁵⁸ and measured *omrB*
430 promoter activity in cells grown in low magnesium vs. no-stress media. Interestingly, we observe
431 an increase of 2.5-fold in EnvZ-regulated transcription under stress in wild-type cells (Figure 9B).
432 The significantly high activity of *omrB* reporter in low Mg^{2+} conditions suggests that upregulation
433 of Yoal protein levels may mediate this effect (Figures 4C, S4). Upon deletion of *yoal*, the *omrB*
434 transcription reduced by \sim 1.4-1.5-fold in both low and high Mg^{2+} media, indicating that Yoal affects
435 EnvZ-OmpR activity regardless of stress. Together with our observation that Yoal-SPA levels
436 increase under low Mg^{2+} stress (Figures 4C, S4B), the higher transcriptional activity of the EnvZ-
437 regulated promoter can be attributed to the positive interaction of Yoal with EnvZ. To further
438 support the idea that an increase in Yoal levels is responsible for modulating the activity of the
439 EnvZ-OmpR system, we examined *omrB* transcription upon plasmid overexpression of Yoal in
440 wild-type *E. coli* cells. We observed a greater than 6-fold increase in P_{omrB} -*lacZ* reporter activity
441 in cells expressing Yoal on a plasmid (Figure S8A) relative to the control cells carrying an empty
442 vector. We also tested two other reporters to genes *ompF*, P_{ompF} -YFP and *ompC*, P_{ompC} -CFP⁵⁹,
443 and found a \sim 2-fold change in their transcriptional activities (Figures S8B, C), consistent with the
444 activation of EnvZ^{40,60,61}. In summary, these findings suggest that Yoal interacts with and
445 stimulates EnvZ activity, potentially enhancing OmpR-P levels.

446
447

448 Discussion

449 In this study, we identified 17 small proteins (\leq 50 amino acids) induced by low magnesium
450 stress from approximately 150 documented small proteins in *E. coli*, using Ribo-RET^{37,38}. Only
451 3 of these proteins, MgrB, MgtS, and PmrR were previously studied in the context of
452 magnesium starvation^{27,31,33}. For all the stress-induced small proteins except one (Yoal),
453 expression from the genomic locus was verified *in vivo* (Table S5). In a previous report when
454 cells were grown in a rich medium, expression of Yoal tagged with SPA epitope from a genomic
455 translation fusion was undetected²¹. Interestingly, our observation of cells grown in low Mg^{2+}
456 detected a robust expression of Yoal expression using the same construct, highlighting the
457 condition-specific expression of this protein (Figures 4C, S4B). Using RNA-Seq, we found that

458 many of these stress-induced proteins are transcriptionally activated in low magnesium media.
459 Further, we identified the regions upstream of these small ORFs responsible for transcriptional
460 regulation using operon fusions and reporter assays. Few hits, including Yoal and YobF, do not
461 show a change in transcription levels under low Mg²⁺, suggesting that they are regulated post-
462 transcriptionally.

463
464 Lacking any functional or phenotypic information for most of these stress-induced proteins, we
465 began their characterization by predicting their subcellular localization using bioinformatic tools
466 (Tables 1, S6). By epitope tagging and imaging or western blotting, we empirically determined
467 that 9 of the 17 shortlisted stress-induced small proteins localize to the membrane (Figure S5).
468 These membrane-bound small proteins may interact with, stabilize, or fine-tune the activities of
469 large transmembrane proteins, such as sensor kinases, channel proteins, and drug efflux pumps,
470 thereby mediating cellular adaptation and stress responses¹⁴. Among the 9 candidates, three
471 proteins – MgrB, MgtS, and PmrR are shown to have specific membrane protein targets, as
472 described earlier. To further investigate their biological roles, we performed targeted deletion and
473 protein overexpression analysis.

474
475 Loss-of-function analysis of small proteins can be a useful approach to understanding their
476 physiological roles. For example, deleting *mgrB* causes hyperactivation of the PhoQP two-
477 component system, leading to cell division inhibition and filamentation of cells grown under low
478 magnesium stress²⁸. In clinical isolates of *Klebsiella pneumoniae*, the absence of MgrB-mediated
479 PhoQ inhibition has been linked to acquired colistin resistance^{30,62}. In the current work, we found
480 deletion phenotypes associated with the following small proteins—PmrR, YobF, YriA/B, and Yqhl
481 —leading to an early entry into the stationary phase and reduced yield at saturation under
482 magnesium limitation. Based on plasmid complementation, growth defects caused by PmrR and
483 YobF are specific to the respective small proteins. The overlapping ORFs of YriA and YriB present
484 a unique challenge in interpreting the $\Delta yriAB$ growth phenotype observed under low magnesium
485 stress. While expressing the native *yriAB* region partially rescues the defect, individual expression
486 of YriA or YriB fails to do so; however, modifying YriB by mutating the out-of-frame YriA start
487 codon fully restores growth, emphasizing that the loss of YriB is a critical factor in the observed
488 phenotype. In the case of Yqhl, the deletion phenotype could not be fully complemented by
489 plasmid-encoded small protein, pointing to putative cryptic ORF or regulatory factors within the
490 small ORF that warrant further investigation.

491
492 We also analyzed overexpression phenotypes associated with each of the stress-induced small
493 proteins to gain insights into cellular pathways or components functionally linked to these proteins.
494 For instance, overexpression of MgtS is shown to activate the PhoRB two-component system by
495 regulating the phosphate transporter, PitA³². Similarly, DinQ overexpression disrupts membrane
496 potential and depletes intracellular ATP levels⁴⁵. We observed overexpression phenotypes for 7
497 of the 17 small proteins induced under magnesium stress—MgtS, MgrB, PmrR, YmiA, Yoal,
498 YkgS, and DinQ—resulting in either a long lag phase or slow growth (Figure 7). These
499 overexpression phenotypes are specific to each small protein as we see control plasmids carrying
500 a mutated start codon do not cause growth defects (Figure 8A). Analysis of the cell morphology
501 during the lag and exponential phases of growth revealed interesting variations in cell sizes and

502 volumes that reflect how cells adapt to the overexpression of these small proteins. Remarkably
503 for one of the small proteins, PmrR, both the deletion and overexpression caused growth defects
504 under low magnesium stress. It is unclear how cells balance small protein levels when needed
505 during specific conditions vs. when not needed, say in the absence of stress. In this context,
506 complementation of the $\Delta pmrR$ strain with plasmid-encoded PmrR restored normal growth under
507 magnesium limitation but caused a growth defect under no-stress condition (Figure 6A). This
508 suggests that PmrR levels may be controlled precisely in cells undergoing stress, as both its
509 absence and its overexpression disrupt the balance necessary for cellular homeostasis.

510
511 In *Salmonella enterica*, PmrR has been shown to inhibit the inner membrane protein LpxT, which
512 plays a role in lipopolysaccharide modification^{33,34,36}. We explored whether the growth defects
513 associated with PmrR deletion and overexpression in *E. coli* are mediated through its interaction
514 with LpxT. Interestingly, expressing PmrR in a $\Delta lpxT$ background did not alleviate the growth
515 defects regardless of stress (Figure S9A). It is possible that higher LpxT levels, resulting from the
516 absence of PmrR, could lead to growth defects. However, both single deletions ($\Delta lpxT$ and
517 $\Delta pmrR$) and the double deletion ($\Delta pmrR \Delta lpxT$) exhibited growth defects under magnesium stress
518 (Figure S9B). This result suggests that the growth defect upon *pmrR* deletion is independent of
519 PmrR's interaction with LpxT. PmrR may be involved in other pathways or regulatory mechanisms
520 where it has additional interacting partners. It is not uncommon for small proteins to have multiple
521 binding partners, as seen with MgtS, which interacts with both MgtA and PitA^{31,32}. Whether PmrR
522 associates with other targets contributing to phenotypes observed here warrants further
523 investigation.

524
525 Finally, we would like to discuss the interesting case of the small membrane protein Yoal,
526 whose transcription is controlled by the PhoR-PhoB signaling system⁴⁷⁻⁴⁹. Yoal levels are quite
527 low or undetectable when cells are grown in a rich medium or specific stress conditions^{21,22}.
528 However, we find that Yoal protein levels strongly increase under magnesium limitation and it
529 becomes detectable (Figure 4C). Overexpression of Yoal leads to a long delay in growth
530 (Figures 7, 8A, S4B). This phenotype is abolished in a *phoQ* mutant (Figure 8C), hinting at an
531 unknown PhoQ-dependent factor that may contribute to the growth defect observed upon Yoal
532 overexpression. Through a bacterial two-hybrid assay, we found a direct interaction of Yoal with
533 the EnvZ sensor kinase (Figure 9A), which controls osmoregulation⁴⁰ suggesting that this small
534 protein acts as a connector between the PhoR-PhoB and EnvZ-OmpR signaling networks.
535 Activation of EnvZ by Yoal is supported by the data from our transcriptional reporter analysis of
536 promoters regulated by the EnvZ-OmpR system (Figures 9B, S8). Modulation of sensor kinases
537 via small protein connectors appears to be a general theme that many small membrane proteins
538 of unknown function may fall under¹⁴. For instance, under acidic conditions, a 65-amino acid
539 protein SafA is activated by the EvgS-EvgA two-component system, which subsequently
540 interacts with and activates the PhoQ-PhoP system⁶³. In a second example, an 88-amino acid
541 protein PmrD connects the PmrB-PmrA and PhoQ-PhoP signaling systems in *Salmonella*^{64,65}.
542 Another 127-amino acid protein MzrA is known to bind and activate EnvZ, which links the CpxA-
543 CpxR and EnvZ-OmpR pathways in *E. coli*⁵⁷. Taken together, our findings outlining the
544 transcriptional regulation of Yoal by PhoB (Figure 4A), PhoQ-dependent growth phenotype
545 upon Yoal overexpression (Figure 8C), and Yoal's physical interaction with EnvZ and

546 modulation of EnvZ-regulated promoter activity (Figures 9B, S8) reveal how this 34-amino acid
547 small protein acts as a connector between distinct two-component signaling systems – PhoRB,
548 PhoQP, and EnvZ-OmpR to integrate diverse stress responses (Figure 9C).

549
550 Most small proteins (≤ 50 amino acids in prokaryotes) are of unknown function and little is known
551 about when/if they are expressed in the cell and what their physiological relevance is. Small
552 proteins are thought to fine-tune the activity and/or stability of larger target molecules in the cell,
553 adding a layer of gene regulation. This type of conditional regulation by small proteins may be
554 especially important when cells are grown in non-ideal or stressful environments. Overall, our
555 work describing the identification of condition-specific small proteins and targeted phenotypic
556 characterization provides a deeper understanding of small protein regulation and the
557 physiological consequences of altering their expression levels, laying a foundation for
558 deciphering their functions and regulatory mechanisms under relevant stress conditions.

559
560

561 Materials and methods

562

563 **Media and growth conditions.**

564 Bacterial cultures were grown in either Luria-Bertani (LB) Miller medium (IBI Scientific) or
565 minimal A medium⁶⁶ supplemented with 0.2% glucose, 0.1% casamino acids, and the indicated
566 concentration of MgSO_4 , with aeration at 37°C, unless otherwise specified. In general, overnight
567 cultures are grown in supplemented minimal A medium containing 1 mM MgSO_4 and
568 antibiotic(s) as applicable. Saturated cultures are diluted 1:500 into fresh medium. For low Mg^{2+}
569 stress, no Mg^{2+} was added when overnight cultures were diluted into fresh medium. Media
570 containing 10 mM Mg^{2+} was used as the no-stress condition. For phosphate stress experiments,
571 MOPS minimal medium (Teknova) supplemented with 0.2% glucose, 0.1% casamino acids, and
572 either 1 mM K_2HPO_4 (no-stress) or no K_2HPO_4 (phosphate stress). For routine growth on solid
573 medium, LB containing 1.5% bacteriological-grade agar (VWR Lifesciences) was used. For
574 antibiotic selection, carbenicillin, kanamycin, and chloramphenicol were added at a final
575 concentration of 50-100 $\mu\text{g ml}^{-1}$, 25- 50 $\mu\text{g ml}^{-1}$, and 6-25 $\mu\text{g ml}^{-1}$. b-isopropyl-D-thiogalactoside
576 (IPTG) was used at a final concentration of 500 μM , unless otherwise specified. Arabinose was
577 used at final concentrations of 0.5% and 30 mM for pBAD24-based vectors and pMR120-based
578 vectors, respectively, to induce protein expression when needed.

579

580 **Strains, plasmids, and cloning.**

581 See supplemental information for details of the strains (Table S1), plasmids (Table S2), and
582 primers (Table S3) used in this study. All the strains used were derived from *Escherichia coli* K-
583 12 MG1655. Strain JW2162 was a gift from Dr. Bryce Nickels; plasmids pEB52, pAL38, pAL39,
584 pSMART, pMR120 and strains AML67, MDG147, TIM92, TIM100, and TIM202 were gifts from
585 Dr. Mark Goulian. Strains GSO195, GSO219, GSO225, GSO232, and GSO317 were gifts from
586 Dr. Gisela Storz. Strains JM2110 and JM2113 were kind gifts from Dr. Maude Guillier.

587

588 A modified version of the method described by⁶⁷ was used to generate in-frame gene deletions
589 corresponding to each small protein. Briefly, the entire small open reading frame (sORF)

590 (including the stop codon) was deleted from the MG1655 genome and substituted with a
591 kanamycin resistance cassette. While constructing the gene deletions, we avoided deleting any
592 overlapping annotated ORFs, non-coding RNAs, or putative regulatory regions in the vicinity of
593 the target gene. In the case of *yriA-yriB* (*yriAB*), the two small protein ORFs significantly
594 overlapped; therefore, we deleted the combined *yriAB* ORF. Detailed information on the
595 genomic coordinates of deleted regions can be found in Table S1. Genomic deletions and
596 reporter constructs carrying the kanamycin resistance cassettes were transferred between
597 strains using P1_{vir} transduction⁶⁶ and validated by PCR. When necessary, the kanamycin
598 cassette was excised from the chromosome by FLP-mediated recombination using pCP20, as
599 previously described⁶⁸. PCR and loss of antibiotic resistance confirmed the excision of the
600 kanamycin cassette.

601
602 Plasmids expressing translational fusions of GFP-A206K to the small open reading frames
603 (sORFs), specifically pPJ1-13 and pSV25, were generated through inverse PCR⁶⁹ using pAL39
604 as a template. Plasmids encoding C-terminally 6XHis-tagged-YobF and -PmrR (sORF-GGSG
605 linker-6XHis) were created using inverse PCR with pEB52 as a template, resulting in pSV14
606 and pSV26, respectively. Plasmids encoding untagged small proteins (pSV30-pSV37, pSV58,
607 pSV60, and pJS1-pJS7) were also generated by inverse PCR with pEB52 as a template. pSV54
608 was generated by inverse PCR using pBAD24 as a template. Plasmids pSV38-pSV45, pSV55,
609 pSV59, and pSV61, encoding variants of sORFs with start codon substitutions to stop codons
610 (TAA), were generated by site-directed mutagenesis with inverse PCR using templates pSV35,
611 pSV34, pJS7, pJS4, pSV36, pJS5, pSV54, pSV37, and pSV60 as applicable. For pSV39-
612 pSV42, another in-frame start codon within five amino acids of the annotated start was also
613 substituted to TAA. All constructs were verified by Sanger sequencing. To generate pSV47, *yoal*
614 was PCR amplified from the MG1655 genome, digested with *XbaI/EcoRI* restriction enzymes,
615 and then cloned into pUT18C at *XbaI/EcoRI* sites.

616
617 To examine the transcriptional regulation of the sORFs and identify the putative regulatory
618 regions, ~200-500 bp upstream region of the start codon of each sORF was selected for cloning
619 (see details in Table 2). If a given sORF was suspected to be part of an operon, two putative
620 regions were chosen for analysis, the first directly upstream of the gene and a second region
621 upstream of the entire operon. Each DNA segment was amplified from MG1655 genomic DNA
622 by PCR and inserted upstream of the *yfp* reporter gene in pMR120 into the restriction sites
623 (*XhoI/BamHI* to construct pSV16, while *EcoRI-BamHI* sites were used for the remaining
624 constructs). The pMR120 vector also includes a *cfp* reporter regulated by a constitutive *tetA*
625 promoter, serving as an internal plasmid control. The resulting plasmids (pPJ14, pPJ16-pPJ20,
626 pPJ23, pSV16-21, pSV24, pSV28, pSV29) were confirmed by Sanger sequencing and
627 transformed into MG1655 and TIM202 (Δ *phoQ*). Note that pSMART (Lucigen), pMR120, and
628 the derivatives are single-copy plasmids when transformed into *E. coli*⁷⁰. *E. cloni* replicator cells
629 (Lucigen) are transformed with these plasmids and cultured with arabinose induction to increase
630 the copy number for plasmid isolation.

631

632 **Translation initiation profiling (Ribo-RET) and RNA-Seq.**

633 Overnight cultures of wild-type *E. coli* grown in supplemented minimal A medium and 1 mM
634 MgSO₄ were diluted 1:500 in 100 ml of fresh medium and the specified concentrations of
635 MgSO₄. Once the cultures reached an OD₆₀₀ of ~0.4, they were split into two 50 ml conical
636 tubes. Cells from one tube were pelleted and submitted to Genewiz for RNA-Seq, while the cells
637 from the second tube were used for Ribo-RET. For Ribo-RET, cells were treated with
638 retapamulin at a final concentration of 100 µg ml⁻¹ for 5 minutes at 37°C with continuous
639 shaking. After retapamulin treatment, cells were filtered through a nitrocellulose membrane with
640 a pore size of 0.45 µm, quickly scraped off the surface, and flash-frozen in liquid nitrogen. The
641 resulting samples were processed, and libraries were prepared as described previously⁴¹ with
642 the following modifications. Specifically, RNA fragments of 15-30 nucleotides (nt) in length were
643 isolated by performing a tight gel size selection, and rRNA depletion was performed using the
644 RiboCop for Gram-negative bacteria kit (Lexogen). The resulting RNA fragments were then
645 pooled, and libraries were prepared, with a final library structure consisting of a 5' adapter – 4
646 random bases – insert – 5 random bases – sample barcode – 3' adapter. The randomized
647 bases serve as Unique Molecular Identifiers (UMIs) for deduplication. The multiplexed library
648 was sequenced on an Illumina HiSeq 4000 with a paired end reading of 150 bases (PE150
649 runs) at a sequencing depth of 40-60 million raw reads per sample.

650

651 **Sequencing data analysis.**

652 The raw sequencing data for this project has been deposited in the GEO database with the
653 accession number GSE276379. An R package with codes for data processing and analysis has
654 been made available on GitHub ([https://github.com/yadavalli-lab/Small-proteins-induced-under-](https://github.com/yadavalli-lab/Small-proteins-induced-under-low-magnesium-stress-in-E.coli)
655 [low-magnesium-stress-in-E.coli](https://github.com/yadavalli-lab/Small-proteins-induced-under-low-magnesium-stress-in-E.coli)). The raw sequencing data was first demultiplexed, and the
656 adaptors were removed using cutadapt⁷¹. The reads were then depleted of rRNA and tRNA and
657 deduplicated using umi_tools dedup⁷². The Ribo-RET reads were aligned to the *E. coli* MG1655
658 genome NC_000913.3 (RefSeq assembly accession: GCF_000005845.2) using hisat2⁷³, and
659 the ribosome density was assigned to the 3' end of the reads. Initiation peak density was
660 calculated for the annotated translation start sites by summing up the normalized reads (reads
661 per million, RPM) within 4 to 20 nt downstream of the first nucleotide in the start codon. For
662 transcript quantification of RNA-Seq reads, Kallisto⁷⁴ was used. DESeq2⁷⁵ was used for the
663 differential expression analysis of gene expression in the RNA-Seq data. Normalization of the
664 data for differential expression was carried out using the "apeglm" method⁷⁶. Fold-changes
665 were estimated by comparing two replicates of the low Mg²⁺ dataset to two replicates of the no-
666 stress dataset, with filtering criteria set at more than two-fold changes and a p-value below 0.05.

667

668 **Measurement of growth (optical density) and fluorescence using a microplate reader.**

669 The optical density (OD₆₀₀) measurements for small protein overexpression and deletion
670 constructs were performed in a clear flat bottom 96-well microplate (Corning) containing 150 µL
671 culture per well using a microplate reader (Agilent BioTek Synergy Neo2S). Fluorescence
672 measurements for transcriptional reporters were carried out similarly, using black-walled, clear-
673 flat bottom 96-well microplates (Corning). Saturated cultures grown in supplemented minimal A
674 or MOPS minimal medium were normalized to the same initial OD₆₀₀ values across all samples
675 and diluted 1:50 in fresh medium containing specified concentrations of MgSO₄ or K₂HPO₄,
676 along with appropriate antibiotics and inducers, as indicated. The microplates were incubated at

677 37 °C with double orbital shaking before the real-time measurements of optical density (at 600
678 nm), YFP (excitation: 500 nm; emission: 540 nm), or CFP (excitation: 420 nm; emission: 485
679 nm) were acquired every hour for 20-24 hours.

680

681 **Microscopy, and image analysis.**

682 For localization of GFP-tagged small proteins, cells were grown to an OD₆₀₀ of ~0.2 (~3 hours)
683 and then rapidly cooled in an ice slurry. Streptomycin was added to a final concentration of 250
684 µg ml⁻¹. GFP fluorescence was captured with a 475 nm excitation wavelength and a 540 nm
685 emission filter, with an exposure time of 30 ms and 20% intensity. While the exposure times
686 were typically set to these specified values, slight variations were occasionally made to optimize
687 imaging based on the levels of fluorescence expression observed.

688

689 For overexpression analysis, overnight cultures were diluted 1:500 into supplemented minimal
690 medium containing no added MgSO₄, 50 µg ml⁻¹ carbenicillin, and either 500 µM IPTG or 0.5%
691 arabinose, as indicated. Cells were grown to the lag phase (OD₆₀₀ ~0.005), then rapidly cooled
692 in an ice slurry before phase contrast microscopy. For empty vector control and start codon
693 variants, we captured lag phase micrograph images approximately 30-40 minutes post-
694 inoculation and -induction. For MgtS, MgrB, PmrR, Yoal, and YmiA overexpression, lag phase
695 measurements were performed at 2-3 hours, and for YkgS and DinQ, at 1-hour post-inoculation
696 and -induction. To concentrate the cells, 1 mL of the culture was pelleted and resuspended in
697 30-50 µL minimal A salt solution. For exponential phase analysis (OD₆₀₀ between 0.2-0.4) in
698 cultures carrying empty vector control and start codon variants images were taken at ~3-4
699 hours. In the case of cells expressing MgtS, MgrB, PmrR, Yoal, and YmiA, images were taken
700 at 16-18 hours, and YkgS and DinQ at 6-8 hours. Microscope slides were prepared using 1%
701 agarose pads as described previously²⁸, with approximately 5-7 µL of the resuspended cells. A
702 40 ms exposure time and 20% intensity were applied to capture the phase-contrast images.

703

704 For single-cell fluorescence measurements of wild type and Δ yoal cells carrying *P_{omrB}-mScarlet*
705 reporter, overnight cultures were diluted 1:500 into fresh supplemented minimal A medium
706 containing either 10 mM or no added MgSO₄. Cells were grown to exponential (OD₆₀₀ ~0.3 for
707 both stress and no stress conditions) and stationary phase (OD₆₀₀ ~1.0 for stress condition and
708 ~3.5 for no stress condition), and then rapidly cooled in an ice slurry. Streptomycin was added
709 to a final concentration of 250 µg ml⁻¹. Fluorescence was measured using the mCherry channel
710 with an excitation wavelength of 570 nm and an emission filter of 645 nm. The exposure time
711 was set to 100 ms, with an intensity of 20%. Background fluorescence was determined by
712 imaging MG1655 cells grown under the same conditions.

713

714 For single-cell fluorescence measurements upon Yoal overexpression (to measure reporter
715 activities of *P_{ompF}-yfp* and *P_{ompC}-cfp*), the overnight cultures were diluted 1:500 into fresh
716 supplemented minimal A medium containing 10 mM MgSO₄. Cells were grown to exponential
717 phase (OD₆₀₀ between 0.2-0.4), then rapidly cooled in an ice slurry, and streptomycin was added
718 at 250 µg ml⁻¹. YFP fluorescence was measured using a 500 nm excitation wavelength and a
719 535 nm emission filter. CFP fluorescence was measured using a 435 nm excitation wavelength
720 and a 480 nm emission filter. The exposure time for YFP and CFP measurements was set to 50

721 ms, respectively, with an intensity of 20%. The background fluorescence was determined by
722 imaging MG1655/pEB52 cells grown under the same conditions.

723
724 Image visualization and acquisition were performed using a Nikon Ti-E epifluorescence
725 microscope equipped with a TI2-S-HU attachable mechanical stage. The images were captured
726 with a Prime 95B sCMOS camera from Teledyne Photometrics with 1x1 binning. All image
727 acquisition was managed using Metamorph software (Molecular Devices), version 7.10.3.279. A
728 minimum of 50 cells per replicate were analyzed for cell volume and single-cell fluorescence
729 quantification. The distributions of cellular volume were assumed to follow the sphero-cylindrical
730 shape of *E. coli*. The volume was calculated using the formula $V = \frac{\pi}{4} * W^2 * \left(L - \frac{W}{3}\right)$. Images
731 were analyzed, and the cellular length, width, and fluorescence intensity were quantified using
732 ImageJ⁷⁷ and MicrobeJ plugin⁷⁸.

733

734 **Preparation of membrane and cytoplasmic fractions.**

735 Membrane and cytoplasmic protein fractions were prepared as described previously²⁶. Briefly,
736 saturated cultures of MG1655/pSV14 (PmrR-GGSG-6XHis), MG1655/pSV26 (YobF-GGSG-
737 6XHis), and MG1655/pEB52 (empty vector) were diluted 1:500 in 4 ml of LB media containing
738 100 $\mu\text{g ml}^{-1}$ carbenicillin and grown at 37 °C. After 4 hours of growth, 0.5 mM IPTG was added
739 to the cultures, and the cells were harvested after 2 hours of induction. Cell pellets were
740 resuspended in 50 μl of cold resuspension buffer containing 20% sucrose, 30 mM Tris pH 8.0,
741 and 1X protease inhibitor cocktail (Sigma, cOmplete, EDTA-free). Then, 50 μl of 10 mg ml^{-1}
742 lysozyme, freshly prepared in 0.1 M EDTA pH 7.0, was added to the cell suspension, and the
743 mixture was incubated on ice for 30 minutes. Next, 1 ml of 3 mM EDTA pH 7.5 was added to
744 each sample, followed by sonication (10s pulse, 10s gap, 6 times) using VCX-130 Vibra-Cell
745 Ultrasonic sonicator (Fisher Scientific). Samples were briefly centrifuged at 6,000 rpm for 10
746 minutes at 4 °C to remove cellular debris, and the supernatant was collected and spun at
747 21,000 $\times g$ for 30 minutes at 4 °C. The supernatant from this step is separated and stored as the
748 cytoplasmic fraction. The pellet, representing the membrane fraction, was resuspended in a
749 storage buffer containing 20 mM Tris pH 8.0/20% glycerol. For strains carrying genomic Yoal-
750 SPA translational fusion, cultures were grown to an OD₆₀₀ of ~0.2-0.4 before harvesting, and
751 membrane and cytoplasmic fractions were prepared as mentioned above.

752

753 **Western blot analysis.**

754 The membrane and cytoplasmic fractions were resuspended in 4X Laemmli sample buffer
755 containing 5% 2-mercaptoethanol and no dyes. The samples were heated at 70 °C for 10
756 minutes and run on a 12% Bis-Tris gel (NuPage, Invitrogen) using MES running buffer
757 (Invitrogen) at 160V for 80 minutes. After electrophoresis, proteins were transferred to a PVDF
758 membrane (AmershamTM HybondTM) with a 0.2 μm pore size using a semi-dry transfer cell (Bio-
759 Rad Trans-Blot SD). The membranes were blocked with 5% w/v milk in Tris-buffered saline, pH
760 7.4, with 1% Tween 20 (TBS-T). Primary antibodies – mouse M2 anti-FLAG (Sigma-Aldrich) and
761 rabbit anti-6XHis tag antibody (Rockland) were used at 1:1000 dilution to detect SPA- and
762 6XHis-tagged constructs, respectively. IRDye 800CW goat anti-mouse and IRDye 680RD
763 donkey anti-rabbit antibodies (LiCOR) were used for secondary detection. SeaBlueTM Plus2 Pre-

764 stained Protein Standard (Invitrogen) was used as a ladder to visualize lower molecular weight
765 protein bands. The proteins were visualized using a LI-COR Odyssey imager.

766

767 **Bacterial two-hybrid and β -galactosidase reporter gene assays.**

768 For the bacterial two-hybrid assay, several clones from the transformation plate were picked to
769 reduce heterogeneity⁵⁵ and inoculated in LB containing 100 $\mu\text{g ml}^{-1}$ ampicillin and 50 $\mu\text{g ml}^{-1}$
770 kanamycin. Cultures were grown overnight at 30°C with shaking. The cultures were then diluted
771 1:1000 into LB supplemented with 500 μM IPTG and grown at 30°C until OD₆₀₀ reached
772 between 0.2-0.4, after which β -galactosidase activities (Miller units) were measured as
773 previously described⁷⁹.

774

775 For the β -galactosidase reporter gene assay, strains carrying P_{omrB}-lacZ with either pSV34
776 (pYoal) or pEB52 (empty vector) were grown overnight in LB medium with 100 $\mu\text{g ml}^{-1}$
777 ampicillin. The next day, the cultures were diluted 1:100 in the same medium and grown at 37
778 °C. After 3 hours of growth, 0.5 mM IPTG was added to the cultures, and the cells were
779 harvested after 2 hours of induction. For high-throughput measurements in a 96-well format, the
780 protocol was modified as previously described⁷⁹.

781

782 **Resource availability**

783

784 **Lead contact**

785 Further information and requests for resources and reagents should be directed to and will be
786 fulfilled by the lead contact, Srujana S. Yadavalli (sam.yadavalli@rutgers.edu).

787

788 **Materials availability**

789 Requests for strains and plasmids generated in this study should be directed to and will be
790 fulfilled by the lead contact, Srujana S. Yadavalli (sam.yadavalli@rutgers.edu).

791

792 **Data and code availability**

793 Next-generation sequencing data generated in this study are deposited at Gene Expression
794 Omnibus (GEO) with accession number GSE276379. All data generated or analyzed during this
795 study are included in the manuscript and its supporting files. Source data files for all figures
796 have been provided. The code used for data processing and analysis is available in a series of
797 R Markdown documents hosted on GitHub ([https://github.com/yadavalli-lab/Small-proteins-
798 induced-under-low-magnesium-stress-in-E.coli](https://github.com/yadavalli-lab/Small-proteins-induced-under-low-magnesium-stress-in-E.coli)).

799

800

801 **Acknowledgments**

802 The authors would like to thank Drs. Mark Goulian, Bryce Nickels, Gisela Storz, and Maude
803 Guillier for generously sharing strains and plasmids. We thank the past and present members of
804 the Yadavalli, Storz, and Shah Labs for their helpful discussions. We also thank Drs. Gisela
805 Storz and Mark Goulian for critical feedback on the manuscript.

806 Funding: S.S.Y. is supported by the National Institutes of Health - National Institute of General
807 Medical Sciences (NIH-NIGMS) ESI-MIRA R35 GM147566 and institutional start-up funds from
808 Rutgers. P.S. was supported by NIH/NIGMS grant R35 GM124976 and start-up funds from the
809 Human Genetics Institute of New Jersey at Rutgers University. S.V. is supported by the
810 Waksman Institute Busch Predoctoral fellowship (2022-24). The funders did not play any role in

811 the study design, data collection and analysis, decision to publish, or preparation of the
812 manuscript.

813
814

815 Author contributions

816 S.V., P.S., and S.S.Y. conceived the experiments. All authors planned the experiments,
817 designed the methodology, and performed data analysis. S.V., P.S., and S.S.Y. wrote the initial
818 draft of the manuscript, P.S. contributed to revisions and editing, and all authors reviewed the
819 final manuscript.

820
821

822 Declaration of interests

823 S.S.Y. consults for and collaborates with Designs for Vision, Inc. P.S. a director at an RNA-
824 therapeutics startup, and consults for Designs for Vision, Inc. S.V. declared that no competing
825 interests exist.

826
827

828 References

829

830 1. Carlberg, C., and Molnar, F. (2016). *Mechanisms of Gene Regulation* 2nd ed. (Springer).

831 2. Goulian, M. (2010). Two-component signaling circuit structure and properties. *Curr. Opin.*
832 *Microbiol.* 13, 184–189.

833 3. Stock, A.M., Robinson, V.L., and Goudreau, P.N. (2000). Two-component signal
834 transduction. *Annu. Rev. Biochem.* 69, 183–215.

835 4. Laub, M.T. (2014). The role of two-component signal transduction systems in bacterial
836 stress responses. In *Bacterial Stress Responses* (ASM Press), pp. 45–58.

837 5. Baumberg, S. ed. (1999). *Prokaryotic Gene Expression*. Preprint at Oxford University
838 PressOxford, <https://doi.org/10.1093/oso/9780199636044.001.0001>
839 <https://doi.org/10.1093/oso/9780199636044.001.0001>.

840 6. Banerjee, T. (2022). Regulation of Gene Expression in Prokaryotes. In *Genetics*
841 *Fundamentals Notes* (Springer Nature Singapore), pp. 569–596.

842 7. Tollerson, R., 2nd, and Ibba, M. (2020). Translational regulation of environmental
843 adaptation in bacteria. *J. Biol. Chem.* 295, 10434–10445.

844 8. Cain, J.A., Solis, N., and Cordwell, S.J. (2014). Beyond gene expression: the impact of
845 protein post-translational modifications in bacteria. *J. Proteomics* 97, 265–286.

846 9. Gottesman, S. (2019). Trouble is coming: Signaling pathways that regulate general stress
847 responses in bacteria. *J. Biol. Chem.* 294, 11685–11700.

848 10. Storz, G., Vogel, J., and Wassarman, K.M. (2011). Regulation by small RNAs in bacteria:
849 expanding frontiers. *Mol. Cell* 43, 880–891.

- 850 11. Seshasayee, A.S.N., Sivaraman, K., and Luscombe, N.M. (2011). An overview of
851 prokaryotic transcription factors : a summary of function and occurrence in bacterial
852 genomes. *Subcell. Biochem.* *52*, 7–23.
- 853 12. Burton, A.T., Zeinert, R., and Storz, G. (2024). Large Roles of Small Proteins. *Annu. Rev.*
854 *Microbiol.* <https://doi.org/10.1146/annurev-micro-112723-083001>.
- 855 13. Hemm Matthew R., Weaver Jeremy, Storz Gisela, Lovett Susan T., and Hinton Deborah
856 (2020). *Escherichia coli* Small Proteome. *EcoSal Plus* *9*.
857 <https://doi.org/10.1128/ecosalplus.ESP-0031-2019>.
- 858 14. Yadavalli, S.S., and Yuan, J. (2022). Bacterial Small Membrane Proteins: the Swiss Army
859 Knife of Regulators at the Lipid Bilayer. *J. Bacteriol.* *204*, e0034421.
- 860 15. Gray, T., Storz, G., and Papenfort, K. (2022). Small Proteins; Big Questions. *J. Bacteriol.*
861 *204*, e0034121.
- 862 16. Pueyo, J.I., Magny, E.G., and Couso, J.P. (2016). New peptides under the s(ORF)ace of
863 the genome. *Trends Biochem. Sci.* *41*, 665–678.
- 864 17. Patraquim, P., Mumtaz, M.A.S., Pueyo, J.I., Aspden, J.L., and Couso, J.-P. (2020).
865 Developmental regulation of canonical and small ORF translation from mRNAs. *Genome*
866 *Biol.* *21*, 128.
- 867 18. Sberro, H., Fremin, B.J., Zlitni, S., Edfors, F., Greenfield, N., Snyder, M.P., Pavlopoulos,
868 G.A., Kyrpides, N.C., and Bhatt, A.S. (2019). Large-Scale Analyses of Human Microbiomes
869 Reveal Thousands of Small, Novel Genes. *Cell* *178*, 1245-1259.e14.
- 870 19. Kastenmayer, J.P., Ni, L., Chu, A., Kitchen, L.E., Au, W.-C., Yang, H., Carter, C.D.,
871 Wheeler, D., Davis, R.W., Boeke, J.D., et al. (2006). Functional genomics of genes with
872 small open reading frames (sORFs) in *S. cerevisiae*. *Genome Res.* *16*, 365–373.
- 873 20. Steinberg, R., and Koch, H.-G. (2021). The largely unexplored biology of small proteins in
874 pro- and eukaryotes. *FEBS J.* *288*, 7002–7024.
- 875 21. Hemm, M.R., Paul, B.J., Schneider, T.D., Storz, G., and Rudd, K.E. (2008). Small
876 membrane proteins found by comparative genomics and ribosome binding site models.
877 *Mol. Microbiol.* *70*, 1487–1501.
- 878 22. Hemm, M.R., Paul, B.J., Miranda-Ríos, J., Zhang, A., Soltanzad, N., and Storz, G. (2010).
879 Small stress response proteins in *Escherichia coli*: proteins missed by classical proteomic
880 studies. *J. Bacteriol.* *192*, 46–58.
- 881 23. Groisman, E.A., and Chan, C. (2021). Cellular Adaptations to Cytoplasmic Mg²⁺ Limitation.
882 *Annu. Rev. Microbiol.* *75*, 649–672.
- 883 24. Groisman, E.A., Duprey, A., and Choi, J. (2021). How the PhoP/PhoQ System Controls
884 Virulence and Mg²⁺ Homeostasis: Lessons in Signal Transduction, Pathogenesis,
885 Physiology, and Evolution. *Microbiol. Mol. Biol. Rev.* *85*, e0017620.
- 886 25. Groisman, E.A. (2001). The pleiotropic two-component regulatory system PhoP-PhoQ. *J.*
887 *Bacteriol.* *183*, 1835–1842.

- 888 26. Yadavalli Srujana S., Goh Ted, Carey Jeffrey N., Malengo Gabriele, Vellappan Sangeevan,
889 Nickels Bryce E., Sourjik Victor, Goulian Mark, Yuan Jing, and Silhavy Thomas J. (2020).
890 Functional Determinants of a Small Protein Controlling a Broadly Conserved Bacterial
891 Sensor Kinase. *J. Bacteriol.* *202*, e00305-20.
- 892 27. Lippa, A.M., and Goulian, M. (2009). Feedback inhibition in the PhoQ/PhoP signaling
893 system by a membrane peptide. *PLoS Genet.* *5*, e1000788.
- 894 28. Yadavalli, S.S., Carey, J.N., Leibman, R.S., Chen, A.I., Stern, A.M., Roggiani, M., Lippa,
895 A.M., and Goulian, M. (2016). Antimicrobial peptides trigger a division block in *Escherichia*
896 *coli* through stimulation of a signalling system. *Nat. Commun.* *7*, 1–10.
- 897 29. Patel, V., and Matange, N. (2021). Adaptation and compensation in a bacterial gene
898 regulatory network evolving under antibiotic selection. *Elife* *10*.
899 <https://doi.org/10.7554/eLife.70931>.
- 900 30. Cannatelli, A., Giani, T., D'Andrea, M.M., Di Pilato, V., Arena, F., Conte, V., Tryfinopoulou,
901 K., Vatopoulos, A., Rossolini, G.M., and COLGRIT Study Group (2014). MgrB inactivation
902 is a common mechanism of colistin resistance in KPC-producing *Klebsiella pneumoniae* of
903 clinical origin. *Antimicrob. Agents Chemother.* *58*, 5696–5703.
- 904 31. Wang, H., Yin, X., Wu Orr, M., Dambach, M., Curtis, R., and Storz, G. (2017). Increasing
905 intracellular magnesium levels with the 31-amino acid MgtS protein. *Proc. Natl. Acad. Sci.*
906 *U. S. A.* *114*, 5689–5694.
- 907 32. Yin, X., Wu Orr, M., Wang, H., Hobbs, E.C., Shabalina, S.A., and Storz, G. (2019). The
908 small protein MgtS and small RNA MgrR modulate the PitA phosphate symporter to boost
909 intracellular magnesium levels. *Mol. Microbiol.* *111*, 131–144.
- 910 33. Kato, A., Chen, H.D., Latifi, T., and Groisman, E.A. (2012). Reciprocal control between a
911 bacterium's regulatory system and the modification status of its lipopolysaccharide. *Mol.*
912 *Cell* *47*, 897–908.
- 913 34. Hong, X., Chen, H.D., and Groisman, E.A. (2018). Gene expression kinetics governs
914 stimulus-specific decoration of the *Salmonella* outer membrane. *Sci. Signal.* *11*.
915 <https://doi.org/10.1126/scisignal.aar7921>.
- 916 35. Chen, A.I., Albicoro, F.J., Zhu, J., and Goulian, M. (2021). Effects of regulatory network
917 organization and environment on PmrD connector activity and polymyxin resistance in
918 *Klebsiella pneumoniae* and *Escherichia coli*. *Antimicrob. Agents Chemother.* *65*.
919 <https://doi.org/10.1128/AAC.00889-20>.
- 920 36. Tian, X., Manat, G., Gasiorowski, E., Auger, R., Hicham, S., Mengin-Lecreulx, D., Boneca,
921 I.G., and Touzé, T. (2021). LpxT-Dependent Phosphorylation of Lipid A in *Escherichia coli*
922 Increases Resistance to Deoxycholate and Enhances Gut Colonization. *Front. Microbiol.*
923 *12*, 676596.
- 924 37. Meydan, S., Marks, J., Klepacki, D., Sharma, V., Baranov, P.V., Firth, A.E., Margus, T.,
925 Kefi, A., Vázquez-Laslop, N., and Mankin, A.S. (2019). Retapamulin-Assisted Ribosome
926 Profiling Reveals the Alternative Bacterial Proteome. *Mol. Cell* *74*, 481-493.e6.

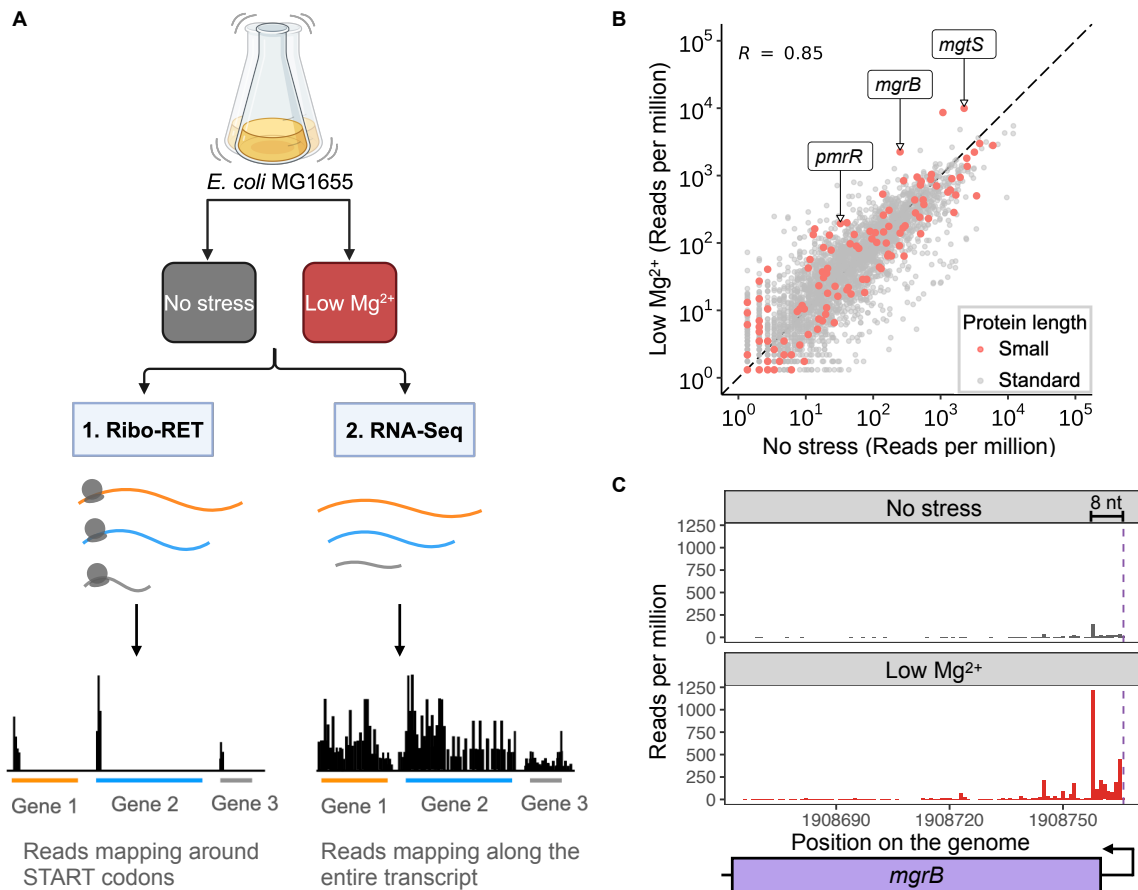
- 927 38. Weaver, J., Mohammad, F., Buskirk, A.R., and Storz, G. (2019). Identifying Small Proteins
928 by Ribosome Profiling with Stalled Initiation Complexes. *MBio* 10.
929 <https://doi.org/10.1128/mBio.02819-18>.
- 930 39. Gardner, S.G., and McCleary, W.R. (2019). Control of the *phoBR* Regulon in *Escherichia*
931 *coli*. *EcoSal Plus* 8. <https://doi.org/10.1128/ecosalplus.ESP-0006-2019>.
- 932 40. Kenney, L.J., and Anand, G.S. (2020). EnvZ/OmpR Two-Component Signaling: An
933 Archetype System That Can Function Noncanonically. *EcoSal Plus* 9.
934 <https://doi.org/10.1128/ecosalplus.ESP-0001-2019>.
- 935 41. Cope, A.L., Vellappan, S., Favate, J.S., Skalenko, K.S., Yadavalli, S.S., and Shah, P.
936 (2022). Exploring Ribosome-Positioning on Translating Transcripts with Ribosome Profiling.
937 *Methods Mol. Biol.* 2404, 83–110.
- 938 42. Glaub, A., Huptas, C., Neuhaus, K., and Ardern, Z. (2020). Recommendations for bacterial
939 ribosome profiling experiments based on bioinformatic evaluation of published data. *J. Biol.*
940 *Chem.* 295, 8999–9011.
- 941 43. Kato, A., Tanabe, H., and Utsumi, R. (1999). Molecular characterization of the PhoP-PhoQ
942 two-component system in *Escherichia coli* K-12: identification of extracellular Mg²⁺-
943 responsive promoters. *J. Bacteriol.* 181, 5516–5520.
- 944 44. Hobbs, E.C., Astarita, J.L., and Storz, G. (2010). Small RNAs and small proteins involved in
945 resistance to cell envelope stress and acid shock in *Escherichia coli*: analysis of a bar-
946 coded mutant collection. *J. Bacteriol.* 192, 59–67.
- 947 45. Weel-Sneve, R., Kristiansen, K.I., Odsbu, I., Dalhus, B., Booth, J., Rognes, T., Skarstad,
948 K., and Bjørås, M. (2013). Single transmembrane peptide DinQ modulates membrane-
949 dependent activities. *PLoS Genet.* 9, e1003260.
- 950 46. Rubin, E.J., Herrera, C.M., Crofts, A.A., and Trent, M.S. (2015). PmrD is required for
951 modifications to *Escherichia coli* endotoxin that promote antimicrobial resistance.
952 *Antimicrob. Agents Chemother.* 59, 2051–2061.
- 953 47. Fitzgerald, D.M., Stringer, A.M., Smith, C., Lapierre, P., and Wade, J.T. (2023). Genome-
954 Wide Mapping of the *Escherichia coli* PhoB Regulon Reveals Many Transcriptionally Inert,
955 Intragenic Binding Sites. *MBio* 14, e0253522.
- 956 48. Yoshida, Y., Sugiyama, S., Oyamada, T., Yokoyama, K., and Makino, K. (2012). Novel
957 members of the phosphate regulon in *Escherichia coli* O157:H7 identified using a whole-
958 genome shotgun approach. *Gene* 502, 27–35.
- 959 49. Chekabab, S.M., Jubelin, G., Dozois, C.M., and Harel, J. (2014). PhoB activates
960 *Escherichia coli* O157:H7 virulence factors in response to inorganic phosphate limitation.
961 *PLoS One* 9, e94285.
- 962 50. Pontes, M.H., and Groisman, E.A. (2018). Protein synthesis controls phosphate
963 homeostasis. *Genes Dev.* 32, 79–92.
- 964 51. Möller, S., Croning, M.D., and Apweiler, R. (2001). Evaluation of methods for the prediction
965 of membrane spanning regions. *Bioinformatics* 17, 646–653.

- 966 52. Ikeda, M., Arai, M., Okuno, T., and Shimizu, T. (2003). TMPDB: a database of
967 experimentally-characterized transmembrane topologies. *Nucleic Acids Res.* *31*, 406–409.
- 968 53. Käll, L., Krogh, A., and Sonnhammer, E.L.L. (2004). A combined transmembrane topology
969 and signal peptide prediction method. *J. Mol. Biol.* *338*, 1027–1036.
- 970 54. Feilmeier, B.J., Iseminger, G., Schroeder, D., Webber, H., and Phillips, G.J. (2000). Green
971 fluorescent protein functions as a reporter for protein localization in *Escherichia coli*. *J.*
972 *Bacteriol.* *182*, 4068–4076.
- 973 55. Battesti, A., and Bouveret, E. (2012). The bacterial two-hybrid system based on adenylate
974 cyclase reconstitution in *Escherichia coli*. *Methods* *58*, 325–334.
- 975 56. Karimova, G., Pidoux, J., Ullmann, A., and Ladant, D. (1998). A bacterial two-hybrid system
976 based on a reconstituted signal transduction pathway. *Proc. Natl. Acad. Sci. U. S. A.* *95*,
977 5752–5756.
- 978 57. Gerken, H., Charlson, E.S., Cicirelli, E.M., Kenney, L.J., and Misra, R. (2009). MzrA: a
979 novel modulator of the EnvZ/OmpR two-component regulon. *Mol. Microbiol.* *72*, 1408–
980 1422.
- 981 58. Bastet, L., Korepanov, A.P., Jagodnik, J., Grondin, J.P., Lamontagne, A.-M., Guillier, M.,
982 and Lafontaine, D.A. (2024). Riboswitch and small RNAs modulate *btuB* translation
983 initiation in *Escherichia coli* and trigger distinct mRNA regulatory mechanisms. *Nucleic*
984 *Acids Res.* *52*, 5852–5865.
- 985 59. Batchelor, E., Silhavy, T.J., and Goulian, M. (2004). Continuous control in bacterial
986 regulatory circuits. *J. Bacteriol.* *186*, 7618–7625.
- 987 60. Alphen, W.V., and Lugtenberg, B. (1977). Influence of osmolarity of the growth medium on
988 the outer membrane protein pattern of *Escherichia coli*. *J. Bacteriol.* *131*, 623–630.
- 989 61. Batchelor, E., Walthers, D., Kenney, L.J., and Goulian, M. (2005). The *Escherichia coli*
990 CpxA-CpxR envelope stress response system regulates expression of the porins *ompF* and
991 *ompC*. *J. Bacteriol.* *187*, 5723–5731.
- 992 62. Olaitan, A.O., Morand, S., and Rolain, J.-M. (2014). Mechanisms of polymyxin resistance:
993 acquired and intrinsic resistance in bacteria. *Front. Microbiol.* *5*, 643.
- 994 63. Eguchi, Y., Itou, J., Yamane, M., Demizu, R., Yamato, F., Okada, A., Mori, H., Kato, A., and
995 Utsumi, R. (2007). B1500, a small membrane protein, connects the two-component
996 systems EvgS/EvgA and PhoQ/PhoP in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.*
997 *104*, 18712–18717.
- 998 64. Kox, L.F., Wösten, M.M., and Groisman, E.A. (2000). A small protein that mediates the
999 activation of a two-component system by another two-component system. *EMBO J.* *19*,
1000 1861–1872.
- 1001 65. Kato, A., and Groisman, E.A. (2004). Connecting two-component regulatory systems by a
1002 protein that protects a response regulator from dephosphorylation by its cognate sensor.
1003 *Genes Dev.* *18*, 2302–2313.

- 1004 66. Miller, J.H., and Others (1992). *A Short Course in Bacterial Genetics: A Laboratory Manual*
1005 *and Handbook for Escherichia Coli and Related Bacteria* (CSHL Press).
- 1006 67. Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in
1007 *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* 97, 6640–6645.
- 1008 68. Cherepanov, P.P., and Wackernagel, W. (1995). Gene disruption in *Escherichia coli*: TcR
1009 and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance
1010 determinant. *Gene* 158, 9–14.
- 1011 69. Ochman, H., Medhora, M.M., Garza, D., and Hartl, D.L. (1990). Amplification of flanking
1012 sequences by inverse PCR. *PCR protocols: A guide to methods and applications*, 219–227.
- 1013 70. Godiska R, Patterson M, Schoenfeld T, and Mead DA (2005). Beyond pUC: Vectors for
1014 Cloning Unstable DNA. In *DNA Sequencing: Optimizing the Process and Analysis*, J.
1015 Kieleczawa, ed. (Jones & Bartlett Learning), pp. 55–76.
- 1016 71. Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing
1017 reads. *EMBnet.journal* 17, 10–12.
- 1018 72. Smith, T., Heger, A., and Sudbery, I. (2017). UMI-tools: modeling sequencing errors in
1019 Unique Molecular Identifiers to improve quantification accuracy. *Genome Res.* 27, 491–
1020 499.
- 1021 73. Kim, D., Paggi, J.M., Park, C., Bennett, C., and Salzberg, S.L. (2019). Graph-based
1022 genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat. Biotechnol.* 37,
1023 907–915.
- 1024 74. Bray, N.L., Pimentel, H., Melsted, P., and Pachter, L. (2016). Near-optimal probabilistic
1025 RNA-seq quantification. *Nat. Biotechnol.* 34, 525–527.
- 1026 75. Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and
1027 dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550.
- 1028 76. Zhu, A., Ibrahim, J.G., and Love, M.I. (2019). Heavy-tailed prior distributions for sequence
1029 count data: removing the noise and preserving large differences. *Bioinformatics* 35, 2084–
1030 2092.
- 1031 77. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years
1032 of image analysis. *Nat. Methods* 9, 671–675.
- 1033 78. Jiang, C., Brown, P.J.B., Ducret, A., and Brun, Y.V. (2014). Sequential evolution of bacterial
1034 morphology by co-option of a developmental regulator. *Nature* 506, 489–493.
- 1035 79. Thibodeau, S.A., Fang, R., and Joung, J.K. (2004). High-throughput beta-galactosidase
1036 assay for bacterial cell-based reporter systems. *Biotechniques* 36, 410–415.
- 1037
- 1038
- 1039

1040 Figures and tables

1041
1042



1043

1044

1045 **Figure 1. Identification of small proteins induced under low magnesium stress in *E. coli***

1046 **by translation-initiation profiling (Ribo-RET).** (A) Schematic diagram showing the Ribo-RET

1047 and RNA-Seq experimental setup used to identify small proteins induced by low magnesium

1048 stress. Wild-type *E. coli* K-12 MG1655 cells were grown in supplemented minimal A medium

1049 containing MgSO₄ at either 10 mM (no stress) or ~1 μM (no added magnesium, low Mg²⁺

1050 stress). (B) Scatterplot showing the correlation between the Ribo-RET reads mapping to the

1051 annotated start sites of proteins expressed under low Mg²⁺ stress and no stress. The red dots

1052 represent annotated small proteins of ≤ 50 amino acids in length, and the gray dots represent

1053 proteins > 50 amino acids long. Small proteins, MgrB, MgtS, and PmrR, known to be induced

1054 under magnesium starvation, are highlighted. Pearson's coefficient, $r = 0.85$. (C) Ribo-RET data

1055 for a representative small protein MgrB induced under low Mg²⁺ stress. A purple dashed line

1056 indicates the translation start site, and the 8 nucleotide (nt) indicates the distance between the

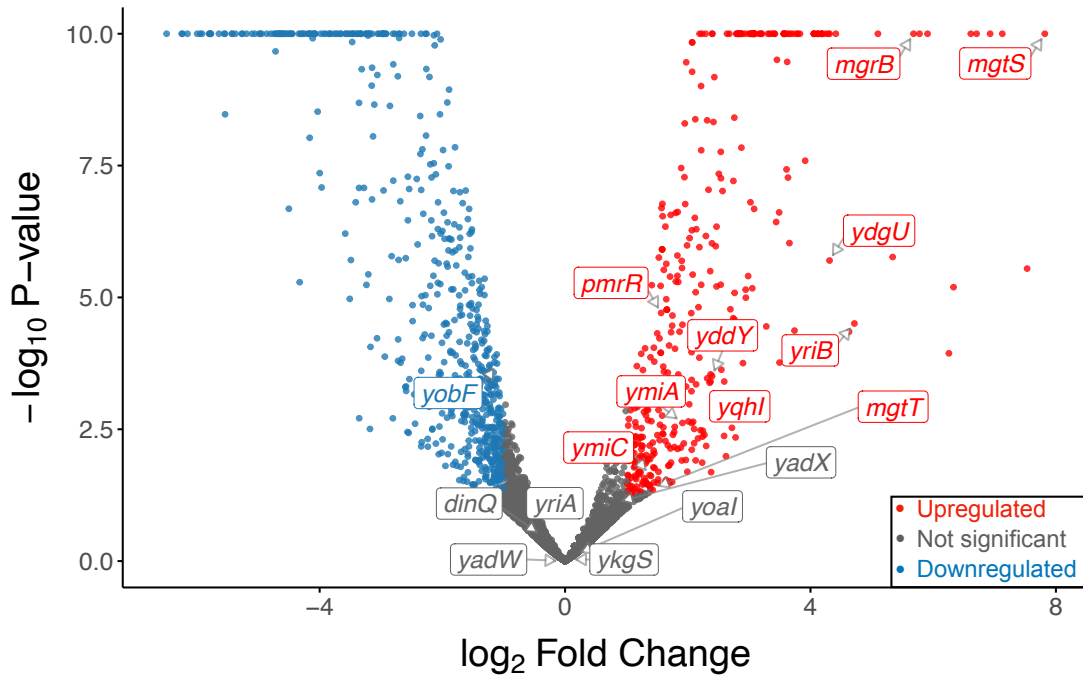
1057 highest ribosome density peak and the first nucleotide of the start codon. (Also see Tables 1,

1058 S4, Figures S1 and S2)

1059

1060

1061



1062

1063

1064

1065

1066

1067

1068

1069

1070

1071

1072

1073

1074

1075

1076

1077

1078

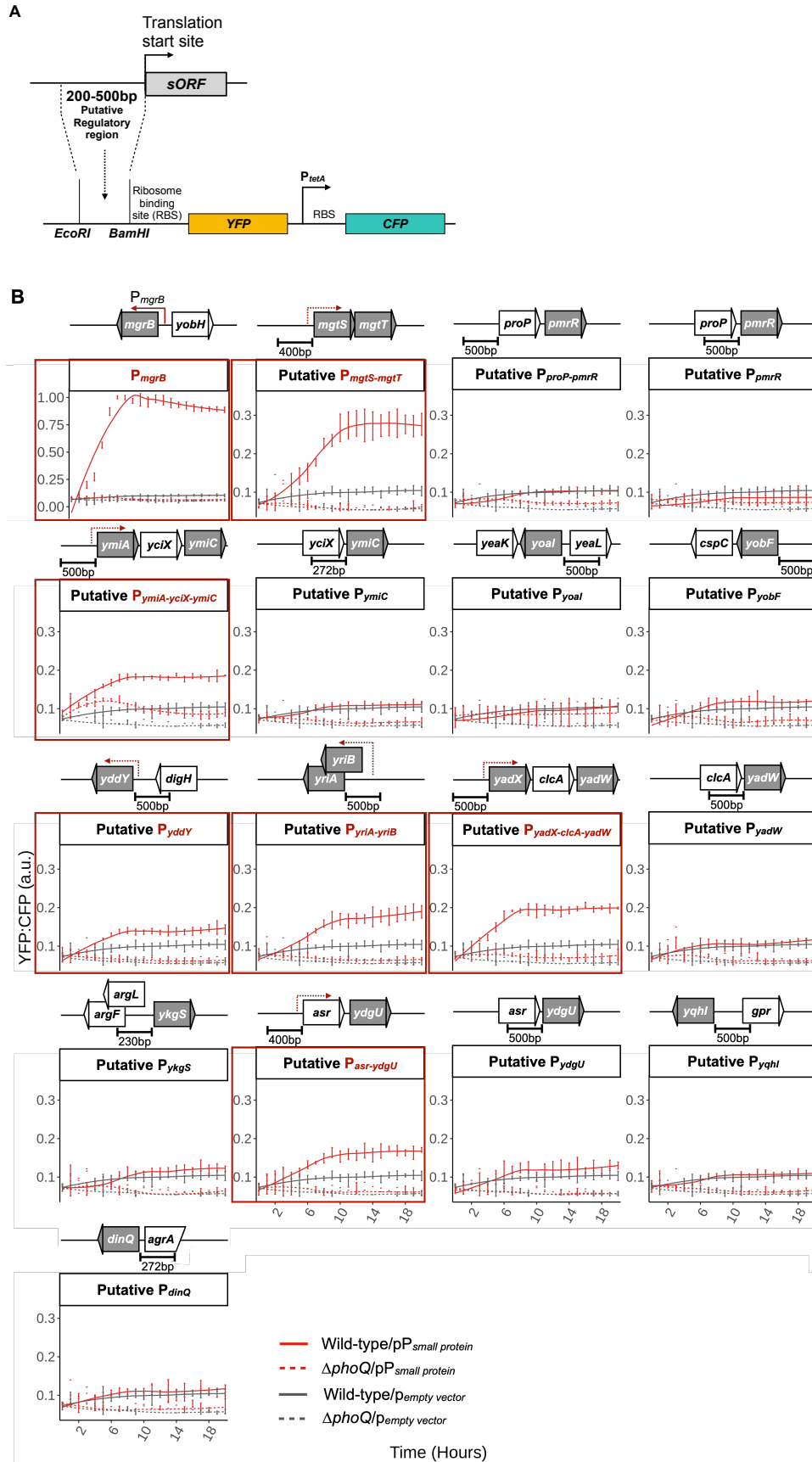
1079

1080

1081

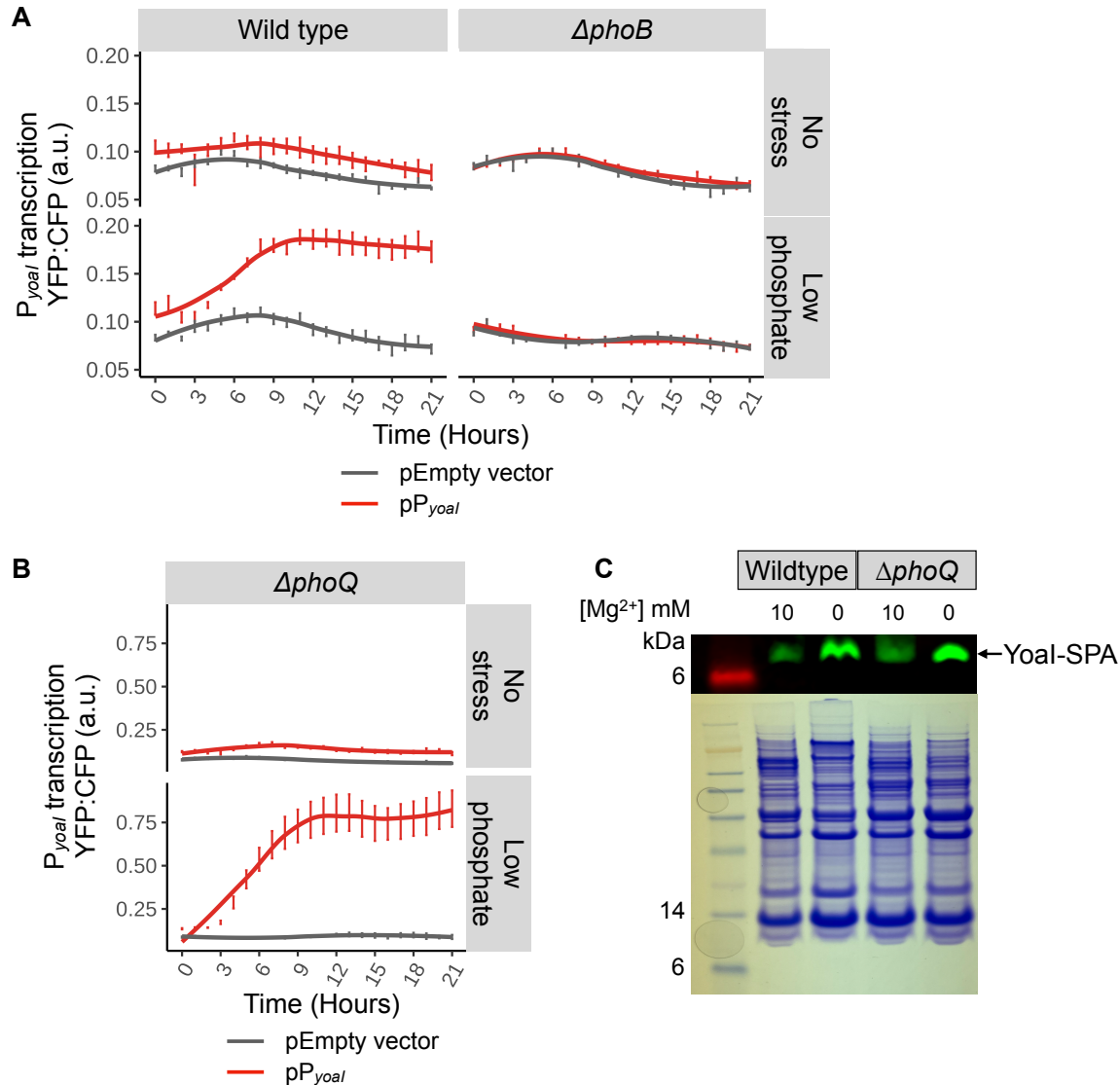
Figure 2. RNA-Seq analysis of transcripts representing small proteins induced under low magnesium stress. Volcano plot illustrating differential expression of small proteins induced under low magnesium stress from RNA-Seq data. Transcripts that are either upregulated or downregulated are highlighted in red or blue, respectively, and those with no significant change are represented in gray. The criteria for significance include a fold change threshold of >2 and a p-value of <0.05 . The $-\log_{10}$ P-value of *mgrB* and *mgtS* were 75 and 140 respectively. To prevent distortion from their exceptionally high values dominating the plot, the transformed p-values were capped at 10 to enhance visualization. Data represent differential expression analysis from two independent replicates. (Also see Tables 2, S4, and Figure S3)

1082
1083



1084

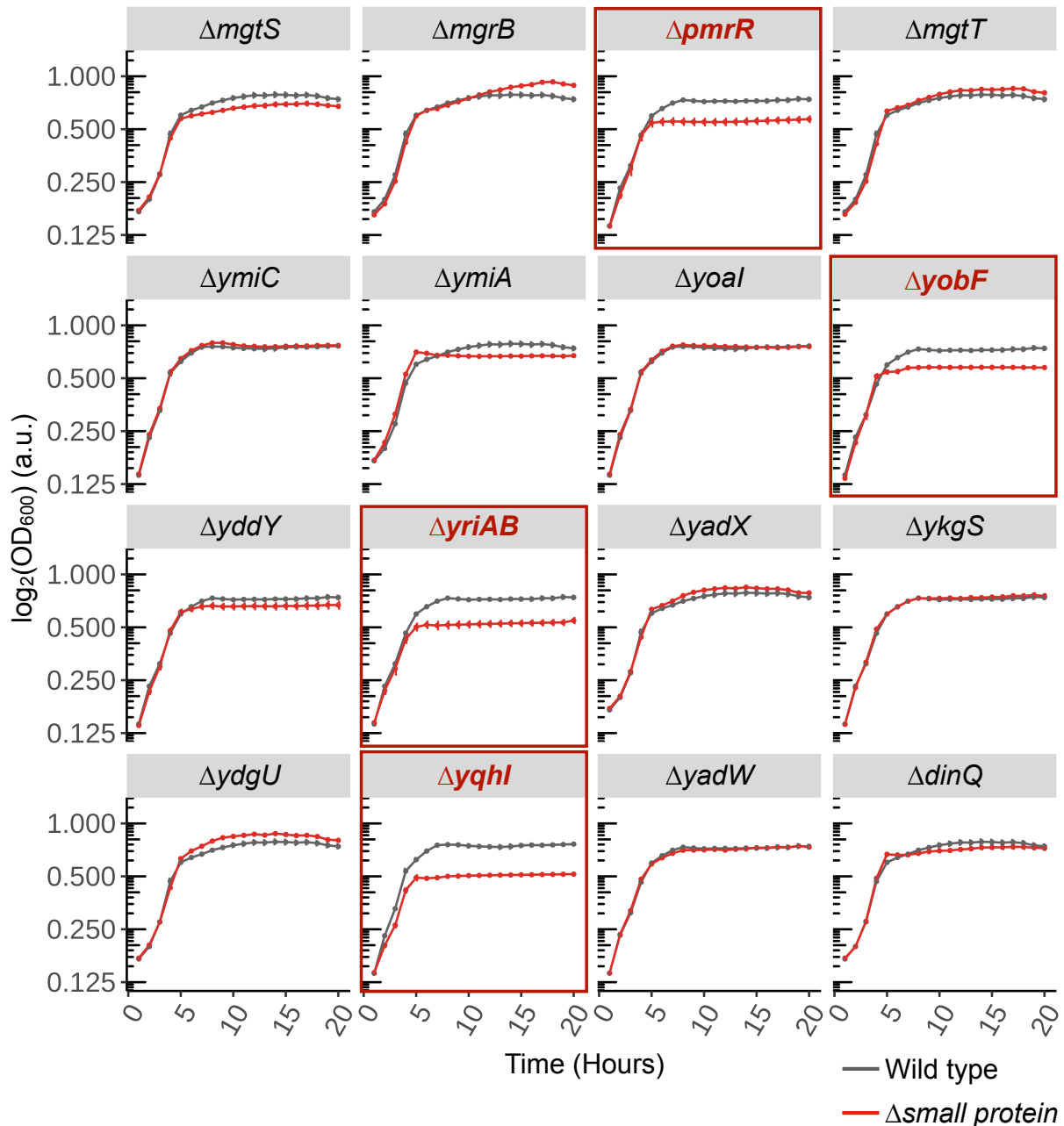
1085 **Figure 3. Transcriptional regulation of small proteins under low magnesium stress.** (A)
1086 Schematic representation of the transcriptional reporter fusion construct. Regions (200-500 bp)
1087 upstream of the small open reading frames of interest were amplified from the MG1655 genome
1088 and cloned into a YFP-encoding reporter plasmid (pMR120) resulting in plasmids pPJ14,
1089 pPJ16-pPJ20, pPJ23, pSV16-pSV21, pSV24, pSV28-pSV29 (See Table 2 for genomic
1090 coordinates of the regulatory regions tested here and Table S2 for plasmid details). (B)
1091 Measurement of transcriptional reporter activity (YFP:CFP) for the indicated small proteins
1092 under low Mg²⁺ (no added magnesium). The solid and dashed lines represent the wild-type *E.*
1093 *coli* MG1655 and Δ *phoQ* cells, respectively, harboring plasmids with either transcriptional fusion
1094 for a putative regulatory region of a small protein with *yfp* (red line) or *yfp* only (gray line). The
1095 schematic above each plot depicts the arrangement of the small protein-encoding genes (shown
1096 in gray), including the putative regulatory regions and operons where applicable. Transcriptional
1097 reporters showing an increased activity compared to the empty vector control (pMR120) are
1098 highlighted in red. The cultures were grown in a supplemented minimal medium with 6 μ g ml⁻¹
1099 chloramphenicol. The data represent averages and standard errors of the means for four
1100 independent replicates. (Also see Table 2)
1101
1102
1103
1104
1105



1106
1107

1108 **Figure 4. Regulation of Yoal expression during magnesium limitation.** (A-B) Transcriptional
1109 activity of *yoal* was measured as a function of YFP reporter fluorescence in *E. coli* MG1655,
1110 $\Delta phoB$ (SV48), and $\Delta phoQ$ (TIM100) strains under phosphate stress as indicated. The red and
1111 gray lines represent cells carrying plasmid-encoded P_{yoal} -*yfp* transcriptional reporter (pPJ18)
1112 and *yfp*-only control vector (pMR120), respectively. The cultures were grown in a supplemented
1113 MOPS minimal medium with 6 $\mu\text{g ml}^{-1}$ chloramphenicol with either 1 mM K_2HPO_4 for no stress
1114 condition, or no added K_2HPO_4 for phosphate stress. The data illustrate average YFP:CFP
1115 fluorescence with standard error of the means, derived from four independent replicates. (C)
1116 Detection of Yoal protein in *E. coli* wild-type (GSO317) and $\Delta phoQ$ (SV60) strains containing
1117 *yoal*-SPA genomic translational fusion. Cells were grown in supplemented minimal A medium,
1118 with MgSO_4 added at the specified concentration (10mM or no added MgSO_4). Membrane
1119 fractions were analyzed by western blotting using M2 anti-FLAG antibodies (top) and by
1120 Coomassie Brilliant Blue staining (bottom). The data represent results from two independent
1121 replicates.

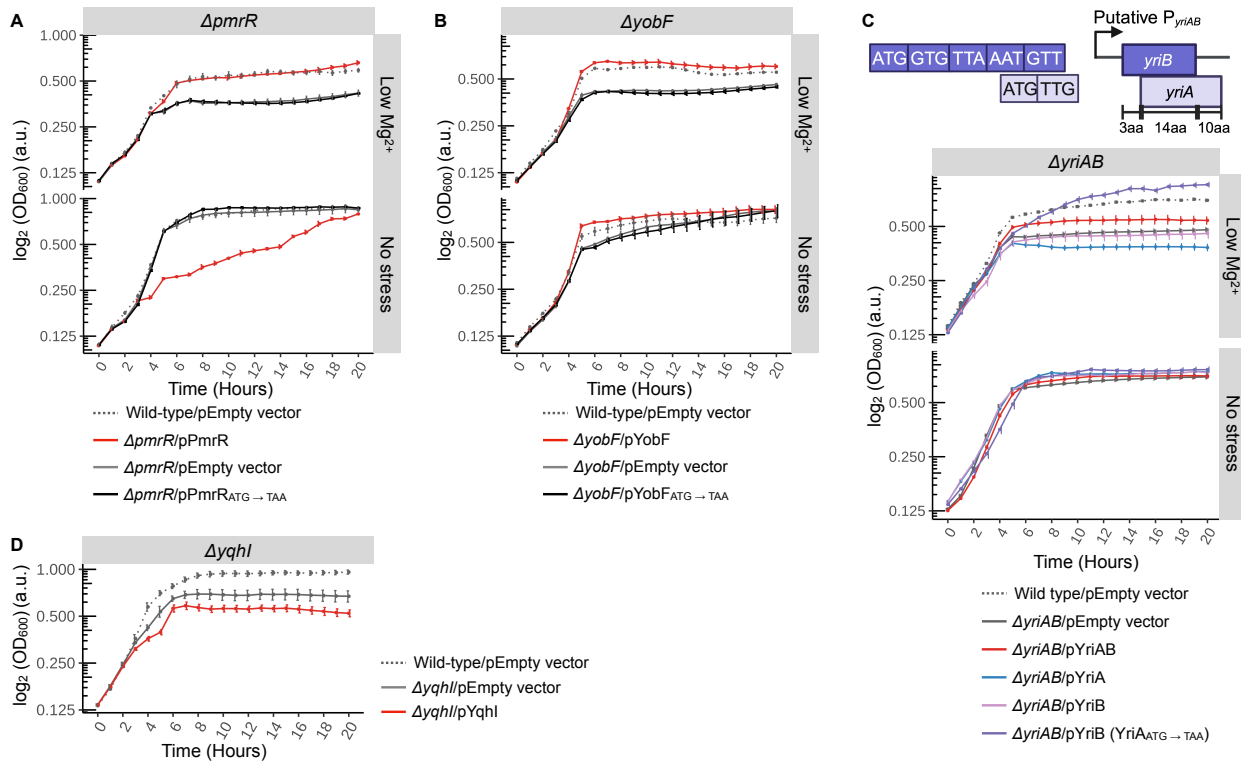
1122
1123



1124
1125

1126 **Figure 5. Effect of deleting genes encoding low Mg^{2+} stress-induced small proteins on**
1127 **bacterial growth.** Growth curves of wild-type *E. coli* MG1655 (gray) and mutants corresponding
1128 to the deletions of small protein-encoding genes (red) (strains SV64, AML67, SV35, SV31,
1129 SV41, SV57, SV59, SV33, SV29, SV25, SV39, SV43, SV58, SV37, SV27, and SV56, please
1130 see Table S1 and methods for strain details). Deletion mutants showing reduced growth yield
1131 compared to wild-type cells are highlighted in red. The cultures were grown in supplemented
1132 minimal A medium with no added magnesium and $50 \mu\text{g ml}^{-1}$ carbenicillin. Data represent
1133 averages and standard errors of means for four independent cultures.

1134



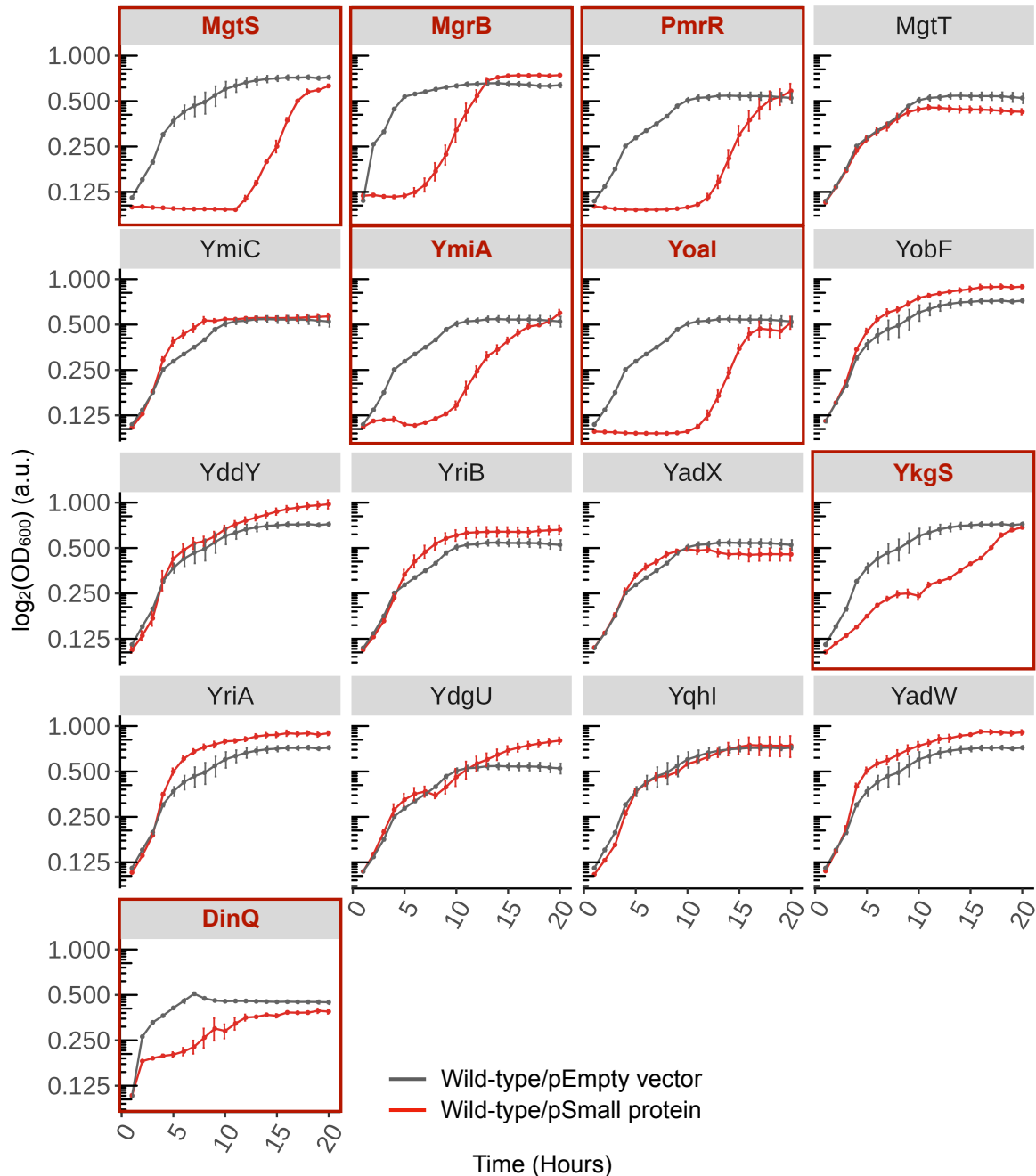
1135

1136

1137 **Figure 6. Complementation of growth phenotypes associated with *pmrR*, and *yobF***
 1138 **deletions.** (A-B) Growth curves of $\Delta pmrR$ (SV35), and $\Delta yobF$ (SV34) deletion strains carrying
 1139 an empty vector (pEB52, solid gray line) or complemented with plasmids encoding small
 1140 proteins pPmrR (pSV35) and pYobF (pJS5) (red line) or their variants pPmrR_{ATG→TAA} (pSV38),
 1141 and pYobF_{ATG→TAA} (pSV45) (black line), as indicated. Wild-type MG1655 cells containing the
 1142 empty vector are included as a control (dashed gray line). (C) Schematic of the overlapping
 1143 genes *yriAB* followed by the growth curves of gene deletion $\Delta yriAB$ (SV25) complemented with
 1144 pYriAB (pSV58), pYriA (pJS1), pYriB (pSV37), pYriB (YriA_{ATG→TAA}) (pSV59), or an empty vector
 1145 (pEB52, as indicated). (D) Growth curves of gene deletion $\Delta yqhl$ (SV37) carrying an empty
 1146 vector (pEB52), or complemented with a plasmid encoding Yqhl. Wild-type cells containing an
 1147 empty vector (pEB52, dashed gray line) are included as a control. For panels A-D, cultures were
 1148 grown in supplemented minimal A medium containing 50 $\mu\text{g ml}^{-1}$ carbenicillin and either no
 1149 added magnesium or 10 mM MgSO_4 as indicated. 500 μM IPTG was used for induction, except
 1150 for PmrR derivatives (pSV35 and pSV38), where 12.5 μM IPTG was used. Data represent
 1151 averages and standard errors of means for four independent cultures.

1152

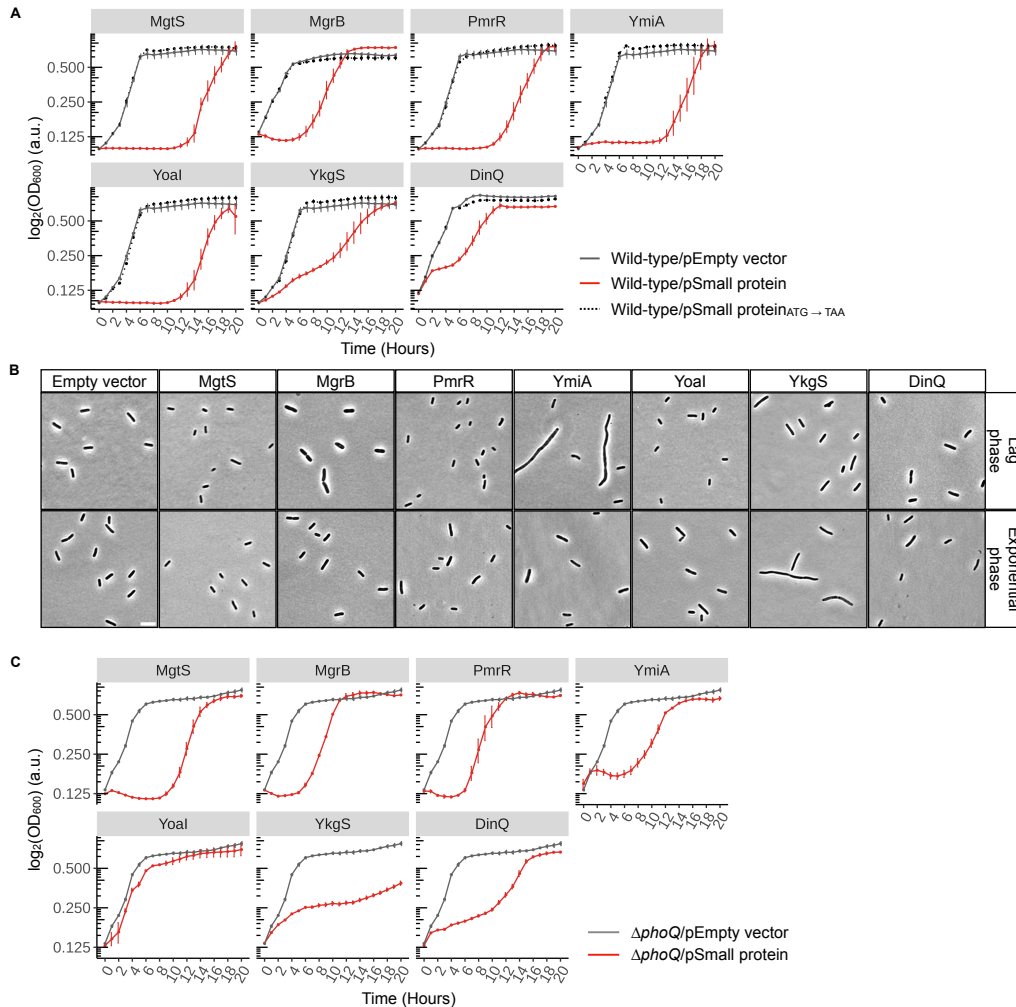
1153



1154
1155

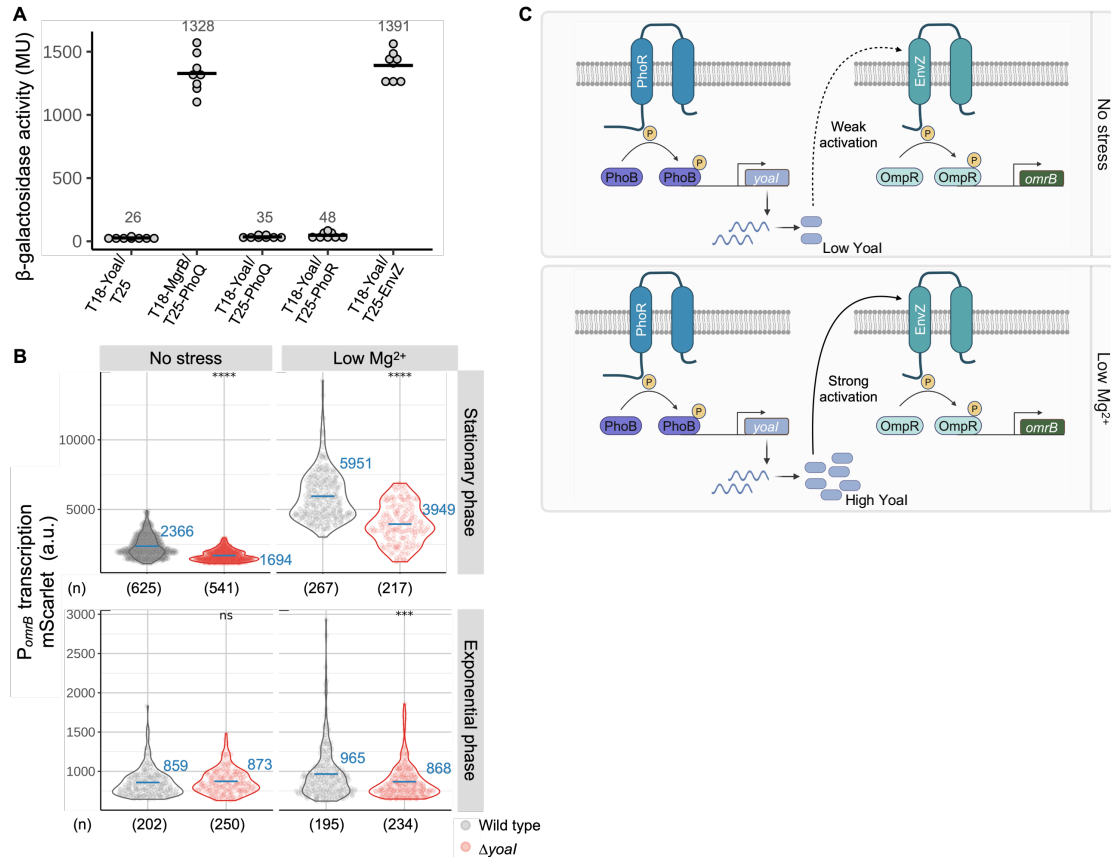
1156 **Figure 7. Effect of overexpression of low Mg^{2+} stress-induced small proteins on growth.**

1157 Growth curves of *E. coli* MG1655 cells expressing each of the 17 stress-specific small proteins
1158 (encoded by plasmids pSV30-37, pSV54, pSV60, or pJS1-7; please see Table S2 for details)
1159 are represented in red, while cells carrying the empty vector (pEB52 and pBAD24) are shown in
1160 gray. The cells were grown under low Mg^{2+} stress (no added magnesium). Small proteins whose
1161 expression causes a growth defect or delay are highlighted in red. The cultures were grown in
1162 supplemented minimal A medium with $50 \mu\text{g ml}^{-1}$ carbenicillin. $500 \mu\text{M}$ IPTG was used as an
1163 inducer for all P_{trc} -based vectors, in the case of pSV54 and pBAD24, 0.5% arabinose was used.
1164 Data represent averages and standard error of means for four independent cultures.



1165
 1166

1167 **Figure 8. Analysis of growth defect and cell size changes associated with overexpression**
 1168 **of small proteins, MgtS, MgrB, PmrR, YkgS, Yoal, YmiA and DinQ.** (A) Growth curves of *E.*
 1169 *coli* MG1655 cells expressing the indicated small proteins (red line) and empty vector control
 1170 (pEB52 or pBAD24, gray line). The start codon (ATG) of each small protein is mutated to a stop
 1171 codon (TAA), and the growth curves of cells expressing these constructs are depicted in dotted
 1172 black. Plasmids pSV34-36, pSV38-42, pSV54-55, pSV60-61, or pJS4, pJS7 were used as
 1173 applicable, please see Table S2 for details. Data represent averages and standard error of
 1174 means for four independent cultures. (B) Representative phase contrast micrographs of the
 1175 cells expressing small proteins during lag (top panel) and exponential phase (bottom panel)
 1176 after induction, scale bar = 5 μm. Data is representative of three independent cultures. (C)
 1177 Growth curves of Δ*phoQ* (TIM202) cells expressing the indicated small proteins (red line) and
 1178 empty vector control (pEB52 or pBAD24, gray line). Plasmids pSV34-36, pSV54, pSV60, pJS4,
 1179 and pJS7 were used as applicable, please see Table S2 for details. Data represent averages
 1180 and standard error of means for four independent cultures. For panels A-C, the cultures were
 1181 grown in supplemented minimal A medium containing no added magnesium, 50 μg ml⁻¹
 1182 carbenicillin, and 500 μM IPTG for all P_{trc}-based vectors, except for pSV54 and pBAD24, where
 1183 0.5% arabinose was utilized instead. (Also see Figures S6 and S7)



1184
1185

1186 **Figure 9. Effect of Yoal expression on the EnvZ-OmpR system.** (A) BACTH assays were
1187 conducted using cells harboring T18 and T25 fusion proteins of *B. pertussis* adenylyl cyclase, in
1188 a *cyaA* mutant BACTH host strain, SAM85, which contains *lac*^R. β -gal activity was measured to
1189 assess interactions between T18-Yoal (pSV47) and T25-linked PhoQ (pAL27), PhoR (pSY68),
1190 or EnvZ (pKK14). T25 alone was used as a negative control, and T18-MgrB (pAL33) combined
1191 with T25-PhoQ (pAL27) was used as a positive control. The cultures were grown in LB medium
1192 containing 50 $\mu\text{g ml}^{-1}$ kanamycin, 100 $\mu\text{g ml}^{-1}$ carbenicillin, and 500 μM IPTG. Each circle
1193 represents a biological replicate, with the mean values from two independent experiments
1194 indicated, expressed in Miller units (MU). (B) Transcriptional activity of *omrB* was measured as
1195 a function of mScarlet reporter fluorescence in *E. coli* MG1655 (SV97), and Δ yoal (SV95)
1196 strains at the stationary phase (top panel) and exponential phase (bottom panel). Each circle
1197 corresponds to a single cell. Δ yoal cells are depicted in red, while wild-type cells are in gray.
1198 The cultures were grown in supplemented minimal A medium containing either 10mM (no
1199 stress) or no added magnesium (Mg^{2+} stress). Data are derived from three independent
1200 replicates and the number of cells analyzed is indicated by (n). Mean fluorescence is shown
1201 with blue bars and is noted in blue text. P-values indicate the results of a t-test when Δ yoal cells
1202 were compared to the wild-type cells, **** $P \leq 0.0001$, *** $P \leq 0.001$, and “ns” = $P > 0.05$. (C)
1203 Schematic representation of small membrane protein Yoal as a connector of the two-component
1204 systems, PhoR-PhoB and EnvZ-OmpR. Yoal is transcriptionally controlled by the PhoR-PhoB
1205 system, under low Mg^{2+} Yoal protein levels increase and it binds sensor kinase EnvZ and
1206 modulates signaling through the EnvZ-OmpR pathway. (Also see Figure S9)

1207
1208
1209
1210

Table 1. Summary of the 17 small proteins induced under low magnesium stress in *E. coli*. Small proteins with at least a 3-fold increase in reads mapping to the translation initiation site under low Mg²⁺ relative to no stress are indicated below. The analysis of transcriptional regulation, localization, and characterization of deletion and overexpression phenotypes is summarized.

Small protein	Transcriptional Regulation		Membrane localization	Phenotypic characterization	
				Deletion	Overexpression
MgtS	+	PhoQP-dependent, in an operon <i>mgtS-mgtT</i>	Y	No effect	Growth defect (prolonged lag phase) & decrease in cell size during lag and exponential phase
MgrB	+	PhoQP-dependent	Y	Increase in cell length, and change in colony morphology (larger, rough, translucent colonies) ²⁸	Growth defect (prolonged lag phase) & increase in cell size during lag phase
PmrR	+	BasSR-dependent ^{33,35}	Y	Growth defect (lower yield at saturation)	Growth defect (prolonged lag phase) & decrease in cell size during lag phase
MgtT	+	PhoQP-dependent, in an operon <i>mgtS-mgtT</i>	N	No effect	No effect
YmiC	+	PhoQP-dependent, in an operon <i>ymiA-yxiX-ymiC</i>	Y	No effect	No effect
YmiA	+		Y	No effect	Growth defect (prolonged lag phase) & increase in cell length during lag phase
Yoal	-	PhoRB-dependent ⁴⁷⁻⁴⁹	Y	No effect	Growth defect (prolonged lag phase) & decrease in cell size during lag phase
YobF	-		Y	Growth defect (lower yield at saturation)	No effect
YddY	+	PhoQP-dependent	N	No effect	No effect
YriB	+	PhoQP-dependent, in an operon <i>yriA-yriB</i>	N	Growth defect (lower yield at saturation)	No effect
YadX	+	PhoQP-dependent, in an operon <i>yadX-clcA-yadW</i>	N	No effect	No effect
YkgS	-		N	No effect	Growth defect (slow growth) & increase in cell length during exponential phase
YriA	+	PhoQP-dependent, in an operon <i>yriA-yriB</i>	N	No effect	No effect
YdgU	+	PhoQP-dependent, in an operon <i>asr-ydgU</i>	Y	No effect	No effect
Yqhl	+		N	No effect	No effect
YadW	+	PhoQP-dependent, in an operon <i>yadX-clcA-yadW</i>	N	No effect	No effect
DinQ	-		Y	No effect	Growth defect (slow growth)

1211
1212
1213

+ indicates transcriptional upregulation under magnesium stress vs. no stress; - indicates no change in transcriptional activity under magnesium stress vs. no stress; 'Y' indicates proteins predicted to contain membrane helices using TMHMM⁵¹ and Phobius⁵³ and shown to localize to the membrane; 'N' indicates a lack of membrane helices based on bioinformatic prediction

1214 **Table 2. Analysis of transcriptional regulation of low magnesium stress-induced small proteins.** Putative regulatory regions
 1215 corresponding to the stress-induced small proteins and their dependence on PhoQ-PhoP signaling system.
 1216

Small protein	Putative region(s) of interest	Length of region (bp)	Genomic location in <i>E. coli</i> MG1655	PhoQP-dependence	Low Mg ²⁺ stress-specific expression reported in
MgrB	P _{mgrB}	500	1908767 - 1909266	Yes	²⁷
MgtS, MgtT	P _{mgtS-mgtT}	405	1622241 - 1622645	Yes	³¹ , This study
ProP, PmrR	P _{proP-pmrR}	500	4330002 - 4330501	No	This study
PmrR	P _{pmrR}	500	4331616 - 4332115	No	This study
YmiA, YmiC	P _{ymlA-ymlX-ymlC}	500	1334648 - 1335147	Yes	This study
YmiC	P _{ymlC}	272	1335300 - 1335571	No	This study
Yoal	P _{yoal}	500	1874183 - 1874682	No	This study
YobF	P _{yobF}	500	1907592 - 1908091	No	This study
YddY	P _{yddY}	500	1567220 - 1567719	Yes	This study
YriA	P _{yriA-yriB}	500	3640697 - 3641196	Yes	This study
YriB					
YadX, YadW	P _{yadX-clcA-yadW}	500	174548 - 175047	Yes	This study
YadW	P _{yadW}	500	176052 - 176551	No	This study
YkgS	P _{ykgS}	230	290280 - 290509	No	This study
YdgU	P _{asr-ydgU}	400	1670976 - 1671375	Yes	This study
	P _{ydgU}	500	1671277 - 1671776	No	This study
Yqhl	P _{yqhl}	500	3147741 - 3148240	No	This study
DinQ	P _{dinQ}	272	3647789 - 3648060	No	This study

Supporting Information (Vellappan *et al.*, 2024)

Table S1. List of strains used in the study

Strain	Genotype	Source or Reference
AML67	MG1655 $\Delta mgrB::(FRT\text{-}kan\text{-}FRT) \Delta lacZ \lambda_{att::}(P_{mgtA}\text{-}lacZ\ cat)$	1
<i>E. cloni</i>	$F^- mcrA \Delta(mrr\text{-}hsdRMS\text{-}mcrBC) endA1 recA1 \phi 80 lacZ \Delta M15 \Delta lacX74 araD139 \Delta(ara\text{-}leu)7697 galU galK rpsL nupG tonA (attL\ araC\text{-}PBAD\text{-}trfA250 bla attR) \lambda^-$	Lucigen Corporation
<i>E. coli</i> K-12 MG1655	$F^- \lambda^- ilvG^- rfb\text{-}50 rph\text{-}1$	<i>E. coli</i> Genetic Stock Center, CGSC# 7740
GSO195	MG1655 $\Delta dinQ::(FRT\text{-}kan\text{-}FRT)$	2
GSO219	MG1655 $\Delta ydgU::(FRT\text{-}kan\text{-}FRT)$	2
GSO225	MG1655 $\Delta ymiA::(FRT\text{-}kan\text{-}FRT)$	2
GSO232	MG1655 $\Delta yoal::(FRT\text{-}kan\text{-}FRT)$	2
GSO317	MG1655 $yoal\text{-}SPA::kan$	3
JM2110	MG1508 $mhpR\text{-}P_{LtetO\text{-}1\text{-}omrB\text{-}104+8}\text{-}lacZ_{+28} \Delta mini\text{-}\lambda\text{-}Tet$	Guillier lab stock (unpublished)
JM2113	OK510 $argG\text{-}[TT1\text{-}P_{LtetO\text{-}1\text{-}omrB\text{-}104+8}\text{-}mScarlet_{+4}\text{-}FRT\text{-}nptII\text{-}FRT\text{-}TT2]\text{-}yhbX, \Delta mini\text{-}\lambda\text{-}Tet$	Guillier lab stock (unpublished)
JW2162	BW25113 $\Delta lpxT::(FRT\text{-}kan\text{-}FRT)$	4
MDG147	MG1655 (seq) $\Phi(ompF^+\text{-}yfp^+)30 \Phi(ompC^+\text{-}cfp^+)31$	5
MG1508	MG1655 $mal::lacI^R, mini\text{-}\lambda\text{-}Tet, mhpR\text{-}P_{LtetO\text{-}1}\text{-}cat\text{-}sacB\text{-}lacZ$	6
OK510	MG1432 $argG\text{-}[TT1\text{-}P_{LtetO\text{-}1}\text{(no -10)}\text{-}sacB\text{-}cat\text{-}mScarlet\text{(no ATG)}\text{-}FRT\text{-}nptII\text{-}FRT\text{-}TT2]\text{-}yhbX$	7
SAM85	$F^+ lacI^R \Delta M15 Tn10(Tet^R), cya\text{-}99 araD139 galE15 galK16 rpsL1 (Str^R) hsdR2 mcrA1 mcrB1$. Strain prepared by conjugation: BTH101 (F-) x XL1-Blue (F+).	8
SV25	MG1655 $\Delta yriAB::(FRT\text{-}kan\text{-}FRT)$, coordinates deleted: 3,640,612 - 3,640,699	This study
SV27	MG1655 $\Delta yadW::(FRT\text{-}kan\text{-}FRT)$, coordinates deleted: 176,552 - 176,594	This study
SV29	MG1655 $\Delta yddY::(FRT\text{-}kan\text{-}FRT)$, coordinates deleted: 1,567,219 - 1,567,178	This study
SV31	MG1655 $\Delta mgtT::(FRT\text{-}kan\text{-}FRT)$, coordinates deleted: 1,622,742 - 1,622,816	This study

SV33	MG1655 $\Delta yobF::$ (FRT-kan-FRT), coordinates deleted: 1,907,451 - 1,907,573	This study
SV35	MG1655 $\Delta pmrR::$ (FRT-kan-FRT), coordinates deleted: 4,332,116 - 4,332,178	This study
SV37	MG1655 $\Delta yqhl::$ (FRT-kan-FRT), coordinates deleted: 3,147,597 - 3,147,740	This study
SV39	MG1655 $\Delta yadX::$ (FRT-kan-FRT), coordinates deleted: 175,048 - 175,077	This study
SV41	MG1655 $\Delta ymiC::$ (FRT-kan-FRT), coordinates deleted: 1,335,572 - 1,335,667	This study
SV43	MG1655 $\Delta ykgS::$ (FRT-kan-FRT), coordinates deleted: 290,510 - 290,641	This study
SV47	MG1655 $\Delta lpxT::$ (FRT-kan-FRT)	This study
SV50	MG1655 $\Delta pmrR$	This study
SV54	MG1655 $\Delta lpxT::$ (FRT-kan-FRT) $\Delta pmrR$	This study
SV56	MG1655 $\Delta dinQ::$ (FRT-kan-FRT)	This study
SV57	MG1655 $\Delta ymiA::$ (FRT-kan-FRT)	This study
SV58	MG1655 $\Delta ydgU::$ (FRT-kan-FRT)	This study
SV59	MG1655 $\Delta yoal::$ (FRT-kan-FRT)	This study
SV60	MG1655 $yoal$ -SPA-kan $\Delta lacZ \Delta phoQ$	This study
SV64	MG1655 $\Delta mgtS::$ (FRT-kan-FRT), coordinates deleted: 1,622,646 - 1,622,735	This study
SV91	MG1655 $\Delta yoal$	This study
SV95	MG1655 $argG$ -[TT1- $P_{LtetO-1-omrB-104+8}$ -mScarlet+4-FRT- $nptII$ -FRT-TT2]- $yhbX$, $\Delta mini$ - λ -Tet	This study
SV97	MG1655 $argG$ -[TT1- $P_{LtetO-1-omrB-104+8}$ -mScarlet+4-FRT- $nptII$ -FRT-TT2]- $yhbX$, $\Delta mini$ - λ -Tet $\Delta yoal$	This study
TIM92	MG1655 $\lambda_{att}::$ ($P_{mgrB-yfp}$) $HK_{att}::$ ($P_{tetA-cfp}$)	9
TIM100	MG1655 $\Delta phoQ \lambda_{att}::$ ($P_{mgrB-yfp}$) $HK_{att}::$ ($P_{tetA-cfp}$)	9
TIM202	MG1655 $\Delta lacZ::$ FRT $\Delta phoQ::$ FRT	10
TOP10	$F^- mcrA \Delta(mrr-hsdRMS-mcrBC) \phi 80 lacZ \Delta M15 \Delta lacX74 recA1 araD139 \Delta(ara-leu)7697 galU galK \lambda^- rpsL(StrR) endA1 nupG$	Invitrogen
XL1-Blue	$recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIq \Delta M15 Tn10 (Tetr)]$	11

Φ and Ψ denote transcriptional and translational fusions, respectively

Table S2. List of plasmids used in the study

Plasmid	Relevant genotype	Source or reference
pAL27	pKT25 $P_{lac-cyaA_{T25}}-phoQ$, Kan ^R	1
pAL33	pUT18C $P_{lac-cyaA_{T18}}-mgrB$, Amp ^R	1
pAL38	pEB52 $P_{trc-gfpA206K}-mgrB$, Amp ^R	1
pAL39	pEB52 $P_{trc-gfpA206K}$, Amp ^R	1
pBAD24	pMB1-derived plasmid, <i>araBAD</i> , Amp ^R	12
pCP20	ori(pSC101) <i>rep101(ts) bla</i> λP_R -FLP $\lambda cl(ts)$ cat. Amp ^R , Cam ^R	13
pEB52	pTrc99a with the NcoI site removed, Amp ^R	Goulian lab collection
pJS1	pEB52 $P_{trc-yriA}$. Amp ^R	This study
pJS2	pEB52 $P_{trc-yadW}$. Amp ^R	This study
pJS3	pEB52 $P_{trc-yqhl}$. Amp ^R	This study
pJS4	pEB52 $P_{trc-ykgS}$. Amp ^R	This study
pJS5	pEB52 $P_{trc-yobF}$. Amp ^R	This study
pJS6	pEB52 $P_{trc-yddY}$. Amp ^R	This study
pJS7	pEB52 $P_{trc-mgtS}$. Amp ^R	This study
pKD13	ori(R6K) FRT-kan-FRT, Amp ^R	14
pKD46	ori(pSC101) <i>rep101(ts) P_{araB-gam-betexo} araC</i> , Amp ^R	14
pKK14	pKT25 $P_{lac-cyaA_{T25}}-EnvZ$, Kan ^R	Goulian lab collection
pKT25	<i>ori_{p15A} P_{lac-cyaA_{T25}}-MCS}</i> , Kan ^R	Euromedex
pMR120	A derivative of pSMART VC (Lucigen) containing two copies of the <i>rrnB</i> transcription terminator, $P_{putative promoter-yfpA206K}-P_{tetA}-cfp$, Cam ^R	Goulian lab collection
pPJ1	pEB52 $P_{trc-gfpA206K}-mgtT$, Amp ^R	This study
pPJ2	pEB52 $P_{trc-gfpA206K}-pmrR$, Amp ^R	This study
pPJ3	pEB52 $P_{trc-gfpA206K}-ydgU$, Amp ^R	This study
pPJ4	pEB52 $P_{trc-yoal-gfpA206K}$, Amp ^R	This study
pPJ5	pEB52 $P_{trc-gfpA206K}-yqhl$, Amp ^R	This study
pPJ6	pEB52 $P_{trc-gfpA206K}-yobF$, Amp ^R	This study
pPJ7	pEB52 $P_{trc-gfpA206K}-yriA$, Amp ^R	This study
pPJ8	pEB52 $P_{trc-gfpA206K}-yddY$, Amp ^R	This study

pPJ9	pEB52 $P_{trc-gfpA206K-yadW}$, Amp ^R	This study
pPJ10	pEB52 $P_{trc-gfpA206K-yadX}$, Amp ^R	This study
pPJ11	pEB52 $P_{trc-gfpA206K-yriB}$, Amp ^R	This study
pPJ12	pEB52 $P_{trc-gfpA206K-ymiA}$, Amp ^R	This study
pPJ13	pEB52 $P_{trc-gfpA206K-ymiC}$, Amp ^R	This study
pPJ14	pMR120 putative $P_{pmrR-yfp}$, Cam ^R	This study
pPJ16	pMR120 putative $P_{asr-ydgU-yfp}$, Cam ^R	This study
pPJ17	pMR120 putative $P_{ydgU-yfp}$, Cam ^R	This study
pPJ18	pMR120 putative $P_{yoal-yfp}$, Cam ^R	This study
pPJ19	pMR120 putative $P_{yqhl-yfp}$, Cam ^R	This study
pPJ20	pMR120 putative $P_{yriA-yriB-yfp}$, Cam ^R	This study
pPJ23	pMR120 putative $P_{yadX-clcA-yadW-yfp}$, Cam ^R	This study
pSMART	pSMART VC <i>Bam</i> HI (BAC) with single-copy replication origin <i>ori2 repE IncC parABC, oriV cat</i> Cam ^R	Lucigen Corporation
pSV14	pEB52 $P_{trc-yobF-GGSG-6XHis}$, Amp ^R	This study
pSV16	pMR120 putative $P_{ykgS-yfp}$, Cam ^R	This study
pSV17	pMR120 putative $P_{proP-pmrR-yfp}$, Cam ^R	This study
pSV18	pMR120 putative $P_{yadW-yfp}$, Cam ^R	This study
pSV19	pMR120 putative $P_{ymiC-yfp}$, Cam ^R	This study
pSV20	pMR120 putative $P_{ymiA-yciX-yimiC-yfp}$, Cam ^R	This study
pSV21	pMR120 putative $P_{dinQ-yfp}$, Cam ^R	This study
pSV24	pMR120 putative $P_{yobF-yfp}$, Cam ^R	This study
pSV25	pEB52 $P_{trc-gfpA206K-ykgS}$, Amp ^R	This study
pSV26	pEB52 $P_{trc-pmrR-GGSG-6XHis}$, Amp ^R	This study
pSV28	pMR120 putative $P_{yddY-yfp}$, Cam ^R	This study
pSV29	pMR120 putative $P_{mgtS-mgtT-yfp}$, Cam ^R	This study
pSV30	pEB52 $P_{trc-mgtT}$. Amp ^R	This study
pSV31	pEB52 $P_{trc-ydgU}$. Amp ^R	This study
pSV32	pEB52 $P_{trc-yadX}$. Amp ^R	This study
pSV33	pEB52 $P_{trc-yimiC}$. Amp ^R	This study
pSV34	pEB52 $P_{trc-yoal}$. Amp ^R	This study
pSV35	pEB52 $P_{trc-pmrR}$. Amp ^R	This study
pSV36	pEB52 $P_{trc-yimiA}$. Amp ^R	This study

pSV37	pEB52 P_{trc} - <i>yriB</i> , Amp ^R	This study
pSV38	pEB52 P_{trc} - <i>pmrR</i> _{ATG->TAA} where the start codon ATG is substituted with a stop TAA, Amp ^R	This study
pSV39	pEB52 P_{trc} - <i>yoal</i> _{ATG->TAA} where the start codon ATG is substituted with a stop TAA, Amp ^R	This study
pSV40	pEB52 P_{trc} - <i>mgtS</i> _{ATG->TAA} where the start codon ATG is substituted with a stop TAA, Amp ^R	This study
pSV41	pEB52 P_{trc} - <i>ykgS</i> _{ATG->TAA} where the start codon ATG is substituted with a stop TAA, Amp ^R	This study
pSV42	pEB52 P_{trc} - <i>ymiA</i> _{ATG->TAA} where the start codon ATG is substituted with a stop TAA, Amp ^R	This study
pSV45	pEB52 P_{trc} - <i>yobF</i> _{ATG->TAA} where the start codon ATG is substituted with a stop TAA, Amp ^R	This study
pSV47	pUT18C P_{lac} - <i>cya</i> _{T18} - <i>yoal</i> , Amp ^R	This study
pSV54	pBAD24 P_{araBAD} - <i>dinQ</i> , Amp ^R	This study
pSV55	pBAD24 P_{araBAD} - <i>dinQ</i> _{ATG->TAA} where the start codon ATG is substituted with a stop TAA, Amp ^R	This study
pSV58	pEB52 P_{trc} - <i>yriAB</i> , Amp ^R	This study
pSV59	pEB52 P_{trc} - <i>yriB</i> _{ATG->TAA} where the start codon ATG is substituted with a stop TAA, Amp ^R	This study
pSV60	pEB52 P_{trc} - <i>mgrB</i> , Amp ^R	This study
pSV61	pEB52 P_{trc} - <i>mgrB</i> _{ATG->TAA} where the start codon ATG is substituted with a stop TAA, Amp ^R	This study
pSY68	pKT25 P_{lac} - <i>cya</i> _{T25} - <i>phoR</i> , Kan ^R	8
pTrc99a	<i>lacI</i> ^q , P_{trc} -MCS, Amp ^R	15
pUT18C	pUC19 P_{lac} - <i>cya</i> _{T18} -MCS, Amp ^R	Euromedex

Amp^R = carries a carbenicillin/ampicillin-resistance marker; Cam^R = carries a chloramphenicol-resistance marker; Kan^R = carries a kanamycin-resistance marker

Table S3. List of primers used in the study

Primer	Sequence (5'-> 3')	Use
C-term-pmrR-F1	AGCTTTCTTTATATCTGGTTTGCCACGTACGGCGGC AGCGGCCACCATCACCATCACCATTAAGCTTAATTA GCTGACCTACTAGAGTCG	Cloning of pSV26
C-term-pmrR-R1	GCTGACCACCAGCACGCTGAACACGGTAGTTAAAC TTTCATAAACACGGTTTTTCATGCTTAATTTCTCCTC TTTAATTCTAGGTACCCG	Cloning of pSV26
dinQ_del_screen_1	aagatgggggcaacaaaaaag	Validation of GSO195
dinQ_del_screen_2	ttcactctggcaatgcgcata	Validation of GSO195
dinQ_F1	ATATGAATTCCATCAATGCTAATACCATACTGAACT ATTGCA	Cloning of pSV21
dinQ_R1	ATATGGATCCCCGTTTTCTCCATGCGATGGAG	Cloning of pSV21
dinQ_pBAD_R	CAGCGCAATTAACGCCCTAGAACGATGATTGCTTT ATCAATcatTGAATTCCTCCTGCTAGCCCAA	Cloning of pSV54
dinQ_pBAD_F	CTGGAAGTATCCGCTTTCTGCTTCAGCTTCTGAAC TAAGGTACCCGGGGATCCTCTAG	Cloning of pSV54
dinQ_stop_F	TAAATTGATAAAGCAATCATCGTTCTAGGGG	Cloning of pSV55
dinQ_stop_R	TGAATTCCTCCTGCTAGCCCA	Cloning of pSV55
F_yoal_BATCH	ACTGTCTAGAGatgAACGATCAAATGTTTGTGAGAC AC	Cloning of pSV47
Fp_Inv_PCR_yobF	TAAGCTTAATTAGCTGACCTACTAGAGTCGAC	Cloning of pSV14
JA1	GCGTACGCAATCAAAATCCCCAGCCAATACAacatTTAACACCATg cttaatttctcctctttaattctaggtaccg	Cloning of pSV58
JA2	AGACCGTAGGCCAGATAAGGTGTTTACGCTGATCAGGtaaGCTT AATTAGCTGACCTactagagtcgac	Cloning of pSV58
JS4	taaTTGTATTGGCTGGGGATTTGATTGC	Cloning of pSV59
lpxT_del_F	gacagccgtattgagctgattcc	Validation of JW2162
lpxT_del_R	aaagtatacagaaagtaataagcgggg	Validation of JW2162
mgrB_forward	atgaaaaagtttcgatgggtcgttct	Cloning of pSV60
mgrB_stop3	TAAaaaaagtttcgatgggtcgttctg	Cloning of pSV61

MgtS_del_F	AAAATTAAGGTAAGCGAGGAAACACACCACACCATA AACGGAGGCAAATAattccgggatccgtcgacctgcagttcgaa gttcctatt	Construction of SV64
MgtS_del_R	AACTGTAACAAGGGGCCGGTTAGGTGAGGGATTAT CTCCGTTcatTAGTctgtaggctggagctgcttcaagttcctatact	Construction of SV64
mgtS_del_screen_1	cccgcgctttgtgatttaagtc	Validation of SV64 together with primer mgtT_del_screen2
mgtS_forward_2_correct	TCTGTTTTCTGGCCGCGTATTTTCAGCCACAAATGG GATGACTaaTAAGCTTAATTAGCTGACCTactagagtgcga c	Cloning of pJS7
mgtS_reverse	AAATAAAATTATTCCCAGTACGGCCATAAAAACATTC ATATTACCCAGcatgcttaattctctctttaattctaggtaccg	Cloning of pJS7
mgtS_stop	TAACTGGGTAATTAATAATGTTTTTATGGCCGTACTG GGAATAATTTTATTTTCTGG	Cloning of pSV40
mgtT_del_F	TATTTTCTGGTTTTCTGGCCGCGTATTTTCAGCCACA AATGGGATGACTAAattccgggatccgtcgacc	Construction of SV31
mgtT_del_R	CAAATCTATCCATGCAAGCATcaCCGCCGTTTACT GGCGGTTTTTTTTgttaggctggagctgcttcg	Construction of SV31
mgtT_del_screen1	CACACCACACCATAAACGGAGG	Validation of SV31
mgtT_del_screen2	ACCGGAAGAAATCGCTGCATG	Validation of SV31
mgtT_F	GAGAATTCTCAGCTGCGGTATTTACTGTCCG	Cloning of pSV29
mgtT_R	GGGGATCCTAGTCATCCATTTGTGGCTGAAATAC G	Cloning of pPJ1
mgtT_R1	ATATGGATCCTATTTGCCTCCGTTTATGGTGTGGTG	Cloning of putative pSV29
mgtT-forward	ATGAACGGAGATAATCCCTCACCTAAC	Cloning of pSV30
N_term_pmrR_forward	ATGAAAACCGTGTTTATGAAAGTTTAACTACCG	Cloning of pSV35
pEB52-rev	GCTTAATTTCTCCTCTTTAATTCTAGGTACCCG	When targeting N-terminal-tagged small proteins, this primer, along with a small protein-specific forward primer, removes the tags by binding to the pEB52 backbone

phoB_del_F	cgaaaaagcatgggcgcgatta	Validation of JW0389
phoB_del_R	agggcaggtaaccaaaaaatgcac	Validation of JW0389
pmrR Primer 1	CTGGTGGTCAGCAGCTTTCTTTATATCTGGTTTGCC ACGTA CTGATAAGCTTAATTAGCTGACCTactagagt	Cloning of pPJ2
pmrR Primer 2	CACGCTGAACACGGTAGTTAACTTTTCATAAACACG GTTTTTCATttgtatagttcatccatgccatgtg	Cloning of pPJ2
pmrR_del_F	CTTAATCTCTGACGCGCATACTCTCCTCCAGGTTAA CGGAGGAGAGTGCAattccggggatccgtcgacc	Construction of SV35
pmrR_del_R	GCCTGGGTACGGCTGAAGAAAGAtcaGTACGTGGCA AACCAGATATAAGttaggctggagctgctcg	Construction of SV35
pmrR_del_screen1	GCGAATTGATGAATAAGCTGAAACGG	Validation of SV35
pmrR_del_screen2	CGTTTGTACGTATGGACAGCCG	Validation of SV35
pmrR_stop	TAaAAAAACCGTGTATTATGAAAGTTTAACTACCG	Cloning of pSV38
pmrR1_F	GAGAATTCTGGCTTCTACCTTGCCAGCG	Cloning of pPJ14
pmrR1_R	GGGGATCCTGCACTCTCCTCCGTAACTG	Cloning of pPJ14
pmrR2_F	GAGAATTCCTGACCGCGCTACTATTACC	Cloning of pSV17
pmrR2_R	GGGGATCCAGCTTTCCTCGCAGAGTTGG	Cloning of pSV17
pSMART SV_19	GACCATAACCGAAAGTAGTGAC	Sequencing primer for pMR120 based vectors
R_yoal_BATCH	TACTGAATTCctaGCCGGTCCGTTTCGATAAGAAG	Cloning of pSV47
Rp_Inv_PCR_yobF	ATGGTGATGGTGATGGTGGCCGCTGCCGCCGATTG TTTTCTTCGCCCGCAGG	Cloning of pSV14
SA26R	CATCCGCCAAAACAGCCAAG	Sequencing primer for pEB52 based vectors
yadW Primer 1	TGCCGATGTCGTTTGGAGCAAATATGAGTGATAAG CTTAATTAGCTGACCTactagagt	Cloning of pPJ9
yadW Primer 2	ATTGGGCAAATTCTAACCCAATAATAATCGCCATttgt atagttcatccatgccatgtg	Cloning of pPJ9
yadW_del_F	GCCGCATCAGCCAGCGAGAATACTTGAACGAAATA CCAGGGTATTAGATAattccggggatccgtcgacc	Construction of SV27
yadW_del_R	TCGGTAACTCCAGCGGCAGTGCTACGTCAAtcaCTC ATATTTTGCTCCAAACGttaggctggagctgctcg	Construction of SV27

yadW_del_scre en1	CGGGAAACCGCTATACTCGG	Validation of SV27
yadW_del_scre en2	GGTCAAACCATACTGGAAGCCG	Validation of SV27
yadW_forward	atgGCGATTATTATTGGGTTAGAATTTGCC	Cloning of pJS2
yadW1_F	GAGAATTCTTCCATTTTCGGTTGGTGCACCAA	Cloning of pSV18
yadW1_R	GGGGATCCCAATGATCACTTATTGGTCATACAAAT AAGATGAC	Cloning of pSV18
yadW2_F	GAGAATTCACCATGCGCACCATATCCATGG	Cloning of pPJ23
yadW2_R	GGGGATCCGAAAATCCTTTGCAAAGCGTAATGTTTC AAAT	Cloning of pPJ23
yadX Primer 1	TCATCTTATTTGTATGACCAATAATAAGCTTAATTAG CTGACCTactagagt	Cloning of pPJ10
yadX Primer 2	CGGCATTTTACTCGCACGTTCCATtttgtatagttcatccatgc catgtg	Cloning of pPJ10
yadX_del_F	AGTATAAAAGTTTTGTGCATTTGAAACATTACGCTTT GCAAAGGATTTTCattccgggatccgtcgacc	Construction of SV39
yadX_del_R	AGAGGGAGTATCAGTTTTTCATCCAATGATCACttaTT GGTCATACAAATAtgtaggctggagctgcttcg	Construction of SV39
yadX_del_scre n1	CATGGAGGGTTCCTGATTCGTAG	Validation of SV39
yadX_del_scre n2	GCCATAAACAAAATGGCTAACGGG	Validation of SV39
yadX-forward	ATGGAACGTGCGAGTAAAATGC	Cloning of pSV32
yddY Primer 1	GCACGTTGCGTTTCATTTTGATAAGCTTAATTAGCT GACCTactagagt	Cloning of pPJ8
yddY Primer 2	GAGATCGACTAACTGCACCATtttgtatagttcatccatgccatg tg	Cloning of pPJ8
yddY_del_F	GTCCTAAATCGCTTATTTCTTTTCAGTATATCTTCAT ATTTTCAGGAGAATattccgggatccgtcgacc	Construction of SV29
yddY_del_R	AATACATCTCATAATTCACACCCTTATAAGGCTGG GAAATCAGACGGAAAtgtaggctggagctgcttcg	Construction of SV29
yddY_del_scre n1	CATACGATATTCGCTGTCACCG	Validation of SV29
yddY_del_scre n2	CCGCGGCTTTAGCACGAAT	Validation of SV29
yddY_F	GAGAATTCCTGTATATCGGTATCGCCTTCTATAAAG TGG	Cloning of pSV28

yddY_forward	ATGGTGCAGTTAGTCGATCTCG	Cloning of pJS6
yddY_R	GGGGATCCATTCTCCTGAAATATGAAGATATACTGAAAG	Cloning of pSV28
ydgU Primer 1	ATTTTATGCGCACTGATTACCGCCCGTTTTTATCTTTCCTGATAAGCTTAATTAGCTGACCTactagagt	Cloning of pPJ3
ydgU Primer 2	AAGGATGATCAGAATGAACTCAAAGCGATAACGGCCACCATttgtatagttcatccatgccatgtg	Cloning of pPJ3
ydgU_del_screen_1	gcagcgaagaaacacgccaa	Validation of GSO219
ydgU_del_screen_2	caaagcagacgtcaaactaattgca	Validation of GSO219
ydgU-forward	ATGGTGGGCCGTTATCGCTT	Cloning of pSV31
ydgU1_F	ACTCGAGAATTCGAAACCAACCACTCACGGAAGTCTG	Cloning of pPJ17
ydgU1_R	CCGGGATCCACTACCCTCCGCTAAAGGCG	Cloning of pPJ17
ydgU2_F	ACTCGAGAATTCATACCCGTCCGGACTTATTGCC	Cloning of pPJ16
ydgU2_R	GGGGATCCTGTCATACCCTCAATTTGTTTTTTCATTTAACC	Cloning of pPJ16
ykgS_del_F2	CAGAAGCAGAAAGACATTGGATCGAATTCTACAACCAGGTCGAGTCAGAAattccgggatccgtcgacctgcagttcgaa gttcctatt	Construction of SV43
ykgS_del_R2	CAAAGTCATCGGGCATTATCTGAACATAAAACACTATCAATAAGTTGGAGtgtaggctggagctgcttccaagttcctatact	Construction of SV43
ykgS_del_screen_1	GGCAAACCCGTCCGTGTG	Validation of SV43
ykgS_del_screen_2	AAGTCGCTGTCGTTCTCAA	Validation of SV43
ykgS_F	ATATCTCGAGAAGTGTTTTTGTATAAATCGGACATT TATCCTCG	Cloning of pSV25
ykgS_F1	ATATCTCGAGAAGTGTTTTTGTATAAATCGGACATT TATCCTCG	Cloning of pSV16
ykgS_forward	ATGAGAATGATTGGCCTTCTTTATGATTTTAAGG	Cloning of pJS4
ykgS_R	ATATGGATCCTTCTGACTCGACCTGGTTGTAGAATT CG	Cloning of pSV25
ykgS_R1	ATATGGATCCTTCTGACTCGACCTGGTTGTAGAATT CG	Cloning of pSV16
ykgS_stop	TAAAGATAAATTGGCCTTCTTTATGATTTTAAGGATT ATGCT	Cloning of pSV41

ymiA Primer 1	TGGCGGTTTTTCTTGTTCTGCACTTTTCTGGGTGG TTGTGCGCACTGCTGATTTGGAAAGTGTGGGGATAAT AAGCTTAATTAGCTGACCTactagagt	Cloning of pPJ12
ymiA Primer 2	GCCAGGCTTTACGTTTTTAATTCAGGATCGCGGCGG GGTTCCTGATTTCCAGAAGGCATTGCTAACCTCATttt gtatagttcatccatgccatgtg	Cloning of pPJ12
ymiA_del_screen_1	gattgaattattcactggagacgattcg	Validation of GSO225
ymiA_del_screen_2	tcacttcagcaaacagttctataaaggc	Validation of GSO225
ymiA_F1	ATATGAATTCTCAGCCACAGTACAACCAAATTGG	Cloning of pSV20
ymiA_R1	ATATGATCCATAATCGCCATCACTTATCAGCAAGAC	Cloning of pSV20
ymiA_stop	TAAAGGTTAGCATAACCTTCTGGAAATCAGGAACCC C	Cloning of pSV42
ymiA-forward	ATGAGGTTAGCAATGCCTTCTGGA	Cloning of pSV36
ymiC Primer 1	CTGTGATGCTCTTCTGGGCCGAACCTCTCTGGAT CATTACTCACTGATAAGCTTAATTAGCTGACCTactag agt	Cloning of pPJ13
ymiC Primer 2	AGAAAACGCGCCCATCCAGGACCAATATTTTCATATT TGTGTTGATCATtttgtatagttcatccatgccatgtg	Cloning of pPJ13
ymiC_del_F	ACAGCTTGAGTTATCTCAACACAAAATAATAACCGT TAAGGGTGTAGCCTattccggggatccgtcgacc	Construction of SV41
ymiC_del_R	GAAACCTTGTGGCAAAGCAAATGACAACCCCGCCG CAGCGGGTCAAGGAtgtaggctggagctgcttcg	Construction of SV41
ymiC_del_screen_1	GATGATCAGCCGAAACAATAATTATCATCATTC	Validation of SV41
ymiC_del_screen_2	AAGCCCTGATTCTCTTCAGGGT	Validation of SV41
ymiC_F1	ATATGAATTCCAGGAGCAACTGGAGTCGTC	Cloning of pSV19
ymiC_R1	ATATGGATCCAGGCTACACCCTAACGGTTATTATT TTG	Cloning of pSV19
ymiC-forward	ATGATCAACACAAATATGAAATATTGGTCCTGGA	Cloning of pSV33
yoal Primer 1	ATTGCTGTTGTACTGGTTTTGTCCGTTCTTCTTATCG AACGGACCGGCcgtaaaggagaagaacttttactgg	Cloning pPJ4
yoal Primer 2	GGCAAAAACGATGACGTGATAATCAGTGTCTCGA CAAACATTTGATCGTTcatgcttaattctcctttaattctaggtac	Cloning pPJ4
yoal_del_screen_1	tggttgccgaccctctactc	Validation of GSO232

yoal_del_screen_2	agcctgctactttacgcttgc	Validation of GSO232
yoal_F	GAGAATTCGCGGCTGGCTGCCCATTAA	Cloning of pPJ18
yoal_R	GGGGATCCAGCATGCCCCGGGAGATA	Cloning pPJ18
yoal_stop	taAAACGATCAATAATTTGTCGAGACACTGATTATCA CGTCATCG	Cloning of pSV39
yoal-forward	TAAGCTTAATTAGCTGACCTACTAGAGTCGAC	Cloning of pSV34
yoal-rev	GCCGGTCCGTTTCGATAAGAAGA	Cloning of pSV34
yobF Primer 1	CCTTATATTGGTGCCTCATGCAGTAATGTGTCAGTT TTATCTATGTTATGCCTGCGGGCGAAGAAAACAATC cgtaaaggagaagaacttttactgg	Cloning of pPJ6
yobF Primer 2	CTCGGCAGAGAAGCGGTATTCAACGTCAACGTGTT TACTCAGGACTTCTTTACTGAAAATGCCACAcatgctta atttctctctttaattctaggtac	Cloning of pPJ6
yobF_del_F	AAAGAAGTCCTGAGTAAACACGTTGACGTTGAATAC CGTTCTCTGCCGAattccgggatccgtcgacc	Construction of SV33
yobF_del_R	TTGAACCACTTAACCTGACCTTTAATCTTTGCCATTT GAAAAATTCCttatgtaggctggagctgcttcg	Construction of SV33
yobF_del_screen1	GTGTGCAAAAAAAGTGAAGACGT	Validation of SV33
yobF_del_screen2	CCATTACCCTGGATAGCGGAG	Validation of SV33
yobF_F1	ATATGAATTCTAATTTATTAGGATGTTTACATCGGAT TTGTGATTAAGCG	Cloning of pSV24
yobF_R1	ATATGGATCCAAACAGAACTGTACCTCGTTTAACCC	Cloning of pSV24
yobF_reverse	GATTGTTTTCTTCGCCCGCAGG	Cloning of pJS5
yobF_stop	taaTGTGGCATTTCAGTAAAGAAGTCCTG	Cloning of pSV45
yqhl Primer 1	ATCTCGAAACGTGAGTTCCTCAACCTTGCGGCGAA GTGCGGTAGGCGGGATGACGGCATTAGCGTTGTTT GATAAGCTTAATTAGCTGACCTactagagt	Cloning of pPJ15
yqhl Primer 2	TTTCCCGTGAGCGTAATAGTCATAGTAATCCAGCAA CTCTTGTGGGAAATCTTTGGCGGTTAAACGCGGCA Tttgtatagttcatcctgcatg	Cloning of pPJ15
yqhl_del_F2	AATACGTGCTTATGCTTTGCTTAAAAAACACCAACT GAGGAGTGCAACGaattccgggatccgtcgacctgcagttcgaa gttcctatt	Construction of SV37
yqhl_del_R2	GGTCGGTAAACTCTACCTGAGTCGCCAGCGCATAA TTTGGCTTGAGCAAAtgtaggctggagctgcttcaagttctatac t	Construction of SV37

yqhl_del_screen 1	TGTTTCTAGTTTAGCGATTGCGCCAG	Validation of SV37
yqhl_del_screen 2	GGGCTTCACCAGATAACCCC	Validation of SV37
yqhl_F	GAGAATTCTATTTACGTGAACCGCCAGAGCC	Cloning of pPJ19
yqhl_forward	ATGCCGCGTTTAACCGCC	Cloning of pJS3
yqhl_R	GGGGATCCCGTTGCACTCCTCAGTTGGTG	Cloning of pPJ19
yriA Primer 1	GCAGACCGTAGGCCAGATAAGGTGTTTACGCTGAT CAGGTAATAAGCTTAATTAGCTGACCTactagagt	Cloning of pPJ7
yriA Primer 2	GTACGCAATCAAATCCCCAGCCAATACAACATtttga tagttcatccatgccatgtg	Cloning of pPJ7
yriA_F	TCGAGAATTCAAATCAGGTCAGGCTTAAGTAGCGAC	Cloning of pPJ20
yriA_forward	ATGTTGTATTGGCTGGGGATTTTGAT	Cloning of pJS1
yriA_R	GGGGATCCTTAACACCATCATATTTTCCATCATTAG TGTGATC	Cloning of pPJ20
yriAB_del_F	ACACTATTACAACAGAAAATAACCAGATGATCACAC TAATGATGGAAAATattccggggatccgtcgacc	Construction of SV25
yriAB_del_R	TGTTCCCTGAACGCCCGCATATGCGGGCGTTTTGCT TTTTGGCGCGCCTTGgtgtaggctggagctgcttc	Construction of SV25
yriAB_del_screen 1	GAACGCGTAAGGGATAACGC	Validation of SV25
yriAB_del_screen 2	CTGATCAACACCGTTCACGTTG	Validation of SV25
yriB Primer 1	GATTTTGATTGCGTACGCAGACCGtagTAAGCTTAAT TAGCTGACCTactagagt	Cloning of pPJ11
yriB primer 2	CCCAGCCAATACAACATTTAACACcattttgtatagttcatcca tgccatgtg	Cloning of pPJ11
yriB-forward	atgGTGTAAATGTTGTATTGGCTGGG	Cloning of pSV37

Table S4. List of all proteins with ≥ 3 -fold increase in read counts from Ribo-RET and differentially expressed transcripts from RNA-Seq.

Ribo-RET					RNA-Seq Differential Expression					
gene	size	no_ stress	low_ mg	fold_ change	gene	base_ Mean	log2Fol d Change	lfcS E	pvalue	padj
<i>phnC</i>	standard	0.68	79.66	117.25	<i>mgtS</i>	14972.46	7.82	0.31	2.11E-140	6.60E-137
<i>iraM</i>	standard	3.4	247.31	72.8	<i>ymgL</i>	17.66	7.53	2.75	2.84E-06	2.56E-05
<i>phoR</i>	standard	0.68	44.65	65.71	<i>mgtA</i>	3789.38	7.13	0.39	9.56E-75	9.97E-72
<i>ptrA</i>	standard	1.36	72.66	53.47	<i>ymgK</i>	236.85	6.93	0.62	6.26E-29	5.02E-27
<i>phoE</i>	standard	5.44	242.93	44.69	<i>iraM</i>	205.15	6.71	0.63	4.09E-27	2.78E-25
<i>eptA</i>	standard	5.44	232.43	42.76	<i>mgtL</i>	11342.3	6.61	0.43	1.18E-54	5.27E-52
<i>ydeT</i>	standard	4.76	148.38	31.2	<i>ydeT</i>	28.94	6.33	1.43	6.39E-06	5.33E-05
<i>arnC</i>	standard	0.68	20.13	29.64	<i>ydeS</i>	9.66	6.26	2.76	1.15E-04	7.48E-04
<i>yciX</i>	standard	1.36	38.96	28.67	<i>asr</i>	332.57	5.91	0.48	3.80E-35	4.96E-33
<i>waa H</i>	standard	2.04	58.22	28.56	<i>ybjG</i>	1315.13	5.78	0.35	4.37E-63	3.42E-60
<i>rstB</i>	standard	0.68	18.82	27.7	<i>mgrB</i>	6552.83	5.68	0.31	4.00E-76	6.25E-73
<i>phoQ</i>	standard	2.04	54.71	26.84	<i>phnD</i>	25.86	5.34	1.17	1.71E-06	1.61E-05
<i>ugpB</i>	standard	10.87	288.45	26.53	<i>yebO</i>	1871.52	5.1	0.33	1.23E-55	6.42E-53
<i>phoB</i>	standard	29.21	711.72	24.36	<i>yriB</i>	18.19	4.72	1.24	3.12E-05	2.30E-04
<i>appC</i>	standard	6.79	162.39	23.9	<i>phnC</i>	17.35	4.63	1.25	4.47E-05	3.18E-04
<i>otsB</i>	standard	0.68	14.88	21.9	<i>pstS</i>	274.79	4.41	0.41	1.93E-28	1.48E-26
<i>argD</i>	standard	10.87	199.6	18.36	<i>yfeO</i>	91.2	4.31	0.56	1.21E-15	2.81E-14
<i>cysD</i>	standard	20.38	358.92	17.61	<i>ydgU</i>	24.42	4.31	0.99	2.00E-06	1.85E-05
<i>cbl</i>	standard	2.04	35.02	17.18	<i>dgcZ</i>	61.86	4.29	0.64	3.40E-12	6.33E-11
<i>ynaL</i>	standard	1.36	21.01	15.46	<i>pgpC</i>	397.23	4.24	0.37	2.38E-31	2.26E-29
<i>asr</i>	standard	51.64	796.2	15.42	<i>ygdR</i>	155.39	4.19	0.46	9.92E-21	3.34E-19
<i>yriA</i>	small	2.72	40.71	14.98	<i>alaV</i>	973.94	4.18	0.36	2.73E-32	2.85E-30
<i>glsA</i>	standard	0.68	10.07	14.82	<i>alaU</i>	973.94	4.18	0.36	2.73E-32	2.85E-30
<i>yciW</i>	standard	8.15	119.06	14.6	<i>alaT</i>	973.94	4.18	0.36	2.73E-32	2.85E-30
<i>yohC</i>	standard	8.15	118.18	14.5	<i>metZ</i>	673.96	4.12	0.43	6.41E-23	2.87E-21
<i>yeeE</i>	standard	4.08	58.65	14.39	<i>met W</i>	673.96	4.12	0.43	6.41E-23	2.87E-21
<i>sbp</i>	standard	6.79	96.3	14.17	<i>metV</i>	673.96	4.12	0.43	6.41E-23	2.87E-21
<i>ydgU</i>	small	2.04	27.14	13.31	<i>glyW</i>	9479.84	4.06	0.33	1.60E-35	2.27E-33
<i>narU</i>	standard	1.36	17.95	13.21	<i>glyV</i>	9479.84	4.06	0.33	1.60E-35	2.27E-33
<i>arnB</i>	standard	11.55	151.45	13.11	<i>glyX</i>	9479.84	4.06	0.33	1.60E-35	2.27E-33
<i>argA</i>	standard	16.31	212.29	13.02	<i>glyY</i>	9479.84	4.06	0.33	1.60E-35	2.27E-33
<i>ais</i>	standard	9.51	123	12.93	<i>ais</i>	16.7	4.03	1.15	6.60E-05	4.54E-04
<i>ycaP</i>	standard	1.36	17.51	12.88	<i>arnB</i>	38.15	3.91	0.76	2.56E-08	3.31E-07
<i>phoA</i>	standard	12.23	156.26	12.78	<i>ugpB</i>	80.2	3.83	0.57	1.18E-12	2.26E-11
<i>ydeQ</i>	standard	1.36	17.07	12.56	<i>alaX</i>	648.46	3.79	0.38	2.29E-24	1.21E-22
<i>yncG</i>	standard	2.04	25.39	12.46	<i>alaW</i>	648.46	3.79	0.38	2.29E-24	1.21E-22

<i>ilvB</i>	standard	1.36	16.2	11.92	<i>rttR</i>	21.62	3.74	1.06	4.26E-05	3.06E-04
<i>yoal</i>	small	13.59	161.95	11.92	<i>metY</i>	276.5	3.73	0.43	6.96E-19	2.05E-17
<i>cysP</i>	standard	63.87	758.56	11.88	<i>leuW</i>	4113.99	3.68	0.35	1.34E-27	9.30E-26
<i>yfcG</i>	standard	4.08	48.15	11.81	<i>arnC</i>	29.57	3.66	0.82	9.33E-07	9.24E-06
<i>lysA</i>	standard	2.04	23.64	11.6	<i>rstA</i>	323.01	3.65	0.38	8.71E-23	3.84E-21
<i>artP</i>	standard	43.48	491.11	11.29	<i>msrQ</i>	38.7	3.63	0.72	5.34E-08	6.58E-07
<i>yhbW</i>	standard	1.36	15.32	11.27	<i>arnF</i>	56.91	3.62	0.61	3.42E-10	5.22E-09
<i>dacD</i>	standard	0.68	7.44	10.95	<i>phoA</i>	78.32	3.62	0.55	3.42E-12	6.33E-11
<i>ruvC</i>	standard	0.68	7.44	10.95	<i>ymcF</i>	40.82	3.61	0.71	3.74E-08	4.73E-07
<i>ydbL</i>	standard	1.36	14.88	10.95	<i>dacC</i>	178.45	3.59	0.42	1.11E-18	3.21E-17
<i>argG</i>	standard	20.38	217.98	10.69	<i>ileV</i>	995.19	3.57	0.34	3.75E-26	2.35E-24
<i>argC</i>	standard	6.11	64.78	10.59	<i>ileU</i>	995.19	3.57	0.34	3.75E-26	2.35E-24
<i>ybhP</i>	standard	0.68	7	10.31	<i>ileT</i>	995.19	3.57	0.34	3.75E-26	2.35E-24
<i>yobF</i>	small	12.91	132.63	10.27	<i>phoQ</i>	258.31	3.57	0.38	1.64E-21	6.11E-20
<i>pstS</i>	standard	48.92	490.68	10.03	<i>phoB</i>	66.9	3.56	0.57	4.75E-11	7.87E-10
<i>nadD</i>	standard	1.36	13.57	9.99	<i>eptA</i>	15.82	3.5	1.11	1.72E-04	0.001078186
<i>gadE</i>	standard	2.72	26.7	9.82	<i>arnA</i>	35.6	3.49	0.74	2.45E-07	2.70E-06
<i>ydeS</i>	standard	2.72	26.7	9.82	<i>ydhl</i>	62.43	3.45	0.58	3.12E-10	4.78E-09
<i>bcsB</i>	standard	0.68	6.57	9.66	<i>pdeR</i>	34.61	3.44	0.74	3.72E-07	3.97E-06
<i>pcm</i>	standard	0.68	6.57	9.66	<i>pstC</i>	127.36	3.42	0.46	7.14E-15	1.56E-13
<i>yadW</i>	small	1.36	13.13	9.66	<i>secG</i>	2556.53	3.41	0.38	2.56E-20	8.08E-19
<i>pagP</i>	standard	8.15	75.29	9.23	<i>phoP</i>	729.83	3.38	0.33	5.44E-25	3.27E-23
<i>pstA</i>	standard	2.04	18.82	9.23	<i>slyB</i>	3744.69	3.34	0.3	7.35E-30	6.39E-28
<i>waaQ</i>	standard	2.04	18.82	9.23	<i>hokD</i>	178.33	3.31	0.42	3.78E-16	8.96E-15
<i>aroE</i>	standard	0.68	6.13	9.02	<i>pagP</i>	21.67	3.28	0.91	3.55E-05	2.57E-04
<i>mgrB</i>	small	250.71	2248.97	8.97	<i>yliM</i>	5741.91	3.21	0.32	5.03E-24	2.58E-22
<i>dtpD</i>	standard	2.72	24.07	8.86	<i>yjcB</i>	202.42	3.2	0.4	7.82E-17	1.93E-15
<i>tatD</i>	standard	8.15	71.78	8.8	<i>relE</i>	150.84	3.19	0.44	6.33E-14	1.28E-12
<i>bcr</i>	standard	2.04	17.51	8.59	<i>rstB</i>	99.93	3.16	0.49	8.30E-12	1.48E-10
<i>pstC</i>	standard	44.16	377.31	8.54	<i>ynfB</i>	711.59	3.09	0.34	1.32E-20	4.34E-19
<i>bhc</i>	standard	2.72	23.2	8.54	<i>ybfA</i>	383.07	3.09	0.38	3.59E-17	9.13E-16
<i>nudL</i>	standard	2.72	23.2	8.54	<i>deoR</i>	205.33	3.08	0.4	6.56E-16	1.54E-14
<i>insQ</i>	standard	6.79	56.9	8.38	<i>sraG</i>	43.7	3.08	0.65	2.09E-07	2.33E-06
<i>cysJ</i>	standard	60.47	504.25	8.34	<i>valT</i>	8108.95	3.08	0.33	7.92E-22	3.02E-20
<i>pdeR</i>	standard	8.15	67.41	8.27	<i>valZ</i>	8108.95	3.08	0.33	7.92E-22	3.02E-20
<i>iaaA</i>	standard	30.57	251.69	8.23	<i>valU</i>	8108.95	3.08	0.33	7.92E-22	3.02E-20
<i>yhdN</i>	standard	4.08	32.83	8.05	<i>valX</i>	8108.95	3.08	0.33	7.92E-22	3.02E-20
<i>chaC</i>	standard	5.44	43.33	7.97	<i>valY</i>	8108.95	3.08	0.33	7.92E-22	3.02E-20
<i>mgtT</i>	small	1082.31	8622.95	7.97	<i>tqsA</i>	29.99	3.05	0.76	6.71E-06	5.58E-05
<i>gadX</i>	standard	4.08	31.95	7.84	<i>lysT</i>	11718.26	3.05	0.33	3.00E-21	1.04E-19
<i>rssB</i>	standard	4.08	31.95	7.84	<i>lysW</i>	11718.26	3.05	0.33	3.00E-21	1.04E-19
<i>hisG</i>	standard	13.59	105.93	7.8	<i>lysY</i>	11718.26	3.05	0.33	3.00E-21	1.04E-19

<i>cysW</i>	standard	0.68	5.25	7.73	<i>lysZ</i>	11718.26	3.05	0.33	3.00E-21	1.04E-19
<i>glaR</i>	standard	0.68	5.25	7.73	<i>lysQ</i>	11718.26	3.05	0.33	3.00E-21	1.04E-19
<i>ibsD</i>	small	0.68	5.25	7.73	<i>lysV</i>	11718.26	3.05	0.33	3.00E-21	1.04E-19
<i>ibsE</i>	small	0.68	5.25	7.73	<i>appC</i>	46.53	3.02	0.63	1.57E-07	1.80E-06
<i>ybdR</i>	standard	0.68	5.25	7.73	<i>fabR</i>	293.94	3.02	0.37	4.17E-17	1.05E-15
<i>hisD</i>	standard	9.51	73.1	7.68	<i>csrC</i>	1142.49	3	0.32	6.80E-22	2.80E-20
<i>tqsA</i>	standard	8.15	61.28	7.52	<i>phoR</i>	33.35	2.98	0.72	3.93E-06	3.47E-05
<i>purF</i>	standard	15.63	117.31	7.51	<i>ygcN</i>	30.28	2.95	0.75	1.01E-05	8.10E-05
<i>glcD</i>	standard	8.15	60.84	7.46	<i>yfiS</i>	34.41	2.94	0.74	7.12E-06	5.88E-05
<i>speG</i>	standard	26.5	196.53	7.42	<i>queE</i>	213.58	2.94	0.39	5.47E-15	1.21E-13
<i>ftsQ</i>	standard	1.36	10.07	7.41	<i>yodB</i>	20	2.9	0.92	1.76E-04	0.001100074
<i>ygbE</i>	standard	3.4	24.95	7.34	<i>tadA</i>	238.08	2.89	0.38	6.15E-15	1.36E-13
<i>yqhl</i>	small	2.04	14.88	7.3	<i>arnD</i>	13.79	2.89	1.12	0.001001481	0.005046111
<i>miaA</i>	standard	13.59	96.73	7.12	<i>ybgS</i>	71.99	2.87	0.54	1.44E-08	1.91E-07
<i>speC</i>	standard	19.7	140.07	7.11	<i>metU</i>	243.67	2.87	0.42	6.17E-13	1.21E-11
<i>appB</i>	standard	0.68	4.81	7.09	<i>metT</i>	243.67	2.87	0.42	6.17E-13	1.21E-11
<i>argH</i>	standard	2.72	19.26	7.09	<i>serV</i>	8051.92	2.86	0.35	2.19E-17	5.70E-16
<i>artJ</i>	standard	13.59	96.3	7.09	<i>cspG</i>	125.16	2.85	0.44	1.28E-11	2.26E-10
<i>cobC</i>	standard	2.72	19.26	7.09	<i>ugpA</i>	23.5	2.84	0.85	9.04E-05	6.07E-04
<i>rcdA</i>	standard	2.72	19.26	7.09	<i>gltW</i>	2671.27	2.83	0.34	1.73E-17	4.54E-16
<i>ydgD</i>	standard	4.76	33.7	7.09	<i>gltU</i>	2671.27	2.83	0.34	1.73E-17	4.54E-16
<i>hrpB</i>	standard	4.08	28.89	7.09	<i>gltT</i>	2671.27	2.83	0.34	1.73E-17	4.54E-16
<i>ydgl</i>	standard	29.21	204.85	7.01	<i>gltV</i>	2671.27	2.83	0.34	1.73E-17	4.54E-16
<i>yodB</i>	standard	5.44	37.64	6.93	<i>speG</i>	402.93	2.82	0.35	2.21E-16	5.39E-15
<i>phoH</i>	standard	16.99	116.87	6.88	<i>glnW</i>	5581.76	2.79	0.34	2.38E-17	6.10E-16
<i>ydhJ</i>	standard	23.78	163.27	6.87	<i>glnU</i>	5581.76	2.79	0.34	2.38E-17	6.10E-16
<i>yeb</i>	standard	7.47	50.77	6.79	<i>phnF</i>	9.4	2.78	1.4	0.004516358	0.018545518
<i>W</i>										
<i>dinQ</i>	small	1.36	9.19	6.76	<i>ydhJ</i>	35.29	2.76	0.7	8.20E-06	6.70E-05
<i>yfbP</i>	standard	0.68	4.38	6.44	<i>appB</i>	29.37	2.76	0.75	2.63E-05	1.97E-04
<i>insD</i>	standard	0.68	4.38	6.44	<i>pstA</i>	84.17	2.76	0.5	3.89E-09	5.37E-08
<i>2</i>										
<i>lhr</i>	standard	0.68	4.38	6.44	<i>phoH</i>	157.53	2.74	0.55	6.19E-08	7.50E-07
<i>nhoA</i>	standard	0.68	4.38	6.44	<i>glsA</i>	31.04	2.74	0.74	2.48E-05	1.86E-04
<i>recX</i>	standard	0.68	4.38	6.44	<i>ycgX</i>	11.14	2.72	1.26	0.002969559	0.012959207
<i>ugpA</i>	standard	0.68	4.38	6.44	<i>clcA</i>	33.59	2.7	0.71	1.68E-05	1.29E-04
<i>yach</i>	standard	0.68	4.38	6.44	<i>relB</i>	212.04	2.68	0.39	9.27E-13	1.79E-11
<i>ycaC</i>	standard	2.04	13.13	6.44	<i>miaF</i>	310.63	2.64	0.36	2.15E-14	4.54E-13
<i>yidD</i>	standard	0.68	4.38	6.44	<i>rhsC</i>	20.37	2.59	0.88	3.93E-04	0.002230904
<i>patA</i>	standard	3.4	21.01	6.18	<i>pmrD</i>	10.57	2.59	1.28	0.004234691	0.017596745
<i>yncJ</i>	standard	50.28	310.34	6.17	<i>ymdF</i>	73.54	2.57	0.52	9.59E-08	1.12E-06
<i>rseA</i>	standard	205.86	1264.99	6.14	<i>mlrA</i>	23.28	2.55	0.82	2.35E-04	0.001436063
<i>opgG</i>	standard	13.59	83.17	6.12	<i>mqsA</i>	81.15	2.54	0.5	5.50E-08	6.75E-07
<i>ybiO</i>	standard	1.36	8.32	6.12	<i>zntA</i>	87.62	2.54	0.48	1.74E-08	2.26E-07

<i>yfeO</i>	standard	1.36	8.32	6.12	<i>mdfA</i>	45.72	2.54	0.62	5.74E-06	4.90E-05
<i>hemD</i>	standard	2.04	12.26	6.01	<i>mqsR</i>	84.83	2.51	0.49	4.53E-08	5.69E-07
<i>iscX</i>	standard	12.23	73.54	6.01	<i>thrV</i>	83.75	2.47	0.53	4.55E-07	4.79E-06
<i>mdlA</i>	standard	2.04	12.26	6.01	<i>slp</i>	50.9	2.47	0.6	6.20E-06	5.20E-05
<i>argR</i>	standard	27.18	163.27	6.01	<i>ycfJ</i>	63.77	2.46	0.55	1.07E-06	1.04E-05
<i>pmrR</i>	small	32.61	193.91	5.95	<i>thrU</i>	145.51	2.43	0.42	6.65E-10	9.68E-09
<i>yddY</i>	small	22.42	130.88	5.84	<i>ytjA</i>	131.58	2.42	0.44	4.71E-09	6.41E-08
<i>yeeD</i>	standard	108.03	627.68	5.81	<i>folA</i>	228.35	2.4	0.38	5.26E-11	8.65E-10
<i>apaH</i>	standard	2.72	15.76	5.8	<i>mlaE</i>	239.82	2.4	0.37	1.49E-11	2.60E-10
<i>folM</i>	standard	3.4	19.7	5.8	<i>yddY</i>	25.91	2.4	0.79	3.10E-04	0.001817096
<i>ibsA</i>	small	0.68	3.94	5.8	<i>ycaC</i>	70.21	2.39	0.53	9.42E-07	9.30E-06
<i>insD3</i>	standard	0.68	3.94	5.8	<i>appA</i>	25.61	2.37	0.77	2.88E-04	0.001708407
<i>insD4</i>	standard	0.68	3.94	5.8	<i>yadE</i>	24.1	2.37	0.8	3.94E-04	0.002230904
<i>insD5</i>	standard	0.68	3.94	5.8	<i>ykfl</i>	55.47	2.37	0.56	3.18E-06	2.85E-05
<i>insD6</i>	standard	0.68	3.94	5.8	<i>arfA</i>	24.15	2.36	0.8	4.15E-04	0.002324926
<i>mhpF</i>	standard	0.68	3.94	5.8	<i>yehS</i>	106.85	2.34	0.47	9.09E-08	1.08E-06
<i>xylB</i>	standard	0.68	3.94	5.8	<i>yohC</i>	26.27	2.32	0.77	3.43E-04	0.00200324
<i>ycdK</i>	standard	1.36	7.88	5.8	<i>yjbJ</i>	153.23	2.32	0.42	4.38E-09	5.98E-08
<i>btuE</i>	standard	19.7	113.81	5.78	<i>paoD</i>	10.75	2.3	1.23	0.00655796	0.025024217
<i>yjdC</i>	standard	8.83	48.59	5.5	<i>yiaG</i>	86.74	2.3	0.5	6.84E-07	6.95E-06
<i>glsB</i>	standard	10.19	56.03	5.5	<i>rmf</i>	1448.26	2.29	0.32	2.75E-13	5.45E-12
<i>recJ</i>	standard	6.79	37.21	5.48	<i>ycaP</i>	13.38	2.27	1.07	0.004022703	0.01682759
<i>dosC</i>	standard	16.31	87.54	5.37	<i>yjdN</i>	14.7	2.25	1.02	0.003375377	0.01444809
<i>yecE</i>	standard	2.04	10.94	5.37	<i>symR</i>	13.43	2.24	1.08	0.004360634	0.01804818
<i>cpxP</i>	standard	86.29	461.35	5.35	<i>hemL</i>	436.05	2.23	0.35	1.79E-11	3.08E-10
<i>ampH</i>	standard	38.05	202.22	5.32	<i>glk</i>	152.05	2.22	0.42	1.62E-08	2.12E-07
<i>ycgV</i>	standard	13.59	71.78	5.28	<i>arrS</i>	71.94	2.22	0.54	5.76E-06	4.90E-05
<i>ymgE</i>	standard	19.02	100.24	5.27	<i>ybjX</i>	352.08	2.22	0.38	9.80E-10	1.41E-08
<i>bepA</i>	standard	20.38	105.49	5.18	<i>yagP</i>	15.62	2.21	0.99	0.003308268	0.014180234
<i>ecpA</i>	standard	1.36	7	5.15	<i>yjdD</i>	302.74	2.19	0.35	8.95E-11	1.45E-09
<i>rzoQ</i>	standard	0.68	3.5	5.15	<i>gadW</i>	98.62	2.19	0.49	1.11E-06	1.08E-05
<i>tfaP</i>	standard	0.68	3.5	5.15	<i>zraR</i>	36.16	2.19	0.66	1.39E-04	8.84E-04
<i>thiQ</i>	standard	0.68	3.5	5.15	<i>psiF</i>	55.56	2.17	0.56	1.53E-05	1.19E-04
<i>ybbA</i>	standard	2.04	10.51	5.15	<i>mgrR</i>	14.06	2.17	1.04	0.004721938	0.019238208
<i>ydbH</i>	standard	0.68	3.5	5.15	<i>ydgV</i>	108.03	2.14	0.46	5.08E-07	5.30E-06
<i>pgpC</i>	standard	21.74	108.99	5.01	<i>yohP</i>	29.27	2.14	0.72	4.45E-04	0.002479686
<i>yqgF</i>	standard	2.72	13.57	4.99	<i>ygiW</i>	67.93	2.13	0.52	7.97E-06	6.53E-05
<i>yeaY</i>	standard	63.19	314.72	4.98	<i>pgaD</i>	9.14	2.13	1.38	0.012332021	0.042160671
<i>waaO</i>	standard	7.47	37.21	4.98	<i>ytfK</i>	199.14	2.12	0.38	4.18E-09	5.74E-08
<i>ycdL</i>	standard	16.99	84.04	4.95	<i>sixA</i>	116.56	2.12	0.44	3.09E-07	3.35E-06

<i>csdE</i>	standard	6.11	30.2	4.94	<i>yafW</i>	13.72	2.1	1.05	0.005813937	0.022796754
<i>yeeY</i>	standard	2.04	10.07	4.94	<i>yqhl</i>	17.34	2.1	0.92	0.003119101	0.013443068
<i>yggC</i>	standard	4.08	20.13	4.94	<i>ybaT</i>	19.7	2.09	0.86	0.002281827	0.010453641
<i>waaG</i>	standard	2.04	10.07	4.94	<i>opgB</i>	81.48	2.08	0.49	3.69E-06	3.28E-05
<i>rseB</i>	standard	163.74	808.02	4.93	<i>glnX</i>	1680.13	2.07	0.34	1.46E-10	2.27E-09
<i>ykgS</i>	small	11.55	56.9	4.93	<i>glnV</i>	1680.13	2.07	0.34	1.46E-10	2.27E-09
<i>yfhH</i>	standard	16.31	80.1	4.91	<i>holE</i>	345.7	2.07	0.35	5.26E-10	7.79E-09
<i>yodE</i>	small	40.77	200.03	4.91	<i>yhcN</i>	112.26	2.07	0.44	5.33E-07	5.52E-06
<i>artQ</i>	standard	1.36	6.57	4.83	<i>glcD</i>	12.51	2.07	1.11	0.007735031	0.028847334
<i>xynR</i>	standard	1.36	6.57	4.83	<i>yeeW</i>	19.64	2.06	0.86	0.002416881	0.010944167
<i>yjfF</i>	standard	5.44	26.26	4.83	<i>yciY</i>	33.11	2.06	0.68	3.66E-04	0.002099702
<i>waaP</i>	standard	10.19	49.02	4.81	<i>gadA</i>	55	2.06	0.6	1.09E-04	7.20E-04
<i>opgB</i>	standard	25.14	119.5	4.75	<i>kefB</i>	29.32	2.06	0.71	6.02E-04	0.003210015
<i>rmf</i>	standard	499.37	2361.46	4.73	<i>sibC</i>	62.55	2.06	0.53	1.98E-05	1.51E-04
<i>yafS</i>	standard	4.08	19.26	4.72	<i>corA</i>	126.56	2.03	0.44	7.50E-07	7.55E-06
<i>yegS</i>	standard	8.15	38.52	4.72	<i>ynfC</i>	26.23	2.02	0.74	0.001064516	0.005332848
<i>clcB</i>	standard	4.76	22.32	4.69	<i>yoaC</i>	21.34	2.01	0.82	0.002245505	0.010302324
<i>yhdV</i>	standard	25.82	120.81	4.68	<i>leuP</i>	370.92	1.99	0.44	1.03E-06	1.01E-05
<i>yriB</i>	small	18.34	85.79	4.68	<i>proP</i>	542.82	1.97	0.33	3.48E-10	5.29E-09
<i>tcyJ</i>	standard	86.97	399.19	4.59	<i>ecpA</i>	34.9	1.97	0.66	4.90E-04	0.002672328
<i>gsiA</i>	standard	7.47	34.14	4.57	<i>amE</i>	10.7	1.97	1.18	0.012091365	0.04162143
<i>amyA</i>	standard	15.63	71.35	4.57	<i>hscA</i>	342.73	1.96	0.4	1.70E-07	1.92E-06
<i>hscA</i>	standard	41.44	187.78	4.53	<i>rpoE</i>	319.84	1.95	0.35	5.02E-09	6.80E-08
<i>cysl</i>	standard	21.74	98.05	4.51	<i>amiC</i>	208.92	1.95	0.38	5.25E-08	6.52E-07
<i>fepE</i>	standard	0.68	3.06	4.51	<i>ugpE</i>	16.7	1.94	0.94	0.005879849	0.023026342
<i>folK</i>	standard	0.68	3.06	4.51	<i>ymlA</i>	23.96	1.91	0.78	0.002399407	0.010880791
<i>hisA</i>	standard	0.68	3.06	4.51	<i>yahO</i>	74.6	1.91	0.51	3.29E-05	2.40E-04
<i>hisM</i>	standard	0.68	3.06	4.51	<i>phoU</i>	114.77	1.91	0.44	2.77E-06	2.50E-05
<i>hyaF</i>	standard	0.68	3.06	4.51	<i>ykfG</i>	11.5	1.9	1.13	0.012368798	0.042181183
<i>insD1</i>	standard	0.68	3.06	4.51	<i>pstB</i>	120.34	1.9	0.43	2.03E-06	1.86E-05
<i>moaE</i>	standard	0.68	3.06	4.51	<i>basS</i>	32.19	1.89	0.68	9.49E-04	0.004844557
<i>pdxI</i>	standard	3.4	15.32	4.51	<i>slyA</i>	260.77	1.89	0.36	3.52E-08	4.47E-07
<i>sapF</i>	standard	0.68	3.06	4.51	<i>yhiD</i>	15.9	1.89	0.94	0.007083942	0.026705609
<i>waaF</i>	standard	1.36	6.13	4.51	<i>kefG</i>	73.43	1.87	0.51	4.47E-05	3.18E-04
<i>yafL</i>	standard	0.68	3.06	4.51	<i>yobH</i>	41.89	1.86	0.61	4.13E-04	0.002321485
<i>ycbV</i>	standard	0.68	3.06	4.51	<i>selC</i>	48.44	1.85	0.58	2.89E-04	0.001710781
<i>ydaG</i>	small	1.36	6.13	4.51	<i>fdx</i>	172.13	1.85	0.44	5.09E-06	4.40E-05
<i>yfdX</i>	standard	1.36	6.13	4.51	<i>kbp</i>	41.95	1.84	0.61	4.87E-04	0.002664475
<i>aat</i>	standard	61.83	278.82	4.51	<i>gadF</i>	49.84	1.84	0.63	6.72E-04	0.003539518
<i>livJ</i>	standard	2.04	9.19	4.51	<i>bglA</i>	277.97	1.84	0.38	2.40E-07	2.66E-06
<i>paaK</i>	standard	2.04	9.19	4.51	<i>yodD</i>	16.48	1.83	0.92	0.007466941	0.027947439
<i>trmD</i>	standard	148.11	664.89	4.49	<i>hdeB</i>	216.8	1.83	0.43	3.89E-06	3.44E-05

<i>mgtS</i>	small	2234.61	9955.35	4.46	<i>osmC</i>	176.67	1.83	0.41	1.61E-06	1.52E-05
<i>bfr</i>	standard	13.59	60.4	4.45	<i>yhbE</i>	148.23	1.81	0.43	5.00E-06	4.33E-05
<i>osmF</i>	standard	4.76	21.01	4.42	<i>miaD</i>	217.43	1.8	0.37	2.49E-07	2.73E-06
<i>tomB</i>	standard	27.86	122.12	4.38	<i>cspB</i>	69.69	1.79	0.51	9.40E-05	6.26E-04
<i>dkgB</i>	standard	11.55	50.34	4.36	<i>serW</i>	53.51	1.79	0.58	3.98E-04	0.002250482
<i>bcsG</i>	standard	2.72	11.82	4.35	<i>serX</i>	53.51	1.79	0.58	3.98E-04	0.002250482
<i>ydeP</i>	standard	2.72	11.82	4.35	<i>ortT</i>	31.77	1.79	0.74	0.002848726	0.012607727
<i>ycgZ</i>	standard	20.38	88.42	4.34	<i>yfeK</i>	18.38	1.78	0.86	0.006865127	0.025974585
<i>glk</i>	standard	43.48	188.22	4.33	<i>yafX</i>	24.7	1.78	0.76	0.00357779	0.015231165
<i>cynR</i>	standard	22.42	96.73	4.31	<i>basR</i>	41.62	1.76	0.61	7.62E-04	0.003938452
<i>fxsA</i>	standard	8.83	38.08	4.31	<i>yabl</i>	44.88	1.76	0.58	5.35E-04	0.002873773
<i>yhfG</i>	standard	12.91	55.15	4.27	<i>gadE</i>	27.23	1.76	0.76	0.004028227	0.0168282
<i>amiB</i>	standard	9.51	40.27	4.23	<i>azuC</i>	71.86	1.76	0.62	9.66E-04	0.004912092
<i>ldtC</i>	standard	16.99	71.35	4.2	<i>mdaB</i>	102.97	1.75	0.45	2.21E-05	1.67E-04
<i>rstA</i>	standard	64.54	270.51	4.19	<i>ynfS</i>	90.26	1.75	0.46	3.43E-05	2.50E-04
<i>ahr</i>	standard	9.51	39.83	4.19	<i>ydhR</i>	93	1.73	0.46	4.57E-05	3.23E-04
<i>fbaB</i>	standard	9.51	39.83	4.19	<i>rcnA</i>	13.84	1.73	0.99	0.01356292	0.045147211
<i>bcsF</i>	standard	1.36	5.69	4.19	<i>ysgD</i>	402.58	1.72	0.35	2.73E-07	2.98E-06
<i>chbG</i>	standard	5.44	22.76	4.19	<i>shoB</i>	19.74	1.72	0.83	0.007317217	0.027419846
<i>fadE</i>	standard	2.72	11.38	4.19	<i>ygdI</i>	40.7	1.71	0.6	9.86E-04	0.004994682
<i>ycgB</i>	standard	7.47	31.08	4.16	<i>miaB</i>	155.62	1.71	0.4	4.03E-06	3.53E-05
<i>gsiB</i>	standard	3.4	14.01	4.12	<i>rem</i>	13.58	1.7	1	0.01516317	0.049319708
<i>pxpA</i>	standard	3.4	14.01	4.12	<i>ybaP</i>	80.94	1.7	0.47	8.16E-05	5.53E-04
<i>wecB</i>	standard	2.04	8.32	4.08	<i>mltF</i>	22.43	1.68	0.78	0.006371506	0.02459144
<i>ydeM</i>	standard	2.04	8.32	4.08	<i>yciX</i>	63.21	1.67	0.51	2.67E-04	0.001591423
<i>nimR</i>	standard	10.87	44.21	4.07	<i>leuT</i>	333.1	1.66	0.42	1.71E-05	1.31E-04
<i>psiF</i>	standard	8.83	35.89	4.06	<i>leuV</i>	333.1	1.66	0.42	1.71E-05	1.31E-04
<i>ynjB</i>	standard	9.51	38.08	4	<i>leuQ</i>	333.1	1.66	0.42	1.71E-05	1.31E-04
<i>yiaG</i>	standard	10.19	40.71	3.99	<i>trpT</i>	53.05	1.66	0.55	6.59E-04	0.0034826
<i>uhpB</i>	standard	12.91	51.21	3.97	<i>hdeA</i>	666.9	1.65	0.37	2.31E-06	2.11E-05
<i>ecpR</i>	standard	5.44	21.45	3.95	<i>elaB</i>	154.33	1.65	0.4	1.08E-05	8.61E-05
<i>mntP</i>	standard	23.1	91.04	3.94	<i>letA</i>	25.84	1.64	0.73	0.005293456	0.021180592
<i>msyB</i>	standard	78.13	307.71	3.94	<i>mltD</i>	413.19	1.64	0.34	4.54E-07	4.79E-06
<i>dgcQ</i>	standard	6.11	24.07	3.94	<i>glmY</i>	73.6	1.61	0.49	2.56E-04	0.001541773
<i>ydbJ</i>	standard	99.87	392.19	3.93	<i>maeA</i>	355.15	1.61	0.37	3.00E-06	2.70E-05
<i>alx</i>	standard	7.47	29.33	3.92	<i>ykfF</i>	34.95	1.6	0.64	0.002915101	0.012813055
<i>yraQ</i>	standard	38.05	148.82	3.91	<i>tatD</i>	80.68	1.6	0.48	2.05E-04	0.001258503
<i>ldtA</i>	standard	29.89	116.87	3.91	<i>ynal</i>	32.23	1.6	0.67	0.003919051	0.016504319
<i>rhoL</i>	small	10.87	42.46	3.91	<i>rseA</i>	791.21	1.59	0.33	2.88E-07	3.13E-06
<i>ygiW</i>	standard	6.79	26.26	3.87	<i>glgS</i>	35.1	1.59	0.66	0.003814277	0.016106441
<i>gudX</i>	standard	0.68	2.63	3.87	<i>gcvB</i>	3584.49	1.59	0.32	1.68E-07	1.91E-06
<i>ibsB</i>	small	2.72	10.51	3.87	<i>msyB</i>	63.26	1.58	0.52	6.14E-04	0.003265833

<i>malX</i>	standard	0.68	2.63	3.87	<i>valW</i>	46.96	1.58	0.59	0.001741963	0.008208737
<i>mdtD</i>	standard	0.68	2.63	3.87	<i>aspU</i>	650.02	1.58	0.34	1.23E-06	1.18E-05
<i>moaD</i>	standard	1.36	5.25	3.87	<i>aspV</i>	650.02	1.58	0.34	1.23E-06	1.18E-05
<i>msrP</i>	standard	1.36	5.25	3.87	<i>aspT</i>	650.02	1.58	0.34	1.23E-06	1.18E-05
<i>phnN</i>	standard	0.68	2.63	3.87	<i>ompX</i>	4546.19	1.57	0.32	2.02E-07	2.27E-06
<i>pphA</i>	standard	1.36	5.25	3.87	<i>pmrR</i>	156.83	1.57	0.39	1.97E-05	1.50E-04
<i>recO</i>	standard	1.36	5.25	3.87	<i>tyrV</i>	115.05	1.56	0.44	1.12E-04	7.33E-04
<i>rseC</i>	standard	0.68	2.63	3.87	<i>tyrT</i>	115.05	1.56	0.44	1.12E-04	7.33E-04
<i>sxy</i>	standard	1.36	5.25	3.87	<i>mIaC</i>	223.38	1.56	0.37	6.04E-06	5.08E-05
<i>trmO</i>	standard	2.72	10.51	3.87	<i>emrD</i>	40.84	1.55	0.6	0.0025991	0.011651268
<i>ydhP</i>	standard	18.34	70.91	3.87	<i>ydhP</i>	31.46	1.54	0.67	0.005090086	0.020524328
<i>yfbN</i>	standard	0.68	2.63	3.87	<i>dinJ</i>	131.95	1.54	0.41	5.98E-05	4.14E-04
<i>yjcO</i>	standard	0.68	2.63	3.87	<i>iscR</i>	308.39	1.54	0.51	7.04E-04	0.003669559
<i>pyrR</i>	standard	2.04	7.88	3.87	<i>sra</i>	525.89	1.53	0.34	1.75E-06	1.64E-05
<i>ybjC</i>	standard	8.15	31.52	3.87	<i>yoeB</i>	53.81	1.52	0.54	0.001361759	0.006609666
<i>paaJ</i>	standard	2.04	7.88	3.87	<i>gltS</i>	37.19	1.5	0.62	0.004079394	0.017019232
<i>tmcA</i>	standard	2.04	7.88	3.87	<i>osmY</i>	248.23	1.5	0.4	4.80E-05	3.38E-04
<i>yceM</i>	standard	2.04	7.88	3.87	<i>ugpC</i>	28.39	1.46	0.69	0.009072358	0.032932027
<i>ydel</i>	standard	2.04	7.88	3.87	<i>soxS</i>	136.64	1.45	0.42	1.65E-04	0.001041569
<i>yghB</i>	standard	42.8	164.58	3.85	<i>yejG</i>	207.82	1.45	0.4	8.98E-05	6.04E-04
<i>ymiA</i>	small	138.6	530.51	3.83	<i>ykiD</i>	30.44	1.44	0.66	0.008017026	0.029651623
<i>yibF</i>	standard	7.47	28.45	3.81	<i>cof</i>	36.87	1.43	0.61	0.005648098	0.022314267
<i>yphH</i>	standard	11.55	43.77	3.79	<i>rcnR</i>	28.58	1.43	0.68	0.00981519	0.035219873
<i>curA</i>	standard	22.42	84.92	3.79	<i>rpoS</i>	518.95	1.41	0.33	5.85E-06	4.95E-05
<i>npr</i>	standard	15.63	59.09	3.78	<i>ryfA</i>	56.68	1.41	0.52	0.002227727	0.010235766
<i>ytfF</i>	standard	9.51	35.89	3.77	<i>yqhA</i>	154.26	1.39	0.39	1.37E-04	8.75E-04
<i>cysE</i>	standard	47.56	178.59	3.76	<i>psiE</i>	28.88	1.39	0.68	0.011626599	0.040374065
<i>yedA</i>	standard	7.47	28.01	3.75	<i>glyU</i>	39.24	1.38	0.6	0.006460553	0.024823494
<i>osmY</i>	standard	40.09	150.14	3.75	<i>erpA</i>	165.91	1.35	0.47	0.001365789	0.006615408
<i>gadW</i>	standard	63.19	236.37	3.74	<i>yeaQ</i>	65.54	1.35	0.5	0.002133684	0.009848486
<i>dacC</i>	standard	55.71	208.35	3.74	<i>dkgB</i>	31.73	1.35	0.65	0.011599563	0.040332427
<i>ytiA</i>	standard	3.4	12.69	3.74	<i>yceK</i>	99.9	1.33	0.46	0.00134666	0.006563393
<i>dnaC</i>	standard	9.51	35.45	3.73	<i>adhP</i>	30.59	1.33	0.65	0.012832445	0.043221442
<i>phoP</i>	standard	233.04	864.48	3.71	<i>cspF</i>	36.22	1.32	0.61	0.009769932	0.035138064
<i>hybD</i>	standard	2.72	10.07	3.7	<i>cydH</i>	77.27	1.32	0.47	0.001728634	0.008158215
<i>tatB</i>	standard	2.72	10.07	3.7	<i>yefM</i>	123.88	1.32	0.41	5.11E-04	0.002764991
<i>yebE</i>	standard	12.91	47.27	3.66	<i>yedR</i>	30.32	1.31	0.65	0.014078261	0.046418208
<i>nagA</i>	standard	14.95	54.71	3.66	<i>degP</i>	308.32	1.3	0.36	9.23E-05	6.19E-04
<i>hemF</i>	standard	6.11	22.32	3.65	<i>yqeF</i>	37.96	1.29	0.61	0.011566698	0.040303115
<i>dadX</i>	standard	2.04	7.44	3.65	<i>yebV</i>	154.21	1.29	0.41	5.61E-04	0.003003626
<i>ggt</i>	standard	4.08	14.88	3.65	<i>iscS</i>	431.78	1.29	0.45	0.001488886	0.007167266
<i>yoeB</i>	standard	2.04	7.44	3.65	<i>zapC</i>	130.31	1.28	0.4	5.74E-04	0.003072022

<i>letA</i>	standard	52.99	193.03	3.64	<i>yncL</i>	120.28	1.28	0.42	9.49E-04	0.004844557
<i>ytjA</i>	standard	47.56	172.46	3.63	<i>ohsC</i>	109.96	1.27	0.42	0.001015212	0.005098873
<i>uvrY</i>	standard	27.18	98.05	3.61	<i>yrbN</i>	225.13	1.27	0.37	2.38E-04	0.001449485
<i>yhjD</i>	standard	8.15	29.33	3.6	<i>mdtK</i>	62.12	1.27	0.5	0.00443095	0.018242686
<i>tehA</i>	standard	18.34	65.66	3.58	<i>yghE</i>	41.85	1.27	0.58	0.010335855	0.036693964
<i>adhP</i>	standard	5.44	19.26	3.54	<i>rsxA</i>	53.19	1.26	0.54	0.006530064	0.024978693
<i>cmo</i>	standard	1.36	4.81	3.54	<i>sfsA</i>	54.6	1.25	0.52	0.006450973	0.024823494
<i>B</i>										
<i>pncA</i>	standard	1.36	4.81	3.54	<i>ytiB</i>	51.03	1.25	0.54	0.007595897	0.028362246
<i>uhpC</i>	standard	1.36	4.81	3.54	<i>tusA</i>	75.69	1.25	0.47	0.003008532	0.013056443
<i>insA4</i>	standard	4.08	14.44	3.54	<i>rpIS</i>	741.32	1.24	0.34	1.17E-04	7.62E-04
<i>pmrD</i>	standard	158.3	556.33	3.51	<i>rnIB</i>	45.16	1.23	0.56	0.010036429	0.035808421
<i>slp</i>	standard	19.02	66.53	3.5	<i>blr</i>	68.02	1.23	0.49	0.004412675	0.018201483
<i>yfjD</i>	standard	4.76	16.63	3.5	<i>bfr</i>	69.08	1.23	0.49	0.004582157	0.018766453
<i>talA</i>	standard	13.59	47.27	3.48	<i>ibpA</i>	46.01	1.2	0.55	0.011140268	0.038903904
<i>yhbO</i>	standard	6.79	23.64	3.48	<i>tatE</i>	68.34	1.19	0.48	0.005545779	0.022021246
<i>yidZ</i>	standard	29.89	103.74	3.47	<i>iscX</i>	499.84	1.18	0.4	0.001193108	0.005907016
<i>yecN</i>	standard	42.8	147.95	3.46	<i>obgE</i>	194.26	1.17	0.39	9.99E-04	0.005046111
<i>mep</i>	standard	15.63	53.84	3.45	<i>tolC</i>	692.49	1.17	0.33	2.01E-04	0.001238049
<i>M</i>										
<i>ecnA</i>	small	2.04	7	3.44	<i>iscU</i>	305.42	1.16	0.43	0.002823147	0.012529967
<i>fecC</i>	standard	2.04	7	3.44	<i>frmR</i>	87.67	1.15	0.45	0.004308386	0.01787923
<i>phnD</i>	standard	4.08	14.01	3.44	<i>rseC</i>	118.42	1.15	0.41	0.002334944	0.010619245
<i>ycgY</i>	standard	2.04	7	3.44	<i>eco</i>	76.98	1.15	0.47	0.006182743	0.024121949
<i>ycho</i>	standard	4.08	14.01	3.44	<i>atpl</i>	308.54	1.14	0.35	4.75E-04	0.002606775
<i>ynjD</i>	standard	2.04	7	3.44	<i>rpoZ</i>	113.46	1.14	0.44	0.004444168	0.018273064
<i>thiL</i>	standard	8.83	30.2	3.42	<i>roxA</i>	178.9	1.14	0.38	0.001264669	0.006212163
<i>ybiR</i>	standard	8.83	30.2	3.42	<i>lpxT</i>	53.03	1.14	0.53	0.013268514	0.04421425
<i>ytjB</i>	standard	69.3	236.8	3.42	<i>hdeD</i>	64.47	1.13	0.53	0.012893055	0.043351172
<i>metI</i>	standard	4.76	16.2	3.41	<i>rcnB</i>	64.97	1.13	0.49	0.008988767	0.032666496
<i>ybjS</i>	standard	93.76	318.66	3.4	<i>yffB</i>	84.37	1.12	0.46	0.006420724	0.024741928
<i>dnaK</i>	standard	14.95	50.77	3.4	<i>cdsA</i>	70.95	1.1	0.49	0.011431427	0.03987618
<i>rscC</i>	standard	12.91	43.77	3.39	<i>iscA</i>	310.37	1.1	0.42	0.004388433	0.018139242
<i>phnO</i>	standard	10.87	36.77	3.38	<i>osmB</i>	100.71	1.09	0.43	0.005613039	0.022203793
<i>pspD</i>	standard	40.77	137.44	3.37	<i>lysU</i>	87.48	1.08	0.46	0.008192364	0.030264355
<i>hokD</i>	standard	38.73	130.44	3.37	<i>ecnB</i>	69.09	1.08	0.48	0.010833276	0.038044129
<i>inaA</i>	standard	15.63	52.53	3.36	<i>pepB</i>	120.64	1.08	0.42	0.004873856	0.019754267
<i>pabB</i>	standard	39.41	131.75	3.34	<i>rseB</i>	238.27	1.07	0.36	0.001374135	0.006645545
<i>yfdC</i>	standard	19.7	65.66	3.33	<i>nadD</i>	71.06	1.05	0.47	0.012553295	0.042741306
<i>fumE</i>	standard	25.82	85.79	3.32	<i>ivy</i>	123.06	1.05	0.41	0.005102016	0.020545956
<i>aroC</i>	standard	4.76	15.76	3.31	<i>bax</i>	212.78	1.04	0.36	0.002015903	0.009331005
<i>wecF</i>	standard	16.99	56.03	3.3	<i>bssS</i>	188.72	1.03	0.37	0.002690689	0.011976054
<i>yadX</i>	small	23.78	78.35	3.29	<i>bolA</i>	157.88	1.02	0.4	0.00569691	0.022448028
<i>degP</i>	standard	21.06	69.16	3.28	<i>ydgK</i>	78.89	1.01	0.45	0.012656898	0.042898198

<i>ydhI</i>	standard	14.27	46.84	3.28	<i>lpxB</i>	94.1	1	0.44	0.011029168	0.038645314
<i>ymdF</i>	standard	14.27	46.84	3.28	<i>deaD</i>	606.52	0.99	0.33	0.001428271	0.006886069
<i>hisQ</i>	standard	16.99	55.59	3.27	<i>frmA</i>	117.64	0.96	0.41	0.009881474	0.03533615
<i>yodC</i>	standard	161.7	524.82	3.25	<i>ybiE</i>	142.68	0.96	0.39	0.008000962	0.029627231
<i>abpA</i>	standard	24.46	79.23	3.24	<i>yrbL</i>	258.5	0.94	0.35	0.004091926	0.017048785
<i>ybjG</i>	standard	303.02	980.92	3.24	<i>pheM</i>	167.34	0.93	0.38	0.008333725	0.030642886
<i>yeaG</i>	standard	29.89	96.73	3.24	<i>ybhL</i>	238.82	0.91	0.38	0.008835762	0.032260328
<i>mrcB</i>	standard	48.24	155.83	3.23	<i>hspQ</i>	188.37	0.87	0.38	0.012704292	0.042928435
<i>mrdB</i>	standard	1.36	4.38	3.22	<i>ydiH</i>	403.56	0.86	0.33	0.005399113	0.021548245
<i>acpS</i>	standard	0.68	2.19	3.22	<i>cpxP</i>	205.41	0.86	0.37	0.011798686	0.040793466
<i>amF</i>	standard	0.68	2.19	3.22	<i>thrW</i>	199.73	0.83	0.36	0.013973818	0.046178214
<i>essQ</i>	standard	0.68	2.19	3.22	<i>nlpD</i>	735.03	0.77	0.31	0.008684907	0.031820928
<i>hcr</i>	standard	0.68	2.19	3.22	<i>csrB</i>	2954.91	0.68	0.29	0.014267278	0.046991908
<i>ilvH</i>	standard	0.68	2.19	3.22						
<i>insB2</i>	standard	0.68	2.19	3.22						
<i>insB3</i>	standard	0.68	2.19	3.22						
<i>kdul</i>	standard	0.68	2.19	3.22						
<i>lpxK</i>	standard	2.04	6.57	3.22						
<i>narJ</i>	standard	0.68	2.19	3.22						
<i>sufD</i>	standard	0.68	2.19	3.22						
<i>treA</i>	standard	0.68	2.19	3.22						
<i>yaal</i>	standard	1.36	4.38	3.22						
<i>ydhT</i>	standard	1.36	4.38	3.22						
<i>ydhU</i>	standard	2.04	6.57	3.22						
<i>ynaK</i>	standard	0.68	2.19	3.22						
<i>ynbA</i>	standard	0.68	2.19	3.22						
<i>ypdC</i>	standard	0.68	2.19	3.22						
<i>yqeC</i>	standard	1.36	4.38	3.22						
<i>yqiA</i>	standard	16.99	54.28	3.2						
<i>ybgA</i>	standard	9.51	30.2	3.18						
<i>ydgJ</i>	standard	108.03	342.73	3.17						
<i>ynfC</i>	standard	8.83	28.01	3.17						
<i>yhbQ</i>	standard	74.74	236.8	3.17						
<i>metL</i>	standard	10.87	34.14	3.14						
<i>eamA</i>	standard	14.27	44.65	3.13						
<i>dusC</i>	standard	9.51	29.76	3.13						
<i>relE</i>	standard	4.76	14.88	3.13						
<i>ygdR</i>	standard	307.1	952.03	3.1						
<i>yfeY</i>	standard	57.07	176.84	3.1						
<i>dnaG</i>	standard	17.66	54.71	3.1						
<i>mukE</i>	standard	35.33	108.99	3.08						
<i>slt</i>	standard	15.63	48.15	3.08						

<i>ybiJ</i>	standard	6.11	18.82	3.08
<i>moa</i>	standard	26.5	81.41	3.07
<i>A</i>				
<i>zraR</i>	standard	23.1	70.91	3.07
<i>insH</i>	standard	32.61	99.8	3.06
<i>5</i>				
<i>cvpA</i>	standard	17.66	53.4	3.02
<i>tcdA</i>	standard	30.57	91.92	3.01
<i>ycjX</i>	standard	8.15	24.51	3.01
<i>yigL</i>	standard	4.08	12.26	3.01
<i>ymlC</i>	small	282.63	839.53	3.00

Table S5. Verification of protein expression from the genomic locus for the 17 candidates identified in this study using Ribo-RET.

Small Protein	Small protein expression by genomic tagging first reported in	Media used
MgtS	16	LB
	17	Supplemented N-minimal medium with no added Mg ²⁺
MgrB	16	LB
PmrR	18	N-minimal medium, pH 7.7 with 10 μM Mg ²⁺
MgtT	19	LB
YmiC	20	LB
YmiA	16	LB
Yoal	[This Study]	Supplemented minimal A medium with 10 mM, 1 mM and no added Mg ²⁺
YobF	16	LB
	3	LB + heat shock (45°C)
YddY	20	LB
YriB	19	LB
YadX	19	LB
YkgS	20	LB
YriA	19	LB
YdgU	16	LB
Yqhl	20	LB
YadW	20	LB
DinQ	21	LB

Table S6. Localization, Tag, and Membrane Topology Prediction of Small Proteins

Small Protein	Localization	Epitope tag	Reference	Prediction of transmembrane helix and orientation		
				TMHMM ²²	TMPred ²³	Phobius ²⁴
MgtS	Membrane	C-terminal/FLAG and C-terminal/GFP	16,25	Yes	Predicted helix: 7-25 (19aa); Inside to Outside: N-terminus facing cytoplasm	Cytoplasmic (1-6aa) / Transmembrane (7-25aa) / Non-cytoplasmic (26-31aa)
MgrB	Membrane	C-terminal/FLAG and N-terminal/GFP	1,16	Yes	Predicted helix: 6-24 (19aa); Inside to Outside: N-terminus facing cytoplasm	Positively charged or n-region (1-5aa) / Hydrophobic α -helical or h-region (6-17aa) / Non cytoplasmic (22-47)
PmrR	Membrane	C-terminal/6XHis	This study	Yes	Predicted helix: 5-27 (23aa); Inside to Outside: N-terminus facing cytoplasm	Cytoplasmic (1-8aa) / transmembrane (9-26aa) / non cytoplasmic (27-29aa)
		N-terminal/FLAG-6XHis (<i>S. enterica</i> PmrR)	18			
MgtT	Cytoplasm	N-terminal/GFP	This study	No	NA	Non cytoplasmic
YmiC	Membrane	N-terminal/GFP	This study	Yes	Predicted helix: 8-29 (22aa); Inside to Outside: N-terminus facing cytoplasm	Cytoplasmic (1-8aa) / Transmembrane (9-29aa) / Non-cytoplasmic (30-31aa)
YmiA	Membrane	N-terminal/GFP	This study	Yes	Predicted helix: 22-42 (21aa); Inside to Outside: N-terminus facing cytoplasm	Cytoplasmic (1-20aa) / Transmembrane (21-42aa) / Non-cytoplasmic (43-46aa)

YoaI	Membrane	C-terminal/GFP	This study	Yes	Predicted helix: 10-20 (21aa); Outside to Inside: N-terminus facing periplasm	Non - cytoplasmic (1-5aa) / Transmembrane (6-30aa) / Cytoplasmic (31-34aa)
YobF	Membrane	C-terminal/6XHis	This study	Yes	Predicted helix: 25-41 (17aa); Outside to Inside: N-terminus facing periplasm	Non cytoplasmic
YddY	Cytoplasm	N-terminal/GFP	This study	No	NA	Non cytoplasmic
YriB	Cytoplasm	N-terminal/GFP	This study	No	NA	Non cytoplasmic
YadX	Cytoplasm	N-terminal/GFP	This study	No	NA	Cytoplasmic
YkgS	Cytoplasm	N-terminal/GFP	This study	No	NA	Cytoplasmic
YriA	Cytoplasm	N-terminal/GFP	This study	No	NA	Non cytoplasmic
YdgU	Membrane	N-terminal/GFP	This study	Yes	Predicted helix: 7-26 (20aa); Inside to Outside: N-terminus facing cytoplasm	Cytoplasmic (1-6aa) / Transmembrane (7-26aa) / Non - cytoplasmic (27aa)
YqhI	Cytoplasm	N-terminal/GFP	This study	No	NA	Non cytoplasmic
YadW	Cytoplasm	C-terminal/GFP	This study	No	NA	Non cytoplasmic
DinQ	Membrane	N-terminal FLAG	²¹	Yes	Predicted helix: 1-22 (22aa); Outside to Inside: N-terminal facing periplasm	Non - cytoplasmic (1-5aa) / Transmembrane (6-23aa) / Cytoplasmic (24-27aa)

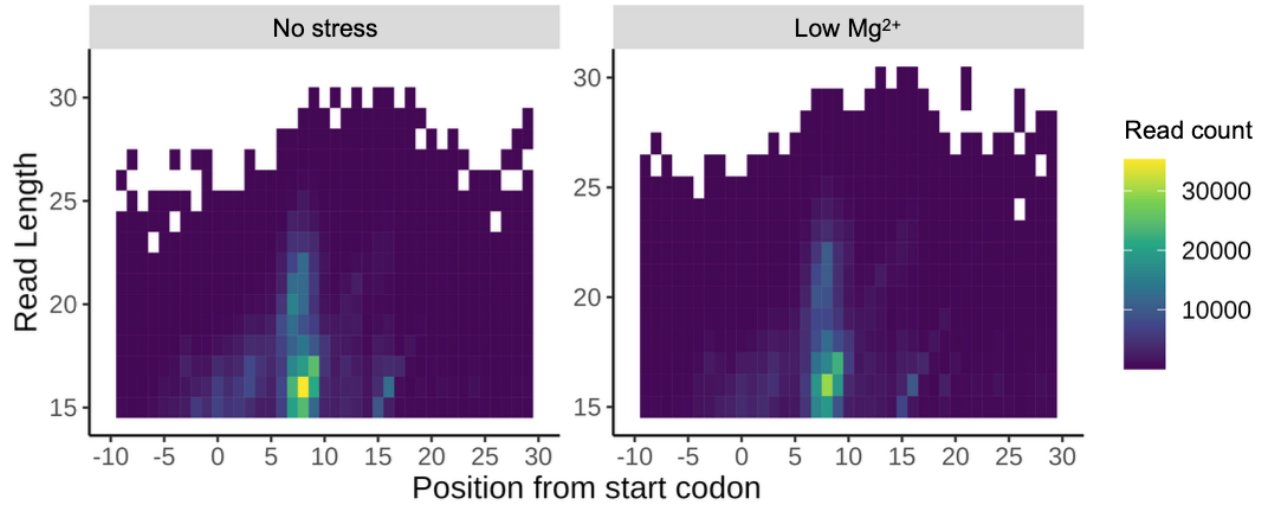


Figure S1. A Ribogrid analysis representing Ribo-RET reads aligned to the genome. The rows represent reads of varying lengths, and the columns indicate the position of the 3'-end of a footprint.

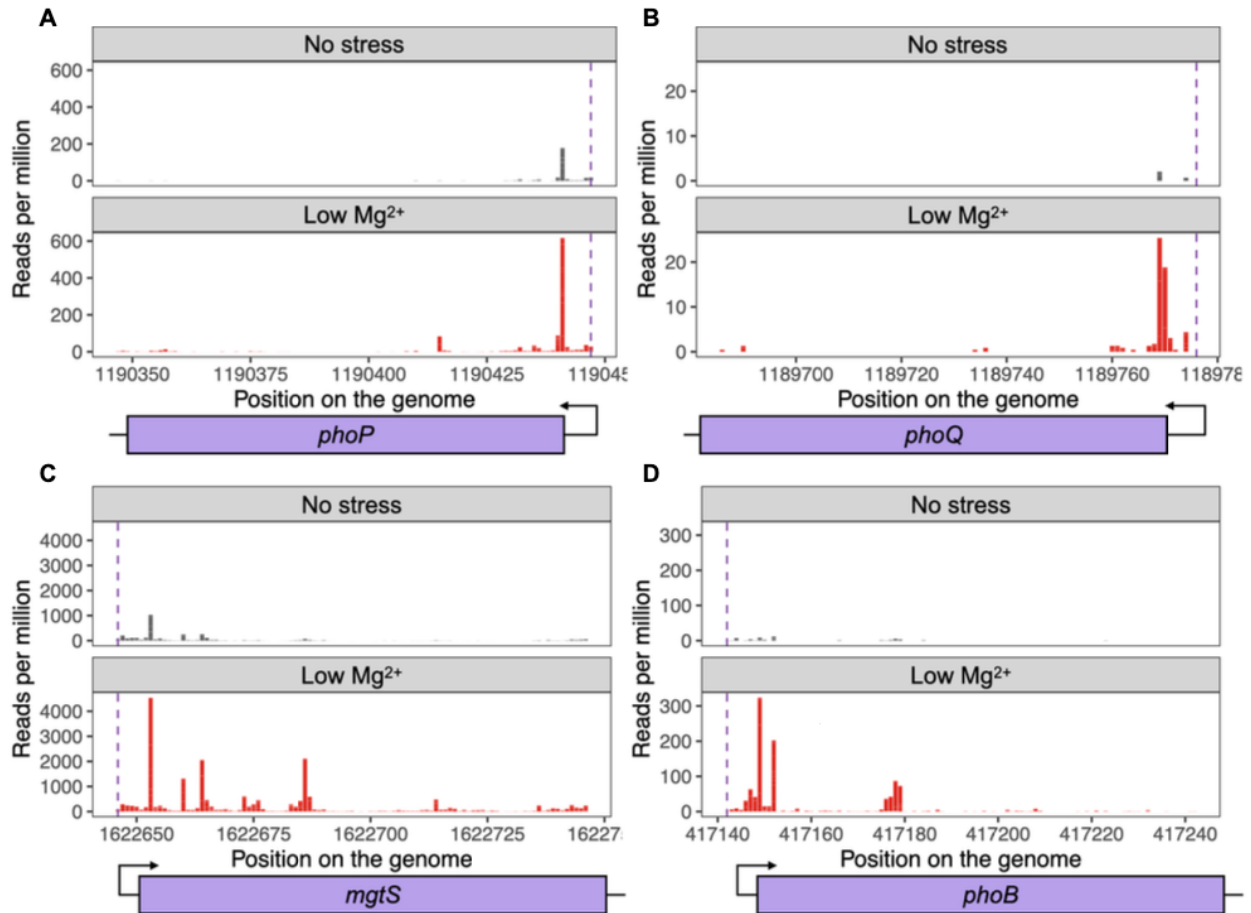


Figure S2. Validation of Ribo-RET in detecting ribosome occupancy on annotated start site of proteins known to be induced under low Mg^{2+} stress.

(A-D) Ribo-RET reads mapping to the translation start site of proteins (A) PhoP, (B) PhoQ, (C) MgtS, and (D) PhoB at no stress condition (10 mM Mg^{2+}) and low Mg^{2+} stress. A purple dashed line indicates the translation start site.

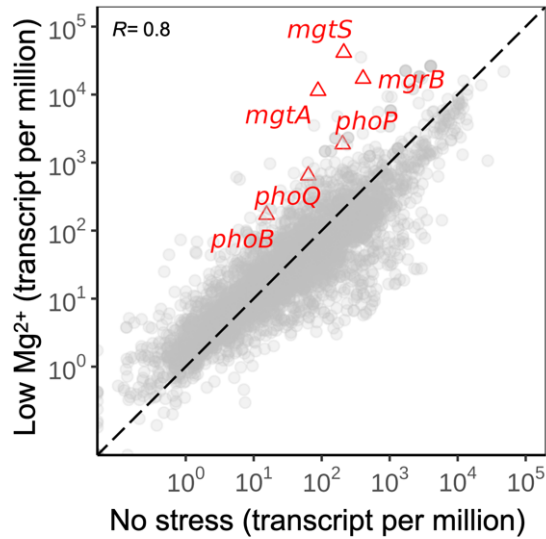


Figure S3. Validation of RNA-Seq in detecting transcripts known to be induced under low Mg^{2+} stress. Scatterplot showing the correlation between the normalized RNA-Seq reads mapping to all the annotated genes under low Mg^{2+} stress and no stress. *mgtS*, *mgrB*, *phoP*, *phoQ*, *mgtA*, and *phoB*, known to be induced under magnesium starvation, are highlighted in red triangle, while the rest of the transcripts are highlighted in gray circles. Pearson's coefficient, $r=0.8$. The data is derived from the average of two biological replicates.

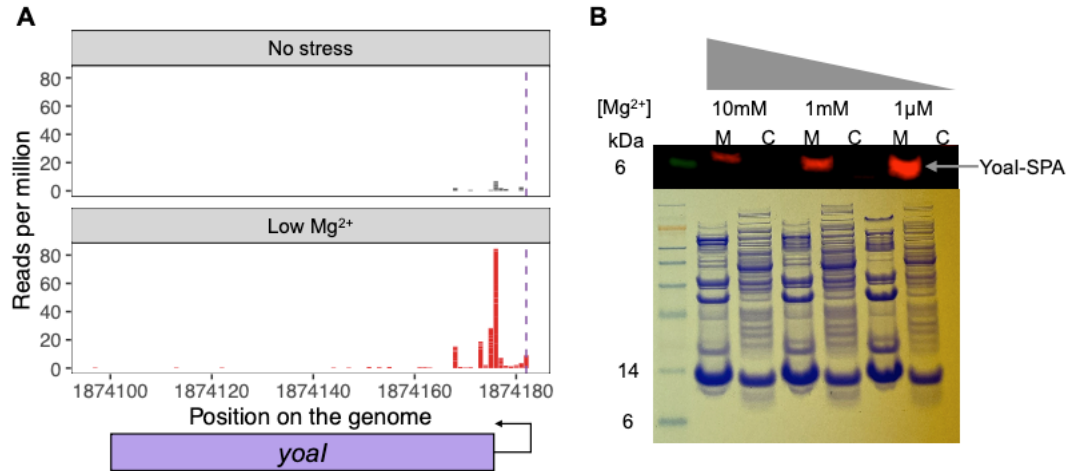


Figure S4. Validation of low magnesium-dependent expression of a small protein, Yoal in the cell. (A) Ribo-RET data for small protein Yoal induced under low Mg^{2+} stress. The translation start site is indicated by a purple dashed line. (B) Validation of Yoal expression using a strain containing *yoal-SPA* genomic fusion (GSO317). Cells were grown in supplemented minimal A medium containing $MgSO_4$ at the indicated concentration. Membrane (M) and cytoplasmic (C) fractions were analyzed by western blotting using M2 anti-FLAG antibodies (top) and by Coomassie Brilliant Blue staining (bottom). The data represents results from two independent replicates.

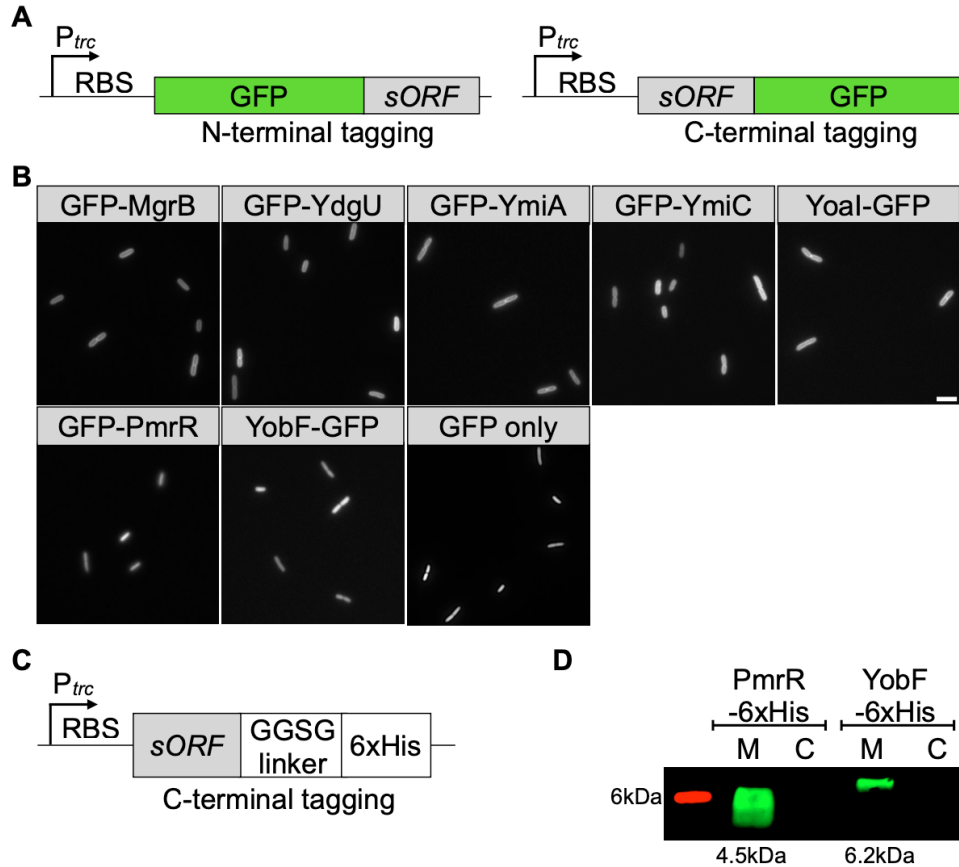


Figure S5. Membrane localization of small proteins identified by epitope tagging. (A)

Schematic representation of GFP-tagged small protein constructs. (B) Fluorescence micrographs of *E. coli* K-12 MG1655 cells expressing GFP-MgrB (pAL38), GFP-YdgU (pPJ3), GFP-YmiA (pPJ12), GFP-YmiC (pPJ13), Yoa1-GFP (pPJ4), GFP-PmrR (pPJ2), YobF-GFP (pPJ6), and GFP only (pAL39). (C) Schematic representation of 6XHis-tagged small protein constructs. (D) Western blot analysis of *E. coli* K-12 MG1655 cells expressing PmrR-6XHis (pSV14) and YobF-6XHis (pSV26), probed with anti-His antibodies. Membrane (M) and cytoplasm (C) fractions are indicated. In Panel B, cells were cultured in supplemented minimal A medium containing 1 mM Mg²⁺ and 50 µg ml⁻¹ carbenicillin. In Panel D, the cells were grown in LB containing 100 µg ml⁻¹ carbenicillin. After four hours of growth, cells were induced with 500 µM IPTG and harvested two hours post-induction. The data represents results from two independent replicates. Scale bar = 5 µm. (Also see Table S6)

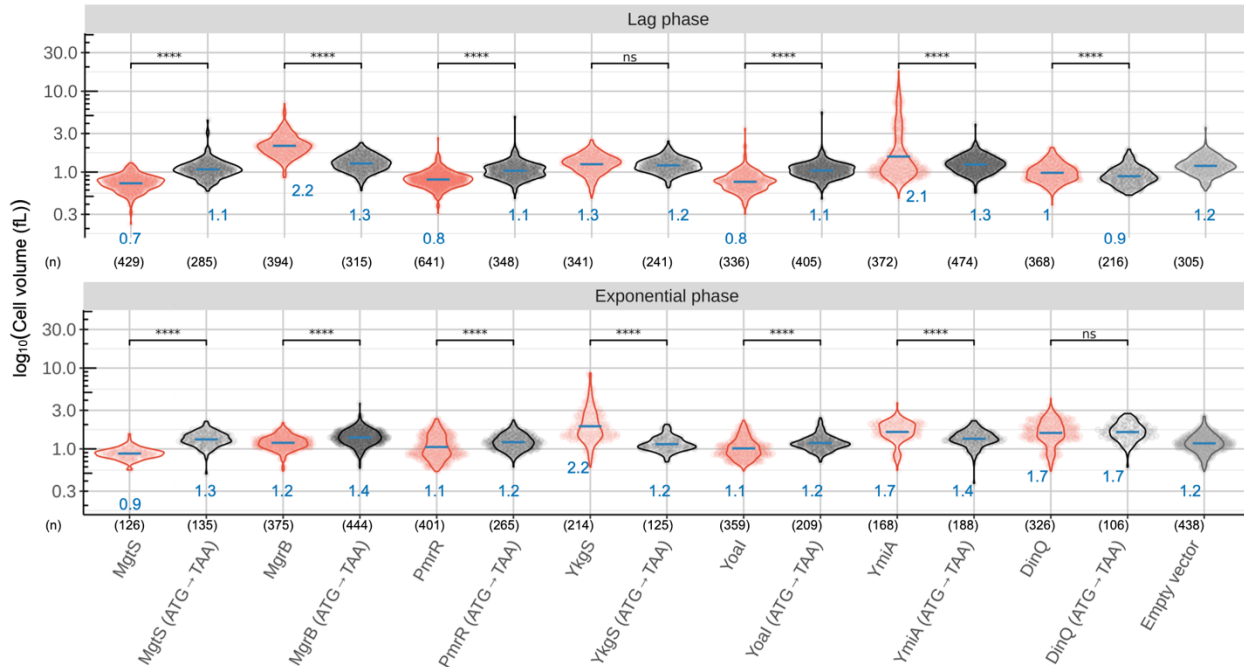


Figure S6. Quantification of cell size changes associated with overexpression of small proteins, MgtS, MgrB, PmrR, YkgS, Yoal, YmiA and DinQ. Cell volume quantification (representative micrograph images are shown in Figure 8B) for the cells expressing small proteins during lag (top panel) and exponential phase (bottom panel) after induction are shown. Each circle corresponds to a single cell. Cells expressing small proteins are shown in red, cells expressing the start codon mutants are depicted in black, and the empty vector (pEB52) control is shown in gray. The cultures were grown in supplemented minimal A medium with no added magnesium. For pSV34-36, pSV38-42, pSV60-61, pJS4, and pJS7, the medium contained $50 \mu\text{g ml}^{-1}$ carbenicillin and $500 \mu\text{M}$ IPTG, while for pSV54-55, 0.5% arabinose was utilized. Data are derived from two independent replicates and the number of cells analyzed is indicated by (n). Mean cell volumes are shown with blue bars and are noted in blue text. P-values indicate the results of a t-test when the strains expressing small protein were compared to their variant with the translation start codon mutated, **** $P \leq 0.001$, and “ns” = $P > 0.05$.

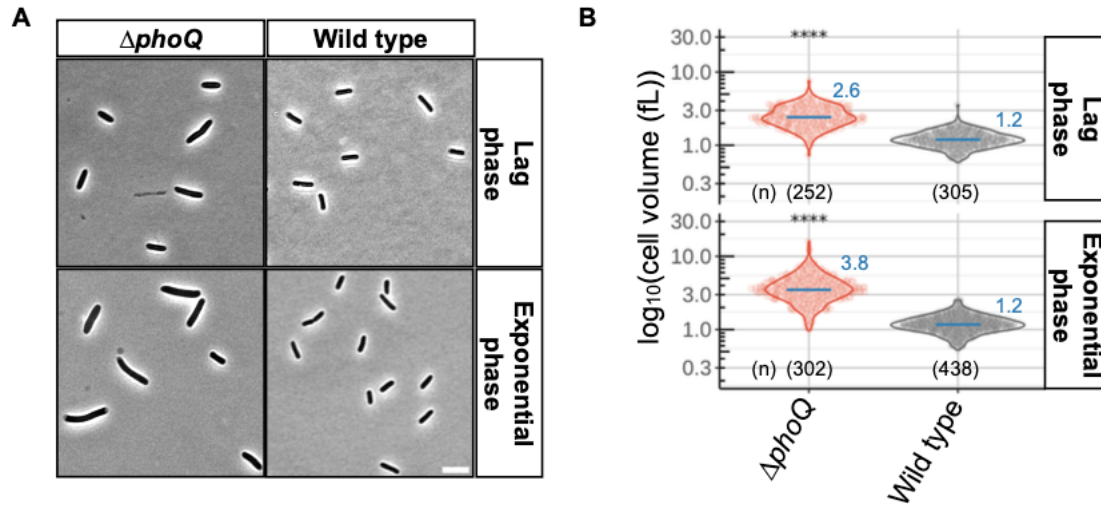


Figure S7. Cell size changes associated with $\Delta phoQ$ under magnesium starvation. (A) Representative phase contrast micrographs of $\Delta phoQ$ (TIM202), and wild type (MG1655) cells expressing an empty vector control (pEB52) during lag and exponential phase, scale bar = 5 μm . Cells were grown in supplemented minimal A medium with no added magnesium, and 50 $\mu\text{g ml}^{-1}$ carbenicillin. (B) Quantification of cell volume for the cells depicted in panel A. Each circle corresponds to a single cell. Cells expressing $\Delta phoQ$ are shown in red and wild type cells are shown in gray. Data are derived from three independent replicates and the number of cells analyzed is indicated by (n). Mean cell volumes are shown with blue bars and are noted in blue text. P-values indicate the results of a t-test when $\Delta phoQ$ cells were compared to the wild-type cells, ****P \leq 0.0001.

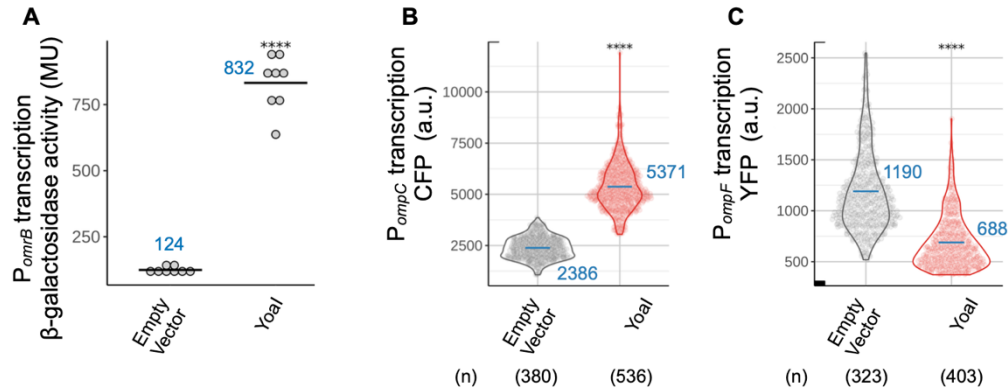


Figure S8. Effect of Yoal overexpression on the EnvZ-OmpR system. (A) Transcriptional activity of *omrB* was measured as a function of *lacZ* expression in wild-type (JM2110) strain during the exponential growth phase upon Yoal (pSV34) and empty vector (pEB52) overexpression. Each circle represents a biological replicate, with the mean values from two independent experiments indicated, expressed in Miller units (MU). The cells were grown in LB containing $100 \mu\text{g ml}^{-1}$ carbenicillin. After three hours of growth, cells were induced with $500 \mu\text{M}$ IPTG, and β -gal activity was measured two hours post-induction. (B-C) Porin expression was measured using CFP fluorescence (corresponding to *ompC*) and YFP fluorescence (corresponding to *ompF*) in wild-type (MDG147) strain during the exponential growth phase upon Yoal (pSV34) and empty vector (pEB52) overexpression. Each circle corresponds to a single cell. Cells overexpressing Yoal are depicted in red, while those with the empty vector are in gray. The cultures were grown in supplemented minimal A medium containing 10mM MgSO_4 (no stress), $50 \mu\text{g ml}^{-1}$ carbenicillin, and $500 \mu\text{M}$ IPTG. Data are derived from three independent replicates and the number of cells analyzed is indicated by (n). Mean fluorescence is shown with blue bars and is noted in blue text. P-values indicate the results of a t-test when cells overexpressing Yoal were compared to the empty vector control, **** $P \leq 0.0001$.

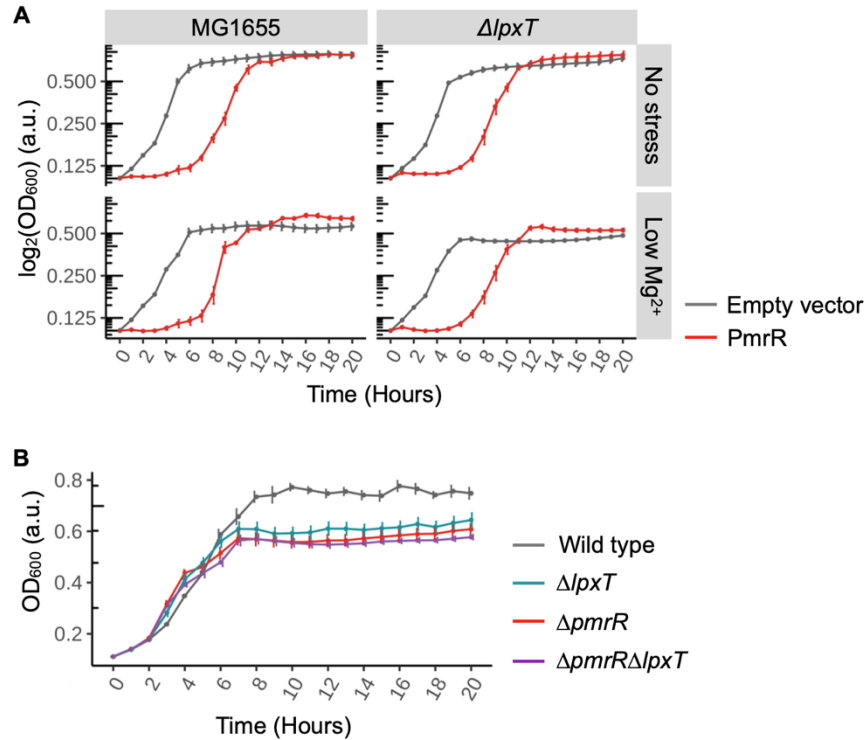


Figure S9. The phenotypes associated with PmrR are LpxT independent. (A) Growth comparison of *E. coli* K-12 MG1655 wild-type and $\Delta lpxT$ strain (SV47) carrying a plasmid encoding the small protein PmrR (pSV35, red line), or an empty vector (pEB52, gray line). The cultures were grown in supplemented minimal A medium containing 50 $\mu\text{g ml}^{-1}$ carbenicillin, 500 μM IPTG, and either 10 mM MgSO_4 (no stress) or no added magnesium (low Mg^{2+}). (B) The plot represents growth curves of wild-type *E. coli* MG1655 (gray) and mutants corresponding to gene deletions $\Delta pmrR$ (SV35, red line), $\Delta lpxT$ (SV47, green line), and $\Delta pmrR \Delta lpxT$ (SV54, purple line). Please see Table S1 for details. The cultures were grown in supplemented minimal A medium with no added magnesium. Data represent averages and standard errors of means for four independent cultures.

References

1. Lipka, A.M., and Goulian, M. (2009). Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. *PLoS Genet.* 5, e1000788.
2. Hobbs, E.C., Astarita, J.L., and Storz, G. (2010). Small RNAs and small proteins involved in resistance to cell envelope stress and acid shock in *Escherichia coli*: analysis of a bar-coded mutant collection. *J. Bacteriol.* 192, 59–67.
3. Hemm, M.R., Paul, B.J., Miranda-Ríos, J., Zhang, A., Soltanzad, N., and Storz, G. (2010). Small stress response proteins in *Escherichia coli*: proteins missed by classical proteomic studies. *J. Bacteriol.* 192, 46–58.
4. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., and Mori, H. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* 2, 2006.0008.
5. Batchelor, E., Silhavy, T.J., and Goulian, M. (2004). Continuous control in bacterial regulatory circuits. *J. Bacteriol.* 186, 7618–7625.
6. Coornaert, A., Chiaruttini, C., Springer, M., and Guillier, M. (2013). Post-transcriptional control of the *Escherichia coli* PhoQ-PhoP two-component system by multiple sRNAs involves a novel pairing region of GcvB. *PLoS Genet.* 9, e1003156.
7. Bastet, L., Korepanov, A.P., Jagodnik, J., Grondin, J.P., Lamontagne, A.-M., Guillier, M., and Lafontaine, D.A. (2024). Riboswitch and small RNAs modulate *btuB* translation initiation in *Escherichia coli* and trigger distinct mRNA regulatory mechanisms. *Nucleic Acids Res.* 52, 5852–5865.
8. Yadavalli Srujana S., Goh Ted, Carey Jeffrey N., Malengo Gabriele, Vellappan Sangeevan, Nickels Bryce E., Sourjik Victor, Goulian Mark, Yuan Jing, and Silhavy Thomas J. (2020). Functional Determinants of a Small Protein Controlling a Broadly Conserved Bacterial Sensor Kinase. *J. Bacteriol.* 202, e00305-20.
9. Miyashiro, T., and Goulian, M. (2007). Stimulus-dependent differential regulation in the *Escherichia coli* PhoQ PhoP system. *Proc. Natl. Acad. Sci. U. S. A.* 104, 16305–16310.
10. Goldberg, S.D., Clinthorne, G.D., Goulian, M., and DeGrado, W.F. (2010). Transmembrane polar interactions are required for signaling in the *Escherichia coli* sensor kinase PhoQ. *Proc. Natl. Acad. Sci. U. S. A.* 107, 8141–8146.
11. Bullock, W.O. (1987). XL1-blue : a high efficiency plasmid transforming *recA* *Escherichia coli* strain with beta-galactosidase selection. *Biotechniques* 5, 376.
12. Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J. Bacteriol.* 177, 4121–4130.
13. Cherepanov, P.P., and Wackernagel, W. (1995). Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158, 9–14.

14. Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* *97*, 6640–6645.
15. Amann, E., Ochs, B., and Abel, K.J. (1988). Tightly regulated tac promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. *Gene* *69*, 301–315.
16. Hemm, M.R., Paul, B.J., Schneider, T.D., Storz, G., and Rudd, K.E. (2008). Small membrane proteins found by comparative genomics and ribosome binding site models. *Mol. Microbiol.* *70*, 1487–1501.
17. Wang, H., Yin, X., Wu Orr, M., Dambach, M., Curtis, R., and Storz, G. (2017). Increasing intracellular magnesium levels with the 31-amino acid MgtS protein. *Proc. Natl. Acad. Sci. U. S. A.* *114*, 5689–5694.
18. Kato, A., Chen, H.D., Latifi, T., and Groisman, E.A. (2012). Reciprocal control between a bacterium's regulatory system and the modification status of its lipopolysaccharide. *Mol. Cell* *47*, 897–908.
19. Weaver, J., Mohammad, F., Buskirk, A.R., and Storz, G. (2019). Identifying Small Proteins by Ribosome Profiling with Stalled Initiation Complexes. *MBio* *10*. <https://doi.org/10.1128/mBio.02819-18>.
20. VanOrsdel, C.E., Kelly, J.P., Burke, B.N., Lein, C.D., Oufiero, C.E., Sanchez, J.F., Wimmers, L.E., Hearn, D.J., Abuikhdair, F.J., Barnhart, K.R., et al. (2018). Identifying New Small Proteins in *Escherichia coli*. *Proteomics* *18*, e1700064.
21. Weel-Sneve, R., Kristiansen, K.I., Odsbu, I., Dalhus, B., Booth, J., Rognes, T., Skarstad, K., and Bjørås, M. (2013). Single transmembrane peptide DinQ modulates membrane-dependent activities. *PLoS Genet.* *9*, e1003260.
22. Möller, S., Croning, M.D., and Apweiler, R. (2001). Evaluation of methods for the prediction of membrane spanning regions. *Bioinformatics* *17*, 646–653.
23. Ikeda, M., Arai, M., Okuno, T., and Shimizu, T. (2003). TMPDB: a database of experimentally-characterized transmembrane topologies. *Nucleic Acids Res.* *31*, 406–409.
24. Käll, L., Krogh, A., and Sonnhammer, E.L.L. (2004). A combined transmembrane topology and signal peptide prediction method. *J. Mol. Biol.* *338*, 1027–1036.
25. Fontaine, F., Fuchs, R.T., and Storz, G. (2011). Membrane localization of small proteins in *Escherichia coli*. *J. Biol. Chem.* *286*, 32464–32474.