JB Accepted Manuscript Posted Online 27 February 2017
J. Bacteriol. doi:10.1128/JB.00057-17

Tail-Anchored Inner Membrane Protein ElaB Increases Resistance to Stress While Reducing Persistence in *Escherichia coli*

Yunxue Guo[1], Xiaoxiao Liu[1], Baiyuan Li[1,2], Jianyun Yao[1,2], Thomas K. Wood[3, 4], and Xiaoxue Wang[1]*

[1]Key Laboratory of Tropical Marine Bio-resources and Ecology, Guangdong Key Laboratory of Marine Materia Medica, RNAM Center for Marine Microbiology, South China Sea Institute of Oceanology Chinese Academy of Sciences, Guangzhou 510301, China, [2]University of Chinese Academy of Sciences, Beijing 100049, China, [3]Department of Chemical Engineering and the [4]Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA 16802-4400, USA

*To whom correspondence should be addressed. Tel: +86 20 89267515; Fax: +86 20 89235490; Email: xxwang@scsio.ac.cn

Running title: ElaB is a stress-related inner membrane protein

Keywords: C-tail anchored membrane protein, oxidative stress, heat shock, persistence

ABSTRACT

Host-associated bacteria, such as *Escherichia coli*, often encounter various host-related stresses such as nutritional deprivation, oxidative stress and temperature shifts. There is growing interest in searching for small endogenous proteins that mediate stress responses. Here, we characterized a small C-tail anchored inner membrane protein ElaB in *E. coli*. ElaB belongs to a class of tail-anchored inner membrane proteins with a C-terminal transmembrane domain but lacking an N-terminal signal sequence for membrane targeting. Proteins from this family have been shown to play vital roles such as membrane traffic and apoptosis in eukaryotes; however, their role in prokaryotes is largely unexplored. Here, we found that transcription of *elaB* is induced in the stationary phase in *E. coli*, and stationary-phase sigma factor RpoS regulates *elaB* transcription by binding to the promoter of *elaB*. Moreover, ElaB protects cells against oxidative stress and heat shock stress. However, unlike membrane peptide toxins TisB and GhoT, ElaB does not lead to cell death, and the deletion of *elaB* greatly increases persister cell formation. Therefore, we demonstrate that disruption of C-tail anchored inner membrane proteins can reduce stress resistance; it can also lead to deleterious effects such as increased persistence in *E. coli*.

IMPORTANCE

Escherichia coli synthesize dozens of poorly understood small membrane proteins containing a predicted transmembrane domain. In this study, we characterized the function of the C-tail anchored inner membrane protein ElaB in *E. coli*. ElaB increases resistance to oxidative stress and heat stress, while inactivation of ElaB leads to high persister cell formation. We also demonstrated that transcription of *elaB* is under the direct regulation of stationary phase sigma factor RpoS. Thus, our study reveals that small inner membrane proteins may have important cellular roles during stress response.

INTRODUCTION

Membrane proteins interact with, or are part of, biological membranes, and they separate the cytoplasm from the extracellular environment in all living cells. These membrane proteins are prevalent as they are encoded by 20 to 30% of all genes in most genomes (1, 2). For *Escherichia coli*, the inner membrane is a phospholipid bilayer and integral inner membrane proteins are mostly α-helical (3). The inner membrane proteins include transporters, channels, receptors, enzymes, and structural membrane-anchoring domains for myriad tasks including energy transduction and cell adhesion (4).

Most proteins are inserted into the membrane by the well-conserved Sec pathway, consisting of a membrane-spanning translocase SecYEG in bacteria. Many accessory proteins aid in protein targeting and insertion, including the signal recognition particle (SRP) and its cognate membrane receptor FtsY (5). To be tagged to the membrane via the Sec pathway, a protein usually has an N-terminal signal sequence for recognition by the SRP. Translation of the N-terminal signal peptide that target proteins to the SRP pathway is required for directing mRNAs to the membrane (6). In *E. coli*, YidC can be specifically cross-linked to the transmembrane domain of newly synthesized peptides during their membrane insertion via translocons (a complex of proteins associated with the translocation of polypeptides across membranes) (7, 8). Several lines of evidence have revealed that in *E. coli*, as well as in mammalian cells, ribosomes translating inner membrane proteins interact cotranslationally with translocons in the membrane, and this interaction is required for the proper insertion of nascent polypeptides into the membrane (6, 9).

C- tail anchored inner membrane proteins, a class of proteins characterized by their lack of N-terminal signal sequence, were first identified in eukaryotes, and they play critical roles in membrane traffic, apoptosis and protein translocation in eukaryotes (10-12). Tail-anchored inner membrane proteins have been found in *Streptomyces coelicolor*, and they are capable of targeting proteins to the membrane in the absence of an N-terminal signal sequence; the C-terminal transmembrane domain is sufficient for membrane targeting (13). By considering the distance of the transmembrane domain from the C-terminus (less than 30 residues) and also the lack of an N-terminal signal peptide, 12 proteins were identified as C-terminal anchored proteins in *E. coli* (14). These 12 proteins include those known to be associated with the inner face of the cytoplasmic membrane, such as

the flagella assembly protein FlK, the TraL protein involved in F pilus formation and enzymes with hydrophobic substrates that are expected to be favored by membrane anchorage (15, 16). Only three out of the 12 C-tailed anchored proteins, YgaM and paralogs ElaB and YqjD, have not been uncharacterized (14). Recently, these three proteins have been shown to be associated with stationary phase ribosomes (17). Complexes in the inner membrane of *E. coli* are often involved in key processes such as energy generation, cell division, signal transduction, and transport (18). Targeting small stress resistance proteins is an emerging area for treating bacterial infections (19-22); however, there are risks in targeting YgaM, ElaB and YqjD since their function is not known.

In this study, we focus on the physiological role of ElaB when cells encounter external stress. The *elaABCD* genes in the *E. coli* genome were previously named without a phenotypic explanation. ElaC was renamed as Rbn and has both endoribonuclease and exoribonuclease activities, while ElaD, previously mis-annotated as a putative sulfatase/phosphatase, is an efficient and specific de-ubiquitinating enzyme (23-25). It has been reported that ElaB is mainly expressed in the stationary phase (17), and we demonstrate here that *elaB* transcription is regulated by the stationary phase sigma factor RpoS. We also provide evidence that ElaB protects cells against heat shock and oxidative stress. However, unexpectedly, disruption of ElaB greatly increases persister cell formation. Thus, ElaB represents a new transmembrane protein that participates in various stress responses.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in **Table 1.** Luria-Bertani (LB) (26) and M9 minimal medium with 0.4% glucose (27) were used as indicated. The Keio collection (28) and the ASKA library (29) were used for deleting and overexpressing single genes. Chloramphenicol (30 μg/mL) was used for maintaining pCA24N-based plasmids, and kanamycin (50 μg/mL) was used for pre-culturing the isogenic knockout mutants.

Construction of plasmids. For the purification of sigma factor RpoS, the coding region of *rpoS* was amplified using BW25113 genomic DNA as template with the primer pair listed in **Table S1**. PCR products were purified using a gel extraction kit (Qiagen, Hilden, Germany), and digested with *Xba*I and *Hind*III and purified with a PCR

product purification kit (Qiagen). The purified PCR products were ligated into the pET28b plasmid and transferred into *E. coli* BL21. The correct construct was verified by DNA sequencing using primer M13**.**

For the promoter activity assay, a 300 nt fragment from -300 to -1 relative to the *elaB* translational start site was amplified by PCR with primers pHGR01-P*elaB*-f and pHGR01-P*elaB*-r (**Table S1**). The PCR product was purified and digested with *Eco*RI and *Hind*III, and then was ligated into pHGR01. The correct vector pHGR01-P*elaB* was verified by DNA sequencing using primer pair pHGR01-f and pHGR01-r listed in **Table S1**. To perform site mutagenesis of the RpoS binding site in the *elaB* promoter in pHGR01-P*elaB* (pHGR01-MP*elaB*), from TTCAGG (-35)…TCTATAGTTA (-10) to AAAAAA (-35)…CCCCCCCCCC (-10), three rounds of PCR were performed. The first round PCR was amplified with pHGR01-P*elaB*-f and M-pHGR01-P*elaB*-r2 (**Table S1**) using the wild-type genomic DNA as template, then the purified PCR product was used as template and the secondary round PCR was amplified with pHGR01-P*elaB*-f and M-pHGR01-P*elaB*-r3 (**Table S1**). The purified products from the secondary PCR were further used as template and amplified with pHGR01-P*elaB*-f and pHGR01-P*elaB*-r. The last round PCR products were further cloned into pHGR01 to make pHGR01-MP*elaB*, and the correct mutation in the *elaB* promoter region in pHGR01-MP*elaB* was validated by DNA sequencing using primers pHGR01-f and pHGR01-r listed in **Table S1.**

Generation of *mCherry* and *gfp* fused strains. To generate *elaB*::*mCherry*, the one step inactivation method (30) was applied to fuse *mCherry*, which encodes a red fluorescence protein, before the stop codon of *elaB* to generate protein ElaB-mCherry. The coding region of *mCherry* without its start codon was amplified by PCR using pmCherry-N1 (Clontech, Mountain View, CA) as template with primers *mCherry*-f and *mCherry*-r. Primer pair *mCherry*-KM-f and KM-r was used to amplify the kanamycin resistance (Km^r) cassette, bordered by FLP recombination target (FRT) sites, from plasmid pKD4. The PCR products were served as templates in overlapping extension PCR with primers *mCherry*-f and KM-r to generate a DNA fragment carrying *mCherry* and Km^r cassettes flanked by about 60 nt regions up- and downstream of *elaB* stop codon. The PCR products were purified using a gel extraction kit (Qiagen), and purified fragments were electroporated into BW25113/pKD46 competent cells. Strain of BW25113 *elaB*::*mCherry* was confirmed by PCR followed by DNA sequencing using primers of

123 conf-f and conf-r. The same procedures were performed to fuse *mCherry* before the stop codon of the *lacZ* gene,
124 as well as for the construction of the *elaB*::*gfp* strain.

125 **Protein localization.** For localization of ElaB using the mCherry-ElaB fusion, strains were cultured to a turbidity
126 at 600 nm of 1.0, washed with 0.85% NaCl, and imaged with fluorescence microscopy (Zeiss Axiophot) using an
127 oil immersion objective (100×). For localization of ElaB by GFP fusion, overnight cultures of BW25113
128 harboring pCA24N-*elaB-gfp* or pCA24N-*gfp* were inoculated into LB with chloramphenicol (30 μg/mL) at a
129 turbidity at 600 nm of 0.1, and 0.5 mM isopropyl-β-d-thiogalactopyranoside (IPTG) was added to induce ElaB-
130 GFP expression for 2 h before imaged.

131 **Fractionation of membrane proteins.** To study the localization of ElaB, proteins from the inner and outer
132 membranes were obtained by differential centrifugation as described previously with modification (17, 31). IPTG
133 (0.5 mM) was added to induce ElaB expression using pCA24N-*elaB* for 3 h. Cell pellets were resuspended in TE
134 buffer (50 mM Tris-HCl at pH 8.0, 5 mM EDTA), and 1 mg/mL lysozyme (Sigma-Aldrich) was added to lyse the
135 cells. Cell debris was removed by centrifugation at 3,200 g at 4°C for 10 min. The supernatant was further
136 centrifuged at 4°C at 500,000 g for 1.5 h, and the pellets (membrane protein factions) were resuspended in 2 mL
137 of TE buffer. An aliquot (1/10) of was then loaded on a 20-mL sucrose step gradient prepared with 10 mL of 70%
138 (wt/vol) sucrose in TE buffer layered with 10 ml of 53% (wt/vol) sucrose in TE buffer to form a gradient, and
139 subjected to centrifugation at 4°C at 400,000 g for 5 h. The upper band (inner membrane proteins) and lower band
140 (outer membrane proteins) were collected from the top of the gradient. After removing sucrose using a filter
141 (Amicon Ultracel-3K; Millipore), the membrane fractions were solubilized in sodium dodecyl sulfate (SDS)
142 sample buffer and were used for Western blot. Cells harboring pCA24N-*ompA* and pCA24N-*macB* prepared at
143 the the same conditions were used as controls.

144 **Western blot analysis.** A total of a 2.5 μg protein was loaded and run using Tricine-SDS-PAGE as described
145 previously (32). Proteins were transferred to a PVDF membrane (Millipore, Bedford, MA, USA) and a Western
146 blot was performed with primary antibodies raised against the His tag (Cell Signaling Technology, Danvers, MA,
147 USA) or GFP (Abmart, Shanghai, China) and horseradish peroxidase-conjugated goat anti-mouse secondary
148 antibodies (Bio-Rad, Richmond, CA, USA).

qRT-PCR. Total RNA was isolated (33) using an RNA isolation kit (Invitrogen, Carlsbad, CA). DNase was applied during the RNA isolation process to avoid contamination of DNA. A total of 50 ng total RNA was used for qRT-PCR using the *Power* SYBR® Green RNA-to-C_T™ *1-Step* Kit and the StepOne™ Real-Time PCR System (Applied Biosystems). Primers were annealed at 60°C, and *rrsG* (34) was used to normalize the data. Relative expression levels of induction or repression of *elaB* under different conditions were calculated as described previously (35).

Electrophoretic mobility shift assay (EMSA). EMSAs were performed as previously described (36, 37). Briefly, DNA fragments were amplified using the primer pairs shown in **Table S1**. PCR products were gel purified with a QIAquick Gel Extraction Kit (Qiagen) and labeled with the Pierce™ biotin 3' end DNA labeling kit (Thermo scientific, Rockford, IL). For the binding reactions, the *E. coli* RNA core polymerase (NEB, Ipswitch, MA, USA) was mixed 1:2 (M:M) with RpoS at room temperature for 5 min to form the holoenzyme before the addition of biotin labeled DNA probes (0.05 pmol). The binding reaction was performed with the nonspecific competitor DNA (poly dI-dC) and NP-40 in buffer containing 10 mM HEPES (pH 7.3), 20 mM KCl, 1 mM $MgCl_2$, and 5% glycerol at 25°C for 3 h. The final mixtures were run on a 6% DNA retardation gel (Invitrogen), transferred to a nylon membrane, and UV cross-linked. Chemiluminescence was performed with the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's protocol.

β-galactosidase activity assay. BW25113 wild-type and Δ*rpoS* cells harboring pHGR01-P*elaB* or pHGR01-MP*elaB* were cultured to a turbidity at 600 nm of 6.0, and 800 µL cultures were diluted with 4 mL PM2 (70 mM $Na_2HPO_4{\cdot}12H_2O$, 30 mM $NaH_2PO_4{\cdot}H_2O$, 1 mM $MgSO_4$ and 0.2 mM $MnSO_4$, pH 7.0) (38). Then 30 µL of toluene and 35 µL of a 0.1% SDS solution were added to 2.5 mL of bacterial suspension to permeabilize cells. The mixtures were vortexed for 10 s and incubated at 37°C for 45 min in a water bath for evaporation of toluene. For the enzymatic reaction, 250 µL of permeabilized cells was added to PM2 supplemented with β-mercaptoethanol (final concentration, 100 mM) to a final volume of 1 mL. The reaction was started by adding 250 µL of 4 mg/mL *o*-nitrophenol-galactoside in PM2. Then 500 µL of 1M Na_2CO_3 was added to stop the reaction and turbidity at 420 nm was measured. The β-galactosidase activity (Miller units) was calculated as described

175 previously (39). For *rpoS* complementation studies, cells carrying pCA24N-*rpoS* were cultured to a turbidity at
176 600 nm 1.0 and expression of RpoS was induced with 0.5 mM IPTG for 2 h, and β-galactosidase activity was
177 measured as above.

178 **Survival assay.** Overnight cultures were diluted to a turbidity at 600 nm of 0.05 in LB, and incubated at 37°C
179 with 250 RPM shaking until cultures reached a turbidity of 1.0. Then 1 mL was collected for a cell viability assay
180 to measure the initial population. For strains using pCA24N-based plasmids, overnight cultures were diluted to a
181 turbidity of 0.05 and grown to a turbidity of 0.5, then 1 mM IPTG was used to induce gene expression for 2 h, and
182 the turbidity was adjusted to 1.0 (34). For the oxidative stress assay, 1 mL cultures were treated with or without
183 20 mM H_2O_2 for 10 mins, and cells were serially diluted in 0.85% NaCl solution and applied as 10 μL drops on
184 LB agar. For the persistence assay, 100 μg/mL ampicillin or 5.0 μg/mL ciprofloxacin were added to cultures with
185 a turbidity of 1.0, followed by incubation for 3 h; cell survival was determined by drop assay. For heat shock
186 stress, cultures at a turbidity of 1.0 were transferred from 37°C to 65°C using a water shaking incubator for 5 min
187 and 10 min.

188 **Determination of MICs**. MICs were determined using BIOFOSUN (Shanghai, China) drug-sensitive test plates.
189 In brief, overnight cultures were diluted to turbidity of 0.05 in LB medium and incubated at 37°C until the
190 turbidity reached 0.1. Cultures were diluted 1:100 in fresh LB medium and 100 μl of the cell suspension
191 inoculated into the wells which were treated with different concentrations of antibiotics. Then the cells were
192 cultured without shaking for 20 h at 37°C. The MIC was determined as the lowest antibiotic concentrations with
193 no visible growth occurred.

194 **Statistical Analysis**. Statistical analysis was performed using SPSS (16.0 version). The differences of the
195 corresponding values among different conditions were tested by one-way analysis of variance (ANOVA)
196 followed by a least significant difference test. A probability level (*p*-value) of less than 0.05 was regarded as
197 statistically significant.

198

199 **RESULTS**

ElaB is an inner membrane protein with conserved C-terminal transmembrane domain. ElaB is a small protein of 101 amino acids. It has one transmembrane domain at the C-terminus, and this domain is conserved in several bacterial species including opportunistic pathogens (**Figure 1**). In contrast, the N-terminus of these proteins shows much less conservation in terms of length and amino acid composition. Two proteins in *E. coli*, YqjD and YgaM, share the same C-terminal transmembrane domain of ElaB. Moreover, the transmembrane domain is very close to the end of the C-terminus and is followed by two to four residues containing one to three arginines (**Figure 1**).

ElaB localization in *E. coli* was first checked by fusing a red fluorescence protein gene *mCherry* to the C-terminal of the *elaB* gene in the chromosome of BW25113 wild-type cells to express the fused protein ElaB-mCherry. As expected, ElaB-mCherry was localized to the cell poles (**Figure 2, upper panel**). To exclude the potential effects of C-terminal mCherry on localization, we also fused the *mCherry* gene to the C-terminal of the *lacZ* gene in the chromosome of BW25113 wild-type cells, and the fused protein LacZ-mCherry was localized in the cytoplasm (**Figure 2, lower panel**). To further check the ElaB localization, ElaB with a green fluorescent protein (GFP) tagged at the C-terminus was produced using pCA24N-*elaB-gfp*. As expected, GFP-fused ElaB was also localized to the cell poles (**Figure S1**). Localization of GFP-fused membrane protein at the cell poles was also observed with previously characterized inner membrane proteins YqjD (40) and YidC (41), suggesting that ElaB should be anchored in the membrane.

To further determine ElaB resides in the inner membrane or outer membrane, the membrane proteins of *E. coli* were fractionated by a sucrose cushion protocol, which takes advantage of the fact that the inner membrane of *E. coli* has a lower density than the outer membrane (17, 31). Different cell fractions were obtained from *E. coli* producing ElaB with an N-terminal His-tag using plasmid pCA24N-*elaB*. A Western blot was conducted to detect the His-tagged ElaB in the membrane fractions using a His-tagged antibody. His-ElaB was only present in inner membrane fraction but not in the outer membrane fraction (**Figure 3A,** lanes 1-4). We also used inner membrane protein MacB (42) and outer membrane protein OmpA (43) as positive controls for membrane protein localization assay. As expected, MacB was found only in the inner membrane fraction (**Figure 3A,** lanes 5-8), while OmpA was largely present in the outer membrane fraction with a small proportion in the inner membrane fraction due to

its high abundance (44) (**Figure 3A,** lanes 9-12).

A previous study showed that both YqjD and ElaB are ribosome-associated proteins and YqjD is also anchored in the inner membrane (17). To see whether there is possibility that some of the ribosome-associated protein ElaB is also present in the soluble fraction, we quantified the levels of GFP-fused ElaB in the soluble and the membrane fractions. More than 90% of the ElaB expressed by pCA24N-*elaB* in the whole cell lysate could be recovered in the membrane fraction with a small proportion present in the soluble fraction (**Figure 3B,** lanes 1-3). Similar results were obtained using GFP-fused ElaB integrated in the chromosome of BW25113 wild-type (**Figure 3B,** lanes 4-6). In *E. coli*, ribosomes translating membrane proteins interact cotranslationally with translocons in the membrane, a process that is essential for proper insertion of nascent polypeptides into the membrane (9). Here, we demonstrate that ElaB, which shares a conserved transmembrane motif with YqjD, is also an inner membrane protein. Unlike YqjD, production of ElaB did not inhibit cell growth or lead to cell lysis (data not shown).

***elaB* is regulated by RpoS.** To determine when ElaB is induced, we first examined *elaB* expression during different growth conditions. Transcription of *elaB* was up-regulated 13.9 ± 0.2 fold when cells entered the stationary phase, and transcription of *elaB* was also up-regulated 9.5 ± 0.6 fold when cells were growing in nutrient-limited minimal medium (M9 minimal medium with 0.4% glucose) compared to LB medium during the exponential growing phase (**Figure 4A**). By contrast, expression of the neighboring upstream gene *elaA* did not change during these growth conditions (data not shown).

To test whether expression of *elaB* is under the direct control of the stationary sigma factor RpoS (σ^{38}), *elaB* expression was first tested in an *rpoS* deletion mutant. As expected, there was no induction of *elaB* in the absence of RpoS in the stationary phase (**Figure 4B**). Moreover, transcription of *elaB* was up-regulated 2.4 ± 0.1 fold by overexpressing *rpoS* via pCA24N-*rpoS* in minimal medium during the exponential growing phase (**Figure 4B**). Next, Virtual Footprint (45) and FGENESB (Softberry, http://www.softberry.com) programs were applied to predict potential RpoS binding sites in the *elaB* promoter. Two RpoS binding sites were identified at 65 nt and 401 nt upstream of the start codon of *elaB*, respectively (**Figure 4C**). The predicted binding sites were also aligned with previous identified consensus sequences for RpoS regulon (37, 46-51), and -10 region in the putative

252 RpoS binding site 2 close to *elaB* start codon showed high similarity to the previously proposed -10 region
253 consensus sequence of $TGN_{0-2}CYATAMT$ (Y stands for C or T and M stands for A or C) (48) or CTATA(c/a)T
254 (50). Moreover, -35 region is 18 nucleotides away from the -10 region in the putative RpoS binding site 2, which
255 is regarded as a functional location (17 ± 2 nucleotides) (51).

256 To determine how RpoS regulates *elaB* transcription, we conducted EMSA with two DNA probes amplified
257 from the promoter of *elaB* (Probe 1 containing putative RpoS binding site1 and Probe 2 containing putative RpoS
258 binding site 2) using purified RpoS (**Figure 3D**) in the presence of *E. coli* core RNA polymerase. As shown in
259 **Figure 4EF,** RpoS only bound and shifted the DNA fragment containing the RpoS binding site close to *elaB* start
260 codon (Probe 2) in a dose dependent manner, but did not bind or shift the DNA fragment containing the RpoS
261 binding site 1 (Probe 1), as well as the mutant RpoS binding site 2 (Probe 3) (**Figure 4C**).

262 To further confirm the regulation of RpoS *in vivo*, we conducted promoter activity assays by fusing the
263 promoter of *elaB* with the *lacZ* gene in pHGR01 to construct the *lacZ* reporter plasmid pHGR01-P*elaB*. As
264 expected, the BW25113 wild-type cells harboring pHGR01-P*elaB* showed significantly higher β-galactosidase
265 activity (1034.2 ± 34.2 miller units, MU) than the Δ*rpoS* cells (268.9 ± 15.9 MU) (**Figure 5A**). Moreover, we
266 performed site-mutagenesis to change the conserved RpoS binding site from TTCAGG (-35
267 region)…TCTATAGTTA (-10 region) to AAAAAA (-35 region)…CCCCCCCCCC (-10 region) (**Figure 4C**) in
268 pHGR01-P*elaB* to construct a mutant *lacZ* reporter plasmid pHGR01-MP*elaB*. Both the wild-type and Δ*rpoS*
269 hosts carrying pHGR01-MP*elaB* had similar β-galactosidase activities, suggesting that RpoS no longer regulates
270 the *elaB* transcription once the conserved binding site is altered (**Figure 5A**). To further confirm the regulation of
271 RpoS on *elaB*, we conducted the complementation experiment of the promoter activity assay by using pCA24N-
272 *rpoS* to overexpress *rpoS* in the Δ*rpoS* host cells. As expected, using pHGR01-P*elaB* as the reporter plasmid,
273 Δ*rpoS* cells overexpressing *rpoS* via pCA24N-*rpoS* showed 8.0 ± 0.9-fold increase in the promoter activity as
274 compared to the empty plasmid pCA24N (937.6 ± 101.5 MU for Δ*rpoS*/pCA24N-*rpoS versus* 117.3 ± 4.0 MU for
275 Δ*rpoS*/pCA24N). However, when pHGR01-MP*elaB* was used as the reporter plasmid, the activity of the mutated
276 promoter of *elaB* did not change in the Δ*rpoS* cells overexpressing *rpoS* or not (70.4 ± 6.7 MU for
277 Δ*rpoS*/pCA24N-*rpoS versus* 83.7 ± 6.2 MU for Δ*rpoS*/pCA24N) (**Figure 5B**). Collectively, these results

278 demonstrate that *elaB* is induced in the stationary phase, and transcription of *elaB* is under the direct control of
279 stationary phase sigma factor RpoS.

280 **ElaB increases survival during heat and oxidative stress.** To determine the physiological role of ElaB, we
281 tested whether *elaB* contributes to survival under stressed conditions using the *elaB* deletion mutant. To test
282 whether ElaB contributes to survival under heat shock, cell viability was determined with and without *elaB* at
283 65°C for 10 min. Survival was greatly reduced (3.3 ± 0.1) × 10^5-fold when *elaB* was deleted (**Figure 6A**), and
284 complementation of the *elaB* mutation increased survival upon heat shock (**Figure 6B**). Similarly, cell survival
285 after treatment with 20 mM H_2O_2 for 10 min was reduced approximately 3.6×10^4-fold when *elaB* was deleted
286 (**Figure 6C**), and complementation of *elaB* mutation increased cell viability 1.2×10^5-fold to levels similar to the
287 wild-type (**Figure 6D**). Collectively, these results show that ElaB increases oxidative and heat stress resistance.

288 **ElaB decreases persister cell formation.** Inner membrane proteins can mediate antibiotic resistance as well as
289 persister cell formation either by damaging the cell membrane or affecting membrane permeability (52-56).
290 Persister cells are phenotypic variants of regular cells, play a major role in the high antibiotic resistance of
291 bacterial biofilms, and are likely responsible for the recalcitrance of chronic infections to antibiotics (57-59). To
292 investigate the role of ElaB in persister cell formation, we first measured the MICs (minimal inhibitory
293 concentrations) of ten commonly used antibiotics for bacterial infections for the Δ*elaB* strain and the wild-type
294 strain. As shown in **Table 2**, changes of MIC values of the ten antibiotics between the two strains were within
295 two-fold. Next, we performed the persister cell assay using high concentrations (>10×MIC) of ampicillin and
296 ciprofloxacin. The numbers of persister cells formed in the Δ*elaB* strain were 4.3 ± 0.5 fold higher after 1 h, and
297 12.6 ± 0.5 fold higher after 3 h when treated with 100 μg/mL ampicillin (**Figure 7A**). Critically, persister cell
298 formation in the Δ*elaB* strain was (1.15 ± 0.1) ×10^3-fold higher than the wild-types cells, when treated with 5
299 μg/mL ciprofloxacin for 1 h. After 3 h, no persister cells was detected in the wild-type strain, while the proportion
300 of persister cell in the Δ*elaB* cells was 0.06 ± 0.01% (**Figure 7B**). Similar to what has been reported earlier (60),
301 ciprofloxacin appears to be more effective in killing regular non-growing cells compared to ampicillin.
302 Additionally, complementation of *elaB* via pCA24N-*elaB* reduced the persister cell formation when compared to
303 Δ*elaB* cells harboring an empty pCA24N plasmid (**Figure 7CD**). Collectively, these results suggest that the

304 inactivation of ElaB greatly increases persister cell formation.

305 **DISCUSSION**

306 Bacterial membranes play essential roles in the response to various stresses. Here we identified a new small
307 inner membrane protein with a C-terminal transmembrane domain in *E.coli*, ElaB, which has a role in multiple
308 stress responses. We found that (i) Expression of *elaB* is up-regulated during the stationary phase, and it is
309 positively regulated by RpoS in a concentration dependent manner by the binding of RpoS to the *elaB* promoter;
310 (ii) ElaB protects cells against oxidative stress and heat shock; and (iii) ElaB decreases persister cell formation. C-
311 tail anchored inner membrane proteins were first identified in eukaryotes, and have been shown to play critical
312 roles in apoptosis and other vital processes (10-12). In *Streptomyces coelicolor,* they may also play an important
313 role (61). Here we provide evidence that the C-tail anchored inner membrane protein ElaB in *E. coli* participates
314 in important cellular processes such as the response to heat stress and oxidative stress.

315 Sigma factor RpoS is a general stress response regulator, and in *E. coli*, environmental stresses such as
316 oxidative stress, stimulate the expression of the *rpoS* gene and thus turn on the expression of the RpoS-controlled
317 regulon (22, 62, 63). Transcription of genes recognized by RpoS through specific sequences in the promoter
318 allows the activation of a 'general stress response', thus protecting cells from harmful conditions (51). In this
319 study, we showed that the previously uncharacterized ElaB is under the direct control of RpoS, and ElaB protects
320 cells fight against oxidative stress. Thus, under stressed condition where RpoS is up-regulated (22), *elaB*
321 transcription should also be induced, illustrating another example of the multifactorial regulation of RpoS. In
322 addition, the upregulation of *elaB* by RpoS has been also previously detected by other groups using DNA
323 microarray studies under stressed conditions (62, 64, 65). Deletion of *rpoS*, as well as the genes that RpoS
324 controls, increases *E. coli* persister formation dramatically to the extent that nearly the whole population became
325 persistent (66). Moreover, biofilms of the *rpoS* mutant were much more resistant to killing by tobramycin than
326 were wild-type *P. aeruginosa* biofilms (67). However, the underlying mechanism is unclear. Here we found that
327 ElaB, activated by RpoS, greatly decreased tolerance to high concentrations of ciprofloxacin by forming more
328 persister cells. Other two identified C-terminal anchored membrane proteins, YqjD and YgaM, are also induced in
329 the stationary phase in *E.coli* (17). However, the physiological functions of both YgaM and YqjD are largely

unknown except that YqjD has been suggested to inhibit cell growth by the inactivation of translational properties of ribosomes (17). We previously found that deletion of the acid stress related genes (e.g., *gadB*) that are activated by RpoS (64) or its transcriptional activator *gadX* greatly increased persister cells formation (66). Nevertheless, these results suggest that the RpoS and genes under the direct control of RpoS are potentially important for the general stress response, but the underlying mechanism of how these genes contribute to the reduction of tolerance to high concentrations of antibiotic while maintaining enhanced resistance to non-antibiotic environmental stresses needs further investigation. Thus, disruption of C-tail anchored inner membrane proteins can reduce stress resistance; it can also lead to deleterious effects such as increased persistence in *E. coli*.

Our blast search indicates that ElaB, as well as YqjD and YgaM, lack N-terminal signal sequences. The high diversity of the N-terminal domains of these proteins, which exhibit no universally conserved sequence characteristics, argues for a membrane targeting mechanism that depends primarily, if not entirely on, the C-terminal domains. Although the 'twin-arginine repeat' or TAT pathway is involved in the secretion of folded proteins (68) and ElaB has two arginines at the end of the C terminus, it lacks the characteristic Z-R-R-φ-X-X sequence (where Z is a polar residues, X-X are hydrophobic residues and φ is any residue) recognized by the TAT system (**Figure 1**). Hence, it is unlikely that ElaB is secreted by the TAT system. In the absence of their N-terminal signal sequence, the C-terminal transmembrane domain of these C-tail anchored proteins identified in *S. coelicolor* is sufficient for membrane targeting (13). However, the underlying targeting pathways has not been elucidated. The identification of this new targeting pathway suggests it may be an important target for antimicrobial agents.

Two small hydrophobic polypeptides TisB (53) and GhoT (52, 54, 69) are toxin components of toxin-antitoxin systems. They both are not under the regulation of RpoS, and are found to increase persister cell formation by reducing cell metabolism to create dormancy. GhoT is a small membrane proteins with two transmembrane domains (residues 7 to 27 and 37 to 57) (52). Additionally, TisB (53) and GhoT damage cell envelopes and lead to cell death when overproduced, but overproduction of ElaB did not cause growth inhibition, indicating that different inner membrane proteins and transmembrane proteins may function differently in the presence of general stress. By using *in vivo* fluorescent imaging and next-generation sequencing, bacterial

356 persister cells, in addition to dormancy (70), employ an ‘‘active defense’’ to pump antibiotics out and reduce
357 intracellular drug concentrations through enhanced efflux activity (55). Since ElaB does not seem to affect the
358 metabolic state of the cell, it is possible that the function of ElaB is involved with efflux pumps either by
359 enhanced pumping out the antibiotic to the outside of the cell or by limiting the uptake of the antibiotic. Although
360 the C-terminal transmembrane domain of ElaB and YqjD is highly conserved, the function of ElaB and YqjD
361 seem different. YqjD inhibits cell growth by binding to the 30S subunit of in the 70S and 100S ribosomes at the
362 N-terminal region (17). However, the N-terminal regions of these two proteins are not highly conserved, and
363 overproduction of ElaB does not lead to growth inhibition or cell lysis. Future studies are needed to elucidate the
364 biochemical properties of these C-tailed inner membrane proteins and how they interact with efflux pumps or
365 other cellular components in prokaryotes.

366 **ACKNOWLEDGEMENTS**

367 We are grateful for the Keio and ASKA strains provided by the Genome Analysis Project in Japan.

368 **FUNDING INFORMATION**

369 This work was supported by the National Science Foundation of China (Grant No. 31290233, 31625001 and
370 31500025), by National Science Foundation of Guangdong Province (2015A030310405), China Postdoctoral
371 Science Foundation funded project (2013M542217 and 2014T70830) and by the Army Research Office
372 (W911NF-14-1-0279). XW is a recipient of the 1000-Youth Elite Program (the Recruitment Program of Global
373 Experts in China).

374 **COMPETING FINANCIAL INTERESTS**

375 The authors declare no competing financial interests.

REFERENCES

1. **Krogh A, Larsson B, von Heijne G, Sonnhammer ELL.** 2001. Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. J Mol Biol **305:**567-580.
2. **Liszewski K.** 2015. Dissecting the structure of membrane proteins.
3. **Koebnik R, Locher KP, Van Gelder P.** 2000. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. Mol Microbiol **37:**239-253.
4. **Saier MH, Jr., Reddy VS, Tsu BV, Ahmed MS, Li C, Moreno-Hagelsieb G.** 2016. The transporter classification database (TCDB): recent advances. Nucleic Acids Res **44:**D372-379.
5. **Shen K, Shan SO.** 2010. Transient tether between the SRP RNA and SRP receptor ensures efficient cargo delivery during cotranslational protein targeting. Proc Natl Acad Sci U S A **107:**7698-7703.
6. **Moffitt JR, Pandey S, Boettiger AN, Wang SY, Zhuang XW.** 2016. Spatial organization shapes the turnover of a bacterial transcriptome. Elife **5**.
7. **Kol S, Nouwen N, Driessen AJ.** 2008. Mechanisms of YidC-mediated insertion and assembly of multimeric membrane protein complexes. J Biol Chem **283:**31269-31273.
8. **Johnson AE, van Waes MA.** 1999. The translocon: A dynamic gateway at the ER membrane. Annu Rev Cell Dev Bi **15:**799-842.
9. **Herskovits AA, Bibi E.** 2000. Association of *Escherichia coli* ribosomes with the inner membrane requires the signal recognition particle receptor but is independent of the signal recognition particle. Proc Natl Acad Sci U S A **97:**4621-4626.
10. **Kalbfleisch T, Cambon A, Wattenberg BW.** 2007. A bioinformatics approach to identifying tail-anchored proteins in the human genome. Traffic **8:**1687-1694.
11. **Pedrazzini E.** 2009. Tail-Anchored Proteins in Plants. J Plant Biol **52:**88-101.
12. **Kriechbaumer V, Shaw R, Mukherjee J, Bowsher CG, Harrison AM, Abell BM.** 2009. Subcellular distribution of tail-anchored proteins in arabidopsis. Traffic **10:**1753-1764.
13. **Craney A, Tahlan K, Andrews D, Nodwell J.** 2011. Bacterial transmembrane proteins that lack N-terminal signal sequences. Plos One **6**.
14. **Borgese N, Righi M.** 2010. Remote origins of tail-anchored proteins. Traffic **11:**877-885.
15. **Karlinsey JE, Pease AJ, Winkler ME, Bailey JL, Hughes KT.** 1997. The *flk* gene of *Salmonella typhimurium* couples flagellar P- and L-ring assembly to flagellar morphogenesis. J Bacteriol **179:**2389-2400.
16. **Frost LS, Paranchych W, Willetts NS.** 1984. DNA-sequence of the F-traale region that includes the gene for F-pilin. J Bacteriol **160:**395-401.
17. **Yoshida H, Maki Y, Furuike S, Sakai A, Ueta M, Wada A.** 2012. YqjD is an inner membrane protein associated with stationary-phase ribosomes in *Escherichia coli*. J Bacteriol **194:**4178-4183.
18. **Luirink J, Yu Z, Wagner S, de Gier JW.** 2012. Biogenesis of inner membrane proteins in *Escherichia coli*. BBA-Bioenergetics **1817:**965-976.
19. **Farr SB, Kogoma T.** 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol Rev **55:**561-585.

20. **Jara LM, Cortes P, Bou G, Barbe J, Aranda J.** 2015. Differential roles of antimicrobials in the acquisition of drug resistance through activation of the SOS response in *Acinetobacter baumannii*. Antimicrob Agents Ch **59:**4318-4320.
21. **Spaniol V, Bernhard S, Aebi C.** 2015. *Moraxella catarrhalis* AcrAB-OprM efflux pump contributes to antimicrobial resistance and is enhanced during cold shock response. Antimicrob Agents Ch **59:**1886-1894.
22. **Battesti A, Majdalani N, Gottesman S.** 2011. The RpoS-mediated general stress response in *Escherichia coli*. Annu Rev Microbiol **65:**189-213.
23. **Dutta T, Deutscher MP.** 2009. Catalytic properties of RNase BN/RNase Z from *Escherichia coli*: RNase BN is both an exo-and endonuclease. J Biol Chem **284:**15425-15431.
24. **Asha PK, Blouin RT, Zaniewski R, Deutscher MP.** 1983. Ribonuclease BN: identification and partial characterization of a new tRNA processing enzyme. Proc Natl Acad Sci U S A **80:**3301-3304.
25. **Catic A, Misaghi S, Korbel GA, Ploegh HL.** 2007. ElaD, a deubiquitinating protease expressed by *E. coli*. PLoS ONE **2:**e381.
26. **Maniatis T, Sambrook J, Fritsch EF.** 1989. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press Cold Spring Harbor, N.Y.
27. **Wang X, Kim Y, Ma Q, Hong SH, Pokusaeva K, Sturino JM, Wood TK.** 2010. Cryptic prophages help bacteria cope with adverse environments. Nat Commun **1:**147.
28. **Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol **2:**2006.0008.
29. **Kitagawa M, Ara T, Arifuzzaman M, Ioka-Nakamichi T, Inamoto E, Toyonaga H, Mori H.** 2005. Complete set of ORF clones of *Escherichia coli* ASKA library (A complete set of *E. coli* K-12 ORF archive): Unique resources for biological research. DNA Res **12:**291-299.
30. **Datsenko KA, Wanner BL.** 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.
31. **Castanie-Cornet MP, Cam K, Jacq A.** 2006. RcsF is an outer membrane lipoprotein involved in the RcsCDB phosphorelay signaling pathway in *Escherichia coli*. J Bacteriol **188:**4264-4270.
32. **Schägger H.** 2006. Tricine-SDS-PAGE. Nat Protocols **1:**16-22.
33. **Ren D, Bedzyk LA, Thomas SM, Ye RW, Wood TK.** 2004. Gene expression in *Escherichia coli* biofilms. Appl Microbiol Biotechnol **64:**515-524.
34. **Wang X, Kim Y, Hong SH, Ma Q, Brown BL, Pu M, Tarone AM, Benedik MJ, Peti W, Page R, Wood TK.** 2011. Antitoxin MqsA helps mediate the bacterial general stress response. Nat Chem Biol **7:**359-366.
35. **Guo Y, Quiroga C, Chen Q, McAnulty MJ, Benedik MJ, Wood TK, Wang X.** 2014. RalR (a DNase) and RalA (a small RNA) form a type I toxin-antitoxin system in *Escherichia coli*. Nucleic Acids Res **42:**6448-6462.
36. **Zhao K, Liu M, Burgess RR.** 2005. The global transcriptional response of *Escherichia coli* to induced sigma 32 protein involves sigma 32 regulon activation

followed by inactivation and degradation of sigma 32 *in vivo*. J Biol Chem **280:**17758-17768.

37. **Lee SJ, Gralla JD.** 2001. Sigma38 (*rpoS*) RNA polymerase promoter engagement via-10 region on nucleotides. J Biol Chem **276:**30064-30071.
38. **Karimova G, Dautin N, Ladant D.** 2005. Interaction network among *Escherichia coli* membrane proteins involved in cell division as revealed by bacterial two-hybrid analysis. J Bacteriol **187:**2233-2243.
39. **Frias JE, Flores E.** 2015. Induction of the Nitrate Assimilation nirA Operon and Protein-Protein Interactions in the Maturation of Nitrate and Nitrite Reductases in the *Cyanobacterium Anabaena* sp Strain PCC 7120. J Bacteriol **197:**2442-2452.
40. **Li G, Young KD.** 2012. Isolation and identification of new inner membrane-associated proteins that localize to cell poles in *Escherichia coli*. Mol Microbiol **84:**276-295.
41. **Urbanus ML, Fröderberg L, Drew D, Björk P, de Gier J-WL, Brunner J, Oudega B, Luirink J.** 2002. Targeting, insertion, and localization of *Escherichia coli* YidC. J Biol Chem **277:**12718-12723.
42. **Daley DO, Rapp M, Granseth E, Melén K, Drew D, Von Heijne G.** 2005. Global topology analysis of the *Escherichia coli* inner membrane proteome. Science **308:**1321-1323.
43. **Freudl R, Schwarz H, Stierhof Y, Gamon K, Hindennach I, Henning U.** 1986. An outer membrane protein (OmpA) of *Escherichia coli* K-12 undergoes a conformational change during export. J Biol Chem **261:**11355-11361.
44. **Fontaine F, Fuchs RT, Storz G.** 2011. Membrane localization of small proteins in *Escherichia coli*. J Biol Chem **286:**32464-32474.
45. **Münch R, Hiller K, Grote A, Scheer M, Klein J, Schobert M, Jahn D.** 2005. Virtual Footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. Bioinformatics**:**4187-4189.
46. **Peano C, Wolf J, Demol J, Rossi E, Petiti L, De Bellis G, Geiselmann J, Egli T, Lacour S, Landini P.** 2015. Characterization of the *Escherichia coli* sigma(S) core regulon by Chromatin Immunoprecipitation-sequencing (ChIP-seq) analysis. Sci Rep **5**.
47. **Maciag A, Peano C, Pietrelli A, Egli T, De Bellis G, Landini P.** 2011. In vitro transcription profiling of the sigmaS subunit of bacterial RNA polymerase: re-definition of the sigmaS regulon and identification of sigmaS-specific promoter sequence elements. Nucleic Acids Res **39:**5338-5355.
48. **Lacour S, Landini P.** 2004. sigma(S)-dependent gene expression at the onset of stationary phase in *Escherichia coli*: Function of sigma(S)-dependent genes and identification of their promoter sequences. J Bacteriol **186:**7186-7195.
49. **Typas A, Hengge R.** 2006. Role of the spacer between the-35 and-10 regions in sigma(s) promoter selectivity in Escherichia coli. Mol Microbiol **59:**1037-1051.
50. **Gaal T, Ross W, Estrem ST, Nguyen LH, Burgess RR, Gourse RL.** 2001. Promoter recognition and discrimination by EsigmaS RNA polymerase. Mol Microbiol **42:**939-954.
51. **Landini P, Egli T, Wolf J, Lacour S.** 2014. sigmaS, a major player in the response to environmental stresses in *Escherichia coli*: role, regulation and mechanisms of promoter recognition. Env Microbiol Rep **6:**1-13.

52. **Wang X, Lord DM, Cheng H-Y, Osbourne DO, Hong SH, Sanchez-Torres V, Quiroga C, Zheng K, Herrmann T, Peti W, Benedik MJ, Page R, Wood TK.** 2012. A new type V toxin-antitoxin system where mRNA for toxin GhoT is cleaved by antitoxin GhoS. Nat Chem Biol **8:**858-861.
53. **Dörr T, Vulić M, Lewis K.** 2010. Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. PLoS Biol **8:**e1000317.
54. **Cheng HY, Soo VW, Islam S, McAnulty MJ, Benedik MJ, Wood TK.** 2014. Toxin GhoT of the GhoT/GhoS toxin/antitoxin system damages the cell membrane to reduce adenosine triphosphate and to reduce growth under stress. Environ Microbiol **16:**1741-1754.
55. **Pu YY, Zhao ZL, Li YX, Zou J, Ma Q, Zhao YN, Ke YH, Zhu Y, Chen HY, Baker MAB, Ge H, Sun YJ, Xie XS, Bai F.** 2016. Enhanced efflux activity facilitates drug tolerance in dormant bacterial cells. Mol Cell **62:**284-294.
56. **Gerdes K, Semsey S.** 2016. Pumping persisters. Nature **534:**41-42.
57. **Wood TK, Knabel SJ, Kwan BW.** 2013. Bacterial persister cell formation and dormancy. Appl Environ Microbiol **79:**7116-7121.
58. **del Pozo JL, Patel R.** 2007. The challenge of treating biofilm-associated bacterial infections. Clin Pharmacol Ther **82:**204-209.
19. **Lewis K.** 2007. Persister cells, dormancy and infectious disease. Nat Rev Microbiol **5:**48-56.
60. **Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K.** 2004. Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett **230:**13-18.
61. **Caraney A.** 2012. Small molecule interrogation of *Streptomyces coelicolor* growth, development and secondary metabolism. Doctor thesis**:**155.
62. **Weber H, Polen T, Heuveling J, Wendisch VF, Hengge R.** 2005. Genome-wide analysis of the general stress response network in *Escherichia coli*: σ^S-dependent genes, promoters, and sigma factor selectivity. J Bacteriol **187:**1591-1603.
63. **Hengge R.** 2009. Proteolysis of sigma(S) (RpoS) and the general stress response in *Escherichia coli*. Res Microbiol **160:**667-676.
64. **Patten CL, Kirchhof MG, Schertzberg MR, Morton RA, Schellhorn HE.** 2004. Microarray analysis of RpoS-mediated gene expression in *Escherichia coli* K-12. Mol Genet Genomics **272:**580-591.
65. **Ito A, May T, Kawata K, Okabe S.** 2008. Significance of *rpoS* during maturation of *Escherichia coli* biofilms. Biotechnol Bioeng **99:**1462-1471.
66. **Hong SH, Wang XX, O'Connor HF, Benedik MJ, Wood TK.** 2012. Bacterial persistence increases as environmental fitness decreases. Microb Biotechnol **5:**509-522.
67. **Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teltzel GM, Lory S, Greenberg EP.** 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. Nature **413:**860-864.
68. **Lee PA,Tullman-Ercek D, Georgiou G.** 2006. The bacterial twin-arginine translocation pathway. Annu Rev Microbiol **60:**373-395.
69. **Wang X, Lord DM, Hong SH, Peti W, Benedik MJ, Page R, Wood TK.** 2013. Type II toxin/antitoxin MqsR/MqsA controls type V toxin/antitoxin GhoT/GhoS. Environ Microbiol **15:**1734-1744.

70. **Rotem E, Loinger A, Ronin I, Levin-Reisman I, Gabay C, Shoresh N, Biham O, Balaban NQ.** 2010. Regulation of phenotypic variability by a threshold-based mechanism underlies bacterial persistence. Proc Natl Acad Sci U S A **107:**12541-12546.

71. **Fu HH, Jin M, Ju LL, Mao YT, Gao HC.** 2014. Evidence for function overlapping of CymA and the cytochrome bc(1) complex in the Shewanella oneidensis nitrate and nitrite respiration. Environ Microbiol **16:**3181-3195.

566 **Table 1.** Bacterial strains and plasmids used in this study. Cm^R and Km^R indicate chloramphenicol
567 and kanamycin resistance, respectively. P and MP indicate promoter and mutant promoter,
568 respectively.
569

Bacterial strains/Plasmids	Description	Source
BW25113 *E. coli* K12 strains		
wild-type	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR*514 Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$ *rph*-	(28)
Δ*elaB*	Δ*elaB* Δkm^R	(28)
Δ*rpoS*	Δ*rpoS* Δkm^R	(28)
elaB::*mCherry*	*mCherry* was fused before *elaB* stop codon in wild-type strain	this study
lacZ::*mCherry*	*mCherry* was fused before *lacZ* stop codon in wild-type strain	this study
elaB::*gfp*	*gfp* was fused before *elaB* stop codon in wild-type strain	this study
Plasmids		
pCA24N	Cm^R; *lacI*^{q},	(29)
pCA24N-*elaB*	Cm^R; *lacI*^{q}, P$_{T5\text{-}lac}$::*elaB*	(29)
pCA24N-*gfp*	Cm^R; *lacI*^{q}, with *gfp* gene	(29)
pCA24N-*elaB-gfp*	Cm^R; *lacI*^{q}, P$_{T5\text{-}lac}$::*elaB*, with *gfp* gene	(29)
pCA24N-*ompA*	Cm^R; *lacI*^{q}, P$_{T5\text{-}lac}$::*ompA*	(29)
pCA24N-*macB*	Cm^R; *lacI*^{q}, P$_{T5\text{-}lac}$::*macB*	(29)
pCA24N-*rpoS*	Cm^R; *lacI*^{q}, P$_{T5\text{-}lac}$::*rpoS*	(29)
pET28b-*rpoS*	Km^R, *lacI*^{q}, pET28b P$_{T7\text{-}lac}$::*rpoS* with C-terminal His-tagged	this study
pHGR01	Km^r, R6K *ori*, promoterless-*lacZ* reporter vector	(71)
pHGR01-P*elaB*	Fused *elaB* promoter with *lacZ* in pHGR01	this study
pHGR01-MP*elaB*	RpoS binding site in *elaB* promoter was mutant as pointed	this study

570
571

572 **Table 2**. MICs (minimal inhibitory concentrations) of wild-type BW25113 and the Δ*elaB* mutant for
573 ten antibiotics.
574

Antibiotics	MICs value (μg/mL)	
	BW25113	Δ*elaB*
Ampicillin	4	4
Polymyxin B	0.5	0.5
Cefoxitin	8	4
Ceftazidime	0.5	<0.25
Imipenem	<0.125	<0.125
Cefotaxime	<0.25	<0.25
Cefepime	<0.5	<0.5
Ciprofloxacin	<0.125	<0.125
Gentamicin	<0.5	<0.5
Tetracycline	2	2

575
576

FIGURE LEGENDS

Figure 1. Conserved C-terminal transmembrane domain in ElaB. Alignment of amino acid sequences among ElaB and other hypothetical membrane proteins from different bacterial species was performed based on Dense Alignment Surface method (http://www.sbc.su.se/~miklos/DAS). The predicted transmembrane motif is underlined.

Figure 2. ElaB is an inner membrane anchored protein. Coexpression of the mCherry fusion protein ElaB or LacZ in BW25113 at a turbidity at 600 nm of 1.0. mCherry fluorescence (middle panels) is overlaid with the phase contrast images (left panels) in the same field to get merged images (right panels). Three independent cultures were used and only one representative figure is shown here.

Figure 3. ElaB localization using Western blot. (A) Cell lysates and fractions containing the inner and outer membrane proteins were obtained as described in the Methods. The His-tag antibody was used to determine ElaB levels when overexpress *elaB* using pCA24N-*elaB*. Cells producing inner membrane protein MacB and outer membrane protein OmpA were also used as positive controls. (B) Expression of ElaB via pCA24N and chromosomal ElaB-GFP, and its levels in soluble fractions and membrane fractions were determined. GFP antibody was used to determine ElaB levels when coexpressed *gfp* and *elaB* in chromosome

Figure 4. ElaB is induced during the stationary phase, and *elaB* is regulated by RpoS. (A) Cells grown to the exponential phase (OD_{600} 0.7) and stationary phase (OD_{600} 6.0) were harvested, and total RNA was isolated. The transcriptional level of *elaB* was determined using qRT-PCR and calculated using $2^{-\Delta\Delta Ct}$. The expression of *elaB* was normalized to that of *rrsG*. The relative expression of *elaB* in the stationary phase was compared to that in the exponential phase, and the relative expression in M9+Glu medium was normalized to that in LB medium. **(B)** e*laB* expression is induced by RpoS. The Δ*rpoS* cells grown to the exponential phase (OD_{600} 0.7) and stationary phase (OD_{600} 6.0) were harvested, and the transcriptional level of *elaB* was determined as in (A). The cells harboring pCA24N-*rpoS* were induced with 1 mM IPTG for 1 h at OD_{600} 1.0, and cells were harvested, and total RNA was isolated. The expression of *elaB* was determined, and vector pCA24N was used as a negative control. Three independent cultures for each strain were used in A, B, and the data are shown as means ± standard deviations. For statistical analysis, $p< 0.01$ is marked as **. **(C)** The promoter region of *elaB* and the sequences of the two probes each containing one putative RpoS binding site. The numbers indicate the locations relative to the start codon A of *elaB*. Two predicted binding sites of RpoS are marked. The "-10" and "-35" regions are highlighted in green and light blue. The ribosome binding site (RBS) is also highlighted in gray. The start codon of *elaB* is shown in red letters. The black arrows pointed to the mutated sequence of "-10" and "-35" regions in RpoS binding site 2. **(D)** Purification of RpoS. A total of 20 μg protein was loaded for each lane. M indicates protein marker. **(E)** RpoS binds to Probe 2 in a concentration-dependent manner with addition of RNA core polymerase (lanes 1-6). The addition of an excess amount of unlabeled probe reduced the binding of RpoS to the labeled probe in a concentration-dependent manner (lanes 7-9). **(F)** RpoS could not bind to Probe 1 (lanes 1-9) or the mutant RpoS binding site 2 (Probe 3, lanes 10-18) under the same conditions.

Figure 5. Promoter activity of *elaB* is induced by RpoS. (A)Wild-type BW25113 (WT) and Δ*rpoS* harboring pHGR01-P*elaB* and pHGR01-Mp*elaB* in the stationary phases were collected, and β-galactosidase activities were tested. **(B)** Δ*rpoS*/pHGR01-P*elaB* and Δ*rpoS*/pHGR01-MP*elaB* cells expressing RpoS were induced with 0.5 mM IPTG for 2 h at OD_{600} 1.0, and β-galactosidase activities were tested. The pCA24N vector was used as

a negative control. Three independent cultures for each strain were used. For statistical analysis, $p< 0.01$ is marked as **.

Figure 6. ElaB increases survival during heat and oxidative stress. (A) Wild-type BW25113 (WT) and Δ*elaB* cells were grown until the turbidity reached 1.0, and were treated with heat stress (65 °C for 5 min and 10 min), then cell survival (%) was tested. **(B)** Overnight cultures were diluted and until the turbidity reached 0.5, then 1 mM IPTG was added for 2 h. Then OD_{600} was adjusted to 1.0, and cell survival was determined as (A). **(C)** Cells as (A) were treated with 10 mM H_2O_2 for the indicated times, then cell survival was assayed. **(D)** Cells were collected as (B) and cell survival was determined after treated with 10 mM H_2O_2. Each assay was performed with three independent cultures, and one standard deviation is shown. Significant changes are marked with an asterisk for $p < 0.05$ and two asterisk for $p <0.01$.

Figure 7. ElaB decreases persistence. Survival of wild-type BW25113 (WT) and Δ*elaB* cells was determined with 100 μg/mL ampicillin **(A)** and 5 μg/mL ciprofloxacin at the indicated time points (**B**). Overnight cultures were diluted and cultured until the turbidity reached 1.0 before adding ampicillin or ciprofloxacin in A and B. Cell survival (CFU/mL) upon expressing *elaB* via pCA24N-*elaB* in the wild-type and Δ*elaB* cells respectively was determined with 100 μg/mL ampicillin **(C)** and 5 μg/mL ciprofloxacin at the indicated time points (**D**) Overnight cultures were diluted and cultured until the turbidity reached 0.5, then 1 mM IPTG was added for 2 h. Then, the turbidity was adjusted to 1.0, the cells were treated with antibiotics, and persistence was determined at the indicated time points. Empty plasmid pCA24N was used as a negative control. Three independent cultures of each strain were evaluated in A-D.