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1 Ribosome-inactivation by a class of widely distributed C-tail anchored membrane proteins

2

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16

17 **Summary**

18 Ribosome hibernation is a commonly used strategy that protects ribosomes under unfavorable
19 conditions and regulates developmental processes. Multiple ribosome-hibernation factors have
20 been identified in all domains of life, but due to their structural diversity and the lack of a
21 common inactivation mechanism, it is currently unknown how many different hibernation
22 factors exist. Here, we show that the YqjD/ElaB/YgaM paralogs, initially discovered as
23 membrane-bound ribosome binding proteins in *E. coli*, constitute an abundant class of
24 ribosome-hibernating proteins, which are conserved across all proteobacteria and some other
25 bacterial phyla. Our data demonstrate that they inhibit *in vitro* protein synthesis by interacting
26 with the 50S ribosomal subunit. *In vivo* cross-linking combined with mass spectrometry reveals
27 their specific interactions with proteins surrounding the ribosomal tunnel exit and even their
28 penetration into the ribosomal tunnel. Thus, YqjD/ElaB/YgaM inhibit translation by blocking
29 the ribosomal tunnel and thus mimic the activity of antimicrobial peptides and macrolide
30 antibiotics.

31

32 **Key words:** YqjD, ElaB, YgaM, translation; stress response, antimicrobial peptides, macrolide
33 antibiotics.

34

35 **Introduction**

36 Unicellular organisms, such as bacteria, are constantly challenged by fluctuations in
37 their immediate environment. Consequently, bacteria have developed sophisticated strategies
38 for sensing environmental conditions and for converting this information into metabolic
39 responses¹⁻³. In bacteria, these responses are primarily controlled by transcriptional regulators
40 that adjust gene expression in response to intra- and extracellular cues⁴⁻⁷. These transcriptional
41 control mechanisms are complemented by multiple post-transcriptional response strategies,
42 such as stress-induced adaptation of ribosome biogenesis and protein synthesis⁸⁻¹¹. Important
43 players of this adaptation are ribosome-inactivating proteins, which are present in all domains
44 of life and which can inactivate ribosomes either reversibly or irreversibly¹⁸⁻²². Examples are
45 RNA-specific N-glycosidases, such as the plant toxin ricin or the bacterial Shiga toxin, which
46 inactivate ribosomes by depurinating adenine nucleotides within the sarcin-ricin RNA loop of
47 the 23S rRNA in bacteria or the 28S rRNA in eukaryotes^{23,24}. This irreversible ribosome
48 inactivation primarily serves as a defense mechanism against predators or competitors, while
49 reversible ribosome inactivation causing transient ribosome hibernation is used as an important
50 strategy for stress adaptation and for regulating developmental processes^{1,20,25-29}. The *E. coli*
51 ribosome-modulation factor (RMF) and the hibernation-promoting factor (HPF) are well
52 studied bacterial hibernation factors^{30,31}. RMF binds to the 30S ribosomal subunit where it
53 interacts with the ribosomal protein bS1 and induces the dimerization of two 70S ribosomes to
54 an instable 90S dimer, which is subsequently converted into the stable 100S dimer by HPF³²⁻
55³⁶. In stationary *E. coli* cells, approx. 40 to 80% of all ribosomes appear to exist as silent 100S
56 ribosomes^{32,37,38}. Silencing ribosomes protects them against RNase-dependent degradation³⁹
57 and adjusts the overall protein synthesis to available nutrients^{19,26,31}. Upon nutrient re-supply,
58 the 100S ribosomes are converted within minutes to active 70S ribosomes^{18,40}. While RMF is
59 only found in γ -proteobacteria, HPF homologues are found in almost all bacteria²⁰. *E. coli*
60 contains additional putative ribosome-inactivating proteins, such as the short HPF-paralog

61 RaiA (ribosome-associated inhibitor A, also referred to as YfiA)³⁶, Sra (stationary-phase-
62 induced ribosome associated)^{19,41,42} or RsfS (ribosome silencing factor S)⁴³, which are like RMF
63 and HPF soluble proteins that are primarily expressed during stationary phase²².

64 Furthermore, *E. coli* contains three paralogous membrane-anchored proteins, YqjD,
65 ElaB and YgaM that are shown or predicted to interact with ribosomes during stationary phase.
66 YqjD, ElaB and YgaM belong to the small number of C-tail anchored membrane proteins in *E.*
67 *coli*⁴⁴⁻⁴⁶. Their production is controlled by the stationary phase specific σ -factor RpoS¹ and
68 YqjD was shown to interact with 70S and 100S ribosomes during stationary phase⁴⁵. However,
69 whether these proteins influence ribosomal activity is not known. Here, we show that
70 YqjD/ElaB/YgaM constitute a widely distributed class of membrane-bound ribosome
71 hibernation factors. YqjD and ElaB inactivate ribosomes by binding to proteins surrounding the
72 ribosomal tunnel exit and even partially protrude into the ribosomal tunnel. Thus, they
73 inactivate ribosomes likely by blocking the ribosomal tunnel, a mechanism that is also observed
74 for macrolide antibiotics and some antimicrobial peptides.

75

76 **Results**

77

78 **YqjD, ElaB and YgaM paralogs are present in many bacterial phyla**

79 YqjD, ElaB and YgaM are largely uncharacterized proteins with a predicted C-terminal
80 transmembrane domain (TM) (Fig. 1A), and they are suggested to be involved in tethering
81 ribosomes to the cytoplasmic membrane^{44,45}. Based on Alphafold2 structural predictions⁴⁷,
82 they are largely α -helical proteins with a conserved helix-breaking proline residue immediately
83 following the transmembrane domain (Fig. 1B). Our bioinformatic analyses show that the
84 paralogs exhibit significant sequence conservation with 37% sequence identity between YqjD
85 and ElaB and 42% sequence identity between YqjD and YgaM (Fig. 1B). The sequence identity
86 between ElaB and YgaM is 34% and their similarity is 38%.

87 YqjD, ElaB, and YgaM contain DUF883 domains⁴⁸ (IPR043604: DUF883, N-terminal
88 domain, IPR043605: DUF883, C-terminal domain, and the overlapping IPR010279: Inner
89 membrane protein YqjD/ElaB domain as defined in the InterPro database) that are widespread
90 in different prokaryotic phyla (Figs. 1A & 1C, Suppl. Fig. S1). Using a sequence similarity
91 network analysis of proteins matching IPR010279 (which encompasses the N- and C-terminal
92 DUF883 regions), we identified DUF883-containing proteins mainly in Pseudomonadota (9281
93 proteins), with some homologs found in the PVC group (Planctomycetota-Verruromicrobiota-
94 Chlamydiota (300)), Spirochaetota (130), the delta/epsilon subdivisions (26), Acidobacteriota
95 (19), Campylobacterota (18), Bdellovibrionota (12), and Thermodesulfobacteriota (11) (Fig.
96 1C). Although some eukaryotic DUF883-containing proteins are predicted, the absence of
97 DUF883-like proteins among closely related eukaryotes suggests that these few eukaryotic
98 proteins could possibly be the result of bacterial contamination in the genome assemblies. In
99 contrast, three separate species from the Archaeal genus *Methanocalculus* contain DUF883
100 proteins, suggesting that these rare archaeal DUF883 proteins are more likely to be genuine.

101 Across the YqjD/ElaB/YgaM family, the helix-breaking proline and several glycine
102 residues within the TM helix are highly conserved (Fig. 2A). Analysis of the YqjD, ElaB and
103 YgaM subfamilies reveals the presence of a conserved tryptophan residue that follows the
104 conserved proline, and C-terminal double arginine motifs for YqjD and ElaB, or an arginine-
105 lysine motif for YgaM (Fig. 2A). These putative interfacial residues may stabilize a particular
106 transmembrane orientation ⁴⁹. The TM region is predicted to be slightly longer for YqjD,
107 followed by ElaB, and then YgaM that has the shortest predicted TM helix but the longest C-
108 terminal periplasmic tail (Fig. 1A). Patches of conserved residues among each
109 YqjD/ElaB/YgaM subfamily were also identified (Fig. 2A) as well as residues that have been
110 maintained since the last common ancestor (Suppl. Fig. S2 & S3).

111 In addition to the taxonomic diversity, our data show that the YqjD/ElaB/YgaM family
112 can be divided into multiple distinct similarity clusters (Fig. 1C, Suppl. Fig. S3). At the
113 similarity threshold used to build the network, YqjD, ElaB and YgaM are found in each of three
114 distinct regions of the network connected to a large cluster dominated by sequences from
115 Pseudomonadota, with numerous smaller protein clusters representing more divergent
116 sequences (Fig. 1C). Based on phylogenetic reconstruction of representatives from each cluster,
117 the family is relatively complex with multiple possible instances of gene duplication, gene loss,
118 and horizontal gene transfer (Fig. 2B, Suppl. Fig. 2 & 3). Based on the conserved presence of
119 *yqjK*-like and/or *yqjE*-like genes, next to genes encoding DUF883 throughout the family (*i.e.*,
120 forming syntenic blocks) (Fig. 2B, Suppl. Fig. S3), YqjD likely represents the archetype of the
121 DUF883 family. The *yqjK* and *yqjE* genes encode for predicted membrane proteins of unknown
122 function. The ElaB and YgaM subfamilies appear to have evolved by subsequent duplication
123 events in Enterobacterales (Suppl. Fig. S2). One early duplication resulted in the YqjD clade
124 and the common ancestor of YgaM/ElaB. A second subsequent duplication event resulted in
125 separate YgaM and ElaB clades (Suppl. Fig. S2). The relatively recent duplication events are
126 evident at the amino acid level, which show significant sequence conservation (Figs. 1B & 2A).

127

128 **Growth-phase dependent expression of the paralogous proteins YqjD, ElaB and YgaM**

129 Synthesis of YqjD, ElaB and YgaM is regulated by the σ -factor RpoS and the
130 transcriptional regulator CsrA^{45,50}, indicating that they are produced during stationary phase⁴⁵.
131 In case of YqjD and ElaB, this is supported by mass spectrometry data, which show increased
132 abundance during stationary phase (Supp. Fig. S3C)^{22,51}. In contrast, the abundance of YgaM
133 was generally lower and MS-data did not reveal increased steady-state levels of YgaM in
134 stationary phase (Supp. Fig. S3C)⁵¹. For validating the growth-phase dependent protein levels
135 of YqjD, ElaB and YgaM, we generated peptide antibodies for western blotting with the
136 corresponding knock-out strains as controls. YqjD was detected in wild-type cells after 4h of
137 growth (corresponding to approx. OD₆₀₀ = 3.0) and its levels stayed constant up to 12h (OD₆₀₀
138 ~ 5.0) (Fig. 3A). ElaB was also detected after 4h and remained largely unchanged up to 12h
139 (Fig. 3B). The endogenous YgaM levels could not be reliably detected with the available
140 peptide antibodies, which probably reflects its low copy number (Suppl. Fig. S3C)⁵¹. In
141 summary, synthesis of YqjD and ElaB starts at late exponential/early stationary phase and their
142 steady-state levels remain stable for several hours.

143 For monitoring the importance of YqjD/ElaB/YgaM for growth and survival during
144 stationary phase, we performed a long-term growth experiment by counting the viable cell
145 number over 60h on LB-medium for wt and the *yqjD/elaB/ygaM*-deletion strains. The initial
146 growth rate of all four strains was largely comparable, with the exception of the $\Delta ygaM$ strain,
147 which showed a reduced growth in comparison to the wt (Fig. 3C). Importantly, while the cell
148 number of wild type cells stayed almost constant up to 48h of growth before the cell number
149 declined, the three deletion strains showed a rapid decline already after 20h (Fig. 3C). This
150 supports a particular function of YqjD/ElaB/YgaM during the transition into and during the
151 stationary phase.

152 Although the generated peptide antibodies recognized YqjD and ElaB in *E. coli* cell
153 extracts, the detection required either a very long exposure (YqjD) or the antibodies recognized
154 additional low-molecular weight bands (ElaB) (Figs. 3A & B). In addition, YgaM could not be
155 reliably detected in whole cell extracts. We therefore constructed copies of *yqjD*, *elaB* and *ygaM*
156 with an N-terminal Xpress-His₆-tag in the arabinose-inducible pBAD24 vector⁵². Wild-type
157 cells expressing these constructs were then grown in the presence of arabinose and fractionated
158 by differential centrifugation. All three proteins were recognized by α -His antibodies
159 exclusively in the inner membrane vesicle (INV) fraction, but not in the outer membrane
160 fraction (OMV). As controls, α -YidC and α -OmpC antibodies detected the localization of the
161 inner membrane protein YidC and the outer membrane protein OmpC, respectively. As an
162 additional control, INV of wild-type cells without plasmid were analyzed. This confirms that
163 YqjD, ElaB and YgaM are inner membrane proteins (Fig. 3D).

164

165 **YqjD, ElaB and YgaM tether the large ribosomal subunit to the membrane**

166 Whether the YqjD levels influenced the amount of ribosomes bound to INVs was probed
167 with antibodies against ribosomal proteins. Antibodies against the 30S ribosomal protein uS5
168 did not reveal large differences in the amount of uS5 bound to wild type INVs, INVs from the
169 *yqjD*-expressing strain or the $\Delta yqjD$ strain (Fig. 4A). In contrast, INVs from the *yqjD*-
170 expressing strain contained increased levels of the 50S ribosomal protein uL2 in comparison to
171 wild-type INVs or $\Delta yqjD$ INVs. Sucrose gradient purified wild-type ribosomes, containing 70S,
172 50S and 30S particles, and antibodies against the inner membrane protein YidC served as
173 controls.

174 We also tested the localization of the 30S and 50S subunits in INVs of the *elaB* and
175 *ygaM* overexpressing strains. As seen for YqjD, the amounts of uS5 in these INVs were
176 comparable to wild-type INVs, but the levels of uL2 were increased (Fig. 4B). These data
177 indicate that YqjD, ElaB and YgaM preferentially tether the 50S ribosomal subunit to the

178 membrane, but not the 30S subunit. However, this does not exclude that YqjD/ElaB/YgaM
179 initially bind to 70S ribosomes, which then dissociate during INV preparation.

180 The interaction between YqjD and ribosomes was further studied in an *in vitro* approach
181 using purified YqjD and sucrose gradient-purified ribosomes isolated from cells grown to either
182 exponential or stationary phase. This revealed binding of YqjD to both types of ribosomes,
183 although binding to stationary phase ribosomes was slightly less efficient (Suppl. Fig. S4A). *E.*
184 *coli* ribosomes isolated via sucrose-gradient centrifugation are almost exclusively non-
185 translating^{11,53}, showing that YqjD primarily interacts with non-translating ribosomes.

186 If YqjD inactivates ribosomes, high concentrations of YqjD should reduce the number
187 of translation-competent ribosomes and increase chloramphenicol sensitivity, because
188 chloramphenicol targets translating ribosomes by inhibiting their peptidyltransferase activity.
189 The minimal inhibitory concentration of chloramphenicol is in the range of 20-30 $\mu\text{g/ml}$ ⁵⁴,
190 however, *yqjD*-expressing cells were already inhibited at 0.75 $\mu\text{g/ml}$ (Suppl. Fig. S4B). This
191 hypersensitivity of *yqjD*-expressing cells against chloramphenicol supports a possible role of
192 YqjD in ribosome hibernation. We did not observe increased chloramphenicol resistance in the
193 $\Delta yqjD$ strain, which is likely explained by the surplus of ribosomes over YqjD in *E. coli* cells.

194

195 **YqjD prevents translation *in vitro***

196 For validating whether YqjD interferes with translation, we employed a coupled *in vitro*
197 transcription/translation system, consisting of a purified cell extract (CTF, cytosolic translation
198 factors) and purified ribosomes⁵⁵. Adding increasing amounts of purified YqjD to the *in vitro*
199 system inhibited synthesis of mannitol permease (MtlA), which is frequently used as model
200 membrane protein for *in vitro* studies⁵⁶ (Fig. 4C). This was different for two additional
201 ribosome-interacting proteins, YchF and SecA^{11,57,58}, which did not inhibit MtlA synthesis
202 (Figs. 4C & 4D). YqjD also inhibited *in vitro* synthesis of the cytosolic protein YchF and the
203 secretory protein OmpA (Fig. 4E), although the inhibitory effect was slightly less pronounced

204 than the inhibition of MtlA synthesis. This could relate to differences in the *in vitro* translation
205 speed due to codon usage or mRNA length⁵⁹. Finally, a concentration dependent inhibition of
206 MtlA synthesis was also observed with ElaB, although full inhibition required higher
207 concentrations than in the case of YqjD (Supp. Figs. S5A & B).

208 If YqjD stoichiometrically inactivates *E. coli* ribosomes, this effect should depend on
209 the ribosome concentration in the *in vitro* assay. The *in vitro* system routinely contains 10-30
210 nM ribosomes⁵⁵, although there are variations in the translational activities between different
211 ribosome preparations. MtlA synthesis was analyzed at different ribosome concentrations in
212 the absence or presence of 30 nM YqjD. In the absence of YqjD, MtlA synthesis increased with
213 increasing ribosome concentrations and reached saturation at approx. 20 nM ribosomes (Fig.
214 4F). In the presence of YqjD, synthesis was strongly reduced up to approx. 10 nM ribosomes,
215 but then gradually increased (Fig. 4F). At approx. 20 nM ribosomes, MtlA synthesis was
216 detectable even in the presence of YqjD. These data demonstrate that YqjD prevents translation
217 by inactivating ribosomes.

218

219 **Ribosome-inactivation by YqjD depends on the N-terminus and on dimerization**

220 Previous data had indicated that YqjD interacts via its N-terminus with ribosomes⁴⁵ and
221 we therefore tested MtlA synthesis in the presence of N-terminally truncated YqjD variants. *In*
222 *vitro* MtlA synthesis was almost completely blocked by the addition of 5 nM full-length YqjD
223 (Fig. 5A). In contrast, gradually deleting N-terminal amino acids diminished YqjD's ability to
224 prevent MtlA synthesis (Fig. 5A), demonstrating that the N-terminus is required for ribosome
225 inactivation. Surprisingly, deleting the C-terminal transmembrane domain (Δ TM-YqjD) also
226 significantly reduced the inhibitory effect of YqjD on translation (Fig. 5A). The *in vitro*
227 translation assays were performed in the presence of purified YqjD, but in the absence of
228 membranes, demonstrating that membrane tethering of ribosomes cannot be responsible for
229 impaired translation in our assay. Adding the purified transmembrane helix of YqjD to the *in*

230 *vitro* synthesis assay did also not reduce MtlA synthesis (Fig. 5B), indicating that the TM is
231 only indirectly involved in ribosome inactivation. Whether the lack of the TM influenced
232 ribosome binding was analyzed by incubating purified YqjD, Δ TM-YqjD and Δ N(12)-YqjD
233 with purified ribosomes. In comparison to full-length YqjD, both truncated variants showed
234 reduced ribosome binding (Suppl. Fig. S6A), but the truncation of the N-terminus had a stronger
235 effect than the deletion of the C-terminus. Thus, even in the absence of the TM, YqjD retains
236 some ribosome binding activity but completely fails to inactivate ribosomes.

237 YqjD contains several glycine residues in its transmembrane domain (Fig. 2A). Such
238 glycine motifs have been shown to promote homo-dimer formation⁶⁰ and indeed, YqjD shows
239 a strong propensity for dimer formation, which are stable even on SDS-PAGE (Fig. 5C).
240 Dimerization of ElaB and YgaM was less pronounced, but this could simply reflect reduced
241 stability of the dimer on SDS-PAGE (Suppl. Fig. S5C). YqjD dimerization was not influenced
242 by the His-tag, because the tag-free YqjD showed a comparable amount of dimer (Suppl. Fig.
243 S6B). Interestingly, YqjD dimerization was completely diminished when the C-terminal TM
244 was deleted (Fig. 5C), while the N-terminal truncations had no influence on dimerization (Figs.
245 5C & 5D). Thus, dimerization of YqjD depends on the TM and the lack of ribosome inactivation
246 by Δ TM-YqjD could reflect its failure to dimerize. This was further tested by constructing YqjD
247 variants, in which the native TM was replaced by the N-terminal TM of either FtsQ or YfgM
248 or by the C-terminal TM of the mitochondrial protein Fis1. These variants showed the same
249 inhibitory effect on MtlA synthesis as wild-type YqjD (Suppl. Fig. S6C), and also formed SDS-
250 resistant dimers, although dimer stability on SDS-PAGE was reduced in comparison to the
251 native YqjD (Suppl. Fig. S6D). Thus, the nature of the TM is not important for ribosome
252 inactivation. Instead, the data rather indicate that the TM is required for YqjD dimerization,
253 which in turn promotes ribosome inactivation via the N-terminus.

254 Dimerization could induce ribosome inactivation by reducing the topological flexibility
255 of the N-terminus, which potentially also explains the conservation of the proline residue in

256 immediate vicinity to the TM of YqjD. Proline residues can act as α -helix breaking amino acids,
257 which promote rigid body motions of helical segments ⁶¹. This is visible in the Alphafold
258 structural prediction of YqjD (Fig. 1B), which shows that the proline residue induces a strong
259 kink after the TM. The importance of the proline residue was tested by replacing it with either
260 glycine, which has a lower helix-breaking propensity than proline, or with alanine, which
261 stabilizes the α -helix. The YqjD(P₈₀G) variant prevented MtlA synthesis to a lesser extent than
262 wild-type YqjD (Fig. 5E), and the inhibitory activity was even further reduced in the
263 YqjD(P₈₀A) variant (Fig. 5E). In summary, ribosome inactivation by YqjD is likely dependent
264 on the correct orientation of its N-terminus, which is compromised when the C-terminal TM is
265 deleted or when the conserved proline residue is mutated.

266

267 **YqjD contacts ribosomal proteins close to the peptide tunnel**

268 For determining the YqjD-ribosome contacts in more detail, we used an *in vivo* site-
269 directed cross-linking approach. The UV-sensitive phenylalanine derivative para-benzoyl-L-
270 phenylalanine (pBpa) was site specifically inserted at the N-terminal positions 10 and 39 of
271 YqjD, using an amber-suppressor tRNA and a cognate tRNA synthetase ^{62,63}. *E. coli* cells
272 expressing either YqjD(L10pBpa) or YqjD(I39pBpa) were then grown on LB-medium in the
273 presence or absence of pBpa. The two YqjD variants were only visible in the presence of pBpa,
274 demonstrating the successful insertion of pBpa into YqjD (Fig. 6A). The two YqjD variants
275 also showed the same propensity for dimerization as the wild-type YqjD (Fig. 6A). When
276 YqjD(L10pBpa) or YqjD(I39pBpa) expressing *E. coli* cells were exposed to UV-light, multiple
277 additional bands were recognized by α -Xpress antibodies in the UV-treated sample, but not in
278 the sample not exposed to UV-light (Fig. 6B). Some additional bands were also visible in UV-
279 treated wild-type cells, lacking pBpa (Fig. 6B). These pBpa-independent cross-linking products
280 are likely the result of UV-induced radical formation of aromatic amino acids that favor non-
281 specific protein-protein and protein-nucleic acid cross-links ^{64,65}. The cross-linked material was

282 then further enriched by affinity chromatography and probed with α -Xpress antibodies. This
283 revealed UV-dependent cross-linked bands at approx. 40 kDa and 55 kDa for YqjD(L10pBpa)
284 and YqjD(I39pBpa), which were not visible for wild-type YqjD lacking pBpa (Fig. 6C). For
285 YqjD(L10pBpa) additional weaker bands at approx. 110 kDa were also visible.

286 Proteins surrounding the ribosomal tunnel exit on the 50S subunit serve as hot spots for
287 ribosome-interacting proteins^{57,66,67}. As YqjD likely interacts with the 50S ribosomal subunit
288 (Fig. 4A), the cross-link samples were analyzed with antibodies against the ribosomal proteins
289 uL22 and uL29, which are located next to the tunnel exit. uL22 has a MW of 12 kDa and the α -
290 uL22 antibodies recognized several UV-dependent bands; the most prominent migrated just
291 below the 25 kDa marker band and could reflect a cross-link between monomeric YqjD and
292 uL22 (Fig. 6D, upper panel). At approx. 35 kDa, three UV-dependent bands were observed for
293 both YqjD(L10pBpa) and YqjD(I39pBpa), which could reflect a cross-link between dimeric
294 YqjD and uL22 (Fig. 6D, upper panel). The migration of a single cross-linking product in
295 multiple species on SDS-PAGE is often observed⁶⁸⁻⁷⁰ and reflects different three-dimensional
296 shapes of the covalently linked proteins.

297 The α -uL29 antibodies also recognized several UV-dependent bands, which
298 corresponded in size to crosslinks between the 7.3 kDa uL29 and monomeric and dimeric YqjD.
299 (Fig. 6D, lower panel). Overall, the sequence similarity of ribosomal proteins and the low
300 specificity of the available antibodies did not allow for an unambiguous identification of cross-
301 links between YqjD and 50S ribosomal proteins. The samples after cross-linking were therefore
302 analyzed by mass spectrometry. This identified two UV-dependent uL22 cross-linking products
303 of 23 kDa and 38 kDa, respectively, which were observed for both YqjD(L10pBpa) and
304 YqjD(I39pBpa) (Fig. 6E). This demonstrates that both monomeric and dimeric YqjD is cross-
305 linked to uL22. The MS analyses also revealed two uL29 cross-linking products of
306 YqjD(L10pBpa) at 21 kDa and 37 kDa, while for YqjD(I39pBpa) only one cross-linking
307 product at 35 kDa was detected (Fig. 6E). In addition, the MS revealed single cross-linking

308 products between uL23 and YqjD(I39pBpa) and between uL24 and YqjD(I39pBpa) (Suppl.
309 Fig. 7A), which were not detected by antibodies. Proteins uL23 and uL24 are, like uL29 and
310 uL22, located in close vicinity to the ribosomal tunnel exit and form a platform for multiple
311 ribosome-interacting proteins^{66,67,71,72}. In summary, these data demonstrate that YqjD interacts
312 with 50S ribosomal proteins that surround the ribosomal tunnel exit.

313

314 **ElaB prevents translation by mimicking anti-microbial peptides**

315 The contact between YqjD and the ribosomal proteins uL22 and uL23 is intriguing,
316 because both proteins are not only exposed to the ribosomal surface but also contact the interior
317 of the ribosomal tunnel via β -hairpin loops. Subunit uL22 together with uL4 forms a central
318 constriction within the ribosomal tunnel, which serves as a binding site for macrolide antibiotics
319 and antimicrobial peptides⁷³⁻⁷⁵. The intra-tunnel loop of uL23 is located closer to the tunnel
320 exit and acts as a nascent chain sensor that binds to the protein targeting factors SRP and SecA
321^{57,66,67}. Thus, it appeared possible that YqjD interferes with translation by inserting into the
322 ribosomal peptide tunnel and as such mimics antimicrobial peptides^{23,74,76,77}, or eukaryotic
323 ribosome hibernation factors^{29,78}.

324 For monitoring whether YqjD reaches into the peptide tunnel of the ribosome,
325 ribosomes that contained the cross-linker pBpa at the tip of the intra-tunnel β -hairpin loop of
326 uL23 (position 71) or at position 52 of the surface-exposed globular domain of uL23 were
327 generated, isolated^{57,66} and incubated *in vitro* with purified YqjD, followed by UV-exposure.
328 As a control, these ribosomes were incubated with purified SRP, which was previously shown
329 to contact both uL23 residues⁶⁶. While UV-dependent cross-links to Ffh, the protein component
330 of the *E. coli* SRP⁷⁹, were observed from both residue 71 and residue 52 (Fig. 7A, left panel),
331 no cross-links between uL23 to YqjD were visible in this *in vitro* approach (Fig. 7A, right
332 panel).

333 We therefore switched to an *in vivo* approach. *E. coli* $\Delta rplW$ cells, which lack the uL23-
334 encoding *rplW* gene on the chromosome but contain either the plasmid-encoded
335 uL23(E52pBpa) or the uL23(G71pBpa) variant, were grown to exponential or stationary phase
336 and then UV-exposed. The *rplW* (uL23) gene is essential in *E. coli*, and cells containing these
337 plasmid-encoded versions were only able to grow in the presence of pBpa, demonstrating that
338 these pBpa-containing ribosomes are functional, which is in agreement with previous reports
339 ^{57,66}. After cell lysis, cells were fractionated into the soluble ribosome fraction and the crude
340 membrane fraction, which were then decorated with α -YqjD antibodies. However, the low
341 specificity of these antibodies did not allow us to detect specific cross-linking bands (Suppl.
342 Fig. 7B). In contrast, when the same material was decorated with α -ElaB antibodies, we found
343 weak UV-dependent cross-links at approx. 20 kDa for both position 52 and 71 of uL23 (Fig.
344 7B, indicated by *), fitting to the size of a ElaB-uL23 cross-link. Importantly, these cross-links
345 were only visible in cells grown to stationary phase, but not exponentially grown cells. This
346 observation is explained by the increased production of native ElaB when cells enter stationary
347 phase (Fig. 3B). As ElaB is a membrane protein (Fig. 3D), the cross-linked band was detected
348 only in the membrane fraction, but not in the soluble ribosome fraction. The cross-linking
349 product is rather weak, but this experiment was executed in the presence of native ElaB, which
350 is greatly sub-stoichiometric to ribosomes *in vivo*. Nevertheless, due to the non-specific
351 recognition of many proteins by the α -ElaB peptide antibody (Fig. 7B, indicated by +) and the
352 lack of detectable YqjD-uL23 cross-links, additional studies need to further validate that ElaB
353 and potentially also YqjD/YgaM inactivate ribosomes by penetrating into the ribosomal peptide
354 tunnel. This would then indicate that YqjD/YgaM/ElaB mimic the strategy of antimicrobial
355 peptides ⁷⁴ and eukaryotic ribosome-hibernation factors, such as Dap1b ²⁹ and Mdf2 ⁷⁸.
356 However, considering that the YqjD dimerization appears to be important for ribosome
357 inactivation, the N-termini of a YqjD dimer will not be able to reach deeply enough into the

358 tunnel to reach the peptidyltransferase center of the ribosome. Still, the proximal vestibule of
359 the ribosomal tunnel is likely wide enough to accommodate two N-termini^{80,81}.

360

361 Discussion

362 Ribosome biogenesis and protein synthesis are the most energy consuming cellular
363 processes and they are therefore strictly regulated in response to nutrient availability and stress
364 conditions^{9,13,22,82,83}. This saves energy and reduces the overall production of damage-prone
365 proteins, while a basal level of protein synthesis is maintained. Complementary to these
366 strategies are ribosome-inactivating mechanisms, which provide the fast and reversible means
367 for shutting down the activity of already assembled ribosomes^{20,21,27,30,41,84-86}. Due to the high
368 structural diversity of hibernation factors and the lack of common mechanisms by which they
369 interfere with ribosomal activity^{19,31}, it is currently unknown how many different hibernation
370 factors exist in bacteria. Recently, a novel hibernation factor, called Balon, was identified in
371 the cold-adapted bacterium *Psychrobacter urativorans* and shown to occupy the ribosomal A-
372 site in complex with EF-Tu⁸⁸. Balon homologues were found in 23 out of 27 bacterial phyla,
373 but are absent in *E. coli*⁸⁸.

374 Previous studies had identified YqjD as an *E. coli* ribosome-interacting protein, which
375 inhibited cell growth when over-produced⁴⁵. It was therefore suggested that YqjD and its
376 paralogs ElaB and YgaM might act as membrane-bound ribosome-hibernation factors in *E. coli*
377^{1,18,50}. This was experimentally verified in our study, which demonstrates that YqjD and ElaB
378 inhibit *in vitro* protein synthesis in a dose-dependent manner. Our study also identified
379 YqjD/ElaB/YgaM proteins in many bacterial species, suggesting that they potentially represent
380 a widely distributed family of ribosome hibernation factors. Although sequence similarity can
381 be a poor predictor of biological function, sequence similarity combined with gene neighbor
382 conservation (*yqjE*- and *yqjK*-like genes) suggests that the function of these proteins could be
383 conserved across and outside of the Pseudomonadota phylum. Since there have clearly been
384 multiple instances of gain/loss of paralogs and, potentially, horizontal gene transfer, functional
385 tailoring has occurred in different lineages and species. However, whether such tailoring has

386 affected molecular and/or biological functions of any individual uncharacterized protein is as-
387 of-yet unknown.

388 Based on sucrose-gradient centrifugation, YqjD was suggested to bind to the 30S
389 ribosomal subunit⁴⁵. However, our data indicate that YqjD/ElaB/YgaM preferentially interact
390 with the 50S ribosomal subunits and tether them to the *E. coli* membrane. Furthermore, by *in*
391 *vivo* cross-linking combined with mass spectrometry, we demonstrate that the N-terminus of
392 YqjD interacts with the ribosomal proteins uL22, uL23, uL24 and uL29, which encircle the
393 ribosomal peptide tunnel exit on the 50S ribosomal subunit. Thus, it is possible that YqjD can
394 bind to both ribosomal subunits.

395 Previous studies have revealed the importance of the ribosomal peptide tunnel as
396 binding site for chaperones and targeting factors.^{57,66,67,89-91} Eukaryotic dormancy factors^{29,78}
397 or antimicrobial peptides and macrolide antibiotics^{74,92} also target the ribosomal peptide tunnel.
398 Intriguingly, the YqjD paralog ElaB, inserts into the ribosomal tunnel and contacts the β -hairpin
399 loop of uL23, which was previously identified as an intra-tunnel nascent chain sensor^{57,66}. The
400 uL23 β -hairpin loop is located in the lower section of the ribosomal tunnel, which is generally
401 wide enough to allow proteins to enter^{91,93}. This has been demonstrated for SRP^{66,94}, SecA⁵⁷
402 and the cytosolic loops of SecY^{95,96}. On the other hand, antimicrobial peptides, such as oncocin
403 or bactenecin, have been shown to contact the A-site tRNA binding pocket and the A-site
404 crevice, demonstrating that they deeply insert into the ribosomal tunnel^{97,98}. Whether ElaB is
405 also able to contact the upper section of the tunnel is currently unknown. While ElaB can enter
406 the ribosomal tunnel, we did not observe this for YqjD. Thus, we can currently not exclude that
407 ElaB and YqjD inhibit protein synthesis by different mechanisms, as also deduced from the low
408 sequence conservation of their respective N-termini, differences in dimer stability and lower
409 ribosome inactivation potential of ElaB in comparison to YqjD.

410 The importance of YqjD's N-terminus for ribosome binding was already shown
411 previously⁴⁵ and we demonstrate here that the N-terminal truncated YqjD is impaired in

412 ribosome inactivation. Surprisingly, deleting the C-terminal TM of YqjD also significantly
413 reduced ribosome inactivation. The transmembrane domain of YqjD contains several conserved
414 glycine residues, which are often involved in dimerization or oligomerization^{60,99}. In support
415 of this, our data show that the propensity of YqjD to form stable dimers even on SDS-PAGE is
416 strictly dependent on the TM. The lack of ribosome inactivation when the dimerization-
417 promoting TM of YqjD is missing could indicate that the YqjD-ribosome interaction is
418 primarily avidity-driven. Thus, each YqjD has only a low affinity for ribosomes, but high
419 affinity ribosome binding is achieved when two or more YqjD monomers oligomerizes.
420 Avidity-driven interactions with the ribosome are not unusual and have been shown for example
421 for trigger factor dimers¹⁰⁰. This probably also explains the importance of the conserved proline
422 residue. Proline-kinked α -helices have been suggested to form cage- or funnel-like structures
423¹⁰¹, and their reduced topological flexibility could help to orient multiple N-termini in close
424 proximity to the ribosome. The reduced ribosome binding of the Δ TM-YqjD variant, supports
425 such an avidity-driven interactions. Still, it is surprising that although Δ TM-YqjD retains some
426 residual ribosome binding activity, it completely fails to inactivate ribosomes. Thus, it is
427 possible that ribosome inactivation depends on the simultaneous binding of both N-termini of
428 the YqjD dimer to a single ribosome, but this needs to be further explored (Fig. 8).

429 C-tail anchored membrane proteins such as YqjD/ElaB/YgaM are generally rather rare
430 in *E. coli*⁴⁶, and the benefit of having membrane-anchored hibernation factors in addition to
431 several soluble hibernation factors is not entirely clear^{18,20,22}. YqjD and its paralogs are
432 primarily located at the cell poles^{44,102}, where a large majority of ribosomes is also located¹⁰³⁻
433¹⁰⁵. Thus, ribosome hibernation is promoted by co-localizing YqjD and a large majority of
434 ribosomes at the cell poles during stationary phase. The determinants for the polar localization
435 of YqjD still need to be further explored, but it has been suggested that specific interactions
436 between YqjD's TM and phosphatidic acid-rich membrane clusters are involved¹⁰². This would
437 then explain why YqjD/ElaB/YgaM require a TM. However, it is also possible that the

438 oligomerization combined with ribosome binding, is sufficient to restrict YqjD's diffusion in
439 the membrane and to tether it to the cell poles. The polar localization also explains why
440 moderate overexpression of *yqjD* is tolerated without drastic growth defects, because only those
441 ribosomes located at the cell poles would be inhibited.

442 In conclusion, the membrane-localization of YqjD/ElaB/YgaM, their enrichment at the
443 cell pole and their mechanism of ribosome inactivation by interacting with the ribosomal tunnel
444 define them as a novel and widely distributed class of bacterial ribosome-hibernation factors.

445

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455

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460

461 **Declaration of interest:** The authors declare no competing interests.

462 **Figure Legends:**

463

464 **Figure 1. YqjD, ElaB and YgaM represent a widely distributed class of C-tail anchored**

465 **membrane proteins.** (A) Sequence alignment of YqjD, ElaB and YgaM. The locations of
466 DUF883 regions are shaded according to the color key. The predicted C-terminal
467 transmembrane regions (TM) of each protein are indicated by the green box. (B) AlphaFold2-
468 predicted structural models for YqjD, ElaB and YgaM. The cartoons are colored according to
469 model confidence, *i.e.* pLDDT that corresponds to the model's prediction of its score on the
470 local Distance Difference Test. Lines at the bottom are used to indicate pairwise amino acid
471 similarity and identity. Dotted lines encircle the DUF883 regions (colored according to the key).
472 Green boxes are used to indicate the location of predicted TM regions. (C) Sequence similarity
473 network representing proteins containing DUF883. Nodes are colored by phylum according to
474 the color key. Clusters of proteins containing at least six nodes are labeled. Dotted lines are
475 used to delineate the clusters labelled "SSN cluster" in Fig. 2B. Node information can be found
476 in Supplemental data file S1.

477

478

479 **Figure 2. YqjD, ElaB and YgaM represent three distinct subfamilies conserved in**

480 **Enterobacterales.** (A) AlphaFold2-predicted models of YqjD, ElaB and YgaM colored
481 according to sequence conservation in the multiple sequence alignment of proteins from the
482 YqjD, ElaB and YgaM subfamilies. Regions of higher conservation, as well as the
483 transmembrane (TM) region (green box), are shown as sequence logos. Colors represent the
484 conservation values according to the keys. (B) Phylogenetic reconstruction of representative
485 DUF883-containing proteins in beta- and gamma-proteobacteria under maximum likelihood.
486 The "SSN cluster" column corresponds to clusters 3, 4, 6 and 7 in Fig. 1. A complete
487 phylogenetic reconstruction of all clusters is shown in Suppl. Fig. S3A. Taxonomic

488 classification for each node is given according to the color key. The protein labels for each leaf
489 list the organism, followed by the cluster number corresponding to Fig. 1C, followed by the
490 paralog number and followed by the total number of identified paralogs for that organism. If
491 the paralog number is “0”, then that organism has a single DUF883-containing protein, where
492 as “1” indicates 1 of N paralogs. Bootstrap values greater than 0.5 are represented with a
493 normalized purple circle according to the key. The YqjD, ElaB and YgaM clades are shaded
494 with a light blue. For each protein in the gene neighborhood is given. Genes are colored
495 according to shared domains; light pink genes encode proteins with no identified domain. All
496 genes are shown as transparent, except for those genes and homologs listed in the key.

497

498 **Figure 3. YqjD, ElaB, and YgaM are inner membrane proteins and produced in late**
499 **exponential phase.** (A) *E. coli* wild-type cells BW25113 and the corresponding $\Delta yqjD$ strain
500 were grown on LB-medium and at the indicated times, 1×10^8 cells were precipitated by 5%
501 trichloroacetic acid (TCA) and loaded on a 15 % SDS-PAGE. The membrane after western
502 blotting was decorated with peptide antibodies against YqjD and OmpC as loading control. (B)
503 As in (A), but wild type and $\Delta elaB$ cells were analyzed with peptide antibodies raised against
504 ElaB. α -YidC antibodies served as a control. (C) Long-term growth experiment of wt, $\Delta yqjD$,
505 $\Delta elaB$, and $\Delta ygaM$ cells on LB medium. Cell counting was performed by the QUANTOM Tx™
506 Microbial Cell Counter using the QUANTOM viable staining kit, which is an image-based
507 automatic cell counting system that detects individual viable bacterial cells. Indicated are viable
508 cells/ml over time. (D) *E. coli* BW25113 cells expressing His-tagged versions of YqjD, ElaB
509 or YgaM in plasmid pBAD24 were grown to exponential phase and subsequently fractionated
510 after cell breakage. Aliquots of the different fractions were then separated by SDS-PAGE and
511 decorated after western blotting with α -His antibodies. Antibodies against the inner membrane
512 protein YidC and the outer membrane protein OmpC served as controls. S30 and P30
513 correspond to the supernatant and pellet after a 30.000 x g centrifugation step following cell

514 breakage. The S30 supernatant was then further separated via a 150.000 x g centrifugation into
515 the S150 supernatant and the P150 pellet, the latter was further separated via sucrose gradient
516 centrifugation into the inner membrane vesicle fraction (INV) and the outer membrane fraction
517 (OMV). INVs of the wild type *E. coli* BW25113 served as control. In YgaM-producing cells,
518 α -His antibodies recognized a double band, but it was not further analyzed whether this reflects
519 partial proteolysis.

520

521 **Figure 4. YqjD, ElaB and YgaM are ribosome-inactivating proteins.** (A) The inner
522 membrane fractions (INV) of wild type cells (wt), wild type cells containing pBAD-YqjD and
523 $\Delta yqjD$ cells were separated on SDS-PAGE and after western blotting decorated with antibodies
524 against the 30S ribosomal protein uS5 or the 50S ribosomal protein uL2. Antibodies against the
525 inner membrane protein YidC and sucrose-gradient purified wild type ribosomes served as a
526 control. (B) The INV fractions of wt cells and wt cells containing either pBAD-YqjD, pBAD-
527 ElaB or pBAD-YgaM were processed and controlled as in (A). (C) YqjD was solubilized from
528 *E. coli* membranes, purified via affinity chromatography and added to a cell-free *E. coli in vitro*
529 transcription/translation system containing 30 nM ribosomes⁵⁵. *In vitro* synthesis of the model
530 protein mannitol permease (MtlA) was performed in the presence of ³⁵S-labelled cysteine and
531 methionine. MtlA synthesis in the presence of detergent-containing buffer served as reference
532 (0 nM YqjD). As further controls, the effect of the purified ribosome-interacting proteins YchF
533 and SecA on MtlA synthesis in the *in vitro* system was analyzed. For these controls, detergent-
534 free buffer was used as reference. Samples were separated by SDS-PAGE and analyzed by
535 phosphorimaging. (D) Quantification of four independent experiments as shown in (C). The
536 amount of MtlA synthesized was quantified using a phosphorimager and the *Image1/Fiji*
537 software and synthesis in the absence of YqjD, YchF, or SecA, respectively, was set to 100%.
538 The values on the X-axis refer to the final concentration (nM) of the added protein (YqjD,
539 YchF, or SecA) in the cell-free transcription/translation system. (E) As in (C) and (D), but *in*

540 *in vitro* synthesis of the outer membrane protein OmpA and the cytosolic protein YchF were also
541 tested in the presence of increasing YqjD concentrations. (F) *In vitro* protein synthesis of MtlA
542 was performed either in the absence of YqjD (-YqjD) or in the presence of 30 nM YqjD (+
543 YqjD) and increasing concentrations of sucrose-gradient purified ribosomes. Quantification
544 after SDS-PAGE and phosphorimaging was performed as in (D) and (E) and MtlA synthesis at
545 40 nM ribosomes was set to 100%. The error bars indicate the SD.

546

547 **Figure 5: Both N- and C-terminus of YqjD are required for inhibiting protein synthesis.**

548 (A) *In vitro* MtlA synthesis was performed as described in the legend to Fig. 4 and analyzed in
549 the absence of YqjD (control) and in the presence of 5 nM wild-type YqjD, or YqjD variants
550 that were N-terminally truncated or lacked the C-terminal 18 amino acids (Δ TM-YqjD). MtlA
551 synthesis in the absence of YqjD was set to 100%. Shown are the mean values of 3 independent
552 experiments and the error bar reflect the SD. (B) The TM helix of YqjD was chemically
553 synthesized and added in increasing concentrations to the MtlA synthesizing *in vitro* translation
554 system. Samples were separated by SDS-PAGE and analyzed by phosphorimaging as in (A).
555 (C) Purified YqjD, Δ N(12)-YqjD and Δ TM-YqjD were denatured in loading dye at 56 °C,
556 separated on SDS-PAGE and probed with α -Xpress antibodies. (D) As in (C), but Δ N(30),
557 Δ N(45) and Δ N(60) were analyzed and compared to full-length YqjD (wt). (E) Purified YqjD
558 and its variants in which the proline residue at position 80 was replaced by either glycine or
559 alanine were added in different concentrations to the MtlA synthesizing *in vitro* translation
560 system. After SDS-PAGE and phosphorimaging, the MtlA synthesis in the absence of YqjD
561 was set to 100%. Shown are the quantifications of three independent experiments and the error
562 bars reflect the SD. The values on the X-axis refer to the final concentration (nM) of the added
563 protein (YqjD, YqjD(P₈₀G), or YqjD(P₈₀A)) in the *in vitro* transcription/translation system.

564

565 **Figure 6: YqjD interacts via its N-terminus with proteins of the ribosomal peptide tunnel.**

566 (A) Cells producing either wild-type YqjD or its variants containing the amber stop codons at
567 position 10 or 39 for inserting the UV-sensitive amino acid derivative para-benzoyl-L-
568 phenylalanine (pBpa) were grown in the presence or absence of pBpa. After TCA precipitation
569 of whole cells, the material was separated by SDS-PAGE. After western blotting, the samples
570 were decorated with antibodies against the N-terminal Xpress tag. Indicated are the YqjD
571 monomer and dimer. (B) As in (A), but *E. coli* cells were UV-exposed for inducing the cross-
572 link reaction. After TCA precipitation and SDS-PAGE, the material was decorated with α -
573 Xpress antibodies. (C) As in (B), but after UV exposure of whole cells, YqjD and its cross-
574 linked partner proteins were affinity purified via its His-tag and analyzed after SDS-PAGE by
575 immune detection with Xpress antibodies. (D) The material in (C) was probed with antibodies
576 against the ribosomal protein uL22 (upper panel) or uL29 (lower panel). Indicated are putative
577 cross-links between YqjD and uL22 or uL29, respectively. (E) Affinity-purified YqjD and its
578 cross-linked partner proteins as in (C) were separated on SDS-PAGE and the gel lanes were
579 sliced into multiple slices, which were separately processed and analyzed by mass spectrometry.
580 Shown are the normalized intensities of uL22 and uL29 peptides in the -UV and +UV treated
581 samples of wild-type YqjD, YqjD(I39) and YqjD(L10).

582

583 **Figure 7: ElaB enters the ribosomal peptide tunnel *in vivo*.** (A) *In vitro* site directed cross-

584 linking was performed with ribosomes containing the cross-linker pBpa either at the surface-
585 exposed position 52 of uL23 or at position 71 within the ribosomal peptide tunnel. Ribosomes
586 were purified via sucrose-gradient centrifugation and incubated for 20 min at 30 °C with
587 equimolar amounts (500 nM) of purified signal recognition particle (SRP) or a 10-fold excess
588 of purified YqjD. After incubation, the sample was exposed to UV light and then separated by
589 SDS-PAGE and decorated with antibodies against Ffh, the protein component of the *E. coli*
590 SRP (A, left panel) or antibodies against the Xpress tag of YqjD (A, right panel). Wild-type

591 ribosomes (no pBpa) and samples without ribosomes served as controls. Indicated are the cross-
592 links to SRP from the surface exposed uL23 residue E52 and the intra-tunnel residue G71. (B)
593 *In vivo* site-directed cross-linking was performed with *E. coli* cells producing pBpa-containing
594 ribosomes. The *E. coli* $\Delta rplW$ (uL23) deletion strain expressing uL23(E52pBpa) or
595 uL23(E71pBpa) was grown to exponential or stationary phase and UV-exposed. After UV
596 exposure of whole cells, cells were fractionated into the soluble ribosome fraction and the crude
597 membrane fraction. These fractions were then separated by SDS-PAGE and probed with
598 peptide antibodies against the native ElaB. The potential cross-links between ElaB and
599 uL23(G71pBpa) and uL23(E52pBpa) are labelled by (*). Note, that the antibody non-
600 specifically recognizes multiple bands, which are indicated by (+).

601

602 **Figure 8: Hypothetical model for ribosome inactivation by YqjD.** The C-tail anchored
603 membrane protein YqjD shows a strong propensity for dimerization and interacts via its N-
604 terminus with the ribosomal proteins uL22, uL23, and uL29. In addition, YqjD contacts uL24
605 (not shown). These proteins encircle the ribosomal peptide tunnel exit and form an important
606 platform for ribosome-interacting proteins. As a consequence of YqjD binding to ribosomes,
607 ribosomes are inactivated. N-terminally truncated YqjD variants fail to bind to ribosomes and
608 are unable to prevent translation. Deletion of the C-terminal transmembrane domains prevents
609 YqjD dimerization and ribosome inactivation, although this Δ TM-YqjD variant still shows
610 ribosome binding, Thus, it is possible that for full ribosome inactivation, both N-termini of the
611 YqjD dimer need to simultaneously bind to a single ribosome, but this needs to be further
612 validated. Note, that the YqjD paralog, ElaB, can even protrude into the ribosomal tunnel.

613 **STAR Methods**

614 **RESOURCE AVAILABILITY**

615

616 *Lead contact*

617 Further information and requests for resources and reagents should be directed to the lead

618 contact, Hans-Georg Koch (Hans-Georg.Koch@biochemie.uni-freiburg.de)

619 **Materials availability**

620 All plasmids are available upon request, subject to a material transfer agreement (MTA), from

621 Hans-Georg Koch (Hans-Georg.Koch@biochemie.uni-freiburg.de)

622 **Data and Code availability**

- 623
- All data reported in this paper will be shared by the lead contact upon request.
 - The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE ¹⁰⁶ partner repository with the dataset identifier PXD052307 and 10.6019/PXD05230. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.
- 627

628

629 **Experimental model and subject details**

630 All bacterial strains used in this study are derived from wild type *E. coli* K-12 strain ¹⁰⁷⁻

631 ¹¹⁰. *E. coli* strains BW25113 or MC4100 served as wild-type strains and were routinely grown

632 on LB medium ¹¹¹ unless stated otherwise. The $\Delta yqjD$ (JW3069), $\Delta elaB$ (JW2261) and $\Delta ygaM$

633 (JW2647) strains were obtained from the Keio collection and were purchased via Horizon
All plasmids were constructed by using the

634 Discovery Ltd. (Cambridge, UK). Plasmids were propagated in *E. coli* DH5 α ¹⁰⁷. Proteins were
Clon-assembly protocol. Using 100 ng vector and a λ 3 vector / insert

635 expressed in *E. coli* strains BW25113, BL21, BL21(DE3) or C43(DE3) (Novagen/Merck,

636 Darmstadt, Germany) for purification.

HiFi DNA

637 assembly Master Mix

638

639 . The sequence of all plasmids was confirmed by sequencing. All primers used in this
640 study are listed in Supplementary Table S2.

641 ratio. For mutagenesis, the NEB Site directed mutagenesis kit using the manufacture's protocol

642 ^{was used} **METHOD DETAILS**

643 **Construction of YqjD, ElaB, and YgaM encoding plasmids**

644 Plasmids encoding *yqjD*, *elaB* or *ygaM* with an N-terminal His-tag in a pBAD24 backbone
645 (pBADHisA) were obtained from BioCat GmbH (Heidelberg, Germany). The YqjD variants
646 were generated by using the Q5 site-directed mutagenesis kit (NEB, Frankfurt, Germany) and
647 the following primer pairs. 1F/1R for the $\Delta N(12)$ -YqjD (deletion of the first 12 residues at the
648 N-terminus region of YqjD), 2F/2R for the ΔTM -YqjD (deletion of the 18 residues
649 (corresponding to the TM) at the C-terminus region of YqjD), 3F/3R for generation of
650 YqjD(P₈₀A) (the highly conserved Proline residue at position 80 in YqjD substituted to Alanine)
651 and 4F/4R for generating YqjD(P₈₀G) (the highly conserved Proline residue at position 80 in
652 YqjD substituted to Glycine). The construction of the YqjD amber-stop codon variants for site-
653 directed cross-linking used the primer pair 5F/5R for YqjD(L10pBpa) and 6F/6R for
654 YqjD(I39pBpa). Additional YqjD truncations were generated and included the $\Delta N(30)$ -YqjD
655 (deletion of the first 30-residues at the N-terminus region of YqjD) using primer pair 7F/7R,
656 primers 8F/8R for $\Delta N(45)$ -YqjD (deletion of the first 45-residues at the N-terminus region of
657 YqjD), 9F/9R for $\Delta N(60)$ -YqjD (deletion of the first 60-residues at the N-terminus region of
658 YqjD). Deletion of the 6xHis and the Xpress-epitope tags attached to the N-terminus region of
659 YqjD were deleted using primer pair 10F/10R. The nucleotide sequences of all primers is listed
660 in Supp. Table S2. YqjD variants in which the nucleotide sequence encoding the YqjD-TM
661 motif was substituted with the nucleotide sequence of the TM-domains of YfgM, FtsQ, or Fis1
662 were obtained from BioCat GmbH (Heidelberg, Germany). BioCat GmbH (Heidelberg,
663 Germany) also synthesized the YqjD-TMD peptide.

664

665 **Viable cell staining and counting**

666 The assay was performed using the QUANTOM Tx™ Microbial Cell Counter and the
667 QUANTOM™ Viable Cell Staining Kit obtained from BioCat GmbH (Heidelberg, Germany)
668 The kit stains live bacterial cells to be counted. The optical density of the cell-culture was
669 determined using a spectrophotometer at each time point and an aliquot corresponding to
670 approx. 1×10^8 cells were collected. The cells were washed, the culture media was completely
671 removed and the cells were resuspended in the QUANTOM™ Viable Cell Dilution Buffer.
672 10.0 μ L of the cell culture was taken into a clean 1.5mL Eppendorf-tube and 2.0 μ L of the
673 QUANTOM™ Viable Cell Staining Dye was added and mixed gently and carefully. The cells
674 were then incubated at 37°C for 30 minutes in the dark. Thereafter 8.0 μ L QUANTOM™ Cell
675 Loading Buffer I was added and mixed gently without creating bubbles. 5.0 μ L of this mixture
676 was loaded onto a QUANTOM™ M50 Cell Counting Slide and centrifuged at 300 x rcf for 10
677 minutes in a QUANTOM™ Centrifuge at room-temperature. The slide was then inserted into
678 the QUANTOM Tx™ cell counter and cells were counted with the light intensity level set to
679 either 7 or 9. The obtained viable cell numbers were then blotted against time.

680

681 **Purification of YqjD, ElaB, YchF, SecA and SRP**

682 The *E. coli* BW25113 strain expressing either ElaB, YqjD or its variants from the
683 pBADHisA vector or *E. coli* BL21(DE3) expressing pET19b-SecA-His⁵⁶ or *E. coli* $\Delta ychF$
684 (JW1194, Km^S)¹¹² expressing pBAD24-YchF-His were all grown on LB-medium
685 supplemented with 100 μ g mL⁻¹ ampicillin to an optical density at 600 nm (OD₆₀₀) of 0.5 and
686 then induced with the corresponding inducers. For ElaB, YqjD and its mutants, as well as for
687 YchF production, cells were induced with 0.02% arabinose for 1-2 hours, while for SecA
688 production the cells were induced with 0.5 mM IPTG for 3-4 hours. Cell growth was stopped

689 on ice for 10-15 minutes and cells were harvested at 7,460 x g for 15 minutes using a JLA-
690 9.100 rotor (Beckman Coulter).

691 The cell pellet was resuspended in HKM buffer (25 mM HEPES, 200 mM KCl, 10 mM
692 MgCl₂ x 6 H₂O and 10% glycerol, pH 7.5). Just before the cell lysis, 5 mM β-mercaptoethanol
693 (β-ME), 0.5 mM PMSF (Carl Roth, Karlsruhe, Germany) and cOmplete™ EDTA-free Protease
694 Inhibitor Cocktail (1 tablet/ 50.0 mL cell culture, Sigma Aldrich, Germany) were added and the
695 cell mixture homogenized using an IKA homogenizer (T 10 basic Ultra TURRAX®). Cells were
696 then lysed by passing them 2-3 cycles through either Emulsiflex C3 (Avestin) or Maximator
697 type HPL6 (Maximator GmbH) at 800-1000 bar (11,603 - 14,503 psi). The broken cells were
698 cleared using a Sorvall RC6 (Thermo Scientific) at 30,000 x g for 30 minutes set at 4°C in an
699 SS34 rotor and the cell debris discarded. The S30 (cleared cell lysate or supernatant) was further
700 centrifuged at 183,700 x g for 2½ hours in a Ti50.2 rotor using Sorvall WX-90 Ultra Series
701 (Thermo Scientific) set at 4°C. For membrane proteins, the supernatant (S150) was discarded,
702 and the crude membrane pellet was homogenized using a Dounce homogenizer in solubilization
703 buffer (HKM buffer plus 1.0% n-dodecyl-β-D-maltoside (DDM, Carl Roth), 5 mM β-ME, 0.5
704 mM PMSF and cOmplete™ EDTA-free Protease Inhibitor Cocktail). Solubilization was
705 performed for 1 h at 4 °C. The solubilized membranes were centrifuged using a Sorvall RC6
706 centrifuge (Thermo Scientific) at 30,000 x g for 15 minutes at 4°C in an SS34 rotor. The
707 solubilized materials were added directly to the equilibrated TALON® resin (TaKaRa;
708 Clontech) and incubated for 2.0 hours on a rotating shaker. For cytosolic proteins, the S150
709 (supernatant after the ultracentrifugation at 183,700 x g for 2½ hours in a Ti50.2 rotor at 4°C)
710 was directly added to the equilibrated TALON® resin and incubated for 2.0 hours on a rotating
711 shaker. The resin was washed 3-times, for 15 minutes each with washing buffer (HKM buffer
712 with 0.03% DDM and 5 mM imidazole pH 8.0). The washed TALON® material was centrifuged
713 using an Eppendorf 5804R centrifuge at 2,937 x g for 5 min at 4°C. The TALON® resin were
714 then transferred into 15 mL polypropylene columns and proteins were eluted with elution buffer

715 (HKM buffer with 0.03% DDM) containing initially 20 mM imidazole pH 8.0 and then 200
716 mM imidazole. The eluted proteins were buffer-exchanged using PD-10 desalting columns
717 Disposable (Sigma Aldrich; Merck) against storage buffer (50 mM HEPES, 50 mM potassium
718 acetate, 10 mM magnesium-acetate and 1.0 mM DTT pH 7.5). The buffer was supplemented
719 with 0.03% DDM if membrane proteins were to be stored. The concentrations were determined
720 by either BCA assays or A_{280} , while the purity was established on an SDS-PAGE. The proteins
721 were aliquoted, frozen in liquid nitrogen and kept at -80 °C for further usage.

722 For the purification of SRP, its protein component Ffh was expressed from pTrc99a-
723 His-Ffh in *E. coli* BL21¹¹³. Cells were induced at an OD_{600} of 0.5 with 1 mM IPTG for 3 h,
724 harvested, washed and lysed with the Emulsiflex Homogenizer (Avestin Europe, Mannheim,
725 Germany). The lysate was cleared at 30,000 x g for 20 min at 4 °C in an SS-34 rotor and loaded
726 on buffer-equilibrated (25 mM HEPES-KOH, 1 M NH₄-acetate, 10 mM Mg-acetate, 1 mM β -
727 mercaptoethanol, 15% glycerol, 5 mM imidazole, pH 7.6) Talon beads for 1 h. After several
728 washing steps with the equilibration buffer, Ffh was eluted with the same buffer containing 200
729 mM imidazole, re-buffered into HT buffer (50 mM HEPES/KOH pH 7.5, 100 mM potassium
730 acetate pH 7.5, 10 mM magnesium acetate pH 7.5, 1 mM DTT, 50% glycerol) and stored at -
731 20 °C. A reconstitution of Ffh with 4.5S RNA is usually not required, as Ffh has sufficient 4.5S
732 RNA bound^{113,114}.

733

734 **Ribosome and membrane purification**

735 High-salt washed wild-type 70S ribosomes were purified from strains MC4100 or BW25113.
736 Ribosomes bearing pBpa at uL23 were purified from MC4100 $\Delta rplW::kan$ containing pCDF-
737 L23(E52pBpa) or L23(G71pBpa)⁶⁶ and pSup-BpaRS-6TRN⁶³ for pBpa incorporation. The
738 cells were propagated in S150-medium consisting of 1% (g/w) yeast extract, 1% (g/w) tryptone-
739 peptone, 41 mM KH₂PO₄, 166 mM K₂HPO₄ and 1% (g/w) glucose. Except for wt ribosomes,
740 the medium was supplemented with 50 μ g/mL streptomycin (Sigma Aldrich) and 0.5 mM

741 IPTG. For pBpa incorporation, 35 $\mu\text{g}/\text{mL}$ chloramphenicol (Sigma Aldrich) and 0.5 mM pBpa
742 (Bachem, Bubendorf, Switzerland) were added to the medium. When the cell density reached
743 OD_{600} of 1.6-1.8, the growth was stopped on ice for 10-15 min, and the cells were harvested,
744 washed and homogenized with the Emulsiflex C3. The lysate was cleared at 30,000 x g for 30
745 min and the crude ribosomes were collected at 184,000 x g in a Ti50.2 rotor for 2.5 h.
746 Ribosomes were dissolved in high-salt buffer (50 mM Triethanolamine acetate; 1M potassium
747 acetate; 15 mM magnesium acetate; 1 mM DTT; pH 7.5) and purified through a 1.44 M sucrose
748 cushion at 344,000 x g for 1 h. 70S ribosomes were isolated via centrifugation through a 0.29-
749 1.17 M sucrose gradient at 29,000 rpm for 17 h in a TH-641 swing-out rotor (Thermo Fisher
750 Scientific). The ribosomal fractions were withdrawn from the gradient, concentrated at 344,000
751 x g for 1h in a TLA120.2 rotor and resuspended in CTF-buffer at pH 7.5 with 1 mM DTT. The
752 isolation of ribosomes from stationary phase *E. coli* cells followed the same protocol, but *E.*
753 *coli* cells were grown for 24 h up to an optical density of about 6.0.

754 For membrane isolation, cells were grown to approx. OD_{600} = 1.5-1.8 on LB medium,
755 harvested and resuspended in INV buffer (50 mM triethanolamine acetate, pH 7.5, 250 mM
756 sucrose, 1 mM EDTA, 1 mM DTT) supplemented with 0.5 mM PMSF and cComplete™ EDTA-
757 free Protease Inhibitor Cocktail. Next, the samples were lysed as described above and the cell
758 debris was removed by centrifugation at 30,000 x g for 30 minutes in an SS34 rotor. The
759 supernatant (S30) was further centrifuged at 184,000 x g for 2,5 hours at 4 °C in a Ti50.2 rotor
760 and the pellet containing the crude bacterial membranes was dissolved in INV buffer, loaded
761 onto a 10-30% sucrose gradient and the inner membrane fraction (inverted inner membrane
762 vesicles, INV) and the outer membrane fraction were separated as described ¹¹⁵.

763

764 **Ribosome binding assays**

765 For ribosome-YqjD binding assays, 10 nM of the purified wild-type YqjD was
766 incubated with varying concentrations of sucrose-gradient purified *E. coli* ribosomes in 50 mM

767 HEPES, 50 mM potassium acetate, 10 mM magnesium-acetate, pH 7.5, 1.0 mM DTT, 0.03%
768 DDM and 1.0 mM spermidine. Ribosomes were isolated as described above from cells grown
769 to either exponential (2.5 h) or stationary phase (24 h) and prepared in high salt buffer. After
770 mixing the components, they were incubated at 30 °C for 30 minutes. The reaction mix was
771 overlaid onto a 30% sucrose cushion (in the above buffer) in TLA 120.2 rotor tubes and
772 centrifuged at 344,000 x g for 2.0 hours. The supernatant was carefully collected into a separate
773 tube, while the pellet was also resuspended and collected in a second tube. The proteins were
774 precipitated by adding 10% TCA, denatured and separated by SDS-PAGE, followed by western
775 blotting.

776

777 ***In vitro* protein synthesis**

778 For *in vitro* protein synthesis, a purified transcription/translation system composed of
779 cytosolic translation factors (CTF) and high salt washed ribosomes⁵⁵ was used. The ³⁵S-
780 Methionine/³⁵S-Cysteine labeling mix was obtained from Hartmann Analytics (Braunschweig,
781 Germany). After 30 min at 37 °C, the *in vitro* reaction was directly precipitated with 10%
782 trichloroacetic acid (TCA). Next, the samples were denatured at 56 °C for 10 minutes in 25 µl
783 of TCA loading dye (prepared by mixing one part of Solution III (1M dithiothreitol) with 4
784 parts of Solution II (8.3% SDS (w/v), 0.083 M Tris-Base, 30% glycerol and 0.03%
785 Bromophenol blue) and 5 parts of Solution I (0.2 M Tris, 0.02 M EDTA pH 8)) and analyzed
786 on SDS-PAGE and by phosphor imaging.

787

788 **Immune detection and antibodies**

789 For immune detection after SDS-PAGE, samples were electro blotted onto
790 Nitrocellulose 0.45 µm membranes (GE Healthcare) with a current of 750 mA for 2.5 hours in
791 a tank buffer system (transfer-buffer: 20 mM Tris, 150 mM Glycine, 20% Ethanol (v/v), 0.02%
792 SDS (w/v)). Membranes were blocked with 5% milk powder in T-TBS buffer for at least 1 h.

793 Polyclonal antibodies against YidC and Ffh were raised in rabbits against the complete and
794 SDS-denatured protein^{55,116}. Monoclonal antibodies against the His6-tag were purchased from
795 Thermo Scientific and from Roche. Antibodies against the Xpress epitope tag were purchased
796 from Invitrogen Life technologies. Peroxidase-coupled goat anti-rabbit and goat-anti mouse
797 antibodies from SeraCare were purchased via medac GmbH (Wedel, Germany) and were used
798 as secondary antibodies with ECL (GE Healthcare)^{55,117}. Antibodies against *E. coli* ribosomal
799 proteins were raised in sheep and were a gift from Richard Brimacombe (Max-Planck-Institut
800 für Molekulare Genetik, Berlin).

801 Peptide antibodies against YqjD (MSKEHTTEHLRAEL), ElaB (VLRSSGDP-
802 ADQKYV) and YgaM (GSDAKGEAEAARSK) were raised in rabbits by GeneScript (Leiden,
803 Netherlands).

804

805 ***In vivo* and *in vitro* cross-linking**

806 p-benzoyl-l-phenylalanine (pBpa) for cross-linking was obtained from Bachem
807 (Bubendorf, Switzerland). For site-directed *in vivo* cross-linking C43(DE3) cells containing the
808 amber-stop codon variants of YqjD on a plasmid and pEVol were cultured overnight in LB
809 medium at 37 °C. 10 ml of the overnight culture were used for inoculation of 1000 ml LB
810 medium supplemented with 1 ml pBpa (final concentration 0.5 mM, dissolved in 1 M NaOH),
811 100 µg/µl of ampicillin and 25 µg/µl of chloramphenicol. The cultures were further incubated
812 at 37 °C until they reached the early exponential growth phase (OD₆₀₀ = 0.5-0.8) and induced
813 with 0.02% arabinose. After induction, the cultures were grown for 1-2 hours at 37 °C, cooled
814 down on ice for 10-15 minutes and harvested by centrifugation at 7,460 x g in a JLA 9.1000
815 rotor for 15 minutes. The cell pellets were resuspended in PBS buffer (137 mM NaCl, 2.7 mM
816 KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄), harvested again as above, resuspended in 10 ml
817 PBS buffer, and divided into two multi-well plates. One plate was exposed to UV light (365nm)

818 on ice for 30 minutes (UV chamber: BLX-365, from Vilber Lourmat) while the other plate was
819 kept in the dark. After UV irradiation, the cell suspension was transferred to SS34 tubes and
820 cells were collected by centrifugation at 11,950 x g for 15 minutes. Each cell pellet was
821 resuspended in 10 ml of lysis buffer (25 mM HEPES, 200 mM KCl and 10 mM MgCl₂ x 6H₂O)
822 and 10% glycerol, pH 7.5), including protease inhibitors (0.5 mM PMSF and cComplete™
823 EDTA-free Protease Inhibitor Cocktail) and YqjD was purified as described above.

824 For *in vivo* crosslinking with the uL23-pBpa variants, the strain MC4100Δ*rpIW::kan*
825 containing pCDF-L23(E52pBpa) or L23(G71pBpa)^{57,66} and pSup-BpaRS-6TRN⁶³ was used
826 and the LB medium additionally supplemented with 50 μg/mL streptomycin and 0.5 mM IPTG.
827 Cells were either grown to OD₆₀₀ = 1 (exponential phase) or OD₆₀₀ = 4.5 (stationary phase),
828 cooled, harvested, UV exposed and then lysed in CTF buffer (50 mM triethanolamine acetate
829 pH 7.5, 50 mM potassium acetate pH 7.5, 5 mM magnesium acetate pH 7.5, 1 mM DTT) as
830 described above. Subsequently, bacterial membranes were prepared, and crude ribosomes
831 purified as before. 500 μg of total protein for each sample was TCA-precipitated, separated by
832 SDS-PAGE and analyzed by Western blot.

833 For *in vitro* crosslinking, purified *E. coli* 70S ribosomes (500 nM) were combined with
834 purified SRP (500 nM) or purified YqjD (5 μM), mixed 20 min at 30 °C in CTF buffer in a total
835 volume of 50 μl and crosslinked by UV exposure using a Biolink 365 nM-crosslinking chamber
836 (Vilber-Lourmat) for 30 min on ice. Samples were then TCA-precipitated, separated on PAGE,
837 and analyzed by western blot.

838

839 **Bioinformatic analyses**

840 DUF883-containing proteins were identified by searching the UniProt database¹¹⁸ for
841 the InterPro domain IPR010279¹¹⁹. The sequence similarity network was built using the EFI-
842 EST webtool¹²⁰ using IPR010279 with an alignment score of 20. Nodes were collapsed at 95%
843 identity. The network was visualized with Cytoscape v3.10.1 using the Prefuse Force Directed

844 OpenCL Layout. Nodes were colored based on the phylum of the representative sequence.
845 Phylogenetic trees were built using MAFFT for multiple sequence alignments on the CIPRES
846 Science Gateway ^{23,121} with default parameters, and either the IQ-Tree web server ^{122,123} with
847 LG+F+G4 (the best-fit model according to the Bayesian information criterion, BIC) ¹²⁴ and
848 ultrafast bootstrap (1000 replicates) ¹²⁵ for the tree in Suppl. Figure S3 (and extracted subtrees
849 in Fig. 2B), or with FastTreeMP on XSEDE ¹²⁶ for the tree in Suppl. Fig. 2A. The resulting
850 trees were visualized and annotated in iTol ¹²⁷. Sequences for the tree in Suppl. Fig. S3A were
851 selected based on the clusters identified in the SSN, such that each numbered cluster contained
852 at least two representatives, and for any given organism, all paralogs were included. In this way,
853 some clusters are represented by more than 2 sequences. For the tree in Suppl. Fig. S2A, YqjD,
854 YgaM and ElaB were used to search UniRef90 clusters in the UniProt database with blastp; the
855 top 250 UniRef90 clusters were collected and duplicates removed. The representative
856 sequences for the resulting UniRef90 clusters were used to build the tree.

857 TM regions were predicted using DeepTMHMM ¹²⁸. AlphaFold2-predicted structures
858 were downloaded from the Alpha Fold Protein Structure Database ¹²⁹. ChimeraX ¹³⁰ was used
859 to visualize structural models and perform conservation analysis using AL2CO ¹³¹.

860

861 **Mass spectrometry**

862 For identification of UV-crosslinked proteins, gel lanes from SDS-PAGE of UV-
863 illuminated or control samples analysis were cut in 26 slices which were individually subjected
864 to in-gel protein digestion using trypsin as previously described ¹¹. Peptide mixtures were
865 desalted using self-packed C18 Stop And Go Extraction tips¹³² using two layers of 1.0 × 1.0
866 mm C18 Empore Disks (3M, St. Paul, USA) and directly analysed by liquid chromatography-
867 tandem mass spectrometry (LC-MS/MS). For chromatographic separation an Ultimate 3000
868 RSLCnano system was coupled online to an Orbitrap Elite mass spectrometer (Thermo Fisher

869 Scientific, Bremen, Germany). Peptides were washed and preconcentrated on nanoEase™ M/Z
870 Symmetry C18 pre-columns (20 mm x 180 µm inner diameter; Waters) at a flow rate of 10
871 µl/min and separated using nanoEase™ M/Z HSS C18 T3 columns (25 cm x 75 µm inner
872 diameter; pore size, 100 Å; particle size, 1.8 µm; Waters) at a flow rate of 0.3 µl/min and 40°C.
873 Peptide elution was controlled with a binary solvent system consisting of 0.1% (v/v) FA
874 (solvent A) and 0.1% (v/v) FA/50% (v/v) MeOH/30% (v/v) ACN (solvent B) using a gradient
875 of 7 - 65% solvent B in 30 min, 65 - 80% in 5 min, and 3 min at 80%, interfaced online with
876 the Nanospray Flex ion source with PST-HV-NFU liquid junction (MS Wil, The Netherlands)
877 and fused silica emitters (EM-20-360; MicrOmics Technologies LLC). MS instruments were
878 operated in data-dependent mode with parameters as follows: mass range of m/z 370 - 1,700 for
879 MS1 scans with a resolution of 120,000 (at m/z 400), a target value (AGC) of 1×10^6 ions, and
880 a maximum injection time (IT) of 200 ms. For MS2 scans, up to 15 most intense precursor
881 peptides with a charge ≥ 2 were selected for collision induced dissociation (CID) in the linear
882 trap with a normalized collision energy of 35%, activation time 10 sec, q -value=0.25, a
883 resolution of 35,000, AGC of 5×10^4 ions, a max. IT of 150 ms, and dynamic exclusion for 45
884 s. Mass spectrometric raw data were processed using MaxQuant (version 2.0.2.0;¹³³) searching
885 against the *E.coli*-specific database from UniProt (release 2022_01). Database searches were
886 performed with tryptic specificity and a maximum number of two missed cleavages. Mass
887 tolerances were set to 4.5 ppm for precursor ions and 0.5 Da for fragment ion matchings.
888 Carbamidomethylation of cysteine residues was considered as fixed modification, oxidation of
889 methionine and N-terminal protein acetylation were set as variable modifications. The options
890 'match between runs' and 'iBAQ' were enabled. Proteins were identified with at least one unique
891 peptide and a false discovery rate of 0.01 on both peptide and protein level.

892

893 **Data quantification and statistical analyses**

894 Western blot and autoradiography samples were analyzed by using the *ImageQuant* (GE
895 Healthcare) or *ImageJ/ Fiji* plug-in software (NIH, Bethesda, USA). All experiments were
896 performed at least three-times as independent biological replicates and representative
897 gels/blots/images are shown. When data were quantified, at least three independent biological
898 replicates with several technical replicates were performed. Mean values and SEM values were
899 determined by using either Excel (Microsoft Corp.) or GraphPad Prism (GraphPad Prism Corp.
900 San Diego).

901

902 **Supplemental information**

903 Table S1: Excel file containing additional data too large to fit in a PDF, related to the YqjD
904 sequence similarity network nodes in Figs 1, 2, S1, S2.

905 Document S1: Table S2 and Figures S1-S7, related to Figs. 1-8.

906

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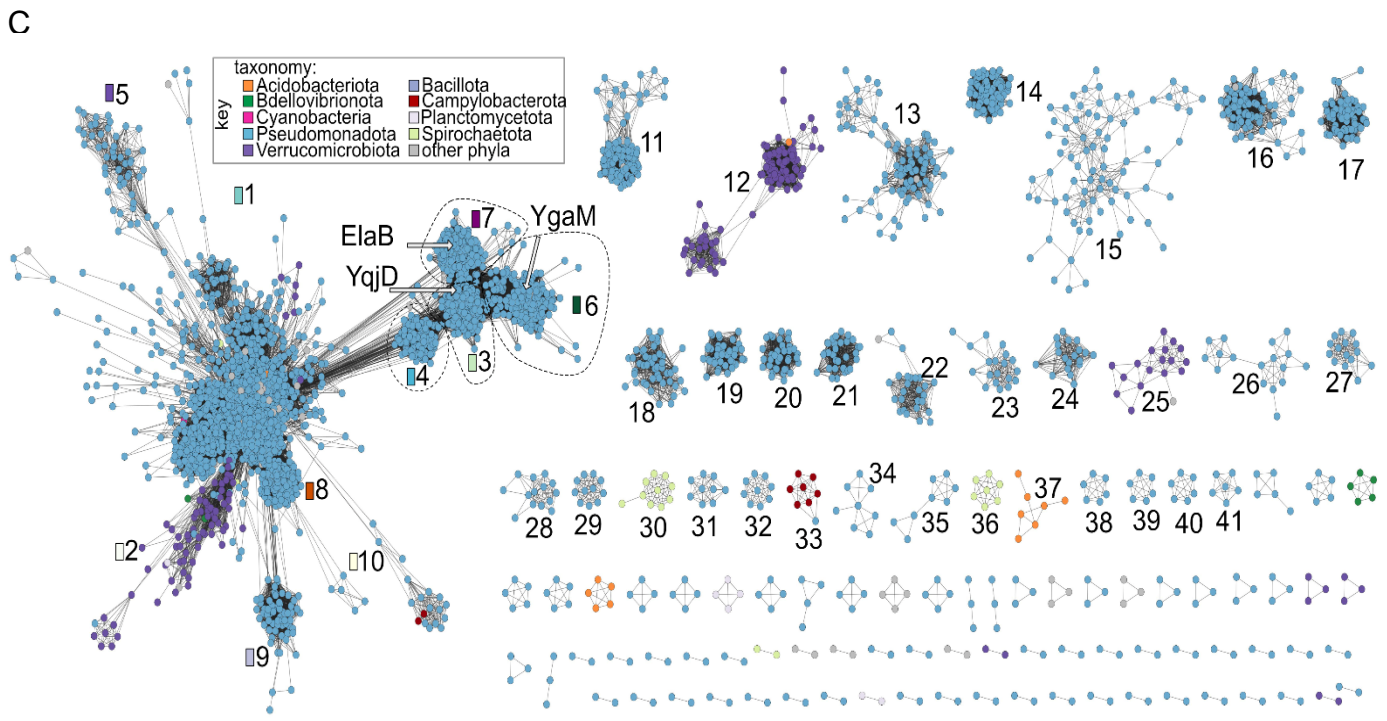
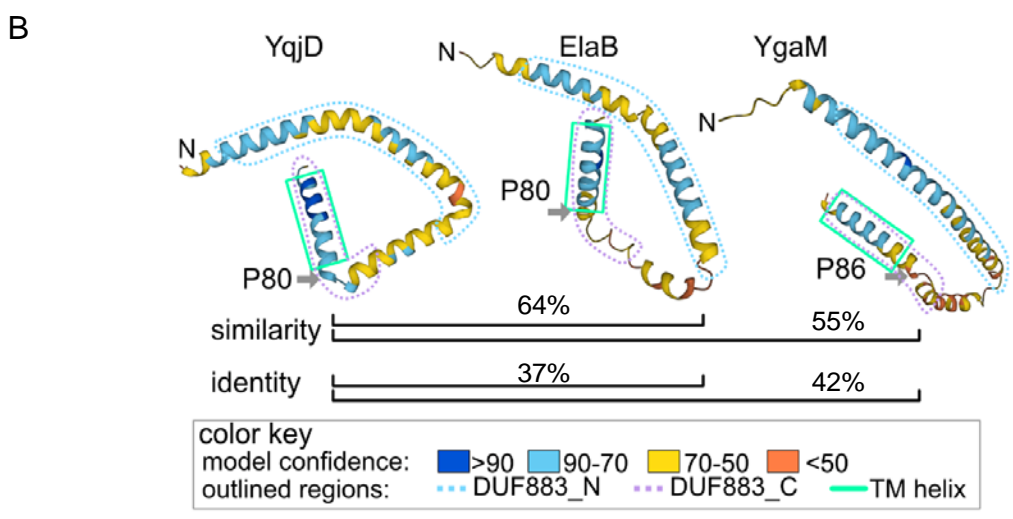
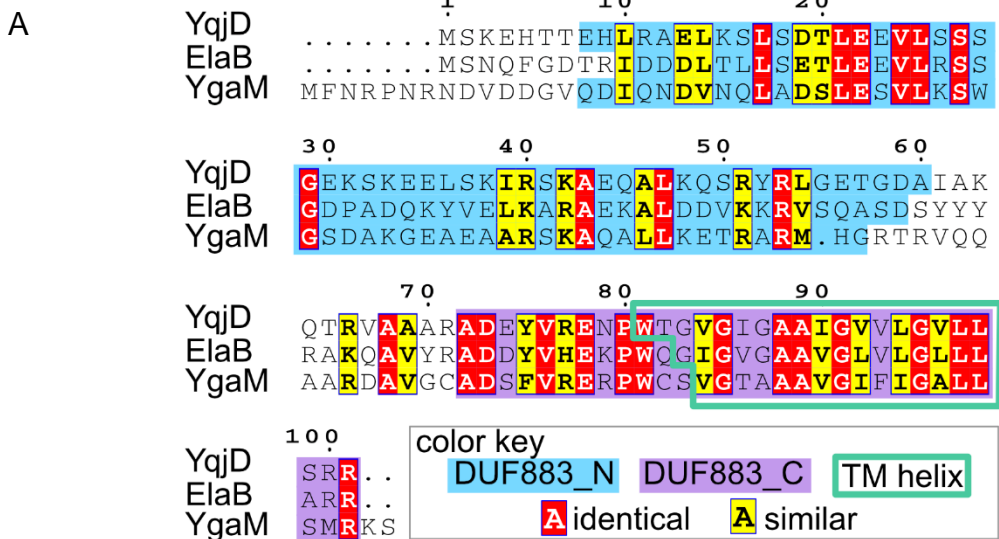
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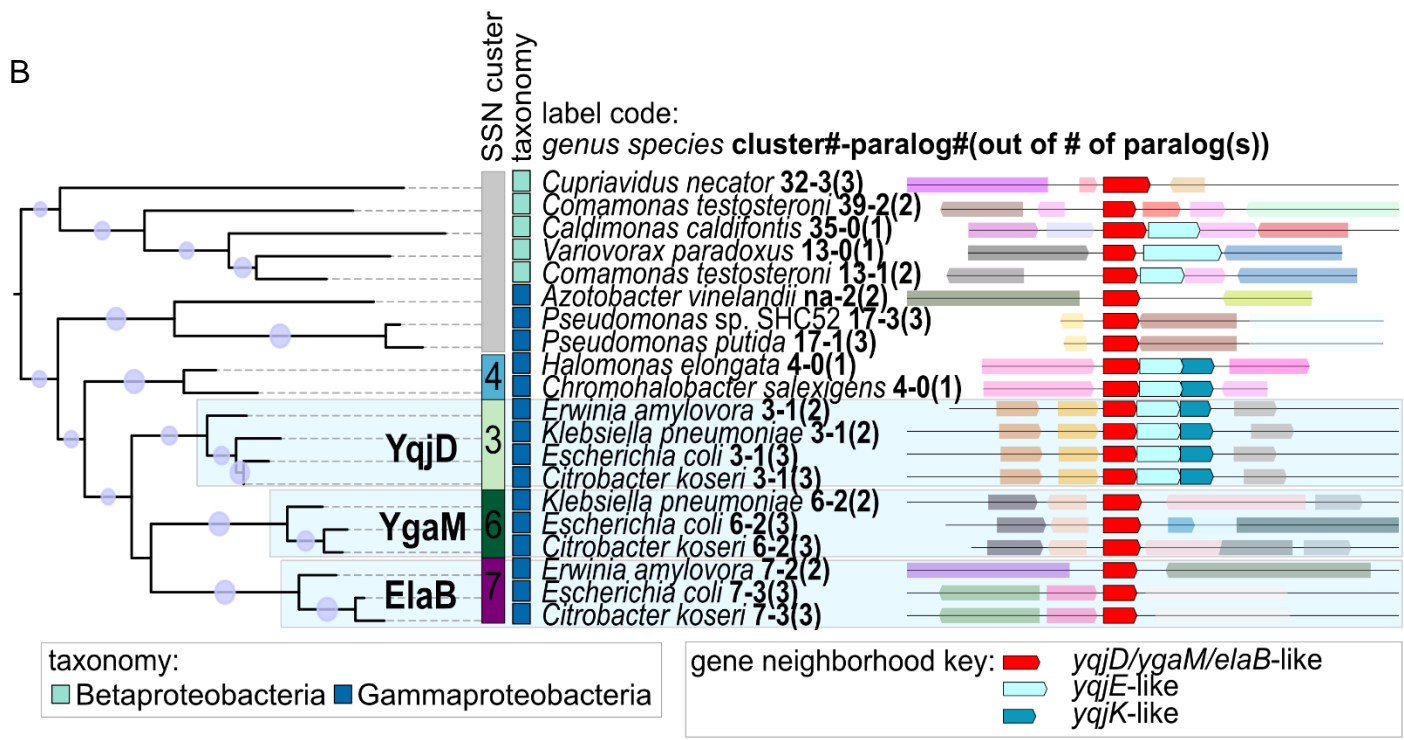
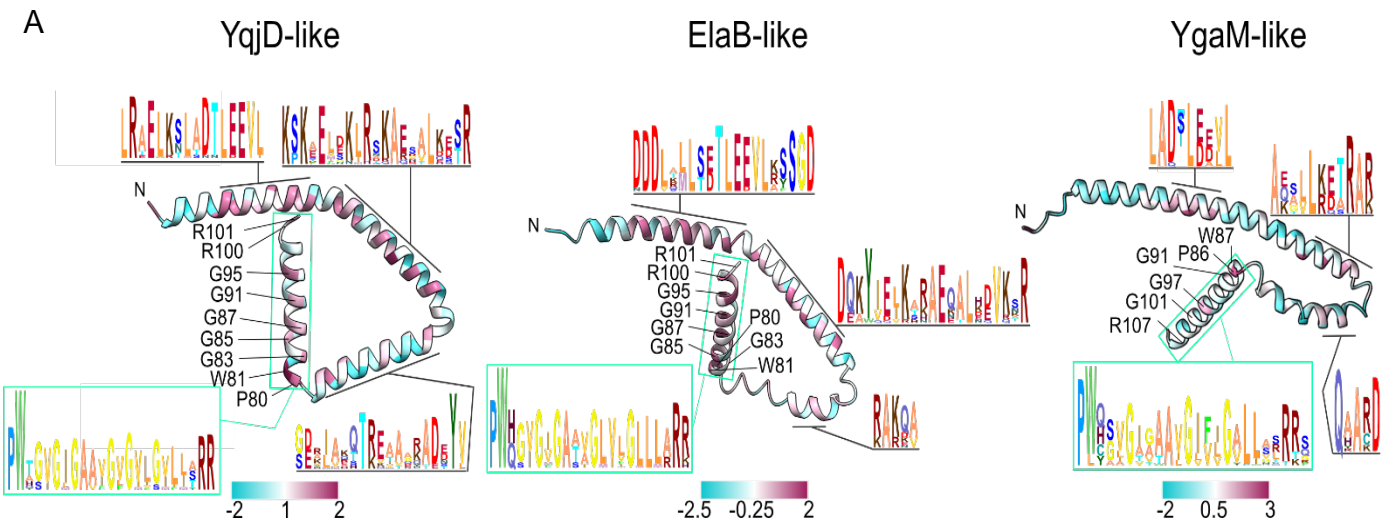
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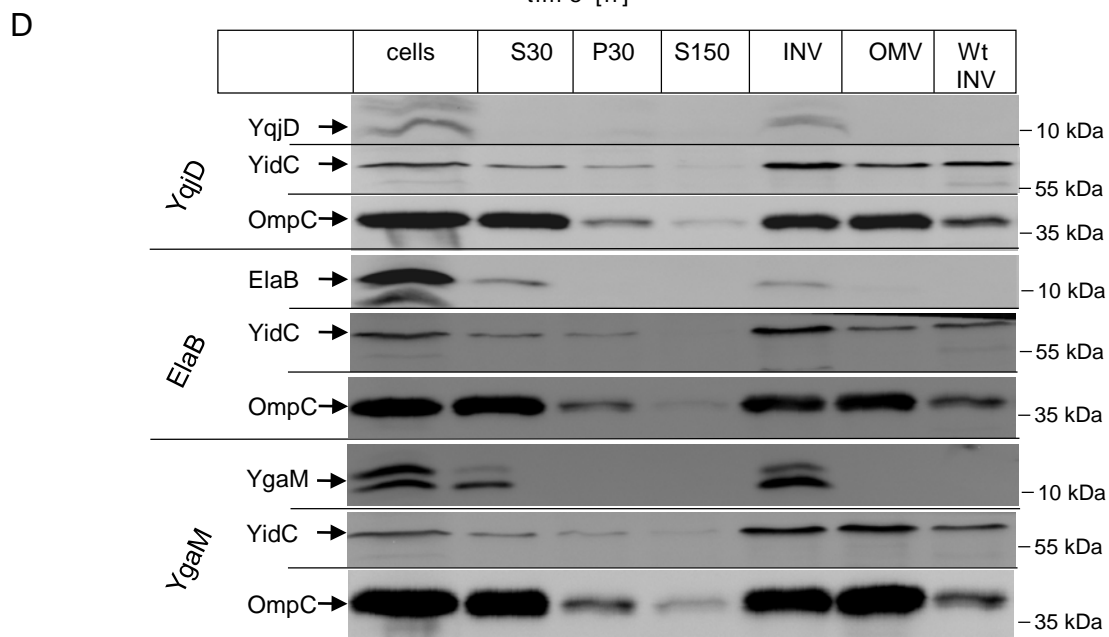
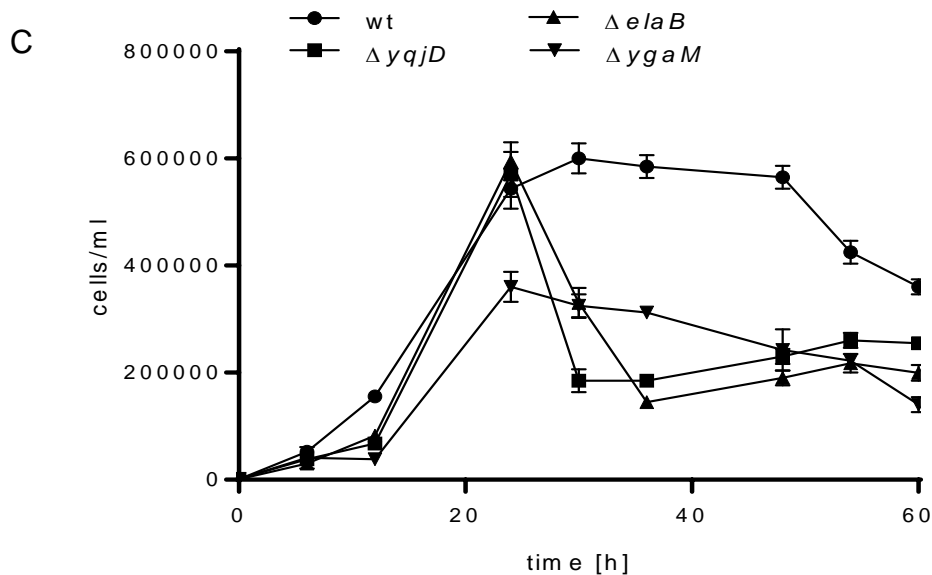
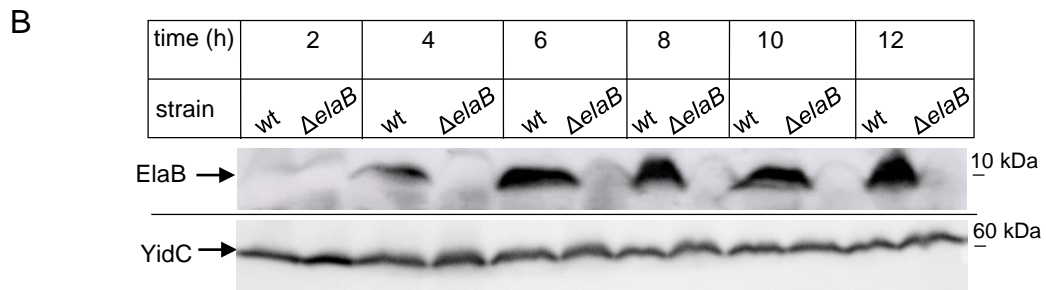
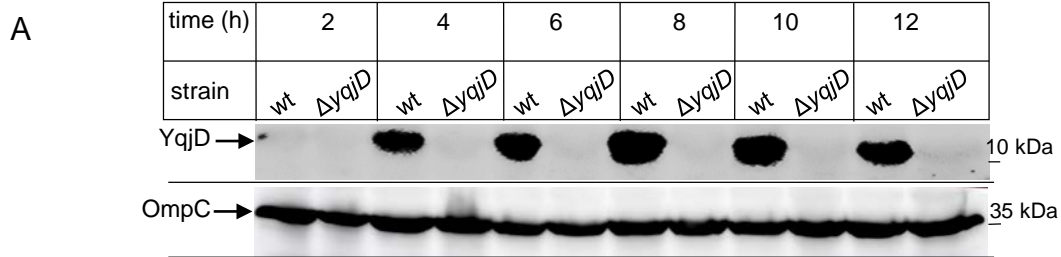
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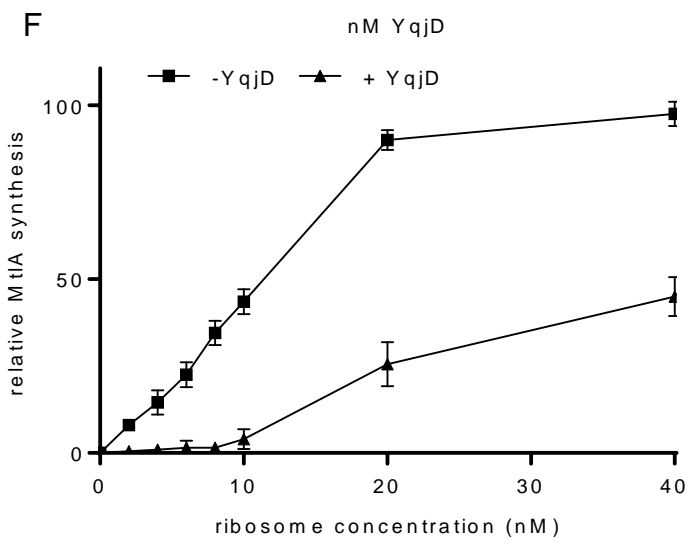
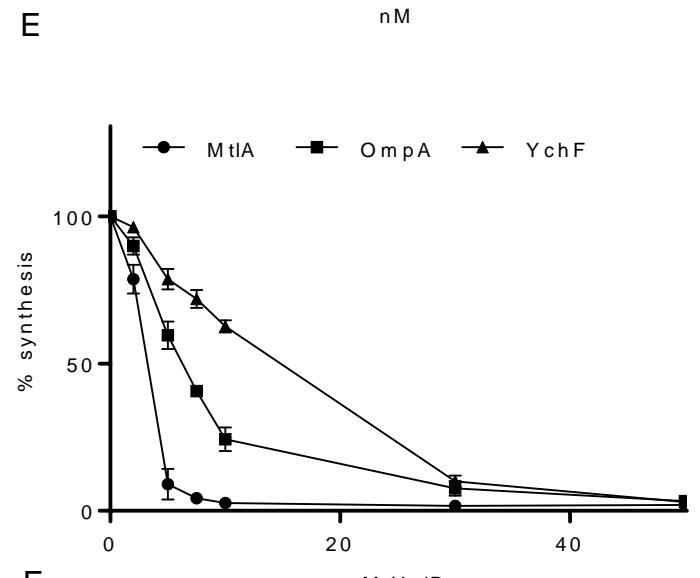
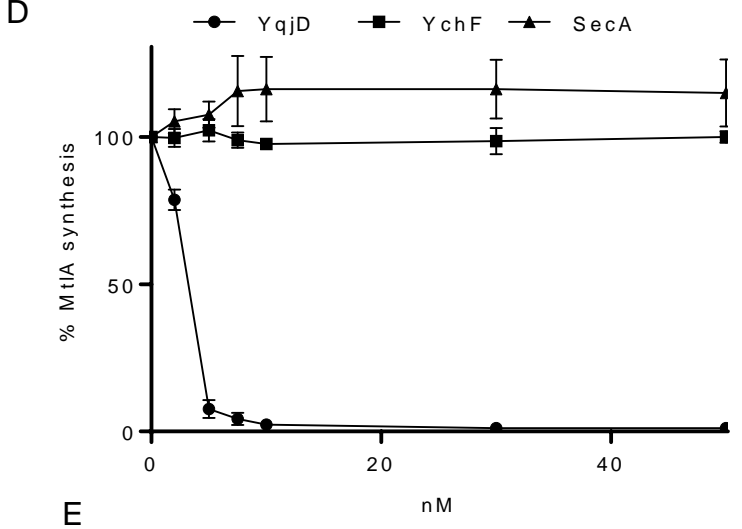
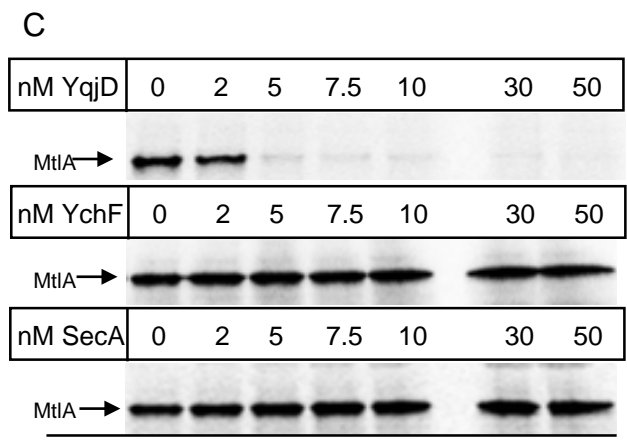
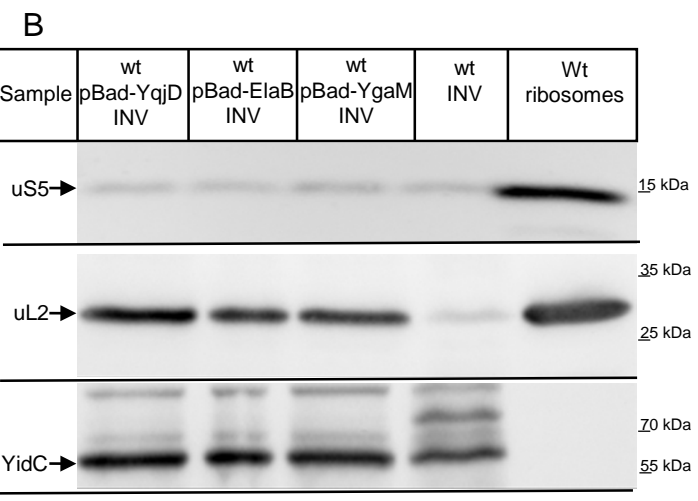
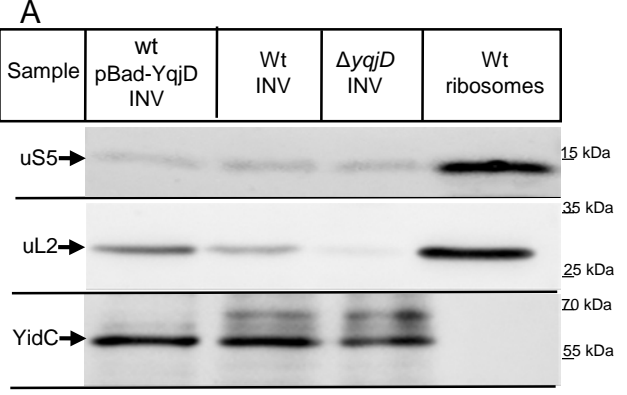
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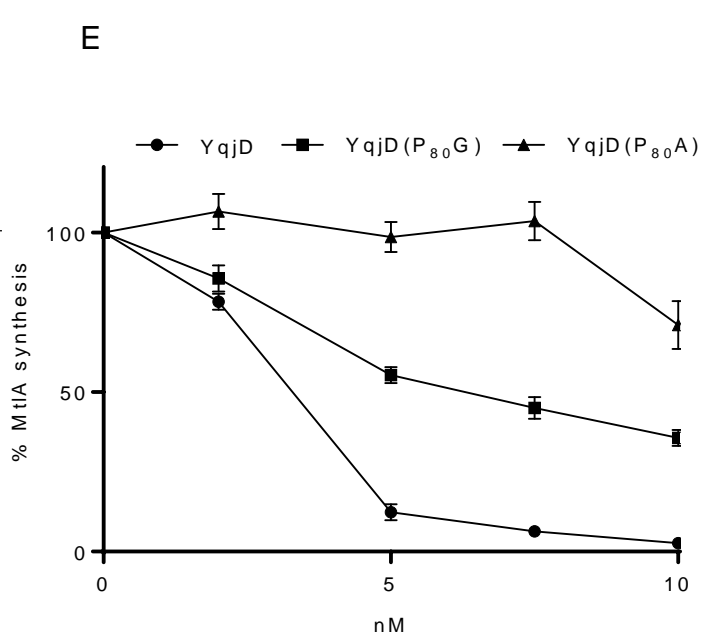
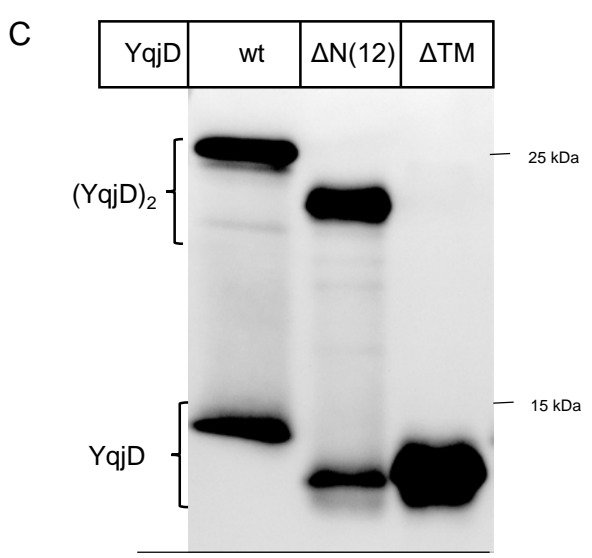
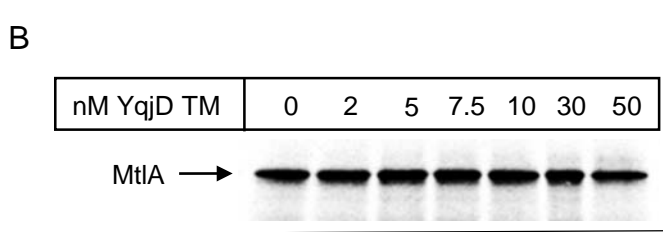
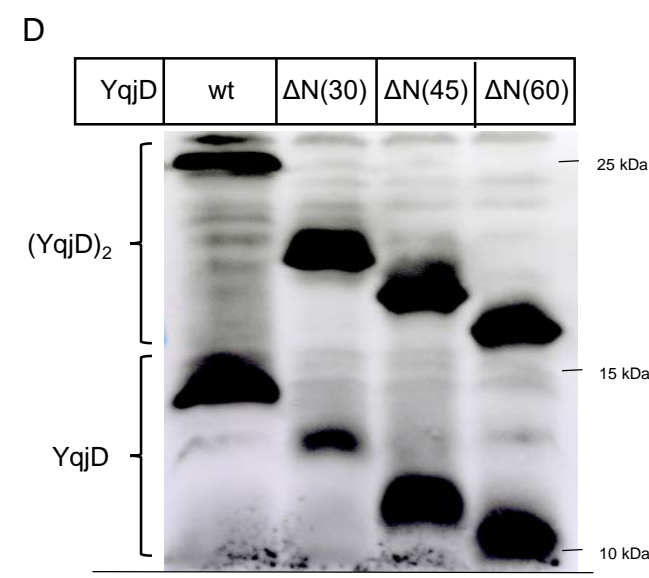
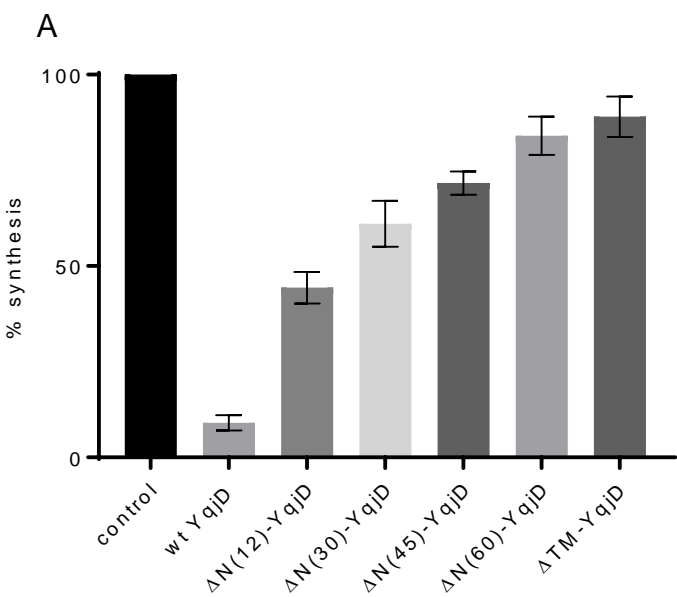




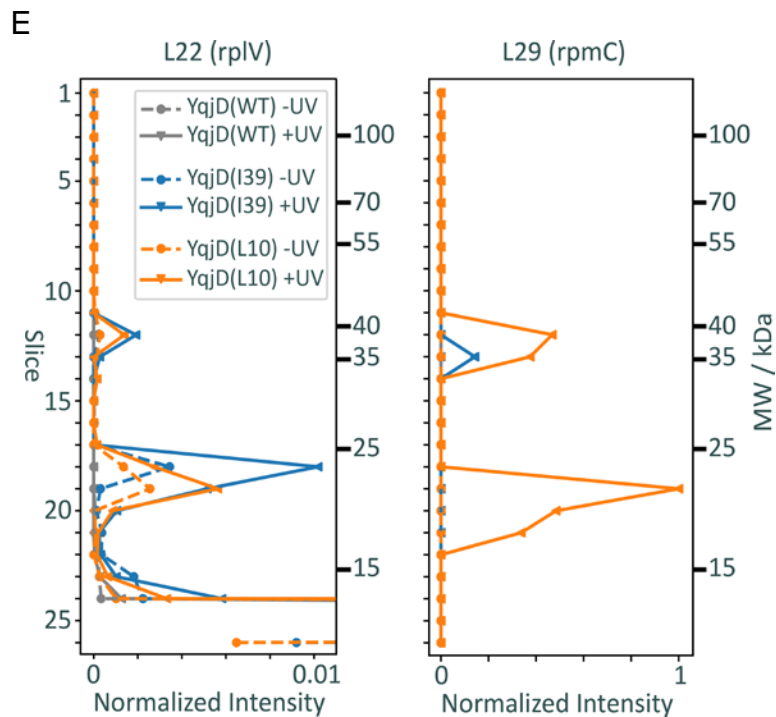
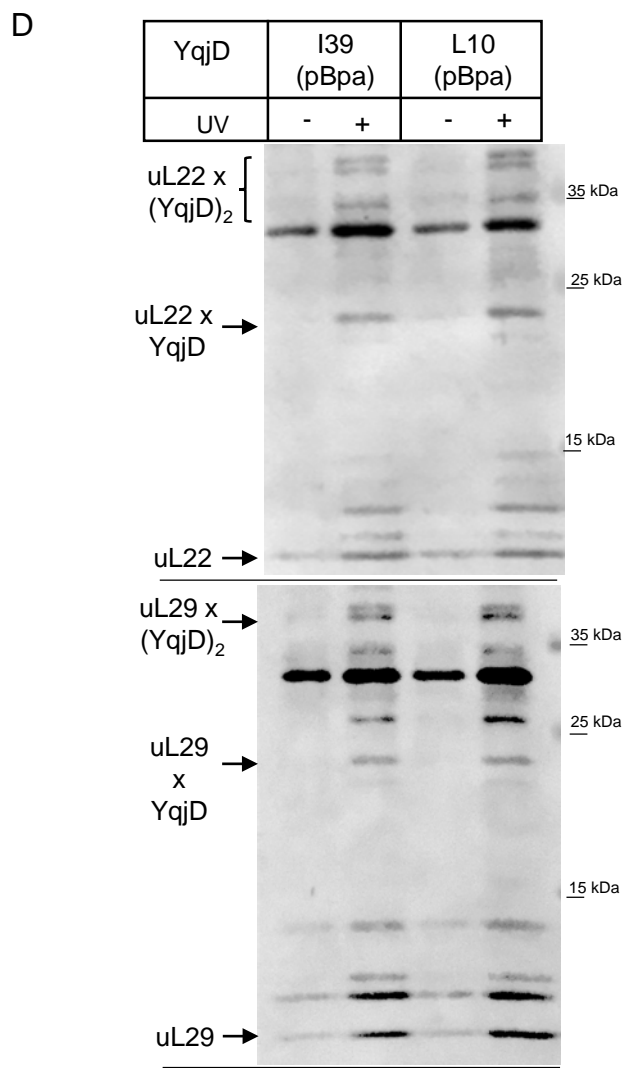
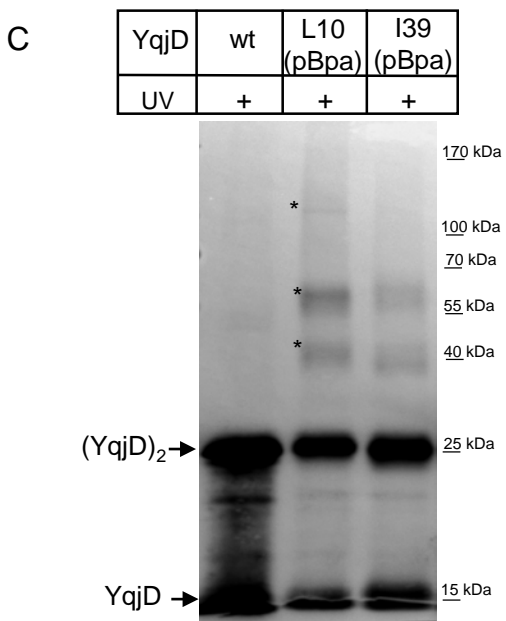
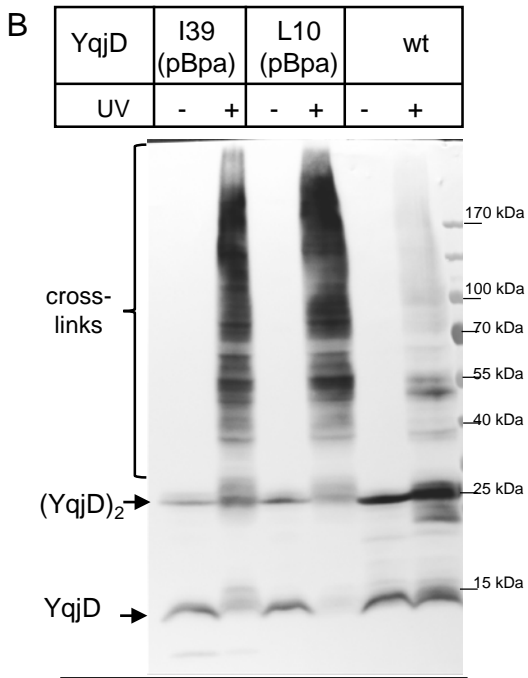
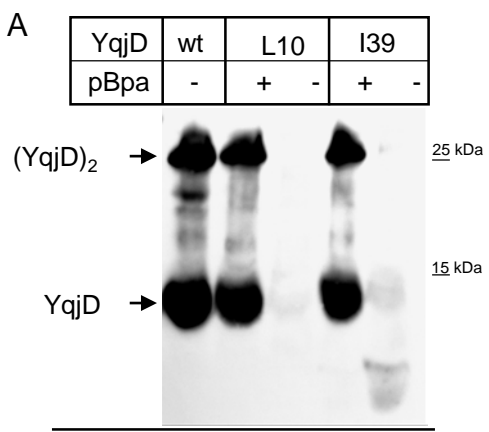
(Njenga et al., Fig. 2)



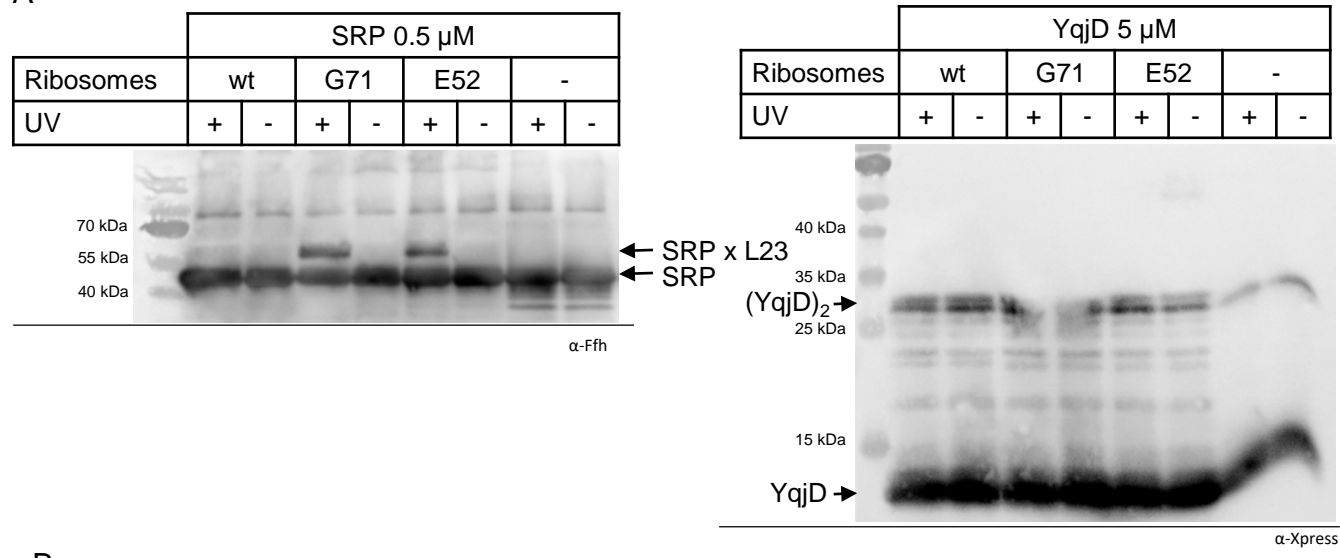




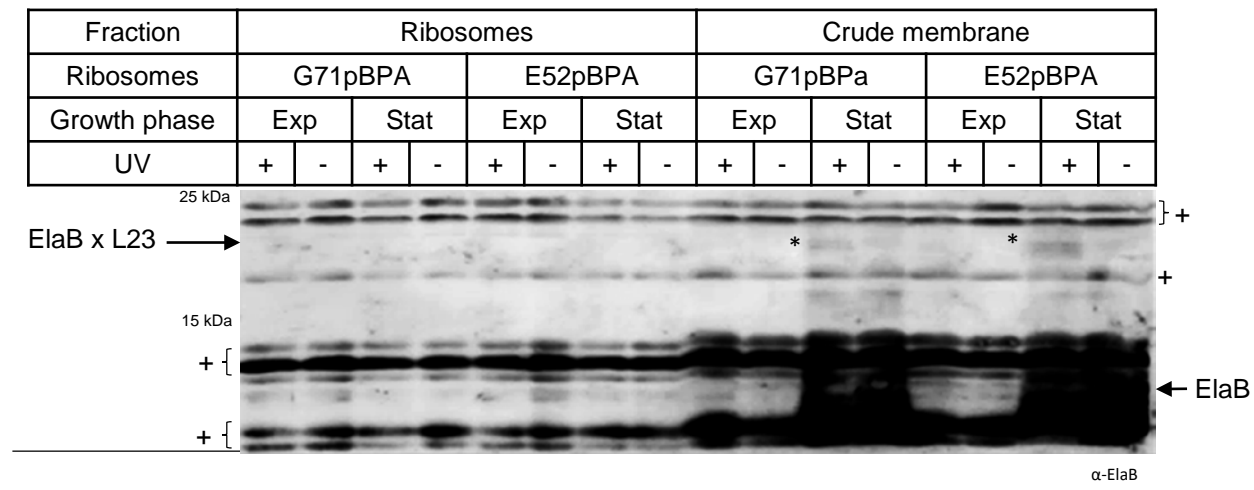
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A



B

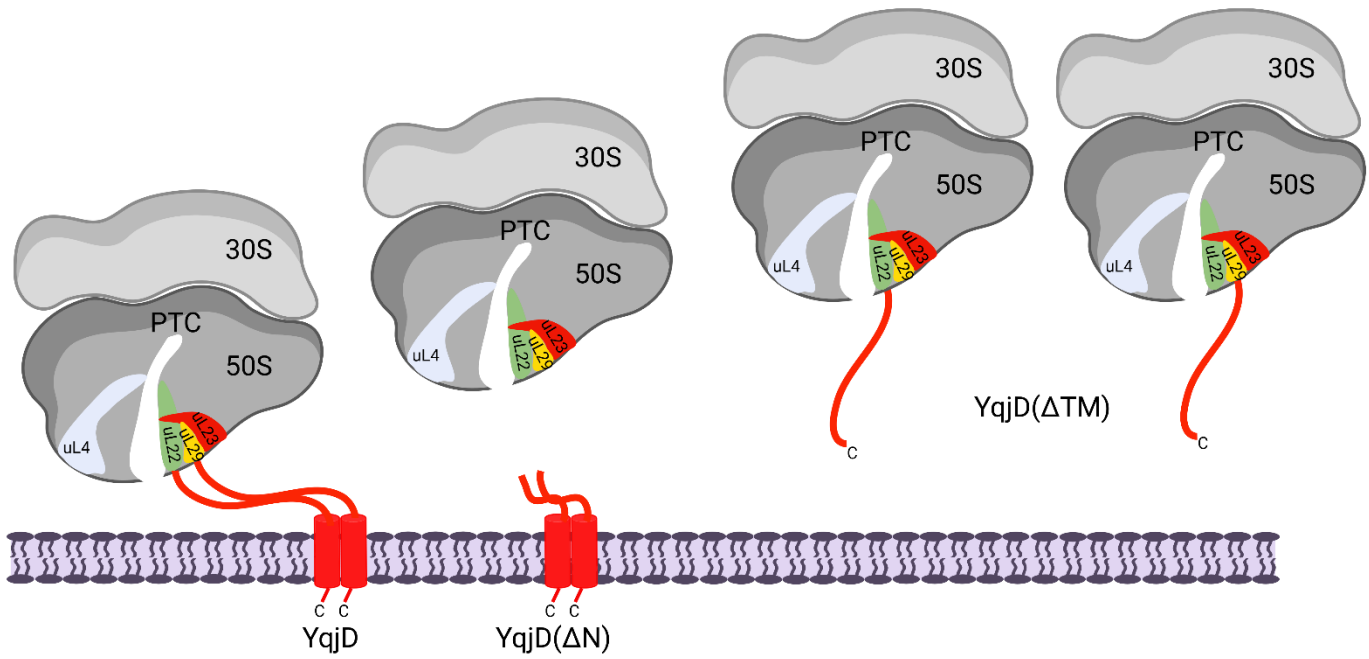


(Njenga et al., Fig. 7)

Ribosome binding & inactivation

No Ribosome binding & no inactivation

Ribosome binding but no inactivation



(Njenga et al., Fig. 8)