

1 **What is the link between stringent response, endoribonuclease encoding Type II Toxin-**
2 **Antitoxin systems and persistence?**

3

4 Bhaskar Chandra Mohan Ramisetty^{1, 2#}, Dimpy Ghosh² and Maoumita Roy Chowdhury²,
5 Ramachandran Sarojini Santhosh².

6

7 ¹Department of Biochemistry and Molecular Biology, University of Southern Denmark,
8 5230 Odense M, Denmark.

9 ²School of Chemical and Biotechnology, SASTRA University, Thanjavur, Tamil Nadu,
10 INDIA, 613401.

11

12 # Corresponding author: Bhaskar Chandra Mohan Ramisetty,
13 School of Chemical and Biotechnology,
14 SASTRA University, Thanjavur,
15 Tamil Nadu, INDIA 613401
16 Phone: +91 4362 304000
17 Fax: +91 4362 264120
18 Mobile: +91 8122609945
19 Email: ramisettybcm@biotech.sastra.edu
20 bcmr25@gmail.com

21

22

23

24 **Running title:** The role of endoribonuclease TAS in bacterial persistence.

25

26 BCMR designed the research, analysed the data and wrote the paper; all primer extension
27 assays were done by BCMR at University of Southern Denmark; BCMR, DG and MRC
28 performed the remaining research at SASTRA University.

29

30 **Keywords:** ppGpp, inorganic polyphosphate, polar effects, fitness.

31

32

33

34

35 **Abstract**

36 Persistence is a transient and non-inheritable tolerance to antibiotics by a small fraction of a
37 bacterial population. One of the proposed determinants of bacterial persistence is Toxin-
38 Antitoxin systems (TAS) which are also implicated in a wide range of stress-related
39 phenomena. In a report (Maisonneuve E, Castro-Camargo M, Gerdes K. 2013. Cell
40 154:1140-1150) an interesting link between ppGpp mediated stringent response, TAS and
41 persistence was proposed. It is proposed that accumulation of ppGpp enhances the
42 accumulation of inorganic polyphosphate which modulates Lon protease to degrade
43 antitoxins. The decrease in the concentration of antitoxins supposedly activated the toxin to
44 increase in the number of persisters during antibiotic treatment. In this study, we show that
45 inorganic polyphosphate is not required for Lon-dependent degradation of YefM, the
46 antitoxin of YefM/YoeB TAS. The $\Delta 10$ strain, an *Escherichia coli* MG1655 derivative in
47 which the ten TAS are deleted, is more sensitive to Ciprofloxacin and Ampicillin compared
48 to wild-type MG1655. Furthermore, we show that the $\Delta 10$ strain has relatively lower fitness
49 compared to the wild type and hence, we argue that the implications based on this strain are
50 void. We conclude that there is no direct and specific link between stringent response and the
51 regulation of TAS. The link between TAS and persistence is inconclusive due to altered
52 fitness of $\Delta 10$ strain and hence requires thorough inspection and debate.

53

54

55 **Importance**

56 A model connecting stringent response, endoribonuclease encoding Type II Toxin-Antitoxin
57 systems (TAS) and persistence is widely propagated. It states that “accumulation of ppGpp
58 results in accumulation of inorganic polyphosphate which modulates Lon protease to degrade
59 antitoxin rendering toxins free to induce persistence”. This work presents a contradiction to
60 and challenges the model. Experimental evidence, literature survey as well as rationale are
61 provided to show that inorganic polyphosphate is not required for the degradation of YefM,
62 the antitoxin in YefM/YoeB TAS. The $\Delta 10$ strain is relatively more sensitive to
63 Ciprofloxacin and Ampicillin as well as has lowered fitness. This is likely because of the
64 polar effects on the adjacent genes caused by the genetic manipulation of multiple TAS loci.

65

66

67

68

69 **Introduction**

70 Toxin–antitoxin systems (TAS) are operons consisting of two or three adjacent genes which
71 code for a toxin, which has the potential to inhibit one or more cellular processes and an
72 antitoxin. The antitoxin forms a complex with the toxin and suppresses the lethality of the
73 toxin. Prokaryotic DNA sequence database mining showed that TAS are abundant in
74 bacterial and archaeal chromosomes often in surprisingly high numbers (Anantharaman and
75 Aravind, 2003; Pandey and Gerdes, 2005; Shao et al., 2011). Based on the antitoxin gene
76 products, either RNA or protein, TAS are divided into 5 types (Goeders and Van Melderen,
77 2014) of which Type II are the most predominant and well characterized. Type II TAS
78 encode two proteins referred to as toxin and antitoxin. They are the predominant type
79 encoded by bacterial genomes and plasmids. The toxin has the potential to inactivate vital
80 cellular targets while the antitoxin has the potential to sequester toxins off the cellular
81 targets by forming a toxin-antitoxin complex (Gerdes et al., 2005; Yamaguchi and Inouye,
82 2011). Toxins and antitoxins also have the autoregulatory function wherein the TA complex
83 binds to the operator present upstream of the TA operon and results in repression. The
84 antitoxin is highly unstable and its relative concentration plays a critical role in
85 transcriptional autoregulation as well as regulation of toxin activity (Gerdes et al.,
86 2005; Yamaguchi and Inouye, 2011). The decrease in antitoxin concentration is a prerequisite
87 for transcriptional activation of TAS. The significance of TAS multiplicity on prokaryotic
88 genomes and their physiological role is highly debated (Magnuson, 2007). Many plasmids
89 also encode TAS whose gene products have the ability to inhibit the growth of the cells
90 cured of TA-encoding plasmids and thereby increase the population of plasmid-containing
91 cells (Gerdes et al., 1986).

92

93 Chromosomal TAS were first discovered in studies dealing with stringent response and
94 later in persistence. Stringent response, a response elicited in cells under amino acid
95 starvation, is characterized by accumulation of ppGpp alarmone catalyzed by RelA upon
96 stimulation by uncharged tRNA at the ribosomal A site (Lund and Kjeldgaard,
97 1972; Haseltine and Block, 1973; Cashel et al., 1996; Wendrich et al., 2002). Accumulation
98 of ppGpp modulates RNA polymerase resulting in reduction of rRNA synthesis and thus
99 prevents frivolous anabolism (Barker et al., 2001; Artsimovitch et al., 2004). Several
100 mutants deficient/altered in stringent response were shown to be mutants of *relBE*
101 (Mosteller and Kwan, 1976; Diderichsen et al., 1977), a TAS encoding an antitoxin (RelB)
102 and a ribosome dependent endoribonuclease toxin (RelE) (Gotfredsen and Gerdes,

103 1998;Christensen et al., 2001). Persistence, a phenomenon of non-inheritable antibiotic
104 tolerance, is the second instance in which genes belonging to the TA family were
105 recognized. Some mutants, high persister mutants (*hip*), of *Escherichia coli* formed more
106 number of persisters than the wild type. These *hip* mutations mapped to the *hipA* locus
107 (Moyed and Bertrand, 1983) which is now recognized as a genuine TAS encoding HipA
108 toxin and HipB antitoxin (Korch et al., 2003;Germain et al., 2013;Kaspy et al., 2013). A
109 recent study shows an attractive link between TAS, stringent response and persistence;
110 ppGpp, through inorganic polyphosphate (polyP), activates TAS resulting in induction of
111 persistence (Maisonneuve et al., 2011;Maisonneuve et al., 2013). The crucial link between
112 ppGpp and TA-mediated persistence is the essentiality of polyP for the degradation of
113 antitoxins. During stringent response, polyP accumulates due to ppGpp-mediated inhibition
114 of exopolyphosphatase (PpX) (Kuroda et al., 1997). The presence or absence of polyP
115 determines the substrate specificity of Lon protease (Kuroda et al., 2001). Maisonneuve et
116 al., 2013 have shown that polyP is essential for Lon-dependent degradation of YefM and
117 RelB antitoxins resulting in increased persistence (Fig 1). YefM is the antitoxin encoded by
118 *yefM/yoeB* TAS, a well-characterized Type II TAS. YoeB, the toxin, is a ribosome-
119 dependent endoribonuclease (Christensen-Dalsgaard and Gerdes, 2008;Feng et al., 2013)
120 that cleaves mRNA. YefM forms a complex with YoeB resulting in inhibition of
121 endoribonuclease activity of YoeB (Cherny et al., 2005;Kamada and Hanaoka, 2005) and
122 also in mediating transcriptional autorepression (Kedzierska et al., 2007).

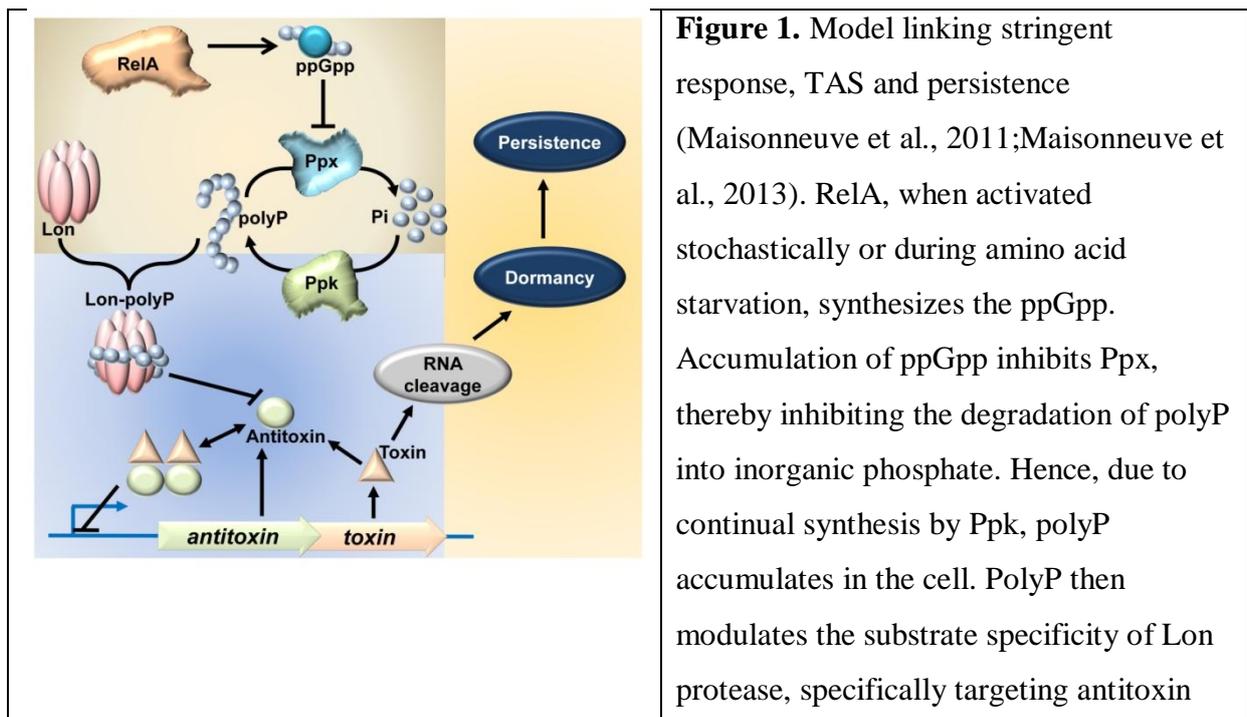


Figure 1. Model linking stringent response, TAS and persistence (Maisonneuve et al., 2011;Maisonneuve et al., 2013). RelA, when activated stochastically or during amino acid starvation, synthesizes the ppGpp. Accumulation of ppGpp inhibits Ppx, thereby inhibiting the degradation of polyP into inorganic phosphate. Hence, due to continual synthesis by Ppk, polyP accumulates in the cell. PolyP then modulates the substrate specificity of Lon protease, specifically targeting antitoxin

	proteins for degradation. This is hypothesized to render the toxin free to act on its target RNA and confer persistence.
--	--

123

124 Interestingly, in earlier studies using semi-quantitative primer extension, Prof. Kenn Gerdes'
125 group has concluded that transcriptional regulation of *relBE* and *mazEF* systems is
126 independent of ppGpp (Christensen et al., 2001; Christensen et al., 2003). Hence, in this
127 study we analyzed the essentiality of polyP in degradation of YefM antitoxin by studying
128 the promoter activity of *yefM/yoeB* loci and endoribonuclease activity of chromosomally
129 encoded YoeB. The endoribonuclease encoding TAS are horizontally transferring genes and
130 most them in *E. coli* are integrated between genes with significance in bacterial stress
131 physiology (Fiedoruk et al., 2015; Ramisetty and Santhosh, 2016). We speculated that
132 deletion of the ten TAS could alter the physiology of the mutant strain. This study is aimed to
133 evaluate the link between three vital aspects of bacterial stress physiology; endoribonuclease
134 encoding TAS, stringent response, and persistence.

135

136 **Materials and methods**

137 **Strains, plasmids and growth conditions.**

138 *Escherichia coli* MG1655, $\Delta 5$ (MG1655 derivative with 5 TAS deletions $\Delta relBE \Delta mazF$
139 $\Delta dinJ yafQ \Delta yefM/yoeB \Delta chpB$) (Christensen et al., 2004), MG1655 $\Delta yefM/yoeB$ (SC36)
140 (Christensen et al., 2004), MG1655 $\Delta relA \Delta spoT$ (Christensen et al., 2001), MG1655
141 $\Delta ppk ppx$ (Kuroda et al., 1997) and $\Delta 10$ strains (MG1655 derivative with 10 TAS deletions
142 $\Delta relBE, \Delta chpB, \Delta mazF, \Delta dinJ/yafQ, \Delta yefM/yoeB, \Delta yafNO, \Delta hicAB, \Delta higBA, \Delta prlf/yhaV,$
143 and $\Delta mqsRA$). pBAD33 (Guzman et al., 1995) and pBAD-*lon* (Christensen et al., 2004) (*lon*
144 gene cloned downstream of arabinose inducible promoter) were used for overexpression
145 experiments. The cloning of *ppk* gene downstream of arabinose inducible promoter yielded
146 pBAD-*ppk* (Mikkel Girke Jorgensen, unpublished). The cultures in all the experiments were
147 grown in Luria Bertani broth, at 37 °C, with 180 rpm shaking in a shaker unless specified
148 otherwise.

149 **Databases**

150 EcoGene 3.0 (Zhou and Rudd, 2013) and RegulonDB (Salgado et al., 2013) were followed
151 for nucleotide sequences, protein sequences, and regulatory information wherever required.

152 **Primer extension**

153 Samples of 25 ml experimental cultures were collected at 0, 10, 30 and 60 minutes and cells
154 were harvested by centrifugation at 4 °C. Total RNA was isolated using hot phenol method
155 and quality was analyzed by agarose gel electrophoresis. ³²P labelled primers, YefMPE-2
156 (5'-GGCTTTCATCATTGTTGCCG-3') and lpp21 (5'-
157 CTGAACGTCAGAAGACAGCTGATCG-3'), were used in primer extension experiments
158 involving *yefM/yoeB* promoter activity and YoeB-dependent mRNA cleavage site mapping
159 respectively. Reverse transcription was carried out on 10 µg of total RNA, purified from
160 samples at designated time points, using AMV-reverse transcriptase. Sequencing reactions
161 were carried out similarly with Sanger's dideoxynucleotide method.

162 **Growth curve**

163 12 hour old overnight cultures were grown to mid log phase in tubes in LB medium at 37 °C.
164 The culture was rediluted 100 folds in LB medium and 2 µL of the diluted cultures were
165 inoculated into 200 µL of LB in microtitre plate wells in triplicates. The microtitre plates
166 were incubated at 37 °C with 170 rpm shaking. Optical density at 595 nm was measured in a
167 96 well microtitre plate reader (BioradTM), every one hour, for 8 hours.

168 **Maximal CFU/ml in optimal conditions in 12 hours.**

169 Overnight cultures were inoculated into tubes containing 3 ml LB broth and grown at 37 °C
170 with 170 rpm shaking for 12 hours. 10 µL of the culture was diluted appropriately and plated
171 on LB plates and incubated overnight. Colonies were counted and colony forming units per
172 ml were determined according to the dilution factor. The CFU/ml of MG1655 was taken as
173 100% for LB medium and culture conditions.

174 **Biofilm assay**

175 Overnight cultures were diluted 100 fold in fresh LB tubes. 2 µL of inoculum was added into
176 200 µL of LB broth in 96 well microtitre plates. The plates were incubated at 37 °C for 16,
177 24, 48 and 72 hours at 37 °C. After the specified time points the plates were washed with
178 PBS to remove floating cells. 125 µL of 1% crystal violet was added to each well and left for
179 20 minutes. The plates were washed with water twice and the dye was re-dissolved by adding
180 90% ethanol. The re-dissolved crystal violet was taken into new wells, to avoid biofilm
181 interference, and readings were taken at 595 nm and readings were represented as the amount
182 of biofilm formation. Experiments were carried out independently thrice in quadruplicates.
183 Error bars indicate standard error.

184 **Antibiotic sensitivity test**

185 Conventional disc diffusion method was used to measure relative sensitivity of the strains.
186 100µL of diluted (100 fold) overnight cultures was spread on LB agar (height – 5 mm)

187 contained in plates with diameter 9.5 cm. Premade antibiotic discs with defined
188 concentrations (purchased from HiMediaTM) were placed on the agar plates after 20 minutes.
189 ERY = Erythromycin (15 µg), GEN = Gentamycin (10 µg), TET = Tetracycline (30 µg), NA
190 = Nalidixic acid (30 µg), AMP = Ampicillin (10 µg), CLM = Chloramphenicol (30 µg), VA =
191 Vancomycin (10 µg), CIP = Ciprofloxacin (5 µg). The plates were incubated overnight at 37
192 °C. Diameters of the zones of inhibition were measured and the graph was plotted. The bars
193 represent averages of three independent experiments done in triplicates. Error bars indicate
194 standard error.

195 **Persistence assay**

196 Exponentially growing cells of MG1655 and $\Delta 10$ were exposed to various antibiotics at the
197 specified concentrations (Ciprofloxacin 1 µg/ml, Ampicillin 100 µg/ml, Erythromycin 100
198 µg/ml, Kanamycin 50 µg/ml and Chloramphenicol 100 µg/ml). After 4 hours of antibiotic
199 treatment, cells were harvested, serially diluted and plated. After 24 hours of incubation
200 number of viable cells was counted. Percentage of survival after antibiotic treatment for $\Delta 10$
201 strain is compared with the wild type MG1655 strain. The bars represent averages of three
202 independent experiments done in triplicates. Error bars indicate standard error.

203 AMP=Ampicillin (100 µg/ml), CIP=Ciprofloxacin (1 µg/ml), CLM=Chloramphenicol (100
204 µg/ml), KAN=Kanamycin (50 µg/ml), ERY=Erythromycin (100 µg/ml).

205

206 **Results and discussion**

207

208 **Inorganic polyphosphate is not required for transcriptional upregulation of *yefM/yoeB*** 209 **loci.**

210 The transcriptional upregulation of *yefM/yoeB* loci, or any typical TAS, is inversely
211 proportional to the relative concentration of YefM. This is because TA proteins autoregulate
212 their promoter/operator; transcription of TA operon is inversely proportional to the
213 concentration of antitoxin. Hence, any transcriptional activation from *yefM/yoeB* operon
214 indicates a decrease in antitoxin concentration. Therefore, quantification of the TA mRNA is
215 a good indicator of antitoxin concentration in the cell. To test the essentiality of polyP in
216 Lon-dependent degradation of YefM in vivo, we employed semi-quantitative primer
217 extension (Christensen et al., 2001; Christensen et al., 2003) of YefM mRNA. Although
218 indirect, this assay has the advantage of a holistic transcriptional regulatory scenario of TAS
219 without employing any genetic manipulations within the TA circuitry, thus avoiding artifacts.
220 To test the role of ppGpp and polyP in the regulation of *yefM/yoeB* system, we performed

221 amino acid starvation experiments using serine hydroxymate (SHX) and analyzed the
222 transcription of *yefM/yoeB* loci using semi-quantitative primer extension using a YefM
223 mRNA-specific primer. Exponentially growing *E. coli* strains MG1655 (Wild type), *Alon*,
224 *ΔppkΔppx*, and *ΔrelAΔspoT*, were treated with 1 mg/ml of SHX to induce serine starvation.
225 *ΔppkΔppx* and *ΔrelAΔspoT* strains are deficient in accumulating polyP and ppGpp,
226 respectively (Xiao et al., 1991;Crooke et al., 1994). In the wild type strain, we found a
227 dramatic increase (16 fold) in the transcription of *yefM/yoeB* loci while in *Alon* strain there
228 was no change (Fig 2A). Interestingly and importantly, we found a higher level of
229 transcription of *yefM/yoeB* loci in *ΔrelAΔspoT* as well as *ΔppkΔppx* strains indirectly
230 indicates that ppGpp or polyP is not required for YefM degradation during amino acid
231 starvation. To further investigate the essentiality of polyP we also carried out overexpression
232 of Lon protease in MG1655 and *ΔppkΔppx* strains to know the role of polyP in the regulation
233 of *yefM/yoeB* system and found that transcription of *yefM/yoeB* increased similarly in both
234 MG1655 and *ΔppkΔppx* strains (Fig 2B). These observations corroborate the earlier findings
235 that the transcriptional regulation of *relBE* (Christensen et al., 2001) and *mazEF* systems
236 (Christensen et al., 2003) during SHX-induced starvation is independent of ppGpp but
237 dependent on Lon protease. In fact, RelA dependent accumulation of ppGpp was shown to be
238 inhibited by chloramphenicol treatment (Svitil et al., 1993;Boutte and Crosson, 2011) and yet
239 the *relBE* and *mazEF* TAS were shown to be upregulated upon addition of chloramphenicol
240 (Christensen et al., 2001;Christensen et al., 2003).

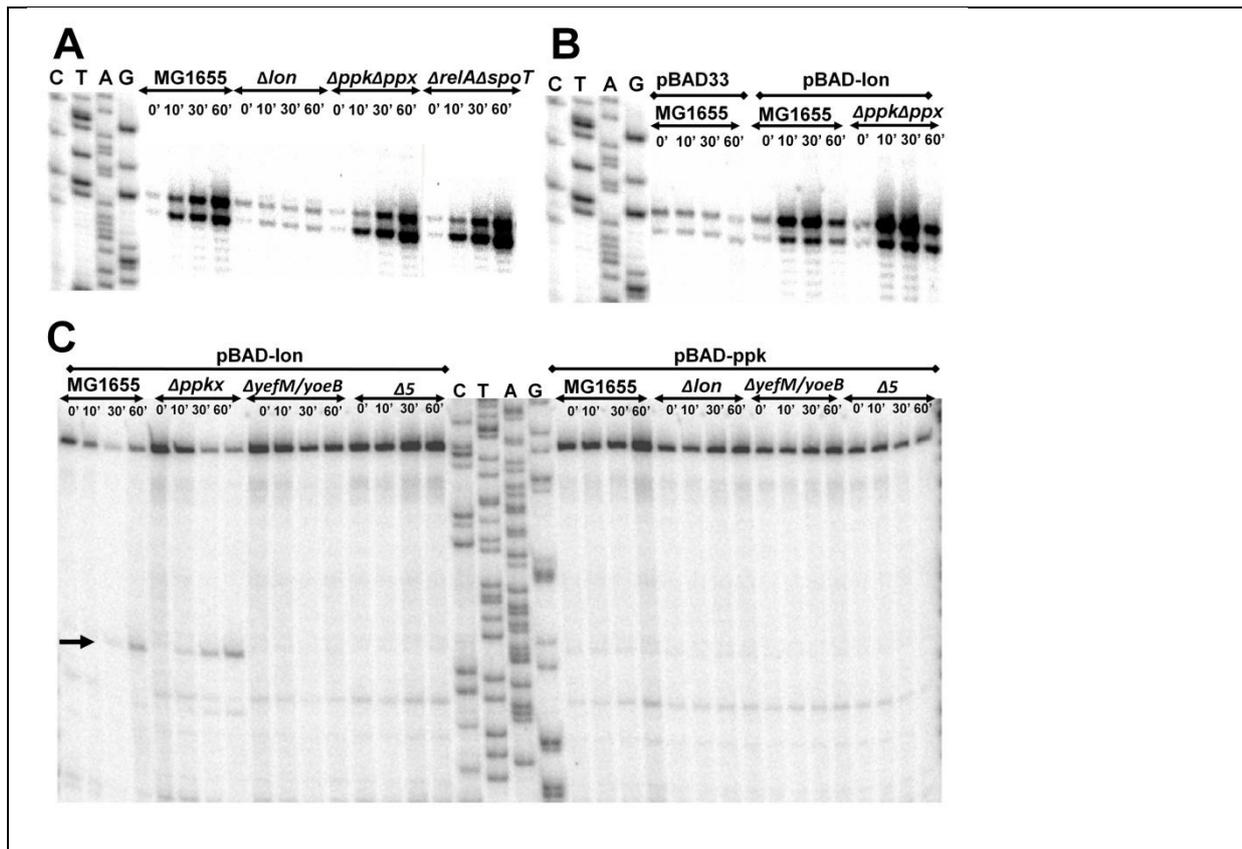


Figure 2. A. Exponentially growing (0.45 of OD₄₅₀) cultures of MG1655, *Δlon*, *Δppkppx* and *ΔrelAΔspoT* were treated with 1 mg/ml of serine hydroxymate. Total RNA was isolated at 0, 10, 30 and 60 minutes and semi-quantitative primer extension was performed using YefM mRNA-specific primer (YefMPE-2). B. MG1655 and *Δppkppx* strains were transformed with pBAD-*lon* plasmids with a control of pBAD33 harbouring MG1655. Overnight cultures were diluted and grown to 0.45 OD₄₅₀ in LB medium supplemented with glycerol as carbon source at 37 °C. Lon overexpression was induced by addition of 0.2 % arabinose. Samples were collected at indicated time intervals and semi-quantitative primer extension performed as described in Materials and methods. C. YoeB-dependent cleavage upon overexpression of *lon* is independent of polyP. MG1655, *Δppkppx* (*Δppkx*), *ΔyefM/yoeB* and *Δ5* strains were transformed with pBAD-*lon* and pBAD-*ppk* was transformed into MG1655, *Δlon*, *ΔyefM/yoeB* and *Δ5*. The transformants were grown in LB media supplemented with 2 % glycerol to mid-exponential phase (0.45 of OD₄₅₀). 0.2 % arabinose was added to induce expression of *lon* or *ppk*. Samples were collected at 0, 10, 30 and 60 minutes and primer extension was carried out using Lpp mRNA-specific primer (lpp21) for cleavage site mapping. YoeB-dependent cleavage, indicated by an arrow, is in accordance with results from Christensen, *et al*, 2004.

241

242 Transcriptional upregulation of *yefM/yoeB* operon does not necessarily mean that YoeB is
 243 free to cleave its target mRNA. To date, chromosomal YoeB-dependent mRNA cleavage has
 244 been observed only upon ectopic overproduction of Lon protease (Christensen *et al.*, 2004).
 245 The ectopic overexpression of Lon degrades YefM, leaving YoeB free to manifest its
 246 endoribonuclease activity. Since it was reported that Lon-mediated degradation of YefM is
 247 dependent on polyP (Maisonneuve *et al.*, 2013), it is interesting to see if polyP is essential to

248 render YoeB free by promoting the degradation of YefM. First, we overexpressed Lon
249 protease in WT, *AppkAppx*, *ΔyefM/yoeB* (MG1655 derivative with *yefM/yoeB* deletion) and *Δ5*
250 (MG1655 derivative in which 5 TAS are deleted) strains and mapped for cleavage sites in Lpp
251 mRNA by primer extension as reported in earlier studies (Christensen et al., 2004). We found
252 that Lpp mRNA is cleaved at the second codon of AAA site in WT and *AppkAppx* strains but
253 not in *ΔyefM/yoeB* and *Δ5* strains (Fig 2C). We overexpressed *ppk* in exponentially growing
254 cultures of wild type, *Δlon*, *ΔyefM/yoeB* and *Δ5* strains. We could not detect any YoeB-
255 dependent cleavage of Lpp mRNA upon ectopic overexpression of *ppk* in any of the strains.
256 This implies that YoeB-specific cleavage is independent of polyP—meaning that activation
257 of YoeB as a result of YefM degradation is independent of polyP. Hence, our results establish
258 that polyP is not required for the transcriptional activation of *yefM/yoeB* loci and
259 endoribonuclease activity of YoeB which imply that polyP is not required for Lon-mediated
260 degradation of YefM. Within the scope of the experiments, it can be argued that translation
261 and Lon protease are the only regulators of YefM concentration. There is no experimental
262 evidence that polyP is required to degrade all the ten antitoxins in any of the studies. Mere
263 decrease in persister formation in *Δ10* and *AppkAppx* strains, but not in wild type, upon *relA*
264 overexpression made Maisonneuve et al., 2013 to assume that degradation of the other
265 antitoxins in *E. coli* (ChpS, DinJ, MazE, MqsA, HicB, PrlF, YafN, HigA) was also dependent
266 on polyP (Maisonneuve et al., 2013). It is likely a fallacious assumption because “polyP-
267 dependent TAS regulation model” (Maisonneuve et al., 2013) fails to explain how all the ten
268 significantly divergent antitoxins of *E. coli* MG1655 could be the substrates of ‘polyP-
269 modulated Lon’ protease. It is to be noted that YefM is degraded even in MC4100 strain
270 (*relA1* mutant strain) (Cherny et al., 2005) which is deficient in accumulating ppGpp during
271 amino acid starvation (Metzger et al., 1989). It may also be noted that antitoxins like YafN,
272 HigA and MqsA (YgiT) were shown to be degraded by both Lon and Clp proteases
273 (Christensen-Dalsgaard et al., 2010). Furthermore, based on studies on “delayed relaxed
274 response” (Christensen and Gerdes, 2004), the half-life of RelB in MC1000 strain is
275 approximately 15 minutes and RelB101 (A39T mutant of RelB) is less than 5 minutes. It is
276 interesting to notice that RelB101 is degraded even in a *Δlon* strain, indicating that some
277 other proteases may also cleave RelB101 (Christensen and Gerdes, 2004). These literature
278 evidences indicate that changes in primary structures of antitoxins could drastically alter their
279 protease susceptibility and specificities. Recently, it was reported that a few TAS could
280 induce persistence even in the absence of ppGpp (Chowdhury et al., 2016). PolyP was shown
281 to inhibit Lon protease *in vitro* (Osbourne et al., 2014) and is reported to act as a chaperone

282 for unfolded proteins (Kampinga, 2014) which may have significant implications in bacterial
283 stress physiology, however, is not essential for the degradation of YefM. To our rationale,
284 since Endoribonuclease encoding TAS propagate through horizontal gene transfer
285 mechanisms (Ramisetty and Santhosh, 2016), minimal dependence on host genetic elements
286 maybe preferable for TAS regulation. Within the scope of our experiments conducted in this
287 study and based on literature, it is appropriate to state that polyP is not essential for Lon-
288 mediated proteolysis of YefM. Although we do not have a ready explanation for this
289 fundamental contradiction, we do not rule out His-tag interference in the proteolysis assays
290 performed by Maisonneuve et al., 2013.

291

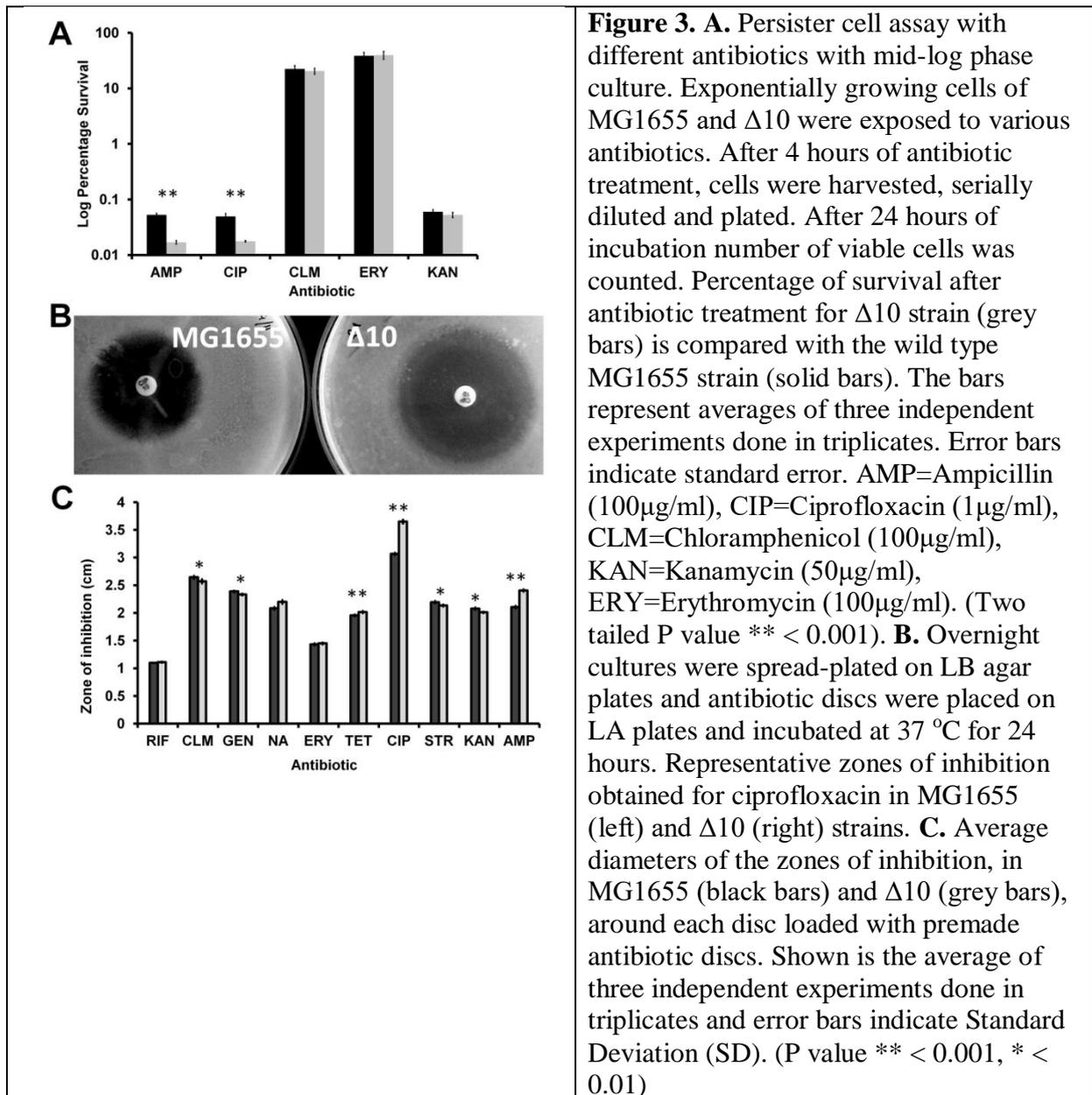
292 **Persistence of MG1655 and Δ 10 strains to Ampicillin, Ciprofloxacin, Erythromycin,**
293 **Chloramphenicol and Kanamycin.**

294 The induction of persistence (Korch and Hill, 2006; Butt et al., 2014) by over expression of
295 toxins was challenged and shown that even proteins unrelated to toxins, during controlled
296 over expression, induced persistence (Vazquez-Laslop et al., 2006). Maisonneuve et al., 2011
297 reported that Δ 10 strain (*E. coli* MG1655 derivative in which ten endoribonuclease TAS
298 were deleted) formed lesser persisters compared to wild type strain when challenged with
299 Ciprofloxacin and Ampicillin. Since there were no reports (during the time these experiments
300 were carried out) on the TAS mediated persistence during treatment with other antibiotics, we
301 performed similar experiments to determine persistence to treatment of logarithmically
302 growing cultures of MG1655 and Δ 10 strains to Ciprofloxacin (1 μ g/ml), Ampicillin (100
303 μ g/ml), Erythromycin (100 μ g/ml), Kanamycin (50 μ g/ml) and Chloramphenicol (100
304 μ g/ml). We found that with Ampicillin and Ciprofloxacin, Δ 10 strain formed significantly
305 lesser persisters compared to the wild type MG1655 (\approx 65 fold) (Fig 3A). However, we could
306 not find significant difference in the number of persisters formed by Δ 10 and MG1655 with
307 Chloramphenicol, Erythromycin and Kanamycin. If toxin induced dormancy or metabolic
308 regression results in bacterial persistence, it is expected that similar persistence is observed
309 with inhibitors of translation. This is because all the toxins in the study are translational
310 inhibitors by virtue of their endoribonuclease activity. At least with Kanamycin, since it is a
311 bactericidal antibiotic, we expected persistence conferred by TAS. However, wild type and
312 Δ 10 strains formed equal number of persisters upon treatment with Kanamycin. Similar
313 observation (Shan et al., 2015) with Genetamycin, another aminoglycoside antibiotic,
314 wherein significant persistence was not observed (Wood, 2016), corroborates our findings.

315

316 **Relative hypersensitivity of MG1655 and Δ 10 strains to Ciprofloxacin and Ampicillin.**

317 In light of the above observations, we were curious about the degree of sensitivity to various
318 antibiotics. We determined the sensitivity of the MG1655 and Δ 10 strains to various
319 antibiotics by disc diffusion method. We observed that zone of inhibition of MG1655 with
320 Ciprofloxacin (10 μ g) was 3.6 cm (averages) while that of Δ 10 strain was 3 cm (Fig 3B).
321 With Ampicillin (10 μ g), the zones of inhibition for MG1655 and Δ 10 strain were 2.45 cm
322 and 2.1 cm, respectively. With Nalidixic acid, the zones of inhibition for MG1655 and Δ 10
323 strain were 1.98 cm and 1.78 cm, respectively. We did not find any significant difference
324 with the other antibiotics at the concentrations used. This indicates that Δ 10 strain is more
325 sensitive to Ciprofloxacin and Ampicillin relative to MG1655 (Fig. 3B&C). Our observation
326 could also mean that TAS confers a certain degree of ‘resistance’ to antibiotics like
327 Ciprofloxacin and Ampicillin. However, so far there are no reports that TAS confer antibiotic
328 resistance. In light of current understanding of the role of TAS in persistence (Maisonneuve
329 et al., 2011;Maisonneuve et al., 2013), this is an important observation. We noticed a
330 difference in sensitivities of these strains to Ciprofloxacin and Ampicillin but not to
331 transcription and translation inhibitors. In fact, it was reported that the Δ 10 strain has lower
332 minimal inhibitory concentration (MIC) for Ciprofloxacin (5.0 ± 0.35 ng/ml) compared to
333 wild type (Ciprofloxacin 5.3 ± 0.45 ng/ml). Similarly, MIC of Δ 10 strains was reported as
334 3.2 ± 0.27 μ g/ml relative to MG1655 3.4 ± 0.42 μ g/ml (Maisonneuve et al., 2011). Although
335 considered as insignificant by the authors, in our view, it is irrational to infer persistence of
336 two strains with marked difference in antibiotic sensitivities. This is vital because it deals
337 with persistence to antibiotics and most of the experiments implicating TAS in persistence
338 are carried out using either Ciprofloxacin or Ampicillin. It would also mean that Δ 10 strain is
339 inherently sensitive to Ciprofloxacin and Ampicillin. Although it is difficult to explain this
340 observed sensitivity at this point of time, we do not rule out the possibility of artefacts due to
341 genetic manipulations during the construction of Δ 10 strain.

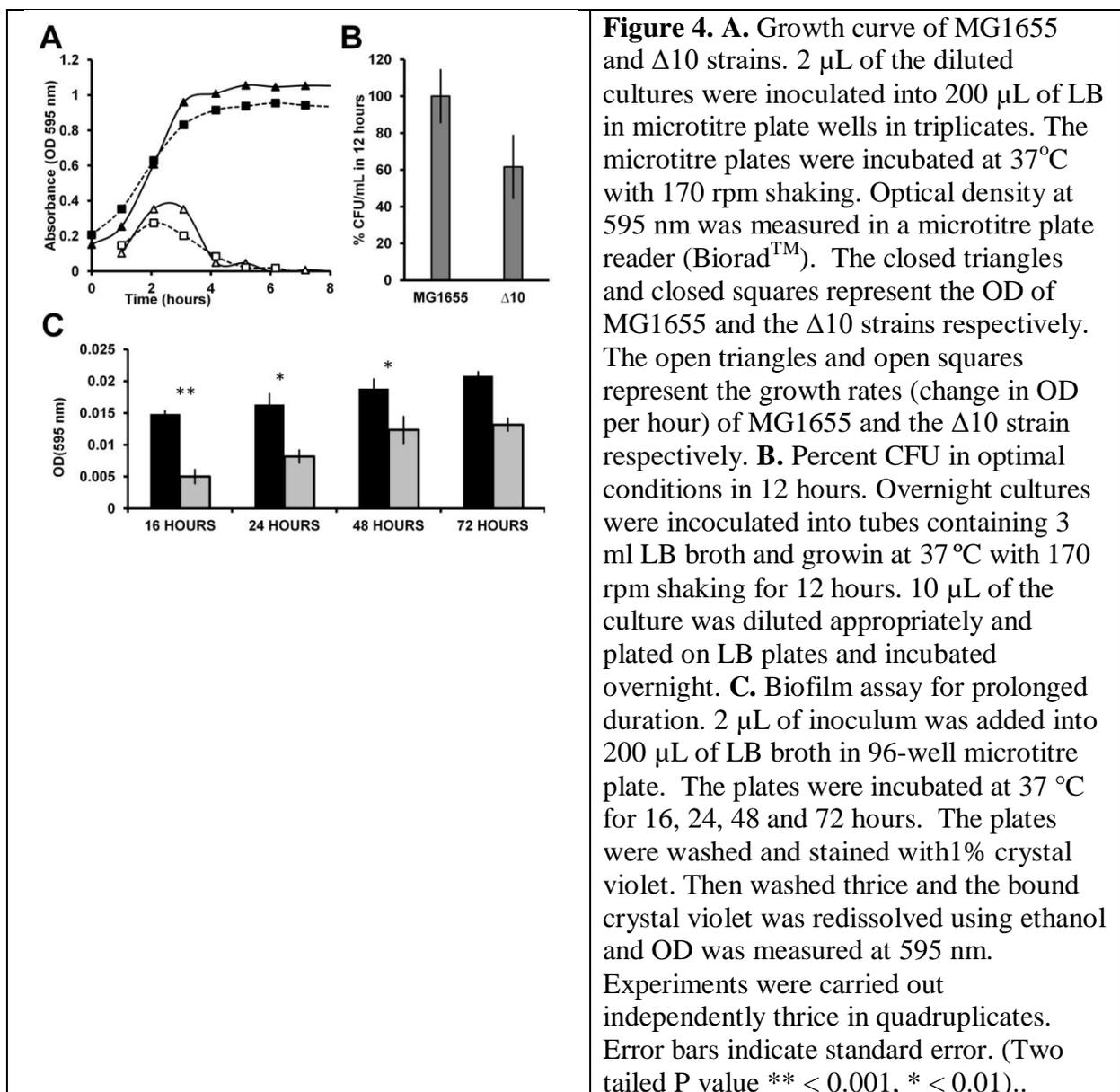


342

343 Deletions of 10 TAS, as in $\Delta 10$ strain, causes loss of fitness.

344 Recently we have shown that TAS are horizontally transferring genes and are integrated
 345 within the intergenic regions between important ‘core’ genomic regions (Ramisetty and
 346 Santhosh, 2016). Maisonneuve et al., 2011 reported that $\Delta 10$ strain formed lesser persisters
 347 compared to wild type strain when challenged with Ciprofloxacin and Ampicillin. Hence, we
 348 speculated that deletion of ten TAS could compromise the expression of flanking genes due
 349 to polar effects resulting in decreased fitness of the $\Delta 10$ strain. We analyzed the differences
 350 in between *E. coli* MG1655 and $\Delta 10$ strains (Maisonneuve et al., 2011) fitness by growth
 351 curve, maximal colony forming units (CFU) per ml in stationary phase and biofilm
 352 formation. We observed that the maximum growth rate (change in OD/hour) of MG1655 was

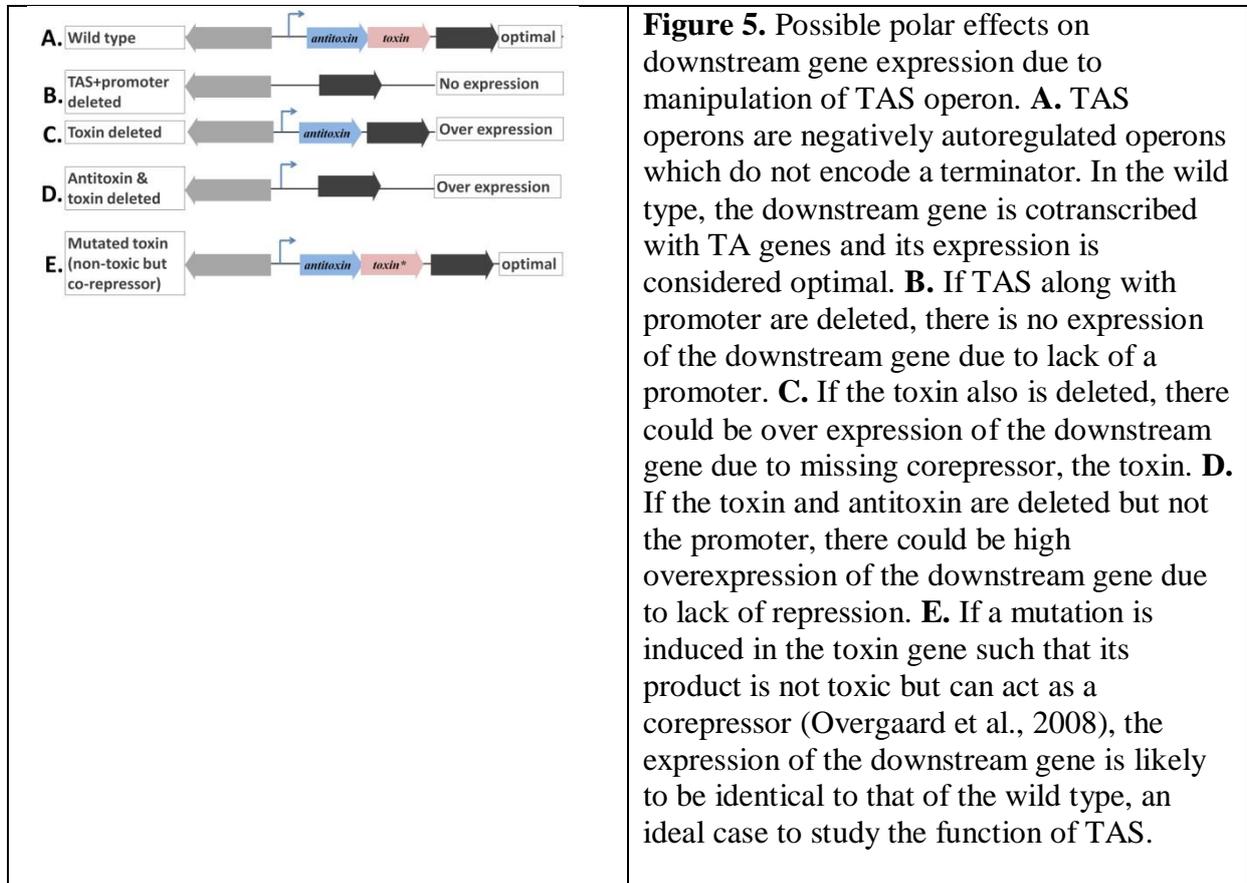
353 0.35 while that of $\Delta 10$ strain was 0.27 (Fig. 4A). During the 8 hour growth curve study in 96
354 well microtitre plates, the maximum absorbance at 595 nm was 1.05 for MG1655 while it
355 was 0.95 for $\Delta 10$ strain (Fig. 4A). We also noticed that the optical density of the overnight
356 cultures of $\Delta 10$ strain grown in tubes was consistently lower than that of MG1655. These
357 observations indicated that the $\Delta 10$ strain may have metabolic deficiencies. To confirm this
358 further we determined the CFU/ml of both the strains after 12 hours of growth in tube
359 containing LB medium at 37 °C with 170 rpm. We observed that MG1655 yielded 7.99×10^{12}
360 CFU/ml while the $\Delta 10$ strain yielded 4.92×10^{12} CFU/ml which is $\approx 40\%$ lesser than the
361 CFU/ml of wild type (Fig 4B). In a given set of conditions, the difference in the CFU/ml of
362 two strains of a species is an indication of difference in their respective fitness.



363

364 We then performed biofilm assay for a prolonged period to determine any differences
365 between these strains in their ability to form biofilms. We found that MG1655 formed
366 consistently more biofilm, represented as absorbance of redissolved crystal violet, compared
367 to the $\Delta 10$ strain at all the time intervals analyzed (16, 24, 48 72 hours). At 16 hours, $\Delta 10$
368 strain formed 66% lesser biofilm compared to MG1655 strain. Upon prolonged incubation,
369 after 72 hours, $\Delta 10$ strain formed 35% lesser biofilm relative to wild type (Fig 4C). These
370 observations reinforce the notion that the $\Delta 10$ strain is not as healthy as the wild type. The
371 wild type would have the highest fitness when grown in conditions closest to their natural
372 habitats. Mutants with one or more genetic deficiencies would lose their fitness to varying
373 degrees. In this case, $\Delta 10$ strain has significantly lower fitness compared to the wild type
374 likely due to the effects of deletions. The loss of fitness could be attributed to two aspects; (i)
375 to the loss of TAS function and (ii) the polar effects on the adjacent genes due to deletion of
376 TAS. One could argue that TAS are responsible for higher growth rate, higher CFU/ml in 12
377 hours as well as higher biofilm formation. However, a qualified counter argument is that the
378 polar effects due to deletion of the ten TAS, and not necessarily the loss of TAS function,
379 might have caused the metabolic deficiency. This is due to inadvertent interference with
380 coding and/or regulatory sequences of the bordering regions. In our view, it is most likely
381 that the expression of the bordering genes is compromised resulting in decreased fitness of
382 $\Delta 10$ strain. It should be noted that TAS are horizontally transferring genes (Ramisetty and
383 Santhosh, 2016) and are integrated within the bacterial core genome adjacent, and/or in close
384 proximity, to important genes. As summarized in the Table 1, most of the genes that are
385 immediately downstream of TA genes have important functions in bacterial physiology as
386 enzymes (*yafP*, *fadH*) or transcriptional factors (*ydcR*, *agaR*) or in nucleotide metabolism
387 (*mazG*, *yeeZ*, *ppa*) or in membrane metabolism (*yafK*, *hokD*, *ygiS*) (Table 1). It must be noted
388 that the minimal composition of a horizontally transferring TAS consists of a
389 promoter/operator and TA ORFs but is not composed of a terminator (Ramisetty and
390 Santhosh, 2016). Hence, the downstream gene is cotranscribed with the TA genes because
391 there is no promoter or terminator in the intergenic region between TA operon and the
392 downstream gene, e.g. *relBEF* (Gotfredsen and Gerdes, 1998) and *mazEFG* (Gross et al.,
393 2006) (Table 1). The spacers between the adjacent genes range from 9 to 218 bps, which is
394 inclusive of the operator/promoter regions if any. Hence, the TA genes are highly linked to
395 the downstream genes physically as well as transcriptionally. It is highly plausible that the
396 artificial deletion of TA genes could cause polar effect on the expression of one or more of
397 these bordering genes which is likely to result in loss of fitness (summarized in Fig. 5).

398 Therefore, confirmation that there are no polar effects on the expression and/or the reading
399 frames of the adjacent genes due to deletion of TA genes is essential. Attribution of the
400 observed phenotypes solely to TAS may result in faulty interpretations and mislead the
401 research community.



402

403 In the past reverse genetic studies on TAS, several deletion strains have compromised the
404 general bacterial physiology (Gross et al., 2006;Tsilibaris et al., 2007) resulting in misleading
405 interpretations. Construction of MC4100 $\Delta mazEF$ (Aizenman et al., 1996;Tsilibaris et al.,
406 2007;Ramisetty et al., 2016) and $\Delta 5$ strain (Tsilibaris et al., 2007) strains have resulted in
407 inadvertent interference in the coding regions of bordering genes. In a Tn-seq based genetic
408 screen to find the molecular determinants of persisters during treatment with Gentamycin, no
409 TAS has been found. Furthermore, in spite of having several Tn inserts in *lon* gene, *lon*
410 mutations did not affect the persister formation frequency (Shan et al., 2015).

411

412 Toxins can induce metabolic stasis and hence we do think that TAS have the potential to
413 induce persistence. However, more systemic studies should be carried out to definitively
414 prove the function of TAS in persistence. As of now, with the current knowledge, we contend
415 that chromosomal endoribonuclease encoding TAS, under their canonical autoregulatory

416 mechanisms, may not be directly involved in persistence. We disregard ectopic
417 overexpression of toxins' role in persistence because it is not necessarily specific as
418 controlled over expression of non-toxin proteins can also induce such persistence (Vazquez-
419 Laslop et al., 2006). Similarly, we disregard the implications derived by using deletion strains
420 such as $\Delta 10$ strain with lower fitness.

421

422 **Conclusions**

423 The observations presented in this study, along with exhaustive literature review, establish
424 that transcriptional control of *yefM/yoeB* is independent of polyP and ppGpp. PolyP is not
425 required for Lon dependent degradation of YefM. $\Delta 10$ strain is relatively hypersensitive to
426 Ciprofloxacin and Ampicillin which is probably the cause for decreased persister formation
427 upon treatment with Ciprofloxacin and Ampicillin. $\Delta 10$ strain has lower metabolic fitness
428 compared to wild type which also strengthens this notion. Hence the role of endoribonuclease
429 encoding chromosomal TAS in persistence is inconclusive. Hence we refute the model
430 presented by Maisonneuve et al., 2013. Extreme caution and evaluation should be exercised
431 during deletion of horizontally transferring genes like TAS and evaluated for the polar effects
432 on the downstream genes.

433

434 **Acknowledgements:**

435 We thank Prof. Kenn Gerdes for the gracious donation of the *E. coli* strains and plasmids
436 used in this study. All primer extension work was carried out in Prof. Kenn Gerdes' lab while
437 at University of Southern Denmark, Odense, Denmark by BCMR. The remaining work was
438 supported by Prof. T. R. Rajagopalan grants and Central Research Facility sanctioned by
439 SASTRA University, Thanjavur, India.

440

441 **Declaration of conflict of interest:**

442 The authors declare that there is no conflict of interest.

443

444 **References:**

445 Aizenman, E., Engelberg-Kulka, H., and Glaser, G. (1996). An *Escherichia coli*
446 chromosomal "addiction module" regulated by guanosine [corrected] 3',5'-
447 bispyrophosphate: a model for programmed bacterial cell death. *Proc Natl Acad Sci U*
448 *S A* 93, 6059-6063.
449 Anantharaman, V., and Aravind, L. (2003). New connections in the prokaryotic toxin-
450 antitoxin network: relationship with the eukaryotic nonsense-mediated RNA decay
451 system. *Genome Biol* 4, R81.

- 452 Artsimovitch, I., Patlan, V., Sekine, S., Vassylyeva, M.N., Hosaka, T., Ochi, K., Yokoyama,
453 S., and Vassylyev, D.G. (2004). Structural basis for transcription regulation by
454 alarmone ppGpp. *Cell* 117, 299-310.
- 455 Barker, M.M., Gaal, T., Josaitis, C.A., and Gourse, R.L. (2001). Mechanism of regulation of
456 transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation in
457 vivo and in vitro. *J Mol Biol* 305, 673-688.
- 458 Boutte, C.C., and Crosson, S. (2011). The complex logic of stringent response regulation in
459 *Caulobacter crescentus*: starvation signalling in an oligotrophic environment. *Mol*
460 *Microbiol* 80, 695-714.
- 461 Butt, A., Higman, V.A., Williams, C., Crump, M.P., Hemsley, C.M., Harmer, N., and Titball,
462 R.W. (2014). The HicA toxin from *Burkholderia pseudomallei* has a role in persister
463 cell formation. *Biochem J*.
- 464 Cashel, M., Gentry, D.R., Hernandez, V.J., and Vinella, D. (1996). *The stringent response*.
465 Washington, DC: American Society for Microbiology Press.
- 466 Cherny, I., Rockah, L., and Gazit, E. (2005). The YoeB toxin is a folded protein that forms a
467 physical complex with the unfolded YefM antitoxin. Implications for a structural-
468 based differential stability of toxin-antitoxin systems. *J Biol Chem* 280, 30063-30072.
- 469 Chowdhury, N., Kwan, B.W., and Wood, T.K. (2016). Persistence Increases in the Absence
470 of the Alarmone Guanosine Tetrphosphate by Reducing Cell Growth. *Sci Rep* 6,
471 20519.
- 472 Christensen-Dalsgaard, M., and Gerdes, K. (2008). Translation affects YoeB and MazF
473 messenger RNA interferase activities by different mechanisms. *Nucleic Acids Res* 36,
474 6472-6481.
- 475 Christensen-Dalsgaard, M., Jorgensen, M.G., and Gerdes, K. (2010). Three new RelE-
476 homologous mRNA interferases of *Escherichia coli* differentially induced by
477 environmental stresses. *Mol Microbiol* 75, 333-348.
- 478 Christensen, S.K., and Gerdes, K. (2004). Delayed-relaxed response explained by
479 hyperactivation of RelE. *Mol Microbiol* 53, 587-597.
- 480 Christensen, S.K., Maenhaut-Michel, G., Mine, N., Gottesman, S., Gerdes, K., and Van
481 Melderren, L. (2004). Overproduction of the Lon protease triggers inhibition of
482 translation in *Escherichia coli*: involvement of the *yefM-yoeB* toxin-antitoxin system.
483 *Mol Microbiol* 51, 1705-1717.
- 484 Christensen, S.K., Mikkelsen, M., Pedersen, K., and Gerdes, K. (2001). RelE, a global
485 inhibitor of translation, is activated during nutritional stress. *Proc Natl Acad Sci U S A*
486 98, 14328-14333.
- 487 Christensen, S.K., Pedersen, K., Hansen, F.G., and Gerdes, K. (2003). Toxin-antitoxin loci as
488 stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are
489 counteracted by tmRNA. *J Mol Biol* 332, 809-819.
- 490 Crooke, E., Akiyama, M., Rao, N.N., and Kornberg, A. (1994). Genetically altered levels of
491 inorganic polyphosphate in *Escherichia coli*. *J Biol Chem* 269, 6290-6295.
- 492 Diderichsen, B., Fiil, N.P., and Lavalley, R. (1977). Genetics of the *relB* locus in *Escherichia*
493 *coli*. *J Bacteriol* 131, 30-33.
- 494 Feng, S., Chen, Y., Kamada, K., Wang, H., Tang, K., Wang, M., and Gao, Y.G. (2013).
495 YoeB-ribosome structure: a canonical RNase that requires the ribosome for its
496 specific activity. *Nucleic Acids Res* 41, 9549-9556.
- 497 Fiedoruk, K., Daniluk, T., Swiecicka, I., Sciepek, M., and Leszczynska, K. (2015). Type II
498 toxin-antitoxin systems are unevenly distributed among *Escherichia coli* phylogroups.
499 *Microbiology* 161, 158-167.
- 500 Gerdes, K., Christensen, S.K., and Lobner-Olesen, A. (2005). Prokaryotic toxin-antitoxin
501 stress response loci. *Nat Rev Microbiol* 3, 371-382.

- 502 Gerdes, K., Rasmussen, P.B., and Molin, S. (1986). Unique type of plasmid maintenance
503 function: postsegregational killing of plasmid-free cells. *Proc Natl Acad Sci U S A* 83,
504 3116-3120.
- 505 Germain, E., Castro-Roa, D., Zenkin, N., and Gerdes, K. (2013). Molecular mechanism of
506 bacterial persistence by HipA. *Mol Cell* 52, 248-254.
- 507 Goeders, N., and Van Melderen, L. (2014). Toxin-antitoxin systems as multilevel interaction
508 systems. *Toxins (Basel)* 6, 304-324.
- 509 Gotfredsen, M., and Gerdes, K. (1998). The *Escherichia coli* *relBE* genes belong to a new
510 toxin-antitoxin gene family. *Mol Microbiol* 29, 1065-1076.
- 511 Gross, M., Marianovsky, I., and Glaser, G. (2006). MazG -- a regulator of programmed cell
512 death in *Escherichia coli*. *Mol Microbiol* 59, 590-601.
- 513 Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. (1995). Tight regulation,
514 modulation, and high-level expression by vectors containing the arabinose pBAD
515 promoter. *J Bacteriol* 177, 4121-4130.
- 516 Haseltine, W.A., and Block, R. (1973). Synthesis of guanosine tetra- and pentaphosphate
517 requires the presence of a codon-specific, uncharged transfer ribonucleic acid in the
518 acceptor site of ribosomes. *Proc Natl Acad Sci U S A* 70, 1564-1568.
- 519 Kamada, K., and Hanaoka, F. (2005). Conformational change in the catalytic site of the
520 ribonuclease YoeB toxin by YefM antitoxin. *Mol Cell* 19, 497-509.
- 521 Kampinga, H.H. (2014). Chaperoned by prebiotic inorganic polyphosphate molecules: an
522 ancient transcription-independent mechanism to restore protein homeostasis. *Mol Cell*
523 53, 685-687.
- 524 Kaspy, I., Rotem, E., Weiss, N., Ronin, I., Balaban, N.Q., and Glaser, G. (2013). HipA-
525 mediated antibiotic persistence via phosphorylation of the glutamyl-tRNA-synthetase.
526 *Nat Commun* 4, 3001.
- 527 Kedzierska, B., Lian, L.Y., and Hayes, F. (2007). Toxin-antitoxin regulation: bimodal
528 interaction of YefM-YoeB with paired DNA palindromes exerts transcriptional
529 autorepression. *Nucleic Acids Res* 35, 325-339.
- 530 Korch, S.B., Henderson, T.A., and Hill, T.M. (2003). Characterization of the *hipA7* allele of
531 *Escherichia coli* and evidence that high persistence is governed by (p)ppGpp
532 synthesis. *Mol Microbiol* 50, 1199-1213.
- 533 Korch, S.B., and Hill, T.M. (2006). Ectopic overexpression of wild-type and mutant *hipA*
534 genes in *Escherichia coli*: effects on macromolecular synthesis and persister
535 formation. *J Bacteriol* 188, 3826-3836.
- 536 Kuroda, A., Murphy, H., Cashel, M., and Kornberg, A. (1997). Guanosine tetra- and
537 pentaphosphate promote accumulation of inorganic polyphosphate in *Escherichia*
538 *coli*. *J Biol Chem* 272, 21240-21243.
- 539 Kuroda, A., Nomura, K., Ohtomo, R., Kato, J., Ikeda, T., Takiguchi, N., Ohtake, H., and
540 Kornberg, A. (2001). Role of inorganic polyphosphate in promoting ribosomal protein
541 degradation by the Lon protease in *E. coli*. *Science* 293, 705-708.
- 542 Lund, E., and Kjeldgaard, N.O. (1972). Metabolism of guanosine tetraphosphate in
543 *Escherichia coli*. *Eur J Biochem* 28, 316-326.
- 544 Magnuson, R.D. (2007). Hypothetical functions of toxin-antitoxin systems. *J Bacteriol* 189,
545 6089-6092.
- 546 Maisonneuve, E., Castro-Camargo, M., and Gerdes, K. (2013). (p)ppGpp controls bacterial
547 persistence by stochastic induction of toxin-antitoxin activity. *Cell* 154, 1140-1150.
- 548 Maisonneuve, E., Shakespeare, L.J., Jorgensen, M.G., and Gerdes, K. (2011). Bacterial
549 persistence by RNA endonucleases. *Proc Natl Acad Sci U S A* 108, 13206-13211.

- 550 Metzger, S., Schreiber, G., Aizenman, E., Cashel, M., and Glaser, G. (1989). Characterization
551 of the *relA1* mutation and a comparison of *relA1* with new *relA* null alleles in
552 *Escherichia coli*. *J Biol Chem* 264, 21146-21152.
- 553 Mosteller, R.D., and Kwan, S.F. (1976). Isolation of relaxed-control mutants of *Escherichia*
554 *coli* K-12 which are sensitive to glucose starvation. *Biochem Biophys Res Commun*
555 69, 325-332.
- 556 Moyed, H.S., and Bertrand, K.P. (1983). *hipA*, a newly recognized gene of *Escherichia coli*
557 K-12 that affects frequency of persistence after inhibition of murein synthesis. *J*
558 *Bacteriol* 155, 768-775.
- 559 Osbourne, D.O., Soo, V.W., Konieczny, I., and Wood, T.K. (2014). Polyphosphate, cyclic
560 AMP, guanosine tetraphosphate, and c-di-GMP reduce in vitro Lon activity.
561 *Bioengineered* 5, 457-461.
- 562 Overgaard, M., Borch, J., Jorgensen, M.G., and Gerdes, K. (2008). Messenger RNA
563 interferase RelE controls *relBE* transcription by conditional cooperativity. *Mol*
564 *Microbiol* 69, 841-857.
- 565 Pandey, D.P., and Gerdes, K. (2005). Toxin-antitoxin loci are highly abundant in free-living
566 but lost from host-associated prokaryotes. *Nucleic Acids Res* 33, 966-976.
- 567 Ramisetty, B.C., Raj, S., and Ghosh, D. (2016). *Escherichia coli* MazEF toxin-antitoxin
568 system does not mediate programmed cell death. *J Basic Microbiol*.
- 569 Ramisetty, B.C., and Santhosh, R.S. (2016). Horizontal gene transfer of chromosomal Type II
570 toxin-antitoxin systems of *Escherichia coli*. *FEMS Microbiol Lett* 363.
- 571 Salgado, H., Peralta-Gil, M., Gama-Castro, S., Santos-Zavaleta, A., Muniz-Rascado, L.,
572 Garcia-Sotelo, J.S., Weiss, V., Solano-Lira, H., Martinez-Flores, I., Medina-Rivera,
573 A., Salgado-Osorio, G., Alquicira-Hernandez, S., Alquicira-Hernandez, K., Lopez-
574 Fuentes, A., Porron-Sotelo, L., Huerta, A.M., Bonavides-Martinez, C., Balderas-
575 Martinez, Y.I., Pannier, L., Olvera, M., Labastida, A., Jimenez-Jacinto, V., Vega-
576 Alvarado, L., Del Moral-Chavez, V., Hernandez-Alvarez, A., Morett, E., and Collado-
577 Vides, J. (2013). RegulonDB v8.0: omics data sets, evolutionary conservation,
578 regulatory phrases, cross-validated gold standards and more. *Nucleic Acids Res* 41,
579 D203-213.
- 580 Shan, Y., Lazinski, D., Rowe, S., Camilli, A., and Lewis, K. (2015). Genetic basis of persister
581 tolerance to aminoglycosides in *Escherichia coli*. *MBio* 6.
- 582 Shao, Y., Harrison, E.M., Bi, D., Tai, C., He, X., Ou, H.Y., Rajakumar, K., and Deng, Z.
583 (2011). TADB: a web-based resource for Type 2 toxin-antitoxin loci in bacteria and
584 archaea. *Nucleic Acids Res* 39, D606-611.
- 585 Svitil, A.L., Cashel, M., and Zyskind, J.W. (1993). Guanosine tetraphosphate inhibits protein
586 synthesis in vivo. A possible protective mechanism for starvation stress in
587 *Escherichia coli*. *J Biol Chem* 268, 2307-2311.
- 588 Tsilibaris, V., Maenhaut-Michel, G., Mine, N., and Van Melderen, L. (2007). What is the
589 benefit to *Escherichia coli* of having multiple toxin-antitoxin systems in its genome? *J*
590 *Bacteriol* 189, 6101-6108.
- 591 Vazquez-Laslop, N., Lee, H., and Neyfakh, A.A. (2006). Increased persistence in *Escherichia*
592 *coli* caused by controlled expression of toxins or other unrelated proteins. *J Bacteriol*
593 188, 3494-3497.
- 594 Wendrich, T.M., Blaha, G., Wilson, D.N., Marahiel, M.A., and Nierhaus, K.H. (2002).
595 Dissection of the mechanism for the stringent factor RelA. *Mol Cell* 10, 779-788.
- 596 Wood, T.K. (2016). Combatting bacterial persister cells. *Biotechnol Bioeng* 113, 476-483.
- 597 Xiao, H., Kalman, M., Ikehara, K., Zemel, S., Glaser, G., and Cashel, M. (1991). Residual
598 guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be
599 eliminated by *spoT* null mutations. *J Biol Chem* 266, 5980-5990.

- 600 Yamaguchi, Y., and Inouye, M. (2011). Regulation of growth and death in *Escherichia coli*
601 by toxin-antitoxin systems. *Nat Rev Microbiol* 9, 779-790.
602 Zhou, J., and Rudd, K.E. (2013). EcoGene 3.0. *Nucleic Acids Res* 41, D613-624.
603