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1 **Cross-Regulations between Bacterial Toxin-Antitoxin Systems:** 2 **Evidence of an Interconnected Regulatory Network?**

3
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9
10 **Keywords:** Toxin-Antitoxin; Cross-regulation; Cross-activation

11 **Abstract**

12
13 Toxin-antitoxin (TA) systems are ubiquitous among bacteria and contain stable toxins whose
14 toxicity can be counteracted by RNA or protein antitoxins. They are involved in multiple
15 functions that range from stability maintenance for mobile genetic elements to stress
16 adaptation. Bacterial chromosomes frequently have multiple homologues of TA system loci,
17 and it is unclear why there are so many of them. In this review, we focus on cross-regulations
18 between TA systems, which occur between both homologous and non-homologous systems,
19 from similar or distinct types, whether encoded from plasmids or chromosomes. In addition to
20 being able to modulate RNA expression levels, cross-regulations between these systems can
21 also influence their toxicity. This suggests the idea that they are involved in an interconnected
22 regulatory network.

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33 **Toxin-Antitoxin Systems Are Abundant in Bacterial Genomes**

34 Toxin-antitoxin (TA) systems are widespread genetic modules that encode both a stable
35 toxic protein, whose overexpression can lead to growth arrest or cell death, and a
36 corresponding labile antitoxin, which neutralizes the toxin's activity during bacterial growth
37 [1]. TA systems are classified into six types depending on the antitoxin's nature and mode of
38 action. While the toxins are all proteins, the antitoxins can be either non-coding RNAs (in
39 type I and III systems) or low-molecular weight proteins (in types II, IV, V, and VI) (**Box 1**).
40 **One exception is the DarTG system, a type II/IV TA hybrid system, where the DarG antitoxin**
41 **interacts with and inhibits expression of the DarT toxin, but also act on the target of DarT**
42 **[2,3].** Antitoxins are unstable and more susceptible to degradation by proteases than toxins,
43 leaving toxins to interfere with cellular housekeeping processes such as replication,
44 translation, and cell division [4,5]. The first discovery of TA systems was in the 1980s on
45 *Escherichia coli* plasmids. These systems prevent plasmid loss during cell division via post-
46 segregational killing [6,7]. In this process, if the daughter cell does not receive a TA system
47 plasmid, the labile antitoxin is degraded, and the stable toxin expresses its toxicity, killing the
48 cells that lack the plasmid. Meanwhile, the bacteria that do inherit the plasmid are unaffected
49 by toxin activation because of *de novo* and continuous antitoxin synthesis. Since their initial
50 discovery, homologues of these plasmid-encoded TA systems have been identified in the
51 chromosomes of many bacteria, including human pathogens such as *Mycobacterium*
52 *tuberculosis*, *Staphylococcus aureus*, and enterobacteria. The biological role of chromosomal
53 TA systems is still elusive even if significant scientific advances have been made to discover
54 it [8]. In addition to maintaining the stability of genetic elements, they are also involved in
55 many other functions, including growth adaptation as a response to environmental stresses or
56 persister cell formation [9-14]. However, contradictory papers have been published and the
57 involvement of TA systems in persister cell formation is now questioned [15-19].

58 **The number of expressed TA loci vary extensively between species (see Table 1). The**
59 **abundance of both homologous and non-homologous TA systems in the bacterial genomes**
60 **and, for some of them, their sequence identity, their sequence homology or their similar**
61 **mechanisms and functions suggest that redundancy and/or cross-regulations between them is**
62 **very likely to occur and interfere with bacterial physiology [20].** In this review, we discuss the
63 cross-regulations that have been reported between type I or II TA systems in various bacteria,
64 and also those between TA systems of different types. Particular attention is given to cross-

65 regulations that influence the RNA levels of other TA pairs, and to how these regulations
66 affect the overall toxicity of the systems.

67

68 **Cross-regulations between Type I TA Systems**

69 Many type I TA homologues were identified when either looking for small RNAs or
70 after sequence comparisons searching for conserved toxin amino acid residues and antitoxin
71 nucleotide sequences (**Table 1**). Within the *E. coli* 0157:H7 genome, 26 loci of type I TA
72 systems (including 14 *hok* and 7 *ibs* genes) were found [21], and recently 7 more from TxpA
73 and Fst families were identified in the *S. aureus* MW2 genome [22]. Below, we describe the
74 putative cross-regulations between homologous type I TA systems in both Gram-negative and
75 -positive bacteria.

76

77 ***Cross-Regulations between Type I TA Systems in Gram-Negative Bacteria***

78 The *ibs/Sib* type I TA systems named *ibsA to-E/SibA to-E* are expressed from five
79 homologues on the *E. coli* K12 genome (**Figure 1A, Table 1**) [23]. Ibs peptides contain 18 or
80 19 amino acids and are located at the *E. coli* membrane. Their overexpression is lethal to *E.*
81 *coli* because the cell envelope is disrupted. This toxicity can be repressed by the Sib RNA
82 antitoxins through base-pairing with *ibs* toxins open reading frames [23]. Sib RNA antitoxins
83 contain two domains, TRD1 and TRD2, that interact with *ibs* mRNA [24]. Only the SibA and
84 SibE TRD2 sequences are identical, and a *sibA*-deleted strain is viable even when *ibsA* toxin
85 expression is induced, which suggests that SibE might prevent toxicity by recognizing *ibsA*
86 mRNA (**Figure 1A**) [23]. Additional experiments should be performed to highlight any direct
87 interactions between SibE and *ibsA* mRNA, and to explore the impact of these interactions on
88 *ibsA* mRNA levels and toxicity. Thus, for that system, the current evidences for cross-
89 regulations are weak.

90 The *zor/Orz* system is another type I TA system which occurs in multiple homologues
91 on the *E. coli* chromosome (**Table 1**). It is expressed from two gene pairs, *zorO/OrzO* and
92 *zorP/OrzP*. An overproduction of ZorO is toxic for *E. coli*, but its toxicity is neutralized in an
93 RNase III-dependent manner by the OrzO RNA antitoxin [25]. OrzO and OrzP antitoxins are
94 structural homologues, with OrzP having a 15-nucleotide (nt) domain at the 5'-end that is
95 predicted to interact with *zorO* mRNA, while the OrzO has an 18-nt sequence. However,
96 toxicity induced by ZorO during growth is only rescued by OrzO, demonstrating the tight
97 specificity of the *zorO/OrzO* system. A 3-nt variable region 'V1' was identified in OrzO and

98 OrzP, and since an OrzP V1 mutant represses *zorO* expression and toxicity, V1 was been
99 shown to be responsible for the OrzO RNA antitoxin's specificity for the *zorO* target mRNA
100 [25]. So in fact, just mutating the three nts located in the mRNA toxin interaction domain is
101 sufficient to confer specificity to two antitoxin homologues.

102 Five homologues of the *aapA*/IsoA TA systems are found in *H. pylori* strains (**Table 1**)
103 [26,27]. Overexpression of the 30-amino acid peptide encoded by *aapA1* induces cell death,
104 and *aapA1* toxin translation is prevented by mRNA folding, RNA processing, and by the
105 IsoA1 RNA antitoxin [27]. Despite the fact that IsoA1 and IsoA3 have similar secondary
106 structures in solution, footprint assays did not detect interactions between *aapA1* and IsoA3
107 RNA, which suggests that toxin neutralization is enabled by the tight specificity between
108 *aapA* and IsoA RNA [27].

109 As for the *hok*/Sok and *ldr*/Rdl TA systems, all that is known is that they are abundant
110 in enterobacterial genomes (**Table 1**). For *hok*/Sok homologues, many are non-functional due
111 to mutations, insertions or large rearrangements in *E. coli* [28-30]. Moreover, it was
112 demonstrated that *sokE* and *sokX* loci can be expressed without *hok* transcripts, suggesting
113 that these antitoxins could possess additional roles beyond toxicity neutralization [28,31].
114 However, nobody has yet looked for cross-regulations between the multiple homologues, so
115 this could warrant investigation [21,28,32].

116

117 ***Cross-Regulations between Type I TA Systems in Gram-Positive Bacteria***

118 Two type I TA systems, *sprA*/SprA_{AS} and *sprG*/SprF, are found in two and four
119 homologues respectively in the *S. aureus* genome (**Table 1**) [33]. The overexpression of toxic
120 membrane peptides encoded by *sprA* and *sprG* RNA triggers *S. aureus* stasis or death [34-37].
121 SprA toxins expression are prevented in *trans* by SprA_{AS} *cis*-RNA antitoxins when they pair
122 at the internal ribosome binding site (RBS) and thus inhibit toxin translation [34,35]. By
123 contrast, SprF antitoxins prevent toxin translation by interacting in *cis* with their overlapping
124 regions [36,37]. Despite high sequence identities, growth kinetics between non-cognate pairs
125 demonstrate a lack of cross-regulation between the two *sprA*/SprA_{AS} TA homologues and
126 between the four *sprG*/SprF TA homologues (**Figure 1B-C**) [35,37]. However, *sprG3*-SprF1
127 and *sprG2*-SprF4 RNA complexes were detected *in vitro*, which might suggest that cross-
128 regulation could occur *in vivo* between the *sprG3*/SprF1 and *sprG2*/SprF4 RNA pairs. RNA
129 level variations during *S. aureus* growth were observed by northern blot analysis in strains
130 overexpressing SprF1 (which decreased *sprG2* and *sprG3* RNA levels); *sprG2* (decreased
131 SprF1, SprF2, and SprF3); *sprG2*/SprF2 (increased *sprG1*); or SprF4 (decreased *sprG1* and

132 *sprG2*) [37]. The *sprG4* toxin is not expressed in *S. aureus* HG003 because *sprG4* lacks an
133 upstream promoter sequence, suggesting that SprF4's primary function is to interact with and
134 regulate *sprG2* mRNA expression. Together, these results show that in each *sprG*/SprF TA
135 system, SprG toxicity control is specific to the cognate antitoxin, but that cross-regulations
136 between the different *sprG*/SprF TA systems can modulate the RNA levels of homologues
137 (**Figure 1C**). Further experiments are needed to investigate the physiological relevance of this
138 sort of network in the *S. aureus sprG*/SprF TA pairs.

139

140 So in all type I TA systems found in multiple homologues, cross-regulations have only
141 been documented between homologous systems, with no studies yet published on non-
142 homologous cross-regulation. In Gram-negative bacteria, toxicity-affecting cross-regulations
143 were only been observed between the *zorO*/OrzO and *zorP*/OrzP systems (in site-directed
144 mutagenesis assays), and they were suggested for *ibsA*/SibE. A lack of cross-regulation of
145 toxicity in Gram-positive bacteria does not however exclude possible regulation at the post-
146 transcriptional level, as demonstrated to exist in the *sprG*/SprF TA systems.

147

148 **Cross-Regulations between Type II TA Systems**

149 Type II TA systems are also abundant in bacterial genomes (**Table 1**). In *M.*
150 *tuberculosis*, 79 systems were identified after research in the Toxin-Antitoxin database
151 (<http://bioinfo-mml.sjtu.edu.cn/TADB/>) and through a homologue search of the *M.*
152 *tuberculosis* genomes for toxin and antitoxin protein sequences from each of eight major type
153 II families (CcdBA, HigBA, HipBA, MazEF, ParDE, RelBE, VapBC, and Doc/PhD). These
154 type II systems include 50 from the VapBC family and 10 from the MazEF family, likely the
155 result of extensive gene duplication events (**Table 1**) [38-41]. Cross-regulations between type
156 II TA systems are more documented than for the type I systems **as type II antitoxins and**
157 **toxins possess global regulatory functions. Type II antitoxins possess a DNA-binding domain**
158 **and act as transcriptional regulator via their interactions with TA gene promoters. Most type**
159 **II toxins are endoribonucleases (RNases) that inhibit bacterial translation by cleaving mRNAs**
160 **when loaded onto translating ribosomes (in the cases of RelE, MazE, Kid, and MqsR), or by**
161 **targeting tRNAs (VapC, TacT, and AtaT) [12,42,43].** In this section, we describe the cross-
162 regulations between homologous or non-homologous type II TA systems. In fact, cross-
163 regulations between type II TA systems can occur both between systems from the same

164 replicon (whether encoded by plasmids or chromosomes) and between systems that have
165 different origins.

166

167 *Cross-Regulations between Type II TA Systems in Gram-Negative Bacteria*

168

169 *Cross-Regulations between Homologous Type II TA Systems*

170 Cross-regulations can occur between homologues where one is encoded by
171 chromosomes and the other by plasmids. Investigations were conducted on the CcdAB TA
172 system expressed from the F plasmid and the chromosome of the pathogenic *E. coli* O157:H7
173 strain, and it was shown that the plasmid CcdB toxin is not neutralized by the chromosome-
174 encoded CcdA antitoxin [44,45]. Other examples of studies between differently-encoded
175 systems in *E. coli* include the *par* locus located on pR100 plasmid with the chromosome-
176 encoded MazEF TA system, but they turned out to be specific [46,47]. In *E. coli*,
177 overproduction of the MazE antitoxin (a homologue of chromosomal Kis) rescues the toxicity
178 induced by Kid toxin overexpression in the presence of a mutated version of the Kis antitoxin
179 [46]. This cross-regulation between MazE and Kid probably occurs due to structural
180 similarities between the antitoxins as a result of a protein-protein complex formation [48].

181 The first study of cross-regulations between homologous chromosome-encoded type II
182 TA systems in Gram-negative bacteria was described in *Caulobacter crescentus*, an α -
183 proteobacterium. There are four homologues of the ParDE and RelBE TA systems (**Table 1**)
184 [49]. ParD and RelB proteins act as antitoxins against ParE and RelE toxicity. Overexpression
185 of the ParE1 toxin inhibits cell division, causing filamentation, but cell growth is unaffected.
186 There is no cross-regulation between the ParDE and RelBE homologues [49].

187 Another example of specificity between homologous type II TA systems was reported
188 in *E. coli* O157:H7 for the two *paaR-paaA-parE* systems which contain the PaaR regulator,
189 the PaaA antitoxin protein, and the ParE bactericidal toxin [50,51]. ‘Plate toxicity’ assays
190 prove that no cross-regulation occurs between these two homologues, as each PaaA antitoxin
191 specifically counteracts the toxicity of its associated ParE toxin [51].

192 At the amino acid sequence level, Polom *et al.* investigated the specificity of TA
193 interactions between plasmid-encoded Axe-Txe and chromosome-encoded YefM-YoeB
194 homologues. No cross-interactions were detected between the wild-type proteins. However, a
195 single N83Y amino acid substitution which converts a Txe-specific residue to a YoeB-
196 specific one will allow functional interaction between the mutated Txe toxin and the YefM
197 antitoxin, resulting in reversal of the toxicity [52]. Using the same approach in *Haemophilus*

198 *influenzae*, Walling and Butler showed that VapC1 and VapC2 toxicity is neutralized by a
199 VapB1 T47W mutated antitoxin that has an additional tryptophan residue which is involved
200 in the VapB2-VapC2 interaction [53]. These results indicate that type II antitoxin specificity
201 can be decreased by a single mutation at an essential residue.

202

203 ***Cross-Regulations between Non-Homologous Type II TA Systems***

204 Smith *et al.* studied cross-regulations between the CcdAB TA system produced by F
205 plasmid, and the *parD* locus from the R1 plasmid in *E. coli*, a system which contains the Kis
206 antitoxin and Kid toxin [54]. CcdB and Kid toxins have different targets, with CcdB targeting
207 DNA gyrase and Kid targeting RNA *via* an RNase action. Despite the different cellular
208 targets, these toxins both have a similar structural fold, leading the authors to conclude that
209 cross-regulations are possible. Kid toxicity is rescued by the Kis cognate antitoxin and
210 increased by the CcdA antitoxin, while conversely, the overexpression of Kis antitoxin
211 inhibits CcdB toxicity, although less efficiently than CcdA. By testing *in vitro* the RNase
212 activity of Kid onto CopT RNA in presence of CcdA antitoxin, Smith *et al.* showed an
213 unexpected and unexplained stimulation of the RNase activity of Kid by CcdA. Direct
214 interactions between toxins and antitoxins from non-homologous complexes were also
215 demonstrated, suggesting that cross-regulations between the *ccd* and *parD* systems are
216 possible (**Figure 2A**) [54].

217 Cross-regulations between non-homologous type II TA systems of different origins
218 were described in *E. coli* between the Phd/Doc module, which participates in P1 plasmid-
219 prophage maintenance [55], and the chromosome-encoded RelBE system. In this case, the
220 Doc toxin associates with Elongation Factor Tu (EF-Tu) on the 30S ribosomal subunit to
221 repress translation elongation, and the Phd antitoxin neutralizes this toxicity [56,57]. Garcia-
222 Pino *et al.* showed that Doc induction increases mRNA cleavage by the RelE toxin, probably
223 via the degradation of RelB antitoxin by Lon proteases coupled to an inhibition of global
224 translation in bacteria [58]. Moreover, Winther and Gerdes demonstrated that pMYSH6000
225 VapC overexpression in *Shigella flexneri* induces Lon proteases, causing YefM antitoxin
226 degradation. Consequently, YoeB toxin cleaves the mRNA at the stop-codons (**Figure 2B**)
227 [59].

228 In 2017, Yao *et al.* characterized the ParE_{So}/CopA_{So} type II TA pair involved in CP4So
229 prophage maintenance in *Shewenella oneidensis* [60,61]. ParE_{So} toxicity is inhibited by the
230 CopA_{So} antitoxin both *via* direct ‘protein-protein’ interactions and by repressing transcription
231 of the TA operon thanks to a DNA-binding domain. The DNA motif of the promoter involved

232 in this auto-regulation is also detected in the promoter of the *pemK_{SO}/pemI_{SO}* TA system
233 found in the *S. oneidensis* pMR-1 megaplasmid. Since CopA_{SO} also represses *pemK_{SO}/pemI_{SO}*
234 transcription, a single antitoxin can cross-inhibit two TA systems [61]. A mutated *pemI_{SO}-*
235 *pemK_{SO}* promoter was synthesized to challenge the antitoxin's ability to bind native and
236 mutated promoters, and, as expected, promoter activity is not repressed with the mutated
237 promoter. Although *parE_{SO}* is prevalent in *Shewenella* strains, its toxicity is sometimes
238 inactivated by nonsense mutations. In some of these strains, the CopA_{SO} antitoxin has 'orphan
239 antitoxin' homologues [61], but these may be involved in other undetermined cross-regulatory
240 functions.

241 Cross-regulations can also occur between non-homologous type II TA systems that are
242 encoded by chromosomes. Cross-regulations between TA systems were investigated within
243 the *V. cholerae* N16961 superintegron, which has 18 type II TA systems belonging to the Hig,
244 Rel, Par and PhD-Doc families that contribute to its stability [62]. Among all these systems,
245 no cross-regulations occur during toxin induction in the presence of a non-cognate antitoxin
246 [62].

247 **Transcriptional cross-activations between non-homologous type II TA systems were detected**
248 **into the *E. coli relBEF* operon which is activated by the ectopic expression of MazF, MqsR,**
249 **HicA, or the HipA toxins, each that inhibits translation, probably by the global depletion of**
250 **the antitoxins leading to a derepression of the TA operons [63].** Moreover, RelE toxin
251 expression induces the transcription of several TA operons, as occurs with *mazEF* during
252 amino acid starvation [63], and this may occur to counteract RelE toxicity. Kasari *et al.* also
253 investigated cross-activations between TA systems in a strain deleted for the genes encoding
254 Lon, ClpP and HslV proteases. In that protease-deficient strain, the overexpression of MazF
255 and MqsR induced *relBEF* transcription and an increase of specific RNA fragments
256 corresponding to the *relBEF* mRNA 3' portion. These RNA fragments may serve as templates
257 for RelB translation [63]. Therefore, type II TA systems activation would be due to antitoxin
258 depletion when protein synthesis is reduced but also to selective accumulation of toxin-
259 encoding fragments upon mRNA cleavages.

260

261 ***Cross-Regulations between Type II TA Systems in Gram-Positive Bacteria***

262

263 ***Cross-Regulations between Homologous Type II TA Systems***

264 In the *S. aureus* chromosome, two *yefM-yoeB* type II homologues were characterized
265 and named '*yefM-yoeB-sa1*' and '*yefM-yoeB-sa2*' (also known as '*axe-txe1*' and '*axe-txe2*')

266 (Table 1) [64,65]. As with YoeB from *E. coli*, *S. aureus* YoeB associates with ribosomes and
267 cleaves target mRNAs near their translation initiation sites [66,67]. Despite a partial
268 conservation of the amino acid sequences, no cross-regulations occur between the YefM-
269 YoeB-sa1 and YefM-YoeB-sa2 type II systems, there is no toxin neutralization and no
270 transcriptional auto-regulation of the TA modules [64].

271 In the *M. tuberculosis* genome, RelBE TA system and two homologues, RelFG, and
272 RelJK exist, and these are expressed in human macrophages during infection (Table 1) [68].
273 A two-hybrid assay of ‘protein-protein’ interactions showed direct interactions between the
274 RelB and RelF antitoxins and the RelE, RelG, and RelK toxins. EMSAs demonstrated that, on
275 the *relBE* promoter, RelG has similar role than RelE and stimulate the DNA-binding activity
276 of the RelB antitoxin [68]. Moreover, the non-cognate RelB antitoxin rescues the growth
277 inhibition induced by overproduction of the RelG toxin, although this is not the case for the
278 RelE toxin and RelF antitoxin pair, and for the RelK toxin and RelB antitoxin pair. Overall,
279 these experiments demonstrate that cross-regulations between the RelBE homologues in *M.*
280 *tuberculosis* do exist (Figure 2C) [68].

281

282 *Cross-Regulations between Non-Homologous Type II TA Systems*

283 Various combinations of non-homologous type II TA cross-regulations were analysed
284 with toxicity assays and *in vitro* interaction experiments in *M. tuberculosis* [69]. Several
285 examples of such interactions were identified, even among different families. For instance,
286 the two MazF toxin homologues, MazF-mt1 and MazF-mt3, both interact with the VapB
287 antitoxins. These interactions were investigated in the *E. coli* heterologous host, in which the
288 non-cognate VapB24 and VapB25 antitoxins reduce MazF-mt1 toxicity when co-expressed.
289 Moreover, MazE-mt1, MazE-mt3, VapB24, and VapB25 antitoxins all inhibit MazF-mt3
290 toxicity [69].

291 Since the VapBC TA systems are a major TA family expressed by *M. tuberculosis*, RT-
292 qPCR assays were performed to monitor the effects of the ectopic expression of the VapC
293 toxin on the RNA levels of other toxin homologues [70]. The overexpressions of VapC3, 11,
294 13, 20, 22, 27, and 33 all resulted in 8- to 16-fold increases in *vapC15* mRNA levels. In
295 addition, the RNA levels of *higB1* and *mazF6* were increased by the ectopic expression of
296 VapC3, 11, 15, 20, 22, 27, and by VapC11, 13, 15, 20, 22, and 44, respectively [70]. This
297 proves that non-homologous toxins are cross-activated by different VapC protein isoforms in
298 *M. tuberculosis* (Figure 2D). In fact, the toxin ribonuclease activity of VapC1, 19, 27, 29 and
299 39 is specific for the UAGG sequence [71]. EMSAs on the VapBC29 TA complex and the

300 *vapBC1* and *vapBC27* promoters showed that there are no cross-regulation between
301 VapBC29, VapBC1, and VapBC27 [71]. Despite RNA level modulations, it is unknown if
302 cross-regulations can modulate toxicity.

303

304 To conclude, among all type II TA systems, cross-regulations have been documented
305 between both homologous and non-homologous TA systems in Gram-negative and -positive
306 bacteria. This is so even for those belonging to different replicons (encoded by either
307 chromosomes or plasmids), although most of the time homologous TA systems from different
308 origins cannot neutralize each other. Indeed, from an evolutionary standpoint, plasmid
309 maintenance is impossible if a chromosome-encoded antitoxin can neutralize the expression
310 of a plasmid-encoded toxin, as for the chromosome-encoded MazE antitoxin that inhibits
311 toxicity of the plasmid-encoded Kid toxin [43,45]. However, when we look at all the
312 independent research results, we see that plasmid-encoded toxins seem to be able to activate
313 chromosome-encoded toxins (demonstrated for the plasmid-encoded Doc toxin with the
314 chromosomal RelF toxin [55], and also for the plasmid-encoded VapC toxin with the
315 chromosomal YoeB toxin [56]), thus further increasing cell toxicity. It is important to note
316 that for the overexpression of type II toxins that target translation (MazF, MqsR, HicA, HipA,
317 RelE, Doc and VapC), cross-activations could be a side effect of translation inhibition
318 [55,56,60,67,68]. Cross-regulations can also occur between an antitoxin and a toxin from non-
319 homologous TA systems, to prevent toxicity [58,65,66]. Future investigations on cross-
320 regulations will reveal additional regulatory mechanisms, such as the ADP-ribosylation of TA
321 genes by the DarTG system [2,3]. Moreover, new homologous type II TA systems such as the
322 *Staphylococcus*-specific family TsaAT, TsbAT, TscAT, and TsdAT are still being
323 investigated experimentally [72].

324

325 **Cross-Regulations between Different Types of TA Systems**

326 Cross-regulation between different types of TA systems was initially discovered
327 between the MqsR/MqsA type II and GhoT/GhoS type V systems. In its active form, the
328 GhoT toxin is located at the bacterial membrane and induces a ghost-cell phenotype. This is
329 rescued by the GhoS antitoxin when it specifically cleaves and inactivates the *ghoT* mRNA
330 [73]. The MqsA antitoxin represses stationary-phase sigma factor RpoS and influences
331 various stress responses [74], while 5'-GCU sites are targeted by MqsR RNase mRNA
332 cleavages [75,76]. MqsR regulation of *ghoST* mRNA stability was investigated, and *ghoT*

333 mRNA is enriched in the presence of the MqsR toxin. On the other hand, introducing a
334 cleavage site recognized by MqsR into the *ghoT* mRNA will reduce GhoT toxicity and cell
335 death, because MqsR degrades *ghoT* mRNA (**Figure 3A**) [77]. Only 14 mRNA transcripts in
336 *E. coli* lack an MqsR-induced 5'-GCU cleavage site, and the *ghoS* mRNA is an MqsR target
337 [78,73]. During a stress such as antibiotic presence, the MqsR toxin degrades the *ghoS*
338 antitoxin, allowing GhoT toxin translation and ghost-cell formation. This is the first
339 description of a TA system being regulated by another type of TA system (**Figure 3A**) [77].

340 Cross-regulation between different TA system types was also observed in V583, a
341 clinical isolate of *Enterococcus faecalis*. A locus encoding two adjacent TA modules was
342 discovered in its genome. One module is related to the type I TxpA family, and the other to
343 the type II MazEF family [79]. In *E. coli*, this newly predicted MazEF-like system turned out
344 to be a type II TA system, as MazF is toxic when RNA degradation is triggered, but this effect
345 is counteracted by the MazE antitoxin. In fact, the MazEF complex presents dual regulatory
346 activity, both repressing *mazEF* operon expression and activating RatA RNA antitoxin
347 transcription [80]. Indeed, overexpression of *mazEF* *in trans* induces ~2.5 fold increase of
348 *mazEF* and RatA RNA levels, in comparison to a control strain with the empty vector. A
349 common sequence encompassing the -10 boxes, the transcription start sites (TSSs) and the 5'
350 transcribed region was identified in the P_{*mazEF*} and P_{*ratA*} promoters (called *drm1* for P_{*mazEF*} and
351 *drm2* for P_{*ratA*}). In a double mutant generated to abolish transcription initiation of *txpA* and
352 *ratA* by mutagenesis of the -10 boxes ('-10_{txpARatA}'), the RatA RNA level was increased as
353 compared to an isogenic strain, suggesting that the interaction of MazEF complex with the
354 *drm2* sequence activates RatA transcription [80]. This is a case of cross-regulation between a
355 type II antitoxin and a type I TA module (**Figure 3B**).

356 To summarize, in this section we reported on two examples of cross-regulation between
357 different types of TA systems: between type II and V, and between type I and II. In the
358 second example from *E. faecalis*, cross-regulation is favored by the genomic proximity of the
359 two TA systems. **Cross-regulations between three homologous type IV TA systems were also**
360 **described in *E. coli* [81].** Given the abundance of these systems in bacterial genomes, cross-
361 regulations between different TA system types remains a topic worthy of further exploration,
362 and these examples may present just the tip of the iceberg.

363

364 **Concluding Remarks**

365 In this review, we present recent data on TA systems which act individually or within a
366 gene network. This network contains cross-regulations between homologous and non-
367 homologous TA systems that may or may not be of the same types and origins. These subtle
368 cross-regulations are specific to each bacterial species, and must play a fundamental role in
369 species such as *M. tuberculosis*, *E. coli*, and *S. aureus* where a high number of TA systems
370 are found. **This implies the existence of sophisticated networks in bacteria between all the TA**
371 **systems to control RNA levels and/or toxicity (Figure 4, Key Figure).** However, in cases
372 where cross-talks were described, it is either under overexpression conditions and/or with
373 mutated antitoxins. **Therefore, the biological relevance of all these interactions is unclear,**
374 **especially because the roles of TA systems in bacterial physiology is debated [8,19].**
375 However, TA diversity, abundance, and cross-interaction were all selected during continuous
376 host-pathogen warfare and/or partnerships in order to increase fitness. A deeper understanding
377 of the many and varying functions of TA systems will require better characterization of their
378 cross-interactions, as well as the identification of the functions of each TA module or RNA in
379 the pair. Type II antitoxins are global regulators of gene expression via their DNA-binding
380 domains, responding to a plethora of environmental stimuli (**Figure 4**). Type II toxins are
381 endoribonucleases inducing depletion of type II antitoxins by translation inhibition but also
382 lead to the accumulation of toxin-encoding mRNA fragments upon cleavages (**Figure 4**). This
383 toxin production constitutes a possible positive feedback loop which contributes to a complex
384 regulatory network that controls bacterial growth. Moreover, small RNAs are widely found in
385 bacteria, regulating many RNA and/or protein targets. This raises an interesting question.
386 RNA antitoxins from type I and III TA systems counteract their respective toxins, but what if
387 they also have sRNA regulatory functions? Cross-regulations can arise between an antitoxin
388 and several toxins from different systems [43,45,58,65,66], and they can both improve
389 toxicity regulation and, conversely, titrate non-cognate antitoxins. Instead of individual
390 entities, we should be considering TA systems as members of an intricate and connected gene
391 networks. In the near future, we predict a much better understanding of the many important
392 effects of TA systems in bacterial physiology, as they are being actively investigated
393 worldwide.

394

395 **Box 1: Classification of the Six Types of Toxin-Antitoxin Systems**

396 **Type I** toxin and antitoxin genes are independently transcribed from their own
397 promoters. Their antitoxins are unstable *cis*- or *trans*-encoded antisense sRNAs that interact
398 with toxin-encoding mRNAs by pairing, thereby inhibiting toxin mRNA translation and/or

399 inducing its degradation (**Figure IA**). Under stress conditions, the RNA antitoxin pool is
400 reduced by specific RNases which in turn induce toxin translation. Most type I toxins are
401 amphipathic peptides with a predicted α -helical transmembrane domain, and they induce pore
402 formation (TisB, HokB) or nucleoid condensation (DinQ, Fst, LdrD, BsrG), that cause
403 membrane potential and/or cell division defects. Some toxins (SymE and RalR) act within the
404 cytosol and can cleave nucleic acids [82].

405 **Type II** genes are in the same operon and co-transcribed [12]. These antitoxins have
406 two domains: a structured N-terminal one with DNA-binding motifs for binding the TA
407 promoter and thus repressing transcription; and a C-terminal domain involved in toxin
408 neutralization by protein-protein interactions. Antitoxins are susceptible to cellular proteases,
409 allowing the activity of stable toxins (**Figure IB**). These toxins function by inhibiting various
410 things: DNA gyrase action (CcdB, ParE); EF-Tu activity during translation (Doc); tRNA
411 synthetase aminoacylation (HipA); or synthesis of tRNA (VapC, AtaT, TacT) or
412 peptidoglycan precursor (PezT). However, most type II toxins are RNases, and either
413 ribosome-independent (MazF, Kid, PemK, MqsR) or ribosome-dependent (RelE, YoeB,
414 HigB) [12].

415 **Type III** toxin and antitoxin genes are co-transcribed and in the same operon. A short,
416 imperfect palindromic repeat between the genes forms a transcription terminator which
417 controls the relative levels of toxin and antitoxin RNA. These toxins, such as ToxN from the
418 ToxIN system, are all RNases that process the RNA antitoxin precursor into smaller RNAs
419 and cleave host RNAs. ToxI antitoxin forms a secondary pseudoknot structure that binds to
420 and blocks the toxin's active site (**Figure IC**) [83,84]. This system induces an abortive phage
421 infection by degrading phage RNAs, promoting the suicide of the phage-infected cells.

422 In **type IV** systems, the antitoxins and toxins do not interact. *E. coli* YeeV and CptA
423 toxins prevent cell division by binding to MreB and FtsZ cytoskeletal proteins, inhibiting their
424 polymerization. YeeU and CptB antitoxins counterbalance this toxic activity by stabilizing
425 MreB and FtsZ cytoskeletal filaments (**Figure ID**) [85].

426 The only **type V** module so far reported is the *ghoST* system in *E. coli* and *Shigella spp.*
427 Here, the GhoS antitoxin is an RNase that inhibits GhoT toxin translation by mRNA cleavage
428 during growth (**Figure IE**) [73]. Under stress conditions, *ghoS* mRNA is degraded by type II
429 MqsR, resulting in the translation of GhoT, a hydrophobic peptide that disrupts cell
430 membranes in the same way as type I toxins.

431 The most recent detection of a **type VI** TA system was in *C. crescentus* [85]. Here, the
432 SocA antitoxin is a proteolytic 'adaptor' protein that inhibits SocB toxicity by promoting its

433 degradation by the ClpXP protease (**Figure IF**). SocB interferes with replication elongation
434 by binding to the DnaN β clamp [86].

435

436 **Figure I: Overview of the Various Types of TA Systems.** Toxins (T) are red and antitoxins
437 (A) are blue. SD points to the Shine-Dalgarno sequence.

438

439 **Box 2: Outstanding Questions**

440 • Are cross-regulations between homologous and non-homologous toxin-antitoxin (TA)
441 systems exceptions or the rule?

442 • At which extent TA systems are interconnected?

443 • Are there cross-regulations involving Type III, IV and VI TA systems?

444 • Can RNA antitoxins and type II antitoxins be considered as global regulators with
445 targets other than their toxin?

446 • How cross-regulations between bacterial TA systems affect their function?

447

448 **Figure legends**

449 **Figure 1: The Absence or Presence of Cross-Regulations between Some Non-Cognate**

450 **Type I TA Systems.** (A) Putative cross-regulations between the five homologues of the

451 *ibs/Sib* system in *Escherichia coli*. An RNA complex forms and creates putative crosstalk

452 between the *ibsA* toxin mRNA and the non-cognate *SibE* RNA antitoxin, allowing *E. coli*

453 growth. (B) No cross-regulation occurs between the homologues of the *sprA/SprA_{AS}* system

454 in *Staphylococcus aureus*. The *SprA_{AS}* cognate antitoxin specifically neutralizes *sprA* toxicity,

455 and RNA complexes and cross-regulations between non-cognate *sprA/SprA_{AS}* RNA pairs are

456 lacking. (C) Cross-regulations between the four homologues of the *sprG/SprF* system in *S.*

457 *aureus*. There are *in vitro* RNA complexes between *SprF1* and *sprG3*, and between *SprF4* and

458 *sprG2*. Cross-regulations occur *in vivo* between *SprF1* and *sprG2* or *sprG3*; between *sprG2*

459 and *SprF1*, *SprF3*, or *sprG1*; and between *SprF4* and *sprG1* or *sprG2*. These cross-regulations

460 are effective in controlling *sprG/SprF* RNA levels and in allowing *S. aureus* growth, but they

461 cannot counteract *SprG* toxicity.

462

463 **Figure 2: Examples of Cross-Regulations between Homologous and Non-homologous**

464 **Type II TA Systems.** The red arrows indicate events related to the presence of cross-

465 regulations. (A) Cross-regulations between the non-homologous, plasmid-encoded *CcdAB*

466 and Kis/Kid TA systems in *Escherichia coli*. The complex identified between the Kis
 467 antitoxin and CcdB toxin enables Kis to decrease CcdB's DNA cleavage, a process mediated
 468 by DNA gyrase, although less efficiently than it decreases with the CcdA-CcdB complex. In
 469 contrast, CcdA cannot neutralize Kid toxicity. (B) Cross-regulations between the plasmid-
 470 encoded VapBC TA system and the chromosome-encoded YefM-YoeB TA systems in *Shigella*
 471 *flexneri*. VapC induction inhibits translation and activates the Lon protease. This protease
 472 degrades the YefM antitoxin, freeing YoeB toxins to cleave mRNA, which in turn promotes
 473 *yefM-yoeB* operon transcription and repopulation of the YefM antitoxin pool. (C) Cross-
 474 regulations between the three homologues of the RelBE TA systems in *Mycobacterium*
 475 *tuberculosis*. Non-cognate antitoxins and toxins form three *in vitro* protein complexes: RelB-
 476 RelG, RelF-RelE, and RelB-RelK. Only RelB neutralizes RelG toxicity and thus permits *M.*
 477 *tuberculosis* growth. On the contrary, RelF and RelB cannot prevent RelE and RelK toxicity,
 478 respectively, triggering *M. tuberculosis* death. (D) Cross-regulations between non-
 479 homologous chromosome-encoded TA systems in *M. tuberculosis*. The induction of several
 480 VapC toxins causes increased *higB1* and *mazF6* mRNA toxin transcript levels, suggesting that
 481 VapC activates HigB1 and MazF6 toxicity.

482

483 **Figure 3: Cross-Regulations between Non-Homologous Toxin-Antitoxin Systems**
 484 **Belonging to Different Types.** The red arrows indicate events related to the presence of
 485 cross-regulations. P_{ghoST} , P_{mqsRA} , $P_{mazEF1-2}$, P_{txpA} and P_{ratA} are TA promoters. T, toxin; A,
 486 antitoxin. (A) Cross-regulations between the *ghoST* type V TA system and the *mqsRA* type II
 487 system in *Escherichia coli*. Cross-regulation event leads to cleavage of the *ghoS* mRNA by
 488 the MqsR toxin, allowing GhoT toxicity. (B) Schematic model of the cross-regulation
 489 between the *mazEF* type II TA system and the *txpA/RatA* type I system in *Enterococcus*
 490 *faecalis*. The red arrow indicates the activator role played by the MazE-MazF complex in
 491 RatA RNA antitoxin transcription allowing RatA to bind *txpA* mRNA and neutralize toxicity.
 492 Drm sequences shared by the P_{mazEF} (*drm1*) and P_{ratA} (*drm2*) promoters are required to fulfil
 493 cross-regulation and allow MazE-MazF complex binding onto *drm2*, activating RatA
 494 transcription.

495

496 **Figure 4 (Key Figure): Overview of the Main Cross-Regulation Events Found in the**
 497 **Toxin-Antitoxin Network.** Red arrows indicate cross-regulations between TA systems. Type
 498 I TA systems (blue) can interact with their homologues, sometimes via RNA complex
 499 formation, to regulate the RNA levels of other type I TA systems. However, type II TA

500 systems (green) appear to be the main actors in a network of possible cross-regulations. By
501 forming complexes and taking advantage of the DNA-binding domain in type II antitoxins,
502 type II TA systems can activate the expression of type I antitoxins and of other homologous or
503 non-homologous type II systems. Type II toxins can also induce the accumulation of non-
504 homologous type II toxin-encoding mRNA fragments and the degradation of type V antitoxins
505 (orange). P, promoter sequence.

506

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516

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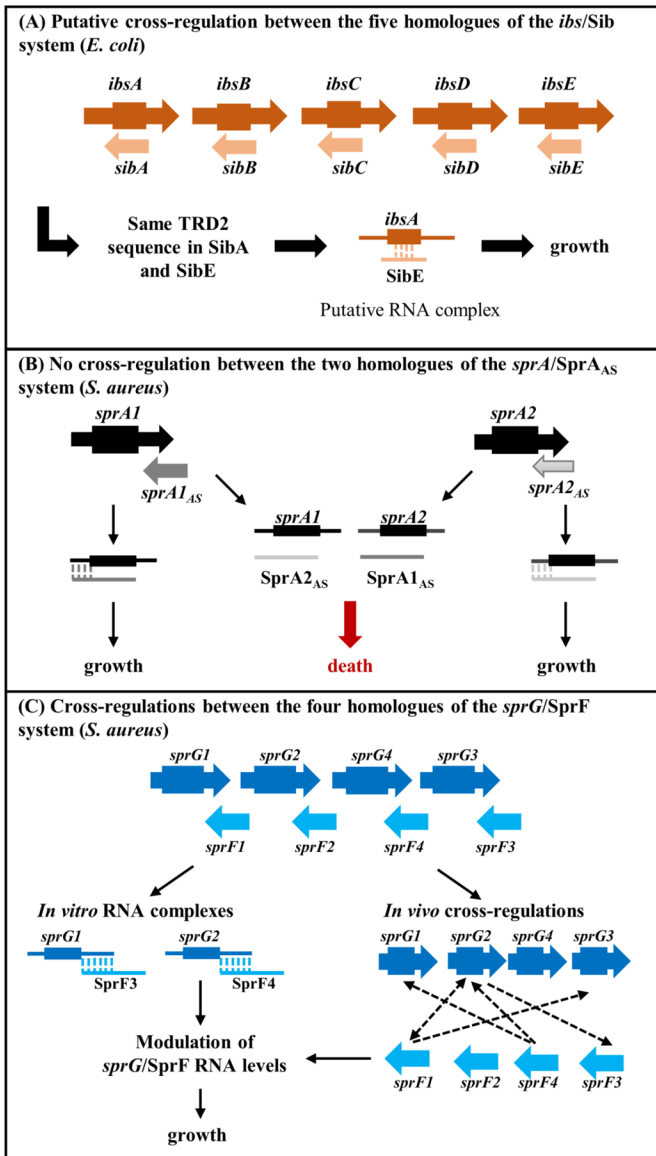


Figure 1

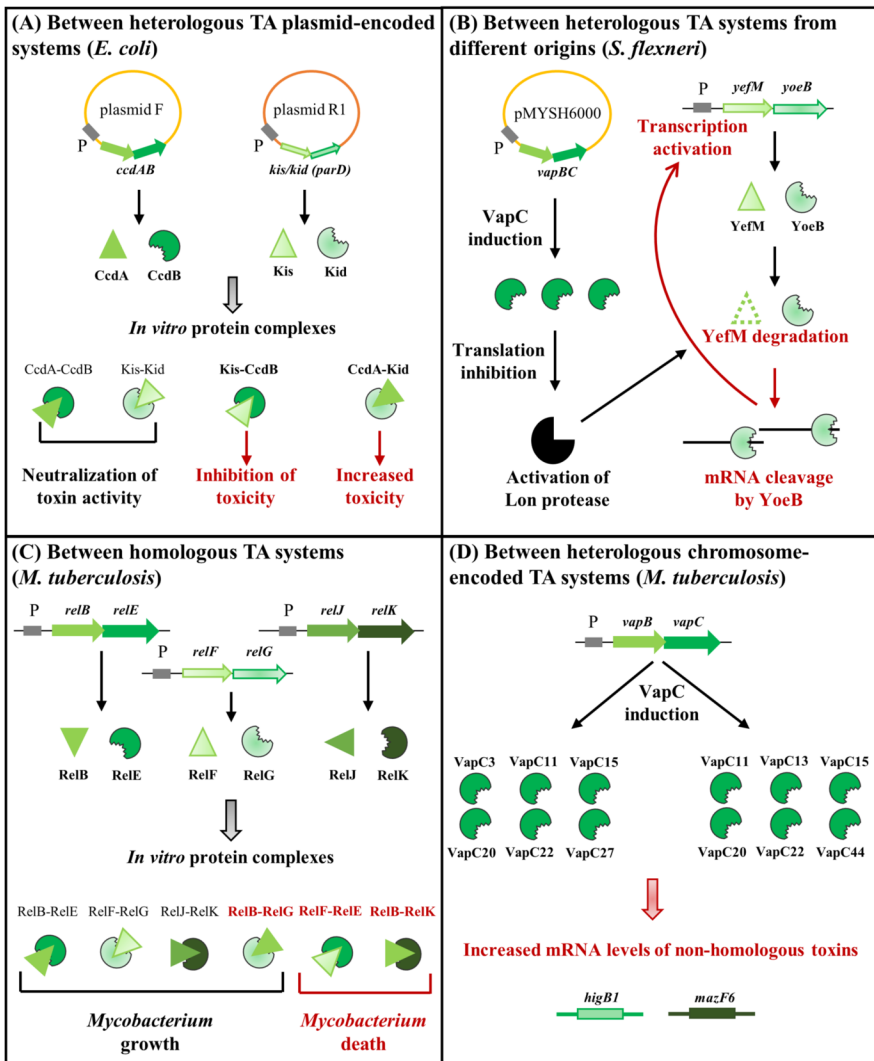


Figure 2

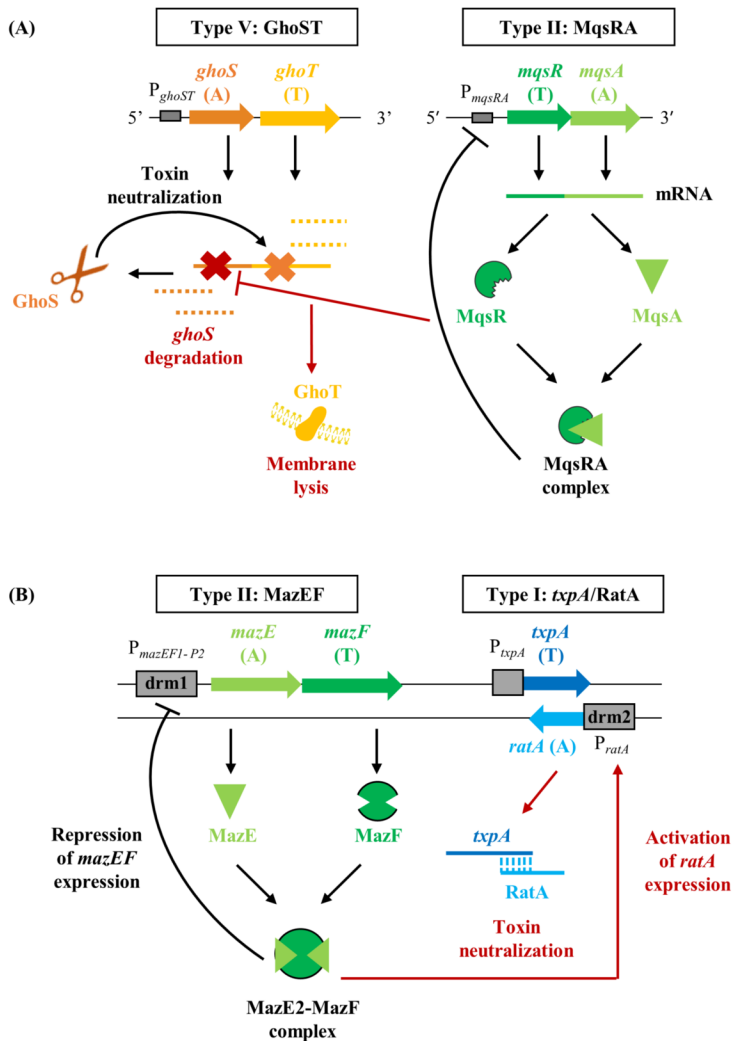


Figure 3

Table 1: Multiple-copy Type I and II toxin-antitoxin systems discussed here

TA system	Bacteria	Location	Copy number	References
Type I TA systems				
Gram-negative bacteria				
<i>ibs/Sib</i>	Enterobacteria	Chromosome	Up to 7	[23]
<i>zor/Orz</i>	<i>Escherichia coli</i>	Chromosome	2	[25]
<i>aapA/IsoA</i>	<i>Helicobacter pylori</i>	Chromosome	6	[26,27]
<i>hok/Sok</i>	Enterobacteria	Plasmid R1, chromosome	Up to 15	[28]
<i>ldr/Rdl</i>	<i>E. coli, Salmonella, Shigella</i>	Chromosome	Up to 10	[32,23]
Gram-positive bacteria				
<i>sprA/SprA_{AS}</i>	<i>Staphylococcus aureus</i>	Chromosome	2	[33-35]
<i>sprG/SprF</i>	<i>S. aureus</i>	Chromosome	4	[33,36,37]
Type II TA systems				
Gram-negative bacteria				
ParDE	<i>Caulobacter crescentus</i>	Chromosome	4	[49]
RelBE	<i>C. crescentus</i>	Chromosome	4	[49]
PaaR-PaaA-ParE	<i>E. coli</i> O157:H7	Chromosome	2	[50,51]
VapBC	<i>Haemophilus influenzae</i>	Chromosome	2	[53]
HigBA	<i>Vibrio cholerae</i> N16961	Chromosome	2	[62]
ParDE	<i>V. cholerae</i> N16961	Chromosome	3	[62]
RelBE	<i>V. cholerae</i> N16961	Chromosome	4	[62]
Gram-positive bacteria				
VapBC	<i>Mycobacterium tuberculosis</i>	Chromosome	Up to 50	[39-41]
MazEF	<i>M. tuberculosis</i>	Chromosome	Up to 10	[39-41]
YefM-YoeB	<i>S. aureus</i>	Chromosome	2	[64,65]
RelBE	<i>M. tuberculosis</i> H37Rv	Chromosome	3	[68]