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Linking bacterial type I toxins with their actions

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Abstract

Bacterial type I toxin-antitoxin systems consist of stable toxin-encoding mRNAs whose expression is counteracted by unstable RNA antitoxins. Accumulating evidence suggests that these players belong to broad regulatory networks influencing overall bacterial physiology. The majority of known transmembrane type I toxic peptides have conserved structural characteristics. However, recent studies demonstrated that their mechanisms of toxicity are diverse and complex. To better assess the current state of the art, type I toxins can be grouped into two classes according to their location and mechanisms of action: membrane-associated toxins acting by pore formation and/or by nucleoid condensation; and cytosolic toxins inducing nucleic acid cleavage. This classification will evolve as a result of future investigations.

Keywords:

1- Introduction

Toxin-antitoxin (TA) systems are widespread in bacterial and archaeal genomes. In these systems, the expression of a stable toxin-encoding messenger RNA (mRNA) leads to stasis or cell death unless an unstable antitoxin counteracts its effects. TA systems were first identified on plasmids, where they ensure plasmid maintenance through post-segregational killing (PSK) mechanisms [1]. TA systems were later identified on chromosomes, sometimes in multiple copies, and on phage-like elements. Although in many cases their functions are unknown, they are involved in programmed cell death, bacterial growth control, defense against the intrusion of foreign genetic material, and persistence [2]. TAs are classified into five types according to the antitoxin's nature and mechanisms of action. Here, we focus exclusively on type I TA systems, characterized by RNA antitoxins that inhibit toxic peptide synthesis [1]. For the four other types, please refer to the review by Goeders and Van Melderen [3].

Type I TA loci can be transmitted by vertical gene transfer or in specific lineages by gene duplications [4]. RNA antitoxins can act in *cis* or in *trans*. The majority of the type I antitoxins identified so far are *cis*-acting antitoxins (e.g., Sib and SprF1 antitoxins [5,6●]), meaning that they overlap and perfectly match the toxin-encoding mRNA. The *trans*-acting antitoxins (e.g., IstR1 and OhsC [5]) are located away from the toxin locus and share often limited sequence complementarities. An exception to this in which a RNA antitoxin controls toxin translation *via* its non-overlapping sequence was recently described in *Staphylococcus aureus* [7]. TA RNA duplex formation can either result in toxin mRNA degradation (e.g., RatA [8]) or, more commonly, result in toxin translation inhibition (e.g., IstR1 [9]), or in the combination of the two regulatory mechanisms (e.g., SR4 [10] and SprF1 [6●]) [11].

Most type I toxins identified to date have conserved structural features: they generally contain less than 60 amino acids are hydrophobic and have a putative α-helical transmembrane domain, except for SymE and RalR [12,13●]. Despite these similarities, the biological functions and mechanisms of action of type I toxins can be very diverse. Therefore, inspired by Jahn and

28 collaborators [14●●], type I TA systems were cataloged in this review based on the toxins'

29 currently-known mechanisms of action, and are summarized here in Table 1. Many studies

30 reporting type I toxin functions were based upon episomal overproduction, and deletion of the

31 modules was not often associated with a phenotype. For this reason, it remains difficult to

32 distinguish between the primary and secondary molecular targets of type I toxins.

33

1/ Membrane-associated type I toxins

35 Some membrane-associated type I toxins have been reported to disrupt membrane integrity

36 through pore formation or *via* nucleoid condensation. However, for many type I toxins, either no, or

37 only one of these mechanisms has been investigated experimentally.

a- Peptides inducing membrane permeation through pore-formation

39 Some type I toxins, similar to the phage holin proteins, are membrane-associated peptides that

40 form pores, resulting in a drop in potential and ATP synthesis. Thus, their overexpression leads to

41 cell death, evidenced by the appearance of "ghost" cells with translucent centers and cell material

42 located at the poles. However, in physiological conditions, these pore-forming peptides are

43 involved in diverse biological functions (see the summary in Figure 1).

44 The Hok toxic peptide in *Escherichia coli*, expressed from the *hok/sok* (or *parB*) locus of the R1

45 plasmid [1], is an example of pore-forming peptide. It was the first type I TA system discovered on

46 plasmids that confers their maintenance by PSK [15]. *hok/sok* homologs were identified in the

47 chromosomes of Gram-negative bacteria, suggesting their involvement in other functions [37,38].

48 In response to nutrient starvation, the stringent response alarmone (p)ppGpp interacts with Obg

49 GTPase, resulting in the induction of the *hokB* toxin-encoding mRNA through an unknown

50 mechanism [16●●]. This moderateoverexpression leads to membrane depolarization responsible

51 for a switch to a persistent state. Persister cells are a bacterial subpopulation that enters into a

52 dormant state and thus becomes tolerant to antibiotics. While the deletion of *hokB* locus has no

53 effect on persistence, possibly due to system redundancies, the overexpression of *hokB* mRNA is

essential for Obg-mediated persistence. Another recent study [17●] has demonstrated the involvement of the *hok/sok* locus in bacterial lag phase expansion. This allows cells to adapt to unfavorable conditions such as elevated temperatures or the presence of antibiotics. In low cell density cultures, the system favors bacterial survival regardless of growth-limiting conditions and could complement existing or defective SOS response mechanism, an inducible DNA repair system [17●]. However, in *E. coli*, some of the *hok/sok* copies can be inactivated by insertion sequences [38].

TisB translated from the "*tisB-istR1*" locus is another pore-forming peptide. The TisB toxic peptide is small, hydrophobic, and spontaneously binds membranes [39]. Chemically-synthesized TisB peptides are assembled as stable transmembrane dimers. The charges on the amphiphilic TisB helix suggest that antiparallel dimers could be assembled by salt bridges, and these narrow channels could enable protons to pass across the hydrophobic membranes. Another study [20] revealed that the positively-charged TisB pores induce selective membrane permeation for hydroxyl anions. In turn, protons and anions passages dissipate the proton motive force (PMF). The TisB pore is so narrow that it is impermeable to intracellular water-soluble components, allowing cell survival. As a consequence, *tisB* mRNA overexpression causes a drop in ATP levels and an increase of cell death even though in physiological conditions it induces a drop in the PMF and the formation of persister cells [20]. By triggering a dormant state, TisB leads to the shutdown of the major antibiotic targets and induces multidrug tolerance (e.g., in β-lactams targeting peptidoglycan synthesis, in aminoglycosides inhibiting translation, and in ciprofloxacin reducing DNA replication by inhibiting topoisomerase activity). This phenotype has only been observed in exponentially-growing *E. coli* cells, corresponding to a higher expression of the genes involved in the SOS response [19]. Persister formation by the SOS-induced TisB toxin involves two strategies for survival: DNA repair activation; and entrance into a dormant state [19].

   b- Peptides inducing nucleoid condensation

The *par* locus, or the RNAI-RNAII TA system (see Figure 2), is expressed from the pAD1 plasmid of *Enterococcus faecalis* and encodes the Fst toxin. The *par* homologs have been detected in low GC Gram-positive bacteria [22]. The Fst toxic peptide possesses a hydrophobic transmembrane domain that is essential for toxicity and conserved within the Fst/Ldr superfamily. This is followed by an unstructured C-terminal tail, probably located in the cytoplasm [22]. Studies focusing on *fst* mRNA overexpression have shown short- and long-term effects of the toxin. Firstly, *fst* mRNA overexpression causes an abnormal nucleoid condensation in *E. faecalis*, *S. aureus*, *Bacillus subtilis,* and *E. coli.* Alteration of peptidoglycan synthesis, cell division and septum placements were also described in *E. faecalis* and *B. subtilis*, resulting in unequal DNA distribution among daughter cells [23,40]. While the early effects of *fst* toxin-encoding mRNA overexpression are at the chromosomal level, its major impact is the membrane stress that induces the transcription of numerous target genes [23]. The majority of these targeted transcripts encode membrane-associated proteins. These proteins include ABC transporters such as glucan, manganese, and glycine-betaine family transporters which are associated with resistance to numerous antibiotics [41]. Transporter overexpression is harmful to the bacteria, probably through depletion of the cellular ATP pool and/or perturbed membrane integrity, as the Fst-induced responses are diverse and nonspecific. This late expression of the transporters may be a response to the Fst-induced chromosomal structure impairment. *fst* toxin-encoding mRNA overexpression also induces cell membrane permeation, without "ghost" cell formation, and the arrest of macromolecular synthesis [42]. RNAI-RNAII is the only type I PSK system described so far in Gram-positive bacteria [23], although numerous *par* homologs have been detected in many bacterial chromosomes [22].

Interestingly, overexpression of the *fst-Sm*/srSm TA system in *Streptococcus mutans* causes a significant decrease in the number of persister cells [34]. Moreover, in the opportunistic pathogen *E. faecalis,* a link was recently identified between the expression of a *par* homolog and virulence [43●●]. The mutant lacking the *par* homolog is more virulent in insect and mouse models, more efficiently colonizes mouse organs, and survives better inside macrophages and upon oxidative

106   stress. The authors suggest that these effects could be involved in the transition from

107   commensalism to virulence [34,43●●].

108   In *E. coli*, overproduction of the LdrD protein, which belongs to the Fst/Ldr superfamily [4], leads to

109   nucleoid condensation [25], suggesting a conserved mechanism of action. Additionally, microarray

110   analysis suggests that overexpression of *ldrD* mRNA upregulates genes involved in the purine

111   metabolism pathway, and reduces cAMP levels [25]. Interestingly, overexpression of the *ldrD*

112   mRNA homolog *ldrA* inhibits ATP synthesis and consequently DNA replication, transcription, and

113   translation, eventually inducing cell growth arrest [44].

114   The *E. coli* DinQ toxic peptide is a small transmembrane peptide located in the inner membrane.

115   Its expression is controlled by AgrB antisense RNA and the repressor LexA which downregulates

116   numerous genes involved in the SOS response [45]. Ectopic overexpression of *dinQ* mRNA

117   induces membrane depolarization [21]. In an *agrB* mutant strain, two-fold constitutive

118   overexpression of *dinQ* mRNA leads to an increase in UV sensitivity associated with a decrease in

119   intracellular ATP and a delay in nucleoid decompaction and extension after UV irradiation.

120   Moreover, elevated levels of *dinQ* mRNA in this mutant have a role in the repair of UV-induced

121   DNA damage through inhibition of conjugal recombination [21]. Thus, the DinQ toxin uses several

122   mechanisms to modulate the *E. coli* UV response.

123   Temperature-sensitive *bsrG*/SR4 system located within the SPβ prophage in *B. subtilis* is probably

124   involved in prophage maintenance within the bacterial chromosome. Indeed, overexpressed *bsrG*

125   mRNA leads to the accumulation of toxic peptides that inhibit cell growth by targeting the

126   cytoplasmic membrane [26]. Nevertheless, *bsrG* mRNA overexpression interferes neither with

127   membrane permeability nor with energy supply [14●●]. *bsrG* mRNA overexpression stimulates

128   fatty acid biosynthesis, which induces invaginations of the cytoplasmic membrane, leading to

129   abnormal membrane topology, distorted cell division planes, and a reduced cell size. This also

130   results in a delocalization of the cell wall synthesis machinery, including the cytoskeletal

131   component MreB normally associated to the membrane and oriented perpendicularly to the longer

132   cell axis. With the assistance of autolytic enzymes, this disturbance of cell wall synthesis

133 machinery leads to cell lysis. Moreover, BsrG toxin induces nucleoid condensation but

134 chromosome segregation and replication are not affected. The nucleoid condensation, in turn,

135 induces a significant global inhibition of transcription and translation [14••].

136 **c- Membrane-associated peptides with a mechanism to determined**

137 There are many other membrane-associated type I toxins with the mechanism of action still to be

138 determined (see Table 1). For many systems, the mechanism was not addressed. For the others,

139 information to classify toxins according to their mechanisms of action is insufficient.

140 Among them, in the human pathogen *S. aureus*, two type I TA systems are located within a

141 pathogenicity island (PI) and express membrane toxic peptides whose overexpression causes

142 bacterial cell death. PepA1, translated from the "*sprA1/sprA1$_{AS}$*" locus, is induced upon acidic and

143 oxidative stresses [46]. PepG1$_{31}$ and PepG1$_{44}$ are both translated from the "*sprG1/sprF1*" locus.

144 Extracellular addition of either the three chemically-synthesized peptides or of membrane extracts

145 prepared from *S. aureus* cells overexpressing PepG1$_{44}$ and PepG1$_{31}$ will trigger the lysis of both

146 competing bacteria and human erythrocytes [6•,7]. NMR structures and dynamic simulations

147 indicate that synthetic PepA1 possesses an α-helical structure and can insert into the membrane.

148 These toxic peptides probably damage bacterial membranes and erythrocytes through pore-

149 formation, membrane disruption due to a detergent-like effect, or by interference with membrane-

150 associated functions, nevertheless we cannot exclude a role of these toxins in nucleoid

151 condensation [46].

152

153 **2/ Cytoplasmic type I toxins that cleave nucleic acids**

154 While a large number of membrane-associated type I toxins have been described, the only

155 reported to be located in the cytoplasm have been SymE and RalR. Contrary to the other type I

156 toxins, SymE and RalR lack putative transmembrane segments and cleave nucleic acids.

157

158 **a- RNA cleavage**

159 SymE is a 113-amino acid peptide that, like the MazE type II antitoxin, contains an AbrB [47].

160 SymE and its homologs constitute a distinct family within the AbrB superfamily. Indeed, all of the

161 other AbrB family members are transcription factors acting as antitoxins, while the SymE

162 homologs have become toxic proteins. SymE toxin synthesis is downregulated additively at

163 multiple levels: at the transcriptional level by LexA; at the mRNA stability level by the *cis*-encoded

164 SymR antitoxin RNA and at the translational level by SymR and Lon proteases [12]. In *E. coli*, the

165 SymE protein co-purifies with ribosomal proteins, and its overexpression drastically affects colony-

166 forming ability and protein synthesis [12]. Overexpression of *symE* mRNA decreases levels of all

167 tested mRNAs and regulatory RNAs except its antitoxin SymR. Moreover, after *symE* mRNA

168 induction, distinct shorter mRNA fragments were observed. These results suggest that SymE acts

169 as an endoribonuclease and cleaves mRNAs that should not be translated [12,48]. The DNA

170 damaging agent mitomycin C induces overexpression of *symE* mRNA at both the mRNA and

171 protein levels, suggesting a role for SymE toxin in simultaneously recycling damaged RNAs and

172 DNAs during the SOS response.

173

174   **b- DNA cleavage**

175 The "*ralR/ralA*" locus is located on the *E. coli rac* prophage. *ralR* mRNA encodes a 64-amino acid

176 type I toxin, and its overexpression leads to cell growth inhibition, while the RalA antitoxin requires

177 the Hfq chaperone to stabilize it [13●]. *In vitro* DNA cleavage assays have shown that purified

178 RalR toxin is a non-specific endonuclease that cleaves methylated or non-methylated DNAs.

179 Furthermore, *in vitro* RNA cleavage assays do not reveal any RNA-targeting cleavage activity.

180 Finally, overproduction of *ralR* mRNA causes a filamentous growth resulting from the SOS

181 response following DNA degradation. The positive effects of the *ralR*/RalA TA system on cell

182 physiology and fosfomycin resistance were confirmed through monitoring the growth and

183 metabolic activity of wild-type and *ralR/ralA* knockout strains in the presence of fosfomycin, an

184 inhibitor of bacterial cell wall biogenesis [13●].

185

2- Concluding remarks and perspectives

When examined together, the effects of some type I toxins on bacterial physiology challenge the concept that membrane-associated type I toxins act solely by pore formation. This is why we propose an updated nomenclature with two classes of type I toxins. These classes are based on peptide location and their mechanisms of action: (i) membrane-associated toxins act either by pore formation and/or nucleoid condensation; and (ii) cytoplasmic toxins that catalyze nucleic acid cleavages. Table 1 summarizes the type I toxins according to the current understanding of their mechanisms of actions, and underlines the two different classes. Our nomenclature only includes the modes of action that are currently well-described, and will thus evolve based on future discoveries, but it provides a solid basis for better understanding of the TA systems. In particular, it would be appropriate to investigate whether, like many other antibacterial peptides, certain type I toxic peptides could induce a membrane disruption *via* the "carpet" mechanism (where peptides bind to the surface of the membrane and provoke a detergent-like effect) [49]. Moreover, the effect of the pore-forming peptides on the nucleoid condensation is usually left unexplored, so it is tempting to speculate that some may act through both mechanisms. Another important challenge would be to determine whether there is heterogeneity in toxin expression within a cell population, along with an exploration of the various triggers and signaling pathways that induce toxin expression. New hypotheses about the biological functions of toxic peptides will arise from a better understanding of their mechanisms and the identification of their molecular targets. Finally, TA systems should be considered as tiny modules embedded in larger regulatory networks where all of the different players interact. Indeed, recent discoveries implying crosstalk between type I and II TAs [50] suggest that numerous interactions involving different TA systems are yet to be discovered.

The unique characteristics of TA loci make them of particular interest for biotechnological applications. TAs can be considered lead compounds for the design of new antimicrobials, plasmid maintenance tools, vaccines, and selective reporter genes [51,52]. Moreover, toxins on their own can also have various applications. Indeed, selective cloning vectors containing variants

213 of IbsC, a type I *E. coli* toxic peptide, have been engineered [53]. In addition, chemical

214 modifications of PepA1 toxin dramatically increased its antibacterial potential and its stability in

215 human serum while considerably reducing its human cell toxicity, implying that toxins can be

216 transformed into potent antibiotics [27]. The DinQ toxin is another promising candidate for the

217 development of anti-cell-envelope antibiotics, notably against *E. coli* infection [54]. These

218 strategies could be applied to other toxic peptides, notably for the design of new antibiotics, thus

219 providing alternatives to the burgeoning issue of bacterial resistance to the drugs presently in use.

Figure captions

Table 1: Overview of the type I TA classes based on the mechanisms of action of toxins.

Figure 1: Reported functions for type I pore-forming toxins.

Some type I toxins expressed in response to stresses (yellow lightning) or after plasmid loss (dark grey) are located within membranes (grey), where they form pores. These pores decrease the membrane potential, cell energy levels and proton motive force (PMF), eventually leading to cell growth arrest or cell lysis of a population fraction. Plasmid maintenance, persistence, and stress adaptation were among the biological functions identified for type I pore-forming toxins located in the membrane.

Figure 2: Fst toxin: an example of a "domino effect" post-segregational killing (PSK).

Within *E. faecalis*, PSK bacterial maintenance of the pAD1 plasmid (dark grey) involves the *par* locus. After bacterial division, RNAI toxin mRNA and RNAII antitoxin are both expressed within the daughter cells carrying the plasmid (at left). They can form a stable RNA duplex that prevents toxin mRNA translation without effecting bacterial growth. Within the daughter cells lacking plasmids (at right), the unstable RNAII antitoxin is degraded and does not downregulate RNAI toxin translation. The toxic peptide is expressed, migrates, and accumulates at the membrane. After 15 minutes, this induces an aberrant nucleoid condensation, altered synthesis of peptidoglycan, abnormal cell division and septum placement, resulting in unequal DNA distribution among the daughter cells. After 60 minutes, probably in response to these initial triggers, Fst toxin induces transcription of numerous target genes including transporters, and results finally in cell death [23],[42].

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Class	Type I toxin mechanisms of action	Mechanisms of action discovered through:	TA systems	Locations	Biological functions (discovered through:)	Refs
Membrane-associated toxins	Pore formation	Overexpression[3]	*hok*/Sok[1]	- *E. coli* R1 plasmid - Numerous Gram- genomes	- Plasmid maintenance (ectopic expression[2]) - Obg-mediated persistence (deletion) - Stress response to temperatures, antibiotics, SOS, etc. (ectopic expression[2])	[15] [16••] [17•]
		Chemically synthesized[4]	*tisB*/IstR1[1]	- *E. coli* chromosome	- SOS-mediated persistence by inducing DNA repair and dormancy (deletion)	[18-20]
	Nucleoid condensation	Overexpression[3]	*dinQ*/AgrB	- *E. coli* chromosome	- SOS response (overexpression[3]) - Conjugal recombination (deletion)	[21]
		Overexpression[3]	*RNAI*/RNAII[1]	- *E. faecalis* pAD1 plasmid	- Plasmid maintenance (ectopic expression[2])	[22,23]
		Overexpression[3]	*ldrD*/RdlD[1]	- *E. coli* chromosome	- Involved in purin metabolism and cAMP levels (overexpression[3])	[24] [25]
		Overexpression[3]	*bsrG*/SR4[1]	- *B. subtilis* SPβ prophage	- Unknown	[14••] [26]
	To be confirmed	Chemically synthesized[4]	*sprA1*/SprA1[AS][1]	-*S. aureus* vΣαβ pathogenicity island	- Acidic and oxidative stress responses (overexpression[3]) - Competing bacterial and human erythrocyte lysis (chemical synthesis[4])	[7] [27]
		Chemically synthesized[4]	*sprG1*/SprF1[1]	- *S. aureus* SaPIn3 pathogenicity island	- Competing bacterial and human erythrocyte lysis (chemical synthesis[4] and membrane extraction)	[6•]
			pnd	- *E. coli* R483 plasmid	- Plasmid maintenance (ectopic expression[2])	[28] [29,30]
			srnB	- *E. coli* F plasmid	- Plasmid maintenance (ectopic expression[2])	[31] [30]
			ibsC/SibC[1]	- *E. coli* chromosome	- Unknown	[32]
			shoB/OhsC	- *E. coli* chromosome	- Heat and oxidative stress response (overexpression[3]) - Induction of carbohydrate transport expression (overexpression[3])	[33] [32]
			zorO/OrzO[1]	- *E. coli* chromosome	- Unknown	[4] [32]
			fst-Sm/SrSm[1]	- *S. mutans* chromosome	- Inhibits persistence (mild-over expression[2])	[34]
			txpA/RatA[1]	- *B. subtilis* skin phage-like element	- Cell metabolic state? (endogenous[5])	[8] [35] [36]
Cytoplasmic toxins	RNA cleavage	Overexpression[3]	*symE*/SymR[1]	- *E. coli* chromosome	- SOS response (endogenous[5]) - Recycling damaged RNAs (overexpression[3])	[12]
	DNA cleavage	Chemically synthesized[4]	*ralR*/RalA	- *E. coli* rac prophage	- Fosfomycin resistance (deletion)	[13•]

[1] TA systems expressed in other bacteria and/or in other copies located in the bacterial genome.

[2] Plasmid maintenance identified using plasmid other than the plasmid of origin expressing the TA locus.

[3] Biological functions identified when the TA systems are highly expressed using overexpression plasmids with endogenous or inducible promoters.

[4] *In vitro* analysis with chemically synthesized peptides.

[5] The environmental conditions induce a modulation of the toxin mRNA expression in the wild-type strain.

Unstable RNA antitoxin

Stable toxin-encoding mRNA

External stresses

Plasmid

Toxin-encoding mRNA translation

Toxic peptide

Pore membrane peptide

↘ Membrane potential (PMF)

↘ Cellular energy levels

Plasmid maintenance

Persistence

Stress adaptation

pAD1

Unstable RNA II

par locus

Stable RNA I

E. faecalis

pAD1

par locus

**sRNA/mRNA translationally
inactive complex**

Plasmid maintenance

RNA II degraded

RNA I toxin translation

**Nucleoid
condensation**

Fst

15'

**Alteration of PG
synthesis**

Transporters ↗

60'

**Unequal DNA distribution
during cell division**

Bacterial death