Plasmid 93 (2017) 30–35

Contents lists available at ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Review

Novel toxins from type II toxin-antitoxin systems with acetyltransferase activity

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ARTICLE INFO

Keywords:
Horizontal gene transfer
GNAT
Acetylation
Toxin-antitoxin
Deacetylation

ABSTRACT

Type II toxin-antitoxin (TA) systems are widespread in bacterial and archeal genomes. These modules are very dynamic and participate in bacterial genome evolution through horizontal gene transfer. TA systems are commonly composed of a labile antitoxin and a stable toxin. Toxins appear to preferentially inhibit the protein synthesis process. Toxins use a variety of molecular mechanisms and target nearly every step of translation to achieve their inhibitory function. This review focuses on a recently identified TA family that includes acetyltransferase toxins. The AtaT and TacT toxins are the best-characterized to date in this family. AtaT and TacT both inhibit translation by acetylating the amino acid charged on tRNAs. However, the specificities of these 2 toxins are different as AtaT inhibits translation initiation by acetylation of the initiator tRNA whereas TacT acetylates elongator tRNAs. The molecular mechanisms of these toxins are discussed, as well as the functions and possible evolutionary origins of this diverse toxin family.

1. Introduction

Bacterial toxin-antitoxin (TA) systems are small modules composed of a stable toxic protein and an unstable antitoxin. The nature and mode of action of the antitoxin are at the basis for TA system classification (for recent reviews, (Goeders and Van Melderen, 2014; Goeders et al., 2016; Hayes and Van Melderen, 2011; Berghoff and Wagner, 2017; Page and Peti, 2016)). Antitoxins can be RNA either inhibiting translation of the cognate toxin (type I) or its activity (type III). Antitoxins can also be proteins and display diverse activities. Type II antitoxins neutralize their cognate toxins by forming a tight antitoxin-toxin complex. Type IV antitoxins protect the target from interactions with the toxin. In type V and VI systems, antitoxins specifically cleave the toxin mRNA or serve as an adaptor to direct toxin to proteolysis, respectively.

This review focuses on type II TA systems. These systems are generally composed of 2 genes organized as an operon, with the antitoxin gene being located upstream the toxin gene. Notable exceptions are the *higBA* and *hipBA* modules in which the gene order is swapped (see below). The antitoxin is generally composed of 2 distinct domains, with a DNA-binding domain (e.g. RHH, HTH, AbrB-like) at the amino-terminus and a toxin-interacting domain at the carboxy-terminus. Antitoxins alone or in complex with cognate toxins have the capacity to bind to palindromic operator sequences in the operon promoter and thereby regulate TA system expression. In some TA modules the toxin is directly involved in the regulatory process acting as a transcription co-repressor or activator depending on the levels of toxin, in a mechanism known as conditional cooperativity (for review, (Loris and Garcia-Pino, 2014)).

With the increase of bacterial genome sequences and data mining, the paradigm for canonical type II TA systems has evolved (Leplae et al., 2011; Anantharaman and Aravind, 2003). It appears that the genetic organization of these systems is more modular than previously thought. Type II TA systems can be organized in 'reverse' order with the toxin gene preceding that of the toxin (Budde et al., 2007; Christensen-Dalsgaard et al., 2010; Jorgensen et al., 2009). In a few examples, a third component can be part of the TA operon. This comes today in 2 flavors – systems in which transcriptional regulation function is ensured by a regulator located upstream the antitoxin and toxin genes (Hallez et al., 2010; de la Hoz et al., 2000) have been described as well as TAC (toxin-antitoxin-chaperon) systems in which the third gene of the operon encodes a SecB chaperone homologue that is needed for antitoxin folding and activity (Sala et al., 2014).

Type II systems were originally discovered on plasmids in the late 80s. The function of these modules is to ensure plasmid maintenance in growing bacterial populations. They act by killing daughter-bacteria devoid of a plasmid copy. This phenomenon was termed post-segregational killing (Gerdes et al., 1986) and later on addiction (Yarmolinski,

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http://dx.doi.org/10.1016/j.plasmid.2017.08.005
Received 4 August 2017; Received in revised form 24 August 2017; Accepted 28 August 2017
Available online 20 September 2017
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Table 1
Eight super-families of type II toxins: activity and process inhibition.

Toxin super-family	Toxin	Activity	Inhibition	References
ParE/RelE	RelE	Ribosome-dependent mRNA cleavage	Translation elongation	(Pedersen et al., 2003)
	ParE	DNA-gyrase inhibition	Replication, SOS induction	(Jiang et al., 2002)
CcdB/MazF	CcdB	DNA-gyrase inhibition	Replication, SOS induction	(Bernard and Couturier, 1992)
	MazF	Free mRNAs cleavage	Translation initiation	(Zhang et al., 2003)
	MazF*mt6*M. tb	Helix/loop 70 of domain IV of 23S rRNA	Translation elongation	(Schifano et al., 2013)
	MazF*mt9*M. tb	Anticodon and D loop of elongator tRNAs	Translation elongation	(Schifano et al., 2016)
Fic	Doc	Phosphorylation of EF-Tu elongation factor	Translation elongation	(Castro-Roa et al., 2013)
	Fic	DNA-gyrase and TopoIV adenylylation	DNA topology	(Harms et al., 2015)
PIN	Enteric VapC, Leptospiral VapC	Anticodon stem-loop of initiator tRNA	Translation initiation	(Winther and Gerdes, 2011; Lopes et al., 2014)
	M.tb. VapC20, VapC26	cleavage	Translation elongation	
	Other mycobacterial VapCs	Sarcin–Ricin loop of 23S rRNA	Translation elongation	(Winther et al., 2013; Winther et al., 2016)
		Anticodon stem-loops of elongation tRNAs		(Winther et al., 2016)
HipA	HipA	Phosphorylation of Glutamyl-tRNA-synthetase	Translation elongation	(Germain et al., 2013; Kaspy et al., 2013)
YafO	YafO	Ribosome-dependent mRNA cleavage	Translation elongation	(Zhang et al., 2009)
Zeta	Zeta	Phosphorylation of UDP-Glc-NAc	Peptidoglycan synthesis	(Mutschler et al., 2011)
AtaT/TacT	AtaT	Acetylation of initiator tRNA	Translation initiation	(Jurenas et al., 2017)
	TacT	Acetylation of elongation tRNAs	Translation elongation	(Cheverton et al., 2016)

M.tb.: *Mycobacterium tuberculosis*.

1995). As mentioned above, antitoxins are unstable as compared to toxins. In plasmid-free daughter cells, since antitoxin is not replenished, the toxin will be liberated from the antitoxin-toxin complex. The free toxin will then act on its target to inhibit cell growth and eventually cause cell death. This will lead to apparent plasmid stabilization by reducing the frequency of plasmid-free cells. Another hypothesis proposes that these systems increase fitness of their host replicons by outcompeting competitor plasmids from the same incompatibility group (Cooper and Heinemann, 2000). Moreover, mathematical models developed on restriction-modification systems, but transposable to TA systems, predict that the addiction phenomenon allows propagation of these modules in bacterial populations independently of their original frequencies (Mochizuki et al., 2006). This might provide a rationale explanation for the high prevalence of these systems. Multiple studies have emphasized the abundance of type II TA systems in bacterial chromosomes (see e.g. (Leplae et al., 2011; Anantharaman and Aravind, 2003; Guglielmini and Van Melderen, 2011; Pandey and Gerdes, 2005; Ramisetty and Santhosh, 2016; Coray et al., 2017)). It is commonly accepted that these systems move through horizontal gene transfer and integrate into bacterial chromosomes as part of genomic islands (phages, transposons) or constitute genomic islets on their own. The role of these systems when located in chromosomes remains an interesting debate in the field (for reviews, (Diaz-Orejas et al., 2017; Kedzierska and Hayes, 2016; Lobato-Marquez et al., 2016)). The role in phages, transposons and genomic islands is most likely reminiscent of the addiction function: they serve to maintain these integrated mobile elements (Wozniak and Waldor, 2009; Huguet et al., 2016; Yao et al., 2015). This is best exemplified by TA systems encoded by superintegron gene cassettes (Escudero et al., 2015). These addiction cassettes might prevent the loss of silent cassettes and favor the formation of large integron arrays. Other systems, whether they are located in chromosomes or on plasmids, might serve as defense mechanisms against invasion by phages (Abortive infection, Abi) (Koga et al., 2011) or against plasmid establishment (anti-addiction) (Saavedra De Bast et al., 2008). On a more controversial side, type II systems have been recently associated to persistence and antibiotic tolerance in *E. coli* (Maisonneuve et al., 2011). The basis of the mainstream persistence model relies on TA-induced dormancy, a physiological state that allows bacteria to tolerate stress such as antibiotics or intracellular conditions. Activation of toxins inhibiting translation leads to such a dormant state. However, contradictory data questioning this model have been published recently (Ramisetty et al., 2016; Van Melderen and Wood, 2017; Shan et al., 2017). Nevertheless, TA systems have been shown to be involved in *Salmonella typhimurium* intracellular persistence in macrophages, the natural cell type infected by this pathogen (Helaine et al., 2014) as well as in fibroblast and epithelial cells (Lobato-Marquez et al., 2015). In line with this finding, TA systems were reported to be associated with virulence in other bacterial pathogens (for recent reviews, (Kedzierska and Hayes, 2016; Lobato-Marquez et al., 2016)). However, the precise molecular mechanisms of activation of TA systems in the different in vivo models remain to be elucidated.

2. Diversity of molecular mechanisms of type II toxins

Remarkably, activity of type II TA toxins appears to be mainly directed towards translation (for reviews, (Goeders and Van Melderen, 2014; Page and Peti, 2016; Guglielmini and Van Melderen, 2011)). Toxins targeting nearly every step of the translation process and using diverse molecular mechanisms have been described. In the context of TA-induced dormancy, inhibition of translation is certainly sufficient to halt cell metabolism and growth and does not preclude returning to a normal physiological state upon toxin neutralization. In addition, inhibition of protein synthesis is less harmful than other type of activities such as inhibition of replication or peptidoglycan synthesis. Thus, toxins inhibiting translation might have been selected preferentially during evolution rather than other more ‘toxic’ activities (Guglielmini and Van Melderen, 2011).

Seven main super-families of toxins have been defined based on amino acid sequence similarity and three-dimensional structures (Table 1). Our group, as well as others, recently identified and characterized a novel toxin super-family, presenting novel toxin-fold and activity (see below) (Cheverton et al., 2016; Jurenas et al., 2017).

Commonly, toxins from the same super-family share very low sequence similarity indicating functional divergence in the time of evolution (Leplae et al., 2011). Accordingly, different members, although sharing a similar fold, show distinct activities and targets. In the CcdB/MazF super-family, CcdB-type of toxins are DNA-gyrase inhibitors leading to replication inhibition and SOS induction while MazF toxins are endoribonucleases cleaving free mRNAs with relaxed specificity, elongator tRNAs or the 23S rRNA (Bernard and Couturier, 1992; Zhang et al., 2003; Schifano et al., 2013). Divergence is also observed within the RelE/ParE super-family. While RelE toxins are endoribonuclease cleaving mRNAs in a ribosome-dependent manner, also with relaxed specificity (Goeders et al., 2013; Feng et al., 2013), ParE toxins are thought to inhibit DNA-gyrase (Jiang et al., 2002). This leads to replication inhibition and SOS system induction as observed in the case of CcdB, although the precise molecular mechanism is likely to be different since mutants resistant to CcdB are sensitive to some of the ParE toxins (Yuan et al., 2010).

Toxins with the Fic-fold also display different activities and targets.

Typically, Fic-fold enzymes perform adenylylation, an example of toxin presenting such activity towards DNA gyrase and topoisomerase IV has been shown (Harms et al., 2015). On the contrary, the well-studied Doc toxin from Fic family phosphorylates the translation elongation factor EF-Tu (Castro-Roa et al., 2013).

Toxins from the PIN super-family although being endoribonucleases have different specificities. Some VapC toxins are cleaving 23S rRNA and other the stem loop of initiation tRNA or of various elongation tRNAs as well as the sarcin-ricin loop of the 23S rRNA (Winther and Gerdes, 2011; Lopes et al., 2014; Winther et al., 2013, 2016). Finally, HipA, YafO and Zeta toxins appear to have more specific activities. While YafO and HipA are translation inhibitors, Zeta inhibits peptidoglycan synthesis. YafO cleaves mRNAs in a ribosome-dependent manner as RelE toxins, although without sharing any similarity at the sequence and fold-level (Zhang et al., 2009). HipA is a kinase, distinct from Doc, that phosphorylates glutamyl-tRNA synthetase (Germain et al., 2013; Kaspy et al., 2013). Zeta toxins are phosphorylating and inactivating the essential intermediate uridine diphosphate-*N*-acetylglucosamine (UNAG) for peptidoglycan synthesis (Mutschler et al., 2011). In addition to UNAG inactivation, Zeta toxin was reported to alter various cellular responses in stress conditions (Tabone et al., 2014). It is likely that homologous toxins of these families with different activities and/or specificities are awaiting discovery.

3. Novel type II toxins with GNAT-fold

Novel type II TA systems have been identified recently in bacteria (Lobato-Marquez et al., 2015; Cheverton et al., 2016; Jurenas et al., 2017; McVicker and Tang, 2016; Iqbal et al., 2015). Interestingly, they comprise a novel super-family of toxins presenting a GNAT-fold (GCN5-related *N*-acetyltransferases). Molecular mechanisms of action have been characterized in details for the AtaT and TacT toxins (Cheverton et al., 2016; Jurenas et al., 2017). Both toxins are targeting translation by a novel mechanism, which relies on acetylation of tRNAs. Interestingly, these 2 homologous toxins show different specificities, AtaT targets translation initiation whereas TacT targets elongation (Table 1, see below).

GNAT is a large family of enzymes typically catalyzing acetylation using acetyl-Coenzyme A (Ac-CoA) as acetyl donor (for recent reviews, (Salah Ud-Din et al., 2016; Favrot et al., 2016)). This family is conserved and ubiquitous in all domains of life targeting a vast variety of substrates, from proteins to small metabolites and antibiotics.

Protein acetylation as post-translational modification comes in two flavors, i.e. acetylation of ε-amine group of lysines and acetylation of free α-amine group of first amino acid in peptide chains. εN-acetylation of lysines in bacteria was recently shown to largely result from chemical acetylation by acetyl-phosphate, however several examples of enzymatic reversible acetylation have been reported to play important role in metabolism (Weinert et al., 2013; Hentchel and Escalante-Semerena, 2015). αN-acetylation of proteins although very common in eukaryotes seems to be restricted to only several examples in bacteria (Favrot et al., 2016; Vetting et al., 2008).

Several families of enzymes possessing a GNAT domain are involved in bacterial translation by acetylating ribosomal proteins or tRNAs (Favrot et al., 2016; Ikeuchi et al., 2008). Rim proteins represent one class of such enzymes. Until recently Rim was the only known family that performed αN-acetylation in bacteria. RimJ, RimI and RimL proteins specifically acetylate ribosomal proteins S5, S18 and L12, respectively (Vetting et al., 2008; Cumberlidge and Isono, 1979; Isono and Isono, 1980). Despite the specificity of each Rim towards its target, the role of these modifications in translation remains unclear (Favrot et al., 2016; Vetting et al., 2008). Other families of GNATs are acetylating tRNAs. For example, TmcA acetylates the cytidine in the anticodon (CUA) wobble base of elongator $tRNA^{Met}$ and therefore prevents misreading of the similar AUA codon (Ikeuchi et al., 2008).

A particular class of GNAT concerns the Fem enzyme family. Although GNATs typically use Ac-CoA to transfer acetyl group to their target, Fem-like enzymes instead use amino-acylated tRNA as an amino-acyl donor. These enzymes contribute to cell wall homeostasis and methicillin resistance by catalyzing the addition of specific amino acids to precursors of peptidoglycan (Favrot et al., 2016). Interestingly, Fem enzymes were shown to use amino-acyl tRNAs that bind poorly to elongation factor EF-Tu and therefore do not participate to translation, representing a specialized class of tRNAs (Villet et al., 2007; Giannouli et al., 2009).

These different instances illustrate the versatility of the GNAT fold in terms of the substrates they can process and the functions they are involved with.

We recently discovered a novel canonical type II TA system comprising a GNAT toxin associated to a RHH antitoxin in the chromosome of the pathogenic *E. coli* O157:H7 strain (Jurenas et al., 2017) (Table 1). We have demonstrated that GNAT-fold toxin AtaT forms a typical functional type II TA module with its antitoxin AtaR. Free AtaT toxin causes translation arrest at initiation step, which manifests in accumulation of ribosome assembly intermediates. We have shown that AtaT transfers an acetyl group from Ac-CoA to the free amine group of the initiator tRNA (Met-$tRNA^{fMet}$). In contrast to the initiation compatible formyl-Met-$tRNA^{fMet}$, the affinity of acetyl-Met-$tRNA^{fMet}$ for the initiation factor II (IF2) is severely compromised precluding the formation of the 30S initiation complex (Fig. 1a). Considering that the acetyl modification on the initiator tRNA is likely not reversible (see below) we propose that besides affecting the formation of the 30S initiation complex, the action of AtaT leads to the formation of a dead-end product. By outcompeting the activity of L-methionyl-tRNAfMet-*N*-formyltransferase (FMT) this would lead to the failure of translation initiation.

Another TA system comprising the TacT GNAT-fold toxin in *Salmonella typhimurium* has been reported to inhibit translation (Cheverton et al., 2016). TacT acetylates the free amine group of several elongation tRNAs (Cheverton et al., 2016). The precise mechanism leading to translation inhibition downstream the initial acetylation of tRNAs remains to be uncovered. However, it is likely that acetylation of target tRNAs by TacT prevents interaction with EF-Tu and formation of the ternary complex (Fig. 1B), as it was shown previously for chemically acetylated tRNAs (Janiak et al., 1990).

The degree of sequence identity between AtaT and TacT is low (24%) (Fig. 2), thus it is not surprising that these toxins show different specificity. Moreover, AtaT seems to be highly specific. The enzyme is capable of discriminating between the two tRNAs carrying methionine – initiator and elongator Met-tRNA, in contrast with TacT that appears

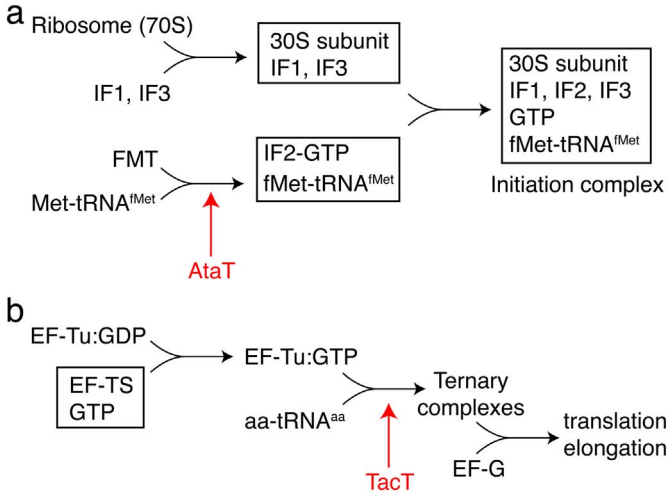


Fig. 1. Mode of action of AtaT and TacT toxins. (A) AtaT acetylates the initiator tRNA thereby preventing its interaction with IF2. (B) TacT acetylates several elongator tRNAs thereby preventing ternary complex formation.

A

	AtaT	AtaTpl	VCA0318	VCA0481	VCA0487	BceGNAT	AtaT2	TacT	GmvT
AtaT		61.9%	28.5%	28.5%	28.0%	28.6%	29.2%	24.2%	26.3%
AtaTpl	61.9%		25.6%	25.6%	26.3%	24.4%	27.9%	20.8%	22.8%
VCA0318	28.5%	25.6%		100.0%	28.2%	31.6%	25.7%	24.9%	26.0%
VCA0481	28.5%	25.6%	100.0%		28.2%	31.6%	25.7%	24.9%	26.0%
VCA0487	28.0%	26.3%	28.2%	28.2%		27.5%	24.3%	27.4%	27.1%
BceGNAT	28.6%	24.4%	31.6%	31.6%	27.5%		29.6%	31.9%	30.1%
AtaT2	29.2%	27.9%	25.7%	25.7%	24.3%	29.6%		43.8%	43.4%
TacT	24.2%	20.8%	24.9%	24.9%	27.4%	31.9%	43.8%		87.0%
GmvT	26.3%	22.8%	26.0%	26.0%	27.1%	30.1%	43.4%	87.0%	

B

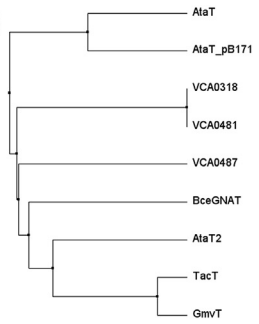


Fig. 2. Homologous GNAT-domain toxins. (A) Percentage of identity between GNAT-domain toxins described in (Cheverton et al., 2016; Jurenas et al., 2017; McVicker and Tang, 2016; Iqbal et al., 2015; Van Acker et al., 2014) and our unpublished data. The identities were retrieved from pairwise sequence alignments performed using EMBOSS Needle tool (EMBL-EBI). (B) Average distance tree using percentage of identity. The tree was generated using Jalview (Waterhouse et al., 2009) based on multiple amino acid sequence alignments performed using Clustal Omega tool (EMBL-EBI). VCA0318, VCA0481 and VCA0487 are toxins form Vibrio cholerae (Iqbal et al., 2015), BceGNAT from *Burkholderia cenocepacia* (Van Acker et al., 2014) and GmvT from the *Shigella flexeneri* pINV virulence plasmid (McVicker and Tang, 2016).

to target several elongation aa-tRNAs.

As mentioned above, toxins from the VapC family also target tRNAs. VapCs cleaving tRNAs at the anticodon loop also show different levels of specificity (Table 1). As an example, VapC4 from *Mycobacterium* appears to be specific for tRNA44 Cys-GCA, but not for other Cys accepting tRNA with the same anticodon such as tRNA40Cys-GCA, while VapC11 cleaved three different tRNAs (Winther et al., 2016).

In comparison to other known GNAT enzymes, AtaT and TacT toxins present unique activity. They recognize tRNA in the manner of TmcA- and Fem-like enzymes, but perform αN- acetylation of an amino acid in a Rim- like fashion. In addition, AtaT is also able to discriminate the tRNA species carrying the same amino acid similar to Fem-like enzymes. Further structural and bioinformatics studies will be required to decipher the evolution of the GNAT enzymes.

4. Mobile GNAT-fold toxins

In addition to AtaRT and TacAT systems, several other GNAT-toxin based TA systems were identified in various bacterial species e.g. in *Burkholderia cenocepacia* (Van Acker et al., 2014), on the *Shigella flexeneri* pINV virulence plasmid (GmvAT system) (McVicker and Tang, 2016) and in the superintegron of *Vibrio cholerae* (Iqbal et al., 2015). The degree of identity between these different toxins varies (Fig. 2) and information regarding activity and specificity of these toxins is not yet available.

We recently identified 2 other GNAT-based TA systems (our unpublished data). We showed that these systems constitute bona fide TA systems (Fig. 3A).

One of these systems, *ataRT*$_2$, is located in the O157:H7 chromosome (as *ataRT*) and appears quite distant from AtaRT since antitoxins and toxins share only 21% and 29% identity, respectively (Fig. 2). However, this system is closely related to the plasmidic GmvAT system and to the *Salmonella* chromosomal TacAT system (around 43% identity) (Fig. 2). A closer homologue of *ataRT* (*ataRT*$_{pl}$) was identified on a pB171-like plasmid. This plasmid and its derivatives are carried by enteropathogenic *E. coli* (EPEC) strains and encode an adherence factor contributing to virulence of EPEC (Levine et al., 1985; Tobe et al., 1999). The AtaR$_{pl}$ and AtaT$_{pl}$ present a high degree of identity with AtaR and AtaT (68.5 and 62%, respectively). Similar systems (more than 60% of sequence identity) are detected in large plasmids from enteropathogenic bacteria (Fig. 4A). They are located close to the replication origin and surrounded by transposition and integration elements (IS, transposase, recombinase, integrase) and other TA systems (*ccd*, *vapBC*) (Fig. 4B).

Interestingly, both the AtatRT and AtaRT$_2$ systems appear to be part of the accessory genome of *E. coli* (our unpublished data). They are detected in EHEC (enterohemorragic *E. coli*) strains but not in classical lab strains such as MG1655 or other *E. coli* strains. In O157:H7 genome, *ataRT* constitutes a small genomic islet, inserted at the branched-chain amino acid transport locus *liv* at the corresponding location of REPv259a/REP269b (Repetitive Extragenic Palindrome) sequences in *E. coli* MG1655 strain. The *ataRT*$_2$ module also forms a small genomic islet located between the *rtcBA* operon encoding the RNA 3′-terminal phosphate cyclase and 3′-5′ RNA ligase and the *malT* transcriptional activator. At the corresponding location in MG1655 genome lies a REP sequence (REPt255) as well. It is worth to mention that REP sequences may act as hot spots of transposition and recombination events (Tobes and Pareja, 2006), opening the possibility that these TA systems 'jumped' into the chromosome from plasmids. This might have happened quite recently since they are not conserved in all *E. coli* strains. Heterogeneous distribution is also observed for other TA systems within the *E. coli* species as well as in bacterial and archeal genomes in general,

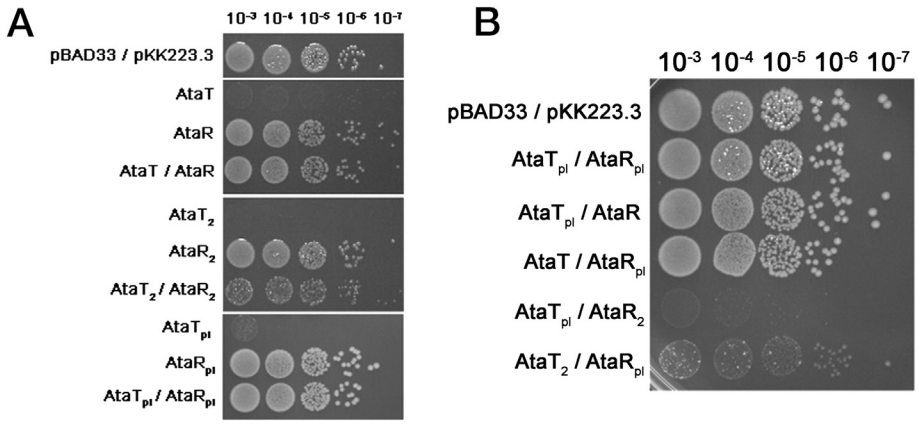


Fig. 3. Cross-talk between the AtaRT, AtaRT$_2$ and AtaRT$_{pl}$ TA systems. MG1655 was co-transformed with pBAD33 derivatives carrying the toxin *ataT* gene as control, the *ataT*$_2$ from O157:H7 or *ataTpl* from pB171 plasmid under the control of the pBAD promoter and pKK223-3 derivatives containing the antitoxin genes, *ataR*, *ataR*$_2$ or *ataR*$_{pl}$ under the control of pTac promoter. Overnight cultures were serially diluted as indicated and spotted on LB agar plates containing 0.2% arabinose and 1 mM IPTG. Plates were incubated for 16 h at 37 °C. (A) AtaRT$_2$ and AtaRT$_{pl}$ constitute functional TA systems. Killing/rescue assays for *ataRT*$_2$ and *ataRT*$_{pl}$ toxin and antitoxin genes, respectively. The *ataRT* system was used as a control. (B) The AtaRT and AtaRT$_{pl}$ systems are able to cross-talk. Killing/rescue assays for *ataT*$_{pl}$ toxin gene with *ataR*$_{pl}$, *ataR* and *ataR*$_2$ antitoxin genes, respectively and *ataR*$_{pl}$ antitoxin gene with *ataT* and *ataT*$_2$ toxin genes, respectively.

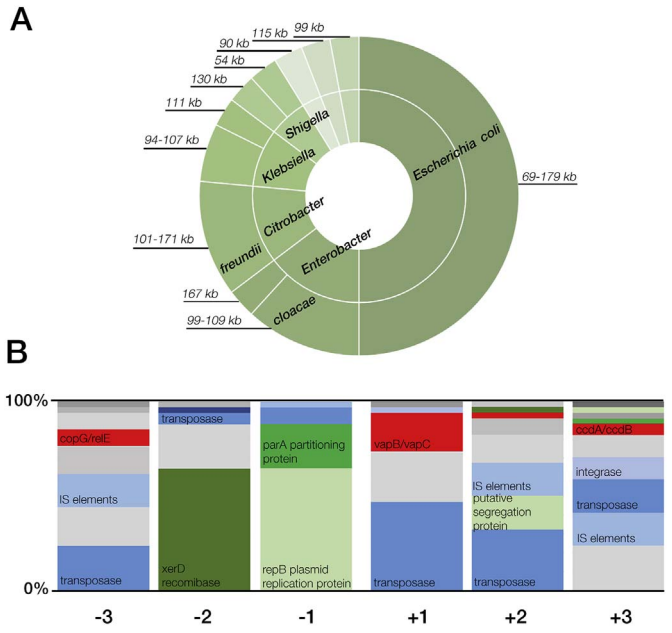


Fig. 4. Distribution and genetic context of *ataRT* module in plasmids. Amino acid sequence of the AtaT toxin from *E. coli* O157:H7 EDL933 strain (Jurenas et al., 2017) was used for TblastN against non-redundant nucleotide collection database using BLOSUM62 matrix and retrieving plasmid sequences coding for homologues sharing minimum 67% amino acid sequence identity with AtaT or $AtaT_{pl}$. (A) Sunburst diagram representing the plasmid size and host distribution (n = 34), different shades represent different genus. (B) Genetic neighborhood of the *ataRT* system on plasmids (n = 34). Green shades illustrate plasmid replication and partitioning genes, the blue shades indicate transposition and integration elements, the red color indicate toxin-antitoxin genes, and the grey shades indicate other functions and hypothetical proteins. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

clearly indicates that these systems are prone to horizontal gene transfer (Leplae et al., 2011; Anantharaman and Aravind, 2003; Guglielmini and Van Melderen, 2011; Pandey and Gerdes, 2005; Ramisetty and Santhosh, 2016; Makarova et al., 2009; Fiedoruk et al., 2015; Mine et al., 2009).

5. Revisiting functions of GNAT-fold toxins

As mentioned above, the TacT toxin from *Salmonella Typhimurium* contributes to intracellular persistence in macrophages (Helaine et al., 2014; Cheverton et al., 2016). Corruption of translation elongation via acetylation of several tRNAs will rapidly halt cell growth. The question of reversibility is essential in this process since bacteria at one point will have to resume growth to propagate. Interestingly, Helaine and coworkers identified the Pth enzyme (peptidyl-tRNA hydrolase) as responsible for reversing the action of TacT. In case of premature translation termination, this essential enzyme hydrolyzes the ester bond between the carboxy-terminus of the nascent peptide chain and the 3′ hydroxyl of tRNA (Sharma et al., 2014). The authors showed that Pth releases acetylated amino acids from tRNAs, effectively uncharging the tRNAs that then become available for acylation by the corresponding aminoacyl-tRNA synthetases. Accordingly, in vivo overexpression of Pth abolishes TacT toxicity and leads to a decrease in persister formation (Cheverton et al., 2016). In addition, deletion of *cobB* (a non specific deacetylase (Starai and Escalante-Semerena, 2004)) abolished the dependency of TacT toxicity on bacterial growth phase and increased the activity of TacT (although *cobB* overexpression did not counteract the action of TacT). This body of data suggests there are several mechanisms implicated in rescuing the cell from the action of TacT in *Salmonella* (Cheverton et al., 2016).

Regarding the toxin AtaT, which acetylates the initiator tRNA, we could not detect any rescuing effect from Pth and CobB (our unpublished data). This suggests that the activity of AtaT might not be reversible, at least not by the mechanisms described for TacT. This might also rule out the possibility that AtaT is involved in persistence. As mentioned in the introduction, chromosomally-encoded TA systems are involved in anti-addiction (Saavedra De Bast et al., 2008). We therefore tested whether AtaR was able to counteract the toxic activity of the plasmid-encoded $AtaT_{pl}$. Our results show that indeed, the chromosomal antitoxin counteracts the plasmidic one. This observation indicates that the AtaRT system might be involved in anti-addiction (Fig. 3B). It is worth mentioning that the $AtaRT_{pl}$ system is absent in the pO157 plasmid commonly found in O157:H7 strains. Interestingly, the more distant $AtaRT_2$ module is a 'stand-alone' module and does not cross-react neither with AtaRT nor $AtaRT_{pl}$ (Fig. 3B, unpublished data). As mentioned above, $AtaRT_2$ is closely related to TacAT and the plasmid-encoded GmvAT system. It was shown that the GmvAT system stabilizes the *Shigella flexeneri* pINV virulence plasmid (McVicker and Tang, 2016). Thus $AtaRT_2$ might also serve as anti-addiction module against plasmid carrying GmvAT-like TA systems.

6. Conclusions

As exemplified above, activities of type II toxins are quite diverse. The newly discovered GNAT-toxins represent a distinct family of toxins with unique activity and targets. The two members of this family characterized so far inhibit translation by acetylating the amine group of amino acid charged on tRNAs. AtaT modifies specifically the methionine charged on the initiator $tRNA^{fmet}$ thereby interfering with formylation and IF2 recognition. This in turn prevents the formation of the 30S initiation complex and inhibits translation. TacT displays relaxed specificity and acetylates several elongator tRNAs.

Another difference is the capacity to reverse the toxin action. While the Pth and CobB enzymes reverse the action of TacT, these enzymes appear unable to reverse AtaT-mediated acetylation. This might highlight important differences in the role of these two toxins. While TacT was clearly shown to be involved in persistence of Salmonella inside macrophages, we hypothesized that AtaT might function as anti-addiction system. GNATs display versatile activities and modify a wide range of molecules, from small metabolites, antibiotics, proteins, tRNAs, etc. AtaT and TacT toxins represent novel acetyltransferase enzymes. How these diversified activities have evolved remains certainly an interesting question in the field.

Acknowledgements

D.J. is a PhD fellow at FNRS (aspirant FNRS). Work in CM2 is supported by the Fonds National de la Recherche Scientifique (FNRS, grant number: T.0147.15F PDR and J.0061.16F CDR and F.4505.16 MIS), the Fonds d'Encouragement à la Recherche ULB (FER-ULB), the Interuniversity Attraction Poles Program initiated by the Belgian Science Policy Office (MICRODEV), Fonds Jean Brachet and Fondation Van Buuren.

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