RNA Biology 12:9, 933–937; September 2015; Published with license by Taylor & Francis Group, LLC

A Type III protein-RNA toxin-antitoxin system from *Bacillus thuringiensis* promotes plasmid retention during spore development

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Keywords: Bacillus cereus group, endoribonuclease, post-segregation killing, RNA antitoxin, sporulation, Type III Toxin-Antitoxin

Members of the *Bacillus cereus sensu lato* group of bacteria often contain multiple large plasmids, including those encoding virulence factors in *B. anthracis*. *Bacillus* species can develop into spores in response to stress. During sporulation the genomic content of the cell is heavily compressed, which could result in counterselection of extrachromosomal genomic elements, unless they have robust stabilization and segregation systems. Toxin-antitoxin (TA) systems are near-ubiquitous in prokaryotes and have multiple biological roles, including plasmid stabilization during vegetative growth. Here, we have shown that a Type III TA system, based on an RNA antitoxin and endoribonuclease toxin, from plasmid pAW63 in *Bacillus thuringiensis* serovar *kurstaki* HD-73 can dramatically promote plasmid retention in populations undergoing sporulation and germination, and we provide evidence that this occurs through the post-segregational killing of plasmid-free forespores. Our findings show how an extremely common genetic module can be used to ensure plasmid maintenance during stress-induced developmental transitions, with implications for plasmid dynamics in *B. cereus* s.l. bacteria.

Introduction

Bacteria of the *Bacillus cereus sensu lato* (s.l.) group (*sensu lato* meaning "in the widest sense") often carry multiple large plasmids, which can define dramatic virulence phenotypes. The *B. cereus* s.l. group includes the etiological agent of anthrax, *Bacillus anthracis*, the insect pathogen *Bacillus thuringiensis*, emetic *B. cereus sensu stricto* strains, and several harmless soil-dwelling species.[1] The need to differentiate the bioterrorism agent *B. anthracis* from other *B. cereus* s.l. species stimulated research into the relationships between these organisms. This revealed that the *B. cereus* s.l. bacteria in fact belong to the same phylogenetic unit, having a lower genome fluidity constant than *Escherichia coli*, and few or no distinguishing genetic features on their main chromosomes.[2,3] Virulence determinants, such as the anthrax toxin and capsule genes, and the delta-endotoxin genes of *B. thuringiensis*, are encoded on large, mobile plasmids. The observation that *B. cereus sensu stricto* bacteria carrying *B. anthracis* virulence plasmids can cause anthrax, but *B. anthracis* strains lacking these plasmids are avirulent[3-5] illustrates the necessity of plasmid-encoded products for pathogenicity. Defining the mechanisms of dissemination and retention of plasmids within the *B. cereus* s.l. group is central to understanding how pathogenesis phenotypes evolve, are maintained, and transferred among members of this group of normally harmless bacteria.

All *Bacillus* species can undergo sporulation – a developmental pathway that produces a highly resilient, dormant cell type – in response to certain types of stress, such as starvation.[6] Spore development begins with an asymmetric cell division and at later stages requires the genomic content of the cell to be heavily compressed.[6] These physical constraints may select against the retention of extrachromosomal elements in the spore, however, sporulation occurs against a backdrop of stress where keeping the extrachromosomal genome (the encoded products of which could provide adaptive advantages on germination) could be beneficial. The function of plasmid maintenance systems in the context of a sporulation cycle has been poorly explored. Inheritance of *B. cereus* s.l. plasmids during sporulation is often attributed to active partitioning systems, however very few of these have been studied specifically during spore development. AlfA of *B. subtilis* is an actin homolog with a role in plasmid partition during sporulation, and increased plasmid retention from 18% to 58%.[7] The same study found that plasmid retention was also enhanced by the developmentally-regulated chromosome remodelling protein RacA, which is thought to tether the plasmid at the forespore pole.[7] The *B. thuringiensis* virulence plasmid pBtoxis encodes a

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Submitted: 06/08/2015; Accepted: 07/12/2015
http://dx.doi.org/10.1080/15476286.2015.1073438

tubulin-like partition protein, TubZ, which increases plasmid retention during sporulation from 69% to 100%.[8] Background loss of naturally-occurring *Bacillus* plasmids during sporulation varies dramatically, with loss rates ranging from 5–95% reported for 4 different plasmids in *B. cereus*.[9] Though difficult to assess from such a small number of studies, it seems that known active partition systems alone are not sufficient to account for the extensive plasmid profiles observed in members of the *B. cereus* s.l. bacteria.

Toxin-antitoxin (TA) systems are a group of near-ubiquitous prokaryotic genetic modules that have multiple biological roles, including plasmid stabilization.[10,11] TA loci are typically arranged as operons, and the encoded antitoxins are unstable in comparison with their toxin partners.[11] TA systems can stabilize plasmids through post-segregational killing (PSK), also called plasmid "addiction," in which the chance loss of the TA-encoding plasmid results in depletion of the unstable antitoxin, thereby releasing the toxin to kill the plasmid-free segregants.[12]

$ToxIN_{Bt}$ is a Type III (protein:RNA) TA system encoded by pAW63; a conjugative, cryptic plasmid of *Bacillus thuringiensis* sr *kurstaki* HD-73.[13,14] Type III TA loci encode a ribonuclease toxin coupled to an antitoxic processed RNA, which suppresses the toxin by forming an inactive protein-RNA complex (**Fig. 1A**).[14-16] Currently, there is no evidence that $ToxIN_{Bt}$ provides protection from bacteriophage, unlike some other Type III TA systems.[14,15,17] $ToxIN_{Bt}$ does, however, promote plasmid maintenance during vegetative growth in *B. subtilis*, and is expressed at moderately high levels under standard laboratory growth conditions, suggesting its biological role may be to stabilize its source plasmid, pAW63.[14,18]

In this study, we have examined the effect of the Type III TA system $toxIN_{Bt}$ of plasmid pAW63 on plasmid inheritance through a sporulation cycle in *Bacillus subtilis*. Our aim was to determine if TA systems could represent a general mechanism to ensure propagation of *B. cereus* s.l. plasmids, including those essential for virulence, in environments that favor sporulation.

Results and Discussion

The effect of $toxIN_{Bt}$ on plasmid retention during sporulation was tested using a medium copy-number pHCMC05-derived $toxIN_{Bt}$ plasmid in the host strain *B. subtilis* YB886, with a frameshifted $toxIN_{Bt}$ derivative encoding a truncated $ToxN_{Bt}$ protein ($toxIN_{Bt}$-FS) as a negative control. Cultures were grown to stationary phase under antibiotic selection and then transferred to sporulation medium, in order to minimise the window for plasmid loss prior to sporulation. Spores were harvested and plated after 18 hours, and individual colonies from the germinated spores were then patch-plated onto selective media to identify those that had retained the plasmid. As shown (**Fig. 1B**), the control plasmid was lost from 58% of cells in the culture following a single round of sporulation and germination. In contrast, the vector encoding functional $toxIN_{Bt}$ was lost from only 6% of cells. Note that the frequency of control plasmid loss during sporulation ($58 \pm 6\%$) is very high in comparison to the loss rate of the same plasmid during vegetative growth (4.75×10^{-3} per cell per generation).[14] A third vector encoding $toxIN_{Bt}$ with a C-terminal hexahistidine tag on $ToxN_{Bt}$ showed an intermediate phenotype and was lost in 25% of the germinated spores. A western blot against $ToxN_{Bt}$-6×His detected the protein in vegetative cells but not spores (data not shown). The difference in the stabilization effects of the native and hexahistidine-tagged $toxIN_{Bt}$ constructs could be due to the reduced toxicity of the tagged protein, as shown in an *E. coli* overexpression assay (**Fig. 1C**). The $toxIN_{Bt}$ locus therefore dramatically enhances plasmid

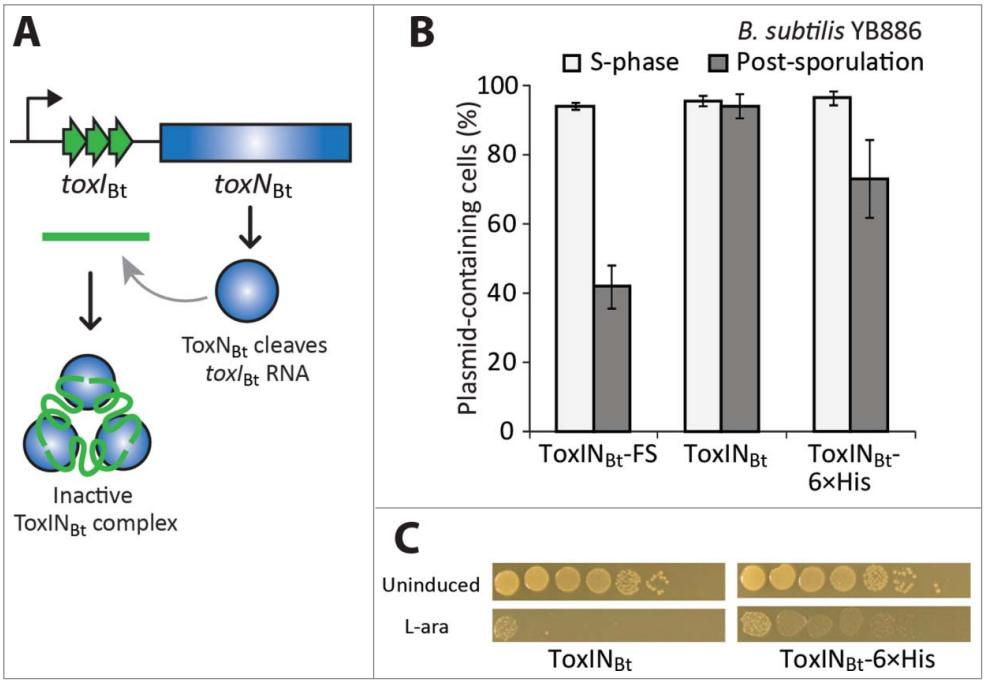

Figure 1. $ToxIN_{Bt}$ promotes plasmid retention during sporulation and germination while reducing sporulation efficiency (A) Schematic of the $toxIN_{Bt}$ system. The antitoxin, $ToxI_{Bt}$, is encoded as a series of repeats upstream of the $toxN_{Bt}$ gene, and both are expressed from a constitutive promoter. The ribonuclease $ToxN_{Bt}$ cleaves $ToxI_{Bt}$ transcript into individual repeats, which then remain bound to their parent enzyme in an inactive, triangular assembly. (**B**) Retention of plasmids in B. subtilis YB886 after one round of sporulation and germination. Results shown are mean ± SD for 2 ($ToxIN_{Bt}$-6×His) or 3 ($ToxIN_{Bt}$, $ToxIN_{Bt}$-FS) biological replicates. S-phase indicates stationary phase cultures. (**C**) Comparison of the toxicity of wild-type and hexahistidine-tagged $ToxN_{Bt}$. Viable counts of E. coli are shown where $ToxN_{Bt}$ expression is repressed with D-glucose, or induced with L-arabinose.

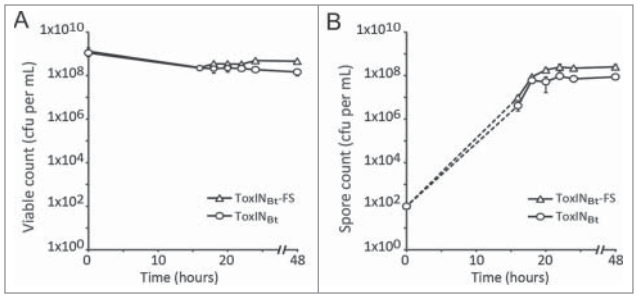

Figure 2. Plasmid-encoded *toxIN*$_{Bt}$ reduces sporulation efficiency. Total (**A**) and heat-resistant (**B**) viable count of *B. subtilis* YB886 cultures containing plasmid pFLS79 (*toxIN*$_{Bt}$-FS; triangles) or pFLS80 (*toxIN*$_{Bt}$; circles) measured over time following transfer to Difco sporulation medium at stationary phase. Results shown are mean ± SD for 3 biological replicates. Dashed lines in the right-hand chart indicate extrapolation to the detection limit. See **Table 1** for calculated sporulation efficiency.

retention during sporulation, and this appears to be through a toxicity-dependent mechanism.

If ToxIN$_{Bt}$ promotes plasmid retention by killing plasmid-free forespores at some point between septation and the formation of the mature spore, it would be expected that cells carrying a *toxIN*$_{Bt}$-encoding plasmid would show reduced sporulation efficiency compared to cells without an addictive plasmid. We tested this possibility by comparing total viable counts and spore counts of *B. subtilis* YB886 strains carrying either a *toxIN*$_{Bt}$ or *toxIN*$_{Bt}$-FS plasmid over the course of a sporulation cycle. Cultures were grown to stationary phase with antibiotic selection then transferred to sporulation medium, and viable cell and spore counts were measured at various time points from 16 to 48 hours following transfer. The total number of spores produced by each strain was then compared to determine whether the *toxIN*$_{Bt}$-containing strain showed reduced sporulation efficiency. As shown (**Fig. 2**; **Table 1**), both *B. subtilis* YB886 strains had an average viable count of $\sim 1.3 \times 10^9$ on transfer to sporulation medium, and no spores were detected at this stage. After 16 hours' incubation in sporulation medium, the control strain had an average spore count of 9.9×10^6 colony forming units (cfu) per mL, and this increased to 2.5×10^8 cfu.mL^{-1} over the course of the experiment. The average spore count of cultures carrying the *toxIN*$_{Bt}$-encoding plasmid was much lower, at 4.2×10^6 cfu.mL^{-1} after 16 hours and 8.8×10^7 cfu.mL^{-1} at the 48 hour endpoint. This trend was consistent across all time points (**Fig. 2**; **Table 1**), and a 2-way ANOVA performed on the data strongly supported the significance of the difference in spore count between the 2 strains ($F = 63.3$, $p = 1.2 \times 10^{-8}$). The overall efficiency of spore formation for bacteria carrying the *toxIN*$_{Bt}$ plasmid, as a proportion of the control strain, was 40.8% (average across all time points). Note that the average efficiency of sporulation in cells carrying a *toxIN*$_{Bt}$ plasmid (40.8%, **Table 1**) is similar to the background retention of the control vector during sporulation (42%, **Table 1**). Overall, our data demonstrate that the presence of *toxIN*$_{Bt}$ on a test plasmid dramatically increases retention of that plasmid in a bacterial population during sporulation and germination, but reduces the proportion of cells in a culture that form a mature spore. These results are consistent with the idea that, for a *toxIN*$_{Bt}$-encoding strain to complete sporulation, a copy of the locus must be partitioned to the forespore. We suggest that *toxIN*$_{Bt}$ promotes plasmid retention in the context of a sporulation cycle through the killing of plasmid-free forespores by ToxN$_{Bt}$, prior to spore maturation (**Fig. 3**). To our knowledge, this is the first indication that a TA system can promote plasmid retention specifically in the context of spore development in *Bacillus*.

Table 1. Effect of *toxIN*$_{Bt}$ on sporulation efficiency of *B. subtilis* YB886

Time (hours)	Average Spore count (cfu.ml^{-1})		Efficiency of sporulation *toxIN*$_{Bt}$ cfu.ml^{-1}/ *toxIN*$_{Bt}$-FS cfu.ml^{-1}
	toxIN$_{Bt}$	*toxIN*$_{Bt}$-FS	
0	*<100*	*<100*	
16	4.2×10^6	9.9×10^6	0.42
18	6.0×10^7	8.7×10^7	0.69
20	5.2×10^7	1.8×10^8	0.29
22	9.4×10^7	2.5×10^8	0.38
24	7.1×10^7	2.2×10^8	0.32
48	8.8×10^7	2.5×10^8	0.35

ToxIN$_{Bt}$ is a Type III TA system, however we envisage that the stabilization phenotype observed here could be mediated by TA systems of other Types, provided they have the differential stability and toxicity required for PSK. Note that several *B. subtilis* Type I TA loci have been proposed to have stabilization activity, and one, *txpA-ratA*, was suggested to specifically prevent the loss of the excised form of the *skn-1* prophage during sporulation though this was not shown experimentally.[19]

The notion that PSK during sporulation could also be mediated by other TA system Types leads to the question of whether enough *B. cereus* s.l. plasmids contain TA loci for these systems to represent a general strategy for promoting plasmid retention in populations of bacteria undergoing sporulation. Besides *toxIN*$_{Bt}$, there are 3 experimentally validated TA systems from *B. cereus* s.l. plasmids.[20,21] However, many more have been predicted through bioinformatic approaches.[16,22,23] Further work is warranted to determine the true prevalence of TA loci in plasmids of *B. cereus* s.l. bacteria, and how these contribute to plasmid dynamics.

The retention of *Bacillus* plasmids has usually been attributed to active partitioning systems, though very few of these systems have been tested during sporulation. The effect of the putative partition system from the *toxIN*$_{Bt}$ source plasmid (pAW63) has not been tested during sporulation, though during vegetative growth this system promotes retention to 87% over 40 generations.[24] In this context, it seems that additional mechanisms must also have contributed to the extensive plasmid profiles observed in members of the *B. cereus* s.l. bacteria. Here we have shown that a TA system can provide a second-line mechanism if partitioning or replication fail, by preventing the maturation of plasmid-free forespores. We propose that TA systems contribute to plasmid dynamics within the *B. cereus* s.l. group by promoting

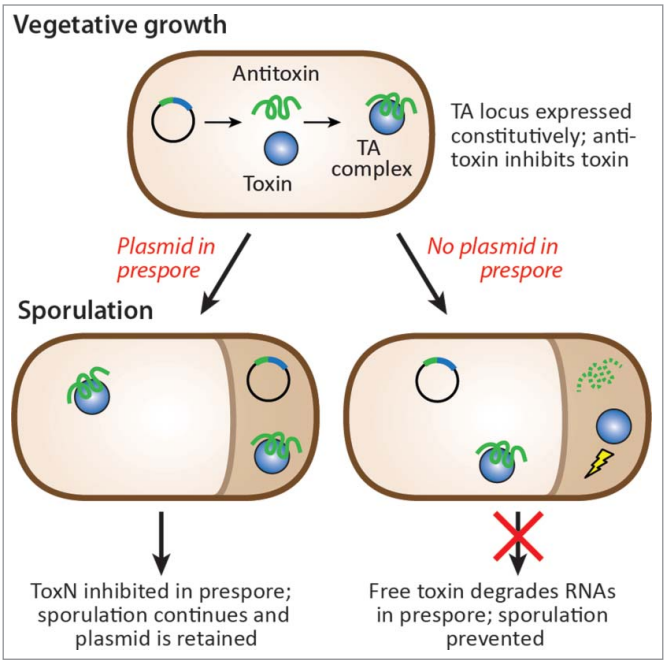

Figure 3. Schematic of *toxIN*$_{Bt}$ mechanism of plasmid stabilization during sporulation. ToxIN$_{Bt}$ is present in vegetative cells as an inactive complex, which is expressed constitutively to replenish the inhibitory ToxI$_{Bt}$. At sporulation, formation of the septum can exclude the *toxIN*$_{Bt}$ plasmid from the prespore (right), which will result in the release of ToxN$_{Bt}$ in the prespore following ToxI$_{Bt}$ degradation. The plasmid-free prespore is thus unable to form a mature spore. If the plasmid is retained in the prespore after the septum forms (left), ToxI$_{Bt}$ is maintained at protective levels during sporulation. The spore can mature and the plasmid is retained.

plasmid retention during stress-induced developmental transitions, with implications for the retention and dissemination of virulence determinants in these bacteria.

Materials and Methods

Bacterial strains and plasmid construction

Strains and plasmids used are listed in **Table 2**. Plasmid pFLS78, which encodes *toxIN*$_{Bt}$-6xHis, was generated by amplifying the *toxIN*$_{Bt}$ locus from *B. thuringiensis* sr *kurstaki* HD-73 genomic DNA using the primers FS105 (5′-CCTTGGTACCGCAGAGAGAGATAAATAA-3′) and FS101 (5′-GGTGCCCGGGTTAATGGTGATGGTGATGGTGTCT CTCACGCCCCATTTG-3′; encodes hexahistidine tag). The resulting PCR product was then cloned into pHCMC05 using the *KpnI*/*SmaI* restriction sites. Plasmid pFLS82, which encodes ToxN$_{Bt}$-6xHis under the control of a p-ARA promoter, was constructed using the primers PF197 (5′-TTTGAATTCGGAGAA-GAAAGTTGACTAATAAAG-3′) and FS77 (5′-GGTGAAG CTTAATGGTGATGGTGATGGTGCGCTCTCTCACGCC CCATTTG-3′) to amplify the *toxN*$_{Bt}$ gene and introduce the C-terminal 6xHis tag, and the PCR product was cloned into pBAD30 using the *EcoRI*/*HindIII* restriction sites. Toxicity tests in *E. coli* DH5α were performed as reported previously.[15]

Plasmid loss assays

Overnight cultures of *B. subtilis* YB886 containing plasmid pFLS78, pFLS79 or pFLS80 were used to inoculate 20 mL LB supplemented with 10μg.mL^{-1} chloramphenicol, and these cultures were grown at 30°C to stationary phase. A sample of each culture was taken at this stage and plated on LB agar, then incubated overnight at 30°C. Stationary phase cultures were harvested by centrifugation and washed twice with 20 mL Difco sporulation medium, then resuspended in 10 mL Difco sporulation medium without added antibiotics and incubated for 18 hours at 30°C. Cultures were harvested by centrifugation and resuspended in sterile phosphate-buffered saline, and then incubated at 70°C for 10 minutes to kill vegetative cells. Spore preparations were then serially diluted in sterile 1xPBS, plated on LB agar and incubated overnight at 30°C. Plasmid-containing cells in the stationary phase cultures, and in the spore preparations, were quantified by patching single colonies grown onto nonselective media onto LB plates containing 10 μg.mL^{-1} chloramphenicol, followed by incubation overnight at 30°C.

Sporulation efficiency tests

Cultures of *B. subtilis* YB886 carrying either pFLS79 or pFLS80 were grown in selective rich medium followed by nonselective Difco sporulation medium, as for the plasmid loss

Table 2. Strains and plasmids used in this study

Strain	Description	Source
Bacillus subtilis YB886	*trpC2 metB10 xin-1* SPβ^{S}	Yasbin et al. 1980 [25]
Escherichia coli DH5α	K-12 strain: F^{-} Φ80*lacZ*ΔM15 Δ*(lacZYA-argF)* U169 *recA*1 *endA*1 *hsdR*17(r_K^- m_K^+) *phoA supE*44 λ^{-} *thi*-1 *relA*1 *gyrA*96	Invitrogen
Plasmid		
pBAD30	*E. coli* overexpression vector, Ap^{R}, p-ARA promoter induced by L-arabinose, repressed by glucose	Guzman et al. 1995 [26]
pFLS78	*toxIN*$_{Bt}$-6×His in pHCMC05, native promoter, Ap^{R} Cm^{R}	This study
pFLS79	*toxIN*$_{Bt}$-FS in pHCMC05, native promoter, Ap^{R} Cm^{R}	Short et al. 2013 [14]
pFLS80	*toxIN*$_{Bt}$ in pHCMC05, native promoter, Ap^{R} Cm^{R}	Short et al. 2013 [14]
pFLS82	*toxN*$_{Bt}$-6×His in pBAD30, Ap^{R}	This study
pHCMC05	*E. coli-Bacillus* shuttle vector, Ap^{R} Cm^{R}	Nguyen et al. 2005 [27]
pTA117	*toxN*$_{Bt}$ in pBAD30, Ap^{R}	Fineran et al. 2009 [15]

assays. At 0, 16, 18, 20, 22, 24 and 48 hours after transfer to Difco sporulation medium, a 0.5 mL sample of each culture was taken and serially diluted and plated for viable counts on plain LB agar. The same sample was then heat-treated at 80°C for 10 minutes to kill vegetative cells, and the treated sample was plated for viable counts on plain LB agar. Plates were incubated overnight at 30°C.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Tim Blower and members of the Salmond lab for useful discussions, and Shue-Li Ong for technical assistance. We also thank the Bacillus Genetic Stock Center (Ohio) for providing plasmids and strains.

Funding

This work was supported by a Commonwealth Scholarship from the Commonwealth Scholarships Commission (UK) and Sir Henry Wellcome Postdoctoral fellowship to FLS, and the Biotechnology and Biological Sciences Research Council (UK).

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