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

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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 avirulent4–5 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
Toxin-antitoxin (TA) systems are a group of near-ubiquitous prokaryotic genetic modules that have multiple biological roles, including plasmid stabilization. TA loci are typically arranged as operons, and the encoded antitoxins are unstable in comparison with their toxin partners. 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.

ToxINBt is a Type III (protein:RNA) TA system encoded by pAW63, a conjugative, cryptic plasmid of *Bacillus thuringiensis* ssp *kurstaki* HD-73. 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). Currently, there is no evidence that ToxINBt provides protection from bacteriophage, unlike some other Type III TA systems. ToxINBt 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.

In this study, we have examined the effect of the Type III TA system toxINBt 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 toxINBt on plasmid retention during sporulation was tested using a medium copy-number pHCMC05-derived toxINBt plasmid in the host strain *B. subtilis* YB886, with a framesshifted toxINBt derivative encoding a truncated ToxINBt protein (toxINBt-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 toxINBt was lost from only 6% of cells. Note that the frequency of control plasmid loss during sporulation (58 ± 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). A third vector encoding toxINBt 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 ToxINBt-6×His detected the protein, as shown in an *E. coli* overexpression assay (Fig. 1C). The toxINBt locus therefore dramatically enhances plasmid stability.

**Figure 1.** ToxINBt promotes plasmid retention during sporulation and germination while reducing sporulation efficiency (A) Schematic of the toxINBt system. The antitoxin, ToxINBt, is encoded as a series of repeats upstream of the toxINBt gene, and both are expressed from a constitutive promoter. The ribonuclease ToxINBt cleaves ToxINBt 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 (ToxINBt-6×His) or 3 (ToxINBt, ToxINBt-FS) biological replicates. S-phase indicates stationary phase cultures. (C) Comparison of the toxicity of wild-type and hexahistidine-tagged toxINBt constructs could be due to the reduced toxicity of the tagged protein, as shown in an E. coli overexpression assay (Fig. 1C). The toxINBt locus therefore dramatically enhances plasmid
either a retention of the control vector during sporulation (42%, Table 1). The difference in spore count between the 2 strains (Fig. 2) showed reduced sporulation efficiency. As shown (Table 1), both *B. subtilis* YB886 strains had an average viable count of ~1.3 × 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 × 10^7 cfu.mL^-1 over the course of the experiment. The average spore count of cultures carrying the *toxIN* encoding plasmid was much lower, at 4.2 × 10^6 cfu.mL^-1 after 16 hours and 8.8 × 10^5 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 × 10^-8). The overall efficiency of sporulation for bacteria carrying the *toxIN* 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* 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* 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* encoding strain to complete sporulation, a copy of the locus must be partitioned to the forespore. We suggest that *toxIN* promotes plasmid retention in the context of a sporulation cycle through the killing of plasmid-free forespores by *ToxIN*, 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 spor development in *Bacillus*.

*ToxIN* 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 *ska-1* prophage during sporulation though this was not shown experimentally. 

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*, there are 3 experimentally validated TA systems from *B. cereus* s.l. plasmids. However, many more have been predicted through bioinformatic approaches. 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* source plasmid (pAW63) has not been tested during sporulation, though during vegetative growth this system promotes retention to 87% over 40 generations. In this context, it seems that additional mechanisms must also have contributed to the extensive plasmid profiles observed in the 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...
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 $se$ kurtaki HD-73 genomic DNA using the primers FS105 (5’-CCTTGGTACCCAGAGAGAATAATAA-3’) and FS101 (5’-GGTGCCCGGTTAATGTGATGTGATGTGCTCTCAACGCCCATTGG-3’) to encode hexahistidine tag. The resulting PCR product was then cloned into pHCMC05 using the KpnI/SmaI restriction sites. Plasmid pFLS82, which encodes ToxNBt-6xHis under the control of a p-ARA promoter, was constructed using the primers PF197 (5’-TTTGAATTCGGAGAAGTTGACTAATAAAG-3’) and FS77 (5’-GGTGCCCTTAATGTGATGTGATGTGCGCTCTCAGCCCTATTG-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 DH5$\alpha$ were performed as reported previously.\(^\text{15}\)

Plasmid loss assays

Overnight cultures of B. subtilis YB886 containing plasmid pFLS78, pFLS79 or pFLS80 were used to inoculate 20 mL LB supplemented with 10 $\mu$g.mL$^{-1}$ chloramphenicol, and the 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 10 minutes at 70°C 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 $\mu$g.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 assays.

Table 2. Strains and plasmids used in this study

| Strain | Description | Source |
|--------|-------------|--------|
| Bacillus subtilis YB886 | trpC2 metB10 xin-1 SPb$^5$ | Yasbin et al. 1980$^{25}$ |
| Escherichia coli DH5$\alpha$ | K-12 strain: F$^{-}$ F' $\Phi$80lacZAM15 (lacZYA-argF)U169 recA1 endA1 hsdR17(k$^{-}m^{-}r^{-}y^{-})$ phoA supE44 thi-1 relA1 gyrA96 | 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$_{Bt}$-6xHis 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 | toxIN$_{Bt}$-6xHis in pBAD30, Ap$^R$ | This study |
| pHCMC05 | E. coli-Bacillus shuttle vector, Ap$^R$, Cm$^R$ | Nguyen et al. 2005$^{27}$ |
| pTA117 | toxIN$_{Bt}$ in pBAD30, Ap$^R$ | Fineran et al. 2009$^{15}$ |
assays. At 0, 16, 18, 20, 22, 24 and 48 hours after transfer to Difco sporation 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.

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