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Genetic Characterization of Two Putative Toxin-Antitoxin Systems on Cryptic Plasmids from *Bacillus thuringiensis* Strain YBT-1520

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A novel putative toxin-antitoxin segregational stability system named *KyAB* system was identified in a novel native plasmid pBMB8240 from *Bacillus thuringiensis* strain YBT-1520, based on sequences homology with other toxin-antitoxin systems, the lethal activity of the KyB putative toxin in *Escherichia coli* and the stabilizing effect of the *kyAB* system in *Bacillus thuringiensis*. Secondarily, the native plasmid pBMB9741 from the same strain was resequenced and the corrected plasmid was named as pBMB7635. Based on sequence homology with the *tasAB* system and the lethal activity of toxin protein in *Escherichia coli*, a *tasAB*-like putative toxin-antitoxin system was identified on pBMB7635.

Keywords: *Bacillus thuringiensis*, small cryptic plasmids, segregational stability toxin-antitoxin system

Toxin-antitoxin (TA) systems contain two components: a stable toxin and an unstable antitoxin that interferes with the lethal action of the toxin [5, 7]. When a plasmid containing a TA system is lost during cell division, the antitoxin degrades quickly, leaving the stable toxin to act on the cell target and cause cell death or growth stasis [7, 10]. TA systems have been extensively studied on low-copy-number plasmids in Gram-negative bacteria as addiction systems [10, 16], and a few TA systems have been described on small plasmids in Gram-positive bacteria [6, 16].

Bacillus thuringiensis is the most widely used bioinsecticide and generally harbors numerous plasmids whose numbers vary from 2 to 12 and sizes from 2 to 600 kb [4, 12, 18]. However, many small plasmids remain cryptic since no function other than their replication machinery or mobilization activity has been associated with them [21]. Here, we found two novel putative TA systems on *B. thuringiensis* small plasmids and this finding may be helpful for further study on TA systems and the role of cryptic plasmids.

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Novel Plasmids pBMB7635 and pBMB8240 were Found from Strain YBT-1520

B. thuringiensis strain YBT-1520 is highly toxic to lepidopteran larvae owing to the presence of the *cry1Aa*, *cry1Ac*, and *cry2* toxin genes [20] and the strain contains at least three large plasmids (>30 kb) and three small plasmids (<15 kb) [22].

Plasmid pBMB9741 (GenBank Accession No. AF202532) from strain YBT-1520 was cloned previously in our laboratory [22]. In this study, the template in all PCR cases are the small plasmids (<15 kb) DNAs of strain YBT-1520. We designed a pair of contiguous primers p3 and p4 (Table 1) to verify the DNA sequence downstream from the *rep* gene. However, we found the actual PCR product was 1,157 bp in length with additional 1,057 bp than the predicted length. Here, we named the plasmid with this 1,057 bp as pBMB7,635 (7,635 bp) (GenBank Accession No. EU130936).

Plasmid pBMB7635 was shotgun sequenced and the presence of the 1,057 bp additional fragment described above was verified. Two ORFs (*orf94* and *orf133*) are present in pBMB7635 but absent in pBMB9741 (Fig. 1). ORF94 shares 100% amino acid identity with the TasA putative antitoxin protein of pGI1, whereas its neighbor ORF133 shares 98% amino acid identity with the TasB putative toxin protein of pGI1 [6]. The *orf94/orf133* system is denoted as the *tasAB*-like system, *orf94* is denoted as the *tasA* gene, and *orf133* is denoted as the *tasB*-like gene.

Based on the shotgun sequence analysis of the small plasmids (<15 kb) in strain YBT-1520 (unpublished data), an 8,240 bp plasmid (named pBMB8240) was found to be a novel cryptic plasmid. In pBMB8240, two contiguous *orf*s (this gene pair was tentatively named *kyAB* for strain *kurstaki* YBT-1520) (Fig. 2) were noticed. The upstream putative protein of 94 amino acids (here named KyA) displays sequence similarities (33% amino acid identity and 54% similarity) with the RF0957 antitoxin from *Rickettsia felis* [15], and the downstream putative protein

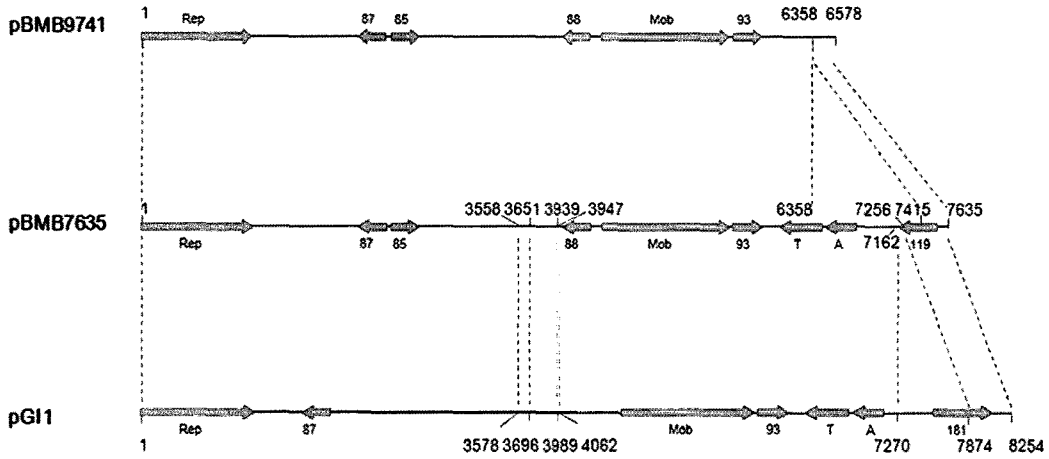

Fig. 1. Schematic representation of the *orfs* present in pBMB7635, pBMB9741, and pGI1 similar plasmids.
The main ORFs in the three plasmids are represented as arrows and the arrowheads show the direction of transcription, and the length of the arrows reflects the relative lengths of the genes. The ORFs are shown by their amino acid size or name (T is putative toxin and A is putative antitoxin) and the conserved areas are shaded. To simplify the description, the three plasmids have the same origin.

of 80 amino acids (here named KyB) displays sequence similarities (30% amino acid identity and 53% similarity) with the StbE toxin (GenBank Accession No. YP_974085) of the *RelE/StbE* addiction module from *Acidovorax sp.* JS42 (unpublished data).

Attempts to Clone the *kyA*, *kyB*, *tasA*, and *tasB*-Like Genes in *E. coli* DH5α

The *kyA*, *kyB*, *tasA*, and *tasB*-like genes were amplified by PCR, respectively (Table 1) and the products were ligated into pET28a. Attempts to clone *kyA* and *tasA* in *E. coli* DH5α were easy and successful. Continuing efforts to clone the *tasB*-like gene in *E. coli* DH5α have not yet been successful. Only four recombinants containing the *kyB* gene were obtained on LB-Kan^R medium and all recombinants harbored mutations in the KyB protein, suggesting the toxic activity of TasB-like and KyB proteins in *E. coli*.

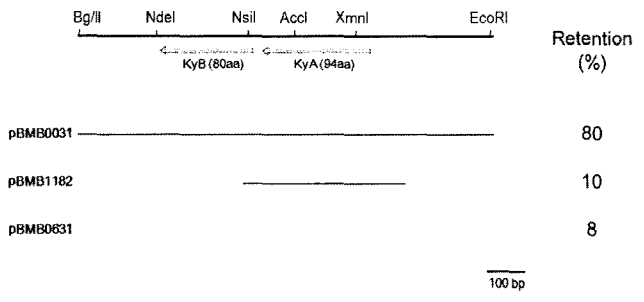

Fig. 2. Genetic organization and stabilizing effect of the *kyAB* system and flanking sequences from pBMB8240 plasmid.
Arrows indicate the length and orientation of ORFs. Lines beneath the map indicate regions cloned in the stability probe vector pBMB0631. After 40 generations in *B. thuringiensis* strain BMB171 without selective pressure, pBMB0631, pBMB0031 (contained *kyAB* system), and pBMB1182 (only contained *kyA* gene) were found to be maintained at levels of 8%, 80%, and 10%, respectively.

The *kyAB* System is a Segregational Stability Region in *B. thuringiensis*

In this study, the segregational stability probe plasmid pBMB0631 was constructed by combining the existing plasmids pEG491 [3] and pDG780 [9]. Plasmid pDG780 contained a replicon and an Amp^R resistance marker for selection in *E. coli* and a Kan^R marker for selection in *Bacillus* [9]. The resulting plasmid pBMB0631 was segregationally unstable in *B. thuringiensis* (8% retention after 40 generations of nonselective growth) in the absence of Kan^R selective pressure at 30°C (Fig. 2).

The *kyAB* system (GenBank Accession No. EU130937), *kyA* gene, and *tasAB*-like system were amplified by PCR and inserted into pBMB0631 to yield pBMB0031, pBMB1182, and pBMB0032, respectively. Only seven recombined plasmids (pBMB0032-1-7) were recovered on selective plates in *E. coli* DH5α, and all of them plus pBMB0031 and pBMB1182 were electroporated into *B. thuringiensis* BMB171, the plasmid-free *B. thuringiensis* strain [11] that was used as the recipient strain in electroporation, separately to test the segregational stability. The apparent stability of plasmids pBMB0031, pBMB0032, pBMB1182, and pBMB0631 was estimated as described previously [19].

Without selective pressure, pBMB0631, pBMB0031, and pBMB1182 were found to be maintained at levels of 8%, 80%, and 10%, respectively (Fig. 2), demonstrating that the *kyAB* system was a novel functional plasmid segregational stability cassette and the *kyA* gene alone did not promote plasmid segregational stability in *B. thuringiensis*. Furthermore, KyA displays sequence similarities (57% amino acid identity and 76% similarity) with the small polypeptide encoded by plasmid pLS1, which belongs to the CopG protein family (GenBank Accession No. AAB19359), suggesting that the putative antitoxin KyA might belong to the MetJ/Arc/CopG family [1, 14, 17].

Table 1. Oligonucleotide primers used in PCR assays to clone different segments of the two putative TA systems from strain YBT-1520.

Primer names	Segment	Oligonucleotide primers[a]
P1	P12, stability region from pBMB8240	CCCG**GAATTC**GGATAATAAGGCGATTGAA
P2		CCG**AGATCT**AGCCAATTTCATAACTTGCT
P3	1,990–2,089 on pBMB9741	TCCTCTTGTGCTACTGTTTG
P4		GGCATCAATTTCCGTGCGGA
P5	*kyA*, 770–486[C] on P12	ATT**GAATTC**ATGGAAGAATTAAATAAAAA
P6		ATT**AAGCTT**TTACAAACTAAACTTAGCTA
P7	*kyB*, 460–218[C] on P12	ATT**GAATTC**ATGGATGATGCATTAACAAA
P8		ATT**AAGCTT**CTAATGTTTTTGTCTCTTTA
P9	*tasA*, 6,765–6,481[C] on pBMB7635	ATT**GAATTC**ATGACAGCAAACACTCGCCA
P10		ATT**AAGCTT**TTATCTGTTTACCAAATCTT
P11	*tasB*-like, 6,443–6,042[C] on pBMB7635	ATT**GAATTC**ATGACAATTTATATAACAG
P12		ATT**AAGCTT**CTAATTTAAAGGTAATTTTG
P13	*kyA* and flanking sequences, 858–433[c] on P12	CCG**GAATTC**ATAAATTTAGCCGAAAAGGC
P14		CCG**GGATCC**CTAAAATTTTTGTTAATGCA
P15	5,635–7,123 on pBMB7635, containing *tasAB*-like system	CCG**GGATCC**GGTACTGTAAAGGAATTGGC
P16		CCG**GAATTC**GTAACTCCCTTTACACTTTC

[a]Letters in bold refer to restriction sites.
[C]Indicates complementary sequences.

Without selective pressure, the seven plasmids pBMB0032-1–7 maintained at a frequency of 8%, 7%, 9%, 9%, 10%, 8%, and 7%. None of them exhibited remarkably higher levels of segregational stabiliy than the empty vector pBMB0631. After sequencing, all seven recombined plasmids harbored mutations in the TasB-like putative toxin. The results incline to assume that the TasB-like protein is lethal in *E. coli* as TasB protein [6]. Owing to being unable to clone the *tasB*-like putative toxin gene of pBMB7635 in *E. coli*, a deletion mutation (above 1,057 bp fragment) could have happened when cloning of pBMB9741.

Comparisons of *kyAB*, *tasAB*-Like, and *tasAB* Putative TA Systems

It is commonly accepted that TA systems occur in large, low-copy-number plasmids, whereas small plasmids pBMB7635, pBMB8240, and pGI1 have putative TA systems too. Toxin-antitoxin genes typically overlap such as the *Axe-Txe* system [8], but the *tasAB* [6], *kyAB*, and *tasAB*-like putative TA systems are not the case.

In this study, the DNA sequence of the *tasB*-like putative toxin gene of pBMB7635 was obtained by PCR product sequencing and plasmid shotgun sequencing, whereas the sequence of pGI1 was determined by subcloning and subsequently sequencing of the several partially overlapping fragments of this 8 kb molecule [2, 13]. The differences between pBMB7635 and pGI1 perhaps resulted from the different sequencing methods.

KyA putative antitoxin was functional and could counteract the functional toxin in both Gram-negative and Gram-positive backgrounds. However, the *tasAB*-like or *tasAB* systems [6] could not be cloned in *E. coli*, suggesting the TasA protein failed to inhibit the lethal activity of the cognate toxin, and the *tasAB*-like system appeared to be functional but unregulated in *E. coli*. YBT-1520 is the first *B. thuringiensis* strain found to contain two putative TA systems on two small plasmids, and moreover, the two putative TA systems might have different precise mechanisms of toxic actions. The toxic mechanisms remain to be elucidated.

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