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ORIGINAL ARTICLE

Distribution of 2-kb miniplasmid pBMB2062 from *Bacillus thuringiensis kurstaki* YBT-1520 strain in *Bacillus* species

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Abstract A multi-copy and small plasmid pBMB2062 from *Bacillus thuringiensis kurstaki* YBT-1520 strain was cloned and characterized and its distribution was analyzed using dot-blot analysis with the *ORF1* fragment as a probe. *Bacillus* species of 84 serotypes were evaluated. The pBMB2062 plasmid was found to be present in commercial *B. thuringiensis kurstaki* (H3abc) and *aizawai* (H7) insecticides of various serotypes, and one *Bacillus cereus* UW85 strain (produced Zwittermicin fungicide and Cry toxin synergist). The sequences of 7 pBMB2062-like plasmids from randomly selected *Bacillus* species (positive signal in the dot-blot analysis) were highly conserved. Two open reading frames (ORFs), *ORF1* and *ORF2*, were present in this plasmid. *ORF1* was found to be necessary for plasmid replication, whereas *ORF2* did not play a role in replication or stability. Based on its sequence homology, *ORF2* was a putative solitary antitoxin. Furthermore, the copy number of the replicon of pBMB2062 was higher than those of *ori*1030 and *ori*44 based on the thermogenic data, and *ori*2062 could drive the stable replication of a recombinant plasmid (11 kb total size) in *B. thuringiensis*.

Keywords *Bacillus thuringiensis* · Multicopy plasmid · Plasmid replication · Replicon

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Introduction

Bacillus thuringiensis of various subspecies are well-known insecticides that produce Cry toxins specifically active against lepidopteran, dipteran or coleopteran insects (Schnepf et al. 1998). *Bacillus thuringiensis* strains generally harbor many plasmids whose numbers vary from 2 to 12 and sizes from 2 to 600 kb (Tang et al. 2006), and the genes encoding the Cry proteins are often located on large conjugative plasmids that are larger than 45-kb (Sylvain et al. 2004). Thus far, 10 small *B. thuringiensis* plasmids (<15 kb) including p2055 (pHD2) from *B. thuringiensis kurstaki* HD-1 DiPel insecticide (McDowell and Mann 1991), p2058 from *B. thuringiensis kurstaki* HD-3a3b Futura insecticide (Marin et al. 1992), pGI1 and pTX14-1 (Andrup et al. 2003) that replicate via the rolling-circle replicating (RCR) mechanism have been sequenced and characterized. Further, plasmid pHT1030 (Lereclus et al. 1988), pBMBt1 from *B. thuringiensis darmstadiensis* (Loeza-Lara et al. 2005) and 4 large plasmids, pAW63, pBMB67, pBT9727 and pBtoxis, from *B. thuringiensis israelensis* (Wilcks et al. 1999; Liu et al. 2007; Rasko et al. 2005; Berry et al. 2002) that predictably replicate via the theta mechanism have also been sequenced and characterized. It is reasonable to speculate that these plasmids might play an important role in the physiology of the host cells. However, unlike the toxin-coding large plasmids such as pBtoxis producing Cry4 and Cyt toxins, the function of many small plasmids remains cryptic. *Bacillus thuringiensis kurstaki* YBT-1520 contains at least 6 detectable indigenous plasmids (Zhang et al. 2007) and the smallest of these resident plasmids named pBMB2062 (Fig. 1) caught our interest due to its high copy number and the fact that it contained only 2 of the predicted open reading frames (ORFs). It is also interesting to compare

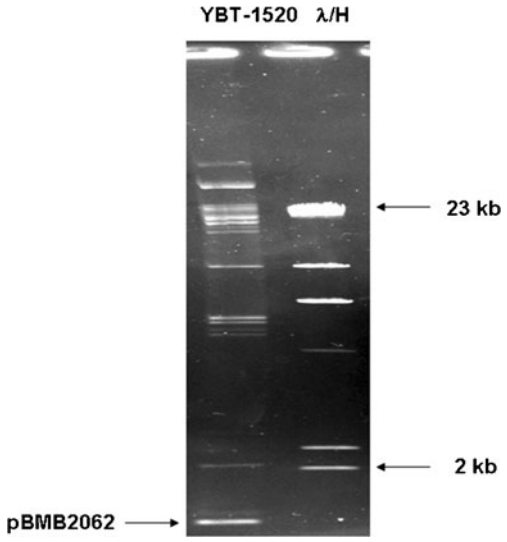

Fig. 1 Agarose gel electrophoresis of plasmids profile from strain *Bacillus thuringiensis* YBT-1520. 0.8 % agarose gel was used for the analysis. M: Lambda/*Hin*dIII linear DNA marker. There were several plasmids in YBT-1520 strain with size range from about 2 kb to larger than 23 kb; the smallest band (*arrow*) was recovered, cloned, sequenced and named as pBMB2062 with the size of 2,062 bps

pMBM2062 of highly active YBT-1520 strain with p2055 or p2058 presented in commercial DiPel or Futura insecticide. This paper describes the distribution and diversity of 2-kb miniplasmid among all the subspecies or serotypes of *B. thuringiensis* and one *B. cereus* and the replication function of the 2 ORFs.

Generally, the replicons from the large plasmids were stable but their copy number was low, and the copy number of the replicons from the small plasmids was high, but they were less stable due to their mode of replication (Viret et al. 1991; Leonhardt and Alonso 1991). In this study, however, we observed that the multicopy replicon *ori*2062 could stably drive the replication of at least a 9-kb foreign fragment in *B. thuringiensis*. The plasmid pBMB2062 is so small that it only contains 2 ORFs: a Rep gene, and another gene that is either involved in regulatory functions or integrated with a toxin from another TA system family, based on the bioinformation analyses. The presence of the miniplasmid in *B. thuringiensis* strains might be important, but its implications remain unclear. In this paper, we attempt to discuss the potential functions of pBMB2062 in *B. thuringiensis* strains based on the sequence conservation, wide distribution, and high plasmid copy number (PCN) of pBMB2062-like plasmids in other *B. thuringiensis* strains.

Materials and methods

Bacterial strains and DNA isolation

Bacillus thuringiensis strain YBT-1520 is highly toxic to lepidopteran larvae due to the presence of the *cry1Aa*, *cry1Ac*, and *cry2* toxin genes (Sun et al. 2000a), and the strain contains at least three large plasmids (>30 kb) and three small plasmids (<15 kb) (Zhang et al. 2007). The *B. thuringiensis* strains named with initial Arabic numbers and those with an initial T were kindly provided by the *Bacillus* Genetic Stock Center (BGSC) or the Pasteur Institute, respectively. Strains from our collection were marked YBT, CT or BMB. *B. cereus* UW 85 (Handelsman et al. 1990) was also used to screen the distribution of the miniplasmid.

All cultures were grown in LB medium containing ampicillin (Amp) and kanamycin (Kan) (Sigma) at 100 and 25 $\mu g ml^{-1}$, respectively. The plasmid pDG780 was used as a *B. thuringiensis* plasmid replicon cloning vector that carried a replicon and an ampicillin-resistance gene for selection in *Escherichia coli* and a kanamycin-resistance gene for selection in *Bacillus* (Guerout-Fleury et al. 1985). Total and plasmid DNA from the *B thuringiensis* and *E. coli* strains were isolated using the alkaline lysis method described by Sambrook and Russell (2001).

Cloning and sequence analysis of the 2-kb plasmid

The smallest plasmid, namely pBMB2062, was recovered from gel, digested with *Hin*cII or *Hin*dIII, and subcloned into the *E. coli* vector pDG780 for sequencing. Two recombinant plasmids were sequenced and resulted in two identical sequences, so we could clone this and other miniplasmids by cutting only with *Hin*cII. The amino acid sequence of pBMB2062 (>70 amino acids) were compared with sequences in the National Center for Biotechnology Information (NCBI) database using the ORF FINDER software, BLASTN and BLASTP algorithms from the NCBI website (http://www.ncbi.nlm.nih.gov/).

Construction of recombinant plasmids

The plasmid pBMB2062 was gel-purified, digested with *Hin*cII (the fragment was named *ori*2062), and then inserted into the *Sma*I site of pDG780 to generate the plasmid pBMB0401. *Orf1* and *orf2* derivatives were constructed as follows. The fragment (1.8-kb) containing an *ORF2* gene deletion was amplified from pBMB2062 by using the primers P1 and P2 (P1: 5′ TTGAAGTAAC GGTTATGTTT 3′ and P2: 5′ CGAACCATTTCACTC ATCTAATC 3′) and then inserted into the *Sma*I site of pDG780, yielding plasmid pBMB0402. Plasmid pBMB0403 was similarly constructed by inserting a 1.3-kb PCR fragment containing an *ORF1* gene deletion amplified from pBMB2062 by using the primers P3 and P4 (P3: 5′ GCCTATCCAAATAAACTTGCA 3′ and P4: 5′ ATCAC TTGGCGGTGGACCCCA 3′) (Fig. 2) into the *Sma*I site of pDG780. Plasmid pBMB0404 (11 kb) was obtained by

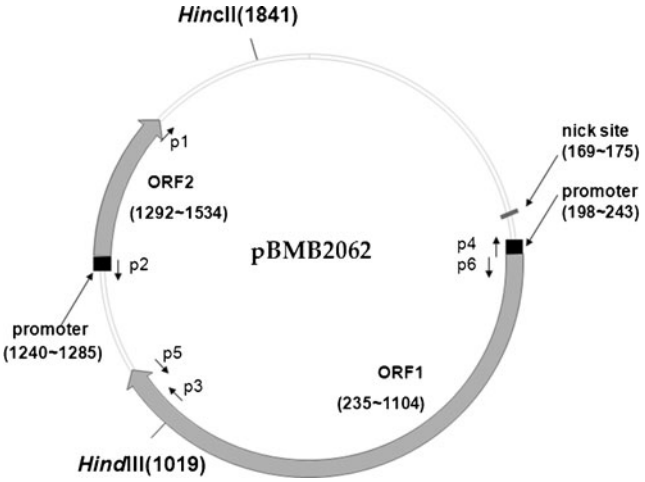

Fig. 2 Physical map and genetic organization of pBMB2062. The *outer circle* represents the small plasmid pBMB2062, and its ORFs are represented as *solid arrows* showing the direction of transcription with the length of the arrow reflecting the relative lengths of the genes. The nick sites and promoters are indicated with *arrows*. P1 (nt at positions 1,273–1,292 on pBMB2062) and P2 (nt at positions 1,520–1,539 on pBMB2062) are the primers used to generate a fragment containing a deletion mutant of the *ORF2* gene that was used to construct plasmid BMB0402. P3 (nt at positions 1,025–1,044 on pBMB2062) and P4 (nt at positions 216–235 on pBMB2062) are the primers used to generate a fragment containing a deletion mutant of the *ORF1* gene that was used to construct plasmid BMB0403. P5 (nt at positions 1,085–1,104 on pBMB2062) and P6 (nt at positions 235–254 on pBMB2062) are the primers used to generate the *ORF1* fragment. To simplify the description, pBMB2062 starts at the same origin as p2055 and p2058 in Figs. 2 and 3

inserting the 4.1-kb *cry1Ac10* (GenBank AJ002514) fragment cloned from strain YBT-1520 into the *Hin*cII site of pBMB0401.

We constructed 3 *B. thuringiensis* strains containing the recombinant plasmids, pBMB0452B, pBMB0462B, or pBMB0472B, that were driven by the replication origins *ori*44 from *B. thuringiensis kurstaki* HD263 (Baum and Gilbert 1991), *ori*1030 from *B. thuringiensis thuringiensis* LM2 (Lereclus et al. 1988), or *ori*2062, respectively. By using the microcalorimetric technique, the *ori* copy numbers per cell of these strains were compared (Lin et al. 2004). The 3 strains were constructed and preserved in our laboratory (Lin et al. 2005). All constructs were confirmed using restriction enzyme and DNA sequence analyses.

Transformation of *E. coli* and *B. thuringiensis*

Transformation of *E. coli* was carried out using $CaCl_2$-treated competent cells, as described by Sambrook and Russell (2001). Transformation of *B. thuringiensis* BMB171 strain (plasmid-free mutant; Li and Yu 1999) was performed by electroporation with the Bio-Rad Gene Pulser set as previously described (Shao and Yu 2004).

Stability test

The stability of the recombinant plasmids, pBMB0401, pBMB0402, and pBMB0404 in *B. thuringiensis* BMB171, was tested under nonselective conditions at 30 °C as described by Sanchis et al. (1997). One hundred single colonies of each recombinant were transferred onto LB plates and LB plates with kanamycin (50 μg/mL). Plasmid stability was estimated as the number of resistant colonies after about 40 generations. Each experiment was repeated three times.

Dot-blot analysis

Dot-blot analysis of the total DNA from 108 strains was performed according to the routine protocol (Sambrook and Russell 2001). A DNA fragment containing *orf1* was amplified from pBMB2062 by using primers P5 and P6, and it was used as a probe that was then labeled with ^{32}P-labeled nucleotides using the random primer method. As the positive and negative controls, 10 ng total DNA of strain YBT-1520 and strain BMB171 were used, respectively.

Nucleotide sequence accession number

The DNA sequence described in this paper has been deposited in the GenBank nucleotide sequence database under the accession numbers DQ269014 for orf1 of pBMB2062-like plasmid (H3abc strain), DQ269015 for orf1 of pBMB2062-like plasmid (H21 strain) and DQ269016 for orf1 of pBMB2062-like plasmid (MGR strain), AF050161 for pBMB2062 plasmid (YBT-1520 strain), HM991869 for pBMB2062-like plasmid (HD-1 strain), and DQ185138–DQ185143 for other related plasmids.

Results

Cloning and sequence analysis of plasmid pBMB2062

On comparing the plasmid profiles of several *B. thuringiensis* strains, we found a 2-kb plasmid that produced a band of considerable intensity. This plasmid was gel-purified, digested with *Hin*cII or *Hin*dIII, and subcloned into the *E. coli* vector pDG780, respectively. The plasmid was 2,062 bp in size by nucleotide sequencing and PCR conformation and was named pBMB2062). It is shown that the average G+C content of pBMB2062 was 34.8 %, i.e., within the range characteristic for *B. thuringiensis* species, and contained 2 ORFs (coding region larger than 70 amino acids). Promoter sequences were found upstream of these ORFs at positions 198–243 bp and 1,240–1,285 bp. The nick site sequence of pBMB2062 was similar to that of the

rolling-circle replication (RCR) plasmid pT181 (Khan 2003), suggesting that pBMB2062 belongs to the RCR group.

The amino acid sequence of pBMB2062-ORF1 (289 amino acids) exhibited 21 % identity with the replication initiation protein from *B. thuringiensis israelensis* ATCC 35646 (GenBank, ZP_00742790). ORF2 (80 amino acids) exhibited 31 % identity with the putative antitoxin of toxin-antitoxin from *Rickettsia heilongjiangensis* 054 (Duan et al. 2011). Toxin-antitoxin (TA) loci are usually organized into operon-contained toxin and antitoxin gene, and the toxic effect of the long-lived toxin is continuously inhibited by the short-lived antitoxin only when the whole system is maintained (Gerdes 2000). However, the pBMB2062-ORF1 did not share identity with any toxin proteins in the database. As reported earlier, a significant number of genes encoded potential toxins or antitoxins with no partner mapping in their vicinity (Sevin and Barloy-Hubler 2007), and, if pBMB2062-ORF1 was an antitoxin protein, it might be a solitary antitoxin and help other plasmid stabilize in the host strain.

Stability assay and copy number comparison

To determine whether *ORF1* and *ORF2* are required for plasmid replication, the 1.8-kb PCR fragment (containing intact *ORF1* but disrupt *ORF2*) and 1.3-kb PCR fragment (containing intact *ORF2* but disrupt *ORF1*) were inserted into the vector pDG780, generating pBMB0402 and pBMB0403, respectively. After transferring the pBMB0402 and pBMB 0403 plasmids into *B. thuringiensis kurstaki* BMB171, a recombinant strain BMB0402B bearing pBMB0402 was recovered on LB agar plates containing kanamycin, while no transformant containing pBMB0403 was obtained. This result suggested that *ORF1* was required for pBMB2062 replication in *B. thuringiensis*, whereas *ORF2* was not.

The three recombinant plasmids, pBMB0401, pBMB0402 and pBMB0404, were used to test the segregation stability of *ori*2062. In the absence of positive selection with kanamycin for 40 generations, the frequency of cells retaining these plasmids was 98, 100, and 98 %, respectively. This result clearly demonstrated that *ori*2062 could stably drive the replication of at least a 9-kb foreign fragment (pBMB0404) in *B. thuringiensis* BMB171. Furthermore, the 1.8-kb minimal replicon only contained *orf1* but without *orf2* was sufficient for replication in *B. thuringiensis* BMB171.

All the above results indicated that *ori*2062 could be used to construct shuttle vectors, like pBMB0401, in *B. thuringiensis*. The plasmid pBMB0401 was of interest because of its small size of 6 kb, high copy number, stable replication in both *E. coli* and *B. thuringiensis*, and multiple cloning sites. This *ori*2062-based vector could stably drive the replication of a 9-kb foreign fragment (recombinant plasmid pBMB0404) in *B. thuringiensis* as described above.

Distribution of pBMB2062 in *B. thuringiensis*

In *B. thuringiensis* plasmids extraction, some other strains were shown to contain this miniplasmid. In order to test the distribution of pBMB2062 in *B. thuringiensis* strains covered all the serotypes, dot-blots using the *ORF1* fragment as a probe were performed. Positive signals were obtained in 44 *Bacillus* strains and 64 strains provided no signal to the probe, see details in Table 1. It showed that 41 % strains contained the *ORF1* of pBMB2062 (44 of 108 tested strains covering 84 *B. thuringiensis* serotypes) and 30 strains contained the *ORF1* of pBMB2062 in the 84 tested *B. thuringiensis* strains with standard serotype (36.1 %). This suggested that the occurrence of pBMB2062 was not serotype-specific and that this plasmid was randomly distributed in *B. thuringiensis* strains plus *B. cereus* UW 85

The 2-kb small plasmids of 6 randomly selected strains (H2, H4ac, H9, H32, H56 and YBT-833) from the above 44 isolates were recovered from gels, digested with *Hin*cII, and subcloned into pDG780 for sequencing. The nucleotide sequences were subsequently deposited in the GenBank database under the accession numbers DQ185138–DQ185143.

Discussion

Two similar plasmids, a 2,055-bp plasmid (named p2055 here) from *B. thuringiensis kurstaki* HD1-DIPEL (McDowell and Mann 1991) and a 2,058-bp plasmid (named p2058 here) from *B. thuringiensis kurstaki* HD-3a3b (Marin et al. 1992), have been previously published. There are some minor sequence differences among the two plasmids and pBMB2062; however, the differences led to *ORF1* disruption and frame-shifts in p2055 and p2058, compared to ORF1 in pBMB2062 (Fig. 3).

The DNA sequences of the 2-kb plasmids from strains of H9, H32 and YBT-833 were identical to that of pBMB2062, and the DNA sequences of 2-kb plasmids from strains of H2, H4ac and H56 showed 1 bp difference each to pBMB2062, but the differences did not lead to differences in the coding sequences and the amino acid sequences of ORF1 in these 6 plasmids were 100 % identical. These results suggested that the 2-kb plasmids were widely distributed in *B. thuringiensis* strains and that their sequences are highly conserved. Moreover, the RT-PCR results confirmed that the proposed mRNA of *ORF1*-pBMB2062 (870 bp) does exist in reality (Sun et al. 2000b). Considering the above, there was a large probability that incorrect sequencing could occur in p2055 and p2058.

It was suggested that the miniplasmid pBMB2062 demonstrated a high copy number as it produced a band of considerable intensity as compared to the other plasmids extracted from *B. thuringiensis* YBT-1520 (Fig. 1). In our laboratory, the real-time PCR method was used to determine

Table 1 Distribution of pBMB2062 in *Bacillus thuringiensis* strains covered all the serotypes plus one *B. cereus* strain by using dot-blots method with the *ORF1*-pBMB2062 fragment as probe

	Strain names
Strains showed positive signal	*Bacillus thuringiensis* 4B1 (H2), 4D1 (H3abc), HD-1 (H3abc), YBT-1406 (H3abc), YBT-1463(H3abc), YBT-1520 (H3abc), 4F1 (H4ac), CT-155 (H4ac), CT-256 (H4ac), 4G1 (H5ab), 4H1 (H5ac), YBT-1416 (H7), 4AA1 (H8ab), 4Z1 (H8ac), 4L1 (H9), 4BF1 (H10ac), 4O1 (H12), 4Y1 (H19), 4AM1 (H20ab), 4X1 (H21), 4AL1 (H25), 4AG1 (H26), 4AC1 (H27), 4CF1 (H28ac), 4AE1 (H29), 4AD1 (H31), 4AF1 (H32), 4AK1 (H33), YBT-833 (H33), 4AW1 (H37), 4BD1 (H40), 4AR1 (H42), 4BJ1 (H47), 4BL1 (H49), 4BS1 (H55), 4BT1 (H56), 4CB1 (H64), T68 (H68), T69 (H69), CT-43 (no flagellum), YBT-031 (unknown serotype), YBT-9603 (unknown serotype), MGR (unknown serotype) and *B. cereus* strain UW85
Strains showed no signal	*Bacillus thuringiensis* 4A1 (H1), HD-a2 (H1), BMB171 (H3abc, plasmid-free mutant), 4C1 (H3ac), 4AP1 (H3ade), 4E1 (H4ab), 4I1 (H6), 4J1 (H7), 4K1 (H8ab), YBT-1765 (H8ab), 4AZ1 (H8bd), 4M1 (H10ab), YBT-1532 (H10ab), 4N1 (H11ab), 4U1 (H11ac), 4P1 (H13), 4Q1 (H14), YBT-007 (H14), YBT-008 (H14), IPS78/11 (H14), 4R1 (H15), 4S2 (H16), 4V1 (H17), 4W1 (H18ab), 4CA1 (H18ac), 4BA1 (H20ac), 4AN1 (H22), 4AT1 (H23), 4BE1 (H24ab), 4AX1 (H24ac), 4AJ1 (H28ab), T30 (H30), 4AD1 (H31), 4AH1 (H34), 4AQ1 (H35), 4AV1 (H36), 4AY1 (H39), 4BB1 (H41), 4BC1 (H43), 4AU1 (H44), 4BG1 (H45), 4BH1 (H46), 4BK1 (H48), 4BM1 (H50), 4BN1 (H51), 4BP1 (H52), 4BQ1 (H53), 4BR1 (H54), 4BU1 (H57), 4BV1 (H58), 4BW1 (H59), 4BX1 (H60), 4BY1 (H61), 4BZ1 (H62), T63 (H63), 4CC1 (H65), 4CD1 (H66), 4CE1 (H67), T70 (H70), T71 (H71), 4T1 (no flagellum), YBT-1514 (unknown serotype), BMB999-7 (unknown serotype) and YBT-1345 (unknown serotype).

that pBMB2062 had the highest PCN (172) in strain YBT-1520 (Zhong et al. 2011).

Moreover, we could exploit microcalorimetry to study the relationship between heat output and gene expression. In microcalorimetry mensuration, a strain containing a plasmid with higher copy number would generate less heat than a strain containing a plasmid with lower copy number, and a strain with lower heat output would produce more protein (Lin et al. 2004, 2005). In this study, we also analyzed the microcalorimetric characteristics of *ori*2062. Three recombinant plasmids, pBMB0472B, pBMB1206R and pBMB1205, were conducted by *ori*2062, *ori*1030 and *ori*44, respectively. The total heat flow rate O_{total} of BMB0472B was 26.01 J, which was less than that of the other 2 strains. The only difference among the 3 strains was with regard to their plasmid replicons. The origin *ori*1030 derives from the 15-kb plasmid pHT1030 with a copy number of 4 (Arantes and Lereclus. 1991), and *ori*44 that derives from a 44-MD plasmid (Baum and Gilbert 1991) probably produces a lower copy number than *ori*1030. Therefore, based on the thermogenic data, we deduced that the copy number of *ori*2062 was higher than those of *ori*44 and *ori*1030 and that the replication effect of *ori*2062 in *B. thuringiensis* strain was better than those of *ori*44 and *ori*1030.

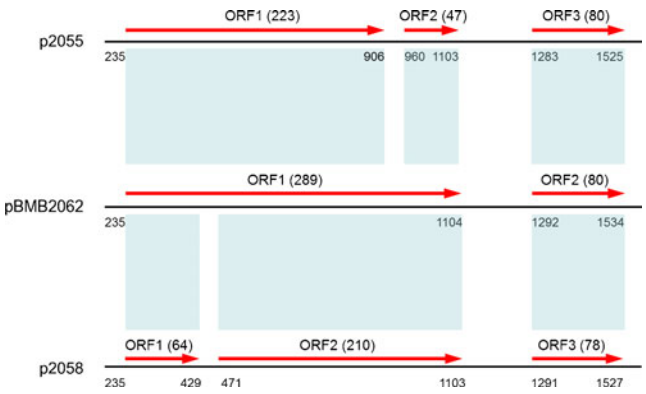

Fig. 3 Schematic representation of the *ORF* genes present in the 3 miniplasmids. The main ORFs in p2055, p2058, and pBMB2062 are represented as *arrows*, the *arrowheads* show the direction of transcription, and the length of the arrows reflects the relative lengths of the genes, as shown in the sketch map. In the pBMB2062-ORF1 area (289 amino acids), the *ORF1* of p2055 is divided into 2 parts that separately code for 233 and 47 amino acids. On the other hand, p2058 divides the pBMB2062-*ORF1* area into 2 parts that separately code for 64 and 210 amino acids. The areas that share the same amino acid sequences are *shaded*

Based on the thermogenic data, we confirmed that the copy number of *ori*2062 was higher than that of *ori*44 and *ori*1030. It was demonstrated that *ori*2062 was useful in genetically manipulating *B. thuringiensis* to produce novel combinations of insecticidal proteins. The fact that *ori*2062 could drive the stable replication of at least a 9-kb foreign fragment suggested that the plasmid pBMB2062 possessed a strong replication ability.

The stable maintenance of multicopy plasmids replicating by the rolling circle mechanism in the host is controlled by the availability of the *rep* gene-encoded initiator of replication protein (Rep), which introduces a site- and strand-specific nick at the origin of replication, *dso* (Gomis-Ruth et al. 1998). Our observation is consistent with the proposal that *pBMB2062-orf1* is sufficient for stable replicating in *B. thuringiensis* strains, independently the *orf2*. So, there is one model regarding the function of ORF2 based on its sequence similarity: a solitary antitoxin and function as a trans-element to play a role in reducing the toxin activity from different systems since some antitoxins can interact with non-cognate toxin proteins in vivo (Grady and Hayes 2003). The YBT-1520 strain contained at least 7 stable plasmids, and we were inclined to believe that pBMB2062 might be considered to act as a selfish plasmid that ensure the inheritance of itself by preventing the proliferation of pBMB2062-free progeny. The

searching of the actual toxin inhibited by ORF2 is now within experimental reach.

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