

Cloning and Characterization of pBMB9741, a Native Plasmid of *Bacillus thuringiensis* subsp. *kurstaki* Strain YBT-1520

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Abstract. A native plasmid of *Bacillus thuringiensis* subsp. *kurstaki* strain YBT-1520 named pBMB9741 has been successfully cloned, sequenced, and characterized. Twelve open reading frames of at least 50 amino acids were identified. BLAST search indicated that three of them encode conserved proteins involved in conjugative mobilization, replication initiation, and transcription regulation. The *orf6* located within a 2.2-kb minimal replication region was predicted to encode a replication protein. An homologous study of the *orf6* product suggested that this plasmid might engage a rolling-circle replication mechanism. Unlike many other plasmids that adopt a rolling-circle model to replicate, pBMB9741 demonstrated strong segregation stability. When tested at 28°C, 37°C, and 42°C, this plasmid maintained 100% stability in a variety of strains, including wild-type strains of *B. thuringiensis* and *B. cereus*, as well as plasmidless mutants of *B. thuringiensis* subsp. *kurstaki* and subsp. *israelensis*.

The Gram-positive bacterium *Bacillus thuringiensis* is widely used as a biocontrol agent, and its primary toxic components are δ -endotoxins. More than 200 types of δ -endotoxin gene have been cloned and sequenced so far and most of these genes are located on plasmids. The plasmid profiles of most *B. thuringiensis* strains are rather complex, with the sizes of their molecular weight varying from 2 to 200 kb and the number of plasmids ranging from 1 to over 10 in most strains [13]. Large plasmids normally display a θ -type replication mechanism in this bacterium, such as p43 (~65 kb) and p60 (~96 kb)[5]. Small plasmids in *B. thuringiensis* usually replicate in a rolling-circle mechanism. Plasmids such as pTX14-1, pTX14-2, pTX14-3, pGI1, pGI2, and pGI3 belong to this group [3]. The size of plasmid, however, is not the only criterion for predicting its replication mechanism. For example, pHT1030, a plasmid 15 kb in size, replicates with the θ -type mechanism [8].

Bacillus thuringiensis strains are well known for their ability to stably maintain their plasmids during cell division, providing us a unique model for studying the mechanisms of plasmid replication, incompatibility and conjugation. *B. thuringiensis* subsp. *kurstaki* YBT-1520 was originally isolated by our group and demonstrated high insecticidal activity against lepidopteran larvae [14]. At least six naturally occurring plasmids have been discovered in strain YBT-1520, with sizes of ~170 b, ~90, ~60, ~12, ~6.6, and ~2. In hope of deciphering the genetic connections among these plasmids and the toxicity of their host cells, efforts have been taken to clone and sequence these plasmids one by one. The smallest one, pBMB2062 (GenBank accession No. AF050161), had been cloned and completely sequenced [15]. This study presents the sequence and the replication characteristics of the second smallest plasmid, pBMB9741.

Materials and Methods

Bacterial Strains and Media. *Escherichia coli* DH5 α was used as the host strain for plasmid amplification. *B. thuringiensis* wild-type strain BMB1 (lab collection) as well as plasmidless strains BMB171 (subsp.

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Table 1. Plasmids involved in this study

Plasmid	Description	Source
pHT3101	<i>ori.Ec, amp^r, ori.Bt, erm^r</i>	Ref. 8
pBMB1105	Derivative of pHT3101, <i>ori.Ec, amp^r, erm^r</i>	This study
pBMB1197	Linear pBMB9741 inserted in pBMB1105	This study
pBMB1198	Linear pBMB9741 inserted in pBMB1105 in opposite direction	This study
pR-1600	1.6-kb <i>HindIII–HindIII</i> fragment of pBMB9741 inserted in pBMB1105	This study
pR-2260	2.26-kb <i>EcoRI–ClaI</i> fragment of pBMB9741 inserted in pBMB1105	This study
pR-2450	2.45-kb <i>StyI–FspI</i> fragment of pBMB9741 inserted in pBMB1105	This study
pR-2570	2.57-kb <i>ClaI–KpnI</i> fragment of pBMB9741 inserted in pBMB1105	This study
pR-2700	2.7-kb <i>EcoRI–SalI</i> fragment of pBMB9741 inserted in pBMB1105	This study
pR-3300	3.3-kb <i>SalI–KpnI</i> fragment of pBMB9741 inserted in pBMB1105	This study
pR-3400	3.4-kb <i>EcoRI–FspI</i> fragment of pBMB9741 inserted in pBMB1105	This study
pR-3740	3.74-kb <i>HindIII–KpnI</i> fragment of pBMB9741 inserted in pBMB1105	This study
pD-81	Derivative of pBMB1198, with a deletion of a 5.5-kb fragment	This study
pD-82	Derivative of pBMB1198, with a deletion of a 3.4-kb fragment	This study
pD-83	Derivative of pBMB1198, with a deletion of a 2.6-kb fragment	This study
pD-84	Derivative of pBMB1198, with a deletion of a 0.9-kb fragment	This study
pD-87	Derivative of pBMB1198, with a deletion of a 0.7-kb fragment	This study
pD-71	Derivative of pBMB1197, with a deletion of a 5.3-kb fragment	This study
pD-78	Derivative of pBMB1197, with a deletion of a 4.5-kb fragment	This study
pD-712	Derivative of pBMB1197, with a deletion of a 4.2-kb fragment	This study
pD-714	Derivative of pBMB1197, with a deletion of a 1.9-kb fragment	This study
pD-719	Derivative of pBMB1197, with a deletion of a 1.2-kb fragment	This study
p9741-Rep	2.2-kb replication region of pBMB9741 inserted into pBMB1105	This study

kurstaki [9] and IPS-78/11 (subsp. *israelensis*), and *B. cereus* strain UW030 (kindly provided by Dr. Handelsman, University of Wisconsin) were used as the host strains for plasmid stability tests. BHIG medium (20% brain heart infusion, 1% tryptone, 0.2% glucose, 0.5% NaCl, 0.25% NaH₂PO₄, 0.15% glycerol) was used to prepare *B. thuringiensis* and *B. cereus* competent cells, and NSM medium (0.8% nutrient broth, 0.7 mM CaCl₂, 0.05 mM MnCl₂, 1 mM MgCl₂) [4] was used as the selective medium after electroporation. When required, antibiotics [ampicillin (100 µg/mL; Sigma); erythromycin (25 µg/mL; Sigma)] were added.

DNA Manipulations. Restriction enzymes, *Taq* polymerase, and T4 DNA ligase were purchased from Roche Applied Science (Germany), protease K was purchased from Promega (Madison, WI), Klenow enzyme was purchased from New England Biolabs (Ipswich, MA), and lysozyme was purchased from Sigma-Aldrich (St. Louis, MO). Nylon membrane (GE, Waukesha, WI) and DIG DNA Labeling and Detection Kit (Roche Applied Science) were used in Southern hybridization. DNA fragments were isolated from the agarose gel by using QIAquick Gel Extraction Kit (QIAGEN). Plasmids were isolated from *E. coli* strains with Plasmid Miniprep Kit (Bio-Rad) for general analysis and with QIAGEN Plasmid Miniprep Kit (QIAGEN) for DNA sequencing. These materials were used as specified by the manufacturers. Plasmids propagated in *B. thuringiensis* and *B. cereus* strains were extracted as described by Voskuil and Chambliss [16].

Plasmids Construction. Plasmids involved in this study are described in Table 1 and Fig. 1. Vector pHT3101 [8; kindly provided by Dr. Lereclus, Institut Pasteur] was double digested with *HpaI* and *NdeI* to remove the replication region of *B. thuringiensis*, then blunted at the 3' end, and self-ligated to generate plasmid pBMB1105 (3.751 kb), which was then used to clone pBMB9741. Total plasmid DNA of *B. thuringiensis* YBT-1520 was extracted and digested with *EcoRI*. After ligation to pBMB1105, predigested also with *EcoRI*, the ligation

mixture was transformed into *B. cereus* strain UW030. One erythromycin-resistant colony bearing an ~10.3-kb recombinant plasmid named pBMB1197 was obtained (Fig. 2a). Plasmid pBMB1197 carried a ~6.6-kb *EcoRI–EcoRI* insertion, which was later confirmed to be the linearized form of pBMB9741 (Fig. 2b). The entire fragment of pBMB9741 was cut off from pBMB1197 by *EcoRI* digestion and was reintroduced back to the same site in an alternative orientation to create pBMB1198.

Subclones pR-1600, pR-2260, pR-2450, pR-2570, pR-2700, pR-3300, pR-3400, and pR-3740 were constructed by single or double restrictive digestion of pBMB1197. Subclones pD-71, pD-78, pD-712, pD-714, and pD-719 were derived from pBMB1197 by DNA deletion approaches. Briefly, pBMB1197 was partial cleaved with *DNaseI* in the presence of Mn²⁺ to obtain the single-cut plasmid, followed by incubation with *PstI* to delete the region between *PstI* and *DNaseI* cutting sites (Fig. 2a). After Klenow treatment and self-ligation, the ligation mixture was subjected to reaction with *XbaI*, which has a single cleavage site between the *EcoRI* and *PstI* sites (Fig. 2a), to linearize intact pBMB1197 DNA molecules. Clones that had the 5' end of pBMB9741 sequence being deleted would not be linearized and therefore had higher transformation efficiency. After *XbaI* treatment, the DNA was used to transform *E. coli* DH5 α , and a series of deletion subclones were obtained after screening based on their sizes. With the similar approach, 3'-end deletion subclones pD-81, pD-82, pD-83, pD-84, and pD-87 were generated from pBMB1198. These subclones were used for both DNA sequencing and replication functionality analyses.

DNA Sequencing. Both strands of series of subclones of pBMB9741 were sequenced with a DNA sequencing kit (Perkin-Elmer) and ABI PRISM 377 DNA sequencer (Perkin-Elmer). The sequencing primers were M13 universal primers and specific primers synthesized by GENSET OLIGOS (Singapore). The DNASTAR software package from LASERGENE (Madison, WI, USA) was used to analyze the sequences.

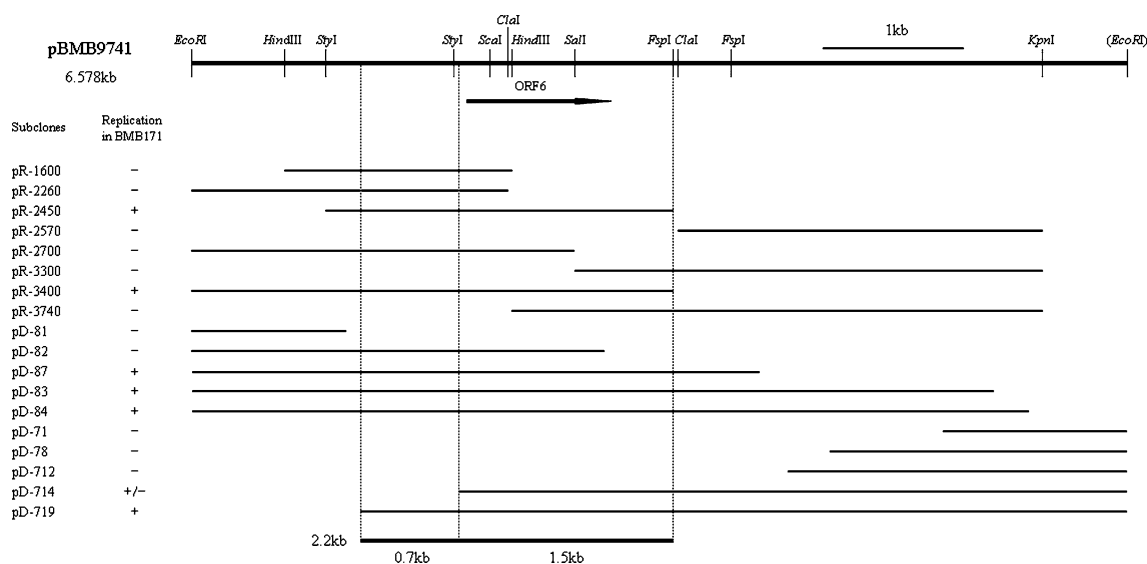


Fig. 1. Diagrams of pBMB9741 and the relevant plasmids. Plasmids able to replicate in BMB171 are marked with “+” in the middle column, and plasmids unable to do so are indicated with “-.” Plasmid that could be transferred into strain BMB171 but was quickly lost during cell propagation is marked with “+/-.” ORF6 is the only open reading frame identified in this 2.2-kb region, which is responsible for the replication of this plasmid.

Polymerase Chain Reaction Amplification. Both genomic DNA and total plasmid DNA of *B. thuringiensis* strain YBT-1520 were used as templates to detect whether the cloned fragment is a complete circular plasmid. A-40F (5'-CAAGGGGAAGAGTTTCCCTTG) and A-38R (5'-GTCGCTAGAAACACCACGCTC) were used as the amplification primers. A half microliter of genomic DNA or 1 μ L of total plasmid DNA was used as the template in a typical 100- μ L reaction. Reactions started with denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 50 s. After a final extension of 72°C for 7 min, the reaction mix was stored at 15°C. The amplicons were analyzed with 1% agarose gel electrophoresis.

Two primers containing *EcoRI* recognition site at the 5' end were designed to clone the 2.2-kb minimal replication region of pBMB9741: Rep-F (5'-ccggaattcTGAAAATGTTGTATTAGTATC) and Rep-R (5'-ccggaattcGCACACTATAGGTTTTTAACT). After amplification of pBMB1197 DNA with the two primers, the amplicon was digested with *EcoRI* and was inserted into pBMB1105 to construct p9741-Rep.

Transformation of *B. thuringiensis* and *B. cereus*. *Bacillus thuringiensis*- and *B. cereus*-competent cells were prepared as follows. *B. thuringiensis* and *B. cereus* strains were inoculated into 10 mL of BHIG medium and incubated at 30°C overnight with shaking at 250 rpm. Two microliters of seed cultures were transferred into 100 mL of fresh BHIG medium and were incubated at 37°C for about 1.5–2 h [final optical density (OD_{600nm}) = 0.2–0.3]. Harvested cell cultures were kept on ice for 5 min and centrifuged. After washing with 50 mL of ice-cold 0.5 M sucrose–1 mM HEPES for three to five times, the cells were resuspended in a mixture of 4 mL of 0.5 M sucrose–1 mM HEPES and 1 mL of autoclaved 50% glycerol and were stored at –70°C after quick freezing in liquid nitrogen. For electroporation, 2-mm cuvettes were used. Suitable donor DNA was added to 100 μ L of competent cells and was pulsed at 2.5 kV with Electroporation System Electro Cell Manipulator® 600 (BTX Inc., CA). After 4 h of recovery, the cells were plated on NSM medium supplemented with 25 μ g/mL erythromycin and were subject to incubation at 30°C overnight.

Assay for Segregation Stability. Plasmid pBMB1197, which contains the complete sequence of plasmid pBMB9741 plus an erythromycin-resistant gene, was transformed into a variety of *B. thuringiensis* and *B. cereus* strains to test its segregation stability at different temperatures: 28°C, 37°C, and 42°C. Overnight cultures of each recombinant strain was inoculated into 10 mL of Lurie–Bertani (LB) medium and incubated at selected temperatures without antibiotics. Cell cultures were transferred to fresh LB medium every 12 h. During each transfer, suitable dilutions of the culture were plated on nonselective medium and incubated at the same temperature to obtain single colonies. Three hundred colonies were then picked and tested for their growth on medium supplemented with erythromycin. The plasmid stability was estimated by the ratio of erythromycin-resistant colonies to total colonies. Each test was performed in triplicate.

Results and Discussion

Confirmation and Sequencing of Plasmid pBMB9741. When analyzing the size of the plasmid pBMB1197, we noticed that the size of the inserted fragment was very close to that (~6.6 kb) of the second smallest plasmid of strain, YBT-1520. To determine whether the cloned fragment was an entire plasmid, after sequencing the 5' and 3' ends of the cloned fragment, two primers were designed to amplify the rest of the plasmid from templates of YBT-1520 genomic DNA and total plasmids DNA. A-38R was annealed with one strand at the 3' end and A-40F was annealed with the complementary strand at the 5' end (Fig. 2b). polymerase chain reaction (PCR) amplification results and consequent sequencing of the amplicons

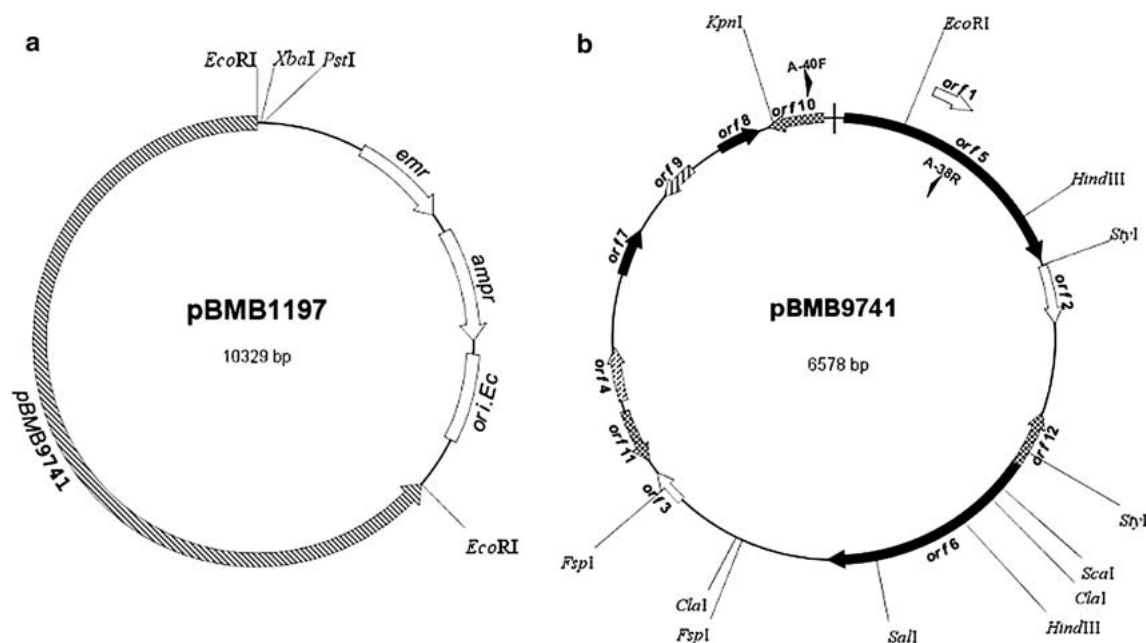


Fig. 2. Restriction and genetic maps of pBMB1197 (a) and pBMB9741 (b). All ORFs larger than 50 amino acids are depicted in arrows; directions of the arrows indicate transcriptional direction of the predicted ORFs, and styles of the arrows represent different reading frames. Primer A-38R and A-40F are shown in small arrows.

demonstrated that the cloned fragment was indeed a complete circular plasmid, because the sequence of the amplicons of size 724 bp was exactly the same as the combined locus of the 3' and 5' ends of the cloned fragment (Fig. 3a). Southern hybridization analysis with the 6.6-kb cloned fragment as probe confirmed that the cloned fragment was truly the second smallest plasmid of strain YBT-1520 (Fig. 3b).

Restriction analysis revealed that the cloned plasmid contains single sites of *EcoRI*, *KpnI*, *ScaI*, and *Sall*, as well as double sites of *HindIII*, *ClaI*, *StyI*, and *FspI*. Based on construction of derivatives of pBMB1197, complete sequences of both strands of the cloned plasmid were determined. The results showed that the cloned plasmid was 6578 bp in size, with the GC content of 32.4%, characteristic of *B. thuringiensis* plasmids [6]. The complete nucleotide sequence was submitted to GenBank under accession number AF202532.

Structural Organization of pBMB9741. Analysis of the coding capacity of pBMB9741 revealed 12 potential open reading frames (ORFs) encoding at least 50 amino acids (Fig. 2b): eight ORFs locate on one strand and four on the complementary strand. The potential modules or domains of each ORF were searched against NCBI's Conserved Domain Database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Search results revealed that ORF5 (bases 51–1262) contained a

Mob_Pre domain, ORF6 (bases 2274–3317) had a plasmid rolling-circle replication initiator protein domain, and ORF7 (bases 5244–5471) possessed a predicted transcriptional regulator domain. No conserved domain was identified in the other nine ORFs, namely ORF1 (bases 403–570), ORF2 (bases 1294–1575), ORF3 (bases 4081–4254), ORF4 (bases 4637–4894), ORF8 (bases 6012–6224), ORF9 (complementary strand, bases 5664–5852), ORF10 (complementary strand, bases 6263–6529), ORF11 (complementary strand, bases 4322–4855) and ORF12 (complementary strand, bases 2000–2275). BLAST search results are in agreement with the above domain searching results.

Determination of Replication Protein of pBMB9741.

To locate the minimal replication origin of pBMB9741, a series of deletion derivatives of pBMB9741 was constructed in *E. coli* and subsequently tested in *B. thuringiensis* plasmidless strain BMB171 for their replication capacities. Recombinant plasmids pR-2450, pR-3400, pD-87, pD-83, pD-84, and pD-719, which contain a common 2.2-kb region, were able to replicate in BMB171. In contrast, plasmids pR-1600, pR-2260, pR-2570, pR-2700, pR-3300, pR-3740, pD-81, pD-82, pD-71, pD-78, and pD-712, which lack the complete sequence of the 2.2-kb region, failed to replicate in BMB171 (Fig. 1). These

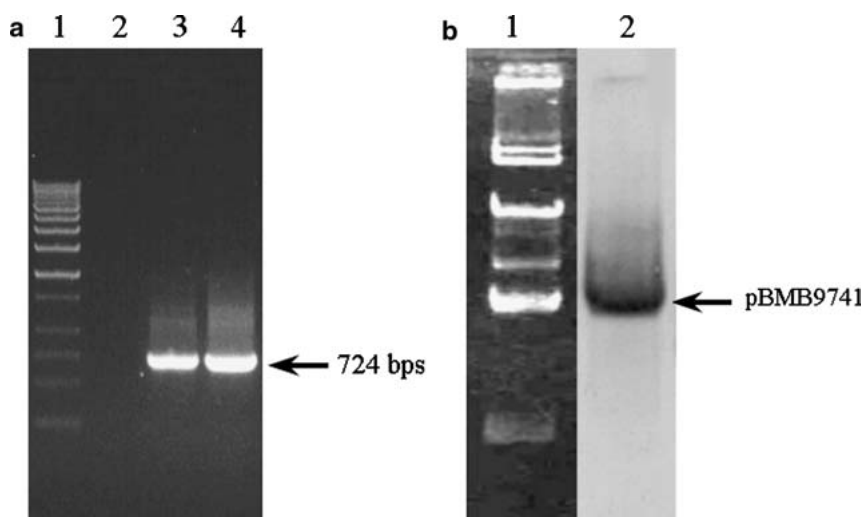


Fig. 3. (a) PCR detection of the integrity of pBMB9741; 1% agarose gel was used. Lane 1, DNA ladder (from top to bottom: 12, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1.5, 1, 0.75, 0.5, 0.25 kb); lane 2, negative control (without template DNA); lane 3, template with total plasmid DNA of strain YBT-1520; lane 4, template with genomic DNA of strain YBT-1520. The arrow indicates the 724-bp PCR products. (b) Resource detection of pBMB9741. Total plasmid DNA of YBT-1520 was hybridized with DIG-labeled 6.6-kb cloned fragment; 0.6% agarose gel was used. Lane 1, plasmids profile of strain YBT-1520; lane 2, Southern blotting of pBMB9741. The arrow indicates the position of pBMB9741.

Table 2. Comparison of *orf6*/ORF6 with other *B. thuringiensis* plasmid replication gene/protein

Plasmid name	Size (bp)	GenBank Accession No.	<i>rep</i> gene size (bp)	<i>rep</i> gene identity (%)	Rep protein size (aa)	Rep protein identity (%)	Ref.
pHD2	2,055	S43931	672	26.2	223	12.5	13
PBMB2062	2,062	AF050161	870	25.3	289	11.7	15
PUIBI-1	4,671	AF516904	420	24.5	139	10.7	11
pTX14-1	5,415	U67921	1,014	69.4	337	72.8	3
pBMBt1	6,700	AY822042	600	28.7	199	13.0	10
pTX14-2	6,829	AY138808	615	26.7	204	14.1	3
pTX14-3	7,649	X56204	639	26.8	212	13.6	1, 2
pGI1	8,254	AY138809	1,047	95.5	348	97.1	3
pGI2	9,672	X13481	855	25.0	284	11.3	12
pGI3	11,365	Y11173	924	25.2	307	12.4	7
PBMB165	20,286	DQ242517	1,557	25.2	518	11.8	17
p43	~65,000	M60513	2,828	25.6	942	12.1	5
p60	~96,000	M60475	2,290	26.1	762	12.6	5
p44	~66,000	M60465	2,249	25.7	749	12.5	5

findings suggest that this ORF6-containing 2.2-kb region is required for replication of pBMB9741, and ORF6 could be the replication protein of the plasmid.

To further test this idea, this 2.2-kb region was introduced to pBMB1105. The resulting plasmid p9741-Rep could successfully replicate in the *B. thuringiensis* plasmid-free strain BMB171, indicating that ORF6 was fully functional in initiating plasmid propagation in *B. thuringiensis* cells. Based on above observations, we conclude that the replication protein of pBMB9741 is ORF6.

The similarity of *orf6*/ORF6 with other published *B. thuringiensis* plasmids replication gene/protein was compared by sequence alignment and the result is shown in Table 2. The pBMB9741 *rep* gene demonstrated high homology to two plasmids engaging the rolling-circle replication mechanism, pTX14-1 and pGI1 [3], with

respective 69.4% and 95.5% identities on DNA sequence level and 72.8% and 97% identities on the deduced gene products sequence level. These suggest that pBMB9741 might also be a rolling-circle replicating plasmid.

Stability of pBMB9741 in *B. thuringiensis* and *B. cereus* at Different Temperatures. To determine the stability of pBMB1197, which bears an erythromycin-resistant gene on a pBMB9741 backbone, recombinant strains were constructed by introducing pBMB1197 to *B. thuringiensis* subsp. *kurstaki* plasmidless strain BMB171, *B. thuringiensis* subsp. *israelensis* plasmidless strain IPS-78/11, *B. thuringiensis* wild-type strain BMB1, which contains at least five native plasmids, and *B. cereus* strain UW030, which harbors a 2-kb plasmid. These strains were cultivated at 28°C, 37°C, and 42°C without antibiotics. The ratio of erythromycin-resistant colonies

to total colonies was surveyed every 12 h to determine the stability of pBMB9741 replication origin in the host strain without selective pressure. After transferring 12 times (equivalent to 196–200 generations), all recombinant strains were still erythromycin resistant, although the total numbers of surviving cells decreased when they were cultivated at 37°C and 42°C, temperatures higher than their optimal growth temperature (28°C). For each of the above four recombinant strains, plasmid profiles after generations of growth were exactly the same as the plasmid patterns before cultivation. All of the above suggests that pBMB9741 could be safely maintained during cell divisions in both *B. thuringiensis* and *B. cereus* strains at a range of temperatures, making it a suitable tool in *Bacillus* cell engineering.

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