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Cloning and Molecular Characterization of a Novel Rolling-Circle Replicating Plasmid, pK1S-1, from *Bacillus thuringiensis* subsp. *kurstaki* K1

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***Bacillus thuringiensis*, an entomopathogenic bacterium belonging to the *B. cereus* group, harbors numerous extra-chromosomal DNA molecules whose sizes range from 2 to 250 kb. In this study, we used a plasmid capture system (PCS) to clone three small plasmids from *B. thuringiensis* subsp. *kurstaki* K1 which were not found in *B. thuringiensis* subsp. *kurstaki* HD-1, and determined the complete nucleotide sequence of plasmid pK1S-1 (5.5 kb). Of the six putative open reading frames (ORF2-ORF7) in pK1S-1, ORF2 (MobK1) showed approximately 90% aa identity with the Mob-proteins of pGI2 and pTX14-2, which are rolling circle replicating group VII (RCR group VII) plasmids from *B. thuringiensis.* In addition, a putative origin of transfer (*oriT*) showed 95.8% identity with those of pGI2 and pTX14-2. ORF3 (RepK1) showed relatively low aa identity (17.8~25.2%) with the Rep protein coded by RCR plasmids, however. The putative double-strand origin of replication (*dso*) and single-strand origin of replication (*sso*) of pK1S-1 exhibited approximately 70% and 64% identities with those of pGI2 and pTX14-2. ORF6 and 7 showed greater than 50% similarities with alkaline serine protease, which belongs to the subtilase family. The other 2 ORFs were identified as hypothetical proteins. To determine the replicon of pK1S-1, seven subclones were contructed in the *B. thuringiensis* ori-negative pHT1K vector and were electroporated into a plasmid cured *B. thuringiensis* strain. The 1.6 kb region that included the putative ORF3 (Rep1K), *dso* and ORF4, exhibited replication ability. These findings identified pK1S-1 as a new RCR group VII plasmid, and determined its replication region.**

***Keywords*:** *Bacillus thuringiensis* K1, plasmid capture system (PCS), pK1S-1, rolling circle replication, RCR group VII

Bacillus thuringiensis, a Gram-positive and spore-forming soil bacterium, produces parasporal inclusions during sporulation that contain insecticidal crystal proteins. The parasporal inclusions from *B. thuringiensis* have been used as one of the most successful biocontrol agents for suppression of agriculturally and medically important insect pests (Schnepf *et al.*, 1998). Plasmids play a crucial role in bacterial evolution and adaptation by mediating the horizontal exchange of genetic materials and providing advantageous functions to their recipients (Andrup *et al*., 2003). Bacterial plasmids often confer advantages to the host, or encode features that favor their own maintenance and survival. For many plasmids, however, no functions other than maintenance have been described, and they are referred to as cryptic plasmids (Lereclus *et al*., 1982).

Strains of *B. thuringiensis* usually exhibit a complex plasmid profile of up to 17 plasmids, ranging from 2 to 250 kb in size (Lereclus *et al.*, 1982; Carlton and Gonzales, 1985; McDowell and Mann, 1991). Because most of the crystal protein genes are encoded on large plasmids, interest has been predominantly focused on these large molecules (Kronstad *et al.*, 1983; Carlton and González, 1985). So far, several sequences of small plasmids from *B. thuringiensis* strains have been reported (Mahillon and Seurinck, 1988; McDowell and Mann, 1991; Lereclus and Arantès, 1992; Hoflack *et al.*, 1997; Andrup *et al.*, 2003). However, most of these small plasmids are referred to as cryptic (Andrup *et al.*, 2003), since no apparent functions have been attributed to them except for replication ability. Lereclus *et al.* (1989) constructed a shuttle vector for *B. thuringiensis* by employing a replication origin fragment from the small cryptic plasmid pHT1030 of *B. thuringiensis*.

We have previously reported the novel *B. thuringiensis* strain, Bt K1, which belongs to *B. thuringiensis* subsp. *kurstaki* (H3a3b3c) and shows a high level of insecticidal effects against *Plutella xylostella* and *Spodoptera exigua* larvae (Li *et al.*, 2002). In this study, we cloned three small plasmids from *B. thuringiensis* K1 using plasmid capture system (PCS), which proved to be a fast and convenient method to clone plasmids from *B. thuringiensis* strain and genomic segments from polydnavirus (Choi *et al.*, 2004, 2005). The complete nucleotide sequence of one plasmid, pK1S-1, was analyzed and characterized, and its replication region was identified.

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Table 1. Bacterial strains and plasmids

Strains	Relevant characteristics	Reference
Escherichia coli		
JM109	*recA1, endA1, gyrA96, thi-1, hsdR17(rK⁻mk⁺), e14⁻(mcrA⁻), supE44, relA1, Δ(lac-proAB)/F' [traD36, proAB⁺, lacI^q, lacZΔM15]*	TaKaRa
TOP10	*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-*	Invitrogen
Bacillus thuringiensis (Bt)		
subsp. *kurstaki* K1	Host of plasmid pK1S-1, pK1S-3, pK1S-5	Li *et al.* (2002)
subsp. *kurstaki* HD-1	Comparative strain for southern hybridization	McDowell and Mann (1991)
subsp. *israelensis* 4Q7	Host for screening origin of replication, plasmid cured	*Bacillus* Genetic Stock Center, Ohio State Univ., Columbus, Ohio
Plasmids		
pPCS-S	pUC ori, R6KγOri, Amp^r, Tet^r, Tn7L, Tn7R, Bt plasmid cloning	Choi *et al.* (2005)
pHT1K	pUC ori, Bt ori, Amp^r, Erm^r	Kang *et al.* (2005)
pK1S-1	5,475 bp; Bt K1	This study
pK1S-3	Approximately 8 kb; Bt K1	This study
pK1S-5	Approximately 9 kb; Bt K1	This study
pHTORF1	0.7 kb region containing the putative *sso* and *oriT* in pHT1KΔBt ori	This study
pHTORF2N	1.1 kb region containing the N-terminal part of putative *mobK1* in pHT1KΔBt ori	This study
pHTORF23	1.1 kb region containing the C-terminal part of putative *mobK1* and *repK1* in pHT1KΔBt ori	This study
pHTORF3	0.4 kb region containing the putative *repK1* in pHT1KΔBt ori	This study
pHTORF34	1.6 kb region containing the putative *repK1*, *dso* and ORF4 in pHT1KΔBt ori	This study
pHTORF56	1.0 kb region containing the putative ORF5 and 6 of pK1S-1 in pHT1KΔBt ori	This study
pHTORF7	1.1 kb region containing the putative ORF7 of pK1S-1 in pHT1KΔBt ori	This study

Materials and Methods

Bacterial strains and plasmid

E. coli strain JM109 (TaKaRa, Japan) and TOP10 (Invitrogen, USA) were used throughout the experiment. For isolation of plasmid DNAs, *B. thuringiensis* strains were grown at 28°C for 5 h with vigorous shaking in Spizizen-yeast (SPY) medium (Nickerson *et al.*, 1974). Plasmid DNAs were prepared using a QIAGEN Midi Prep kit (QIAGEN, Germany) according to the manufacturer's protocol. *B. thuringiensis* subsp. *israelnesis* 4Q7 strain (*Bacillus* genetic stock center, Ohio State University, Columbus, Ohio, USA) was used as a recipient for electroporation with the pHTORF subclones. Bacterial strains and plasmids used in this study are listed in Table 1.

Cloning of small plasmid DNAs using pPCS-S

We used a plasmid capture system (PCS), based on Tn7 transposition, to clone double stranded circular DNA molecules in *E. coli* cells, as previously described (Choi *et al.,* 2005). The PCS donor-S consists of a pUC19 origin of replication and an ampicillin resistance marker between Tn7L and Tn7R regions. Both origin of replication and marker gene of this donor-S are transferred into target plasmid DNAs by *in vitro* transposition and the transposed DNAs can replicate in *E. coli* cells by transformation. Finally, we can acquire *E. coli* cells harboring the cloned target plasmids.

Donor vectors (pPCS-S) were digested with *Hin*dIII and *Sph*I to eliminate unreacted donor molecules; the resulting fragment (donor-S) was about 3.8 kb in size. The transposition reaction was performed according to the procedure of the Genome Priming System (New England Biolabs, USA). One microliter of donor-S (40 ng/μl) was mixed with 1 μg of Bt K1 plasmid DNA. TnsABC* transposase (New England Biolabs) was added to each transposition reaction and the total mixture was pre-incubated at 37°C for 10 min. The transposition reaction was initiated by adding 1 μl of the start solution and incubated at 37°C for 1 h. The reaction was stopped by heat incubation (75°C, 10 min). The reacted DNA was transformed into competent *E. coli* JM109 cells and transformed cells were selected by plating on ampicillin-supplemented (50 μg/ml) nutrient agar plates.

Southern hybridization analysis

Southern hybridization to total plasmid DNA of *B. thuringiensis* strains was performed according to the manufacturer's instruction (Boehringer Mannheim, Germany). Total plasmid DNAs of *B. thuringiensis* strains were separated on 0.7% agarose gels. The gels were treated for 15 min in 0.25 N HCl and transferred to Hybond N^+ filters (Amersham Pharmacia, Biotech, Sweden) in 0.2 N NaOH as transfer buffer. *Sal*I-digested small plasmid clones from *B. thuringiensis* strains were used as probes and labeled with digoxigenin using a DIG DNA labeling kit (Boehringer Mannheim). Prehybridization, hybridization, washing and detection procedures were as described by the manufacturer.

Sequence analysis of pK1S-1

For sequencing of pK1S-1, a shotgun library was established

Table 2. Comparison of putative Mob protein and *oriT* region of plasmid pK1S-1 with other plasmids of *B. thuringiensis* in RCR VII group

Plasmid name	Size (bp)	GenBank accession no.	Mob protein size (aa)	Mob protein identity (%)	*oriT* coordinates / size (bp)	*oriT* identity (%)	Reference
pK1S-1	5,475	EF406356	441	-	700-723 / 24	-	This study
pGI2	9,672	X13481	445	89.7	7726-7749 / 24	95.8	Mahillon and Seurinck (1988)
pTX14-2	6,829	AY138808	445	89.3	2014-2127 / 24	95.8	Andrup *et al.* (2003)
pTX14-3	7,649	X56204	285	15.8	6698-6726 / 29	41.7	Andrup *et al.* (1994)

and their sequences were determined on an ABI sequencer Model 3700 (PE-Applied Biosystem, USA). We obtained the nucleotide sequence of pK1S-1 with 6-fold average. The complete nucleotide sequences of pK1S-1 has been assigned GenBank accession number EF406356.

Potential open reading frames (ORFs) were established with GLIMMER 2.1 (Delcher *et al.*, 1999) and ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). To analyze of the ORFs, we used BLAST (Altschul *et al.*, 1997) searches against the NCBI non-redundant protein database and the Swiss-Prot protein database to determine their homologies. To further investigate the function of each protein, we used Inter-Pro-Scan against the InterPro protein family database, which included PROSITE, PRINTS, Pfam, Pro-Dom, SMART, TIGRFAMs, PIRSF, and SUPERFAMILY (Mulder *et al.*, 2005). Multiple alignments and sequence identities of amino acids and DNA sequences were performed with MegAlign (ver. 7.1.0) of Lasergene (DNASTAR, USA).

Construction of pHTORF subclones

The minimal *E. coli-B. thuringiensis* shuttle vector pHT1K (Kang *et al.*, 2005) contains an *E. coli* and *B. thuringiensis* origin of replications (pUC ori and Bt ori, respectively), ampicillin and erythromycin resistant genes. To eliminate the *B. thuringiensis* origin of replication from pHT1K, inverse PCR was performed with the primers, Fw-1K-Inv-Xho and Re-1K-Inv. The amplified 3 kb fragment (pHT1KΔBt ori) was digested with restriction enzymes, *Xba*I and *Xho*I, and then ligated with PCR fragments consisting of seven regions of pK1S-1 amplified with specific primer pairs. The pHTORF subclones were designed based on the predicted ORF regions and their sequence analysis results. All PCRs were conducted with *pyrobest*™ DNA polymerase (TaKaRa). Subclones are listed in Table 1, and primer sequences are listed in Supplementary Table 2.

Electroporation into *B. thuringiensis* 4Q7

The pHTORF subclones were introduced into *B. thuringiensis* 4Q7 by electroporation using a slightly modified protocol commonly used for the transformation of *B. thuringienisis* (Lereclus *et al.*, 1989). In order to generate electro-competent *B. thuringiensis* 4Q7 cells, a 10-fold dilution of an overnight *B. thuringiensis* culture was incubated in 10 ml Brain heart infusion (BHI) medium for 12 h at 30°C. Cells were harvested, washed with 10 ml ice-cold ddH_2O three times, and re-suspended in 1 ml of ice-cold 40% PEG 6000 (w/v). Competent cells (200 μl) were mixed with 1 μg subclone DNA and single-shocked in an 0.2 cm electroporation cuvette (Bio-Rad, USA) using a Bio-Rad Gene Pulser and the following conditions: volts, 2.3 kV; resistance, 500 Ω; capacitance, 25 μF. The electroporated cells were recovered in 3 ml BHI medium for 1 h at 30°C and plated on erythromycin-supplemented (25 μg/ml) BHI agar plates. Transformed *Bacillus* colonies were examined at 24 h post-transformation.

Results and Discussion

Cloning of small plasmids from *B. thuringiensis* K1

Strain *B. thuringiensis* K1 (Bt K1) shows different plasmid patterns from *B. thuringiensis* subsp. *kurstaki* HD-1 (Bt HD-1) (Fig. 1A); the typically low molecular plasmid (2 kb) of Bt HD-1 was not found in Bt K1. We therefore attempted to clone small plasmids from Bt K1 using the pPCS-S (S-donor). The pPCS-S proved to be a fast and convenient method to clone plasmids from *B. thuringiensis* strains (Choi *et al.*, 2004). Through transposition of PCS-S donor to plasmid DNAs of Bt K1, three distinct plasmids were cloned (Fig. 1 and 2). The sizes of the cloned plasmids, named pK1S-1, pK1S-3, and pK1S-5, were approximately 5.5 kb, 8 kb, and 9 kb, respectively (Fig. 2).

Southern blot analysis located the three plasmids in different positions in total plasmid DNA of strain Bt K1, but

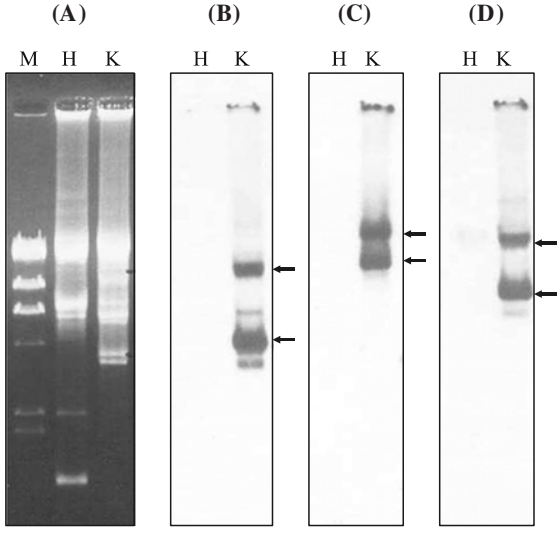

Fig. 1. Electrophoresis (A) and Southern hybridization (B, C, D) of total plasmid DNAs from *B. thuringiensis* K1 and *B. thuringiensis* subsp. *kurstaki* HD-1. Probes were the Dig-labeled 5.4 kb fragment of pK1S-1 (B), 8.0 kb fragment of pK1S-3 (C) and 9.0 kb fragment of pK1S-5 (D), digested with *Sal*I. Lanes: M, Lambda DNA digested with *Hin*dIII; H, *B. thuringiensis* subsp. *kurstaki* HD-1; K, *B. thuringiensis* K1.

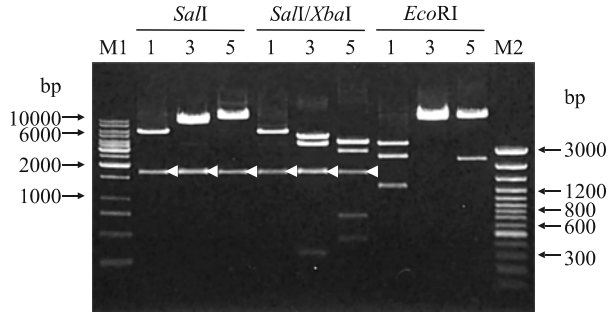

Fig. 2. Restriction endonuclease digestion pattern of the small plasmid DNAs of *B. thuringiensis* K1 cloned by PCS-S donor. Lanes: 1, pK1S-1; 3, pK1S-3; 5, pK1S-5; M1, 1 kb DNA Ladder; M2, 100 bp DNA Ladder. White arrowheads indicate the PCS-S donor.

did not detect them in strain Bt HD-1 (Fig. 1). To test whether or not Bt K1 had the 2,055 bp pHD2 of Bt HD-1 (McDowell and Mann, 1991), PCR analysis was performed using pHD2-specific primers. A PCR fragment of about 1.5 kb in length was detected in Bt HD-1, but not detected in Bt K1 (data not shown). Thus, Bt K1 had three small plasmids whereas Bt HD-1 had only one small plasmid, pHD2 (McDowell and Mann, 1991).

Sequence analysis of pK1S-1 cloned from Bt K1

The complete nucleotide sequence of pK1S-1 was determined. The plasmid pK1S-1 was 5,475 bp in length and the overall GC content was 32.9%, within the range characteristic for *B. thuringiensis* (Claus and Berkeley, 1984). Seven ORFs were originally identified. However, ORF1 was not designated, because part of ORF1 overlapped with putative single-strand origin (*sso*) and origin of transfer (*oriT*) exhibiting high similarity with known plasmids. That locus was designated as *sso* and *oriT*. The six remaining putative ORFs account for as much as 60.9% of the total pK1S-1 sequence, with an average ORF length of 556 bp. The PCS-S donor was inserted into position 1826 GTCGA 1830 in ORF2 (Fig. 3). According to overall sequence analysis, pK1S-1 had nucleotide similarities with pGI2, pTX14-2, and pTX14-3 plasmids from *B. thuringiensis*, which belong to the rolling-circle replication (RCR) group VII and have no similarity with any θ-replication plasmid (Mahillon and Seurinck, 1988; Andrup *et al.*, 2003). Because the new plasmid pK1S-1 appeared to correspond to RCR group VII plasmids, we compared pK1S-1 with the mobilization and replication features of those plasmids (Database of Plasmid Replicons at http://www.essex.ac.uk/bs/staff/osborn/DPR_home.htm).

The location, size and characteristics of the ORFs are given in Fig. 3 and Supplementary Table 1. Of the six predicted ORFs, 4 ORFs could be assigned putative functions and 2 ORFs coded for hypothetical proteins (Supplementary Table 1).

The ORF2 gene from pK1S-1 displayed high similarity to the Mob protein (Table 2 and Supplementary Fig. 1), which confers the ability to be mobilized by the conjugative plasmid pGI2 from *B. thuringiensis* subsp. *thuringiensis* H1.1 (Andrup *et al.*, 2003). Two conserved motifs of the Mob protein, motif2 and motif3 defined by Ilyina and Koonin (1992), were present in the ORF2 sequence (Supplementary Fig. 1). The biological function of ORF2 has not yet been verified experimentally, but its close relationship (89.7% identity) to Mob2 from pGI2 argues for a similar role.

In the general model for mobilization mediated by a coresident conjugative plasmid, the origin of transfer (*oriT*) represents the site on the mobile plasmid where the transfer process is initiated by a specific single-stranded cleavage event (Fig. 4A). The protein responsible for the cleavage is the plasmid-encoded Mob protein. The nucleotide sequences of the *oriT* region of numerous plasmids from both Gram-negative and Gram-positive bacteria have been determined (Meijer *et al.*, 1998; Zechner *et al.*, 2000). Five families of *oriT* core sequences have been defined through comparison of a wide variety of transfer origins (Lanka and Wilkins, 1995; Guzman and Espinosa, 1997), and a high degree of similarity exists among *oriT* sequences from RCR plasmids, with the sequence from pMV158 as the paradigm. Based on similarities to previously described *oriT* regions with respect to location (upstream from the *mob* gene), sequence consensus, and potential to form stem-loop structures, a putative *oriT* region from pK1S-1 was identified (Fig. 4A and Table 2). The *oriT* region was located upstream of the start codon of the ORF2 gene (*mobK1*) and showed 95.8% identities with the *oriTs* of pGI2 and pTX14-2 (Andrup *et al.*, 2003).

There are several examples of plasmids with extended abilities to intensify their dissemination and broaden their host range: some plasmids have several *sso*, each functioning in different hosts (Kramer *et al.*, 1995; Suzuki *et al.*, 1997), and others harbor transposable elements allowing co-integrate formation and possible transfer via conduction (Mahillon and Chandler, 1998). It has been suggested that bacteria can be regarded as one multicellular organism sharing a

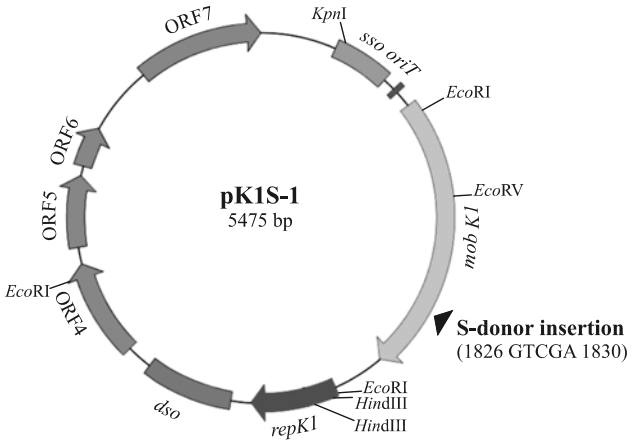

Fig. 3. Genetic map of pK1S-1. Putative *mobK1* and *repK1* genes, ORFs, double-strand origin (*dso*), single-strand origin (*sso*), origin of transfer (*oriT*) and restriction sites are indicated. The orientation of arrows indicate the direction of transcription. Arrowhead indicates PCS-S donor insertion position.

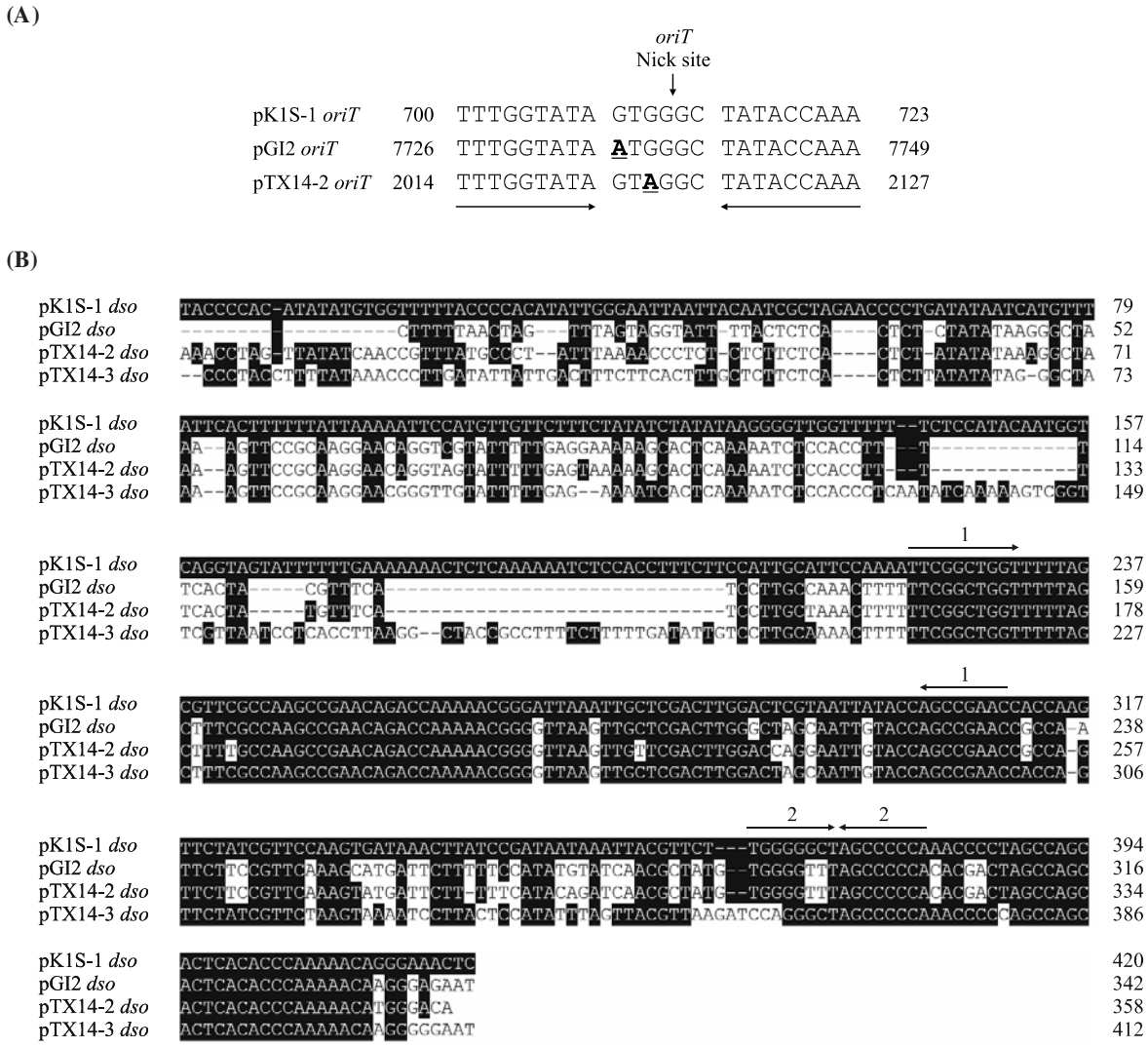

Fig. 4. Alignment of putative *oriT* region (A) and double-strand origin (B) of pK1S-1 with those of rolling circle replicating group VII plasmids (pGI2, pTX14-2 and/or pTX14-3). The stem region of *oriT* (A) and the inverted repeat regions of *dso* (B) are indicated by arrows. Underlined letters (A) indicate different base from pK1S-1.

worldwide gene pool (Levy, 1998) or, even further, that the whole biosphere is a global organism in which genes flow (De la Cruz and Davies, 2000). A mobile gene pool allows bacteria to share the cost of maintaining various potential and useful functions in the population. Presumably, all available recombination mechanisms (homologous, illegitimate, site-specific, and transpositional) contribute to the creation of new mobile elements. Apparently these modules, and maybe even sub-modules, should be regarded as separate evolutionary units. In the pK1S-1 sequence, a putative *sso* was located upstream of the *mob*K1-*oriT* region, and it exhibited 71.5% and 71.1% identities with those of pTX14-1 and pGI2, respectively (Table 3 and Supplementary Fig. 2). This putative *sso* of pK1S-1 seems to be an *ssoT*-type se-

Table 3. Comparison of putative *sso*, Rep protein, *dso* and regions of plasmid pK1S-1 with other plasmids of *B. thuringiensis* in RCR VII group

Plasmid name	*sso* coordinates / size (bp)	*sso* identity (%)	Rep protein size (aa)	Rep protein identity (%)	*dso* coordinates / size (bp)	*dso* identity (%)
pK1S-1	363-639 / 277	-	135	-	2881-3300 / 420	-
pGI2	7385-7665 / 281	71.1	204	17.8	5571-5951 / 342	63.6
pTX14-2	1762-2042 / 281	71.5	204	20.7	5426-5783 / 358	63.9
pTX14-3	6405-6644 / 240	66.5	212	25.2	1570-1981 / 412	61.3

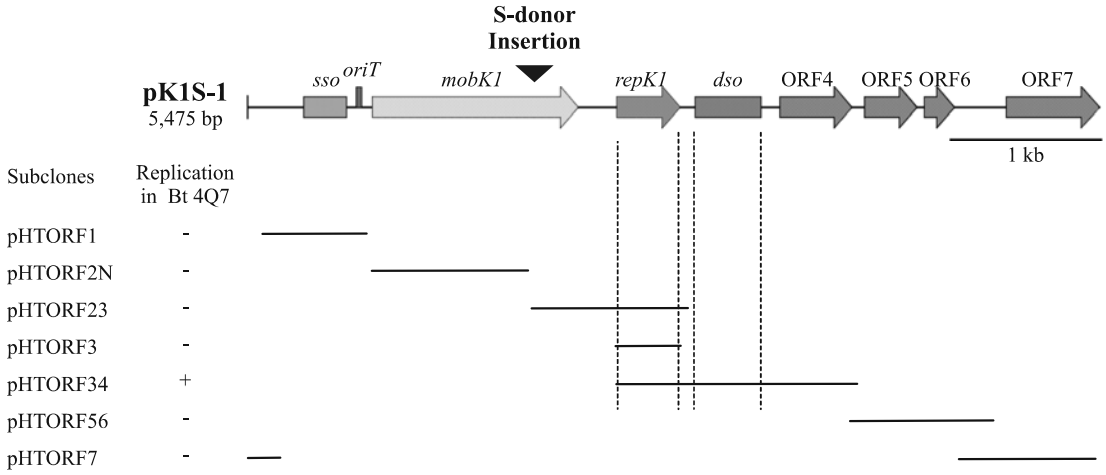

Fig. 5. Physical map of pK1S-1 and the relevant plasmids. The predicted ORFs are shown by block arrows indicating the direction of transcription. The ability or inability of the constructs to replicate in *B. thuringiensis* 4Q7 are marked with "+" or "-" in the middle column.

quence (Andrup *et al.*, 2003).

ORF6 and 7 were closely related to alkaline serine protease, which belongs to the subtilase family (Supplementary Table 1). Since there are no report of subtilase-like genes in RCR group VII, including pGI2, pTX14-2, and pTX14-3, further studies on the role of the putative proteases are needed.

The 420 bp GC-rich region (coordinates 2881-3300) located in the interspace (2384-3421) between ORF3 (RepK1) and ORF4, showed 63.9% and 63.6% identities with double-strand origin (*dso*) regions of pTX14-2 and pGI2, respectively (Table 3 and Fig. 3; Andrup *et al.*, 2003). Therefore, this interspace region between ORF3 and ORF4, might be the *dso* region of pK1S-1. The *dso* sites of the RCR group VII plasmids are located downstream from the Rep proteins, as is the putative *dso* of pK1S-1. In other RCR groups, the *dso* region is found upstream of, or inside of, their rep genes (Andrup *et al.*, 2003). Additionally, inverted repeat sequences that are typical in the *dso* of several RCR plasmids existed on the *dso* locus of pK1S-1 (Fig. 4B). To date, all RCR plasmids contain a *rep* gene encoding a replication protein (Rep) that is essential for the initiation of replication. The identities between the Rep proteins of RCR group VII plasmids were more than 30% (Andrup *et al.*, 2003). However, ORF3 (RepK1), located upstream of the putative *dso* region, exhibited low similarities with Rep protein genes of RCR group VII plasmids (25.2% with pTX14-3, 20.7% with pTX14-2, and 17.8% with pGI2) (Table 3 and Supplementary Fig. 3).

Determination of replication region

To locate the minimal replicon of pK1S-1, a set of subclones were constructed and transformed into plasmid-cured *B. thuringiensis* 4Q7 strain. These subclones were designed based on ORF regions and covered all sequences of pK1S-1. The region and relevant characteristics of subclones are listed in Table 1 and Fig. 5. Following transformation by electroporation, only the transformant harboring pHTORF34 replicated in *B. thuringiensis* 4Q7 (Fig. 5). To verify stable transformation, we extracted plasmid DNAs from the *B. thuringiensis* transformant, and confirmed pHTORF34 DNA by PCR analysis and sequencing (Supplementary Fig. 4). This result demonstrated that both putative ORF3 (RepK1) and *dso* regions play a key role in replication; the ORF3 region (pHTORF3) alone could not replicate in *B. thuringiensis*. This result is consistent with the previous findings that the minimal replicon, responsible for leading strand replication, consists of the gene encoding the initiator Rep protein and the *dso* site where leading-strand replication begins (Del Solar *et al.*, 1998). According to BLAST result of ORF4, it was only highly matched with *B. thuringiensis* hypothetical proteins. A study on role of ORF4 for replication will be needed.

In conclusion, we cloned three plasmids from *B. thuringiensis* K1 and analyzed the complete sequence of pK1S-1. In this plasmid, a putative *mobK1-oriT* dissemination cassette was observed, and Rep proteins and a *dso* locus were identified as the replicon of pK1S-1. This supports classification of pK1S-1 from *B. thuringiensis* K1 as a new member of RCR group VII.

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