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Sequencing and Characterization of Plasmid pUIBI-1 from *Bacillus thuringiensis* Serovar *entomocidus* LBIT-113

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Abstract. Plasmid pUIBI-1 from *Bacillus thuringiensis* svr. *entomocidus* was sequenced and its replication mechanism analyzed. Sequence analysis revealed that pUIBI-1 contains 4671 bp and a 32% GC content. Plasmid pUIBI-1 also includes at least seven putative open reading frames (ORFs) encoding for proteins ranging from 5 to 50 kDa. ORF-1 encodes for a putative 16-kDa Rep protein, which lacks homology with proteins of similar function. ORF2 encodes for a protein of 50 kDa and shows homology with Mob proteins of plasmids pLUB1000 from *Lactobacillus hilgardii* (32.2%) and pGI2 from *B. thuringiensis* (33.7%). Detection of single-stranded DNA (ssDNA) intermediates indicated that pUIBI-1 replicates by the rolling-circle replication mechanism, as demonstrated by S1 treatment and Southern hybridization under non-denaturing conditions.

The Gram-positive bacterium *Bacillus thuringiensis* is well known for the insecticidal activity of its crystal (Cry) proteins produced during sporulation [10]. Strains of *B. thuringiensis* usually exhibit a complex plasmid profile of up to 17 plasmids, ranging from 2 to 250 kb in size [3]. Because most of the Cry proteins are encoded by genes found in high MW plasmids, interest has been focused mainly on these plasmids, in spite of the accumulating literature about the small, multi-copy plasmids of Gram-positive bacteria [7, 14]. Most of these plasmids form the family of highly interrelated RCR plasmids, so called because of their rolling-circle replication mechanism and the occurrence of an ssDNA intermediate during the replication process [7, 12, 13]. The RCR plasmids contain at least a double-strand replication origin (*dso*), which initiates the replication, and a single-strand origin (*sso*), which starts the generation of the new double strand from the ssDNA intermediate. All the process is mediated by a self-encoded Rep protein [7].

So far, only five sequences of small plasmids from *B. thuringiensis* have been reported and, except for one (pHT1030) [16], all use the RCR mechanism. Among these, pGI2 is a 9672-bp plasmid that contains the transposon Tn4430 and a modular organization [9, 20]. Also, pHD2, a 2-kbp plasmid from *B. thuringiensis* serovar (svr.) *kurstaki,* harbors only two ORFs and belongs to the pT181 family [21]. The plasmid pTX14-3, isolated from *B. thuringiensis* svr. *israelensis,* contains two ORFs that encode for the replication protein (Rep) and a mobilization protein (Mob) [2]. Both pGI2 and pTX14-3 plasmids display a distinctive homology, suggesting that both belong to a new family of RCR plasmids. On the other hand, genetic characterization of the RCR plasmid pGI3 from *B. thuringiensis* svr. *thuringiensis* H1.1 defined another new family of rolling-circle replicons, along with pSTK1 from *Bacillus stearothermophilus* [8].

Recently, a strain of *B. thuringiensis* svr. *entomocidus* (LBIT-113) was characterized, showing a parasporal crystal with a distinctive morphology as a major peculiarity [17]. Another feature of this strain was its plasmid pattern, composed of only three elements of 110, 54, and 4.7 kb. This report shows the cloning, sequence analysis, and characterization of the smallest plasmid (pUIBI-1) from this strain.

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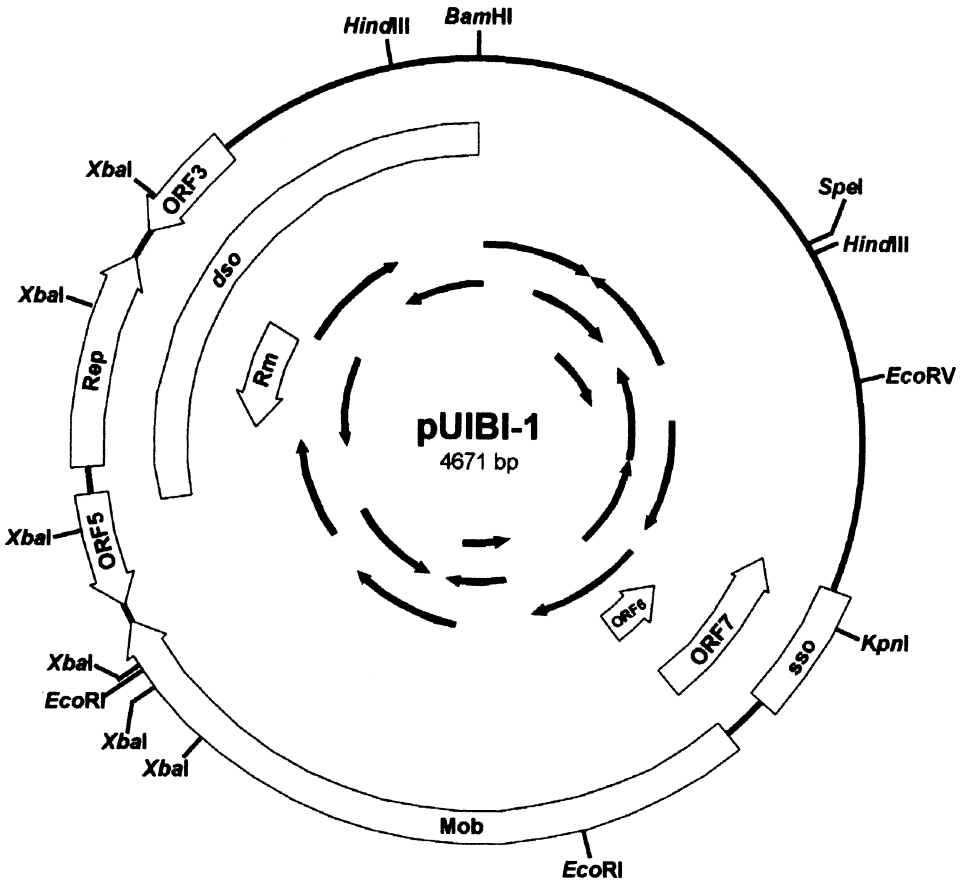

Fig. 1. Sequencing strategy, physical map, and genetic organization of the pUIBI-1 plasmid. Sequencing strategy is indicated by solid arrows. Hollow arrows indicate the orientation and location of putative ORFs, including those that probably code for Rep, Rm, and Mob proteins. Hollow bars indicate both replication origins (*dso* and *sso*). Restriction sites were determined by sequence analysis and corroborated by enzyme digestion.

Materials and Methods

Cloning of pUIBI-1. Plasmid pUIBI-1 from the atypical strain LBIT-113 of *B. thuringiensis* svr. *entomocidus* [17] was extracted as described previously [18] and purified by CsCl gradient centrifugation. Plasmids were visualized by electrophoresis in agarose gels, and pUIBI-1 was purified by Gene Clean (Bio 101 Inc.). A partial restriction map of pUIBI-1 was constructed by single and double restriction enzyme digests (New England Biolabs Inc., Boehringer Mannheim, and Bethesda Research Laboratories). Plasmid pUIBI-1 was cloned in two separate *Eco*RI fragments (3.8 and 0.9 kbp each), with the vector pBluescript KS (+) (Stratagene). White, single-cell colonies of *E. coli* DH5αF' electroporated transformants were analyzed by colony blot Southern hybridization, by using the pUIBI-1 plasmid as a probe. Minipreps from selected transformants were digested with *Eco*RI and visualized in agarose gels to corroborate the size of inserts.

DNA sequencing. Both strands of inserts were sequenced by the dideoxy-chain termination method. Subclones of the 3.8-kb insert and specific primers were used for sequencing following the strategy described in Fig. 1. No subcloning was required for the 900-bp insert. Sequencing was accomplished with [^{35}S]dATP and the Sequenase Version 2.0 kit (US Biochemical). Sequences were analyzed with the DNAsis 2.0 software (Hitachi) and the BLAST's URL.

Detection of ssDNA intermediates. Plasmids from strain LBIT-113 were digested in 100 μl plasmid DNA (ca. 2 μg), 11 μl of 10× S1 buffer, and 100 U of S1 nuclease (Boehringer) (active on ssDNA), at 37°C for 20 min. Digested and non-digested plasmids were electrophoresed in agarose gels and transferred to Nylon membranes (Hybond N^+, Amersham), under both denaturing and non-denaturing conditions, as described previously [25]. The 3.8-kb *Eco*RI fragment of pUIBI-1 was non-radioactively labeled (Genius kit, Boehringer) and used as a probe. Probe hybridized blotted DNA in 6× SSC (1× = 0.15 M NaCl, 0.015 M sodium citrate) overnight at 65°C. Membranes were washed twice at room temperature in 2× SSC, 0.1% SDS; and twice at 65°C in 0.5× SSC, 0.1% SDS, 15 min each. Kodak X-Omat XAR-5 films were exposed to membranes.

Results and Discussion

Cloning of pUIBI-1. The physical map of plasmid pUIBI-1 is shown in Fig. 1. Unique restriction sites were detected with enzymes *Eco*RV, *Spe*I, and *Bam*HI. However, attempts to clone plasmid pUIBI-1 by using these sites failed because, for some reason still unknown, all transformants showed plasmid constructs that varied greatly in size. According to previous reports [7, 12, 13], when RCR plasmids are used as cloning vectors, inserts

Table 1. Open reading frames detected in the analysis of the plasmid pUIBI-1 sequence. N: coding sequence at the sense strand; C: coding sequence at the complementary strand. M.W.: Predicted molecular weight of the putative protein

ORF	Location[a]	Amino acids	M.W. (kDa)	Prediction[b]
ORF1N (Rep)	3451–3870	140	16	−
ORF2N (Mob)	1809–3137	442	50.4	+
ORF3C	4144–3926	72	8.1	±
ORF4C (Rm)	3890–3546	114	13	±
ORF5C	3399–3175	75	8.8	+
ORF6C	1864–1667	66	7.5	−
ORF7C	1829–1452	126	14.1	−

[a] According to the reported sequence in GenBank (ORFs in the complementary strand follow the same number progression, as those in the sense strand).
[b] Feasibility of each ORF as a coding region, as predicted by Fickett's method.

show high instability, and constructs with inserts containing the total sequence of pUIBI-1 are analogous to using this plasmid as a cloning vector. In the end, cloning of pUIBI-1 was easier and successful in two separate *Eco*RI fragments.

Nucleotide sequence of pUIBI-1. Once both *Eco*RI fragments of plasmid pUIBI-1 were completely sequenced, nucleotides totaled 4671 bp. Nucleotide progression starts at the first base of the *Bam*HI site, as submitted to the GenBank database (Accession No. AF516904). The G+C content of pUIBI-1 was 32%, similar to the proportion reported for this species (from 33.5% to 40.1%). The physical map of pUIBI-1 (Fig. 1) shows the restriction sites, as determined by sequence analysis and corroborated by digestion of DNA (data not shown). Sequence comparison found the highest homology with some regions of plasmids pGI2 of *B. thuringiensis* svr. *thuringiensis* and pTX14-3 of *B. thuringiensis* svr. *israelensis* (see below).

ORFs of pUIBI-1. Sequence analysis of pUIBI-1 found seven potential ORFs with a coding capacity of more than 50 codons, four of them with the potential to code for proteins of more than 100 amino acids. Table 1 shows some properties of the seven putative ORFs and their products. Fickett's analysis [6] recognized only ORF2 and ORF5 as actual coding regions; however, ORF4 showed high homology with other known sequences (GenBank). This result reveals the limitations of Fickett's prediction, especially when ORFs are overlapped or uncommon codons are used.

ORF2 showed 37% homology with the *mob2* gene of plasmid pGI2 from *B. thuringiensis* svr. *thuringiensis* H1.1, and 34% homology with the *mob14-3* gene of plasmid pTX14-3 from *B. thuringiensis* svr. *israelensis.* Mob proteins are involved in the mobilization of small plasmids between cells [23] and are bound to plasmids through a co-integration site named RS_A, which overlaps

Table 2. Comparison of RS_A sites from different plasmids of Gram-positive bacteria. RS_A: recognition sites used by Mob proteins (see text). Dist: Distance from the promoter region (−10) to the starting Mob protein codon (SC). NT: nucleotides; SD: Shine-Delgarno sequence. Box indicates the total extension of the RS_A sites.

Plasmid	Species	RS_A	-10	Dist.	SC
pUIBI-1	*B.thuringiensis*	AATTTGGTATAACGTGC	TAGACC	64NT SD ATG	
pGI2	*B. thuringiensis*	AATTTGGTATAATGGGC	TATACC	61NT SD ATG	
pTX14-3	*B. thuringiensis*	TTACGTTAATTGCTTTG	TAGACT	27NT SD ATG	
pUB110	*S. aureus*	TATAAAGTATAGTGTGT	TATACT	59NT SD ATG	
pE194	*S. aureus*	AAATAAGTCTAGTGTGT	TAGACT	38NT SD ATG	
pT181	*S. aureus*	AAATAAGTCTAGTGTGT	TAGACT	35NT SD ATG	
pLAB1000	*L. hilgardii*	CATAAAGTATAGTGTGT	TATACT	23NT SD ATG	
pMV158	*S. agalactiae*	TATAAAGTATAGTGTGT	TATACT	15NT SD ATG	

the promoter of the Mob coding region and shows several inverted repeats. Table 2 shows a comparison of RS_A region sequences of several plasmids from Gram-positive bacteria.

Although sequences are conserved to some extent, the RS_A of plasmid pGI2 shares the highest homology with that of pUIBI-1 (87.5% homology). However, the distance between the RS_A site and the starting codon of Mob proteins is highly variable. Also, it is known that mobilization of small plasmids in *B. thuringiensis* svr. *israelensis* has been associated with a cell aggregation mechanism [1], genetically controlled by a factor associated to a 135-MDa plasmid [11]. Interestingly, preliminary observations indicate that strain LBIT-113 shows this aggregation behavior, which may be associated with the plasmid transfer mechanism.

On the other hand, ORF4 showed 24% homology with genes expressing proteins involved in replication

and maintenance (*rm*), coded by plasmids pE5 from *Staphylococcus aureus,* pIM13 from *B. subtilis,* and pNE131 from *S. epidermidis* [15, 22, 24]. A phylogenetic analysis of these sequences showed that the Rm protein from pUIBI-1 probably evolved separately (data not shown). Besides, ORF1 showed no significant homology with any known gene, but it may code for a Rep protein, owing to its location within the putative *dso* of pUIBI-1 (Fig. 1) (see below). Also, a possible promoter was located upstream of ORF1, according to a consensus sequence for promoters of *B. subtilis.*

***Dso*.** The region between bases 3363 and 4670, which includes ORF1, reveals a 49.3% homology with the *dso* of plasmid pTX14-3 of *B. thuringiensis* svr. *israelensis* (Fig. 1). There are two ORFs within the *dso,* including the putative Rep protein. This observation is common among RCR plasmids and is related to the replication mechanism [4]. These replication origins are normally larger than the ORF for Rep proteins, because they contain regulatory *cis* elements.

***Sso*.** Also, a 253-bp region of pUIBI-1 shows significant homology with the *sso* of plasmids pTX14-3 (73%) and pGI2 (66%) of *B. thuringiensis.* This region (bases 1460 to 1716) contains four potential stem-and-loop structures, very similar to those of plasmids pTX14-3 and pGI2. Out of the four types of *sso* (*ssoA, ssoT, ssoU,* and *ssoW*) known in RCR plasmids [14], pUIBI-1 belongs to the *ssoT* type and consequently is highly related to the pBAA1 plasmid family [5]. Palindromic structures 1 and 2 of pBAA1 are almost identical to the structures 2 and 3 of pUIBI-1, respectively. Also, the *sso* of pUIBI-1 is located within a 253-bp region, just like other *sso* regions, which vary from 200 to 300 bp [7]. Also, the *sso*'s of pUIBI-1, pTX14-3, and pGI2 are located upstream of the Mob coding region [2, 19, 20].

Additionally, comparison of RCR plasmids from different families indicates an exchange of genetic material among them (http://www.essex.ac.uk/bs/staff/osborn/DPR_home.htm). So, pUIBI-1 may be the result of several events of recombination, which could explain the relatedness (or lack of it) of its different regions, with different plasmids: its *sso* with that of the pBAA1 plasmid; its Mob gene with the *mob2* gene of pGI2; and the lack of homology with any of the known Rep genes.

Detection of ssDNA intermediates. For additional support of the hypothesis that pUIBI-1 replicates by the rolling-circle process, location of ssDNA replication intermediates was performed by Southern blotting under native conditions, combined with S1 nuclease digestion of ssDNA. Figure 2a shows an agarose gel with plasmid DNA of strain LBIT-113, both undigested and digested with S1 nuclease.

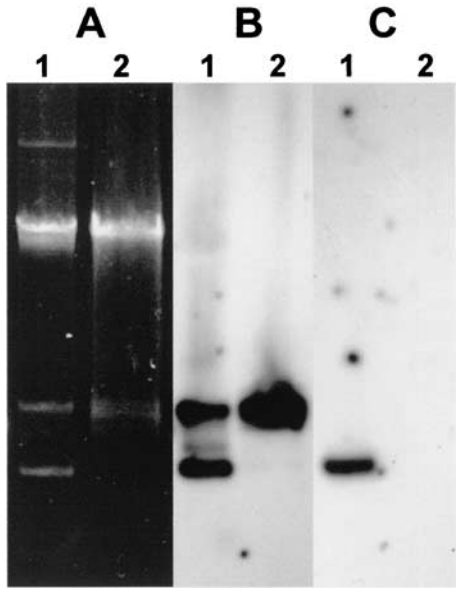

Fig. 2. Detection of ssDNA intermediates of plasmid pUIBI-1. (A) Agarose gel; (B) Southern blot, transferred under denaturing conditions; (C) Southern blot, transferred under native conditions. Lane 1, undigested plasmids of *B. thuringiensis* svr. *entomocidus* LBIT-113. Lane 2, plasmids of *B. thuringiensis* svr. *entomocidus* LBIT-113, digested with S1 nuclease. The 3.8-kb *Eco*RI fragment of pUIBI-1 was used as a probe.

When plasmids were transferred to membranes under denaturing (alkaline) conditions, all conformational structures of pUIBI-1 were detected, by using the largest *Eco*RI fragment of pUIBI-1 as a probe (Fig. 2b, lane 1). However, when pUIBI-1 was digested with S1 nuclease, one band disappeared (Fig. 2b, lane 2), indicating its ssDNA nature. Additionally, when plasmids were transferred under non-denaturing conditions to membranes (hybridization occurred only on ssDNA), only one band of pUIBI-1 was detected (Fig. 2c, lane 1). This band matches with that which disappeared when plasmid was digested with S1 nuclease. As expected, no bands were detected when samples were digested with the S1 nuclease (Fig. 2c, lane 2). These results indicate the presence of a pUIBI-1 ssDNA intermediate in the plasmid preparations of LBIT-113.

In summary, according to information obtained from the sequence analysis of pUIBI-1 and from the experimental work, this plasmid is an RCR plasmid that uses the rolling-circle mechanism to replicate; it is related to the pBAA1 plasmid family, although part of its gene content is more related to other RCR plasmids; and its putative Rep protein has no relationship with any other

protein of this kind. The role of low-molecular-weight plasmids of *B. thuringiensis* is still vague, and more attention should be paid in the future.

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