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Cloning of Small Plasmids from *Bacillus thuringiensis* Subsp. *israelensis* Using Plasmid Capture System

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Recently, we have developed an easy, simple and convenient circular DNA cloning system named plasmid capture system (PCS). To investigate usefulness of PCS in cloning of plasmids from *Bacillus thuringiensis* strains, PCS donors, pPCS-S and pPCS-L were applied to clone plasmids of *B. thuringiensis* subsp. *israelensis* by *in vitro* transposition using TnsABC* transposase. In result, 3 small plasmids were cloned, and these were consistent with pTX14-1, pTX14-2 and pTX14-3 reported previously from *B. thuringiensis* subsp. *israelensis*. Therefore, the PCS can be successfully applied to clone small plasmids from *B. thuringiensis* strains.

Key words: *Bacillus thuringiensis*, Plasmid, Plasmid capture system

Introduction

Bacillus thuringiensis, gram-positive, spore-forming soil bacterium produces parasporal inclusions consisted with insecticidal crystal proteins during sporulation. The parasporal inclusions from *B. thuringiensis* has been used as one of the most successful biological control agents for suppression of agriculturally and medically important insect pests (Schnepf *et al.*, 1998; Glare and O'Callaghan, 2000). 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 Gonzáles Jr, 1985; McDowell and Mann, 1991). Because most of the crystal protein genes are encoded in high MW plasmids, interest has been focuses predominantly on these large plasmids (Kronstad *et al.*, 1983; Carlton and Gonzáles Jr, 1985). In addition, other functions such as conjugative functions (Battisiti *et al.*, 1985; Reddy *et al.*, 1987; Jensen *et al.*, 1996; Wilcks *et al.*, 1998), a heat-stable exotoxin (Ozawa and Iwahana, 1986), a transposon (Lereclus *et al.*, 1986), a temperate phage (Kanda *et al.*, 1989) and insertion sequences (Mahillon *et al.*, 1994) have been attributed to these large plasmids.

So far, nine sequences of small plasmids from five *B. thuringiensis* strains have been reported, and these include pGI1, pGI2 and pGI3 from *B. thuringiensis* subsp. *thuringiensis* H1.1 (Mahillon and Seurinck, 1988; Hoflack *et al.*, 1997; Andrup *et al.*, 2003), pHD2 from *B. thuringiensis* subsp. *kurstaki* HD1-DIPEL (McDowell and Mann, 1991), pTX14-1, pTX14-2 and pTX14-3 from *B. thuringiensis* subsp. *israelensis* (Boe *et al.*, 1991; Madsen *et al.*, 1993; Andrup *et al.*, 1994, 1995, 2003), pHT1030 from *B. thuringiensis* subsp. *thuringiensis* LM2 (Lereclus and Arantès, 1992) and pUIBI-1 from *B. thuringiensis* subsp. *entomocidus* LBIT-113 (López-Meza *et al.*, 2003). Most of these small plasmids, except for pHT1030, form the family of RCR plasmids because of their rolling-circle replication mechanism (Khan, 1997, 2000). Since no striking functions have been attributed to these small plasmids, most of them referred to as cryptic (Andrup *et al.*, 2003).

However, it have been very difficult to clone these small plasmids as a single fragment using traditional restriction endonuclease digestion/ligation because of not only rare unique restriction sites, but also some unknown reasons

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(Andrup *et al.*, 2003; López-Meza *et al.*, 2003). Therefore, it was needed complex and tiring process such as sub-cloning or PCR amplification to clone and analyze sequences of these small plasmids. Recently, we have developed a convenient cloning system based on Tn7 transposition in order to clone circular DNA segments in *Escherichia coli* cell and designated plasmid capture system (PCS). The principle of PCS was that a donor containing an *E. coli* origin of replication for amplification and an antibiotic resistant gene for selection between Tn7 left (Tn7L) and right (Tn7R) end, can be inserted into target circular DNA molecule by transposition reaction using transposase and the reacted DNA can be cloned and amplified in *E. coli*. In this study, we verified the usefulness of PCS in the cloning of small plasmid DNAs from *B. thuringiensis*.

Materials and Methods

Bacterial strains and plasmids

E. coli strain JM109 (Takara, Japan) was used throughout the experiment. *B. thuringiensis* subsp. *israelensis* was grown at 30°C with vigorous shaking in spizizen-yeast (SPY) medium (Nickerson *et al.*, 1974). The plasmid DNA of *B. thuringiensis* subsp. *israelensis* was isolated using plasmid midi-prep. Kit (Qiagen, Germany) with additional lysozyme treatment according to manufacturers instruction.

Tn7 transposition *in vitro* and southern hybridization

In transposition reaction, 1 μl of S-donor (40 ng/μl) or L-donor (40 ng/μl) was mixed up with 1 μg of *B. thuringiensis* subsp. *israelensis* plasmid DNA. TnsABC* transposase (New England Biolabs, UK) was added to each transposition reaction and mixture was pre-incubated at 37°C for 10 min. The final reaction mixture was completed by adding 1 μl of start solution and incubated at 37°C for 1 h. After that, transposition reaction was stopped by heat incubation (37°C, 10 min). The reacted DNA was transformed to the competent *E. coli* JM109 cells (Takara, Japan) and the transformed cells were selected by plating on antibiotic (ampicillin or kanamycin) added nutrient agar plate. Transposition reaction was analyzed by southern hybridization on *B. thuringiensis* subsp. *israelensis* plasmid DNA with probes, *Sal*I-digested origin of replication and antibiotic resistant gene cassettes. Probes were labeled with digoxigenin using a DIG DNA labeling kit (Boehringer Mannheim, Germany).

Sequence analysis and Blast search

The cloned DNAs of viable colonies was analyzed by restriction endonuclease pattern and sequencing analysis. The partial DNA sequences of plasmid clones of *B. thuringiensis* subsp. *israelensis* were determined on an ABI sequencer Model 377 (ABI system) and the obtained sequence was analyzed using BlastN search.

Results and Discussion

In vitro transposition of plasmids from *B. thuringiensis* subsp. *israelensis*

Previously, we newly developed PCS in order to clone circular DNAs. In the PCS, an *E. coli* origin of replication coupled with an antibiotic resistant gene between Tn7R and Tn7L might be transposed into target circular form DNAs such as plasmids by *in vitro* transposition using TnsABC* transposase. As the result, circular target DNAs could be easily cloned as a single molecule into *E. coli* without time-consuming and troublesome restriction digestion/ligation. Two different PCS donors, L-form and S-form were separately constructed according to the size of transposed target DNA. The pPCS-S (S-donor) consists of a pUC ori and an ampicillin resistant gene and the pPCS-L (L-donor) consists of a mini-F replicon and a kanamycin resistant gene.

We supposed that the PCS could be efficiently applied to clone the plasmids from *B. thuringiensis* strains. To investigate the efficiency of *in vitro* transposition of PCS donors, Southern hybridization was carried out using S- and L-donor probes against plasmid DNAs from *B. thuringiensis* subsp. *israelensis*, which were separately transposed with S- and L-donor, respectively. While no transposition was observed under the conditions without PCS donors and/or TnsABC* transposase, various sizes of plasmid DNAs were transposed by both S- and L-donors in the presence of TnsABC* transposase (Fig. 1).

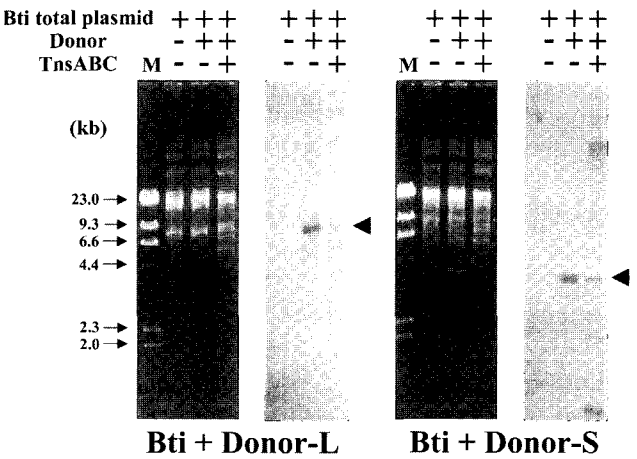

Fig. 1. Southern hybridization of *Bacillus thuringiensis* subsp. *israelensis* plasmid DNAs transposed with pPCS-S and pPCS-L. Arrowheads indicate the L-donor and S-donor, respectively.

The mosquito toxic isolate *B. thuringiensis* subsp. *israelensis* has been reported to contain three small plasmids pTX14-1 (5.4 kb), pTX14-2 (6.8 kb) and pTX14-3 (7.6 kb). The complete sequences of these small plasmids, along with analysis of replication and mobilization functions, have been reported previously (Boe *et al.*, 1991; Madsen *et al.*, 1993; Andrup *et al.*, 1994, 1995, 2003). In addition, *B. thuringiensis* subsp. *israelensis* harbors four large plasmids including the 128 kb pBtoxis encoding the Cry and Cyt toxins (Ben-Dov *et al.*, 1999; Berry *et al.*, 2002) and the 350 kb pXO16 encoding an aggregation-mediated conjugation system (Andrup *et al.*, 1993; Jensen *et al.*, 1995) and a linear molecule pGIL01 (Verheust *et al.*, 2003). These various size profiles (5.4 ~ 350 kb) of plasmids from *B. thuringiensis* subsp. *israelensis* were suitable to evaluate the efficacy of PCS to clone plasmids from *B. thuringiensis* strains. The Southern hybridization results suggested that the PCS donors could effectively transpose omnifarious plasmids of *B. thuringiensis* strains.

Cloning and sequence analysis of plasmids from *B. thuringiensis* subsp. *israelensis*

Transformation of transposed plasmid DNAs into *E. coli* cells yielded three distinct clones in both case of S-donor and L-donor (Fig. 2). Sequence analysis of these clones revealed that each of three clones were consistent with pTX14-1, pTX14-2 and pTX14-3, respectively, in both case of S-donor and L-donor. All plasmids cloned in this study corresponded only to small plasmids from *B. thuringiensis* subsp. *israelensis*. Theoretically, the pUC ori of S-donor could amplify the circular DNA less than 30 kb and the transposed target DNA may exist as high copy number in *E. coli*. Whereas, the mini-F replicon of L-donor could amplify the circular DNA ranging from 30 to 200 kb and the transposed target DNAs may exist as low copy number in *E. coli* cells (Kim *et al.*, 1992; Shizuya *et al.*, 1992). The absence of large plasmid clones in the case of L-donor might be resulted from the transformation method used in this study. We transformed the plasmids by "heat shock" using chemically treated competent cells, but it has been reported that the transformation efficiency declines linearly with increasing plasmid size in this method (Hanahan, 1983).

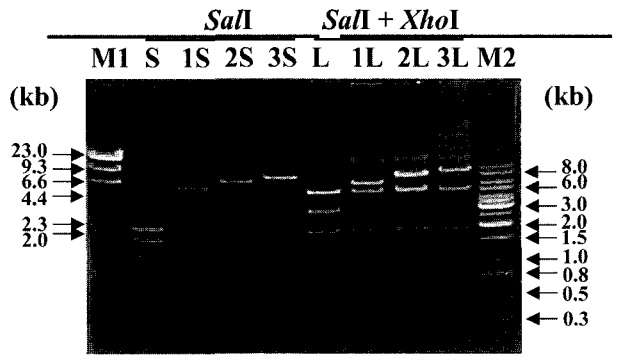

Fig. 2. Restriction endonuclease digestion pattern of *Bacillus turingiensis* subsp. *israelensis* plasmid DNAs transposed with pPCS-S and pPCS-L. Lanes: M1, Lambda DNA digested with *Hind*; M2, 1 kb DNA ladder; S, pPCS-S; L, pPCS-L; 1S, 2S and 3S, *B. thuringiensis* subsp. *israelensis* plasmid DNA transposed with pPCS-S; 1L, 2L and 3L, *B. thuringiensis* subsp. *israelensis* plasmid DNA transposed with pPCS-L.

As the results, we cloned three small plasmids from *B. thuringiensis* subsp. *israelensis* using PCS, and the PCS was proved to be a novel fast and convenient method to clone plasmids from *B. thuringiensis* strains, especially for small plasmids. Increasing information on small plasmids from *B. thuringiensis* strains were accumulated including transposon Tn*4430*, gene encoding the Rep protein, double-strand origin of replication (*dso*), single-strand origin of replication (*sso*), genes implicated in conjugative mobilization (Mob-genes and origin of transfer (*oriT*)) and ORF encoding a polypeptide containing a central domain with repetitive elements similar to eukaryotic collagen (*bcol*) (Andrup *et al.*, 2003; López-Meza *et al.*, 2003). The PCS as rapid and easy cloning system will provide new insights to the study on small plasmids from *B. thuringiensis* strains. Moreover, the PCS could be applied to clone other circular DNA molecules originated from diverse organisms.

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