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The plasmid pBMBt1 from *Bacillus thuringiensis* subsp. *darmstadiensis* (INTA Mo14-4) replicates by the rolling-circle mechanism and encodes a novel insecticidal crystal protein-like gene

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Abstract

This work describes a novel rolling-circle replicating (RCR) plasmid pBMBt1 from *Bacillus thuringiensis* subsp. *darmstadiensis* (INTA Mo14-4) encoding an insecticidal crystal protein-like gene. pBMBt1 (6700 bp) contains three ORFs and their putative transcription initiation sites and Shine-Dalgarno sequences were localized. ORF1 encodes a 34.6 kDa protein which showed identity with the protein CryC53 from *B. thuringiensis* subsp. *cameroun* (24.6%), the Cry15Aa insecticidal crystal protein from *B. thuringiensis* subsp. *thompsoni* (21.9%) and the Mtx3 protein from *Bacillus sphaericus* (27.8%). The ORF2 (52.3 kDa) showed a 74% identity with the Mob protein coded by pUIBI-1 from *B. thuringiensis* subsp. *entomocidus* and 64% identity with the Mob protein of pBMY1 from *Bacillus mycoides*; both Mob proteins belong to the pMV158 superfamily. To evaluate the Mob protein, the plasmid pHTMob14-4 was constructed. This plasmid shows transfer frequencies of 9.1×10^{-6} in *B. thuringiensis* subsp. *israelensis* (4Q7Gm^{R}). The ORF3 (23.6 kDa) gene product is homologous to the Rep protein from the plasmid pBMYdx of *B. mycoides* (37.6%). A putative double-strand origin with significant homology to that of *B. thuringiensis* plasmids, and an *sso*A-type single-strand origin were also identified. Detection of single-stranded pBMBt1 DNA replicating intermediaries suggests that replication occurs via the rolling-circle mechanism.

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1. Introduction

During the sporulation the Gram positive bacterium *Bacillus thuringiensis* produces delta endotoxin proteins coded by *cry* genes and are toxic against insects (Höfte and Whiteley, 1989). A common characteristic of many strains of *B. thuringiensis* is the presence of a complex arrangement of plasmid DNA. The number and size of these plasmids (2–250 kb) vary considerably among strains (González and Carlton, 1980; Lereclus et al., 1982; McDowell and Mann, 1991). In contrast to the abundant information related to the *cry* genes coding for the delta endotoxins located on large plasmids, little information has been obtained from these plasmids and their contribution to the biology of their host.

In *B. thuringiensis*, most small plasmids (<15 kb) have been considered as "cryptic," because their functions are unknown. The functions attributed to these widely distributed plasmids have been the replication and mobilization. Most small, multicopy plasmids from Gram-positive bacteria replicate by an asymmetric rolling-circle mechanism producing single-stranded DNA intermediates (Khan, 1997). Based on similarities of replication proteins (Rep) these plasmids are classified in at least 17 Groups (http://www.essex.ac.uk/bs/staff/osborn/DPR/DPR_RCRdata.htm).

To date, several sequences contained in small RCR plasmids of *B. thuringiensis* have been reported. Plasmids pHD-3a3b and pHD2 from *B. thuringiensis* subsp. *kurstaki*, have functions related specifically with the replication and maintenance and belong to RCR Group I (Marin et al., 1992; McDowell and Mann, 1991). Sequence analysis of the plasmids pTX14-1, pTX14-2, and pTX14-3 isolated from *B. thuringiensis* subsp. *israelensis*, revealed three main ORFs which encode for a Rep protein, a mobilization protein (Mob), and a collagen-like-protein (Andrup et al., 2003; Madsen et al., 1993). pTX14-1 belongs to RCR Group III and is mobilized by conjugative plasmid (pXO16) (Andrup et al., 1994; Boe et al., 1991). pTX14-2 shows significant homology to members of the RCR Group VII (pTX14-3 and pGI2) and contains a collagen-like gene (Andrup et al., 2003). On the other hand, pTX14-3 (RCR Group VII) shows sequences encoding the proteins involved in replication and mobilization and a sequence encoding a putative protein with high similarity to various collagens (Andrup et al., 1994).

Molecular characterization of pGI1, pGI2, and pGI3 plasmids isolated from *B. thuringiensis* subsp. *thuringiensis* strain H1.1 revealed interesting characteristics. pGI1 contains three ORFs that encode the Rep protein, a Mob protein and a poison component of a poison-antidote system found in the P1 bacteriophage and belongs to RCR Group III (Andrup et al., 2003; Mahillon et al., 1988). pGI2 (RCR Group VII) is the second smallest of these plasmids and harbors three ORFs. The first ORF encodes for the Rep protein, the second encodes for a Mob protein and the third contains the transposon Tn*4430* encoding a TnpI recombinase, a member of the phage integrase family of site-specific recombinases, similar to plasmids pHT1000 and pHT1030 of *B. thuringiensis* (Hoflack et al., 1999). pGI3, the largest of the three plasmids, belongs to RCR Group VI, and contains at least 11 putative ORFs. The ORF6 encodes for the Rep protein (Andrup et al., 2003; Hoflack et al., 1997). Recently, the pUIBI-1 plasmid from *B. thuringiensis* subsp. *entomocidus* was shown to contain at least seven ORFs. Among the ORFs characterized, the ORF1 encodes for a Rep protein (lacks homology with Rep proteins) and the ORF2 encodes a Mob protein (López-Meza et al., 2003).

Although most of the low molecular weight plasmids in *B. thuringiensis* are classified as RCR, so far, there are no reports about the presence of *cry* genes in them. *cry* genes have been found in large plasmids, which duplicate their genetic material through the theta mechanism. The plasmid pBtoxis from *B. thuringiensis* contains several *cry* genes; however, its replication mechanism has not been characterized (Berry et al., 2002).

Several isolates of *B. thuringiensis* from Argentina in a nationwide screening program were