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Two novel delta-endotoxin gene families *cry26* and *cry28* from *Bacillus thuringiensis* ssp. *finitimus*.

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Abstract Genes *cry26Aa1* and *cry28Aa1* were cloned from *Bacillus thuringiensis* ssp. *finitimus* strain B-1166 VKPM. This strain forms insecticidal crystal bodies either outside or inside the exosporium. The deduced amino acid sequence of the *cry26Aa1* gene product included seven residues determined to be an N-terminal part of a chymotrypsin-treated delta-endotoxin isolated from the same strain. Earlier this protein was detected in both free and spore-associated types of crystals [Revina et al., Biokhimia (1999) in press]. Neither BtI nor BtII promoter sequences were found upstream of the open reading frames in both genes. Southern hybridization has shown that the surroundings of both genes at least 3 kb upstream and downstream of the open reading frames are unique. We suggest that the protein Cry26Aa1 in both types of crystal bodies is synthesized under the control of one and the same genomic locus.

Key words: *Bacillus thuringiensis* ssp. *finitimus*; Exosporium; Cry26Aa1; Cry28Aa1

1. Introduction

Bacillus thuringiensis (BT) strains produce delta-endotoxins ranging from 50 to 140 kDa and displaying highly specific entomocidal activity toward various insect taxa. Delta-endotoxins are usually produced in BT cells during sporulation forming large parasporal crystal inclusions. Number, size and location of the crystals vary between subspecies. A crystal is usually formed outside the exosporium and is released after mother cell lysis [1]. In BT ssp. *finitimus* the parasporal inclusion is formed inside the exosporium and remains attached to the spore after mother cell lysis [2]; mechanisms of delta-endotoxin accumulation have not yet been explained.

Some strains of BT ssp. *finitimus* form both types of parasporal inclusions [2]. Debro et al. [3] showed that free and spore-associated inclusions of BT *finitimus* were immunologically distinct and had non-overlapping protein compositions. The ability to form spore-associated crystals was shown to correlate with the presence of a large plasmid [3] thus suggesting that the plasmid was carrying all the genes essential for crystal formation within the exosporium. The type of crystal may be related to the features of delta-endotoxin itself or may depend on other regulatory or structural proteins.

Recently we reported [4] the distribution of a number of delta-endotoxins between the free and spore-associated crystals of BT *finitimus*. Two stable lines were isolated from BT *finitimus* strain B-1166 VKPM: one of them formed only spore-associated and the another produced only free delta-endotoxin crystal bodies. At least three different Cry proteins were found in crystal bodies of this strain and its derivatives. One of them was found in smaller amount in the spore-associated crystals, while the others were equally represented in both types of inclusion bodies [4]. This result is contradictory to the data of Debro et al. [3]. Protein location may depend on both strain features and Cry protein properties, therefore cloning and analysis of BT *finitimus cry* genes may be beneficial for understanding the spatial distribution of BT crystals.

2. Materials and methods

2.1. Bacterial strain

Strain B-1166 VKPM of BT ssp. *finitimus* (BT *finitimus* 1166) was used [4].

2.2. Antibodies and protein assay

Rabbit antiserum was raised against a mixture of BT *finitimus* 1166 true toxins obtained by chymotrypsin processing of parasporal inclusions [4]. The antiserum was pre-exhausted with crude extract of *Escherichia coli* NM522 and purified by affinity chromatography on immobilized BT *finitimus* 1166 delta-endotoxin mix as described before [7].

2.3. Genomic bank construction and screening

BT *finitimus* 1166 total DNA was isolated as described by Delecluse et al. [5] and partially digested with *Sau*3A. DNA fragments exceeding 5 kb in size were recovered from an agarose gel and ligated into the *Bam*HI-linearized pUK21 vector [6] treated with CIAP. *E. coli* NM522 was transformed with ligation mix and plated on LB agar medium supplemented with kanamycin and IPTG. About 3000 clones were screened with the rabbit antiserum and two colonies were selected for further analysis.

2.4. Sequencing

Sequencing was performed using the Taq DNA polymerase modification of Sanger's method [7] with a set of overlapping subclones ensuring complete sequencing of both strands of the cloned fragments.

2.5. Expression of the cloned genes

The cloned genes were expressed in *E. coli* NM522 as a host in standard LB broth supplemented with 20 μg/ml kanamycin and 0.1 mM IPTG [7]. Cell cultures were grown overnight at 30°C. Western blot analysis was performed following the standard protocol [7].

2.6. Southern blot analysis

Southern blot analysis was performed according to the standard protocol [7].

3. Results and discussion

3.1. Cloning and sequence analysis

Two independent clones pF1 and pF2 were selected by screening of the genomic bank of BT *finitimus* 1166 with antiserum. Sequence analysis of the 6930 bp fragment in pF1 and the 4896 bp fragment in pF2 revealed a single long open read-

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PII: S0014-5793(99)00650-X

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Block 1
Cry1Aa    YQVPLLSVYVQAANLHLSVLRDVSVFGQRW
Cry26Aa   FEVLLLVVYTQAANLHLLSLRDAYIYGAEW
Cry27Aa   YDTLLLSCYTEAANLHLNLLHQGVQFADQW
          . ..**..*..****** *..   ..  *

Block 2
Cry1Aa    WVRYNQFRRELTLTVLDIVALFSNYDSRRYPIRTVSQLTREIYTN
Cry26Aa   WFQYNRFRREMTLSVLDVIALFPAYDVKMYPIPYNFQLTREVYTD
Cry27Aa   WDAYNTYRREMTLIVLDLVATFPFYDIRRFPRGVELELTREVYTS
          *  ** .****** *** .*.*. ** ...*.    .****.**

Block 3
Cry1Aa  FSWQHRSAEFNNIIPSSQITQIPLTKSTNLGSGTSVVKGPGFTGGDI
Cry26Aa FSWTHSSVDFDNYVYPTKITQLPATKGYNV----SIVKEPGFIGGDI
Cry27Aa LGWTHNSVNSESVSSQNLITQIPLVKAYEVTN-NSVIRGPGFTGGDL
        ..*.* *. . .      ***.*..* ...    *....***.***.

Block 4
Cry1Aa    QRYRVRIRYAS
Cry26Aa   QKYRFRVRYAT
Cry27Aa   KKYAISLFYAA
          ..*. . .**

Block 5
Cry1Aa    YIDRIEFVP
Cry26Aa   YLAGIEIIP
Cry27Aa   LIDKLEFKP
          ... .*. *

Block 6
Cry1Aa  LKTDVTDYHIDQVSNLVECLSDEFCLDEKQELSEKVKHAKRLSDERNLLQDPNF
Cry26Aa LKIDVTDYQIDQAANLVECISGDLYAKEKIVLLRAVKFAKQLSQSQNLLSDPEF
Cry27Aa LKMDVTDYHIDQVANLVECISDDLYAKEKIKFTPCIKFAKQLSQARNLLSDPNF
        ** *****.***..*****.*......**. .   .*.**.**. .***.**.*

Block 7
Cry1Aa    YPTYLYQKIDESKLKAYTRYQLRGYIEDSQDLE
Cry26Aa   FPTYLYQKIDESTLKPYTRYQLRGFVEGSENLD
Cry27Aa   FPTYLYQKIDESLLKPYTRYQLRGFVEGSQDLE
          .********** **.********..*.*..*.

Block 8
Cry1Aa    HFSLDIDVGCTDLNEDLGVWVIFKIKTQDGHARLGNLEFLEEKPL
Cry26Aa   AFSFHIDTGTVDSTENLGIWVAFKISELDGSAIFGNLELIEVGPL
Cry27Aa   AFSFHIDTGTTDNNRNLGIWIIFKIATPDGYATFGNLELIELGPL
          .**..**.*..* ...**.*..*** . ** * .****..* .**
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Fig. 1. Alignment of amino acid sequences in eight conserved blocks of Cry1Aa1, Cry26Aa1 and Cry28Aa1 proteins. Identity in all three sequences is marked with an asterisk; in two out of three sequences it is marked with a dot.

ing frame (ORF) in each of them. Both deduced amino acid sequences were found to be most homologous to those of the Cry1–Cry9 group (35–42% identity). However, they appeared different enough to warrant two new primary ranks as *cry26Aa1* (pF1 insertion, GenBank accession number AF122897) and *cry28Aa1* (pF2 insertion, GenBank accession number AF132928).

Cry26Aa1 and Cry28Aa1 are more similar in the C-terminal than in the N-terminal moiety (64% and 36% identity, respectively). The alignment of conserved blocks of both proteins in comparison with Cry1Aa is shown in Fig. 1.

A sequence of seven amino acid residues determined in the N-terminus of the major chymotrypsin-processed Cry protein of BT *finitimus* 1166 [4] corresponded to that occurring within the deduced amino acid sequence of Cry26Aa1 (Fig. 2). This protein was shown to be equally distributed between both types of BT *finitimus* 1166 parasporal inclusions, spore-associated and free ones [4].

The cloned fragment harboring the *cry26Aa1* gene was

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1     5          45      ⇓    55    60
MNSEEMNH----QSYGEFNMDNFGESEPFID----
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Fig. 2. N-terminal amino acid sequence of Cry26Aa1. Amino acid residue numbers are marked. The sequence determined by Edman degradation of the major chymotrypsin-processed Cry protein of the BT *finitimus* 1166 [4] is underlined. The arrow marks a site of chymotrypsin hydrolysis usually coinciding with a site of delta-endotoxin maturation in the midgut of insect larvae.

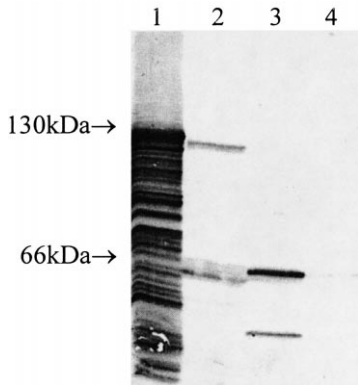

Fig. 3. Expression of cloned toxins. Western blot analysis of SDS-PAGE of crude SDS extraction of *E. coli* NM522 harboring recombinant plasmids: (1) pF1 (Cry26Aa1), (2) pF2 (Cry28Aa1), (3) pF1-N (truncated Cry26Aa1 with deleted C-terminal part), (4) pUK21, the negative control plasmid. Molecular weight of the products was estimated to be 130 kDa for pF1, 125 kDa for pF2, and 65 kDa for pF1-N.

lacking in BtI and BtII or any other conserved BT promoter sequences. However, efficient production of the Cry26Aa1 protein implies efficient transcription of the *cry26Aa1* gene in the BT *finitimus* 1166 strain. Near the *cry26Aa1* ORF a ribosome binding sequence (GGAGG) was found.

The cloned fragment harboring the *cry28Aa1* gene was also lacking in BtI and BtII promoter sequences. A putative vegetative promoter sequence TTGCAA(N)$_{15}$TAAGCC similar to that of *cry3Aa* was located 280 bp upstream of the

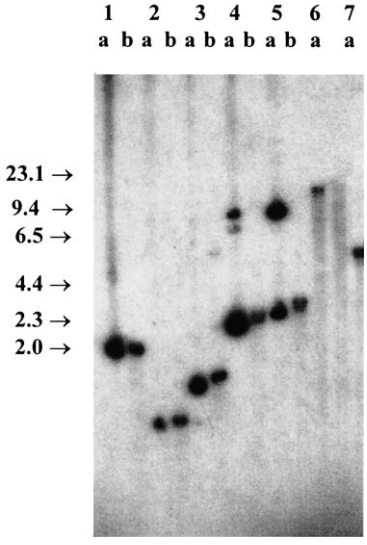

Fig. 4. Southern hybridization of BT *finitimus* 1166 genomic DNA (BT 1166 DNA). The *Pau*I-*Pst*I DNA fragment containing the 5′-terminus of the *cry26Aa1* gene was used as a probe. The genomic DNA was digested with seven pairs of restriction endonucleases. The plasmid DNA harboring the full length cloned insertion (pF1 DNA) digested with the same enzymes was loaded onto the adjacent lane as a control if both restriction sites were present in the cloned fragment. Lanes: (1a) pF1 DNA/*Pau*I+*Eco*RV; (1b) BT 1166 DNA/*Pau*I+*Eco*RV; (2a) pF1 DNA/*Pau*I+*Xba*I; (2b) BT 1166 DNA/*Pau*I+*Xba*I; (3a) pF1 DNA/*Pau*I+*Pst*I; (3b) BT 1166 DNA/*Pau*I+*Pst*I; (4a) pF1 DNA/*Pau*I+*Afl*II; (4b) BT 1166 DNA/*Pau*I+*Afl*II; (5a) pF1 DNA/*Pau*I+*Eco*72I; (5b) BT 1166 DNA/*Pau*I+*Eco*72I; (6a) DNA/*Pau*I+*Bam*HI; (7a) BT 1166 DNA/*Pau*I+*Hin*dIII.

cry28Aa1 ORF. Near the *cry28Aa1* ORF a putative ribosome binding sequence AAAGG complementary to the 3′-terminal region of 16S rRNA was found.

Recombinant plasmids pF1 and pF2 provided efficient expression of Cry26Aa1 and Cry28Aa1 in *E. coli* cells (Fig. 3). Cry26Aa1 was also expressed in a truncated form of 506 N-terminal amino acid residues (Fig. 3).

3.2. Examination of regions flanking the cry26Aa1 gene in the BT finitimus genome

The lack of conventional BT promoters suggests a number of Cry26Aa1 alleles differing in genomic surrounding and allowing differential control of expression.

The restriction map of the *cry26Aa1* upstream flanking region in the BT *finitimus* 1166 genome was studied by Southern hybridization. The DNA fragment containing the 5′-terminus of the *cry26Aa1* gene was used as a probe. Total BT *finitimus* 1166 genomic DNA samples were digested with seven pairs of restriction endonucleases; only one hybridized fragment was observed by examination of the region within 7.5 kb from the translation start in each case (Fig. 4). The downstream flanking region also had a unique restriction map (data not shown). This suggests that the protein Cry26Aa1 in both spore-associated and free types of crystals is synthesized under the control of one and the same genomic locus.

Southern hybridization also demonstrated unique surroundings in the BT *finitimus* genome for at least 3 kb both upstream and downstream of the *cry28Aa1* ORF (data not shown).

An expression control of the cloned *cry* genes remains to be studied. The lack of the typical BtI or BtII promoters may be essential for the specific distribution of delta-endotoxins in BT *finitimus*.

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