Mol Gen Genet (1994) 242:365–368

MGG

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Use of an operon fusion to induce expression and crystallisation of a *Bacillus thuringiensis* δ-endotoxin encoded by a cryptic gene

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Received: 9 November 1992 / Accepted: 5 August 1993

Abstract. A δ-endotoxin gene previously cloned from *Bacillus thuringiensis* subsp. *galleriae* has been shown by a combination of restriction mapping and DNA sequence analysis to be a *cryIIB* clone; in common with other *cryIIB* genes it was found to lack a functional promoter. Addition of a promoter resulted in expression of the gene in *Bacillus thuringiensis* but did not result in the formation of the crystalline inclusions normally associated with such toxins. Inclusion formation was only observed when the gene was incorporated into an operon containing a gene known to be involved in the crystallisation of another δ-endotoxin.

Key words: CryIIB – δ-Endotoxin – Cryptic gene – Operon – Chaperone

Introduction

During sporulation *Bacillus thuringiensis* (BT) synthesizes insecticidal proteins, known as δ-endotoxins, that accumulate as cytoplasmic inclusion bodies (Höfte and Whiteley 1989). The δ-endotoxin genes are usually located on plasmids and are transcribed from promoters recognised by sporulation-specific sigma factors (Brown and Whiteley 1990). In the search for improved biopesticides the reintroduction of cloned toxin genes into BT provides a way of investigating the effects of novel gene combinations on insecticidal activity (Crickmore et al. 1990b; Lecadet et al. 1992). The introduction of cloned genes into BT also provides a system with which to study the factors regulating the expression of δ-endotoxin genes. Using such a system, it has been discovered that two δ-endotoxin genes, *cytA* and *cryIIA*, require an accessory protein to be co-expressed in order that their products may form crystalline inclusions (Adams et al. 1989; Crickmore et al. 1990a; Crickmore and Ellar 1992). In the case of *cryIIA* this accessory protein, believed to act as a molecular chaperone, is found as the second gene in an operon in which *cryIIA* is the third and most distal gene (Widner and Whiteley 1989; Crickmore and Ellar 1992). CryIIB is a very similar toxin to CryIIA and has been independently cloned from BT subsp. *kurstaki* HD1 on two occasions (Widner and Whiteley 1989; Dankocsik et al. 1990). Both groups of workers found that the gene was not expressed in HD1, although the gene product was functional when synthesized in various expression systems. A third gene in the *cryII* family, *cryIIC*, is also found as the distal gene in an operon preceded by two open reading frames that are similar, but not identical, to *orf1* and *orf2* in the *cryIIA* operon. Unlike CryIIA, these *orfs* do not appear to have a role in the formation of CryIIC inclusions (Wu et al. 1991).

In this paper we confirm that a *cryII* gene previously cloned from BT subsp. *galleriae* 916 (Ahmad et al. 1989) is a *cryIIB* gene and, as in HD1, appears to be cryptic. By placing the first two genes of the *cryIIA* operon upstream of this *cryIIB* gene we have established that the molecular chaperone ORF2 is also required for the formation of CryIIB inclusions.

Materials and methods

Plasmids and bacterial strains. The plasmid containing the *cryIIB* gene (pKL5) consisted of a 6.7 kb *Hin*dIII fragment (previously estimated at 5.8 kb) cloned into pUC12, as described by Ahmad et al. (1989). The shuttle vector pSV2, the promoter fusion vector $pSVP_{27}$, the various *cryIIA* constructs and the acrystalliferous strain of BT subsp. *israelensis*, IPS78/11, have all been described previously (Crickmore and Ellar 1992).

Site-directed mutagenesis. Introduction of an *Xba*I site into plasmids encoding *cryIIA* and *cryIIB* was achieved using the Altered Sites Mutagenesis kit (Promega). The oligonucleotide used (5′-TTTTAATATATTTCTAGA-TATTTAAGGAGG-3′) was annealed to single-stran-

Communicated by H. Hennecke

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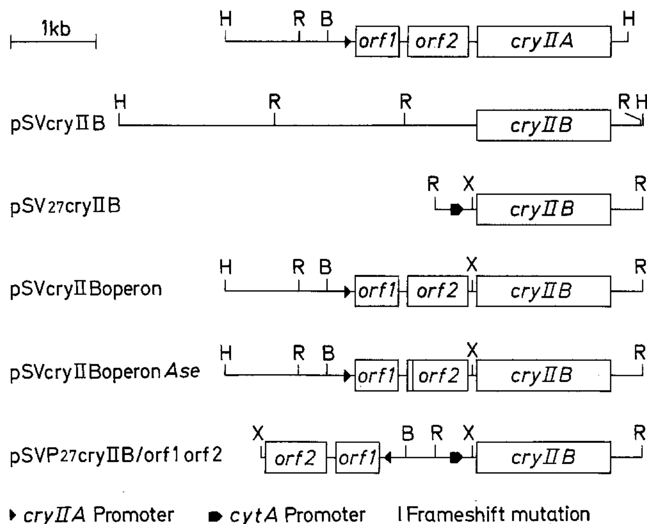

Fig. 1. Construction of CryIIB expression vectors. The top line represents the *cryIIA* operon, the following lines show the constructions described in the text and used to express CryIIB in IPS78/11. All the fragments shown were cloned into the shuttle vector SV2. H, *Hin*dIII; R, *Eco*RI; B, *Bam*HI; X, *Xba*I

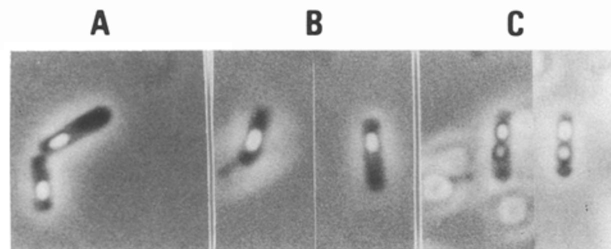

Fig. 2A–C. Light micrographs showing sporulating cells of IPS78/11 containing pSV2 (**A**), pSVP$_{27}$cryIIB (**B**) and pSVcryIIBoperon (**C**). Magnification, 1800×

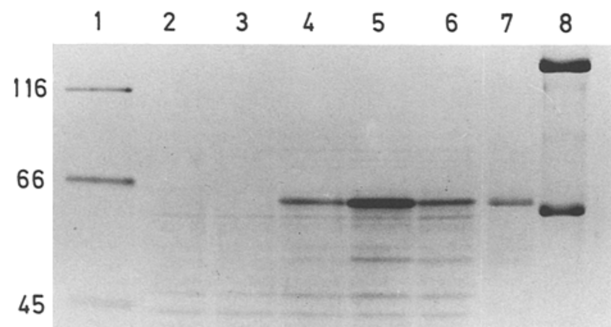

Fig. 3. Expression of CryIIB in IPS78/11. Coomassie-stained SDS-polyacrylamide gel comparing the levels of expression of CryIIB from the vectors shown in Fig. 1. Transformants were grown and harvested, and proteinacous material precipitated with trichloroacetic acid as described previously (Crickmore et al. 1990b). Samples representing equal volumes of spore/crystal mixes were compared. Lane 1, molecular mass standards; lane 2, pSV2; lane 3, pSVcryIIB; lane 4, pSVP$_{27}$cryIIB; lane 5, pSVcryIIBoperon; lane 6, pSVcryIIBoperonAse. Lanes 7 and 8 contain purified inclusion bodies from IPS78/11 (pSVP$_{27}$cryIIB/orf1orf2) and *Bacillus thuringiensis* subsp. *galleriae* 916, respectively

ded template derived from the plasmid pSELECT, into which the target sequences had been cloned. Plasmid pSelB40 contained the 5.0 kb *cryIIA*-encoding *Hin*dIII fragment (Crickmore and Ellar 1992), and plasmid pSelKL5 contained a 3.2 kb *cryIIB*-encoding *Eco*RI fragment identified by further restriction mapping of pKL5. The mutagenised plasmids were termed pSelB40X and pSelKL5X, respectively.

Construction of the expression vectors. The plasmid pSVcryIIB was obtained by subcloning the 6.7 kb *Hin*dIII fragment from pKL5 into pSV2. Plasmid pSVP$_{27}$cryIIB was constructed by inserting the *cryIIB* gene, on a 2.4 kb *Xba*I fragment from pSelKL5X, downstream of the *cytA* promoter in pSVP$_{27}$. The construction of pSVcryIIBoperon involved first subcloning *cryIIB*, on a 2.4 kb *Xba*I-*Eco*RI fragment, into pSV2, after which the proximal part of the *cryIIB* operon was added as a 3.0 kb *Hin*dIII-*Xba*I fragment from pSELB40X. The frameshift mutation (which resulted in termination of ORF2 after 14 amino acids) was substituted on a 1.8 kb *Bam*HI-*Xba*I fragment from pSVB40XAse (Crickmore and Ellar 1992). Plasmid pSVP$_{27}$cryIIB/orf1orf2 was constructed by first subcloning *orf1* and *orf2* (from pSelB40X, on an *Eco*RI-*Xba*I fragment) into pSV2, and then adding the P$_{27}$cryIIB fusion (from pSVP$_{27}$cryIIB) on an *Eco*R1 fragment. Graphic representations of all the above constructs are shown in Fig. 1.

Results

Identification of the cryII *clone*

The *cryII* gene from BT subsp. *galleriae* 916 was cloned on a *Hin*dIII fragment that was significantly smaller than the 9 kb *cryIIB*-encoding fragment from HD1 (Widner and Whiteley 1989), yet an internal 3.2 kb *Eco*RI fragment showed a marked similarity to the restriction map deduced from the published sequences of *cryIIB* (Widner and Whiteley 1989; Dankocsik et al. 1990). In order to confirm the identity of the clone, a portion was sequenced, including the first 150 bp of the coding region, which proved to be identical to the published *cryIIB* sequences.

Expression of the cryIIB *gene in* Bacillus thuringiensis

In HD1, *cryIIB* is found as a cryptic gene in that it is not expressed at any point in the cell cycle (Widner and Whiteley 1989). To establish whether the clone from subsp. *galleriae* 916 was also cryptic, the *Hin*dIII fragment was subcloned onto an *E. coli* /*B. thuringiensis* shuttle vector (pSV2) and the resulting plasmid (pSVcryIIB) was introduced into an acrystalliferous BT strain by electroporation. No CryIIB protein could be detected in this transformant (see Fig. 3, lane 3). In previous studies we have used the promoter from the *cytA* toxin gene of BT subsp. *israelensis* to express toxin

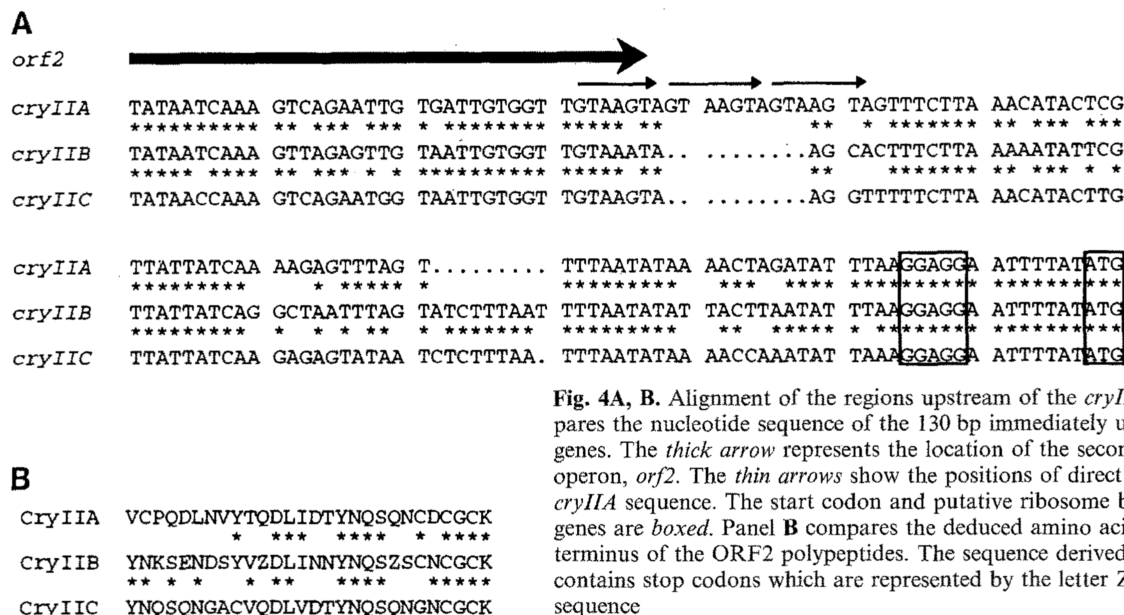

Fig. 4A, B. Alignment of the regions upstream of the *cryII* genes. Panel **A** compares the nucleotide sequence of the 130 bp immediately upstream of the *cryII* genes. The *thick arrow* represents the location of the second gene in the *cryIIA* operon, *orf2*. The *thin arrows* show the positions of direct repeats identified in the *cryIIA* sequence. The start codon and putative ribosome binding site of the *cryII* genes are *boxed*. Panel **B** compares the deduced amino acid sequence of the C-terminus of the ORF2 polypeptides. The sequence derived from the *cryIIB* clone contains stop codons which are represented by the letter Z in the amino acid sequence

genes lacking a functional promoter (Ward and Ellar 1988; Crickmore and Ellar 1992). A plasmid was thus constructed (pSVP$_{27}$cryIIB) in which the *cytA* promoter was placed immediately upstream of the *cryIIB* gene, and again introduced into IPS78/11. Microscopic examination of sporulating cells of this transformant (Fig. 2B) did not reveal any of the inclusions typically found with other BT toxins, yet when the cultures were analysed by SDS-PAGE it was clear that the toxin was being synthesised (Fig. 3, lane 4). This observation was identical to that made on CryIIA, where an accessory factor is required for inclusion formation, though not for expression per se. Since CryIIA and CryIIB are very similar toxins it was possible that the presence of this accessory factor might also be neccessary for the CryIIB protein to form inclusions. The plasmid pSVcryIIBoperon was thus constructed in which the *cryIIB* gene was fused downstream of the promoter and first two genes of the *cryIIA* operon. Microscopic examination of IPS78/11 transformants revealed the presence of large, regular, extrasporal inclusions in almost every cell (Fig. 2C). Purification of these inclusions on a discontinuous sucrose gradient followed by SDS-PAGE analysis showed that they consisted predominantly of a single protein of 63–65 kDa, which co-migrated with the major band seen in Fig. 3, lane 5. With CryIIA it was found that if a frameshift mutation was introduced into the second gene of the operon *(orf2)* then inclusion body formation was abolished (Crickmore and Ellar 1992). The same result was obtained when this mutation was introduced into pSVcryIIBoperon, although, as with *cryIIA*, a significant amount of toxin was still synthesized (Fig. 3, lane 6). In order to establish whether the *orf2* gene is required in cis, pSVP$_{27}$cryIIB/orf1orf2 was constructed, in which *cryIIB* and *orf1orf2* have different promoters and are transcribed in opposite directions. Cells of IPS78/11 containing this plasmid produced inclusions apparently identical to those produced by cells carrying pSVcryIIBoperon.

Comparison of the subsp. galleriae *916 CryII proteins*

Figure 3 also shows a comparison between the CryII protein present in the native strain (lane 8) with that encoded by pSVP$_{27}$cryIIB/orf1orf2 (lane 7). It can be seen that the cloned *cryIIB* gene product migrates more slowly than the native CryII toxin. The N-terminus of this native CryIIB toxin was determined and revealed the sequence MNTVLNNGRN, which is identical to the deduced amino acid sequence of the *cryIIC* gene cloned by Wu et al. (1991).

Discussion

Although the two toxins CryIIA and CryIIB are very similar and display 87% identity in amino acid sequence, their genetic environments are completely different. The *cryIIA* gene is found as the most distal gene in a functional three-gene operon. As well as not being part of an operon, *cryIIB* also appears to lack a functional promoter. Expression of CryIIB from the 9.0 kb *Hin*dIII fragment was observed in *E. coli* by Widner and Whiteley (1989), yet no expression was observed by Dankocsik et al. (1990) using the same fragment, also in *E. coli*. This could be explained if expression of the gene required a promoter provided by the cloning vector which may have been present, in the correct orientation, only in the former case. Dankocisk et al. (1990), achieved expression of CryIIB in an acrytalliferous strain of BT subsp. *kurstaki* by fusing the structural gene to a promoter derived from the BT *cryIIIA* toxin gene. Although irregularly shaped particles were occasionally observed, no regular inclusions appeared to be synthesized within the cells. We report a similar finding in that a functional promoter is sufficient to achieve expression but not inclusion formation. In order to achieve the latter, expression of the second gene in the *cryIIA* operon, *orf2,* was also re-

quired. We have previously speculated that the gene product of *orf2* acts as a molecular chaperone to CryIIA, and is directly involved in laying down toxin as crystalline inclusions (Crickmore and Ellar 1992). It seems likely that ORF2 can function similarly for CryIIB, furthermore we have demonstrated that ORF2 can still exert its effect when expressed in trans.

Alignment of the nucleotide sequences upstream of *cryIIA, cryIIB* and *cryIIC* reveals a high degree of similarity between *cryIIB* and *cryIIA/C* in the 130 bp stretch immediately upstream of the structural genes (Fig. 4). Beyond this, there is little similarity between *cryIIB* and the other two clones (data not shown). This 130 bp region includes the C-terminus of the *orf2* gene, suggesting that *cryIIB* may once have been part of an operon similar to those that include *cryIIA* or *cryIIC*. Figure 4 also compares a translated section of the *cryIIB* upstream region with the deduced amino acid sequences of the *cryIIA* and *cryIIC* ORF2 polypeptides, where homologies can clearly be seen. Based on these comparisons, the *cryIIB* clone seems to be more closely related to *cryIIC* than to *cryIIA*. In particular, the *cryIIA* clone alone contains an additional 10 basepairs immediately after the end of the *orf2* gene in a section consisting of three tandem direct repeats of the heptamer GTAAGTA (or TAAGTAG).

Acknowledgements. We would like to thank Jillian Webster for excellent technical assistance, and Dr. Len Packman for performing the N-terminal sequence analysis. We acknowledge the financial support of E.I. DuPont de Nemours and Co. and the Wellcome Trust for help with synthesizing the oligonucleotides.

References

Adams LF, Visick JE, Whiteley HR (1989) A 20-kilodalton protein is required for efficient production of the *Bacillus thuringiensis* subsp. *israelensis* 27-kilodalton crystal protein in *Escherichia coli*. J Bacteriol 171:521–530

Ahmad W, Nicholls C, Ellar DJ (1989) Cloning and expression of an entomocidal protein gene from *Bacillus thuringiensis galleriae* toxic to both lepidoptera and diptera. FEMS Microbiol Lett 59:197–202

Brown KL, Whiteley HR (1990) Isolation of the second *Bacillus thuringiensis* RNA polymerase that transcribes from a crystal protein gene promoter. J Bacteriol 172:6682–6688

Crickmore N, Ellar DJ (1992) Involvement of a possible chaperonin in the efficient expression of a cloned CryIIA δ-endotoxin gene in *Bacillus thuringiensis*. Mol. Microbiol 6:1533–1537

Crickmore N, Bone EJ, Ellar DJ (1990a) Genetic manipulation of *Bacillus thuringiensis:* towards an improved pesticide. Aspects Appl Biol 24:17–24

Crickmore N, Nicholls C, Earp DJ, Hodgman TC, Ellar DJ (1990b) The construction of *Bacillus thuringiensis* strains expressing novel entomocidal δ-endotoxin combinations. Biochem J 270:133–136

Dankocsik C, Donovan WP, Jany CS (1990) Activation of a cryptic crystal protein gene of *Bacillus thuringiensis* subspecies *kurstaki* by gene fusion and determination of the crystal protein insecticidal specificity. Mol Microbiol 4:2087–2094

Höfte H, Whiteley HR (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol Rev 53:242–255

Lecadet MM, Chaufaux J, Ribier J, Lereclus D (1992) Construction of novel *Bacillus thuringiensis* strains with different insecticidal activities by transduction and transformation. Appl Environ Microbiol 58:840–849

Ward ES, Ellar DJ (1988) Cloning and expression of two homologous genes of *Bacillus thuringiensis* subsp. *israelensis* which encode 130-kilodalton mosquitocidal proteins. J Bacteriol 170:727–735

Widner WR, Whiteley HR (1989) Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki* possess different host range specificities J Bacteriol 171:965–974

Wu D, Cao XL, Bai YY, Aronson AJ (1991); Sequence of an operon containing a novel δ-endotoxin gene from *Bacillus thuringiensis*. FEMS Microbiol Lett 81:31–36