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Influence of the 20-kDa protein from *Bacillus thuringiensis* ssp. *israelensis* on the rate of production of truncated Cry1C proteins

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Abstract

The potential role of a molecular chaperone on the rate of production of extensively altered *Bacillus thuringiensis* Cry1C proteins was investigated. Analysis of the proteins produced by the recombinant *B. thuringiensis* strains showed that the truncated proteins were produced at a low rate. Expression of the 20-kDa protein gene from *B. thuringiensis* ssp. *israelensis* in tandem with the truncated-*cry1C* genes led to the production of a greater amount of proteins. The formation of inclusion bodies, however, did not occur even when the 20-kDa protein gene was expressed.

Keywords: *Bacillus thuringiensis*; Cry1C; Molecular chaperone; Protein stability

1. Introduction

The soil bacterium *Bacillus thuringiensis* is characterized by the production upon sporulation of highly specific insecticidal proteins which accumulate into parasporal inclusion bodies commonly referred to as crystals. Several types of crystal proteins have been reported with differences in structure and specificity [1]. Naturally occurring variations [2] as well as modification of the sequence of Cry1 proteins by point mutation, deletion or domain swapping have been shown to be detrimental to toxicity [3], binding [3,4], protein stability [5], solubility or crystal formation [3]. Although hybrids showing an enlarged host range have been created in the laboratory [6], negative effects of the modifications on protein stability and production are still limiting factors and research should be undertaken to stabilise and improve the rate of production of promising altered proteins.

We report here evidence that the impact of extensive modifications of the protease-resistant domain of Cry1C, which are detrimental to the rate of production of protein and to the formation of crystal, could be partially compensated by the use of a molecular chaperone. We show that expression of the 20-kDa protein gene from *Bacillus thuringiensis* ssp.

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israelensis in the recombinant strains helps to increase the rate of production of modified Cry1C proteins although these proteins still cannot accumulate into visible crystals.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli DH5α and *B. thuringiensis* ssp. *kurstaki* HD1 cry⁻B were used as host strains. *cry1C* was subcloned from p60.5G31 [7] and the 20-kDa protein gene construct was isolated from pWF45 [8].

2.2. Standard procedures

PCR reactions were performed with Vent DNA polymerase for 25 cycles: 94°C for 1 min, 42°C for 1 min and 72°C for 2 min. PCR products were verified by DNA sequencing. SDS-PAGE analyses of protein contents were conducted under standard conditions [9] and proteins were alternatively transferred onto PVDF membranes.

2.3. Construction of truncated cry1C toxin genes

The *cry1C* gene was mutated to remove the toxin-encoding moiety and to create an *Eco*RV site and a *Bam*HI site, respectively, at the first codon and at the last codon of the toxin-encoding domain to yield the clone pBT1C-4 (Fig. 1A). Four PCR fragments, PCR-1, PCR-2, PCR-3 and PCR-4 of 372, 690, 1030 and 1386 bp, respectively, were amplified from the toxin-coding region of *cry1C* and an *Sma*I and a *Bam*HI site were created at the 5′ and 3′ ends of the PCR products, respectively, except for the PCR-1 product where an *Eco*RV site was created instead of an *Sma*I site (Fig. 1B). Truncated *cry1C* genes were reconstructed by in-frame cloning of the PCR products PCR-1 to PCR-4 into pBT1C-4 using the *Eco*RV and *Bam*HI cloning sites to yield pMB-1 to pMB-4, respectively (Fig. 1C). Modified *cry1C* genes were isolated as 2.6-, 3.0-, 3.3- and 3.7-kb *Sst*I-*Sal*I fragments and cloned separately in the shuttle vector pHT 3101 [10].

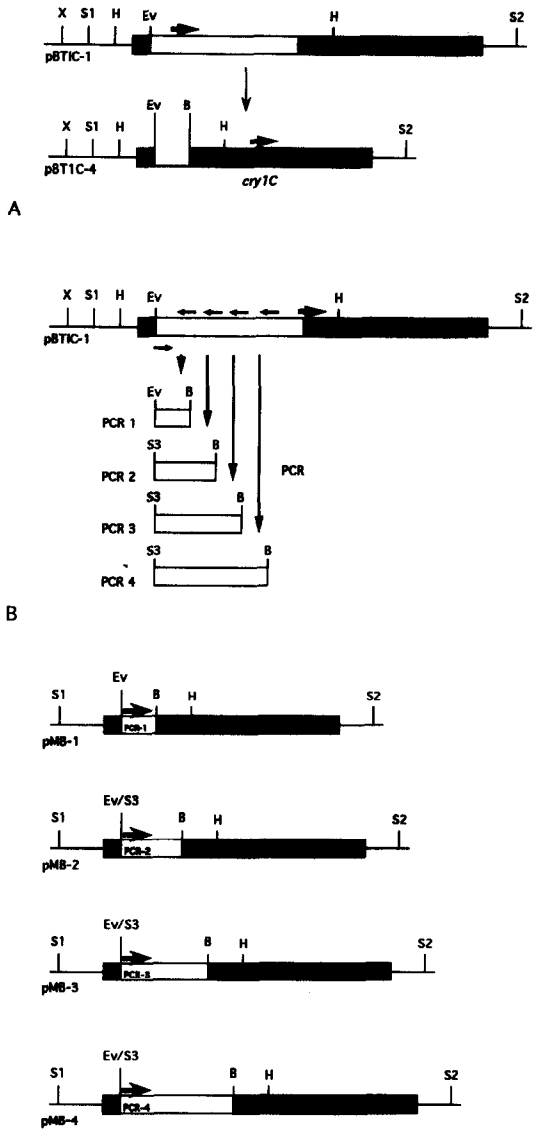

Fig. 1. Restriction maps of constructs. Figures are not to scale. Large arrows indicate the orientation of the gene. Small arrows indicate the position and orientation of PCR primers. (A) Map of pBT1C-4. (B) Obtaining of truncated toxin-encoding fragments. (C) Maps of the pMB series of constructs. Restriction enzymes: B, *Bam*HI; EV, *Eco*RV; H, *Hin*dIII; S1, *Sal*I; S2, *Sst*I; S3, *Sma*I, X, *Xho*I.

2.4. Insertion of the 20-kDa protein gene in tandem with truncated cry1C genes

The 20-kDa protein gene was isolated from

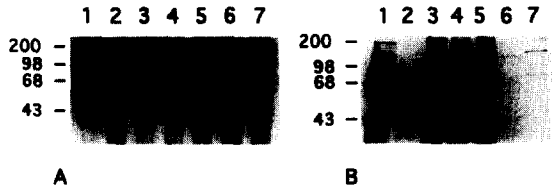

Fig. 2. Electrophoretic analysis of the protein contents of recombinant *B. thuringiensis* strains. (A) Analysis of the protein contents of recombinant bacteria expressing the modified *cry1C* gene alone. Lanes: (1) molecular weight markers; (2) BT-pHT; (3) BT-1; (4) BT-2; (5) BT-3; (6) BT-4; (7) BT-1C. (B) Analysis of the protein contents of recombinant bacteria expressing the modified *cry1C* gene in tandem with the 20-kDa protein gene. Lanes: (1) molecular weight markers; (2) BT-pHT/20; (3) BT-1/20; (4) BT-2/20; (5) BT-3/20; (6) BT-4/20; (7) BT-1C.

pWF45 as a 2.2-kb *Afl*II/*Pvu*II fragment. After filling in the *Afl*II cohesive ends with Klenow enzyme, the fragment containing both the 20-kDa protein gene and the *cry1Ac* promoter was cloned in the *Sma*I site of pBC KS+. To complement the constructs containing the different versions of truncated *cry1C* gene, the 20-kDa protein gene was inserted as an approx. 2-kb *Sal*I fragment to yield the plasmids pMB-1/20, pMB-2/20, pMB-3/20 and pMB-4/20.

3. Results and discussion

The various versions of the truncated *cry1C* gene as well as the shuttle vector pHT3101 [10] and the full length native *cry1C* gene were introduced into the crystal minus *B. thuringiensis* strain Cry$^-$B as previously described [10] to yield the recombinant *B. thuringiensis* strains, BT-1, BT-2, BT-3, BT-4, BT-pHT and BT-1C, respectively. Observation through light microscopy of the recombinant bacteria at sporulation showed that BT-pHT (a control strain bearing only the shuttle vector), BT-1, BT-2, BT-3 and BT-4 did not produce any visible inclusion body whereas BT-1C, expressing the native *cry1C* gene, produced large typical bipyramidal crystals (data not shown). The recombinant *B. thuringiensis* clones BT-1/20, BT-2/20, BT-3/20 and BT-4/20 bearing the 20-kDa protein from *B. thuringiensis* ssp. *israelensis* in tandem with the various versions of the truncated *cry1C* gene as well as the control strain BT-pHT/20 bearing only the 20-kDa protein gene did not produce any inclusion body at sporulation. Only the control strain BT-1C made inclusion bodies.

Analysis of the protein contents of the recombinant strains on SDS-polyacrylamide gels showed no major band except for proteins from BT-1C which showed a major band of approx. 130 kDa (Fig. 2A). In contrast, SDS-PAGE analysis of the protein contents of the recombinant strains BT-1/20, BT-2/20, BT-3/20, BT-4/20 and BT-1C showed a major band migrating at the position corresponding to the expected protein sizes (Fig. 2B). Immunodetection of blotted proteins using polyclonal antibodies raised against purified Cry1C endotoxin showed clearly the presence of modified or native Cry1C proteins corresponding to the expected size in BT-1, BT-2, BT-3, BT-4, and BT-IC as well as in BT-1/20, BT-2/20, BT-3/20, BT-4/20. BT-pHT and BT-pHT/20 did not produce any detectable protein. Detection by the anti-Cry1C antibodies confirmed that the proteins were actually produced and that the major bands observed on SDS-PAGE were actually the truncated recombinant proteins (Fig. 3).

The results reported here indicate that the 20-kDa protein from *B. thuringiensis* ssp. *israelensis* could interact positively with a crystal protein, i.e.

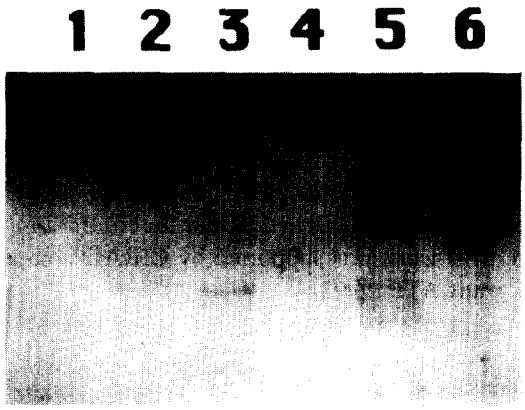

Fig. 3. Immuno-analysis of the protein contents of recombinant *B. thuringiensis* strains with polyclonal antibodies raised against Cry1C. Only the immuno-analysis of strains bearing both the truncated *cry1C* gene and the 20-kDa protein gene is shown. The same result (i.e. detection of the truncated proteins) was obtained with the strains shown in Fig. 2A containing the same constructs but in the absence of the 20-kDa protein gene. Lanes: (1) BT-pHT/20; (2) BT-1/20; (3) BT-2/20; (4) BT-3/20; (5) BT-4/20; (6) BT-1C.

Cry1C, different from the proteins with which it is naturally associated [11–13]. Wu and Federici [14] suggested that the 20-kDa protein could act as a molecular chaperon therefore interacting with Cyt1A upon synthesis and protecting the cell from the cytolytic activity. This protein also allows Cyt1A and Cry11A to accumulate into large crystals [11,14]. Considering that this protein was shown to be active at the post-translational level [15], the increased amount of truncated proteins may be a consequence of protection from endogenous proteases.

Modification of the host range or level of toxicity by mutagenesis on the protease-resistant domain has been suggested and although hybrids showing an extended host range have been actually created, several were shown to be unstable [5]. Since the tertiary structure of a crystal protein and of its various domains is most likely the result of an important selective pressure [16], it is therefore not surprising that modification of sequences in an attempt to improve the toxins through genetic engineering results in a lack of stability and a low rate of production. The ability of the 20-kDa protein to compensate in part the detrimental effect of the extensive deletions made in Cry1C and to increase the rate of production suggests that this protein or other molecular chaperones might prove useful for the development of new proteins through genetic engineering.

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