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Fusion proteins with both insecticidal and neomycin phosphotransferase II activity

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Hybrid proteins consisting of N-terminal fragments of increasing length of a *Bacillus thuringiensis* insecticidal protein (Bt2) fused to neomycin phosphotransferase II (NPTII) were produced in *Escherichia coli*. The minimum fragment required for insect toxicity is comprised of the first 607 amino acids of Bt2. Fusion proteins not containing this minimum fragment were non-toxic. The NPTII activity of the different non-toxic hybrid proteins varied considerably but was not correlated with the length of the Bt2 fragment. Fusion proteins including the minimum toxic fragment of Bt2 exhibited insecticidal and NPTII activity comparable to that of the individual proteins. This was largely independent of the fusion point within Bt2. Our data suggest that the conformation of the Bt2 polypeptide exerts an important influence on the enzymatic activity of the fused NPTII protein. The combination of insecticidal activity and a dominant selectable trait into one protein offers important advantages for the generation of insect resistant transgenic plants.

δ-Endotoxin; Kanamycin; Hybrid gene; Bifunctional protein; Protein conformation; (*Bacillus thuringiensis*)

1. INTRODUCTION

Artificial fusion proteins have proven to be very useful in molecular biology. Fusions with easily assayable enzymes like β-galactosidase, thymidine kinase, alkaline phosphatase and neomycin phosphotransferase have been used to study gene regulation [1,2], secretion of proteins [3], the topology of proteins in membranes [4] and intracellular transport processes [5]. Also, chimeric proteins have been used to analyse the function of different domains and their mutual interactions [6,7]. Moreover, the design of artificial multifunctional proteins may have important potential applications: e.g. hybrid proteins consisting of a peptide hormone and the enzymatically active domain of a toxin have been shown to exhibit a cell-specific cytotoxicity [8]. The bioprocessing industry might take advantage of artificial bifunctional enzymes that perform sequential catalytic steps of a biochemical pathway [9]. Another application of fusion proteins is their use to select for efficient expression of transformed genes in eucaryotic cells. The expression level of foreign genes introduced into the eucaryotic genome is influenced by their site of insertion [10]. Cotransformation of a selectable marker gene facilitates the recognition of cells carrying the desired gene but does not ensure its expression, even when both genes are closely linked on the same DNA fragment [11]. A translational fusion of a selectable marker gene to the gene of interest will allow one to select for transformants expressing high levels of the desired gene through direct selection for the phenotype conferred by the marker gene. This however requires that the fusion protein exhibits the biological activities of both constituent proteins.

Neomycin phosphotransferase II (NPTII) encoded by the *neo* gene of transposon Tn5 confers resistance in bacteria [12] and in eucaryotic cells

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[13] towards aminoglycosides like kanamycin (Km), neomycin (Nm) or G418. In addition this selectable marker enzyme can retain its activity when foreign polypeptides are fused to its N-terminus [14].

We constructed fusions between a *Bacillus thuringiensis* insect toxin and NPTII and evaluated whether they both exhibited insecticidal and NPTII activity. δ-Endotoxins of *B. thuringiensis* are insecticidal proteins produced as parasporal crystalline inclusions during sporulation (review [15]). A typical example is Bt2, a 130 kDa protoxin cloned from *B. thuringiensis* strain *berliner* 1715 [16]. Bt2 has potent insecticidal activity against a number of lepidopteran insects. It is a protoxin which dissolves in the alkaline environment of the insect midgut and is proteolytically degraded into a 60 kDa toxic core. This toxic fragment was localized between amino acids 29 and 607 of the protoxin molecule [16].

This paper describes the analysis of fusion proteins between different N-terminal parts of Bt2 and NPTII. Some fusion proteins have both insect toxicity and NPTII activity at levels comparable to that of the individual parental proteins. We also provide evidence that the NPTII activity of these hybrid proteins is largely dependent on the conformation of the Bt2 moiety.

In a recent study, we have used such bifunctional fusion proteins to select transformed plants expressing *B. thuringiensis* toxin at levels sufficient to protect them from insect damage [17].

2. MATERIALS AND METHODS

2.1. *Bacterial strains, plasmids and growth conditions*

The *E. coli* strains used were K12ΔH1ΔTrp, carrying a thermosensitive cI857-repressor [18], and K12ΔH1ΔTrp(pRKM63), which expresses the *neo* gene of Tn5 [19]. pLBKM25 was constructed as in [16].

2.2. *DNA manipulations*

All molecular cloning procedures were performed according to Maniatis et al. [20]. DNA sequencing was done according to Maxam and Gilbert [21]. The *bt2-neo* fusion genes were derived from plasmid pLBKm25 (fig.1; [16]).

2.3. *Preparation of* E. coli *extracts for an in vitro NPTII assay and toxicity tests*

20 ml LB was inoculated with 0.2 ml of a saturated culture and grown at 38°C for approx. 90 min. The cells were lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.9, 50 mM EDTA, 15% sucrose, 0.2 mg/ml phenylmethylsulphonyl fluoride and 1 mg/ml leupeptin) by a treatment with 1 mg/ml lysozyme for 30 min at 0°C, followed by sonication at 0°C for 20 s. The extracts were centrifuged at 20000 × *g* for 10 min, the supernatants were divided in aliquots and stored at −70°C until use.

2.4. *In vitro NPTII assay*

This was performed according to Reiss et al. [22]. Extracts of 4×10^8 cells for all clones and 10^7 cells for K12ΔH1ΔTrp(pRKM63) were loaded. Fragments of the filter corresponding to the intact fusion proteins were cut out and the radioactivity counted. The amount of NPTII enzyme and fusion protein present in the cell extracts was estimated from a Western blot with a rabbit anti-NPTII antiserum, with a dilution series of NPTII as standard.

2.5. *Immunological techniques*

Rabbit antisera were raised against Bt2 protein, purified from the recombinant *E. coli* strain K514(pGI502) as described [16]. Monoclonal antibodies 4.8, 5D11 and 10E3 were raised and characterized by Höfte et al. (in preparation). ELISA assays were done as described [16].

2.6. *Toxicity assays*

Toxicity tests on 3rd instar larvae of *Pieris brassicae* were performed as described in [16].

3. RESULTS

3.1. *Construction of* bt2-neo-*fusions and their expression in* E. coli

A random series of *bt2-neo* gene-fusions was constructed starting from plasmid pLBKm25. This plasmid [14] (fig.1) contains the *bt2* gene behind the λ P_L promotor. The *neo* gene, preceded by a unique *Xho*I site is fused at position 3244 of *bt2*, giving rise to the junction sequence indicated in fig.1B. pLBKM25 was digested with *Kpn*I, treated with *Bal*31, ligated to *Xho*I-linkers, digested with

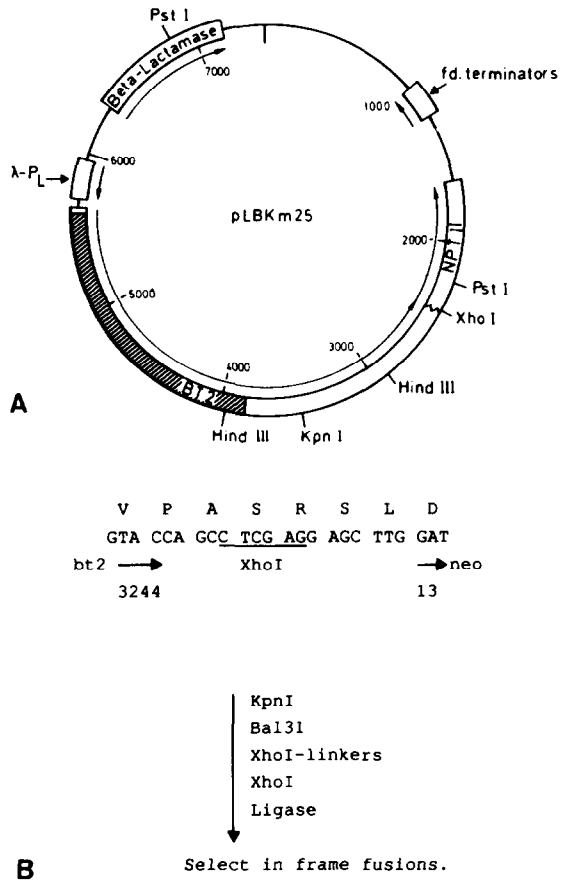

Fig.1. Plasmid pLBKm25 (A) and the strategy used for the construction of *bt2-neo* fusion-genes (B). The junction sequence between *bt2* and *neo* in pLBKm25 is shown. The numbers indicate the bp position in the Bt2 sequence. The hatched region in the plasmid corresponds to the sequence encoding the active toxin.

*Xho*I and self-ligated. *E. coli* transformants expressing in-frame fusions were selected for Km-resistance on 50 μg/ml Km. The fusion positions in the *bt2* gene were determined using appropriate restriction digests, with an accuracy of $\pm$ 20 bp (fig.2). For clones 6, 10 and 11, the exact fusion position was determined by DNA sequencing. Eleven plasmids containing *bt2* segments varying between 1430 and 2174 bp were selected for further analysis (fig.2).

The fusion proteins encoded by these plasmids were produced in *E. coli* strain K12ΔH1ΔTrp containing the temperature sensitive cI857 repressor. Cultures were induced at 38°C to activate the λ P_L promotor. The presence of the hybrid gene products was determined in the soluble and the pellet fraction of cell extracts in Western blots with anti-Bt2 and anti-NPTII antibodies (not shown). All fusion proteins were of the expected molecular mass and for about 50% present in a soluble form. We have used the soluble fraction of the *E. coli* extracts in further experiments (fig.3).

3.2. *Insect toxicity of the fusion proteins*

Cell extracts were tested for toxicity against larvae of *P. brassicae* (table 1). All clones expressing Bt2 fragments smaller than 596 amino acids were non-toxic (clones 1–6). The *bt2-neo*-fusions containing 633 ($\pm$ 3) or more codons of *bt2*, encoded polypeptides which were as toxic as the intact Bt2 protein (table 1). This is in agreement with the localization of the C-terminus of the minimum toxic fragment of Bt2 between codon 599 and 607 [16]. Our results indicate that fusing toxic Bt2 fragments to the NPTII enzyme does not influence their insect toxicity.

3.3. *NPTII activity in clones expressing fusion proteins*

The minimum inhibitory Km concentration for the *E. coli* clones expressing fusion proteins was determined by plating the cultures on media con-

Table 1

Toxicity of Bt2-NPTII fusion proteins against *Pieris brassicae* (3rd instar larvae), expressed in percentage mortality after 5 days, and reactivity with 3 mabs

Clone	Plasmid	Toxicity (ng/larva)			Reactivity with mabs		
		250	50	10	4.8	5D11	10E3
1	pS20	n.d.	4	8	+	–	–
2	pS23	n.d.	8	12	+	–	–
3	pLBKm865	n.d.	10	8	+	–	–
4	pS14	n.d.	12	14	+	–	–
5	pS33	n.d.	4	10	+	–	–
6	pS16	n.d.	10	8	+	–	–
7	pS13	100	100	56	+	+	+
8	pS11	100	98	60	+	+	+
9	pS9	100	87	63	+	+	+
10	pLBKm860	100	94	94	+	+	+
11	pLBKm23	100	100	65	+	+	+
Bt2 (purified)		100	100	80	+	+	+

taining 20, 50, 100, 200 and 400 μg/ml of Km. The in vivo resistance level of the different clones varied dramatically and was not directly correlated to the size of the *bt2* segment (fig.2).

The specific NPTII activity of individual proteins in the *E. coli* extracts was determined by an in situ NPTII assay, after separation of the proteins on a non-denaturing gel [22].

Fig.3b shows that in the *E. coli* clones NPTII activity was expressed in intact fusion proteins as well as in smaller polypeptides. In addition a good correlation was observed between the NPTII activity expressed by the different polypeptides present in the *E. coli* extracts and the in vivo Km resistance of the corresponding bacteria. The 5 clones expressing toxic polypeptides (7–11) behaved very similar in the in situ assay: the fusion proteins exhibited a comparable NPTII activity (figs 2,3). In these clones we also observed NPTII activity in smaller polypeptides, increasing in size from clones 7 to 11. Hence these fragments were most likely specific proteolytic degradation products generated through cleavage at the same position in the Bt2 sequence. Indeed, it has been shown that Bt2 contains a proteolytic site around amino acid 610 [16], a position which is present in the toxic fusion proteins.

The non-toxic clones showed an unexpected pattern of NPTII activity. In clones 1, 4, 5 and 6, the fusion proteins exhibited little or no NPTII activity. Accordingly, the Km resistance of clones 1, 4 and 6 was also very low (fig.2). The high Km resistance of clone 5, was solely due to NPTII activity exhibited by a smaller polypeptide. This protein could arise through either degradation of the fusion protein or more likely, through secondary translation initiation within the *bt2* sequence. Indeed, in clone 5 the *neo* gene is fused very close to an in-frame ATG preceded by a fortuitous ribosome binding site (fig.4A). This ATG-codon could serve as a secondary translation initiation codon. In clone 4, which produced little if any smaller polypeptides with NPTII activity, this ATG-codon has been removed. A similar observation has been reported for hybrid genes derived from the *Eco*RI endonuclease [24]. Clones 3 and 4

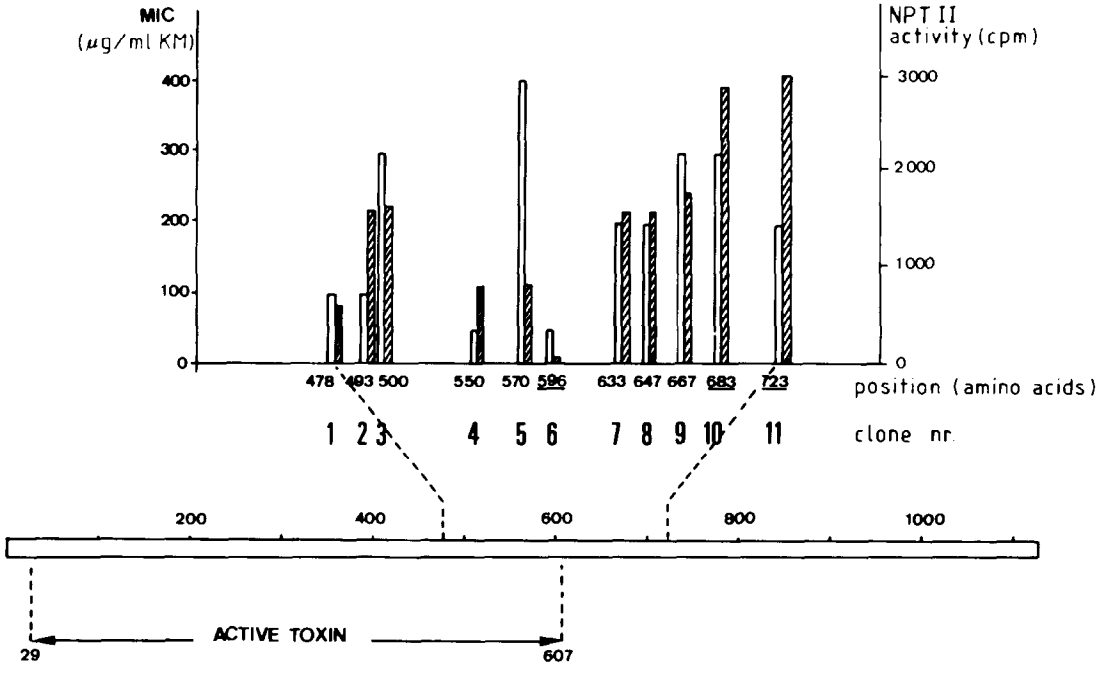

Fig.2. Fusion position in the Bt2 amino acid sequence (± 3 amino acids), and the minimum inhibiting concentration on Km for the *E. coli* clones containing the different fusion genes. The hatched bars represent the in situ NPTII activity of the intact fusion proteins. Numbers are underlined when the exact position is determined through DNA sequencing.

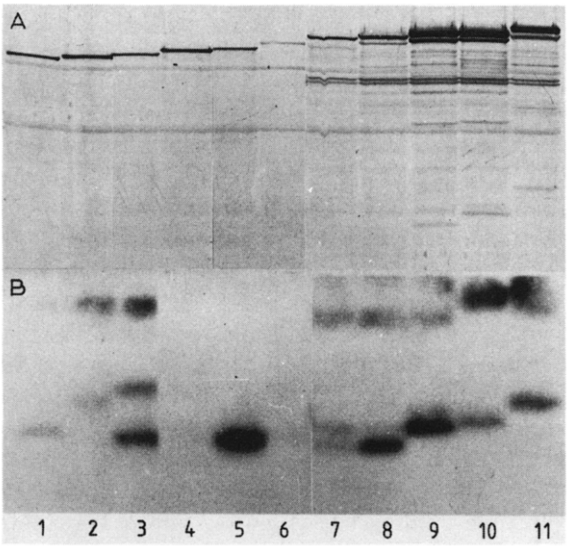

Fig.3. Western blot with a rabbit anti-Bt2 serum (A) and NPTII assay (B) of *E. coli* clones containing *bt2-neo* fusion-genes with *bt2* fragments of decreasing size. The numbers are as in table 1. The quantitation of the NPTII activity of the intact fusion proteins is shown in fig.2.

behaved clearly different. Here an important part of the NPTII activity was associated with the intact fusion proteins.

In summary, the fusion proteins containing the toxic fragment were insecticidal and showed NPTII activity. The non-toxic hybrid proteins of clones 1, 4, 5 and 6 showed little or no NPTII activity whereas those of clones 2 and 3 were clearly active.

3.4. *The conformation of the Bt2 domain in the fusion proteins*

We previously identified 2 monoclonal antibodies (mabs), 5D11 and 10E3, that recognize conformational determinants on Bt2 (Höfte et al., unpublished). These determinants disappear upon removal of 8 C-terminal amino acids from the smallest toxic fragment. However, the C-terminal residues are not a part of the epitopes of mabs 5D11 and 10E3. Also, the epitope of 10E3 contains, or is closely associated with, amino acids at the N-terminus of the toxin. This suggests that 5D11 and 10E3 recognize epitopes associated with a global 3-D structure which is disturbed upon removal of the 8 C-terminal residues. To investigate if fusion to NPTII influences the conformation of the Bt2 toxin, we determined in ELISA, the reactivity of mabs 5D11, 10E3 and 4.8 with extracts of *E. coli* clones containing the fusion proteins (table 1). Mab 4.8 recognizes a sequential determinant between residue 89 and 163 of Bt2 and served as a control. All toxic fusion proteins reacted with the 3 mabs whereas extracts containing the non-toxic proteins were only recognized by

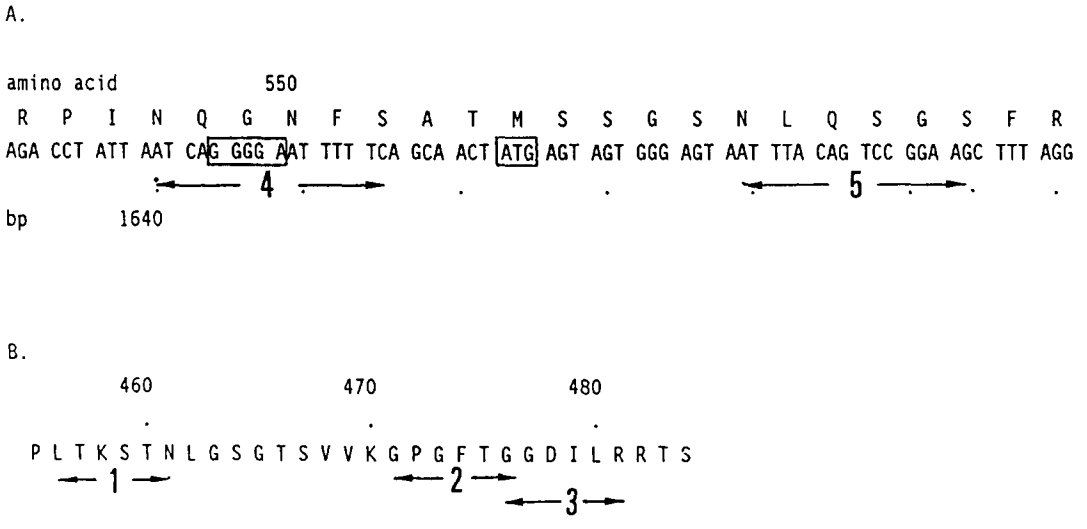

Fig.4. DNA and deduced amino acid sequence of *bt2* around the fusion position to *neo*. (A) In clones 4 and 5. The putative internal secondary translation initiation site and its SD sequence are boxed. (B) In clones 1, 2 and 3.

mab 4.8. This suggests that the fusion to NPTII did not induce a major disturbance in the global 3-D structure of the toxin.

4. DISCUSSION

This study shows that fusion proteins exhibiting both insect toxicity and NPTII activity can be constructed.

The toxicity of such fusion proteins only depends on the presence of the minimum toxic fragment of Bt2. This is not unexpected since Bt2 is a protoxin which is very efficiently processed into the active toxin by insect midgut proteases. Apparently, this also occurs for fusion proteins containing the toxic fragment, provided that the proteolytic site in Bt2 remains accessible.

The NPTII activity of the toxic fusion proteins was not affected by the presence of the Bt2 domain. Moreover, the reactivity of the toxic fusion proteins with mabs 5D11 and 10E3 suggested that the 3-D structure of the Bt2 toxin was not disturbed by the fusion to NPTII. Hence, we assume that in the toxic hybrid proteins, both the Bt2 and the NPTII domains were independently folded, which is in accordance with the high NPTII activity of these fusion proteins.

The situation for the non-toxic fusions was very different. The intact fusion proteins of clones 1, 4, 5 and 6 showed little or no NPTII activity, whereas those of clones 2 and 3 were clearly active.

We presume that in clones 1, 4, 5 and 6 a disturbance of the tertiary structure of the Bt2 moiety also imposed a change in the folding of the NPTII domain with a consequent loss of its activity. This idea was substantiated by previous experiments with monoclonal antibodies which demonstrate that the removal of 8 amino acids from the C-terminus of the minimum toxic fragment induces a dramatic conformational change through the whole molecule (Höfte et al., unpublished). The NPTII activity of the non-toxic fusion proteins of clones 2 and 3 could indicate that here the NPTII enzyme is fused at a large loop region in the toxin molecule, which is flexible enough to allow an independent folding of the NPTII domain. In clones 2 and 3 the fusion position to NPTII indeed is located in a region rich in Gly (fig.4B) allowing the flexibility typical for loop structures.

Reiss et al. [14] showed that the addition of long protein fragments to the N-terminus of NPTII almost invariably interfered with its activity. In these cases the presence of active proteolytic fragments accounted for the observed NPTII activity and Km resistance. Our data clearly show that even very long protein segments (up to 700 amino acids) can be fused to the N-terminus of NPTII without disturbing its activity, on condition that these polypeptides fold into an independent structure which does not interfere with the NPTII enzyme.

The applicability of such proteins was exemplified in a recent study in which the hybrid genes of clones 10 and 11 were expressed in tobacco plants. The selection on high levels of Km indeed delivered transformed plant cells expressing sufficient amounts of fusion protein to confer resistance to lepidopteran larvae [17].

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