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New *Bacillus thuringiensis* toxin combinations for biological control of lepidopteran larvae

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ABSTRACT

Cyt1Aa from *Bacillus thuringiensis israelensis* is known by its synergistical activity with *B. thuringiensis* and *Bacillus sphaericus* toxins. It is able to improve dipteran specific toxins activity and can prevent or overcome larval resistance to those proteins. The objective of the current study was to investigate the possible improvement of larvicidal activity of *B. thuringiensis kurstaki* expressing heterogeneous proteins Cyt1A and P20. *cyt1A98* and *p20* genes encoding the cytolytic protein (Cyt1A98) and the accessory protein (P20), respectively, were introduced individually and in combination into *B. thuringiensis kurstaki* strain BNS3. Immunoblot analysis evidenced the expression of these genes in the recombinant strains and hinted that P20 acts as molecular chaperone protecting Cyt1A98 from proteolytic attack in BNS3. The toxicities of recombinant strains were studied and revealed that BNS3pHT*p20* exhibited higher activity than that of the negative control (BNS3pHT*Blue*) toward *Ephestia kuehniella*, but not toward *Spodoptera littoralis*. When expressed in combination with P20, Cyt1A98 enhanced BNS3 activity against *E. kuehniella* and *S. littoralis*. Thus, Cyt1Aa protein could enhance lepidopteran Cry insecticidal activity and would prevent larval resistance to the most commercialized *B. thuringiensis kurstaki* toxins.

1. Introduction

Lepidopteran larvae found worldwide, attack different cultivated and wild plants and cause serious economic losses. Particularly, the Egyptian cotton leafworm *Spodoptera littoralis* attack more than 60 different types of plants such as cotton, clover, maize, wheat, rice, and barley [1,2]. Also the Mediterranean flour moth, *Ephestia kuehniella* (Lepidoptera: Pyralidae) is one of the major pests which destroy stored grain products in industrial flour mills [3]. These insects have developed resistance to a wide variety of highly toxic chemical insecticides due to routine use in spraying programs. Therefore, potential alternatives are needed. The entomopathogenic bacterium *Bacillus thuringiensis kurstaki* is widely used in commercial formulations for controlling various agricultural against lepidopteran pests [4] and their *cry* genes are intensively expressed in transgenic plants especially cotton and maize. These factors bring up some cases of resistance. Tabashnik et al. [5] have reported the resistance of diamondback moth, *Plutella xylostella*, to *B. thuringiensis kurstaki* in the field. However, Cry1Ac toxin from *B. thuringiensis kurstaki* combined with Cyt1Aa protein from *B. thuringiensis israelensis* could overcome the onset resistance of this lepidopteran insect [6]. Cyt1Aa protein is the major component (45–50%) of *B. thuringiensis israelensis* crystals [7]. It is endowed with a low insecticidal activity, but, it interacts synergistically with *B. thuringiensis* dipteran-specific toxins [7–10] and with mosquito larvicidal toxins from *B. sphaericus* and avoids the resistance of dipteran to those proteins [11–13]. Moreover, Cyt1Aa reduces the resistance of *Chrysomela scripta* larvae to Cry3A protein [14]. Co-expressed with a combination of Cry1Ac and/or Cry1Ca in *Escherichia coli*, Cyt1A enhances their activity against *Helicovera armigera* but not against *Pectinophora gossypiella* and *S. littoralis* [15]. The ability of Cyt1Aa to synergize with many toxins is probably due to the multiple mode of action of this protein [16]. In fact, it has cytolytic activity without requiring specific receptors; it interacts directly with membrane lipids [17]. For efficient expression, Cyt1Aa needs the 20-kDa helper protein [18,19]. The latter, found in *B. thuringiensis israelensis*, is encoded as the third ORF of the *cry11A* operon. Many reports suggested that this helper protein increases the expression levels probably by post-translational mechanism stabilization [20,21]. The *p20* gene expressed into wild strains of *kurstaki* subsp. does not increase Cry2A production, but it doubles Cry1A protoxin production [22]. In addition, it was demonstrated that P20 enhances the Cry1Ac and Cry3A crystal size and the toxicity of Cry1Ac against *H. armigera* but not Cry3A toxicity against *Leptinotarsa decemlineata* [23,24].

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Table 1
Plasmids and strains used in this study.

Plasmids and strains	Description	References
Plasmids		
pMOS*Blue*		Amersham, Biosciences, France
pBleuscript II SK (+/−)		Stratagene, La Jolla, CA, U.S.A.
pHT*cry1Ia*	Shuttle vector, derivative of pHT3101	[30]
pMOS*cyt1A98*	Derivative of pMOS*Blue* containing *cyt1A98*	[26]
pMOS*p20*	Derivative of pMOS*Blue* containing *p20*	This work
pHT*p20*	Derivative of pHT*Blue* containing *p20*	This work
pMOS*cyt1A98C*	Derivative of pMOS*Blue* containing *cyt1A98* amplified using D33 an D24 primers	This work
pMOS*cyt1A98H*	Derivative of pMOS*Blue* containing *cyt1A98* amplified using D31 an D24 primers	This work
pBS*cyt1A98*	Derivative of pBluescript vector containing *cyt1A98*	This work
pBS*p20-cyt1A98*	Derivative of pBluescript vector containing *p20* and *cyt1A98*	This work
pHT*cyt1A98*	Derivative of pHT*Blue* containing *cyt1A98*	This work
pHT*p20-cyt1A98*	Derivative of pHT*Blue* containing *p20* and *cyt1A98*	This work
Strains		
MOS*Blue* cells	*E. coli* (*end*A1 hsdR17 (r_{k12}-m^{+}_{k12}) *supE44thi*-1 *rec*A1 gyrA96 *rel*A1 lac [F' *pro*$A^{+}B^{+}$ $lacI^{q}ZΔM15$: Tn10 (Tc^{R})])	Amersham, Paris, France
BNS3	*Bacillus thuringiensis kurstaki* serotype H3a, H3b and H3c harboring *cry1Aa, cry1Ac*, *cry2Aa* and *cry1Ia*-type genes	[27–31]
H14	*B. thuringiensis israelensis*	*Bacillus* Stock Centre (U.S.A.)
BUPM98	*B. thuringiensis israelensis*	[26]
BUPM97	*B. thuringiensis israelensis*	[25]
HD1CryB	plasmid-cured *B. thuringiensis kurstaki*	*Bacillus* Stock Centre (U.S.A.)
BNS3Cry^{-}	plasmid-cured BNS3 strain	[28]
BNS3pHT*Blue*	BNS3 containing pHT*Blue* plasmid	[30]
BNS3pHT*p20*	BNS3 containing pHT*p20* plasmid	This work
BNS3pHT*cyt1A98*	BNS3 containing pHT*cyt1A98* plasmid	This work
BNS3pHT*p20-cyt1A98*	BNS3 containing pHT*p20-cyt1A98* plasmid	This work
HD1CryBpHT*p20-cyt1A98*	HD1CryB containing pHT*p20-cyt1A98* plasmid	This work

In order to find a combination of various *B. thuringiensis* delta-endotoxins which interact synergistically and overcome or prevent insects from developing resistance, BNS3 strain, devoid of Cyt1A and P20 proteins, was engineered to co-express those proteins individually and in combination. The obtained strains were tested *in vivo* against *E. kuehniella* and *S. littoralis* larvae in order to determine the effect of P20 and Cyt1A98 proteins on larvicidal activity of *B. thuringiensis kurstaki* toxins against those lepidopteran larvae.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

BUPM97 and BUPM98 are *B. thuringiensis* subsp. *israelensis* strains, isolated from a Tunisian soil sample, in the laboratory [25,26], by the method of Jaoua et al. [27]. BNS3 strain is *B. thuringiensis kurstaki* serotype H3a, H3b and H3c and has an insecticidal activity against larvae of several Lepidoptera [28–31]. HD1CryB (an acrystalliferous strain of *B. thuringiensis kurstaki*) and H14 (a crystalliferous strain of *B. thuringiensis israelensis*) were obtained from the *Bacillus* Stock Centre at Ohio State University (Columbus, OH, U.S.A.) (Table 1). Plasmid pMOS*Blue* (Amersham, Biosciences, France) and pBleuscript II SK (+/−) (Stratagene, La Jolla, CA, U.S.A.) were used as cloning vectors. The following plasmids were hosted in *E. coli* strain named MOSBlue cells (Table 1). The *E. coli–B. thuringiensis* pHT*cry1Ia* shuttle vector [30], a derivative of pHT3101 (Table 1), was used for gene cloning and expression in *B. thuringiensis*.

Luria-Bertani medium [32] was used for growth of *E. coli* and *B. thuringiensis*. T3 medium was used for sporulation and delta-endotoxin production by *B. thuringiensis* strains [33]. In bioassays, *B. thuringiensis* strains were grown in a liquid medium as described by Zouari et al. [34]. Luria-Bertani and T3 media were supplemented with 60 μg ampicillin/ml and 50 μg erythromycin/ml for transformed *E. coli* and *B. thuringiensis* strains, respectively.

2.2. Construction of recombinant plasmids

The oligonucleotides used in the present study were synthesized by the "Centre de Génétique Moléculaire, CNRS, GENSET, Orsay, France". All PCR products were generated using a high-fidelity *pfu* DNA polymerase (Amersham) and sequenced (data not shown) using a taq Dye Deoxy terminator cycle Sequencing kit and a 3,700 ABI Prism DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer's instructions.

Primers D29 and D30 (Table 2) used for amplification of *p20* gene contained restriction sites of *Cla*I and *Hin*dIII, respectively. DNA extracted from BUPM97 strain has served as template. The blunt-end PCR product (0.6 kb) corresponding to *p20* open reading frame was purified from agarose gel with MiniElute Gel Extraction Kit (Qiagen S. A. France) and cloned in pMOS*Blue* vector generating pMOS*p20*. The pHT*cry1Ia* vector was digested by *Eco*RI restriction enzyme to obtain 2 fragments (1 kb and 9.6 kb). The 9.6 kb fragment

Table 2
List of primers designed and used in this study.

Primers	Description[a]
D29-*d*	5′AATTATA**ATCGAT**ATTTAG3′
D30-*r*	5′AAGGTTA**AAGCTT**CCGATTA3′
D33-*d*	5′ACTT**ATCGAT**AGGAGTTGTT3′
D24-*r*	5′TAAATAGAGCTCCTAAGATT3′
D31-*d*	5′TTCTAAT**AAGCTT**AAGGAGT3′

d, direct primer; *r* reverse primer.
[a] The enzyme restriction sites are bold-faced.