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Expression of the Insecticidal Protein Gene *cry*1C of *Bacillus thuringiensis* in Plant-Colonizing Nitrogen-Fixing Bacteria

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ABSTRACT

The NMO10 bacterial isolate was isolated from the rhizosphere of cotton plants. This isolate showed high potentiality to fix nitrogen and a high ability to colonize the phyllosphere of cotton plants. Based on this, plasmid pHTNC3 harboring the *cry*1C gene was used to transform NMO10. The transformed bacterial isolate (tNMO10) had a dual action both in the biocontrol of the Lepidopteron insect *Spodoptera littoralis* and as a bio-fertilizer. The presence of an expressed 135 kDa Cry1C protein in the protein pattern of tNMO10, microscopic examination of the bipyramidal crystals that characterize the Cry1C protein and immuonoblot analysis indicated efficient expression of the *cry*1C gene in the heterologous host NMO10. Bioassays against the neonate larvae of the cotton leaf worm *S. littoralis* revealed that the protein preparations from the tNMO10 were toxic.

Keywords: biocontrol, biofertilizer, cotton plants, phyllosphere, plasmid pHTNC3, *Spodoptera littoralis*

INTRODUCTION

In the present study, attempts were made to develop a new delivery system for the insecticidal protein of *Bacillus thuringiensis* (*Bt*) by transferring the *cry*1C gene into the nitrogen-fixing bacterial isolate NMO10 that was isolated from the rhizosphere of cotton (*Gossypium barbadense* var. 'Giza 75') (Feibo and Omar 1998). This soil bacterium, a Gram-positive, endospore-forming diazotrophic organism found in association with plants, is capable of atmospheric di-nitrogen reduction. The effect of this bacteria ranges from increased shoot/root ratio, increased seedling emergence to higher yield. NMO10 is a good colonizer of the cotton phylloplane. Thus, obtaining bacteria that are a strong and potent bioinsecticide as well as biofertilizer was the principal aim of this work.

The most abundant and successful microorganism used as a biopesticide is *Bt*, is characterized for containing a variety of plasmids encoding insecticidal proteins named delta (δ)-endotoxins (Hofte and Whiteley 1989). These proteins are assembled into parasporal crystalline inclusion bodies. Commercial preparations of *Bt* have been used for many years in insect control programmes and have shown no toxicity against non-targeted animals and plants. *Bt* was available to control Lepidoptera, using a highly potent strain (*Bt* var. 'Kurstaki') (Herms *et al*. 1997; Lozzia and Manachini 2003). It still constitutes the basis of many *Bt* formulations. The efficient use of these preparations is hindered by several field conditions (Cohen 1991), photo inactivation by UV-light, temperature, dew or rain are the major environmental factors affecting stability and efficacy of entomopathogenic toxins (McGuire and Shasha 1990). Reaching root- and stem-dwelling insects is another problem with conventional *Bt* biopesticide.

One approach to overcome these problems is to introduce the *cry* gene into the plant chromosome where the plant cell expresses the Cry proteins, although the success of this approach has certain restrictions, one of which is that target insects are perpetually exposed to toxins and this creates a strong selection pressure for the development of toxin resistance (Jenkins and McCarty 1997).

A second approach relies on the expression of Cry proteins in different genera of microorganisms which naturally colonize plants and express Cry proteins at sufficient levels to protect the plant. The introduction of *cry* genes into plant-associated microorganisms has been successfully developed, as this is much quicker and more cost-effective than producing transgenic plants (Bora *et al*. 1994). Moreover, the use of an endophytic bacterium was seen as possible solution to the problem of inaccessibility of conventional *Bt*-based products to the interior regions of plant.

MATERIALS AND METHODS

All reagents used in this work were from Fisher Biotech (Fisher Scientific, NJ, USA).

Bacterial strains, plasmids and growth conditions

An Egyptian isolate NMO10 was isolated from cotton rhizosphere (Fiebo and Omar 1998) and selected for a molecular study on the basis of its nitrogen-fixing activity. The bacterial strain *Bt* subsp. *entomocidus* was used as a positive control for Cry1C crystal protein, which it naturally produces (Kalman *et al*. 1995). The recombinant strain *Bt*NC3 (Ibrahim *et al*. 2008) was used as a source for the plasmid pHTNC3. All bacterial strains used were grown on T3 media (tryptone 3 g/L, tryptose 2 g/L, yeast extract 1.5 g/L, sodium phosphate buffer 50 mM, Mn Cl_2 0.005 g/L, in I L dH_2O) at 30°C for 72 h for spores; and they were grown on LB media (Trypton 10 g/L, yeast 5 g/L, sodium chloride 5 g/L) at 30°C overnight for vegetative cells. pHTNC3 was isolated from bacterial strain *Bt*NC3 by the alkaline lysis method (Sambrook *et al*. 1989).

Transformation

NMO10 bacterial cells were transformed by electroporation as described by Chang *et al*. (1992). 500 ng of pHTNC3 DNA was added to 0.8 ml of NMO10-competent cells and placed in sterile, pre-chilled electroporation cuvettes (0.4 cm interelectrode gap) and held on ice for 5 min. Electroporation was carried out with a

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Bio-Rad Gene pulser at field strength 2.5 KV, resistance 150 Ω and capacitance 25 µf. The electroporated cells were added to 1.5 ml of LB medium, incubated for 1 h at 37°C, plated on LB medium containing 100 µg/ml kanamycin and then incubated at 30°C for 24 to 48 h. One of the kanamycin-resistant (Kan^r) transformants was analyzed for the presence of pHTNC3.

Expression of *cry*1C toxin gene in transformed NMO10 cells

Total cellular proteins of sporulated bacterial cells from the transformed NMO10 (tNMO10) and the parental bacterial isolate were prepared. Those bacterial cells were grown on T3 medium for 72 h in an incubator shaker at 30°C. SDS-PAGE was carried out as described by Laemmli (1970). The transformed cells were collected by centrifugation and treated with sample buffer that was composed of 50 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.0025% (w/v) bromophenol blue) and boiled at 100°C for 5 min. Samples were applied to 10% polyacrylamide gels and run at 200 V for 45 min at room temperature in a mini protein Biorad cell. Protein bands on gels were visualized with Coomassie brilliant blue R-250.

Western blot

The presence of Cry1C δ-endotoxin was detected in crude extracts of transformed cells by western blot (immunoblot) analysis (Lampel *et al*. 1994). Total cellular proteins were prepared and solubilized by boiling in sample buffer and separated by electrophoresis on 10% polyacrylamide gels. The gels were electrophoretically blotted onto pre-wet PVDF membrane. The membranes were blocked in blocking buffer containing 1% bovine serum albumin (BSA), then membranes were incubated in blocking buffer contained the toxin (Cry1C) for 2 h. Anti-truncated 65 k Da from *Bt kur*-HD-1 serum (1-1000 dilution) was used as primary antibody and was incubated overnight with membranes in blocking buffer at 4°C. The membranes were incubated with alkaline phosphate conjugated secondary antibody (1-1000 dilution) (Phototope®-Star, Western Blot Detection Kit, for Western Blotting anti-Rabbit IGg, Biolabs, New England). CDP-chemiluminescent substrate was used and the emitted light was captured on X-ray film.

Microscopic examination

Microscopic examination of different transformed (tNMO10) bacterial cells compared to the parental isolate (NMO10) was examined for the presence of bipyramidal crystals that characterized the presence of Cry1C toxin protein. A smear of sporulated culture, that was grown in T3 media on a shaking incubator for 72 h at 30°C, was taken, spread on a clean slide and stained with spore-stain solutions. The slide was stained first with malachite green for 20 min over a boiling water bath, and then the slide was rinsed with water, dried and stained with safranine for 5 min.

Polymerase chain reaction and oligonucleotide primers

Two pairs of specific primers to *cry*1C gene were used to check for the transformation of *cry*1C into bacterial cells. The two primers were IAF and IAR (Ragev *et al*. 1996). IAF, 5'- ACG GAGGATCCATATGGAGGAAAATAATCAAAATC-3' and IAR, 5'-CTCTTGGATCCTAACGGGTATAAGCTTTTAATTTC-3', give a 2.2 kbp PCR product. The reaction conditions were performed according to Regev *et al*. (1996), where the PCR mixture was in a total volume of 25 µl containing 1 µg of total DNA, 50 pmol of each primer, 0.2 mM deoxy nucleoside triphosphates, 2.5 µl of *Taq* polymerase (RTS *Taq* DNA Polymerase, recombinant GIBCO BRL, USA), 2.5 µl of 10X enzyme buffer and 2.5 µl $MgCl_2$. The amplification reaction was carried out using 35 cycles of 94°C (45 sec), 48°C (45 sec) and 72°C (120 sec) and then a 7-min termination at 72°C. The same reaction conditions were used with a second pair of primers IAF and ICR, 5'-TTATTCCTCCATAAGGAG TAATTCC-3' (Ibrahim *et al*. 2008), that give a 3.7 kbp PCR product. The *gln*B-specific pair of primers that define the nitrogen regulatory gene were used to detect that gene in both the NMO10 isolate and the transformed tNMO10. *Gln*B up 5'-GCCATCATT AAGCCGTTCAA-3' and *gln*B do 5'-AAGATCTTGCCGTCG CCGAT-3'. The reaction conditions were as described by Potrich *et al*. (2001) and a 250-bp PCR product was amplified by this pair of primers.

Bioassay

Bacterial isolates were grown until sporulation in liquid T3 media for 72 h. Cultures were centrifuged and the pellets were washed once with Tris-HCl pH 8.00 containing 1 M NaCl, and lyophilized. The dried cells were used directly for the bioassay. A stock concentration of 1000 ppm was made by dissolving 1 g of lyophilized cells in 1000 ml H_2O (Dulmage 1971). 500, 400, 250, 100, 75, 50, 25 and 10 ppm were added to the surface of solidified artificial medium (dry powdered Lima beans 150 g, dry yeast 15 g, ascorbic acid 3 g, Nipagin 3 g, agar-agar 6 g and 600 ml dd H_2O) (Loutfy 1973) and kept for 2 h at room temperature. 10 neonate larvae of *S. littoralis* were added to each cup, the mortality was recorded every 24 h until 72 h.

RESULTS

The aim of this study was to introduce the *Bt* toxin gene (*cry*1C) into plant-colonizing bacteria to protect the toxin protein from environmental factors that affect its activities and also to prolong its efficiency.

Electroporation

The shuttle vector pHT7593 harboring a copy of the entire *cry*1C gene represented in a 4.1 kbp *Hind*III DNA fragment was cloned into the *Hind*III sites of pHT7593 (Ibrahim *et al*. 2008) and was used to transform the bacterial isolate NMO10. The transformed bacterial cells resulting from electroporation were selected primarily according to their growth on 100 µg/ml Kan plates.

Expression of the *cry*1C gene in NMO10 bacterial isolate

SDS-PAGE analysis of protein from sporulated cultures from transformed NMO10 (tNMO10), the parental isolate NMO10, and from recombinant bacterial cells containing Cry1C as a positive control revealed the presence of a 135 kDa protein in both tNMO10 and the positive control but it was absent in the parental isolate NMO10. The 135 kDa protein produced by tNMO10 and the positive control, recombinant bacteria, was confirmed as the Cry1C protein by immunoblot analysis as shown in **Fig. 1A, 1B**. An antibody-antigen immunoreaction was detected between anti-60 kDa toxin antiserum and antigen expressed by tNMO10. Reactions were also performed with the parental isolate. The recombinant proteins from tNMO10 and from the recombinant bacteria containing the Cry1C toxin protein gave sharp bands where the homologous antiserum reacted strongly to its respective homologous toxin protein.

PCR

The presence of the target gene (*cry*1C gene) in the transformed tNMO10 bacterial strain was confirmed by PCR. Two pair of primers, IAF + IAR and IAF + ICR, specific to the *cry*1C gene, were used to amplify 2.2 and 3.7 kbp fragments, respectively (**Fig. 2A**). On the other hand, a specific pair of primers for detection of the *gln*B gene (the nitrogen regulatory gene) which amplifies a 250 bp fragment from the *gln*B gene was used with the parental isolate NMO10. The expected PCR product that appeared in **Fig. 2B** indicates the presence of the *gln*B gene in that isolate. This test was used to show that the parental isolate is a nitrogen-fixing bacterium.

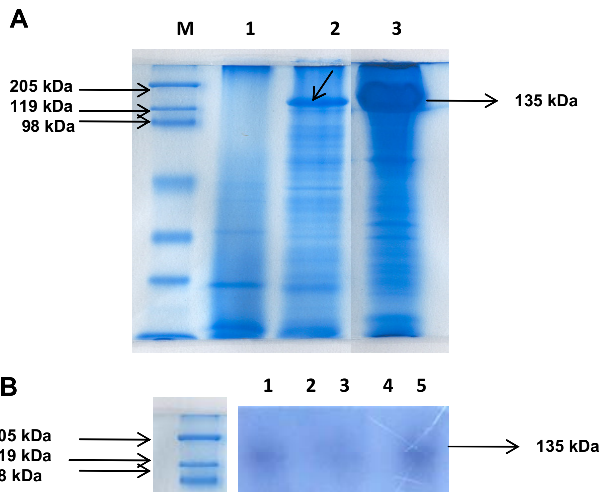

Fig. 1 SDS-PAGE and immunodetection of crystal proteins in transformant cells obtained from tNMO10 compared to the parental isolate NMO10 and the positive control Cry1C. (**A**) 25 µl samples of total cellular proteins were run on 10% polyacrylamide gels. The gels were stained with Coomassie blue. Lane M: Pre-stained broad range SDS-PAGE standards. Lane 1: the parental isolate NMO10; Lane 2: tNMO10 (arrow indicates the 135 kDa Cry1C protein expressed in tNMO10); Lane 3: positive control, recombinant bacteria containing Cry1C. (**B**) Western blot analysis of the proteins synthesized by the transformed bacteria tNMO10, a recombinant bacteria containing Cry1C as positive control and the parental isolate NMO10 with polyclonal antibodies for the *B. thuringiensis* crystal protein after SDS-PAGE. Lanes 1 and 3: tNMO10. Lanes 2 and 4: parental isolate NMO10 with no reaction. Lane 5: recombinant bacteria containing Cry1C as the positive control. The protein marker found on the left was cut from the gel before transfer onto the membrane and stained with Coomassie blue.

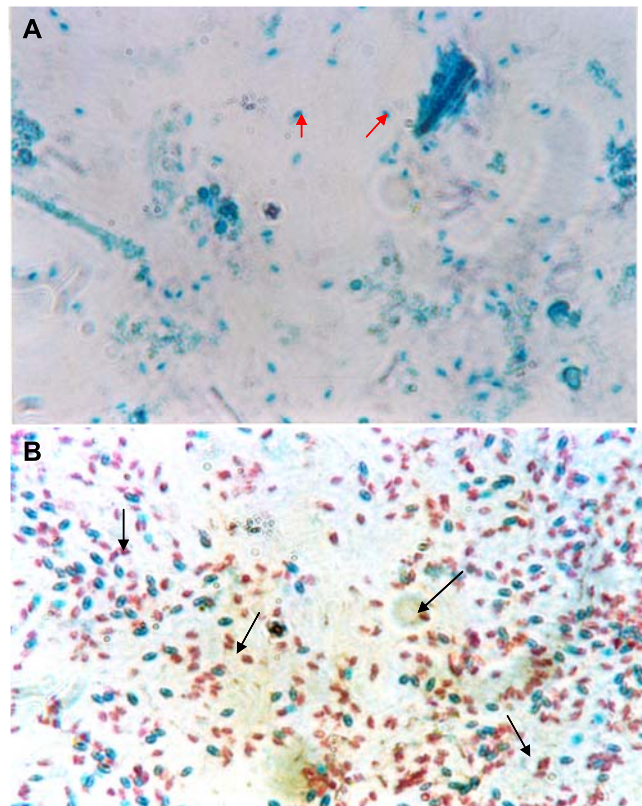

Fig. 3 Microscopic examination of sporulating cells from both tNMO10 and parental isolate NMO10. (**A**) Sporulating cells of parental isolate NMO10 which was grown on T_3 media at 30°C for 72 h (red arrows indicate spores). (**B**) Sporulating cells from tNMO10 grown on T_3 media at 30°C for 72 h. The photo shows the spores and bipyramidal crystals as a result of the expression of the *cry*1C gene (black arrows indicate crystals).

Light microscopy

Microscopic examination of spore-crystal stained smears from transformed bacteria tNMO10 and from the parent isolate NMO10 were illustrated. **Fig. 3A** shows the spores from sporulating cells of the isolate NMO10, and **Fig. 3B** shows the bipyramidal crystal in the transformed bacteria tNMO10. The data revealed and confirmed the expression of *cry*1C in the transformed bacterial isolate.

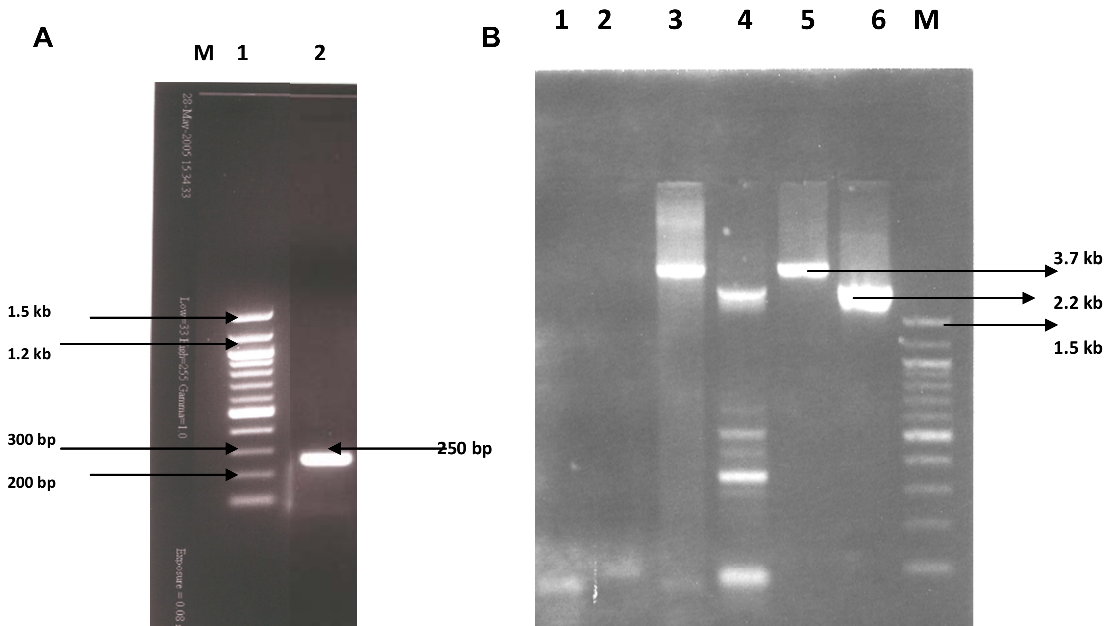

Fig. 2 Agarose gel electrophoresis for amplified PCR products from DNA of transformed tNMO10 and parental isolate NMO10. (**A**) The primer set IAF+IAR which gives a 2.2 kb product and IAF+ICR that gives a 3.7 kb PCR product for the *cry*1C gene were used. Lanes 1 and 2: parental isolate NMO10 with IAF+ICR and IAF+IAR, respectively (no PCR products). Lanes 3 and 4: tNMO10 with IAF+ICR and IAF+IAR, respectively where the 2.2 and 3.7 kb expected PCR products are found. Lanes 5 and 6: *cry*1C (positive control) with IAF+ICR and IAF+IAR, respectively. M: 100 bp DNA ladder marker. (**B**) The primer set *gln*B up and *gln*B do that gives a 250-bp PCR product with the *gln*B gene. Lane 1: *gln*B up and do specific primers with the parental isolate NMO10. M: 100 bp DNA ladder marker.

Table 1 The LC_{50} values in ppm of toxins used against the cotton leaf worm *S. littoralis*.

Strain / toxin	LC_{50} (ppm)	95% confidential limits	Slope/SE
tNMO10 Cry1C	103.0655	66.12-158.901	1.945 ± 0.498
Bt Entomocidus Cry1C	202.95	130.355-333.309	1.480 ± 0.515

[a] Bioassays were performed on spore-crystal preparations from T_3 liquid cultures.
[b] LC_{50} is a concentration of toxin required to kill 50% of 1st instar larvae
[c] LC_{50}s were calculated by probit analysis.
[d] Probit model is $Y= a + b * x$
where Y = probit value, a = intercept, probit value for x =0, b = slope, regression coefficient of y on x, x = log (dose)
[e]SE is the standard error

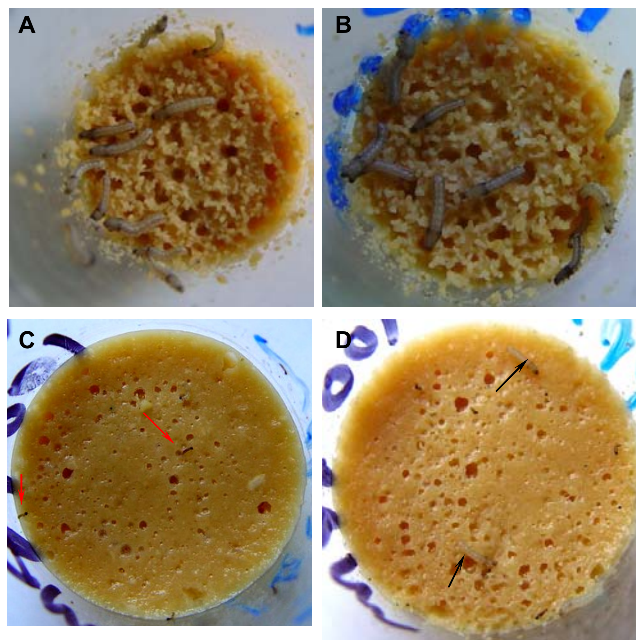

Fig. 4 The effect of tNMO10 toxicity compared with that of *Bt entomocidus*. (**A**) Negative control where no toxin was added. (**B**) Parental isolate NMO10 had no toxicity at 2000 ppm where it looked like the negative control. (**C**) Toxicity of tNMO10 with 100% mortality at 100 ppm (red arrows indicate dead larvae). (**D**) Toxicity of *Bt entomocidus* in which some larvae were still alive at 100 ppm (black arrows indicate living larvae).

Insecticidal activity

Bioassays against neonatal larvae were performed. In the toxicity assays, the insecticidal activity of Cry1C proteins synthesized by the transformed bacteria tNMO10 was compared with that of the crystal protein Cry1C isolated from the native *entomocidus* strain. **Table 1** show that the recombinant toxin, displayed a low $LC_{50,}$ it was nearly half of the LC_{50} of *Bt entomocidus*. The parental isolate NMO10 showed no mortality at all and it was like negative control, where no toxins were added. Using the propet analysis the data were analyzed in **Table 1**. **Fig. 4B** shows how the parental isolate had no effect on larvae while the transformed tNMO10 caused 100% mortality at 100 ppm (**Fig. 4C**).

DISCUSSION

In recent years, the need for environmentally safe pesticides has encouraged the replacement of these chemicals with biological approaches, which are friendlier to the environment. Strategies are being developed to control a variety of phytopathogenic agents including the development of transgenic plants expressing δ-endotoxin of *Bt*. This has led to the production of insect-resistant *Bt*-transformed lines of tobacco, cotton, corn, potatoes, maize, tomatoes and others (Frutos *et al.* 1999; Ferry *et al.* 2006; Gatehouse 2008; Anilkumar *et al.* 2008). *Cry* genes have been transferred into other plant-associated microorganisms to improve the stability and efficacy by obtaining maximum insect control on aerial and subterranean surfaces of plants (Downing *et al.* 2000). Thanabalu *et al.* (1992) tried *Caulobacter crescentus* as an ideal carrier for biological toxins instead of *Bt*. Graham *et al.* (1986) also tried to clone the *Bt* δ-endotoxin gene into *Pseudomonas fluoprescens*, which colonizes roots to protect toxin from environmental factors that affect its efficiency. Manasherob *et al.* (2002) tried to express the *Bt* toxin in nitrogen-fixing bacteria *Anabaena* to protect toxin from damage by UV-B, a sunlight component. Also, Bainton *et al.* (2004) modified a strain of *Pseudomonas* with dual biocontrol mechanisms. Theoduloz *et al.* (2003) expressed *Bt* toxin in *B. subtilis* and *B. licheniformis* that naturally colonize the phylloplane of tomato. All these attempts and progress would suggest that microorganisms that naturally colonize the plant phylloplane could be a source of new microbial expression systems of a δ-endotoxin gene of *Bt*. Furthermore; we showed that the delivery of *Bt* toxin genes to other microorganisms seems to be a practical and quicker alternative instead of the production of transgenic plants. In our study insertion of plasmid *Bt*NC3 that harbors the *cry*1C toxin gene into the plant-colonizing bacterium NMO10 was accomplished by electroporation. PCR was used to detect the presence of the *cry*1C gene in the transformed strain tNMO10 (**Fig. 2A**). PCR was also used to study the presence of the *gln*B gene, the nitrogen regulatory gene, in the parental isolate NMO10 and data in **Fig. 2B** shows that NMO10 contained the *gln*B gene. This was a confirmation, at the molecular level, that our isolate NMO10 is a nitrogen-fixing bacterium. The expression of the cloned *cry*1C gene was examined and analyzed on SDS-PAGE, and the results showed that the cloned *cry*1C gene was highly expressed in the isolate NMO10. Immunoblot analysis revealed the success of the reaction of antiserum with the 135 kDa protein from tNMO10, indicating clearly that Cry1C is present and well expressed in the transformed strain (**Fig. 2B**). The microscopic examination of sporulating cells of tNMO10 showed bipyramidal crystals (**Fig. 3B**), emphasizing the high expression of Cry1C toxin protein in the heterologous bacterial isolate tNMO10. Expression of the *cry*1C gene and its toxic effect were examined by determining the activity of the tNMO10 strain against the cotton leaf worm *S. littoralis* in an artificial diet bioassay. The activity was measured by mortality of the *S. littoralis* larvae compared with activity of the *Bt entomocidus* strain, which is a native donor of *cry*1C. Data in **Table 1** and **Fig. 4** show that the activity of tNMO10 against *S. littoralis* larvae was two times higher than that of the native strain and the LC_{50} of tNMO10 was half that of *entomocidus*. Moreover, the toxin proteins of tNMO10 showed quick mortality when readings were made every 24 h. On the other hand, the toxin proteins from *entomocidus* had a slow effect on the larvae; however, after three days (72 h), the real effect became apparent. This activity indicates that our strain tNMO10 is a promising strong bioinsecticidal agent. In conclusion, the cloned *cry*1C gene in NMO10 bacteria synthesized a polypeptide of 135 kDa that cross reacted with anti-truncated 65 kDa from *Bt* HD-1 serum. The transformed tNMO10 showed higher toxicity to *S. littoralis* larvae than that of the *Bt entomocidus* strain which contained native Cry1C. Thus our results revealed that we have a strong biopesticide as well as a biofertilizer strain.

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