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A Novel Negative Regulatory Factor for Nematicidal Cry Protein Gene Expression in *Bacillus thuringiensis*

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A 3-kb HindIII fragment bearing the *cry6Aa2* gene and the adjacent and intergenic regions was cloned from *Bacillus thuringiensis* strain YBT-1518. Two open reading frames (ORFs), namely, *orf1* (termed *cry6Aa2*) and *orf2* that were separated by an inverted-repeat sequence were identified. *orf1* encoded a 54-kDa protein that exhibited high toxicity to the plant-parasitic nematode *Meloidogyne hapla*. The *orf2* expression product was not detected by SDS-PAGE, but its mRNA was detected by RT-PCR. The *orf2* coexpressed with *orf1* at a high level in the absence of the inverted-repeat sequence, whereas, the expression level of *orf1* was decreased. When *orf2* was mutated, the level of *orf1* expression was enhanced obviously. In conclusion, the inverted-repeat sequence disturbs *orf2* expression, and the *orf2* downregulates *orf1* expression. This is an example of novel negative regulation in *B. thuringiensis* and a potential method for enhancing the expression level of *cry* genes.

Keywords: *Bacillus thuringiensis*, nematicidal *cry* gene, expression, negative regulation

Bacillus thuringiensis is a well-known Gram-positive, aerobic, and spore-forming soil bacterium that produces parasporal crystal inclusions during the sporulation phase of its growth cycle. The crystals comprise 1 or more protoxins (delta-endotoxins), which are specifically toxic to insects (*Lepidoptera*, *Diptera*, and *Coleoptera*), nematodes, mites, and protozoa [22]. Because of its insecticidal activity, *B. thuringiensis* is extensively used as an efficient and safe bacterial insecticide worldwide. The insecticidal Cry proteins are encoded by *cry* genes. Since the cloning of the first Cry protein gene by Schnepf and Whiteley [23], more than 300 *cry* genes encoding variable Cry proteins have been cloned and characterized from *B. thuringiensis* [8].

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The Cry toxin has been classified as belonging to the Cry1 to Cry51 families on the basis of its amino acid sequence homology [8]. Many of the published *cry* genes encode nematicidal activity protein toxins that can be classified into 2 groups based on their amino acid sequence homology. One group that includes Cry5, Cry12, Cry13, Cry14, and Cry21 is clearly related to the commercially important Cry1A, Cry3A, and Cry4A insecticidal toxins and includes proteins with 4 of the 5 protein motifs conserved in the main family of Cry proteins. The other group includes only Cry6A and Cry6B, and their amino acid sequences show no significant similarity to Cry5 or other members of the main family of *B. thuringiensis* Cry proteins. These protein toxins contain none of the 5 conserved sequence motifs and are likely to be structurally different from the other Cry toxins [16, 22, 25]. Both 2 group toxin groups have been described in patents regarding nematicidal *B. thuringiensis* Cry toxins [25]; however, only sparse data have been presented.

The Cry protein synthesized by *B. thuringiensis* during the stationary phase can account for 20–30% of the dry weight of the sporulated cells. The high-level expression of the *cry* gene in *B. thuringiensis* is due to the many events occurring at the transcriptional, posttranscriptional, and posttranslational levels, such as effective promoter function, stable mRNA, coexpression, and even assistance by accessory proteins [1, 2, 6]. For example, the 2 accessory proteins found in the *cry11A* operon in the *B. thuringiensis* subsp. *israelensis*, namely, helper proteins P20 (20 kDa) and P19 (19 kDa), were believed to have the effect of promoting Cry11A production [9, 26]. Recently, we reported that P20 has been proven to be effective in enhancing the expression of other *cry* genes in plasmid-cured acrystalliferous mutants [24]. However, no negative regulation was detected in Cry protein gene expression in *B. thuringiensis*.

The *B. thuringiensis* strain YBT-1518, which produces "rice-shaped" protein crystals with 2 components of molecular masses 54 kDa and 45 kDa, is toxic to the root-knot nematode, which is one of the most important nematodes worldwide

and causes notable agricultural yield reduction every year [21]. In this study, the operon responsible for the synthesis of the nematicidal Cry protein Cry6Aa2 was cloned and its expression regulation was characterized. This study provides new insight into the regulation of *cry* gene expression and is an effective method for enhancing the expression of *cry* genes in *B. thuringiensis* by eliminating disadvantageous factors.

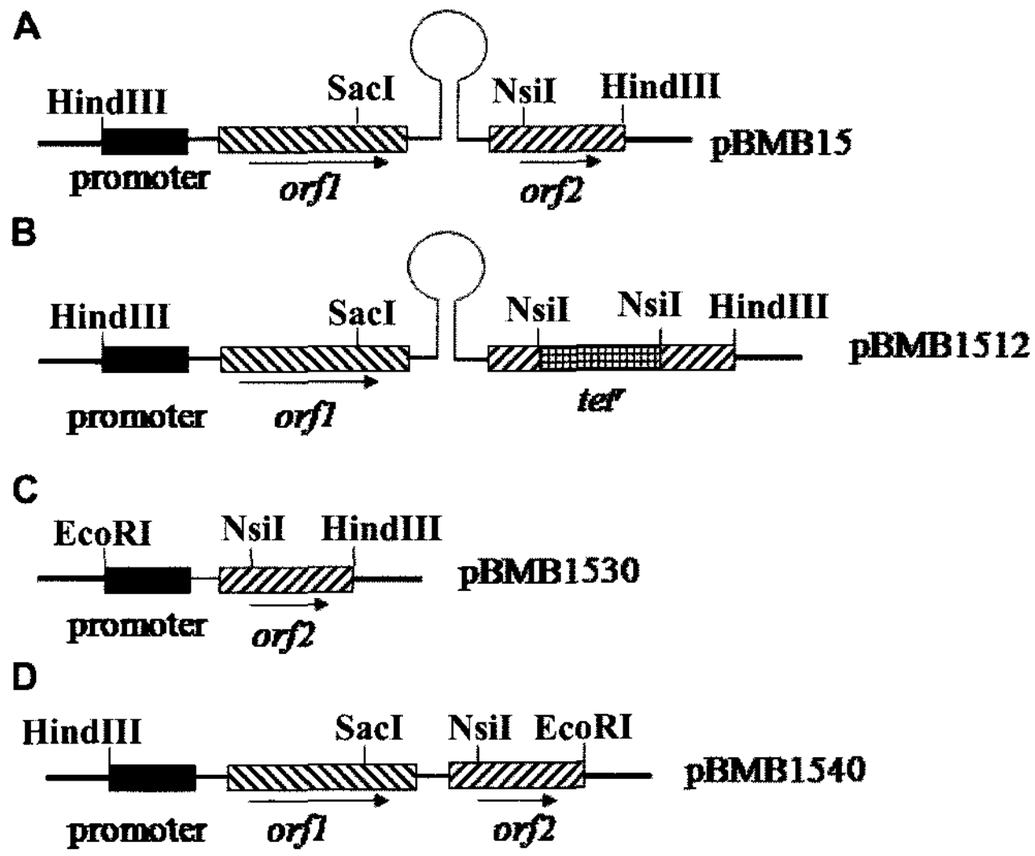

Fig. 1. Restriction maps of the cloned HindIII fragment on the recombinant plasmids.
Figures are not to scale. The position and direction of the transcription of the genes are indicated. Black bar, vector pHT304.

Materials and Methods

Strains and Plasmids

The *B. thuringiensis* strain YBT-1518 was isolated by our group in China. The acrystalliferous *B. thuringiensis* strain BMB171 (H_{3a3b}) was used as the recipient strain for electroporation. The *Escherichia coli* host strain TG1 (*supE*, *hsdΔ*5, *thi*, Δ (*lac-proAB*)/F [*traD*36, *proAB*$^+$, *lacI*^{q}, *lacD*ΔM15]) was used for plasmid amplification. pMD18-T Simple Vector (Takara Company, Amp^r) was used to clone the PCR product. The plasmid pHT304 (Amp^r, Erm^r) was used as the *E. coli-B. thuringiensis* shuttle vector [4]. pEMB0361 (Amp^r, Kan^r), which harbors a temperature-sensitive replicon, is an *E. coli-B. thuringiensis* shuttle vector and was used for double crossover.

DNA Manipulation

E. coli and *B. thuringiensis* plasmid DNAs were extracted by alkaline lysis [3, 26]. Total DNA from *B. thuringiensis* was extracted as described by Kalman *et al.* [12]. The preparation of *E. coli* competent cells and their transformation were carried out by the standard procedure [20]. Transformation of the Cry$^-$ strain BMB171 was performed by electroporation using the BIO-RAD MicroPulser Electroporation Apparatus, as previously described by Lenin *et al.* [14].

Construction of Recombinant Plasmid

PCR was performed with primer pairs derived from various *cry* genes. A 700-bp fragment was amplified from YBT-1518 with the primer pair derived from *cry6A* as follows: p1 (forward): 5'-CCGATGATGATGCAATAGCT-3'; p2 (reverse): 5'-CTAACCAAG-CATCAGAAGCG-3'. Using the 700-bp fragment as a probe, a 2,941-bp HindIII fragment was screened using the YBT-1518 total DNA library. Insertion of the 2,941-bp HindIII fragment into pHT304 yielded the recombinant plasmid pBMB15 (Fig. 1A). In order to assay the function of *orf2*, an *orf2* interruption mutant was constructed by inserting a tetracycline-resistant gene into pBMB15 at the NsiI site within *orf2*, resulting in pBMB1512 (Fig. 1B). To confirm *orf2* expression, the recombinant plasmid pBMB1530 was constructed by ligating the *orf1* promoter to *orf2*. The promoter upstream of *orf1* was amplified from pBMB15 with the primer pair of p3 (forward: 5'-AGCGAATTCATTTTTTTCACATATAAACTAT-3') and p4 (reverse: 5'-TATTGTTCATTTTCATACCACCTTTTTAAT-3'). *Orf2* was amplified from pBMB15 with the primer pair of p5 (forward: 5'-TGGTATGAAAATGAACAATAATTCTAAATT-3') and p6 (reverse: 5'-GTCAAGCTTTTAGCATCCTTGTTTTTTAAAG-3'). The 2 amplified fragments were ligated by SOE (splicing by overlap extension) PCR with the primer pair of p3 and p6 and cloned into pHT304 at the EcoRI and HindIII sites, yielding pBMB1530 (Fig. 1C). To confirm that *orf2* expression is associated with the stem-loop, the recombinant plasmid pBMB1540 was constructed by deleting the stem-loop from pBMB15. The 3'-end of *orf1* and intact *orf2* were amplified with the primer pairs of p7 (forward: 5'-TTGGAGCTCAAATAGAAAATC-3') and p8 (reverse: 5'-AGATTCCTCCAACAATTAATTATTATACCA-3'); p9 (forwared: 5'-TTGGAGGAATCTTAAATGAACAA-3') and p10 (reverse: 5'-CTGGAATTCTTAGCATCCTTGTTTTTTAAAG-3'), respectively. The 2 amplified fragments were ligated with the primer pair of p7 and p10 by SOE-PCR and then ligated with the 5'-end of the cloned 2.9-kb fragment (containing the region upstream of *orf1* and its promoter) at the SacI site. Therefore, when the stem-loop was deleted (from 1,675 bp to 1,740 bp), *orf1* and *orf2* were under the control of the *orf1* promoter. This fragment was cloned into the vector pHT304 at the HindIII and EcoRI sites, resulting in pBMB1540 (Fig. 1D).

Construction of the *orf2* Knockout Mutant of Strain YBT-1518

To obtain the *orf2* mutant of strain YBT-1518, the mutant plasmid pEMB0635 was constructed (Fig. 2A). A 2,425 bp fragment containing the downstream of *orf1* and intact *orf2* was amplified from plasmid pBMB15 by using the primer pair of p10 and p11 (forward): 5'-GTCTCTAGAATGAAAATGATTATTGATAG-3'. The fragment was cloned into pMD18-T Simple Vector to generate the plasmid pEMB0632. A 1,214-bp fragment harboring the erythromycin resistance gene was obtained from pDG646 by digestion with PstI and NsiI [11], and this fragment was cloned into pEMB0632 at the PstI site located within *orf2* to generate the plasmid pEMB0633. A 3,639-bp fragment containing the erythromycin resistance gene flanked with the regions upstream and downstream of *orf2* was obtained from pEMB0633 by digestion with EcoRI and XbaI and ligated into the plasmid pEMB0631 between the EcoRI and XbaI sites. The resulting plasmid pEMB0635 was transformed into strain YBT-1518 by electroporation. The transformants were cultivated in LB medium (containing 10 μg/ml erythromycin) for 8 h, and then cultivated at 42°C for 4 days. Erythromycin-resistant (25 μg/ml) but kanamycin-sensitive (50 μg/ml) colonies were harvested. The correct mutant strain was named BMB0658; its genotype was confirmed as double crossover by PCR amplification for upstream and downstream

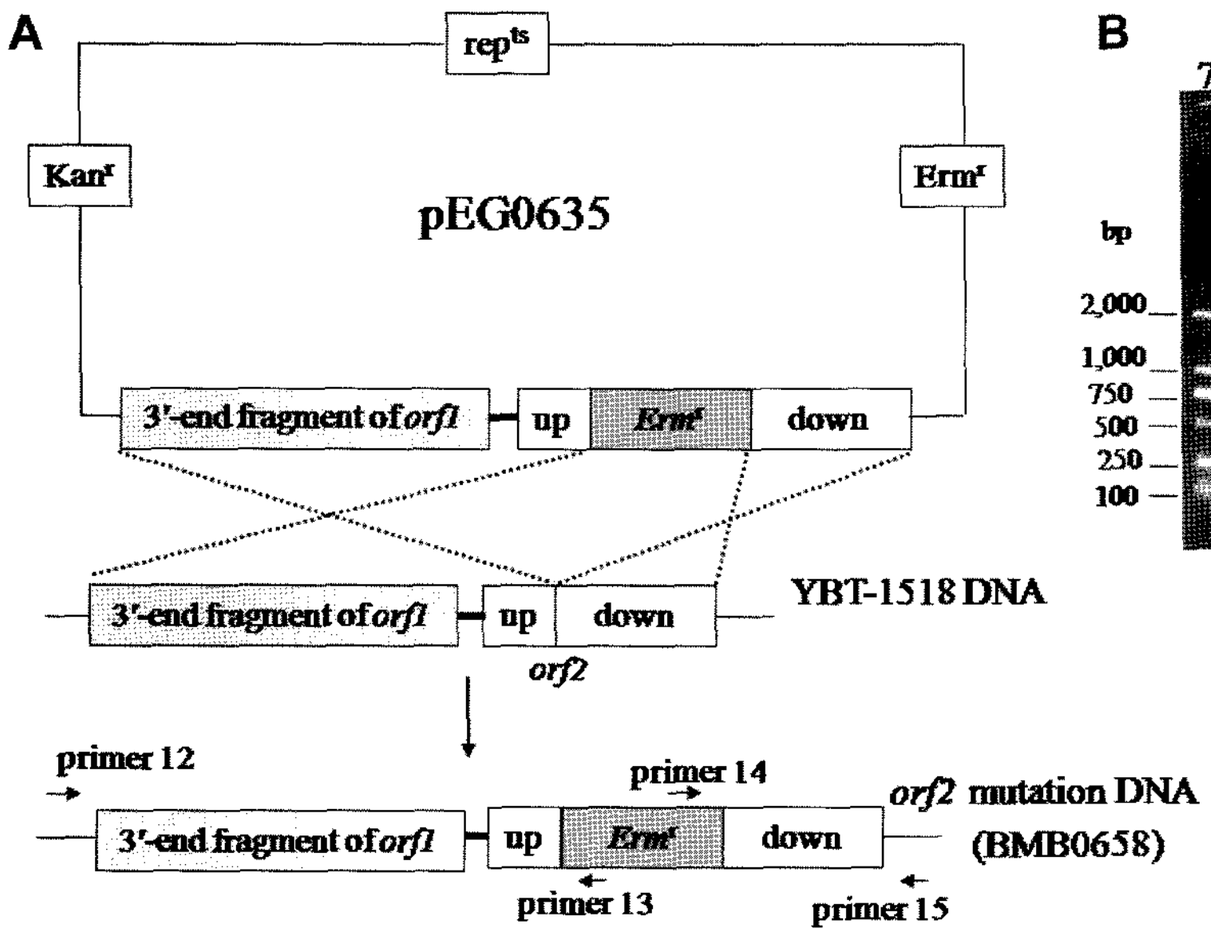

Fig. 2. The *orf2* gene mutation.

A. A sketch map of the *orf2* gene mutation obtained by double crossover. **B**. PCR confirmation of the *orf2* gene mutation. Lane 1, amplification of a 2.43-kb fragment upstream of the erythromycin-resistant gene; lane 2, 2.43-kb fragment digested with NsiI (1,857 bp+580 bp); lane 3, 2.43-kb fragment digested with AccI (1,349 bp+1,088 bp); lane 4, amplification of a 1.04-kb fragment downstream of the erythromycin-resistant gene; lane 5, 1.04-kb fragment digested with NdeI (674 bp+373 bp); lane 6, 1.04-kb fragment was digested with SalI (880 bp+167 bp); lane 7, DL (DNA Ladder) 2,000.

of the erythromycin resistance gene with the primer pair of p12 (forward: 5'-GTTTCATACAACCCTCCATT-3'), p13 (reverse: 5'-AACTGCCATTGAAATAGACC-3'); and p14 (forward: 5'-TAGTT-TTGGTCGTAGAGCACA-3'), p15 (reverse: 5'-ACAAATAGCACA-ATTCATCG-3') (Fig. 2A). Furthermore, the amplified upstream and downstream fragments were digested with AccI and NsiI, and SalI and NdeI, respectively, to confirm the presence of the *orf2* mutant in YBT-1518.

SDS-PAGE and Protein Quantification

All the *B. thuringiensis* strains were cultivated in PM medium [18] at 28°C with constant shaking until the cells were completely lysed (25 μg/ml erythromycin was added for the recombinant strains). To quantify the expression of the Cry6Aa2 protein, all SDS-PAGE samples were processed and loaded strictly parallel as described by Shao *et al.* [24]. The density of every strain culture was adjusted to OD_{600}=0.8, and 2 ml was harvested by centrifugation, and washed 3 times each with ice-cold 1 M NaCl and double-distilled water (ddH_2O). To every sample, 30 μl of ddH_2O and 30 μl of 2× sample buffer were added, and 5 μl of sample was loaded in each lane. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10%) was performed as described by Laemmli [13]. The relative amount of the 54-kDa Cry6Aa2 protein on the SDS-PAGE gel was assayed using the Dense-Scanning Quantification program of the Bioimage System according to the manufacturer's instructions and by using the Cry6Aa2 protein produced by YBT-1518 as the standard.

Preparation of the Crystal Protein Sample for Bioassay in the Nematode

Bt strains were cultivated in PM medium, and the mixture of spores and crystals was washed as mentioned above. The pellet was resuspended in 50 mM Na_2CO_3 (pH 9.5) containing 3% β-mercaptoethanol (v/v) and incubated at 37°C for 60 min. The supernatant was collected by centrifugation. We added 1/20 (v/v) 4 M NaAc-HAc (pH 4.0) to the supernatant, followed by incubation on ice for 1 h. The Cry protein pellet was collected by centrifugation and washed 3 times with ddH_2O. A concentrated Cry protein solution was obtained by dissolving the pellet in 50 mM Na_2CO_3 (pH 9.5), and served as the bioassay sample for *M. hapla*. Protein concentration was determined by the method described by Lowry *et al.* [15] using BSA (bovine serum albumin) as the standard.

Microscopic Observations

B. thuringiensis strains were grown in PM medium. The crystals and spores were observed under a transmission electron microscope (TEM) [17].

Nematode Rearing and Bioassay

The eggs of *M. hapla* were harvested from the root-knot of infected tomato, disinfected by treating with 1% NaClO for 5–10 min, and subsequently washed with sterile water. The 2nd stage juveniles were hatched from the eggs that were on a sheet of water-saturated filter paper at 18–25°C and used to test the toxicity of the Cry toxin.

Forty 2nd stage *M. hapla* larvae were individually placed into each well of 96-well microtiter plates. The wells contained a known concentration of the Cry6Aa2 toxin, and tetracycline and chloramphenicol (30 μg/ml each) to prevent microorganism growth [7]. The final volume in each well was 120 μl. The Cry6Aa2 toxin was diluted to at least 5 different concentrations by 2-fold dilutions with at least 5 replications. BSA (20 μg/ml) was used as the control. The nematodes were incubated in a humid chamber at 25°C for 5 days and scored under a dissecting microscope. The dead nematodes were counted. Death was assayed by examining the larvae for movement when the well was disturbed with a pipette [16]. Then, the toxicity (LC_{50}) of the Cry6Aa2 to the 2nd stage juveniles of *M. hapla* was evaluated.

Preparation of the RNA of the *B. thuringiensis* Strains and RT-PCR
The *B. thuringiensis* strains were cultivated in G-Tris medium [5] at 28°C, and the cells were harvested by centrifugation at 4°C at the sporulation stage (approximately 13 h). The total RNA was extracted as previously described by Naresh *et al.* [19] and visualized by formaldehyde agarose gel electrophoresis. RT-PCR was performed with the primer pair of 5 and 6 according to protocols of the reverse transcriptase manufacturer (Takara Company).

Southern Hybridization
The total DNA of strain YBT-1518 was digested with different restriction enzymes, separated by electrophoresis in a 0.8% agar gel, and transferred to a nylon membrane (Hybond N^+; Amersham). The 700-bp fragment amplified with the primer pair of p1 and p2 was labeled with DIG according to the kit manufacturer's protocol (Roche Company), and used as probe. Prehybridization and hybridization were carried out as described by Sambrook *et al.* [20].

Nucleotide Sequence Accession Number
The nucleotide sequence obtained in this study has been deposited in the GenBank database under the accession number AF499736.

RESULTS

Cloning the Nematicidal Crystal Protein Gene *cry6Aa2* from the *B. thuringiensis* Strain YBT-1518

The *B. thuringiensis* strain YBT-1518 produces "rice-shaped" parasporal protein crystals (Fig. 3A) that comprise 2 proteins of molecular masses 54 kDa and 45 kDa (Fig. 4, A, lane 1). A bioassay showed that the purified 54-kDa Cry protein exhibits nematicidal activity against the plant-parasitic nematode *M. hapla* with an LC_{50} value of 7.43 μg/ml (Table 1).

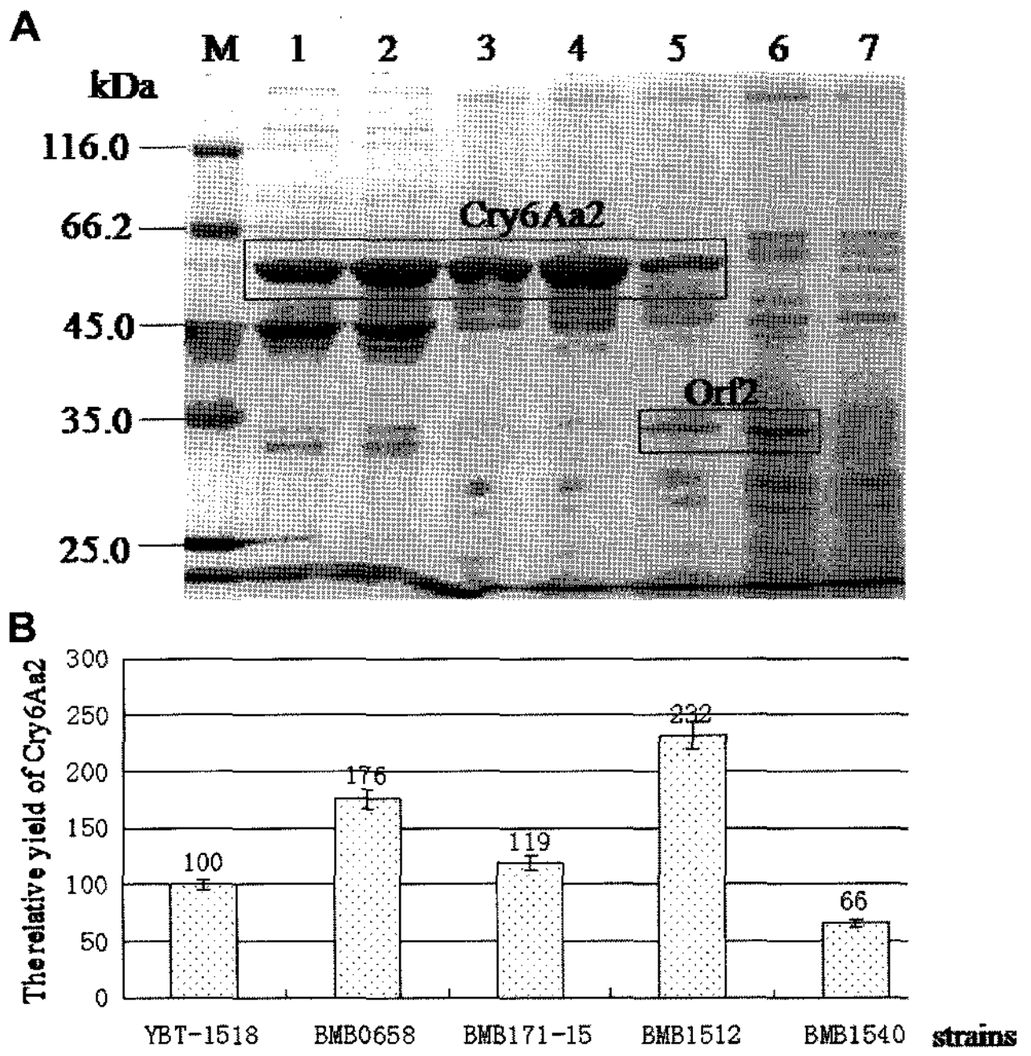

Fig. 4. Analysis of Cry6Aa2 production in the wild-type, mutant, and engineered strains of *B. thuringiensis* by SDS-PAGE.
A. SDS-PAGE results. M, standard protein marker; lane 1, YBT-1518; lane 2, BMB0658; lane 3, BMB171-15; lane 4, BMB1512; lane 5, BMB1540; lane 6, BMB1530; lane 7, vector pHT304 in the acrystalliferous strain BMB171. **B**. Quantification of the 54-kDa Cry6Aa2 with the Dense-Scanning Program. Error bars denote standard deviation from means.

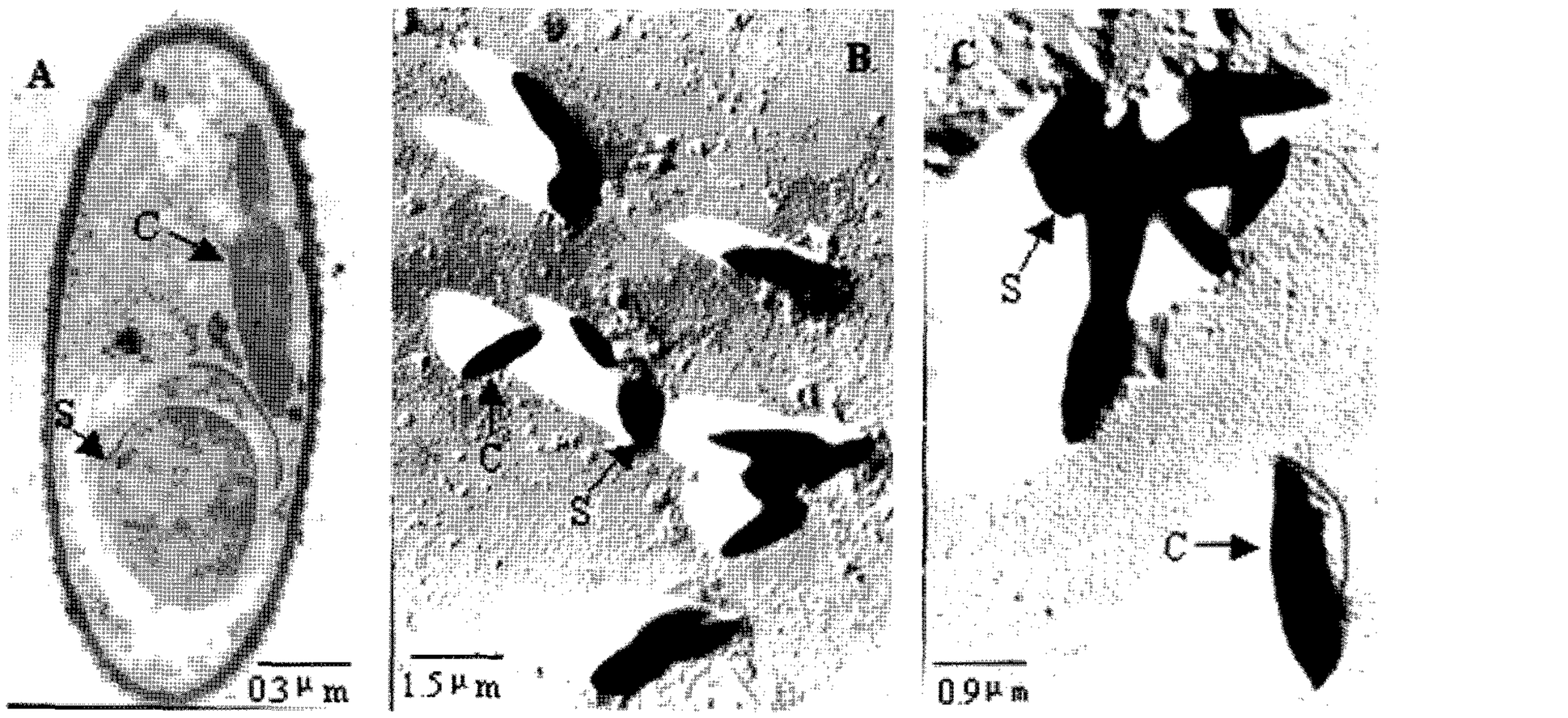

Fig. 3. Microscopic observation of the formation of crystal inclusions.
A. Transmission electron micrograph (TEM) of the crystal inclusions in wild strain YBT-1518 observed using ultrathin sections; **B**. TEM of the parasporal crystals and spores in the recombinant strain BMB171-15; **C**. TEM of the parasporal crystals and spores in the recombinant strain BMB1512. C, crystal; S, spore. *cry6Aa2* was expressed in the wild strain YBT-1518 and the recombinant strains BMB171-15 and BMB1512; it encoded for "rice-shaped" protein crystals.

Table 1. Toxicity of the Cry protein encoded by *cry6Aa2* toward *Meloidogyne hapla*.

Strain	LC_{50} (μg/ml)	Regression equation	R^2
YBT-1518	7.55	y=1.0297x+4.0961	0.9896
BMB171-15	7.43	y=1.1067x+4.0359	0.9658

PCR detection with the primer pair of p1 and p2 showed that a *cry6A*-type gene is present in YBT-1518. Southern blotting indicated that the gene is located within the 3-kb HindIII fragment. The positive transformant (containing recombinant plasmid pBMB15) was screened from the YBT-1518 total DNA HindIII-fragment library by PCR and dot blotting. Sequence analysis showed that the cloned HindIII fragment in pBMB15 is 2,941 bp in size and contains 2 ORFs - *orf1* (from 243 bp to 1,670 bp) and *orf2* (from 1,756 bp to 2,661 bp) - and a putative promoter in the region upstream of *orf1* (Fig. 1A). Between the 2 ORFs, an SD (Shine-Dalgarno) sequence (GGAGG, from 1,743 bp to 1,747 bp) and an inverted-repeat sequence (from 1,674 bp to 1,713 bp) were observed (Fig. 5A). Sequence analysis showed that the inverted-repeat sequence in this stem-loop is AT rich (A+T%=77.8), and the calculated free energy ($\Delta G=25^{\circ}C$) is comparatively low (–27.5 kcal/mol) (Fig. 5B). This implies that the stem-loop is a regulator of the expression, not a terminator of the *cry* gene. We observed 3 rich "AT" direct-repeat sequences (from 1,618 bp to 1,668 bp) located at the 3'-end of *orf1*. The direct-repeat sequences contained a TATAAT box and were separated by CGG (Fig. 5A). This indicated that the region from 1,618 bp to 1,668 bp may be the *orf2* promoter and involved in *orf2* expression.

BLAST analysis indicated that the *orf1* shares 98% base sequence identity with the republished *cry6Aa1*, and ORF1 protein shares 100% amino acid sequence identity with Cry6Aa1, and hence, it was named Cry6Aa2. No protein in GenBank with obvious similarity to ORF2 was found.

Cry6Aa2 Expression and Nematicidal Activity

The plasmid pBMB15 was introduced into BMB171 by electroporation, yielding recombinant strain BMB171-15. "Rice-shaped" parasporal crystals with a morphology similar to that of the crystals of strain YBT-1518 were observed by electron microscopy (Fig. 3B). SDS-PAGE revealed the presence of a protein band with molecular mass consistent with that of the band observed in strain YBT-1518 (Fig. 4A, lane 3). This implies that *orf1* is responsible for the synthesis of the 54-kDa protein.

The toxicity of the Cry proteins from YBT-1518 (54-kDa and 45-kDa) and BMB171-15 (54-kDa) against the 2nd stage juvenile of *M. hapla* was assayed. A bioassay showed that the Cry proteins from strains YBT-1518 and BMB171-15 were toxic to the *M. hapla* juveniles with LC_{50} values of 7.43 μg/ml and 7.55 μg/ml, respectively (Table 1).

The Stem-Loop Downregulates *orf2* Expression

The product of *orf2* could not be detected in strains YBT-1518 and BMB171-15 (Fig. 4A, lanes 1 and 3). Is there any possibility that the stem-loop between *orf1* and *orf2* depresses *orf2* expression? To confirm our hypothesis, *orf2* was subcloned into vector pHT304 at the HindIII site under the control of the *orf1* promoter, and it generated the recombinant plasmid pBMB1530 (Fig. 1C). The plasmid

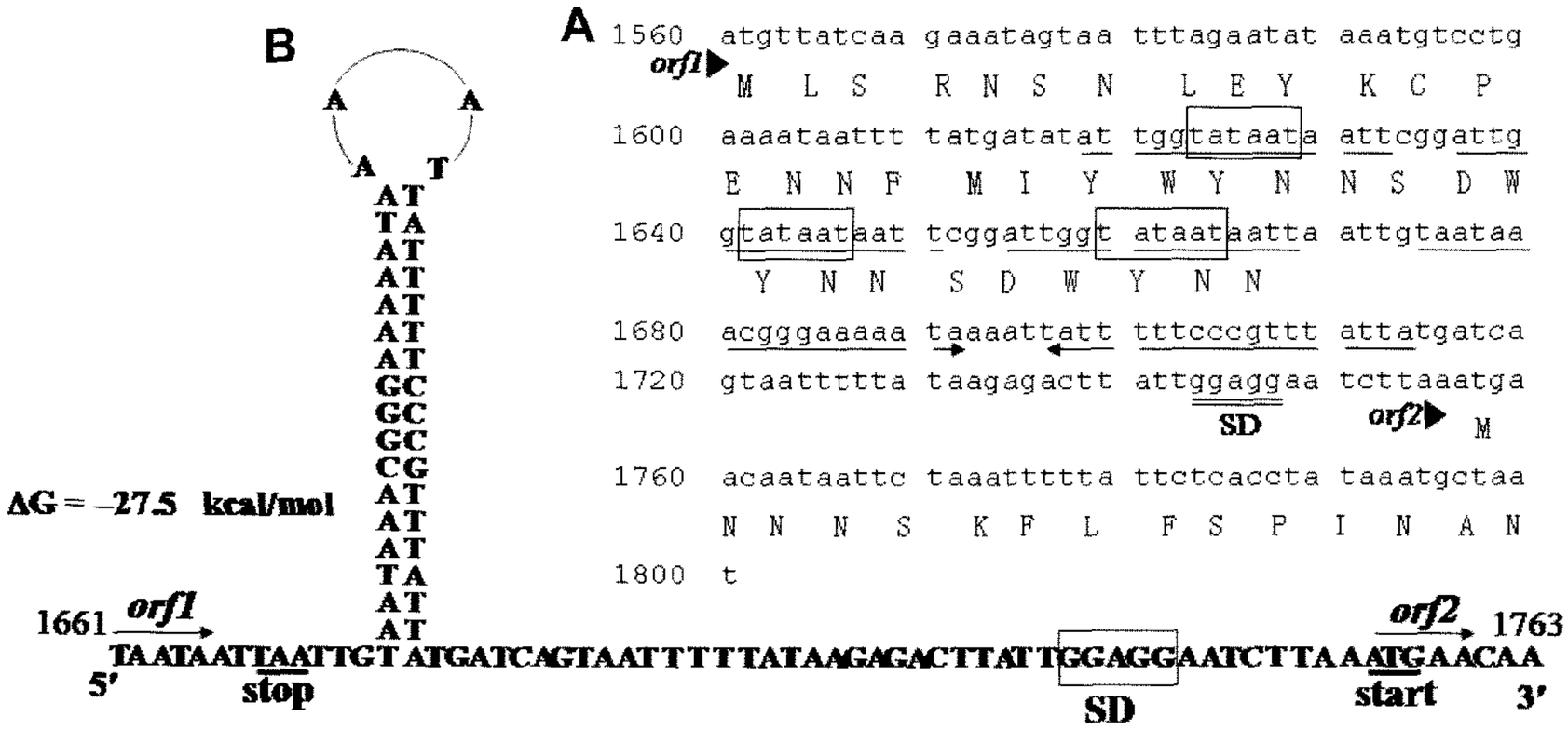

Fig. 5. Structure of the stem-loop and flanking fragment.

A. Nucleotide and amino acid sequence of the stem-loop and flanked fragment. Three direct-repeat sequences are underlined, and 3 TATAAT sequences are boxed. The inverted-repeat sequences are underlined by arrows. The SD (Shine-Dalgarno) sequence is indicated with a double underline. **B**. Structure of the stem-loop. The SD sequence is boxed. The stop codon of *orf1* and the start codon of *orf2* are underlined. The calculated free energy ($\Delta G=25^{\circ}C$) is –27.5 kcal/mol. The inverted-repeat sequence was rich in AT (A+T%=77.8); this indicated that the stem-loop was a regulator of expression and not a terminator of the *cry* gene.

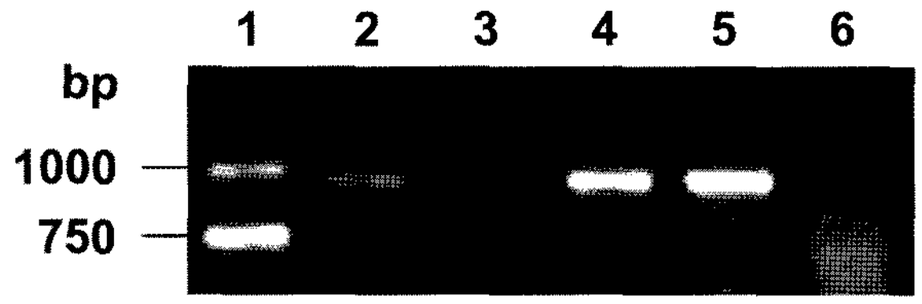

Fig. 6. Agarose gel electrophoresis analysis of *orf2* mRNA obtained by RT-PCR from *B. thuringiensis* strains in the sporulation stage.
Lane 1, DNA ladder; lanes 2, 3, 4, 5, and 6, total RNA amplified by RT-PCR from the strains YBT-1518, BMB0658, BMB171-15, BMB1530, and BMB1512 in the sporulation stage, respectively. The amplified *orf2* cDNA indicated that *orf2* was expressed in the operon.

pBMB1530 was introduced into BMB171 to yield the recombinant strain BMB1530. A protein band of molecular mass approximately consistent with that of the *orf2* encoding protein was observed by SDS-PAGE (Fig. 4A, lane 6). The stem-loop was deleted from pBMB15, and subsequently, the obtained recombinant plasmid pBMB1540 was introduced into BMB171. SDS-PAGE showed that *orf1* and *orf2* were coexpressed (Fig. 4A, lane 5). The "rice-shaped" crystal with similar morphology to BMB171-15 was observed by optical microscopey (data not shown).

The above data suggested that *orf2* could be expressed alone or coexpressed with *orf1* without the stem-loop. Therefore, it could be concluded that the lack of a detectable *orf2*-encoded protein in strains YBT-1518 and BMB171-15 was associated with the inverted-repeat sequence.

RT-PCR was performed to reveal whether *orf2* could be expressed or not at the transcriptional level. As shown in Fig. 6, a DNA fragment of the expected size of approximately 900 bp was amplified from YBT-1518 and BMB171-15, which are similar to the positive control BMB1530, but not amplified from the 2 *orf2* mutated strains BMB0658 and BMB1512. This confirmed that under the repression of the stem-loop, *orf2* continued to be expressed in the operon at the transcriptional level, although its expression product yield was too low to be detected by SDS-PAGE.

Orf2 Expression Downregulates *orf1* Expression

An *orf2* interruption mutant plasmid pBMB1512 was constructed to detect the expression relationship between *orf1* and *orf2*, and was subsequently introduced into strain BMB171. SDS-PAGE revealed that the resulting strain BMB1512 did produce "rice-shaped" protein crystals (Fig. 3C) with a molecular mass of 54 kDa (Fig. 4A, lane 4). The expression yield was clearly enhanced when compared with that of strain BMB171-15 (Fig. 4A). Quantification analysis showed that the protein yield of Cry6Aa2 in BMB1512 was 1.95-fold of that in BMB171-15 (Fig. 4B). This suggested that *orf2* negatively regulates *orf1* expression. In strain BMB1540, which has improved *orf2* expression, the Cry6Aa2 protein yield decreased to approximately 55% of that in BMB171-15 (Fig. 4B).

To further confirm *orf2* negative regulation to *orf1*, *orf2* was knocked out from YBT-1518 by double crossover and generated the *orf2* mutant strain BMB0658 (Fig. 2A). The mutant was confirmed by PCR and restriction enzyme digestion (Fig. 2B). Quantification analysis of the Cry6Aa2 protein showed that the yield of the Cry6Aa2 protein produced by BMB0658 was 1.76-fold of that of the protein produced by YBT-1518 (Fig. 4B).

Discussion

An operon containing a structural gene (*orf1*), a regulatory sequence (stem-loop), and a regulatory gene (*orf2*) was cloned and characterized. *orf1* is responsible for synthesis of the nematicidal activity Cry protein. The regulatory sequence acts as a *cis*-factor and results in low level *orf2* expression. *orf2*, a trans-factor, negatively regulates *orf1* expression and shares no homology with any sequence. This study provided new insight into the regulation of *cry* gene expression. It is presumed that high-level *cry6Aa2* expression is due to the presence of 2 negative regulatory factors - *orf2* and stem-loop.

The *orf2* encodes a soluble cytoplasmic protein to regulate the *orf1* expression, but it was at so low level that its expression product could not be detected in the cultures of YBT-1518 and BMB171-15. When the *orf2* expressed at high levels in BMB1530 and BMB1540, the ORF2 accumulated in the host cell and formed inclusions, and was detected from the mixture of spores and crystal. How the *orf2* downregulates the expression of *orf1* is not clear. We hypothesize that the ORF2 may bind the inverted-repeat sequence and affect the stability or *orf1* mRNA, and then downregulate the expression of *orf1*. The regulation mode of *orf2* needs further study.

The bioassay indicated that the toxicity of strains YBT-1518 and BMB171-15 was almost the same, which indicated that the 45-kDa protein produced by strain YBT-1518 might be toxic to the nematode. The N-terminal sequence of the 45-kDa protein indicated that it is a novel protein (data not shown).

The expression yield of pesticidal proteins is directly related to the application of *B. thuringiensis*. Thus, achieving a high yield of *B. thuringiensis* toxin can promote the application of *B. thuringiensis* in insect biocontrol. Eliminating disadvantageous factors is an effective method of enhancing the Cry protein yield in *B. thuringiensis*. For instance, the *nprA* gene that encodes for neutral protease A has been deleted from the *B. thuringiensis* chromosome, and this led to increased production of the Cry1Bb and Cry3Bb full-length proteins [10]. In this study, the Cry6Aa2 toxin yield was enhanced by deleting the negative regulatory

factor *orf2*. This could provide an effective method of modifying the strain YBT-1518 to enhance Cry6Aa2 yield and increase its application potential in plant-parasitic nematode control.

Acknowledgments

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