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Screening of *cry*-type promoters with strong activity and application in Cry protein encapsulation in a *sigK* mutant

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Abstract To optimize the expression of *cry* genes in a *Bacillus thuringiensis sigK* mutant failing in crystal releasing, the transcriptional activity of the *cry* promoters *cry1A*, *cry3A*, *cry4A*, and *cry8E* was compared using *lacZ* gene fusions. A beta-galactosidase assay indicated that the *cry8E* promoter showed the highest transcriptional activity. A novel *Escherichia coli-B. thuringiensis* shuttle vector pHT315-8E21b was constructed for *cry* gene expression using the *cry8E* promoter and the multiple cloning sites from vector pET21b, based on vector pHT315. SDS-PAGE analysis showed that the expression of the *cry1Ac* gene directed by the *cry8E* promoter was increased by approximately 2.4-fold over the expression directed by the *cry3A* promoter. The *cry1Ba* gene was expressed in the *sigK* mutant with the constructed vector pHT315-8E21b. Normal bipyramidal crystals encapsulated in mother cell were observed by transmission electron microscopy (TEM). The encapsulated Cry1Ba protein expressed in the *sigK* mutant showed activity against *Ostrinia furnacalis* and *Plutella xylostella* similar to that of the released Cry1Ba protein expressed in the acrystalliferous strain HD73 and can be protected from inactivation by UV light. All these results suggest that the *cry8E* promoter can be an efficient transcriptional element for *cry* gene expression in *sigK* mutants and can be utilized for the construction of a genetically engineered strain.

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Introduction

Bacillus thuringiensis (Bt) is a Gram-positive bacterium that produces highly specific insecticidal crystal proteins encoded by the *cry* or *cyt* genes, also called δ-endotoxin, during sporulation. These insecticidal proteins are specifically toxic to the larvae of *Lepidoptera*, *Coleoptera*, *Diptera*, and nematodes and are more environmentally friendly than conventional pesticides. Thus, Bt-based biopesticides have become the most used microbial insecticides worldwide. Nonetheless, to date, Bt-based biopesticides have captured only ~2 % of the pesticide market (Bravo et al. 2011; Huang et al. 2007; Bradley et al. 1995). The low persistence of Bt agents after application due to environmental stresses, such as UV radiation, temperature, and rain, particularly the sunlight-mediated inactivation of the toxins, has become a conspicuous influencing factor for the further development of Bt-based biopesticides (Leong et al. 1980; Marianne et al. 1991). Some methods have been developed to increase the stability of toxins in the environment, such as microencapsulation (Yang et al. 2012b), melanin (Ruan et al. 2004), and genetic modification (Sanchis et al. 1999; Bravo et al. 1996). Of these, the genetic modification of the *sigK* mutant has proved to be an efficient way to prevent the inactivation of the crystal by sunlight due to the failure to release the crystal in the mother cell (Sanchis et al. 1999; Yang et al. 2013). However, the expression of some *cry* genes and the comparison of transcriptional activity among *cry* genes have not been clarified for the *sigK* mutant.

The products of *B. thuringiensis cry* genes are largely synthesized and accumulate as crystalline inclusions in the mother cell, processes that are dependent on highly ordered

programs for the expression of the corresponding genes, which are regulated by a series of sigma factors in a signaling cascade (Kroos et al. 1999; Agaisse and Lereclus 1995). Usually, these *cry* genes are classified as sporulation dependent or sporulation independent based on their transcriptional mechanisms (Kroos et al. 1999; Schnepf et al. 1998). The *cry3A* gene, a typical example of a sporulation-independent *cry* gene, is controlled by σ^A and is expressed during vegetative growth (Agaisse and Lereclus 1994a). In contrast, most *cry* genes (e.g., *cry1A*, *cry1B*, *cry1C*, *cry2A*, *cry4A*, *cry4B*, *cry11A*, *cry18Aa*, *cry34*, and *cry40*) are sporulation-dependent genes controlled by σ^E, σ^K, or both (Bravo et al. 1996; Kroos et al. 1999; Brown 1993; Brizzard et al. 1991; Yoshisue et al. 1993; Zhang et al. 1998; Dervyn et al. 1995). Although the *cry8Ea1* gene also belongs to the sporulation-dependent type, its transcription is controlled by two promoters, P_{orf1} and P_{cry8E}, which are located upstream of the *orf1* gene in the intergenic region mapping between *orf1* and *cry8Ea1* and are controlled by σ^E and σ^H, respectively (Du et al. 2012).

In this paper, we analyzed the influence of the *sigK* gene deletion on the activity of the *cry1Ac*, *cry3A*, *cry4A*, and *cry8E* gene promoters in *B. thuringiensis* and compared the transcriptional activity among these promoters in the *sigK* mutant (HDΔsigK). A high-level expression vector, pHT315-8E21b, and a *sigK* mutant engineered strain of *B. thuringiensis*, ΔsigK$^-$-8E1Ba, were constructed using the *cry8E* promoter. We demonstrated that the *cry8E* promoter is a high-efficiency transcriptional element for *cry* gene expression in the *sigK* mutant and can be utilized for the construction of genetically engineered strains.

Material and methods

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* JM109 was used for the cloning experiments, and SCS110 was used to produce nonmethylated plasmid DNA for *B. thuringiensis* transformations (Macaluso and Mettus 1991). *B. thuringiensis* subsp. *kurstaki* HD73 and the acrystalliferous *sigK* mutant strain HDΔsigK$^-$ were used as the recipient strains to measure promoter activities (Du and Nickerson 1996). All the *B. thuringiensis* strains were grown with 220-rpm rotary agitation at 30 °C in Schaeffer's sporulation medium (SSM); *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) medium (1 % NaCl, 1 % tryptone, and 0.5 % yeast extract) (Schaeffer et al. 1965). The antibiotic concentrations used for bacterial selection were the following: 100 μg/ml ampicillin (for *E. coli*), 5 μg/ml erythromycin (for *B. thuringiensis*), and 200 μg/ml kanamycin (for *B. thuringiensis*).

DNA manipulation and transformation. Plasmid DNA was extracted from *E. coli* by the standard alkaline lysis procedure with a Plasmid Miniprep Kit (Axygen, Hangzhou, China). Restriction enzymes and T4 DNA ligase were used according to the manufacturer's instructions (Takara Biotechnology Corporation, Dalian, China). PCR was performed with high-fidelity DNA polymerase (TOYOBO). The DNA fragments were separated on 0.7 % agarose gels after digestion and extracted from the gels using a DNA gel extraction kit (Axygen, Hangzhou, China). Standard procedures were used for *E. coli* transformation (Sambrook and Russell 2001), and Bt cells were transformed by electroporation as previously described (Lereclus et al. 1989).

Screening of acrystalliferous mutant strain HDΔsigK$^-$. To obtain a acrystalliferous *sigK* mutant HDΔsigK$^-$ as receipt strain, we cure the plasmid pHT73 harboring *cry1Ac* gene from a *sigK* mutant strain HDΔsigK, which was obtained from strain HD73 with *sigK* gene deletion (Du et al. 2011). The *sigK* mutant strain HDΔsigK was grown in BP medium (1 % tryptone, 0.5 % NaCl, 0.36 % Na_2HPO_4, and 0.15 % KH_2PO_4) with 200 μg/ml kanamycin at 42 °C for 48 h with 220 rpm. The 2-ml culture was inoculated into 50-ml fresh BP medium with kanamycin at 45 °C for 12 h with 220-rpm rotate agitation. Then, the cells were plated on LB agar medium without antibiotics and grown at 30 °C for 48 h. The resulting single colonies were screened and identified by PCR amplification with primers *cry1Ac*-5/*cry1Ac*-3 and pHT73-5/pHT73-3 (Du and Nickerson 1996). Further identification of the acrystalliferous mutant was performed by SDS-PAGE and optical microscopy.

Construction of *cry* gene promoters with *lacZ* fusions. For determining the transcription activity of *cry* gene promoters, a high-copy-number plasmid pHTlac was constructed. The *lacZ* fragment was amplified by using the plasmid pHT304-18Z (Agaisse and Lereclus 1994a) as template, and the fragment then was digested and ligated into the *Hin*dIII and *Pst*I sites of the *B. thuringiensis-E. coli* shuttle vector pHT315 (Arantes and Lereclus 1991) to produce the recombinant plasmid pHTlac harboring the promoterless *lacZ* gene. The promoter fragments of *cry1Ac*, *cry3A*, *cry4A*, and *cry8E* were amplified from *B. thuringiensis* HD73, Bt22, Bti, and Bt185 genomic DNA using the specific primer pairs according to previously studies LP1Ac-5/LP1Ac-3, LP3A-5/LP3A-3, LP4A-5/LP4A-3, and LPorf1-5/LP8E-3 (with 5′-*Kpn*I and 3′-*Xba*I) (Table 2), respectively. P_{cry1Ac} is a 382-bp fragment upstream of the *cry1Ac* translational start codon (access number AAB46989.1) (Wong et al. 1983). P_{cry3A} is a 570 bp upstream of the *cry3A* translational start codon, and the access number is CAB41411.1 (Agaisse and Lereclus 1994b). P_{cry4A} is a 826 bp upstream of the *cry4A* translational start codon, and the access number is CAD30148.1 (Yoshisue et al. 1993). P*cry8E* is a 1,352-bp fragment upstream of the *cry8E* translational start codon, and the access number is AY329081.1 (Du

Table 1 Strains and plasmids used in this study

Strains or plasmids	Characteristics	Reference
B. thuringiensis strains		
HD73	Wild-type strain containing plasmid pHT73 carrying *cry1Ac* gene	Du and Nickerson (1996)
HD73$^-$	Acrystalliferous mutant strain	Lereclus et al. (1989)
HDLP*cry1Ac*	HD73$^-$ strain containing plasmid pHTP*cry1Ac*	Wang et al. (2012)
HDLP*cry3A*	HD73$^-$ strain containing plasmid pHTP*cry3A*	Wang et al. (2012)
HDLP*cry4A*	HD73$^-$ strain containing plasmid pHTP*cry4A*	Wang et al. (2012)
HDLP*cry8E*	HD73$^-$ strain containing plasmid pHTP*cry8E*	Wang et al. (2012)
ΔsigKLP*cry1Ac*	*sigK* mutant strain containing plasmid pHTP*cry1Ac*	This work
ΔsigKLP*cry3A*	*sigK* mutant strain containing plasmid pHTP*cry3A*	This work
ΔsigKLP*cry4A*	*sigK* mutant strain containing plasmid pHTP*cry4A*	This work
ΔsigKLP*cry8E*	*sigK* mutant strain containing plasmid pHTP*cry8E*	This work
UV17	Bt strain carrying *cry1Ba* gene	Wang et al. (2006)
HDΔsigK	HD73 Mutant, *sigK* gene was disrupted by homologous recombination	Du et al. (2011)
HDΔsigK$^-$	Acrystalliferous *sigK* mutant strain	This work
Bt22	Bt strain carrying *cry3A* gene	Wang et al. (2008)
Bt185	Bt strain carrying *cry8Ea1* gene	Shu et al. (2009)
Bti	Bt strain carrying *cry4A*,*cry4B*,*cry11A* and *cytA* gene	BGSC
HD8E-1Ac	HD73$^-$ strain containing plasmid pHT315-8E-1Ac	This work
HD3A-1Ac	HD73$^-$ strain containing plasmid pSXY-422b-1Ac	This work
ΔsigK$^-$-8E1Ba	HDΔsigK$^-$ strain containg plasmid p8E1Ba	This work
HD$^-$-8E1Ba	HD73$^-$ strain containg plasmid p8E21b-1Ba	This work
Plasmids		
pHTlac	pHT315 harboring the promoterless *lacZ* gene	Wang et al.
pHTP*cry1Ac*	pHTlac carrying *cry1Ac* promoter	Wang et al. (2012)
pHTP*cry3A*	pHTlac carrying *cry3A* promoter	Wang et al. (2012)
pHTP*cry4A*	pHTlac carrying *cry4A* promoter	Wang et al. (2012)
pHTLP*cry8E*	pHTlac carrying *cry8E* promoter	Wang et al. (2012)
pET-21b	Containing ampicillin resistance gene	Novagen
pHT315	Amp^r, Erm^r, *E. coli*-Bt shuttle vector	Arantes and Lereclus (1991)
pSXY-422b	pHT315 carrying *cry3A* promoter and multiple clone sites (MCS) from pET-21b	Wang et al. (2006)
pHT315-8E21b	pHT315 carrying *cry8E* promter and multiple clone sites (MCS) from pET-21b	This work
pHT315-8E-1Ac	pHT315-8E21b containing *cry1Ac* gene	This work
pSXY-422b-1Ac	pSXY-422b containing *cry1Ac* gene	This work
p8E1Ba	pHT315-8E21b carrying *cry1Ba* gene	This work

BGSC Bacillus Genetic Stock Center

et al. 2012). The PCR products were digested by restriction endonuclease *Kpn*I and *Xba*I and then ligated into the vector pHTlac. The recombinants, pHTP*cry1Ac*, pHTP*cry3A*, pHTP*cry4A*, and pHTP*orf1-cry8E*, were separately introduced into HD73 and the *sigK* mutant.

β-Galactosidase assay. *B. thuringiensis* strains containing *lacZ* fusions were grown in SSM at 30 °C with 220 rpm. Samples of 2 ml were removed at 1-h intervals from T_1 to T_{12} (T_0 indicates the end of the exponential growth phase; T_n indicates n hours of the postexponential phase). The cells were harvested by centrifugation, and the pellets were stored at −20 °C. The β-galactosidase-specific activities were determined as previously described (Yang et al. 2012a) and are expressed as Miller units (Miller 1972). The values reported are the means of at least three independent assays.

Construction of an *E. coli-B. thuringiensis* shuttle vector. A *B. thuringiensis-E. coli* shuttle vector was constructed to express the *cry* gene under the control of the *cry8E* gene promoter. The 1,352-bp promoter fragment of the *cry8E* gene was obtained as described previously (Du et al. 2012), and the 213-bp expression sequence from plasmid pET-21b was amplified using pET21b-5/pET21b-3 as primers. The 1,565-bp overlapping fragment was amplified using the mixture of both the above fragments as a template and LPorf1-5 and pET21b-

Table 2 Primers and sequences

Primer	Sequence(5′–3′)[a]	Restriction site
1Ba-5	ATTACTGGATCCG TTGACTTCAA ATAGGAA	*Bam*HI
1Ba-3	ACTGTCGACCTATTCTTCC ATGA	*Sal*I
1Ac-5	CGCGGATCCCATGGATAACAATCCG AACATCAAT	*Bam*HI
1Ac-3	ACGCGTCGACCTATTCCTCCATAAGG AGTAATTC	*Sal*I
LPorf1-5	AACTGCAGAATGCACCTCCAATTGTT AATTATGT	*Pst*I
LP8E-3	GCTCTAGACTATTTTCCTCCTATACATA CACAAGAT	*Xba*I
pET21b-5	TGTATAGGAGGAAAATAGATGGCTAG CATGACTGGT	
pET21b-3	TCCCCCGGGCAAAAAACCCCTCAAG ACCC	*Sma*I
LP1Ac-5	GGGGTACCATTCAAGGTGAATTGCAG GTAAATG	*Kpn*I
LP1Ac-3	GCTCTAGAAAGTTACCTCCATCTCTT TTATTAAGAT	*Xba*I
LP3A-5	GGGGTACCGGGAGCTTAATTAAAGAT AATATCTTTG	*Kpn*I
LP3A-3	GCTCTAGATTTTCTTCCTCCCTTTCTTA TCAT	*Xba*I
LP4A-5	GGGGTACCAAGGTCAACTCAAATTAA GGGCAC	*Kpn*I
LP4A-3	GCTCTAGAATTTGTTCCTCCCATACT CAATTTAGAT	*Xba*I
pHT73-5	CTCTTTATTTTCAATTTTTCGAAGT	
pHT73-3	ATGGAAAAAGATTTATTTGAAGATG	

[a] Restriction enzyme sites are underlined

3 as primers. The overlapping PCR product was digested with *Sma*I and ligated into the *Eco*RI and *Hin*dIII sites of the *B. thuringiensis-E. coli* shuttle vector pHT315 by blunt-end ligation to produce the recombinant plasmid pHT315-8E21b, which carries the promoter of the *cry8E* gene, a multiple cloning site (MCS), and the T7 terminator (Fig. 3a).

Construction of the expression plasmid of *cry1Ba* with the *cry8E* promoter. The *cry1Ba* fragment was amplified from *B. thuringiensis* UV17 chromosomal DNA (Wang et al. 2006) using the primers 1Ba-5/1Ba-3 (with 5′-*Bam*HI and 3′-*Sal*I). The *Bam*HI-*Sal*I fragment of *cry1Ba* was then ligated into the *B. thuringiensis-E. coli* shuttle vector pHT315-8E21b (Fig. 3a), which harbors the *cry8E* gene promoter. The resulting plasmid was introduced into the HD73$^-$ and HDΔsigK$^-$ strains to produce the corresponding strains HD$^-$-8E1Ba and ΔsigK$^-$-8E1Ba.

SDS-PAGE analysis of Cry protein production. Different *B. thuringiensis* strains were grown at 30 °C with 220 rpm in SSM medium. After complete autolysis, 2.0-ml samples were centrifuged at 12,000×*g* for 10 min, and the cells were suspended in 0.5 ml Tris–HCl (50 mM, pH 8.0). The bacterial cells were then ruptured with a Mini-Beadbeater. Samples of 100 μl were mixed with 5× loading buffer and boiled for 10 min for subsequent total protein quantitation and SDS-PAGE. The total protein quantitation was determined using Pierce 660 nm Protein Assay Reagent (Thermo Scientific). ImageJ software (National Institutes of Health) was used to determine the intensity of protein bands.

Irradiation of *B. thuringiensis* samples with a solar simulator. *B. thuringiensis* samples were irradiated with a solar analyzer (XT5409-XPC80) from Xutemp, which delivered a spectrum equivalent to that of sunlight passing through the Earth's atmosphere. The products (60 μg of toxins) were sprayed onto 2.5- by 7.5-cm glass plates and air-dried (Sanchis et al. 1999). The coated plates were irradiated for 4 and 8 h using a solar analyzer. The analyzer uses a xenon lamp emitting from 280 to 800 nm at 100 klx. During irradiation, the glass plates were maintained at 25 °C. The irradiated toxins were recovered and then bioassayed against *Plutella xylostella* and *Ostrinia furnacalis*, as described below. For each irradiated sample, a control sample from the same batch was sprayed onto glass plates, air-dried, and recovered under the same conditions as for the irradiated plates.

Bioassay of insecticidal activity. Biological assays were performed by using free ingestion techniques and neonates. The protein concentration in the samples was estimated using a Total Protein Quantity Kit (Thermo Scientific, Beijing, China).

1. 50 % lethal concentration (LC_{50}). Insecticidal activities were tested by exposing first instar larvae (the Asian corn borer, *O. furnacalis*) or second instar larvae (diamondback moth, *P. xylostella*) to an artificial diet incorporating one of seven dilutions of each preparation in water (Xue et al. 2008). Total seven toxin concentrations (0.1, 0.5, 1, 2, 4, 8, and 16 μg/ml) were used to determine LC_{50} values. The test for each concentration was performed in triplicate. The diet given to *O. furnacalis* was uniformly distributed into 48-well trays, with 400 mg in each tray. One first instar larva was placed in each of the 48 wells. The 300-mg diet provided to *P. xylostella* was uniformly distributed into 9- by 9-cm plastic Petri dishes, and 20 second instar larvae were placed in each dish. All tests used water as the control. The number of surviving larvae was recorded after 7 days for *O. furnacalis* and 3 days for *P. xylostella*. The LC_{50} was calculated using probit analysis (Finney 1971).

2. Mortality of samples after solar irradiation. The samples after solar radiation for 8 h, as described above, and control samples were diluted to 16 μg/ml and mixed into the diet of neonate larvae of *P. xylostella* and *O. furnacalis*. The concentration (16 μg/ml) which is the highest concentration among the seven dilution concentrations that were assayed to determine LC_{50} values was used to detect the mortality of samples in consideration of the loss of toxin activity after solar irradiation. The subsequent procedure was identical as the above,

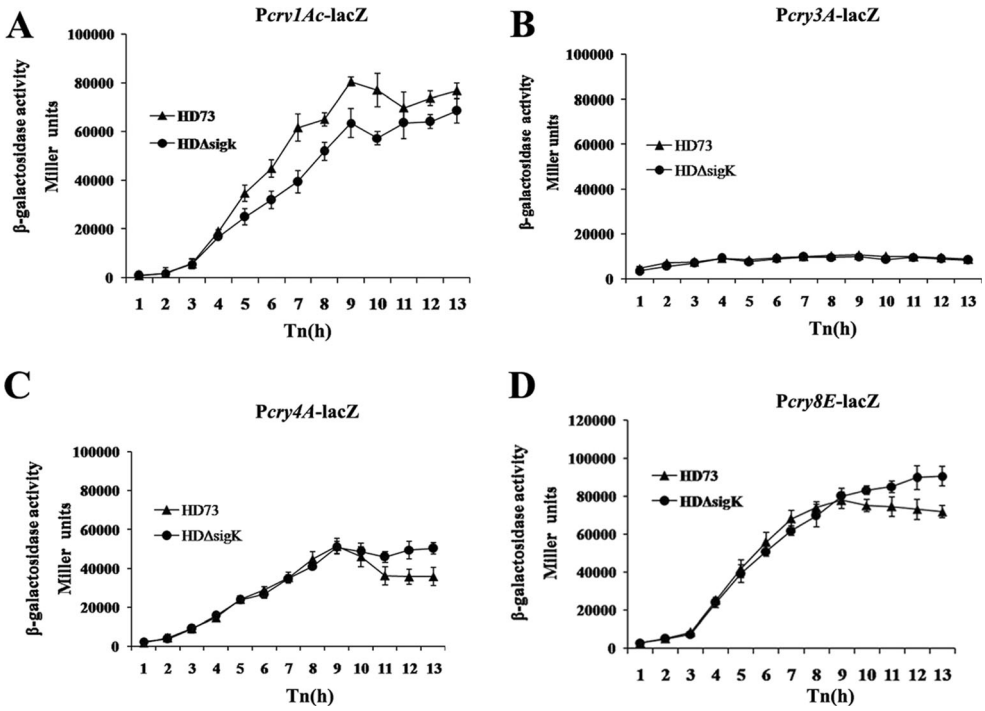

Fig. 1 The effect of the *sigK* mutation on the transcriptional activity of the *cry* promoter. The promoter-directed β-galactosidase synthesis of three clones was determined at the indicated times after growing the cells in SSM at 30 °C. Each value represents the mean of at least three independent replicates. **a** *cry1Ac* promoter, **b** *cry3A* promoter, **c** *cry4A* promoter, **d** *cry8E* promoter

and the mortality of each sample was calculated as previously described (Raymond et al. 1993).

Results

Transcriptional analysis of four *cry* gene promoters. The promoter activities of the *cry1Ac*, *cry3A*, *cry4A*, and *cry8E* genes were analyzed by constructing transcriptional fusions with the *lacZ* gene. β-Galactosidase assays indicated no difference of the activity of the P_{cry1Ac} promoter in HD73 and HDΔsigK from T_0 to T_3. However, the transcriptional activity of P_{cry1Ac} in strain HD73 was higher than that in strain HDΔsigK after T_3, and the highest transcriptional activity was obtained at T_9 in both strains (Fig. 1a). The β-galactosidase activities of P_{cry3A} were detected at T_1, and there was no significant change in transcriptional activity during sporulation (Fig. 1b). The transcriptional activity of P_{cry4A} in strain HDΔsigK was higher than that in strain HD73 after T_9 (Fig. 1c). Before T_9, the transcriptional activity of the *cry8E* promoter in strain HDΔsigK was similar to that in strain HD73. Thereafter, the β-galactosidase activity in the *sigK* mutant steadily increased, but it decreased in strain HD73 (Fig. 1d).

The transcriptional activities of the four *cry* gene promoters were also compared in both HD73 and HDΔsigK. The order of promoter activity, from high to low, was P_{cry8E}, P_{cry1A}, P_{cry4A}, and P_{cry3A} in both strains (Fig. 2a, b).

The comparison of expression efficiency of shuttle vectors with different promoters. To compare the efficiency of the *E. coli-B. thuringiensis* shuttle vector with respect to the expression of the *cry* gene, the intact fragment of the *cry1Ac* gene obtained by PCR amplification was inserted into the *Bam*HI and *Sal*I sites of the recombinant plasmid pHT315-

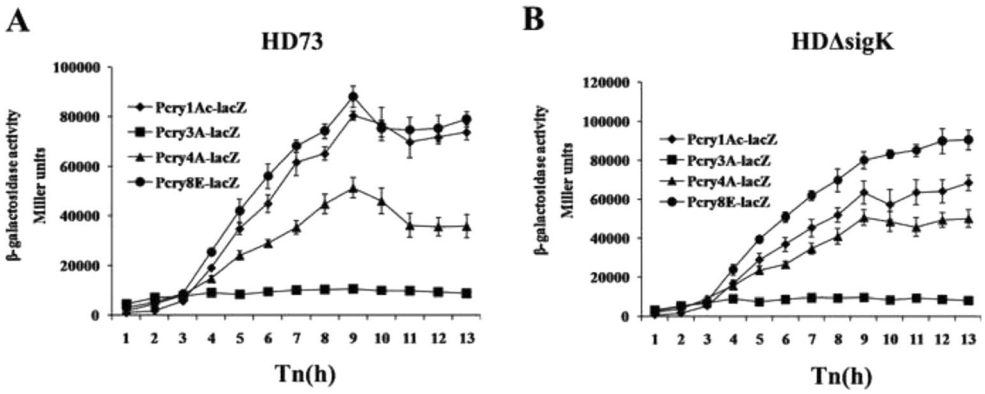

Fig. 2 Comparison of the transcriptional activity among different *cry* gene promoters. The promoter-directed β-galactosidase synthesis of three clones was determined at the indicated times after growing the cells in SSM at 30 °C. Each value represents the mean of at least three independent replicates. **a** *cry* promoters in wild-type strain HD73, **b** *cry* promoters in *sigK* mutant

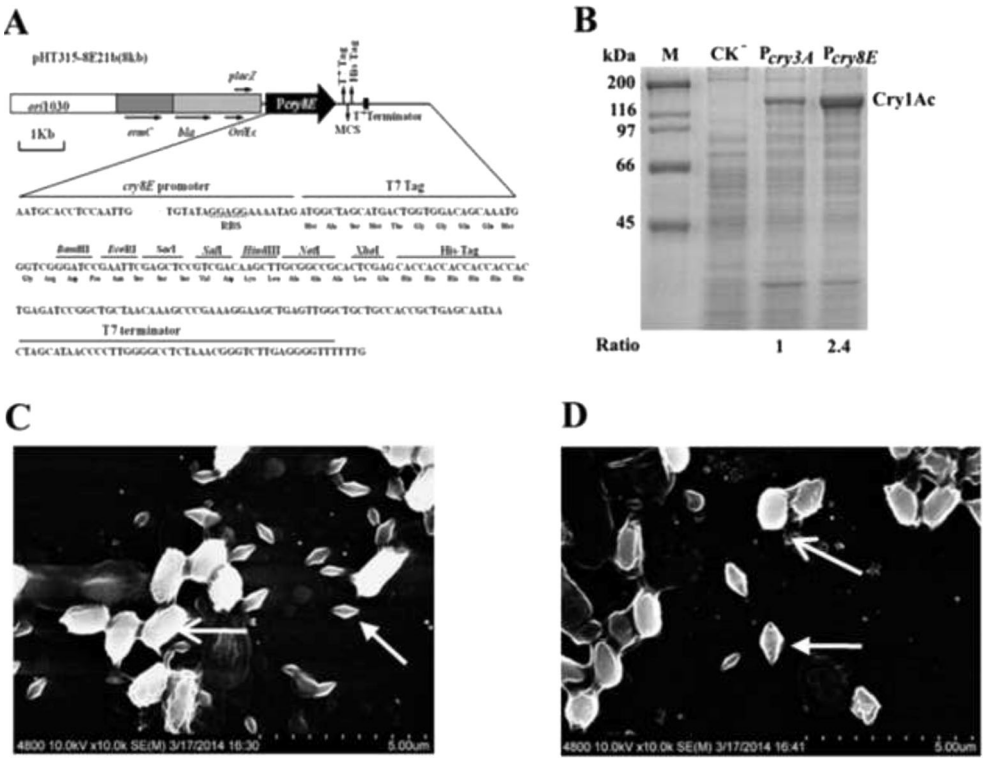

Fig. 3 Construction of a highly efficient expression vector, pHT315-8E21b, expressing the *cry* gene. **a** Physical map of *B. thuringiensis-E. coli* shuttle plasmid pHT315-8E21b. The expression region includes the *cry8Ea* promoter, a T7 tag, an MCS, a His-tag, and a T7 terminator. **b** SDS-PAGE analysis of Cry1Ac expression in strains HD3A-1Ac and HD8E-1Ac. CK^-:recipient strain $HD73^-$. Ratio means the relative value of Cry1Ac production against the Cry1Ac production directed by the P*cry3A* promoter, which is defined as 1. **c** SEM pictures of Bt strains HD3A-1Ac. **d** SEM pictures of Bt strains HD8E-1Ac. The oval inclusions marked by *hollow arrow* indicate spores, and the bipyramidal inclusions marked by *solid arrow* indicate protein crystals

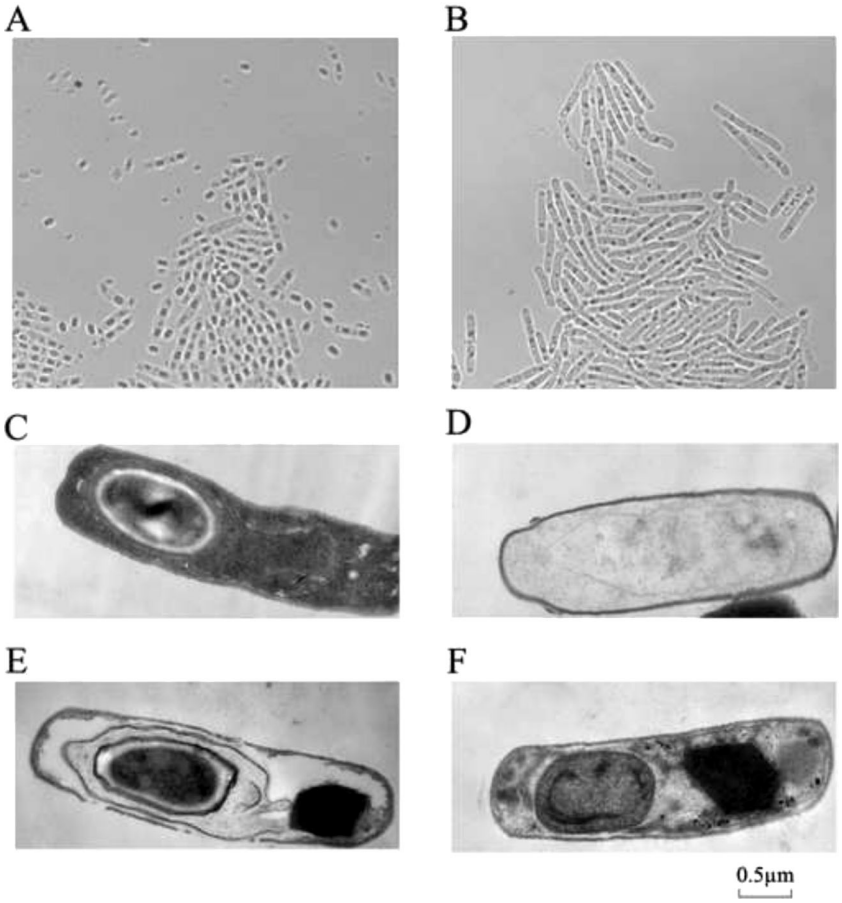

Fig. 4 CLSM and TEM pictures of Bt strains HD^--8E1Ba and $\Delta sigK^-$-8E1Ba. **a**, **b** CLSM photos of strains HD^--8E1Ba and $\Delta sigK^-$-8E1Ba grown after 24 h in SSM medium at 30 °C, respectively. **c**–**f** TEM pictures of different Bt strains grown for 48 h in SSM medium at 30 °C. **c** $HD73^-$, **d** HD$\Delta sigK^-$, **e** HD^--8E1Ba, **f** $\Delta sigK^-$-8E1Ba

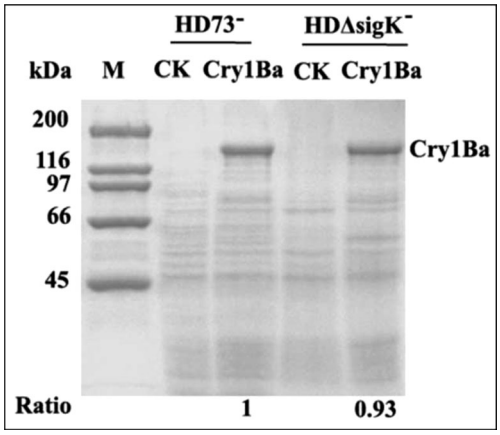

Fig. 5 SDS-PAGE analysis of Cry1Ba production in the *sigK* mutant and HD73⁻. *M* protein molecular weight marker, CK1, recipient strain HD73⁻; CK2, recipient strain HDΔsigK⁻; Cry1Ba, recipient strains containing the vector p8E1Ba. Ratio means the relative value of Cry1Ba production against the Cry1Ba production from untreated HD⁻-8E1Ba, which is defined as 1

8E21b and the previously used plasmid pSXY-422b, which also derived from pHT315 containing the *cry3A* gene promoter and pET-21b MCS site (Wang et al. 2006). The two expression plasmids were introduced into the acrystalliferous mutant HD73⁻, and the resulting strains were designated HD8E-1Ac and HD3A-1Ac. The SDS-PAGE result showed that the expression of the *cry1Ac* gene was significantly improved in HD8E-1Ac compared to the expression in HD3A-1Ac (Fig. 3b), with an increase of approximately 2.4-fold.

The expression and bioassay of encapsulated Cry1Ba in the *sigK* mutant. The acrystalliferous *sigK* mutant strain HDΔsigK⁻ was obtained by culturing the *sigK* mutant strain HDΔsigK at various temperatures (42, 45 °C) and screening via PCR amplification. To test the expression of the *cry* gene in the *sigK* mutant by the pHT315-8E21b vector, a *cry1Ba3* gene, which has broad-spectrum insecticidal activity against three orders, *Lepidoptera*, *Coleoptera*, and *Diptera* (Zhong et al. 2000), and no cross-resistance with the Cry1A-resistant and Cry1C-resistant strain of the diamondback moth (*P. xylostella*) (Liu et al. 2001), was introduced into this vector to generate p8E1Ba. Then, the p8E1Ba vector was transformed into HD73⁻ and HDΔsigK⁻ to obtain the strains HD⁻-8E1Ba and ΔsigK⁻-8E1Ba, respectively. The formation of crystal proteins was examined by optical microscopy and electron microscopy (Fig. 4). Large bipyramidal crystals were observed in both the HD⁻-8E1Ba and ΔsigK⁻-8E1Ba strains, but the crystals were released from the HD⁻-8E1Ba strain (Fig. 4a, e); in contrast, those from the *sigK* mutant strain ΔsigK⁻-8E1Ba were encapsulated within the cell wall (Fig. 4b, f), and no mature phase refractile spores were observed (Fig. 4f). SDS-PAGE analysis showed that both the ΔsigK⁻-8E1Ba and HD⁻-8E1Ba strains produced the 130-kDa protein Cry1Ba and there were no 130-kDa proteins expressed in the recipient strains (Fig. 5). The expression of *cry1Ba* in the *sigK* mutant strain ΔsigK⁻-8E1Ba was similar to that of the control strain HD⁻-8E1Ba.

A bioassay of the activity of the HD⁻-8E1Ba and ΔsigK⁻-8E1Ba strains against *O. furnacalis* and *P. xylostella* was performed. The results showed that ΔsigK⁻-8E1Ba had a similar toxicity to *P. xylostella* and *O. furnacalis* as the control strain HD⁻-8E1Ba, in which expressed Cry1Ba proteins are released (Table 3). The LC_{50} values to *P. xylostella* and *O. furnacalis* of strain ΔsigK⁻-8E1Ba were close to those of strain HD⁻-8E1Ba.

Encapsulation of Cry proteins in a *sigK* mutant increases UV resistance. Because the *sigK* mutation resulted in no spore formation and no crystal release, it has been developed to protect crystal inactivation against UV light (Sanchis et al. 1999). The effect of encapsulation of the Cry1Ba protein in the *sigK* mutant on UV resistance was also analyzed in this study. Both *B. thuringiensis* strains HD⁻-8E1Ba (expressing Cry1Ba protein in the HD73 strain) and ΔsigK⁻-8E1Ba (expressing Cry1Ba protein in the *sigK* mutant) were irradiated for 4 and 8 h under a xenon lamp emitting from 280 to 800 nm at 100 klx, and the bioassays against *P. xylostella* and *O. furnacalis* were performed. The results indicated that the corrected mortality due to HD⁻-8E1Ba was significantly lower than that due to ΔsigK⁻-8E1Ba after irradiation of 8 h, whereas the mortality due to these strains was almost the same before irradiation. The loss percentage of toxicity against *P. xylostella* was 61.1 % for HD⁻-8E1Ba and 16.1 % for ΔsigK⁻-8E1Ba. The loss percentage of toxicity against *O. furnacalis* was 70.8 % for HD⁻-8E1Ba and 11.9 % for ΔsigK⁻-8E1Ba (Table 4).

Discussion

The transcription of four *cry* promoters in the *sigK* mutant was analyzed in this study. The σ^K factor was previously reported

Table 3 Bioassay results of strains HD⁻-8E1Ba and ΔsigK⁻-8E1Ba effect on Asian corn borer and diamondback moth

Samples	*Ostrinia furnacalis*		*Plutella xylostella*	
	LC_{50} (μg/ml)	95 % Confidence limits	LC_{50} (μg/ml)	95 % Confidence limits
HD⁻-8E1Ba	2.41	1.30–3.70	0.80	0.65–0.97
ΔsigK⁻-8E1Ba	2.88	2.35–3.47	0.71	0.54–0.92

Table 4 Comparison of activity loss of strains HD⁻-8E1Ba and ΔsigK⁻-8E1Ba after UV radiation on Asian corn borer and diamondback moth[a]The percent loss is calculated as $[(\text{Corrected mortality})_{\text{sun 0 h}} - (\text{Corrected mortality})_{\text{sun 8 h}}] / (\text{Corrected mortality})_{\text{sun 0 h}}$

Samples	UV radiation(h)	*Ostrinia furnacalis*		*Plutella xylostella*	
		Corrected mortality (%)	% Loss (P)[a]	Corrected mortality (%)	% Loss (P)[a]
HD-8E1Ba	0	91.1	70.8	95.1	61.1
	8	26.6		37.0	
ΔsigK-8E1Ba	0	93.3	11.9	91.3	16.1
	8	82.2		76.6	

to control the expression of several *cry* genes, including *cry1A*, *cry1B*, *cry1C*, *cry4A*, *cry11A*, and *cry18A* (Bravo et al. 1996; Brown 1993; Brizzard et al. 1991; Yoshisue et al. 1993; Zhang et al. 1998; Dervyn et al. 1995; Yoshisuea et al. 1997). It is not surprising that the activity of the *cry1A* promoter decreased in the *sigK* mutant after T_3 because BtII of the two overlapped *cry1Ac* promoters is σ^K dependent, a result that is similar to the result reported by Yang et al. (2012a). Our results also showed that the transcriptional activation of the *cry3A* promoter in the *sigK* genetic background was similar to that in the wild type, which was consistent with results reported by Salamitou et al. (1996). The *cry4A* and *cry8E* genes have been reported to be primarily controlled by the sporulation factor σ^E (Du et al. 2012; Yoshisuea et al. 1997). It is very interesting that the activities of both the *cry4A* and *cry8E* promoters increased in the *sigK* mutant after T_9 (Fig. 1c, d). These data suggest that these two promoters were negatively regulated in the same pattern at the late stage of sporulation.

Although transcriptional analyses of many *cry* genes have been reported (Brown 1993; Brizzard et al. 1991; Yoshisue et al. 1993; Zhang et al. 1998; Dervyn et al. 1995; Yoshisuea et al. 1997; Yang et al. 2012a; Salamitou et al. 1996), it is surprising that little comparison of their promoter activities has been performed. Here, we proved that the *cry8E* promoter is the strongest of the four tested promoters in both the *sigK* mutant and the wild-type strain HD73. The *cry3A* promoter is controlled by σ^A and is expressed during vegetative growth. The STAB-SD sequence that stabilizes the *cry3* transcript-ribosome complex has been used to enhance the yields of the Cry proteins (Park et al. 1998; Park et al. 1999). The vectors based on the *cry3A* promoter have been widely utilized to express Cry proteins and construct genetically engineered strains (Sanchis et al. 1996; García-Gómez et al. 2013). In this study, a constructed vector, pHT315-8E21b, containing the *cry8E* promoter proved to be more efficient at expressing the *cry* gene than a *cry3A* promoter-related vector. Our results suggest that the pHT315-8E21b vector is an efficient expression system for *cry* gene expression and the *cry8Ea* promoter can be a potential expression element for developing genetically engineered Bt strains. The *cry8E* promoter was also found to be the strongest promoter in the *sigK* mutant, which cannot release crystals during the late stage of sporulation and has been successfully used to develop a genetically engineered strain against the UV inactivation of crystals by Sanchis et al. (1999). We also confirmed that the encapsulation of the Cry1Ba protein increased its UV resistance in this study. These findings provide an efficient expression system for the future development of more powerful bioinsecticides based on *B. thuringiensis*.

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