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A strong promoter of a non-*cry* gene directs expression of the *cry1Ac* gene in *Bacillus thuringiensis*

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Abstract

Bacillus thuringiensis bacteria show insecticidal activities that rely upon the production of insecticidal crystal proteins, which are encoded by *cry* or *cyt* genes and can target a variety of insect pests. It has been shown that *cry1Ac* is the only *cry* gene in *B. thuringiensis* subsp. *kurstaki* HD73 (*B. thuringiensis* HD73) and its expression is controlled by both σ^E and σ^K. Here, we report a novel σ^E-dependent strong promoter of a non-*cry* gene (*HD73_5014*), which can direct strong *cry1Ac* gene expression in *B. thuringiensis* HD73. We constructed an *E. coli-B. thuringiensis* shuttle vector (pHT315-P_{5014}-*1Ac*) for *cry1Ac* gene expression, using the *HD73_5014* gene promoter. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis showed that expression of the *cry1Ac* gene directed by the *HD73_5014* gene promoter was at the same level as that directed by the previously known strongest *cry* promoter, P_{cry8E}. However, this strain did not form typical bipyramidal crystals in mother cells, as observed by transmission electron microscopy and atomic force microscope. The strain with Cry1Ac protein expression under the control of the *HD73_5014* gene promoter (P_{5014}-*cry1Ac*) showed insecticidal activity against *Plutella xylostella* similar to that under the control of the *orf1cry8E* gene promoter (P_{cry8E}-*cry1Ac*). Collectively, these results suggest that the *HD73_5014* gene promoter, as a non-*cry* gene promoter, would be an efficient transcriptional element for *cry* gene expression. These data also show the possibility for improving Cry production by searching for transcriptional elements in not only *cry* genes, but also non-*cry* genes.

Keywords Non-*cry* gene promoter · P_{5014} · *cry1Ac* · *Bacillus thuringiensis*

Introduction

Bacillus thuringiensis is a gram-positive, spore-forming bacterium that belongs to the *Bacillus cereus* (Bc) group (Skerlova et al. 2014). It is characterized by forming parasporal crystal proteins and spores during the stationary phase of its growth cycle (Bravo et al. 2011). These crystal proteins, encoded by *cry* or *cyt* genes, possess highly specialized insecticidal activities against numerous insect species, including *Lepidoptera*, *Coleoptera*, and *Diptera* (Schnepf et al. 1998). The insecticidal specificity of *B. thuringiensis* strains toward different insects is determined by these *cry* and *cyt* genes. Due to its insecticidal and environmentally friendly properties, *B. thuringiensis* has been used commercially as a biocontrol agent worldwide, accounting for approximately 50% of the ever-growing biopesticide market (Gerwick and Sparks 2014; Lacey et al. 2015).

To date, approximately 825 *cry* and *cyt* genes have been discovered (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/).The *cry* genes are expressed during the

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stationary phase, and their products generally accumulate in the mother cell compartment to form a crystal inclusion that can account for 20 to 30% of the dry weight of the sporulating cells (Schnepf et al. 1998). The *cry* genes expressed during the stationary phase are classified as sporulation-dependent and sporulation-independent, based on how their expression is regulated (Agaisse and Lereclus 1995; Schnepf et al. 1998; Kroos et al. 1999). A typical example of a sporulation-independent *cry* gene is the *cry3Aa* gene, which is weakly but constitutively expressed during vegetative growth under the control of the sigma factor, σ^A (Desouza et al. 1993; Agaisse and Lereclus 1994). However, most *cry* genes (e.g., *cry1Aa*, *cry1Ba*, *cry1Ac*, *cry2Aa*, *cry4Aa*, *cry4Ba*, *cry11Aa*, and *cry15Aa*) are sporulation-dependent, which are active during the sporulation stages (Brizzard et al. 1991; Brown 1993; Yoshisue et al. 1993; Dervyn et al. 1995; Bravo et al. 1996; Zhang et al. 1998; Kroos et al. 1999). These sporulation-dependent *cry* genes are expressed in the mother cell and the crystals accumulate there. This progress is dependent on highly ordered cell programs and is regulated by a series of sigma factors (e.g., σ^E and σ^k) in signaling cascades (Agaisse and Lereclus 1995; Schnepf et al. 1998; Kroos et al. 1999). For example, transcription of the double promoter-containing gene *cry1Aa* is controlled by both σ^E and σ^K, which bind to the BtI and BtII promoters during the early-to-middle and middle-to-late sporulation phases, respectively (Wong et al. 1983; Brown and Whiteley 1988, 1990; Bravo et al. 1996; Saxild et al. 1996). Another sporulation-dependent *cry* gene, *cry8Ea1* (also named *cry8E*), is transcribed from two promoters, namely P_{orf1} (located upstream of the *orf1* gene) and P_{cry8E} (located in the intergenic region between *orf1* and *cry8Ea1*), which are regulated by σ^E and σ^H, respectively (Du et al. 2012).

Despite the fact that *B. thuringiensis* has been used as an insecticidal agent worldwide, it has only captured approximately 2% of the total insecticides market owing to the relatively low yield and instability of the crystal protein in the field (Bradley et al. 1995; Huang et al. 2007; Bravo et al. 2011). Some methods have been developed to increase the yield, titer, and stability of insecticidal crystal proteins. For example, it has been reported that Cry1Ac production was enhanced when the gene was controlled by the heterologous *cry8E* gene promoter P_{cry8E}, compared with that in wild-type strain (HD73) (Li et al. 2013). Interestingly, it has been reported that a non-*cry* gene promoter can also successfully drive *cry1Ac* expression. ExsY is a basal layer protein in the exosporium of *B. anthracis* spores (Boydston et al. 2006). The promoter of the *exsY* gene (P_{exsY}) can direct *cry1Ac* gene expression during the late sporulation stage although it leads to a lower Cry protein yield (Zheng et al. 2014). Those studies provide us alternative strategies of improving Cry production and efficacy of Bt strains as insecticidal agents. Nevertheless, the pick of non-*cry* gene promoters and the efficiency of using those promoters to direct *cry* gene expression and Cry protein production remain to be further investigated.

In this study, we identified a novel strong promoter from a gene encoding a putative DeoR family transcriptional regulator (P_{5014}, as it is encoded by *HD73_5014*). We showed that this non-*cry* promoter can efficiently drive *cry1Ac* gene expression in *B. thuringiensis* to a level comparable to that from the strong *cry* gene promoter P_{cry8E}. A crystal protein-producing strain, HD$^-$$P_{5014}$-*1Ac*, in which *cry1Ac* is expressed under the *HD73_5014* gene promoter, was constructed. We found that HD$^-$$P_{5014}$-*1Ac* was similar to the wild-type strain (HD73), both in terms of Cry1Ac production and insecticidal activity against *P. xylostella* larvae. More importantly, when introduced into a *sigK* mutant, we obtained a higher yield of encapsulated crystals since the non-cry gene promoter P_{5014} is *sigK*-independent. Encapsulated crystals showed better resistance to environmental factors such as UV light inactivation and better efficacy when applied in the field in previous studies (He et al. 2017). Our work suggested that the non-*cry* gene promoter P_{5014} showed good prospects in constructing engineered *B. thuringiensis* strains for application in agriculture.

Materials and methods

Bacterial strains, plasmids, and growth conditions

B. thuringiensis subsp. kurstaki HD73 (BGSC strain number BGSC 4D4, hereafter designated as HD73), the acrystalliferous mutant strain HD73$^-$ (Lereclus et al. 1989) and their derivatives were grown at 30 °C in Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) or on solid LB medium supplemented with 1.5% agar. Schaeffer's sporulation medium (Schaeffer et al. 1965) (SSM; 0.8% nutrient broth, 1 mM $MgSO_4$, 13.4 mM KCl, 0.5 mM NaOH, 1 mM $Ca(NO_3)_2$, 0.01 μM $MnCl_2$, and 1 μM $FeSO_4$) was used to measure promoter activities. *Escherichia coli* JM109 was used for molecular cloning experiments, and *E. coli* ET1 was used for producing non-methylated plasmid DNA for *B. thuringiensis* transformations (Macaluso and Mettus 1991; Wang et al. 2006); both strains were grown at 37 °C in LB medium. When required, antibiotics were added at the following concentrations for growth of *B. thuringiensis*: 5 μg/ml erythromycin and 50 μg/ml kanamycin. For growth of *E. coli*, 100 μg/ml ampicillin was added when needed. The HD73 complete genomic sequence had been submitted to NCBI database (GenBank Accession No. NC_020238.1), all gene sequences could be found in this database. The bacterial strains and plasmids used in this study are summarized in Table 1.

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant details[a]	Reference or source
B. thuringiensis strains		
HD73	Wild-type strain containing plasmid pHT73 carrying *cry1Ac* gene	(Du and Nickerson 1996) Strain No. BGSC 4D4
HD73$^-$	Acrystalliferous mutant strain	(Lereclus et al. 1989)
BT185	Bt strain carrying *cry8Ea1* gene	(Shu et al. 2009)
HDΔ*sigK*	HD73 mutant, *sigK* gene was deleted by homologous recombination	(Du et al. 2011)
HDΔ*sigE*	HD73 mutant, *sigE* gene was deleted by homologous recombination	(Du et al. 2011)
HDP$_{5014}$	HD73 strain containing plasmid pHT304-P$_{5014}$	This study
HDP$_{0588}$	HD73 strain containing plasmid pHT304-P$_{0588}$	This study
HDP$_{1453}$	HD73 strain containing plasmid pHT304-P$_{1453}$	This study
HDP$_{4734}$	HD73 strain containing plasmid pHT304-P$_{4734}$	This study
HDP$_{4944}$	HD73 strain containing plasmid pHT304-P$_{4944}$	This study
HDP$_{0992}$	HD73 strain containing plasmid pHT304-P$_{0992}$	This study
HDΔ*sigK*P$_{5014}$	*sigK* mutant containing plasmid pHT304-P$_{5014}$	This study
HDΔ*sigE*P$_{5014}$	*sigE* mutant containing plasmid pHT304-P$_{5014}$	This study
HD(P$_{cry8E}$-*lacZ*)	HD73 strain containing plasmid pHTP$_{orf1\text{-}cry8E}$	(Du et al. 2012)
HD8E-1Ac	HD73$^-$ strain containing plasmid pHT315-8E-*1Ac*	(Zhou et al. 2014)
HD$^-$ P$_{5014}$-1Ac	HD73$^-$ strain containing plasmid pHT315-P$_{5014}$-*1Ac*	This study
sigK$^-$P$_{5014}$-1Ac	*sigK*$^-$ strain containing plasmid pHT315-P$_{5014}$-*1Ac*	This study
sigK$^-$	Acrystalliferous *sigK* mutant strain	(Zhou et al. 2014)
Plasmids		
pHT304-18Z	*E. coli*-Bt shuttle vector with promoter-less *lacZ* reporter, Amp^R, Erm^R	(Agaisse and Lereclus 1994)
pHT315	Amp^R, Erm^R, *E. coli*-Bt shuttle vector	(Arantes and Lereclus 1991)
pHTP$_{orf1\text{-}cry8E}$	pHT304-18Z carrying P$_{orf1\text{-}cry8E}$, Amp^R, Erm^R	(Du et al. 2011)
pHT304-P$_{5014}$	pHT304-18Z carrying P$_{5014}$, Amp^R, Erm^R	This study
pHT304-P$_{0588}$	pHT304-18Z carrying P$_{0588}$, Amp^R, Erm^R	This study
pHT304-P$_{1453}$	pHT304-18Z carrying P$_{1453}$, Amp^R, Erm^R	This study
pHT304-P$_{4734}$	pHT304-18Z carrying P$_{4734}$, Amp^R, Erm^R	This study
pHT304-P$_{4944}$	pHT304-18Z carrying P$_{4944}$, Amp^R, Erm^R	This study
pHT304-P$_{0992}$	pHT304-18Z carrying P$_{0992}$, Amp^R, Erm^R	This study
pHT315-P$_{5014}$-*1Ac*	pHT315 containing P$_{5014}$-*cry1Ac* gene	This study
pHT315-8E-*1Ac*	pHT315 containing P$_{orf1\text{-}cry8E}$-*cry1Ac* gene	(Zhou et al. 2014)

[a] Antibiotic resistance cassettes are indicated as follows: Erm^R, erythromycin resistance; Amp^R, ampicillin resistance

DNA manipulation and transformation

Plasmid DNA was extracted from *E. coli* cells with a Plasmid Miniprep Kit (Axygen, Beijing, China). Restriction enzymes and T4 DNA ligase (Takara Biotechnology Corporation, Dalian, China) were used according to the manufacturer's instructions. PCR was performed with the high-fidelity PrimeSTAR HS DNA polymerase (Takara Biotechnology Corporation, Beijing, China) or *Taq* DNA polymerase (BioMed, Beijing, China). DNA fragments were purified from 1% agarose gels, using a AxyPrep DNA Gel Extraction Kit (Axygen, Beijing, China). Standard procedures were followed for *E. coli* transformation (Sambrook and Russell 2015). *B. thuringiensis* cells were transformed by electroporation as previously described (Lereclus et al. 1989).

RNA-Seq analysis

Total RNA was extracted from HD73 cells grown in SSM medium until T_7 stage (7 h after the end of the exponential phase) following the previously published method (Du et al. 2012). Briefly, 1 ml of Bt cells were harvested by centrifugation (14,000×*g*, 1 min at 4 °C), and the pellets were resuspended in 1 ml of cold TRI-Reagent (Invitrogen, San Diego,

CA). The RNA was extracted with the Qiagen Easy RNA kit according to the manufacturer's instructions. The residual DNA was removed using RNase-free DNase I (New England BioLabs). The pure total RNA was used for RNA-Seq analysis. Briefly, rRNA (including 16S and 23S) was removed from 4 μg of total RNA by Microbexpress™ (Ambion), and the left RNA was chemically fragmented. The sequence library construction is carried out according to ScriptSeq: trademark: mRNA-Seq Library Preparation Kit (Illumina). In brief, the fragmented RNA is reverse-transcribed into cDNA using the SuperScript double-stranded cDNA synthesis kit (Invitrogen) with the addition of SuperScript III reverse transcriptase (Invitrogen) and random primers containing a tagging sequence at their 3′ ends. This was followed by RNase A (Roche, Germany) treatment, phenol-chloroform extraction, and ethanol precipitation. The resulting cDNAs were ligated to a 5′ DNA/DNA adaptor and the di-tagged cDNA was purified by PAGE gel. The size of the inserted fragment is ~ 150–250 bp. The purified products were PCR amplified to in 18 cycles using a high-fidelity DNA polymerase. PCR products were purified using the PAGE gel. cDNAs were sequenced using a single flow cell of the Illumina Hiseq 2000. FPKM = cDNA fragments/(mapped reads (millions)×transcript length (kb)).

Construction of the gene promoter with the *lacZ* reporter gene

To compare the transcriptional activity of the target genes, the promoter sequences of the chosen genes were amplified from HD73 genomic DNA (GenBank Accession No. NC_020238.1) using different primers (Table 2). The length of the amplified fragment (P_{5014}) is 710 base pairs upstream of the *HD73_5014* translational start codon. The amplified fragments were digested with *Pst*I and *Bam*HI, followed by ligation into the linearized pHT304-18Z plasmid, which harbors a promoterless *lacZ* gene (Agaisse and Lereclus 1994). The recombinant plasmids were introduced into HD73 cells by electroporation using a Gene Pulser II apparatus (Bio-Rad, USA). The resulting strains were selected on the agar plates supplemented with erythromycin (validation is done by sequencing).

Construction of the P_{5014}-Cry1Ac fusion

Overlapping PCR was performed to generate the P_{5014}-*cry1Ac* fusion. The primer pairs, pHT5014-5, pHT5014-3 and pHT1Ac-5, pHT1Ac-3, were used to amplify P_{5014} and the *cry1Ac* ORF (GenBank Access Number: AAB46989.1) (Wong et al. 1983), respectively. Long-fragment PCR was conducted with the pHT5014-5/pHT1Ac-3 primer set, using the PCR products as templates. The generated P_{5014}-*cry1Ac* fusion product was then digested with *Sal*I and *Sph*I and integrated into the pHT315 vector, which was also digested with *Sal*I and *Sph*I, resulting in pHT315-P_{5014}-*1Ac*. The plasmid was verified by Sanger sequencing, followed by introduction into HD73$^-$ (acrystalliferous mutant strain by curing of the plasmid carrying the *cry1Ac* gene) (Lereclus et al. 1989) and HD*sigK*$^-$ strain (acrystalliferous *sigK* mutant strain) (Zhou et al. 2014). Transformants were selected on erythromycin agar plates at 30 °C. In addition, the pHT315-8E-1Ac plasmid, HD8E-1Ac, and Δ*sigK* strains were used as controls (Table 1) (Zhou et al. 2014).

Assays of β-galactosidase activities

To assay β-galactosidase activities, the *B. thuringiensis* strains were grown in SSM medium at 30 °C with shaking (220 rpm). Two milliliters of culture were collected at 1-h intervals from T_1 to T_{12} (T_0 indicates the end of the exponential growth phase and T_n indicates n hours after T_0). Cells were centrifuged (14,000×g, 1 min) and pellets were stored at – 20 °C ready for use. β-galactosidase activities were measured as previously described (Yang et al. 2012) and expressed as Miller units (Miller 1972). Values are reported as the mean and standard error of at least three independent assays.

SDS-PAGE analysis of Cry protein production

B. thuringiensis cells were grown to T_{24} in 50 ml of fresh SSM at 30 °C with shaking (220 rpm), and the cells were centrifuged at 4 °C for 10 min at 8000 rpm, followed by freeze-drying for ~ 48 h until the pellets became lyophilized powders. An appropriate volume of double-distilled water was added to each sample to adjust them to equivalent bacterial biomass concentrations (mg/ml). The pellets were thoroughly vortexed for 20 s and resuspended. Then, the cells were disrupted with a Mini-BeadBeater (Biospec Products, Inc., Bartlesville, OK, USA) in a 2-ml centrifuge tube containing approximately 200 μg of glass beads (0.1 mm diameter). After a low-speed (1000×g) centrifugation, the total cell lysates were transferred to a new centrifuge tube. The mixture of 40 μl of cell lysates and 10 μl of 5× loading buffer was boiled for 10 min, followed by centrifugation at 12000×g for 1 min at 4 °C, then the cell lysates were analyzed to detect Cry protein production by SDS-PAGE and Coomassie Brilliant Blue staining. Protein band intensities were determined using ImageJ software (Version 1.6.0_24, National Institutes of Health) by comparison to bovine serum albumin (BSA).

Western blot analysis

Western blot experiments were performed after the samples were separated by SDS-PAGE (4% polyacrylamide stacking gel, 10% polyacrylamide separating gel) as previously described (Wang et al. 2006). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The protein-

Table 2 Oligonucleotide primers used in this study

Primer name	Sequence(5′–3′)[a]	Restriction site
P5014-5	AACTGCAGCTTTCCATCGCGAATATCC	*Pst*I
P5014-3	CGGGATCCCGAGTAGTTGTAGGTTTC	*Bam*HI
P0588-5	CGGGATCCTGATTTCGCTTTCGGTTGACC	*Bam*HI
P0588-3	AACTGCAGGCCTTGATAACGACCTGATTT	*Pst*I
P1453-F	CGGGATCCTGCACATTCTTGTAGTTCATGTATA	*Bam*HI
P1453-R	AACTGCAGATCGACGAGGCGATTCAAGGT	*Pst*I
P4734 -F	CGGGATCCAACTAACAAAGAAGCAGCAATCAC	*Bam*HI
P4734-R	AACTGCAGCGAATATGATGCCAATCATGG	*Pst*I
P4944-F	CGGGATCCAACACCAAACTCTTGAGCGATTTCG	*Bam*HI
P4944-R	AACTGCAGGGAAGCAGTTCACTTCCATAGTCCA	*Pst*I
P0992-F	CGGGATCCTTCTTGAGCGATTTCGTACTTCA	*Bam*HI
P0992-R	AACTGCAGGCGTTAAACCATCTACTGCACTTAA	*Pst*I
Porf1-5	AACTGCAGAATGCACCTCCAATTGTTAATTATGT	*Pst*I
P8E-3	CGGGATCCCTATTTTCCTCCTATACATACACAAGAT	*Bam*HI
pHT5014-5	GCGTCGACCTTTCCATCGCGAATATCCG	*Sal*I
pHT5014-3	CGGATTGTTATCCATGAGAAAACCACTCCT	
pHT1Ac-5	AGGAGTGGTTTTCTCATGGATAACAATCCG	
pHT1Ac-3	ACATGCATGCCTATTCCTCCATAAGGAG	*Sph*I
P5014Race	CGCGGCAAGGGCTATACACGAGTTCATT	

[a] Restriction enzyme sites are underlined

laden PVDF membranes were probed with a primary antibody against Cry1Ac (Beijing Protein Innovation Inc., Beijing, China) at a dilution of 1:5000. Antibody binding was detected with HRP-conjugated goat anti-mouse IgG (CWBiotech, Beijing, China). Visualization was performed as previously described (Ni et al. 2016).

Determination of the transcriptional start site

Total RNA was extracted from HD73 cells grown in SSM until T_7 stage, and reverse transcription PCR was conducted as previously described (Du et al. 2012). We used the SMARTer RACE (switching mechanism at the 5′ end of the RNA transcript-rapid amplification of cDNA ends) cDNA amplification kit (Clontech, Mountain View, CA) to determine the transcription start site, following the manufacturer's instructions. P5014Race, located ~200 bp downstream of the *HD73_5014* start codon (TTG), was designed as the specific reverse primer. NestRace was the forward primer provided in the kit (Clontech, Mountain View, CA). P5014Race and NestRace were used as specific primers for amplifying the 5′ end of P_{5014} cDNA. The sequences of the primers used in this study are shown in Table 2.

Observation of cell shape and crystal structure

Transmission electron microscopy (TEM) and atomic force microscopy (AFM) were employed to analyze the *B. thuringiensis* cell shape and structure of the insecticidal crystals. Samples were prepared for TEM as follows. Briefly, the cells were grown until T_{24} in SSM at 30 °C. Twenty-milliliter samples were harvested by centrifugation, followed by adding 1 ml of 3% glutaraldehyde solution. After performing a series of procedures (fixation, dehydration, replacement, soaking, embedding, sectioning, and staining), the cell shape was observed as previously described (Liu et al. 2008; Weigel and Glazebrook 2010). For AFM experiments, the strains were grown until T_{24} or a later stage to release the crystals. The MultiMode 8 SPM instrument was operated according to the guidelines of the manufacturer (Bruker, Germany).

Bioassay of insecticidal activities

Biological assays were performed as described by (Zhou et al. 2014), using equivalent bacterial biomass concentrations for the HD$^-$$P_{5014}$-*1Ac*, HD8E-*1Ac*, HD73, and HD73$^-$ strains. Briefly, insecticidal activities were tested by exposing second instar larvae (diamondback moth, *P. xylostella*) to an artificial diet incorporating 1 of 7 dilutions (bacterial lyophilized powder concentrations of 1.25, 2.5, 5, 10, 20, 40, and 80 μg/ml) of each preparation in water (Xue et al. 2008). A 6-cm-diameter cabbage leaf disc was pretreated with a gradient bacterial concentration as described above and then transferred to a new plastic culture dish, after which 30 s instar larvae were placed in each dish. The numbers of surviving larvae were counted

Table 3 RNA-Seq data for the top 20 genes with high expression at late sporulation phase (T_7)

No.	Gene ID	Relative expression level[a]	Annotation
1	HD73_0588	40873.15	Small, acid-soluble spore protein K
2	HD73_6004	39824.43	Pesticidal crystal protein Cry1Ac
3	HD73_4734	35557.62	Putative forespore-specific protein
4	HD73_1453	31127.24	Spore coat protein
5	HD73_4944	26293.69	Small acid-soluble spore protein
6	HD73_0992	24544.49	Small acid-soluble spore protein
7	HD73_5014	20343.18	DeoR family transcriptional regulator
8	HD73_0593	19203.71	Small, acid-soluble spore protein gamma-type
9	HD73_0587	19058.02	Hypothetical protein
10	HD73_5860	12809.66	Superoxide dismutase
11	HD73_5537	12290.43	DUF1657 domain-containing protein
12	HD73_2153	12085.26	Small acid-soluble spore protein
13	HD73_4056	11916.65	Spore coat protein E
14	HD73_5804	8961.88	Spore coat protein GerQ
15	HD73_1729	6583.09	YpzI family protein
16	HD73_1738	5272.76	Stage IV sporulation protein A
17	HD73_4645	5156.66	Sporulation sigma factor SigK
18	HD73_3939	5102.29	Anti-terminator HutP
19	HD73_4703	4834.34	Hypothetical protein
20	HD73_4111	4635.42	Transcriptional repressor CodY

[a] FPKM = cDNA fragments/(mapped reads (millions)×transcript length (kb))

after 3 days, and the LC_{50} was calculated by probit analysis. The test for each concentration was performed in triplicate.

Results

The *HD73_5014* gene promoter showed high activities during sporulation

cry1Ac, the only *cry* gene in HD73, is sporulation-dependent since it is regulated by the sporulation sigma factor K (SigK) (Schnepf et al. 1998). The *cry1Ac* gene was expressed after entry into sporulation phase and the transcriptional activity was maintained at a high level from T_6 (Zhou et al. 2014). We aimed to decouple high expression of *cry1Ac* at the late sporulation stage from its dependence on SigK. We assumed that some promoters of the genes with high transcriptional activity at the late sporulation phase might be applied to direct *cry1Ac* gene expression efficiently in a SigK-independent fashion. To obtain a potential strong promoter with this feature, we analyzed gene-expression levels from microarray data (NCBI Gene Expression Omnibus Accession No. GSE 48410) at the late sporulation phase (here, we chose T_7) of the *B. thuringiensis* HD73 strain (Peng et al. 2015) and selected 100 candidate genes with top-level transcriptional activity (Table S1). Then, RNA-Seq data (SRA accession: SRP127175; Table 3) were utilized to check the relative transcriptional levels of those candidate genes at the late sporulation phase (T_7). We obtained seven candidate genes with high transcriptional activity both in the microarray data (Table S1) and RNA-Seq data (value > 20,000). The top seven genes with relatively high transcriptional activity, from high to low, are *HD73_0588*, *HD73_6004 (cry1Ac)*, *HD73_4734*, *HD73_1453*, *HD73_4944*, *HD73_0992*, and *HD73_5014* (Table 3). To confirm the high transcriptional activity of these candidate genes (except for *cry1Ac*), we fused each of the corresponding promoter regions to the *lacZ* gene to create the *lacZ* reporter fusion and constructed individual strains bearing each of those reporter fusions (see Materials and Methods, Fig. 1a, b). The assays of the β-galactosidase activity demonstrated that the transcriptional activity of P_{5014} was the highest (Fig. 1c). In addition, we also found that *HD73_5014* was transcribed at a relatively high level even at T_{-1} (relative expression level is 1881.97, data not shown). Finally, the *HD73_5014* gene, a putative DeoR family transcriptional regulator, was selected since it showed high expression levels at the sporulation stage (Fig. 1c).

It has been reported that the *cry8E* gene promoter (P_{cry8E}) is a strong *cry* promoter that can direct high *cry1Ac* gene expression during the sporulation stages (Zhou et al. 2014). To compare the high transcriptional activity of *HD73_5014* gene promoter (P_{5014}) and the transcriptional levels with P_{cry8E}, we performed β-galactosidase assays using two strains harboring these *lacZ* fusions (see Materials and Methods, Fig. 1b).

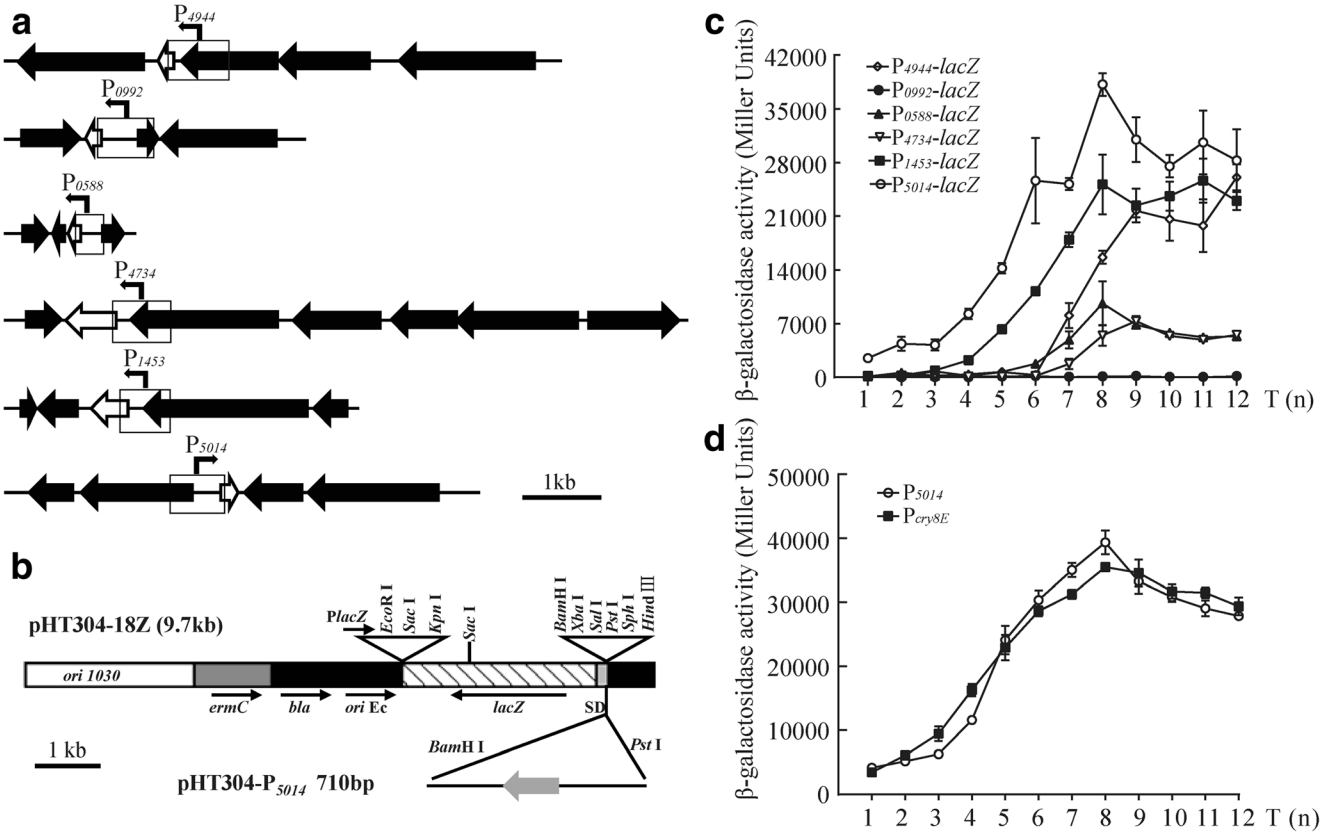

Fig. 1 The promoter of the *HD73_5014* gene showed high transcriptional activity in the sporulation stages. **a** The arrangement of six putative highly transcriptional genes and their promoter regions in HD73 genome. The white open arrows indicate the candidate genes and black open arrows show adjacent genes. **b** Construction of a transcriptional reporter by fusing the *HD73_5014* gene promoter with *lacZ*. **c** Comparison of the transcriptional activities of the 6 putative strong promoters. **d** Comparison of the transcriptional activities between the *HD73_5014* and *cry8E* gene promoters. The β-galactosidase activities of three clones were determined at the indicated times after growing the cells in SSM at 30 °C. Each value represents the mean and standard error of at least three independent replicates

β-Galactosidase assays indicated that the transcriptional activities of both P_{5014} and P_{cry8E} were high during the sporulation stages, especially from T_6 to T_{12} (Fig. 1d). The transcriptional activities of both promoters were similar, and both increased until they peaked at T_8 and then decreased (Fig. 1d). In general, the transcriptional activity of P_{5014} was comparable to that of P_{cry8E}, demonstrating that P_{5014} is also a strong promoter during the sporulation stages.

The *HD73_5014* gene promoter functions in a σ^E-dependent manner

We wanted to investigate the transcriptional regulation of *H73_5014* in sporulation stages. We started by analyzing the *HD73_5014* gene promoter sequence in detail. The transcription start site was confirmed to be a single 5′-end nucleotide residue "A," located 55 nucleotides upstream of the *HD73_5014* translational start codon, based on the sequences of random clones obtained by 5′-RACE (Fig. 2a).

Our analysis of the *HD73_5014* promoter region revealed that − 35 (GCATGA) and − 10 (AGTACAAT) regions (Fig. 2a) were similar to the consensus sequences of σ^E-dependent promoters (KHATANHT…MATANNHT) (Zhang et al. 1998). These findings indicated that the *HD73_5014* promoter might function in a σ^E-depended manner. The *cry1Ac* gene is a sporulation-dependent gene that has two promoters, which are recognized by σ^E and σ^K, respectively (Wong et al. 1983). We aimed to use the *HD73_5014* gene promoter to drive *cry1Ac* gene expression; thus, we were interested in determining whether the *HD73_5014* promoter is controlled by σ^E and σ^K. To that end, we introduced the previously constructed pHT304-P_{5014} plasmid containing the P_{5014}-*lacZ* fusion into the wild-type strain (HD73), the *sigE* mutant (HDΔsigE), and the *sigK* mutant (HDΔsigK) and then compared the β-galactosidase activities of these three strains. In the *sigE* mutant, the P_{5014}-*lacZ* fusion was expressed at a much lower level, confirming that the *HD73_5014* promoter is controlled by σ^E (Fig. 2b). The transcriptional activity of P_{5014} increased rapidly during T_1 to T_8 in both the wild-type and *sigK* mutant strains (Fig. 2b). However, the transcriptional activity of P_{5014} in the wild-type strain was lower than that in the *sigK* mutant from T_9 to T_{12} (Fig. 2b), consistent with the transcriptional activity of P_{cry8E} observed previously (Du et al. 2012). Our results confirmed that the *HD73_5014* gene promoter functions

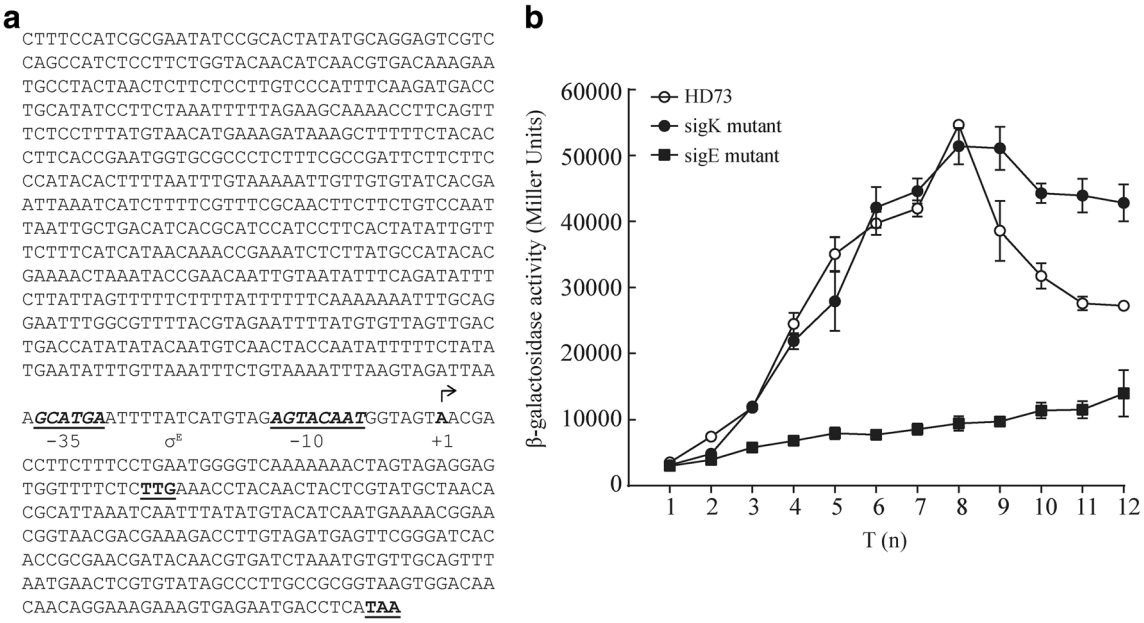

Fig. 2 The activity of the *HD73_5014* gene promoter relies on σ^E. **a** Analysis of the promoter of the *HD73_5014* gene in *B. thuringiensis* HD73. The − 35 and − 10 motifs and the transcription start site (+ 1) are annotated. The underlined TTG is the start codon of *HD73_5014* gene. **b** Analyses of *HD73_5014* gene promoter activities in the wild-type HD73, the *sigK* mutant, and the *sigE* mutant strains. Promoter-directed β-galactosidase expression in three clones was determined at the indicated times after growing the cells in SSM at 30 °C. The values shown represent the mean and standard error of at least three independent replicates

specifically in a σ^E-, but not σ^K-, dependent manner during sporulation. Thus, using the *HD73_5014* gene promoter to direct expression of *cry1Ac* would allow us to achieve high expression and presumably high yield of crystal proteins independent on SigK.

The *HD73_5014* gene promoter directed *cry1Ac* expression and crystal accumulation

To test whether *HD73_5014* promoter could effectively direct Cry1Ac expression in *B. thuringiensis*, we first constructed the pHT315-P_{5014}-1Ac plasmid (Fig. 3a), which contains the P_{5014}-*cry1Ac* fusion. The reaction product Cry1Ac on pHT315-P_{5014}-1Ac is fused protein of N-terminal seven amino acids (1–7) for HD73_5014 and Cry1Ac (Fig. 3a). In parallel, the pHT315-8E-*1Ac* (Zhou et al. 2014) plasmid was used as a control. Subsequently, these two plasmids were introduced into the acrystalliferous HD73$^-$ strain, which could not produce the Cry protein (Lereclus et al. 1989), resulting in the HD$^-$$P_{5014}$-*1Ac* and HD8E-*1Ac* strains (Zhou et al. 2014), respectively. The pHT315-P_{5014}-1Ac plasmid was also introduced into HD*sigK*$^-$ strain (Zhou et al. 2014), resulting in the Δ*sigK*$^-$$P_{5014}$-*1Ac* strain. The formation of crystal proteins was examined by TEM and high-resolution AFM (Fig. 4). In contrast to the HD73$^-$ strain (Fig. 4d), HD$^-$$P_{5014}$-*1Ac* (Fig. 4a), HD8E-*1Ac* (Fig. 4b), and HD73 (Fig. 4c) all could produce clear crystalline inclusions. Interestingly, HD$^-$$P_{5014}$-*1Ac* (Fig. 4a) and HD8E-*1Ac* (Fig. 4b) could not form typical bipyramidal crystals during the sporulation stage, while the typical bipyramidal crystalline inclusion was clear in HD73 (Fig. 4c). For further confirmation, we examined the crystal formation by AFM. Compared with the perfect bipyramidal crystals produced by HD73 (Fig. 4g), we saw imperfect bipyramidal crystals in both the HD$^-$$P_{5014}$-*1Ac* (Fig. 4e) and HD8E-*1Ac* strains (Fig. 4f), some of which had a rounded, blunt shape. These results suggested that the regulation of Cry1Ac expression significantly affected bipyramidal crystal formation.

The Cry protein was detected by performing SDS-PAGE (Fig. 3b). The results showed that HD$^-$$P_{5014}$-*1Ac*, HD8E-*1Ac*, and HD73 all produced the ~ 130-kDa protein Cry1Ac with approximately the same yield, and no ~ 130-kDa proteins were expressed in the control strain HD73$^-$. In the Δ*sigK* strain, the production of Cry1Ac was dramatically decreased as that the native promoter of *cry1Ac* gene was partially σ^K dependent (Zhou et al. 2014), whereas the *sigK*$^-$$P_{5014}$-1Ac produced ~ 130-kDa Cry1Ac in a large amount (Fig. 3d), indicating that P_{5014} could efficiently direct Cry1Ac expression. This result suggested that P_{5014} may be applied to achieve high yield of the crystals in the *sigK* gene deletion background.

We also performed immunoblot analysis to investigate the specific Cry1Ac proteins produced in different strains using antibodies against Cry1Ac. Protein was extracted from stationary phase cells grown in SSM and tested for Cry1Ac. As expected, ~ 130-kDa Cry1Ac proteins were found produced by the HD$^-$$P_{5014}$-*1Ac*, HD8E-*1Ac*, and HD73 strains, but not by HD73$^-$ (Fig. 3c). Thus, all results showed that the promoter

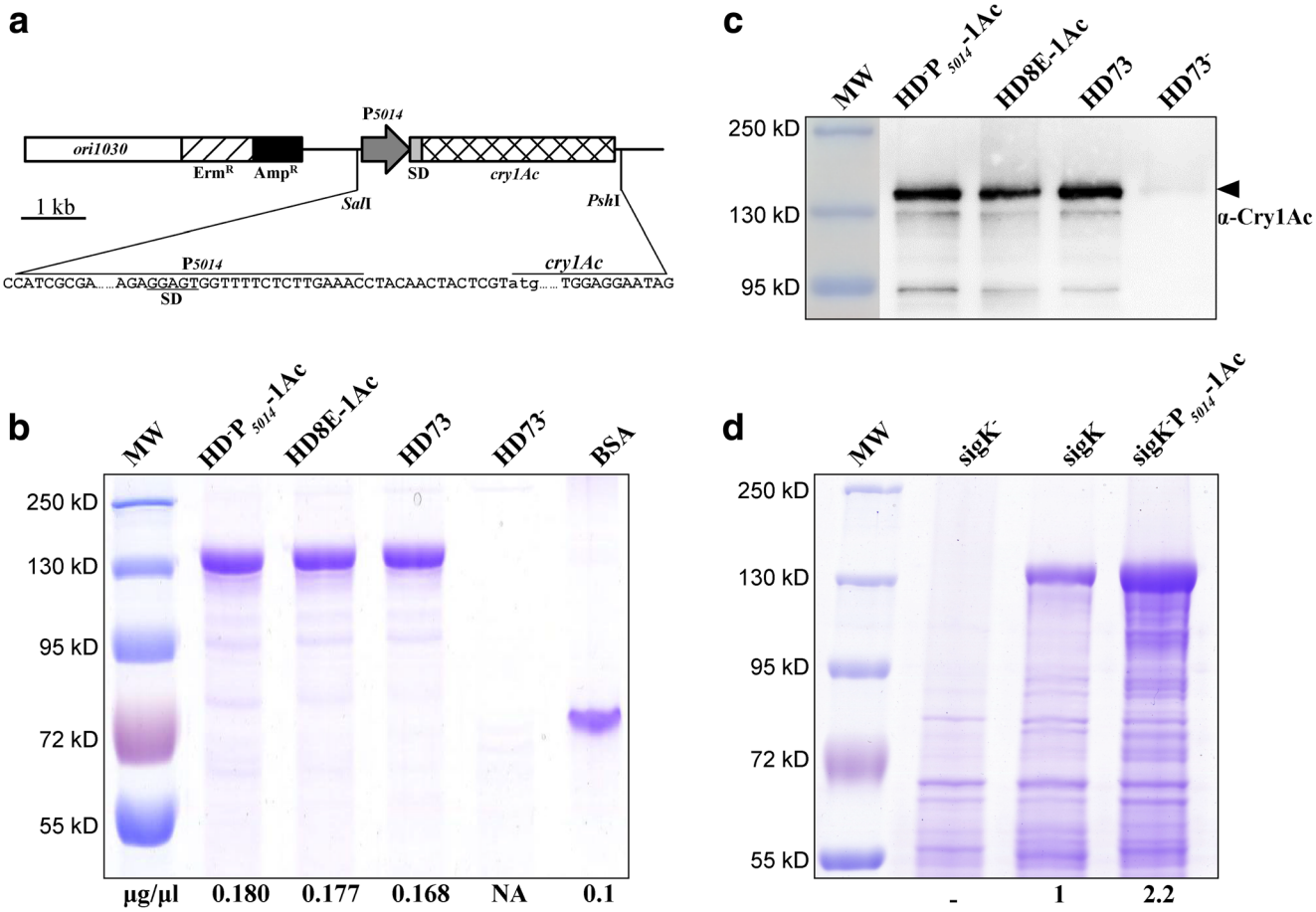

Fig. 3 The *HD73_5014* promoter drives Cry1Ac expression with high efficiency in *B. thuringiensis*. **a** Physical map and expression region sequence of pHT315-P_{5014}-*1Ac*. **b** SDS-PAGE analysis of Cry1Ac expression in the HD$^-$$P_{5014}$-1Ac, HD8E-1Ac, HD73, and HD73$^-$ strains. The numbers below indicate the Cry1Ac yield in the four strains after normalization to 0.1 μg/μl BSA. **c** Comparison of Cry1Ac levels detected by western blotting using a specific antibody against Cry1Ac. Shown is a comparison of Cry1Ac levels from HD73$^-$ with those of HD$^-$$P_{5014}$-*1Ac*, HD8E-*1Ac*, and HD73 in liquid shaking SSM during stationary phase. The triangle points to the Cry1Ac-specific band. **d** SDS-PAGE analysis of Cry1Ac expression in strains *sigK*$^-$, HDΔ*sigK*, and *sigK*$^-$$P_{5014}$-1Ac. The relative protein yield of Cry1Ac was calculated with Image J software

of the *HD73_5014* gene could direct *cry1Ac* gene expression correctly and efficiently.

The *HD73_5014* promoter drove high expression of the insecticidal Cry1Ac protein in *B. thuringiensis*

HD$^-$$P_{5014}$-*1Ac* did not produce typical bipyramidal crystals that the wild-type strain (HD73) did, although we saw obvious Cry1Ac protein accumulation in HD$^-$$P_{5014}$-*1Ac* compared with HD73$^-$ (Figs. 3 and 4). Thus, we determined the toxicity of Cry1Ac protein driven by *HD73_5014* promoter to *P. xylostella* in comparison to that of HD8E-*1Ac* (Zhou et al. 2014). The second instar *P. xylostella* larvae were fed cabbage leaves pretreated with HD$^-$$P_{5014}$-*1Ac*, HD8E-*1Ac*, HD73, or HD73$^-$. The toxicity assay was carried out accordingly (see Materials and Methods). Our results showed that the LC_{50} value of HD$^-$$P_{5014}$-*1Ac* against *P. xylostella* was close to those of HD8E-*1Ac* and HD73 (Table 4), indicating that crystals from HD$^-$$P_{5014}$-*1Ac* were capable of killing *P. xylostella* effectively.

Discussion

A principal contribution of this investigation to the literature is the identification of a new strong non-*cry* gene promoter, P_{5014}, which could effectively drive *cry1Ac* expression. This could be important since decoupling high expression of the *cry* genes from their native promoters is particularly useful in engineering Bt-based biological pesticides as we discuss next. The promoter of *HD73_5014* among six candidate genes showed the highest transcriptional activity at T_7 stage by using the *lacZ* reporter fusions (Fig. 1c), which differs from the RNA-Seq and microarray data (Table 3 and S1). One possibility is that RNA-Seq and microarray are the general methods useful for identification of novel transcripts. Differential

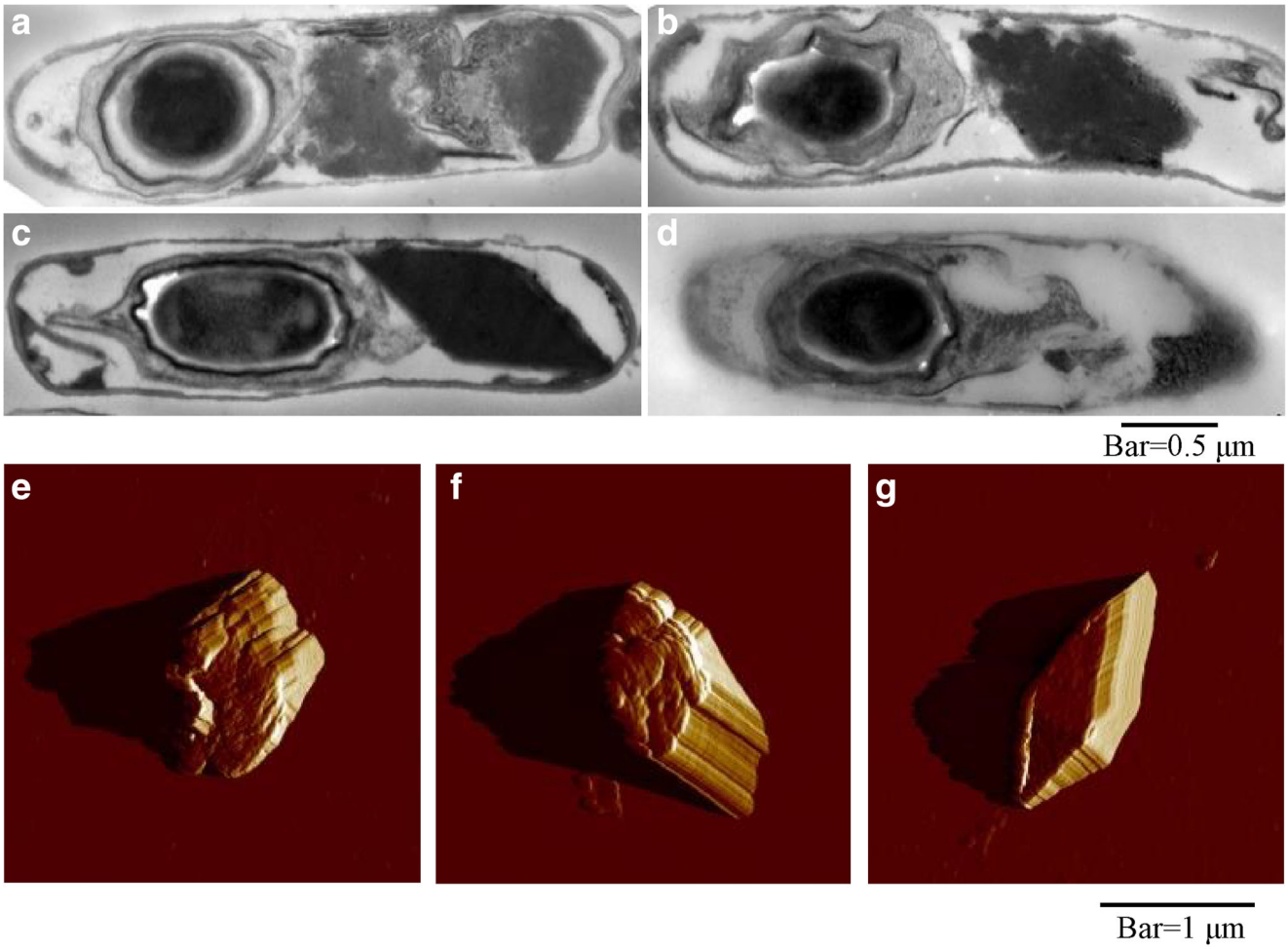

Fig. 4 Cry1Ac expression under different promoters in *B. thuringiensis* strains. **a**–**d** TEM images of different *B. thuringiensis* cells grown for T_{24} in SSM medium at 30 °C. **a** HD^{-}P$_{5014}$-*1Ac* strain. **b** HD8E-*1Ac* strain. **c** HD73, a wild-type strain. **d** HD73^{-}, a negative control strain. **e**–**g** AFM pictures of Cry1Ac crystals in different *B. thuringiensis* strains. **e** HD^{-}P$_{5014}$-*1Ac* strain. **f** HD8E-*1Ac* strain. **g** HD73, a wild-type strain

expression of the genes of interest based on RNA-Seq or microarray has to be further confirmed by other methods such as qRCR or the *lacZ* reporter fusion (Chen et al. 2014). The promoter of the *HD73_5014* gene is a good candidate for directing the Cry1Ac expression since P$_{5014}$ maintains a very high transcriptional activity from exponential phase to stationary phase (Fig. 1c, d).

We showed that the Cry1Ac protein directed by P$_{5014}$ in the engineered strain is functionally similar to that directed by the strong *cry* gene promoter P$_{cry8E}$. This conclusion is supported by the following evidence. Firstly, SDS-PAGE and western blot analysis showed that the Cry1Ac proteins expressed under the *HD73_5014* promoter, the *cry8E* promoter, and the native *cry1Ac* promoter were the same in molecular weight and similar in quantity (Fig. 3). Also, like the wild-type strain, the P$_{5014}$-driven Cry1Ac-producing strain strongly accumulated crystal proteins in the mother cell (Fig. 4). Secondly, the P$_{5014}$-controlled Cry1Ac protein showed similar insecticidal activity against *P. xylostella* as did the P$_{cry8E}$-controlled Cry1Ac and Cry1Ac from the wild-type strain (Table 4).

Table 4 Insecticidal activities of *B. thuringiensis* strains against *P. xylostella*

Strain	LC$_{50}$ (μg/ml)	95% confidence limits
HD50141Ac-315	17.1	12.77–22.93
HD8E1Ac-315	21.63	18.74–25.09
HD73	20.63	18.3–23.19
HD73^{-}	NA	NA

In *B. subtilis*, DeoR (deoxyribonucleoside regulator) is homologous to the sorbitol operon regulator family of metabolic regulators, and contains a C-terminal effector-binding domain and an N-terminal DNA-binding domain (Skerlova et al. 2014). DeoR negatively regulates expression of enzymes involved in the catabolism of deoxyribonucleosides and deoxyribose (Zeng et al. 2000). It is located immediately upstream of the *dra-nupC-pdp* operon, which encodes three enzymes required for deoxyribonucleoside and deoxyribose utilization (Saxild et al. 1996). By sequence analysis, we found that a

similar *dra-nupC-pdp* gene cluster exists in *B. thuringiensis* HD73 genome (Accession No. NC_020238.1). The gene located immediately upstream of this operon is *HD73_2060*, sharing 78% identity with *deoR* of *B. subtilis* 168, indicating that *HD73_2060* is the ortholog of *B. subtilis deoR*. However, *HD73_5014* is quite different from *deoR*, even though its product was annotated as the DeoR family transcriptional regulator. The *HD73_5014* encoded protein is predicted to be 73 amino acids in length and comprises primarily the HTH domain (from amino acid 11 to 49). It shares a very low sequence identity with DeoR (only about 6%) and may not be related with deoxyribonucleoside regulation. *HD73_5014* did not attract much attention in previous studies and its function remains unclear. We plan to determine the function of the *HD73_5014* gene in our follow-up studies.

Many previous studies have focused on improving the yield and stability of insecticidal crystal proteins even after exposure to multiple environmental stresses (Sanchis et al. 1999; Yang et al. 2013; Zhou et al. 2014). One approach taken is to use strong non-*cry* gene promoters to direct expression of the *cry* gene. But only the gene promoters with high transcriptional activity are useful to be selected for directing *cry* gene expression. P_{cry8E} is a relatively strong promoter of the *cry8Ea1* gene and is regulated by both σ^E and σ^H (Du et al. 2012). This promoter was capable of increasing Cry1Ac protein yield in *B. thuringiensis*, especially in the *sigK* mutant (Li et al. 2013; Zhou et al. 2014). This engineered strain is of clear advantage since the P_{cry8E}-driven Cry1Ac expression is at high levels even in the *sigK* mutant while crystals are encapsulated because of lack of mother cell lysis at the end of sporulation due to the *sigK* deletion mutation. Previous work has shown that encapsulation allows better protections from environmental stresses and more sustained insecticidal activities of the crystals when applied in the field (He et al. 2017).

Here, we report that P_{5014} is another candidate of non-*cry* gene promoters with high transcriptional activity and could effectively direct high Cry1Ac expression, similar to the known so far the strongest *cry* gene promoter P_{cry8E} (Fig. 1d and 3). Our results indicated that the *cry* gene expression driven by P_{5014} was higher than that driven by most *cry*-type gene promoters, supporting the idea that non-*cry*-type gene promoter could be an alternative for directing crystal protein expression in the engineered Bt strains. This is particularly useful in the *sigK* mutant background. The deletion of *sigK* gene leads to the encapsulation of Cry1Ac inside the mother cell; however, the Cry1Ac production was dramatically decreased due to the dependence of the *cry1Ac* gene expression on SigK (Fig. 3d). In this study, we showed that the σ^K-independent promoter P_{5014} could direct Cry1Ac expression efficiently in the $sigK^-$ strain, providing an alternative solution of producing high quantity of Cry1Ac in the *sigK* deletion background.

Interestingly, the Cry1Ac protein produced under the control of the P_{5014} and P_{cry8E} promoters could not assemble perfectly to form typical bipyramidal crystals (Fig. 4e–g). This is the first report describing the abnormal 3-dimensional crystal shape of Cry1Ac. Exactly how expression from the *HD73_5014* gene promoter (or P_{cry8E}) led to abnormal assembly of the Cry1Ac protein remains unknown. We speculated one reason was that the Cry1Ac protein expressed under P_{5014} was fused additional seven amino acids of HD73_5014 at N-terminal portion (Fig. 3a). However, Cry1Ac proteins produced from the three strains were very similar in terms of the quantity and insecticidal activity against *P. xylostella* (Table 4). Our data appeared to indicate that the abnormal crystal shape of Cry1Ac did not affect its biological activity. However, since we only tested the insecticidal activity against *P. xylostella* under laboratory conditions, it remains to be determined whether different promoter-driven crystals have the same or different performance in the field.

Taken together, our data provide a good example of Cry1Ac expression using a strong non-*cry* gene promoter in an engineered *B. thuringiensis* strain. The *HD73_5014* gene promoter might also be useful for expressing other *cry* genes. In addition, non-*cry* gene promoters maybe capable of increasing Cry protein yield and insecticidal activity in engineered *B. thuringiensis* strains. The data generated in this study enable us to propose new strategies for improving other biopesticides in future studies and for applications in agriculture.

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Author contributions XZ, JZ, DS, and FS designed the experiments. XZ performed the experiments. TG, QP, and FS analyzed the results. LS analyzed the RNA-Seq data. TG, YC, and FS wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors

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