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Broadening the toxin specificity to control *Spodoptera litura* and *Aedes aegypti* by co-expressing the *cry*2Ab and *cry*4Ba genes from *Bacillus thuringiensis*

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ABSTRACT

Bacillus thuringiensis produces crystal toxins that are specifically toxic to several orders of insect larvae. The Cry2Ab toxin from *B. thuringiensis* subsp. *kurstaki* and the Cry4Ba toxin from *B. thuringiensis* subsp. *israelensis* are highly toxic to lepidopteran and dipteran insect larvae, respectively. The high specificity of Cry toxins leads to a narrow spectrum of toxicity. To broaden the insect specificity, we constructed a recombinant bacterial strain that co-expressed both *cry*2Aa and *cry*4Ba genes from *B. thuringiensis*. The 2.1 kb *cry*2Ab and 3.5 kb *cry*4Ba genes were successfully ligated into the pUC19 plasmid vector under the *lac*Z promoter. The *cry*2Ab and *cry*4Ba genes were expressed as approximately 65-kDa and 130-kDa proteins, respectively. Insecticidal activity assays revealed that the *Escherichia coli* expressing both Cry toxins showed larvicidal activity against both *Spodoptera litura* (lepidopteran insect) and *Aedes aegypti* (dipteran insect) larvae, indicating a broadened range of targeted insects. This finding shows for the first time that genetic manipulation of a bacterial strain can be used to control both an agricultural pest and a human disease vector.

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INTRODUCTION

The use of *Bacillus thuringiensis* (*Bt*), a Gram-positive spore-forming bacteria, as a biological agent has increased because of its higher specificity and lower resistance compared with broad-spectrum chemicals. During the sporulation phase, *B. thuringiensis* produces crystalline inclusion bodies containing δ-endotoxins that are specifically toxic to several orders of insect larvae such as Lepidoptera (butterflies and moths), Diptera (mosquitoes and flies), and Coleoptera (beetles) [1]. The two main families of *B. thuringiensis* δ-endotoxins include Cry (crystal) and Cyt (cytotoxic) toxins [2]. To date, more than 700 Cry toxins and 30 Cyt toxins have been discovered in various *B. thuringiensis* subspecies and classified based on their amino acid sequence identity into 73 Cry groups (Cry1-Cry74) and 3 Cyt groups (Cyt1-Cyt3) [for more information: http://www.btnomenclature.info/].

Each strain of *B. thuringiensis* produces Cry toxins that tend to be specifically toxic to one order of insect. For example, *B. thuringiensis* subsp. *israelensis* produces at least four toxins, including Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa toxins, which are toxic to dipteran insect larvae such as mosquitoes and black flies [3]. In contrast, the *B. thuringiensis* subsp. *kurstaki* produces Cry1Aa, Cry1Ab, Cry2Aa and Cry2Ab, which are highly toxic to lepidopteran insect larvae. Synergistic effects of the *B. thuringiensis* Cry toxins in a specific species of insect are frequently reported. The phenomena of synergism in toxicity was reported for the combination of Cry1Aa and Cry1Ac proteins, which are toxic to the maize stem borer (lepidopteran insect) [4], and for the combination of the Cry4Ba and Cyt2Aa, which are toxic to *A. aegypti* and *Culex quinquefasciatus* larvae (Dipteran insect) [5].

The 130-kDa Cry4Ba toxin has been shown to be toxic to *A. aegypti* and *Anopheles dirus* but not to *C. quinquefasciatus* [6]. The 65-kDa Cry2Ab toxin has been previously reported to be toxic only to lepidopteran insects and not dipteran insect larvae [7]. However, a recent report revealed that Cry2Ab was also toxic to

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dipteran insect larvae including *A. gambiae* but not to *A. aegypti* or *C. pipiens* [8]. The high specificity of *B. thuringiensis* Cry toxins leads to a narrow larvicidal activity spectrum. Broadening the insect spectrum by combining *B. thuringiensis* Cry toxins, which are specific to different insect orders, has rarely been reported. Yue *et. al*. integrated the coleopteran-specific *cry*3A gene into the chromosomal DNA of the lepidopteran-toxic *B. thuringiensis* subsp. *kurstaki*, resulting in high toxicity of the recombinant strain to both *Plutella xylostella* (lepidopteran) and *Rhyllodecta vulgatissima* (coleopteran) insect larvae [9].

In Thailand, *A. aegypti* is a primary carrier for Dengue fever, a major threat for public health, whereas *Spodoptera litura* is an insect pest for agricultural crops. Using biological methods such as *B. thuringiensis* to control these insect species is an alternative to using chemicals. In this study, we constructed a recombinant bacterial plasmid that expressed *B. thuringiensis* toxins, which are toxic to the insect larvae of both species. Here, we showed for the first time that co-expression of the *cry*4Ba and *cry*2Ab genes could broaden the host-range specificity against *A. aegypti* and *S. litura*.

MATERIALS AND METHODS

Bacterial strains:

B. thuringiensis subsp. *kurstaki* and subsp. *israelensis* supplied by the Thailand Institute of Scientific and Technological Research (TISTR) culture collection were used as the source for the *cry*2Aa and *cry*4Ba genes, respectively. *E. coli* strain JM109 was used as a host for recombinant protein expression.

Total DNA extraction from B. thuringiensis:

Total DNA containing both genomic and plasmid DNA was extracted and purified from *B. thuringiensis* subsp. *kurstaki* and subsp. *israelensis* according to the following steps. A loopful of *B. thuringiensis* was transferred to 3 ml of LB medium and cultured overnight in an incubator shaker (200 rpm, 37°C). One and a half milliliter of the cell culture was transferred to a microtube, and the cells were harvested by centrifugation at 9,000 x g for 10 min. The cell pellet was resuspended in 1.5 ml of pre-cooled buffer (10 mM Tris·HCl, pH 8.0; 150 mM NaCl; and 10 mM EDTA, pH 8.0). The cell suspension was then centrifuged, resuspended in 0.7 ml of lysis buffer (20 mM Tris·HCl, pH 8.0; 10 mM EDTA; 50 mM NaCl; and 4 mg/ml of lysozyme), vortexed thoroughly and incubated for 20 min at 70°C. After cooling on ice for 2 min, 0.7 ml of water-saturated phenol was added to the lysate, the sample was centrifuged at 15,000 x g, and the upper phase was transferred to a fresh microtube. Then, 0.6 ml of chloroform : isoamyl alcohol (24:1) was added to the sample, and the sample was vortexed and phase-separated by centrifugation at 15,000 x g. The upper phase was collected, 5 µl of RNaseA (10 mg/ml) was added, and the samples were incubated for 15 min at 37 °C. Total DNA was precipitated with 95% EtOH, and the DNA pellet was dissolved with 100 µl of TE buffer (10 mM Tris·HCl, pH 7.6 and 1 mM EDTA, pH 8.0)

Construction of recombinant plasmids harbouring the cry2Aa and cry4Ba genes:

A pair of *cry*2Ab and *cry*4Ba gene specific primers (Table 1) (Biobasic) were used to amplify the 2.1 kb *cry*2Ab gene from *B. thuringiensis* subsp. *kurstaki* and the 3.5 kb *cry*4Ba gene from *B. thuringiensis* subsp. *israelensis*. PCR was performed using the high-fidelity Phusion® DNA polymerase (NEB). The 2.1-kb PCR products of the *cry*2Ab gene and 3.5-kb PCR products of the *cry*4Ba gene were double digested with *Kpn*I-*Bam*HI and *Bam*HI-*Hind*III, respectively. After digestion, the PCR products of the *cry*2Ab and *cry*4Ba genes were gel-purified using the PCR product cleaning kit (Vivantis) prior to sequential ligation into the *Kpn*I-*Bam*HI and *Bam*HI-*Hind*III sites of pUC19 vector, respectively. The recombinant plasmid harbouring both of the *cry* genes was transformed into *E. coli* JM109. Plasmid DNA was extracted from selected clones and analysed by restriction enzyme digestion. The nucleotide sequences of both genes were verified by DNA sequencing (Macrogen).

Table 1: Primers used to amplify *cry*2Ab gene from *B. thuringiensis* subsp. *kurstaki* and *cry*4Ba gene from *B. thuringiensis* subsp. *israelensis* for the recombinant plasmid pCry4B-2A construction

Gene	Primer name	Sequence	Restriction enzyme	Product size
*cry*2Ab	*cry*2Ab-F	5′-AAAAGGATCC*GCGTATAACAAAAGTGAGAA*-3′	*Bam*HI	2.1 kb.
	*cry*2Ab-R	5′-AAAAGGTACC*TTAATAAAGTGGTG(A/G)AA(T/G)(A/T)TTAG*-3′	*Kpn*I	
*cry*4Ba	*cry*4Ba-F	5′-AAAAAGCTT*GAATTGTTCATAGGAATCCG* -3′	*Hin*dIII	3.5 kb.
	*cry*4Ba-R	5′-AAAAGGATCC *TTAATCACTCGTTCATGCAAAT*-3′	*Bam*HI	

* Bold letters indicate an additional recognition site
* Underline signifies an added nucleotides
* Double underline signifies stop codon
* Italic letter denotes gene-specific sequence

Co-expression of the cry2Ab and cry4Ba genes:

The transformant harbouring the recombinant plasmids was grown at 37°C with shaking (200 rpm) in 100 ml LB broth containing 100 µg/ml ampicillin until the OD_{600} reached 0.3-0.4. The expression of the *cry*2Ab and *cry*4Ba genes under the *lac*Z promoter was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM for 4 h. The induced cells were harvested by centrifugation at 9,000 x g for 10 min, and the expression of the recombinant proteins was analysed by sodium dodecyl sulphate-(10% w/v) polyacrylamide gel electrophoresis (SDS–PAGE).

Western blot analysis:

Proteins from the recombinant strain that were separated by polyacrylamide gel electrophoresis were transferred onto nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM glycine, and 0.04% SDS in 20% methanol). The nitrocellulose membranes were incubated with blocking solution (5% skimmed milk in 1x PBS buffer) for 30 min. The membranes were then incubated with either rabbit anti-Cry4B or Cry2A primary antibody (1:1000 in blocking solution) for 1 h. After three washes with PBS-Tween (0.1% tween 20 in PBS), the membranes were incubated with alkaline phosphatase-conjugated anti-rabbit IgG antibody (Amresco) (1:5000 in blocking solution). After washing once with PBS-Tween and three times with PBS, the immunoreactive bands were visualised by the addition of 25 mg/ml of 5-bromo-4-chloro-3-indolylphosphate (BCIP) and 50 mg/ml of nitro blue tetrazolium (NBT) in 0.1 M carbonate buffer (pH 9.2).

Larvicidal activity assay:

The *E. coli* cells expressing the Cry2Ab and Cry4Ba toxins were tested for their larvicidal activity against *S. litura* (lepidopteran insect) and *A. aegypti* (dipteran insect) larvae and compared with the toxicity of *B. thuringiensis* subsp. *kurstaki* and subsp. *israelensis*. The larvicidal activity assays were performed at room temperature. To determine the toxicity against *S. litura* larvae, *B. thuringiensis* and the induced *E. coli* cells were individually spread on the surface of an artificial diet with varying concentrations (10^3-10^8 cells/piece [approximately 1 cm^2]) in a 12-well plate, and one second-instar *S. litura* larva was added to each well. The mortality was recorded after incubation for 24 h. Thirty larvae were used in each experiment, and three independent replications were performed. For the toxicity assay against *A. aegypti* larvae, bioassays were performed using 2-day-old *A. aegypti* mosquito larvae (supported by the Ministry of Public Health, Thailand). The bioassays were performed in 1 ml of cell suspension containing either *E. coli* expressing Cry toxins or *B. thuringiensis* (10^3-10^8 cells suspended in distilled water) in a 48-well microtitre plate containing 10 *A. aegypti* larvae per well. A total of 100 larvae were tested for each experiment, and three independent replications were performed. *E. coli* cells containing the pUC19 vector were used as a negative control. Mortality was recorded after incubation for 24 h. The lethal concentration 50 (LC_{50}) was calculated using the Probit analysis in the SPSS v.16.0 software.

RESULTS AND DISCUSSION

Construction and characterisation of the recombinant plasmids containing the cry2Ab and cry4Ba genes:

In Thailand, we face serious problems concerning the outbreak of *A. aegypti*, a vector of Dengue fever, and *S. litura*, an important agricultural pest insect. Microbial control using *B. thuringiensis* is an alternative way to control the insect larvae. Previous reports have shown that the Cry2Ab toxin from *B. thuringiensis* subsp. *kurstaki* and the Cry4Ba toxin from *B. thuringiensis* subsp. *israelensis* are highly toxic against *S. litura* and *A. aegypti*, respectively [5, 10]. To achieve a broad spectrum of larvicidal activity against these insects, both the *cry*2Ab and *cry*4Ba genes were chosen for incorporation into a single constructed plasmid. The 2.1 kb *cry*2Ab gene from *B. thuringiensis* subsp. *kurstaki* and the 3.5 kb *cry*4Ba gene from *B. thuringiensis* subsp. *israelensis* were amplified, and the PCR products were then sequentially ligated into the pUC19 plasmid, resulting in the recombinant plasmid pCry4B-2A (Fig. 1A). The recombinant plasmid was analysed by restriction enzyme digestion, which revealed that the size of the recombinant plasmid was approximately 8.3 kb (Fig. 1B) and composed of 2.7 kb pUC19, 2.1 kb *cry*2Ab and 3.5 kb *cry*4Ba fragments (Fig. 1C). Nucleotide sequences of the 2.1 kb and 3.5 kb fragments showed 100% identity to the *cry*2Ab and *cry*4Ba genes, respectively (data not shown). This result indicated that the *cry*2Ab and *cry*4Ba genes were successfully ligated into the pUC19 and could be used in further experiments.

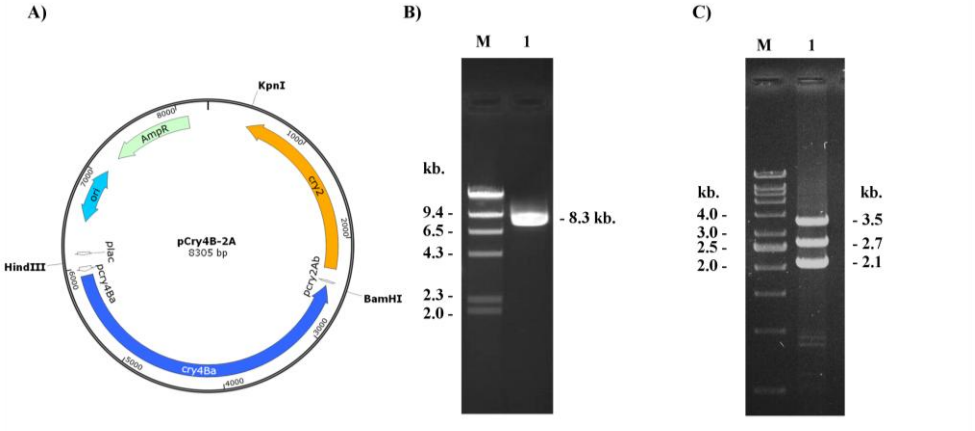

Fig. 1: (A) Map of the recombinant plasmid pCry4B-2A containing the *cry*4Ba gene from *B. thuringiensis* subsp. *israelensis* and the *cry*2Ab gene from *B. thuringiensis* subsp. *kurstaki*. (B) The restriction digestion analysis of the recombinant plasmids with *Bam*HI and (C) with *Hin*dIII, *Bam*HI and *Kpn*I. Lane M represents the standard DNA marker.

Co-expression of the cry2Ab and cry4Ba genes:

Previous works have revealed that the *cry*2Ab and *cry*4Ba genes under the control of their native promoter could be expressed in *E. coli* [6, 11]; however, the expression level was lower than using *lac*Z and T7 promoters [5, 11]. In this work, the *cry*2Ab and *cry*4Ba genes were cloned together downstream of the *lac*Z promoter in the pUC19 vector. After induction with 1 mM IPTG, the induced cells were analysed for the expression of the Cry2Ab and Cry4Ba toxins. SDS-PAGE analysis showed the expression of approximately 65-kDa and 130-kDa bands corresponding to Cry2Ab and Cry4Ba, respectively (Fig. 2A), indicating that both Cry toxins were expressed in the *E. coli* cells. It should be noted that the expression level of the *cry*4Ba gene was lower than that of the *cry*2Ab gene. Because the molecular weight of Cry2Ab is about half that of the Cry4Ba protein, production of Cry2Ab should produce twice as much Cry2Ab as Cry4Ba. This agrees with a previous report that demonstrated that the Cry4Ba gene showed lower expression than proteins of lower molecular weight [5, 12, 13]. The expression of the Cry2Ab and Cry4Ba proteins was further confirmed by Western blot assay. The immunoreactive bands revealed the expression of both toxins in the recombinant strain (Fig. 2B, 2C).

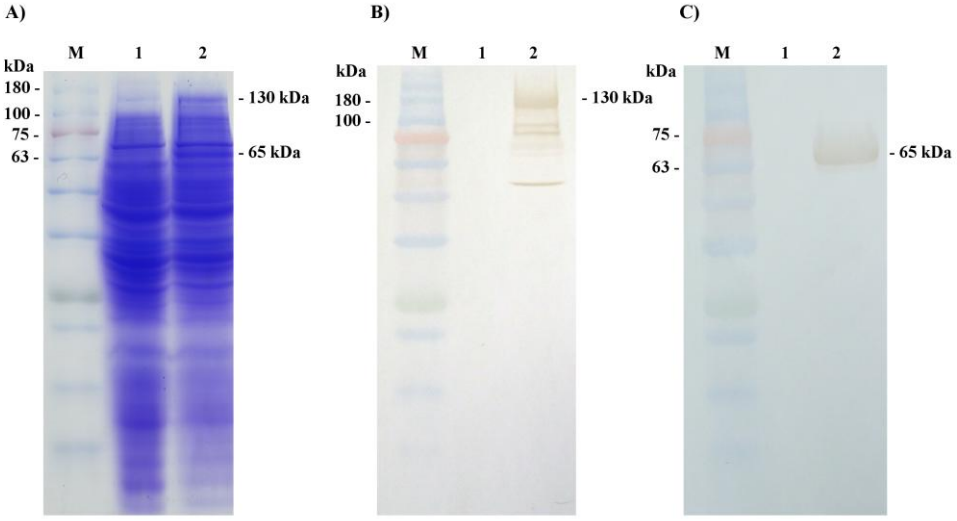

Fig. 2: (A) Expression analysis of the recombinant *cry*4Ba and *cry*2Ab genes in *E. coli*; lane 1 is uninduced and lane 2 is induced with 1 mM IPTG. *E. coli* cells containing the recombinant plasmid (pCry4B-2A) were analysed by SDS-PAGE (10% gel). Approximately 10^7 cells were loaded in each lane. (B) The immunoblot analysis for the expression of the *cry*4Ba gene using an anti-Cry4Ba antibody and (C) the immunoblot analysis for the expression of the *cry*2Ab gene using an anti-Cry2Ab antibody. Lane M represents the standard protein marker.

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Larvicidal activity assay:

The larvicidal activity of the *E. coli* cells expressing the Cry2Ab and Cry4Ba toxins was performed against *S. litura* and *A. aegypti* larvae. The bioassay results clearly showed that the recombinant strain exhibited a broad range of toxicity against both *S. litura* and *A. aegypti* larvae with a lethal concentration 50 (LC_{50}) of approximately 2.1 x 10^7 cells/ml and 1.4 x 10^5 cells/ml, respectively. As expected, the larvicidal activity of the recombinant *E. coli* cells was lower than that of *B. thuringiensis* subsp. *kurstaki* and *israelensis* (Table 2), which are the original sources of the *cry* genes in this study. This result might be due to the lower expression levels of the recombinant Cry toxins in *E. coli* compared with expression in their native host; this idea is supported by a report that revealed that the Cry1B gene that showed approximately 1000-fold greater expression in *Bacillus* than in *E. coli* [14]. In addition, it is also possible that the lower expression level is due to the expression of multiple heterologous genes in an *E. coli* strain that usually had lower expression levels comparing with that of a single gene. Tanapongpipat *et al*. (2003) reported that a *cry*4Ba gene showed high expression levels as a single gene in *E. coli* compared with the expression of multiple genes [6]. The increased larvicidal activity of *B. thuringiensis* compared with the recombinant *E. coli* expressing the *cry* genes might be due to the synergistic effect of toxins produced in each strain of *B. thuringiensis*. Previous reports confirm that synergism in the toxicity of Cry4Ba and Cyt toxins in *B. thuringiensis* subsp. *israelensis* against *A. aegypti* and between Cry1Ac and Cry2Ab in *B. thuringiensis* subsp. *kurstaki* against *Earias vitella* has been observed [5, 15]. However, no synergistic effect has been reported for the combination of the Cry4Ba and Cry2Ab toxins.

Table 2: Larvicidal activity assay against *S. litura* and *A. aegypti* larvae

Strain	LC_{50} (cells / ml)	
	S. litura	*A. aegypti*
B. thuringiensis subsp. *kurstaki*	3.9 x 10^4	Non-toxic[a]
B. thuringiensis subsp. *israelensis*	Non-toxic[a]	1.0 x 10^3
E. coli + pCry4B-2A	2.1x10^7	1.4 x 10^5

[a]No toxicity was detected when using a concentration of 1 x 10^8 cells/ml

Conclusion:

The results from this work revealed for the first time that broadening the toxicity spectrum against agricultural insect pests and a mosquito vector could be achieved by co-expressing the *cry*2Ab and *cry*4Ba genes in *E. coli*. Plans for future studies include improving the larvicidal activity of this recombinant strain by optimising the expression of the insecticidal toxins.

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