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Original Article

Isolation, toxicity and detection of *cry* gene in *Bacillus thuringiensis* isolates in Krabi province, Thailand

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Abstract

One hundred twenty one isolates of *Bacillus thuringiensis* were isolated from 91 soil samples collected in the national park and wildlife sanctuary in Krabi province. All isolates of *B. thuringiensis* were tested for their insecticidal activity against *Spodoptera litura*, *S. exigua* and *Plutella xylostella* larvae. Seven isolates of *B. thuringiensis* named JCPT7, JCPT16, JCPT18, JCPT64, JCPT68, JCPT74 and JCPT89 exhibited toxic activities against the insects, more than 90% mortality. The detection of *cry* gene of these isolates was done by a method based on polymerase chain reaction (PCR). The PCR result indicated that *cry*1Ab, *cry*1Ac, *cry*1C, *cry*1D, *cry*1I, *cry*9A, *cry*9B and *cry*2A were on chromosomal DNA and *cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1C, *cry*1D, *cry*1I and *cry*2A were on plasmid DNA. This study has introduced the promising *B. thuringiensis* isolates collected from soil samples which could be developed as an effective biocontrol agent for Lepidopterous pest.

Keywords: *Bacillus thuringiensis*, toxicity, *cry* gene, biopesticide

1. Introduction

The Gram-positive soil bacterium *Bacillus thuringiensis* produces parasporal crystalline inclusion bodies which contain one or more Cry protein that can be toxic for a number of insects, especially the important insect pest. Cry protein are codified by *cry* genes and, up todate, more than 200 *cry* genes have been described and classified into a large number of groups and subgroups due to their amino acid sequence homology (http://www.lifesci.sussex.ac.uk /Home/ Neil_ Crickmore/Bt/)

B. thuringiensis shows genetic diversity with different toxic potential mostly due to plasmid exchange between strains (Thomas *et al*., 2001). In each habitat may contain a novel *B. thuringiensis* strain awaiting discovery which is toxic on a target insect group. Therefore, *B. thuringiensis* strains have been collected from different environments and characterized to evaluate their toxic potential against various insect orders (Uribe *et al*., 2003).

In this work, we isolated *B. thuringiensis* from Krabi province, selected the high efficacy isolates against Lepidopterous pest, *Spodoptera litura*, *S. exigua* and *Plutella xylostella*, and investigated their *cry* genes using PCR techniques.

2. Materials and Methods

2.1 Sample collection and isolation

Soil samples were collected from a national park and wildlife sanctuary in Krabi province. They were taken by scraping off soil surface with sterile spatula and then obtaining a 200 g sample, 1 inch below the surface. These samples were kept in plastic bags.

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2.2 Isolation of *B. thuringiensis*

Isolation of *B. thuringiensis* was done according to the method previously described by Attathom *et al*. (1994). Approximately 0.5 g of each sample was suspended in 4.5 ml of sterile distilled water and mixed vigorously by vortexing. Ten-fold serial dilutions with sterile distilled water were plated on Nutrient Agar (NA). Bt-like colonies, which are usually described as cream-colored and have the appearance of a fried egg on a plate, were labeled and subcultured. Gram positive reaction and catalase production were observed in 24-hour cultures. Endospore and crystal protein were observed after 72 hours under a phase-contrast microscope. Morphological differences among crystal proteins were examined by scanning electron microscope following the method described by Attathom *et al.* (1994). The Bt index was calculated for each sample as the number of isolates of *B. thuringiensis* / number of isolates of sporulated bacilli. After identification as *B. thuringiensis*, the colonies were inoculated onto nutrient agar and stored as stock culture for insecticidal activity test.

2.3 Insect Bioassay

The toxicities of different *B. thuringiensis* isolates were screened on neonate larvae of *Spodoptera litura* and *S. exigua* and second-instar larvae of *Plutella xylostella* as described by Chanpaisaeng *et al*. (1996). In the first screening, the bioassay was performed with highly concentrated of spore-crystal suspension. For each isolate, 30 neonate larvae were used. Mortality was observed after 3 days. Bioassay was replicated with the most active isolates and LC_{50} values were determined for the most active isolates. Three dilutions were used for each isolate and 30 neonate larvae were used for each dilution. Mortality was observed after 3 days and the LC_{50} were obtained by probit analysis (Finney, 1971)

2.4 *cry* gene detection by PCR technique

Genomic DNA and plasmid DNA were extracted following the method described by Harwood and Cutting (1990) and Birnboim and Doly (1979), respectively. PCR amplication was carried out in a 25 ml reaction volume; 1 ml of DNA, 1x buffer deoxynucleoside triphosphate (0.2mM each), oligonucleotide primer (0.2mM each) and *Taq* polymerase (5U). The 24 specific primer pairs as described by Juárez-Pérez *et al*. (1997) were used for identification of *cry*1 *cry*2 *cry*5 *cry*13A and *cry*14 gene families.

Template DNA was preheated at 94°C for 5 min. Then it was denatured at 94°C for 1 min, annealed to primers at 45°C for 45 sec and extensions of PCR products were achieved at 72°C for 2 min. The PCR was done for 30 cycles. The PCR products were separated on a 1% agarose gel.

3. Result and Discussion

3.1 Isolation of *B. thuringiensis*

Ninety-one soil samples were isolated for *B. thuringiensis*. The result revealed that *B. thuringiensis* was ubiquitously distributed in the soil of national park and wildlife sanctuary in Krabi province. A total of 121 isolates were obtained from 859 bacterial colonies examined (Table 1). These colonies produced both spore and crystal protein.

Crystal morphology were classified into four groups; 62 (51.24%) isolates had bipyramidal crystal protein in exosporium, 35 (28.93%) isolates had bipyramidal which varied greatly in size, 20 (16.53%) isolates had polymorphic crystals, composed of a mixture of cubidal, round, amorphous and bipyramidal crystal, and 4 (3.35%) isolates had small bipyramidal crystal protein (Figure 1)

B. thuringiensis isolates were present in samples from all sites except Khao Pra Bang Khram Wildlife Sanctuary. *B. thuringiensis* was isolated from all of the samples from Than Bokkhorani National Park (the highest Bt index, 0.380), a heterogeneous territory which gives unique geographical feature and abundant biological resource. We could not find *B. thuringiensis* isolates from Khao Pra Brag Kram Wildlife Sanctuary. This area might be disturbed by travellers and type of soil samples were sandy soil which limited survival of spores, containing smaller amount of nutrient and water. Hossain *et al*. (1997) reported that properties of the soil could affect the abundance and distribution of *B. thuringiensis* and demonstrated that the *B. thuringiensis* population presence

Table 1. Distribution of *B. thuringiensis* isolates from soil sample collected in Krabi province

Location	Number of samples		Number of colonies		Bt index
	Examined	With Bt	Examined	With Bt	
Khoa Pla Bang Kram Wildlife Sanctuary	10	0	108	0	0.000
Plai Praya Wildlife Sanctuary	17	1	110	1	0.009
Huayto Waterfall	20	5	201	6	0.030
Tung Ta Lee Wildlife Sanctuary	12	1	145	2	0.015
Than Bokkhorani National Park	20	9	163	62	0.380
Hat Noppharat Thara-Phi Phi National Park	10	9	142	50	0.352
Total	91	32	859	121	0.14

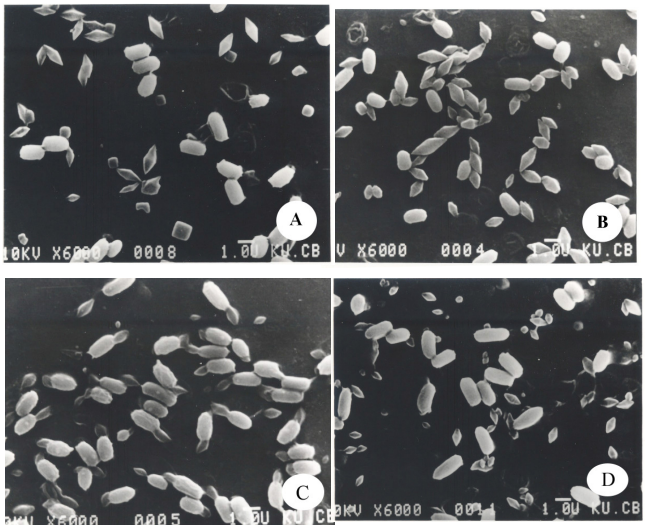

Figure 1. Scanning electron microscopy of crystal protein. A; isolates had bipyramidal crystal protein in exosporium, B; isolates had bipyramidal which varied greatly in size, C; isolates had polymorphic crystals, composed of a mixture of cubidal, round, amorphous and bipyramidal crystal, and D; isolates had small bipyramidal crystal protein

in the soil samples were affected negatively by soil sand percentage. Soil was very important source of *B. thuringiensis* strain providing a large genetic resource for its use in the development of bioinsecticide to control insect pests that have not previously reported to be susceptible to *B. thuringiensis* (Quesada-Morage *et al*., 2004).

The Bt index from Krabi province ranged from 0.009 to 0.380 in the soil samples studied. Very different values were frequently reported in several studies to range from 0 to 0.2 in United States (DeLucca *et al*., 1981), 0.2 to 0.5 in New Zealand (Chilcott and Wigley, 1993) and 0.75 in Bangladesh (Hossain *et al.,* 1997). Vilas-Bôas amd Manoel (2004) suggested the Bt index may be a consequence of biotic environmental factor, e.g., microorganism in the soil, the type of insect commonly found in the area, or the vegetal top besides, abiotic factors such as the pH, texture, oxygen and nutrient availability, temperature, and humidity. For this reason they avoided comparing their result with those of other authors, as they considered that the procedures of collection of sample, storage, processing, and identification of the bacteria are influence by the experience of the working group and this may influence the result.

3.2 Insect bioassay

According to the LC_{50} values, the most effective isolates against *P. xylostella* was the JCPT74 that showed a value of LC_{50} $1.13x10^{-2}$ spore/ml (Table 2). The LC_{50} values were lower than previously reported using same methodology in this study. Chanpaisaeng *et al*. (2001) was founded the high effectiveness of *B. thuringiensis* in killing Diamondback moth named JC150 (*B. thuringiensis* subsp. *galleriae*) and JC590 (*B. thuringiensis* subsp. *kurstaki*) which showed LC_{50} at $1.140x10^{4}$ and $4.470x10^{3}$ spores/ml, respectively. Chowanadisai *et al*. (1995) found highly effective isolates for controlling aedinine mosquitoes larvae, namely S-KB1802, S-KB1001 and S-KB2701, which showed LC_{50} at $1.28x10^{2}$, $3.59x10^{2}$ and $9.80x10^{2}$ spores/ml, respectively. The LC_{50} of high toxicity Bt isolates obtained from this study is lower than the previous report as described above. This indicated the potential of this isolates as bioinsecticide against *S. litura, S. exigua* and *P. xylostella.*

Table 2. LC_{50} of *Bacillus thuringiensis* isolates against second instar of *Plutella xylostella*

Isolates	LC_{50}
JCPT 7	4.08×10^{-2}
JCPT 16	4.19×10^{-1}
JCPT 18	4.11×10^{-3}
JCPT 64	5.26×10^{-1}
JCPT 68	2.06×10^{-1}
JCPT 74	1.13×10^{-2}
JCPT 89	2.98×10^{-1}

3.3 Detection of *cry* gene by PCR technique

Identification of *cry* gene was performed with a set of primers amplifying *cry*1, *cry*2, *cry*5, *cry*13A and *cry*14 families which produced toxic proteins to Lepidoptera (Crickmore *et al*., 1998). Results are shown in Table 2. *cry*1Aa *cry*1I and *cry*2A genes were present in all isolates but one (JCPT64) found only *cry*9A and *cry*9B genes. The toxicity of effective isolates can be explained by the production of the *cry* genes that have been reported by many researchers. Tabashnik *et al*. (1994) reported that Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry1C, Cry1F and Cry2A were highly toxic to *P. xylostella*. Porcar *et al*. (2000) reported that Cry1C, Cry1D, Cry1E, Cry1F and Cry9C were highly toxic towards *S. exigua, S. littoralis* and *S. frugiperda*. Song *et al*., (2003) reported that Cry1I was toxic to *P. xylostella*, *Ostrinia furnacalis* and *Maluca testulalis.*

JCPT64 isolates did not amplify for *cry*I genes but detected *cry*9A and *cry*9B genes. Nevertheless JCPT64 had high toxicity to *P. xylostella* because Cry9A and Cry9B were toxic to various Lepidoptera, such as being highly toxic to spruce budworm (*Choristeneura furniferano*) (van Frankenhuyzen *et al*, 1997). Jansen *et al*. (1997) reported Cry9 was toxic to *S. litura, S. exigua, H. armigera* especially *P. xylostella* that is resistant to *cry*1 gene groups. The toxicity of *B. thuringiensis* did not depend on *cry* gene content only because factors other than Cry proteins may contribute to toxicity as well as spore interaction with crystal protein and the other soluble toxins such as β-exotoxin (Porcar *et al*., 2000).

Martinez *et al*. (2004) suggested that the biological activity of a strain cannot be fully predicted on the basis of

Table 3. *cry* gene in *Bacillus thuringiensis* isolates

Isolates	*cry* gene
JCPT7	*cry*1Aa, *cry*1C, *cry*1D, *cry*1I, *cry*2A
JCPT16	*cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1I, *cry*2A
JCPT18	*cry*1Aa, *cry*1Ab, *cry*1C, *cry*1D, *cry*1I, *cry*2A
JCPT64	*cry*9A, *cry*9B
JCPT68	*cry*1Aa, *cry*1Ab, *cry*1C, *cry*1D, *cry*1I, *cry*2A
JCPT74	*cry*1Aa, *cry*1Ab, *cry*1C, *cry*1D, *cry*1I, *cry*2A
JCPT89	*cry*1Aa, *cry*1Ac, *cry*1D, *cry*1I, *cry*2A

its *cry* gene content alone. The relative proportion of the Cry proteins produced, their interaction, and the possible presence of undetected crystal proteins, such as *cry*1I genes are all important. Some investigators have reported that many *cry*1I genes are silent in *B. thuringiensis* strain because these genes are often located downstream of *cry*1 genes and a strong *cry*1 transcriptional terminator is present in the interval sequence between the *cry*1 and *cry*1I gene (Gleave *et al.,* 1993). So PCR information does not allow for direct correlation between PCR information and the biological activity of strain. For quantification of the toxicity, insect bioassay is the only way to assess the potential of a strain for pest control. However, insect bioassay involves high costs and long time in insect mass rearing while PCR technique is the most widely used efficient and rapid determination of the presence or absence of a sequence and it is highly sensitive, relatively fast, and can easily be used on a routine basis (Ceron *et al*., 1994). Thus, for prediction of insecticidal activity by PCR must always be corroborated by bioassay in order to assess the potential of promising isolates as biopesticides.

4. Conclusion

Among 91 soil samples collected from a national park and wildlife sanctuary in Krabi province, *B. thuringiensis* was isolated from 121 isolates. Seven isolates, namely JCPT7, JCPT16, JCPT18, JCPT68, JCPT74 and JCPT89, showed high toxicity to *S. litura, S. exigua* and *P. xylostella*. The detection of *cry* genes in these isolates was done by PCR technique and showed that *cry*1Ab, *cry*1Ac, *cry*1C, *cry*1D, *cry*1I, *cry*9A, *cry*9B and *cry*2A were on chromosome and *cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*1C, *cry*1D, *cry*1I and *cry*2A were on plasmid DNA. The increase in the number of *B. thuringiensis* collections has led to an increase in the discovery of new *B. thuringiensis* isolates with insecticidal activity against a diverse range of insect or with increased insecticidal activity. This should provide a large genetic resource for the utilization of *B. thuringiensis* as a microbial insecticide or for the incorporation of *cry* gene into other organisms for insect pest control.

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