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CHARACTERIZATION OF *CRY2*-TYPE GENES OF *BACILLUS THURINGIENSIS* STRAINS FROM SOIL-ISOLATED OF SICHUAN BASIN, CHINA

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ABSTRACT

Sichuan basin, situated in the west of China, is the fourth biggest basin in China. In order to describe a systematic study of the *cry2*-type genes resources from *Bacillus thuringiensis* strains of Sichuan basin, a total of 791 *Bacillus thuringiensis* strains have been screened from 2650 soil samples in different ecological regions. The method of PCR-restriction fragment length polymorphism (PCR-RFLP) was used to identify the type of *cry2* genes. The results showed that 322 *Bacillus thuringiensis* strains harbored *cry2*-type genes and four different RFLP patterns were found. The combination of *cry2Aa/cry2Ab* genes was the most frequent (90.4%), followed by *cry2Aa* (6.8%) and *cry2Ab* alone (2.5%), and only one novel type of *cry2* gene was cloned from one isolate (JF19-2). The full-length of this novel gene was obtained by the method of thermal asymmetric interlaced PCR (Tail-PCR), which was designated as *cry2Ag1* (GenBank No. ACH91610) by the Bt Pesticide Crystal Protein Nomenclature Committee. In addition, the result of scanning electron microscopic (SEM) observation showed that these strains had erose, spherical, bipyramidal, and square crystal. And the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated that these strains harbored about one to three major proteins. These strains exhibited a wide range of insecticidal spectrum toxic to *Aedes aegypti* (Diptera) and *Pieris rapae Linnaeus,* 1758 (Lepidoptera). Particularly, JF19-2 contained *cry2Ag* gene had the highest insecticidal activity. All these researches mentioned above revealed the diversity and particularity of *cry2*-type gene resources from *Bacillus thuringiensis* strains in Sichuan basin.

Key words: *Bacillus thuringiensis*; PCR-RFLP; SDS-PAGE; novel *cry2*-type gene

INTRODUCTION

Bacillus thuringiensis (Bt) is a typical aerobic, Gram-positive bacterium. During sporulation, Bt produces one or more insecticidal crystal proteins, Cry and Cyt, encoded by the *cry* and *cyt* genes, respectively (20). Up to July 2009, the Cry toxins had been classified into 59 families (i.e., Cry1 to Cry59) based on their amino acid sequence homology

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(http://www.biols.susx.ac.uk/Home/Neil-Crickmore/Bt/index.html). Among them, Cry1-type protein exhibits highly specific toxin to Lepidoptera insects and has been widely applied in transgenic plants. However, there are also some problems such as narrow insecticidal spectrum and insect resistance (2). Isolation and screening novel Bt strains, and cloning novel insecticidal genes are efficient ways to resolve these problems.

The *cry2* genes mainly encode 65-70 kDa proteins, which occur as cuboidal inclusions in many Bt strains (9; 18). Some researchers reported that Cry2-type proteins were different from Cry1-type not only in structure, but also in pesticidal mechanism (8; 16; 21), which can be used as the beneficial gene resource for insect-resistant transgenic plants (4). Many PCR-based methods have been developed to detect *cry2*-type genes (3; 14; 24). Sauka et al. (19) have established a PCR-RFLP method to describe the distribution of *cry2*-type genes profiles from Argentina and one novel *cry2*-type gene was found in their collections. However, to our knowledge, there was only two work described the distribution of *cry2* genes of China (24; 25) and no systematic research on the type of *cry2* genes.

Sichuan basin, the fourth biggest basin in China, is a special area with complicated geomorphology, characteristic of mountain, gorge, highland, hurst, glacier, and plain, and contains a rich and unique biodiversity (13). These distinctive features and diversity of insects provide the opportunity of isolating novel entomopathogenic bacteria, so it is most possible that some novel *cry2* genes or special Bt strains may be found. Our lab had surveyed the distribution of *cry* gene in Sichuan basin (25). In the present study, we specially identify the distribution of *cry2* genes and the type of *cry*2 genes in this basin. Furthermore, a novel *cry2*-type gene was cloned and designated as *cry2Ag1*. In addition, Bt strains harbored *cry2* genes were further characterized by SEM observation, SDS-PAGE analysis, and the testing of insecticidal activity. The results of insecticidal activity showed that these strains exhibited a wide range of insecticidal spectrum toxic to Dipteran (*Aedes aegypti*) and Lepidoptera (*Pieris rapae Linnaeus*, 1758) pests.

MATERIALS AND METHODS

Bacillus thuringiensis strains and Plasmids

In total, 2650 soil samples were collected from different regions with unique geographical features in Sichuan basin and 791 isolates were identified as Bt based on the production of parasporal crystals (Table 1; 25).These Bt strains were cultured at 30 on Luria-Bertani (LB) medium plates {1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0, and 1.5% (w/v) agar}.

The plasmid pGEM-T (Tiangen) and *E. coli* DH5α were used for DNA manipulation

Identify the distribution of *cry2* genes and the type of *cry2* genes in this basin. Bt strains were cultured at 30 for 24 h on LB. A loopfull of Bt cells was transferred to 0.1 ml of H_2O, frozen for 20 min at -70°C, and then boiled for 10 min in water to lyse the cells. Cells were briefly spun (10,000g at 4°C for 10 s), and 15 µl of supernatant was collected for PCR amplification. Based on the conserved regions of each class of *cry2*-type genes, the primers, II (+): 5'-TAAAGAAAGTGGGGAGTCTT-3' and II (-): 5'-AACTCCATCGTTATTTGTAG-3', were used for PCR-RFLP as described by Sauka et al. (19). The expected restriction fragment sizes of the known *cry2*-type genes were determined by *in silico* digestion of their available sequences in the Bt toxin nomenclature website *(http://www.biols.susx.ac.uk/Home/Neil-Crickmore/Bt)* with the software 'DNAStar' (Table 2). The PCR products were digested with *Dde* I enzyme (Takara). PCR products with novel RFLP patterns was cloned to pGEM-T and sequenced by Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd.

Characterization of the strains by scanning electron microscopy (SEM)

Bt strains were grown on LB at 30°C for 72h, and then the spore-crystal mixture was placed on aluminium stubs, which was fixed in 1% OsO_4. Then the sample was sputter-coated with gold in IB-5 ion coater (HITACHI) for 5 min. The SEM

was taken on Zeiss 950 digital scanning microscope at a voltage of 12 kV.

SDS-PAGE analysis. Bt strains were grown in liquid LB medium for 72h (30°C, 220 rpm). Concentrated Bt strain suspensions on disruption buffer were boiled for 5 min, cells were spun at 10,000 x g for 8 min at 4°C, and the supernatant was used to SDS-PAGE analysis as described by Ibarra *et al.* (10).

Cloning the full-length sequence of *cry2*-type gene and sequence analysis

According to the sequencing results, two specific primers: SP1: 5'-GAGACAGGAAGTTGGGCATT-3' and SP2: 5'-AG AAATAAATGTTCGTGTTTGATT-3', and one degenerate primer 5'-GGAGGNNNNNNNWWTG-3' were designed to obtain the full-length of novel *cry* genes using Tail-PCR with the following conditions: 5 min denaturation at 94; 15 cycles of 2 cycles of 94 for 30 sec, 52 for 50 sec, and 72 for 2 min and 1 cycle of 94 for 30 sec, 33 for 50 sec and 72 for 2 min; extension at 72 for 7 min. PCR products were then sequenced. Sequence homology was determined using the NCBI nucleotide-nucleotide BLAST and protein-protein BLAST online services at http://www.ncbi.nlm.nih.gov/BLAST.

Evaluation of insecticidal activity

These strains were tested against *Pieris rapae Linnaeus,* 1758 (Lepidoptera), and *Aedes aegypti* (Diptera). These larvae used in this study were reared in our lab. The bioactivity assay against *Pieris rapae Linnaeus,* 1758 was performed as described by Song *et al.* (22). The first-instar larvae were placed into 100 ml dechlorinated water.

Six concentrations (1μg/ml to 100μg/ml) of the spore-crystal complexes were incorporated into their artificial diet. Insecticidal activity of mosquitoes was assayed as described by Ibarra *et al.* (10). Early four-instar larvae were placed in 100 ml dechlorinated water. Ten concentrations (0.0625 μg/ml to 32 μg/ml) of the spore-crystal complex were added. Larvae were incubated at 28 and examined after 24h. Thirty larvae were used for each treatment. Each treatment was replicated three times. The mean 50% lethal concentration (LC_{50}) was estimated by the software SPSS10.0.

RESULTS

Identification of the distribution of *cry2* genes and the type of *cry2* genes by PCR-RFLP. In the total 791 Bt isolates, 322 amplification products approximately 1.5 kb were obtained using the primers II (+) and II (-), which indicated that these Bt strains contained *cry2* genes. Based on different topographic feature and vegetation, the isolation rates of strains containing *cry2* genes were different (Table 1), which revealed that the distribution of Bt strains with *cry2* gene are diversity in different typically ecological regions.

Table 1. The geographical features of collecting locations and *cry2*-type gene profiles of Bt in Sichuan basin.

Soil sample source	I	II	III	IV	Rate*	Rate#	*cry2* gene profile			
							2Aa/2Ab	*2Aa*	*2Ab*	Novel
Forest	**Mountain[1]**	**850**	**287**	**115**	**40.1**					
	Glacier[2]	**250**	**25**	**3**	**12.5**	**40.5**	**173**	**13**	**2**	**1**
	Hurst[3]	**127**	**45**	**24**	**53.3**					
	Gorge[4]	**320**	**110**	**47**	**42.7**					
Grassland	**Highland[5]**	**105**	**20**	**2**	**10.0**	**11.4**	**2**	**2**	**0**	**0**
	Glacier[2]	**90**	**15**	**2**	**13.3**					
Farmland	**Hurst[3]**	**153**	**35**	**21**	**60.0**					
	Highland[6]	**215**	**98**	**47**	**47.9**	**44.6**	**116**	**7**	**6**	**0**
	Plain[7]	**540**	**156**	**61**	**39.1**					
Total Number		**2650**	**791**	**322**			**291**	**22**	**8**	**1**

Note: I, Site Characteristics; II, Soil Samples; III, Bt Isolations; IV, Bt with *cry2*; [1], include Zhonggong Mountain, Jinfeng Mountain, Muchuan Mountain; [2], Hailuo Glacier; [3], include over twenty hursts distributed in different orientation of Sichuan Basin; [4], Bifeng Gorge; [5], Kangding Highland; [6], Luding Highland; [7], Chengdu Plain; *, Rate of Bt strains harbored *cry2* genes form the same geographical features; #, Rate of Bt strains harbored *cry2* genes from different vegetation.

RFLP analysis of the PCR products demonstrated that four different kinds of PCR-RFLP profiles (digested with *Dde* I) have been detected (Figure 1). Three RFLP profiles were in agreement with the predicted fragment sizes of *cry2Aa* and *cry2Ab*, and one RFLP profile (about 1 kb, 0.35 kb and 0.15 kb) was different from the predicted fragment (Figure 1, Table 2), which revealed that a novel *cry2*-type gene could be found in the this Basin. Overall, the combination of *cry2Aa/cry2Ab* genes was detected in 291 Bt isolates (90.4%), the *cry2Aa* and *cry2Ab* genes alone were found in 22 (6.8%) and 8 (2.5%) Bt isolates, respectively, and the novel PCR-RFLP profile was only found in JF19-2 (Figure 1; Table 1, 2). The PCR product with novel PCR-RFLP profile was cloned into pGEM-T vector and transformed into *E. coli* DH5α. Then the positive clones were sequenced and the sequences were analyzed with 'BLAST' (*http://www.ncbi.nlm.nih.gov/BLAST/*). The result showed that the sequence had maximum 92 % homologous to *cry2Ab1,* which proved that a novel *cry2A*-type gene was found in this basin.

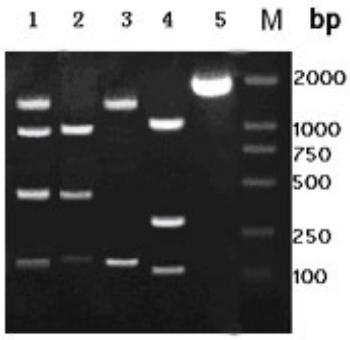

Figure 1. PCR-RFLP patterns of *cry2*-type genes and PCR products of novel *cry* genes.

Note: Lane 1-4, PCR-RFLP patterns of *cry2Aa/cry2Ab*, *cry2Aa, cry2Ab,* and a novel *cry2A*-type gene (obtained from strain JF19-2), respectively; Lane 5, PCR product of *cry2Ag1*; M, D2000 ladder marker.

Table 2. Expected restriction fragment sizes of digested *cry2* genes

Gene	Fragment size (bp) with *Dde* I
cry2Aa	972, 450, 134
cry2Ab	1386, 134, 36
cry2Ac	915, 252, 162, 131, 36, 27
cry2Ad	663, 414, 309, 134, 36
cry2Af	417, 369, 298, 254, 156, 36

Characterization by SEM and SDS-PAGE analysis

Bt strains harboring *cry2*-type genes produced erose, bipyramidal, square, and spherical crystal inclusions under the phase contrast microscopy and scanning electron microscopy observation (Fig. 2A).The SDS-PAGE analysis of their spore-crystal suspensions revealed that there were four different protein profiles, which had one or two major protein bands with the molecular weights ranged from about 60 kDa to 130 kDa (Fig. 2B). All the results showed that there were diversity among potential novel Cry toxins profiles in Sichuan basin and some strains may contain other Cry proteins besides Cry2-type proteins.

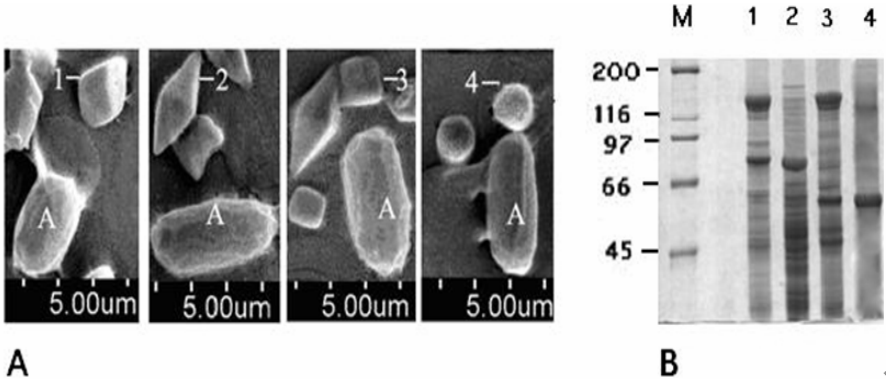

Figure 2. SEM observation and SDS-PAGE analysis of the spore-crystal suspensions of selected Bt strains. (A): SEM observation of the spore-crystal. Note: 1, erose crystal inclusions; 2, bipyramidal crystal inclusions; 3, square crystal inclusions; 4, spherical crystal inclusions; A, spores. FIG.2 (B): SDS-PAGE analysis of the spore-crystal. Note: Lane M, protein Marker; Lane 1-4: Four different protein profiles of Bt strains harboring *cry2*-type genes (lane 1, Ywc5-4; lane 2, JF19-2; lane 3, Bts; lane 4, A1).

Cloning of the full-length sequence of the novel *cry2A*-type gene

In order to obtain the full-length of the novel gene, Tail-PCR upstream and downstream strategy was performed. Then a fragment about 3,285 bp was obtained, which contains the open reading frame of 1,905 nucleotides encoding a polypeptide of 635 amino acid residues with a predicted molecular mass of 70 kDa and isoelectric point of 9.18. Sequence alignment analysis revealed that it corresponds to a putative Cry protein and has maximum 92% identical to Cry2Ab1. According to the nomenclature principles of insecticidal crystal protein from Bt strains, this gene was a holotype *cry* gene, which was designated as *cry2Ag1* (GenBank No. ACH91610) by the Bt Pesticide Crystal Protein Nomenclature Committee.

Insect toxicity assay

The result of bioassay showed that the tested Bt strains were toxic to Lepidopteran and Dipteran pests (Table 3). Particularly, the JF19-2, which contained *cry2Ag* gene, had the highest insecticidal effects against *Aedes aegypti* and *pieris rapae Linnaeus,* 1758.

Table 3. Dose-response insecticidal activities against *A.aegypti* and *Pieris rapae Linnaeus,* 1758

Strains	***cry2*-gene types**	***A.aegypti***		***Pieris rapae Linnaeus***	
		LC_{50}(μg/ml)	**95% CI* (μg/ml)**	**LC_{50}(μg/ml)**	**95% CI* (μg/ml)**
Rpp39	*cry2Aa*	>100[#]		19.15	13.2-27.5
Ywc5-4	*cry2Ab*	23.42	19.86-25.53	13.24	11.02-16.48
Bts	*cryAa/cry2Ab*	17.65	12.64-22.47	13.37	12.09-17.95
JF19-2	*cry2Ag*	2.541	1.707-3.432	7.51	3.42-15.68

Note: "[#]", at this concentration, no mortality was obtained; CI*, confidence interval.

DISCUSSION

In this paper, the presence of certain *cry2*-type genes was analyzed in 791 Bt isolates from different regions and vegetation. And the strains harbored *cry2*-type genes were characterized by the methods of SDS-PAGE and SEM, the results of which indicated that these Bt strains contained various Cry proteins, and also reflected on their insecticidal activity. It suggested the diversity of *Bacillus thuringiensis* strains with *cry2*-type genes in Sichuan basin, China. The results are useful for understanding the distribution of *cry2*-type genes and the features of Bt strains containing *cry2*-type genes in Sichuan basin, which may have important meanings in theories and practices.

Several researches on distribution of *cry* genes have been described in Asia (3; 5; 6; 17). But no systematic research about *cry2* genes in China or basins was done, so it is of interest to determine the distribution feature of *cry2* genes and identify the type of *cry2* genes. In this paper, the presence of *cry2*-type genes was analyzed in 791 Bt isolates from different regions and vegetation in Sichuan basin as described by Sauka et al. (19). The rates of strains containing *cry2*-type gene from different vegetation and ecological region were different (Table 1), which revealed that the distribution of these strains are diversity in different typically ecological regions.

Sichuan basin contains four different types of *cry2* genes, such as the combination of *cry2Aa* and *cry2Ab, cry2Aa, cry2Ab,* and a novel *cry2A*-type gene (Fig. 1). The combination of *cry2Aa/cry2Ab* genes were the most frequent, followed by *cry2Aa* and *cry2Ab* alone, and a novel *cry2*-type gene alone was only in JF19-2 (Table 1), which was conformed to the distribution features of Argentina (19). But Ben-Dov et al. (3) found that Bt isolates from Israel, Kazakhstan and Uzbekistan containing *cry2Ab* alone were the most frequent, followed by *cry2Aa/cry2Ab* and *cry2Ab/cry2Ac*, which revealed that the distribution of *cry2* genes in Sichuan basin was unique compare with other countries of Asian. The profile, *cry2Aa/cry2Ab* genes, was the most frequent kind in this study

(Table 1), which is consistent with a low diversity in the *cry2* content of the isolates from our collection. It is possible that this combination of genes is common in nature, but the biological significance of this association has to be the still studied. And the strains harbored *cry2*-type genes were also characterized by the methods of SDS-PAGE and SEM. The results reveal that Cry2-type toxins profiles harbored in Bt strains in Sichuan basin were diversity and some strains might contain other *cry* genes (Fig. 2B), which suggests that these strains may be promising Bt resource for the controlling of pests. Specially, we detected the insecticidal crystal protein genes harbored in strain JF19-2 by using all the primers based on the conserved regions of each class of *cry* and *cyt* genes as previously described by Sauka *et al.* (19) and Su (22). But only one PCR product about 1.5 kb was obtained using the primers II (+)/II (-) (data not shown), which revealed that this strain may contain only one *cry2* gene. It is consistent with the result of SDS-PAGE analysis (Figure 2B).

The insecticidal activity assay on some Bt strains harbored Cry2 proteins were performed. The results showed that these strains exhibited a wide range of insecticidal spectrum toxic to Dipteran and Lepidopteran pests (Table 3). Especially, JF19-2 exhibited highly larvicidal activity against *Aedes aegypti* (Diptera), and *Pieris rapae Linnaeus,* 1758 (Lepidoptera) (Table 3), which conformed to the fact that Bt strains, contained *cry2A*-type genes, are successfully used as commercial products to control Dipteran and Lepidopteran pests in agriculture and medicine (12; 20). Therefore, the strain JF19-2 with *cry2Ag1*, appears to be an alternative for controlling mosquitoes and crop pests, managing resistance development in insects, and insect-resistant transgenic plants in the future, so it will be worthwhile to make clear the insecticidal activity and insecticidal spectrum of the protein Cry2Ag1 in strain JF19-2. These works mentioned above are in the process of researching.

However, many different *cry* genes have been cloned up to now and many insecticidal toxins have been successfully used for controlling pests, a significant number of pests are not controlled with the available Cry proteins and some insects have developed resistance against some Bt toxins (7; 15). Cry1-type proteins have been widely applied in transgenic plants, but the problem of narrow insecticidal spectrum and insect resistance have been observed due to long time and high concentration using of the single Bt toxins (2). In addition, the threat of secondary pests may result in the need of transgenic plants with high insecticidal activity and wide insecticidal spectrum. Cry2A is toxic to several of the main Lepidopteran pests such as yellow stem borer and striped stem borer (1;11). Furthermore, biochemical studies showed that Cry2A did not share binding sites with Cry1A in Brush Border Membrane Vesicles from Lepidopteran pests (1;11). Therefore, the isolation of new Bt strains and novel Cry2-type toxins are crucial to solve these problems. In this study, a new insecticidal crystal protein gene (*cry2Ag1*) omitted in our previous study, was found and cloned, which may not only enriched the available categories of insecticidal genes but also provided the potential candidate for the control of pest worldwide and beneficial gene resource for insect-resistant transgenic plants in the future.

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