Annals of Microbiology, **59 (1)** 1-8 (2009)

Characterization of insecticidal crystal protein *cry* gene of *Bacillus thuringiensis* from soil of Sichuan Basin, China and cloning of novel haplotypes *cry* gene

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Received 26 May 2008 / Accepted 12 January 2009

Abstract - Sichuan Basin situated in the west of China, the fourth-largest basin of China, is a special area with complicated geomorphology (mountain, pasture, gorge, virgin forest, highland, hurst, glacier, and plain), and contains a rich and unique biodiversity. In order to describe a systematic study of *cry* gene resources from *Bacillus thuringiensis* (Bt) strains of different ecological regions in Sichuan Basin, a total of 791 Bt strains have been screened from 2650 soil samples. The analysis of the *cry* genes was based on the method of PCR-restriction fragment length polymorphism (PCR-RFLP). *cry1, cry2, cry3, cry4/10, cry9, cry30,* and *cry40*-type genes were found in this basin. Strains containing *cry1* genes were the most abundant in our collection (66%), and twenty-one different *cry1*-type gene combinations were found. Bt strains harboring *cry2* genes were the second most abundant (39.5%), and the strains containing *cry3*, *cry9*, *cry4/10*, *cry30,* and *cry40* genes were found in 2.5, 3.5, 4.2, 4.2, and < 1%, respectively. Furthermore, several novel haplotypes *cry* genes were found, and the full-length sequences of three novel *cry* genes were obtained, which were designated as *cry52Ba1*, *cry54Aa1*, *cry30Fa1* by the *B. thuringiensis* Pesticide Crystal Protein Nomenclature Committee, respectively. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) assay of 80 strains which did not produce any PCR products indicated that these strains may harbour potentially novel Cry proteins. All these researches mentioned above revealed that the diversity and particularity of *cry* gene resources from *B. thuringiensis* strains in Sichuan Basin.

Key words: *Bacillus thuringiensis*; *cry* gene; PCR-RFLP; SDS-PAGE; novel haplotypes genes.

INTRODUCTION

Bacillus thuringiensis (Bt) is a typical aerobic, Gram-positive, and endospore-forming bacterium, which produces insecticidal crystal proteins distinguished as distinctively shaped crystals under phase-contrast microscopy (Cry proteins or δ-endotoxins) during sporulation (Hofte and Whiteley, 1989; Schnepf *et al.,* 1998). Many Cry protein genes have been cloned, sequenced, and named. These Cry proteins are classified into different categories according to the homology of their amino acid sequences (Crickmore *et al.*, 1998). To date, there were over 390 kinds of such protein genes had been isolated and cloned. These genes can be classified into 55 groups, 106 subgroups, 168 classes and 390 subclasses according to encoded amino acid sequence similarities (http://www.biols.susx.ac.uk/Home/Neil-Crickmore/Bt). Although many toxins have been found from Bt strains, only a few of them have been used to control some pests. Moreover, some insects have developed resistance against some Bt toxins (McGaughey, 1985). In order to solve these problems, isolation of new strains and toxins are crucial. Many scholars had isolated some novel variations from Bt in the world (Bravo *et al.,* 1998; Iriarte *et al.*, 2000; Arango *et al.,* 2002; Merdan and Labib, 2003; Uribe *et al.,* 2003; Chen *et al.,* 2004; Vilas-Boas *et al.,* 2004; Luo *et al.,* 2005).

Recently, PCR amplification restriction fragment length polymorphism (RFLP) has been exploited to identify *cry*-type genes. The PCR-RFLP system is a facile method to detect both known and unknown *cry* genes existing in Bt strain (Kuo and Chak, 1996; Song *et al.*, 1998, 2003).

Sichuan Basin, the fourth-largest basin of China, situated in the west of China, is a special area with complicated geomorphology, characteristic of mountain, pasture, gorge, virgin forest, highland, hurst, glacier, and plain, and contains a rich and unique biodiversity (Liu *et al*., 2006). These distinctive features and diversity of insects provide the opportunity of isolating novel entomopathogenic bacteria, so it is the most possible that some novel *cry* genes or special Bt strains might be found (Ibarra *et al*., 2003). In order to describe a systematic study of *cry* gene resources from *Bacillus thuringiensis* (Bt) strains of different ecological regions in Sichuan Basin, we collected 2650 soil samples from different ecological regions in Sichuan Basin to isolate

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Bt strains and study on their characteristics of *cry* genes; and we wanted to seek for new type insecticidal genes to enrich the available categories of insecticidal genes.

MATERIALS AND METHODS

Screening for *Bacillus thuringiensis* strains from soil samples. In total, 2650 soil samples were collected from different regions with unique geographical features in Sichuan Basin (Table 1). The characteristics of the sites and soils were also mentioned in Table 1. Soil samples were collected from the top to a depth of 10 cm below the surface, and then kept in a 25 °C air-conditioned room. Soil sample (10 g) was added to 50 ml sodium acetate medium (3.4% sodium acetate, 0.5% beef extract, 1% tryptone, 400 µg/ml gentamycin sulphate, 400 µg/ml benzylpenicillin sodium, pH 7.0) in a conical flask and incubated at 30 °C, 220 rpm, for 4 h. And then the suspension was continually heated at 65 °C for 15 min. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) were plated onto Luria-Bertani (LB) agar, and incubated at 30 °C for 72 h. Colonies with Bt-like morphology were observed by phase contrast microscopy to verify the presence of crystal inclusions (Ohba and Aizaw, 1986; Yang *et al.,* 2000).

Scanning electron microscopy (SEM). All the Bt strains were grown in LB medium at 30 °C until parasporal crystal were formed, and then the spore-crystal mixture was collected by centrifugation for 10 min at 10000 rpm at 4 °C. The pellets were resuspended in sterile distilled water. Spore-crystal mixture was placed on aluminium stubs, which was fixed in 1% OsO_4. Then the samples were sputter-coated with gold in IB-5 ion coater (HITACHI) for 5 min. The SEM micrographs were taken on a Zeiss 950 digital scanning microscope (DSM) at a voltage of 12 kV.

Chemical reagents and enzymes. Chemical reagents were purchased from Sigma. *Taq* DNA polymerase were purchased from Promega, and restriction enzymes were obtained from New England Biolabs, Inc., and MBI Ferments, respectively.

Identification of *cry* gene by PCR-restriction fragment length polymorphism. The Bt strains were incubated overnight at 30 °C with agitation at 220 rpm in LB liquid medium. A 1 ml volume of bacterial cells was collected after centrifugation, and the pellet was resuspended in 100 µl of purified water, boiled 5 min, and spun at 14000 rpm for 5 min. The supernatant was collected and used as a template for PCR amplification. Based on the conserved regions of each class of *cry* genes, the *cry1*-type gene primers for PCR-RFLP were used as previously described by Kuo and Chak (1996); the *cry1I* gene primers for PCR-RFLP were used as previously described by Song *et al.* (2003); other *cry* genes primers were used as previously described by Song *et al.* (1998) and Su (2005) (Table 2). PCR was carried out for 30 cycles at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min, with an additional step of extension at 72 °C for 10 min. A restriction endonuclease reaction was performed in a 20 µl volume containing the PCR products (0.5-1.0 µl) and 0.5 U restriction endonucleases, as described by Sambrook *et al.* (2002). With the aim of identifying subtypes of *cry1* genes from Bt isolates collected by our laboratory, we used a PCR-RFLP identification system of *cry1*-type genes developped by Liu (2003) and Song *et al*. (2003). The expected restriction fragment sizes of the known *cry4/10* and *cry30* genes were determined by *in silico* digestion of their available sequences in the Bt toxin nomenclature website with the software 'DNAStar' (Table 2).

Partial sequence analysis of novel *cry* gene and the cloning of full *cry* gene. PCR products were analyzed by electrophoresis in 1% agarose gels, and then were recovered with a Tiangen Midi Purification Kit as described by the manufacturer (Tiangen Biotech). The recovered PCR products were cloned into pGEM-T vector and transformed into *Escherichia coli* DH5α competent cells. Positive clones were selected by

TABLE 1 - The geographical features of collecting locations and results of *Bacillus thuringiensis* isolation

Site[a]	Site characteristics	SC[b]	SN[c]	TAI[d]	NBI[e]	IR[f] (%)	*cry* genes present in each ecosystem						
							1	2	3	4/10	9	30	40
I	Mountain, forest	i	385	590	124	21	115	82	1	9	3	9	-
II	Mountain, forest	ii	285	410	78	19	68	28	2	3	2	3	-
III	Mountain, forest	iii	320	478	110	23	66	47	1	-	3	-	3
IV	Glacier, forest	iv	250	500	25	5	12	3	5	10	3	10	-
V	Highland, farmland	v	215	1400	98	7	50	47	2	-	3	-	-
VI	Virgin forest	vi	180	425	85	20	60	5	2	5	4	5	-
VII	Pasture, grassland	vii	195	350	35	10	33	4	2	-	3	-	-
VIII	Plain, farmland	viii	540	1114	156	14	39	61	4	6	-	6	-
IX	Hurst, farmland	ix	280	615	80	13	79	45	1	-	7	-	-
Total			2650	5889	791	13.4*	522	312	20	33	28	33	3
%							66[#]	39.5[#]	2.5[#]	4.2[#]	3.5[#]	4.2[#]	< 1[#]

[a] I: Zhou Gong, II: Meng Ding, III: Bi Feng, IV: Hai Luo, V: Lu Ding, VI: Mu Chuan, VII: Kang Ding, VIII: Cheng Du, IX: other Cities.

[b] SC: soil characteristics. i: cinnamon soil, ii: cinnamon soil, iii: gray forest soil, iv: alpine meadow soil, v: irrigation-silted soil, vi: black soil, vii: meadow soil, viii: irrigation-silted soil, ix: purple soil and irrigation-silted soil.

[c] SN: sample number.

[d] TAI: total aerobic sporeformers isolations.

[e] NBI: number of Bt isolations.

[f] IR: isolation rate of Bt isolations in total aerobic sporeformers isolations from the same site.

* : Average rate of Bt isolations in all aerobic sporeformers isolations, [#]: The rate of strain harboured the same *cry* gene in all Bt isolations, -: Not amplified with any primers.

TABLE 2 - The partial primers for identification of *cry* genes and expected restriction fragment sizes of digested *cry4/10* and *cry30*-type genes

Target gene	Primer name	Primer sequence (5′ to 3′)	AT[a] (°C)	Expected size (bp)	Fragment size (bp)	References
cry1	K5un2 K3un2	AGGACCAGGATTTACAGGAGG GCTGTGACACGAAGGATATAGCCAC	53	about 1500	not test	Kuo and Chak, 1996
	K3un3 K5un3	CCTCCTGTAAATCCTGGTCCT CAATGCGTACCTTACAATTGTTTAAGTAAT	54	about 1400	not test	
cry1I	S5uni S3uni	GCTGTCTACCATGATTCGCTTG CAGTGCAGTAACCTTCTCTTGC	53	about 1500	not test	Song *et al.,* 2003
cry2	S5un2 S3un2	GGAAGAACTACTATTTGTGATGC AATAGTTTGAATTACCGCGAGC	53	about 1200	not test	Song *et al.*, 1998; Su, 2005
cry3	S5un3 S3un3	CGAACAATCGAAGTGAACATGATAC CATCTGTTGTTTCTGGAGGCAAT	53	about 1380	not test	the same as above
cry4Aa	S5un4 S3un4	GTGTCAAGAGAACCAACAGTATG ACTAAGTCTCCTCCTGTATGACCAG	53	about 1500	270, 273, 276, 732*	the same as above
cry4Ba	S5un4 S3un4	the same as above the same as above	53 53	about 1500	69, 470, 910*	the same as above
cry10Aa	S5un4 S3un4	the same as above the same as above	53 53	about 1400	371, 511, 585*	the same as above
cry9	S5un9 S3un9	AGGACCAGGATTTACAGGAGG CCCAATGCGAAAGAACTAAG	53	about 1550	not test	the same as above
cry30Aa	S5un30 S3un30	AAGATTGGCTCAATATGTGTC GATTATCAGGATCTACACTAG	52	about 1400	146, 459, 820[#] 116, 136, 392, 792^	the same as above
cry30Ba	S5un30 S3un30	the same as above the same as above	52	about 1400	60, 603, 759[#] 237, 389, 796^	the same as above
cry30Ca	S5un30 S3un30	the same as above the same as above	52	about 1400	762, 775[#] 355, 387, 795^	the same as above
cry40	S5un40 S3un40	GTATGTCAGATGAACATTCTC GCTAGCATATCCTTGCATAC	53	about 1350	not test	the same as above

[a] AT: annealing temperature.

*: Digested with *BstE* II+*Dra* I, [#]: digested with *Dra* I, ^: digested with *Msp*I.

alpha-complementation, and at last PCR identification were performed (Sambrook *et al.,* 2002). The sequencing was done by Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd. According to the known partial sequences, six specific primers were designed (Table 3). Thermal asymmetric interlaced-PCR (Tail-PCR) strategy was carried out to obtain the unknown sequence of novel genes using specific primers and degenerate primer (Liu and Whittier, 1995). PCR was carried out in a 50 µl volume, containing 1 µl template DNA, 0.4 mmol/L deoxynucleotide triphosphates, 0.2 µmol/l specific and degenerate primers, 1 U LA polymerase (Takara), and reaction buffer. PCR reaction were performed under the following conditions: 5 min of denaturation at 94 °C, followed by 15 cycles of (2 cycles of at 94 °C for 30 s, 56 °C for 50 s, and 72 °C for 2 min) and (1 cycle of at 94 °C for 30 s, 33 °C for 50 s and 72 °C for 2 min), then followed by an extra step of extension at 72 °C for 7 min. The amplification products were ligated into the cloning vector pGEM-T vector and sequenced.

Isolation of crystal proteins. The Bt strains were grown in 100 ml LB liquid medium at 30 °C, 220 rpm until parasporal crystal were formed. Concentrated Bt strains suspensions on disruption buffer were boiled for 5 min; cells were spun at 10000 rpm for 8 min at 4 °C , and the supernatant was used as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of total proteins (Ibarra *et al*., 2003). SDS-PAGE analysis was performed as described by Sambrook *et al.* (2002).

TABLE 3 - The specific primers and degenerate primer sequences of the Tail-PCR used in this study

Primer name	Primer sequence (5′ to 3′)	AT[a] (°C)	Target *cry*-like genes and isolate
SP1	TTTTGCCTGTTTTACTTCATCCC	60	*cry40*-like gene from BM59-2
SP2	GAGAAACCGAGGGTACATTTTAT	58	
SP3	GCTGATCCTGGACCAGGCATAGC	68	*cry4/10*-like from BtMc28
SP4	AACTTATCAAGCAGGTAGAA	63	
SP5	TACTTCCCACAAACCCTCTTACTC	63	*cry30*-like from BtMc28
SP6	GTCAAACTGGAGTGCTGGGGTA	64	
DP[b]	GGAGGNNNNNNNNWTG	39	

[a] AT: annealing temperature, [b] DP: degenerate primer.

RESULTS

Bacillus thuringiensis strains of Sichuan Basin

In total, 5889 aerobic sporeformers strains have been isolated from 2650 soil samples collected from different typically ecological regions in Sichuan Basin. The characteristics of sites and soils were mentioned in Table 1. Basing on the production of parasporal crystals, 13.4% of the isolates were identified as *Bacillus thuringiensis* (Table 1). These *B. thuringiensis* strains produced bipyramidal, square, round, and irregular crystal inclusions observed under the phase contrast microscopy and scanning electron microscopy (Fig. 1). The isolation rates from the gray forest soil in Bi Feng (23%), the cinnamon soil in Zhou Gong (21%), the black soil in Mu Chuan (20%), and the cinnamon soil in Meng Ding (19%) were higher than these from other soils (Table 1). The Independent-Samples T Test analysis of the isolation rates from the soil of forest and the farmland showed rate from the soil of forest was higher than that of farmland (T-value, 1.690; P-value, 0.135; 95% Confidence Interval of the Difference; SPSS 10.0).

Determination of the *cry* genes content from Bt in Sichuan Basin by PCR-RFLP

In total, 30 pairs of primers were used for identifying *cry*-type genes from 791 Bt strains. Seven *cry*-type genes, *cry1, cry2, cry3, cry9, cry4/10, cry30,* and *cry40*-type, were identified. The strains containing *cry1*-type genes were the most abundant in our collection (66%), the strains harboring *cry2*-type gene were also highly abundant (39.5%), but *cry3, cry4/10, cry9,* and *cry30*-type genes were less abundant, found from 2.5 to 4.2% of the strains. *cry1, cry2, cry3,* and *cry9*-type genes were found from both the soil of farmland and forest with different soil characteristics (Table 1). Only 3 strains harbouring *cry40*-type gene screened from Bi Feng. Thirty-three strains, mainly distributed in five forest ecology regions, harboured both *cry4/10* and *cry30*-type genes (Table 1). Finally, 80 Bt strains did not give any PCR products when assayed with all the primers, suggesting that they may contain potentially novel Cry toxins.

Identification of specific *cry1*-type genes from *Bacillus thuringiensis* strains

The Cry proteins encoded by *cry1*-type genes have special insecticidal activities against *Lepidoptera* (http://www.biols.susx.ac.uk/Home/Neil-Crickmore/Bt/; Su, 2005), so we further identified the subtypes of *cry1* genes. The PCR amplified products were digested with *Pst* Ⅰ/*Xba* Ⅰ, *Pst* Ⅰ/*Eco*R Ⅰ, and *Bsp*119 I/*Ban* I, respectively. Six distinct *cry*1-type genes, including *cry1A, cry1B, cry1C, cry1E, cry1F, and cry1I,* were found from the 522 isolates, which constituted twenty-one different *cry1*-type genes combinations (Table 4). The most common profiles were *cry1Ac, cry1Aa,* and *cry1Aa+cry1Ab+cry1Ac* with the frequencies 15.3, 13.2 and 10.5%, respectively. Strains containing *cry1Ab*, *cry1Aa+cry1Ac, cry1Aa+cry1Ac+cry1Ia, cry1Aa+cry1Ab+cry1Ia, cry1Aa+cry1Ab, cry1Aa+cry1Ia* were also frequently observed, which frequencies ranged from 5.17 to 8.62%. Other *cry1*-type gene combinations, such as *cry1Aa+cry1Ab+cry1Ca*, *cry1Aa+cry1Ab+cry1Ac+cry1Ia*, *cry1Aa+cry1Ac+cry1Cb*, *cry1Aa+cry1Ea,* and *cry1Aa+cry1Fa*, were less abundant. In Zhou Gong and Cheng Du, 16 types of *cry1*-type genes combination profiles were found, followed by 15 types in Bi Feng, 14 in Lu Ding and Mu Chuan, 12 in Meng Ding and Kang Ding, and 10 in other cities. There were only 7 types of *cry1*-type gene combination profiles found in Hai Luo. All these results indicated a high diversity of the *cry1*-type gene content in Sichuan Basin.

Identification of specific *cry4/10*-type and *cry30*-type genes from *Bacillus thuringiensis* strains

The PCR products obtained using primers S5un4/S3un4 and S5un30/S3un30 were digested with corresponding endonucleases to identify the profiles of *cry4/10*-type and *cry30*-type genes

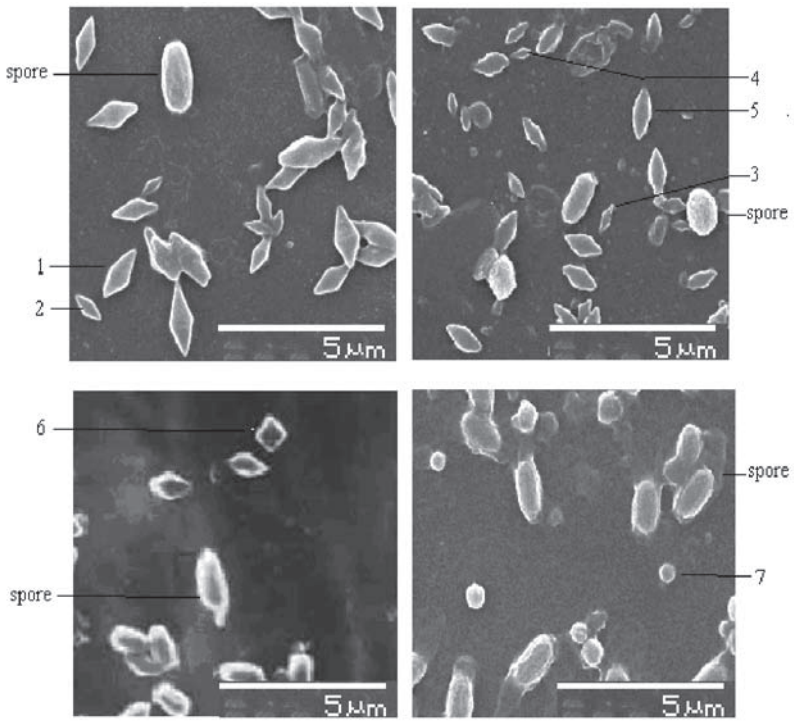

FIG. 1 - Scanning electron microscopy observation of the spore and crystal mixtures produced by *Bacillus thuringiensis*. 1-4: bipyramidal crystal inclusions, 5: irregular crystal inclusions, 6: square crystal inclusions, 7: round crystal inclusions.

TABLE 4 - The distribution of *cry1*-type gene profiles in different ecology areas of Sichuan Basin

*cry*1-type gene profiles	Site[a]										
	I	II	III	IV	V	VI	VII	VIII	IX	N[b]	F[c] (%)
cry1Ac	30	8	7	-	3	10	4	12	6	80	15.33
cry1Aa	15	11	13	2	10	-	-	7	11	69	13.22
cry1Aa+cry1Ab+cry1Ac	8	9	7	2	8	9	2	6	4	55	10.54
cry1Ab	12	9	7	-	4	3	3	-	7	45	8.62
cry1Aa+cry1Ac	9	7	6	1	2	4	3	5	5	42	8.05
cry1Aa+cry1Ac+cry1Ia	6	9	2	2	1	6	4	-	6	36	6.90
cry1Aa+cry1Ab+cry1Ia	-	4	8	1	7	6	-	2	3	31	5.94
cry1Aa+cry1Ab	8	-	3	-	-	4	-	-	12	27	5.17
cry1Aa+cry1Ia	1			-			3	2	6	27	5.17
cry1Aa+ cry1Ab+cry1Ca	6	-	-	1	6	4	3	-	4	24	4.60
cry1Aa+cry1Ab+cry1Ac+cry1Ia	5	2	4	-	1	1	2	1	3	19	3.64
cry1Ab+cry1Ac	5	1	-	-	-	-	3	1	3	13	2.49
cry1Aa+cry1Ca+cry1Ia	1	-	1	-	2	4	2	-	2	12	2.30
cry1Ac+cry1Ca	-	2	-	1	1	2	1	-	3	10	1.91
cry1Aa+cry1Ca	2	1	1	-	2	-	1	1	1	9	1.72
cry1Aa+cry1Ac+cry1Cb	2	3	1	-	2	-	-	-	-	8	1.53
cry1Aa+cry1Ba+cryAb	-	-	2	-	-	2	-	-	1	5	0.96
cry1Aa+cry1Ab+cry1Ba	1	-	1	-	1	-	-	-	-	3	0.57
cry1Ba	1	-	2	-	-	-	-	-	-	3	0.57
cry1Aa+cry1Ea	-	-	-	-	-	2	-	-	-	2	0.38
cry1Aa+cry1Fa	-	-	-	-	-	2	-	-	-	2	0.38

[a] I: Zhou Gong, II: Meng Ding, III: Bi Feng, IV: Hai Luo, V: Lu Ding, VI: Mu Chuan, VII: Kang Ding, VIII: Cheng Du, IX: other Cities.

[b] N: number of strains.

[c] F: frequency.

-: Not containing the type gene.

(Fig. 2, Fig. 3). The PCR-RFLP patterns of the PCR products from the strain BtMC28 (primers S5un4/S3un4, digested with *BstE* II+*Dra* I) revealed that there were four main bands; however, the PCR products obtained from other strains (ZG108-36, MD7-5, HS18-1, HS15-3, HS70-8) using the same primers could not be digested by *Bst*E II+*Dra* I (Fig. 2), which were different from the expected fragments of the *cry4Aa*, *cry4Ba* and *cry10Aa* digested with same enzymes (Table 2). In addition, other PCR products obtained using primers S5un30/S3un30 were also digested by corresponding endonucleases as described in Table 2, while the patterns of restriction fragment length polymorphism showed that the restriction fragments were different from those of the known genes (Table 2, Fig. 3). All these results explained that these fragments of *cry* genes were likely to be novel genes.

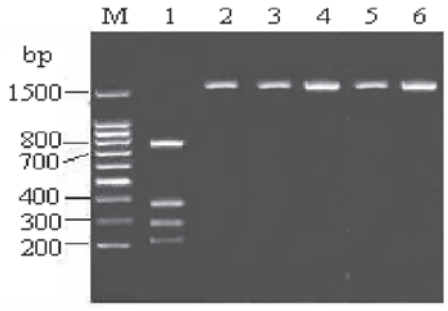

FIG. 2 - PCR-RFLP patterns of *cry4/10*-type genes in different strains digested with *BstE* II+*Dra* I. Lanes 1-6: PCR-RFLP patterns of PCR product of BtMC28 (210, 280, 380, 780 bp), ZG108-36, MD7-5, HS18-1, HS15-3, HS70-8; lane M, 100 bp marker.

Partial sequence analysis of novel *cry* gene and cloning of novel haplotypes *cry* gene

PCR product of *cry40*-type gene (obtained with S5un40/S3un40, BM59-2), *cry4/10* (S5un4/S3un4, BTMC28), and *cry30*-type (S5un30/S3un30, BTMC28) were sequenced, which had maxi-

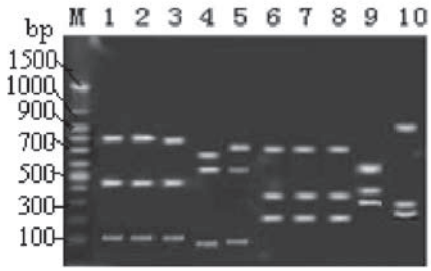

FIG. 3 - PCR-RFLP patterns of *cry30* genes digested with *Dra* I and *Msp* I. Lanes 1-5: PCR-RFLP patterns of PCR product of BTMC28 (800, 450, 100 bp), ZG108-36 (800, 450, 100 bp), MD7-5 (800, 450, 80 bp), HS18-1(700, 550, 80 bp), HS15-3 (750, 550, 90 bp) digested by *Dra*I; lanes 6-10: PCR-RFLP pattern of PCR product of BTMC28 (750, 350, 200 bp), ZG108-36 (750, 400, 350 bp), MD7-5 (750, 350, 200 bp), HS18-1 (550, 400, 300 bp), HS15-3(950, 400, 250 bp) digested with *Msp*I; lane M: 100 bp marker.

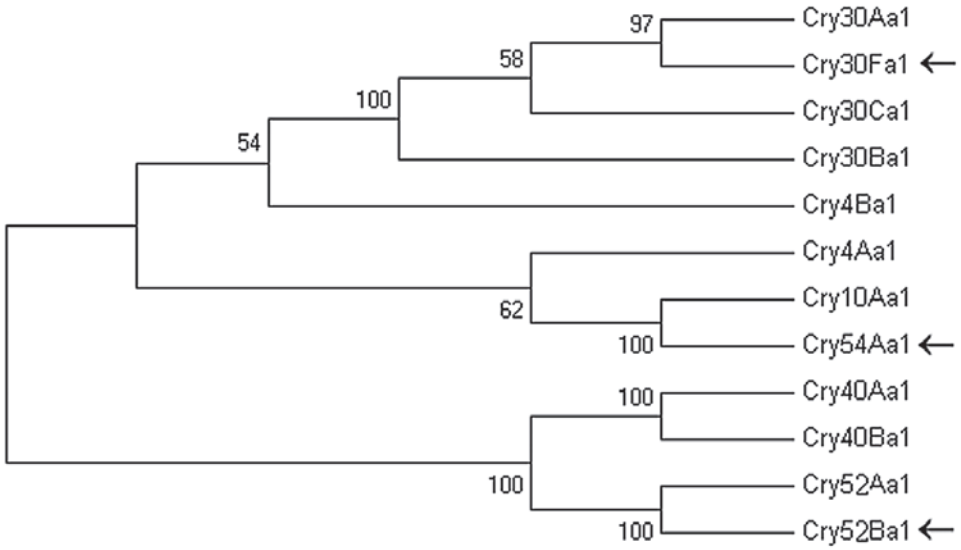

FIG. 4 - Phylogenetic analysis of Cry proteins. Neighbor-joining phylograms were constructed after sequence alignment of the deduced amino acid sequences of selected known toxins and Cry54Aa1, Cry30Fa1, and Cry52Ba1 protein by using the CLUSTAL W program. The tree was generated using Tree Explorer. Sequences were obtained from the NCBI database: Cry4Aa1 (Y00423), Cry4Ba1 (X07423), Cry10Aa1 (M12662), Cry30Aa1 (AJ251978), Cry30Ba1 (BAD00052), Cry30Ca1 (BAD67517), Cry40Aa1 (BAB72018), Cry40Ba1 (BAC77648), and Cry52Aa1 (EF613489). The arrow indicates the position of the novel Cry sequence. Numbers at nodes represent the percentages of bootstrap resamplings based on 500 replicates.

mum 82, 40, and 72% amino acid sequence homology with Cry40 ORF2 protein, Cry10Aa1, and Cry30Aa1, respectively. These results further showed that novel genes could be found. The full-length sequences of these *cry* genes were obtained using a method of Tail-PCR strategy and the primers indicated in Table 3. Two novel *cry* genes harboured in BTMC28 encode a polypeptide of 674 and 687 amino acid with a molecular weight of 76 and 77 kDa, respectively; the novel *cry* gene harboured in BM59-2 encodes a polypeptide of 703 amino acid with a molecular weight of 79 kDa. Amino acid sequence alignment analysis revealed that the three novel Cry proteins had maximum 38, 74, and 71% identical amino acid sequence to Cry10Aa1, Cry30Aa1, and Cry52Aa1, respectively. The phylogenetic analysis of the deduced amino acid sequences of selected known toxins and the three novel Cry proteins exhibited that the three Cry proteins and Cry10Aa1, Cry30Aa1, and Cry52Aa1 might have evolved from a common ancestor, respectively (Fig. 4). According to the nomenclature principles of insecticidal crystal protein from *B. thuringiensis*, these novel *cry* genes belong to novel haplotypes genes, which were designated as *cry54Aa1* (GenBank accession: EU339367), *cry30Fa1* (GenBank accession: EU751609), and *cry52Ba1* (GenBank accession: FJ361760) by the *B. thuringiensis* Pesticide Crystal Protein Nomenclature Committee, respectively.

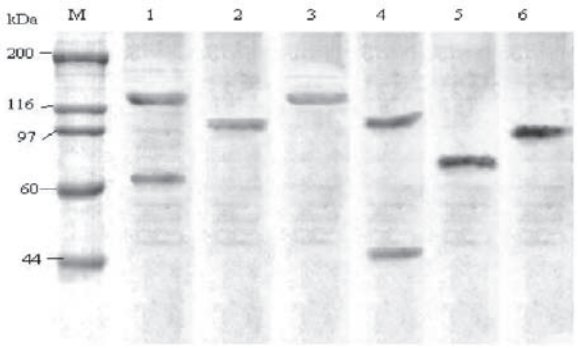

FIG. 5 - The SDS-PAGE of spore-crystal suspensions of selected strains. Lanes 1-6: A1, CW3, MD7-2, ZG157-4, JF19-8, YWC9-4; lane M: protein marker.

Study on the insecticidal crystal proteins

The SDS-PAGE of spore-crystal suspensions of *B. thuringiensis* strains which did not give any PCR products assayed with all the primers were shown in Fig. 5. All the strains had one or two major protein bands, with the molecular weights ranged from 40 to 130 kDa. This result revealed that these Bt strains might contain potential novel *cry* genes. These 80 strains have six different protein profiles (Fig. 5), suggesting that there are diversity between potential novel Cry toxins profiles in Sichuan Basin.

DISCUSSION

The characterization of Bt strain collections may help in the understanding the role of Bt in the environment and the distribution of *cry* genes. Several Bt strain collections have been described (Ohba and Aizaw 1986; Ben-Dov *et al*., 1997; Chen *et al*., 2004; Luo *et al.*, 2005; Su, 2005; Gholamreza *et al.*, 2008) in Asia. These strains were from different countries, mainly Israel, Kazakhstan, Iran, Uzbekistan, Korea, China, and Japan. None of these collections have included samples from Sichuan Basin and none of systematic study of *cry* gene resources in basin were done, so that we isolated 791 Bt strains from different regions with unique geographical features in Sichuan Basin (Table 1). In the present study, the feature of Bt strains distribution and *cry* gene types were determined (Table 1, Table 4). Moreover, several novel haplotypes genes were found in Sichuan Basin. All these results indicated that Sichuan Basin have plentiful and special Bt resources.

The coverage rates of forest and vegetation in Sichuan Basin are 28 and 70%, respectively, which might be beneficial for the growth of Bt, so the average isolation rate (13.4%) was higher than that of other place in China (Yang *et al.*, 2000; Su, 2005).

The isolation rate of forest is higher than that of farmland in this study, it should be possible that there were more farming activities in the farmland than in the forest, which might affect the growth of Bt (Liu *et al.*, 2006). The soils of Hai Luo, Lu Ding, and Kang Ding were leanness, so the isolation rates were as low as 5, 7, and 10%, respectively, which conformed to the distribution features of Bt (Su, 2005).

Several PCR-based methods for identification of *cry* genes from Bt strains have been developed (Carozzi *et al.,* 1991; Bourque *et al.,* 1993; Ben-Dow *et al.,* 1997). Exclusive PCR and PCR-RFLP can identify both known and novel genes (Carozzi *et al.,* 1991; Kuo and Chak*,* 1996; Ben-Dow *et al.*, 1997; Song *et al.*, 1998). We determined the presence of *cry* genes in Sichuan Basin which contains seven *cry* genes types, such as *cry1, cry2, cry3, cry9, cry4/10, cry30,* and *cry40*-type*.* The types of *cry* genes in Sichuan Basin were different from those of other places in Asia (Ben-Dov *et al*., 1997; Chen *et al*., 2004; Luo *et al.*, 2005; Su, 2005; Gholamreza *et al.*, 2008). The *cry1*-type genes (66%) were the most frequently found in this collection, conformed to the collections of Mexican, Israel, Kazakhstan, Uzbekistan, and Taiwan (Kuo and Chak, 1996; Ben-Dow *et al.,* 1997; Bravo *et al.,* 1998). In Iran, the strains containing *cry2*-type genes were the most abundant (Gholamreza *et al.*, 2008). The second most abundant *cry* gene in Sichuan Basin was *cry2*-type gene (39.5%), similar to that of other places in China (Luo *et al.*, 2005; Su, 2005). However, the second most abundant genes in Israel, Kazakhstan, and Uzbekistan were all *cry4*-type gene while *cry24*-type, and *cry3*-type genes were the second most abundant genes in Iran, and Mexican, respectively (Ben-Dow *et al.,* 1997; Bravo *et al.,* 1998; Gholamreza *et al.*, 2008). In Israel, Kazakhstan, Uzbekistan, and Iran, *cry3*-type genes were absent (Ben-Dow *et al.,* 1997; Gholamreza *et al.*, 2008), but we found *cry3*-type genes exist in China (Table 1; Luo *et al.*, 2005). These results indicated that *cry* genes harboured in Sichuan Basin have particularity and similarity compared with other places. In addition, no PCR products were obtained from some strains with all primers. However, these strains could produce crystal inclusions. This result suggested that these Bt strains may harbor potentially novel Cry proteins as well as strains with combinations of less frequently observed *cry* genes. Therefore, the primers of PCR-RFLP should be redesigned to identify these novel *cry* genes.

In this research, we also found that *cry1, cry2, cry3,* and *cry9*-type genes existed in the soil of farmland and forest with different soil characteristics; *cry40*-type gene was only screened from the gray forest soil of Bi Feng, and *cry4/10* and *cry30*-type genes mainly distributed in the forest ecology regions (Table 1). These results indicated the characterization of *cry* genes in Sichuan Basin seemed to be geographically related. The Cry4, Cry10, and Cry30 protein toxins have been showed to be highly insecticidal activities against insects of *Diptera* numerously living in the forest (Federici *et al.*, 2003; Ibarra *et al*., 2003). In this study, the *cry4/10* and *cry30*-type genes was mainly discovered from five forest ecology regions, with the exception of one farmland ecology region (Table 1). We wonder whether different geographical and climatic environments bring about a different ecology of insects, consequently resulting in the existence of relevant Cry proteins gene.

Because the *cry1* gene was the most frequent in this collection and the Cry1 protein has special insecticidal activities against *Lepidoptera*, we further identified the subtypes of *cry1* gene as previously reported (Liu, 2003; Song *et al.,* 2003). Twenty-one different *cry1*-type gene profiles were detected. These results clearly indicated that the Bt of Sichuan Basin contained a great variety of *cry1*-type gene combinations. And the distribution of *cry1*-type genes was different in ecology region of Sichuan Basin (Table 4). For example, *cry1Aa+cry1Ea* and *cry1Aa+cry1Fa* were only found in Mu Chuan, *cry1Ba* was only screened from Zhong Gong and Bi Feng.

By the method of the PCR-RFLP, we found that reported strains exhibited an RFLP pattern different from that of known *cry4/10* and *cry30,* suggesting novel *cry4/10* and *cry30*-type genes harboured in the reported strains (Fig. 2, Fig. 3). Sequence analysis further indicated that putative novel *cry* genes presented by PCR-RFLP were haplotypes *cry* genes, which also proved that PCR-RFLP can identify not only known *cry* genes but also novel genes, again (Song *et al.,* 2003; Su, 2005). Notwithstanding many different *cry* genes cloned up to now, it is still necessary to search for more toxins, since a significant number of pests are not controlled with the available Cry proteins. The revised Cry toxin nomenclature is available on World Wide Web (http://www.biols.susx.ac.uk/Home/Neil-Crickmore/Bt/), we found that the proteins toxic against lepidopteron belong to the Cry1, Cry9, and Cry2 groups; toxins active against coleopteran insects are the Cry3, Cry7, and Cry8 proteins as well as the Cry1B and Cry1I proteins, which have dual activity. The Cry5, Cry12, Cry13, and Cry14 proteins are nematicidal, and the Cry2, Cry4, Cry10, Cry11, Cry16, Cry17, Cry19, Cry24, Cry25, Cry27, Cry30, Cry39, Cry40, Cry44 and Cyt proteins are toxic for dipteran insects. Through the results obtained in the present work, showed in Table 1 and Table 4, we found that Cry toxin harboured in Sichuan Basin may have insecticidal activities against pests of lepidopteran, coleopteran, and dipteran. Although many insecticidal toxins have been successfully used for controlling pests, some insects have developed resistance against some Bt toxins, isolation of new strains and toxins are crucial to solve the problem (McGaughey, 1985; Georghiou and Wirth, 1997; Becker, 2000), hence, the Cry52Ba1, Cry54Aa1, and Cry30Fa1 toxins and other Cry toxins found in this research have potential interests for insect resistance management.

Acknowledgments

This study was supported by a grant from the National High Technology Research and Development Program of China (No. 2006AA02Z189).

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