RESEARCH

CHILEANJAR
CHILEAN JOURNAL of AGRICULTURAL RESEARCH

APPLICATION OF OLIGONUCLEOTIDE MICROARRAY FOR THE DETECTION AND GENOTYPING OF *CRY* GENES IN *Bacillus thuringiensis*

Liu Xu-Guang[1,2], Song Fu-Ping[1], Wen Si-Yuan[3], Wang Sheng-Qi[3], Huang Da-Fang[4], and Zhang Jie[1*]

ABSTRACT

We have developed a parallel, rapid, high-throughput oligonucleotide microarray-based assay for the reliable detection and genotyping of three *cry* genes (*cry1*, *cry2* and *cry9*) in *Bacillus thuringiensis* (Bt). After the non-polymerase chain reaction (PCR), amplified Bt genomic DNA were fluorescent-labeled using a random primer. The corresponding oligonucleotide probes were designed for the different *cry* genes that can hybridize Bt genomic DNA after cluster analysis and were printed on glass slides. This microarray has unambiguously detected and identified the *cry* genes in 10 isolates and reference Bt. Our data demonstrates that the microarray assay is simple and rapid for the detection and genotyping of genes. This type of assay is also a potentially valuable tool for identification and characterization of bacterial functional genes in general.

Keywords: oligonucleotide microarray, *cry* genes, genotyping, random primer labeling, bioinformatics.

INTRODUCTION

Bacillus thuringiensis (Bt) is capable of producing highly poisonous crystal proteins (also called β-endotoxin) that kill Lepidoptera insects and other agricultural pests. More than 375 crystal protein genes have already been cloned and sequenced. Based on amino acid sequence similarity, Crickmore (2003) categorized these endotoxins into 55 groups (1st level), 106 subgroups (2nd level), 173 types (3rd level), and 375 subtypes (4th level). Bt has become the most effective biopesticide in the biological control of agricultural pests. With the development of biotechnology, new β-endotoxin genes are continually being isolated and cloned. Along with the development in the study of toxin structure and functions, genetic modification of the toxin genes has increased the effectiveness and specific targeting ability of these endotoxins and widened the application perspective. Identification of Bt resources is essential to predict the pesticide effect, rapid screening for desirable isolates, and cloning of novel toxin genes.

[1]Institute of Plant Protection (CAAS), State Key Laboratory for Biology of Plant Diseases and Insect Pests, Beijing 100094, China. *Corresponding author (jzhang@ippcaas.cn).
[2]Lianyungang Teachers College, Lianyungang 222206, China.
[3]Beijing Institute of Radiation Medicine Science, Beijing 100850, China.
[4]Institute of Biotechnology, Chinese Academy of Agricultural Sciences, Beijing 100081, China.
Received: 28 October 2008.
Accepted: 22 April 2009.

Current detection and identification methods of Bt *cry* genes are based on polymerase chain reaction (PCR), including PCR-restriction fragment length polymorphism (RFLP) (Song *et al*., 2003), PCR-single-strand conformation polymorphism (SSCP), PCR-sequencing, and Multiplex PCR. These methods are rapid and sensitive. However, they become complicated, tedious, and time-consuming for analyzing multiple genes.

The development of DNA microarray has enabled the rapid, parallel, and high throughput detection and identification of microbes (Gabig and Wegrzyn, 2001; Bodrossy *et al*., 2003). DNA microarray is not only manufactured by light-directed *in situ* synthesis, but is also produced by DNA probe fixation (PCR products, synthetic oligonucleotides) on solid surfaces. This microarray is then hybridized with nucleic acid samples. Information about the nucleotide sequences can be rapidly and accurately obtained on a large scale (Small *et al*., 2001; Hwang *et al*., 2003). In this study, we report the development of an oligonucleotide microarray that can be used to detect and identify *cry1*, *cry2*, and *cry9* genes in Bt.

MATERIALS AND METHODS

Strains and culture media

The 10 Bt strains used (G03, HD-73, and Ly30, etc.) are collections from the authors' laboratory. These strains are cultured in Luria-Bertani (LB) medium

(containing tryptone, yeast extract, sodium chloride, and microbiologically tested agar).

Total DNA isolation

Bacillus thuringiensis strains were activated in LB at 30 ºC overnight. Activated bacteria were diluted in LB (1:100), incubated at 37 ºC for 4 h, and shaken (230 rpm) until OD_{600} = 2.0. Cells were collected by centrifugation and then resuspended in solution I (0.3 mol L^{-1} sucrose, 25 mmol L^{-1} Tris-HCl [pH 8.0], 25 mmol L^{-1} EDTA, 50 mg mL^{-1} lysozyme). For each 100 mL culture, 3 mL of solution I was used. Suspension was incubated at 37 ºC for 1 to 2 h and then solution II was added (0.1 mol L^{-1} NaCl, 0.1% sodium dodecyl sulphate [SDS], 0.1 mol L^{-1} Tris-HCl [pH 8.0]). Bacterial cells were disrupted by repeated freeze-thaw until the solution became clear. Lysate was extracted twice with phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). DNA was precipitated by adding 1/10 volume of 3 mol L^{-1} sodium acetate (pH adjusted to 5.2 with glacial acetic acid) and 2 × volume of absolute ethanol. Precipitate was separated by spinning, washed twice with 70% ethanol, once with absolute ethanol, and dried in a vacuum. DNA was resuspended in ultrapure water, analyzed with 0.7% agarose gel electrophoresis (5 V cm^{-1}), and quantified with UV spectrophotometry (DU® 640, Beckman Coulter, Fullerton, California, USA).

Oligo probe design and synthesis

Nucleotide sequences for *cry1*, *cry2*, and *cry9* were retrieved from the National Center for Biotechnology Information (NCBI, Bethesda, Maryland, USA) and saved in the local BioEdit database. Probes were designed by Vector NTI, Array Designer 2.0 software after eliminating non-coding sequences in these genes.

Oligonucleotide probes were synthesized with the conventional phosphoramidite method with ABI 8089 (USA). Oligos were released by incubation with concentrated ammonium at 55 ºC for 15 h followed by column purification. Probes were linked by a spacer (PEG-phosphoramidite) to modified 3'-amine.

Chip manufacture

In the microarray, each *cry* gene was distinguished by three corresponding oligonucleotide probes (Figure 1, Table 1). Probes were mixed with a printing solution (3 × SSC [sodium chloride/sodium citrate], 0.01% SDS) concentrated to 40 µmol L^{-1} and printed on glass slides with Cartisan (Cartesian Technologies, Delaware, USA). Each print has a volume of about 0.5 nL. The distance between two spots was 500 µm and spot diameter was 200 μm. Each spot was printed twice. Printing temperature was controlled at 23 ºC and relative humidity was 90%. Slides were kept at room temperature for at least 24 h before hybridization. Sixty-three probes and empty controls were arranged in a 10 × 13 array. The location of the probes is listed in Table 1. A1A2 was the empty control and M9M10 was the negative control.

Random labeling of genomic DNA (Brown, 2000)

Genomic DNA was quantified by spectrophotometry, labeled with high-concentration Klenow enzyme and random primer. Labeling volume was 50 μL containing 5 μL of 10× buffer, 30 μg of genomic DNA, 5 μL of 10 × dNTP mix (dATP, dGTP, dCTP each 1.2 mmol L^{-1}, 0.6 mmol L^{-1} dTTP), 3 µL of Cy3 or Cy5 dUTP (Amersham), 5 µL of hexamer primer, 50 U μL^{-1} of Klenow DNA polymerase (Invitrogen or New England Biolabs [NEB]). Reaction mix was incubated at 37 ºC for 2 h, inactivated in boiling water for 10 min, and chilled on ice. Labeled DNA was analyzed with 0.7% agarose gel electrophoresis and Typhoon 9410 scanner (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The probe was precipitated and suspended in 2 μL ultrapure water and 8 μL hybridization buffer (6 × SSC, 0.5% SDS, 5 × Denhart's reagents, 0.1 µg μL^{-1} salmon sperm DNA).

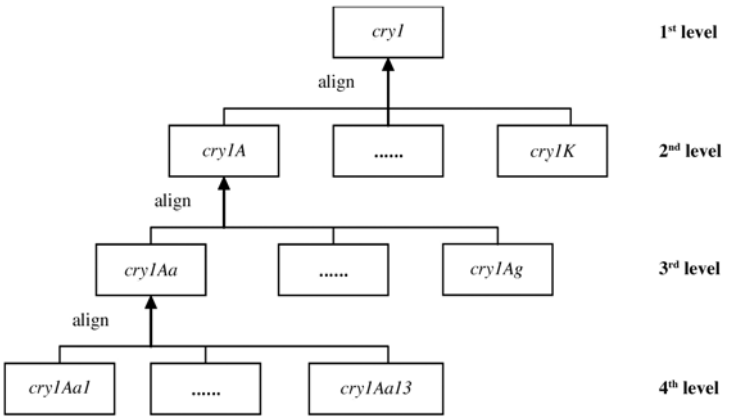


Figure 1. Strategy for designing a probe to detect and determine the genotype of Bt *cry1*, *cry2*, and *cry9* genes.

Table 1. Probes for detection and typing of Bt cry1, cry2, and cry9 genes.

Name	Sequence (5'-3')	Length	Tm	GC%	Location (row-column)
01Aa	GCCTCCCTAACGACCAACTTGCCTTCCACTATATATAGAC	40	69.5	47.5	A3,A4
01Ab1[a]	CGTTTCTCTATTTCCGAACTATGATAGTAGAACGTATCCA	40	65	37.5	A5,A6
01Ab2[a]	GATATGACGGAGCCTATGAAAGCAATTCTTCTGTACCAGC	40	68.6	45	A7,A8
01Ac	CGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAACG	46	74.4	52.2	A9,A10
01Ad	GCTGAGTATGCGCCAGTTTATGAGGAAGCATATATAGATGG	41	68	43.9	B1,B2
01Ae	GGAACTTTCATCGTGGATAGTGTAGAATTATTCCTCATGGAAG	43	67	39.5	B3,B4
01Af	GCAATTACATTATCAGTTCAGAGAGTGTCATGTGTGGACAAAGGTCGG	48	71.6	43.8	B5,B6
01Ag	CACTGTTTCTTCTCCTTGTTCTCTTGATACATGCTAGGTCGA	42	68.9	42.9	B7,B8
01A	TCAATGACATGAACAGTGCCCTTACAACCGCTATTCCTCT	40	70.4	45	B9,B10
01Ba	GTAAAAGCATCCGAACTTCCTCAAGGTACCACTGTTGTTA	40	68.4	42.5	C1,C2
01Bb	GAATCTTCATTCAGGTGTTACTGTTGTTGGAGGGCCAGGA	40	70.7	47.5	C3,C4
01Bc	ACTGAGAATCAAGGTACAAGAACGTGCGGTAATGAATGTGGT	42	70.5	42.9	C5,C6
01Bd	ACTGGTGGAGACGTAGTTAGATTGAATAGGAATAATGGTAAT	42	65.8	35.7	C7,C8
01Be	GCTATGACACGCGTGTTTATCCAATGAATACCAGTGCTCAA	41	70.1	43.9	C9,C10
01Bf	GGTTATCTCACATAGGGCTCATTTCACAATCTAGGGTGCA	40	69.1	45	D1,D2
01B	CGCATCAACAGTCCAAACGGGTATTAACATAGCTGGTAGAA	41	69.5	43.9	D3,D4
01Ca	GCACGAGTTATAGTATTAACAGGAGCGGCATCCACAGGAG	40	70.5	50	D5,D6
01Cb	TCTGGCAGTCAAGGTGCATCAATAAGTATTAGTAATAATGT	41	65.4	34.1	D7,D8
01C	CTGGATTTGAAGTACCCCTTTTATCCGTTTATGCTCAAGCGGC	43	71.3	46.5	D9,D10
01Da	AGGTCTATGTTAGAGCGTTTAGCGACTGGGAGAAAGATCCTACT	44	71	45.5	E1,E2
01Db	AAAATTTAGAGGGATCTCGATTGTCAGATTGGGTTGTATAT	41	65.3	34.1	E3,E4
01D	CTCGTTCGTTCGCTCATACAACACTCTTCACTCCAATAACCT	42	70.4	45.2	E5,E6
01Ea	TACTGTAGCAACAAACATCGCCTTGGAGATTAGTCGTCTG	40	69.3	45	E7,E8
01Eb	AGTAACGTAGCAGCAGAAATCGGCTTGGGGCTTAGTCGTCT	41	73.6	51.2	E9,E10
01E	ACGCGGGAAGTATATACAGATCCGGTAATTAATATAACTGA	41	65.7	36.6	F1,F2
01Fa	ACATTCCCAATGAGCCAGAGTAGTTTCACAGTAGGTGCTGA	41	71.1	46.3	F3,F4
01Fb	GCTGTAAAAGCACACAATCTTCATTCGGGTTCTACGGTTG	40	69.6	45	F5,F6
01F	GGGAGGACACTTAGTTAGTTCACGAAATACGGCTGGTAACC	41	70.4	48.8	F7,F8
01Ga	ACGACAAGAAATAACAGAGTTAGAAAGGAATAGAGCGACTGC	42	68.1	40.5	F9,F10
01Gb	CGCAAGAGGGCAAGCAATTCAGAGACTAGTAGGGTTTGGAAGG	43	72.9	51.2	G1,G2
01G	ACCGTTAGTAAAGGCTTTCAATCTTCATTCAGGTGCCACG	40	70.1	45	G3,G4
01Ha	AGCAAATAGCAGTGGTGTATTTAGGCATTTACCGCAACCTTCGT	44	71.9	43.2	G5,G6
01Hb	ACCTTAGGAGCTTTAAGAGTGACCCTTACAGGACAATTACCACAA	45	70.4	42.2	G7,G8
01H	CTTGGAGATAGTTATGAAGTGTATATTGAGTCGCTTAGAGAATGG	45	66.5	37.8	G9,G10
01Ia	ACACAAATGTATCCAATTAAAACTACAGCCCAACTTACAAGA	42	66.2	33.3	H1,H2
01Ib	GCACTTTCAGACTTGAGAGGATTAGGTGATGCTTTAGCCGTC	42	70.8	47.6	H3,H4
01Ic	GATATCGAGTTAATGGTATTAGATTTAGTTCGCGTATTCCCAAGC	45	67.2	37.8	H5,H6
01Id	TCCACATTTTATAACCGCCAATCTAGTCAAACACAGGAAT	40	67.1	37.5	H7,H8
01Ie	TTGGATTAAAAATCGCAACAACGCAAGGGCTACAAGTGTT	40	69.7	40	H9,H10
01I	TTCAGCAACTATGAATAGAGGAGAGGACTTAGACTATAAA	40	64	35	I1,I2
01Ja	AGATCCTGATAACGAAGCGGCTAAAAGTAGAGTAATTGAT	40	66.3	37.5	I3,I4
01Jb	TTTCACCCCCGCCATCTGCAAGATACAGAAATCCTGGAAC	40	72.1	50	I5,I6
01Jc	AATGCGGTACAGCGTTATCGAATTAGAATGCGTTATGCGGC	41	71.7	46.3	I7,I8
01Jd	ACTCATTTCCGAATCCCATTTCAGGCAAACACTCCACAAAGGT	43	72	44.2	I9,I10
01J	GGATGGGGAGAGGATATTACCTGATATCGATCCACTCGAAGTTTC	45	70.4	46.7	J1,J2

Continuation Table 1.

01Ka	CGTGGATGGATAGGAAGCACTGGTATTAGCATTCAAGGAGGCG	43	72.7	51.2	J3,J4
01-2[b]	GAGGCAGAATATGATTTAGAAAGAGCACAAAAGGCGGTGAATG	43	70.5	42	J5,J6
01-3[b]	GGTCGTGGCTATATCCTTCGTGTCACAGCGTACAAAGAGGGATATGGAGA	50	74.8	50	J7,J8
01-4[b]	GAAGTTTATATAGATCGAATTGAATTTGTTCCGGTAGAAGTAAC	44	64.2	31.8	J9,J10
02Aa	CACAACTTTAATTGCAGCACGGTCCTCCCTCCTTTATCAA	40	70	45	K1,K2
02Ab	TGGAGGAATTTCGTCTGGTGATATAGGTGCATCTCCGTTT	40	70	45	K3,K4
02Ac	GAGTGTCATCTAGCCGCATAGGTCAAGCTAATCTTAATCA	40	67.7	42.5	K5,K6
02Ad	CGAATTATGTATTATCCGGCTTTAGTGGGGCTAGTCTTTT	40	66.9	40	K7,K8
02A	ACGCTTAGAGGGAATGGAAATAGTTACAATCTTTATTTAAGAGT	44	65.4	31.8	K9,K10
09Aa	TTACTGAACTAATCTCTGGACAACATACGACTGCTACACAA	41	67.4	39	L1,L2
09Ba	TGAGTTACCGAGCCTAGACCCAAATGAACCTATCAGTAGAAGT	43	70	44.2	L3,L4
09Ca	CGTGGAGGGGTTTCTATCGGTGATGTTAGATTAGGGAGCACAATGA	46	72.7	47.8	L5,L6
09Da	TCTCAGAAGCAGTAAAGGGTAGGGCATTGGATGACCTAACTGGA	44	72.4	47.7	L7,L8
09Ea	CGAAGAACGGACGGTGGTGCAGTTGGAACGATTAGAGCTAATGT	44	73.7	50	L9,L10
09Eb	AGCAGTAGTAGGGACAGCAGCGGATCATTTAACGGGATTACACGA	45	73.7	48.9	M1,M2
09E	AGTTACGGTCAGATTACCTCTAACACACGTTTATTCAATACGACT	45	68.4	37.8	M3,M4
9	GGATTGCTGTGAAGCGGCTCAAACACATGAGTTTTCTTCCTATATT	46	71	41.3	M5,M6
vip3Aa[c]	GCTTAAATGATCTTATCGCACAGGGAAACTTAAATACAGA	40	65	35	M7,M8
lec[d]	TTGCCAGCTTCGCCGCTTCCTTCAACTTCACCTTCTATGC	40	75.2	52.5	M9,M10

[a]For the detection and typing of *cry1Ab*; [b]For the detection and typing of *cry1*; [c]For the detection of *vip3Aa;* [d]For the detection of LECTIN (negative control); Tm: DNA melting temperature; GC%: guanine-cytosine content.

Hybridization and washing

The chip was pretreated with hybridization buffer at room temperature for 1 h, then hybridized with 10 μL probe. It was placed in a box and incubated at 56 ºC for 2 h, and washed with buffers A (1 × SSC, 0.2% SDS), B (0.2 × SSC), C (0.1 × SSC) for 1 min each. It was finally spun dried (Wen *et al*., 2003).

Microarray scanning and data analysis

The microarray was scanned with GenePix 4000 (Axon Instrument, Foster City, California, USA) at 530 nm excitation, 585 nm (Cy3) and 650 nm (Cy5) emission. PMT was 700 to 800. Power level was 33% to100%. Image resolution was 10 μm in 16 bit tiff format. Signal intensity was the mean value of the two spots after background subtraction.

RESULTS

Oligonucleotide probes for gene detection and typing

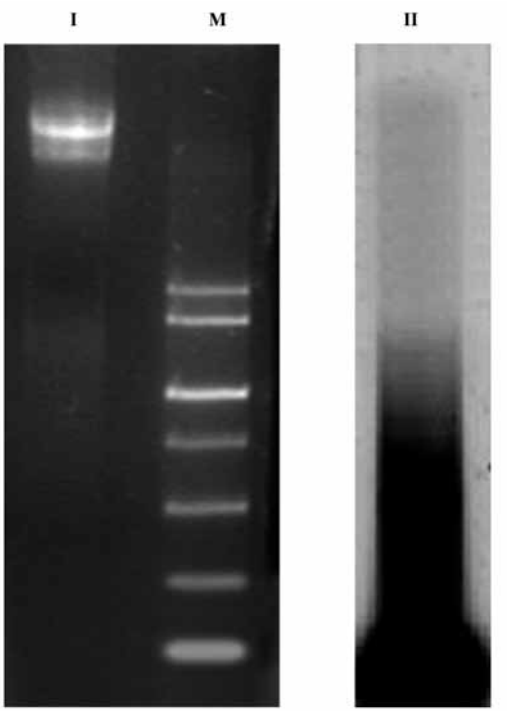
Nucleotide sequences (131 entries) of *cry1*, *cry2,* and *cry9* were retrieved from the NCBI database. After removing non-coding sequences, they were deposited in a local Bioedit database for local BLAST. Sequences were aligned with the strategy described in Figure 1 using ClustalW from the Vector NTI package (Invitrogen, Carlsbad, California, USA). Thirteen sequences, *cry1Aa1~cry1Aa13,* were first aligned and a consensus sequence *cry1Aa* was obtained. Probes were designed by Array Designer 2.0 based on the consensus sequence. Melting temperature of the probe was 70 ± 5 ºC, sizes were 40to 50 bp, hairpin maximum ΔG was -3.0 kcal mol^{-1}, self-dimer maximum ΔG was -6.0 kcal mol^{-1}, and the run/repeat (dinucleotide) maximum length was 4 bp. A specific oligonucleotide probe, 01Aa, for *cry1Aa* gene detection and typing was obtained after the probe sequences were searched with local BLAST to ensure specificity. Those with E value smaller than 1e^{-10} and specificity score greater than 70 were selected (Liu *et al*., 2004). Similarly, consensus sequences of *cry1Ab*… *cry1Ag* were obtained and corresponding oligonucleotide probes 01Ab…01Ag obtained. After consensus, *cry1A* was obtained by aligning the sequences of *cry1Aa*… *cry1Ag*, and the 01A probe was obtained. In the same way, we got the consensus sequences *cry1B*…*cry1K* and the 01B…01K probes. Furthermore, we got a consensus sequence *cry1* by aligning the sequences of *cry1A*… *cry1K,* and 01 probes were obtained.

In this method, the *cry1Aa1* gene could be distinguished by three probes: 01, 01A, and 01Aa, and we called this method TPPS (Three-Ply Position System).

We designed a total of 63 probes. Information regarding the probes (sequences, Tm, size, GC%, and location on chip) is listed in Table 1.

Random labeling of genomic DNA

Genomic DNA was fragmented during fluorescent labeling. By controlling the amount of random primer, the labeled probe was concentrated in a smear band of 1000 bp ~ 150 bp. Since the non-fluorescent bands cannot be registered in the Typhoon scanner, the smear band (II) image in Figure 2 is different from the other two lanes.



I: Genomic DNA; II: Labeling product; M: Marker DGL2000.

Figure 2. Random labeling of prepared products and total DNA.

This figure shows that genomic DNA was randomly fragmented and successfully labeled with fluorescent dye.

Hybridized fluorescent images

Figure 3 shows the hybridized fluorescent images with partial Bt of Ly30, G03, and HD-73. The *cry* genes in these strains were examined with Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP).

The fluorescence signal was weaker than that obtained from the PCR-based probe because of the small size of the oligonucleotide probe. After optimizing hybridization parameters, *cry* genes in the different strains can be appropriately distinguished with the corresponding probes. The result of chip hybridization is consistent with PCR-RFLP (Yao *et al*., 2003).

DISCUSSION

Nowadays, for an important agricultural pest such as Lepidoptera insects, Bt is widely and effectively used as a critical biopesticide in biocontrol. It is significantly necessary to screen a Bt isolate with high toxicity and clone its novel insecticidal toxin genes to construct genetically engineered bacteria and transgenic plants. Among the insecticidal toxin genes against Lepidoptera, the most important are the *cry1, cry2,* and *cry9* genes. In this study, we used an oligonucleotide microarray to detect and identify *cry1, cry2,* and *cry9* genes in Bt.

At this time, methods for the detection and identification of Bt *cry* genes are based on PCR. One method is multiplex PCR (Bourque *et al*., 1993; Jaoua *et al*., 1996) and another is the two-step multiplex PCR (Ceron *et al*., 1995; Juarez-Perez *et al*., 1997). These methods, which include more than one primer, are applied

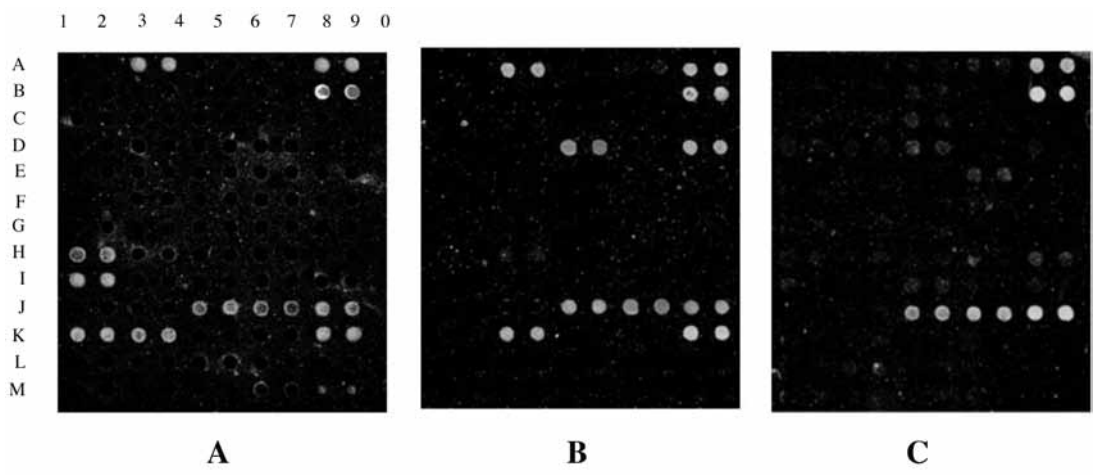


Figure 3. Fluorescent images of microarray hybridized with Ly30 (A), G03 (B), and HD73 (C). Ly30 contains *cry1Aa*, *cry1Ac*, *cry1Ia*, *cry2Aa*, and *cry2Ab*. G03 contains *cry1Aa*, *cry1Ac*, *cry1Ca*, and *cry2Ab*. HD-73 contains *cry1Ac*.

to detect known *cry* sequences. However, it becomes less reliable than separate PCR (Lin *et al*., 2000). Another method is PCR-RFLP can identify known and unknown *cry* genes with only several pairs of primers. However, it becomes complicated, tedious, and time-consuming for analyzing multiple genes. It also ignores some variances in the conserved region (Yu *et al*., 1998). More importantly, PCR does not allow the rapid, parallel, and high throughput detection and identification of microbes. F. Song identified 14 known *cry* genotype with three universal primers and the corresponding enzymes, in the 3rd –level (Song *et al*., 2003); scholars from Israel obtained *cry* genes by PCR using five universal primers, and then identified 20 *cry* genotypes (Ben-Dov *et al*., 1997) by using specific primers. In this study, 45 known *cry* genotypes can be identified by probes. It fully reflects that the DNA microarray is an advantage.

The design and selection of probes are essential for the quality and application of a microarray. It is particularly important to use bioinformatic tools to explore the details of related genes in designing and optimizing probes.

The probes should be able to not only detect, but also correctly classify *cry* genes. There are currently 375 known *cry* sequences. The similarity among the 4th level sequences is greater than 95%. It is not possible to design an oligonucleotide probe that is able to correctly detect and type all *cry* genes at this level. As a result, we designed probes to distinguish sequences at the 1st, 2nd, and 3rd levels with a reasonable specificity level. At the 3rd level, some sequences have a 75% similarity. It is difficult to design proper specificity probes Consequently, we relaxed the criteria, such as Tm, length, E value, and specificity score, and even designed two separate probes for some sequences (for example, *cry1Ab*). It is not possible to find one or two probes that could detect all *cry1* genes so we designed three probes which contain almost all the *cry1* genes (Liu *et al*., 2004).

In this study, the detection and typing of *cry* genes were based on TPPS. Any *cry* gene in the NCBI can hybridize with three corresponding probes. If a new *cry* gene exists in a strain, it will be unable to hybridize with all corresponding three-tier probes. Analysis of the hybridization profile can reveal the new *cry* gene. If a strain has more than one type of new *cry* genes (i. e. *cry1Aa* and *cry1A*), this method will be unable to reveal them (Jaroslaw *et al*., 2005).

Fluorescence labeling of genomic DNA is another key step of chip-based detection. It is not possible to design one or a few sets of primers for labeling all *cry* genes simultaneously by PCR amplification because there are a large number of diverse *cry* genes. Thus, conventional PCR-based chip detection is not applicable here.

Labeling of genomic DNA usually employs random primed nucleotide synthesis. This method can generate short fluorescent-labeled DNA. Due to its complexity, genomic DNA was first sheared with an ultrasonic processor (Vibra-Cell) before labeling. Therefore, it is not necessary to sonicate DNA prior to labeling. Since a large amount of genomic DNA is used for labeling, a high concentration of Klenow DNA polymerase should be used. If not, low efficiency of labeling results. In addition, the amount of random primer should be optimized so that the product is 1000 bp or shorter. A large DNA fragment could cause the hybridization signal to decrease due to the spatial effect.

Since the random labeling method does not have a selective amplification step of target genes, the content of target genes is very low, leading to a low hybridization signal on the oligo chip. To counteract these drawbacks, it is necessary to use several dozen or even one hundred micrograms of genomic DNA to label them. In addition, the labeled target has to be concentrated and purified before hybridization. High PMT and power settings are necessary during array scanning. These operations lead to high background and increased difficulty level (Volokhov *et al*., 2002; Wang *et al*., 2003). Sometimes inconsistent results may be obtained using a chip to detect genes in the same strain. However, it may be reduced by a higher hybridization temperature. This inconsistency may be caused by random primed DNA labeling which requires multiple trials to determine the *cry* genes in Bt.

Although sensitivity and consistency need to be improved, our study demonstrates that hierarchical oligonucleotide microarray is a high throughput assay capable of realistically reflecting all functional genes without PCR amplification of the target fragment (Wu *et al*., 2001; Zhou, 2003). Compared to the PCR method, the chip-based assay has more application prospects.

CONCLUSIONS

Our study demonstrates that hierarchical oligonucleotide microarray is a high throughput assay capable of realistically reflecting all functional genes without PCR amplification of the target fragment, although the sensitivity and consistency need to be improved.

ACKNOWLEDGEMENTS

This study was supported by 973 Project (Nº 2009CB118902, 2007CB109203), and 863 Plan (Nº 2006AA10A212), China.

RESUMEN

Aplicación de microarreglo de oligonucleótidos en la detección y tipificación génica de genes *cry* en *Bacillus thuringiensis*. Se desarrolló un ensayo paralelo, rápido, de alto rendimiento, basado en microarreglo de oligonucleótidos para la detección y tipificación génica confiable de tres genes *cry* (*cry1*, *cry2* y *cry9*) en *Bacillus thuringiensis* (Bt). Después de la no reacción de cadena polimerasa (PCR), el ADN genómico de Bt amplificado se marcó con fluorescencia usando primer al azar. Las correspondientes sondas de oligonucleótido fueron diseñadas por los diferentes genes *cry* en ADN genómico de Bt que pueden ser hibridados después de análisis cluster y se imprimieron en portaobjetos de vidrio. Este microarreglo ha detectado e identificado sin ambigüedad los genes *cry* en 10 aislamientos y Bt de referencia. Nuestros datos demuestran que el microarreglo es simple y rápido para la detección y tipificación génica de genes. Este tipo de ensayo es además una herramienta potencialmente valiosa para identificación y caracterización de genes funcionales bacterianos en general.

Palabras clave: microarreglo de oligonucleótidos, genes *cry*, tipificación génica, marcación con primer al azar, bioinformática.

LITERATURE CITED

Ben-Dov, E., A. Zaritsky, E. Dahan, Z. Barak, R. Sinai, Manasherob, *et al*. 1997. Extended screening by PCR for seven *cry*-group genes from field-collected strains of *Bacillus thuringiensis*. Appl. Environ. Microbiol. 63:4883-4890.

Bodrossy, L., N. Stralis-Pavese, J.C. Murrell, S. Radajewski, A. Weilharter, and A. Sessitsch. 2003. Development and validation of a diagnostic microbial microarray for methanotrophs. Environ. Microbiol. 5:566-582.

Bourque, S.N., J.R. Valéro, J. Mercier. M.C. Lavoie, and R.C. Levesque. 1993. Multiplex polymerase chain reaction for detection and differentiation of the microbial insecticide *Bacillus thuringiensis*. Appl. Environ. Microbiol. 59:523-527.

Brown, P. 2000. Genomic DNA labeling protocol. Available at http://cmgm.stanford.edu/pbrown/protocols/4_genomic.html (accessed October 2007).

Ceron, J., A. Ortiz, R. Quintero, L. Guereca, and A. Bravo. 1995. Specific PCR primers directed to identify *cry I* and *cryIII* genes within a *Bacillus thuringiensis* Strain collection. Appl. Environ. Microbiol. 61:3826-3831.

Crickmore, N. 2003. Full list of delta-endotoxins. Available at http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html (accessed October 2006).

Gabig, M., and G. Wegrzyn. 2001. An introduction to DNA chips: Principles, technology, applications and analysis. Acta Biochim. Pol. 48:615-622.

Hwang, T.S., J.K. Jeong, M. Park, H.S. Han, H.K. Choi, and T.S. Park. 2003. Detection and typing of HPV genotypes in various cervical lesions by HPV oligonucleotide microarray. Gynecol. Oncol. 90:51-56.

Jaoua, S., N. Zouari, S. Tounsi, and R. Ellouz. 1996. Study of the δ-endotoxins produced by three recently isolated strains of *Bacillus thuringiensis*. FEMS Microbiol. Lett. 145:349-354.

Jaroslaw, L., B. Alejandra, B. Roland, and M. Luke. 2005. Assessment of *cry1* Gene Contents of *Bacillus thuringiensis* Strains by Use of DNA Microarrays. Appl. Environ. Microbiol. 71:5391-5398.

Juárez-Perez, V.M., M.D. Ferrandis, and R. Frutos. 1997. PCR-Based approach for detection of novel *Bacillus thuringiensis cryV* genes. Appl. Environ. Microbiol. 63:2997-3002.

Lin, Y., Z.P. Huang, J.W. Chen, B.W. Huang, and X. Guan. 2000. Strategies and advances on PCR identification for insecticidal crystal protein genes of *Bacillus thuringiensis*. Chin. J. Agric. Biotechnol. 8:56-58 (in Chinese).

Liu, X.G., F. Song, S.Y. Wen, S.Q. Wang, D.F. Huang, and J. Zhang. 2004. Study of microarray detection method of *cry* genes from *Bacillus thuringiensis*. Sci. Agric. Sin. 37:987-992 (in Chinese).

Small, J., D.R. Call, F.J. Brockman, T.M. Straub, and D.P. Chandler. 2001. Direct detection of 16S rRNA in soil extracts by using oligonucleotide microarrays. Appl. Environ. Microbiol. 67:4708-4716.

Song, F., J. Zhang, A. Gu, Y. Wu, L. Han, K. He, *et al*. 2003. Identification of *cry1I*-type genes from *Bacillus thuringiensis* strains and characterization of a novel *cry1I*-type gene. Appl. Environ. Microbiol. 69:5207-5211.

Volokhov, D., A. Rasooly, K. Chumakov, and V. Chizhikov. 2002. Identification of Listeria species by microarray-based assay. J. Clin. Microbiol. 40:4720-4728.

Wang, D., H. Gao, R. Zhang, X. Ma, Y. Zhou, and J. Cheng. 2003. Single nucleotide polymorphism discrimination assisted by improved base stacking hybridization using oligonucleotide microarrays. Biotechniques 35:300-302, 304, 306.

Wen, S.Y., H. Wang, O.J. Sun, and S.Q. Wang. 2003. Rapid detection of the known SNPs of CYP2C9 using oligonucleotide microarray. World J. Gastroenterol. 9:1342-1346.

Wu, L., D.K. Thompson, G. Li, R.A. Hurt, J.M. Tiedje, and J. Zhou. 2001. Development and evaluation of functional gene arrays for detection of selected genes in the environment. Appl. Environ. Microbiol. 67:5780-5790.

Yao, J., J. Zhang, F. Song, C. Li, X. Liu, and D. Huang. 2003. Cloning and expression of *cry1Ac* gene from a novel *Bacillus thuringiensis* Ly30 strain. Chin. J. Agric. Biotechnol. 11:516-519 (in Chinese).

Yu, J.X., Y. Pang, J.Y. Liu, Y.J. Zhang, and M.Q. Xu. 1998. Screening of novel crystal protein genes from soil-collected *Bacillus thuringiensis* by PCR-RFLP. p. 43. *In* 7th International Colloquium on Invertebrate Pathology and Microbial Control and 4th International Conference on *Bacillus thuringiensis*, Sapporo, Japan. August 23-28. Society for Invertebrate Pathology, Marceline, Missouri, USA.

Zhou, J.Z. 2003. Microarrays for bacterial detection and microbial community analysis. Curr. Opin. Microbiol. 6:288-294.