Microbiological Research **165** (2010) 376—383

Available online at www.sciencedirect.com

ScienceDirect

Microbiological Research

www.elsevier.de/micres

Determination and distribution of *cry*-type genes in halophilc *Bacillus thuringiensis* isolates of Arabian Sea sedimentary rocks

Deeba Noreen Baig*, **Samina Mehnaz**

School of Biological Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan

Received 14 July 2009; received in revised form 12 August 2009; accepted 16 August 2009

KEYWORDS
Bacillus thuringiensis;
Cry genes;
Bio-insecticide;
Marine environment

Summary

Bacillus thuringiensis produces insecticidal crystal during its sporulation phase. In this study, marine sediments from Arabian Sea along coastal area of Pakistan were examined for the occurrence of *B. thuringiensis*. On the basis of morphological and biochemical properties, 31 out of 200 colonies were assigned to *B. thuringiensis*. Isolated strains were characterized on the basis of *cry* genes profile. PCR approach was used to analyze the presence of different crystal toxin encoding genes with six pairs of universal primers that could detect the *cry1*, *cry4*, *cry7*, *cry8*, *cry9*, and *cry10* genes. Strains containing *cry1* genes were the most abundant in our collection (49.5%). Seventeen different profiles of *cry* genes were identified, i.e., twelve harboring two *cry* genes while five profiles of more than two *cry* genes. The characterization of these strains provided useful information on the ecological patterns of distribution of *B. thuringiensis* and opportunities for the selection of new strains to develop novel bio-insecticidal products.

Introduction

Bacillus thuringiensis is a gram-positive, facultative anaerobe and spore-forming bacterium. It produces different insecticidal toxic proteins in para-sporal crystals during the stationary phase of its growth cycle (Rowe and Margaritis 1987). These proteins are toxic to a wide variety of insects which attack on economically important crops. This makes the bacterium an environment friendly biopesticide for control of insects/pests in agriculture forestry, veterinary and medicine (Schnepf et al. 1998). An extensive study has been done on the occurrence of *B. thuringiensis* in different ecological zones. It has been reported that *B. thuringiensis* can be present in many different

*Corresponding author. Tel.: +92 42 3334308303; fax: +92 42 9230980.
E-mail address: deeba.noreen@yahoo.com (D.N. Baig)

0944-5013/$ - see front matter © 2009 Published by Elsevier GmbH.
doi:10.1016/j.micres.2009.08.003

habitats such as soil, stored product dust, insect cadavers, grains, agricultural soils, olive tree related-habitats, plant and aquatic environments (Martin and Travers 1989; Meadows et al. 1992; Bel et al. 1997; Ben-Dov et al. 1997; Bravo et al. 1998; Iriarte et al. 1998). In fact, each habitat may contain a novel *B. thuringiensis* strain awaiting discovery which has a toxic effect 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 (Chak et al. 1994; Bravo et al. 1998; Uribe et al. 2003).

Several isolates of *B. thuringiensis* have been used as biological pesticides against different insect orders such as Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera and Acari (Feitelson et al. 1992; Cannon 1996). In addition, some strains of *B. thuringiensis* have been found to be toxic to nematodes and protozoa (Edwards et al. 1988; Feitelson et al. 1992). The lack of mammalian toxicity of Cry proteins has resulted in an increase in the use of *B. thuringiensis* as an insecticide and intensified the search for new strains with different toxic activities. Entomopathogenic activity of *B. thuringiensis* depends on the presence of crystal (Cry) and vegetative insecticidal proteins (Vip). The genes coding for Cry proteins are mostly carried on plasmids ranging from 3 to 150 Mda (Aronson 2002).

Different methods are used for the screening of *cry* genes such as polymerase chain reaction (PCR), southern blotting, serotyping and bioassay to predict the toxicity of *B. thuringiensis* isolates; among all, PCR-based techniques have been proposed most efficient and rapid (Juarez-Perez et al. 1997; Porcar and Juarez-Perez 2003). However, it is important to mention that this method cannot distinguish between expressed and silent genes. All previously reported data clearly indicate that *B. thuringiensis* is among the predominant spore former in all natural environments. However, there has been little information on the distribution of this organism in marine environment. In this report we present the isolation, biochemical characterization of *B. thuringiensis* and detection of *cry* genes in the marine sediments of Arabian Sea along the coastal area of Pakistan.

Materials and methods

Sample collection

Sand, soil, sea shells and stone particles were collected from 5 cm depth from Hawks Bay area of Cape Mount along the Arabian Sea and were placed into plastic bags aseptically. Sampling was done from the bottom of sedimentary rocks which are frequently exposed to marine water.

Bacterial strains

B. thuringiensis subsp. *kurstaki* (HD1), *B. thuringiensis* subsp. *aizawai* (HD133), *B. thuringiensis* subsp. *kurstaki* (HD73), *B. thuringiensis* subsp. *israelensis* (HD500) were kindly provided by Dr. Daniel R. Zeigler from the *Bacillus* Genetic Stock Center (Ohio, USA) and *B. thuringiensis* subsp. *morrisoni* (HD12), *B. thuringiensis* subsp. *dakota* (HD511), *B. thuringiensis* subsp. *tolworthi* (HD537), *B. thuringiensis* subsp. *kenyae* (HD29) and *B. thuringiensis* (IPS78) were kindly provided by Prof. M. Akhtar from the Department of Biochemistry, University of Southampton (UK) and used as reference strains. Two standard *B. thuringiensis* strains; HD-1 and HD-73; were used as reference for the confirmation of biochemical analysis of marine isolates.

Isolation of *Bacillus thuringiensis*

Thirty samples were processed for the isolation of *B. thuringiensis*, by sodium acetate selective method. The crushed homogenized samples (1 g) were incubated in 10 ml of 0.3 M sodium acetate (pH 6.8) at 37 °C for 4 h in 100 ml flask with 250 rpm agitation. Two ml of the each incubated sample was heated at 80 °C for 10 min to eliminate non-sporulated microbes that germinated. The surviving spores were diluted 10–1000 folds in sodium acetate buffer (pH 6.8), and 300 μl of each was spread on T3 agar plates; g/ Liter; (tryptone 3; tryptose 2; yeast extract 1.5; sodium di-hydrogen phosphate 6.9; di-sodium hydrogen phosphate 8.9; $MnCl_2$ 0.005; agar 15) and incubated at 37 °C to grow until sporulation. For each sample 10–14 well isolated colonies representing different morphologies were picked and purified on T3 agar plates. Each culture was grown on T3 agar plate and colonies dispersed in sterile distilled water were examined with a phase contrast microscope for crystal production and morphology.

Phenotype and biochemical analysis

In total, two hundred pure cultures were obtained and thirty five cultures showing *Bacillus* like colonies were selected. All of these strains were identified as *B. thuringiensis*, on the basis of

morphological, physiological and biochemical properties.

Biochemical characterization includes growth on sabouraud dextrose (Difco), at pH 9.6, tyrosine decomposition, casein, starch and gelatin hydrolysis, citrate utilization, nitrate reduction, indole production, presence of cytochrome oxidase, gas production, phenyl deamination, and motility, according to the diagnostic scheme described in Bergey's manual of determinative bacteriology (Holt et al. 1994). Four isolates were not included in the biochemical characterization due to their Gram negative appearance in phase contrast microscopy.

Cry genes identification

The detection of *cry* genes combinations in *B. thuringiensis* isolates was performed by the PCR amplification with the primers listed in Table 1. All crystal proteins producing isolates were screened by six pairs of general primers for the *cry1*, *cry4*, *cry7*, *cry8*, *cry9* and *cry10* genes, as described by Ceron et al. (1995), Ben-Dov et al. (1997) and Alberola et al. (1999). Primers were selected on the basis of their specific *cry* genes targeted to specific insects most prevalent in the local environment.

Total DNA (chromosomal and plasmid) of marine and reference strains of *B. thuringiensis* were isolated from overnight grown cultures on agar plates. A loopful culture was harvested and suspended in 0.2 ml of autoclaved H_2O in a 1.5 ml microfuge tube and kept at −80 °C for 15 min. 200 μl detergent solution (20 mM Tris-Cl pH 8.8, 100 mM KCl, 0.016% Nonidet P40, 3 mM $MgCl_2$, 2% Triton X100) and 6 μl (5 mg ml^{-1}) of Proteinase K was added into suspended culture and incubated at 45 °C and 94 °C for 30 and 10 min, respectively. Phenol: chloroform extraction was followed by ethanol precipitation. Finally, the washed dried DNA pellet was suspended in 100 μl of TE buffer (pH 8). DNA was quantified by resolving on 1% agarose gel and UV spectrum using Biospec Shimadzu CPS 240 A.

500 ng DNA template was mixed with reaction buffer containing 200 μM deoxynucleotide triphosphate mix, 0.5 μM of forward (+) and reverse (−) primers, 1.5 mM $MgCl_2$ and 2.5 U μl^{-1} of Taq DNA polymerase (Fermentas). Amplifications were carried out in a DNA thermal cycler (Applied Biosystem). For all *cry* genes, an initial denaturation step was applied at 94 °C for 1 min and followed by denaturation at 94 °C for 1 min, annealing at 54 °C (for *cry1*) and 60 °C (for *cry4*, *cry7*, *cry8*, *cry9* and *cry10*) for 1 min, extension at 72 °C for 1 min. Thirty-five cycles were carried out for the amplification of *cry* gene fragments. Finally, an extra extension step was applied at 72 °C for 10 min. After amplifications, 10 μl of each PCR product were electrophoresed on 1% agarose gel in TAE buffer (40 mM Tris-Acetate, 1 mM EDTA; pH 8) at 80 V for 40 min. Gels were visualized in a gel documentation system.

Sub-screening of twelve *cry1* positive isolates with *cry1* gene specific primers (*cry1A*, *cry1C*, *cry1D*, *cry1F*, *cry1G*, *cry1H*, *cry1I*, *cry1K*) was done. For the sub-screening of isolates, HD-1, HD-12, HD-29, HD-73 and HD-133 were used as positive control.

Results

Isolation of *B. thuringiensis* from marine samples

Microscopic and biochemical analyses resulted in the selection of thirty one *B. thuringiensis* strains out of 200 cultures. The colony morphology of the selected strains varied, i.e., off-white, light yellow, light pink color, smooth or slightly wrinkled margin, rounded, oval shape, 0.1-2.0 mm diameter. Twenty six isolates showed growth on medium supplemented with 0.01% lysozyme. All strains showed rich growth in the presence of sabouraud dextrose, 10% sodium chloride, when provided as medium supplement, individually. All strains were positive for growth at pH 9.6, tyrosine decomposition, casein, starch and gelatin hydrolysis, citrate utilization, nitrate reduction, indole production, presence of cytochrome oxidase and negative for motility, gas production and phenyl deamination.

PCR-based detection of *cry* genes in *B. thuringiensis*

Each set of primers produced a PCR product with a unique molecular weight. Results showed the expected sizes of PCR products, i.e., 276, 439, 264, 430, 354 and 623 bp for *cry1*, *cry4*, *cry7*, *cry8*, *cry9* and *cry10* genes, respectively as mentioned in Table 1.

The presence of more than one *cry* gene in the isolates was used as a parameter to categorize them into three groups; (i) single *cry* gene; (ii) two *cry* genes and (iii) more than two *cry* genes. In the first group seventeen isolates; namely MKAD-4, MKAD-5, MKAD-24 (*cry1*), MKAD-19, MKAD-22, MKAD-23, MKAD-25, MKAD-26, MKAD-29, MKAD-30

Table 1. Primers used for the detection of different *cry* genes in the local isolates of *Bacillus thuringiensis*.

Primer pair	Sequence (5′→3′)	Gene recognized	T_m (°C)	Product size (bp)	Source
Un1(d) Un1(r)	(+) 23mer 5′CATGATTCATGCGGCAGATAAAC3′ (−) 23mer 5′TTGTGACACTTCTGCTTCCCATT3′	*cry1*	55.2 54.5	276	Ben-Dov et al. (1997)
Un4(d) Un4(r)	(+)24mer 5′GCATATGATGTAGCGAAACAAGCC3′ (−)25mer 5′ GCGTGACATACCCATTTCCAGGTCC3′	*cry4*	52.2 50.2	439	Ben-Dov et al. (1997)
Un7	(+)31mer 5′TGATCCAGCAACTATAACACGAGTGATAGAT3′ (−)33mer 5′CACTTAACGCAATGGTTAGAATATTCAGAGATA3′	*cry7*	61.4 60.4	264	Alberola et al. (1999)
8A	(+)23mer 5′AACGTAATTCGTCCGCCACACTT 3′ (−)27mer 5′TTTCAGCTACCGGTACAGTTCTATCTA3′	*cry8*	58.6 54.3	430	Alberola et al. (1999)
Un9	(+)24mer 5′CGGTGTTACTATTAGCGAGGGCGG3′ (−)24mer 5′GTTTGAGCCGCTTCACAGCAATCC3′	*cry9*	60.2 58.2	354	Ben-Dov et al. (1997)
Un10	(+)32mer 5′TATTGTTGGAGTTAGTGCAGGTATTATTGTAG3′ (−)27mer 5′TATTCCATGTTGCGTTAGTATTAGTTC3′	*cry10*	57.1 52.6	623	Alberola et al. (1999)
CJI-1 CJI-2	(+)22mer 5′cgtagaagaggaaatctatcca3′ (−)19mer 5′tatcggtttctgggaagta3′	*cry1Ac*	50.7 48.4	272	Ceron et al. (1995)
1Ca	(+)33mer 5′ATTGGGGAGGACATCGAGTAATATCTAGCCTTA3′ (−)32mer 5′ACTCCTGTGGATGCCGCTCCTGTTAATACTAT3′	*cry1C*	63.8 65.3	670	Alberola et al. (1999)
1Db	(+)27mer 5′GTCGCTAAACGCTCTAACATAGACCTT3′ (−)34mer 5′GTTTAAGTAATCCTGATGCGATATTACTAGATGC3′	*cry1D*	56.9 59.6	802	Alberola et al. (1999)
1Fb	(+)28mer 5′GCTTATACTATTGTTAATATAAATGCGC3′ (−)28mer 5′GTATCAGCACCTAVTGTGAAACTACTCT3′	*cry1F*	52.8 53.0	245	Alberola et al. (1999)
1Ga	(+)34mer 5′TTGCAGGTAATATAAATTTTGGTTTAAGTATAGC3′ (−)30mer 5′TCCCTCAATTGGAATTTCATCAATAGTATC3′	*cry1G*	57.9 59.0	419	Alberola et al. (1999)
1Ha	(+)28mer 5′CACGCGCTTTCTTTTGGAGTCACTTCTC3′ (−)34mer 5′GTTCTCTTAATATATTCTGTAAATTCGCATTCAA3′	*cry1H*	64.9 58.5	731	Alberola et al. (1999)
1Ia	(+)28mer 5′TTGAAAATGTCTGAGTATGAAAATGTAG3′ (−)27mer 5′GTTCGACTTGACGGTTATAAAATGTTG3′	*Cry1I*	52.5 56.4	621	Alberola et al. (1999)
1K	(+)26mer 5′AAAACGCAATTATTCGACAACCTCAC3′ (−)26mer 5′GCCCACCAGCCTAATGATACAGTTCG3′	*cry1K*	58.6 59.8	246	Alberola et al. (1999)

(*cry4*), MKAD-27 (*cry7*), MKAD-8 and MKAD-31 (*cry8*), MKAD-15 (*cry9*) and MKAD-10, MKAD-18, MKAD-28 (*cry10*) were positive for single *cry* gene. In the second group six isolates were harboring the two *cry* genes, i.e., MKAD1, MKAD2 harbored *cry1*and *cry8* gene, MKAD6 and MKAD17 harbored *cry1* and *cry7*, MKAD8 contained *cry7* and *cry8* and MKAD14 contained *cry8* and *cry9*. In the third

group, MKAD3, MKAD-12 and MKAD-13 showed presence of *cry1*, *cry8* and *cry9* genes, MKAD11 indicated the presence of *cry1*, *4*, *7 and 9*; MKAD 21 indicated the presence of *cry4*, *9* and *10*. The profiles of *cry* genes indicated that MKAD-9, MKAD-11 and MKAD13 isolates were unique as they exhibited most diversified combinations of *cry* genes active against Lepidopteran, Dipteran and Coleopteran insects. Two isolates MKAD16 and MKAD20 showed amplification with *cry4* and *cry7* when assayed with universal primers (Figure 1, lanes 18 and 33) but the size of the amplified products were higher than expected. The sequences of these amplified products did not show homology with any *cry* gene reported sequences.

Out of twelve, eight isolates were found to be positive for *cry1* subtypes (Figure 2). Seven isolates showed presence of single *cry1* subtype but one isolate (MKAD-3) exhibited amplification for two *cry1* subtypes (*cry1F* and *cry1K*). All of these isolates did not show amplification with the *cry1A*, *cry1G* and *cry1H* gene specific primers. MKAD-24 and MKAD-12 were positive for *cry1C* and *cry1D*, respectively. MKAD-3 and MKAD-6 isolates were positive for *cry1F*, MKAD-1 and MKAD-5 were positive for *cry1I* and MKAD2, MKAD3 and MKAD4 showed amplification with *cry1K*. The profiling pattern of native isolates for *cry1* subtypes genes was entirely different from the pattern of standard strains.

Discussion

Keeping in view the presence of crystalloferous protein in *B. thuringiensis* isolates we conducted the study on PCR based detection of *cry* genes in the marine isolates to identify highly toxic halophilic *B. thuringiensis* strains. It is clear from the data that *B. thuringensis* was frequently present in the marine environment of Hawks Bay, Cape Town. These bacterial strains survived in highly saline environment. This character may lead to carry novel insecticidal genes proving high toxicity against insects especially storage insects of sea food. Reports of *Bacillus* from marine sources rarely mentioned the presence of *B. thuringiensis* isolates (Maeda et al. 2000).

The identification of known *cry* genes in the *B. thuringiensis* strains is important, since the specificity of the action is known for many of the Cry toxins. This fact allows the possibility of selecting novel native strains which can be used in the control of those target insects which are able to survive in the highly saline environment. PCR technique was introduced by Carozzi et al. (1991) to identify the *cry* genes in order to predict insecticidal activity. The native marine strain collection was characterized to identify *cry1*, *cry4*, *cry7*, *cry8*, *cry9* and *cry10* with six pairs of universal primers (Un1, Un4, Un7, 8A, Un9 and Un10) that were selected from highly conserved regions among each group of genes. The *cry* gene content of the marine *B. thuringiensis* strains is shown in Figure 1. Strains containing *cry1* genes were the most abundant in our collection (12 strains, representing 38.7%). The frequency of *B. thuringiensis* strains harboring *cry4*, *cry7* was 32%, *cry8*, *cry9* 22.6% and *cry10* genes 13%, in the total collection of marine isolates.

The presence of different *cry* genes in the same *B. thuringiensis* strain has been reported earlier. Aronson (2002) and Ben-Dov et al. (1997) reported the presence of *cry1* genes and *cry3*, *cry8*, or *cry7* genes in the same *B. thuringiensis* strain. Bravo et al. (1998) observed Lepidopteran-active *cry1* genes and Coleopteran-active *cry3A*, *cry3Ba* and *cry7A* genes in the same strain. They suggested that such *B. thuringiensis* strains which harbors more than one *cry* gene, have a high frequency of genetic information exchange.

In our marine culture collection most of the isolates (55%) harbor single *cry* gene, 19.35% harbor two types, 9.6% harbor three types of *cry* genes and 9.6% isolates showed diversified profile with five types of *cry* genes. The *cry1* genes were the most frequently found among the thirty one marine *B. thuringiensis* isolates (Figure 3B and C). Among all *B. thuringiensis* isolates only two isolates were considered to be negative for the six sets of universal primers used. However, these strains produced crystal inclusions, suggesting that they may contain other Cry toxins. These isolates may have had some other *cry* genes not identified by the gene specific primers used in this study.

In our strain collection, the distribution and frequency of *cry1* genes were very high (38.7%) but it was low as compared to previous reports. According to Bravo et al. (1998), occurrence of *B. thuringiensis* strains containing *cry1* genes is the abundant (49.5%) in Mexican strain collection. However, strains harboring *cry4* and *cry3* genes were the second most abundant.

Among the profiles of *cry1* genes of our isolates, *cry1K* was the most frequently found (25%) and the second abundant genes were *cry1F* (16.6%) and *cry1I* (16.6%). Chak et al. (1994) reported that *cry1A* genes were the most abundant (21.26%) in soil samples collected from different areas of Taiwan, followed by the *cry1D* (0.086%) and *cry1C*

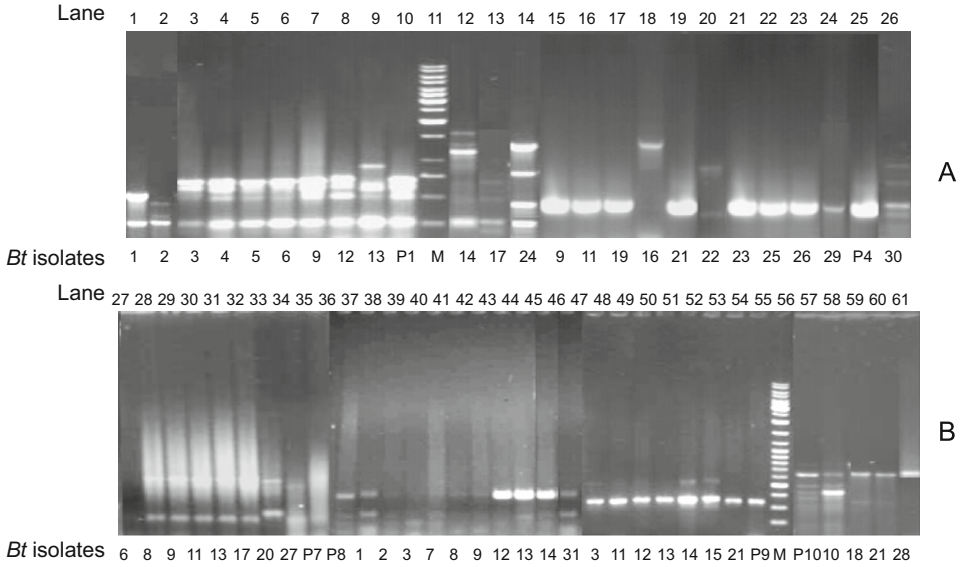

Figure 1. PCR analysis of crystal protein positive *B. thuringiensis* isolates. PCR products of native marine isolates: (A) *cry1*, lanes 1–10; *cry4*, lanes 12–26 (B) *cry7*, lanes 27–35; *cry8*, lanes 36–47; *cry9*, lanes 48–55 and *cry10*, lanes 57–61. Lane M: 1 kb DNA MW marker; [a]P1: HD-73 (276 bp); [a]P4: IPS78 (439 bp); [a]P7: HD-511 (264 bp); [a]P8: HD-537 (430 bp); [a]P9: HD-29 (354 bp); [a]P10: IPS78 (623 bp). [a] Positive control strains.

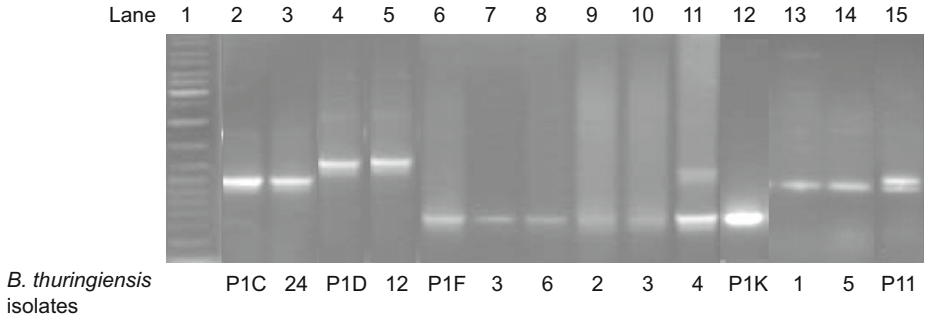

Figure 2. PCR analysis of *cry1* subtype *B. thuringiensis* positive isolates. PCR products for *cry1C* (670 bp), lanes 2 and 3; *cry1D* (802 bp), lanes 4 and 5; *cry1F* (246 bp), lanes 6–9; *cry1K* (246 bp), lanes 8–12 and *cry1I* (621 bp), lanes 13–15. Lane 1: 1 kb molecular weight marker sizes indicated on left. Lane 2: [a]P1C, lane 3: MKAD-24, lane 4: [a]P1D, lane 5: MKAD-12, lane 6: [a]P1F, lane 7: MKAD-3, lane 8: MKAD-6, lane 9: MKAD-2, lane 10: MKAD-3, lane 11: MKAD-4; lane 12: P1K, lane 13: MKAD-1 and lane 14: MKAD-5. [a] Positive control strains.

(0.075%) genes. Four out of 225 strains harbored *cry4* genes, and *cry3*, *cry1B*, *cry1E* or *cry1F* genes were not detected in any strain. Song et al. (2003) showed that *cry1I*-type genes appeared in 95 of 115 (82.6%) *B. thuringiensis* isolates and 7 of 13 standard strains. They found a novel *cry1I*-type gene in one standard strain and six isolates.

The PCR screening is a rapid method for detecting and differentiating *B. thuringiensis* strains by their PCR product profiles and for predicting their insecticidal activities in order to direct them for subsequent toxicity assays against Lepidoptera, Coleoptera and Diptera. The *B. thuringiensis* isolates displaying new profiles that containing combinations of *cry* genes should be further characterized and developed for integration with other toxicity assays against Coleoptera, Diptera and other Lepidoptera insects. Ben-Dov et al. (1997) also suggested that a strain may contain a novel gene with sequences annealing to the primers for known genes but different sequences in other regions defining a new insecticidal activity. This limitation can be resolved by a set of specific primers through the sequence of a particular gene (Kalman et al. 1993).

This is the first report about the occurrence of *B. thuringiensis* in marine sedimentary environment of Arabian Sea. These strains are good candidates for use as biocontrol agents with a wider spectrum of action.

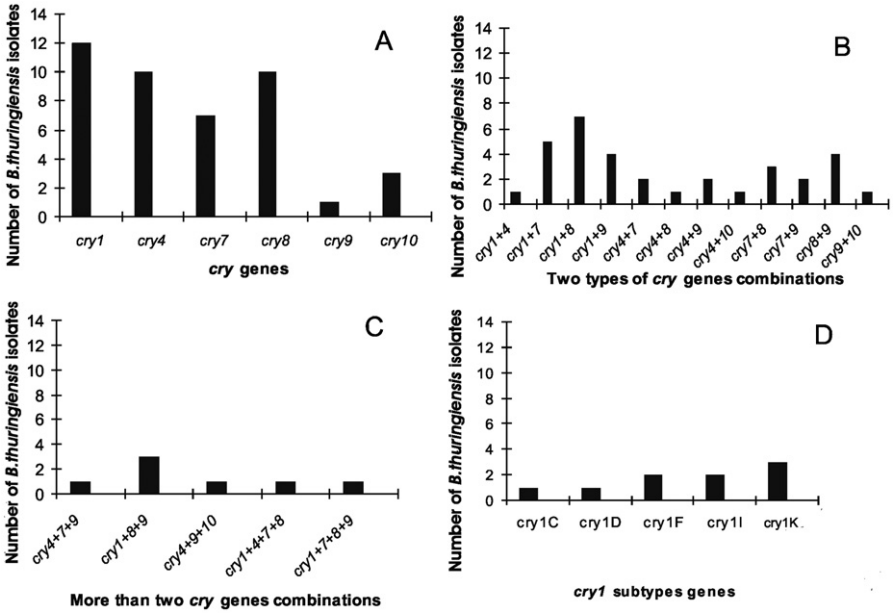

Figure 3. Distribution of different *cry* genes in the marine *B. thuringiensis* isolates. (A) Distribution of single *cry* gene, (B) distribution of two *cry* genes, (C) distribution of more than two *cry* genes and (D) distribution of *cry1* subtype genes.

References

Aronson A. Sporulation and d-endotoxin synthesis by *Bacillus thuringiensis*. Cell Mol Life Sci 2002;59: 417–425.

Alberola TM, Aptosoglou S, Arsenakis M, Bel Y, Delrio G, Ellar DJ, et al. Inseticidal activity of strains of *Bacillus thuringiensis* on larvae and adults of *Bactrocera oleae* Gmelin (Dipt Tephritidae). J Invert Pathol 1999;74: 127–136.

Bel YF, Granero TM, Alberola J, Sebastian M, Ferre J. Distribution, frequency and diversity of *Bacillus thuringiensis* in olive tree environments in Spain. Syst Appl Microbiol 1997;20:652–8.

Ben-Dov EQ, Zaritsky A, Dahan E, Barak Z, Sina R, Manasherob R, et al. Extended screening by PCR for seven *cry* group genes from field collected strains of *Bacillus thuringiensis*. Appl Environ Microbiol 1997; 63(12):4883–90.

Bravo A, Sarabia S, Lopez L, Ontiveros H, Abarca C, Ortiz A, et al. Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. Appl Environ Microbiol 1998;64:4965–72.

Carozzi NB, Kramer VC, Warren GW, Evola S, Koziel MG. Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Appl Environ Microbiol 1991;57:3057–61.

Cannon RJC. *Bacillus thuringiensis* use in agriculture. A molecular perspective. Biol Rev 1996;71:561–636.

Ceron J, Ortiz A, Quintero R, Guereca L, Bravo A. Specific PCR primers directed to identify *cry I* and *cry III* genes within a *Bacillus thuringiensis* strain collection. Appl Environ Microbiol 1995;61:3826–31.

Chak KF, Chao DC, Tseng MY, Kao SS, Tuan S, Feng TY. Determination and distribution of *cry*-type genes of *Bacillus thuringiensis* isolates from Taiwan. Appl Environ Microbiol 1994;60:2415–20.

Edwards DL, Payne J, Soares GG. Novel isolates of *Bacillus thuringiensis* having activity against nematodes. European Patent Application, EP 1988.

Feitelson LS, Payne L, Kim L. *Bacillus thuringiensis*: insects and beyond. J Biotech 1992;10:271–5.

Holt G, Krieg NR, Sneath P, Williams ST. Bergey's manual of determinative bacteriology. Baltimore, USA: Williams and Wilkins; 1994.

Iriarte J, Bel Y, Ferrandis MD, Andrew R, Murillo J, Caballero P. Environmental distribution and diversity of *Bacillus thuringiensis* in Spain. Syst Appl Microbiol 1998;21:97–106.

Juarez-Perez VM, Ferrandis MD, Frutos R. PCR-based approach for detection of novel *Bacillus thuringiensis cry* genes. Appl Environ Microbiol 1997;63:2997–3002.

Kalman S, Kiehne KL, Libs JL, Yamamoto T. Cloning of a novel *cryIC*-type gene from a strain of *Bacillus thuringiensis* subsp. *galleriae*. Appl Environ Microbiol 1993;59:1131–7.

Martin PAW, Travers RS. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl Environ Microbiol 1989;55:2437–42.

Maeda M, Mizukie E, Nakamura Y, Hatano T, Ohba M. Recovery of *Bacillus thuringiensis* from marine sediments of Japan. Curr Microbiol 2000;40:418–22.

Meadows MP, Ellis DJ, Butt J, Jarrett P, Burges HD. Distribution, frequency, and diversity of *Bacillus thuringiensis* in an animal feed mill. Appl Environ Microbiol 1992;58:1344–50.

Porcar M, Juarez-Perez V. PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes. FEMS Microbiol Rev 2003;26:419–32.

Rowe GE, Margaritis A. Bioprocess development in the production of bioinsecticide by *Bacillus thuringiensis*. CRC Critical Rev Biotechnol 1987;6:87–127.

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, et al. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 1998;62:775–806.

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

Uribe D, Martinez W, Ceron J. Distribution and diversity of *cry* genes in native strains of *Bacillus thuringiensis* obtained from different ecosystems from Colombia. J Invertebr Pathol 2003;82:119–27.