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

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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.

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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...
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. _kenya_ (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 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; tryptoase 2; yeast extract 1.5; sodium di-hydrogen phosphate 6.9; di-sodium hydrogen phosphate 8.9; MnCl₂ 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 H2O 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% MgCl2, 2% Triton X100) and 6 µl (5 mg ml−1) of Protease 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 mM deoxynucleotide triphosphate mix, 0.5 µM of forward (+) and reverse (−) primers, 1.5 mM MgCl2 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...
(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

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<th>Table 1. Primers used for the detection of different cry genes in the local isolates of Bacillus thuringiensis.</th>
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<td>Primer pair</td>
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<td>-----------------</td>
</tr>
<tr>
<td>Un1(d)</td>
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<td>Un1(r)</td>
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<td>Un4(r)</td>
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<td>Un7</td>
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<tr>
<td>(−)33mer 5′CACTTAAACGCAATGGTTAGAATATCCAGAGATA3′</td>
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<td>8A</td>
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<td>(−)27mer 5′TTTGACACTTCTGCTCATCTA3′</td>
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<td>Un9</td>
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<tr>
<td>(−)24mer 5′GTTTGAGCCGCTTCACAGCAATCC3′</td>
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<td>CJII-1</td>
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<td>CJII-2</td>
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<td>(−)34mer 5′GTCTAGAATCTCTGATGCGATTACAGATGC3′</td>
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<td>1Fb</td>
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<tr>
<td>(−)28mer 5′GCTACGACCACTAVGGAACACTCT3′</td>
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<td>1Ha</td>
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<td>(−)34mer 5′GTTCTCTTAATATATTGCTGAAATTACCA3′</td>
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<td>1a</td>
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<td>1K</td>
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<td>(−)26mer 5′GCCCACCAGCCTATAGTACAGTTCG3′</td>
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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. thuringiensis 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.
(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 cry11-type genes appeared in 95 of 115 (82.6%) B. thuringiensis isolates and 7 of 13 standard strains. They found a novel cry11-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.
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