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PCR-based methods to discriminate *Bacillus thuringiensis* strains

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Abstract - *Bacillus thuringiensis* is a Gram positive bacterium that is used for the production of biopesticides. The toxic action of different strains and serovars of *B. thuringiensis* can be extremely selective towards specific pests, or, in contrast, it can affect a wide variety of non-target organisms such as insects, vertebrates or humans. A reliable characterization of the cultivated strains is of primary importance for the biopesticide industry, in order to assess the contamination of the final product with strains with different pesticide actions or that might be dangerous for human health. The aim of this study was to develop useful methods for the typing of different *B. thuringiensis* strains using two PCR-based methods, RAPD and Rep-PCR with BOXA1R and ERIC2 primers. The molecular fingerprints obtained using ERIC2-PCR showed a reliable ability to discriminate *Bacillus thuringiensis* strains.

Key words: *Bacillus thuringiensis*, RAPD PCR, Rep-PCR.

INTRODUCTION

Over the past 20 years, the negative effects of pesticides have led to the development and production of alternative methods of controlling harmful insects. Nowadays, most biopesticides are represented by *Bacillus thuringiensis* (Glare and O'Callaghan, 2000): in 1986 over 410 commercial formulations of this bacterium had been patented in the USA (Aronson *et al.*, 1986). The wide diffusion of these products is related to the production of *B*. *thuringiensis* insecticidal crystal proteins (ICPs), which are considered to be non-pathogenic for birds, reptiles, amphibians and humans. The wide range of action of *B*. *thuringiensis* is the result of the high variability of the *cry* genes that code for about 170 different ICPs (Levinson *et al.,* 1990; Lereclus *et al.,* 1993), and that are produced by different strains and serovars. Strains of *B. thuringiensis* serovar *kurstaki*, for example, can be divided in two different groups characterized by specific activities against *Lepidoptera*, and *Lepidoptera* and *Diptera* (Nicholls *et al.*, 1989). Furthermore, it has been shown that under laboratory experimental conditions the plasmids carrying *cry* genes are interchangeable between *B. thuringiensis*, *Bacillus cereus* and *Bacillus anthracis* (Battisti *et al.*, 1985; Wiwat *et al.*, 1990). In industrial production, the high bacterial density within the biofermentors makes something frequent the interchange of genetic material between different strains (Deseo Kovacs and Rovesti, 1992). Moreover, the contamination with bacteria belonging to different serovars can result in a lack of specificity and effectiveness of the commercial product, thus representing a serious and expensive problem for the industry. The ability to constantly monitor and trace the production process and to specifically and rapidly assess the presence of undesired microrganisms allows intervention before excessive economic losses.

Different methods that have been used for the identification of *B. thuringiensis*, such as H-antigen serotyping (Lecadet *et al.*, 1999) and the analysis of the genes coding for the δ-endotoxins (Höfte and Whiteley, 1989), are not suitable for the monitoring of the production since they are extremely time consuming. In contrast, PCR-based techniques are fast and effective for bacterial typing and monitoring (Caetano-Anolles *et al*., 1991; Priest *et al*., 1994; Damgaard *et al*., 1996; Daffonchio *et al*., 1998).

In the present study, two molecular methods based on PCR have been tested in order to evaluate their suitability for the characterization of *B. thuringiensis* strains. To assess the discriminating abilities of different PCR primers and methods, reference strains belonging to five diverse *B. thuringiensis* serovars were analysed. Furthermore the suitability of the different primers was then tested to characterize four natural strains identified by phenotypic and physiological traits as *Bacillus thuringiensis*. Besides, the molecular fingerprints obtained using ERIC2-PCR were investigated by cluster analysis to assess phylogenetic consistency.

MATERIALS AND METHODS

Bacterial strains. The reference strains of *Bacillus thuringiensis* and the *Bacillus* spp. isolates used in this study are listed in Table 1.

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TABLE 1 – *Bacillus thuringiensis* reference strains and *Bacillus* spp. isolates used in this study

Reference strains[a]	Code
Bacillus thuringiensis serovar *finitimus*	HD19
Bacillus thuringiensis serovar *kurstaki*	HD1
Bacillus thuringiensis serovar *kurstaki*	HD73
Bacillus thuringiensis serovar *aizawai/pacificus*	HD133
Bacillus thuringiensis serovar *israelensis*	HD500
Bacillus thuringiensis serovar *morrisoni*	tenebrionis
Natural isolates [b]	**Code**
Bacillus spp.	B01
Bacillus spp.	B02
Bacillus spp.	B03
Bacillus spp.	B04

[a] Reference strains obtained from *Bacillus* Genetic Stock Center, Department of Biochemistry, Ohio State University; [b] Natural isolates obtained from Dipartimento di Scienze Ambientali Agrarie e Biotecnologie Agroalimentari, Università di Sassari.

DNA isolation and PCR conditions. Two different methodologies for DNA isolation and recovery have been used and their respective yield and rapidity compared. The first method was based on the Marmur protocol (1961). Briefly *Bacillus thuringiensis* strains were grown in 5 ml of Nutrient Broth medium (Oxoid, Basingstroke, Hampshire) and incubated at 30°C for 12 h on a rotary shaker at 150 rpm. Cells from 1.5 ml of the overnight cultures were centrifuged at 4,000 rpm for 5 min and the cell pellets resuspended in 1 ml of sterile dH_2O and centrifuged again. The pellet was finally resuspended in 200 μl of solution 1 (Tris-HCl 10mM, EDTA 1 mM, Sucrose 0.35 M) added with 7 mg/ml of lysozyme. This solution was incubated at 37 °C for 40 min, added to 300 μl of solution 2 (100 mM Tris-HCl, 0.3M NaCl, 20 mM EDTA, 2% SDS), incubated at 60 °C for 20 min with proteinase K (10 mg/ml), added of 600 μl phenol:chloroform:isoamylic alcohol (25:24:1) and centrifuged at 14,000 rpm for 15 min at 4 °C. Aliquots of 300 µl from the aqueous layer were transferred to a tube containing 1 volume of isopropanol, centrifuged at 14,000 rpm for 15 min at 4 °C, the isopropanol was discarded and the pellet washed with 0.5 ml of 70 % ethanol. The ethanol was discarded and the pellet vacuum dried for 15 min and finally resuspended in 100 µl of TE buffer. The second method was based on the Hoffman and Winston (1987) procedure for DNA isolation from *Escherichia coli* or yeast. Briefly *Bacillus thuringiensis* strains were grown as described above. Aliquots of 1.5 ml from the overnight cultures were centrifuged at top speed and the cell pellets resuspended in 200 µl of lysis buffer [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)], 200 µl of phenol:chloroform:isoamylic alcohol (25:24:1) and 0.3 g of acid-washed glass beads added. The mixtures were vigorously vortexed for 2 min and subsequently centrifuged for 5 min at top speed. Aliquots of 200 µl from the aqueous layer were transferred to a tube containing 1 volume of isopropanol, centrifuged at 14,000 rpm for 15 min at 4 °C, the isopropanol was discarded and the pellet washed with 0.5 ml of 70 % ethanol. The ethanol was discarded and the pellet vacuum dried for 15 min and finally resuspended in 100 µl of TE buffer.

In both protocols, RNA was eliminated by adding 1 μl RNase (10 mg/ml), at 37 °C for 15 min. The DNA yield and quality were subsequently determined following gel electrophoresis on 1% agarose gel (100 V, 400 mA for 70 min), ethidium bromide staining and spectrophotometric analysis. The stained gels were photographed using Chemi Doc UV transilluminator (BioRad, Mi Italy).

Fifty nanograms of DNA were used for PCR reactions. The primers used to generate PCR fingerprints are listed in Table 2. The PCR conditions employed were as follows: BOXA1R, 1 cycle at 95 °C for 7 min, 35 cycles at 94 °C for 3 min, 50 °C for 1 min, 68 °C for 8 min, and final elongation at 68 °C for 8 min; the PCR reaction mixture (25 µl) contained: 4 mM $MgCl_2$, 0.2 μM primer BOX A1R, 0.2 mM dNTPs mix, 0.2 μl *Taq* DNA polymerase (5U/μl). ERIC2, 1 cycle at 95 °C for 2 min, 45 cycles at 94 °C for 3 min, 50 °C for 1 min, 72 °C for 2 min; the PCR reaction mixture (25 µl) contained: 3 mM $MgCl_2$, 0.2 μM primer ERIC2, 0.2 mM dNTPs mix, 0.2 μl *Taq* DNA polymerase (5U/μl). RAPD1254, 1 cycle at 94 °C for 2 min followed by 45 cycles at 95 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min, and a final elongation at 72 °C for 10 min; the PCR reaction mixture (25 µl) contained: 6 mM $MgCl_2$, 0.2 μM primer 1254, 0.2 mM dNTPs mix, 0.2 μl *Taq* DNA polymerase (5U/μl). The PCR products were examined following gel electrophoresis on 1.8 % agarose gel (100 V, 400 mA for 70 min) and ethidium bromide staining. DNA bands were visualized using Chemi Doc (BioRad, Mi Italy).

Digital images of the gels were further analysed using Quantity One image analysis software (BIO-RAD Laboratories Inc., California). Binary matrixes obtained from strain fingerprints have been subjected to cluster analysis using the Minitab statistical programme Release 1.4 Statistical Software (Minitab Inc. State College, Pennsylvania).

Metabolic and phenotypic analyses. *Bacillus* isolates were grown on Nutrient Broth medium (Oxoid, Basingstroke, Hampshire) and subsequently used for metabolic and phenotypic analyses. The cultures were examined for the shape of sporangia and the presence of parasporal crystals under phase contrast microscopy (Forsyth and Logan, 2000). The four natural isolates were identified according to Bergey's Manual of Bacteriology (Claus and Berkeley**,** 1986). The characters considered were: cell morphology; catalase reaction; anaerobic growth; acid production from D-glucose, L-arabinose, D-xylose and D-mannitol; gas production from glucose; casein, gelatine and starch hydrolysis; growth in

TABLE 2 – Oligonucleotide primers used to generate PCR fingerprints

Primer	Sequence	Description	Reference
1254	5'CCGCAGCCAA3'	RAPD-PCR	Miteva *et al.*, 1999
BOXA1R	5'CTACGGCAAGGCGACGCT3'	Rep-PCR	Versalovic *et al.*, 1991
ERIC2	5'AAGTAAGTGACTGGGGTGAGCG3'	Rep-PCR	Versalovic *et al.*, 1991

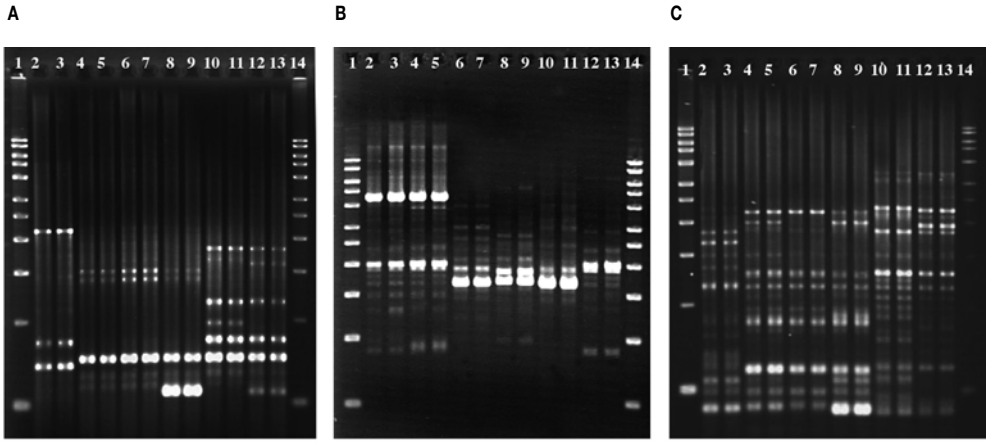

FIG. 1 – PCR amplification patterns obtained with the primer BOXA1R (A), RAPD1254 (B) and ERIC2 (C) primers. The DNA from the samples 2-4-6-8-10-12 was isolated according to Marmur (1961); the DNA from the samples 3-5-7-9-11-13, according to Hoffman and Winston (1987). Lanes 1 and 14: 1 kB ladder, lanes 2 and 3: *Bacillus thuringiensis* serovar *finitimus*, lanes 4 and 5: *B. thuringiensis* serovar *kurstaki* (HD1), lanes 6 and 7: *B. thuringiensis* serovar *kurstaki* (HD73), lanes 8 and 8: *B. thuringiensis* serovar *aizawai*, lanes 10 and 11: *B. thuringiensis* serovar *morrisoni,* lanes 12 and 13: *B. thuringiensis* serovar *israelensis*.

Nutrient Broth medium at pH 5.7 and 6.8; growth at 5, 10, 30, 40, 50 and 65 °C; growth in Nutrient Broth medium at 5, 7 and 10% NaCl; resistance to lysozyme; reduction of nitrate to nitrite. Moreover, strains were tested using API 50CHB and 20E (BioMérieux, Paris).

RESULTS AND DISCUSSION

Electrophoresis and spectrophotometric analyses revealed that both methods of DNA extraction and recovery allowed to obtain not degraded DNA suitable for PCR reaction. Substantial differences in time required to complete the procedures were evident. Marmur-based protocol required about 3 h while the method based on Hoffman and Winston required about 30 min. We found this latter the most suitable procedure for our purposes. Indeed, even with so few steps, compared to the DNA isolation method described for Gram positive bacteria (Marmur, 1961), it allowed to obtain pure DNA suitable for PCR analysis. Moreover, the PCR reactions carried out with different primers definitely showed that the amplification patterns were not affected by the DNA isolation method (Fig. 1A, B, C). The analysis of PCR-fingerprints showed that a total of 17 DNA bands with different molecular weight were generated using BOXA1R (Fig. 1A), 29 with RAPD 1254 (Fig. 1B), and 33 with ERIC2 (Fig. 1C). The use of the primer BOXA1R, designed to amplify interspersed and repeated DNA elements (Versalovic *et al*., 1994), was not entirely satisfactory. In particular, this primer did not show any discriminatory power in the recognition of the two strains *B. thuringiensis* serovar *kurstaki* notably HD1 and HD73. This is a significant limitation since these two strains have different pesticide activities (Geiser *et al*., 1986). On the contrary the use of ERIC2 and 1254 allowed the differentiation of all the six *B. thuringiensis* reference strains.

To further investigate the suitability of the DNA extraction procedure after Hoffman and Winston (1987), as well as the strain-specific discriminatory power of the different oligonucleotides primers, we extended our study to four natural *Bacillus* isolates. An identification of these latter, based on metabolic and phenotypic profiles, was first achieved to establish the species. On the basis of the characters comparison related to the metabolic and phenotypic profiles (Table 3), B01, B02, B03 and B04 (Table 1) were identified as *B. thuringiensis.* According to Logan and Berkeley (1984) *B. thuringiensis* and *B. cereus* showed the same metabolic profile when tested with API 50CHB and 20E, where the only difference is the presence of parasporal crystals which have been observed in our case. Based on these findings the isolates were assigned to the *Bacillus thuringensis* species. To characterize at molecular level the four isolates, Hoffman and Winston procedure for DNA isolation was used and then PCR performed using BOXA1R, RAPD1254 and ERIC2. BOXA1R and RAPD1254 PCR amplification profiles did not discriminate between the isolates (Fig. 2A, 2B). On the contrary the ERIC2 primer allowed the unambiguous recognition of all of these isolates as different strains (Fig. 2C). Globally ERIC2 showed to be a suitable oligonucleotide primer for the molecular characterization or distinction among *Bacillus thuringensis* strains.

Rep-PCR revealed as a useful tool for evaluating infra specific diversity of different bacterial species e.g. *Yersinia enterocolitica* and *Sinorhizobium meliloti* (de Bruijn, 1992; Niemann *et al*., 1999; Sachdeva and Virdi, 2004). Despite in some case cluster analysis applied to Rep-PCR fingerprints was able to show a clear distinction between strains belonging to different species (Zribi *et al*., 2004), this was not our case. In particular no consistency was observed when cluster analysis was applied to the binary matrixes obtained from ERIC2 fingerprintings. In particular the ERIC2 profile of *B. thuringensis* serovar *kurstaki* HD1 was found more similar to the *B. thuringensis* serovar *finitimus* HD19 and the *B. thuringensis* serovar *aizawai* HD133 and not to the closer *B. thuringensis* serovar *kurstaki* HD73. For this reason the use of ERIC2 does not seem to be suitable for philogenetic analyses (Fig. 3).

TABLE 3 – Metabolic and phenotypic features of *Bacillus* isolates and type strain

Tests	B01	B02	B03	B04	ATCC 10792[a]
Gram staining	+	+	+	+	+
Sporangium swollen	–	–	–	–	–
Parasporal crystal	+	+	+	+	+
Production of catalase	+	+	+	+	+
Anaerobic growth	+	+	+	+	+
Acid production from					
D-glucose	+	+	+	+	+
L-arabinose	–	–	–	–	–
D-xylose	–	–	–	–	–
D-mannitol	–	–	–	–	–
Gas production from glucose	–	–	–	–	–
Growth temperatures at					
5 °C	–	–	–	–	–
10 °C	+	+	+	+	+
30 °C	+	+	+	+	+
40 °C	+	+	+	+	+
50 °C	–	–	–	–	–
65 °C	–	–	–	–	–
Growth in NaCl					
5%	+	+	+	+	+
7%	+	+	+	+	+
10%	+	+	+	+	+
Growth at pH					
5.7	+	+	+	+	+
6.8	+	+	+	+	n.d.[b]
Resistance to lysozyme	+	+	+	+	n.d.
Hydrolysis of starch	+	+	+	+	n.d.
Hydrolysis of casein	+	+	+	+	n.d.
Hydrolysis of gelatin	+	+	+	+	n.d.
Reduction of nitrate to nitrite	+	+	+	+	n.d.

[a] *Bacillus thuringiensis* Berliner (Claus and Berkeley, 1986); [b] not determined.

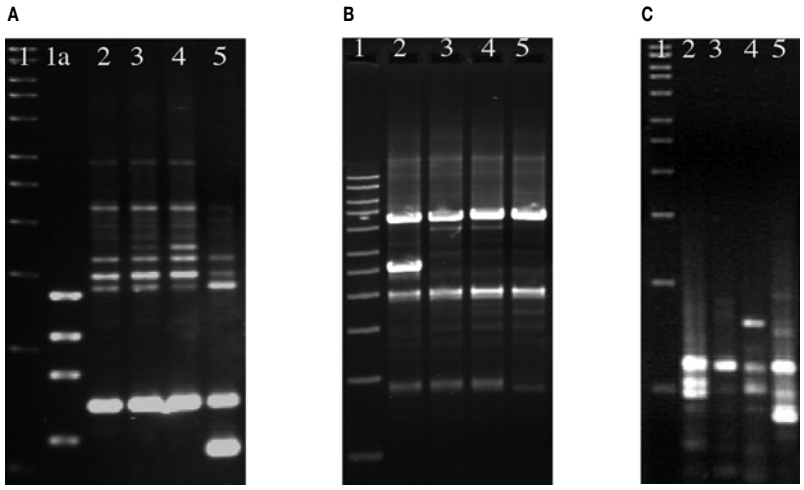

FIG. 2 – PCR amplification patterns of the isolates obtained with the primer BOXA1R (A), RAPD1254 (B) and ERIC2 (C) primers. Lane 1: 1kb ladder, lane 1a: 50 bp ladder, lanes 2, 3, 4 and 5: B01, B02, B03 and B04 respectively.

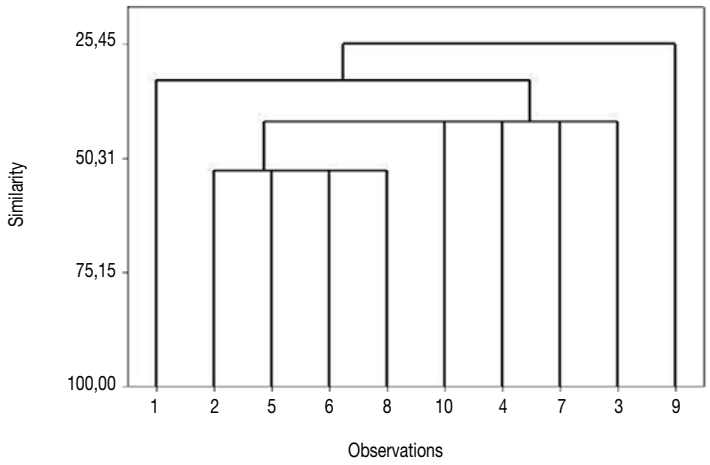

FIG. 3 – The dendrogram obtained from the ERIC2 PCR amplification pattern. Observations: 1, B01; 2, B02; 3, B03; 4, B04; 5, *Bacillus thuringiensis* serovar *kurstaki* (HD1); 6, *B. thuringiensis* serovar *finitimus*; 7, *B. thuringiensis* serovar *kurstaki* (HD73); 8, *B. thuringiensis* serovar *aizawai*; 9, *B. thuringiensis* serovar *morrisoni*; 10, *B. thuringiensis* serovar *israelensis.*

CONCLUSIONS

The purpose of this study was to find out an efficient method for the discrimination of *B. thuringiensis* reference strains and natural isolates. The methods used for the DNA isolation and ERIC2-PCR analysis allowed to obtain a unique molecular profile for all the tested *B. thuringiensis* strains. However, they do not give any information on the strain pesticide activity, and they cannot be used for serovar identification. The ability of a method to consistently discriminate between different strains might represent a useful tool for the monitoring of the production of *B. thuringiensis*-based pesticides at industrial level. In this context the results from this studies can be considered promising.

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